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Human Skin Cancers

Pathways, Mechanisms, Targets and Treatments

Edited by Miroslav Blumenberg

HUMAN SKIN CANCERS - PATHWAYS, MECHANISMS, TARGETS AND TREATMENTS

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http://dx.doi.org/10.5772/68061
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First published in London, United Kingdom, 2018 by IntechOpen
eBook (PDF) Published by IntechOpen, 2019
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Human Skin Cancers - Pathways, Mechanisms, Targets and Treatments
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p. cm.

Print ISBN 978-1-78923-094-9

Online ISBN 978-1-78923-095-6

eBook (PDF) ISBN 978-1-83881-278-2

Meet the editor

Miroslav Blumenberg, PhD, was born in Subotica and received his BSc in Belgrade, Yugoslavia. He completed his PhD at MIT in organic chemistry; this he followed up with two postdoctoral study periods at Stanford University. Since 1983 he has been a faculty member of the RO Perelman Department of Dermatology, NYU School of Medicine, where he is a co-director of a training grant in cutaneous biology. Dr. Blumenberg's research is focused on the epidermis, expression of keratin genes, transcription profiling, keratinocyte differentiation, inflammatory diseases and cancers, and most recently the effects of the microbiome on skin. He has published over 100 peer-reviewed research articles and graduated numerous PhD and post-doctoral students. Dr. Blumenberg lives in New York, USA, with his wife and two children.

Contents

Preface

The annual incidence of all forms of human skin cancer are increasing, representing a growing public concern. Human skin cancers are by far the most common type of tumors and so represent a significant health burden to the society: it is estimated that the annual cost is $8.1 billion for all skin cancers combined, of which some $3.3 billion is devoted to melanoma[1]. Nearly 5 million people are treated in the United States each year for various skin cancers, with melanoma, the deadliest, killing nearly 9,000 people yearly[2]. Skin cancer is a disease of the elderly and it has been estimated that half of all Americans who live to age 65 develop skin cancer at least once.

The deadliest skin cancer is unquestionably melanoma. If detected early it is curable by resection, however at later stages it is often deadly[3]. Melanoma stages 0 – 4 are described as: Stage 0: Melanoma in situ, the cancer is only in the epidermis. Stage 1: Up to 2 millimeters (mm) deep, not spread to lymph nodes or other sites. Stage 2: Melanoma thicker than 1 mm, may be thicker than 4 mm, not spread to lymph nodes or other sites, may or may not be ulcerated. Stage 3: Spread to local lymph nodes or nearby lymphatics, but not spread to distant sites. The primary cancer may be thicker than 4 mm, and it may be ulcerated. Stage 4: Metastatic melanoma has reached distant lymph nodes or metastasized to additional organs; most commonly liver, lungs, bones and brain are affected by these metastases.

It is estimated that 50% of all melanomas have an activating mutation in the *BRAF* gene, which promotes unrestrained melanocyte proliferation[4]. Novel drugs have been developed to specifically target mutated BRAF proteins. Two such drugs, vemurafenib and dabrafenib, have been approved by FDA for the treatment of late-stage melanomas. MEK is a BRAF effector protein, it acts downstream from BRAF, and therefore is another attractive target for melanoma therapy. Drugs that block MEK proteins, trametinib (Mekinist) and cobimetinib (Cotellic),have been shown to shrink some melanomas with mutated BRAF changes. Generally, they are used in combination therapy, with BRAF inhibitors, because they are expected to delay the development of resistance to BRAF-targeting monotherapies[5].

A very promising new approach for melanoma treatment is immunotherapy, an approach that boosts patient's own immune system to identify and defeat cancer cells more effectively. These drugs work by eliminating the restraints from the body's immune system. Pembrolizumab (Keytruda) and nivolumab (Opdivo) inhibit PD-1, a protein that prevents immune cells from attacking other cells in the body[6]. Blocking PD-1boosts the immune response against melanoma cells, leading to diminished tumors and prolonged lives. These drugs often cause serious side effects. Ipilimumab (Yervoy) also boosts the immune response, but it targets a different protein, CTLA-4, another immunity check point[7].

Alternatively, interferon-alfa and interleukin-2 are proteins that boost the immune system in a general way[8]. They were shown to shrink advanced melanomas in about 10% to 20% of patients and may be given together.

Interferon-alfa is sometimes be used as adjuvant therapy after surgery to delay the melanoma recurrence.

Public is urged to use the simple ABCDE rule when noticing the presence of malignant melanoma. These stand for asymmetry of the lesion - A, border, irregular - B, color, non-uniform - C, diameter >6 mm - D and evolving, changing in size, shape, or color - E [9].

Less deadly but more common than melanoma common human skin cancers are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC, the most common type of skin cancer, is caused by ultraviolet part of the sunlight, Usually it occurs on sun-exposed areas, such as the face, neck, or forearms. BCC is usually treated with surgical excision often using Mohs micrographic surgery to spare the nonmalignant tissue[10].

SCC, the second most common skin cancer, also occurs in chronically sun-exposed areas. Environmental agents, including papillomaviruses, can be contributing risk factors. SSCs may present as an ulcer, lump or red patch on the skin with scaling or crusting. Common in elderly, SCC often originates in actinic keratosis lesions. Squamous cell carcinoma is usually surgically treated, using Mohs micrographic surgery. Treating actinic keratosis may be a preventive procedure for SCC.

In this volume we focus on pathways, mechanisms, targets and treatments of human skin cancers, with particular emphasis on the new developments in the research on melanomas.

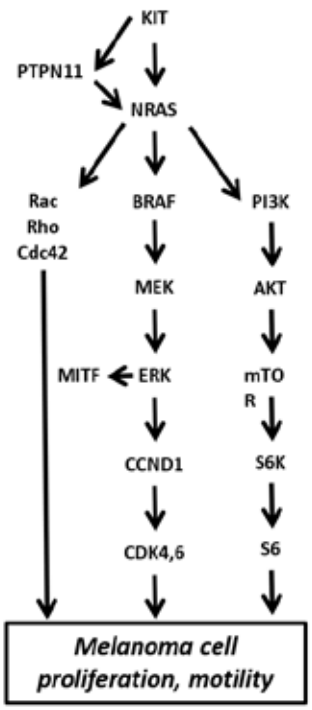

Figure 1. The important signaling pathways causing melanoma and potential objects for targeted therapies. Adapted from reference[11]

KIT is a proto-oncogene receptor tyrosine-protein kinase (CD117).

PTPN11 is a non-receptor tyrosine-protein phosphatase.

NRAS Neuroblastoma RAS viral oncogene homolog.

BRAF proto-oncogene serine/threonine-protein kinase.

MEK, ERK are members of a cascade of protein kinases that convey signal from cell surface to the nucleus pathway.

CCND1 encodes cyclin D1, a cyclin that regulates subunit of CDK4 or CDK6.

CDK4, 6 are cell division protein kinases whose activity is required for G1/S transition of the cell cycle.

RAC, Rho and **Cdc42** are small GTPase of the Rho family that regulate cytoskeleton in cell adhesion and migration.

MITF is a lineage-specific transcription factor found in melanocytes.

PI3K is phosphatidylinositol-3-kinase, an enzyme involved in cell proliferation, survival, differentiation, and motility.

AKT (a.k.a. PKB) is a serine/threonine protein kinase that regulates glucose metabolism, apoptosis, proliferation and cell migration.

mTOR is a member of the PI3K-related kinases family regulates cell proliferation, motility, survival and other processes.

S6K is the Ribosomal protein **S6** kinase; phosphorylation of S6 stimulates ribosomal protein synthesis.

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Section 1

Melanoma: Pathways and Mechanisms

Chapter 1

Epigenetics in Melanoma Development and Drug Resistance

Heinz Hammerlindl and Helmut Schaider

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70983

Abstract

Melanomas, which originate from melanocytic cells, mainly develop in the skin but can also arise at other body sites. The disease accounts for approximately 90% of deaths related to cutaneous tumors with late stage metastatic melanoma having a very poor prognosis of 6–9 month median survival for untreated patients. Research in the last decades resulted in ground-breaking discoveries of melanoma genetics and biology. High frequency mutations in genes like *BRAF*, *NRAS* and *KIT*, which lead to hyper-activation of the MAPK signaling pathway, drive melanoma progression. Targeting the MAPK signaling pathway has successfully been translated into effective therapies that significantly improve patient survival. Despite the unquestionable importance of such genetic events, the involvement of epigenetic alterations for melanoma development, and resistance to aforementioned therapies is becoming increasingly apparent. In this chapter, epigenetic alterations commonly found in melanoma are introduced, with a focus on histone and DNA modifications and their relevance for melanoma development, progression and therapy response. Detailed knowledge about this emerging aspect of melanoma research will help to understand the plastic nature of melanoma and set the foundation for novel treatment strategies that target aberrant gene regulation on genetic and epigenetic levels.

