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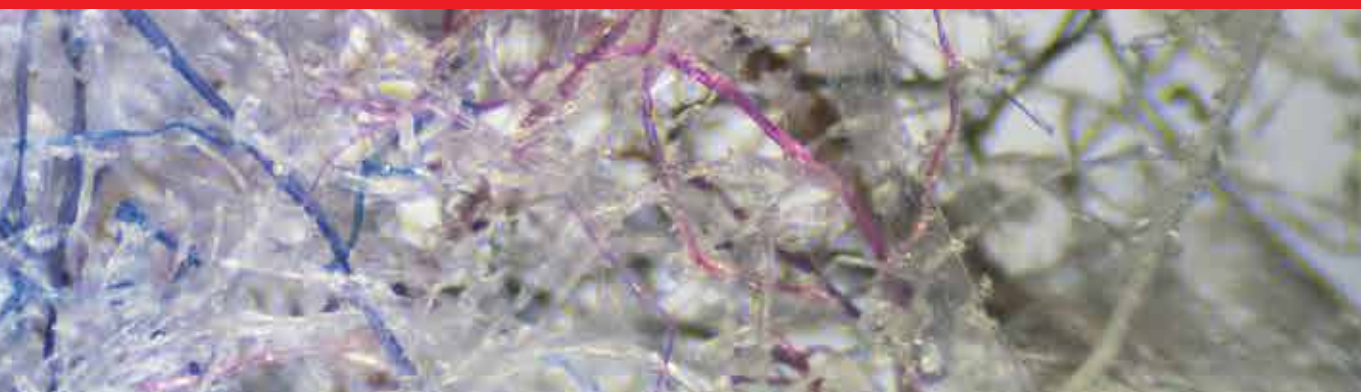
Advances in the Molecular Understanding of Colorectal Cancer

Edited by Eva Segelov



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Contributors
Daphne Day, Cameron Naidu, Sophia Frentzas, Maja Green, Eva Segelov, Matthew Dun, Tiffany Gould, Peter Pockney, Muhammad Jamaluddin, Brett Nixon, Rodney Scott, Joel Petit, Simon King, Timothy Price, Lee-Jen Luu, Avani Athauda, Ian Chau, Martin Berger, Rania Georges, Hassan Adwan, Oliver Sieber, Anuratha Sakthianandeswaren, Polly Sabljak, Meg Elliott, Michelle Palmieri, Tao Lu, Antja-Voy Hartley, Matthew Martin

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Meet the editor

Eva Segelov (MBBS, PhD, FRACP) is Professor and Director of Oncology at Monash University and Monash Health, Melbourne, Australia. She is a Medical Oncologist with specialist interest in the diagnosis and management of upper and lower gastrointestinal cancers, particularly colorectal cancer, neuroendocrine tumours and anal cancer. Prof. Segelov is the international lead on the investigation initiated ICECREAM and ASCOLT academic international trials and is the Australian lead for many others. She has published over 110 articles, expert reviews and book chapters and is a frequently invited speaker at national and international conferences. She is a European Society of Medical Oncology (ESMO) faculty member for the Gastro-intestinal Group and the CUP, Endocrine Tumours and Others Group and Associate Editor of the Journal of Global Oncology (ASCO). Eva is a Board member of the Australasian Gastro-Intestinal Trials Group, Chair of Gastrointestinal Cancer of the Clinical Oncological Society of Australia and co-founder of the international neuroendocrine research collaborative group CommNETS.

Contents

Preface

Modern molecular technology has revolutionized the procurement of information from cancer tissue and blood samples. With this has come a rapid increase in our understanding of the molecular pathogenesis of cancer, at a genomic, transcriptomic and proteomic level. The challenge is to translate this into clinically useful information that will impact on cancer care; conversely, to use knowledge from the clinical behaviour of cancers to direct fruitful laboratory-based research that will yield new treatment targets, prognostic and/or predictive factors, therapy resistance markers and other relevant molecular information. Colorectal cancer, although highly curable if detected early, still has one of the poorest prognoses of any cancers once metastatic. Understanding the molecular profile of colorectal cancer is essential for all clinicians who interact with patients with colorectal cancer, as well as for translational scientists looking for areas most likely to yield clinically applicable information that can guide current treatment pathways as well as direct areas for future progress.

This book comprises a series of expert reviews on aspects of colorectal cancer that influence current therapy and/or enhance understanding of tumourogenesis, metastatic processes and therapeutic targets and treatment. A broad array of topics is covered with contributions from across the globe.

The book is divided into two sections: Molecular Classification and Biomarkers. The first chapter describes recent advances in molecular classification of colorectal cancer, detailing the clinically relevant profiles of deficient mismatch repair and *RAS/RAF* and other mutations, as well as describing the development and limitation of the Consensus Molecular Subtypes. Chapter Two examines how experimental results have the potential to impact on patient management. This is followed by a chapter dedicated to the understanding of *BRAF* mutant colorectal cancer, incorporating molecular and clinical results in this important subgroup of patients with a very poor prognosis once metastatic disease is present.

The four chapters in the Biomarkers section explore the 'hot topics' of the clinical utility of circulating tumour DNA (is it ready for "prime time"?); the therapeutic application of epigenetic markers that are of particular prominence in colorectal cancer; the detailed story behind the evolution of predictive markers for the anti-EGFR monoclonal antibody class of therapies in clinical use and finally, the current and potential value of proteomics.

This field is changing rapidly and as such, this book is a clear and concise snapshot of where we have come from, where we are now and where we are heading, in the search for improved outcomes for patients with colorectal cancer. I would like to thank my laboratory and clinical teams at Monash University and Monash Health for their support during this project, as well as all the contributors for sharing their expertise in a timely and responsive manner. I sincerely thank the editorial and production team at IntechOpen for their prompt and professional assistance.

The proposal for this book was one of ten selected from 288 applicants for the IntechOpen Women in Science 2018 Book Collection. I am grateful to IntechOpen for providing this opportunity to women in science and very proud to have been chosen to bring this important topic into press.

Professor Eva Segelov MBBS, PhD, FRACP
Monash University and Monash Health,
Melbourne, Australia

Section 1

Molecular Classification

Chapter 1

Advances in Molecular Subclassification of Colorectal Cancer

Avani Athauda and Ian Chau

Abstract

This chapter will highlight the advances made in our understanding of the molecular landscape of colorectal cancer (CRC) via the development of molecular subclassification systems and their potential predictive and prognostic utility. Firstly, the comprehensive integrative analysis of 224 colorectal cancer samples performed by The Cancer Genome Atlas (TCGA) Research Network will be described highlighting the potential therapeutic targets identified. The development of molecular subclassification systems primarily via gene expression profile analysis by independent groups will also be described, and their potential clinical and prognostic associations will also be discussed. The chapter will then go on to describe the four consensus molecular subtypes of colorectal cancer which were proposed by an international consortium who applied unsupervised clustering techniques to the independent classification systems previously described. The clinical and prognostic associations of these four subtypes have been explored, and these findings will be discussed. Finally, the utility of molecular subclassification in colorectal cancer will be briefly explored.

Keywords: molecular subclassification, consensus molecular subtypes, gene expression profiles, colorectal cancer

1. Introduction

Colorectal cancer (CRC) was one of the earliest molecularly characterised solid tumours. Vogelstein et al. initially described the stepwise manner of adenoma formation to carcinoma via the accumulation of genetic and epigenetic events in the late 1980s [1]. This model provided insight into how driver alterations in the main oncogenes (KRAS, NRAS, BRAF and PI3K) and tumour suppressor genes (APC, TP53 and PTEN) were implicated in the biology of CRC [2]. The accumulation of these genetic mutations leads to carcinogenesis through deregulation of key pathways involved in cell proliferation, differentiation and apoptosis. It is now known that abnormalities of the Wnt signalling pathway are almost ubiquitous in sporadic CRC and usually arise from mutations of the APC gene [3].

Further to this, genetic and epigenetic exploration of CRC subsequently identified significant molecular heterogeneity in this disease. This was clinically evident by the differing responses to systemic therapy and varying clinical course of patients with the same stage of tumour. Biomarker discovery in CRC has arisen

through the analysis of responders and nonresponders to targeted agents and the subsequent discovery of RAS mutations conferring resistance to anti-epidermal growth factor receptor (EGFR) therapies. More recently, our deeper understanding of the underlying biology of CRC has also revealed that clonal, stromal and immune characteristics of tumours are important when considering therapeutic targets. The ongoing need to accurately define molecularly distinct subgroups and identify the underlying genetic drivers as well as novel therapeutic targets within each subgroup in order to rationalise drug development continues to be of paramount importance in CRC.

2. Early molecular characterisation of colorectal cancer

It is now well established that the majority of sporadic CRC cases (85%) exhibit chromosomal instability (CIN) with changes in chromosome number and structure such as deletions, gains, translocations and amplifications. CIN is associated with inactivating mutations or losses in the APC tumour suppressor gene which occurs early in the adenoma-carcinoma sequence [3]. The remaining 15% of sporadic CRCs demonstrate microsatellite instability (MSI) through changes in the number of repeats or length of microsatellites. MSI arises through defective DNA mismatch repair (MMR) mechanisms caused by epigenetic silencing of the MLH1 gene by promotor hypermethylation [4]. Epigenomic studies have shown that MSI tumours have a high CpG island methylator phenotype (CIMP-H) which involves aberrant methylation of CpG-rich gene promoter regions. This leads to silencing of expression of critical tumour suppressor genes such as MLH1, thereby leading to the development of CRC [5]. Familial syndromes, such as Lynch syndrome/hereditary non-polyposis colorectal cancer syndrome (HNPCC), occur through germline mutational inactivation of genes encoding MMR proteins, namely, MLH1, MSH2, PMS2 and MSH6.

Clinicopathological features and the mutational status of CRC tumours differ according to the above classification. Sporadic MSI-high (MSI-H) tumours are more likely to be right-sided (proximal), poorly differentiated, mucinous and associated with tumour-infiltrating lymphocytes (TILs) and have higher rates of BRAF mutation, whereas microsatellite-stable (MSS) tumours are more frequently left-sided (distal) and have higher rates of KRAS mutation [5].

It has been shown that MSI status has both a prognostic and a predictive role in CRC. MSI-H tumours have better stage-adjusted survival (in stages I–III) when treated with surgery alone and do not derive as much benefit from adjuvant fluorouracil-based chemotherapy as MSS tumours do [4]. In advanced disease, MSI-H tumours are associated with a worse prognosis, and this is due to their association with activating BRAF mutations [6]. It has more recently been shown that MSI status also predicts for significant response and benefit from anti-PD1 antibodies with MMR-deficient tumours exhibiting higher response rates and longer progression-free survival (PFS) than MMR-proficient tumours [7].

3. Integrative molecular analysis of colorectal cancer

It is clear that anatomical factors and common DNA alterations are helpful in identifying subtype characteristics in CRC, but they alone are inadequate to define the boundaries between the different molecular entities that comprise CRC. In recent years, many studies have begun to exploit microarray technology to investigate gene expression profiles (GEPs) in CRC; however, no single signature

has proven clinically meaningful, especially in regard to predicting prognosis, and studies have been poorly reproducible due to the high molecular heterogeneity that exists in this disease.

In 2012, The Cancer Genome Atlas (TCGA) Research Network produced a comprehensive integrative analysis of 224 colorectal cancer tumour samples with paired normal samples in order to improve our understanding of the biology of this disease and identify potential therapeutic targets [8]. In addition, independent scientific groups also attempted to define intrinsic subtypes of CRC using GEPs in the hope that this will refine the molecular classification of CRC and facilitate clinical translation [9–14]. The findings of all of these independent analyses are discussed below.

3.1 The Cancer Genome Atlas (TCGA) comprehensive analysis of colorectal cancer

The comprehensive analysis of CRC undertaken by TCGA Research Network included tumours whose clinical and pathological characteristics reflected the usual breadth of features of CRC patients. Tumours were split into two main groups by mutation rate: those that were hypermutated (16%) and those that were non-hypermutated (84%) which seems to match the previously described MSI and CIN groups. The hypermutated group was then subdivided into those caused by defective MMR (dMMR) with a mutation rate of 12–40 mutations/Mb (approximately 13%) and those with an extremely high mutation rate of >40 mutations/Mb (approximately 3%)—the ultramutated group.

Initially, TCGA researchers considered colon and rectal tumours as separate entities due to their known anatomical and therapeutic differences. However, it was found that similar patterns of genomic alteration (copy number, expression profile, DNA methylation and miRNA changes) were seen in both types of tumours, so they were subsequently analysed together within the non-hypermutated group.

Thirty-two genes were identified to be recurrently mutated, and, after removal of non-expressed genes, the hypermutated and non-hypermutated groups had 15 and 17 recurrently mutated genes, respectively (see **Table 1**).

It was found that the tumour suppressor genes ATM and ARID1A displayed a disproportionately high number of frameshift or nonsense mutations. As expected, KRAS and NRAS mutations were activating oncogenic mutations at codons 12, 13 or 61, whereas the other genes had inactivating mutations. BRAF mutations were the classical V600E-activating mutations [8]. Given the differences in recurrently

Hypermutated group		Non-hypermutated group	
ACVR2A (63%)	CASP8 (29%)	APC (81%)	TCF7L2 (9%)
APC (51%)	CDC27 (29%)	TP53 (60%)	FAM123B (7%)
TGFBR2 (51%)	FZD3 (29%)	KRAS (43%)	SMAD2 (6%)
BRAF (46%)	MIER3 (29%)	TTN (31%)	CTNNB1 (5%)
MSH3 (40%)	TCERG1 (29%)	PIK3CA (18%)	KIAA1804 (4%)
MSH6 (40%)	MAP7 (26%)	FBXW7 (11%)	SOX9 (4%)
MYOB1 (31%)	PTPN12 (26%)	SMAD4 (10%)	ACVR1B (4%)
TCF7L2 (31%)		NRAS (9%)	GP6C (4%)
		EDNRB (3%)	

Table 1.
Significantly mutated genes in non-hypermutated and hypermutated groups.

mutated genes between hypermutated and non-hypermutated cancers, it appears that these tumours progress through different sequences of genetic events.

Interestingly, the recent data published by Jones et al. has identified that non-V600 BRAF-mutated advanced CRC represents a molecular subtype with distinct characteristics (which are different to $BRAF^{V600E}$-mutated CRC) and an excellent prognosis [15]. These patients may not require the aggressive chemotherapy treatment that is beneficial to classical BRAF-mutated patients. It is not yet clear whether non-V600 BRAF-mutated cancers harbour the same resistance to anti-EGFR therapies as cancers with $BRAF^{V600E}$ mutations, but higher frequency of concomitant RAS mutations in this subgroup will have to be taken into account.

TCGA analysis provided further confirmation on the pathways previously known to be deregulated in CRC. The vast majority of tumours in both groups (93% of non-hypermutated and 97% of mutated tumours) had deregulated Wnt signalling, predominantly via inactivation of APC. The MAPK signalling pathway was also commonly activated, as was the PI3K signalling pathway. Inactivation of the TGF-β inhibitory pathway was also seen, resulting in increased activity of MYC. Almost all of the analysed tumours, irrespective of location or mutation levels, exhibited changes in MYC transcriptional targets, highlighting the important role of MYC in CRC development. New findings identified by TCGA included recurrent mutations in FAM123B, ARID1A and SOX9 and very high levels of overexpression of the Wnt ligand-receptor gene FZD10. The SOX9 gene is associated with intestinal stem cell differentiation and has not previously been shown to be implicated in CRC. It has been shown to facilitate β-catenin degradation [16], and its transcription is suppressed by Wnt signalling which is activated by extrinsic Wnt ligands. These findings suggest a number of potential therapeutic targets in CRC, namely, Wnt signalling inhibitors and small molecule β-catenin inhibitors, which are beginning to show initial promise [17–19]. In addition, overexpression of the genes ERBB2 and IGF2, which are involved in regulating cell proliferation, were identified thus indicating potential therapeutic opportunities of inhibiting the products of these genes.

mRNA expression profiles of a subset of 189 TCGA samples separated the colorectal tumours into three clusters. One significantly overlapped with CIMP-H tumours and was enriched for hypermutated tumours, thereby representing a MSI/CIMP subgroup. The two other groups were representative of a CIN and an invasive phenotype subgroup.

3.2 Intrinsic subtypes of colorectal cancer identified by independent groups

Three molecular CRC subtypes were also identified by Roepman and colleagues ((A) MMR-deficient epithelial, (B) proliferative epithelial and (C) mesenchymal) using unsupervised clustering of whole genome data from 188 CRC tumour samples [9]. These intrinsic subtypes were subsequently validated in a cohort of 543 patients with stage II–III disease. In addition to identifying these subtypes with phenotypes matching those identified via TCGA, prognostic features and chemotherapy benefit characteristics were also investigated in this study. The dMMR subtype A (22%) was found to be epithelial-like and displayed a strong MSI phenotype linked to dMMR and a high mutational rate including activating BRAF mutations. Type A patients exhibited the best prognosis with minimal benefit from adjuvant 5-FU chemotherapy. The mesenchymal subtype C (16%) tumours exhibit epithelial-to-mesenchymal transition and show dMMR characteristics. These patients showed a poor baseline prognosis and no benefit from adjuvant 5-FU chemotherapy which is probably linked to their mesenchymal phenotype and low proliferative activity. The proliferative epithelial subtype B (62%) is almost exclusively MSS, BRAF wild type

and MMR proficient. They exhibit a relatively poor baseline prognosis but receive the most benefit from adjuvant chemotherapy. This study focused on stages II and III CRC; therefore, further validation of the subtype classification and its clinical relevance on a larger set of stage IV tumours is warranted.

In addition, De Sousa E Melo and colleagues also identified three similar subtypes using over 1100 CRC tumour samples: chromosomal instable (subtype A), microsatellite instable (subtype B) and a third subtype (subtype C) which is largely microsatellite stable and contains relatively more CIMP-H carcinomas but cannot be identified on the basis of characteristic mutations [10]. This third subtype is therefore similar to the third subtype described in the studies above. This subtype was found to be associated with a very unfavourable prognosis as well as resistance to anti-EGFR targeted therapy. It is thought to relate to sessile-serrated adenomas due to a very similar GEP involving upregulation of genes involved in matrix remodelling and epithelial-mesenchymal transition (EMT) which was seen in both. This study therefore suggests that sessile-serrated adenomas and tumours belonging to subtype C possess high malignant potential and need to be clinically managed as such [10].

Further groups have also used GEPs to identify more than three intrinsic subtypes of CRC using large numbers of tumour samples. The biological relevance of the subtypes has been investigated in regard to treatment response and prognosis. Marisa and colleagues utilised a large multicentre cohort of tumour samples from patients with stage I–IV CRC, of which 556 fulfilled RNA quality requirements for GEP analysis [11]. These samples were split into a discovery set (n = 443) and a validation set (n = 1029) which also included 906 samples from eight public datasets. Unsupervised hierarchical clustering was applied to gene expression data which form the discovery subset to identify six molecular subtypes (C1–C6) with distinct clinicopathological features, molecular alterations, enrichments of supervised gene expression signatures and deregulated signalling pathways. In addition to identifying a deficient MMR subtype (C2), three CIN subtypes were shown (C1, C5 and C6): one with downregulated immune pathways (C1), one with upregulation of Wnt pathway (C5) and one displaying a normal-like GEP (C6). The remaining two were comprised of a KRAS mutant subtype (C3) and a cancer stem cell subtype (C4).

As expected, BRAF mutation was associated with the C2 subtype but was also frequent in the C4 CIMP-H, poor prognosis subtype. Although TP53 and KRAS mutations were found in all subtypes, the C3 subtype was highly enriched for KRAS mutant tumours suggesting a specific role for this mutation in this subtype of CRC. The biological relevance of these six subtypes is highlighted by their differing prognoses with the C4 and C6 subtypes being independently associated with the shortest relapse-free survival (RFS). However, the robustness of this gene signature as a prognostic classification requires further confirmation as some established prognostic factors in CRC, such as tumour grade and number of nodes examined, were not available for a significant proportion of cases and thus were not included in the analysis.

Schlicker et al. performed genome-wide mRNA expression profiling on 62 primary CRC samples using an unsupervised iterative approach [12]. Two main groups were identified (type 1 mesenchymal and type 2 epithelial) which were then split into five subtypes which were validated in independent published datasets comprising over 1600 samples. This subtype stratification was successfully aligned to several CRC cell line panels, and it was found that the GEPs defining the subtypes were well represented in these cell lines. Pharmacological response data showed that type 2 cell lines were more sensitive to treatment with aurora kinase inhibitors in keeping with the high levels of expression of aurora kinase A seen in the samples of this subtype. Additional data suggested that subtype 1.2 cell lines were most sensitive to inhibition of Src and also showed a higher sensitivity to inhibition of proteins on the PI3K pathway, GSK3β, PI3K and TOR than subtype 2.1 [12].

Budinska et al. performed unsupervised clustering of 1113 CRC samples based on gene models and distinguished at least five different gene expression CRC subtypes which they called surface crypt-like (A), lower crypt-like (B), CIMP-H-like (C), mesenchymal (D) and mixed (E) [13]. These subtypes showed distinct biological motifs and morphological features as well as differences in prognosis. The subtypes were validated in an independent dataset of 720 CRC expression profiles. Subtype C was enriched for both MSI and BRAF mutations, and its characteristics were in keeping with the described CIMP-H phenotype and hypermutated tumours found in TCGA analysis. This subtype had one of the best outcomes for RFS but the worst outcome in survival after relapse (SAR). Once again, KRAS mutations were found in all subtypes, and this supports the emerging theory that KRAS mutant CRCs are highly heterogeneous and that the oncogenic role of KRAS varies with the specific mutation and molecular background of the tumour in which it occurs [20]. Subtypes C and D were associated with the worst overall survival (OS)—for subtype D this was primarily due to early relapse associated with high EMT gene expression and low proliferation-associated gene expression, and for subtype C, it was the result of short SAR.

Subtypes B and E highly expressed canonical Wnt signalling target signatures, whereas subtypes A and D and normal samples expressed low levels of this signature. This was in concordance with the corresponding high percentages of β-catenin-positive nuclei seen in subtypes B and E and converse low percentages seen in subtypes A and D. This analysis is in support of the data suggesting that the colon stem cell signature, under the condition of silenced canonical Wnt target genes, is associated with a higher risk of recurrence (subtype D) [21].

Sadanandam and colleagues performed an analysis of GEPs from 1290 CRC samples using consensus-based unsupervised clustering. The resultant clusters were then correlated with response to cetuximab using a dataset annotated with therapeutic response to cetuximab in 80 patients [14]. The results of these studies identified five clinically relevant CRC subtypes which were named according to genes preferentially expressed in each. The transit-amplifying subgroup was found to contain two groups which differed in cetuximab sensitivity, so it was split into cetuximab-sensitive and cetuximab-resistant, thereby making six subgroups in total. These sub-subtypes showed the best response to cetuximab and increased sensitivity to cMET inhibition, respectively.

Additionally, response to standard chemotherapy with FOLFIRI (5-FU and irinotecan) was also investigated, and the analyses suggested that stem-like subtype tumours, both in the adjuvant and metastatic settings, and inflammatory-subtype tumours in the adjuvant setting may best be treated with FOLFIRI [14]. The transit-amplifying sub-subtypes and the goblet-like subtype were not likely to respond to FOLFIRI in the adjuvant setting, thereby potentially sparing some patients from toxicity of futile treatment. These findings obviously warrant further retrospective and prospective validation, but in unselected CRC patients, FOLFIRI chemotherapy has not shown a survival benefit in the adjuvant setting.

3.3 Outcomes of integrative molecular analysis in CRC

As is evidenced above, up to six molecular subtypes of CRC have been identified by these independent groups, but only superficial similarities exist between the studies. The main characteristics of these subtypes are summarised in **Table 2**. Two subtypes have been repeatedly identified (microsatellite instability enriched and high expression of mesenchymal genes), but full consistency amongst the others has not been achieved probably due to the underlying biological complexity of this cancer and the significant

Subtype	Major subtype category	Subtype characteristics	Prevalence	Ref
MSI/CIMP-H	MSI	Enriched for hypermutated tumours	30%	[8]
CIN	Epithelial		30%	
Invasive	Mesenchymal		40%	
A-type	MSI	Hypermutated, dMMR Good prognosis	22%	[9]
B-type	Epithelial	MSS, BRAF WT, pMMR High proliferative activity Relatively poor baseline prognosis Most benefit from adjuvant chemotherapy	62%	
C-type	Mesenchymal	Undergone EMT Low proliferative activity Poor baseline prognosis No benefit from adjuvant chemotherapy	16%	
CCS1	Epithelial	Mainly left sided KRAS and TP53++	49%	[10]
CCS2	MSI	Mainly right sided dMMR/MSI-H	24%	
CCS3	Mesenchymal	BRAF and KRAS++ Poor prognosis Upregulation of genes involved in matrix remodelling and EMT	27%	
C1: CIN immune down	Epithelial	CIN+++ KRAS and TP53++ Immune system and EMT down regulated	21%	[11]
C2: dMMR	MSI	dMMR/CIMP+++ BRAF++, KRAS++ Immune system and proliferation upregulated	19%	
C3: KRAS mutated	Epithelial	KRAS+++ Immune system and EMT down regulated	13%	
C4: Cancer stem cell	Mesenchymal	KRAS++ Proliferation down regulated EMT upregulated	10%	
C5: CIN Wnt up	Epithelial	CIN+++ KRAS and TP53++ Wnt pathway upregulated	27%	
C6: CIN normal	Mesenchymal	CIN+++ Proliferation down regulated EMT upregulated	10%	
1.1	Mesenchymal	Activation of MAPK, TGFβ and calcium signalling	19%	[12]
1.2	MSI	Activation of immune system-related pathways Highly enriched for MSI-H tumours	15%	
1.3	Mesenchymal	High expression of transporter genes	11%	
2.1	Epithelial	Activation of immune system-related pathways	23%	
2.2	Epithelial	High expression of genes on chromosomes 13q and 20q	32%	

Subtype	Major subtype category	Subtype characteristics	Prevalence	Ref
Surface crypt	Epithelial	KRAS+ Upregulated top colon crypt, secretory cell and metallothioneins	26%	[13]
Lower crypt	Epithelial	Upregulated top colon crypt, proliferation, Wnt Longest SAR	30%	
CIMP-H	MSI	MSI+, BRAF+ Upregulated proliferation, immune, metallothioneins Shortest SAR	11%	
Mesenchymal	Mesenchymal	Upregulated EMT/stroma, CSC, immune	19%	
Mixed	Mesenchymal	P53+ Upregulated EMT/stroma, immune, top colon crypt, Chr20q, CSC	14%	
Inflammatory	MSI	Comparatively high expression of chemokines and interferon-related genes Intermediate prognosis	18%	[14]
Goblet	Epithelial	High mRNA expression of goblet-specific MUC2 and TFF3, Good prognosis May not benefit from adjuvant chemotherapy	14%	
Enterocyte	Epithelial	High expression of enterocyte-specific genes Intermediate prognosis	18%	
Cetuximab-sensitive transit amplifying	Epithelial	Higher levels of EGFR ligands known to predict cetuximab response Good prognosis	32%	
Cetuximab-resistant transit amplifying		Overexpressed FLNA (regulates expression and signalling of cMET receptor), cell lines more sensitive to cMET inhibition Good prognosis		
Stem-like	Mesenchymal	High expression of Wnt signalling targets plus stem cell, myoepithelial and mesenchymal genes and low expression of differentiation markers Worst prognosis May benefit from adjuvant chemotherapy Most benefit from FOLFIRI	18%	

Table 2.
Intrinsic molecular subtypes of CRC based on gene expression profiles.

overlap of features between subgroups. Methodological differences in the processing and analysing of samples have also contributed to these inconsistencies.

In addition, the majority of samples from these datasets have been derived from primary tumours, so their applicability to advanced disease also needs to be considered as the molecular makeup of primary tumours versus metastases may vary, especially in response to the tumour microenvironment and immune cell infiltrate. Altogether, this has meant that the usefulness of these subclassification systems in clinical practice has been limited.

4. The consensus molecular subtypes of colorectal cancer

More recently, in order to resolve inconsistencies in subclassification systems and to aid clinical translation, the CRC research community formed an international consortium dedicated to large-scale data sharing and analytics [22]. After analysing the independent transcriptomic-based classification systems (which comprised 18 CRC datasets and 4151 patients in total) and using unsupervised clustering techniques, four robust consensus molecular subtypes (CMSs) with distinguishing features were proposed. Tumours with mixed features (approximately 13%) were thought to represent a transition phenotype or intratumoural heterogeneity. **Table 3** summarises the main biological, molecular, clinical and prognostic associations of the four consensus subtypes.

In regard to genomic aberrations, CMS1 samples were hypermutated and encompassed the majority of MSI-H tumours. This group also displayed widespread hypermethylation and low prevalence of SCNAs. CMS2 and CMS4 subgroups displayed higher CIN via high SCNA counts. CMS3 samples consisted of fewer SCNAs than other CIN tumours, a significant proportion (30%) of hypermutated tumours

	CMS1 **MSI immune**	**CMS2** **Canonical**	**CMS3** **Metabolic**	**CMS4** **Mesenchymal**
Percentage of samples	**14**	**37**	**13**	**23**
Biological characteristics	MSI high CIMP high Hypermutation SCNA low	SCNA high	Distinctive profile: Mixed MSI status SCNA low CIMP low	SCNA high
	Overexpression of proteins involved in DNA damage repair Widespread hypermethylation status	Higher chromosomal instability	Intermediate levels of gene hypermethylation	Higher chromosomal instability
Molecular features	BRAF mutations Activation of RTK and MAPK pathways		KRAS mutations Activation of RTK and MAPK pathways	
	Immune infiltration and activation Strong activation of immune evasion pathways	Wnt and Myc activation	Metabolic deregulation	Stromal infiltration TGF-β activation Angiogenesis
Clinical features	Females Right sided tumours Higher grade	Left sided tumours		More advanced stages
Prognostic features	Better relapse-free survival Worse survival after relapse	Better survival after relapse		Worse relapse-free and overall survival

Table 3.
The four consensus molecular subtypes of CRC.

and intermediate levels of gene hypermethylation [22]. Despite clear enrichment of certain gene mutations within CMS groups, such as high rates of BRAF mutation in CMS1 and KRAS mutations in CMS3, no single genetic aberration was found to be limited to one subtype, and no subtype was defined by a single molecular event. Further integrative genomic analysis did not draw any clear associations either, highlighting the poor genotype–phenotype correlation in this cancer.

Further exploration of gene expression data revealed insight into the underlying biology of the subtypes: CMS1 samples showed strong immune activation and infiltration with CD4+ T helper cells, CD8+ cytotoxic T cells and natural killer (NK) cells along with strong activation of immune evasion pathways. CMS2 showed marked upregulation of Wnt and MYC downstream targets and higher expression of oncogenes EGFR, ERBB2, insulin-like growth factor 2 (IGF-2), insulin receptor substrate 2 (IRS-2) and transcription factor hepatocyte nuclear factor 4α (HNF4α). CMS3 samples showed enrichment for multiple metabolism signatures which are keeping with the described notion that activating KRAS mutations induce prominent metabolic adaptation [23, 24]. CMS4 tumours showed upregulation of genes associated with epithelial-mesenchymal transition (EMT), such as transforming growth factor β (TGF-β) and integrins, as well as stromal invasion.

4.1 Clinical and prognostic associations of the consensus molecular subtypes

Associations between CMS subgroups and clinical features and prognosis were also investigated and showed that CMS1 tumours were more common in females, more likely to be right-sided and of higher histopathological grade. Conversely, CMS2 tumours were more likely to be left-sided and present at more advanced stages. CMS4 tumours show the worst OS and RFS even after adjustment for BRAF and KRAS mutations and MSI status. CMS1 tumours display good survival but very poor SAR in keeping with known data of MSI tumours associated with $BRAF^{V600E}$ mutations. CMS2 and CMS3 subgroups display intermediate survival, but a superior survival following relapse was noted in the CMS2 subgroup.

Further prognostic associations of the CMS subtypes have been explored via retrospective analysis of large clinical trial datasets, as have their association with biological therapies. 392 KRAS wild-type samples from the CALGB 80405 dataset were analysed via a NanoString platform to determine their CMS subtype classification, and this was correlated with survival [25]. It was found that CMS1 tumours treated with bevacizumab had significantly longer OS compared to those treated with cetuximab. CMS2 tumours treated with bevacizumab had a trend towards shorter OS than those treated with cetuximab. A meta-analysis of six randomised trials, including the CRYSTAL and FIRE-3 datasets, also confirmed the improvement in PFS and OS of left-sided tumours (CMS2) treated with anti-EGFR antibodies compared to no significant benefit for right-sided tumours (CMS1) [26]. No survival differences were found for left- or right-sided tumours treated with bevacizumab. This suggests sidedness of the primary tumour that determines efficacy of biological therapies, and this can possibly be explained by the biological differences of tumours from different sides of the bowel: left-sided tumours overexpress the EGFR ligands amphiregulin (AREG) and epiregulin (EREG) and also display amplifications of markers of cetuximab sensitivity, whereas right-sided tumours show reduced expression of EGFR ligands [27].

4.2 Clinical utility of the consensus molecular subtypes

Much hope was placed upon the CMS classification system allowing stratification of patients for clinical trials to validate the prognostic and predictive value of the subgroups and enable translation into clinical care. Although CMS classification

has enabled refinement of the large 'non-MSI' group of CRC patients and provided a tool for systemic interrogation, there is some data which suggests that critical clinical information which predicts for outcome is still not distinguishable under this classification system. For example, a separate analysis of the CALGB 80405 dataset identified that sidedness of the primary tumour was still an independent prognostic factor over and above CMS subtype [28].

The association with treatment outcomes of the CMS subtypes, especially in the metastatic setting, still requires further exploration and validation. Kim et al. found that colorectal cancer assigner (CRCA) is subtyping more clearly defined oxaliplatin benefit group than CMS subtyping did prior to their analysis of the NSABP-C07 trial [29]. It is also important to consider the 13% of samples which could not be classified into CMS subtypes and the need to better characterise samples of mixed phenotypes and the clinical implications of this.

The challenge of reproducibility of this classification system which requires complex transcriptomic, proteomic and genomic analyses is also an issue, and its implementation is not feasible in many centres in its current form. There has been some work already undertaken to develop a robust and practical classifier based on immunohistochemistry (IHC) which appears promising but requires prospective validation [30].

All in all, the clinical utility and widespread reproducibility of this classification system in CRC is still to be determined, and it is likely that, with further characterisation, we may see additional subtyping of the four described subtypes in the future.

5. Conclusions

Much progress has been made in our understanding of the complex underlying biology of CRC which leads to heterogeneous drug responses and outcomes. Comprehensive integrative molecular analysis has led to the identification of molecularly distinct subgroups within this disease, and the consensus molecular subtypes have enabled some refinement of these subgroups. However, widespread reproducibility and confirmation of the clinical utility of CMS classification still need to be addressed. There are vast amounts of data being generated from molecular classification systems, and this needs to be prospectively integrated into clinical trial design in order to confirm biomarkers of resistance and response as well as to allow rational combinations of therapies to be explored. The ultimate goal is to streamline biomarker and drug codevelopment and recruit patients to innovative clinical trials of targeted agents to which they are more likely to respond based on the underlying molecular makeup of their tumours.

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Conflict of interest

AA has no conflicts of interest.

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Author details

Avani Athauda and Ian Chau*
The Royal Marsden NHS Foundation Trust, London and Surrey, United Kingdom

*Address all correspondence to: Ian.chau@rmh.nhs.uk

References

[1] Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic alterations during colorectal-tumor development. New England Journal of Medicine. 1988;**319**:525-532. DOI: 10.1056/NEJM198809013190901

[2] Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990;**61**:759-767. DOI: 10.1016/0092-8674(90)90186-I

[3] Müller MF, Ibrahim AEK, Arends MJ. Molecular pathological classification of colorectal cancer. Virchows Archiv. 2016;**469**:125-134. DOI: 10.1007/s00428-016-1956-3

[4] Marzouk O, Schofield J. Review of histopathological and molecular prognostic features in colorectal cancer. Cancers. 2011;**3**:2767-2810. DOI: 10.3390/cancers3022767

[5] Sanchez JA, Krumroy L, Plummer S, Aung P, Merkulova A, Skacel M, et al. Genetic and epigenetic classifications define clinical phenotypes and determine patient outcomes in colorectal cancer. British Journal of Surgery. 2009;**96**:1196-1204. DOI: 10.1002/bjs.6683

[6] Tran B, Kopetz S, Tie J, Gibbs P, Jiang Z-Q, Lieu CH, et al. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Cancer. 2011;**117**:4623-4632. DOI: 10.1002/cncr.26086

[7] Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 blockade in tumors with mismatch-repair deficiency. The New England Journal of Medicine. 2015;**372**:2509-2520. DOI: 10.1056/NEJMoa1500596

[8] Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;**487**:330-337. DOI: 10.1038/nature11252

[9] Roepman P, Schlicker A, Tabernero J, Majewski I, Tian S, Moreno V, et al. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. International Journal of Cancer. 2014;**134**:552-562. DOI: 10.1002/ijc.28387

[10] De Sousa E Melo F, Wang X, Jansen M, Fessler E, Trinh A, de Rooij LPMH, et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. Nature Medicine. 2013;**19**:614-618. DOI: 10.1038/nm.3174

[11] Marisa L, de Reyniès A, Duval A, Selves J, Gaub MP, Vescovo L, et al. Gene expression classification of colon cancer into molecular subtypes: Characterization, validation, and prognostic value. PLoS Medicine. 2013;**10**:e1001453. DOI: 10.1371/journal.pmed.1001453

[12] Schlicker A, Beran G, Chresta CM, McWalter G, Pritchard A, Weston S, et al. Subtypes of primary colorectal tumors correlate with response to targeted treatment in colorectal cell lines. BMC Medical Genomics. 2012;**5**:66. DOI: 10.1186/1755-8794-5-66

[13] Budinska E, Popovici V, Tejpar S, D'Ario G, Lapique N, Sikora KO, et al. Gene expression patterns unveil a new level of molecular heterogeneity in colorectal cancer. The Journal of Pathology. 2013;**231**:63-76. DOI: 10.1002/path.4212

[14] Sadanandam A, Lyssiotis CA, Homicsko K, Collisson EA, Gibb WJ, Wullschleger S, et al. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. Nature Medicine. 2013;**19**:619-625. DOI: 10.1038/nm.3175

[15] Jones JC, Renfro LA, HO A-S, Schrock AB, Rankin A, Zhang BY, et al. Non-V600 BRAF mutations define a clinically distinct molecular subtype of metastatic colorectal cancer. Journal of Clinical Oncology. 2017;**35**:2624-2630. DOI: 10.1200/JCO.2016

[16] Topol L, Chen W, Song H, Day TF, Yang Y. Sox9 inhibits Wnt signaling by promoting beta-catenin phosphorylation in the nucleus. The Journal of Biological Chemistry. 2009;**284**:3323-3333. DOI: 10.1074/jbc.M808048200

[17] Sack U, Walther W, Scudiero D, Selby M, Aumann J, Lemos C, et al. S100A4-induced cell motility and metastasis is restricted by the Wnt/β-catenin pathway inhibitor calcimycin in colon cancer cells. Molecular Biology of the Cell. 2011;**22**:3344-3354. DOI: 10.1091/mbc.E10-09-0739

[18] Ewan K, Pajak B, Stubbs M, Todd H, Barbeau O, Quevedo C, et al. A useful approach to identify novel small-molecule inhibitors of Wnt-dependent transcription. Cancer Research. 2010;**70**:5963-5973. DOI: 10.1158/0008-5472.CAN-10-1028

[19] Lau T, Chan E, Callow M, Waaler J, Boggs J, Blake RA, et al. A novel tankyrase small-molecule inhibitor suppresses APC mutation-driven colorectal tumor growth. Cancer Research. 2013;**73**:3132-3144. DOI: 10.1158/0008-5472.CAN-12-4562

[20] Singh A, Sweeney MF, Yu M, Burger A, Greninger P, Benes C, et al. TAK1 inhibition promotes apoptosis in KRAS-dependent colon cancers. Cell. 2012;**148**:639-650. DOI: 10.1016/j.cell.2011.12.033

[21] de Sousa E Melo F, Colak S, Buikhuisen J, Koster J, Cameron K, de Jong JH, et al. Methylation of cancer-stem-cell-associated Wnt target genes predicts poor prognosis in colorectal cancer patients. Cell Stem Cell. 2011;**9**:476-485. DOI: 10.1016/j.stem.2011.10.008

[22] Guinney J, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, et al. The consensus molecular subtypes of colorectal cancer. Nature Medicine. 2015;**21**:1350-1356. DOI: 10.1038/nm.3967

[23] Ying H, Kimmelman AC, Lyssiotis CA, Hua S, Chu GC, Fletcher-Sananikone E, et al. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. Cell. 2012;**149**:656-670. DOI: 10.1016/j.cell.2012.01.058

[24] Brunelli L, Caiola E, Marabese M, Broggini M, Pastorelli R. Capturing the metabolomic diversity of KRAS mutants in non-small-cell lung cancer cells. Oncotarget. 2014;**5**:4722-4731. DOI: 10.18632/oncotarget.1958

[25] Lenz H-J, Fang-Shu O, Venook AP, Hochster HS, Niedzwiecki D, Goldberg RM, et al. Impact of consensus molecular subtyping (CMS) on overall survival (OS) and progression free survival (PFS) in patients (pts) with metastatic colorectal cancer (mCRC): Analysis of CALGB/SWOG 80405 (Alliance). Journal of Clinical Oncology. 2017;**35**(15_suppl):3511. DOI: 10.1200/JCO.2017.35.15_suppl.3511

[26] Arnold D, Lueza B, Douillard J-Y, Peeters M, Lenz H-J, Venook A, et al. Prognostic and predictive value of primary tumour side in patients with RAS wild-type metastatic colorectal cancer treated with chemotherapy and EGFR directed antibodies in six randomized trials†. Annals of Oncology. 2017;**28**:1713-1729. DOI: 10.1093/annonc/mdx175

[27] Dienstmann R, Vermeulen L, Guinney J, Kopetz S, Tejpar S, Tabernero J. Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer. Nature Reviews Cancer. 2017;**17**:79-92. DOI: 10.1038/nrc.2016.126

[28] Venook AP, DIF N. Impact of primary (1°) tumor location on overall survival (OS) and progression-free survival (PFS) in patients (pts) with metastatic colorectal cancer (mCRC): Analysis of CALGB/SWOG 80405 (Alliance). Journal of Clinical Oncology. 2016;**34**(15_suppl):3504-3504

[29] Kim SR, Song N, Yothers G, Gavin PG, Allegra CJ, Paik S, et al. Tumour sidedness and intrinsic subtypes in patients with stage II/III colon cancer: Analysis of NSABP C-07 (NRG Oncology). British Journal of Cancer. 2018;**118**:629-633. DOI: 10.1038/bjc.2017.448

[30] Trinh A, Trumpi K, De Sousa E Melo F, Wang X, de Jong JH, Fessler E, et al. Practical and robust identification of molecular subtypes in colorectal cancer by immunohistochemistry. Clinical Cancer Research. 2017;**23**: 387-398. DOI: 10.1158/1078-0432.CCR-16-0680

Chapter 2

Experimental Results Help Shape the Development of Personalized Medicine in Colorectal Cancer

Rania B. Georges, Hassan Adwan and Martin R. Berger

Abstract

With estimated 700,000 deaths each year, colorectal carcinoma (CRC) continues to be the fourth leading cause of cancer-related deaths worldwide. Fortunately, the mortality of CRC is considered to be most avertable; hence, it is essential to develop new approaches for more accurate and early diagnosis of primary as well as metastatic CRC, including genetic and biomarker tests. In this regard, the intercellular junctions and the insulin-like growth factor (IGF) axis attract increasing attention, since they are involved in several stages of cancer and for their vital role in regulating cell survival and growth; furthermore, constituents of intercellular junctions and of the IGF axis could be used as tumor and/or metastasis markers, which are becoming the focus of increasing research activities. Our experimental results highlight the importance of gene expression changes in the tight junction proteins claudins, and in the IGF-binding proteins IGFBP3 and IGFBP7. They show additionally that claudins and IGFBPs cannot be simply defined in terms of favoring or antagonizing cancer progression but have additional properties and activities, which become apparent only in the context of liver colonization. Furthermore, their intensive modulation during the initial phase of liver colonization may suggest them as early metastasis-related markers.

Keywords: colorectal cancer, liver metastasis, personalized medicine, influence on treatment, claudins, IGFBPs, tumor cell reisolation, metastasis marker

1. Introduction

With estimated 700,000 deaths each year, colorectal carcinoma (CRC) continues to be the fourth leading cause of cancer-related deaths in both sexes worldwide [1]. The 5-year relative survival rate for stage IV metastatic CRC is about 11%, while in stage I this number rises to nearly 90%. These figures reflect the fact that despite the high incidence and mortality rate of CRC, its mortality is among the most avertable ones. In addition, the fact that liver metastasis is the cause of most deaths from CRC [2], underlines the significance of (early) metastasis prevention. In other words, it is of great importance to develop new approaches for more accurate and early diagnosis of primary CRC on one hand and of its metastasis on the other; including screening programs as well as genetic, molecular and biomarker tests.

Colorectal cancer progression is driven by increasing or recurring growth of the primary carcinoma as well as hematogenic and lymphatic spread. For hematogenic spread, the liver is most important as it constitutes the first vascular bed in which disseminating CRC cells can be trapped after their dissemination. Hence, this organ is affected in up to 10–20% of CRC patients at the time of presentation. Another 20–25% will develop overt liver metastasis during the course of their illness [3, 4].

The main purpose of our experimental studies was first to develop a suitable model for investigating the efficacy of novel drugs [5–7]. One of the few well-characterized animal models for hepatic CRC benefits from the rat CC531 cell line. After injecting the cells, liver metastases develop and their growth has been frequently used for studying effects of various anti-cancer treatments [8–10].

The second aim was to identify genes, which are instrumental in the survival and metastasis formation of disseminated CRC cells. In addition, we reasoned that there are genes, which are necessary for the primary tumor as well as those, which are essential for metastasis initiation and formation. We furthermore hypothesized that the latter genes would be modulated in expression during the cells' colonization of the liver. Consequently, temporal changes in gene expression of CRC cells homing into the liver were investigated using an *in vivo* rat model, which is characterized by a definite metastatic proliferation-onset in rat liver after intra-portal inoculation of CC531 rat colorectal cancer cells. This model relies on the successful reisolation of CC531 cells at various time intervals after their injection into the mesenteric vein of syngeneic rats and allows exploring the chronological modulation of gene expression, from the very beginning of cancer cell homing into the liver to their final colonization of the whole organ. Based on this procedure, a cDNA microarray was performed to analyze gene expression profiles of several thousand genes in the reisolated CC531 cells. Upon analysis of microarray's data, candidates from gene families being significantly up- or down-regulated were chosen for further study by using different *in vitro* models. These candidate genes included claudins and insulin like-growth factor binding proteins. It was hoped that the emerging genes or their products would be useful as target of a specific therapy or as a biomarker.

The National Institutes of Health defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [11]. From a therapeutic point of view, genome variations are recognized as the main cause of variable response to and side effects of drugs and a "one size fits all" approach is not the best solution any more. The individual's genetic and molecular makeup will be devoted to improve and develop more specific and "personal" diagnostic and therapeutic approaches.

Claudins (CLDNs) are tight junction (TJ) proteins that serve an intercellular adhesion function. The aberrant expression of individual claudins is well documented in different stages of various human cancers [12]. In addition, some claudins were proven to be useful as biomarkers [13, 14].

The insulin-like growth factor (IGF) axis attracts increasing attention since it is involved in several stages of cancer [15–17], and for its vital role in regulating cell survival and growth [18, 19] as well as the possible use of constituents of this axis as tumor and/or metastasis markers, which is becoming the focus of increasing research activities.

The insulin-like growth factors IGF-I and –II orchestrate their roles through the interaction with other members in this system, namely their receptors IGF-IR and -IIR, their binding proteins (IGFBPs) and the IGFBP proteases including matrix metalloproteinases (MMPs), cathepsins, and kallikreins [20]. Type I receptor mediates the growth promoting effects of IGFs [21], which are further modulated by 6 binding

proteins (IGFBPs 1–6) with high affinity for IGFs [22] as well as at least 4 IGFBPs with low affinity [23, 24], also known as IGFBP-related proteins (IGFBP-rp-1-4).

Based on the observation that the increased expression of IGFBPs attenuates the proliferative and anti-apoptotic effects of IGFs, they have been long considered as tumor suppressors, mostly due to their IGF-dependent roles. Interestingly, however, in addition to these IGF-dependent actions, IGFBPs were found to exert IGF-independent effects, as was reported for IGFBP1 [25], IGFBP3 [26–28], IGFBP5 [29] and IGFBP7 (or IGFBP-rp1) [30–32].

In this report, we have used the technique of cancer cell reisolation from rat liver, which permitted to monitor for the first time the expression profile of numerous candidate genes in a time-dependent manner. Based on these results we summarize our knowledge on claudins and IGFBP members and delineate their potential as tumor and/or metastasis markers.

2. Results

2.1 Modulation of selected genes in reisolated CC531 tumor cells

For identifying genes, which enable tumor cells to metastasize and colonize the liver, the CC531 cells were reisolated from rats, which had been implanted intraportally with these tumor cells. After various periods following tumor cell implantation, the CC531 cells were reisolated with a specific technique [33]. In subsequent experiments, these cells were used for mRNA and protein isolation and the mRNA screened by cDNA microarray and RT-PCR, and the proteins by Western blot.

As shown in **Table 1**, the microarray analysis revealed a significantly increased expression of insulin like growth factor binding proteins (*Igfbp3* and *Igfbp7)* and significantly decreased expression levels of claudins (*Cldn1* and *Cldn4*) in the beginning of liver colonization (days 3 and 6 after tumor implantation). These results were further confirmed by RT-PCR (for all four genes) and Western blot (for the two claudins and igfbp7) (**Figure 1**).

2.2 Effects of genes' knockdown in colorectal cancer cells

To investigate the knockdown effect(s) of each gene on various functions of colorectal cancer cell lines, siRNA experiments for transient knockdown were performed.

Gene	Time point of cell reisolation (days)[a]						
	3	6	9	14	21	14 (*in vitro*)	22 (*in vitro*)
Igfbp3	6.88[b]	13.62	6	18.03	17.29	1.56	*0.9*
Igfbp7	90.02	101.57	38.62	49.13	42.03	19.46	1.47
Cldn1	0.16	0.12	0.31	0.63	0.55	1.29	1.53
Cldn4	0.15	0.09	0.87	1.11	1.37	1.47	1.11

[a]*The day of tumor cell implantation was counted day 0.*
[b]*The number denotes the fold change in expression versus an in vitro control.*

Table 1.
Gene expression profiles from members of two gene families, chosen from the microarray analysis of reisolated CC531 cells.

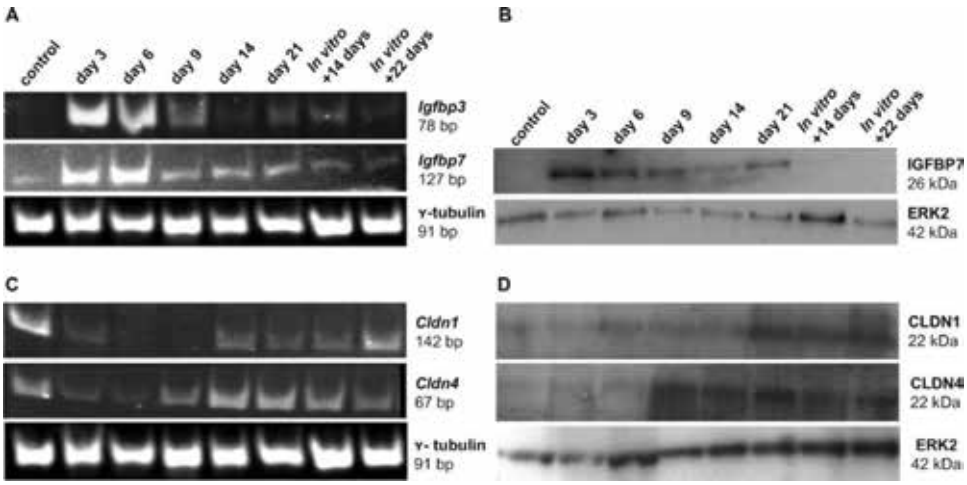

Figure 1.
Expression of Igfbp3, Igfbp7, cldn1, and cldn4 in reisolated CC531 cells. (A and C): Expression of Igfbp3 and Igfbp7 (A) and of Cldn1 and Cldn4 (C) in reisolated CC531 cells as shown by RT-PCR compared to the expression of the housekeeping gene γ-tubulin. (B and D): Expression of the proteins IGFBP7 (B), CLDN1 and CLDN4 (D) in the reisolated CC531 cells as shown by western blot compared to the expression of ERK2 loading control. 1st lane: CC531 cells (control); 2nd, 3rd, 4th, 5th and 6th lanes: CC531 cells (reisolated from rat livers after 3, 6, 9, 14 and 21 days, respectively); 7th and 8th lanes: CC531 cells (reisolated after 21 days and cultured in vitro for further 14 and 22 days, respectively).

Colorectal cancer cells (CC531, Caco2 or SW480) cultured in 6-well-plates were transfected with specific siRNA (200 nM) or negative control using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The cells were harvested at 24, 48 and 72 h after treatment.

As shown for overview in **Table 2** (for details see **Figures 2–4**), knockdown of *igfbp3* or *igfbp7* was induced in cell lines with relevant expression only and caused significantly reduced proliferation rates (**Figure 2A–C**). Similarly, colony formation (**Figure 3A–C**) of CRC cells was diminished. Finally, cell migration was reduced in SW480 cells (**Figure 4B**), but not in CC531 (**Figure 4A**) and Caco2 (**Figure 4C**) cells.

Interestingly, different effects were noticed after *cldn1* or *cldn4* knockdown in CC531 cells. No significant effect on cell proliferation was observed, while a significant inhibition of colony formation and significant stimulation of cell migration resulted from the siRNA knockdown of each claudin (**Table 2**).

Target gene	Cell proliferation	Cell migration	Colony formation
Igfbp3 (in SW480)	↓*	↓*	↓*
Igfbp7 (in CC531 or Caco2)	↓*	ns	↓*
Cldn1 (in CC531)	ns	↑*	↓*
Cldn4 (in CC531)	ns	↑*	↓*

↓ and ↑* denote significant ($p < 0.05$) inhibition or stimulation of the investigated cell function (proliferation, migration or colony formation), respectively; ns denotes a nonsignificant effect.*

Table 2.
Overview of the siRNA knockdown effects of insulin like growth factor binding proteins 3 and 7 (Igfbp3 and 7) and claudins 1 and 4 (cldn1 and 4) on cellular functions (cell proliferation, migration and colony formation) of colorectal cancer cell lines (SW480, Caco2 and CC531).