Keywords: biomarker, drug resistance, histone modifications, DNA methylation, melanoma, targeted therapy

1. Introduction

The grim prognosis for metastatic melanoma patients and the steadily increasing rates of melanoma incidents, that are projected to continuously rise within the next decades [1], represent a challenge for healthcare systems worldwide and highlight the importance of developing and optimizing prevention strategies, diagnostic approaches and treatment regimes.

After many years of research with unsatisfying treatment options and poor clinical outcomes, last decade has seen major advances in the therapy of metastatic melanoma driven by the revolutionizing discoveries of driver mutations and immune escape mechanisms that contribute to the aggressive nature of this disease. Drugs, developed to specifically exploit these mechanisms, administered either alone or in combination, have been shown to be clinically effective treatment strategies significantly increasing survival rates of patients [2–5]. Despite these recent ground-breaking advances in melanoma therapy, no currently available treatment options are curative in the majority of responding patients nor do all patients with $BRAF^{V600E}$ mutations respond to targeted therapies. Melanoma and targeted inhibition of oncogenic $BRAF^{V600E}$ became the poster child of an exciting initial therapy success followed by long-term resistance, which has also been experienced with other promising novel treatment strategies like immune checkpoint inhibitors targeting CTLA-4 and PD-1. The benefit of these new therapies is limited by the emergence of resistance, ultimately leading to tumor relapse. While the importance of genetic alterations for the development of disease and therapy resistance is unquestionable, it turns out that epigenetic remodeling is a fundamental feature of tumor development and adaption to therapy.

This chapter will briefly introduce the concept of epigenetics focusing on epigenetic alterations, especially changes in histone and DNA modifications during melanoma development and the emergence of therapy resistance. Detailed investigations into these changes will greatly contribute to our understanding of the heterogeneous and adaptive nature of melanoma. A thorough perception of how epigenetic drivers are modulating the genetic landscape will be the foundation for the development of new treatment strategies beyond pathway and immune checkpoint inhibitors.

2. Epigenetic changes

It has long been recognized that chromatin contains information beyond the primary DNA sequence. This information that is stored "on top of" the genetic information is highly dynamic and influences gene expression patterns and phenotypes without altering the nucleotide sequence while maintaining heritability to somatic daughter cells and in some cases even offspring *via* the germline. Multiple epigenetic mechanisms have been identified including ATP-dependent chromatin remodeling, the non-coding RNAs and different histone variants [6]. Here, we focus on the two most well studied aspects of epigenetic gene regulation, DNA methylation and histone modifications.

2.1. DNA methylation

The most well studied form of epigenetic information is stored by direct covalent chemical modification of the DNA itself. Cytosine residues in CpG dinucleotides are methylated at the fifth position generating 5-methyl cytosine (5-mC) (**Figure 1**) without affecting Watson-Crick base pairing and sequence information [7]. This modification is consistently found in most eukaryotic model systems [8]. Generally speaking, DNA methylation is associated with

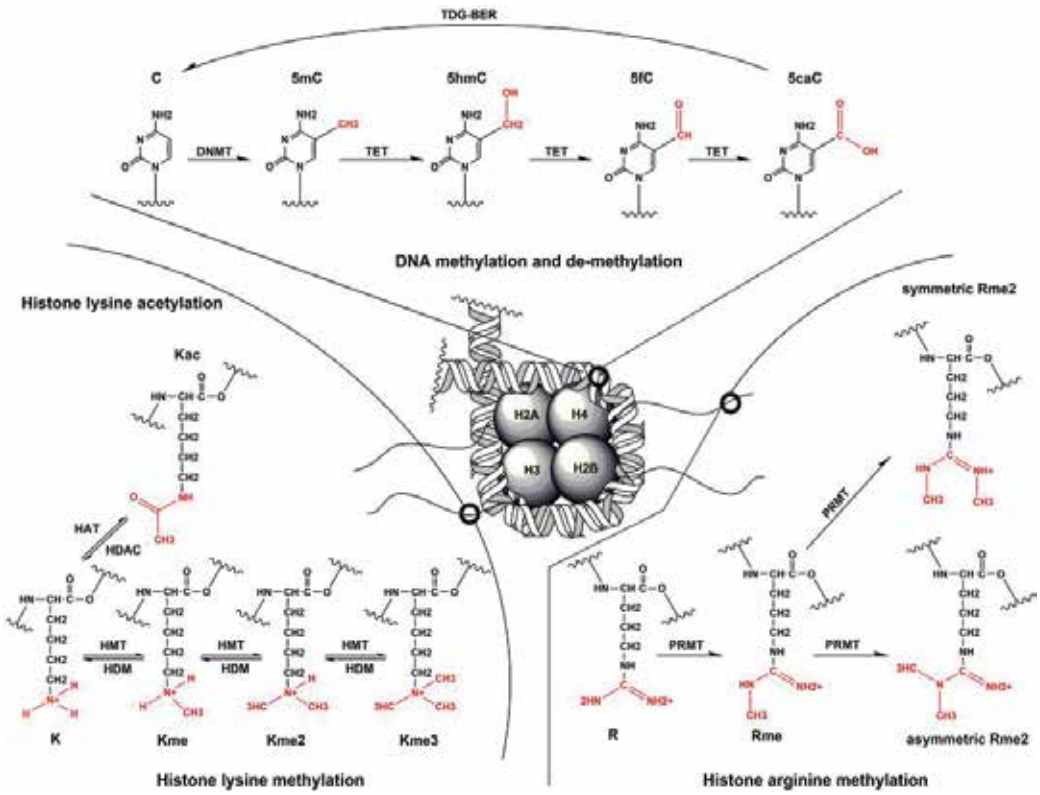

Figure 1. Schematic representation of DNA methylation and histone acetylation/methylation. The basic structural unit of eukaryotic DNA is the nucleosome. A nucleosome includes 147 bp of DNA that is wrapped around a histone octamer consisting of two copies of each core histone protein H2A, H2B, H3 and H4. The N-terminal tails of the histones protrude from the core particle and are subject to posttranslational modifications. Lysine residues (K) of several histone tails can be either acetylated (Kac) by histone acetyltransferases (HAT) or mono-, di- or tri-methylated (Kme, Kme2 or Kme3) by histone methyltransferases (HMT). These modifications can be reversed by corresponding histone deacetylases (HDAc) or histone demethylases (HDM). Additionally, arginine residues (R) can be either mono- or di-methylated (Rme or Rme2), whereby di-methylation can be presented either symmetrically or asymmetrically. Cysteine residues (C) can be directly methylated by DNA methyltransferases (DNMT) resulting in 5-methylcytosine (5mC), which can be further processed as part of active DNA demethylation to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by ten-eleven translocation (TET) proteins. DNA demethylation is then completed by thymine DNA glycosylase (TDG)-dependent base excision repair (BER).

transcriptional repression [9] and established by DNA methyltransferases namely DNMT1, DNMT3A and DNMT3B. While DNMT1 is responsible for the maintenance of DNA methylation, DNMT3A and DNMT3B catalyze the *de novo* synthesis of 5-mC [10]. Conversely, 5-mC can be removed either by replication-dependent dilution or active DNA de-methylation by ten-eleven translocation (TET) proteins. TET proteins are Fe(II)/α-ketoglutarate-dependent dioxygenases that catalyze the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which eventually is removed via base excision repair (BER) to restore un-methylated cytosine (**Figure 1**) [11]. Aberrant DNA methylation is a wide spread phenomenon in all cancers [12] suggesting an important role in malignant transformation.

2.2. Histone modifications

Regulatory epigenetic information is also embedded in the basic structure of chromatin and the nucleosome. The nucleosome core particle comprises 147 bp of DNA that is wrapped around an octamer of histone proteins consisting of two copies of H2A, H2B, H3 and H4 (**Figure 1**). Histones, especially the N-terminal tails, are subject to a multitude of posttranslational modifications including acetylation, methylation, phosphorylation, sumoylation, ubiquitylation or O-GlcNAcetylation with new modifications continuously identified [13]. The genome can be classified in transcriptionally active "open" euchromatin and transcriptionally inactive "closed" heterochromatin. Histone lysine acetylation affects this "open" and "closed" states by converting the charge of the affected residue at the histone tail, which decreases the histone/DNA interactions, increases DNA accessibility and therefore facilitates transcription and replication [14]. Alternatively, histone modifications can act as binding motives for transcription factors and other histone-modifying enzymes. For example, bromodomains specifically recognize acetylated lysine residues and are an important part of many chromatin-associated proteins [14]. The second very prominently studied histone modification is methylation of lysine or arginine residues. In contrast to acetylation, methylation can be present in different forms. Lysine residues can be mono-, di- or tri-methylated, while arginine residues can be mono-methylated or symmetrically or asymmetrically di-methylated, neither of which affects the charge of the amino acid side chain (**Figure 1**) [15]. Instead, methylated histone residues are recognized by a plethora of protein domains including plant homeodomain (PHD) zinc fingers, chromodomains, Tudor domains or WD40 repeats [16]. While histone acetylation is generally associated with active transcription, histone methylation has more diverse functions depending on the location of the modification. For example, H3K4me3 or H3K36me3 are usually found in active gene promoters whereas H3K9me3 or H3K27me3 are linked to transcriptional repression [13]. Histone modifications are generally reversible and dysregulation of either 'writers' (e.g. histone acetyltransferase or histone methyltransferases) or 'erasers' (e.g. histone deacetylase or histone demethylases) are attributed to the pathogenesis of human diseases [17].

3. Epigenetics in melanoma initiation and development

High-throughput DNA sequencing enabled detailed investigations into the genetic makeup of cancer and revealed hundreds of genes that are frequently mutated in melanoma [18]. Among these, a set of driver mutations has been identified that allows melanocytes to proliferate excessively, to overcome senescence and to divide indefinitely, resulting in their transformation into melanoma [19]. Despite the undeniable importance of genetic events, detailed knowledge of the molecular mechanisms of tumor initiation is still absent. This is due to the fact that such events, like epigenetic changes, are challenging to observe because models that represent individual stages of melanomagenesis are required. Nevertheless, the importance of epigenetic dysregulation in melanoma development becomes increasingly apparent, which is emphasized by the high frequency of mutations found in epigenetic regulators [20].

3.1. DNA methylation in melanoma development

One model used to investigate epigenetic alterations during melanoma development utilizes sequential cycles of anchorage blockade to transform mouse melanocytes resulting in cell lines that show different degrees of aggressiveness and *in vivo* tumor growth potential, to mimic different stages of melanomagenesis [21]. Investigating DNA and histone modifications in this model showed substantial epigenetic changes as global DNA methylation was decreased while multiple histone modifications including H4K16ac, H3K4me3, H3K27me3 and H3K9me3 were increased [21]. These findings are consistent with data from melanoma cell lines showing global hypo-methylation compared to melanocytes [22–24] with 11 out of 14 types of repetitive DNA elements being hypo-methylated [22]. Considering that repetitive DNA sequences constitute more than 45% of the human genome [25], changes of their methylation patterns affect the readout of global DNA methylation the most. Demethylation of these repetitive DNA elements has been reported to negatively influence chromatin organization, increase genetic instability or result in gene deregulation, all of which can promote tumorigenesis [26–28]. Microarray analysis of 27 common benign nevi and 22 primary invasive melanomas that covered 1505 CpG sites of regulatory regions of 807 cancer-related genes identified 26 CpG sites, associated with 22 genes that showed significant methylation differences. Of these 26 CpG sites, 19 showed significant hypomethylation with 7 hypermethylated [29].

While it appears that global DNA methylation levels are decreased during melanocyte transformation, many gene-specific CpG islands are hypermethylated. Comparing 24 primary cutaneous melanomas and 5 benign nevi using the Infinium BeadChip technology covering 27,578 CpG loci in the promoter regions of 14,495 genes identified 106 hypermethylated and 44 hypomethylated CpG islands. Among the 106 hypermethylated genes, *MAPK13*, which encodes the p38 isoform, has been found to have tumor suppressive functions as retrovirus-mediated overexpression of this gene displayed cytostatic effects and reduced melanoma growth *in vitro* [30]. Another interesting target gene that has been found to be regulated by DNA hypermethylation is the master regulator of pigmentation, MITF [31]. Lauss, et al. showed that hypermethylation of CpG islands belonging to *MITF* or MITF target genes correlated with decreased expression in metastatic melanoma tumors and melanoma cell lines. Melanoma cell lines that show intrinsically low *MITF* expression displayed CpG hypermethylation while cell lines that show high endogenous *MITF* levels were characterized by hypomethylation of these CpG islands. Accordingly, treatment with the DNA methyltransferase inhibitor 5′-Aza-2′-Deoxycytidine resulted in re-expression of MITF in MITF low cell lines. However, this re-expression was not sufficient to induce expression of the MITF target gene MLANA, suggesting that DNA methylation is involved but not sufficient to regulate MITF pathway activity in melanoma [31].

Differences in DNA methylation between melanocytes and melanoma can also be attributed to mutant BRAF, the most frequently mutated gene in melanoma [32]. Knockdown of BRAF in BRAF-mutant melanoma cell lines resulted in profound alterations of the methylation landscape with changes in gene expression affecting proliferation and invasion. Furthermore knockdown of BRAF significantly decreased DNMT1 and EZH2 expression suggesting that

$BRAF^{V600E}$-mediated pathway activation has a profound influence on the epigenetic landscape [33]. Analyzing $BRAF^{V600E}$ and $BRAF^{WT}$ samples from The Cancer Genome Atlas (TCGA) revealed that $BRAF^{V600E}$ correlates with global DNA hypomethylation. Primary melanoma samples showed a significantly decreased expression of *DNMT3A*, which is mainly responsible for *de novo* DNA methylation. Interestingly, *DNMT3A* expression was not found to be decreased in BRAF mutant BRAF wild type metastatic melanoma samples, suggesting that downregulation of this DNA methylatransferase is a transient event that might be important for melanoma initiation but not for metastatic spread and maintenance of global DNA hypomethylation [34]. Furthermore, Fang, et al. showed that $BRAF^{V600E}$ drives DNA hypermethylation and gene silencing of specific target genes in a v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G (MAFG) dependent manner in colorectal cancer and melanoma [35]. Specifically, MAFG is phosphorylated by the BRAF downstream kinase ERK1. This phosphorylation increases protein stability by reducing polyubiquitination and subsequent proteasomal degradation. MAFG then binds target gene promoters and recruits co-repressors including DNMT3B, ultimately resulting in DNA hypermethylation and gene silencing [35]. Unsupervised clustering of DNA methylation data from metastatic melanoma samples and TCGA melanoma samples identified three subgroups of melanoma tumors with differential methylation patterns [36]. These clusters did not correlate with either *BRAF*- or *NRAS*-mutation status nor primary tumor features like Breslow thickness or Clark's level, suggesting that factors beyond BRAF regulate DNA methylation in melanoma. The hypermethylated cluster (MS1) was associated with cell proliferation while the cluster with the lowest methylation levels (MS3) was associated with immunity, indicating a fundamental role of DNA methylation on melanoma and the microenvironment [36].

3.2. Histone modifications during melanoma development

Remarkable insights into the importance of histone modifications for melanoma development have been revealed using a zebrafish model in which the human $BRAF^{V600E}$ gene is under the control of the *mitfa*-promoter crossed onto a p53 loss-of-function background. This model develops nevi that eventually progress into melanoma [37]. Like in most genetically engineered animal models, only a small fraction of genetically identical melanocytes transform into melanoma, highlighting the importance of molecular events beyond genetic alterations to drive melanoma development. To address this problem and investigate melanoma initiation in more detail, Kaufman, et al. developed a triple transgenic zebrafish model (p53/BRAF/crestin:EGFP) in which a crestin/enhanced green fluorescent protein (crestin:EGFP) allows the visualization of neural crest stem/progenitor cells, the precursors of melanocytes [38]. Melanomas, which developed in these animals reestablish crestin:EGFP expression indicating that these cells reverse into a neural crest progenitor state. Knockout of sox10, a master regulator of neural crest identified and regulated by acetylation of histone 3 lysine 27 (H3K27Ac), significantly delayed melanoma onset. H3K27Ac in super enhancers at the *SOX10* locus was also found to be enriched in human melanoma cell lines indicating that epigenetic regulation of SOX10 expression is an important step of melanoma initiation [38].

Several histone-modifying enzymes have been shown to function aberrantly and contribute to melanoma progression. The H3K9me3-specific histone methyltransferase SET domain bifurcated 1

(SETDB1) is recurrently amplified within a region of chromosome 1 and shows a high expression in melanoma compared to nevi or normal skin [39]. Using the same zebrafish model as described above (*mitfa*-promoter crossed into a p53 loss-of-function background), Ceol, et al. identified that *SETDB1* amplification accelerates melanoma onset and increased invasivness. This was found to be independent of SETDB1 enzyme activity. Instead SETDB1 is part of a multimeric H3K9 methyltransferase complex including the H3K9me3 methyltransferase SUV39H1. Overexpression of SUV39H1 in the same zebrafish model also resulted in accelerated melanoma onset suggesting analogue functions of the entire H3K9 methyltransferase complex influencing melanoma development, at least partially by abrogating oncogene-induced senescence [39].