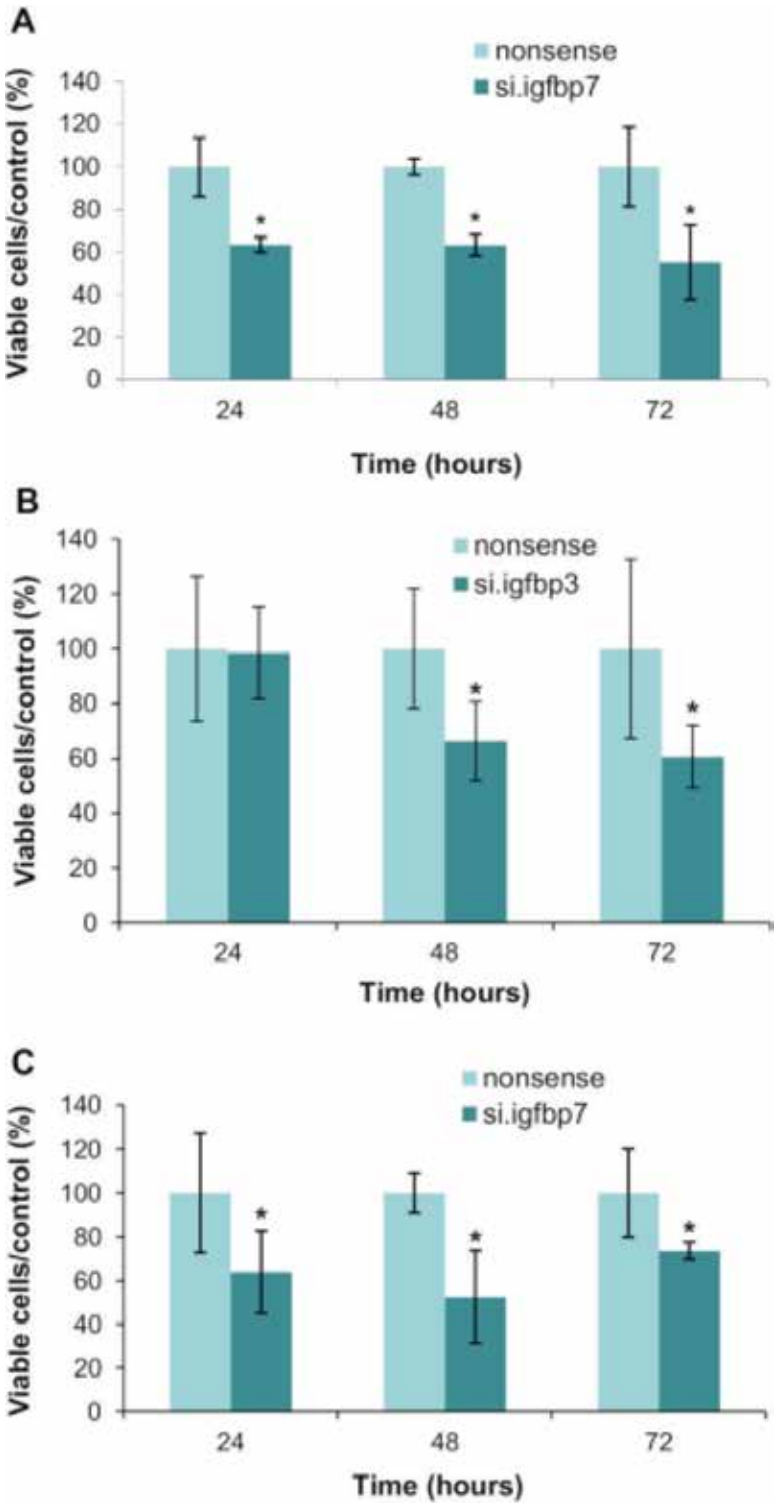

Figure 2.
Effects of Igfbp7 or Igfbp3 knockdown on proliferation of colorectal cancer cells. (A) Reduced proliferation of rat CC531 colorectal cancer cells after si.Igfbp7 treatment. (B) Reduced proliferation of human SW480 colorectal cancer cells after si.Igfbp3 treatment. (C) Reduced proliferation of human Caco2 colorectal cancer cells after si.Igfbp7 treatment. Data (n = 3) are shown as means ± S.D. in percentage of nonsense-treated cells. Asterisk (*) denotes a significant difference to controls (p < 0.05). Abbreviations: CF; colony formation.

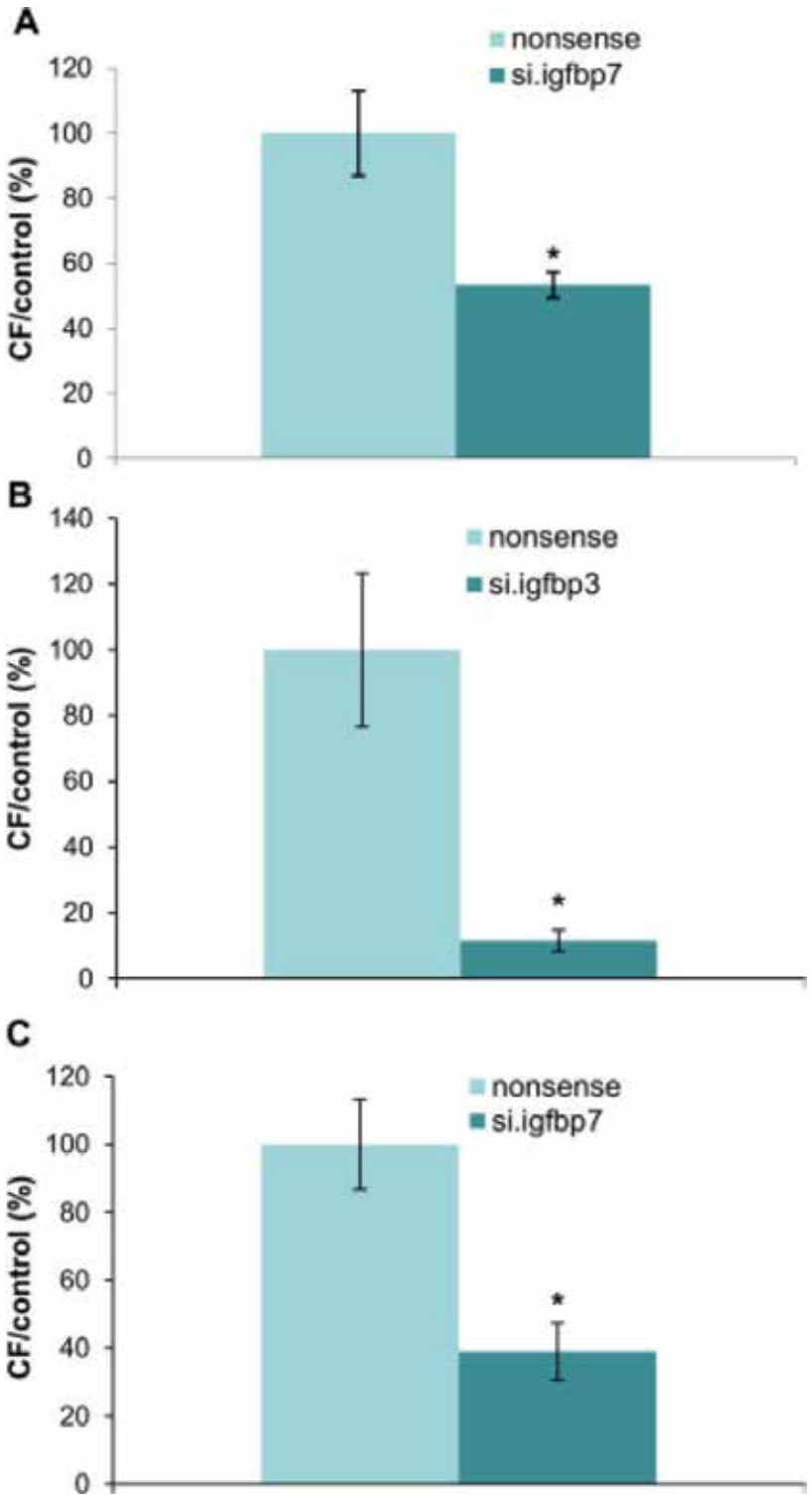

Figure 3.
Effects of Igfbp7 or Igfbp3 knockdown on colony formation of colorectal cancer cells. (A) Inhibited colony formation of rat CC531 colorectal cancer cells after si.Igfbp7 treatment. (B) Inhibited colony formation of human SW480 colorectal cancer cells after si.Igfbp3 treatment. (C) Inhibited colony formation of human Caco2 colorectal cancer cells after si.Igfbp7 treatment. Data (n = 3) are shown as means ± S.D. in percentage of nonsense-treated cells. Asterisk (*) denotes a significant difference to controls ($p < 0.05$). Abbreviations: CF; colony formation.

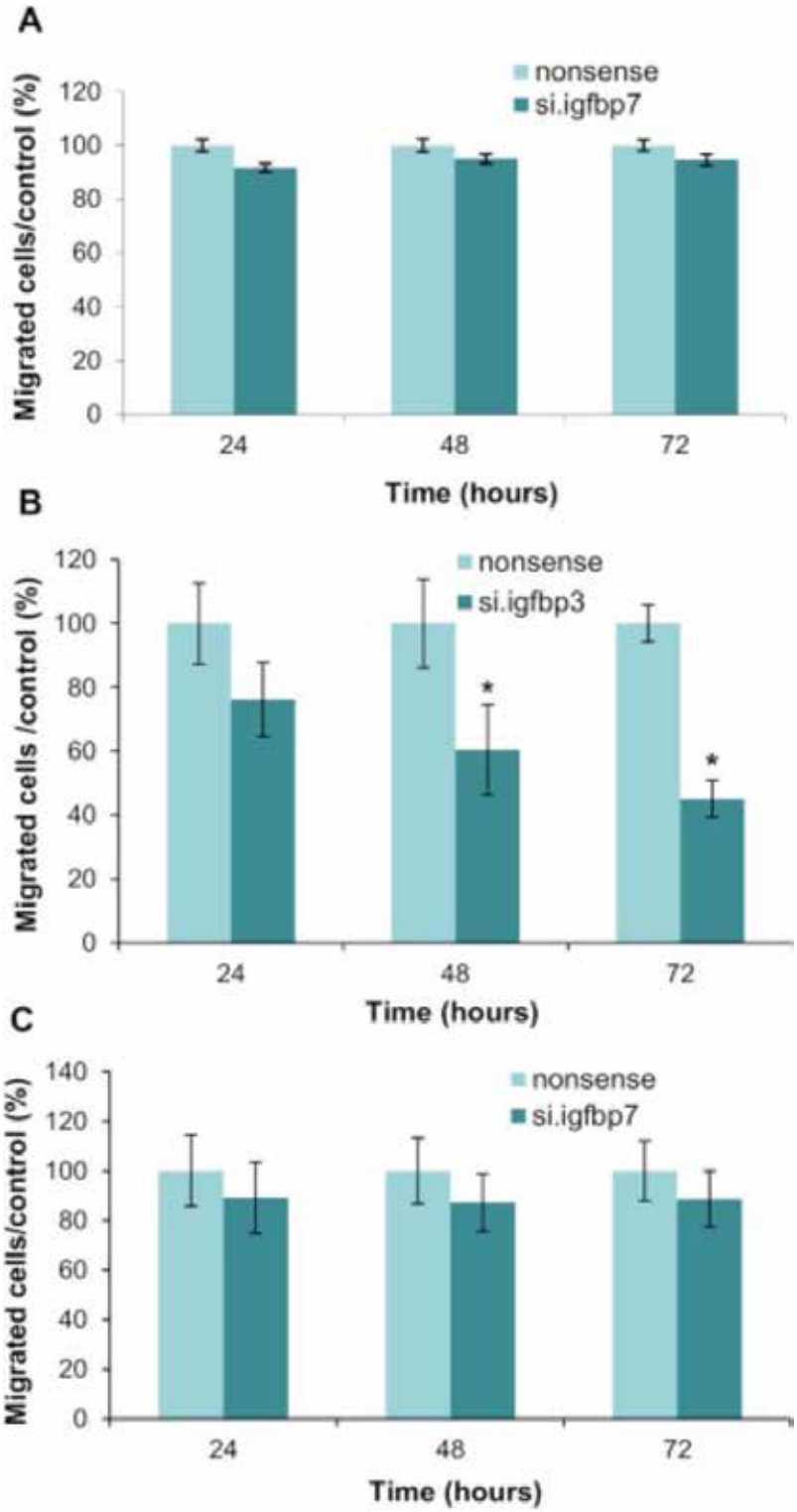

Figure 4.
Effects of Igfbp7 or Igfbp3 knockdown on migration of colorectal cancer cells. (A) Migration of rat CC531 colorectal cancer cells after si.Igfbp7 treatment. (B) Reduced migration of human SW480 colorectal cancer cells after si.Igfbp3 treatment. (C) Migration of human Caco2 colorectal cancer cells after si.Igfbp7 treatment. Data (n = 3) are shown as means ± S.D. in percentage of nonsense-treated cells. Asterisk (*) denotes a significant difference to controls ($p < 0.05$). Abbreviations: CF; colony formation.

3. Discussion

"If it were not for the great variability among individuals, medicine might as well be a science and not an art" with these words the famous Canadian physician Sir William Osler anticipated and acknowledged the concept of personalized medicine since the nineteenth century.

Personalized medicine aims to optimize and tailor preventive and therapeutic approaches in favor of the best outcome for each patient, by using genetics, proteomics, and biological information, including biomarkers [34]. It attempts to sub-categorize patients into different groups according to their "molecular make up", i.e. using biomarkers.

Here we have used a rat model of liver metastasis to identify genes with importance for organ colonization, which could be used as biomarkers or therapeutic targets.

The selected model for this experiment is characterized by a defined metastatic proliferation-onset in rat liver after intra-portal inoculation of CC531 cancer cells. Consequently, this allows exploring the chronological modulation of gene expression, from the very beginning of cancer cell homing into the liver to their final colonization of the whole organ. The technique of cancer cell reisolation from rat liver permitted, for the first time, monitoring the expression profile of numerous candidate genes from the whole genome in a time-dependent manner.

The initial observation of these studies was based on cDNA microarray analysis of CC531 colon cancer cells, which allowed selecting candidates from gene families with significant up- or down-regulation. These candidates were further analyzed by different *in vitro* studies.

We have been focusing on the detection and evaluation of biomarkers for the last decades [33, 35–43]. From the several gene groups, which were highly modulated in expression during liver colonization we focused on two families. Both were characterized by dramatic initial changes in expression, with claudins being down modulated and IGFBPs being up-regulated.

The first group (claudins; CLDNs) form the structural backbone of tight junctions (TJs), one type of cell-cell adhesion, and comprise at least 27 members of integral transmembrane proteins ranging in size from 20 to 34 kDa [12]. In recent years, the up- or down-modulated expression of several claudins has been associated with the progression of various cancers in humans, even in a tissue specific manner [12].

Interestingly, individual claudins are being used as therapeutic targets [44, 45] as well as diagnostic biomarkers [13, 14], making them a very interesting molecule to be investigated and characterized.

The second group of insulin like growth factor binding proteins (IGFBPs) differs in its importance from claudins, as they belong to the insulin-like growth factor (IGF) axis, which has a vital role in regulating cell survival and growth and is involved in several stages of cancer.

The expression of the two IGFBP genes, igfbp3 and igfbp7, was intensely upregulated at the beginning of liver colonization (days 3 and 6 after tumor implantation). Subsequently, however, this increased expression returned gradually to normal, hence we assume that the up-regulation of IGFBPs is essential for dissemination and homing of tumor cells into the liver during early metastasis formation. This strongly suggests that the tumor/metastasis microenvironment has a crucial impact on the regulation of igfbp3 and igfbp7. Furthermore, these results along with previous studies [28, 46–48] show that the balanced expression of IGFBP3 and IGFBP7 is very essential for several cellular functions as both, up- and down-regulation of these two genes were related to malignant properties. This further suggests that the aberrant expression of these two genes can be an early indicator of CRC progression.

Further verification of their value arises from studies that demonstrate involvement of the IGF axis in several stages of cancer and for its vital role in regulating cell and tissue survival, growth and differentiation [18, 19]. In addition, the possible use of constituents of this axis as tumor and/or metastasis markers is becoming the focus of increasing research activities [49–51]. Most *in vitro* studies, reported a tumor suppressor function of IGFBP3 and IGFBP7 through IGF-dependent and/or independent mechanisms [27, 31, 52, 53]. At variance to these studies, our experiments on silencing *IGFBP3* and *IGFBP7* in three CRC cell lines uniformly show reduced proliferation, colony formation, and for *IGFBP3*, also reduced migration. Our observations are in agreement with few reports, which related IGFBP3 and IGFBP7 to growth promoting functions [28, 54]. Accordingly, these and our results support the idea that IGFBP3 and IGFBP7 are multi-functional.

IGFBP3 is well known in the literature: It is the predominant IGFBP in plasma, hence plays a crucial role in regulating the bioavailability of plasma IGFs, and it is expressed locally in most tissues including the intestine [55]. Additionally, IGFBP3 induces apoptosis and inhibits proliferation in human colon [56], prostate [57], breast [58], and lung cancer cells [26] *in vitro* and in experimental CRC animal models [59]. Furthermore, wild type p53 can induce IGFBP3 expression [60], thus enhancing the p53-dependent apoptotic response of CRC cells to DNA damage [56]. Reduced levels of IGFBP3 and elevated circulating levels of IGF-I were associated with increased risk of prostate [61], breast [62], and colorectal [63, 64] cancers. Nevertheless, this association was not confirmed in all conducted studies [65–67]. It was observed that TGF-β can induce IGFBP3 and mediates its proliferative response in aggressive CRC cells, which exempts the studies reporting a tumor suppressor function of IGFBP3 [28].

In contrast to IGFBP3, the regulation and functions of IGFBP7 are less investigated. This gene was originally cloned as a gene, which is down-regulated in meningioma cell lines [68]. IGFBP7 is usually expressed by colonic mucosa [65], however both, up- and down-regulation patterns were recorded in the context of cancer [46, 47]. Lately, it was shown that IGFBP7 is a direct p53 target and the DNA methylation mediated-epigenetic silencing of IGFBP7 was associated with the absence of p53 mutations in CRC [30]. Until now, *in vitro* experiments demonstrated a negative effect of IGFBP7 on the growth of various cancer cells, including cervical carcinoma (HeLa) [69], osteosarcoma (Saos-2) [69], and breast [70]. Furthermore, in human CRC cell lines, expression of IGFBP7 was detectable in Caco2 and SW480 cells only, whereas its expression in invading tumor cells associated with poor prognosis in CRC patients [71]. In addition, immunohistochemistry and RT-PCR showed IGFBP7 over-expression in CRC tissues as compared to the respective normal tissues [36].

As also known from the literature, several members of the IGF axis were found to be prognostic markers for various tumor types, including IGFBP5 and IGFBP7 for nonsmall cell lung cancer (NSCLC) [72], IGFBP2 and IGFBP3 as compensatory biomarkers for CA19-9 in early-stage pancreatic cancer [51], IGF-1 for metastatic uveal melanoma [49] and IGF-IR for glioblastoma [50]. In addition, a recent study showed that certain single nucleotide polymorphisms (SNPs) in IGF1R and IGF2R were associated (positively or inversely, respectively) with adenomas in Caucasian, but not in African American CRC patients [73]. Similarly, specific SNPs in the IGF-l gene were suggested as risk assessment markers of gastrointestinal cancers [74]. All these studies emphasize the crucial role of the patient's genetic background in tailoring the therapeutic approach to fit the "size" of this particular patient.

With regard to using the above family members as prognostic marker, the past experience should be considered. Contrasting with our expectations and reports from the literature, no significant correlation was found between the increased expression levels of four known tumor progression-associated genes (Opn, Tgf-β,

Mmp-2 and Cox-2) and the prognostic value of these genes in CRC patients [43]. This raises an important to answer question, i.e. what would be the best procedure to apply personalized medicine effectively and reliably? Could it be a minimum number of (bio)-markers for each cancer type to be tested, or should it be a complex approach as high throughput genome sequencing, as it is increasingly performed?

Ideally, a few markers would be better suited regarding costs of analysis and time until a patient can benefit from the results. However, there are currently only few markers which succeeded to be applied accordingly. Therefore, even more methods are being approached to assess the specific changes inherent to the full genome.

4. Conclusion and perspectives

Here we show a new high throughput approach in exploring genes relevant to CRC progression in terms of liver metastasis. Our method has yielded initial results related to the importance of claudins and IGFBP in liver colonization. Nevertheless, we reason that other genes, which result from this model, might be even more valuable. For instance, one of the very important and interesting gene families that resulted from this model, which is extensively investigated, is the endothelin system with all its components (endothelins, their converting enzymes and their receptors). Several members of this system could prove useful as tumor/metastasis markers. Future experiments will show whether such a functional model can compete or complement, at least partially, other techniques, including whole genome sequencing.

Conflict of interest

The authors confirm that there are no conflicts of interest.

Author details

Rania B. Georges[1*], Hassan Adwan[2] and Martin R. Berger[1]

1 German Cancer Research Center (DKFZ), Heidelberg, Germany

2 German University of Cairo (GUC), Cairo, Egypt

*Address all correspondence to: r.georges@dkfz-heidelberg.de

References

[1] Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global patterns and trends in colorectal cancer incidence and mortality. Gut. 2017;**4**:683-691. DOI: 10.1136/gutjnl-2015-310912

[2] Valderrama-Trevino AI, Barrera-Mera B, Ceballos-Villalva JC, Montalvo-Jave EE. Hepatic metastasis from colorectal cancer. Euroasian Journal of Hepato-Gastroenterology. 2017;**2**:166-175. DOI: 10.5005/jp-journals-10018-1241

[3] Bismuth H, Adam R, Levi F, Farabos C, Waechter F, Castaing D, et al. Resection of nonresectable liver metastases from colorectal cancer after neoadjuvant chemotherapy. Annals of Surgery. 1996;**4**:509-520. discussion 520-502

[4] Fong Y, Fortner J, Sun RL, Brennan MF, Blumgart LH. Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer: Analysis of 1001 consecutive cases. Annals of Surgery. 1999;**3**:309-318. discussion 318-321

[5] Wittmer A, Khazaie K, Berger MR. Quantitative detection of lac-Z-transfected CC531 colon carcinoma cells in an orthotopic rat liver metastasis model. Clinical & Experimental Metastasis. 1999;**5**:369-376

[6] Seelig MH, Leible M, Sanger J, Berger MR. Chemoembolization of rat liver metastasis with microspheres and gemcitabine followed by evaluation of tumor cell load by chemiluminescence. Oncology Reports. 2004;**5**: 1107-1113

[7] Eyol E, Boleij A, Taylor RR, Lewis AL, Berger MR. Chemoembolisation of rat colorectal liver metastases with drug eluting beads loaded with irinotecan or doxorubicin. Clinical & Experimental Metastasis. 2008;**3**:273-282

[8] Marinelli A, van Dierendonck JH, van Brakel GM, Irth H, Kuppen PJ, Tjaden UR, et al. Increasing the effective concentration of melphalan in experimental rat liver tumours: Comparison of isolated liver perfusion and hepatic artery infusion. British Journal of Cancer. 1991;**6**: 1069-1075

[9] Oldenburg J, Begg AC, van Vugt MJ, Ruevekamp M, Schornagel JH, Pinedo HM, et al. Characterization of resistance mechanisms to cis-diamminedichloroplatinum(II) in three sublines of the CC531 colon adenocarcinoma cell line in vitro. Cancer Research. 1994;**2**:487-493

[10] Veenhuizen RB, Marijnissen JP, Kenemans P, Ruevekamp-Helmers MC, t Mannetje LW, Helmerhorst TJ, et al. Intraperitoneal photodynamic therapy of the rat CC531 adenocarcinoma. British Journal of Cancer. 1996;**11**: 1387-1392

[11] Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. Clinical Pharmacology & Therapeutics. 2001;**3**:89-95. DOI: 10.1067/mcp.2001.113989

[12] Osanai M, Takasawa A, Murata M, Sawada N. Claudins in cancer: Bench to bedside. Pflügers Archiv/European Journal of Physiology. 2017;**1**:55-67. DOI: 10.1007/s00424-016-1877-7

[13] Benczik M, Galamb A, Koiss R, Kovacs A, Jaray B, Szekely T, et al. Claudin-1 as a biomarker of cervical cytology and histology. Pathology Oncology Research. 2016;**1**:179-188. DOI: 10.1007/s12253-015-9990-z

[14] Szabo I, Kiss A, Schaff Z, Sobel G. Claudins as diagnostic and prognostic markers in gynecological cancer. Histology and Histopathology. 2009;**12**:1607-1615. DOI: 10.14670/HH-24.1607

[15] Chhabra Y, Waters MJ, Brooks AJ. Role of the growth hormone-IGF-1 axis in cancer. Expert Review of Endocrinology and Metabolism. 2011;**1**:71-84. DOI: 10.1586/eem.10.73

[16] Samani AA, Yakar S, LeRoith D, Brodt P. The role of the IGF system in cancer growth and metastasis: Overview and recent insights. Endocrine Reviews. 2007;**1**:20-47. DOI: 10.1210/er.2006-0001

[17] LeRoith D, Roberts CT Jr. The insulin-like growth factor system and cancer. Cancer Letters. 2003;**2**:127-137. DOI: 10.1016/S0304-3835(03)00159-9

[18] Stewart CE, Rotwein P. Growth, differentiation, and survival: Multiple physiological functions for insulin-like growth factors. Physiological Reviews. 1996;**4**:1005-1026

[19] Bustin SA, Jenkins PJ. The growth hormone-insulin-like growth factor-I axis and colorectal cancer. Trends in Molecular Medicine. 2001;**10**:447-454. DOI: S1471-4914(01)02104

[20] Rajah R, Katz L, Nunn S, Solberg P, Beers T, Cohen P. Insulin-like growth factor binding protein (IGFBP) proteases: Functional regulators of cell growth. Progress in Growth Factor Research. 1995;**2**(4):273-284

[21] Grimberg A, Cohen P. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. Journal of Cellular Physiology. 2000;**1**:1-9

[22] Baxter RC, Binoux MA, Clemmons DR, Conover CA, Drop SL, Holly JM, et al. Recommendations for nomenclature of the insulin-like growth factor binding protein superfamily. The Journal of Clinical Endocrinology and Metabolism. 1998;**9**:3213

[23] Hwa V, Oh Y, Rosenfeld RG. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocrine Reviews. 1999;**6**:761-787

[24] Kostecka Y, Blahovec J. Insulin-like growth factor binding proteins and their functions (minireview). Endocrine Regulations. 1999;**2**:90-94

[25] Peterkofsky B, Gosiewska A, Wilson S, Kim YR. Phosphorylation of rat insulin-like growth factor binding protein-1 does not affect its biological properties. Archives of Biochemistry and Biophysics. 1998;**1**:101-110

[26] Lee KW, Cohen P. Nuclear effects: Unexpected intracellular actions of insulin-like growth factor binding protein-3. The Journal of Endocrinology. 2002;**1**:33-40. DOI: 10.1677/joe.0.1750033

[27] Baxter RC. Signalling pathways involved in antiproliferative effects of IGFBP-3: A review. Molecular Pathology. 2001;**3**:145-148

[28] Kansra S, Ewton DZ, Wang J, Friedman E. IGFBP-3 mediates TGF beta 1 proliferative response in colon cancer cells. International Journal of Cancer. 2000;**3**:373-378

[29] Nam TJ, Busby W Jr, Clemmons DR. Insulin-like growth factor binding protein-5 binds to plasminogen activator inhibitor-I. Endocrinology. 1997;**7**:2972-2978

[30] Suzuki H, Igarashi S, Nojima M, Maruyama R, Yamamoto E, Kai M, et al. IGFBP7 is a p53-responsive gene

specifically silenced in colorectal cancer with CpG island methylator phenotype. Carcinogenesis. 2010;**3**:342-349. DOI: 10.1093/carcin/bgp179

[31] Ruan WJ, Lin J, Xu EP, Xu FY, Ma Y, Deng H, et al. IGFBP7 plays a potential tumor suppressor role against colorectal carcinogenesis with its expression associated with DNA hypomethylation of exon 1. Journal of Zhejiang University. Science. B. 2006;**11**:929-932. DOI: 10.1631/jzus.2006.B0929

[32] Ahmed S, Yamamoto K, Sato Y, Ogawa T, Herrmann A, Higashi S, et al. Proteolytic processing of IGFBP-related protein-1 (TAF/angiomodulin/mac25) modulates its biological activity. Biochemical and Biophysical Research Communications. 2003;**2**:612-618. DOI: 10.1016/j.bbrc.2003.09.058

[33] Georges R, Bergmann F, Hamdi H, Zepp M, Eyol E, Hielscher T, et al. Sequential biphasic changes in claudin1 and claudin4 expression are correlated to colorectal cancer progression and liver metastasis. Journal of Cellular and Molecular Medicine. 2012;**2**:260-272. DOI: 10.1111/j.1582-4934.2011.01289.x

[34] Schleidgen S, Klingler C, Bertram T, Rogowski WH, Marckmann G. What is personalized medicine: Sharpening a vague term based on a systematic literature review. BMC Medical Ethics. 2013;**55**. DOI: 10.1186/1472-6939-14-55

[35] Schimanski CC, Linnemann U, Berger MR. Sensitive detection of K-ras mutations augments diagnosis of colorectal cancer metastases in the liver. Cancer Research. 1999;**20**:5169-5175

[36] Shao L, Huang Q, Luo M, Lai M. Detection of the differentially expressed gene IGF-binding protein-related protein-1 and analysis of its relationship to fasting glucose in Chinese colorectal cancer patients. Endocrine-Related Cancer. 2004;**1**:141-148. DOI: 10.1677/erc.0.0110141

[37] Conzelmann M, Linnemann U, Berger MR. K-ras codon 12 and 13 mutations are correlated with differential patterns of tumor cell dissemination in colorectal cancer patients. International Journal of Oncology. 2004;**6**:1537-1544

[38] Schimanski CC, Zimmermann T, Schmidtmann I, Gockel I, Lang H, Galle PR, et al. K-ras mutation status correlates with the expression of VEGFR1, VEGFR2, and PDGFRalpha in colorectal cancer. International Journal of Colorectal Disease. 2010;**2**:181-186. DOI: 10.1007/s00384-009-0843-7

[39] Schimanski CC, Mohler M, Zimmermann T, Gockel I, Lang H, Arbogast R, et al. Detection of hepatic micrometastases in the context of adjuvant chemotherapy and surgery for hepatic metastases. Deutsche Medizinische Wochenschrift. 2010;**21**:1078-1082. DOI: 10.1055/s-0030-1253703

[40] Georges R, Adwan H, Zhivkova M, Eyol E, Bergmann F, Berger MR. Regulation of osteopontin and related proteins in rat CC531 colorectal cancer cells. International Journal of Oncology. 2010;**2**:249-256

[41] Schimanski CC, Moehler M, Gockel I, Zimmermann T, Lang H, Galle PR, et al. Expression of chemokine receptor CCR5 correlates with the presence of hepatic molecular metastases in K-ras positive human colorectal cancer. Journal of Cancer Research and Clinical Oncology. 2011;**7**:1139-1145. DOI: 10.1007/s00432-011-0980-6

[42] Allami RH, Graf C, Martchenko K, Voss B, Becker M, Berger MR, et al. Analysis of the expression of SDF-1 splicing variants in human colorectal cancer and normal mucosa tissues. Oncology Letters. 2016;**3**:1873-1878. DOI: 10.3892/ol.2016.4139

[43] Uhlmann ME, Georgieva M, Sill M, Linnemann U, Berger MR. Prognostic value of tumor progression-related gene expression in colorectal cancer patients. Journal of Cancer Research and Clinical Oncology. 2012;**10**:1631-1640. DOI: 10.1007/s00432-012-1238-7

[44] Hashimoto Y, Fukasawa M, Kuniyasu H, Yagi K, Kondoh M. Claudin-targeted drug development using anti-claudin monoclonal antibodies to treat hepatitis and cancer. Annals of the New York Academy of Sciences. 2017;**1**:5-16. DOI: 10.1111/nyas.13337

[45] Morin PJ. Claudin proteins in human cancer: Promising new targets for diagnosis and therapy. Cancer Research. 2005;**21**:9603-9606. DOI: 10.1158/0008-5472.CAN-05-2782

[46] How HK, Yeoh A, Quah TC, Oh Y, Rosenfeld RG, Lee KO. Insulin-like growth factor binding proteins (IGFBPs) and IGFBP-related protein 1-levels in cerebrospinal fluid of children with acute lymphoblastic leukemia. The Journal of Clinical Endocrinology and Metabolism. 1999;**4**:1283-1287

[47] Komatsu S, Okazaki Y, Tateno M, Kawai J, Konno H, Kusakabe M, et al. Methylation and downregulated expression of mac25/insulin-like growth factor binding protein-7 is associated with liver tumorigenesis in SV40T/t antigen transgenic mice, screened by restriction landmark genomic scanning for methylation (RLGS-M). Biochemical and Biophysical Research Communications. 2000;**1**:109-117

[48] Leibowitz BJ, Cohick WS. Endogenous IGFBP-3 is required for both growth factor-stimulated cell proliferation and cytokine-induced apoptosis in mammary epithelial cells. Journal of Cellular Physiology. 2009;**1**:182-188. DOI: 10.1002/jcp.21748

[49] Frenkel S, Zloto O, Pe'er J, Barak V. Insulin-like growth factor-1 as a predictive biomarker for metastatic uveal melanoma in humans. Investigative Ophthalmology & Visual Science. 2013;**1**:490-493. DOI: 10.1167/iovs.12-10228

[50] Maris C, D'Haene N, Trepant AL, Le Mercier M, Sauvage S, Allard J, et al. IGF-IR: A new prognostic biomarker for human glioblastoma. British Journal of Cancer. 2015;**5**:729-737. DOI: 10.1038/bjc.2015.242

[51] Yoneyama T, Ohtsuki S, Honda K, Kobayashi M, Iwasaki M, Uchida Y, et al. Identification of IGFBP2 and IGFBP3 as compensatory biomarkers for CA19-9 in early-stage pancreatic cancer using a combination of antibody-based and LC-MS/MS-based proteomics. PLoS One. 2016;**8**:e0161009. DOI: 10.1371/journal.pone.0161009

[52] Ma Y, Lu B, Ruan W, Wang H, Lin J, Hu H, et al. Tumor suppressor gene insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) induces senescence-like growth arrest in colorectal cancer cells. Experimental and Molecular Pathology. 2008;**2**:141-145. DOI: 10.1016/j.yexmp.2008.04.005

[53] Mohan S, Baylink DJ. IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms. The Journal of Endocrinology. 2002;**1**:19-31. DOI: 10.1677/joe.0.1750019

[54] Jiang W, Xiang C, Cazacu S, Brodie C, Mikkelsen T. Insulin-like growth factor binding protein 7 mediates glioma cell growth and migration. Neoplasia. 2008;**12**:1335-1342. DOI: 10.1593/neo.08694

[55] Winesett DE, Ulshen MH, Hoyt EC, Mohapatra NK, Fuller CR, Lund PK. Regulation and localization of the insulin-like growth factor system in small bowel during altered nutrient

status. The American Journal of Physiology. 1995;**4**(Pt 1):G631-G640

[56] Williams AC, Collard TJ, Perks CM, Newcomb P, Moorghen M, Holly JM, et al. Increased p53-dependent apoptosis by the insulin-like growth factor binding protein IGFBP-3 in human colonic adenoma-derived cells. Cancer Research. 2000;**1**:22-27

[57] Hong J, Zhang G, Dong F, Rechler MM. Insulin-like growth factor (IGF)-binding protein-3 mutants that do not bind IGF-I or IGF-II stimulate apoptosis in human prostate cancer cells. The Journal of Biological Chemistry. 2002;**12**:10489-10497. DOI: 10.1074/jbc.M109604200

[58] Gill ZP, Perks CM, Newcomb PV, Holly JM. Insulin-like growth factor-binding protein (IGFBP-3) predisposes breast cancer cells to programmed cell death in a non-IGF-dependent manner. The Journal of Biological Chemistry. 1997;**41**:25602-25607. DOI: 10.1074/jbc.272.41.25602

[59] Kirman I, Poltoratskaia N, Sylla P, Whelan RL. Insulin-like growth factor-binding protein 3 inhibits growth of experimental colocarcinoma. Surgery. 2004;**2**:205-209. DOI: 10.1016/j.surg.2004.04.020

[60] Ogino S, Meyerhardt JA, Kawasaki T, Clark JW, Ryan DP, Kulke MH, et al. CpG island methylation, response to combination chemotherapy, and patient survival in advanced microsatellite stable colorectal carcinoma. Virchows Archiv. 2007;**5**:529-537. DOI: 10.1007/s00428-007-0398-3

[61] Chan JM, Stampfer MJ, Ma J, Gann P, Gaziano JM, Pollak M, et al. Insulin-like growth factor-I (IGF-I) and IGF binding protein-3 as predictors of advanced-stage prostate cancer. Journal of the National Cancer Institute. 2002;**14**:1099-1106

[62] Krajcik RA, Borofsky ND, Massardo S, Orentreich N. Insulin-like growth factor I (IGF-I), IGF-binding proteins, and breast cancer. Cancer Epidemiology, Biomarkers & Prevention. 2002;**12**:1566-1573

[63] Palmqvist R, Hallmans G, Rinaldi S, Biessy C, Stenling R, Riboli E, et al. Plasma insulin-like growth factor 1, insulin-like growth factor binding protein 3, and risk of colorectal cancer: A prospective study in northern Sweden. Gut. 2002;**5**:642-646

[64] DeLellis K, Rinaldi S, Kaaks RJ, Kolonel LN, Henderson B, Le Marchand L. Dietary and lifestyle correlates of plasma insulin-like growth factor-I (IGF-I) and IGF binding protein-3 (IGFBP-3): The multiethnic cohort. Cancer Epidemiology, Biomarkers & Prevention. 2004;**9**:1444-1451

[65] Durai R, Yang W, Gupta S, Seifalian AM, Winslet MC. The role of the insulin-like growth factor system in colorectal cancer: Review of current knowledge. International Journal of Colorectal Disease. 2005;**3**. DOI: 203-220. 10.1007/s00384-004-0675-4

[66] Serel TA, Kecelioglu M. Serum insulin-like growth factor is not a useful marker of prostate cancer. BJU International. 2000;**4**:559-560

[67] Kaaks R, Toniolo P, Akhmedkhanov A, Lukanova A, Biessy C, Dechaud H, et al. Serum C-peptide, insulin-like growth factor (IGF)-I, IGF-binding proteins, and colorectal cancer risk in women. Journal of the National Cancer Institute. 2000;**19**:1592-1600

[68] Murphy M, Pykett MJ, Harnish P, Zang KD, George DL. Identification and characterization of genes differentially expressed in meningiomas. Cell Growth & Differentiation. 1993;**9**:715-722

[69] Kato MV. A secreted tumor-suppressor, mac25, with activin-binding activity. Molecular Medicine. 2000;**2**:126-135

[70] Wilson HM, Birnbaum RS, Poot M, Quinn LS, Swisshelm K. Insulin-like growth factor binding protein-related protein 1 inhibits proliferation of MCF-7 breast cancer cells via a senescence-like mechanism. Cell Growth & Differentiation. 2002;**5**:205-213

[71] Adachi Y, Itoh F, Yamamoto H, Arimura Y, Kikkawa-Okabe Y, Miyazaki K, et al. Expression of angiomodulin (tumor-derived adhesion factor/mac25) in invading tumor cells correlates with poor prognosis in human colorectal cancer. International Journal of Cancer. 2001;**4**:216-222

[72] Shersher DD, Vercillo MS, Fhied C, Basu S, Rouhi O, Mahon B, et al. Biomarkers of the insulin-like growth factor pathway predict progression and outcome in lung cancer. The Annals of Thoracic Surgery. 2011;**5**:1805-1811. DOI: 10.1016/j.athoracsur.2011.06.058. discussion 1811

[73] Ochs-Balcom HM, Vaughn CB, Nie J, Chen Z, Thompson CL, Parekh N, et al. Racial differences in the association of insulin-like growth factor pathway and colorectal adenoma risk. Cancer Causes & Control. 2014;**2**:161-170. DOI: 10.1007/s10552-013-0318-6

[74] Ong J, Salomon J, te Morsche RH, Roelofs HM, Witteman BJ, Dura P, et al. Polymorphisms in the insulin-like growth factor axis are associated with gastrointestinal cancer. PLoS One. 2014;**3**:e90916. DOI: 10.1371/journal.pone.0090916

Chapter 3

BRAF Mutation and Its Importance in Colorectal Cancer

Lee-Jen Luu and Timothy J. Price

Abstract

BRAF mutation is seen in nearly one in ten patients with advanced colorectal cancer. Despite major improvements in survival for advanced colorectal cancer overall, patients with BRAF mutation continue to have a very poor prognosis often with median survival of less than 12 months. It is important for clinicians to be aware of this subgroup as the treatment approach should be different. Treatment options beyond standard chemotherapy are crucial to achieve better outcomes and the role of anti-EGFR therapy alone remains controversial. Current trials assessing combinations of molecular targeted agents have seen some promise. This chapter explores the background of BRAF mutation and current treatment strategies.

Keywords: BRAF, colorectal cancer, V600E mutation

1. Introduction

The RAS/RAF/MEK/ERK signalling cascade, also known as the MAPK (mitogen-activated protein kinase) pathway, is involved in cell proliferation, differentiation, survival and apoptosis [1]. It receives input from multiple sources including internal metabolic stress and DNA damage pathways and altered protein concentrations as well as through signalling from external growth factors, cell-matrix interactions and communication from other cells [2]. This allows for a nodal point for therapeutic targeting, however, dysregulation of this pathway can also increase malignant behaviour [3].

Multiple signals activate RAS (KRAS, NRAS and HRAS), a family of GTPases. This, in turn, activates downstream RAF protein kinases (ARAF, BRAF and CRAF). The dominant substrates of RAF kinases are the MAPK/ERK kinases, MEK1 and MEK2. ERKs phosphorylate a variety of substrates, including multiple transcription factors that regulate several key cellular activities (**Figure 1**).

Mutations in RAS and RAF are the most common oncogenes in human cancer [4]. The focus of this chapter will be on BRAF mutations in colorectal cancer, in particular the V600E mutation, the clinical significance, molecular and clinical pathogenesis as well as treatment, now and into the future.

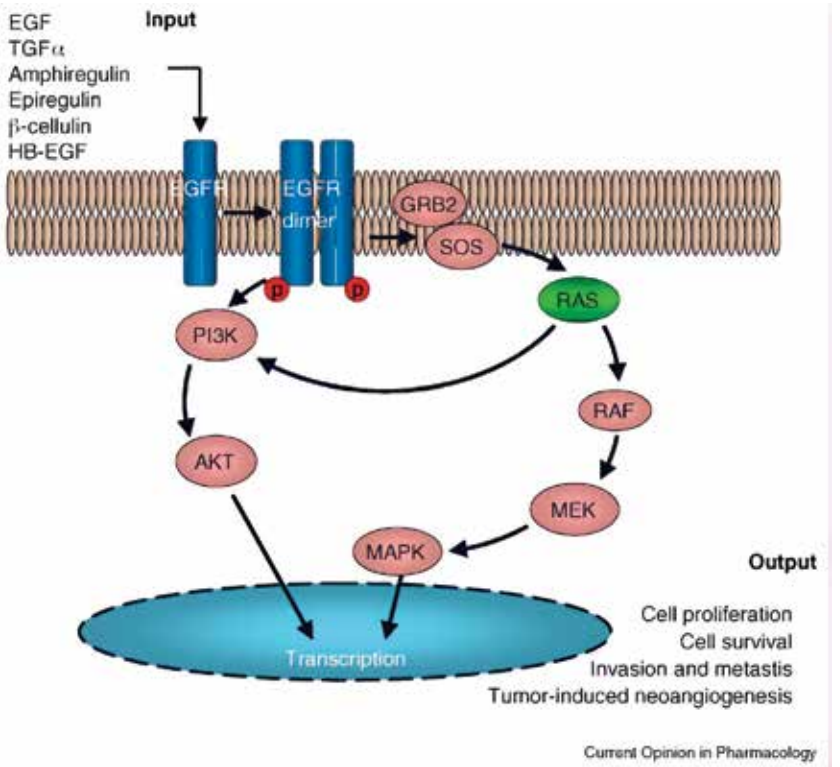

Figure 1.
The MAPK pathway [80].

2. BRAF

The RAF protein is made of three conserved regions: CR1, CR2 and CR3. CR1 and CR2 are situated in the N terminus. CR1 acts as the main binding domain for RAS; CR2 is the regulatory domain. CR3 is situated in the C terminus and functions as the catalytic kinase domain. CR3 contains two regions important for RAF activation: the activation segment and the regulatory region [5]. Of the RAF family of protein kinases, BRAF is the most frequently mutated and remains the most potent activator of MEK.

The BRAF protooncogene, which encodes for the BRAF protein kinase, is located on chromosome 7 (q34) and is composed of 18 exons. There have been more than thirty BRAF mutations identified to date, occurring in various frequencies. The most common is BRAF V600E mutation (MT), which corresponds to a thymine to adenine transversion at position 1799, resulting in the substitution of valine by glutamate at position 600 of the protein [5]. This lies within the activating segment of the kinase domain. It renders BRAF constitutionally active, increasing kinase activity relative to BRAF wild-type (WT) by 10 times [6]. Because of this, co-mutations in the MAPK signalling cascade offers no selective advantage for developing tumours and therefore BRAF mutations are mutually exclusive with KRAS or NRAS mutations [7].

The V600E mutation accounts for more than 85% of BRAF mutations in melanoma, more than 50% of the mutations in non-small cell lung cancer and more than 95% of mutations in cholangiocarcinoma and hairy cell leukaemia. It accounts for more than 90% of BRAF mutations in colorectal cancer (CRC) [8]. Other BRAF mutations include R461I, I462S, G463E, G463V, G465A, G465E, G465V, G468A, G468E, N580S, E585K, D593V, F594L, G595R, L596V, T598I, V599D, V599E (V600E), V599K, V599R, V600K, and A727V [9].

3. Prevalence and clinical features of BRAF MT CRC

BRAF mutations have been found in 7–10% of patients with metastatic CRC [7, 10]. BRAF MT CRC has been associated with a particular phenotype in multiple studies and meta-analysis and specifically pertaining to the BRAF V600E mutation. BRAF tumours are more prevalent in women and in patients >70 years of age. BRAF is not associated with age at diagnosis of less than 60 years [11]. BRAF mutation is more prevalent in proximal colon tumours and is rarely found in the left colon [7]. Histopathology also differs, with 60% of BRAF MT tumours being poorly differentiated and a higher rate of mucinous pathology [12]. There is an association with larger primary tumours. BRAF MT CRC is also associated with a high rate of peritoneal metastases and less lung and liver-limited disease [13–15]. In contrast, most non-V600 mutations were more likely to be lower grade and left-sided tumours with a greater overall survival [16, 17], except for codon 601/597 mutations which behave similarly to V600E MT CRC [18].

4. The serrated neoplastic pathway

The pathogenesis of CRC is a heterogeneous and complex process. The classic model of adenoma-carcinoma sequence was initially described by Vogelstein and accounts for approximately 80% of sporadic CRC [19]. Mutation of the tumour suppressor gene, APC, occurs early in the process and additional mutations and chromosomal instability leads to neoplastic progression [20].

The serrated neoplastic pathway is an alternative model of CRC pathogenesis with distinct morphologic and molecular characteristics. It is estimated about 20% of CRC develop via this pathway. These lesions develop from aberrant crypt foci and hyperplastic polyps (HP) into traditional serrated adenoma (TSA) and sessile serrated adenoma (SSA), with malignant potential. BRAF mutation occurs early in the pathway, shown to be present in HP, hyperplastic adenomas and SSA [21].

SSA are also characterised by the CpG island methylator phenotype (CIMP) [22]. A cytosine nucleotide followed by a guanine nucleotide (CpG dinucleotide) can be found in dense clusters (CpG islands) in the promoter regions of approximately half of all genes [23]. Aberrant hypermethylation of these CpG islands can lead to silencing of tumour suppressor genes that, in turn, lead to carcinogenesis. CIMP can be described as high, low or negative. Hypermethylation of the mismatch repair gene *MLH1* results in microsatellite instability (MSI) in sporadic CRC [24].

MSI is implicated in 15% of sporadic CRC and >95% of Hereditary Non Polyposis Colorectal Cancer (HNPCC), also known as Lynch syndrome. It is caused by deficiency of the DNA mismatch repair (MMR) system, composed of multiple interacting proteins including MSH2, MLH1. The majority of sporadic MSI high CRC is due to the hypermethylation of the mismatch repair gene *MLH1* [25]. Sporadic MSI high CRC is also associated with BRAF mutation. BRAF mutations have been observed in 30–50% of MSI high CRC compared with 10% in microsatellite stable tumours [26, 27]. Germline mutations in 1 of 4 mismatch repair genes (MLH1, MSH2, MSH6 and PMS2) account for the majority of cases of HNPCC. BRAF mutations rarely occur in patients with germline mutations in MMR genes [28].

5. Prognostic significance of BRAF mutation

BRAF MT CRC is strongly associated with inferior survival compared with BRAF WT disease. Randomised control trials of first line treatment of metastatic CRC demonstrate differences in OS of up to 12 months, shown in **Table 1**.

Study	Treatment regimen	Key outcomes in BRAF MT disease	Prognostic finding
MRC FOCUS [76]	5-FU/irinotecan or 5-FU/ oxaliplatin	HR for OS 1.82 (P = 0.0002)	BRAF predicts poor OS but no difference in PFS
MRC COIN [43]	Fluoropyrimidine/ oxaliplatin ± cetuximab	OS 8.8 vs. 20.1 months	Median OS was significantly shorter in patients with BRAF, KRAS or NRAS mutations than in patients with WT KRAS, NRAS, and BRAF tumours, irrespective of treatment (P < 0.0001)
OPUS [77]	FOLFOX ± cetuximab	Median OS, 20.7 months with cetuximab + FOLFOX	Small numbers precluded definitive conclusions
CRYSTAL [78]	FOLFIRI ± cetuximab	Median PFS (cetuximab + FOLFIRI vs. FOLFIRI), 8.0 vs. 5.6 months (HR, 0.934; P = 0.87) Median OS, 14.1 vs. 10.3 months (HR 0.908; P = 0.74)	BRAF MT was strong indicator of poor prognosis
NORDIC-VII [48]	FLOX ± cetuximab	BRAF MT had lower ORRs than BRAF WT (20 vs. 50%; P < 0.001)	BRAF mutations was a strong negative prognostic factor
PRIME [42]	FOLFOX ± panitumumab	Median PFS: Panitumumab + FOLFOX vs. FOLFOX, 6.1 vs. 5.4 months Median OS: Panitumumab + FOLFOX vs. FOLFOX, 10.5 vs. 9.2 months	BRAF mutation was a negative prognostic factor
CAIRO2 [10]	Capecitabine + oxaliplatin + bevacizumab vs. CAPOX + bevacizumab + cetuximab	Lower median PFS, 5.9 and 6.6 months in BRAF-MT vs. 12.2 and 10.4 months in BRAF WT tumours with CAPOX + bevacizumab and CAPOX + bevacizumab + cetuximab, respectively Lower median OS, 15.0 and 15.2 months in BRAF MT vs. 24.6 and 21.5 months in BRAF WT with CB and CBC, respectively	BRAF mutation was a negative prognostic marker
AGITG MAX [79]	Capecitabine ± bevacizumab	Median OS, 20.8 months in BRAF WT vs. 8.6 months in BRAF MT tumours	BRAF mutation was a marker of poor prognosis irrespective of treatment

Table 1.
BRAF mutation as a prognostic factor in clinical studies of first-line treatment of metastatic CRC.

Venderbosch et al. reported a pooled analysis of the CAIRO, CAIRO2, COIN and FOCUS studies examining mismatch repair and BRAF status [29]. BRAF MT was associated with a poor prognosis with OS of 11.4 vs. 17.2 months, and PFS of 6.2 vs. 7.7 months compared with BRAF WT. This analysis also found dMMR to indicate poor prognosis, despite significant evidence to show that MSI-high tumours confer a better prognosis. However, it is concluded that as there is no interaction between BRAF MT and dMMR, the poor prognostic value of dMMR is likely driven by BRAF MT. There was no difference in OS or PFS between dMMR BRAF MT and pMMR BRAF MT tumours. In a study examining RAS and BRAF mutations, BRAF patients had the worst overall survival. The median OS for WT, KRAS, NRAS and BRAF patients were 49.2, 36.2, 30.1 and 22.5 months, respectively [30].

Similarly, BRAF MT has been shown to be a negative prognostic factor in stage II and III disease. Data from the PETACC-3 was extracted, with KRAS, BRAF and MSI status examined [31]. MSI-high tumours were associated with better prognosis. BRAF MT was not prognostic of PFS. The MSI-high status appeared to attenuate the negative prognostic effect of BRAF MT on OS; BRAF MT is a negative prognostic factor in MSS CRC. However, more recently, a meta-analysis of 1164 patients with MSI-high non-metastatic CRC has shown that BRAF V600E mutation does correlate with adverse overall survival, but not disease recurrence [32].

Survival following metastasectomy is also worse for BRAF MT mCRC as demonstrated in a meta-analysis of patients undergoing resection of liver metastases. It showed the BRAF mutation was negatively associated with OS (HR 3.055, P = 0.00004) [33].

In contrast, non-V600E BRAF mutations have a different prognosis. BRAF codons 594 and 596 mutations, when compared with V600E BRAF mutations, are more frequently rectal, non-mucinous with no peritoneal spread. In a study of 10 patients, all BRAF 594 and 596 tumours were microsatellite stable. OS was significantly longer (62 vs. 12.6 months, P = 0.002) [34]. Jones et al. identified 208 metastatic CRC patients out of 9643 with non-V600E mutations. When compared with V600E BRAF mutation patients, those with non-V600E mutations were found to be younger, more likely male, and had lower grade tumours. In addition, median OS was significantly longer compared with both V600E BRAF mutant and BRAF wild-type patients (60.7 vs. 11.4 vs. 43 months respectively) [35]. This has also been demonstrated in a retrospective study of 98 patients, 6 of whom had non-V600E BRAF mutations. Although only a small sample size, OS was significantly better compared with V600E BRAF MT patients (P = 0.38) [17].

6. Treatment of BRAF-mutation CRC

6.1 Standard treatment

Doublet chemotherapy remains the standard of care for metastatic BRAF MT CRC in patients with appropriate performance status [36, 37]. First-line chemotherapy options include 5 fluorouracil, leucovorin and oxaliplatin (FOLFOX), 5-fluorouracil, leucovorin and irinotecan (FOLFIRI) and capecitabine plus oxaliplatin. A retrospective study reported no difference in median PFS between irinotecan-based or oxaliplatin-based chemotherapy regimens in the first line for BRAF-MT CRC [38].

A more intensive triplet chemotherapy regime has been proposed based on 5 fluorouracil, leucovorin, oxaliplatin and irinotecan with bevacizumab (FOLFOXIRI+bev). A phase II trial of FOLFOXIRI and bevacizumab in the metastatic CRC population

showed a statistically significant benefit to progression free survival and trend towards improved overall survival at the expense of greater incidence of grade three toxicities [39]. An exploratory analysis of the BRAF-MT cohort (25 patients in a pooled population) reported a median PFS of 11.8 months, median OS of 24.1 months and an impressive response rate of 72%, including one patient with complete response [40]. This was followed up by the open label phase III TRIBE study comparing FOLFIRI plus bevacizumab with FOLFOXIRI with bevacizumab [41]. In the molecular subgroup analysis, 28 out of 391 cases were BRAF mutant. There was a trend towards benefit in overall survival (19.0 months in the FOLFOXIRI plus bevacizumab arm vs. 10.7 months in the FOLFIRI plus bevacizumab arm, HR 0.54); however, this was not statistically significant. This was also seen in median PFS (7.5 vs. 9.5 months, HR 0.57) and best overall response (56 vs. 42%). While not statistically significant, this regime has been proposed in the first line setting for BRAF-MT mCRC patients with good performance status given the overall survival data.

6.2 EGFR inhibitors

The epidermal growth factor receptor (EGFR) is involved in signalling upstream of the RAS-RAF-MEK-ERK pathway. Monoclonal antibodies directed against EGFR, cetuximab and panitumumab have shown to be effective in metastatic CRC; however, KRAS mutation is a negative predictor of EGFR treatment response and upfront testing is recommended before starting treatment [36, 37].

As previously discussed, KRAS and BRAF mutations are mutually exclusive. Given the common signalling pathway, BRAF mutation has also been proposed to be a negative predictive marker of EGRF antibody treatment response. In the first line setting, the PRIME study evaluated the addition of panitumumab to FOLFOX. In BRAF-MT tumours, panitumumab added no benefit to survival (HR 0.9, P = 0.76) [42]. Similarly, the phase III MRC COIN trial showed no benefit in the addition of cetuximab to first-line oxaliplatin based chemotherapy, irrespective of KRAS or BRAF mutation status [43]. In the second line setting, the PICCOLO study reported no effect of panitumumab in combination with irinotecan on PFS, but a significant negative effect on OS (HR 1.84, P = 0.029). Cetuximab was also evaluated against best supportive care in the phase III CO.17 trial [44]. For BRAF MT tumours, there were no responses and no change to survival in the sample size of 13 (HR 0.84, P = 0.81).