Another deregulated histone-modifying enzyme during melanoma development is the H3K27me3-specific histone methyltransferase enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2). EZH2 and H3K27me3 have been found to be elevated in aggressive melanoma cell lines and metastatic tumor samples. The expression of tumor suppressors RUNX3 and E-cadherin was found to be suppressed by EZH2 dependent H3K27me3 [40]. Accordingly, EZH2 is a major factor for melanoma initiation and progression. Knockout of EZH2 in a genetically engineered $NRAS^{Q61K}$ melanoma mouse model reduced the number of melanomas and prevented metastasis formation [41]. Mechanistically, this was mediated by EZH2 target genes including deoxycytidine kinase (*DCK*), adenosylmethionine decarboxylase 1 (*AMD1*) and WD repeat domain 19 (*WDR19*) which are suppressed in H3K27me3-dependent manner [41]. A possible explanation for EZH2 facilitating melanoma development could be by enabling senescence evasion. Knockdown of EZH2 in melanoma cells reestablished a senescence phenotype partially by reactivating p21/CDKN1A transcription, which was found to be independent of H3K27me3. Instead, at the transcriptional start site of p21/CDKN1A, H3K14ac was increased as a result of decreased recruitment of histone deacetylase 1 (HDAC1), which correlated with transcriptional activation [42]. This is in line with reports that EZH2, as part of the Polycomb repressive complex 2 (PRC2), is able to recruit histone deacetylases, which shows functional synergy with H3K27me3 in mediating target gene silencing [43]. Later, it was found that the non-canonical NF-kB pathway regulates EZH2 expression by direct binding of NF-kB2 to the EZH2 promoter. Inhibition of NF-kB2 induced a senescence-like phenotype, which was reversible upon EZH2 re-expression [44]. Not suprisingly, pharmacological inhibition of EZH2 has been shown to impair melanoma growth *in vitro* and *in vivo* [41, 44, 45] and has emerged as a interesting target in multiple cancer types [46].

A more specific example how histone modifications promote melanoma progression is found in the case of the telomerase reverse transcriptase (TERT). Recently, activating *TERT* promoter mutations result in new transcription factor binding sites within the promoter, have been identified in up to 71% of all melanomas, which equals or even exceeds the frequency of *BRAF* and *NRAS* mutations, suggesting a key role for this genetic alteration in melanoma development [47]. Interestingly, mutations in the *TERT* promoter frequently co-occur with BRAF mutations [48–50]. In fact it was found that MAPK pathway inhibition decreased H3K4me3 and H3K9ac in the mutant *TERT* promoter region. This resulted in loss of RNA polymerase II (Pol II) recruitment and decreased *TERT* transcription. Mechanistically, ERK2 directly binds mutant *TERT* promoters and inhibits HDAC1 repressor complex recruitment, which results in active *TERT* transcription [51].

A systematic overview of the epigenomic landscape of two phenotypically distinct melanocyte cell models that are characterized by low or high tumorigenicity showed distinct chromatin states associated with melanomagenesis. Specifically, chromatin state transitions characterized by loss of histone acetylation marks like H3K27Ac, H2BK5Ac and H4K5Ac and di-/trimethylation of H3K4 in regulatory domains associated with signaling pathways important for melanoma including phosphatidylinositol 3-kinase (PI3K), interferon (IFN) γ-, LKB1-, TRAIL- and platelet-derived growth factor (PDGF)-mediated signaling was observed, again emphasizing the link between epigenetic changes and melanoma development and progression [52].

Especially, loss of H3K4 methylation seems to be a key factor for melanoma growth and the highly problematic intratumor heterogeneity frequently observed in melanoma [53]. The histone 3 K4 demethylases jumonji/ARID1 (JARID1/KDM5B/PLU-1/RBP2-H1) defines a subpopulation of slow cycling melanoma cells, which is important for continuous growth of melanoma tumors. Interestingly, this subpopulation was found to be highly dynamic, as isolated KDM5B-positive and negative melanoma cells give rise to a heterogenous population consisting of both subpopulations [54] which highlights the variable nature of the epigenetic landscape in melanoma.

3.3. Epigenetic modifications as biomarkers and prognostic factors in melanoma

Because of the profound differences in DNA methylation patterns between melanocytic nevi and melanoma, several studies have investigated the suitability of DNA methylation as a predictive biomarker in melanoma. Unsupervised hierarchical clustering of 27 common benign nevi and 22 primary invasive melanomas resulted in separation of the two sample cohorts. Specifically, 22 genes were identified that significantly distinguished melanomas from nevi whereas 14 of these genes were validated in a separate set of 25 melanomas and 29 nevi [29] suggesting that analysis of differential DNA methylation patterns could be used as melanoma biomarkers. Later on Gao, et al. investigated the methylation differences of common nevi, dysplastic nevi, primary melanomas and metastatic melanomas and established a diagnostic algorithm based on promoter methylation patterns of *CLDN11, CDH11, PPP1R3C* which was able to distinguish dysplastic nevi from melanomas with a specificity of 89% and sensitivity of 67% [55]. DNA methylation changes, however, are not limited to melanoma development (nevi *versus* primary melanoma) but are also apparent in melanoma progression (primary melanoma *versus* metastatic melanoma). DNA methylation profiling using Illumina Infinium Human Methylation 450 K Beadchips of 14 normal nevi, 33 primary melanomas and 28 melanoma metastases identified gene promoters that were hypermethylated during melanoma development or melanoma progression [56]. Promoter methylation of several identified genes including *HOXA9, MEOX2, RBP1, TFAP2B, TWIST1* and *AKT3* were shown to be suitable biomarkers to distinguish between nevi, primary and metastatic melanoma. AKT3 and TFAP2B protein expression was also confirmed as biomarkers suitable for staining by immunohistochemistry. Furthermore, Wouters, et al. were able to correlate hypomethylation of *MEOX2, OLIG3* and *PON3* promoter hypomethylation with increased overall free survival [56]. Another major player in melanoma development that has been shown to be regulated by DNA methylation is Phosphatase and Tensin Homolog (*PTEN*). *PTEN* inactivating mutations or deletions have been found in 12% of the

TCGA melanoma cohort [57]. However, loss of PTEN expression is a more frequent event as reduced expression has been observed in approximately 50% of stage IIIB/C melanomas with a complete loss in 20–25% of all samples which correlated with decreased overall survival [58]. Accordingly, *PTEN* promoter methylation was found in 60.7% in the TCGA melanoma cohort and was an independent predictor for impaired patient survival [59]. Besides gene-specific DNA methylation, hypomethylation of repetitive DNA elements has also been associated with patient survival. Two studies report contradicting findings. Sigalotti, et al. analyzed cell lines isolated from 42 stage IIIC patients and reported that hypomethylation of 2 out of 3 CpG sites within Long Interspersed Nucleotide Element-1 (LINE-1) sequences correlated with improved prognosis and 5 year overall survival [59]. In contrast, Ecsedi, et al. extracted genomic DNA from primary melanoma and found that hypomethylation of 6 CpG sites associated within LINE-1 sequences in 46 primary melanomas correlated with decreased relapse-free survival of the corresponding patients and was also found to be associated with increased metastatic capacity [60]. A possible explanation for these contradictory results could be fundamental differences in the way how samples were analyzed. Sigalotti, et al. isolated and cultured melanoma cells from primary tumor tissue which might have affected the DNA methylome leading to the observed differences.

Besides changes in DNA methylation (5-mC), genome wide loss of the DNA demethylation intermediate 5-hydroxymethylcytosine (5-hmC) has recently been found to be a hallmark of melanoma [61]. Specifically, it has been shown that 5-hmC levels are progressively lost in melanoma compared to benign nevi, which was accompanied by decreased expression of TET family members and IDH2. Re-establishing 5-hmC by overexpression of TET2 reduced tumor growth and invasion suggesting an important function for 5-hmC in melanoma pathology. Accordingly, high levels of 5-hmC were found to negatively correlate with Breslow depth and predict better survival [61]. These findings were confirmed later on and suggest that 5-hmC analysis by immunohistochemistry could be a promising candidate as a prognostic biomarker in melanoma [62].

Presumed correlations between histone modifications and melanoma progression with prognosis have not been investigated compared to DNA methylation. This is in part because of technical challenges eminent by direct assessment of histone modifications [63]. Martinez, et al. performed immunohistochemical analyses of 10 benign nevi, 25 primary cutaneous melanomas without metastases, 19 primary cutaneous melanomas with metastases and 33 metastatic melanomas using an antibody specifically detecting H3K79 trimethylation and H3T80 phosphorylation (H3K79me3T80ph). They found a significant increase of H3K79me3T80ph in melanoma compared to nevi seemingly identifying a subset of primary melanomas with metastatic potential [64]. Another strategy to utilize histone modifications as biomarkers and prognostic factors that avoids the technical difficulties of direct assessment of histone modifications is to investigate the expression levels of histone-modifying enzymes. Along this line, it has been reported that the expression of the H3K27-specific histone methyltransferase EZH2 is increased during melanoma progression. However, only metastatic melanomas showed a significant increase compared to nevi [65]. Accordingly, analyses of EZH2 expression of TCGA melanoma samples showed a significantly shorter survival of patients with high EZH2 expression. Additionally, EZH2 high patients developed distant metastases faster, suggesting

a role for EZH2 in metastasis formation [41]. In contrast to EZH2, KDM5B has been found to be significantly downregulated during melanoma development. About 70% of the investigated nevi samples showed a KDM5B expression compared to 10 and 30% in primary and metastatic melanoma samples, respectively [66].

To our knowledge and despite the wealth of epigenetic changes that differentiate melanocytes and melanoma, no epigenetic biomarkers are used in the clinic to date.

4. Impact of epigenetic modifications on melanoma therapy

4.1. Acquired drug resistance, an obvious problem in melanoma therapy

Despite tremendous advances in developing innovative cancer therapies within the last few years, mechanisms for treatment failure are still not fully understood. Targeted inhibition of oncogenic $BRAF^{V600E}$ melanomas became the poster child of exciting initial therapeutic responses unfortunately followed by long-term resistance. Development of therapy resistance is the major obstacle for the successful use of targeted therapies, where almost all patients, who respond initially, are relapsing, irrespectively of single or combined inhibition of the MAPK pathway [67]. Furthermore, 15–20% of mutant *BRAF* tumors do not respond to targeted therapy in the clinical setting [68], suggesting the presence of pre-existing resistance mechanisms. Resistance to MAPK pathway inhibition has been shown to involve emergence of genetic mutations in *RAS* or *MEK*, amplification of mutant *BRAF* or alternative BRAF splicing [69, 70]. However, such genetic resistance mechanisms are absent in approximately 40% of patient samples, indicating the involvement of other mechanisms contributing to therapy failure [67]. Among these mechanisms, the upregulation of CRAF [71] or the SOX10-mediated activation of TGF-β that results in increased EGFR and PDGFRβ expression [72] that have been reported to mediate non-genetic resistance. Elevated EGFR and PDGFRβ levels have been shown to be reversible after discontinuing BRAF and MEK inhibitor treatment, while expression of EGFR or treatment with TGF-β resulted in a slow cycling drug-resistant phenotype [72]. This observation reflects findings by our group [73] and others [74, 75] of reversible multidrug-tolerant slow-cycling state following stressors like drug treatment. Beside failure of BRAF inhibition, a recent study found that dynamic and recurrent non-genomic alterations following chronic BRAF inhibitor treatment also affect tumor immunity possibly resulting in cross resistance to anti PD-1 therapy [76].

Even though immunotherapies like IL-2, adoptive T-cell transfer or antibodies that block CTLA-4 or PD-1 have shown long-term responses in some patients [77–80], many patients eventually relapse as melanoma cells escape immune surveillance. Genetic mechanisms like loss or mutation of specific antigens or parts of the major histocompatibility complexes that are involved in antigen presentation, have been attributed to immune evasion [81]. More recently, loss of function mutations in interferon-receptor signaling and in antigen presentation have been linked to resistance to PD-1 inhibition in three of four investigated patients [82]. Beside these genetic alterations that cause immunotherapy resistance, the expression of several melanoma antigens is linked to the dynamically regulated expression of NGFR

[83] or can be reversibly lost in response to inflammation [84]. Another study found a correlation between a mesenchymal transcription signature, including WNT5A and ROR2, with resistance to anti-PD-1 therapy in metastatic melanoma [85] suggesting the involvement of epithelial-mesenchymal transition in immunotherapy failure.

In the following paragraphs, the current knowledge about epigenetic mechanisms contributing to drug resistance in melanoma is summarized.

4.2. Epigenetic alterations and targeted therapy

One of the most clinically relevant observations that point towards non-genetically regulated drug resistance is the concept of drug holidays, which describes the phenomenon of intermittent treatment schedules or treatment interruption. This delays the emergence of resistance. One of the first reports describing the benefit of treatment interruption was a case study of a patient diagnosed with an adenocarcinoma of the lungs. After initial chemotherapy, the patient enrolled in a phase I study of the orally active epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib. After 18 month of drug response, the disease eventually progressed and was treated with a different combination of chemotherapy. One year after discontinuation of the initial treatment, gefitinib re-treatment resulted in a significant response [86]. Similar observations were further reported for patients treated with BRAF or BRAF/MEK inhibitors in which re-treatment with BRAF inhibitors resulted in a significant response after disease progression during an earlier treatment with BRAF or BRAF/MEK inhibitors [87]. A multi-institutional retrospective study later on found that 43% of patients that received re-treatment with BRAF inhibitors after disease progression and treatment interruption showed a clinically significant response [88]. Studies using vemurafenib-naive, primary human-patient-derived melanoma xenograft mouse models showed that vemurafenib resistance could be delayed by intermittent dosing schedules compared to continuous treatment [89].

The reversibility of drug resistance observed in clinical settings matches well with findings of slow cycling subpopulations that have been found to allow for reversible drug tolerance *in vitro.* One of the first reports of such a drug-tolerant subpopulation showed that a very small fraction of cancer cells including melanoma survives treatment with drug concentrations 100-fold higher than the IC50 [74]. These surviving cells were found to be mainly quiescent and in G1 arrest, they eventually continued growth in the presence of the drug. Importantly, drug withdrawal re-sensitized these drug-tolerant cells and re-established the same cellular heterogeneity as found in the initial sensitive population. Mechanistically, the surviving drug-tolerant cells exerted an altered chromatin state with increased expression levels of the histone demethylase KDM5A (JARID1A/RBP2) and concomitantly reduced levels of H3K4me2/3. RNAi-mediated knockdown of KDM5A confirmed that this histone demethylase is important for the establishment of the reversible drug-tolerant state [74]. This observation of an epigenetically regulated mainly G1 arrested state surviving exposure to high drug concentrations is similar to the previously mentioned slow cycling KDM5B^{high} subpopulation that is important for continuous melanoma growth [54]. KDM5B^{high} cells have been found to be enriched upon drug treatment and resemble a slow cycling drug-tolerant state in melanoma

as shRNA-mediated knockdown of KDM5B increased sensitivity to different drugs [90]. In accordance with the dynamic nature of KDM5A and KDM5B positive subpopulations, we have observed that chronic exposure to external stressors, rather than specific drug treatment, initiates an innate cellular response whereupon cells adopt a slow cycling, multidrug-tolerant phenotype [91]. Continuous exposure of melanoma cells to sub lethal BRAF inhibitor concentrations for 12 days initiated a cellular transformation and not the selection of a pre-existing subpopulation, which resulted in a slow cycling, mainly G1 arrested phenotype. These so called induced drug-tolerant cells (IDTCs) were unresponsive to 20-fold higher BRAF inhibitor concentrations as well as multiple other drugs including the MEK inhibitor GSK1120212 or cisplatin. As demonstrated for the KDM5A^{high} subpopulation [74], IDTCs re-gained drug sensitivity upon 7 days of drug withdrawal. On the molecular level IDTCs displayed elevated expression of drug efflux genes including *ABCB5*, *ABCA5*, *ABCB8* and *ABCB4*, as well as melanoma stem cell markers *NGFR*, *SOX10*, *CD44*, *SOX2* and *SOX4*, suggesting the transition into an undifferentiated state [91]. These molecular changes were accompanied by a profound decrease of histone marks H3K4me3, H3K27me3 that were decreased and H3K9me3, which was increased. Accordingly, expression of several histone-modifying enzymes including the H3K27-specific demethylases, KDM6A, KDM6B and the H3K4-specific demethylases, KDM1B, KDM5A and KDM5B was increased at the IDTC state [91]. Interestingly, a similar transition into an H3K4me3^{low}/H3K27me3^{low}/H3K9me3^{high} state was triggered by hypoxia and nutrient starvation and IDTCs generated by these stressors exhibited tolerance to BRAF inhibitors or cisplatin treatment, suggesting an epigenetically regulated drug-independent generic stress response that allows cells to cope with difficult environmental conditions [91]. Similar to our proposed IDTCs, a slow cycling, reversible NGFR^{high} state that displays features of de-differentiation has also been described, which has been shown to be susceptible to inhibition of epigenetic modifiers as bromodomain inhibitors, that block recognition of acetylated histones, suppressed the slowly cycling NGFR^{high} state [92].

Multiple studies proposed strategies to target the slow cycling drug-tolerant phenotype. Sharma, et al. showed that the KDM5A^{high} subpopulation that emerged after exposure to very high drug concentrations was susceptible to histone deacetylase (HDAC) inhibitors [74] because KDM5A is associated with histone decatylases during removal of histone modification marking active transcription [93]. HDAC inhibitors induced apoptosis in this subpopulation and combination of HDAC inhibitors with other drugs prevented the emergence of acquired resistance. Interestingly, HDAC inhibitors have to be present during the cytotoxic treatment as pre-treatment with histone deacetylase inhibitors followed by exposure to cytotoxic drugs alone was not sufficient to block acquired resistance [74]. This is important as it suggests that drug resistance is not mediated by a pre-existing subpopulation that carries intrinsic resistance mechanisms like additional mutations that can be eradicated, but by a dynamically regulated adaptive response that allows cancer cells to withstand unfavorable and toxic conditions. Roesch, et al. found that the KDM5B^{high} population enriched upon drug treatment in melanoma is dependent on oxidative phosphorylation as several members of the electron transport chain, including NADH dehydrogenase, ubiquinol cytochrome c reductase, cytochrome c oxidase and ATP synthase are highly expressed in these cells [90]. They further described that inhibition of the mitochondrial respiratory chain using oligomycin,

rotenone or phenformin blocked endogenous KDM5B expression and decreased the drug-induced enrichment of KDM5B^{high} cells. Furthermore, combination of orally available NADH dehydrogenase inhibitor phenformin with BRAF inhibitor vemurafenib increased the tumor suppressive effects *in vivo* [90]. In the same year, Yuan, et al. showed AMPK-dependent synergistic cytotoxicity of combining BRAF inhibitors and phenformin which also suppressed the emergence of a drug-resistant phenotype [94].