Given the small numbers of BRAF-MT patients in these trials, there have been a number of meta-analyses evaluating the BRAF mutation as a predictive marker of EGFR therapy. Therkildsen et al. reviewed KRAS, NRAS, BRAF, PIK3CA and PTEN mutations in patients with KRAS exon 2 wild-type patients. Of the 1267 patients in 17 studies treated with either cetuximab or panitumumab in both first line and subsequent like therapies, 128 patients had BRAF V600E mutations [45]. There was a significant decrease in overall response rate (17 vs. 45%). BRAF mutation was also linked to shorter PFS (HR 2.95) and OS (HR 2.52) compared to BRAF wild-type tumours.

Pietrantonio et al. examined the impact of cetuximab and panitumumab on PFS, OS and overall response rate (ORR) [46]. This meta-analysis included 9 phase III trials and 1 phase II trial (across first-line, second-line and chemotherapy refractory settings). 463 RAS wild-type/BRAF MT CRC patients were identified. The addition of EGFR antibody therapy did not significantly improve PFS (HR 0.88, P = 0.33), OS (HR 0.91, P = 0.63) and ORR (relative risk 1.31, P = 0.25).

A further meta-analysis was published in 2015 but Rowland et al. [47]. It included 8 randomised control trials that had also been included in the analysis by Pietrantonio et al., but differed by excluding 2 trials; 1 by Tveit et al. [48] due to lack of OS and PFS data and Stintzing et al. [49] as the control arm included

bevacizumab. In addition, the statistical analysis differed as Rowland et al. compared BRAF MT patients with BRAF wild-type. 351 patients were identified with BRAF mutation, of which 330 with the V600E mutation. The HR for PFS was 0.86 for RAS wild-type/BRAF MT compared with 0.62 for RAS wild-type/BRAF wild-type tumours with a test of interaction that nears but does not reach statistical significance (P = 0.07). There was no difference for OS either, the HR for RAS wild-type/BRAF MT tumours was 0.97 compared with 0.81 for RAS wild-type/BRAF wild-type (test of interaction, P = 0.43). It concluded that there was insufficient evidence to definitively state that RAS wild-type/BRAF MT individuals derive a different treatment benefit from EGFR antibodies compared with RAS wild-type/BRAF wild-type patients.

More recently, the triplet chemotherapy regime, FOLFOXIRI, has been studied in combination with panitumumab in the VOLFI trial [50]. This was a randomised phase II trial of patients with RAS WT, unresectable metastatic CRC. 96 patients were included, of which, 16 patients with BRAF MT disease. The primary endpoint was ORR. The addition of panitumumab significantly improved ORR in the overall population (85.7 vs. 54.5%, P = 0.0013), and in the BRAF MT population, there was trend to improved ORR (71.4 vs. 22.2%, P = 0.1262).

Thus, while there exists a significant body of evidence that suggests minimal clinical benefit of EGFR antibody treatment in BRAF MT metastatic CRC, it is not definitive and therefore remains an option for therapy in discussion with the patient. This primarily relates to anti-EGFR as the sole biological agent however anti-EGFR therapy may have a definite role when combined with additional biological agents such as BRAF inhibitors as discussed below.

6.3 BRAF inhibition in mCRC

BRAF represents a therapeutic target in cancer as, unlike KRAS, it is a relatively unidirectional MEK-ERK effector. Inhibition of BRAF with vemurafenib (PLX4032) has been demonstrated to significantly benefit patients with unresectable or metastatic BRAF V600E MT melanoma, improving progression free survival and OS, with a response rate of 48% [51]. In sharp contrast, BRAF inhibition in mCRC is disappointing. An expansion phase II study examined vemurafenib in patients with BRAF MT mCRC who have had at least one line of prior therapy [52]. Of the 21 patients treated, 1 patient had a partial response and 7 other patients had stable disease by RESIST criteria. The median PFS was only 2.1 months and ORR of 5%. Although there were signs of efficacy, the authors concluded that single-agent vemurafenib did not show any meaningful clinical activity in patients with BRAF V600E MT mCRC.

These results were similar to a histology-independent phase II "basket" trial of vemurafenib. 122 patients with BRAF V600 MT malignancies were enrolled into 7 prespecified cohorts, including 37 with mCRC [53]. Vemurafenib, as a single agent, was given to 10 patients with mCRC. Response was poor, with 50% having stable disease and the rest progressing on therapy. The remaining 27 patients with mCRC received combination of vemurafenib and cetuximab, and the results will be discussed later in the chapter.

There are several mechanisms of resistance identified that reduce the efficacy of BRAF inhibition in mCRC. For example Prahallad et al. identified that BRAF inhibition with vemurafenib in mCRC cells causes a rapid activation of EGFR through an ERK-dependent negative feedback loop [54]. Unlike in melanoma, CRC cell lines express high levels of activated EGFR. Blockade of EGFR with either EGFR monoclonal antibodies or small-molecule kinase inhibitors (gefitinib and erlotinib) was showed to work synergistically with BRAF inhibition.

More recently, it has been showed that BRAF inhibition can also lead to up regulation of other receptor tyrosine kinases including human epidermal growth factor receptor (HER) 2 and HER3 [55].

Activation of the phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway has also been implicated in BRAF inhibition resistance [56]. PI3K signalling is activated by direct mutational activation or amplification of PIK3CA and AKT1 or loss of PTEN [57]. Approximately 40% of CRC have been shown to have alterations in 1 of 8 PI3K pathway genes, which are almost always mutually exclusive to each other [58]. Genotyping of BRAF MT CRC has showed concomitant PI3KCA and PTEN mutations [59].

The Wnt/β-catenin pathway is also involved in cell proliferation, differentiation and survival and interacts with the RAS-RAF-MEK-ERK pathway at multiple points. It has been identified as an important step in tumourigenesis and alterations in the Wnt pathway have been identified more frequently in BRAF V600E MT CRC patient samples, potentially representing an alternative pathway of tumour development when BRAF is inhibited [60].

Based on these findings, BRAF inhibition has been combined with a number of different agents in order to attempt to overcome resistance and improve response.

6.4 BRAF and EGFR inhibition

In the fore-mentioned phase II "basket" trial, the effect of vemurafenib and cetuximab evaluated in 27 patients with BRAF V600 MT mCRC. The result was marginally improved compared to single-agent treatment. One patient had a partial response (4% ORR) and 69% had stable disease. Median PFS was 3.7 months and median OS was 7.1 months. A pilot trial with combination panitumumab and vemurafenib included 15 patients with BRAF V600E mCRC who had received at least 1 prior line of therapy [61]. 2 patients had confirmed partial response and 6 patients had stable disease, including 2 patients with stability lasting over 6 months. The treatment was well tolerated with fatigue and rash being the most frequently observed adverse events.

Other combinations of BRAF and EGFR inhibitors have also been investigated including vemurafenib plus erlotinib [62], encorafenib (LGX818, a highly selective ATP-competitive small molecule RAF kinase inhibitor) plus cetuximab [63] and dabrafenib (a small molecule kinase BRAF inhibitor) plus panitumumab [64]. Response rates in these trials range from 4 to 23%. To improve this outcome, the combination has been combined with chemotherapy in the randomised phase 2 SWOG 1406 study [65]. Interim results of this trial were presented in 2017. The combination of irinotecan and cetuximab with or without vemurafenib was examined in 106 patients. Median PFS was significantly improved with the addition of vemurafenib (4.4 vs. 2.0 months, $P < 0.001$). Response rate increased from 4 to 16% ($P = 0.09$). However, there was an increase in grade 3 and 4 adverse events including neutropenia, anaemia and nausea. It was noted that no new safety signals. The data on median OS was immature. Based on these findings, this treatment regime has been included in treatment guidelines [36].

6.5 BRAF and MEK inhibition

BRAF and MEK inhibition has been combined in melanoma with greater efficacy and so has been evaluated in the BRAF MT mCRC population. 43 patients were treated with dabrafenib and trametinib [66]. 1 patient achieved a complete response and 4 patients had a partial response (ORR 12%). 24 patients achieved stable

disease (56%). During-treatment biopsies in 9 patients showed reduced levels of ERK compared with pre-treatment biopsies. It is suggested that combination BRAF and MEK inhibition could be a potential therapeutic backbone for the addition of other agents including EGFR inhibitors.

6.6 BRAF, MEK and EGFR inhibition

Given the role of MEK and ERK in EGFR activation leading to BRAF inhibitor resistance, the triplet combination of BRAF, MEK and EGFR inhibitors have been investigated. Corcoran et al. reported on a trial involving 3 cohorts, dabrafenib and panitumumab (n = 20), dabrafenib, trametinib and panitumumab (n = 91), and trametinib and panitumumab (n = 31) [67].

The ORR for triplet therapy was 21%, compared with 0% with trametinib and panitumumab and 10% with dabrafenib and panitumumab. With the increase in response rate, there was also a corresponding increase in adverse events. 70% of patients on triplet therapy had a grade 3 or 4 adverse event. 18% of patients had an adverse event resulting in study discontinuation, 54% had an adverse event that resulted in dose reduction, and 71% of patients had an adverse event that led to dose interruption or delay. Skin toxicity including rash and dermatitis acneiform occurred in 90% of patients, with 21% having grade 3 or 4 adverse events. Paired pre-treatment and on-treatment biopsies demonstrated that triplet combination produced greater inhibition of ERK than the dabrafenib and panitumumab doublet or the dabrafenib and panitumumab doublet.

It has been suggested that BRAF inhibitors may offset the dermatologic toxicity resulting from MEK or EGFR inhibitors. Mondaca et al. reported on a case of BRAF V600E MT metastatic CRC on clinical trial with dabrafenib, trametinib and panitumumab [68]. Dabrafenib dose reductions for neutropenia were associated with increased skin toxicity, which subsequently improved with increasing the dose. This case highlights the importance of dose intensity of BRAF inhibitors with used in combination regimens.

6.7 Other therapeutic strategies and current trials

Current therapeutic investigations in the BRAF MT mCRC field involve multiple targeted therapies aimed at overcoming acquired resistance to MAPK pathway inhibition.

One such combination is encorafenib, cetuximab and alpelisib. Alpelisib (BYL719) specifically inhibits the alpha subunit of PI3K. A phase 1b dose escalation study included 2 arms, encorafenib plus cetuximab vs. triplet therapy with encorafenib, cetuximab and alpelisib [69]. Triplet therapy was showed to be active with an ORR of 18% and impressively a disease control rate of 92.8%. This combination has been investigated further in a phase 2 trial [63]. 102 patients with refractory BRAF MT CRC were randomised to doublet or triplet therapy. Progression free survival was the primary endpoint. Interim data following 73 events were released and showed no statistical difference between doublet and triplet therapy with HR 0.69 (P = 0.064) and median PFS of 4.2 vs. 5.4 months. Grade 3 and 4 adverse events were higher in the triplet arm, including anaemia and hyperglycaemia. Further investigations with other PI3K inhibitors are currently underway; however, the efficacy of PI3K inhibition remains unclear (NCT01337765, NCT01363232).

Other potential targets include BRAF and AKT inhibition [70], BRAF, EGFR and HER2 inhibition [55], ERK inhibition alone or in combination with BRAF inhibition [71] and Wnt/β-catenin pathway inhibition (NCT02278133) **Table 2**.

ClinicalTrials.gov number	Therapeutic strategy	Agents investigated	Study design	Status
NCT02928224	BRAF + EGFR + MEK inhibition	Encorafenib + cetuximab ± binimetinib	Phase 3, randomised, open label	Recruiting
NCT02906059	Chemotherapy + selective Wee 1 inhibitor	Irinotecan + AZD1775	Phase 1b	Recruiting
NCT01351103	PORCN inhibitor + immunotherapy	LGK974 ± PDR001	Phase 1	Recruiting
NCT01640405	Antiangiogenesis agent + cytotoxic chemotherapy	FOLFOX + bevacizumab vs. FOLFOXIRI + bevacizumab	Phase 3 open label	Active, not recruiting
NCT01750918	BRAF + EGFR + MEK inhibition	Dabrafenib + panitumumab vs. dabrafenib + trametinib + panitumumab Dabrafenib + panitumumab vs. dabrafenib + trametinib + panitumumab vs. 5-fluorouracil-based chemotherapy + monoclonal antibody Trametinib + panitumumab	4 part phase 1/2, open label	Active, not recruiting
NCT01719380	BRAF + EGFR + PI3K inhibition	Encorafenib + cetuximab + alpelisib	Phase 1b/2, open label, dose escalation	Active, not recruiting

Updated 7th November 2018.

Table 2.
Ongoing studies investigating different treatment strategies for BRAF MT mCRC.

Immunotherapy also plays a role in the management of metastatic CRC [36]. Pembrolizumab and nivolumab are immune check point inhibitors against programmed death 1 (PD-1) that have demonstrated significant activity against MSI-high mCRC [72, 73]. Given the strong association between MSI-high and BRAF MT CRC, this represents a possible therapeutic option. The initial trial of pembrolizumab in MSI-high CRC did not include BRAF MT cases; however, a case report does suggest activity in the MSI-high BRAF MT population [74].

Nivolumab and combination nivolumab with ipilimumab (cytotoxic T-lymphocyte associated protein 4 inhibitor) in MSI-high/dMMR CRC was examined in the phase 2 CheckMate 142 study [73, 75]. 12 of the 74 patients receiving nivolumab harboured a BRAF mutation. An objective response was seen in 3 patients (25%) and 9 patients achieved disease control for greater than 12 weeks. The ORR for combination immunotherapy was greater at 55% in patients with MSI-high BRAF MT CRC, and disease control rate of 79%. Safety data was not reported by mutation status, however, appeared manageable, with 32% experiencing a grade 3 or 4 adverse event, most commonly raised AST. Discontinuation due to a treatment related adverse event was 13%.

7. Conclusion

BRAF V600E mutations are present in 7–10% of CRC. It represents a population with poor prognosis and a particular clinical phenotype, being more prevalent in

women, older than 70 years of age, associated with poorly differentiated histology and right-sided tumours. Chemotherapy with the addition of anti-angiogenesis agent remains the current standard of care in the first line metastatic setting. More aggressive, triplet chemotherapy (FOLFOXIRI) may be appropriate in the selected patient. BRAF inhibition has been extensively investigated for second line therapy and beyond and when in combination with EGFR, MEK and PI3K inhibitors have increased response rates, however, PFS and OS remains poor. Ongoing research remains important to improve outcomes in BRAF MT CRC.

Author details

Lee-Jen Luu[1,2] and Timothy J. Price[3,4]*

1 Adelaide Oncology and Haematology, North Adelaide, Australia

2 Royal Adelaide Hospital, Adelaide, Australia

3 The Queen Elizabeth Hospital, Woodville, Australia

4 University of Adelaide, Adelaide, Australia

*Address all correspondence to: timothy.price@sa.gov.au

References

[1] Kolch W. Meaningful relationships: The regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochemical Journal. 2000;**351**(Pt 2): 289-305

[2] Yang SH, Sharrocks AD, Whitmarsh AJ. MAP kinase signalling cascades and transcriptional regulation. Gene. 2013;**513**(1):1-13

[3] Burotto M, Chiou VL, Lee J-M, Kohn EC. The MAPK pathway across different malignancies: A new perspective. Cancer. 2014;**120**(22):3446-3456

[4] Sanz-Garcia E, Argiles G, Elez E, Tabernero J. BRAF mutant colorectal cancer: Prognosis, treatment, and new perspectives. Annals of Oncology: Official Journal of the European Society for Medical Oncology. 2017;**28**(11):2648-2657

[5] Michaloglou C, Vredeveld LC, Mooi WJ, Peeper DS. BRAF(E600) in benign and malignant human tumours. Oncogene. 2008;**27**(7):877-895

[6] Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature. 2002;**417**(6892):949-954

[7] Tie J, Gibbs P, Lipton L, Christie M, Jorissen RN, Burgess AW, et al. Optimizing targeted therapeutic development: Analysis of a colorectal cancer patient population with the BRAF(V600E) mutation. International Journal of Cancer. 2011;**128**(9):2075-2084

[8] BRAF Gene. COSMIC. 2018. Available from: https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=BRAF

[9] Hussain MRM, Baig M, Mohamoud HSA, Ulhaq Z, Hoessli DC, Khogeer GS, et al. BRAF gene: From human cancers to developmental syndromes. Saudi Journal of Biological Sciences. 2015;**22**(4):359-373

[10] Tol J, Nagtegaal ID, Punt CJA. BRAF mutation in metastatic colorectal cancer. The New England Journal of Medicine. 2009;**361**(1):98-99

[11] Clancy C, Burke JP, Kalady MF, Coffey JC. BRAF mutation is associated with distinct clinicopathological characteristics in colorectal cancer: A systematic review and meta-analysis. Colorectal Disease: The Official Journal of the Association of Coloproctology of Great Britain and Ireland. 2013;**15**(12):e711-e718

[12] Ogino S, Brahmandam M, Cantor M, Namgyal C, Kawasaki T, Kirkner G, et al. Distinct molecular features of colorectal carcinoma with signet ring cell component and colorectal carcinoma with mucinous component. Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc. 2006;**19**(1):59-68

[13] Yaeger R, Cercek A, Chou JF, Sylvester BE, Kemeny NE, Hechtman JF, et al. BRAF mutation predicts for poor outcomes after metastasectomy in patients with metastatic colorectal cancer. Cancer. 2014;**120**(15):2316-2324

[14] Gonsalves WI, Mahoney MR, Sargent DJ, Nelson GD, Alberts SR, Sinicrope FA, et al. Patient and tumor characteristics and BRAF and KRAS mutations in colon cancer, NCCTG/Alliance N0147. Journal of the National Cancer Institute. 2014;**106**(7):dju106

[15] Greene C, Atreya CE, McWhirter R, Ikram N, Van Loon K, Venook AP, et al. Differential radiographic appearance of BRAF V600E mutant metastatic colorectal cancer (mCRC) in patients matched by primary tumor

location. Journal of Clinical Oncology. 2016;**34**(4_suppl):554

[16] Jones JC, Kipp B, Leal AD, Voss JS, Hubbard JM, McWilliams RR, et al. Commonality and clinical, pathological, and prognostic characteristics of non-V600E BRAF mutations (BRAFMut) in metastatic colorectal cancers (mCRC) compared to V600 BRAFMut CRCs. Journal of Clinical Oncology. 2016;**34**(15_suppl):3529

[17] Shimada Y, Tajima Y, Nagahashi M, Ichikawa H, Oyanagi H, Okuda S, et al. Clinical significance of BRAF non-V600E mutations in colorectal cancer: A retrospective study of two institutions. Journal of Surgical Research. 2018;**232**:72-81

[18] Schirripa M, Biason P, Cortiula F, Pino MS, Urbano F, Antoniotti C, et al. Clinico-pathological and molecular characterisation of BRAF mutant metastatic colorectal cancer (mCRC): Are all mutations created equal? Journal of Clinical Oncology. 2018;**36**(15_suppl):3590

[19] Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic alterations during colorectal-tumor development. The New England Journal of Medicine. 1988;**319**(9):525-532

[20] Bhalla A, Zulfiqar M, Bluth MH. Molecular diagnostics in colorectal carcinoma: Advances and applications for 2018. Clinics in Laboratory Medicine. 2018;**38**(2):311-342

[21] Chan TL, Zhao W, Leung SY, Yuen ST. BRAF and KRAS mutations in colorectal hyperplastic polyps and serrated adenomas. Cancer Research. 2003;**63**(16):4878-4881

[22] Szylberg Ł, Janiczek M, Popiel A, Marszałek A. Serrated polyps and their alternative pathway to the colorectal cancer: A systematic review. Gastroenterology Research and Practice. 2015;**2015**:573814

[23] Bird AP. CpG-rich islands and the function of DNA methylation. Nature. 1986;**321**:209

[24] Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, et al. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Research. 1997;**57**(5):808-811

[25] Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**(12):6870-6875

[26] Tran B, Kopetz S, Tie J, Gibbs P, Jiang Z-Q, Lieu CH, et al. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Cancer. 2011;**117**(20):4623-4632

[27] Sinicrope FA, Mahoney MR, Smyrk TC, Thibodeau SN, Warren RS, Bertagnolli MM, et al. Prognostic impact of deficient DNA mismatch repair in patients with stage III colon cancer from a randomized trial of FOLFOX-based adjuvant chemotherapy. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology. 2013;**31**(29):3664-3672

[28] Thiel A, Heinonen M, Kantonen J, Gylling A, Lahtinen L, Korhonen M, et al. BRAF mutation in sporadic colorectal cancer and Lynch syndrome. Virchows Archiv: An International Journal of Pathology. 2013;**463**(5):613-621

[29] Venderbosch S, Nagtegaal ID, Maughan TS, Smith CG, Cheadle JP, Fisher D, et al. Mismatch repair status and BRAF mutation status in metastatic colorectal cancer patients: A pooled analysis of the CAIRO, CAIRO2, COIN, and FOCUS studies. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research. 2014;**20**(20):5322-5330

[30] Wang Y, Loree JM, Yu C, Tschautscher M, Briggler AM, Overman MJ, et al. Distinct impacts of KRAS, NRAS and BRAF mutations on survival of patients with metastatic colorectal cancer. Journal of Clinical Oncology. 2018;**36**(15_suppl):3513

[31] Roth AD, Tejpar S, Delorenzi M, Yan P, Fiocca R, Klingbiel D, et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: Results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology. 2010;**28**(3):466-474

[32] Manthravadi S, Sun W, Saeed A. Prognostic impact of BRAF V600E mutation in patients with non-metastatic colorectal cancer with microsatellite instability: A systematic review and meta-analysis. Journal of Clinical Oncology. 2018;**36**(15_suppl):3597

[33] Tosi F, Magni E, Amatu A, Mauri G, Bencardino K, Truini M, et al. Effect of KRAS and BRAF mutations on survival of metastatic colorectal cancer after liver resection: A systematic review and meta-analysis. Clinical Colorectal Cancer. 2017;**16**(3):e153-ee63

[34] Cremolini C, Di Bartolomeo M, Amatu A, Antoniotti C, Moretto R, Berenato R, et al. BRAF codons 594 and 596 mutations identify a new molecular subtype of metastatic colorectal cancer at favorable prognosis. Annals of Oncology. 2015;**26**(10):2092-2097

[35] Jones JC, Renfro LA, Al-Shamsi HO, Schrock AB, Rankin A, Zhang BY, et al. Non-V600BRAF mutations define a clinically distinct molecular subtype of metastatic colorectal cancer. Journal of Clinical Oncology. 2017;**35**(23):2624-2630

[36] Network NCC. Colon Cancer (Version 2.2018). 2018. Available from: https://www.nccn.org/professionals/physician_gls/pdf/colon.pdf

[37] Van Cutsem E, on behalf of the EGWG, Cervantes A, on behalf of the EGWG, Nordlinger B, on behalf of the EGWG, et al. Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of Oncology. 2014;**25**(suppl_3):iii1-iii9

[38] Morris VK, Overman MJ, Jiang Z-Q, Garrett C, Agarwal S, Eng C, et al. Progression-free survival remains poor over sequential lines of systemic therapy in patients with BRAF-mutated colorectal cancer. Clinical Colorectal Cancer. 2014;**13**(3):164-171

[39] Loupakis F, Cremolini C, Masi G, Lonardi S, Zagonel V, Salvatore L, et al. Initial therapy with FOLFOXIRI and bevacizumab for metastatic colorectal cancer. The New England Journal of Medicine. 2014;**371**(17):1609-1618

[40] Loupakis F, Cremolini C, Salvatore L, Masi G, Sensi E, Schirripa M, et al. FOLFOXIRI plus bevacizumab as first-line treatment in BRAF mutant metastatic colorectal cancer. European Journal of Cancer. 2014;**50**(1):57-63

[41] Cremolini C, Loupakis F, Antoniotti C, Lupi C, Sensi E, Lonardi S, et al. FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: Updated overall

survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study. The Lancet Oncology. 2015;**16**(13):1306-1315

[42] Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. The New England journal of medicine. 2013;**369**(11):1023-1034

[43] Maughan TS, Adams RA, Smith CG, Meade AM, Seymour MT, Wilson RH, et al. Addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for treatment of advanced colorectal cancer: Results of the randomised phase 3 MRC COIN trial. Lancet. 2011;**377**(9783):2103-2114

[44] Karapetis CS, Jonker D, Daneshmand M, Hanson JE, O'Callaghan CJ, Marginean C, et al. PIK3CA, BRAF, and PTEN status and benefit from cetuximab in the treatment of advanced colorectal cancer—Results from NCIC CTG/AGITG CO.17. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research. 2014;**20**(3):744-753

[45] Therkildsen C, Bergmann TK, Henrichsen-Schnack T, Ladelund S, Nilbert M. The predictive value of KRAS, NRAS, BRAF, PIK3CA and PTEN for anti-EGFR treatment in metastatic colorectal cancer: A systematic review and meta-analysis. Acta Oncologica. 2014;**53**(7):852-864

[46] Pietrantonio F, Petrelli F, Coinu A, Di Bartolomeo M, Borgonovo K, Maggi C, et al. Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: A meta-analysis. European Journal of Cancer. 2015;**51**(5):587-594

[47] Rowland A, Dias MM, Wiese MD, Kichenadasse G, McKinnon RA, Karapetis CS, et al. Meta-analysis of BRAF mutation as a predictive biomarker of benefit from anti-EGFR monoclonal antibody therapy for RAS wild-type metastatic colorectal cancer. British Journal of Cancer. 2015;**112**(12):1888-1894

[48] Tveit KM, Guren T, Glimelius B, Pfeiffer P, Sorbye H, Pyrhonen S, et al. Phase III trial of cetuximab with continuous or intermittent fluorouracil, leucovorin, and oxaliplatin (Nordic FLOX) versus FLOX alone in first-line treatment of metastatic colorectal cancer: The NORDIC-VII study. Journal of Clinical Oncology. 2012;**30**(15):1755-1762

[49] Stintzing S, Modest DP, Rossius L, Lerch MM, von Weikersthal LF, Decker T, et al. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab for metastatic colorectal cancer (FIRE-3): A post-hoc analysis of tumour dynamics in the final RAS wild-type subgroup of this randomised open-label phase 3 trial. The Lancet Oncology. 2016;**17**(10):1426-1434

[50] Geissler M, Riera-Knorrenschild J, Tannapfel A, Greeve J, Florschütz A, Wessendorf S, et al. mFOLFOXIRI + panitumumab versus FOLFOXIRI as first-line treatment in patients with RAS wild-type metastatic colorectal cancer m(CRC): A randomized phase II VOLFI trial of the AIO (AIO-KRK0109). Journal of Clinical Oncology. 2018;**36**(15_suppl):3509

[51] Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. The New England Journal of Medicine. 2011;**364**(26):2507-2516

[52] Kopetz S, Desai J, Chan E, Hecht JR, O'Dwyer PJ, Maru D, et al. Phase II pilot study of vemurafenib in patients with metastatic BRAF-mutated colorectal cancer. Journal of Clinical Oncology. 2015;**33**(34):4032-4038

[53] Hyman DM, Puzanov I, Subbiah V, Faris JE, Chau I, Blay J-Y, et al. Vemurafenib in multiple nonmelanoma cancers with BRAF V600 mutations. The New England Journal of Medicine. 2015;**373**(8):726-736

[54] Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature. 2012;**483**:100

[55] Herr R, Halbach S, Heizmann M, Busch H, Boerries M, Brummer T. BRAF inhibition upregulates a variety of receptor tyrosine kinases and their downstream effector Gab2 in colorectal cancer cell lines. Oncogene. 2018;**37**(12):1576-1593

[56] Mao M, Tian F, Mariadason JM, Tsao CC, Lemos R Jr, Dayyani F, et al. Resistance to BRAF inhibition in BRAF-mutant colon cancer can be overcome with PI3K inhibition or demethylating agents. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research. 2013;**19**(3):657-667

[57] Yuan TL, Cantley LC. PI3K pathway alterations in cancer: Variations on a theme. Oncogene. 2008;**27**(41):5497-5510

[58] Parsons DW, Wang TL, Samuels Y, Bardelli A, Cummins JM, DeLong L, et al. Colorectal cancer: Mutations in a signalling pathway. Nature. 2005;**436**(7052):792

[59] Ahmed D, Eide PW, Eilertsen IA, Danielsen SA, Eknaes M, Hektoen M, et al. Epigenetic and genetic features of 24 colon cancer cell lines. Oncogene. 2013;**2**:e71

[60] The Cancer Genome Atlas N. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;**487**:330

[61] Yaeger R, Cercek A, O'Reilly EM, Reidy DL, Kemeny N, Wolinsky T, et al. Pilot trial of combined BRAF and EGFR inhibition in BRAF-mutant metastatic colorectal cancer patients. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research. 2015;**21**(6):1313-1320

[62] Desai J, Markman B, Ananda S, Tebbutt NC, Michael M, Solomon BJ, et al. A phase I/II trial of combined BRAF and EGFR inhibition in patients (pts) with BRAF V600E mutated (BRAFm) metastatic colorectal (mCRC): The EViCT (erlotinib and vemurafenib in combination trial) study. Journal of Clinical Oncology. 2017;**35**(15_suppl):3557

[63] Tabernero J, Geel RV, Guren TK, Yaeger RD, Spreafico A, Faris JE, et al. Phase 2 results: Encorafenib (ENCO) and cetuximab (CETUX) with or without alpelisib (ALP) in patients with advanced BRAF-mutant colorectal cancer (BRAFm CRC). Journal of Clinical Oncology. 2016;**34**(15_suppl):3544

[64] Corcoran RB, André T, Yoshino T, Bendell JC, Atreya CE, Schellens JHM, et al. Efficacy and circulating tumor DNA (ctDNA) analysis of the BRAF inhibitor dabrafenib (D), MEK inhibitor trametinib (T), and anti-EGFR antibody panitumumab (P) in patients (pts) with BRAF V600E-mutated (BRAFm) metastatic colorectal cancer (mCRC). Annals of Oncology. 2016;**27**(suppl_6):4550

[65] Kopetz S, McDonough SL, Morris VK, Lenz H-J, Magliocco AM, Atreya CE, et al. Randomized trial of irinotecan and cetuximab with or without vemurafenib in BRAF-mutant metastatic colorectal cancer (SWOG 1406). Journal of Clinical Oncology. 2017;**35**(4_suppl):520

[66] Corcoran RB, Atreya CE, Falchook GS, Kwak EL, Ryan DP, Bendell JC, et al.

Combined BRAF and MEK inhibition with dabrafenib and trametinib in BRAF V600-mutant colorectal cancer. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology. 2015;**33**(34):4023-4031

[67] Corcoran RB, André T, Atreya CE, Schellens JHM, Yoshino T, Bendell JC, et al. Combined BRAF, EGFR, and MEK inhibition in patients with BRAF^{V600E}-mutant colorectal cancer. Cancer Discovery. 2018;**8**(4):428-443

[68] Mondaca S, Lacouture M, Hersch J, Yaeger R. Balancing RAF, MEK, and EGFR inhibitor doses to achieve clinical responses and modulate toxicity in BRAF V600E colorectal cancer. JCO Precision Oncology. 2018;**2**:1-4

[69] van Geel R, Tabernero J, Elez E, Bendell JC, Spreafico A, Schuler M, et al. A phase Ib dose-escalation study of encorafenib and cetuximab with or without alpelisib in metastatic BRAF-mutant colorectal cancer. Cancer Discovery. 2017;**7**(6):610-619

[70] Yang H, Higgins B, Kolinsky K, Packman K, Bradley WD, Lee RJ, et al. Antitumor activity of BRAF inhibitor vemurafenib in preclinical models of BRAF-mutant colorectal cancer. Cancer Research. 2012;**72**(3):779-789

[71] Ahronian LG, Sennott EM, Van Allen EM, Wagle N, Kwak EL, Faris JE, et al. Clinical acquired resistance to RAF inhibitor combinations in BRAF-mutant colorectal cancer through MAPK pathway alterations. Cancer Discovery. 2015;**5**(4):358-367

[72] Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 blockade in tumors with mismatch-repair deficiency. The New England Journal of Medicine. 2015;**372**(26):2509-2520

[73] Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): An open-label, multicentre, phase 2 study. The Lancet Oncology. 2017;**18**(9):1182-1191

[74] Sehdev A, Cramer HM, Ibrahim AA, Younger AE, O'Neil BH. Pathological complete response with anti-PD-1 therapy in a patient with microsatellite instable high, BRAF mutant metastatic colon cancer: A case report and review of literature. Discovery Medicine. 2016;**21**(117):341-347

[75] Overman MJ, Lonardi S, Wong KYM, Lenz H-J, Gelsomino F, Aglietta M, et al. Durable clinical benefit with Nivolumab plus ipilimumab in DNA mismatch repair-deficient/microsatellite instability-high metastatic colorectal cancer. Journal of Clinical Oncology. 2018;**36**(8):773-779

[76] Richman SD, Seymour MT, Chambers P, Elliott F, Daly CL, Meade AM, et al. KRAS and BRAF mutations in advanced colorectal cancer are associated with poor prognosis but do not preclude benefit from oxaliplatin or irinotecan: Results from the MRC FOCUS trial. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology. 2009;**27**(35):5931-5937

[77] Bokemeyer C, Bondarenko I, Hartmann JT, de Braud F, Schuch G, Zubel A, et al. Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: The OPUS study. Annals of Oncology: Official Journal of the European Society for Medical Oncology. 2011;**22**(7):1535-1546

[78] Van Cutsem E, Köhne C-H, Láng I, Folprecht G, Nowacki MP, Cascinu S, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as

first-line treatment for metastatic colorectal cancer: Updated analysis of overall survival according to tumor KRAS and BRAF mutation status. Journal of Clinical Oncology. 2011;**29**(15):2011-2019

[79] Price TJ, Hardingham JE, Lee CK, Weickhardt A, Townsend AR, Wrin JW, et al. Impact of KRAS and BRAF gene mutation status on outcomes from the phase III AGITG MAX trial of capecitabine alone or in combination with bevacizumab and mitomycin in advanced colorectal cancer. Journal of Clinical Oncology. 2011;**29**(19):2675-2682

[80] Raponi M, Winkler H, Dracopoli NC. KRAS mutations predict response to EGFR inhibitors. Current Opinion in Pharmacology. 2008;**8**(4):413-418

Section 2

Biomarkers

Chapter 4

Current Utility and Future Applications of ctDNA in Colorectal Cancer

Daphne Day, Sophia Frentzas, Cameron A. Naidu, Eva Segelov and Maja Green

Abstract

Circulating tumour DNA (ctDNA) shows promise as a minimally invasive biomarker with a myriad of emerging applications including early detection and diagnosis, monitoring of disease and treatment efficacy, and identification of actionable alterations to guide treatment. The potential utility of ctDNA in colorectal carcinoma (CRC) is of particular interest given the limitations of current radiographic imaging and blood-based tumour markers in detecting disease and evaluating therapeutic benefit. While ctDNA has yet to demonstrate clinical utility in CRC, a growing body of research highlights the potential of these novel biomarkers. This chapter provides an overview of the current evidence for employing ctDNA in CRC as well as previewing the future directions that these exciting technologies may take.

Keywords: colorectal carcinoma/cancer, circulating tumour DNA, biomarker

1. Introduction

Ongoing research in oncology aims to generate patient-directed treatment options, targeting each individual's specific cancer molecular profile with therapies most likely to initiate and maintain an effective anti-tumour response [1]. Currently, molecular profiling in colorectal cancer (CRC) relies on direct biopsy of tumour tissue. However, tissue biopsy presents a number of procedural and biological challenges. Firstly, it is an inherently invasive procedure, making recurrent sampling difficult. Secondly, results may be affected by bias owing to tumoural heterogeneity. Tumours are affected by factors such as genomic instability, the surrounding tissue microenvironment and therapeutic effects [2]. These influences create dynamic molecular selection and evolution, resulting in spacial and temporal heterogeneity, which cannot be represented by a single site tissue biopsy, particularly in the case of metastatic disease [3].

Recognition of these limitations has prompted an interest in non-invasive circulating tumour-specific biomarkers. The concept of 'liquid biopsy' originally described the detection and analysis of circulating tumour cells (CTC) in blood, with reference to tissue biopsy. More recently, it has been broadly adapted to describe any tumour-related constituents circulating in body fluids such as CTC, DNA, RNA and exomes [4]. Compared with tissue biopsies, liquid biopsies may

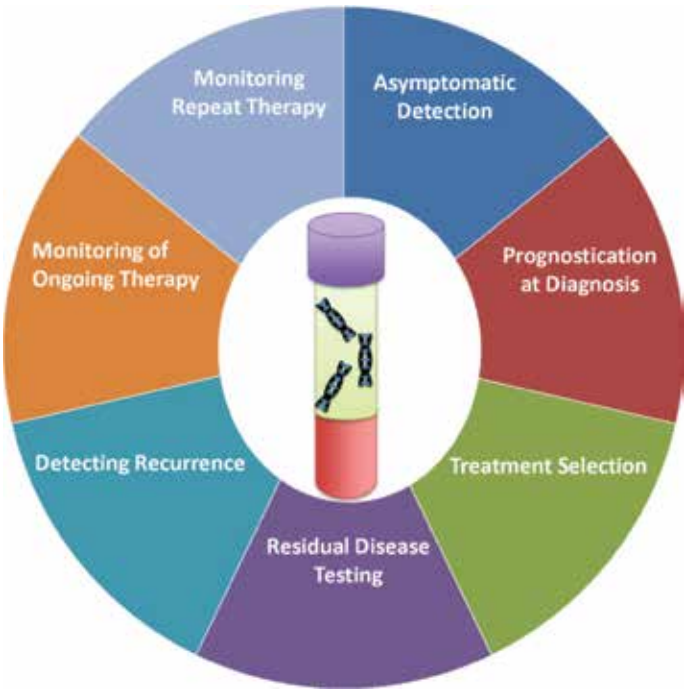

Figure 1.
Emerging ctDNA applications in various CRC management settings.

be better suited for serial surveillance, by reducing procedural time and potential harm. Blood sampling may also provide a more accurate representation of global tumoural heterogeneity, not limited to the site-specific characteristics detected through tissue biopsy [5]. The focus of this review will be directed towards circulating tumour DNA (ctDNA) found in blood samples, which at present, of all the liquid biopsy approaches, has had the greatest clinical impact. CtDNA is thought to be released by tumour cells, containing tumour-specific genetic and epigenetic alterations [6]; and has been found to correlate with tumour stage, burden of disease and response to therapy in CRC [7].

Herein, we provide an overview of ctDNA technologies in use and highlight the emerging clinical applications of ctDNA in various CRC management settings (**Figure 1**). The future directions of this rapidly advancing field will also be explored.

2. ctDNA methodological approaches and technical considerations

Circulating cell-free DNA (cfDNA) was first detected in 1948 by Mendel and Metais in the peripheral blood plasma of healthy and diseased individuals [8]. CfDNA levels can vary between 1 and 10 ng mL^{-1} in plasma and can be affected by physiological conditions such as exercise and acute inflammation [9]. In 1977, Leon et al. found that cfDNA was more elevated in cancer patients compared with healthy subjects, with higher levels correlating with higher burden of disease [10]. In 1989, Stroun et al. discovered that at least part of the plasma DNA in cancer patients originated specifically from cancer cells [11]. In the ensuing decades, knowledge and applications of tumour-derived cfDNA has rapidly evolved due to advances in molecular techniques, and also gave rise to the term, circulating tumour DNA (ctDNA).

A variety of tumour-specific molecular alterations may be identified by ctDNA including mutations, methylation variants, microsatellite alterations, copy number variations and structural changes [12]. Although the exact mechanisms are yet to be elucidated, ctDNA is thought to be released into the blood stream via biological processes such as apoptosis, necrosis, inefficient phagocytosis and active secretion [13, 14]. CtDNA has a short half-life of up to a few hours and accounts for generally only a small fraction of cfDNA, although concentration can vary widely from <0.01 to 90% [12]. The biological and tumoural determinants underlying ctDNA variations both between and within individuals are incompletely understood, but are likely affected by tumour burden, treatment response, circulatory elements, circadian rhythm, cellular turnover and clearance mechanisms [12, 15]. Somatic variants may also be found in healthy individuals, mostly commonly associated with clonal haematopoiesis [5]. Such variability, coupled with the often-low allele frequency of the molecular aberration of interest, demand sensitive and robust detection methods. As we interpret the results of ctDNA studies and consider their clinical relevance, it is prudent to reflect on these biological variables.

2.1 Pre-analytic considerations

Numerous inherent challenges have affected the development of ctDNA pre-analytic and analytic methods. These include variable fragmentation, low abundance in plasma or serum volumes, tumour heterogeneity, and low stability as a result of the aforementioned biological factors [16].

To minimise sample degradation and optimise stability, a number of pre-analytical steps need to be carefully planned. Although there are currently no standardised methodology guidelines on ctDNA collection, storage and processing, the typical workflow is illustrated in **Figure 2**.

2.2 Detection methods

A variety of methods for detecting and characterising ctDNA have been reported. These can be broadly categorised as targeted and non-targeted approaches. Differing performance characteristics, strengths and disadvantages may also facilitate complementary roles of these approaches in molecular analysis. **Table 1** lists examples of described methods. Applying any of these approaches in

Figure 2.
Pre-analytical components in ctDNA analysis. (1) Collection of blood samples (usually 5–10 mL) via phlebotomy. Currently, there is no guidance on the comparative impact of the source of blood draw (for example, peripheral venepuncture or intravascular ports) on ctDNA analysis [5]; (2) samples should be collected in tubes containing anticoagulants compatible with polymerase chain reaction methods, such as ethylenediaminetetraacetic acid (EDTA) [9]; (3) centrifugation of blood to separate cells should be performed promptly. The exact optimal time to centrifugation is not known and may depend on storage conditions and the presence of stabilising agents [16]. Current evidence suggests that plasma is preferred to serum samples, as in the latter case, cfDNA released during white blood cell lysis may lead to a dilutional effect [9]. (4) Processed plasma is then generally stored frozen, often in aliquots; (5) CfDNA is extracted using commercially available kits. There are multiple DNA purification strategies and modifications, which may variably impact on DNA yield and purity [5].

routine clinical practice in a credentialed laboratory would require considerable scaling up, standardisation, and optimisation of methodological efficiency and accuracy, while minimising cost [5].

Earlier approaches such as Sanger sequencing and conventional polymerase chain reaction (PCR)-based methods have limited sensitivity for ctDNA detection, particularly for rare alterations [6]. A number of digital PCR-based approaches were subsequently developed, capable of improved limits of detection (up to 0.001%), low frequency allele detection and nucleic acid quantitation. Commonly used digital PCR methods for ctDNA detection include BEAMing (beads, emulsion, amplification and magnetics) and droplet digital PCR (ddPCR). BEAMing which combines beads in emulsion and flow cytometry, was first described in 2003 [17] and facilitated serial tracking of mutant allele fractions in patients with CRC [9, 18]. This method and its variations are now widely applied in ctDNA analysis. DdPCR involves the massive partitioning of nucleic acids into thousands of droplets to enable highly sensitive and precise detection and quantitation of small concentrations of DNA [19, 20].

Next generation sequencing (NGS) or massively parallel sequencing are broad terms describing a range of high throughput methods capable of the simultaneous analysis of thousands to millions of DNA molecules, and also encompasses both targeted and non-targeted approaches. Targeted sequencing platforms such as safe sequencing system (Safe-SeqS) [21] and tagged-amplicon deep sequencing (TAm-Seq) [22] have the advantage of improved multiplex capability and evaluating a larger number of loci simultaneously in the genomic areas of interest [15]. However, targeted PCR-based or NGS methodologies mostly rely on prior knowledge of molecular changes and cannot identify variants located in areas that are not analysed.

In contrast, non-targeted genome or exome-wide sequencing allows discovery of *de novo* alterations as well as detection of structural changes including rearrangements, gene fusions and copy number alterations [23]. These may be advantageous in patients who do not have accessible tumour tissue for biopsy. Several techniques have been described and used in the ctDNA setting. The personalised analysis of rearranged ends (PARE) method uses paired-end sequencing, and was utilised in a proof-of-principle study to identify unselected genome-wide chromosomal alterations characteristic of tumour DNA in cfDNA in patients with CRC and

Technique types	Technique	Application
PCR-based [26–28]	ARMS-PCR Mutant allele-specific PCR Bi-PAP	Known point mutations
Digital PCR [18–20, 29]	BEAMing DdPCR	Known point mutations
Targeted sequencing [21, 22, 30]	Safe-SeqS TAm-Seq CAPP-Seq	Point mutation Structural changes in specific gene regions
WGS and WES [24, 31, 32]	PARE Digital karyotyping	Copy number variations Structural rearrangements

PCR, polymerase-chain reaction; ARMS, amplification-refraction mutation system; Bi-PAP, bidirectional pyrophosphorolysis-activated polymerisation; BEAMing, beads, emulsion, amplification and magnetics; ddPCR, droplet digital PCR; Safe-Seq, safe-sequencing system; TAm-Seq, tagged-amplicon deep sequencing; WGS, whole-genome sequencing; WES, whole-exome sequencing; PARE, personalised analysis of rearranged ends.

Table 1.
Methods of ctDNA detection.

breast cancer, including copy number changes and rearrangements [24]. Another group demonstrated the feasibility and utility of exome-wide sequencing in ctDNA to identify mutations associated with acquired therapeutic resistance in a small cohort of patients with advanced cancer [25]. Current limitations of non-targeted approaches include lower sensitivity and relatively prohibitive costs impeding routine clinical implementation [24]. Furthermore, due to the relatively large amount of resulting sequencing data, substantial bioinformatic expertise and filters are required to decipher somatic tumoural alterations from the structural variants commonly seen in germline DNA to avoid false positives [15].

3. CtDNA in screening

Five-year survival for CRC patients is highly dependent on the timing of disease detection and tumour stage. CRC screening can achieve early disease detection and treatment, including that of pre-malignant dysplastic lesions, and has been shown to improve CRC-related mortality. However, 60–70% of patients are diagnosed at mid- to late stage CRC despite recent advances in screening methods [33]. Screening approaches used to test asymptomatic people for a presence of unsuspected disease, which have proven efficacy in CRC include endoscopic visualisation and faecal occult blood tests (FOBT). The former is invasive and expensive with associated morbidity, thus affecting patient compliance and acceptance. FOBT measure the presence of haemoglobin in faeces and can be categorised into guaiac-based (gFOBT) and the newer haemoglobin-antibody-based faecal immunochemical test (FIT). FIT is used more commonly in current practice and has largely superseded gFOBT, due to its superior sensitivity for colorectal bleeding, improved analytical characteristics and it is also less likely to be affected by dietary and medication factors [34–37]. In addition, FIT has better acceptance and participation which improves population participation [38]. In a meta-analysis of 19 studies, FIT was found to have high accuracy and specificity, and moderately high sensitivity, although substantial heterogeneity was noted across studies [39].

This prompted the development of faecal-based tests targeting genetic and epigenetic alterations. Cologuard is the first commercially-approved faecal test which combines several technologies including molecular assays for aberrant *NDRG4* and *BMP3* methylation, *β-actin* (a reference gene for human DNA quantity), and *KRAS* mutations; and a haemoglobin immunoassay [40]. The haemoglobin component of the Cologuard test contributes to 80% of the cancer detection in the algorithm. A large randomised clinical trial comparing Cologuard and FIT screening showed that the sensitivity of Cologuard was superior to that of FIT in the detection of CRC and precancerous lesions [40]. However, the higher cost of Cologuard and its lower specificity compared with FIT has limited its adaptation as a screening tool [33].

CtDNA analysis may offer a more convenient screening approach compared with faecal-based tests. The malignant transformation pathway, from adenoma to carcinoma, is driven by mutations such as *APC*, *KRAS* and *TP53* [41]. However, somatic mutational profiles are highly variable between patients. For example, *KRAS* and *BRAF V600E* are seen in approximately 40 and 7% of patients with CRC respectively [42]. To date, the vast majority of cancer patients evaluated with mutation-based blood plasma assays have advanced-stage disease. A challenge in early stage disease is the often-minute fraction of ctDNA present in the total circulating DNA—may be as low as <0.01%—which may be below the limit of detection assays [18]. A study which enrolled 170 patients with positive FOBT investigated differences in *KRAS* mutation levels in plasma and tissue samples [43]. The rate of *KRAS* mutations in plasma (3%) was found to be lower compared with

that observed in matched adenocarcinoma and high-grade intra-epithelial neoplasia tissues (45%) [43]. Although this is a small study, the results suggest that either this particular assay is not sufficiently sensitive, or that ctDNA was found at low or undetectable levels in the population tested.

The detection of epigenetic alterations characterised by aberrant DNA methylations is an alternative approach to mutational ctDNA analysis. Aberrant DNA methylation leads to transcription silencing of tumour suppressor genes, occurs early in CRC carcinogenesis, and may be more commonly seen and consistent in cancer patients compared with somatic mutations. Indeed, DNA methylation profiles in plasma have been used in biomarker development to identify emergence of early CRC by several groups [44]. One of the methylated promotors that has gained a lot of interest is the *Septin 9* gene promoter. Methylation in the *Septin 9* promoter demonstrated high sensitivity in preclinical studies and a small clinical cohort [45, 46], however a large prospective screening study demonstrated a sensitivity profile of only 48% [47]. In addition, the sensitivity to detect advanced adenomas was low (11%) [47]. Despite this, in 2016, the FDA approved the use of the Epi proColon, a plasma-derived *Septin 9* methylation assay, for the screening of CRC. This decision occurred in the setting of encouraging results from a meta-analysis comparing the pooled sensitivity of methylated *Septin 9* with FOBT as a screening tool [48], and the improved sensitivity and specificity results of a modified version of the Epi proColon assay (2.0 version) [49, 50]. Recently, promising results have been reported in the utilisation of two methylation markers in the screening context—*branched-chain amino acid transaminase 1* (*BCAT1*) and *ikaros family zinc finger protein 1* (*IKZF1*)—where methylation of either gene identified close to 70% of CRC with specificity of 92% [51, 52].

To date, ctDNA is yet to demonstrate clinical utility in the CRC screening setting. Challenges lie in minimising false positive readings, whilst developing a test sensitive enough to detect small amounts of ctDNA. For example, normal physiological ageing is associated with the development of somatic mutations in the absence of malignant disease, and false positive readings may also be seen in patients with chronic inflammatory disease. False positive results can lead to unnecessary follow-up procedures and anxiety. Studies examining a large number of healthy control individuals will be essential to evaluate the specificity of potential screening assays. Yet another challenge with ctDNA-based screening is the identification of the underlying tissue of origin. Because the same gene mutations drive multiple tumour types, ctDNA tests based on genomic analysis alone generally cannot identify the anatomical location of the primary tumour.

4. CtDNA in detection of residual disease in early stage CRC

Although surgical resection can cure a high percentage of patients with CRC, tumour recurrence occurs in approximately 30–50% of all patients who undergo curative resection. The majority of these recurrences take place during the first 2 years after surgery and 90% recur within 5 years [53, 54]. Recommendation for adjuvant cytotoxic therapy is based on clinicopathological risk, although it may not be necessary in many patients and toxicity is substantial. Thus, biomarkers that would aid in identifying patients at high risk of recurrence and who would benefit from adjuvant therapies is of utmost importance. Carcinoembryonic antigen (CEA), a blood protein-based tumour marker, is currently used for monitoring CRC treatment and can also be detected at elevated levels in pancreatic, gastric, lung and breast cancers, as well as a number of benign conditions. Whilst CEA is upregulated in the majority of advanced

CRC, the sensitivity for recurrence detection has been shown to be unacceptably low, approximately 30% [55, 56], supporting the need for alternative markers.

It is well known that in CRC, there is high genomic concordance between the primary tumour and its metastases [57]. Therefore, a promising strategy to detect minimal residual disease or even relapsed disease, could be to use ctDNA to track and quantify key genomic aberrations (*APC*, *KRAS*, *BRAF* and *TP53*), which are recognised as playing a role in early CRC, and may persist in metastatic disease [18, 58]. Several studies have shown that peri-operative ctDNA detection is associated with higher rate of recurrence and in some cases, poorer overall survival; albeit with varying detection methodology, sensitivity and specificity [59–63]. Additionally, the aforementioned methylation markers *BCAT1 and IKZF1* have also shown promise in a study of patients with resected stage I–IV CRC, where post-operative positivity for *BCAT1/IKZF1* methylation was more sensitive (68%) for recurrence detection than CEA (32%, $p < 0.05$) and its odds of recurrence given a positive test (14.4, 95% CI: 5–39) was twice that of CEA (6.9, 95% CI: 2–22) [64]. However, they fail to detect advanced adenomas despite their frequent presence in cancer and adenoma tissue [65]. It would be reasonable to speculate that the release of any DNA from neoplasia seems to be a function of progression along the oncogenesis pathway and it is not a simple reflection of whether or not the change is present in tissue.

These preliminary studies support the need for large prospective trials evaluating pre- and post-operative ctDNA-based biomarkers to help predict recurrence and evaluate prognosis. However, it is not yet known whether ctDNA represents the molecular diversity of disease or whether only selective clones (for example, highly apoptotic clones) are secreted into the bloodstream. Furthermore, it is yet to be established whether early detection of recurrence can indeed improve survival outcomes, if treatment is followed soon after.

5. CtDNA in metastatic CRC (mCRC)

For the majority of patients with mCRC, the mainstay of treatment consists of palliative-intent systemic therapy with median overall survival (OS) approaching 24 months. Three classes of cytotoxic agents (fluoropyrimidine, irinotecan and oxaliplatin) and two classes of molecularly-targeted agents (monoclonal antibodies targeting vascular endothelial growth factor [VEGF], and the epidermal growth factor receptor [EGFR]) are currently approved for use in mCRC, although the optimal sequencing and scheduling of these treatments are debated. To optimise their therapeutic ratio and minimise toxicity, effective and accurate means of assessing treatment response are needed. In the following section, we summarise the evidence on the use of ctDNA in mCRC management. These include prognostication, monitoring tumour burden and predicting treatment efficacy, guiding targeted treatment selection, and detecting anti-EGFR therapy resistance.

5.1 CtDNA in mCRC: prognostic value and monitoring tumour burden

The association between the presence or high levels of ctDNA and adverse survival outcomes in mCRC has been demonstrated in several studies [66–69]. For example, in a landmark study, Bettegowda et al. observed a steady decrease in 2-year survival rate as ctDNA concentration increased [69]. Moreover, a systematic review exploring the prognostic role of ctDNA in CRC (mostly mCRC) found that most studies, although not all, demonstrated a negative correlation between ctDNA and disease-free survival and OS [70].

Another application for ctDNA that has been explored in mCRC is correlating longitudinal dynamics during systemic therapy with prediction of treatment response and tumour burden [71]. Currently, anatomical radiographic imaging—particularly computed tomography (CT)—is the chief modality to evaluate therapeutic benefit in mCRC. However, limitations include cost, operator- and reader-dependence, challenges in standardisation and radiographic lag behind clinical changes. Furthermore, changes in tumour size which form the basis of response measurement on CT does not account for changes in tumour density or morphology that may result from response to molecularly targeted agents commonly used in mCRC. CEA is also used in mCRC disease monitoring, usually in-between or in addition to radiology assessments. However, CEA is elevated in only approximately 70–80% of patients with mCRC and has limited sensitivity and specificity in detecting disease progression or treatment response [72].

A study of 53 mCRC patients undergoing standard first-line chemotherapy, found that significant decline in ctDNA levels using Safe-SeqS prior to cycle two chemotherapy was associated with objective radiological response at 8–10 weeks ($p = 0.016$) [73]. This study also found a trend between ctDNA reduction and improved progression-free survival. The more recent PLACOL study in 82 patients receiving chemotherapy for mCRC echoed these findings [7]. PLACOL utilised picodroplet-digital PCR assays based on either genomic or hypermethylation alterations. The investigators found that the baseline ctDNA concentration was prognostic for OS, and that early and deep ctDNA reductions were associated with improved objective response rate and longer survival ($p < 0.001$) [7]. Another recent study using digital PCR found methylation changes over time correlated with tumoural response in patients with mCRC [74].

These studies suggest that early changes in ctDNA during systemic therapy may be predictive for treatment efficacy and prognostic for survival outcomes, thus suggesting a role for serial ctDNA monitoring during palliative treatment with systemic therapy. Indeed, with the advantages of a short half-life reflecting immediate-term changes [18] and high tumour-related specificity, ctDNA monitoring may be complementary to radiological assessments and blood biomarkers currently in use. In clinical circumstances where radiological assessments are indeterminate or ambiguous, such as the lack of measurable disease by imaging criteria or the presence of mixed response, ctDNA dynamics may be of particular value; although ctDNA may not always correspond to imaging findings [75]. Equally, it is also prudent to acknowledge that no current evidence supports the strategy of biomarker-monitoring of palliative therapy and that earlier adaptive treatments will augment survival or quality of life.

5.2 CtDNA in mCRC: genotyping to guide targeted treatment selection

It is now standard of care for patients with mCRC to undergo molecular profiling on their tumour tissue in order to determine *BRAF V600E* and *Rat sarcoma* (*RAS*, particularly exon 2–4 *KRAS*) gene mutational status. This informs clinical decision-making regarding benefit from anti-EGFR therapy. The advantageous role of genotyping with ctDNA has already been established in the field of advanced non-small cell lung cancer. Circulating genetic aberrations of EGFR (for example, exon 19 deletions or exon 21 (L858R) substitution mutations) are now being used in standard practice, as a companion tool, to identify eligible patients for treatment with erlotinib. This technology was approved by the US Food and Drug Administration in 2016 for this indication [76].

In mCRC, a meta-analysis of 21 studies on the diagnostic performance of ctDNA-based *KRAS* gene detection found overall high pooled specificity (96%)

and moderate sensitivity (67%) [77]. Not surprisingly, heterogeneity was noted to be high probably owing to varying molecular techniques, tumour stage and study designs [77]. Although a number of *KRAS* ctDNA assays have demonstrated high agreement (91–93%) with tumour tissue *KRAS* testing and are available for commercial use [78, 79]; at present, tumour tissue testing remains the gold standard to establish *KRAS* mutational status. Given the appreciable discordance rate with tumour tissue genotyping, it is recommended that a negative ctDNA result should trigger tumour tissue variant analysis [5]. As will be discussed under Section 6, ctDNA assays in mCRC may also been utilised to select predictive immune-related biomarkers for immunotherapy selection.

5.3 CtDNA in mCRC: genotyping to monitor for targeted treatment response and resistance

The role of genomic alterations and their evolution in both the development and progression of CRC have culminated in the realisation that serial genotyping of the primary tumour, and its secondaries, is ideally required if we want to succeed in personalising patient care with precision [80]. Unfortunately, patients with mCRC who do not harbour a somatic *RAS* mutation pre-treatment, will typically develop acquired resistance to anti-EGFR therapy in a matter of months after initially showing response. There is a battery of pre-clinical and clinical evidence which points to the acquisition of molecular mechanisms of resistance associated with aberrations in the *RAS-MEK-mitogen-activated protein kinases* (*MAPK*), pathway [2, 42, 81, 82].

Longitudinal ctDNA analysis can be used in this setting with high diagnostic precision to detect both primary resistance and early molecular changes that may confer acquired resistance. Several translational studies have successfully employed ctDNA to illustrate and verify the emergence of *RAS* mutations as a mechanism of acquired resistance to anti-EGFR therapy.

In a phase II trial of anti-EGFR antibody, panitumumab in mCRC, serial prospective plasma analysis detected more emergent *RAS* mutations than serial tissue biopsies, suggesting that the former may be more comprehensive in evaluating global tumoural heterogeneity [83]. In a small retrospective study of 10 mCRC patients who developed resistance to anti-EGFR therapy (cetuximab or panitumumab) in combination with chemotherapy, Misale et al. demonstrated that the onset of the emerging *KRAS* mutations was detected in serum ctDNA analysis as early as 10 months prior to radiological reporting of disease progression [2]. In this study, ctDNA analysis was also explored in a separate cohort of patients who were receiving chemotherapy alone (control group). No acquired *KRAS* mutations were identifiable at disease progression [2]. In the same year, Diaz et al. also demonstrated the feasibility of using serum ctDNA to identify emerging resistance to panitumumab in a prospective cohort of 28 patients [81]. Thirty-eight percent of patients whose tumours were initially *KRAS* wild type developed detectable *KRAS* mutations in their sera, three of whom developed multiple different *KRAS* mutations. These were detected approximately 5 months before radiological progression [81]. Another study subsequently made the intriguing observation that *KRAS* clones can fluctuate under the selective pressure of anti-EGFR therapy implying that there may be a role for 'pulsing' or re-challenging with anti-EGFR therapy [84].

Furthermore, in a prospective study of 108 patients, treated in the third line setting with cetuximab and irinotecan, Spindler et al. investigated the quantitative correlation between plasma cfDNA with tumour-specific plasma mutant *KRAS* levels [85]. This study revealed that (i) the majority of *KRAS* mutations that were detected in tumours were also found in the plasma, (ii) there was a strong correlation between cfDNA and plasma mutant *KRAS* levels, and (iii) high levels of plasma

mutant *KRAS* were associated with 0% disease control rate [85]. More recently, a large retrospective exploratory analysis used BEAMing technology to identify *KRAS*, *PIK3CA* and *BRAF* mutations in the plasma ctDNA of 503 patients who enrolled in the CORRECT trial of regorafenib, a multi-kinase inhibitor in refractory mCRC [66]. Tumour-associated *KRAS* mutations were readily detected with BEAMing of plasma DNA and were identified in 48% of patients who had previously received anti-EGFR therapy and whose archival tumour tissues were KRAS wild type [66].

Beyond *KRAS* mutations and amplifications, acquired genetic aberrations in other genes have been found to potentially lead to anti-EGFR therapy resistance, albeit in smaller subsets of patients. For example, emerging *EGFR* extracellular domain (ECD) mutations which lead to impaired antibody binding were found to be a resistance mechanism to anti-EGFR therapy in approximately 20% of patients. Interestingly, these mutations tend to arise later than *RAS* mutations during therapy, and patients with *EGFR* ECD mutations had greater and more durable response to anti-EGFR therapy than patients with *RAS* mutations [86]. Interestingly, a phase I trial of a third generation EGFR-targeting agent that binds multiple regions of the EGFR ECD demonstrated efficacy in patients with *EGFR* ECD mutations and acquired resistance to prior EGFR blockade [87]. Other genomic alterations linked to acquired resistance to EGFR blockade include *MET and ERBB2* amplifications [88, 89] and mutations in *NRAS*, *BRAF* and *PIK3CA* [6]. *ERBB2* amplification was found in the plasma in four out of eight *RAS* wild type patients who derived no clinical benefit from anti-EGFR treatment, suggesting that it may also be a source of primary resistance [84].

Another innovative study provided proof-of-principle that parallel analysis of patient-derived xenografts and ctDNA allowed the identification of resistance mechanisms to a pan-tropomyosin-related kinase (TRK) inhibitor in mCRC, with validation in preclinical models [90]. In interpreting these translational findings, it is important to note that typically, multiple complex molecular abnormalities emerge rather than a singular clone and an overlap exists between abnormalities associated with primary and secondary resistance [6].

CtDNA genotyping has now paved the way for prospective clinical trials which aim to evaluate a range of targeted agents in mCRC and their resistance mechanisms. However, significant knowledge gaps exist in the field, including lack of standardisation of ctDNA techniques, clinical relevance of minority clones detected (for example, no threshold for *KRAS* allele frequency has been established to predict anti-EGFR therapy resistance) and it remains to be proven that changing treatment strategy according to ctDNA findings improves patient outcomes [6]. Challenges notwithstanding, it is foreseeable that in the near future, ctDNA genotyping may be used longitudinally to (i) identify *RAS* wild type patients with mCRC who may be suitable for anti-EGFR antibodies, (ii) dynamically assess treatment response, (iii) identify patients who are developing acquired resistance, (iv) delineate resistance mechanisms to therapy, and (v) discover new druggable targets.

6. Future directions

Despite growing enthusiasm, ctDNA in CRC remains largely unavailable for clinical application outside of the trial setting. Recently, there has been a surge of research to further investigate the utility of more sensitive and accurate technologies for ctDNA detection and analysis, and to further elucidate its clinical implementation and significance in the various settings of CRC management.

6.1 Advancing ctDNA detection accuracy

Improved sensitivity techniques with the use of targeted-sequencing methods have been developed by several groups [9, 91]. For example, Lanman et al. validated the analytical and clinical use of a novel, ultra-high specific, digital sequencing technique (Guardant360) consisting of 54 clinically actionable cancer genes [91]. In 165 consecutively matched plasma and tumour tissue samples from patients with advanced cancer, this study demonstrated significantly improved sensitivity for Guardant360 in the plasma-derived cfDNA compared to that of tumour tissue. It also demonstrated the clinical success rate of the assay in 1000 consecutive plasma samples in the clinic (assay failure rate of 0.02%) due to its ability to eliminate false positives [91].

Other investigators have combined the use of DNA fragment sequencing by using molecular barcodes with relevant bioinformatics filtering steps to enhance sensitivity and specificity [30, 69, 92, 93]. In a study using cfDNA from mCRC patients, Mansukhani et al. showed that false positive mutation calls could be reduced by 98.6% when incorporating novel molecular barcodes for error correction and by applying custom solution hybrid capture enrichment [93].

6.2 Detecting aberrant DNA methylation

Several studies have explored the use of DNA methylation markers that may have a role in CRC screening and diagnosis, and which in some cases may have similar sensitivity and specificity to the aforementioned *Septin 9* methylation assay (for example, *APC*, *MGMT*, *RASSF2A*, *Wif-1*, *ALX4*, *NEUROG1*) [94–99]. More compelling is the evidence suggesting that the use of a combination of DNA methylation markers—a multigene methylation signature—may enhance sensitivity and specificity compared with single biomarker detection [94, 96]. Such an assay, utilising the methylation of both *BCAT1* and *IKZF1*, has shown promising results in this setting as previously discussed [51, 52, 64, 100].

6.3 Using CTC, extracellular vesicles, and microRNA as adjuncts biomarkers

This chapter has highlighted the recognisable potential for a paradigm shift with the use of ctDNA for the molecular diagnosis and monitoring of CRC, as well as its multiple drawbacks when used in isolation. Notably, ctDNA is largely unable to evaluate biomarkers other than genomic aberrations. An alternative approach is the use of tools such as CTC, extracellular vesicles, and circulating microRNAs (miRNA), in conjunction to ctDNA, to overcome these limitations.

6.3.1 CTC

A significant limitation of utilising CTC as a biomarker in CRC, particularly in early disease, is that they are difficult to detect in the blood due to a large proportion being captured in the liver prior to entering the general systemic circulation [101–103]. Furthermore, there have been a number of heterogenous studies, systematic reviews and meta-analyses which demonstrate conflicting results for the role of CTC as a prognostic biomarker [104–106]. This makes interpretation very challenging. Nonetheless, it is worth noting that detectable ctDNA and CTC as biomarkers are distinct entities and, in isolation, neither can be regarded as optimal surrogates of the multiclonal malignant state in an individual CRC patient. As discussed earlier, ctDNA is likely to be released by apoptotic or necrotic tumour cells, rather than highly proliferative cells, within a multiclonal tumour [103]. However, we do not know whether all clones have the same apoptotic potential, and therefore

detectable ctDNA levels in CRC patients do not always correspond to the ability to detect CTC [102, 107]. As such, it would be worth exploring the concomitant use of both of these biomarkers in a liquid biopsy.

6.3.2 Extracellular vesicles

The clinical utility for these small, membrane-bound cell fragments, which are also thought to originate from apoptotic, necrotic or proliferating tumour cells, has also recently been considered [108]. Depending on their size and content, they fall under the categories of exosomes, microvesicles, and apoptotic bodies. In particular, tumour-derived exosomes are constitutively formed and released from tumour cells and can be found in the peripheral circulation, other body fluids and interstitial spaces. They can contain concentrated forms of RNA, miRNA, long non-coding RNA, nucleic acids, protein and lipids, but only very small amounts of double-stranded DNA [109]. As such, exosome-derived nucleic acids from the serum of CRC patients may be used to identify genetic aberrations from the tumour that are not detectable by ctDNA, and therefore can be used in a complementary fashion with other biomarkers. To date, there is no published data that has alluded to their clinical application in CRC. Intriguingly, exosomes have recently been shown to have roles in cell-cell signalling which may affect tumour growth and development [110].

6.3.3 miRNA

The role of circulating, exosome-free miRNAs as potential diagnostic and prognostic biomarkers in CRC has been extensively investigated over the past 5 years [111–116]. However, owing to extensive heterogeneity between several studies, it has been difficult to gauge their clinical worth in terms of sensitivity and specificity, which has often been described as 'modest'. Encouragingly, recent studies have suggested that by using miRNA panels or signatures, the predictive accuracy of these assays can be significantly enhanced [117, 118]. In mCRC, only a few studies have addressed the role of circulating miRNAs as predictive biomarkers to systemic therapy [119]. Conceptually, miRNA assays could be used in conjunction with ctDNA, or with the aforementioned biomarkers, to facilitate accurate read-outs for improved sensitivity and specificity.

6.4 Detecting microsatellite phenotype

The use of immune-checkpoint inhibitors has drastically changed the therapeutic landscape for several solid tumours, including a mCRC subset that harbours mutations in DNA mismatch repair (MMR) genes (for example, mutations in *MLH1*, *MSH2*, *MSH6*, *PMS2*, or *MLH1* promoter hypermethylation) [120]. On a molecular level, impaired DNA MMR can lead to genomic hypermutability, including uncontrolled expansion or contraction in DNA microsatellite repeats, termed microsatellite instability (MSI); and the consequent development of malignant neoplasms which have an MSI-high (MSI-H) phenotype. The MSI-H phenotype is present in approximately 15% of all primary CRC and may occur as a result of either inherited (hereditary non-polyposis colon cancer or lynch syndrome) or sporadic abnormalities. It is now common for institutions to screen for this in tumour tissue, either by immunohistochemistry for deficient MMR (dMMR) or PCR for MSI [121]. The identification of the MSI-H phenotype in CRC patients has important prognostic and therapeutic implications, both in the adjuvant and advanced settings when considering conventional chemotherapeutic and targeted agents.

More recently, a small phase II clinical trial using pembrolizumab, an anti-programmed cell death protein 1 (PD1) monoclonal antibody in dMMR mCRC

patients, demonstrated high rates of objective response (40%) and progression-free survival, while no responses were seen in proficient MMR patients [122]. Similarly, a phase II study of anti-PD1 antibody, nivolumab in dMMR/MSI-H mCRC showed 31% objective response rate and 69% disease control rate (12 weeks or longer) [123]. In addition to these encouraging results, multiple trials using anti-PD1 agents, with or without other targeted therapies are ongoing (for example, NCT02460198, NCT02563002, NCT02060188), and it is expected that immunotherapy will rapidly become standard of care in dMMR/MSI-H mCRC.

In this setting, liquid biopsy might be useful in providing a potentially faster, cost-efficient, and safer approach compared to tumour biopsy sampling in patients with suspected MSI-H tumours. Therefore, such assays need to be optimised for routine use in the future. The novel ctDNA techniques described earlier in this chapter could be adapted to identify dMMR CRC in several ways, such as change in microsatellite length, loss of heterozygosity, mutations, or hypermethylation of MMR-related genes [124]. However, similar drawbacks regarding their sensitivity and specificity apply in this setting [125]. To overcome these limitations, several groups have developed enrichment techniques which are able to enhance the presence of altered microsatellites with enrichment probes and detect alterations at very low allele frequencies [126].

Moreover, ctDNA in the setting of immunotherapy can also be used (i) as a predictive marker to identify tumour mutational burden or specific response mutations (for example, PTEN loss or activating beta-catenin mutations), (ii) to monitor treatment response or resistance in conjunction with radiological imaging, and (iii) to identify neoepitopes and epigenetic or transcriptomic markers [124]; although the data for such techniques are preliminary at this stage.

6.5 Detecting ctDNA in other body fluids

This chapter has predominantly focused on the utility of ctDNA in the peripheral blood. Multiple studies have also demonstrated the presence of tumour-derived nucleic acids in other body compartments, such as the urine, stool, saliva, cerebrospinal fluid, pleural fluid, and bronchial washings [40, 127–129]. Of course, topography of the primary tumour, and of any disseminated lesions, will have a significant effect on the concentration of ctDNA in different body fluids.

In a small study, Fujii et al. demonstrated the utility of detecting *KRAS* mutations in the urine of mCRC patients who were undergoing systemic treatment. Both NGS and enrichment PCR were used to detect KRAS in the urine, plasma and archival tumour tissue [128]. The results not only suggested good concordance between ctDNA in the urine and mutant *KRAS* in the tumour, but also demonstrated that ctDNA trends in the urine reflected the tumour dynamics in the plasma. As such, this may also represent an alternative approach to monitoring for therapeutic response or resistance.

7. Conclusion

The data generated from basic research, retrospective clinical studies, and limited prospective studies all support the potential role of ctDNA as a biomarker for early disease, minimal residual disease, recurrence, response to therapy, and emerging drug resistance mechanisms in the management of CRC. Nevertheless, multiple challenges need to be overcome before this promising technology can be adopted into routine clinical practice.

Firstly, a crucial question is whether the genomic aberrations detected in ctDNA actually drive tumour progression. It is also still unknown whether ctDNA will ever be able to mirror the heterogeneity or molecular subclones of CRC in a given

Clinical trial identifier	Study title
Curatively treated CRC (recurrence surveillance and prognostication)	
NCT02842203	Use of ctDNA for Monitoring of Stage III Colorectal Cancer
NCT02842203	Circulating Tumour DNA Analysis to Optimise Treatment for Patients With Colorectal Cancer (IMPROVE)
NCT03416478	The Implication of ctDNA in the Recurrence Surveillance of Stage II and III Colorectal Cancer
NCT03312374	ctDNA as a Prognostic Marker for Postoperative Relapse in Early and Intermediate Stage Colorectal Cancer
NCT02997241	Colon Cancer Treatment Decisions and Recurrence Predicting (CCTDRP)
NCT03189576	Measuring Molecular Residual Disease in Colorectal Cancer After Primary Surgery and Resection of Metastases
NCT03038217	Investigation of the Value of ctDNA in Diagnosis, Treatment, and Surveillance of Surgically Resectable Colorectal Cancer
NCT03615170	Application of Circulating Tumour DNA Test in the Diagnosis and Treatment of Patients With Advanced Rectal Cancer
mCRC—monitoring during chemotherapy	
NCT02872779	Correlation Between Circulating Tumour Markers Early Variations and Clinical Response in First Line Treatment of Metastatic Colorectal Cancer (COCA-MACS)
NCT02948985	Evaluation of CTCs Combined With Tumour Marker Detection of Efficacy of Chemotherapy in mCRC
mCRC—RAS testing	
NCT02502656	RAS Mutation Testing in the Circulating Blood of Patients With Metastatic Colorectal Cancer (RASANC)
NCT03227926	Rechallenge With Panitumumab Driven by RAS Dynamic of Resistance (CHRONOS)
NCT03259009	RAS Mutations in ctDNA and Anti-EGFR reINTROduction in mCRC (RASINTRO) (RASINTRO)
mCRC—MSI testing	
NCT03561350	Detect Microsatellite Instability Status in Blood Sample of Advanced Colorectal Cancer Patients by Next-Generation Sequencing
NCT03594448	Detection of MSI in Circulating Tumour DNA of Colorectal Carcinoma Patients
Large multi-disease observational studies	
NCT03517332	Circulating Tumour DNA Exposure in Peripheral Blood
NCT02838836	Tumour Cell and DNA Detection in the Blood, Urine and Bone Marrow of Patients With Solid Cancers
NCT03027401	Clinical Sequencing of Cancer and Tissue Repository: OncoGenomics
Other	
NCT03546569	Tumour Cells, Tumour DNA and Immunological Response in Colonic Stent Placement (CISMO)
NCT03284684	Kinetics of Perioperative Circulating DNA in Cancer Surgery (Periop ctDNA)
NCT02579278	Circulating Tumour DNA (ctDNA) Rectal Cancer and the Relationship to Extramural Venous Invasion (ctDNA Trial)

Table 2.
Currently recruiting and upcoming clinical trials assessing ctDNA in CRC (http://clinicaltrials.gov).

patient. Further clarity is also needed regarding intra-patient variability in ctDNA levels, the dynamics of ctDNA release and ctDNA clearance. Such knowledge will inform the design of future studies, particularly regarding the optimal timing of ctDNA assessment relevant to the appropriate therapeutic intervention.

Secondly, to determine the true value of ctDNA analysis in guiding decision-making, carefully designed and well-controlled prospective trials are needed to address clinically relevant questions for various settings. An important question, for example, is how to utilise ctDNA detection as a biomarker of minimal residual disease after resection of a stage I–III CRC. Can we use this biomarker to make decisions about the necessity, type and duration of adjuvant therapy and guide follow-up or surveillance scheduling? Another question is how to use ctDNA to monitor for the emergence of molecular resistance and can we use this approach to inform us about timely adaptation of further treatment lines? **Table 2** lists selected currently recruiting and upcoming clinical trials assessing the utility of ctDNA in various settings in CRC.

Importantly, pre-analytical considerations, ctDNA detection techniques, and interpretation of results need to be standardised. On review of the current literature, it will be obvious to the reader that there is a high level of heterogeneity amongst various techniques. Consequently, results that are obtained from one study cannot be interpreted in the same way and applied to other techniques. Standardisation will ensure that there is consensus regarding the sensitivity and specificity of utilised techniques and that there are established cut-off levels, for each clinical setting. Finally, it is important to acknowledge that the use of promising novel technologies will have cost implications which may hinder their rapid entry into routine clinical practice.

Glossary

Allele frequency	The relative frequency of a gene variant in a specimen, expressed as a percentage or fraction
CfDNA	Cell-free DNA. DNA fragments found circulating in body fluids, including plasma or serum. CfDNA may come from a variety of sources including tumour cells
CtDNA	Circulating-tumour DNA. A proportion of cfDNA that is tumour-derived
Liquid biopsy	Sampling and analysis of tumour-based material (e.g. CTC, ctDNA, RNA, exosomes) from body fluids such as blood, urine and pleural fluid
NGS	Next generation sequencing (NGS) or massively parallel sequencing are broad terms describing a range of high throughput methods capable of the simultaneous analysis of thousands to millions of DNA molecules
PCR	Polymerase chain reaction. A laboratory technique used to make many copies (amplification) of a specific DNA sequence of interest
Pre-analytical	The pre-analysis phase in the laboratory testing process and may include sample collection, handling, processing, transport and storage. These factors can affect the subsequent analysis outcomes
Clinical utility	The ability of an intervention or test to demonstrate benefit in patient care compared to not using the intervention or test

Author details

Daphne Day[1,2], Sophia Frentzas[1,2], Cameron A. Naidu[2,3], Eva Segelov[1,2] and Maja Green[2*]

1 Department of Medical Oncology, Monash Health, Clayton, Victoria, Australia

2 Department of Oncology, Monash University, Clayton, Victoria, Australia

3 Faculty of Medicine, Nursing and Health Sciences, Monash University, Clayton, Victoria, Australia

*Address all correspondence to: maja.green@monash.edu

References

[1] Li ZZ et al. Mutation profiling in Chinese patients with metastatic colorectal cancer and its correlation with clinicopathological features and anti-EGFR treatment response. Oncotarget. 2016;**7**(19):28356-28368

[2] Misale S et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012;**486**(7404):532-536

[3] Gerlinger M et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. The New England Journal of Medicine. 2012;**366**(10):883-892

[4] Alix-Panabieres C, Pantel K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. Cancer Discovery. 2016;**6**(5):479-491

[5] Merker JD et al. Circulating tumor DNA analysis in patients with cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review. Journal of Clinical Oncology. 2018;**36**(16):1631-1641

[6] Nadal C et al. Future perspectives of circulating tumor DNA in colorectal cancer. Tumour Biology. 2017;**39**(5):1010428317705749

[7] Garlan F et al. Early evaluation of circulating tumor DNA as marker of therapeutic efficacy in metastatic colorectal cancer patients (PLACOL study). Clinical Cancer Research. 2017;**23**(18):5416-5425

[8] Mendel P, Metais P. Les acides nucléiques du plasma sanguin chez l'homme. Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales. 1948;**142**:241-243

[9] Wan JCM et al. Liquid biopsies come of age: Towards implementation of circulating tumour DNA. Nature Reviews. Cancer. 2017;**17**(4):223-238

[10] Leon SA et al. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Research. 1977;**37**(3):646-650

[11] Stroun M et al. Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology. 1989;**46**(5):318-322

[12] Diaz LAJ, Bardelli A. Liquid biopsies: Genotyping circulating tumor DNA. Journal of Clinical Oncology. 2014;**32**(6):579-586

[13] Jahr S et al. DNA fragments in the blood plasma of cancer patients: Quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Research. 2001;**61**(4):1659-1665

[14] Khakoo S et al. Circulating tumour DNA, a promising biomarker for the management of colorectal cancer. Critical Reviews in Oncology/Hematology. 2018;**122**:72-82

[15] Haber DA, Velculescu VE. Blood-based analyses of cancer: Circulating tumor cells and circulating tumor DNA. Cancer Discovery. 2014;**4**(6):650-661

[16] Gorgannezhad L et al. Circulating tumor DNA and liquid biopsy: Opportunities, challenges, and recent advances in detection technologies. Lab on a Chip. 2018;**18**(8):1174-1196

[17] Dressman D et al. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proceedings of the National Academy of Sciences of the United States of America. 2003;**100**(15): 8817-8822

[18] Diehl F et al. Circulating mutant DNA to assess tumor dynamics. Nature Medicine. 2008;**14**(9):985-990

[19] Hindson BJ et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analytical Chemistry. 2011;**83**(22):8604-8610

[20] Sanmamed MF et al. Quantitative cell-free circulating BRAFV600E mutation analysis by use of droplet digital PCR in the follow-up of patients with melanoma being treated with BRAF inhibitors. Clinical Chemistry. 2015;**61**(1):297-304

[21] Kinde I et al. Detection and quantification of rare mutations with massively parallel sequencing. Proceedings of the National Academy of Sciences of the United States of America. 2011;**108**(23):9530-9535

[22] Forshew T et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Science Translational Medicine. 2012;**4**(136):136ra68

[23] Siravegna G et al. Integrating liquid biopsies into the management of cancer. Nature Reviews. Clinical Oncology. 2017;**14**(9):531-548

[24] Leary RJ et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Science Translational Medicine. 2012;**4**(162):162ra154

[25] Murtaza M et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature. 2013;**497**(7447):108-112

[26] Board RE et al. Detection of PIK3CA mutations in circulating free DNA in patients with breast cancer. Breast Cancer Research and Treatment. 2010;**120**(2):461-467

[27] Yamada T et al. Detection of K-ras gene mutations in plasma DNA of patients with pancreatic adenocarcinoma: Correlation with clinicopathological features. Clinical Cancer Research. 1998;**4**(6):1527-1532

[28] Shi J, Liu Q, Sommer SS. Detection of ultrarare somatic mutation in the human TP53 gene by bidirectional pyrophosphorolysis-activated polymerization allele-specific amplification. Human Mutation. 2007;**28**(2):131-136

[29] Taly V et al. Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. Clinical Chemistry. 2013;**59**(12):1722-1731

[30] Newman AM et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nature Medicine. 2014;**20**(5):548-554

[31] Heitzer E et al. Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. Genome Medicine. 2013;**5**(4):30

[32] Chan KC et al. Cancer genome scanning in plasma: Detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. Clinical Chemistry. 2013;**59**(1):211-224

[33] Song LL, Li YM. Current noninvasive tests for colorectal cancer screening: An overview of colorectal cancer screening tests. World Journal of Gastrointestinal Oncology. 2016;**8**(11):793-800

[34] Young GP et al. Advances in fecal occult blood tests: The FIT revolution. Digestive Diseases and Sciences. 2015;**60**(3):609-622

[35] Mandel JS et al. Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. The New England Journal of Medicine. 1993;**328**(19):1365-1371

[36] Hardcastle JD et al. Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. Lancet. 1996;**348**(9040):1472-1477

[37] Kronborg O et al. Randomised study of screening for colorectal cancer with faecal-occult-blood test. Lancet. 1996;**348**(9040):1467-1471

[38] Cole SR et al. A randomised trial of the impact of new faecal haemoglobin test technologies on population participation in screening for colorectal cancer. Journal of Medical Screening. 2003;**10**(3):117-122

[39] Lee JK et al. Accuracy of fecal immunochemical tests for colorectal cancer: Systematic review and meta-analysis. Annals of Internal Medicine. 2014;**160**(3):171

[40] Imperiale TF, Ransohoff DF, Itzkowitz SH. Multitarget stool DNA testing for colorectal-cancer screening. The New England Journal of Medicine. 2014;**371**(2):187-188

[41] Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990;**61**(5):759-767

[42] De Roock W et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: A retrospective consortium analysis. The Lancet Oncology. 2010;**11**(8):753-762

[43] Perrone F et al. Circulating free DNA in a screening program for early colorectal cancer detection. Tumori. 2014;**100**(2):115-121

[44] Dong L, Ren H. Blood-based DNA methylation biomarkers for early detection of colorectal cancer. Journal of Proteomics and Bioinformatics. 2018;**11**(6):120-126

[45] de Vos T et al. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. Clinical Chemistry. 2009;**55**(7):1337-1346

[46] Toth K et al. Free circulating DNA based colorectal cancer screening from peripheral blood: The possibility of the methylated septin 9 gene marker. Orvosi Hetilap. 2009;**150**(21):969-977

[47] Church TR et al. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. Gut. 2014;**63**(2):317-325

[48] Yan S et al. Diagnostic value of methylated Septin9 for colorectal cancer screening: A meta-analysis. Medical Science Monitor. 2016;**22**:3409-3418

[49] Jin P et al. Performance of a second-generation methylated SEPT9 test in detecting colorectal neoplasm. Journal of Gastroenterology and Hepatology. 2015;**30**(5):830-833

[50] Toth K et al. Detection of methylated septin 9 in tissue and plasma of colorectal patients with neoplasia and the relationship to the amount of circulating cell-free DNA. PLoS One. 2014;**9**(12):e115415

[51] Pedersen SK et al. Evaluation of an assay for methylated BCAT1 and IKZF1 in plasma for detection of colorectal neoplasia. BMC Cancer. 2015;**15**:654

[52] Symonds EL et al. A blood test for methylated BCAT1 and IKZF1 vs. a fecal immunochemical test for detection of colorectal neoplasia. Clinical and Translational Gastroenterology. 2016;**7**:e137

[53] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA: A Cancer Journal for Clinicians. 2018;**68**(1):7-30

[54] Goldberg RM et al. Surgery for recurrent colon cancer: Strategies for identifying resectable recurrence and success rates after resection. Eastern Cooperative Oncology Group, the North Central Cancer Treatment Group, and the Southwest Oncology Group. Annals of Internal Medicine. 1998;**129**(1):27-35

[55] Tas F et al. Measurement of serum CA 19-9 may be more valuable than CEA in prediction of recurrence in patients with gastric cancer. American Journal of Clinical Oncology. 2001;**24**(2):148-149

[56] Aloe S et al. Prognostic value of serum and tumor tissue CA 72-4 content in gastric cancer. The International Journal of Biological Markers. 2003;**18**(1):21-27

[57] Brannon AR et al. Comparative sequencing analysis reveals high genomic concordance between matched primary and metastatic colorectal cancer lesions. Genome Biology. 2014;**15**(8):454

[58] Ryan BM et al. A prospective study of circulating mutant KRAS2 in the serum of patients with colorectal neoplasia: Strong prognostic indicator in postoperative follow up. Gut. 2003;**52**(1):101-108

[59] Wang JY et al. Molecular detection of APC, K-ras, and p53 mutations in the serum of colorectal cancer patients as circulating biomarkers. World Journal of Surgery. 2004;**28**(7):721-726

[60] Lee HS et al. Circulating methylated septin 9 nucleic acid in the plasma of patients with gastrointestinal cancer in the stomach and colon. Translational Oncology. 2013;**6**(3):290-296

[61] Bazan V et al. Molecular detection of TP53, Ki-Ras and p16INK4A promoter methylation in plasma of patients with colorectal cancer and its association with prognosis. Results of a 3-year GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study. Annals of Oncology. 2006;**17**(Suppl 7):vii84-vii90

[62] Frattini M et al. Quantitative and qualitative characterization of plasma DNA identifies primary and recurrent colorectal cancer. Cancer Letters. 2008;**263**(2):170-181

[63] Tie J et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Science Translational Medicine. 2016;**8**(346):346ra92

[64] Young GP et al. A cross-sectional study comparing a blood test for methylated BCAT1 and IKZF1 tumor-derived DNA with CEA for detection of recurrent colorectal cancer. Cancer Medicine. 2016;**5**(10):2763-2772

[65] Symonds EL et al. Circulating tumour DNA for monitoring colorectal cancer—A prospective cohort study to assess relationship to tissue methylation, cancer characteristics and surgical resection. Clinical Epigenetics. 2018;**10**:63

[66] Tabernero J et al. Analysis of circulating DNA and protein biomarkers to predict the clinical activity of regorafenib and assess prognosis in patients with metastatic colorectal cancer: A retrospective, exploratory analysis of the CORRECT trial. The Lancet Oncology. 2015;**16**(8):937-948

[67] El Messaoudi S et al. Circulating DNA as a strong multimarker prognostic tool for metastatic colorectal cancer patient management care. Clinical Cancer Research. 2016;**22**(12):3067-3077

[68] Lefebure B et al. Prognostic value of circulating mutant DNA in unresectable metastatic colorectal cancer. Annals of Surgery. 2010;**251**(2):275-280

[69] Bettegowda C et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Science Translational Medicine. 2014;**6**(224):224ra24

[70] Fan G et al. Prognostic value of circulating tumor DNA in patients with colon cancer: Systematic review. PLoS One. 2017;**12**(2):e0171991

[71] Spindler KL et al. Changes in mutational status during third-line treatment for metastatic colorectal cancer—Results of consecutive measurement of cell free DNA, KRAS and BRAF in the plasma. International Journal of Cancer. 2014;**135**(9):2215-2222

[72] Goldstein MJ, Mitchell EP. Carcinoembryonic antigen in the staging and follow-up of patients with colorectal cancer. Cancer Investigation. 2005;**23**(4):338-351

[73] Tie J et al. Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Annals of Oncology. 2015;**26**(8):1715-1722

[74] Barault L et al. Discovery of methylated circulating DNA biomarkers for comprehensive non-invasive monitoring of treatment response in metastatic colorectal cancer. Gut. 2017

[75] Garcia-Saenz JA et al. Tumor burden monitoring using cell-free tumor DNA could be limited by tumor heterogeneity in advanced breast cancer and should be evaluated together with radiographic imaging. BMC Cancer. 2017;**17**(1):210

[76] Bernabe R et al. What do we need to make circulating tumour DNA (ctDNA) a routine diagnostic test in lung cancer? European Journal of Cancer. 2017;**81**:66-73

[77] Hao YX et al. Effectiveness of circulating tumor DNA for detection of KRAS gene mutations in colorectal cancer patients: A meta-analysis. OncoTargets and Therapy. 2017;**10**:945-953

[78] Vidal J et al. Plasma ctDNA RAS mutation analysis for the diagnosis and treatment monitoring of metastatic colorectal cancer patients. Annals of Oncology. 2017;**28**(6):1325-1332

[79] Schmiegel W et al. Blood-based detection of RAS mutations to guide anti-EGFR therapy in colorectal cancer patients: Concordance of results from circulating tumor DNA and tissue-based RAS testing. Molecular Oncology. 2017;**11**(2):208-219

[80] Misale S et al. Resistance to anti-EGFR therapy in colorectal cancer: From heterogeneity to convergent evolution. Cancer Discovery. 2014;**4**(11):1269-1280

[81] Diaz LAJ et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012;**486**(7404):537-540

[82] Bertotti A et al. The genomic landscape of response to EGFR blockade in colorectal cancer. Nature. 2015;**526**(7572):263-267

[83] Siena S et al. Dynamic molecular analysis and clinical correlates of tumor evolution within a phase II trial of panitumumab-based therapy in metastatic colorectal cancer. Annals of Oncology. 2018;**29**(1):119-126

[84] Siravegna G et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nature Medicine. 2015;**21**(7):827

[85] Spindler KL et al. Quantitative cell-free DNA, KRAS, and BRAF mutations in plasma from patients with metastatic colorectal cancer during treatment with cetuximab and irinotecan. Clinical Cancer Research. 2012;**18**(4):1177-1185

[86] Van Emburgh BO et al. Acquired RAS or EGFR mutations and duration of response to EGFR blockade in colorectal cancer. Nature Communications. 2016;**7**:13665

[87] Arena S et al. Emergence of multiple EGFR extracellular mutations during Cetuximab treatment in colorectal Cancer. Clinical Cancer Research. 2015;**21**(9):2157-2166

[88] Mohan S et al. Changes in colorectal carcinoma genomes under anti-EGFR therapy identified by whole-genome plasma DNA sequencing. PLoS Genetics. 2014;**10**(3):e1004271

[89] Bardelli A et al. Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. Cancer Discovery. 2013;**3**(6):658-673

[90] Russo M et al. Acquired resistance to the TRK inhibitor Entrectinib in colorectal cancer. Cancer Discovery. 2016;**6**(1):36-44

[91] Lanman RB et al. Analytical and clinical validation of a digital sequencing panel for quantitative, highly accurate evaluation of cell-free circulating tumor DNA. PLoS One. 2015;**10**(10):e0140712

[92] Newman AM et al. Integrated digital error suppression for improved detection of circulating tumor DNA. Nature Biotechnology. 2016;**34**(5):547-555

[93] Sonia Mansukhani LJB, Moorcraft SY, Davidson M, Woolston A, et al. Ultra-sensitive mutation detection and genome-wide DNA copy number reconstruction by error corrected circulating tumour DNA sequencing. bioRxiv. 2017;**64**(11):1626-1635

[94] Lee BB et al. Aberrant methylation of APC, MGMT, RASSF2A, and Wif-1 genes in plasma as a biomarker for early detection of colorectal cancer. Clinical Cancer Research. 2009;**15**(19):6185-6191

[95] Tanzer M et al. Performance of epigenetic markers SEPT9 and ALX4 in plasma for detection of colorectal precancerous lesions. PLoS One. 2010;**5**(2):e9061

[96] Cassinotti E et al. DNA methylation patterns in blood of patients with colorectal cancer and adenomatous colorectal polyps. International Journal of Cancer. 2012;**131**(5):1153-1157

[97] Herbst A et al. Methylation of NEUROG1 in serum is a sensitive marker for the detection of early colorectal cancer. The American Journal of Gastroenterology. 2011;**106**(6):1110-1118

[98] Lam K et al. DNA methylation based biomarkers in colorectal cancer: A systematic review. Biochimica et Biophysica Acta. 2016;**1866**(1):106-120

[99] Rasmussen SL et al. Hypermethylated DNA, a circulating biomarker for colorectal cancer detection. PLoS One. 2017;**12**(7):e0180809

[100] Pedersen SK et al. A two-gene blood test for methylated DNA sensitive for colorectal cancer. PLoS One. 2015;**10**(4):e0125041

[101] Gkountela S et al. Recent advances in the biology of human circulating tumour cells and metastasis. ESMO Open. 2016;**1**(4):e000078

[102] Diehl F et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proceedings of

the National Academy of Sciences of the United States of America. 2005;**102**(45):16368-16373

[103] Grimm M et al. High phosphorus intake only slightly affects serum minerals, urinary pyridinium crosslinks and renal function in young women. European Journal of Clinical Nutrition. 2001;**55**(3):153-161

[104] Peach G et al. Prognostic significance of circulating tumour cells following surgical resection of colorectal cancers: A systematic review. British Journal of Cancer. 2010;**102**(9):1327-1334

[105] Rahbari NN et al. Meta-analysis shows that detection of circulating tumor cells indicates poor prognosis in patients with colorectal cancer. Gastroenterology. 2010;**138**(5):1714-1726

[106] Yang C et al. Prognostic and clinicopathological significance of circulating tumor cells detected by RT-PCR in non-metastatic colorectal cancer: A meta-analysis and systematic review. BMC Cancer. 2017;**17**(1):725

[107] Kin C et al. Colorectal cancer diagnostics: Biomarkers, cell-free DNA, circulating tumor cells and defining heterogeneous populations by single-cell analysis. Expert Review of Molecular Diagnostics. 2013;**13**(6):581-599

[108] Simpson RJ et al. Exosomes: Proteomic insights and diagnostic potential. Expert Review of Proteomics. 2009;**6**(3):267-283

[109] Gold B et al. Do circulating tumor cells, exosomes, and circulating tumor nucleic acids have clinical utility? A report of the association for molecular pathology. The Journal of Molecular Diagnostics. 2015;**17**(3):209-224

[110] Valadi H et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature Cell Biology. 2007;**9**(6):654-659

[111] Uratani R et al. Diagnostic potential of cell-free and exosomal microRNAs in the identification of patients with high-risk colorectal adenomas. PLoS One. 2016;**11**(10):e0160722

[112] He Y et al. Current state of circulating microRNAs as cancer biomarkers. Clinical Chemistry. 2015;**61**(9):1138-1155

[113] Mohammadi A, Mansoori B, Baradaran B. The role of microRNAs in colorectal cancer. Biomedicine & Pharmacotherapy. 2016;**84**:705-713

[114] Masuda T et al. MicroRNAs as biomarkers in colorectal cancer. Cancers (Basel). 2017:**9**(9)

[115] Toiyama Y et al. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. Journal of the National Cancer Institute. 2013;**105**(12):849-859

[116] Yu W et al. Circulating microRNA-21 as a potential diagnostic marker for colorectal cancer: A meta-analysis. Molecular and Clinical Oncology. 2016;**4**(2):237-244

[117] Vychytilova-Faltejskova P et al. Serum-based microRNA signatures in early diagnosis and prognosis prediction of colon cancer. Carcinogenesis. 2016;**37**(10):941-950

[118] Carter JV et al. A highly predictive model for diagnosis of colorectal neoplasms using plasma microRNA: Improving specificity and sensitivity. Annals of Surgery. 2016;**264**(4):575-584

[119] Xie T et al. MicroRNAs as regulators, biomarkers and therapeutic targets in the drug resistance of colorectal cancer. Cellular Physiology and Biochemistry. 2016;**40**(1-2):62-76

[120] Ryan E et al. The current value of determining the mismatch repair status of colorectal cancer: A rationale for routine testing. Critical Reviews in Oncology/Hematology. 2017;**116**:38-57

[121] Buza N, Ziai J, Hui P. Mismatch repair deficiency testing in clinical practice. Expert Review of Molecular Diagnostics. 2016;**16**(5):591-604

[122] Le DT et al. PD-1 blockade in tumors with mismatch-repair deficiency. The New England Journal of Medicine. 2015;**372**(26):2509-2520

[123] Overman MJ et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): An open-label, multicentre, phase 2 study. The Lancet Oncology. 2017;**18**(9):1182-1191

[124] Cabel L et al. Clinical potential of circulating tumour DNA in patients receiving anticancer immunotherapy. Nature Reviews. Clinical Oncology. 2018;**15**(10):639-650

[125] Hause RJ et al. Classification and characterization of microsatellite instability across 18 cancer types. Nature Medicine. 2016;**22**(11):1342-1350

[126] Ladas I et al. Enhanced detection of microsatellite instability using pre-PCR elimination of wild-type DNA homo-polymers in tissue and liquid biopsies. Nucleic Acids Research. 2018;**46**(12):e74

[127] Millholland JM et al. Detection of low frequency FGFR3 mutations in the urine of bladder cancer patients using next-generation deep sequencing. Research and Reports in Urology. 2012;**4**:33-40

[128] Fujii T et al. Mutation-enrichment next-generation sequencing for quantitative detection of KRAS mutations in urine cell-free DNA from patients with advanced cancers. Clinical Cancer Research. 2017;**23**(14):3657-3666

[129] Li Y et al. Salivary transcriptome diagnostics for oral cancer detection. Clinical Cancer Research. 2004;**10**(24):8442-8450

Chapter 5

Epigenetic Biomarkers and Their Therapeutic Applications in Colorectal Cancer

Antja-Voy Hartley, Matthew Martin and Tao Lu

Abstract

Colorectal cancer (CRC) is one of the most aggressive cancers worldwide and is known to develop through a stepwise process involving the accumulation of several genetic and epigenetic alterations. Furthermore, numerous studies have highlighted the significant role that certain epigenetic enzymes play in CRC pathogenesis, particularly those that govern chromatin components in the promoter regions of tumor suppressors and oncogenes. Here, we delineate the relationship between CRC-associated epigenetic marks, their modifying enzymes, and the classification of CRC into distinct molecular pathways or subtypes. Moreover, we discuss some of the most prominent methyltransferases, demethylases, acetyltransferases, and deacetylases, which have been targeted for preclinical and clinical CRC treatment. Notably, inhibitors against these epigenetic enzymes are a promising new class of anticancer drugs, with several obtaining Food and Drug Administration (FDA) approval for the treatment of blood and solid tumors. By highlighting the epigenetic molecular pathways leading to CRC development as well as providing an update on current CRC epigenetic therapies, this chapter sheds fresh insight into new and emerging avenues for future therapeutics.

Keywords: checkpoint, CIMP, CIN, CRC, demethylase, DNA methylation, DNMT, epigenetics, HAT, HDAC, methyltransferase, MSI

1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths globally and is expected to be responsible for an estimated 1.1 million deaths by 2030 [1]. With this growing global burden, prevention and treatment of CRC remains a significant public health challenge. CRC is thought to originate from sequential accumulation of genetic and epigenetic aberrations [2]. Of the identified genetic mutations, approximately 15 have been characterized as "driver mutations" and are thought to be functionally important during CRC initiation and progression [3, 4]. These include genes affecting critical cellular pathways such as those governing proliferation, apoptosis, migration, adhesion, and DNA damage and repair [3]. Importantly, it is now well established that epigenetic alterations can also serve as major driver events in the pathogenesis of CRC [5–7]. However, unlike genetic mutations, epigenetic modifications consist of heritable changes in gene expression

without DNA sequence changes and are intrinsically reversible by nature. These epigenetic events include alterations in DNA methylation, histone modifications, and non-coding RNAs. Moreover, the reversibility of these modifications makes them attractive molecular targets for anticancer therapeutic interventions [3, 8].

CRC is a highly heterogeneous disease and can be classified into molecularly and pathologically distinct pathways and subtypes [9]. Moreover, these classifications have significantly influenced patient stratification, prognosis, and therapeutic response [9, 10]. In this chapter, we focus on three epigenetic-related primary molecular pathways, namely the microsatellite instability (MSI) phenotype, the chromosomal instability (CIN) phenotype, and the CpG island methylator phenotype (CIMP). Importantly, each pathway reflects the underlying mechanisms of carcinogenesis as marked by certain aberrations such as a defective DNA mismatch repair (MMR) system, which is associated with MSI CRCs [11], or by widespread promoter DNA methylation within CpG islands as is the case with CIMP tumors [11, 12]. On the other hand, the CIN pathway, which manifests in majority of CRC cases (~85%), arises through widespread chromosomal imbalances [9, 13, 14]. We also make mention of the relationship between these defined pathways and the four consensus molecular subtyping classifications, with emphasis on the frequent overlap observed between two or more of the aforementioned pathways.

In the past few decades, several studies have analyzed epigenetic marks, the enzymes mediating these marks, and the extent of their active contribution to CRC tumor development and progression [2, 3]. For instance, several methylation-related enzymes have been found to be clinically relevant to CRC [15]. Among these, some of the most prominent histone methyltransferases (HMTs) that have been targeted for preclinical and clinical treatment of CRC are discussed in Section 4 of this chapter [15, 16]. On the other hand, a comparatively less number of histone demethylases (HDMs) have been validated as pertinent to CRC pathogenesis. As important regulators of colon cell transformation, histone deacetylases (HDAC) have also emerged as prominent markers of early carcinogenic events due to their unique role in maintaining higher-order chromatin structure [17].

In this chapter, we also highlight a few chemical inhibitors relevant to epigenetic therapy. However, we also noted that among the CRC-associated epigenetic enzymes, only a few of them have potent inhibitors available [15]. This suggests that the knowledge concerning targeting these enzymes for CRC is still insufficient and needs further evaluation. For example, only a few DNA methyltransferase (DNMT) and HMT inhibitors have been used in CRC cells [2], and a handful of Food and Drug Administration (FDA)-approved HDAC inhibitors are currently being explored for the treatment of solid tumors including CRC [18].

Unfortunately, the use of such epigenetic-based inhibitors has not been without limitations. Major drawbacks, such as adverse side effects and lack of clinical efficacy, have limited their use as single agents. Therefore, many inhibitors show more promise in combination therapy with chemotherapies suggesting that the full therapeutic potential of epigenetic therapy will perhaps be best realized in combination with other anti-cancer agents [19, 20]. This is also complemented by the recent understanding that there is a strong interplay between immune and cancer cells within the tumor microenvironment [21]. Recent studies in CRC cells have shown promising combinations of epigenetic and immunomodulatory drugs. By reversing expression changes of genes involved in immune suppression and thus enhancing expression of tumor-associated antigens, cancer cells potentially become more sensitive to immune checkpoint inhibitors [22]. These and other discoveries have established a highly promising basis for studies using combined epigenetic and immunotherapeutic agents for treating CRC.

2. Epigenetic modifications in CRC

2.1 Histone modifications

Over the past decade, significant advances in our understanding of the CRC "epigenome" have revealed that most CRC cases harbor alterations in their histone modification states, particularly regarding aberrant histone methylation and acetylation [6, 15, 23]. Importantly, these abnormal histone marks are highly recurrent and have recently been used as biomarkers to predict the clinical outcome in CRC patients [2]. These include changes in the global patterns of specific histone modifications. For example, Tamagawa et al. showed that global changes in histone H3K4me2, H3K9ac, and H3K9me2 in metachronous liver metastasis correlated to overall survival of CRC patients [24]. Specifically, low H3K4me2 levels were shown to correlate with overall poor prognosis [24]. Likewise, other studies have identified reduced levels of H3K9me3 and H4K20me3 as diagnostic biomarkers for CRC in circulating nucleosomes which correlated with poor patient outcome [25]. Conversely, high H4K20me3 and H3K9me3, as well as low nuclear expression of H3K4me3, were associated with a better prognosis for early-stage CRC patients [26].

Furthermore, since reduction or enrichment of these marks frequently occurs at the promoters of key CRC-related oncogenes and tumor suppressors, this results in detrimental changes in gene expression that form the basis of tumorigenesis [15, 27]. For instance, H3K4me3, when found to be elevated in CRC primary tumors and cell lines, resulted in activated *Wingless-type* (*WNT*) signaling and target gene expression via interaction between *SET domain-containing protein 1A* (*SETD1A*) and *β-catenin* [28]. Meanwhile, another study revealed that low H3K4me1/2/3 levels were associated with hypoxia-induced silencing of *MLH1* in SW480 cells, which is a key event in the DNA mismatch defects linked to the development of sporadic CRC [29]. Yokoyama's group also demonstrated a role for the well-recognized repressive mark H3K9me3, revealing that its increased levels in metastatic CRC patient-derived cells correlated with enhanced cell motility [30]. Interestingly, this coincided with repression of *Ataxia-telangiectasia mutated* (*ATM*) and *p53-associated KZNF protein* (*APAK*), leading to a defect in p53-dependent apoptosis [30]. Moreover, enrichment of another repressive mark, H3K27me3, was associated with poor CRC patient prognosis while elevated H3K79me2 was shown to enhance interleukin (IL)-22-induced stemness in CRC cells [31, 32]. Intriguingly, more recent studies have also shown that mutations in specific methylation sites could promote CRC development. For instance, the Shah and Lu groups identified histone 3 lysine 36-to-methionine (H3K36M) substitution mutations in CRC patient samples, which promoted more undifferentiated sarcomas *in vivo* [33, 34]. This suggests that H3K36 methylation potentially constitutes a major tumor suppressive epigenetic mark.

In addition to abnormal methylation, disruption of histone acetylation patterns also contributes to CRC pathogenesis, particularly relating to transcriptional inactivation of tumor suppressor genes and, sometimes, activation of oncogenes. For example, Richon et al. showed that hypoacetylation at the promoter of the tumor suppressor *p21WAF*[1] led to its repression, an effect that was reversed by inhibition of HDAC activity [35]. Conversely, mass spectrometry-based analyses used to quantify global alterations of histone modifications in CRC samples identified H3K27ac as a modification frequently upregulated in CRC [36]. In fact, one study highlighted the effects of aspirin in reducing the enrichment of H3K27ac in the promoters of *inducible nitric oxide synthase* (*iNOS*), *tumor necrosis factor alpha* (*TNF-α*), and *IL-6* [37]. This in turn corresponded to the dramatic reduction of the mRNA and protein levels of these genes, which suppressed inflammatory colitis symptoms and CRC tumor burden [37]. Taken together, these studies emphasize

the differential abundance of key repressive and activating histone methylation and acetylation marks in CRC and suggests their role in regulating genes associated with CRC development and progression.

2.2 DNA methylation

DNA methylation constitutes the first recognized epigenetic alteration in CRC [38]. Usually, global DNA hypomethylation is frequently seen, which occurs gradually and early in the process of CRC carcinogenesis [38]. More precisely, global DNA hypomethylation mainly takes place on cytosine guanine (CpG) dinucleotides within pericentromeric regions. Initially, this hypomethylation in CRC was hypothesized to be associated mainly with widespread oncogene activation but has now been linked predominantly to increased genomic instability [3]. This increased accumulation of chromosome breakage and overall chromosomal instability contributes to a prevalent subtype of CRCs known as the CIN phenotype as we briefly described in Section 1 [39].

Notably, hypomethylation typically occurs in concert with systematic and discrete DNA hypermethylation events at the promoters of genes involved in DNA repair, apoptosis, proliferation, angiogenesis, adhesion, and invasion [38, 40]. DNA hypermethylation is the most extensively characterized epigenetic alteration in CRC, occurring at CpG dinucleotide-dense regions, called CpG islands, which are present in about 60% of genes [6, 41]. Apart from CpG islands, DNA hypermethylation has also been extensively observed within the first exonic/intronic regions of some genes and generally results in transcriptional silencing [42]. Some of the most frequently hypermethylated genes in CRC include *Adenomatous polyposis coli* (*APC*), *Cyclin-dependent kinase inhibitor 2A* (*p16^{INK4a}/CDKN2A*), *Tissue inhibitor of metalloproteinases 3* (*TIMP3*), *O-6-Methylguanine-DNA methyltransferase* (*MGMT*), *Secreted frizzled related protein 1* (*SFRP1*), *Transmembrane protein with epidermal growth factor* (*EGF*) *like and two follistatin like domains 2* (*TMEFF2*), *Heparan sulfate-glucosamine 3-sulfotransferase 2* (*HS3ST2/3OST2*), *Ras association domain family member 1* (*RASSF1A*), and *GATA binding protein 4* (*GATA4*) [43].

Another subtype of CRCs with extensive patterns of promoter methylation, known as the CIMP phenotype as described in Section 1, is also characterized by aberrant DNA methylation at genes with roles in CRC initiation or progression [44]. For instance, using a qPCR-based technique, one group identified genes with the highest percentage of methylation in CRC patients including *Runt related transcription factor 3* (*RUNX3*), *Protocadherin 10* (*PCDH10*), *Secreted frizzled related protein 5* (*SFRP5*), *Insulin-like growth factor 2* (*IGF2*), and *Hepatocyte nuclear factor 1 homeobox A* (*HNF1*) *homeobox B* (*Hnf1b*) [45]. Moreover, these genes were observed to have the most promising biomarker potential because of the frequent gene repression patterns [45]. Other commonly hypermethylated genes, such as *Sex-determining region Y* (*SRY*)*-related HMG-box* (*SOX17*) and *Apoptosis-associated speck-like protein containing a CARD* (*ASC*)*/target of methylation-induced silencing* (*TMS1*), were differentially methylated based on the staging of the disease [46–48], whereas *MLH1*, *p16*, *Death-associated protein kinase 1* (*DAP-kinase*), *Ras association domain family member 2* (*RASSF2A*), and *WNT inhibitory factor 1* (*Wif-1*) were regarded as plasma or serum detection markers for CRC [49].