The IDTC phenotype described by us is characterized by elevated expression of several histone-modifying enzymes showing no specific susceptibility to combinations of BRAF inhibitors with HDAC inhibitors, AKT inhibitors or oligomycin [91]. In accordance with previous studies, knockdown of KDM5B-sensitized melanoma cells to BRAF inhibition, but the surviving cells again displayed the IDTC phenotype. Exposure of established IDTCs to different drugs including MEK, AKT and HDAC inhibitors showed that these compounds effectively suppressed their target pathways within 3 days of treatment. However, slow cycling melanoma cells were able to adapt to this additional stressor and re-activated the respective pathways within 12 days of drug exposure. In the case of HDAC inhibitors, methylation patterns of histone 3 lysine 4 and 9, which have been shown to be co-regulated with histone acetylation via transcriptional regulation of histone methyltransferases and histone demethylases [95, 96] were re-established to resemble the H3K4me3^{low}/H3K9me3^{high} pattern seen in the slow cycling multidrug-tolerant cells [91]. A possible explanation for the discrepancy between the discussed studies in regards to the different strategies to target heterogenous slow cycling populations could be that the KDM5A^{high} or KDM5B^{high} cells are stringently selected subtypes of the slow cycling phenotype whereas IDTCs are characterized by multiple epigenetic modifiers, most likely including multiple subtypes that contribute to the same phenomenon. The dynamic signaling rewiring observed in the IDTC phenotype is reminiscent of the diverse drug resistance mechanisms that have been reported to emerge from slow cycling EGFR inhibitor addicted lung cancer cells [75], which suggests that an adaptive response as described for IDTCs in melanoma might be present in multiple cancer types. One key feature of all slow cycling drug-tolerant cell populations that emerge after 3–12 days of drug exposure is the reversibility upon drug withdrawal. However long-term exposure (90 days) of melanoma cells to BRAF inhibitors resulted in loss of the IDTC markers NGFR as well as KDM5B [91]. Interestingly, these cells displayed no multidrug resistance but maintained resistance to BRAF inhibitors despite drug withdrawal, suggesting the emergence of permanent resistance [91].

4.3. Epigenetic alterations and immunotherapy

Epigenetic regulation is a key mechanism for maintaining immune cell identity and differentiation. For example, CD8 positive cytotoxic T lymphocytes undergo dynamic changes of DNA methylation and histone modification patterns following infection that are important for regulation and maintenance of their differentiation states [97]. Therefore, it is important to consider that epigenetic targeting drugs will not only affect tumor cells but also influence immune cells and other cells of the tumor microenvironment. Herein, the effects of epigenetic alterations within cancer cells, specifically melanoma, and how these changes affect the therapeutic effect of immunotherapy will be discussed.

The most promising immunotherapies currently in clinical use are anti-PD-1 and PD-L1 therapies [98]. Analyses of 52 immunotherapy-naïve stage III melanomas specimens in regard to the PD-L1 expression suggested that PD-L1 negative status is associated with worse prognosis and a poor immune response gene signature. PD-L1 positive melanomas showed a significant association with the TCGA hypomethylation cluster suggesting that upregulation of immune checkpoint inhibitors is found in cancer cells with altered gene expression. Another study showed that treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine activates a viral defense pathway. Expression levels of these viral defense genes grouped different cancers including melanoma into separate categories where high expression was associated with the TCGA immune reactive (IMR) tumors with a good prognosis [99]. Melanoma patients with high levels of the viral defense signature correlated with response to anti-CTLA-4 for more than 6 month and combined treatment of 5-aza-2′-deoxycytidine and anti-CTLA4 immune checkpoint therapy in a B16-F10 mouse melanoma model enhanced tumor responses [99]. Another important factor for the successful immunotherapy response is the expression of tumor-associated antigens [100]. Along this line, it has been shown that the expression of high molecular weight-melanoma associated antigen (HMW-MAA) is regulated by DNA methylation as its expression correlates with promoter methylation. As such it is induced by treatment with 5-aza-2′-deoxycytidine [101].

Multiple studies reported the importance of histone modifications for the regulation of immunogenic factors. For example, H3K4me3 dependent PD-L1 expression has been observed in pancreatic cancer [102] or H3K27me3 and DNA methylation-mediated silencing of Th1-type chemokines CXCL9 and CXCL10 in ovarian cancer cells [103], suggesting an important role for histone modifications in the regulation of immunomodulatory factors across different cancer types. Further evidence of epigenetically regulated PD-L1 expression is provided by studies using HDAC inhibitors in melanoma cell lines. Specifically, treatment with class I HDAC inhibitors resulted in increased acetylation of histone 3 in PD-L1 and PD-L2 promoter regions, which resulted in increased PD-L1 expression *in vitro* and *in vivo* [104].

5. Conclusion

Keeping in mind the wealth of data describing epigenetic alterations during melanoma development and also in relation to the therapeutic response targeting or co-targeting these epigenetic events appears to be a very promising strategy for improving melanoma management. This is especially true in light of the highly heterogeneous and adaptive nature of melanoma which cannot be explained only by stable genetic events. While epigenetic biomarkers have not yet been put to clinical use, there is an overwhelming number of clinical trials utilizing and testing epigenetic drugs in different cancer types. These trials investigate the use of general epigenetic inhibitors targeting histone deacetylases, bromodomain and extra-terminal (BET) proteins (histone acetylation binding proteins) and more specific inhibitors targeting DNMT1, IDH1 and IDH2 (affect TET enzyme function), EZH2, DOT1L (histone H3K79 methyltransferase) or KDM1A [105].

Additionally, epigenetic drugs are tested in combination with already established chemo-, targeted- and immunotherapies. Besides synergistic effects of these drugs, this approach could also result in prevention or reversion of drug resistance, a concept that has already been shown *in vitro* more than 15 years ago [106]. In melanoma, one clinical trial is currently investigating the combination of the BRAF/MEK inhibitors vemurafenib and cobimetinib with the DNA hypomethylating agent decitabine (NCT01876641). However, the main focus in the field appears to be the combination of epigenetic drugs, especially DNA methyltransferase and histone deacetylase inhibitors with immunotherapy, which is currently tested in numerous clinical trials [107] and the outcome of these promising approaches is highly anticipated.

While these current clinical trials hold great promise, improved understanding of detailed epigenetic mechanisms, identification of new key players in epigenetic remodeling and the subsequent development of specific inhibitors, which modulate and target epigenetics have the potential to shape the future of melanoma therapy.

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Chapter 2

LncRNAs as Biomarkers for Melanoma

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70499

Abstract

Melanoma is the most aggressive and serious type of skin cancer. Known for being highly malignant and metastatic, melanoma typically has low survival rates. Prognosis can be improved with an early diagnosis and a good monitoring of the disease. However, current melanoma biomarkers display severe limitations, making them inadequate for early detection of the malignancy. Therefore, it is of urgent matter for us to characterize and establish novel biomarkers with a direct application to daily clinics in order to accurately detect early american joint committee on cancer (AJCC) stages in melanoma patients, efficiently monitor the disease progression, and reliably predict the response to therapies, survival, and likely future recurrence. Long non-coding RNAs (lncRNAs) are a promising biomarker and regulator of tumor progression for many cancers. They are secreted into the bloodstream inside exosomes by a wide range of malignant cells and several of them have actually been validated as promising circulating molecular signatures of other cancer types, but not melanoma. However, in recent years there has been much research into lncRNA melanoma biomarkers, and many of them have been characterized as potentially clinically relevant.

Keywords: melanoma, biomarkers, lncRNAs, cancer, metastatic melanoma

1. Introduction

Melanoma is the most aggressive and serious type of skin cancer. Its propensity for rapid development and ease of metastasis to vital organs such as the brain, lungs, and liver make it so deadly. Additionally, the incidence of melanoma in the United States has been consistently increasing since at least the 1970s [1]. Most importantly, early diagnosis predicts longer survival and better prognosis [2, 3]. This makes efficient and accurate diagnosis of melanoma a priority for clinicians. Thus, the continued exploration for accurate and efficacious biomarkers is a priority among cancer research.

This chapter aims to describe various characterized and novel long non-coding RNAs (lncRNAs) as melanoma biomarkers. We will first explore the shortcomings and problems of current biomarkers and how lncRNAs can serve as the potential future for melanoma markers. We will then look at already characterized lncRNAs such as BRAF-activated non-coding RNA (BANCR), Sprouty 4 (SPRY4), HOX transcript antisense RNA (HOTAIR), Metastasis-associated lung adenocarcinoma transcript (MALAT), and Antisense non-coding RNA in the INK4 locus (ANRIL), as well as current research methods. Finally, we will discuss future perspectives and what we still need to do to adapt lncRNA for use as a melanoma biomarker.

2. The importance of early diagnosis

Melanoma is diagnosed as an AJCC stage I, II, III, or IV. Stages I and II are characterized as melanomas of varying Breslow thicknesses and possible ulceration, but with no lymph node involvement or metastases. Depending on the sub-stage, 5-year survival ranges from 53% to a robust 97% [4]. Stage III is characterized by regional metastases with 5-year survival rates of 40–78% depending on sub-stage [4]. Stage IV is characterized by distant metastases with extremely poor prognosis and 1-year survival rates ranging from 33 to 62% depending on location of metastases and serum LDH level [4].

There have been recent breakthroughs in melanoma treatments for stages IIIC and IV. Common therapies approved by the Food and Drug Administration include both immunotherapy and small molecule targeted therapy. Both immunotherapy drugs such as pembrolizumab (anti-PD-1) [5, 6], nivolumab (anti-PD-1) [7], and ipilimumab (anti-CTLA-4) [7, 8], and small molecules inhibitors such as dabrafenib (BRAF inhibitor) [9], vemurafenib (BRAF inhibitor) [10], and trametinib (MEK inhibitor) [11] have improved patient survival. However, most tumors become drug-resistant shortly after commencing therapy, resulting in disease progression [12, 13]. Unfortunately, our current therapies are more of a temporary stay than a permanent cure.

Thus, the best way to ensure long-term survival is to diagnose the malignancy while it is in its early stages and slow disease progression through surgery and adjuvant therapy. Melanoma biomarkers play an important role in the diagnosis and prediction of the progression of the disease. However, they have severe limitations in regard to precision to detect early stages of melanoma and reliability as a predictor of disease prognosis and treatment response. By understanding the molecular basis of the disease more, we can identify novel biomarkers that can be used to more efficiently diagnose disease which will undoubtedly improve outcomes and quality of life.

3. Current biomarkers in melanoma

According to the National Institutes of Health Biomarkers Definitions Working Group, biomarkers, or "biological markers," are "a characteristic that is objectively measured and

evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [14]. Biomarkers are commonly used in both clinical trials and clinical practice because of its multitude of applications: as diagnostics for identifying patient conditions or diseases, as a tool for staging and characterization of disease, as an indicator of disease progression, or as a predictor of treatment response [14].

Tumor markers are specifically biomarkers of cancer. They are usually proteins that are tumor-derived (produced by the tumor cells) or tumor-associated (produced by the body in response to tumor cells development) [15]. Tumor markers can appear in a variety of samples, but the most commonly used specimens for detection are blood serum and urine. Both are viable for these purposes because of the ease of collection and processing, as well as the secretion of distinctive cancer markers into these fluids.

Proteins are the most common tumor marker because of their central roles in cell signaling and influence on key molecular pathways in various cells and systems of the body. They are often most easily measurable through immunohistochemistry as certain protein levels change between normal and tumor cells. However, many other molecules can also serve as tumor markers. Cell-free circulating tumor DNA (ctDNA) were characterized in the late 1980s and since then, it has been found that ctDNA is correlated with tumor size and disease activity [16, 17]. ctDNA can be analyzed through liquid biopsies and PCR-based assays [18]. Circulating tumor cells (CTCs) are often present in advanced neoplasias that have metastatic potential and can be purified from serum on the basis of different cell surface markers compared to normal blood cells [18, 19]. Serum metabolites are small molecules secreted by tumor cells that can often serve as a signature of the tumor [20–22]. Carbohydrate expression also changes during oncogenic transformation and cancer progression and can serve as a measure of cancer stage [23–25].

This chapter will specifically examine RNA as biomarkers. In recent years, differing transcriptomes among tumor and healthy cells have become a point of emphasis. Several cancers have different noncoding RNA expression profiles in a time- and tissue-dependent manner. Changes in miRNA within a specific tissue has been shown to correlate with disease status including tumor invasiveness and metastatic potential in various cancers such as breast, colorectal, hepatic, lung, pancreatic, and prostate [26]. LncRNAs can also be used as serum tumor markers, which we will examine in detail in the following section.

4. lncRNAs as biomarkers for cancer

Much of our genome codes for RNA with no protein-coding potential. Such RNA is known as noncoding RNA. Long non-coding RNAs (lncRNAs) specifically refer to transcripts longer than 200 bp in length, and can reach up to over tens of kilobases in length. lncRNAs play a vital role in the regulation of many cellular processes especially gene-expression and post-transcriptional activity. This is in part due to their structural versatility and ability to form ribonucleoprotein complexes (RNPs) [27].

It has been noticed that lncRNA expression differs among normal and cancer cells, making it a prime candidate for novel cancer research. Different studies have shown the diverse roles that lncRNAs play in cancer, helping malignant cells proliferate, resist apoptosis, evade growth suppression, maintain genomic instability, and invade and metastasize [28, 29].

What makes lncRNA so valuable as a potential biomarker is its accessibility and detection outside of the cell and in easily collectable biological samples such as blood and urine. LncRNAs are often found in high concentrations in exosomes [30], small cell-derived vesicles 30–100 nm in diameter that are released from the plasma membrane to the extracellular environment. Thus, exosomes contain cytoplasm surrounded by a phospholipid bilayer, along with endosomal compartments known as multivesicular bodies (MVBs) that fuse with the plasma membrane before exosome release [31]. Exosomes contain many particles within the cytoplasm such as proteins and various nucleic acids like mRNA, lncRNA, and miRNA [31]. They are also able to communicate with both the immediate extracellular environment and distant sites for potential metastases in the case of cancer cell exosomes [32]. Additionally, studies have demonstrated that compared to normal cells, cancer cells secrete more and differently constituted exosomes [31, 33, 34].

Exosomes can transfer molecules from cell to cell, and often, they end up in blood or waste to be excreted. It is simple to isolate these vesicles from blood serum or urine, and then analyze its components. Thus, we can use these molecules, including lncRNAs, as a tumor fingerprint to identify potentially tumorigenic cells. The use of exosome particles to identify cancers has already been demonstrated in gastric [35] and pancreatic cancer [36], among others. These circulating lncRNA have already been shown to have great potential as biomarkers for several cancers, which make it all the more promising that some lncRNA can be effective in diagnosing and monitoring melanoma. For example, the expression of the gene $DD3^{PCA3}$ is highly upregulated in prostate cancer cells compared to normal cells [37]. Traditionally, prostate specific antigen (PSA) is a protein biomarker used to test for prostate cancer. However, because the $DD3^{PCA3}$ test has better specificity, it is now used in conjunction with PSA testing (along with TMPRSS2:ERG fusion RNA) to form a more specific test for this cancer. Other examples include MALAT1 in lung cancer, H19 and LINC00152 in gastric cancer, and HOTAIR in colorectal cancer and oral squamous cell cancer [38].

5. Potential lncRNAs as biomarkers for melanoma

Currently, lncRNAs are being used as biomarkers in many different malignancies, as outlined in the previous section. However, there is currently no reported usage of lncRNAs as melanoma biomarkers in a clinical setting. Various lncRNAs are still being tested as potentially viable clinical melanoma biomarkers, including many lncRNA upregulated in and/or used as a tumor marker for other cancers. A brief overview of each is provided here and a summary in **Table 1**. Known mechanisms of action are presented in **Figure 1A–I**.

Melanoma lncRNA	Full name	Function
ANRIL	Antisense non-coding RNA in INK4 locus	Promotes EMT and metastasis
BANCR	BRAF-activated non-coding RNA	Implicated in cell survival, proliferation, and metastasis, expression correlated with disease progression
CASC15	Cancer susceptibility candidate 15	Increases migration and metastatic activity, prognosticator of melanoma stage
HOTAIR	HOX transcript antisense RNA	Promotes EMT and metastasis, prognostic role and serum marker in other cancers
Llme23	–	Inhibits tumor suppressor; plays oncogenic role
MALAT-1	Metastasis-associated lung adenocarcinoma transcript 1	Migration and metastasis, overexpression activates MAPK, Wnt/beta-catenin
SAMMSON	Survival associated mitochondrial melanoma-specific oncogenic non-coding RNA	Promotes cancer cell survival
SLNCR1	–	Promotes EMT and invasion
SNHG5	SnoRNA host gene 5	Melanoma invasion and metastasis
SPRY4-IT1	–	Promotes melanoma cell growth and invasion and blocks apoptosis
UCA-1	Urothelial carcinoma-associated 1	Promotes invasion and metastasis

Table 1. Summary of melanoma lncRNA.

5.1. Antisense non-coding RNA in the INK4 locus (ANRIL)

Antisense non-coding RNA in the INK4 locus (ANRIL) is a 3834 nt lncRNA consisting of 19 exons alternatively spliced in the antisense direction of the p15/CDKN2B-p16/CDKN2A-p14/ARF gene cluster on chromosome 9 in humans. ANRIL interacts with polychrome repressive complexes 1 and 2 (PRC1/2) to reduce the expression of essential tumor suppressor proteins p15INK4b and p16INK4a. It has been shown that ANRIL can promote epithelial to mesenchymal transition (EMT) and metastasis in cancers such as non-small cell lung carcinoma and pancreatic [39, 40]. Although a research group used genome wide association studies (GWAS) to determine several single nucleotide polymorphisms in the ADP ribosylation factor (ARF) locus, including one within the ANRIL sequence, rs1011970, is associated with melanoma, we know little else about ANRIL's potential role in melanoma. As such, further investigation is needed to determine its suitability as a melanoma biomarker.