In summary, these data strongly support the promising utility of DNA methylation as a critical diagnostic marker for CRC. Unfortunately, this has not necessarily translated into their prognostic or predictive use in clinical practice [50]. This can be attributed to significant variability in sensitivity, specificity, and reproducibility between diverse patient cohorts and gene expression platforms, which ultimately impacts the prognostic value of many tests. Currently, two FDA-approved

commercial tests, Epi proColon® and Cologuard, have been used for screening alterations in methylation of common genes, such as *SEPT9*, *NDRG4*, and *BMP3*, for early detection of CRC [51]. However, they also generally lack prognostic value and require improvements in terms of sensitivity and specificity. Several other methylation biomarker assays have also been suggested, but validation in independent and large population screening studies is still needed [52].

2.3 Noncoding RNAs

Another epigenetic regulatory mechanism frequently deregulated in CRC involves the role of noncoding RNA (ncRNAs). Specifically, aberrations of microRNAs (miRNAs) expression, a major class of ncRNAs, are often observed in CRC and are considered to play a major role in tumorigenesis and CRC progression [23, 53]. These observations are consistent with the fact that miRNAs tend to exert oncogenic or tumor-suppressive effects. For example, miRNAs, such as miR-141, miR-200c, miR-145, miR-373, miR-520c, miR-135a, and miR-135b, have all been shown to affect CRC by regulating epithelial differentiation, WNT signaling, and CRC cell migratory and invasive potential [54]. Other miRNAs implicated in CRC include miRNA-124a and miRNA-34b/34c, which were shown to regulate the cell cycle and TP53 pathway, respectively [55]. Several miRNAs are also associated with epithelial-to-mesenchymal transition (EMT) in CRC. miR-15/16, miR-140, and miR-200 family members were shown to be associated with suppression of EMT and tumor cell metastatic potential while miR-21 enhanced this process [56].

Other studies have highlighted that alterations in the expression pattern of miRNAs in CRC were considered diagnostic, prognostic, or chemosensitivity markers [57]. For instance, high levels of miR-320 and miR-498 were correlated with progression-free survival in stage II CRC [58], while miR-21 abundance was associated with poor patient response to 5-fluorouracil adjuvant chemotherapy [59]. On the other hand, induced suppression of miR-21 promoted the sensitization of CRC cells to chemotherapeutic regimens [60, 61]. Another study by Toiyama et al. demonstrated a correlation between elevated serum miR-200c levels and stage IV CRC compared to earlier stages. Furthermore, high serum miR-200c showed a significantly positive correlation with lymph node metastasis, distant metastasis, and prognosis [62]. A comprehensive look of miRNAs as CRC biomarkers is reviewed by several other sources [60].

There is also ample evidence of miRNAs being downregulated in CRC and thus playing tumor-suppressive roles [63]. Arndt et al. showed that reduced levels of miR-133a as well as enrichment of miR-224 were associated with CRC initiation [64]. Moreover, this study and others also revealed that CRC patients at the adenomatous and carcinoma stages consistently exhibited reduced steady-state levels of miR-143 and miR-145 [63, 64]. Another classic example includes the miR-34 family. Transfection of miR-34a into CRC cells led to induction of apoptosis and inhibition of cell proliferation in part by amplifying the p53-mediated apoptotic response [65, 66]. Intriguingly, p53 has been shown to regulate miR-34a, suggesting a positive feedback loop between the two in which miR-34a could partly mediate the tumor-suppressive roles of p53 [55].

Interestingly, miRNA deregulation can also induce aberrant activity of many of the components of the epigenetic machinery [67]. Take *DNMT3A* for example, which has been identified as a miR-143 target and is associated with CRC via downregulation of miR-143 and subsequent increase in *DNMT3A* expression levels [68]. Other examples include miR-140 and miR-449, which have been shown to target and downregulate HDAC1 and 4, respectively, thus exerting their tumor-suppressive effects [69, 70]. Taken together, these findings underscore the importance of miRNAs in exerting both oncogenic and antitumor roles in CRC, which may serve as the basis for the development of novel prognostic and therapeutic markers.

3. Classification of CRC pathways and subtypes using epigenetic features

The development of CRC occurs via aberrations in multiple genetic and epigenetic pathways. These pathways can be defined by three principal molecular phenotypes (**Figure 1**). As aforementioned in Section 1, these include the MSI phenotype, which is characterized by mutations in DNA MMR genes; the CIN phenotype characterized by mutations in *APC*/*Wnt*/*β-catenin* pathway; and a third CIMP, defined by global CpG island hypermethylation, which results in widespread silencing of tumor suppressor genes [9, 71]. Notably, each pathway is characterized by distinct epigenetically related pathological features that drive the process of tumor initiation and development. In this section, we will describe various epigenetic aspects of these CRC phenotypes as well as how the molecular aspects of each pathway have been employed as useful diagnostic and prognostic tools to guide the clinical management of CRC patients. Finally, we also briefly acknowledge how these pathways may overlap within broader systems of subtype classification and highlight some of the current challenges in precisely defining CRC subtypes.

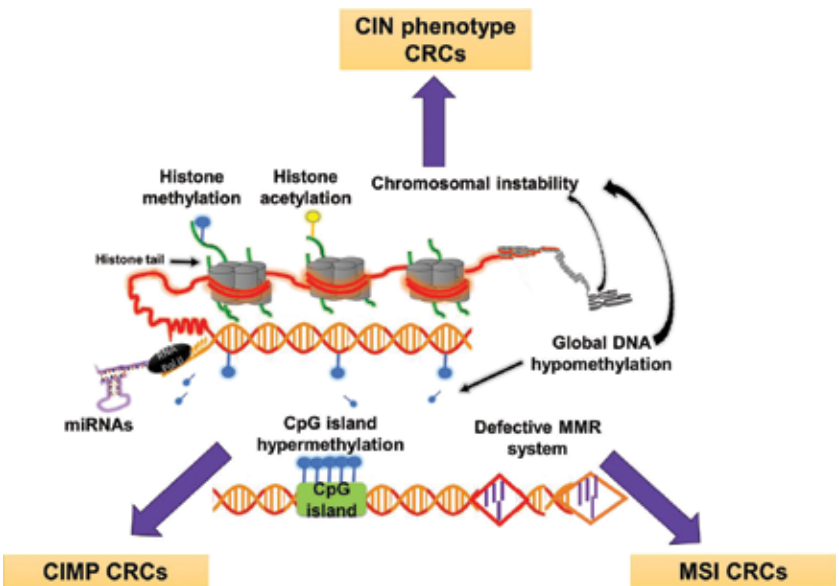

Figure 1.
Schematic depicting epigenetic alterations in CRC and their association with CRC molecular subtypes. The CRC "epigenome" harbors alterations in their histone modification states, particularly regarding aberrant histone methylation (blue dot) and acetylation (yellow dot). These global histone aberrations serve as biomarkers to predict the clinical outcome in CRC patients. DNA methylation is another major epigenetic alteration seen in CRC. Usually, global DNA hypomethylation occurs gradually and early in the process of CRC carcinogenesis. This occurs in concert with systematic and discrete CpG island hypermethylation events at the promoters of genes involved in DNA repair, apoptosis, proliferation, angiogenesis, adhesion, and invasion. This generally results in transcriptional silencing. Aberrations of micro-RNAs (miRNAs) expression, a major class of ncRNAs, are also often observed in CRC and are considered to play a major role in tumorigenesis and CRC progression. This figure also illustrates three main epigenetic-related molecular subtypes of CRC, namely the microsatellite instability (MSI) phenotype, the chromosomal instability (CIN) phenotype, and the CpG island methylator phenotype (CIMP). MSI CRCs arise from a defective DNA mismatch repair (MMR) system, which is associated with frameshift mutations and base pair substitutions in genes. CIMP CRCs are characterized by widespread promoter CpG island hypermethylation, whereas the CIN CRCs arise through widespread imbalances in chromosome number (aneuploidy), global hypomethylation, and loss of heterozygosity (LOH).

3.1 Microsatellite instability phenotype (MSI)

Approximately 15–20% of all CRC cases harbor MSI [72]. Microsatellites are defined as repetitive one to six base pair DNA sequences distributed along coding and noncoding regions of the genome. Importantly, the repetitive nature of these regions makes them particularly susceptible to mismatch errors [72]. Tumors with the MSI phenotype are therefore driven by the inactivation of MMR genes, which are involved in repairing DNA recombination and replication errors as well cellular responses to DNA damage [12]. The net effect of defective MMR machinery is accumulation of single base-pair mismatches, which results in a hypermutable cellular state [72].

MSI tumors are typically classified as MSI-high (MSI-H) or MSI-low (MSI-L). In MSI-H CRCs, usually two or more of the five microsatellite markers show instability, whereas in MSI-L tumors, only one of the five markers shows instability [12]. If none of the markers show instability, however, these are classified as microsatellite stable (MSS) CRC tumors, which account for approximately 80–85% of CRC patients [12]. Although majority of MSI-H tumors sporadically arise, a few are also linked to a familial hereditary syndrome known as Lynch syndrome or hereditary non-polyposis CRC (HNPCC) which account for about 3–5% of all CRC cases [12]. Sporadic MSI tumors are generally affected by epigenetic inactivation at the *MLH1* promoter via CpG island hypermethylation, whereas Lynch syndrome is caused by germline mutations in *MLH1*, *PMS2*, *MSH6*, or *MSH2* [12].

Importantly, unlike MSS, MSI tumors are poorly differentiated and are more often located in the proximal colon. MSI tumors also harbor a mucinous or signet ring type histology and increased number of tumor-infiltrating lymphocytes [72]. Additionally, when MSI was first identified in CRC patients, it was shown that MSI-H patients tended to have a better patient prognosis compared with MSS tumors and had an overall lower tumor stage at diagnosis [12]. Moreover, randomized phase III clinical trials along with several prospective studies have shown that the MMR status of these patients is also predictive of response to adjuvant 5-FU-based chemotherapy [12, 73]. The consensus was that patients with MSI-H tumors did not benefit from adjuvant 5-FU therapy compared to their non-MSI-H counterparts [73]. Furthermore, these data are consistent with other studies that revealed that human CRC cell lines with MSI-H phenotypes displayed resistance to DNA damaging agents, such as 5-FU, which could be overcome by the restoration of normal MMR function [74, 75].

3.2 Chromosomal instability (CIN) phenotype

The acquisition of genomic or chromosomal instability is a key feature in CRC development [39]. In fact, CIN has been found in approximately 85% of CRC cases and is characterized by increased chromosomal losses and gains as well as increased loss of heterozygosity [13, 14]. Although the exact mechanisms underlying CIN remain incompletely understood, it has been attributed to defects in genes related to the DNA damage response, telomere stability, and chromosomal segregation [39]. Unfortunately, standardizing the precise quantitative criteria that define a "CIN-positive" tumor has been challenging due to difficulties in the detection approaches of chromosomal instability [39]. The approaches currently in use include cytometry, karyotyping, and loss of heterozygosity analyses [76].

Along with the typical chromosomal abnormalities, accumulation of a characteristic set of mutations in specific tumor suppressor genes and oncogenes is

also a prominent feature observed in CIN tumors [39]. These include mutations in *APC*, *p53*, Cyclooxygenase-2 (*COX-2*), and *KRAS* as well as 18q alterations [39]. Interestingly, many studies have sought to determine the prognostic value of *KRAS*, *TP53*, or 18q alterations. So far, evidence of increased risk of relapse or mortality in CRC patients with *KRAS* mutations has been presented, but other studies have failed to confirm this correlation [77]. Consequently, some of these putative individual prognostic markers are still undergoing rigorous study. However, several compelling studies indicate that the overall CIN phenotype is associated with a less favorable outcome in patients than those with the MSI phenotype, and unlike MSI tumors, it is not significantly influenced by adjuvant therapy in patients with stage II–III CRC [78].

Several ongoing phase I and II clinical trials are underway to therapeutically target pathways that directly or indirectly initiate and perpetuate CIN. Some of these include small-molecule inhibitors of *COX-2*, *Polo-Like Kinases* (*Plks*), *Eg5*, and *Centromere protein E* (*CENP-E*) [39]. Swanton et al. also showed that CIN-positive tumors are intrinsically resistant to taxanes due to the similarity between pathways that regulate the chromosomal segregation and those implicated in the taxane response [79]. These and other studies have collectively prompted the Chromosomal Instability and Anti-Tubulin Response Assessment (CINATRA) trial to assess whether patients with MSI-positive solid tumors derive benefit from EPO906 (new microtubule stabilizer) compared to patients with CIN-positive cancers [80]. Overall, phase I trials showed encouraging tumor control and response rates in patients with metastatic CRC (mCRC), although the trial was prematurely closed due to toxicity issues [80]. In summary, these data support the role of the CIN pathway in guiding patient stratification and the clinical management of CRC. However, more studies to better define the mechanisms underlying CIN and determine how CIN influences progression will be critical to advance our understanding of the most common form of genetic instability in CRC. Moreover, the feasibility of standardizing detection of CIN-positive tumors and thus be able to target chromosomally unstable cells, will be critical.

3.3 CpG island methylator phenotype (CIMP)

The term "CpG island methylator phenotype," or CIMP, was coined in 1999 by Toyota and Issa to denote the CRC tumor subtypes characterized by widespread promoter DNA hypermethylation at certain tumor suppressor genes [81]. More than 50% of genes have promoters found within CpG islands [44]. Hence, the frequency of CIMP CRCs depends on which promoters are examined for methylation, with some promoters being more beneficial than others for identifying CIMP. Several studies have revealed that this methylation is common at the promoters of a diverse spectrum of genes, including *Phosphatase and tensin homolog* (*PTEN*), *RUNX3*, and *Unc-5 netrin receptor C* (*UNC5C*), making these key genes part of the expression signature profile in the evolution of CIMP CRCs [44]. Moreover, based on a panel of CIMP-specific markers coupled with the *B-Raf proto-oncogene* (*BRAF*) mutational status, CIMP tumors may be further classified according to the fraction of promoters that exceed a certain threshold of DNA methylation as being CIMP high, low, or negative [44]. Although CIMP-high and CIMP-low CRCs are significantly associated with biological sex as well as BRAF and KRAS mutational status, these classifications need additional refinement [44]. Nonetheless, it is reminiscent of the classification of CRCs based on degrees of MSI [82].

Notably, several of the clinicopathological characteristics of CIMP-high tumors have also been correlated to MSI tumors [82, 83]. For example, like MSI, CIMP tumors also represent a clinically distinct group characterized by epigenetic instability, distinct histological and pathological features, and discrete precursor lesions [84].

Pathologically, CIMP tumors also originate similarly to MSI tumors in the proximal colon, with a mucinous and poorly differentiated histological type and are frequently diagnosed in elderly and female patients [84, 85]. However, the determination of which specific methylated loci should be used to define CIMP remains a major challenge in the evaluation of CIMP tumors. Currently, several studies have proposed the classic panel containing the genes *hMLH1* and *p16* as well as Munc-18-interacting (MINT) proteins *MINT1*, *MINT2*, and *MINT31*. This panel has been further developed to contain the genes *Calcium voltage-gated channel subunit alpha1 G* (*CACNA1G*), *Cellular retinoic acid binding protein 1* (*CRABP1*), *IGF2*, *Neurogenin 1* (*NEUROG1*), *RUNX3*, *Suppressor of cytokine signaling 1* (*SOCS1*), *Hypermethylated in cancer 1* (*HIC1*), *IGF-binding protein 3* (*IGFBP3*), and *Werner syndrome ATP-dependent helicase* (*WRN*) [86].

In recent years, the use of DNA hypermethylation of specific genes to predict CRC patient outcome and therapeutic approach has received much attention. Although further validation is warranted, many studies have found a correlation between MSS CIMP+ CRC patients and poor prognosis [87]. Furthermore, the correlation between CIMP status and response to chemotherapy has been investigated. CIMP status predicts poor survival in metastatic MSS CRC patients treated with chemotherapeutic agent 5-FU [88]. Overall, patients who did not receive chemotherapy treatment but had tumors identified as MSS and CIMP had a worse survival outcome [89]. In contrast, two separate studies reported better outcomes for patients with CIMP tumors, a conflict that could be attributed to differences in the criteria used across the studies to define CIMP status. Additionally, this also suggests that the heterogeneous nature of CIMP tumors may warrant further classification [44].

Intriguingly, many studies have also found an association between CIMP status and other important epidemiological factors [90]. For instance, reports of an association between cigarette smoking, obesity, and CIMP showed that the number of cigarettes smoked as well as body mass index (BMI) had a significant relationship to CIMP tumor development [90, 91]. Furthermore, associations of CIMP status with smoking and obesity were evident only for females [90]. Taken together, the above evidences support a critical role of the CIMP pathway in the pathogenesis of CRC, which has also become a significant part of the current management of CRC. In the future, it will also be essential to have a consensus on a standardized panel of loci to define CIMP, similar to that utilized to identify MSI CRCs.

3.4 Consensus molecular subtypes

The three molecular pathways described so far also fall within several consensus classification systems for CRC. These systems vary in terms of the number of proposed subtypes, which can range from three to six depending on the combinations of genetic, epigenetic, clinical, and histopathological parameters used as well as the extent of the overlap between the three molecular pathways. For instance, the Consensus Molecular Subtypes (CMS) consortium has been suggested as one of the most robust classification systems and describes four groups (CMS1–4) based on expression profiling data from multiple studies [92]. While the majority of MSI-H CRCs fall into the CMS1 category, CMS2–4 display higher CIN. However, CMS3 samples have a distinctive profile compared with other CIN tumors. They tend to have lower CIN, higher prevalence of CIMP and close to 30% of the tumors are hypermutated which confers significant overlap with MSI status tumors [93]. Additionally, the Cancer Genome Atlas study also demonstrated that CIMP overlaps with the MSI pathway because of the fact that sporadic MSI-H CRCs usually harbor CIMP-high clinicopathological features [94]. Meanwhile, CMS4 are defined as CIN-heterogeneous tumors with mesenchymal characteristics that occur in later disease stages [93].

It is also noteworthy that many CRC tumors demonstrate mixed characteristics compatible with two or more of these subtypes, which may represent a transition phenotype or intratumoral heterogeneity, while others cannot be precisely classified into any of these pre-defined subgroups [95]. Furthermore, these classifications often lack incorporation of the molecular markers used for traditional TNM staging of CRC [96]. Taken together, these challenges as well as the existing incongruity between the various systems illustrate the need to further refine these consensus classifications by developing more progressive and integrated approaches.

4. The role of major epigenetic enzymes in CRC and therapeutic strategies for targeting them

4.1 Histone methyltransferases and demethylases

As discussed so far, aberrant changes in epigenetic modifications can significantly contribute to CRC progression. It is therefore unsurprising that many of the epigenetic enzymes mediating these modifications are themselves deregulated during the initiation and progression of CRC. Here, we describe the significance of changes in the expression levels of two such families of enzymes that oppose each other in terms of function, namely histone methyltransferases (HMTs) and demethylases (HDMs). Although changes in the expression or activity levels of several methylation-related enzymes have been linked to CRC, in most cases only a limited knowledge regarding the molecular mechanisms by which these enzymes contribute to disease development exists [15]. We summarize current knowledge regarding some of the preclinical validated implications of these enzymes as proof of principle for the employment of epigenetic agents in CRC. We also briefly discuss potential mechanisms of action of these enzymes as well as the advantages of targeting them using combinatorial over monotherapy approaches.

Histone lysine methyltransferases (HKMTs) have been widely studied across multiple solid tumor types including CRC [97]. For instance, studies in a preclinical model of CRC found that increased expression and activity of *SET and MYND domain containing 3* (*SMYD3*), a well-known HKMT, was strongly correlated with tumorigenesis. Moreover, RNAi-mediated depletion of *SMYD3* significantly impaired CRC cell proliferation, indicating a crucial role of *SMYD3* in maintaining CRC malignancy [98]. More recent studies suggest a putative mechanism by which this overexpression might occur by demonstrating that hypomethylation of the *SMYD3* promoter was observed in CRC tumor tissues compared to adjacent normal tissues. Further subgroup clinicopathological analyses showed that this hypomethylation was observed with stage III and IV tumors as defined by moderate to well-differentiated histology and positive lymph node metastasis [99].

Another well-studied HKMT, *enhancer of zeste 2* (*EZH2*), is also frequently deregulated in CRC. Both mRNA and protein levels of *EZH2* were found to be significantly increased in CRC tissues compared to non-cancerous counterparts [16]. Additionally, increased *EZH2* expression was directly correlated with tumor size, metastases, and overall worse disease-free survival of CRC patients [100]. He et al. also showed that siRNA-mediated depletion of *EZH2* inhibited the proliferation and migration of SW620 CRC cells, while inducing apoptosis and G0/G1 cell cycle arrest [101]. Another mechanistic study also revealed that knockdown of *EZH2* significantly reduced CRC cell invasion and *matrix metalloproteinases 2/9* (*MMP2/9*) secretion *in vitro* while promoting increased overall survival and decreased lung metastasis *in vivo* [102]. Furthermore, this *EZH2*-induced CRC cell invasion was mediated by direct binding of *Signal transducer and activator of transcription 3*

(*STAT3*) to the *EZH2* promoter, resulting in downregulation of the vitamin D receptor (VDR) [102]. Interestingly, an association between a missense variant in *EZH2* and risk of CRC was discovered by the Li group. They identified that the presence of the rs2302427 variant showed a significant association with increased CRC susceptibility [103]. Recent studies point to other mechanistic roles of HKMTs in CRC. For example, depletion of *SETD1A*, a member of the trithorax (TrxG) family of HMTs, inhibited CRC cell growth and colony formation in part by decreasing expression of approximately 50% of *Wnt/β-catenin* target genes [28]. Finally, in a mouse model, IL-22-mediated activation of *disruptor of telomeric silencing 1-like* (*DOT1L*) promoted CRC stemness and tumorigenic potential and was considered a predictor of poor survival outcome in CRC patients [32].

Protein arginine methyltransferases (PRMTs), although studied to a lesser extent, have also been shown to play critical roles in CRC malignancy via activation of *Wnt/β-catenin* and NF-κB signaling [104]. *CARM1*, for example, is an important positive modulator of *Wnt/β-catenin* transcription and was found to promote survival and anchorage-independent growth of CRC cells with aberrantly activated *Wnt/β-catenin* signaling [105]. Meanwhile, our lab and others have shown that *protein arginine methyltransferase 5* (*PRMT5*) was overexpressed in CRC cells and patient-derived primary tumors, which correlated with increased cell growth, migration, invasion, and NF-κB activation as well decreased overall patient survival [106–109]. The enzymes catalyzing removal of methylation marks, HDMs, are perhaps the least studied among the enzymes mentioned thus far and only a few have been implicated as playing tumor suppressive or oncogenic roles in CRC. *LSD1*, *KDM4B*, *KDM4C*, and *KDM5B* have all been shown to play pro-tumorigenic roles by promoting CRC cell growth and metastasis, whereas HDMs, such as *JMJD3* and *JMJD1B*, have been implicated as tumor suppressors [15]. Taken together, these data provide strong support for the continued development of selective and potent small-molecule inhibitors against these methylation-modifying enzymes as promising therapeutic agents for CRC.

4.2 Targeting HMTs and HDMs in CRC

Disruption of epigenetic regulation in CRC mediated by deregulated HMTs, DNMTs, and HDMs has garnered increasing interest in recent years. In this section, we aim to review the current status on the development of therapeutic strategies to modulate histone methylation for CRC treatment. The current therapeutics including pre-clinical and clinical agents that target epigenetic enzymes in CRC are listed in **Table 1**. Thus far, more than 20 histone-methylation enzymes have been found to be clinically relevant to CRC, including 17 oncoproteins and 8 tumor suppressors, although their exact mechanisms of action are not fully understood [15]. Furthermore, more than 20 small-molecule inhibitors targeting HMTs, DMNTs, and HDMs have been employed for preclinical or clinical studies. For example, treatment of DLD1 colon cell line and primary CRC cells with a potent HKMT inhibitor EPZ004777 (anti-*DOT1L*) resulted in significant reduction in sphere formation *in vitro*, thus inhibiting cell growth [32]. Other HKMT inhibitors, such as BCI-121 and Chaetocin, have significantly suppressed CRC cell growth and migration by inhibiting *SMYD3* and *SUV39H1*, respectively [98]. Notably, inhibitors against the HKMT *EZH2* have yielded some of the most promising results for treating CRC. DZNep, an indirect *EZH2* inhibitor, induced apoptosis in CRC cell lines and stem cells, while GSK346 impaired the migratory potential of CRC cells and reduced H3K27me3 levels in Colo205 and HT-29 cells (**Table 1**) [110].

Unlike HKMTs, the development of inhibitors against PRMTs has only recently gained prominence in the cancer field, and only a couple of these have made it

Epigenetic drug class	Drug & Target	Description of study design	Agent used in combination with epigenetic drug
DNMT inhibitors	5-Azacitidine: DNMT1	Phase I in solid tumors including mCRC	Erlotinib [20]
		Phase I/II in mCRC	Capecitabine [20]
			Oxaliplatin [20]
	Decitabine: DNMT1	Preclinical and Phase I in solid tumors including mCRC	Carboplatin [20]
			Gefitinib [115]
			Panitumumab [20]
	5-fluoro-2'-deoxycytidine: DNMTs	Phase I in solid tumors including mCRC	Tetra-hydrouridine (THU)[20]
	Guadecitabine: DNMTs	Ongoing Phase II in mCRC [NCT01896856]	Irinotecan [20]
HMT inhibitors	EPZ004777: DOT1L	Preclinical in CRC cells and mouse xenografts	Unknown [32]
	DZNep: EZH2	Preclinical in CRC cells and mouse xenografts	Unknown [101]
	GSK346: EZH2	Preclinical in CRC cells and mouse xenografts	Unknown [110]
HDAC inhibitors	CI-994: HDAC 1, 2, 3, and 8	Phase I in advanced solid tumors including mCRC	Capecitabine [20]
	Entinostat: HDAC1 and HDAC2	Phase I in advanced solid tumors including mCRC	13-cis retinoic acid [20]
			Sorafenib [20]
	Panobinostat: HDAC	Phase I in advanced solid tumors including mCRC	Bevacizumab [20]
	Pheniylbutyrate: HDAC	Phase I in mCRC	5-FU [20]
	Vorinostat: HDAC, HDAC1, HDAC3	Phase I/II in solid tumors including mCRC	Modified FOLFOX6 or Bortezomib [20, 135]
			Pazopanib [20]
			FOLFOX [135]
			Doxorubicin [134, 135]
			Hydroxychloroquine or Regorafenib [134, 135]
	ACY-1215: HDAC6	Preclinical in CRC cells and mouse xenografts	Oxaliplatin [129]
	CG2: HDAC	Preclinical in CRC cells and mouse xenografts	Oxaliplatin 5-FU Irinotecan [128]

Table 1.
Overview of pre-clinical and clinical drugs that target epigenetic enzymes in CRC.

to the clinical trial phase thus far. AMI-1, which inhibits *PRMT1* and *PRMT5*, demonstrated antiproliferative activity in CRC cells and xenograft mouse models [106]. However, further *in vivo* validation studies are needed, and it has not

entered clinical trial yet. Another promising *PRMT5* inhibitor that recently made it to Phase I clinical trials is GSK3326595, which potently inhibited tumor growth *in vitro* and *in vivo* [111]. Trials with GSK3326595 are currently being conducted in adult subjects with relapsed and/or refractory solid tumors (NCT02783300). Additionally, inhibitors targeting HDMs are even fewer in number and have shown limited efficacy in suppressing CRC cell growth. For example, *KDM4A/C* inhibitors were ineffective in blocking HCT116 CRC cell growth when used in isolation [112]. However, they exhibited potent antiproliferative effects in combination with another HDM inhibitor, NCL-2, which targets *LSD1* [113]. These data suggest a potential for synergy between the two classes of HDM inhibitors.

Finally, the use of DNMT inhibitors for CRC treatment has also shown some exciting promise. In studies using CRC cell lines, suppression of *DNMT1* and *DNMT3B* resulted in significant reduction in methylation, which correlated with the re-expression of tumor suppressor genes. This also resulted in induction of apoptosis as well as reduced cell proliferation and stemness [114]. Notably, studies with the *DNMT1* inhibitor, 5-aza-2′-deoxycytidine (decitabine), exhibited its ability to re-sensitize colorectal tumors to both irinotecan and 5-FU, thus becoming a major component of the treatment regimen for CRC in the clinic [19]. Another recent preclinical study showed that combination of the anti-*EGFR* inhibitor, gefitinib and decitabine showed highly synergistic inhibition of CRC cell proliferation and migration [115]. Additional combination regimens are outlined in **Table 1**.

4.3 Acetyltransferases and deacetylases

Acetylation of histones by acetyltransferases (HATs) and removal of these acetyl marks by HDACs are essential events for the maintenance of normal chromatin organization and function [116]. However, as is often the case in cancer, these enzymes are dysregulated, leading to increased chromosomal instability and aberrant gene expression changes [117]. To date, only a handful of HATs have been reported as contributing to the pathogenesis of CRC. Here, we describe the role of a few of these HATs namely *p300/CREB-binding protein* (*p300/CBP*), *GCN5*, *N-Acetyltransferase 10* (*Nat10*), and *Human males absent on the first* (*hMOF*). Assessment of 262 CRC samples from patients receiving 5-FU treatment demonstrated that low expression of *p300/CBP* in CRC tissue was closely associated with poor clinical response to 5-FU based-chemotherapy [118]. Furthermore, low *p300/CBP* expression also correlated with poor disease-free survival and increased early disease progression in the same patients [118]. Mechanistic studies also uncovered that 5-FU induced degradation of *p300/CBP* which was dependent on chaperone-mediated autophagy involving *heat-shock cognate protein 70 kDa* (*hsc70*) and *lysosomal-associated membrane protein 2A* (*LAMP2A*). In short, degradation of *p300/CBP* was found to be relevant to chemoresistance to 5-FU, since blocking this degradation also enhanced 5-FU's cytotoxicity in CRC cells [118].

Conversely, another HAT *GCN5* has been implicated in promoting CRC cell growth via its upregulation rather than downregulation. One study found that *GCN5* overexpression in human colon adenocarcinoma tissues was attributed to the activities of the transcription factors, *c-Myc* and *E2F transcription factor 1* (*E2F1*) [119]. Depletion of *c-Myc* inhibited CRC cell proliferation mainly by downregulating GCN5 transcription, an effect that was rescued by ectopic expression of *GCN5*. However, ectopic overexpression of *E2F1* had the opposite effect by suppressing *GCN5* levels, thus inducing cell death. Furthermore, inhibition of *GCN5* with CPTH2, a HAT inhibitor, also suppressed CRC cell growth, revealing an avenue of great therapeutic potential [119]. Other HATs implicated in CRC include *Nat10* and *hMOF*, which were downregulated in CRC tissues. Particularly, recent studies

showed that *Nat10* downregulation and subcellular redistribution were associated with increased cellular motility and invasion in CRC cells [120]. Meanwhile, low expression of *hMOF* correlated with clinicopathological features of CRC such as lymph node metastasis and advanced tumor stage [121].

In CRC, HDACs are also frequently overexpressed and represent another attractive class of targets for anticancer therapy. *HDAC1–3* and *HDAC5–8* have emerged as some of the most relevant deacetylases in CRC. Although all are highly overexpressed in CRC, only few studies have explored the relevance of this overexpression to disease [23]. For example, knockdown of *HDAC1*, *2*, and *3* reduced the growth of several CRC cells by largely unknown mechanisms [122]. Interestingly however, a mechanistic link between *HDAC2* expression and sensitivity of CRC cells to other anticancer agents was recently established. Alzoubi et al. demonstrated that depletion of *HDAC2* specifically enhanced the combined anti-tumor effect of the pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and the DNA-damaging agents, 5-FU and oxaliplatin, in SW480 and HT29 cells. On the other hand, overexpression of *HDAC2* conferred resistance to these agents, which were independent of the p53 mutational status [123]. In summary, these findings strongly suggest that HATs and HDACs are critical biomarkers for CRC and influence the sensitivity of CRC cells to certain therapeutics as evidenced by their frequent combination with other anticancer agents (**Table 1**).

4.4 Targeting HATs and HDACs in CRC

Like methylation, several studies have demonstrated that inhibitors targeting HATs and HDACs also induce epigenetic alterations that modulate the expression of genes or pathways critical for CRC treatment. One study showed that direct inhibition of *p300/KAT3B* histone acetyltransferase, a coactivator of *β-Catenin* with rimonabant, induced downregulation of *Wnt/β-catenin* target genes in HCT116 cells [124]. Furthermore, rimonabant also significantly reduced tumor growth in HCT116 xenografts [124]. The general HAT inhibitors such as garcinol and anacardic acid have also been shown to inhibit CRC tumor growth in mice as well as sensitize cancer cells to irradiation [125].

Compared to HATs, a far greater number of studies have been dedicated to investigating the efficacy of HDAC inhibitors at both the preclinical and clinical level. Overall, the use of HDAC inhibitors in preclinical solid tumor models has shown some early promise albeit their progress to the clinic has been hindered by serious limitations including ineffective concentrations and cardiac toxicity [126]. In CRC specifically, these inhibitors are mainly being administered as combination therapy with conventional chemotherapy or other agents [127]. In pre-clinical models for instance, treatment of irinotecan-resistant CRC cells with HDAC inhibitors conferred sensitization of these cells to irinotecan, whereas HDAC inhibitor CG2 showed an additive effect when used with irinotecan, 5-FU, or oxaliplatin in HCT116 xenografts [128]. Meanwhile, a small molecular inhibitor of *HDAC6*, ACY-1215, was able to enhance the anticancer activity of oxaliplatin by promoting apoptosis and blocking cell proliferation in CRC cells and xenograft models [129].

4.5 Benefits and pitfalls of epigenetic enzyme inhibitors

Despite their potential, a large gap still remains between the biological activity of epigenetic enzyme inhibitors in preclinical studies and their potential clinical utility. For example, the development of HAT inhibitors poses several challenges [130]. Because of their function in complexes consisting of many proteins which play multiple roles in HAT target specificity, this significantly limits inhibitor use *in vivo* [130].

Additionally, many undesired effects such as reactivity or lack of selectivity between different HAT subtypes are often associated with HAT inhibitors [130]. Hence, studies geared towards the development of more potent and selective inhibitors by carefully studying the catalytic mechanism and enzyme kinetics of various HATs are needed. As far as HDAC inhibitors are concerned, they have shown preferential efficacy against hematological malignancies, and therefore, drugs such as vorinostat (SAHA) and romidepsin (FK228) have achieved FDA approval for the treatment of cutaneous T cell lymphoma [131, 132]. Unfortunately, the evidence regarding HDAC inhibitors efficacy for solid tumors has not been as convincing and encouraging although they are well tolerated at low but not high doses. Currently, adverse side effects and inadequate clinical efficacy are the major limitations to their use, and more efforts are underway to generate specific HDAC inhibitors for solid tumors such as CRC [133]. Nonetheless, a few early phase clinical trials using vorinostat in combination with other chemotherapeutic agents have shown some early promise for mCRC patients. These include combinatorial regimens of vorinostat with 5-FU, leucovorin, and oxaliplatin (FOLFOX) as well as randomized phase II trial studies investigating the efficacy of vorinostat and hydroxychloroquine or regorafenib in refractory mCRC patients [134, 135]. Other regimens are outlined in **Table 1**. Similarly, while DNMT inhibitors have also met with some degree of success for treating blood cancers such as myelodysplastic syndrome (e.g., decitabine and 5-azacitidine), the major drawbacks of these compounds in solid tumors are harsh side effects and transient demethylation, which revert after drug removal [136, 137]. Interestingly, however, some studies have suggested that this transient demethylation that occurs with DNMT inhibition (e.g., 5-azadeoxycytidine) potentially creates a therapeutic window that can be leveraged for epigenetic reprogramming and/or combinatorial therapies with cytotoxic drugs [138].

Other general limitations regarding the use of epigenetic therapy in solid tumors deal with the unfavorable pharmacokinetic properties of these drugs, including instability, toxicity, and short half-life [137]. Some of these invariably contribute in some way to common toxicities associated with HDAC and DNMT inhibitors in CRC including thrombocytopenia, neutropenia, diarrhea, nausea, vomiting, and fatigue [139]. Furthermore, maintaining therapeutically relevant levels of the drugs necessary for clinical benefit is particularly difficult, and as of yet, no FDA-approved epigenetic treatments exist for CRC despite promising preclinical studies. This signifies the overall marginal clinically compelling responses to these agents in CRC patients. To overcome some of these limitations, newer formulations have been made to render these inhibitors more bioavailable, stable, and ultimately usable at lower doses with less toxicity and greater therapeutic efficacy. Examples of these include the oral HDAC inhibitor, entinostat, used in *in vitro* and *in vivo* models of CRC and an orally active formulation of 5-azacitidine, cc-486 [140].

4.6 Emerging immunomodulatory and epigenetic combinatorial therapies

Compared to MSS tumors, there are an exponentially higher number of mutations acquired in MSI-H CRCs. Interestingly, these mutations have the potential to elevate the production of neo-antigens [141]. The result is increased tumor immunogenicity, which is further complemented by the fact that these tumors also harbor a high number of tumor-infiltrating lymphocytes. Within this context, CRC patients with MSI-H represent a subgroup more likely to benefit from immune checkpoint inhibitors compared to those with MSS tumors. Immune checkpoint inhibitors have shown unprecedented benefit across multiple tumor types. These agents specifically target the proteins *programmed death ligand-1* (e.g., durvalumab) and *programmed death-1* (e.g., nivolumab, pembrolizumab) and are administered

as monotherapies or in combination with other anticancer agents. At the present time, several ongoing early and late-phase II and III clinical trials investigating the efficacy of immune checkpoint inhibitors in MSI-H and MSS CRC patients are being extensively explored including pembrolizumab (Keytruda), which recently obtained FDA approval (e.g., NCT01876511 and NCT02060188) [142].

Moreover, the possibility of combining epigenetic therapy and immunotherapy has also been recently explored, and several ongoing clinical trials in CRC investigating the combination strategies of HDACi and DNMTi with checkpoint inhibitors have been undertaken. Specifically, these epigenetic therapies have been shown to augment the effect of checkpoint inhibitors and are currently in early and late phase clinical trials [143]. However, since MSS subtypes represent the larger fraction of CRC cases, the marginal activity displayed by drugs such as pembrolizumab for treating MSS CRCs has been less than encouraging [144]. Hence, overcoming the clinical ineffectiveness of this class of drugs for this subtype remains an important need. Intriguingly, however, recent studies showed that treatment with 5-azacitidine and entinostat in CRC cell lines conferred a shift towards a CIMP+ signature, which would predictively convert them into a more immunogenic state [145]. This increased sensitivity to immunotherapy has prompted a clinical trial evaluating this strategy, with the combination regimen of romidepsin (HDAC inhibitor) and cc-486 with pembrolizumab in MSS-CRC patients (NCT02512172) [145]. Finally, romidepsin was also found to potentiate 5-FU cytotoxicity in HCT-116, HT29, and SW48 cells by inducing apoptosis and cell cycle arrest [146]. Interestingly, MHC class II gene expression was also induced with this combination, once again supporting the possible cooperation of epigenetic therapy with immunomodulatory agents [146]. In summary, the above evidences support a cooperative role between epigenetic and immune therapies, although further efforts to optimize the epigenetic control of immune-related gene expression will be necessary to successfully translate these notions to the clinic.

5. Conclusion and perspectives

In this chapter, we have highlighted the pivotal contribution of epigenetic deregulation, specifically, DNA methylation, histone alterations, and miRNAs to the initiation, progression, and prognosis of CRC. We also underscored the relevance of these epigenetic mechanisms in terms of classifying CRC subtypes as well as their importance in guiding strategies for therapeutic intervention. Moreover, we emphasized the epigenetic enzymes that are involved in these aberrant pathways and presented some up-to-date findings on pre-clinical and clinical trials of epigenetic drugs used as single agents or in combination with conventional anticancer agents in CRC. Furthermore, mounting evidence demonstrates that epigenetic drugs are also capable of altering the immunogenicity of the CRC microenvironment and creating opportunities for potentiating the effects of immune checkpoint inhibitors.

Understandably, drugs targeting the cancer epigenome are also plagued with major challenges including lack of specificity, toxicity, and short half-life. Fortunately, these challenges have facilitated re-evaluation of the dosing and formulation strategies for epigenetic drugs, leading to superior therapeutic drugs with lower toxic profiles. Another underexplored avenue includes targeting less commonly manipulated epigenetic mechanisms such as the use of miRNA mimics [147]. Furthermore, in light of the advent of personalized therapies, more intricate studies are also needed to elucidate the relationship between individual driver genetic mutations and epigenetic alterations, thus providing a pathway-driven basis for developing selective therapeutic strategies. This may call for a more stringent control of gene expression in CRC cells via selective targeting of epigenetic regulatory

enzymes. This includes the prospects of CRISPR/Cas9/Cas13-based genome and RNA editing, which may provide validated starting points for further development towards novel CRC therapeutic agents [148].

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Abbreviations

APAK	ATM and p53-associated KZNF protein
APC	adenomatous polyposis coli
ASC/TMS1 or PYCARD	apoptosis-associated speck-like protein containing a CARD
BET	bromodomain and extra-terminal motif
BMI	body mass index
BRAF	B-Raf proto-oncogene, serine/threonine kinase
CACNA1G	calcium voltage-gated channel subunit alpha1 G
CARM1	coactivator-associated arginine methyltransferase 1
CDKN2A/p16^{INK4a}	cyclin-dependent kinase inhibitor 2A
CENP-E	centromere protein E
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
COX-2	cyclooxygenase-2
CRABP1	cellular retinoic acid binding protein 1
CRC	colorectal cancer
DAP-kinase	death-associated protein kinase 1
DNMT	DNA methyltransferase
DNMT3A	DNA methyltransferase 3A
DOT1L	disruptor of telomeric silencing 1-like
E2F1	E2F transcription factor 1
Eg5	kinesin 5 family member
EMT	epithelial-to-mesenchymal transition
EZH2	enhancer of zeste 2
FDA	Food and Drug Administration
5-FU	5-fluorouracil
GATA4	GATA-binding protein 4
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDM	histone demethylase
HIC1	hypermethylated in cancer 1
HKMT	histone lysine methyltransferase
HKMTs	histone lysine methyltransferases
hMLH1	human mutL homolog 1
hMOF	human males absent on the first
HMT	histone methyltransferase

Hnf1b	HNF1 homeobox B
HNPCC	hereditary non-polyposis colon cancer
HS3ST2 (3OST2)	heparan sulfate-glucosamine 3-sulfotransferase 2
hsc70	heat-shock cognate protein 70 kDa
IGF2	insulin-like growth factor 2
IGFBP3	insulin-like growth factor binding protein 3
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
KDM	lysine demethylase
KRAS	Kirsten rat sarcoma 2 viral oncogene
LAMP2A	lysosomal-associated membrane protein 2A
LOH	loss of heterozygosity
mCRC	metastatic colorectal cancer
MGMT	O-6-methylguanine-DNA methyltransferase
MINT	Munc-18-interacting
miRNA	microRNA
MLH1	mutL homolog 1
MSH2, 6	MutS protein homolog 2, 6
MSI	microsatellite instability
MSS	microsatellite stable
Nat10	N-acetyltransferase 10
NEUROG1	neurogenin 1
nRNA	noncoding RNA
p300/CBP	p300/CREB-binding protein
p53 or TP53	tumor protein 53
PCDH10	protocadherin 10
PD-1	programmed death-1
PDL-1	programmed death ligand-1
Plks	polo-like kinases
PMS2	PMS1 homolog 2, mismatch repair system component
PRMT	protein arginine methyltransferase
PRMT5	protein arginine methyltransferase 5
PTEN	phosphatase and tensin homolog
RASSF1A	ras association domain family member 1
RASSF2A	ras association domain family member 2
RUNX3	runt-related transcription factor 3
SAHA	suberoylanilide hydroxamic acid
SETD1A	SET domain-containing protein 1A
SFRP1	secreted frizzled related protein 1
SFRP5	secreted frizzled related protein 5
SMYD3	SET and MYND domain containing 3
SOCS1	suppressor of cytokine signaling 1
SOX17	SRY-Box 17
STAT3	signal transducer and activator of transcription 3
SUV39H1	suppressor of variegation 3–9 homolog 1
TIMP3	tissue inhibitor of metalloproteinases 3
TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2
TNF-α	tumor necrosis factor alpha
UNC5C	Unc-5 netrin receptor C
Wif-1	WNT inhibitory factor 1
WNT	wingless type
WRN	Werner syndrome ATP-dependent helicase

Author details

Antja-Voy Hartley[1], Matthew Martin[1] and Tao Lu[1,2,3]*

1 Department of Pharmacology and Toxicology, Indiana University School of Medicine, USA

2 Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, USA

3 Department of Medical and Molecular Genetics, Indiana University School of Medicine, USA

*Address all correspondence to: lut@iupui.edu

References

[1] Arnold M et al. Global patterns and trends in colorectal cancer incidence and mortality. Gut. 2017;**66**(4):683-691

[2] Vaiopoulos AG, Athanasoula KC, Papavassiliou AG. Epigenetic modifications in colorectal cancer: Molecular insights and therapeutic challenges. Biochimica et Biophysica Acta (BBA)—Molecular Basis of Disease. 2014;**1842**(7):971-980

[3] Bardhan K, Liu K. Epigenetics and colorectal cancer pathogenesis. Cancers. 2013;**5**(2):676-713

[4] Sjoblom T et al. The consensus coding sequences of human breast and colorectal cancers. Science. 2006;**314**(5797):268-274

[5] Migheli F, Migliore L. Epigenetics of colorectal cancer. Clinical Genetics. 2012;**81**(4):312-318

[6] Lao VV, Grady WM. Epigenetics and colorectal cancer. Nature Reviews. Gastroenterology & Hepatology. 2011;**8**(12):686-700

[7] van Engeland M et al. Colorectal cancer epigenetics: Complex simplicity. Journal of Clinical Oncology. 2011;**29**(10):1382-1391

[8] Datta K, Kolaja K. Epigenetics: Druggable targets for anti-cancer therapies. Current Opinion in Toxicology. 2017;**6**:79-86

[9] Inamura K. Colorectal cancers: An update on their molecular pathology. Cancers. 2018;**10**(1):26

[10] Gavin PG et al. Colon cancer mutation: Prognosis/prediction—Response. Clinical Cancer Research. 2013;**19**(5):1301

[11] Kawakami H, Zaanan A, Sinicrope FA. Microsatellite instability testing and its role in the management of colorectal cancer. Current Treatment Options in Oncology. 2015;**16**(7):30

[12] Kawakami H, Zaanan A, Sinicrope FA. MSI testing and its role in the management of colorectal cancer. Current Treatment Options in Oncology. 2015;**16**(7):30-30

[13] Comprehensive molecular characterization of human colon and rectal cancer. Nature. Cancer Genome Atlas Network; 2012;**487**(7407):330-337

[14] Carethers JM, Jung BH. Genetics and genetic biomarkers in sporadic colorectal cancer. Gastroenterology. 2015;**149**(5):1177-1190.e3

[15] Huang T et al. Targeting histone methylation for colorectal cancer. Therapeutic Advances in Gastroenterology. 2017;**10**(1):114-131

[16] Liu YL et al. Expression and clinicopathological significance of EED, SUZ12 and EZH2 mRNA in colorectal cancer. Journal of Cancer Research and Clinical Oncology. 2015;**141**(4):661-669

[17] Stypula-Cyrus Y et al. HDAC up-regulation in early colon field carcinogenesis is involved in cell tumorigenicity through regulation of chromatin structure. PLoS One. 2013;**8**(5):e64600

[18] Mottamal M et al. Histone deacetylase inhibitors in clinical studies as templates for new anticancer agents. Molecules. 2015;**20**(3):3898-3941

[19] Flis S, Gnyszka A, Flis K. DNA methyltransferase inhibitors improve the effect of chemotherapeutic agents in SW48 and HT-29 colorectal cancer cells. PLoS One. 2014;**9**(3):e92305

[20] Baretti M, Azad NS. The role of epigenetic therapies in colorectal

cancer. Current Problems in Cancer. 2018;**42**(6):530-547

[21] Bhome R et al. The colorectal cancer microenvironment: Strategies for studying the role of cancer-associated fibroblasts. Methods in Molecular Biology. 2018;**1765**:87-98

[22] Dunn J, Rao S. Epigenetics and immunotherapy: The current state of play. Molecular Immunology. 2017;**87**:227-239

[23] Jia Y, Guo M. Epigenetic changes in colorectal cancer. Chinese Journal of Cancer. 2013;**32**(1):21-30

[24] Tamagawa H et al. The global histone modification pattern correlates with overall survival in metachronous liver metastasis of colorectal cancer. Oncology Reports. 2012;**27**(3):637-642

[25] Gezer U et al. Characterization of H3K9me3- and H4K20me3-associated circulating nucleosomal DNA by high-throughput sequencing in colorectal cancer. Tumour Biology. 2013;**34**(1):329-336

[26] Benard A et al. Histone trimethylation at H3K4, H3K9 and H4K20 correlates with patient survival and tumor recurrence in early-stage colon cancer. BMC Cancer. 2014;**14**:531

[27] Kodach LL et al. The role of EZH2 and DNA methylation in the silencing of the tumour suppressor RUNX3 in colorectal cancer. Carcinogenesis. 2010;**31**(9):1567-1575

[28] Salz T et al. hSETD1A regulates Wnt target genes and controls tumor growth of colorectal cancer cells. Cancer Research. 2014;**74**(3):775-786

[29] Lu Y et al. Silencing of the DNA mismatch repair gene MLH1 induced by hypoxic stress in a pathway dependent on the histone demethylase LSD1. Cell Reports. 2014;**8**(2):501-513

[30] Yokoyama Y et al. Cancer-associated upregulation of histone H3 lysine 9 trimethylation promotes cell motility in vitro and drives tumor formation in vivo. Cancer Science. 2013;**104**(7):889-895

[31] Olcina MM et al. H3K9me3 facilitates hypoxia-induced p53-dependent apoptosis through repression of APAK. Oncogene. 2016;**35**(6):793-799

[32] Kryczek I et al. IL-22(+)CD4(+) T cells promote colorectal cancer stemness via STAT3 transcription factor activation and induction of the methyltransferase DOT1L. Immunity. 2014;**40**(5):772-784

[33] Shah MA et al. A global assessment of cancer genomic alterations in epigenetic mechanisms. Epigenetics & Chromatin. 2014;**7**(1):29

[34] Li F et al. The histone mark H3K36me3 regulates human DNA mismatch repair through its interaction with MutSalpha. Cell. 2013;**153**(3):590-600

[35] Richon VM et al. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proceedings of the National Academy of Sciences of the United States of America. 2000;**97**(18):10014-10019

[36] Karczmarski J et al. Histone H3 lysine 27 acetylation is altered in colon cancer. Clinical Proteomics. 2014;**11**(1):24-24

[37] Guo Y et al. The epigenetic effects of aspirin: The modification of histone H3 lysine 27 acetylation in the prevention of colon carcinogenesis in azoxymethane- and dextran sulfate sodium-treated CF-1 mice. Carcinogenesis. 2016;**37**(6):616-624

[38] Suzuki K et al. Global DNA demethylation in gastrointestinal cancer

is age dependent and precedes genomic damage. Cancer Cell. 2006;**9**(3):199-207

[39] Pino MS, Chung DC. The chromosomal instability pathway IN colon cancer. Gastroenterology. 2010;**138**(6):2059-2072

[40] Wu C, Bekaii-Saab T. CpG Island methylation, microsatellite instability, and BRAF mutations and their clinical application in the treatment of colon cancer. Chemotherapy Research and Practice. 2012;**2012**:359041

[41] Hinoue T et al. Genome-scale analysis of aberrant DNA methylation in colorectal cancer. Genome Research. 2012;**22**(2):271-282

[42] Brenet F et al. DNA methylation of the first exon is tightly linked to transcriptional silencing. PLoS One. 2011;**6**(1):e14524

[43] Ashktorab H et al. Toward a comprehensive and systematic methylome signature in colorectal cancers. Epigenetics. 2013;**8**(8):807-815

[44] Mojarad EN et al. The CpG island methylator phenotype (CIMP) in colorectal cancer. Gastroenterology and Hepatology From Bed to Bench. 2013;**6**(3):120-128

[45] Silva TD et al. DNA methylation as an epigenetic biomarker in colorectal cancer. Oncology Letters. 2013;**6**(6):1687-1692

[46] Galamb O et al. Aberrant DNA methylation of WNT pathway genes in the development and progression of CIMP-negative colorectal cancer. Epigenetics. 2016;**11**(8):588-602

[47] Riojas MA et al. Methylation-induced silencing of ASC/TMS1, a pro-apoptotic gene, is a late-stage event in colorectal cancer. Cancer Biology & Therapy. 2007;**6**(11):1710-1716

[48] Zhang W et al. Epigenetic inactivation of the canonical Wnt antagonist SRY-box containing gene 17 in colorectal cancer. Cancer Research. 2008;**68**(8):2764-2772

[49] Lee BB et al. Aberrant methylation of APC, MGMT, RASSF2A, and Wif-1 genes in plasma as a biomarker for early detection of colorectal cancer. Clinical Cancer Research. 2009;**15**(19):6185-6191

[50] Draht MXG et al. Prognostic DNA methylation markers for sporadic colorectal cancer: A systematic review. Clinical Epigenetics. 2018;**10**(1):35

[51] Toth K et al. Detection of methylated SEPT9 in plasma is a reliable screening method for both left- and right-sided colon cancers. PLoS One. 2012;**7**(9):e46000

[52] Mojtabanezhad Shariatpanahi A et al. The importance of stool DNA methylation in colorectal cancer diagnosis: A meta-analysis. PLoS One. 2018;**13**(7):e0200735

[53] Orang AV, Barzegari A. MicroRNAs in colorectal cancer: From diagnosis to targeted therapy. Asian Pacific Journal of Cancer Prevention. 2014;**15**(17):6989-6999

[54] Strubberg AM, Madison BB. MicroRNAs in the etiology of colorectal cancer: Pathways and clinical implications. Disease Models & Mechanisms. 2017;**10**(3):197-214

[55] Okada N et al. A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression. Genes & Development. 2014;**28**(5):438-450

[56] Li J et al. MicroRNA-140 inhibits the epithelial-mesenchymal transition and metastasis in colorectal cancer. Molecular Therapy—Nucleic Acids. 2018;**10**:426-437

[57] Hollis M et al. MicroRNAs potential utility in colon cancer: Early detection,

prognosis, and chemosensitivity. World Journal of Gastroenterology. 2015;**21**(27):8284-8292

[58] Earle JS et al. Association of microRNA expression with microsatellite instability status in colorectal adenocarcinoma. The Journal of Molecular Diagnostics. 2010;**12**(4):433-440

[59] Schetter AJ et al. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. Journal of the American Medical Association. 2008;**299**(4):425-436

[60] Okugawa Y, Toiyama Y, Goel A. An update on microRNAs as colorectal cancer biomarkers: Where are we and what's next? Expert Review of Molecular Diagnostics. 2014;**14**(8):999-1021

[61] Shibuya H et al. Clinicopathological and prognostic value of microRNA-21 and microRNA-155 in colorectal cancer. Oncology. 2010;**79**(3-4):313-320

[62] Toiyama Y et al. Serum miR-200c is a novel prognostic and metastasis-predictive biomarker in patients with colorectal cancer. Annals of Surgery. 2014;**259**(4):735-743

[63] Meng W-J et al. MicroRNA expression profile reveals miR-17-92 and miR-143-145 cluster in synchronous colorectal Cancer. Medicine. 2015;**94**(32):e1297

[64] Arndt GM et al. Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer. BMC Cancer. 2009;**9**:374-374

[65] Zhang Q et al. miR-34a increases the sensitivity of colorectal cancer cells to 5-fluorouracil in vitro and in vivo. American Journal of Cancer Research. 2018;**8**(2):280-290

[66] Slattery ML et al. The p53-signaling pathway and colorectal cancer: Interactions between downstream p53 target genes and miRNAs. Genomics. 2018. pii: S0888-7543(18)30036-3. DOI: 10.1016/j.ygeno.2018.05.006. [Epub ahead of print]

[67] Wang F et al. Reciprocal regulation between microRNAs and epigenetic machinery in colorectal cancer. Oncology Letters. 2017;**13**(3):1048-1057

[68] Ng EK et al. MicroRNA-143 targets DNA methyltransferases 3A in colorectal cancer. British Journal of Cancer. 2009;**101**(4):699-706

[69] Yu L et al. microRNA-140-5p inhibits colorectal cancer invasion and metastasis by targeting ADAMTS5 and IGFBP5. Stem Cell Research & Therapy. 2016;**7**:180

[70] Sun X et al. miR-449a inhibits colorectal cancer progression by targeting SATB2. Oncotarget. 2017;**8**(60):100975-100988

[71] Roelands J et al. Immunogenomic classification of colorectal cancer and therapeutic implications. International Journal of Molecular Sciences. 2017;**18**(10):2229

[72] Gatalica Z et al. High microsatellite instability (MSI-H) colorectal carcinoma: A brief review of predictive biomarkers in the era of personalized medicine. Familial Cancer. 2016;**15**:405-412

[73] Samowitz WS et al. Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level. Cancer Epidemiology, Biomarkers & Prevention. 2001;**10**(9):917-923

[74] Boland CR, Goel A. Microsatellite instability in colorectal cancer. Gastroenterology. 2010;**138**(6): 2073-2087.e3

[75] Arnold CN, Goel A, Boland CR. Role of hMLH1 promoter

hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. International Journal of Cancer. 2003;**106**(1):66-73

[76] Weber JC et al. Allelotyping analyses of synchronous primary and metastasis CIN colon cancers identified different subtypes. International Journal of Cancer. 2007;**120**(3):524-532

[77] Wang C et al. Prognostic significance of microsatellite instability and Ki-ras mutation type in stage II colorectal cancer. Oncology. 2003;**64**(3):259-265

[78] Walther A, Houlston R, Tomlinson I. Association between chromosomal instability and prognosis in colorectal cancer: A meta-analysis. Gut. 2008;**57**(7):941-950

[79] Swanton C et al. Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. Cancer Cell. 2007;**11**(6):498-512

[80] Moorcraft SY et al. Patupilone in patients with pretreated metastatic/locally recurrent colorectal cancer: Results of the phase II CINATRA trial. Investigational New Drugs. 2013;**31**(5):1339-1344

[81] Toyota M et al. CpG island methylator phenotype in colorectal cancer. Proceedings of the National Academy of Sciences of the United States of America. 1999;**96**(15):8681-8686

[82] Hawkins N et al. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. Gastroenterology. 2002;**122**(5):1376-1387

[83] Shen L et al. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. Proceedings of the National Academy of Sciences of the United States of America. 2007;**104**(47):18654-18659

[84] Kang GH. Four molecular subtypes of colorectal cancer and their precursor lesions. Archives of Pathology & Laboratory Medicine. 2011;**135**(6):698-703

[85] Ogino S et al. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: Possible associations with male sex and KRAS mutations. The Journal of Molecular Diagnostics. 2006;**8**(5):582-588

[86] Lee S et al. CpG island methylator phenotype in colorectal cancers: Comparison of the new and classic CpG island methylator phenotype marker panels. Archives of Pathology & Laboratory Medicine. 2008;**132**(10):1657-1665

[87] Ward RL et al. Adverse prognostic effect of methylation in colorectal cancer is reversed by microsatellite instability. Journal of Clinical Oncology. 2003;**21**(20):3729-3736

[88] Shen L et al. Association between DNA methylation and shortened survival in patients with advanced colorectal cancer treated with 5-fluorouracil based chemotherapy. Clinical Cancer Research. 2007;**13**(20):6093-6098

[89] Van Rijnsoever M et al. CpG island methylator phenotype is an independent predictor of survival benefit from 5-fluorouracil in stage III colorectal cancer. Clinical Cancer Research. 2003;**9**(8):2898-2903

[90] Weisenberger DJ et al. Association of the Colorectal CpG Island Methylator phenotype with molecular features, risk factors and family history. Cancer Epidemiology, Biomarkers & Prevention. A publication of the American Association for Cancer Research, cosponsored by the American

Society of Preventive Oncology. 2015;**24**(3):512-519

[91] Samowitz WS et al. Association of smoking, CpG island methylator phenotype, and V600E BRAF mutations in colon cancer. Journal of the National Cancer Institute. 2006;**98**(23):1731-1738

[92] Müller MF, Ibrahim AEK, Arends MJ. Molecular pathological classification of colorectal cancer. Virchows Archiv: An International Journal of Pathology. 2016;**469**(2):125-134

[93] Thanki K et al. Consensus molecular subtypes of colorectal cancer and their clinical implications. International Biological and Biomedical Journal. 2017;**3**(3):105-111

[94] Kim JH et al. Distinct features between MLH1-methylated and unmethylated colorectal carcinomas with the CpG island methylator phenotype: Implications in the serrated neoplasia pathway. Oncotarget. 2016;**7**(12):14095-14111

[95] Guinney J et al. The consensus molecular subtypes of colorectal cancer. Nature Medicine. 2015;**21**(11):1350-1356

[96] Alwers E et al. Associations between molecular classifications of colorectal cancer and patient survival: A systematic review. Clinical Gastroenterology and Hepatology. 2018. pii: S1542-3565(17)31536-7. DOI: 10.1016/j.cgh.2017.12.038. [Epub ahead of print]

[97] Albert M, Helin K. Histone methyltransferases in cancer. Seminars in Cell & Developmental Biology. 2010;**21**(2):209-220

[98] Peserico A et al. A SMYD3 small-molecule inhibitor impairing cancer cell growth. Journal of Cellular Physiology. 2015;**230**(10):2447-2460

[99] Li B et al. SMYD3 promoter hypomethylation is associated with the risk of colorectal cancer. Future Oncology. 2018;**14**(18):1825-1834

[100] Wang CG et al. EZH2 and STAT6 expression profiles are correlated with colorectal cancer stage and prognosis. World Journal of Gastroenterology. 2010;**16**(19):2421-2427

[101] He SB et al. Inhibition of EZH2 expression is associated with the proliferation, apoptosis, and migration of SW620 colorectal cancer cells in vitro. Experimental Biology and Medicine (Maywood, N.J.). 2015;**240**(4):458-466

[102] Lin Y-W et al. Role of STAT3 and Vitamin D Receptor in EZH2-Mediated Invasion of Human Colorectal Cancer. 2013;**230**(3):277-290

[103] Li H et al. A missense variant in EZH2 is associated with colorectal cancer risk in a Chinese population. Oncotarget. 2017;**8**(55):94738-94742

[104] Chung J et al. Protein arginine methyltransferase 5 regulates WNT/β-catenin target gene expression in at multiple levels. Blood. 2016;**128**(22):4106-4106

[105] Ou CY et al. A coactivator role of CARM1 in the dysregulation of beta-catenin activity in colorectal cancer cell growth and gene expression. Molecular Cancer Research. 2011;**9**(5):660-670

[106] Zhang B et al. Targeting protein arginine methyltransferase 5 inhibits colorectal cancer growth by decreasing arginine methylation of eIF4E and FGFR3. Oncotarget. 2015;**6**(26): p. 22799-22811

[107] Prabhu L et al. Adapting AlphaLISA high throughput screen to discover a novel small-molecule inhibitor targeting protein arginine methyltransferase 5 in pancreatic and colorectal cancers. Oncotarget. 2017;**8**(25):39963-39977

[108] Wei H et al. Protein arginine methylation of non-histone proteins and its role in diseases. Cell Cycle. 2014;**13**(1):32-41

[109] Wei H et al. PRMT5 dimethylates R30 of the p65 subunit to activate NF-kappaB. Proceedings of the National Academy of Sciences of the United States of America. 2013;**110**(33):13516-13521

[110] Verma SK et al. Identification of potent, selective, cell-active inhibitors of the histone lysine methyltransferase EZH2. ACS Medicinal Chemistry Letters. 2012;**3**(12):1091-1096

[111] Wang Y, Hu W, Yuan Y. Protein arginine methyltransferase 5 (PRMT5) as an anticancer target and its inhibitor discovery. Journal of Medicinal Chemistry. 2018;**61**(21):9429-9441

[112] Kim TD et al. Pro-growth role of the JMJD2C histone demethylase in HCT-116 colon cancer cells and identification of curcuminoids as JMJD2 inhibitors. American Journal of Translational Research. 2014;**6**(3):236-247

[113] Lin H et al. Small molecule KDM4s inhibitors as anti-cancer agents. Journal of Enzyme Inhibition and Medicinal Chemistry. 2018;**33**(1):777-793

[114] Li S et al. Inhibition of DNMT suppresses the stemness of colorectal cancer cells through down-regulating Wnt signaling pathway. Cellular Signalling. 2018;**47**:79-87

[115] Lou YF et al. Combination of gefitinib and DNA methylation inhibitor decitabine exerts synergistic anti-cancer activity in colon cancer cells. PLoS One. 2014;**9**(5):e97719

[116] Gong F, Miller KM. Mammalian DNA repair: HATs and HDACs make their mark through histone acetylation. Mutation Research. 2013;**750**(1-2):23-30

[117] Yang XJ, Seto E. HATs and HDACs: From structure, function and regulation to novel strategies for therapy and prevention. Oncogene. 2007;**26**(37):5310-5318

[118] Du C et al. 5-fluorouracil targets histone acetyltransferases p300/CBP in the treatment of colorectal cancer. Cancer Letters. 2017;**400**:183-193

[119] Yin YW et al. The histone acetyltransferase GCN5 expression is elevated and regulated by c-Myc and E2F1 transcription factors in human colon cancer. Gene Expression. 2015;**16**(4):187-196

[120] Zhang H et al. GSK-3beta-regulated N-acetyltransferase 10 is involved in colorectal cancer invasion. Clinical Cancer Research. 2014;**20**(17):4717-4729

[121] Cao L et al. Correlation of low expression of hMOF with clinicopathological features of colorectal carcinoma, gastric cancer and renal cell carcinoma. International Journal of Oncology. 2014;**44**(4):1207-1214

[122] Wilson AJ et al. Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. The Journal of Biological Chemistry. 2006;**281**(19):13548-13558

[123] Alzoubi S et al. Synergy between histone deacetylase inhibitors and DNA-damaging agents is mediated by histone deacetylase 2 in colorectal cancer. Oncotarget. 2016;**7**(28):44505-44521

[124] Proto MC et al. Inhibition of Wnt/beta-catenin pathway and histone acetyltransferase activity by Rimonabant: A therapeutic target for colon cancer. Scientific Reports. 2017;**7**(1):11678

[125] Ranjbarnejad T et al. Garcinol exhibits anti-proliferative activities by

targeting microsomal prostaglandin E synthase-1 in human colon cancer cells. Human & Experimental Toxicology. 2017;**36**(7):692-700

[126] Tampakis A, Christina Tampaki E, Kouraklis G. Histone deacetylase inhibitors and colorectal cancer: What is new? 2014;**14**(9):1220-1227

[127] Suraweera A, O'Byrne KJ, Richard DJ. Combination therapy with histone deacetylase inhibitors (HDACi) for the treatment of cancer: Achieving the full therapeutic potential of HDACi. Frontiers in Oncology. 2018;**8**:92

[128] Na YS et al. Effects of the HDAC inhibitor CG2 in combination with irinotecan, 5-fluorouracil, or oxaliplatin on HCT116 colon cancer cells and xenografts. Oncology Reports. 2010;**24**(6):1509-1514

[129] Lee DH et al. The HDAC6 inhibitor ACY1215 enhances the anticancer activity of oxaliplatin in colorectal cancer cells. International Journal of Oncology. 2018;**53**(2):844-854

[130] Wapenaar H, Dekker FJJCE. Histone acetyltransferases: Challenges in targeting bi-substrate enzymes. Clinical Epigenetics. 2016;**8**(1):59

[131] Jain S, Zain J. Romidepsin in the treatment of cutaneous T-cell lymphoma. Journal of Blood Medicine. 2011;**2**:37-47

[132] Geskin LJ. Vorinostat in combination with other agents for therapy of cutaneous T-cell lymphomas: A case series. Blood. 2007;**110**(11):4482

[133] Li Y, Seto E. HDACs and HDAC inhibitors in cancer development and therapy. Cold Spring Harbor Perspectives in Medicine. 2016;**6**(10):a026831

[134] Van Cutsem E et al. Regorafenib for patients with metastatic colorectal cancer who progressed after standard therapy: Results of the large, single-arm, open-label phase IIIb CONSIGN study. The Oncologist. 2018. https://doi.org/10.1634/theoncologist.2018-0072

[135] Fakih MG et al. A phase I, pharmacokinetic, and pharmacodynamic study of vorinostat in combination with 5-fluorouracil, leucovorin, and oxaliplatin in patients with refractory colorectal Cancer. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research. 2009;**15**(9):3189-3195

[136] Hervouet E et al. Specific or not specific recruitment of DNMTs for DNA methylation, an epigenetic dilemma. Clinical Epigenetics. 2018;**10**(1):17

[137] Heerboth S et al. Use of epigenetic drugs in disease: An overview. Genetics & Epigenetics. 2014;**6**:9-19

[138] Lyko F, Brown R. DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. Journal of the National Cancer Institute. 2005;**97**(20):1498-1506

[139] Nervi C, De Marinis E, Codacci-Pisanelli G. Epigenetic treatment of solid tumours: A review of clinical trials. Clinical Epigenetics. 2015;**7**:127

[140] Azad NS et al. Combination epigenetic therapy in metastatic colorectal cancer (mCRC) with subcutaneous 5-azacitidine and entinostat: A phase 2 consortium/stand up 2 cancer study. Oncotarget. 2017;**8**(21):35326-35338

[141] Buckowitz A et al. Microsatellite instability in colorectal cancer is associated with local lymphocyte infiltration and low frequency of distant metastases. British Journal of Cancer. 2005;**92**(9):1746-1753

[142] Le DT et al. PD-1 blockade in tumors with mismatch-repair

deficiency. The New England Journal of Medicine. 2015;**372**(26):2509-2520

[143] Emambux S et al. Results and challenges of immune checkpoint inhibitors in colorectal cancer. Expert Opinion on Biological Therapy. 2018;**18**(5):561-573

[144] Passardi A et al. Immune checkpoints as a target for colorectal cancer treatment. International Journal of Molecular Sciences. 2017;**18**(6). Artical ID: 1324

[145] Abdelfatah E et al. Epigenetic therapy in gastrointestinal cancer: The right combination. Therapeutic Advances in Gastroenterology. 2016;**9**(4):560-579

[146] Okada K et al. Combination of the histone deacetylase inhibitor depsipeptide and 5-fluorouracil upregulates major histocompatibility complex class II and p21 genes and activates caspase-3/7 in human colon cancer HCT-116 cells. Oncology Reports. 2016;**36**(4):1875-1885

[147] Shah MY et al. microRNA therapeutics in cancer—An emerging concept. eBioMedicine. 2016;**12**:34-42

[148] Klann TS et al. CRISPR-Cas9 epigenome editing enables high-throughput screening for functional regulatory elements in the human genome. Nature Biotechnology. 2017;**35**:561

Chapter 6

Predictive Biomarkers for Monoclonal Antibody Therapies Targeting EGFR (Cetuximab, Panitumumab) in the Treatment of Metastatic Colorectal Cancer

Anuratha Sakthianandeswaren, Polly Sabljak, Meg J. Elliott, Michelle Palmieri and Oliver M. Sieber

Abstract

The treatment for patients with metastatic colorectal cancer has progressively improved over the past few decades with the development of more effective anti-cancer drugs and multi-disciplinary management approaches that combine sequential lines of non-cross-resistant drugs and increased use of potentially curative surgery for metastases of the liver and lung. In this setting, the introduction of monoclonal antibody therapies that target the epidermal growth factor receptor (EGFR) (cetuximab and panitumumab) has made an important contribution to improved patient outcomes. However, the efficacy of therapies is generally limited to a small proportion of patients and associated with toxicity and high cost. There is an urgent clinical need for robust predictive biomarkers to guide the effective use of therapy options. In this chapter we review clinical and molecular predictive markers of primary therapy response for metastatic colorectal cancer, focusing on anti-EGFR antibody therapies, discussing both currently approved and emerging biomarkers.

Keywords: metastatic colorectal cancer, epidermal growth factor receptor (EGFR), cetuximab, panitumumab, predictive biomarkers

1. Introduction

Metastatic colorectal cancer (mCRC) remains a major contributor to cancer-related morbidity and mortality worldwide. Among patients diagnosed with colorectal cancer, approximately 20–25% present with distant metastases, while another 20–35% develop metastases following curative-intent treatment for early-stage cancer [1]. The median overall survival for mCRC has improved significantly over the past few decades, increasing from 10 to 12 months for 5-fluorouracil plus leucovorin to currently beyond 30 months [2]. Improvements have been driven by advancements in surgery for metastatic disease, the expansion of chemotherapy options and the introduction of targeted therapies such as monoclonal antibodies against the epidermal growth factor receptor (EGFR) or the vascular endothelial growth factor A (VEGFA) [2]. Presently, there are 11 therapeutics approved by the

United States Food and Drug Administration (FDA) for the treatment of mCRC, including 5-fluorouracil, irinotecan, capecitabine, oxaliplatin, bevacizumab, cetuximab, panitumumab, ziv-aflibercept, regorafenib, ramucirumab, and trifluridine-tipiracil. The expansion of treatment options has resulted in an increased clinical need for predictive biomarkers to guide the effective use of therapy. Only a small proportion of patients will respond to any given therapy, and treatments are associated with significant toxicities and often with high financial costs.

Predictive biomarkers for anti-cancer agents are best developed prospectively as companion diagnostics during the drug development process. However, these can also be developed retrospectively through analysis of samples and data from previously conducted randomized clinical trials. Another avenue for marker discovery are longitudinal studies of patients analyzing the emergence of drug resistant tumor clones, although mechanisms of intrinsic (primary) and acquired (secondary) drug resistance may differ. Predictive markers can provide either drug sensitivity (positive prediction of response) or resistance (negative prediction of response) information depending on the biomarker-drug relationship.

There are many challenges in the biomarker development process, such as the choice of analyte (e.g. urine, blood, tissue), cancer sampling procedures (e.g. circulating tumor cells, primary cancer, metastatic lesions), technology for marker evaluation (e.g. DNA, RNA or protein) and determination of clinically relevant cut-offs. In this chapter, we review development efforts for predictive biomarkers for patients with mCRC focusing on anti-EGFR antibody therapies. Our discussion will concentrate on markers of primary drug resistance; markers of acquired drug resistance have been summarized in recent reviews [3, 4].

2. Anti-EGFR therapy

EGFR is a tyrosine kinase transmembrane receptor that belongs to the ErbB protein family. EGFR-mediated signaling has important roles in cell proliferation, survival and differentiation, and dysregulation is a central driver in multiple malignancies including colorectal cancer [4–6]. EGFR interacts with multiple ligands including epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin (AREG), epiregulin (EPR), betacellulin (BTC), heparin-binding EGF (HB-EGF), epigen (EPN), and neuregulin 1-4 (NRG1-4). Activation of EGFR following ligand binding triggers a variety of signaling cascades, including the RAS/MAPK, PI3K/AKT, PLCγ/PKC, SRC tyrosine kinase and STAT pathways. In addition, ligand binding can induce EGFR translocation to the nucleus where EGFR behaves as a co-transcriptional activator regulating key genes such as Aurora Kinase A (*AURKA*), Cyclin D1 (*CCND1*), Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*) and MYB Proto-Oncogene Like 2 (*MYBL2*).

EGFR is overexpressed in colorectal tumors, with most estimates between 40% and 80% depending on the methods and cut-offs used, highlighting the receptor as a prime drug target in this malignancy [7, 8]. Two monoclonal antibodies targeting EGFR have been clinically approved for the treatment of mCRC including cetuximab (Erbitux®), a chimeric mouse-human IgG_1 antibody, and panitumumab (Vectibix®), a humanized IgG_2 antibody. Both antibodies bind the extracellular domain of EGFR, inhibiting ligand-induced tyrosine kinase activation and leading to EGFR cellular internalization and degradation, thereby preventing the activation of downstream signaling (**Figure 1**). Panitumumab has a higher binding affinity for EGFR than cetuximab [9], and cetuximab is thought to additionally lead to activation of the immune response through antibody-dependent cell-mediated cytotoxicity (ADCC) due to the IgG_1 chimeric antibody structure [10, 11]. With respect to

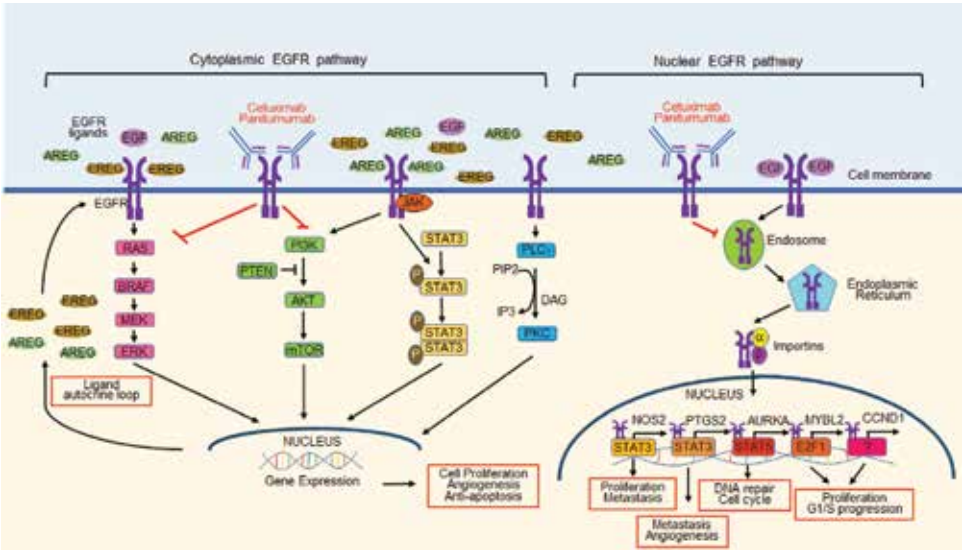

Figure 1.
Targeting of the EGFR signaling pathway with anti-EGFR monoclonal antibodies. EGFR activation is triggered by ligand binding which results in the formation of receptor homo- or hetero-dimers. Receptor autophosphorylation at tyrosine residues within the cytoplasmic tail acts as a docking site for proteins with Src homology2 (SH2) and phosphotyrosine-binding domains (PTB), initiating cellular signaling via the RAS/MAPK, PI3K/AKT, STAT and PLCγ/PKC pathways. Ligand binding can further stimulate EGFR translocation into the nucleus, with nuclear EGFR interacting with transcription factors to drive expression of target genes including NOS2, PTGS2, AURKA, MYBL2 and CCND1. EGFR signaling in tumor cells promotes cell proliferation and survival, and this can be blocked with antibodies against the receptor (cetuximab and panitumumab).

toxicity, panitumumab treatment is associated with significantly lower occurrence of grade 3–4 infusion reactions (allergic reactions) than cetuximab due to its fully humanized nature [12]. Despite these differences, cetuximab and panitumumab showed clinical equivalence in efficacy in refractory patients [12], and both are approved for use in combination with chemotherapy in the first and second line setting or as monotherapy for refractory disease.

In unselected patient populations, the response rate to anti-EGFR therapy is typically less than 30% [13], and for patients who initially respond to treatment most tumors become refractory within 3–12 months [14]. The need to identify biomarkers predictive of EGFR response is therefore vital, and numerous studies have explored resistance mechanisms to EGFR blockade. Findings have unraveled a variety of biomarkers and pathways that are associated with resistance to anti-EGFR therapy. As discussed below, this work has led to the endorsement of predictive testing for tumor *RAS* (*KRAS* and *NRAS*) mutation status and consideration of primary tumor location to guide the use of anti-EGFR therapy. Efforts to discover and validate additional biomarkers is ongoing to further refine treatment delivery are ongoing.

2.1 Current predictive biomarkers for anti-EGFR therapy

2.1.1 KRAS and NRAS mutations

Genes of the RAS type GTPase family, comprising *KRAS*, *NRAS* and *HRAS*, are principal downstream mediators of activated EGFR signaling [15]. In colorectal cancer, *KRAS* and *NRAS* are major oncogenes, with activating mutations found in approximately 40% and 3–5% of cases, respectively [16]. Constitutive downstream signaling through oncogenic RAS proteins activates processes contributing to tumor progression and metastasis, independent of EGFR and other cell surface receptor

kinases [15]. As anticipated from the biological mechanism, mutations in *KRAS* and *NRAS* genes have been found to render tumors insensitive to anti-EGFR therapy.

The majority of *KRAS* mutations (85–90%) in colorectal cancer occur in exon 2 at codons 12 and 13 [16]. Analyses of clinical trials of cetuximab or panitumumab over the last decade have provided conclusive evidence that patients with *KRAS* mutations in exon 2 do not benefit from anti-EGFR therapy when given as a single agent or combined with chemotherapy (**Table 1**) [17–24]. Retrospective analyses of the randomized phase III CO.17 and 20020408 studies which evaluated cetuximab or panitumumab plus best supportive care (BSC) *vs* BSC alone in patients with chemotherapy-refractory mCRC, respectively, found a significant improvement in outcomes for patients with wild-type *KRAS* exon 2 tumors, but no benefit of anti-EGFR therapy in patients who had mutant *KRAS* exon 2 tumors [19, 22]. Similar results for the first-line setting were subsequently reported for both retrospective and prospective analyses of several randomized clinical trials, including the phase II OPUS and phase III PRIME studies which examined cetuximab or panitumumab plus oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) *vs* FOLFOX4 alone, respectively [23, 25], and the phase III CRYSTAL study which assessed the addition of cetuximab to irinotecan, infusional fluorouracil, and leucovorin (FOLFIRI) [18]. Prospective analysis of the randomized phase III 20050181 study which evaluated panitumumab plus FOLFIRI in the second-line setting further confirmed the predictive value of *KRAS* exon 2 mutation status [24].

There is evidence that *KRAS* codon 12 and 13 mutations may exhibit differential biological effects, including variable ratios of these codon mutations between tumor types [16] and weaker *in vitro* transforming activity for *KRAS* codon 13 as compared to codon 12 mutant proteins [26]. Accordingly, some studies have suggested that patients with KRAS glycine (G)-to-aspartate (D) transitions at codon 13 (G13D), the most common codon 13 variant in colorectal cancer, might derive some benefit from anti-EGFR therapy [27, 28]. A retrospective consortium analysis assessing patients with chemotherapy-refractory mCRC treated with cetuximab who participated in multiple clinical trials (CO.17, BOND, MABEL, EMR202600, EVEREST, BABEL and SALVAGE) or who received off-study treatment reported longer overall and progression-free survival among individuals with KRAS G13D-mutated tumors than with other *KRAS*-mutated tumors [27]. An analysis of the updated pooled data sets from the CRYSTAL and OPUS studies also reported that addition of cetuximab to first-line chemotherapy appeared to benefit patients with KRAS G13D-mutant tumors [28]. In contrast, a retrospective analysis of 110 patients treated with cetuximab, found that patients with KRAS G13D mutations were unlikely to respond to therapy [29], and similar findings were reported for a retrospective pooled analysis of three randomized phase III trials evaluating panitumumab therapy (20050203, first line; 20050181, second line; and 20020408, monotherapy) [30]. To resolve this controversy, the randomized phase II ICECREAM study prospectively assessed cetuximab monotherapy and cetuximab plus irinotecan in patients with KRAS G13D-mutated chemotherapy-refractory mCRC. In this study, no statistically significant improvement in disease control was observed for patients with this rare molecular subtype [31].

More recently, several retrospective analyses have indicated that not only *KRAS* exon 2 mutations but also *KRAS* exons 3 and 4 and *NRAS* exons 2, 3, and 4 mutations are negative predictive markers for anti-EGFR therapy [23, 32–36]. These additional mutations are observed in approximately 15–20% of wild-type *KRAS* exon 2 tumors [23, 32]. Reassessment of the randomized OPUS and PRIME studies of cetuximab or panitumumab plus FOLFOX4 *vs* FOLFOX4 alone in the first-line setting found that additional *RAS* mutations predicted a lack of response [23, 34], and corresponding observations were reported for the CRYSTAL study of cetuximab plus FOLFIRI [32]. Accordingly, analyses of single arms of the phase III FIRE-3 study

Study	Treatment arms	Number of patients	PFS (months)	HR PFS	95% CI	p-Value
RAS wild-type						
CRYSTAL	FOLFIRI + C	178	11.4	0.56	0.41–0.76	p < 0.001
	FOLFIRI	189	8.4			
FIRE-3	FOLFIRI + C	199	10.3	0.97	0.88–1.99	0.77
	FOLFIRI +B	201	10.2			
OPUS	FOLFOX + C	38	12	0.53	0.27–1.04	0.0615
	FOLFOX	49	5.8			
PEAK	FOLFOX + P	50	13	0.65	0.44–0.96	0.029
	FOLFOX + B	60	9.5			
PRIME	FOLFOX + P	259	10.1	0.72	0.58–0.90	0.004
	FOLFOX	253	7.9			
20050181	FOLFIRI + P	208	6.4	0.7	0.54–0.90	0.007
	FOLFIRI	213	4.6			
20020408	P + BCS	107	12.3 wks	0.45	0.34–0.59	p < 0.001
	BSC	110	7.3 wks			
CO.17	C + BSC	117	3.7	0.4	0.3–0.54	p < 0.001
	BSC	113	1.9			
RAS mutant						
CRYSTAL	FOLFIRI + C	246	7.4	1.1	0.85–1.42	0.47
	FOLFIRI	214	7.5			
FIRE-3	FOLFIRI + C	n.a	n.a	n.a	n.a	n.a
	FOLFIRI +B	n.a	n.a			
OPUS	FOLFOX + C	92	5.6	1.54	1.04–2.29	0.0309
	FOLFOX	75	7.8			
PEAK	FOLFOX + P	n.a	n.a	n.a	n.a	n.a
	FOLFOX + B	n.a	n.a			
PRIME	FOLFOX + P	272	7.3	1.3	1.07–1.60	p < 0.001
	FOLFOX	276	8.7			
20050181	FOLFIRI + P	299	4.8	0.86	0.71–1.05	0.14
	FOLFIRI	294	4			
20020408	P + BCS	76	7.4 wks	0.99	0.73–1.36	n.r.
	BSC	95	7.3 wks			
CO.17	C + BSC	81	1.8	0.99	0.73–1.35	0.96
	BSC	83	1.8			

Abbreviations: PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; FOL, folinic acid; F, fluorouracil; IRI, irinotecan; OX, oxaliplatin; B, bevacizumab; C, cetuximab; P, panitumumab; n.r., not reported; BSC, best supportive care.

Table 1.
Summary of clinical trials and treatment effects within subgroups defined by RAS status in patients with metastatic colorectal cancer.

evaluating cetuximab plus FOLFIRI and the phase II PEAK study evaluating panitumumab plus modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) in the first-line setting reported a more pronounced survival advantage for the wild-type *RAS* population as compared to the wild-type *KRAS* exon 2 population [36, 37]. Retrospective analysis of the randomized 20050181 study of panitumumab plus

FOLFIRI in the second-line setting further found no benefit of panitumumab addition in patients with *RAS* mutations beyond *KRAS* exon 2 [35]. Low response rates for additional *RAS* mutations were also reported by a European consortium analyzing tumor samples from a large cohort of patients with chemotherapy-refractory mCRC treated with cetuximab and chemotherapy [38].

A systematic review and meta-analysis of nine randomized controlled trials for anti-EGFR therapy comprising a total of 5948 participants evaluated for *RAS* mutations has confirmed tumors without any *RAS* mutations to have significantly superior progression-free (PFS) and overall survival (OS) as compared to tumors with *RAS* mutations. No difference in PFS or OS benefit was evident between tumors with *KRAS* exon 2 mutations and tumors with other *RAS* mutations [33]. Treatment guidelines for mCRC now recommend *RAS* testing prior to start of anti-EGFR antibody therapy to exclude patients with mutated *RAS* [2, 21]. However, *RAS* mutations only account for approximately 35–50% of nonresponsive patients, and the search for additional biomarkers that predict resistance continues to be an active area of research as surveyed below.

2.1.2 Primary tumor location

Colorectal cancers can be broadly grouped by their primary tumor location within the colon [39]. The left-sided colon, comprising the distal third of the transverse colon, splenic flexure, descending colon, sigmoid colon and rectum, are derived from the embryonic hindgut. The right-sided colon, comprising the proximal two-thirds of the transverse colon, ascending colon and caecum, is derived from the embryonic midgut. Baseline differences exist along the colorectal tract such as cell composition and function of the epithelium, the microbiome and gene expression. Strong evidence for the prognostic effect of primary tumor location is available from clinical studies in patients with mCRC, with right-sided tumors exhibiting a worse prognosis [40, 41]. Right- and left-sided cancers differ in their clinical and molecular characteristics: right-sided colon cancers are more likely to be diploid and have high-grade or mucinous histology, DNA mismatch-repair deficiency and microsatellite instability, CpG island methylation, *BRAF*, *TGFBR2* and *PIK3CA* mutations [41, 42], while left-sided cancers often show chromosome instability, *APC*, *KRAS*, *SMAD4* and *TP53* mutations [43]. Right-sided tumors have also been associated with more frequent overexpression of the EGFR ligands, EREG and AREG, and amplification of EGFR and human epidermal growth factor receptor 2 (HER2) [44, 45]. In cohort studies, the classification of tumor sidedness is variable, with right-sided tumors commonly defined as comprising the region from the ceacum to the splenic flexure.

Clinically, primary tumor location was not considered of particular interest in metastatic patients treated with anti-EGFR therapy, until the importance of sidedness as a biomarker was recognized. Retrospective surveys of clinical trials have indicated that while anti-EGFR therapy provides clinical benefit to patients with *RAS* wild-type mCRC, this benefit is specific to patients with left-sided tumors (**Table 2**). In the CRYSTAL and FIRE-3 studies of cetuximab in the first-line setting, patients with *RAS* wild-type left-sided tumors had better outcomes compared to the respective comparators (FOLFIRI alone and FOLFIRI plus bevacizumab), while limited efficacy was observed in patients with RAS wild-type right-sided tumors [46]. Benefit from cetuximab treatment specific to patients with *KRAS* wild-type left-sided tumors was further observed for the randomized phase III CALGB/SWOG 80405 study of cetuximab or bevacizumab with either irinotecan/5-FU/leucovorin (FOLFIRI) or oxaliplatin/5-FU/leucovorin (mFOLFOX6) [47]. Similar results for patients with *RAS* wild-type left-sided as compared to right-sided

Study	Treatment arms	Number of patients	PFS (months)	HR PFS	95% CI	p-Value
Left-sided colorectal cancer						
CRYSTAL	FOLFIRI	138	8.9	0.5	0.34–0.72	<0.001
	FOLFIRI + C	142	12			
PRIME	FOLFOX	159	9.2	0.72	0.57–0.90	n.r.
	FOLFOX + P	169	12.9			
CALGB/SWOG 80405	FOLFOX/FOLFIRI + B	152	11.2	0.84	0.66–1.06	0.15
	FOLFOX/FOLFIRI + C	173	12.7			
FIRE-3	FOLFIRI + B	149	10.7	0.9	0.71–1.14	0.38
	FOLFIRI + C	157	10.7			
PEAK	FOLFOX + P	53	14.6	0.65	0.21–2.0	n.r.
	FOLFOX + B	54	11.5			
Right-sided colorectal cancer						
CRYSTAL	FOLFIRI	51	7.1	0.87	0.47–1.62	0.66
	FOLFIRI + C	33	8.1			
PRIME	FOLFOX	49	7	0.8	0.50–1.26	n.r.
	FOLFOX + P	39	7.5			
CALGB/SWOG 80405	FOLFOX/FOLFIRI + B	78	10.2	1.64	1.15–2.36	0.006
	FOLFOX/FOLFIRI + C	71	7.5			
FIRE-3	FOLFIRI + B	50	9	1.44	0.92–2.26	0.11
	FOLFIRI + C	38	7.6			
PEAK	FOLFOX + P	22	8.7	0.84	0.18–3.79	n.r.
	FOLFOX + B	14	12.6			

Abbreviations: PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; FOL, folinic acid; F, fluorouracil; IRI, irinotecan; OX, oxaliplatin; B, bevacizumab; C, cetuximab; P, panitumumab; n.r., not reported; BSC, best supportive care.

Table 2.
Summary of clinical trials and treatment effects within subgroups defined by primary tumor location in patients with metastatic colorectal cancer.

tumors were reported for panitumumab for analyses of the PRIME (comparator: FOLFOX alone) and PEAK studies (comparator: FOLFOX plus bevacizumab) [48]. A meta-analysis integrating these data for the first-line setting is available [49]. For the second-line setting, a retrospective analysis of FIRE-3 study also found evidence of better outcomes for cetuximab treatment in patients with *KRAS* wild-type left-sided tumors as compared to right-sided tumors (comparator: bevacizumab) [50]. Similar results for panitumumab were reported in a preliminary efficacy analysis of the 20050181 study for *RAS/BRAF* wild-type patients (comparator FOLFIRI) [51]. A retrospective analysis of the CO.17 study in the treatment-refractory setting further observed that only individuals with *KRAS* wild-type left-sided tumors appeared to benefit from cetuximab as compared to BSC [52].

Given the above evidence, NCCN guidelines now recommend the use of anti-EGFR antibody therapies for the treatment of *RAS* wild-type left-sided colon cancers only [53].

2.2 Future predictive biomarkers for anti-EGFR therapy

2.2.1 Skin toxicity

Dermatological toxicities such as papulopustular rash (acneiform eruption), erythema, and skin fissures are common side effects of treatment with anti-EGFR antibodies, as EGFR is involved in the normal development and physiology of the epidermis [54]. Both undifferentiated and proliferating keratinocytes in the basal and suprabasal layers of the epidermis express EGFR, and keratinocytes depend on EGFR to regulate proliferation, differentiation, migration, and survival [55]. The emergence of skin toxicity has therefore been investigated as an on-target marker for anti-EGFR therapy efficacy in patients with mCRC.

Subset analyses of outcomes by skin toxicity severity suggest that improvements in outcome are associated with a higher grade of severity for patients treated with either panitumumab or cetuximab. For example, in the CRYSTAL trial of cetuximab as a first-line therapy, PFS was 11.3 months compared with 5.4 months in patients with G3 and G0-1 skin reactions, respectively [56]. Similarly, the randomized phase III EPIC study of cetuximab plus irinotecan *vs* irinotecan after fluoropyrimidine and oxaliplatin failure in patients with EGFR-expressing mCRC observed a median PFS of 15.6 months for patients who developed G3-4 rash compared to 5.8 months for those with no rash [57]. In the PRIME study of panitumumab plus FOLFOX4 (first line) and 20050181 study of panitumumab plus FOLFIRI (second line) the addition of a targeted agent even appeared detrimental for outcomes in patients with G0-1 skin toxicity as compared to the control arms [58, 59]. Better outcomes in patients with higher-grade skin toxicity were further noted for both arms in a randomized trial of cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer [60]. A meta-analysis by Petrelli *et al* of 14 studies including a total of 3833 patients, found that the occurrence of skin toxicity was a predictive factor for survival (HR 0.51; 95% CI 0.40–0.64) and progression (HR 0.58; 95% CI 0.49–0.68). However, 12 of the studies included patients with either *KRAS* wild-type or mutated tumors, and data on skin toxicity by *KRAS* mutation status remains limited.

Analysis of skin toxicity in the randomized phase III ASPECCT study of panitumumab *vs* cetuximab in chemorefractory wild-type *KRAS* exon 2 mCRC observed improved outcomes in patients with higher grade of severity for both antibodies, although patients with higher-grade skin toxicity had longer median duration of treatment [61]. Two retrospective trial analyses (PRIME and AIO CRC-0104 [cetuximab with CAPOX or CAPIRI, first-line]) suggest that the relationship between skin toxicity and outcome may not only apply to patients with wild-type *RAS* tumors, but perhaps also to patients mutant *RAS* tumors [62, 63]. A recent meta-analysis of skin toxicity identified seven and five studies that reported information on PFS and OS stratified by *KRAS* mutation status, respectively [64]. Improved clinical outcome in the presence of higher grade severity was observed for both patients with wild-type *KRAS* tumors and those with mutant *KRAS* tumors (PFS for wild-type *KRAS*, HR = 0.60, 95% CI (0.51, 0.70); mutant *KRAS*, HR = 0.60, 95% CI (0.45, 0.80), OS for wild-type *KRAS*, HR = 0.54, 95% CI (0.46, 0.65), mutant *KRAS*, HR = 0.64, 95% CI (0.50, 0.81), $P < 0.001$]. However, only mCRC patients with wild-type *KRAS* tumors who suffered grade 2+ skin toxicity derived absolute benefit from anti-EGFR treatment additional to best BSC or chemotherapy (PFS HR = 0.58, 95% CI (0.41, 0.82), OS HR = 0.73, 95% CI (0.61, 0.88)).

These data raise the question whether wild-type *RAS* patients receiving anti-EGFR therapy who do not develop skin toxicity should receive a dose escalation to induce skin toxicity or whether treatment should be discontinued. Further prospective data are needed to establish the clinical value of skin toxicity as a predictive biomarker.

2.2.2 EGFR gene copy number

EGFR is localized on chromosome 7p11.2 which exhibits DNA copy number gain in approximately 35% of colorectal cancers [65]. Based on this observation, *EGFR* gene copy number has been investigated as a predictive biomarker for anti-EGFR therapy in multiple *post hoc* analyses. Study results have been aggregated in three meta-analyses [66-68], which broadly concurred in identifying gain of *EGFR* gene copy number as associated with improved outcomes among patients receiving cetuximab or panitumumab treatment. This association was found to be retained in subgroup analyses for patients with *KRAS* wild-type tumors, with one meta-analysis suggesting that this difference was not present in patients with *KRAS* mutated tumors [69]. However, the methodologies and criteria used for scoring increased EGFR gene copy number were highly inconsistent across different studies, and more research is required to clarify the predictive potential of this biomarker.

2.2.3 Amphiregulin (AREG) and epiregulin (EREG) expression

The EGFR ligands AREG and EREG are overexpressed in colorectal cancer at both the mRNA and protein levels [70, 71], and suppression of *AREG* or *EREG* gene expression reduces the therapeutic efficacy of cetuximab in tumor cell lines [72]. Accordingly, multiple studies have found evidence that the extent of expression of these ligands is related to efficacy of anti-EGFR therapy [71, 73–79].

For example, in the randomized phase III PICCOLO study of panitumumab and irinotecan *vs* irinotecan alone in fluorouracil-resistant mCRC, high messenger RNA (mRNA) expression of *EREG* or *AREG* (defined as either *EREG* or *AREG* in the top tertile for mRNA level) was a predictive marker for anti-EGFR therapy benefit in patients with wild-type *RAS* tumors. In contrast, patients with mutant *RAS* tumors gained no panitumumab therapy benefit regardless of ligand status [80]. Similarly, in the CO.17 study of cetuximab in chemotherapy-refractory mCRC, wild-type *KRAS* patients with high *EREG* gene expression obtained benefit from cetuximab therapy, while no benefit was observed in patients with low *EREG* expression; patients with mutant *KRAS* tumors showed no improvement on anti-EGFR therapy irrespective of *EREG* expression levels [76]. A retrospective analysis of the single-arm phase II NCT 00508404 study of first-line panitumumab plus FOLFIRI similarly also found a higher overall response rate for patients with wild-type *RAS* tumors and high *vs* low *AREG* expression [81]. A meta-analysis including eight studies that used anti-EGFR therapy alone or in combination with chemotherapy reported that *AREG*/*EREG* mRNA overexpression was associated with longer overall survival in patients with wild-type *RAS* tumors who received cetuximab or panitumumab treatment; *AREG* overexpression was further associated with longer PFS. In contrast, *AREG* and *EREG* was found not to have predictive value in patients with mutant *KRAS* tumors [82]. Given these encouraging data, further examination of these ligands in prospective controlled trials appears warranted.

2.2.4 BRAF mutation

The *BRAF* gene encodes a serine-threonine protein kinase that is an integral member of the RAS/MAPK signaling pathway. Approximately 10% of colorectal cancers harbor activating mutations in *BRAF*, with a valine (V) to a glutamic acid (E) substitution at codon 600 (V600E) accounting for more than 95% of alterations [16]. Mutations in *BRAF* are mutually exclusive with *KRAS* mutations in CRC [83]. Patients with mCRC who possess a *BRAF* mutation have significantly poorer prognosis as measured by PFS and OS, and mutational analysis is recommended

for prognostic stratification in guidelines from the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology [84]. The relatively low mutation prevalence and strong association with prognosis in the metastatic setting have hampered conclusive evaluation of *BRAF* status as a predictive biomarker for anti-EGFR therapy in individual trials.

Two meta-analyses of randomized studies of anti-EGFR antibodies have been conducted with inconsistent findings. The first meta-analysis of eight randomized controlled trials published in seven studies concluded that there was insufficient evidence to demonstrate that mCRC patients with wild-type *RAS*/mutant *BRAF* tumors attain a different treatment benefit from anti-EGFR therapy as compared to patients with wild-type *RAS*/wild-type *BRAF* tumors [85]. However, the second meta-analysis of 10 randomized controlled trials from nine reports focusing on wild-type *RAS*/mutant *BRAF* tumors reported that anti-EGFR therapy provided no benefit in these patients, indicating presence of mutation as a marker of drug resistance. Based on these uncertain data, current guidelines for the treatment of mCRC do not recommend *BRAF* mutations as a biomarker for response to anti-EGFR therapy [84].

2.2.5 PIK3CA mutation

Phosphoinositide 3-kinases (PI3K) are a family of heterodimeric lipid kinases which consist of regulatory (p85) and catalytic (p110) subunits. PI3K is a key signaling mediator downstream of EGFR involved in the regulation of cell metabolism, growth, proliferation and survival. The *PIK3CA* gene encodes the catalytic subunit, p110α, which, when mutated in cancer, results in constitutively active PI3K signaling. *PIK3CA* mutations are present in approximately 10–20% of colorectal cancers, with missense mutations in exon 9 (helical domain) and exon 20 (kinase domain) being the most common alterations [86, 87]. Notably, biochemical studies comparing mutant p110α proteins have established that exon 9 and exon 20 substitutions have different mechanisms of action. Exon 9-mutant p110α protein induces cell transformation independently of binding to p85 but requires interaction with RAS-GTP, whereas exon 20-mutant p110α protein is active in the absence of RAS-GTP binding but is dependent on the interaction with p85 [88].

PIK3CA mutations have been investigated as a potential predictor of anti-EGFR therapy efficacy, with studies considering mutation status overall or for exons 9 and 20 separately. Again, conclusive analyses from individual studies have been hampered by the relatively low mutation prevalence, with *PIK3CA* mutations tending to co-occur with *KRAS* mutations [87]. A series of meta-analyses have been conducted to consolidate findings, indicating that *PIK3CA* mutations as a whole are associated with a lack of anti-EGFR therapy response in patients with wild-type *RAS* tumors [89–93]. Some studies further suggest that the predictive power may be confined to exon 20 mutations, although sample size remains limited [90, 91, 94]. However, these meta-analyses have included many of the same studies, as well as observed and acknowledged between-study heterogeneity. Further investigations are needed before definitive conclusions regarding the predictive value of *PIK3CA* mutations for clinical decision making can be drawn, and *PIK3CA* mutational analysis of colorectal carcinoma tissue for therapy selection outside of a clinical trial is currently not recommended [84].

2.2.6 PTEN loss

PTEN is a negative regulator of the PI3K/AKT pathway downstream of EGFR through its lipid phosphatase activity. PTEN is a tumor suppressor gene

in colorectal cancer, with inactivating mutations or loss of protein expression observed in approximately 5% and 30% of sporadic colorectal cancers [87, 95, 96].

With respect to response to anti-EGFR therapy, a number of studies have indicated an association with PTEN loss and lack of response to cetuximab and panitumumab [97–100], although other reports have not identified this relationship [101–103]. There are also data to suggest that some discordance in PTEN protein expression may exist between primary tumors and metastases [104]. Several meta-analyses have considered published findings, supporting the notion that loss of PTEN protein expression and/or mutation are predictive of worse outcomes in patients with wild-type *KRAS* tumors treated with anti-EGFR therapy [91, 92, 105]. However, given a high level of variability in methods for assessment of PTEN expression between studies, including IHC scoring algorithms, and the potential inconsistency in expression between primary and metastatic tumor samples, loss of PTEN expression cannot yet be regarded as a reliable predictive biomarker. Further investigation and prospective large randomized clinical trials are still required to fully confirm the role of PTEN in anti-EGFR therapy resistance.

3. Conclusion

The introduction of multiple chemotherapy and biological therapy options for the treatment of CRC over the past few decades have driven an increased need for predictive biomarkers to select the most appropriate therapy for each patient. Biomarker guided treatment selection is critical to improving patient outcomes, reducing exposure to ineffective lines of treatment that are associated with significant toxicities and costs. For the use of the anti-EGFR antibodies cetuximab and panitumumab, current best clinical practice mandates that assessment of all common mutations in *KRAS* and *NRAS* be undertaken at the time of diagnosis of mCRC. Sidedness is also an important factor and it is recommended to limit anti-EGFR therapy to cases with left-sided primary tumor location [53].

Mutations and amplifications of several genes other than *RAS* have been investigated as potential predictive biomarkers of response to anti-EGFR therapy. These include *EGFR* gene copy number, *BRAF* and *PIK3CA* mutation as well as PTEN loss (mutation and loss of protein expression). The individual frequencies of all of these mutations and amplifications are low and methodologies to determine DNA copy number or protein expression have been highly variable across studies, thus whether these alterations are true biomarkers for anti-EGFR therapy resistance remains uncertain. Expression of the EGFR ligands AREG and EREG are an interesting avenue to explore, but current evidence is insufficient to recommend routine testing in clinical practice. Skin toxicity is a potential predictive marker in wild-type *RAS* patients receiving anti-EGFR therapy, but prospective randomized data are required to demonstrate clinical utility and determine how this information is best used to inform patient management (dose escalation *vs* treatment discontinuation).

While significant progress has been made in identifying predictive biomarkers for anti-EGFR therapy, with *RAS* mutation status and tumor sidedness endorsed as clinical diagnostics, many patients treated with cetuximab and panitumumab as selected by these parameters still do not experience treatment benefit. Further basic biology and clinical studies are clearly warranted to improve our understanding of EGFR signaling to identify novel biomarkers predictive of anti-EGFR therapy response and to develop more refined companion diagnostics.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Anuratha Sakthianandeswaren[1,2], Polly Sabljak[1], Meg J. Elliott[1], Michelle Palmieri[1,2] and Oliver M. Sieber[1,2*]

1 Systems Biology and Personalised Medicine Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

2 Department of Medical Biology, The University of Melbourne, Parkville, Victoria, Australia

*Address all correspondence to: sieber.o@wehi.edu.au

References

[1] Surveillance, Epidemiology, and End Results Program SEER Facts and Figures. Colorectal Cancer. National Cancer Institute. 2018. [cited Jul 20, 2018]. Available from: https://seer.cancer.gov/statfacts/html/colorect.html

[2] Van Cutsem E, Cervantes A, Adam R, Sobrero A, Van Krieken JH, Aderka D, et al. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. Annals of Oncology. 2016;**27**(8):1386-1422

[3] Zhao B, Wang L, Qiu H, Zhang M, Sun L, Peng P, et al. Mechanisms of resistance to anti-EGFR therapy in colorectal cancer. Oncotarget. 2017;**8**(3):3980-4000

[4] Brand TM, Iida M, Wheeler DL. Molecular mechanisms of resistance to the EGFR monoclonal antibody cetuximab. Cancer Biology & Therapy. 2011;**11**(9):777-792

[5] Tebbutt N, Pedersen MW, Johns TG. Targeting the ERBB family in cancer: couples therapy. Nature Reviews. Cancer. 2013;**13**(9):663-673

[6] Sigismund S, Avanzato D, Lanzetti L. Emerging functions of the EGFR in cancer. Molecular Oncology. 2018;**12**(1):3-20

[7] Spano JP, Lagorce C, Atlan D, Milano G, Domont J, Benamouzig R, et al. Impact of EGFR expression on colorectal cancer patient prognosis and survival. Annals of Oncology. 2005;**16**(1):102-108

[8] Goldstein NS, Armin M. Epidermal growth factor receptor immunohistochemical reactivity in patients with American Joint Committee on Cancer Stage IV colon adenocarcinoma: Implications for a standardized scoring system. Cancer. 2001;**92**(5):1331-1346

[9] Yang BB, Lum P, Chen A, Arends R, Roskos L, Smith B, et al. Pharmacokinetic and pharmacodynamic perspectives on the clinical drug development of panitumumab. Clinical Pharmacokinetics. 2010;**49**(11):729-740

[10] Inoue Y, Hazama S, Iwamoto S, Miyake Y, Matsuda C, Tsunedomi R, et al. FcgammaR and EGFR polymorphisms as predictive markers of cetuximab efficacy in metastatic colorectal cancer. Molecular Diagnosis & Therapy. 2014;**18**(5):541-548

[11] Kurai J, Chikumi H, Hashimoto K, Yamaguchi K, Yamasaki A, Sako T, et al. Antibody-dependent cellular cytotoxicity mediated by cetuximab against lung cancer cell lines. Clinical Cancer Research. 2007;**13**(5):1552-1561

[12] Price TJ, Peeters M, Kim TW, Li J, Cascinu S, Ruff P, et al. Panitumumab versus cetuximab in patients with chemotherapy-refractory wild-type KRAS exon 2 metastatic colorectal cancer (ASPECCT): A randomised, multicentre, open-label, non-inferiority phase 3 study. The Lancet Oncology. 2014;**15**(6):569-579

[13] Linardou H, Briasoulis E, Dahabreh IJ, Mountzios G, Papadimitriou C, Papadopoulos S, et al. All about KRAS for clinical oncology practice: Gene profile, clinical implications and laboratory recommendations for somatic mutational testing in colorectal cancer. Cancer Treatment Reviews. 2011;**37**(3):221-233

[14] Van Emburgh BO, Sartore-Bianchi A, Di Nicolantonio F, Siena S, Bardelli A. Acquired resistance to EGFR-targeted therapies in colorectal cancer. Molecular Oncology. 2014;**8**(6):1084-1094

[15] Malumbres M, Barbacid M. RAS oncogenes: the first 30 years. Nature Reviews. Cancer. 2003;**3**(6):459-465

[16] Catalogue of Somatic Mutations in Cancer. Wellcome Trust Sanger Institute. 2018 [cited Jul 20, 2018]. Available from: http://www.sanger.ac.uk/genetics/CGP/cosmic/

[17] Bokemeyer C, Bondarenko I, Hartmann JT, de Braud F, Schuch G, Zubel A, et al. Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study. Annals of Oncology. 2011;**22**(7):1535-1546

[18] Van Cutsem E, Kohne CH, Lang I, Folprecht G, Nowacki MP, Cascinu S, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. Journal of Clinical Oncology. 2011;**29**(15):2011-2019

[19] Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. Journal of Clinical Oncology. 2008;**26**(10):1626-1634

[20] Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, et al. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. Journal of Clinical Oncology. 2009;**27**(12):2091-2096

[21] Allegra CJ, Rumble RB, Hamilton SR, Mangu PB, Roach N, Hantel A, et al. Extended RAS gene mutation testing in metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy: American Society of Clinical Oncology Provisional Clinical Opinion Update 2015. Journal of Clinical Oncology. 2016;**34**(2):179-185

[22] Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. The New England Journal of Medicine. 2008;**359**(17):1757-1765

[23] Douillard JY, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. The New England Journal of Medicine. 2013;**369**(11):1023-1034

[24] Peeters M, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, et al. Final results from a randomized phase 3 study of FOLFIRI {+/−} panitumumab for second-line treatment of metastatic colorectal cancer. Annals of Oncology. 2014;**25**(1):107-116

[25] Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, de Braud F, et al. Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. Journal of Clinical Oncology. 2009;**27**(5):663-671

[26] Guerrero S, Casanova I, Farre L, Mazo A, Capella G, Mangues R. K-ras codon 12 mutation induces higher level of resistance to apoptosis and predisposition to anchorage-independent growth than codon 13 mutation or proto-oncogene overexpression. Cancer Research. 2000;**60**(23):6750-6756

[27] De Roock W, Jonker DJ, Di Nicolantonio F, Sartore-Bianchi A, Tu D, Siena S, et al. Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. Journal of the American Medical Association. 2010;**304**(16):1812-1820

[28] Tejpar S, Celik I, Schlichting M, Sartorius U, Bokemeyer C, Van Cutsem E. Association of KRAS G13D tumor mutations with outcome in patients with metastatic colorectal cancer treated with first-line chemotherapy with or without cetuximab. Journal of Clinical Oncology. 2012;**30**(29):3570-3577

[29] Gajate P, Sastre J, Bando I, Alonso T, Cillero L, Sanz J, et al. Influence of KRAS p.G13D mutation in patients with metastatic colorectal cancer treated with cetuximab. Clinical Colorectal Cancer. 2012;**11**(4):291-296

[30] Peeters M, Douillard JY, Van Cutsem E, Siena S, Zhang K, Williams R, et al. Mutant KRAS codon 12 and 13 alleles in patients with metastatic colorectal cancer: Assessment as prognostic and predictive biomarkers of response to panitumumab. Journal of Clinical Oncology. 2013;**31**(6):759-765

[31] Segelov E, Thavaneswaran S, Waring PM, Desai J, Robledo KP, Gebski VJ, et al. Response to cetuximab with or without irinotecan in patients with refractory metastatic colorectal cancer harboring the KRAS G13D mutation: Australasian gastro-intestinal trials group ICECREAM study. Journal of Clinical Oncology. 2016;**34**(19):2258-2264

[32] Van Cutsem E, Lenz HJ, Kohne CH, Heinemann V, Tejpar S, Melezinek I, et al. Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. Journal of Clinical Oncology. 2015;**33**(7):692-700

[33] Sorich MJ, Wiese MD, Rowland A, Kichenadasse G, McKinnon RA, Karapetis CS. Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized, controlled trials. Annals of Oncology. 2015;**26**(1):13-21

[34] Bokemeyer C, Kohne CH, Ciardiello F, Lenz HJ, Heinemann V, Klinkhardt U, et al. FOLFOX4 plus cetuximab treatment and RAS mutations in colorectal cancer. European Journal of Cancer. 2015;**51**(10):1243-1252

[35] Peeters M, Oliner KS, Price TJ, Cervantes A, Sobrero AF, Ducreux M, et al. Analysis of KRAS/NRAS mutations in a phase III study of panitumumab with FOLFIRI compared with FOLFIRI alone as second-line treatment for metastatic colorectal cancer. Clinical Cancer Research. 2015;**21**(24):5469-5479

[36] Schwartzberg LS, Rivera F, Karthaus M, Fasola G, Canon JL, Hecht JR, et al. PEAK: A randomized, multicenter phase II study of panitumumab plus modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) or bevacizumab plus mFOLFOX6 in patients with previously untreated, unresectable, wild-type KRAS exon 2 metastatic colorectal cancer. Journal of Clinical Oncology. 2014;**32**(21):2240-2247

[37] Stintzing S, Modest DP, Rossius L, Lerch MM, von Weikersthal LF, Decker T, et al. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab for metastatic colorectal cancer (FIRE-3): A post-hoc analysis of tumour dynamics in the final RAS wild-type subgroup of this randomised open-label phase 3 trial. The Lancet Oncology. 2016;**17**(10):1426-1434

[38] De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. The Lancet Oncology. 2010;**11**(8):753-762

[39] Stintzing S, Tejpar S, Gibbs P, Thiebach L, Lenz HJ. Understanding

the role of primary tumour localisation in colorectal cancer treatment and outcomes. European Journal of Cancer. 2017;**84**:69-80

[40] Loupakis F, Yang D, Yau L, Feng S, Cremolini C, Zhang W, et al. Primary tumor location as a prognostic factor in metastatic colorectal cancer. Journal of the National Cancer Institute. 2015;**107**(3):dju427

[41] Nitsche U, Stogbauer F, Spath C, Haller B, Wilhelm D, Friess H, et al. Right sided colon cancer as a distinct histopathological subtype with reduced prognosis. Digestive Surgery. 2016;**33**(2):157-163

[42] Sinicrope FA, Mahoney MR, Yoon HH, Smyrk TC, Thibodeau SN, Goldberg RM, et al. Analysis of molecular markers by anatomic tumor site in stage III colon carcinomas from adjuvant chemotherapy trial NCCTG N0147 (alliance). Clinical Cancer Research. 2015;**21**(23):5294-5304

[43] Rowan A, Halford S, Gaasenbeek M, Kemp Z, Sieber O, Volikos E, et al. Refining molecular analysis in the pathways of colorectal carcinogenesis. Clinical Gastroenterology and Hepatology. 2005;**3**(11):1115-1123

[44] Missiaglia E, Jacobs B, D'Ario G, Di Narzo AF, Soneson C, Budinska E, et al. Distal and proximal colon cancers differ in terms of molecular, pathological, and clinical features. Annals of Oncology. 2014;**25**(10):1995-2001

[45] Lee MS, McGuffey EJ, Morris JS, Manyam G, Baladandayuthapani V, Wei W, et al. Association of CpG island methylator phenotype and EREG/AREG methylation and expression in colorectal cancer. British Journal of Cancer. 2016;**114**(12):1352-1361

[46] Tejpar S, Stintzing S, Ciardiello F, Tabernero J, Van Cutsem E, Beier F, et al. Prognostic and predictive relevance of primary tumor location in patients with RAS wild-type metastatic colorectal cancer: Retrospective analyses of the CRYSTAL and FIRE-3 trials. JAMA Oncology. 2017;**3**(2):194-201

[47] Venook A, Niedzwiecki D, Innocenti F, Fruth B, Greene C, O'Neil B, et al. Impact of primary (1°) tumor location on overall survival (OS) and progression free survival (PFS) in patients (pts) with metastatic colorectal cancer (mCRC): analysis of CALGB/SWOG 80405 (Alliance). Journal of Clinical Oncology. 2016;**34**:3504

[48] Boeckx N, Koukakis R, Op de Beeck K, Rolfo C, Van Camp G, Siena S, et al. Primary tumor sidedness has an impact on prognosis and treatment outcome in metastatic colorectal cancer: results from two randomized first-line panitumumab studies. Annals of Oncology. 2017;**28**(8):1862-1868

[49] Holch JW, Ricard I, Stintzing S, Modest DP, Heinemann V. The relevance of primary tumour location in patients with metastatic colorectal cancer: A meta-analysis of first-line clinical trials. European Journal of Cancer. 2017;**70**:87-98

[50] Modest DP, Stintzing S, von Weikersthal LF, Decker T, Kiani A, Vehling-Kaiser U, et al. Exploring the effect of primary tumor sidedness on therapeutic efficacy across treatment lines in patients with metastatic colorectal cancer: analysis of FIRE-3 (AIOKRK0306). Oncotarget. 2017;**8**(62):105749-105760

[51] Boeckx AT N, Op de Beeck K, Kafatos G, Deschoolmeester V, Rolfo C, et al. Primary tumor sidedness impacts on prognosis and treatment outcome: results from three randomized studies of panitumumab plus chemotherapy versus chemotherapy or chemotherapy plus bevacizumab in 1st and 2nd line RAS/BRAF WT mCRC Ann Oncol. 2016;**27**(suppl 6):vi15-vi42

[52] Brule SY, Jonker DJ, Karapetis CS, O'Callaghan CJ, Moore MJ, Wong R, et al. Location of colon cancer (right-sided versus left-sided) as a prognostic factor and a predictor of benefit from cetuximab in NCIC CO.17. European Journal of Cancer. 2015;**51**(11):1405-1414

[53] Clinical Practice Guidelines in Oncology Colon cancer: National Comprehensive Cancer Network (NCCN). 2018. [cited Jul 20, 2018]. Available from: https://www.nccn.org

[54] Petrelli F, Ardito R, Ghidini A, Zaniboni A, Ghidini M, Barni S, et al. Different toxicity of cetuximab and panitumumab in metastatic colorectal cancer treatment: A systematic review and meta-analysis. Oncology. 2018;**94**(4):191-199

[55] Lacouture ME. Mechanisms of cutaneous toxicities to EGFR inhibitors. Nature Reviews. Cancer. 2006;**6**(10):803-812

[56] AAMPP I. Randomized phase III study of irinotecan and 5-FU/FA with or without cetuximab in the first-line treatment of patients with metastatic colorectal cancer (mCRC): The CRYSTAL trial. Journal of Clinical Oncology. 2007;**25**(18S; June 20 suppl)

[57] Sobrero AF, Maurel J, Fehrenbacher L, et al. EPIC: phase III trial of cetuximab plus irinotecan after fluoropyrimidine and oxaliplatin failure in patients with metastatic colorectal cancer. Journal of Clinical Oncology. 2008 May 10;**26**(14):2311-2319

[58] Sobrero AF, Peeters M, Price TJ, et al. Final results from study 181: Randomized phase III study of FOLFIRI with or without panitumumab (pmab) for the treatment of second-line metastatic colorectal cancer (mCRC). Journal of Clinical Oncology. 2012;**30**(suppl 4):387

[59] Douillard YJSS, Tabernero J, et al. Final skin toxicity (ST) and patient-reported outcomes (PRO) results from PRIME: A randomized phase III study of panitumumab (pmab) plus FOLFOX4 (CT) for first-line metastatic colorectal cancer (mCRC). Journal of Clinical Oncology. 2012;**30**(suppl 4):531

[60] Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. The New England Journal of Medicine. 2004;**351**(4):337-345

[61] Price T, Kim TW, Li J, Cascinu S, Ruff P, Suresh AS, et al. Final results and outcomes by prior bevacizumab exposure, skin toxicity, and hypomagnesaemia from ASPECCT: randomized phase 3 non-inferiority study of panitumumab versus cetuximab in chemorefractory wild-type KRAS exon 2 metastatic colorectal cancer. European Journal of Cancer. 2016;**68**:51-59

[62] Douillard JY, Rong A, Sidhu R. RAS mutations in colorectal cancer. The New England Journal of Medicine. 2013;**369**(22):2159-2160

[63] Stintzing SKC, Laubender RP, et al. Cetuximab-related skin toxicity in metastatic colorectal cancer (mCRC) patients and its correlation with molecular markers: Results from the German AIO CRC 0104 trial. Journal of Clinical Oncology. 2102;**30**(suppl 4):491

[64] Hu J, Zhang Z, Zheng R, Cheng L, Yang M, Li L, et al. On-treatment markers as predictors to guide anti-EGFR MoAb treatment in metastatic colorectal cancer: a systematic review with meta-analysis. Cancer Chemotherapy and Pharmacology. 2017;**79**(2):275-285

[65] Mouradov D, Sloggett C, Jorissen RN, Love CG, Li S, Burgess AW,

et al. Colorectal cancer cell lines are representative models of the main molecular subtypes of primary cancer. Cancer Research. 2014;**74**(12):3238-3247

[66] Shen WD, Chen HL, Liu PF. EGFR gene copy number as a predictive biomarker for resistance to anti-EGFR monoclonal antibodies in metastatic colorectal cancer treatment: A meta-analysis. Chinese Journal of Cancer Research. 2014;**26**(1):59-71

[67] Ketzer S, Schimmel K, Koopman M, Guchelaar HJ. Clinical pharmacokinetics and pharmacodynamics of the epidermal growth factor receptor inhibitor panitumumab in the treatment of colorectal cancer. Clinical Pharmacokinetics. 2018;**57**(4):455-473

[68] Jiang Z, Li C, Li F, Wang X. EGFR gene copy number as a prognostic marker in colorectal cancer patients treated with cetuximab or panitumumab: A systematic review and meta-analysis. PLoS One. 2013;**8**(2):e56205

[69] Yang ZY, Shen WX, Hu XF, Zheng DY, Wu XY, Huang YF, et al. EGFR gene copy number as a predictive biomarker for the treatment of metastatic colorectal cancer with anti-EGFR monoclonal antibodies: A meta-analysis. Journal of Hematology & Oncology. 2012;**5**:52

[70] Li XD, Miao SY, Wang GL, Yang L, Shu YQ Yin YM. Amphiregulin and epiregulin expression in colorectal carcinoma and the correlation with clinicopathological characteristics. Onkologie. 2010;**33**(7):353-358

[71] Khambata-Ford S, Garrett CR, Meropol NJ, Basik M, Harbison CT, Wu S, et al. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. Journal of Clinical Oncology. 2007;**25**(22):3230-3237

[72] Oliveras-Ferraros C, Cufi S, Queralt B, Vazquez-Martin A, Martin-Castillo B, de Llorens R, et al. Cross-suppression of EGFR ligands amphiregulin and epiregulin and de-repression of FGFR3 signalling contribute to cetuximab resistance in wild-type KRAS tumour cells. British Journal of Cancer. 2012;**106**(8):1406-1414

[73] Yoshida M, Shimura T, Sato M, Ebi M, Nakazawa T, Takeyama H, et al. A novel predictive strategy by immunohistochemical analysis of four EGFR ligands in metastatic colorectal cancer treated with anti-EGFR antibodies. Journal of Cancer Research and Clinical Oncology. 2013;**139**(3):367-378

[74] Baker JB, Dutta D, Watson D, Maddala T, Munneke BM, Shak S, et al. Tumour gene expression predicts response to cetuximab in patients with KRAS wild-type metastatic colorectal cancer. British Journal of Cancer. 2011;**104**(3):488-495

[75] Jacobs B, De Roock W, Piessevaux H, Van Oirbeek R, Biesmans B, De Schutter J, et al. Amphiregulin and epiregulin mRNA expression in primary tumors predicts outcome in metastatic colorectal cancer treated with cetuximab. Journal of Clinical Oncology. 2009;**27**(30):5068-5074

[76] Jonker DJ, Karapetis CS, Harbison C, O'Callaghan CJ, Tu D, Simes RJ, et al. Epiregulin gene expression as a biomarker of benefit from cetuximab in the treatment of advanced colorectal cancer. British Journal of Cancer. 2014;**110**(3):648-655

[77] Pentheroudakis G, Kotoula V, De Roock W, Kouvatseas G, Papakostas P, Makatsoris T, et al. Biomarkers of benefit from cetuximab-based therapy in metastatic colorectal cancer: Interaction of EGFR ligand expression with RAS/RAF, PIK3CA genotypes. BMC Cancer. 2013;**13**:49

[78] Saridaki Z, Tzardi M, Papadaki C, Sfakianaki M, Pega F, Kalikaki A, et al. Impact of KRAS, BRAF, PIK3CA mutations, PTEN, AREG, EREG expression and skin rash in ≥2 line cetuximab-based therapy of colorectal cancer patients. PLoS One. 2011;**6**(1):e15980

[79] Llovet P, Sastre J, Ortega JS, Bando I, Ferrer M, Garcia-Alfonso P, et al. Prognostic value of BRAF, PI3K, PTEN, EGFR copy number, amphiregulin and epiregulin status in patients with KRAS Codon 12 wild-type metastatic colorectal cancer receiving first-line chemotherapy with anti-EGFR therapy. Molecular Diagnosis & Therapy. 2015;**19**(6):397-408

[80] Seligmann JF, Elliott F, Richman SD, Jacobs B, Hemmings G, Brown S, et al. Combined epiregulin and amphiregulin expression levels as a predictive biomarker for panitumumab therapy benefit or lack of benefit in patients with RAS wild-type advanced colorectal cancer. JAMA Oncology. 2016

[81] Karthaus M, Hofheinz RD, Mineur L, Letocha H, Greil R, Thaler J, et al. Impact of tumour RAS/BRAF status in a first-line study of panitumumab + FOLFIRI in patients with metastatic colorectal cancer. British Journal of Cancer. 2016;**115**(10):1215-1222

[82] Jing C, Jin YH, You Z, Qiong Q Jun Z. Prognostic value of amphiregulin and epiregulin mRNA expression in metastatic colorectal cancer patients. Oncotarget. 2016;**7**(34):55890-55899

[83] Tie J, Gibbs P, Lipton L, Christie M, Jorissen RN, Burgess AW, et al. Optimizing targeted therapeutic development: Analysis of a colorectal cancer patient population with the BRAF(V600E) mutation. International Journal of Cancer. 2011;**128**(9):2075-2084

[84] Sepulveda AR, Hamilton SR, Allegra CJ, Grody W, Cushman-Vokoun AM, Funkhouser WK, et al. Molecular biomarkers for the evaluation of colorectal cancer: Guideline from the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology. The Journal of Molecular Diagnostics. 2017;**19**(2):187-225

[85] Rowland A, Dias MM, Wiese MD, Kichenadasse G, McKinnon RA, Karapetis CS, et al. Meta-analysis of BRAF mutation as a predictive biomarker of benefit from anti-EGFR monoclonal antibody therapy for RAS wild-type metastatic colorectal cancer. British Journal of Cancer. 2015;**112**(12):1888-1894

[86] Nosho K, Kawasaki T, Ohnishi M, Suemoto Y, Kirkner GJ, Zepf D, et al. PIK3CA mutation in colorectal cancer: Relationship with genetic and epigenetic alterations. Neoplasia. 2008;**10**(6):534-541

[87] Day FL, Jorissen RN, Lipton L, Mouradov D, Sakthianandeswaren A, Christie M, et al. PIK3CA and PTEN gene and exon mutation-specific clinicopathologic and molecular associations in colorectal cancer. Clinical Cancer Research. 2013;**19**(12):3285-3296

[88] Zhao L, Vogt PK. Helical domain and kinase domain mutations in p110alpha of phosphatidylinositol 3-kinase induce gain of function by different mechanisms. Proceedings of the National Academy of Sciences of the United States of America. 2008;**105**(7):2652-2657

[89] Wu S, Gan Y, Wang X, Liu J, Li M, Tang Y. PIK3CA mutation is associated with poor survival among patients with metastatic colorectal cancer following anti-EGFR monoclonal antibody therapy: a meta-analysis. Journal of Cancer Research and Clinical Oncology. 2013;**139**(5):891-900

[90] Huang L, Liu Z, Deng D, Tan A, Liao M, Mo Z, et al. Anti-epidermal

growth factor receptor monoclonal antibody-based therapy for metastatic colorectal cancer: A meta-analysis of the effect of PIK3CA mutations in KRAS wild-type patients. Archives of Medical Science. 2014;**10**(1):1-9

[91] Yang ZY, Wu XY, Huang YF, Di MY, Zheng DY, Chen JZ, et al. Promising biomarkers for predicting the outcomes of patients with KRAS wild-type metastatic colorectal cancer treated with anti-epidermal growth factor receptor monoclonal antibodies: A systematic review with meta-analysis. International Journal of Cancer. 2013;**133**(8):1914-1925

[92] Therkildsen C, Bergmann TK, Henrichsen-Schnack T, Ladelund S, Nilbert M. The predictive value of KRAS, NRAS, BRAF, PIK3CA and PTEN for anti-EGFR treatment in metastatic colorectal cancer: A systematic review and meta-analysis. Acta Oncologica. 2014;**53**(7):852-864

[93] Mohamed A, Twardy B, AbdAllah N, Akhras A, Ismail H, Zordok M, et al. Clinical impact of PI3K/BRAF mutations in RAS wild metastatic colorectal cancer: Meta-analysis results. Journal of Gastrointestinal Cancer. 2018. DOI: 10.1007/s12029-018-0062-y

[94] Mao C, Yang ZY, Hu XF, Chen Q, Tang JL. PIK3CA exon 20 mutations as a potential biomarker for resistance to anti-EGFR monoclonal antibodies in KRAS wild-type metastatic colorectal cancer: A systematic review and meta-analysis. Annals of Oncology. 2012;**23**(6):1518-1525

[95] Zhou XP, Loukola A, Salovaara R, Nystrom-Lahti M, Peltomaki P, de la Chapelle A, et al. PTEN mutational spectra, expression levels, and subcellular localization in microsatellite stable and unstable colorectal cancers. The American Journal of Pathology. 2002;**161**(2):439-447

[96] Goel A, Arnold CN, Niedzwiecki D, Carethers JM, Dowell JM, Wasserman L, et al. Frequent inactivation of PTEN by promoter hypermethylation in microsatellite instability-high sporadic colorectal cancers. Cancer Research. 2004;**64**(9):3014-3021

[97] Frattini M, Saletti P, Romagnani E, Martin V, Molinari F, Ghisletta M, et al. PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. British Journal of Cancer. 2007;**97**(8):1139-1145

[98] Perrone F, Lampis A, Orsenigo M, Di Bartolomeo M, Gevorgyan A, Losa M, et al. PI3KCA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients. Annals of Oncology. 2009;**20**(1):84-90

[99] Sartore-Bianchi A, Martini M, Molinari F, Veronese S, Nichelatti M, Artale S, et al. PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. Cancer Research. 2009;**69**(5):1851-1857

[100] Negri FV, Bozzetti C, Lagrasta CA, Crafa P, Bonasoni MP, Camisa R, et al. PTEN status in advanced colorectal cancer treated with cetuximab. British Journal of Cancer. 2010;**102**(1):162-164

[101] Laurent-Puig P, Cayre A, Manceau G, Buc E, Bachet JB, Lecomte T, et al. Analysis of PTEN, BRAF, and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer. Journal of Clinical Oncology. 2009;**27**(35):5924-5930

[102] Tol J, Dijkstra JR, Klomp M, Teerenstra S, Dommerholt M, Vink-Borger ME, et al. Markers for EGFR pathway activation as predictor of outcome in metastatic colorectal cancer patients treated with or without

cetuximab. European Journal of Cancer. 2010;**46**(11):1997-2009

[103] Ulivi P, Capelli L, Valgiusti M, Zoli W, Scarpi E, Chiadini E, et al. Predictive role of multiple gene alterations in response to cetuximab in metastatic colorectal cancer: a single center study. Journal of Translational Medicine. 2012;**10**:87

[104] Loupakis F, Pollina L, Stasi I, Ruzzo A, Scartozzi M, Santini D, et al. PTEN expression and KRAS mutations on primary tumors and metastases in the prediction of benefit from cetuximab plus irinotecan for patients with metastatic colorectal cancer. Journal of Clinical Oncology. 2009;**27**(16):2622-2629

[105] Wang ZH, Gao QY, Fang JY. Loss of PTEN expression as a predictor of resistance to anti-EGFR monoclonal therapy in metastatic colorectal cancer: evidence from retrospective studies. Cancer Chemotherapy and Pharmacology. 2012;**69**(6):1647-1655

Chapter 7

Finding Needles in Haystacks: The Use of Quantitative Proteomics for the Early Detection of Colorectal Cancer

Tiffany Gould, Muhammad Fairuz B. Jamaluddin, Joel Petit, Simon J. King, Brett Nixon, Rodney Scott, Peter Pockney and Matthew D. Dun

Abstract

Colorectal cancer (CRC) is a common and treatable disease if diagnosed early. Current population screening programs are suboptimal, and consequently, there is a need for the development of new methodologies for early diagnosis of CRC. In the past 10 years, unprecedented technological advancements in the field of mass spectrometry (MS)-based proteomics have progressively increased the sophistication and utility of these investigations, leading to the draft mapping of the human proteome. These exciting studies have shaped our mechanistic understanding of the human genome and begun to provide us with a suite of novel biomarkers to predict the onset, progression and severity of many debilitating diseases. Thus, sophisticated MS workflows coupled with revolutionary protein quantification techniques hold promise for the field of MS-based plasma proteomics, particularly valuable in the context of early stage identification of curable CRC. However, within the last 40 years, no new plasma protein biomarkers of CRC have been translated into clinical practice. Here. we discuss the application of proteomic technologies within the field of CRC, highlighting contemporary MS-based plasma proteomic strategies that could be exploited to deliver on the promise of a panel of sensitive and specific plasma-based biomarkers with which to non-invasively detect early stage CRC.

Keywords: colorectal cancer, colonic adenomas, polyps, proteomics, SWATH, mass spectrometry, isobaric tag

1. Introduction

Colorectal carcinoma (CRC) is a common form of cancer that is estimated to be responsible for approximately 694,000 deaths worldwide each year [1]. It is the third most common form of cancer in males and the second in females, with an estimated 1.4 million new cases diagnosed annually. The natural history of progression of adenomatous polyps to CRC has been well described by the adenoma-carcinoma sequence, a stepwise process which recognizes that the majority CRC arises

from adenomatous polyps (**Figure 1**) [2]. Colon and rectal cancer is staged from radiological, histopathological and intraoperative findings with the TNM (tumor-nodes-metastasis) system [3] or historically with the Dukes staging system [4]. The stage at diagnosis correlates to prognosis; the 5-year survival of patients with stage one disease is 90%, stage two is 71%, stage three 53% and stage four is only 14% [5]. Therefore, diagnosis and treatment of early stage disease is associated with significantly better outcomes than late stage disease.

Screening programs aim to detect asymptomatic patients with early stage disease where there is a conferrable survival benefit. Investigations used for screening require appropriate levels of sensitivity and specificity, this is to ensure adequate probability of disease detection and to reject patients without the disease in question. Fecal immunochemical tests (FIT) stool screening tests suffer from low predictive values for CRC and as such, positive tests can lead to unnecessary investigation with colonoscopy and other modalities. When considering the discovery of a biomarker for clinical use, the test must have both high sensitivity and specificity to capture the appropriate patient cohort without falsely reassuring patients. In addition, it must be specific in early stage disease, where the natural history of the disease can be successfully altered by surgical intervention. Currently, the participation in CRC screening programs is suboptimal, particularly given that early diagnosis and subsequent treatment significantly correlate to improved outcomes. Depending on the country or region, and the screening test offered, participation can be as low as 40% of the target population [10]. In the context of this poor compliance and subsequent effects on patient morbidity and mortality, there has been increased interest in the role of plasma-based biomarkers as a screening tool for the detection of early stage CRC.

Early stage screening for CRC is via stool-based tests or endoscopic or radiological investigations. Stool-based tests include guaiac-based fecal occult blood (FOB) tests or fecal immunochemical tests (FIT) [6, 7]. Other methods include; colonoscopy, computed tomography colonography, flexible sigmoidoscopy or capsule colonoscopy [8]. Currently, FIT testing is the main method of population based screening for average risk patients as it has 83% sensitivity and 93% specificity [9]. However, the FIT test sufferers from low compliance rates [10]. Colonoscopy is also used for screening and diagnosis but it is a procedure associated with risk, with complications estimated to occur between 0.5–2.8 per 1000 procedures and a mortality rate of 0.007% [11]. Patients who participate in screening programs and

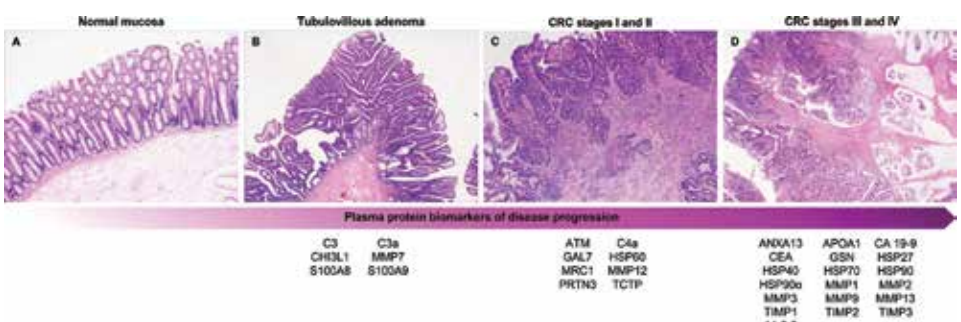

Figure 1.
Summary of plasma protein biomarkers according to disease stage. (A) Normal mucosa, (B) adenomatous polyp, (C) stage one and two adenocarcinoma (D) and stage three and four adenocarcinoma. Beneath each image representing each clinical stage, corresponding plasma protein biomarkers are listed. Each biomarker is placed corresponding to the earliest point where it has been identified. Images (A–C) are 40× objective lens. Image (D) 12.5× objective lens, hematoxylin-eosin staining. After deparaffinization and rehydration of colon sections were counterstained with hematoxylin (Gill's formulation, vector laboratories, California, USA). Imaging was performed on an Axioplan-2 microscope (Carl Zeiss AG, Oberkochen, Germany).

Analyzer	Technology	Advantages	Disadvantages	References
Quadruple	Parallel cylindric magnets to filter ions based on the mass to charge ratio (m/z).	Clinical mass spectra Good reproducibility Lower cost Efficient conversion of precursor to product Preferential for targeted analysis	Poorer resolution Peak heights represented as a function of mass Limited application for pulsed ionization methods	[129, 130]
Ion trap	Combination of radio frequency and direct current (DC) electrical fields which allow ions to be trapped for analysis.	High sensitivity High resolution Multi-stage MS Compact mass analyzer	Poor quantitation Poor dynamic range Affected by space charge effects and ion molecule reactions Collision energy not well defined Many parameters which can affect the quality	[129, 130]
Time of flight	Utilizes different ion velocities allowing separation based on mass.	Fast Can be used for pulsed ionization methods High ion transmission Good mass range	Requires pulsed ionization method or ion beam switching Analyzers used can have limited dynamic range Limited precursor selectivity	[129, 130]
Orbitrap	Utilizes DC between electrodes which results in orbiting ions. The ions oscillate at various frequencies which enable mass-to-charge measurement.	High resolution Accurate-mass detection Good for non-targeted analysis	Decay of coherent ion packets	[129, 130]
Ionization • Surface-enhanced laser desorption/ ionization • Matrix-assisted laser desorption ionization	Uses a laser energy absorbing matrix to create ions from molecules.	High-throughput Fast Minimal fragmentation required Rapid identification Good sensitivity for low abundant proteins	Poorer reproducibility Requires small sample volume Detection limits at attomole level Limited to detection of proteins of relatively low molecular mass Dependent of change in expression profiles	[131, 132]

Table 1.
Overview of common MS analytical technologies.

undertake colonoscopy examination have an estimated 90% decreased incidence of colon cancer than those who do not [12]. Early detection of polypoid disease and subsequent removal of polyps therein prevents progression to CRC [8].

Over the last 2 decades, unprecedented technological advancement in protein-based mass spectrometry (proteomics) has radically changed the landscape of biomarker research [13] (**Table 1**). This has facilitated the characterization of complex cellular proteomes [14–19], research that has identified hundreds of over and under expressed proteins in carcinoma patients using tumor tissue, histological sections, plasma or fecal samples when compared to matched normal tissues [20–24]. Despite this, with the exception of Carcinoembryonic antigen (CEA) and Cancer antigen 19-9 (CA 19-9) [25], no new protein biomarkers have made it into routine clinical practice [21, 26, 27]. In this book chapter, we have sought to present an overview of the diagnostic and prognostic protein biomarkers of early stage CRC to aid in the development of accommodating future screening tools that will continue to increase the rate at which early stage CRC is diagnosed and treated. We also review the use of contemporary proteomic approaches to address many of the long-standing challenges in the field of human CRC plasma proteomics and speculate on the future clinical applications of these technologies (**Figure 2**).

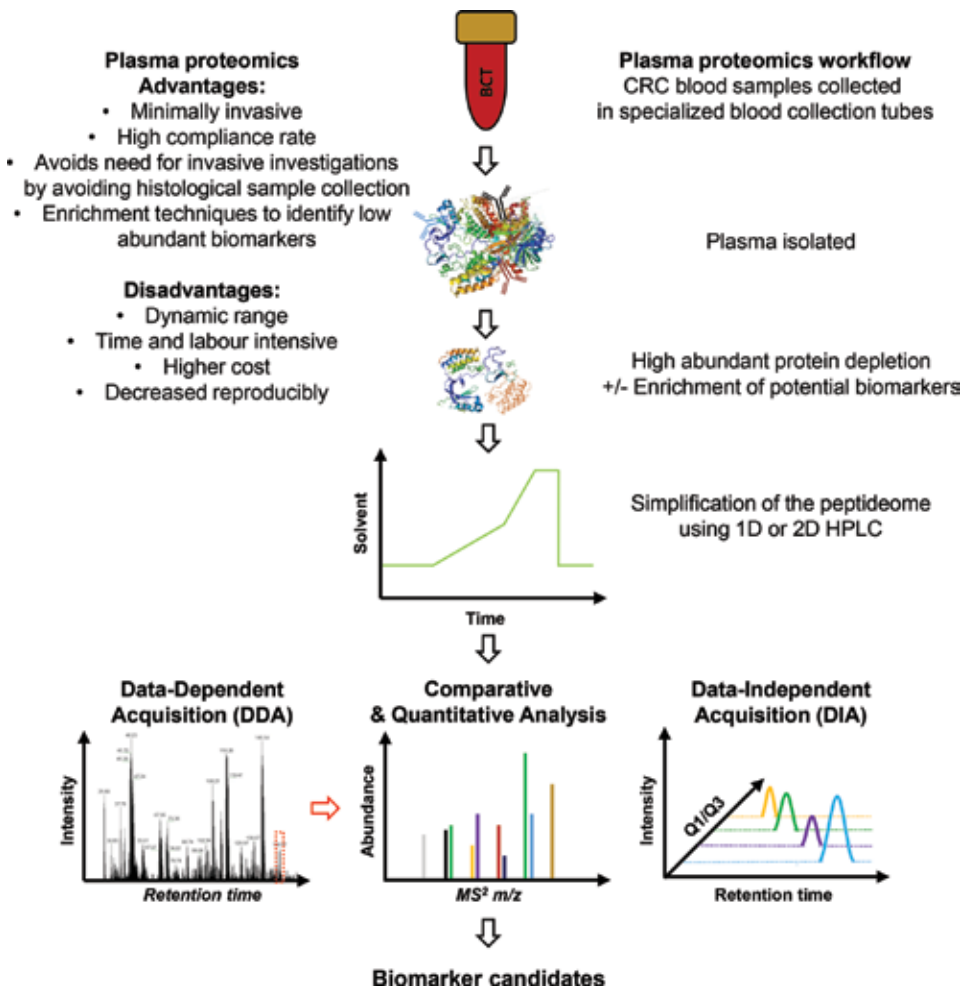

Figure 2.
Overview, and advantages and disadvantages of using gel-free quantitative proteomic approaches for the identification of plasma protein biomarkers of early stage colorectal carcinoma.

2. Proteomic technologies for the identification of plasma proteins of early stage CRC

The use of blood or plasma for screening or diagnosis of CRC is the most attractive non-invasive material available for the identification of clinically relevant protein biomarkers. Most commonly, candidate protein biomarkers of early stage CRC are identified using MS-based proteomics techniques. Below we list the limitations and advantages of the most common sample preparation and proteomics techniques specifically to identify candidate biomarkers in the plasma of early stage CRC. These techniques face a number of limiting factors, which have reduced the utility of proteins revealed by proteomics. Indeed, factors including the extreme dynamic range of proteins within plasma [28], the variability in collection and processing methods [21], preanalytical and analytic processes [29], and the inherent heterogeneity of patient samples [30], have all hindered uniform consent for which biomarkers are the most relevant for use in the setting of early stage disease.

As a small number of highly abundant proteins such as; albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, comprise 90% of the human plasma proteome [31], therefore little capacity is left for the identification of lower abundance proteins to be used as early stage markers of CRC [32]. Researchers have thus turned to immunodepletion strategies to enrich for low abundant proteins, resulting in a 25% increase in identified proteins and 4-fold increased enrichment of non-targeted plasma proteins using peptide isoelectric focusing (IEF), followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [31]. These pioneering studies have paved the way for high-resolution LC-MS/MS studies employed on depleted samples, routinely affording researchers with the capacity to identify 100 s if not 1000 s of plasma proteins during the course of a proteomics investigation [21] (**Figure 2**).

In the context of proteogenomic approaches to biomarker discovery [33], recent studies have also made some progress in reducing variability during collection and processing, revealing the suitability of human plasma proteins for qualitative and quantitative proteomic analysis after collection and storage for up to 48 hours at room temperature in cell free DNA-optimized blood collection tubes [21]. These tubes have been developed to overcome some of the issues that delays in processing time, temperature, and handling contribute to the deterioration of non-protein–based biomarkers [34] and now protein biomarkers [21]. Although not yet in widespread use, future studies may show that these cell stabilization tubes reduce plasma contamination by proteins originating from blood cells during collection and storage, thus increasing the reproducibility of proteomics-based biomarker discovery projects (**Figure 2**).

2.1 Gel-based separation platforms

Two-dimensional electrophoresis (2DE) coupled to mass spectrometry is a very accurate and sensitive method of large-scale protein separation using human CRC tissue [35]. The application of this preparative platform, which facilitates the resolution of protein mixtures on the basis of proteins isoelectric point and molecular weight has been extensively employed using CRC tissue [36–38]. These techniques can be combined with any analytical MS platform to identify changes in protein abundance between samples. Results of these studies are most commonly validated using orthogonal immunological-based techniques using plasma including; ELISA, flow cytometry, immunoblotting. Recently, two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) was employed on early and late stage CRC plasma samples, identifying apolipoprotein A1 (APOA1) as a potential marker

of early stage CRC [39]. Interestingly, this study also showed decreased levels of galectin-7 (GAL-7) in patients with early stage disease compared to healthy controls. CRC tissue examination of GAL-7 revealed 100% negative immunoreactivity implying that it may not might not be originating from the tumor tissues [39].

Gel-based separation approaches coupled to mass spectrometry face significant limitations related to their reproducibility, low sample number capacity, poor resolution of low abundant potential biomarker proteins, poor resolution of highly acidic/basic proteins and of proteins with extreme size or hydrophobicity, and co-migration of multiple proteins in a single spot that renders comparative quantification rather inaccurate [40]. Therefore, more recently researchers have largely focused on gel-free approaches for the identification of biomarkers of early stage CRC.

2.2 Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS)

SELDI-TOF MS also known as ProteinChip® technology, is a high-throughput technique that can purify and identify plasma protein biomarkers [41]. The method offers simplicity as proteins are bound to a solid-phase chromatographic surface, which helps protein isolation from crude mixtures, with non-bound proteins being washed away. The remaining bound proteins are mixed with an energy-absorbing matrix such as sinapinic acid (SPA) or α-cyano-4-hydroxycinnamic acid (CHCA) to induce ionization and desorption of the proteins on the surface of the plate. MALDI-TOF MS is then used to generate a unique mass-to-charge ratio (m/z) of the desorbed molecules, which are analyzed as they fly down the TOF tube and detected as peaks in a mass spectrum [42]. The normalized peak intensity is directly proportional to the concentration of the corresponding protein molecule in the sample.

One of the earliest reports of SELDI-TOF MS for the identification of early stage CRC plasma biomarkers identified a four protein peak m/z profile (m/z: 3191.5, 3262.9, 3396.3 and 5334.4) that was able to discriminate CRC from healthy controls with a sensitivity and specificity exceeding 90% [43]. Furthermore, two additional protein peaks (m/z: 9184.4 and 9340.9) were described as being able to discriminate plasma from patients with primary CRC from those with metastatic CRC [43]. In the same year, a similar study employing SELDI-TOF revealed a set of two protein peaks (m/z: 8132 and 4002) that could discriminate CRC from control again with >90% sensitivity and specificity [44]. This study was followed up some years later using an independent, patient case-control series of blood samples collected at multiple sites. However the latter study failed to discriminate plasma from CRC patients from healthy controls using these two protein peaks. Rather, the study identified two new protein peaks (m/z: 3961 and m/z 5200) in CRC plasma compared to healthy controls, again yielding very high sensitivity and specificity [45]. Drift and intensity of m/z were suggested to be responsible for the variation in reproducibility between the studies, an inherent limitation of SELDI-TOF based biomarker discovery projects, mostly underpinned by the wide dynamic range of human plasma.

2.3 Chromatographic separation platforms

While the analysis of intact proteins by 2DE is likely to continue to play an important role in comparative studies of the CRC tissue proteome, recent technical developments have heralded a new era in proteomics where the emphasis is placed on peptides rather than on whole proteins. Trypsin-based proteomics is now well recognized at the starting point in any proteomics investigation. Hydrolysis of

peptide bonds in proteins is achieved using proteolytic enzymes resulting in the generation of an even more complex peptide mixture. However, the smaller size of peptides makes them much more homogenous structures than proteins. This coupled with the continued maturation of nanoscale chromatographic strategies, and the revolution of electrospray ionization MS (ESI-MS) [46] have meant that the rapid and detailed analysis of the human proteome using tryptic peptides is now common place in the MS laboratories of the world [47] (**Figure 2**).

Tryptic digestion at the whole proteome level increases the complexity of a protein sample, therefore peptide purification techniques including reversed-phase high performance liquid chromatography (RP-HPLC) are key for achieving increased sensitivity through flow separating eluting peptides entering the MS over time. RP-HPLC is most commonly used for one-dimensional (1D) peptide purification in proteomics. In RP-HPLC, peptides are generally retained due to hydrophobic interactions with the stationary silica phase. Polar mobile phases, such as water mixed with acetonitrile, are subsequently used to elute the bound peptides in order of decreasing polarity (increasing hydrophobicity). While reversed phase chromatography can be used as the sole separation procedure for moderately complex peptide mixtures prior to LC-MS/MS analysis, it is generally considered to have insufficient resolution for the analysis of more complex mixtures. This reflects the fact that although an MS instrument can perform mass measurements on several co-eluting peptides, if many peptides co-elute, the instrument cannot fragment them all and valuable information is likely to be irretrievably lost. Therefore, 2D-HPLC fractionation strategies including ion exchange chromatography (IEX), strong cation exchange (SCX), hydrophilic interaction liquid chromatography (HILIC), electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) and hydrophilic strong anion exchange chromatography (hSAX) are commonly employed prior to RP-HPLC. These 2D approaches were used in the draft mapping of the human proteome [48, 49], and continue to be a key preparative step in the successful application of whole proteome based investigations.

2.4 Quantitative proteomics

2.4.1 Isobaric labeling

In addition to their utility in building an in-depth understanding of the CRC plasma proteome, gel-free strategies have also proven to be particularly amenable for use in comparative profiling applications. Indeed, since peptides are inherently less variable than their parent proteins, it has been argued that they constitute a more reliable basis for quantitative comparisons. This property has been exploited for the development of a suite of isobaric-tag based labeling strategies, which facilitate the simultaneous comparison of complex proteomic mixtures using different sample populations. The most common of these approaches used in plasma introduces a isobaric tag covalently bound to the N-terminus and side chain amines of plasma peptides [e.g. isobaric tags for relative and absolute quantitation (iTRAQ) [50] and tandem mass tags (TMT)] [51]. Each of these approaches allows for relative quantification between samples based on the intensities of the reporter produced by precursor-ion fragmentation in the low m/z region of spectra. In each technique, the isobaric tags possess identical chemical properties to ensure similar behavior during chromatographic peptide separation and MS1 applications, but thereafter present as an easily distinguishable mass difference. As such, chromatographic separation platforms have become viable alternatives to 2DE for the differential analysis of complex protein mixtures [52, 53]. The MS operates in Data Dependent Acquisition (DDA) mode so that during each duty cycle the MS cycles

through a short survey scan of the eluting peptides or precursor-ions, then a series of n (~10–15) MS2 scans, during which each of the precursor-ions are isolated, fragmented and their fragment-ions are detected. Database searching is then performed on the MS2 fragmentation spectra and used to identify the sequence of their MS1 parent peak. Limitations in this technology underpin some of the variation seen in MS based biomarker studies since MS2 spectra rarely allow unambiguous identification of the precursor-ions. Nevertheless, the application of quantitative DDA (iTRAQ) to investigate a panel of 10 CRC plasma samples revealed Orosomucoid 2 (ORM2) to be elevated compared to 10 healthy control samples [54]. ORM2 expression was confirmed in CRC tissues compared with corresponding adjacent normal mucosa; however no significant association between ORM2 concentrations and TNM stage or histological grade was shown. Nevertheless, an interesting finding to arise from this work was that plasma levels of ORM2 were higher in patients with inflammatory bowel disease, than in patients presenting with either a normal colorectum, hyperplastic polyps, or adenoma [54]. Thus, ORM2 appears to function in modulating the activity of the immune system, potentially mediating escape from immune recognition; an important first step during transformation.

A recent study by our group assessed whether the plasma samples of CRC patients stored in specialized blood collection tubes (e.g., PAXgene or STRECK; referred to as "BCT"), designed to reduce plasma DNA (pDNA) contamination and enhance low-abundance DNA target detection, was amenable for comparative and quantitative proteomics [21]. Eight patients with Stage I–IIA, and one patient with Stage IIIB were collected pre- and post- resection, in both BCT and EDTA tubes, and subjected to comparative and quantitative analyses using TMT. Of the 641 unique proteins identified across all samples, 184 proteins showed $\pm 0.5 \log_2$ fold-change in peptide abundance pre- versus post-operation. Label-free targeted proteomics validation using parallel reaction monitoring (PRM, discussed below) showed the most well recognized blood marker of CRC, CEA, was significantly more abundant pre- compared to post-operation in patients with early stage disease when collected and stored in BCT prior to MS. The same trend was also seen for gelsolin (GSN), structural maintenance of chromosomes protein 1B (SMC1B), E3 ubiquitin-protein ligase SHPRH (SHPRH), and semaphorin-3C (SEMA3C), highlighting the importance of preanalytical considerations during biomarker investigations using proteomic-based techniques [21].

2.4.2 Label-free quantification

Label-free mass spectrometry has recently emerged as a quantitative tool for the analysis of CRC plasma proteins. In the absence of isobaric-tagged based modifications, this rapid, low-cost technology relies on a workflow in which individual samples are analyzed (e.g. by LC-MS or LC-MS/MS) separately prior to protein quantitation via precursor ion (intact peptide) signal intensity or via spectral counting. The development of high-resolution accurate mass time-of-flight (TOF), and Orbitrap MS facilitates the extraction of precursor ion peaks at the MS1 level, permitting identification based at MS2 level (**Table 1**). The m/z ratios for all ions are detected and their signal intensities at a particular chromatographic retention time recorded. Owing to the tight correlation between signal intensity and ion concentration, relative peptide levels between samples can be determined directly from these peak intensities. Similarly, spectral counting exploits the strong correlation between protein abundance and the number of MS/MS spectra. This approach involves counting the number of peptide-specific spectra identified in different biological samples and the subsequent integration of these data for all measured peptides of the protein(s) that are quantified.

Examples of the application of label-free based proteomic profiling in the context of CRC include comparative analyses of the plasma samples from a cohort of 118 CRC patients compared with 96 healthy controls [55]. This study reported the identification of 373 plasma proteins, with 69 linked to CRC. Of the 69 CRC associated proteins, 2 proteins; Macrophage mannose receptor 1 (MRC1) and S100A9, were verified as being upregulated in CRC by immunoblot analysis and proved effective in identifying CRC from healthy controls with high accuracy, using ELISA analyses [55].

2.4.3 Multiple and parallel reaction monitoring (MRM/PRM)

Targeted proteomics, using multiple (MRM) (also known as; selected reaction monitoring or SRM) [56] or parallel reaction monitoring (PRM) [57] technologies enables absolute quantitation of multiple peptides per chromatographic experiment by exploiting the unique capabilities of triple quadrupole (QQQ) and quadrupole Orbitrap MS and the unique characteristics of the targeted peptides. Analysis is performed by the acquisition of selected events across the chromatographic retention time, of predefined pairs of precursor-ion and product-ion masses for MRM, or individual precursor-ions for PRM. The technique becomes an absolute quantitation tool by spiking isotopically labeled synthetic peptide(s) into the complex sample of interest, which act as internal standards for any peptide(s) of interest. The labeled peptide standards are designed to mimic those generated by tryptic sample digestion, differing by only a few Daltons dependent on the isotopic label used. This enables endogenous and isotope-labeled peptides to be subjected to targeted MS/MS analysis and differentiated by the unique MS2 mass spectra provided by the isotopic label. MRM assay development and optimization are key elements for this method of targeted quantitation. This is somewhat mitigated using PRM-based targeted proteomics, owing to the high-resolution mass accuracy of quadruple Orbitrap MS for precursor-ion selection and the monitoring of all MS2 fragment-ions used for quantitation in parallel.

High-throughput targeted proteomics using MRM in immunodepleted blood plasma has previously been employed to measure the abundance of large numbers of candidate CRC plasma proteins using 137 [23] and 1045 [20] confirmed CRC patients. These powerful studies highlight the capabilities of current MS technologies. Indeed, no less than 187 and 392 candidate marker proteins were simultaneous monitored, respectively. These analyses have aided in the development of candidate panels of plasma protein markers that can be monitored simultaneously to identify CRC in the symptomatic population [20, 23].

2.4.4 SWATH MS

Sequential windowed acquisition of all theoretical fragment-ion mass spectra (SWATH-MS) is a quantitative MS approach heralded as among the most important recent developments in proteomics research [58]. Driven by the recent advances in speed and sensitivity of the new generation of high resolution Triple-TOF MS, these technologies afford the ability not only to determine which proteins are present in the proteome, but also to accurately quantitate without the need for label-based methods, or by limited numbers of targeted peptides. This is due to the lower duty cycle of a Triple-TOF MS compared to an Orbitrap-based mass analyzers [59]. SWATH-MS operates in Data Independent Acquisition (DIA) in which all ions within a selected m/z range are fragmented and analyzed in a second stage of tandem mass spectrometry. In combining the unique advantages of traditional DDA (high-throughput) and MRM (high reproducibility and consistency) technologies,

SWATH-MS can be deployed for both discovery and quantitation of all detectable peptides present in complex biological samples.

SWATH-MS also affords the added advantage that it does not rely on prior knowledge of the precursor peptide ions, instead acquiring information in a DIA manner and thus avoiding laborious assay development. The SWATH-MS workflow involves two key steps beginning with the generation of a spectral library (e.g. via conventional LC-MS/MS) through which acquired peptides are identified. During this acquisition mode, the mass spectrometer is programed to step within 2–4 s cycles through a set of precursor acquisition windows covering the mass range accessible by a quadrupole mass analyzer and also that in which most tryptic peptide precursors should fall (400–1200 m/z). During each cycle, the mass spectrometer fragments peptide precursors and records a complete, high accuracy fragment ion spectrum for all precursors that elute on the chromatograph. This is then followed by acquisition of SWATH-MS data for each sample under analysis, interrogation and matching against the spectral library to identify peptides, and finally extraction of specific peptide ions to enable area-under-the-curve quantitation between samples.

The first SWATH-MS study of CRC plasma also simultaneously assessed protein biomarkers from pancreatic cancer, lung cancer, prostate cancer, and ovarian cancer, all from patients diagnosed with early forms of these diseases. This sophisticated study employed sample enrichment and subsequent detection of tissue-specific secreted protein profiles via SWATH-MS. These data were used to generate a digital representation of the proteins from within each plasma sample that could be queried for the presence and quantity of specific peptides using a targeted data analysis [60]. Tumor specific biomarkers were detected for individual cancer types, as well as a common biomarker Thrombospondin-1 (THBS1), which was significantly altered in the blood of four of five carcinomas (CRC, lung, prostate and ovarian) [61]. These ground breaking studies highlight the potential of the new generation of analytical MS techniques for the detection of early stage.

3. Overview of biomarkers of colorectal adenocarcinoma

Plasma biomarkers used in clinical practice include Carcinoembryonic antigen and Cancer antigen 19-9, however these investigations have limited use in early diagnosis of CRC [6]. A variety of plasma and histological biomarkers of early and late stage CRC including heat shock proteins, matrix metalloproteinases, complement component proteins, Annexins and S100 proteins are discussed and summarized in **Table 2**.

3.1 Carcinoembryonic antigen

Carcinoembryonic antigen (CEA) is a cell-surface high molecular weight glycoprotein important for cell adhesion, discovered in 1965 by Gold and Freedman as a component of human colon carcinoma and foetal tissue [62]. The production of CEA typically ceases at birth and it is present in very low concentrations in healthy patients. It can, however, be elevated in CRC, other types of cancer and non-malignant conditions [63]. CEA is one of the most commonly used biomarkers of CRC worldwide, however its sensitivity for the detection of CRC is not good enough to be useful as a diagnostic tool, with plasma elevation >5 μg/L in Dukes type A, B, C and D reportedly 3, 25, 45 and 65% respectively [63, 64] . Limited evidence supports a role of CEA as a marker of CRC prognosis and recurrence; its sensitivity as an indicator of recurrence is estimated to be 80% [65] and post-operative elevation is particularly sensitive for the detection of hepatic and retroperitoneal metastases.

Protein	Sample type	Workflow	MS platform	Proteins identified	Validation set	References
APO1A	Serum samples: Dukes A And B (N = 24), Dukes C and D (N = 24) and healthy controls (N = 26).	Serum protein enrichment and clean up, lysis buffer and labeling, 2D-DIGE.	Nanoacquity UPLC Q-TOF LS-MS/MS	2419 protein spots detected, 8 proteins up -regulated in early CRC and 2 down regulated. In late stage CRC, 14 proteins up-regulated, 4 proteins were down-regulated.	ELISA: 66 Serum Samples (Early Stage Disease N = 29, Late stage disease N = 19, Healthy controls N = 18). The 5 proteins selected for validation were APO1a, APOe, CFH, SYNJ2, GAL7. IHC: APO1a the only protein consistently identified.	[91]
HSP27	Histological samples: 404 primary tumors.	2DGE for 12 CRC samples. HSP identified, excised and trypsin digested. TMA of 404 CRC samples and 100 controls followed by IHC analysis.	QSTAR Pulsar i hybrid mass spectrometer LC-MS/MS	HSP27	TMA of 315 CRC in an independent cohort.	[77]
HSP40	Histological samples: 50 CRC formalin fixed, 10 frozen CRC.	IHC for the 50 formalin samples using positive and negative controls. Immunoblotting (SDS-PAGE). Immunoblot for 10 frozen samples.	N/A	HSP40 expressed in 14% of IHC samples, 80% of immunoblot HSP70 in 80% of IHC samples, 60% of immunoblot	N/A	[79]
HSP60	Histological samples: 50 CRC Serum: 112 CRC and 90 healthy controls for immunoassay.	Histological samples underwent 2DGE with internal standards. ELISA for HSP60 to compare serum levels.	MALDI TOF-MS	Approx. 1600 gel protein spots. 17 proteins with differential expression.	IMMUNOBLOT demonstrated significant overexpression of HSP60, glutathione-*S*-transferase pi, α-enolase, TCP1β. Leukocyte elastase inhibitor with decreased expression. HSP60 overexpression confirmed with IHC in 20 samples.	[83]

Protein	Sample type	Workflow	MS platform	Proteins identified	Validation set	References
HSP70 HSP 110 BCL	Histological: 81 CRC samples Cell culture lines	SDS-PAGE Immunoblot analysis IHC	N/A	Cell culture: HSP110, HSP70 elevated in highly metastatic cell lines. HSP 90, HSP60, HSP27 variable expression. BCL preferentially elevated in weakly metastatic cell lines. Histological IHC: HSP70, HSP110, Bcl-2 corresponded to cell line results. Bcl-2 positive staining correlated to less invasive cancer and earlier clinical stage.	N/A	[88]
HSP90A	Histological samples: 56 CRC cell culture lines	Immunoprecipitation with IHM-2 antibody Immunoblotting or MS analysis RT-PCR	MALDI-TOF LC-MS/MS	Tumor HSP90α overexpression was correlated with the metastasis and poor prognosis of CRC patients.	N/A	[39]
S100 A9 TCTP	Histological samples: Dukes stage B (n = 28) with paired normal tissue	2DGE, spot excision, trypsin digestion. TMA with normal colon mucosa (n = 50), primary (n = 515) and metastatic CRC (n = 224)	ESI-TRAP LC-MS/MS	1200 protein spots. 45 proteins overexpressed.	15 proteins validated with IHC. The most significantly overexpressed were HSP60, S100A9 and TCTP. 4-3-3b and aldehyde dehydrogenase 1 were identified as having prognostic benefit.	[81]
GSN	Histological samples: 5 CRC	Protein digestion, iTRAQ labeling, fractionation	Q-Star Pulsar LC-MS/MS	802 proteins identified, 82 with differential expression.	Immunoblot and IHC for GSN	[90]
MMP-1	Histological samples: 20 adenoma, 142 CRC.	IHC, in-situ hybridization, RT-PCR	N/A	MMP-1 immunoreactivity only	N/A	[96]

Protein	Sample type	Workflow	MS platform	Proteins identified	Validation set	References
MMP-2 MMP-1,3,9	Histological samples: 72 CRC with matched normal tissue and serum plasma samples	ELISA using commercial kits for MMP-1,2,3,9	N/A	Highly elevated concentrations of MMP-1, MMP-2, MMP-3 and MMP-9 protein expression in tumor tissue compared with tumor-free tissue ($p < 0.0001$). MMP-2 the only significantly increased in plasma.	N/A	[98]
MMP-7	76 Histological samples: Normal mucosa (n = 15) or tubular (n = 32), tubulovillous (n = 16), or villous (n = 13) adenomas.	IHC Grading G0-G3 according to the percentage of strongly stained areas.	N/A	MMP-7 identified in cytoplasm of all adenoma cells. Statistically significant difference in degree of overexpression of the three subtypes of colonic adenomas.	N/A	[103]
MMP-12	Serum samples: 78 CRC, 38 healthy controls.	Protein concentration estimation by commercial Assay kit. Luminex based Assay for MMP-7, MMP-10, MMP-12	N/A	Significant overexpression of MMP-7. MMP-10 and 12 not statically significant. All associated with significantly impaired survival.	N/A	[108]
MMP-13	Histological samples: 249 CRC.	IHC. Monoclonal antibody to MMP-13. Detection using Gelatin Zymography	N/A	MMP-13	N/A	[93]
TIMP-1	Histological: 94 CRC with matched healthy controls	IMMUNOBLOT assay IHC analysis TMA	N/A	Positive TIMP staining in 53.2% CRC samples, 80.6% of lymph node metastasis. Increased levels associated with decreased disease-free survival and overall survival	N/A	[109]

Protein	Sample type	Workflow	MS platform	Proteins identified	Validation set	References
TIMP-2	Histological and Serum samples: 72 CRC patients, 68 healthy controls	Serum: ELISA for TIMP-2 and MMP-2 Histological: IHC	N/A	Serum levels of MMP-2 and TIMP-2 were significantly lower in CRC. IHC demonstrated overexpression for both, immunoreactivity correlated with tumor grade.	N/A	[110]
TIMP-3	Histological samples: 351 CRC patients.	TMA IHC staining of MMP-1,2,7,13 using a commercial kit and TIMP-1,2,3,4 hand stained.	N/A	TIMP-3 the only marker of independent prognostic value	N/A	[111]
A2	Serum samples: 100 patients CRC, 70 healthy controls	ELISA	N/A	A2 levels significantly lower in patients with colon cancer when compared to control subjects	N/A	[113] See also [90]
A3 A4 A11	Serum and cell culture supernatants. Training set: 51 CRC, 26 healthy controls.	725 Candidate proteins identified by Literature search, Shotgun proteomics for Extracellular Vesicle (EV) Proteins	Q Exactive LC-MS/MS	356 EV proteins from Shotgun analysis. 46 proteins selected for SRM analysis for target peptide selection.	SRM analysis: 22 proteins A11, A3, A4, Tenascin-N, Transferring receptor protein 1, GLUT-1, C9, CD88 antigen, 78 kDa glucose-regulation protein, Alpha-1-acid glycoprotein, MMP-9, Angiopoietin-1, CD67 antigen, Mucin-5B, Adapter protein GRB2, A5, Olfactomedin-4, Neutral amino acid transport B(0), Tripeptidyl-peptidase 1, Heat shock-related 70 kDa protein 2, Proteasome subunit alpha type-5, Neutrophil gelatinase-associated lipocalin.	[26]

Protein	Sample type	Workflow	MS platform	Proteins identified	Validation set	References
PRTN3 ATM	Stool samples: 12 CRC, 10 healthy controls	1D-SDS GE. Tryptic peptides excised for MS	LTQ-FT MS LC-MS/MS	830 proteins, 134 increased in CRC (78 significantly more enriched than FIT negative samples).	Validation set identification of 63 of the proteins from discovery set, 33 selected for further validation using SRM. Differentially expressed proteins: Complement component C4B, Glucose-6-phosphate isomerase, Proteinase 3 (PRTN3), Alpha-2-micorglobulin (A2M), A100A8, S100A9, Azurocidin, Ceruloplasmin	[119]
C3	Fecal samples: (n = 315). Three series of patients with CRC, nonadvanced adenomas, advanced adenomas and healthy normal.	Protein extraction, GE, in-gel tryptic digestion. FIT analysis using antibody-based assays.	LTQ-FT Hybrid MS LC-MS/MS	834 proteins identified, 29 statistically increased in CRC including ATM, S100A8, S100A9, and C9	N/A	[118]
C9	Plasma: 69 CRC and 69 healthy control.	Assay for 187 biomarkers identified from a literature search. Sample preparation and trypsin digestion. Targeted MS analysis using MRM.	6490 triple quadrupole mass spectrometer LC-MS/MS	15 transition, 13 protein cross classifier.	MS-MRM assay. Identification of over 50% of the proteins from the discovery set. Proteins: Alpha1-acid glycoprotein, Alpah1-Antitrypsin, Alpha-Amylase 2B, Clusterin, Complement component C9, Mitochondrial Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, Ferritin light chain, Gelsolin, Metalloproteinase inhibitor 1, Osteopontin, Selenium-binding protein 1, Seprase, Spondin-2.	[23]

Protein	Sample type	Workflow	MS platform	Proteins identified	Validation set	References
S100A8 S100A9	Histological: 6 CRC and 6 healthy controls Plasma: 77 CRC, 11 adenoma, 21 healthy controls	2DGE. In gel digestion, protein spots excised for MS	AB 4700 Proteomics Analyzer LC-MS/MS	34 increased and 17 decreased proteins on gel spots, 4 selected for RT-PCR and IMMUNOBLOT analysis: Nm23-H1, S100A8, S100A9, Adenosylhomocysteinase.	Semi-quantitative PCR and IMMUNOBLOT analysis. IMMUNOBLOT analysis preformed for plasma samples. S100A8 and S100A9 were significantly increased in the plasma of CRC and colorectal adenoma patients compared to controls.	[36]

Synopsis of biomarker identification including sample type, pre-analytic workflow and MS technique (where applicable).

Table 2.
Summary of CRC biomarker identification methods.

3.2 Cancer antigen 19-9

Cancer (or Carbohydrate) antigen 19-9 (CA 19-9) is a clinical biomarker used in various diseases. Elevation can occur in benign conditions such as biliary and pancreatic disease, pulmonary disease, renal failure and autoimmune disease as well as malignant conditions of the pancreas, colon, rectum, liver, ovary and lung. CA 19-9 is therefore considered a non-specific biomarker of CRC [66] and is a classical marker for late stage disease and metastasis. For this reason it is not appropriate for use as a screening, or diagnostic, marker of carcinoma [67]. CA 19-9 can be used in tandem with CEA for post-operative monitoring to detect recurrence, or as a prognostic indicator as pre-operative elevation without correspond elevation of CEA is associated with a poorer 5-year survival [68]. When the combination of pre-operative elevation of both CEA and CA19-9 occurs, this is predictive of increased cancer mortality compared to non-elevated pre-operative levels [69].

3.3 Heat shock proteins

Heat shock proteins (HSP) are a type of stress-inducible protein that are present in all organisms [70] and their cells at low levels in normal physiological conditions. They have been functionally linked to cell apoptosis, protein homeostasis, cell growth mediation as play an important role during fertilization [70–76]. HSPs also function as chaperones, and act in protein assembly and unfolding. Various member of the HSP family have been postulated to have roles in antigen presentation and as a chaperones of peptides to major histocompatibility complex class I and class II [75, 76]. HSPs are typically classified into five subunits or families according to their molecular weight; Large HSP (HSP110, glucose-regulated protein 170), HSP90, HSP70, HSP60 and small HSPs (HSP27, HSP40). Significant research has focused around the role of HSPs in disease progression and on their role as therapeutic targets and as biomarkers.

3.3.1 HSP27

HSP27 is a member of the small HSP family, it has an anti-apoptotic role and acts as a chaperone to prevent misfolded protein aggregation. It is considered to be modulated by mitogen-activated protein kinase through phosphorylation. Abnormal HSP27 expression has been demonstrated in various cancer types, including ovarian, prostate, breast and colon cancer, as well as non-malignant conditions such as neurological and cardiovascular disease [76]. The overexpression of HSP27 in histological colon and rectal cancer samples was assessed in a large cohort of 404 patients with 2DE and tandem mass spectrometry (MS/MS) combined with a large validation set using tissue microarrays (TMA). The authors found that overexpression of HSP27 was present in both colon and rectal cancer and associated with poorer cancer-free survival in the rectal cancer cohort [77]. Furthermore the use of immunohistochemistry (IHC) and TMA analytical approaches has revealed that high HSP27 and HSP70 are associated with poorer clinical outcomes in primary resected CRC [78].

3.3.2 HSP40

HSP40 is also a member of the small HSP family and act as co-chaperones to HSP70. This family are further subdivided into DNAJA, DNAJB, and DNAJC; subgroups that have been shown to participate in both tumor progression or conversely, in tumor suppression in different types of cancer [75]. HSP40 overexpression in CRC has been demonstrated (along with HSP70) in 50 histological samples using IHC and immunoblotting [79].

3.3.3 HSP60

HSP60 is a chaperone protein with functions including transport and mitochondrial protein folding. This protein has been associated with a wide range of cancers including prostate, breast, cervical bladder, hepatic and CRC [71]. HSP60 is also elevated in non-cancer conditions such as chronic hepatitis and liver cirrhosis [80]. 2DE coupled to LC-MS–MS/MS using 28 histological adenocarcinoma samples and a 789 patient IHC validation set revealed that HSP60 was overexpressed along with S100A9 and translationally controlled tumor protein ($p < 0.001$ for each) [81]. This study also identified the beta subunit of 14-3-3 as a prognostic marker [69]. Additionally, IHC and immunoblot were used to demonstrate, in histological samples of 44 patients, that HSP60 was elevated in tumor tissues and there was a significant association between HSP60 levels, tumor differentiation, and tumor stage [82]. Comparison of colonic tumor samples and matched normal tissue confirmed overexpression of HSP60 (3.25-fold change ratio) with 2D-DIGE and immunoblotting) [83]. The authors of this study also developed an immunoassay for serum HSP60 detection, confirming a statistically significant elevation in serum HSP60 levels in CRC compared to controls ($P = 0.0001$) [83].

3.3.4 HSP70

HSP70 has 13 subgroup family members. It is associated with cytosolic calcium level homeostasis and, inhibition of HSP70 expression, has been shown to stimulate release of intra-cellular calcium in cell culture. Calcium induces cell death by the caspase dependent mechanism in CRC cell lines, and functions in the stabilization of lysosomes and inhibition of apoptosis [84]. Importantly, in other types of cancer such as pancreatic and prostate cancer, HSP70 has been shown to upregulate cell survival [84]. In a study of 33 CRC patient plasma samples, using ELISA assays, serum levels of HSP70 were significantly elevated ($\geq$2.25 ng/ml) in cancer patients compared to healthy controls, The sensitivity and specificity of elevated serum HSP70 in the CRC group was reported as 96.77% and 96.96% respectively [85]. It has been further demonstrated using ELISA testing that high serum concentration of HSP70 is associated with increased mortality ($p = 0.005$) [86]. Additionally the use of immunostaining has shown that mitochondrial HSP70 overexpression correlates to poor survival ($p = 0.04$) [87]. Independent IHC analyses of 81 primary CRC tissues revealed that HSP70, as well as HSP110, overexpression is associated with highly advanced clinical stages and positive lymph node involvement [88].

3.3.5 HSP90

HSP90 activates Hypoxia-inducible factor-1 and Nuclear Factor-κB which in turn regulate epithelial to mesenchymal transition, invasion and motility of CRC [89]. HSP90 has been shown in various studies to be overexpressed in CRC and may serve as a potential biomarker for CRC. In a small study of histological adenocarcinoma samples with an iTRAQ labeling method and QStar LC-MS/MS approach, a total of 82 altered proteins were found in CRC patients, which included overexpression of HSP90α and significant downregulation of Gelsolin. The results also suggested that HSP70 had decreased expression in the same samples [90]. Further validation using immunoprecipitation, MALDI-TOF-MS and immunoblotting confirmed that HSP90α is overexpressed in tumor cells and is correlated with poor prognosis and metastatic disease [91]. Plasma HSP90α serum levels were also significantly elevated in an analysis of 77 CRC patients compared to controls [92], thus highlighting the potential biomarker utility of this protein.

3.4 Matrix metalloproteinases and their tissue inhibitors

Matrix metalloproteinases (MMPs) are a diverse class of at least 25 zinc-dependent endopeptidases, which have important physiological applications and have also been implicated in the invasion, progression and metastasis of CRC. Accordingly, MMPs have been implicated as therapeutic targets, diagnostic and prognostic biomarkers. MMP subclasses have been demonstrated in various types of cancer, including breast and melanoma, and therefore are not cancer specific biomarkers [93].

3.4.1 MMP1 and MMP13: collagenases

MMP1 functions to degrade type I, II and III collagen. MMP13 is structurally similar to MMP1, and likewise it also cleaves collagens, as well as degrading extracellular matrix proteins including fibrillar collagen, fibronectin, tenascin C and aggrecan core protein 1 [94]. Demonstration by immunostaining of 133 CRC samples showed that MMP1 expression was significantly correlated with hematogenous colorectal metastasis [95]. Increased expression is also associated with poor prognostic factors such as invasion level, lymph node and hepatic metastasis [96]. Similarly, MMP13 overexpression in CRC has also been shown to be associated with poor prognosis [93].

3.4.2 MMP2 and MMP9: gelatinases

The gelatinase group of MMPs also function to degrade the extracellular matrix; their main substrates being collagen and gelatin. Overexpression of MMP2 may promote CRC invasiveness due to its degradation of β1 integrins, thereby enhancing motility and decreasing cell adhesion [97]. Quantification of tumor, normal tissue and plasma samples using ELISA in 72 patients identified upregulation of MMP1, MMP2, MMP3 and MMP9 in carcinoma. MMP2 overexpression was also significantly associated with lymph node metastasis [98].

3.4.3 MMP7: matrilysin

MMP7 promotes tumor invasion by proteolytic cleavage of extracellular matrix proteins such as proMMP2 and proMMP9, and it is also involved in cellular proliferation and apoptosis regulation. Overexpression of MMP7 is found in 80% of CRC [99], and is associated with poor prognosis. This protein has been shown to have a sensitivity of greater than 92% to identify colonic adenomas in mouse models, Additionally mouse models have implicated overexpression of MMP7 in tumourigenesis [100, 101] whilst in humans, MMP7 has been implicated in progression of adenoma to carcinoma. Accordingly, MMP7 has been demonstrated in numerous studies using IHC to be overexpressed in adenoma and various stages of carcinoma [102–104].

3.4.4 MMP12: metalloelastase

MMP12 is predominantly expressed in macrophages and degrades a wide range of substrates. MMP12 levels have been shown to be overexpressed in CRC, however this increased expression is associated with decreased risk of hepatic metastasis and decreased vascular endothelial growth factor expression [105, 106]. It is therefore postulated that MMP12 may have a protective role; a notion supported by a range of pro-tumourigenic effects being recorded following MMP12 inhibition [106, 107]. Conversely, along with MMP7 and MMP10, elevated serum levels of MMP12 have been suggested to be associated with poor CRC prognosis [108].

3.4.5 Tissue inhibitors of metalloproteases

Tissue inhibitors of metalloproteases (TIMPs) have been implicated in tumourigenesis. TIMP1 overexpression is associated with advanced stages of CRC [109]. IHC studies have demonstrated a significant correlation between TIMP2 expression in inflammatory cells, increasing tumor size, lymph node involvement and presence of metastasis [110]. TIMP3 has been described as independent prognostic marker for CRC, where strong cytoplasmic staining has been associated with longer survival in rectal cancer patients [111].

3.5 Annexins

Annexins are phospholipid-binding membrane-binding calcium regulated proteins from a multigene family. They function in membrane processes such as structural control as well as cell transport and as linkers between membranes, or between membranes and cytoskeleton as well as calcium regulated exocytosis. In humans the annexin family consists of subfamilies; A1–A11 and A13 [112]. The sensitivities of annexins A3, A4, and A11 peptides for detecting early-stage CRC have been reported to exceed those of CEA, and as such these peptides are promising biomarkers for early detection of CRC [26].

A shotgun proteomics analysis (LC-MS/MS) of extracellular vesicle proteins with selected reaction monitoring performed on CRC cell culture lines has demonstrated annexin A3, annexin A4, and annexin A11 overexpression, particularly in early stage CRC patients. Reported sensitivities of annexin A3, A4, A11 for stage one disease are in the range of 82.1–85.7%, and for stage two disease between 89.3–96.4% [26], therefore highlighting a potential role for these annexins as an early stage disease biomarker. Notably, the same study reported the sensitivity of CEA for early stage disease to be as low as 38.8% [26]. Importantly, progressive increases in annexin A3 abundance have been shown to strongly correlate with disease progression from normal tissue, to adenoma and finally to carcinoma [26].

Confirmation of Annexin A2 overexpression in a small cohort of histological samples has been described using a 2D-LC-MS/MS approach with iTRAQ labeling; with results being validated with immunoblot and IHC [90]. Conversely a study examining serum levels of Annexin A2 found that the protein was significantly lower in CRC patients compared to healthy controls; Annexin A2 levels were also inversely related to tumor size and stage [113]. In addition to Annexin A2, altered Annexin A4 expression has been demonstrated in CRC via the application of a label free LC-MS/MS approach, with validation in CRC serum samples confirming its overexpression and thus potential as a biomarker of the disease [114]. Annexin A10 is not frequently overexpressed in CRC with an estimated elevation being recorded in only 5.8% of patients. However, it too is associated with poor overall survival and poorer progression-free survival particularly in late stage cancers. As such, Annexin A10 may be considered as a prognostic marker when present [115], similarly annexin A13 expression is associated with lymph node metastasis, however it is not associated with tumor stage or differentiation [116].

3.6 Complement component proteins

3.6.1 Complement component C3

Complement component C3 (C3), and its fragment C3 anaphylatoxin (C3a), overexpression has been demonstrated in fecal, serum and histological samples from CRC patients. C3 is also a component of the innate immune system, with

functions including promotion of phagocytosis, local inflammatory responses and aiding in the adaptive immune response. C3 may also have a role in host cell damage when up regulated and aid in foreign pathogen invasion [117]. C3 overexpression in stool samples of CRC was demonstrated in two different cohort studies [118, 119], the second also showing a down-regulation of Proteinase 3 (PRTN3) and ataxia-telangiectasia mutated protein (ATM). Elevated levels of C3a overexpression were further demonstrated in serum samples using SELDI-TOF-MS and validated with MS and ELISA; the authors reporting a sensitivity of 96% and specificity of 96.21%. They also found C3a to be increased in the serum of 81.6% of adenomas [120].

3.6.2 Complement component C9

Complement component C9 (C9) is a constituent of the complement system that has important functions in the innate immune system. It is a terminal constituent of the membrane attack complex (MAC) and thereby aids in immune system response to cell death [121]. Changes in C9 expression have been described in both fecal and plasma samples, in a series of 315 stool samples using a combination of LC separation, LTQ-FT hybrid MS and QE-Label free-MS; C9 and C3 in addition with S100A8, S100A9 were found to be overexpressed [118]. A UHPLC–LC-MS approach and plasma-based immunoassay using 187 proteins previously described in the literature, demonstrated significant elevation of C9 in CRC plasma samples [23]. Similarly, an analysis of 31 CRC plasma samples revealed overexpression of C9 compared to healthy controls as well as reduced expression of Apolipoprotein AI [122].

3.7 S100 proteins

S100 are a family calcium-binding proteins, which consists of 24 members subdivided into three groups; broadly those with intracellular regulatory functions only, extracellular functions only and those with both intracellular and extracellular functions [123]. The proteins, S100A8 and S100A9, form a heterocomplex that is postulated to function in myeloid differentiation, cell transport, nuclear factor interaction and calcium related phagocytosis [123]. Mouse models have demonstrated accumulation of S100A8/A9 positive cells in areas of dysplasia and adenoma as well as promotion of MAPK and NF-κB activation signaling pathways [124]. IHC staining has demonstrated overexpression of S100A8, S100A9, Adenosylhomocysteinase (AHCY) and Nm23-H1 in CRC tumor cell cytoplasm, in the same study, S100A8 and S100A9 were also significantly increased in the plasma of CRC patients [36]. S100A8 has also been shown to have increased expression at progressive CRC stages (Duke's A-D) compared with controls [125]. Minichromosome maintenance complex component 4 (MCM4) and S100A9 overexpression have also been shown in proximal colonic fluid mouse proteome, using label free MS [126]. The same study identified Chitinase 3 like 1 (CHI3L1) protein overexpression in adenomas and advanced adenomas and CRC, the overexpression was further confirmed to be present in the serum of all three patient subtypes compared to controls [126]. A 2DGE LC-MS/MS based analysis of Dukes stage B CRC also identified S100A9, HSP60 and TCTP as overexpressed proteins. In addition to histological and plasma samples, S100A8 and S100A9 have been shown to be overexpressed in fecal samples also using a LC-MS/MS approach [118, 119]. Additionally, S100A11 has been identified among a cohort of 23 upregulated proteins in CRC samples using a combined targeted LC-MS/MS and SRM approach [22].

4. Conclusions

The development of non-invasive modalities with high patient compliance that can unequivocally detect and diagnose early stage CRC will afford the greatest opportunity for early intervention strategies to treat asymptomatic patients and ultimately improve the survival of patients with CRC. However, current screening methods are inadequate and there remains a pressing need to establish reliable biomarkers of early stage CRC disease. The resolution that is now achievable with advanced quantitative MS-based proteomic workflows and instrumentation hold the promise of unlocking the secrets of early stage disease that could be exploited to prevent or cure CRC. However, the inherent issues that have plagued MS-based biomarker discovery projects over the last 20 years moderate optimism. Sample size, particularly in the early stage setting, coupled with the wide dynamic range of blood plasma and the observed low concentrations of early stage specific individual protein biomarkers and the lack of reproducibility of MS investigations have meant that no new biomarkers of early stage CRC have entered the clinical setting since the discovery of CEA.

Over the coming years, the limitations of most current MS-based biomarker discovery projects will be resolved, mostly thanks to the recent developments in sophisticated techniques and technologies that not only simplify pre-analytical issues but address analytical limitations. Improvements in sample preparation techniques that potentially do away with immunodepletion, or the enrichment techniques that are currently absolutely necessary for the successful implementation of MS-based plasma biomarker investigation, will increase the reproducibility of future projects [127]. Analytic techniques that employ wider MS/MS windows for the simultaneous detection and quantification of low-abundant potential biomarkers using SWATH-MS strategies are important developments that will continue to arm our ever-evolving arsenal of MS technologies and resolve the issue of both detection and quantitation of low-abundant potential marker of early stage disease.

It is likely in the age of proteogenomics, that the greatest increase in resolution of early stage disease markers will come from the high-throughput simultaneous detection and quantification of protein and non-protein based biomarkers. Indeed, the combination of ctDNA and protein biomarkers in patient plasma with resectable pancreatic ductal adenocarcinomas showed a staggering 99.5% specificity, providing hope of early stage diagnosis for one of the most aggressive forms of gastrointestinal cancer [128]. Non-invasive blood tests combining non-protein and protein biomarkers represents an exciting approach for the early detection of any cancer type and holds the greatest potential for the increased survival of CRC patients worldwide.

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Conflict of interest

The authors declare no conflicts of interest.

Acronyms and abbreviations

CA 19-9	cancer/carbohydrate antigen 19-9
CEA	carcinoembryonic antigen
CRC	colorectal cancer
ELISA	enzyme-linked immunosorbent assay
FIT	fecal immunochemical test
HSP	heat shock protein
IHC	immunohistochemistry
iTRAQ	isobaric tags for relative and absolute quantitation
LC	liquid chromatography
LC-MS/MS	liquid chromatography tandem mass spectrometry
MS	mass spectrometry
MMP	matrix metalloproteinase
MRM	multiple reaction monitoring
PRM	parallel reaction monitoring
RP-HPLC	reversed-phase high performance liquid chromatography
TMA	tissue microarray
TMT	tandem mass tag
TNM	tumor, node, metastases, classification for malignant tumors
SRM	selected reaction monitoring
2DE	two-dimensional gel electrophoresis

Author details

Tiffany Gould[1,2], Muhammad Fairuz B. Jamaluddin[2,3], Joel Petit[1,2], Simon J. King[1,2], Brett Nixon[4], Rodney Scott[2,3], Peter Pockney[1,2] and Matthew D. Dun[2,3*]

1 School of Medicine and Public Health, Faculty of Health and Medicine, University of Newcastle, Callaghan, NSW, Australia

2 Priority Research Centre for Cancer Research, Innovation and Translation, Hunter Medical Research Institute Cancer Research Program, Newcastle, NSW, Australia

3 School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, University of Newcastle, Callaghan, NSW, Australia

4 Priority Research Centre for Reproductive Science, School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia

*Address all correspondence to: matt.dun@newcastle.edu.au

References

[1] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: Sources methods and major patterns in GLOBOCAN 2012. International Journal of Cancer. 2015;**136**(5):E359-E386

[2] Day DW, Morson BC. The adenoma-carcinoma sequence. Major Problems in Pathology. 1978;**10**:58-71

[3] Greene FL, Stewart AK, Norton HJ. A new TNM staging strategy for node-positive (stage III) colon cancer: An analysis of 50,042 patients. Annals of Surgery. 2002;**236**(4):416-421 discussion 421

[4] Gerstenmaier JF. Dukes staging system for colorectal cancer. Radiopaedia. 2018

[5] Society AC. Survival Rates for Colorectal Cancer, by Stage. 2018. Available from: https://www.cancer.org/cancer/colon-rectal-cancer/detection-diagnosis-staging/survival-rates.html [Accessed: July 9, 2018]

[6] Robertson DJ, Lee JK, Boland CR, Dominitz JA, Giardiello FM, Johnson DA, et al. Recommendations on fecal immunochemical testing to screen for colorectal neoplasia: A consensus statement by the US multi-society task force on colorectal cancer. Gastroenterology. 2017;**152**(5):1217-1237e3

[7] Schreuders EH, Ruco A, Rabeneck L, Schoen RE, Sung JJ, Young GP, et al. Colorectal cancer screening: A global overview of existing programmes. Gut. 2015;**64**(10):1637-1649

[8] Macrae FA. Colorectal Cancer: Epidemiology, Risk Factors, and Protective Factors. 2018. Available from: https://www.uptodate.com.acs.hcn.com.au/contents/colorectal-cancer-epidemiology-risk-factors-and-protective-factors?search=colorectal%20carcinoma&source=search_result&selectedTitle=3~150&usage_type=default&display_rank=3 [cited 2018]

[9] Government, A. National Bowel Cancer Screening Program. 2018. Available from: http://www.cancerscreening.gov.au/internet/screening/publishing.nsf/Content/bw-gp-infochart [cited 2018]

[10] Government D.o.H.A. National Bowel Cancer Screening Program. Review of the National Bowel Cancer Screening Program (Phase 2) - Final Report, Part One. 2018. Available from: http://www.cancerscreening.gov.au/internet/screening/publishing.nsf/Content/rev-nbcs-prog-pt1~inc-part~interp [cited 2018]

[11] Bretthauer M, Kaminski MF, Loberg M, Zauber AG, Regula J, Kuipers EJ, et al. Population-based colonoscopy screening for colorectal cancer: A randomized clinical trial. JAMA Internal Medicine. 2016;**176**(7):894-902

[12] Cappell MS. Reducing the incidence and mortality of colon cancer: Mass screening and colonoscopic polypectomy. Gastroenterology Clinics of North America. 2008;**37**(1):129-160, vii–viii

[13] Murray HC, Dun MD, Verrills NM. Harnessing the power of proteomics for identification of oncogenic, druggable signalling pathways in cancer. Expert Opinion on Drug Discovery. 2017;**12**(5):431-447

[14] Wilson SR, Vehus T, Berg HS, Lundanes E. Nano-LC in proteomics: Recent advances and approaches. Bioanalysis. 2015;**7**(14):1799-1815

[15] Gallien S, Domon B. Advances in high-resolution quantitative proteomics: Implications for clinical applications. Expert Review of Proteomics. 2015;**12**(5):489-498

[16] Larance M, Lamond AI. Multidimensional proteomics for cell biology. Nature Reviews. Molecular Cell Biology. 2015;**16**(5):269-280

[17] Lesur A, Domon B. Advances in high-resolution accurate mass spectrometry application to targeted proteomics. Proteomics. 2015;**15**(5-6):880-890

[18] Degryse S, De Bock CE, Demeyer S, Govaerts I, Bornschein S, Verbeke D, et al. Mutant JAK3 phosphoproteomic profiling predicts synergism between JAK3 inhibitors and MEK/BCL2 inhibitors for the treatment of T-cell acute lymphoblastic leukemia. Leukemia. 2018;**32**(3):788-800

[19] Dun MD, Chalkley RJ, Faulkner S, Keene S, Avery-Kiejda KA, Scott RJ, et al. Proteotranscriptomic profiling of 231-BR breast cancer cells: Identification of potential biomarkers and therapeutic targets for brain metastasis. Molecular & Cellular Proteomics. 2015;**14**(9):2316-2330

[20] You J, Kao A, Dillon R, Croner LJ, Benz R, Blume JE, et al. A large-scale and robust dynamic MRM study of colorectal cancer biomarkers. Journal of Proteomics. 2018;**187**:80-92

[21] Almazi JG, Pockney P, Gedye C, Smith ND, Hondermarck H, Verrills NM, et al. Cell-free DNA blood collection tubes are appropriate for clinical proteomics: A demonstration in colorectal cancer. Proteomics. Clinical Applications. 2018;**12**(3):e1700121

[22] Uzozie AC, Selevsek N, Wahlander A, Nanni P, Grossmann J, Weber A, et al. Targeted proteomics for multiplexed verification of markers of colorectal tumorigenesis. Molecular & Cellular Proteomics. 2017;**16**(3):407-427

[23] Jones JJ, Wilcox BE, Benz RW, Babbar N, Boragine G, Burrell T, et al. A plasma-based protein marker panel for colorectal cancer detection identified by multiplex targeted mass spectrometry. Clinical Colorectal Cancer. 2016;**15**(2):186-194 e13

[24] Surinova S et al. Prediction of colorectal cancer diagnosis based on circulating plasma proteins. EMBO Molecular Medicine. 2015;**7**(9):1166-1178

[25] Goslin R, O'Brien MJ, Steele G, Mayer R, Wilson R, Corson JM, et al. Correlation of plasma CEA and CEA tissue staining in poorly differentiated colorectal cancer. The American Journal of Medicine. 1981;**71**(2):246-253

[26] Shiromizu T, Jume H, Ishida M, Adachi J, Kano M, Matsubra H, et al. Quantitation of putative colorectal cancer biomarker candidates in serum extracellular vesicles by targeted proteomics. Scientific Reports. 2017;**7**:12782

[27] Leslie A, Carey FA, Pratt NR, Steele RJ. The colorectal adenoma-carcinoma sequence. British Journal of Surgery. 2002;**89**:845-860

[28] Hortin GL, Sviridov D. The dynamic range problem in the analysis of the plasma proteome. Journal of Proteomics. 2010;**73**(3):629-636

[29] Hassis ME, Niles RK, Braten MN, Albertolle ME, Ewa Witkowska H, Hubel CA, et al. Evaluating the effects of preanalytical variables on the stability of the human plasma proteome. Analytical Biochemistry. 2015;**478**:14-22

[30] Crews B, Wikoff WR, Patti GJ, Woo HK, Kalisiak E, Heideker J, et al. Variability analysis of human plasma and cerebral spinal fluid reveals

statistical significance of changes in mass spectrometry-based metabolomics data. Analytical Chemistry. 2009;**81**(20):8538-8544

[31] Tu C, Rudnick PA, Martinez MY, Cheek KL, Stein SE, Slebos RJ, et al. Depletion of abundant plasma proteins and limitations of plasma proteomics. Journal of Proteome Research. 2010;**9**(10):4982-4991

[32] Nibbe RK, Chance MR. Approaches to biomarkers in human colorectal cancer: Looking back, to go forward. Biomarkers in Medicine. 2009;**3**(4):385-396

[33] Faulkner S, Dun MD, Hondermarck H. Proteogenomics: Emergence and promise. Cellular and Molecular Life Sciences. 2015;**72**(5):953-957

[34] Norton SE, Luna KK, Lechner JM, Qin J, Fernando MR. A new blood collection device minimizes cellular DNA release during sample storage and shipping when compared to a standard device. Journal of Clinical Laboratory Analysis. 2013;**27**(4):305-311

[35] Friedman DB, Hill S, Keller JW, Merchant NB, Levy SE, Coffey RJ, et al. Proteome analysis of human colon cancer by two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics. 2004;**4**(3):793-811

[36] Kim HJ, Kang HJ, Lee ST, Lee H, Yu MH, Kim H, et al. Identification of S100A8 and S100A9 as serological markers for colorectal cancer. Journal of Proteome Research. 2009;**8**(3):1368-1379

[37] Choi JW, Liu H, Shin DH, Yu GI, Hwang JS, Kim ES, et al. Proteomic and cytokine plasma biomarkers for predicting progression from colorectal adenoma to carcinoma in human patients. Proteomics. 2013;**13**(15):2361-2374

[38] He Y, Mou Z, Li W, Liu B, Fu T, Zhao S, et al. Identification of IMPDH2 as a tumor-associated antigen in colorectal cancer using immunoproteomics analysis. International Journal of Colorectal Disease. 2009;**24**(11):1271-1279

[39] Lim LC, Looi ML, Zakaria SZ, Sagap I, Rose IM, Chin SF, et al. Identification of differentially expressed proteins in the serum of colorectal cancer patients using 2D-DIGE proteomics analysis. Pathology Oncology Research. 2016;**22**(1):169-177

[40] Abdallah C, Dumas-Gaudot E, Renaut J, Sergeant K. Gel-based and gel-free quantitative proteomics approaches at a glance. International Journal of Plant Genomics. 2012;**2012**:494572

[41] Muthu M, Vimala A, Mendoza OH, Gopal J. Tracing the voyage of SELDI-TOF MS in cancer biomarker discovery and its current depreciation trend - need for resurrection? Trac-Trends in Analytical Chemistry. 2016;**76**:95-101

[42] Hutchens TW, Yip TT. New desorption strategies for the mass-spectrometric analysis of macromolecules. Rapid Communications in Mass Spectrometry. 1993;**7**(7):576-580

[43] Zheng GX, Wang CX, Qu X, Deng XM, Deng BP, Zhang J. Establishment of serum protein pattern for screening colorectal cancer using SELDI-TOF-MS. Experimental Oncology. 2006;**28**(4):282-287

[44] Liu XP, Shen J, Li ZF, Yan L, Gu J. A serum proteomic pattern for the detection of colorectal adenocarcinoma using surface enhanced laser desorption and ionization mass spectrometry. Cancer Investigation. 2006;**24**(8):747-753

[45] Wang Q, Shen J, Li ZF, Jie JZ, Wang WY, Wang J, et al. Limitations in SELDI-TOF MS whole serum proteomic profiling with IMAC surface to specifically detect colorectal cancer. BMC Cancer. 2009;**9**:711-718

[46] Wilm MS, Mann M. Electrospray and taylor-cone theory, doles beam of macromolecules at last. International Journal of Mass Spectrometry. 1994;**136**(2-3):167-180

[47] Mann M, Kelleher NL. Precision proteomics: The case for high resolution and high mass accuracy. Proceedings of the National Academy of Sciences of the United States of America. 2008;**105**(47):18132-18138

[48] Kim MS, Pinto SM, Getnet D, Nirujogi RS, Manda SS, Chaerkady R, et al. A draft map of the human proteome. Nature. 2014;**509**(7502):575-581

[49] Wilhelm M, Schlegl J, et al. Mass-spectrometry-based draft of the human proteome. Nature. 2014;**509**(7502):582-587

[50] Wiese S, Reidegeld KA, Meyer HE, Warscheid B. Protein labeling by iTRAQ: A new tool for quantitative mass spectrometry in proteome research. Proteomics. 2007;**7**(3):340-350

[51] Thompson A, Schafer J, et al. Tandem mass tags: A novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Analytical Chemistry. 2003;**75**(8):1895-1904

[52] Johansson C, Samskog J, Sundstrom L, Wadensten H, Bjorkesten L, Flensburg J. Differential expression analysis of *Escherichia coli* proteins using a novel software for relative quantitation of LC-MS/MS data. Proteomics. 2006;**6**(16):4475-4485

[53] Kaplan A, Soderstrom M, Fenyo D, Nilsson A, Falth M, Skold K, et al. An automated method for scanning LC-MS data sets for significant peptides and proteins, including quantitative profiling and interactive confirmation. Journal of Proteome Research. 2007;**6**(7):2888-2895

[54] Zhang X, Xiao Z, Liu X, Du L, Wang L, Wang S, et al. The potential role of ORM2 in the development of colorectal cancer. PLoS One. 2012;**7**(2):e31868

[55] Fan NJ, Chen HM, Song W, Zhang ZY, Zhang MD, Feng LY, et al. Macrophage mannose receptor 1 and S100A9 were identified as serum diagnostic biomarkers for colorectal cancer through a label-free quantitative proteomic analysis. Cancer Biomarkers. 2016;**16**(2):235-243

[56] Yost RA, Enke CG. Triple quadrupole mass spectrometry for direct mixture analysis and structure elucidation. Analytical Chemistry. 1979;**51**(12):1251-1264

[57] Gallien S, Duriez E, Crone C, Kellmann M, Moehring T, Domon B. Targeted proteomic quantification on quadrupole-orbitrap mass spectrometer. Molecular & Cellular Proteomics. 2012;**11**(12):1709-1723

[58] Gillet LC, Navarro P, Tate S, Rost H, Selevsek N, Reiter L, et al. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: A new concept for consistent and accurate proteome analysis. Molecular & Cellular Proteomics. 2012;**11**(6):O111 016717

[59] Crutchfield CA, Thomas SN, Sokoll LJ, Chan DW. Advances in mass spectrometry-based clinical biomarker discovery. Clinical Proteomics. 2016;**13**:1

[60] Rost HL, Rosenberger G, Navarro P, Gillet L, Miladinovic SM, Schubert OT, et al. OpenSWATH enables automated,

targeted analysis of data-independent acquisition MS data. Nature Biotechnology. 2014;**32**(3):219-223

[61] Sajic T, Liu Y, Arvaniti E, et al. Similarities and differences of blood N-glycoproteins in five solid carcinomas at localized clinical stage analyzed by SWATH-MS. Cell Reports. 2018;**23**(9):2819-2831 e5

[62] Shively JE, Todd CW. Carcinoembryonic antigen A: Chemistry and biology. Cancer Markers. Contemporary Biomedicine. 1980;**1**:295-314

[63] Nicholson BD, Shinkins B, Pathiraja I, Roberts NW, James TJ, Mallett S, et al. Blood CEA levels for detecting recurrent colorectal cancer (protocol). Cochrane Database of Systematic Reviews. 2014;(12):6

[64] Macrae FA, Bendell J. Clinical Presentation, Diagnosis, and Staging of Colorectal Cancer: UpToDate. 2018

[65] Duffy MJ. Carcinoembryonic antigen as a marker for colorectal cancer: Is it clinically useful? Clinical Chemistry. 2001;**47**(4):6

[66] Pavai S, Yap SF. The clinical significance of elevated levels of CA 19-9. Medical Journal of Malaysia. 2003;**58**(5):667-672

[67] Vukobrat-Bijedic Z, Husic-Selimovic A, Sofic A, Bijedic N, Bjelorlic I, Gogov B, et al. Cancer antigens (CEA and CA 19-9) as markers of advanced stage of colorectal carcinoma. Medical Archives. 2013;**67**(6):397-401

[68] Stiksma J, Grootendorst DC, Van Der Linden PW. CA 19-9 as a marker in addition to CEA to monitor colorectal cancer. Clinical Colorectal Cancer. 2014;**13**(4):239-244

[69] Forones NM, Tanaka M. CEA and CA 19-9 as prognostic indexes in colorectal cancer. Hepato-Gastroenterology. 1999;**46**(26):908-908

[70] Dun MD, Aitken RJ, Nixon B. The role of molecular chaperones in spermatogenesis and the post-testicular maturation of mammalian spermatozoa. Human Reproduction Update. 2012;**18**(4):420-435

[71] Dun MD, Mitchell LA, Aitken RJ, Nixon B. Sperm-zona pellucida interaction: Molecular mechanisms and the potential for contraceptive intervention. Handbook of Experimental Pharmacology. 2010;**198**:139-178

[72] Dun M, Smith ND, Baker MA, Lin MJ, Aitken RJ, Nixon B. The chaperonin containing TCP1 complex (CCT/TRiC) is involved in mediating sperm-oocyte interaction. Journal of Biological Chemistry. 2011;**286**(42):36875-36887

[73] Dun M, Anderson AL, Bromfield EG, Asquith KL, Emmett B, McLaughlin EA, et al. Investigation of the expression and functional significance of the novel mouse sperm protein, a disintegrin and metalloprotease with thrombospondin type 1 motifs number 10 (ADAMTS10). International Journal of Andrology. 2012;**35**(4):572-589

[74] Black JD, Rezvani K. Heat shock protein 70s as potential molecular targets for colon cancer therapeutics. Current Medical Chemistry. 2016;**23**(28):3171-3188

[75] Chatterjee S, Burns TF. Targeting heat shock proteins in cancer: A promising therapeutic approach. International Journal of Molecular Sciences. 2017;**18**(9):1978

[76] Ge H, Yan Y, Guo L, Tian F, Wu D. Prognostic role of HSPs in human gastrointestinal cancer: A systematic review and meta-analysis. Oncotargets and Therapy. 2018;**11**:351-359

[77] Tweedle EM, Khattak I, Ang CW, Nedjadi T, Jenkins R, Park BK, et al. Low molecular weight heat shock protein HSP27 is a prognostic indicator in rectal cancer but not colon cancer. Gut. 2010;**59**:1501-1510

[78] Bauer K, Nitsche U, Slotta-Huspenina J, Drecoll E, von Weyhern CH, Rosenberg R, et al. High HSP27 and HSP70 expression levels are independent adverse prognostic factors in primary resected colon cancer. Cellular Oncology. 2012;**35**:197-205

[79] Kanazawa Y, Isomoto H, Oka M, Yano Y, Soda H, Shikuwa S, et al. Expression of heat shock protein (Hsp) 70 and Hsp 40 in colorectal cancer. Medical Oncology. 2003;**20**:157-164

[80] Seigneuric R, Mjahed H, Gobbo J, Joly A-L, Berthenet K, Shirley S, et al. Heat shock proteins as danger signals for cancer detection. Frontiers in Oncology. 2011;**1**:37

[81] O'Dwyer D, Ralton LD, O'Shea A, Murray GI. The proteomics of colorectal cancer: Identification of a protein signature associated with prognosis. PLoS One. 2011;**6**(**11**):e27718

[82] Mori D, Nakafusa Y, Miyazaki K, Tokunaga O. Differential expression of Janus kinase 3 (JAK3), matrix metalloproteinase 13 (MMP13), heat shock protein 60 (HSP60), and mouse double minute 2 (MDM2) in human colorectal cancer progression using human cancer cDNA microarrays. Pathology—Research and Practice. 2005;**201**(12):777-789

[83] Hamelin C, Cornut E, Poirier F, Pons S, Beaulieu C, Charrier JP, et al. Identification and verification of heat shock protein 60 as a potential serum marker for colorectal cancer. The FEBS Journal. 2011;**278**(24):4845-4859

[84] Kuppusamy P, Govindan N, Masthitah MY, Solachuddin JAI. Proteins are potent biomarkers to detect colon cancer progression. Saudi Journal of Biological Sciences. 2017;**24**(6):1212-1221

[85] Gunaldi M, Kocoglu H, Okuturlar A, Gedikbasi A, Karabulut M, Alis H, et al. Heat shock protein 70 is a useful marker for predicting colorectal cancer. Journal of the Balkan Union of Oncology. 2015;**20**(6):1464-1470

[86] Kocsis J, Madaras B, Toth EK, Fust G, Prohaszka Z. Serum level of soluble 70-kD heat shock protein is associated with high mortality in patients with colorectal cancer without distant metastasis. Cell Stress and Chaperones. 2010;**15**(2):143-151

[87] Dundas SR, Lawrie LC, Rooney PH, Murray GI. Mortalin is over-expressed by colorectal adenocarcinomas and correlates with poor survival. Journal of Pathology. 2005;**205**(1):74-81

[88] Hwang TS, Han HS, Choi HK, Lee YJ, Kim Y-J, Han M-Y, et al. Differential, stage-dependent expression of Hsp70, Hsp110 and Bcl-2 in colorectal cancer. Journal of Gastroenterology and Hepatology. 2003;**18**(6):690-700

[89] Nagaraju GP, Long TE, Park W, Landry JC, Taliaferro-Smith L, Farris AB, et al. Heat shock protein 90 promotes epithelial to mesenchymal transition, invasion, and migration in colorectal cancer. Molecular Carcinogenesis. 2014;**54**(10): 1147-1158

[90] Fan NJ, Gao CF, Wang CS, Lv JJ, Zhao G, Sheng XH, et al. Discovery and verification of gelsolin as a potential biomarker of colorectal adenocarcinoma in a Chinese population: Examining differential protein expression using an iTRAQ labelling-based proteomics approach. Canadian Journal of Gastroenterology. 2012;**26**(1):41-47

[91] Chen WS, Lee CC, Huang TS. Identification of heat shock protein 90α as an IMH-2 epitope-associated protein and correlation of its mRNA overexpression with colorectal cancer metastasis and poor prognosis. Colorectal Disease. 2011;**26**(8):1009-1017

[92] Kasanga M, Liu L, Xue L, Song X. Serum heat shock protein 90α have an advantage in diagnosis of colorectal cancer at early stage. Biomarkers in Medicine. 2018;**8**:881-890

[93] Leeman MF, Mckay JA, Murray GI. Matrix metalloproteinase 13 activity is associated with poor prognosis in colorectal cancer. Journal of Clinical Pathology. 2002;**55**(10):758-762

[94] Roy R, Yang J, Moses MA. Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer. Journal of Clinical Oncology. 2009;**27**(31):5287-5297

[95] Sunami E, Tsuno N, Osada T, Saito S, Kitayama J, Tomozawa S, et al. MMP-1 is a prognostic marker for hematogenous metastasis of colorectal cancer. The Oncologist. 2000;**5**(2):108-114

[96] Shiozawa J, Ito M, Nakayama T, Nakashima M, Kohno S, Sekine I. Expression of matrix metalloproteinase-1 in human colorectal carcinoma. Modern Pathology. 2000;**13**(9):925-933

[97] Said AH, Raufman JP, Xie G. The role of matrix metalloproteinases in colorectal cancer. Cancers. 2014;**6**(1):366-375

[98] Langenskiöld M, Holmdahl L, Falk P, Ivarsson M-L. Increased plasma MMP-2 protein expression in lymph node-positive patients with colorectal cancer. International Journal of Colorectal Disease. 2004;**20**(3):245-252

[99] Brabletz T, Jung A, Dag S, Hlubek F, Kirchner T. β-Catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal Cancer. The American Journal of Pathology. 1999;**155**(4):1033-1038

[100] Clapper ML, Hensley H, Chang WC, Devarajan K, Nguyen MT, Cooper HS. Detection of colorectal adenomas using a bioactivatable probe specific for matrix metalloproteinase activity. Neoplasia. 2011;**13**(8):685-691

[101] Wilson CL, Heppner K, Labosky PA, Hogan BL, Matrisian LM. Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. PNAS. 1997;**94**(4):1402-1407

[102] Newell KJ, Witty JP, Rodgers WH, Matrisan LM. Expression and localization of matrix-degrading metalloproteinases during colorectal tumorigenesis. Molecular Carcinogenesis. 1994;**10**(4):199-206

[103] Kirimlioglu H, Kirimlioglu V, Yilmaz S, Sagir V, Coban V, Turkmen E. Role of matrix metalloproteinase-7 in colorectal adenomas. Diggestive Disease and Sciences. 2006;**51**:2068

[104] Zeng Z, Shu W, Cohen A, Guillem J. Matrix metalloproteinase-7 expression in colorectal cancer liver metastases: Evidence for involvement of MMP-7 activation in human cancer metastases. Clinical Cancer Research : An Official Journal of the American Association for Cancer Research. 2002;**8**(1):144-148

[105] Asano T, Tada M, Cheng S, Takemoto N, Kuramae T, Abe M, et al. Prognostic values of matrix metalloproteinase family expression in human colorectal carcinoma. Journal of Surgical Research. 2008;**146**(1):32-42

[106] Decock J, Thirkettle S, Wagstaff L, Edwards DR, et al. Journal of Cellular and Molecular Medicine. 2011;**15**(6):1254-1265

[107] Overall CM, Kleifeld O. Validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. Nature Reviews Cancer. 2006;**6**:227-239

[108] Klupp F, Neumann L, Kahlert C, Diers J, Halama N, Franz C, et al. Serum MMP7, MMP10 and MMP12 level as negative prognostic markers in colon cancer patients. BMC Cancer. 2016;**16**:494

[109] Song G, Xu S, Zhang H, Wang Y, Xiao C, Jiang T, et al. TIMP1 is a prognostic marker for the progression and metastasis of colon cancer through FAK-PI3K/AKT and MAPK pathway. Journal of Experimental & Clinical Cancer Research: CR. 2016;**35**:148

[110] Groblewska M, Mroczko B, Gryko M, Pryczynicz A, Guzińska-Ustymowicz K, Kędra B, et al. Serum levels and tissue expression of matrix metalloproteinase 2 (MMP-2) and tissue inhibitor of metalloproteinases 2 (TIMP-2) in colorectal cancer patients. Tumor Biology. 2014;**4**:3793-3802

[111] Hilska M, Roberts P, Collan YU, Laine VJ, Kössi J, Hirsimäki P, et al. Prognostic significance of matrix metalloproteinases-1, -2, -7 and -13 and tissue inhibitors of metalloproteinases-1, -2, -3 and -4 in colorectal cancer. International Journal of Cancer. 2007;**121**(4):714-723

[112] Rescher U, Gerke V. Annexins—Unique membrane binding proteins with diverse functions. Journal of Cell Science. 2004;**117**:2631-2639

[113] Gurluler E, Guner O, Tumay LV, Kucukmetin NT, Hizli B, Zorluoglu A. Serum Annexin A2 levels in patients with colon cancer in comparison to healthy controls and in relation to tumor pathology. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research. 2014;**20**:1801-1807

[114] Han CL, Chen JS, Chan EC, Wu CP, Yu KH, Chen KT, et al. An informatics-assisted label-free approach for personalized tissue membrane proteomics: Case study on colorectal cancer. Molecular & Cellular Proteomic. 2011;**10**(4):M110.003087

[115] Bae JM, Kim J, Rhee Y-Y, Cho N-Y, Kim T-Y, Kang GH. Annexin A10 expression in colorectal cancers with emphasis on the serrated neoplasia pathway. World Journal of Gastroenterology : WJG. 2015;**21**(33):9749-9757

[116] Jiang G, Wang P, Wang W, Li W, Dai L, Chen K. Annexin A13 promotes tumor cell invasion in vitro and is associated with metastasis in human colorectal cancer. Oncotarget. 2017;**8**(13):21663-21673

[117] Sahu A, Lambris JD. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. Immunological Reviews. 2001;**180**:35-48

[118] Bosch LJW, De Wit M, Pham TV, Coupe VMH, Hiemstra AC, Piersma SR, et al. Novel stool-based protein biomarkers for improved colorectal cancer screening. Annals of Internal Medicine. 2017;**167**(12):855-866

[119] Bosch LJ, De Wit M, Oudgenoeg G, Hiemstra AC, Mongera S, Piersma SR, et al. Stool proteomics reveals new candidate biomarkers for colorectal cancer screening. Gastroenterology. 2012;**1**:S524

[120] Habermann JK, Roblick UJ, Luke BT, Prieto DA, Finlay WJJ, Podust VN, et al. Increased serum levels of complement C3a Anaphylatoxin indicate the presence of colorectal tumors. Gastroenterology. 2006;**131**(4):1020-1029

[121] Hoseok I, Cho J-Y. Chapter three—Lung Cancer biomarkers. In: Advances in Clinical Chemistry. 2015;**72**:107-170

[122] Murakoshi Y et al. Plasma biomarker discovery and validation for colorectal cancer by quantitative shotgun mass spectrometry and protein microarray. Cancer Science. 2011;**102**(3):630-638

[123] Donato R, Cannon B, Sorci G, Riuzzi F, Hsu K, Weber DJ, et al. Functions of S100 proteins. Current Molecular Medicine. 2013;**13**(1):24-57

[124] Ichikawa M, Williams R, Wang L, Vogl T, Srikrishna G. S100A8/A9 activate key genes and pathways in colon tumor progression. Molecular Cancer Research. 2011;**9**(2):133-148

[125] Xu BJ, Li J, Beauchamp RD, Shyr Y, Li M, Washington MK, et al. Identification of early intestinal neoplasia protein biomarkers using laser capture microdissection and MALDI MS. Molecular and Cellular Proteomics. 2009;**8**(5):936-945

[126] Fijneman RJA, De Wit M, Pourghiasian M, Piersma SR, Pham TV, Warmoes MO, et al. Proximal fluid proteome profiling of mouse colon tumors reveals biomarkers for early diagnosis of human colorectal cancer. Clinical Cancer Research. 2012;**18**(9):2613-2624

[127] Krisp C, Molloy MP. SWATH mass spectrometry for proteomics of non-depleted plasma. Methods in Molecular Biology. 2017;**1619**:373-383

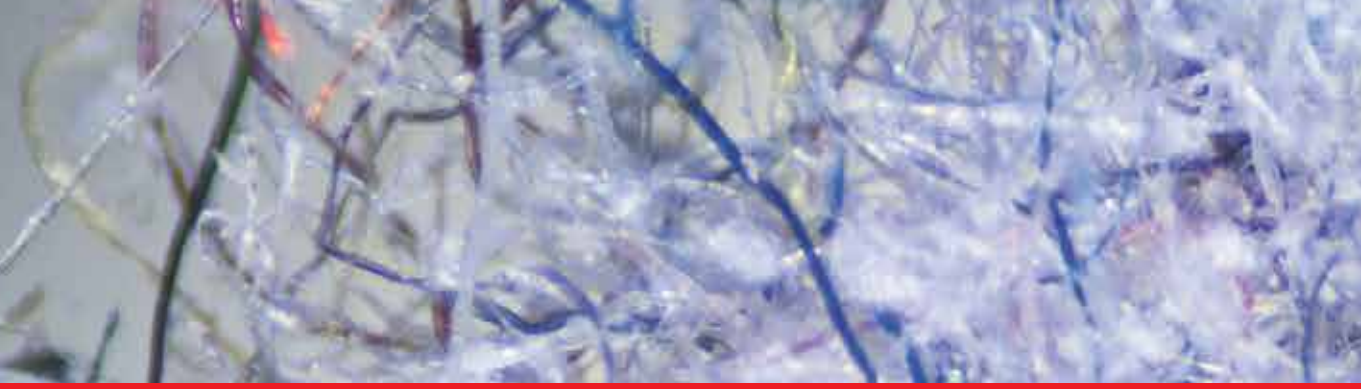
[128] Cohen JD, Javed AA, Thoburn C, Wong F, Tie J, Gibbs P, et al. Combined circulating tumor DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers. Proceedings of the National Academy of Sciences of the United States of America. 2017;**114**(38):10202-10207

[129] Binkley J, Libarondi M. Comparing the capabilities of time-of-flight and quadrupole mass spectrometers. Liquid Chromatography Online. 2010;**8**(3):28-33

[130] Scientific T. Mass analyzer technology overview. MS Learning Centre. 2018

[131] Liu C. The application of SELDI-TOF-MS in clinical diagnosis of cancers. Journal of Biomedicine and Biotechnology. 2011;**2011**:6

[132] Butterfield DA, Sultana R. Proteomics analysis in Alzheimer's disease: New insights into mechanisms of neurodegeneration. Human Brain Proteome. 2004;**61**:159-188



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An understanding of the molecular pathogenesis of colorectal cancer by researchers and clinicians is essential to facilitate progress in improving patient outcomes in this common cancer that still carries a poor prognosis if not identified early. This book covers the major areas of importance in the field, incorporating new knowledge that has arisen due to the advancement of molecular techniques and the ability to correlate molecular changes with clinical behaviour of tumours. Each chapter is a summary written by experts, concisely summarising current data as well as highlighting potential areas for advancement. Appreciating the differences between tumours on a molecular level is the key to developing and delivering precision medicine, and nowhere is this more critically required than in the field of colorectal cancer.

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