5.2. BRAF-activated non-coding RNA (BANCR)

BRAF-activated non-coding RNA (BANCR) is a 693 bp lncRNA transcript highly induced by oncogenic BRAF and overexpressed in melanoma. More than 70% of melanomas contain an

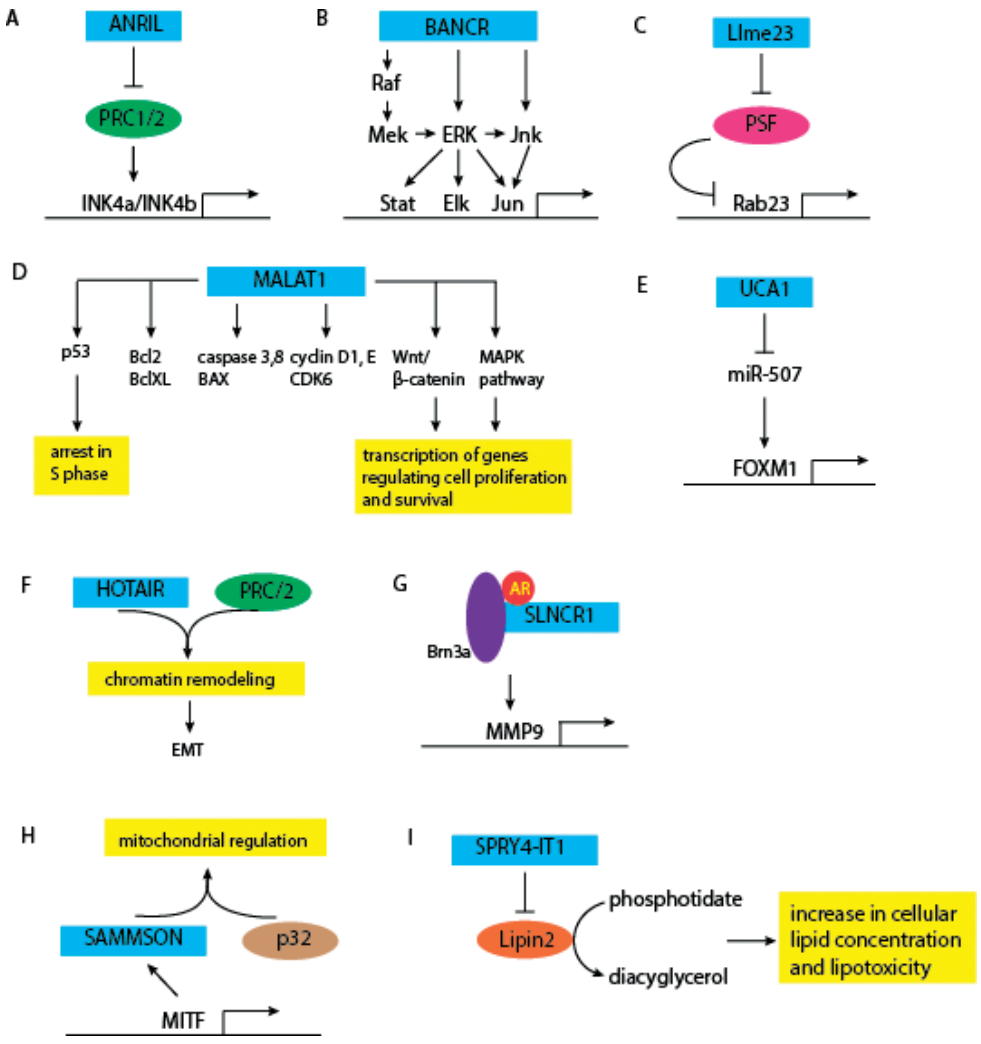

Figure 1. This figure displays the pathways and mechanisms of action of potential melanoma lncRNAs. Growth and proliferation: ANRIL, BANCR, Llme23, MALAT-1, UCA1 (A–E). Invasion and metastases: HOTAIR, SLNCR1 (F–G). Apoptosis: SAMMSON, SPRY4-IT1 (H–I). Mechanism not yet characterized: SNGH5, CASC15.

activating BRAF mutation, of which 90% is the V600E mutant. Mutant BRAF causes the upregulation of various proteins and transcripts implicated in cell survival, proliferation, and metastasis, including BANCR. BANCR is overexpressed in both melanoma cell lines and tissues, with increased expression correlated with disease progression [41, 42]. Flockhart et al. first characterized BANCR as regulating melanoma cell migration [41]. Perhaps unsurprisingly, patients with high levels of BANCR had a much lower survival rate than patients with low levels of BANCR (3-year survival of 40% compared to 71%) [42]. So far, research on BANCR has shown its potential value as both a target for therapy as well as a prognostic measure.

However, there have been few studies to validate its efficacy. Additionally, its presence in serum, which would make it a more viable biomarker, has not yet been demonstrated.

5.3. Cancer susceptibility candidate 15 (CASC15)

Cancer susceptibility candidate 15 (CASC15) is a 530 kb intergenic lncRNA (lincRNA) on chromosome six that was observed to be actively transcribed in metastatic melanoma. Its expression increases migration and metastatic activity, although its mechanism of action and binding partners is currently unknown [43]. A study found that CASC15 was both expressed in melanoma cell lines and upregulated in a mouse xenograft model of brain metastases [44]. Additionally, CASC15 can serve as a good prognosticator of melanoma stage, as levels increase during melanoma progression, with significant expression in advanced stage IV metastases compared to controls and melanoma in situ (MIS) [44]. However, while normal tissue shows virtually undetectable levels of CASC15, other cancers can also show high levels of CASC15 expression, making it less specific than other biomarkers [44]. Nonetheless, if it is indeed possible to isolate CASC15 in blood or urine samples of melanoma patients, which to date has not been done, it can serve as a valuable diagnostic and prognosticator of metastatic melanoma.

5.4. HOX transcript antisense RNA (HOTAIR)

HOX transcript antisense RNA (HOTAIR) is a 2.2 kilobase lncRNA in an intronic region of the HOXC gene locus. HOTAIR is believed to regulate gene expression through chromatin remodeling; it promotes epithelial-mesenchymal transition (EMT) and cancer metastasis by coordinating with polycomb repressive complex 2 (PRC2) to repress the expression of various genes suppressing metastasis [45, 46]. Its net effect in metastatic melanoma is to promote motility, invasion, and metastatic potential [47], evidenced by its upregulation in metastatic tissue. HOTAIR's role as a prognostic factor in breast cancer is well characterized, and it may also serve prognostic roles in gastroenteric tumors and liver metastases [45]. Additionally, its potential as a serum biomarker in other cancers has been documented [48, 49]. It may serve a similar capacity in melanoma, but more research still needs to be done, including verification of its presence in serum.

5.5. Llme23

Llme23 is a 1600 bp lncRNA expressed exclusively in melanoma. It was first discovered through identifying lncRNA binding partners to polypyrimidine tract-binding (PTB) protein-associated splicing factor (PSF) via RNA-SELAX affinity chromatography [50]. PSF is a tumor suppressor repressing the transcriptional activity of multiple proto-oncogenes, including Rab23 [50]. Llme23 interacts with and subsequently blocks the function of PSF, thus inhibiting its tumor suppressor function [50]. Because of its exclusive nature to melanoma cell lines, Llme23 would serve as a highly specific biomarker for melanoma. Before it can be adapted to clinical use, further research is required to determine its secretion and levels in plasma and/or urine.

5.6. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1)

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1), also known as nuclear-enriched transcript 2 (NEAT2) is a well-characterized, roughly 8000 bp lncRNA. It has been extensively studied and shown to have a regulatory function lung cancer cell migration and metastasis [51], as well as similar roles in liver cancer [52], thyroid cancer [53], and neuroblastoma [54]. MALAT-1 targets many tumor suppressor genes, and its overexpression activates MAPK, Wnt/beta-catenin, p53, caspase-3, caspase-8, and the Bax signaling pathway. Its relevance to melanoma has recently come to attention, as Tian et al. found that MALAT-1 is highly expressed in melanoma tumors compared to normal tissues [55]. Moreover, metastatic sites display higher levels of MALAT-1 than primary tumors [55]. MALAT-1 is a promising new biomarker for melanoma, as its presence in serum and urine has already been reported in prostate cancer patients [56, 57].

5.7. Survival associated mitochondrial melanoma-specific oncogenic non-coding RNA (SAMMSON)

Survival associated mitochondrial melanoma-specific oncogenic non-coding RNA (SAMMSON) is located around 30 kb downstream of microphthalmia-associated transcription factor (MITF), an oncogene specific to melanoma. Although SAMMSON and MITF are only co-amplified in around 10% of melanomas, SAMMSON itself was detected in 90% of melanoma samples from the TCGA RNA-seq data set [58]. SAMMSON binds to p32, which regulates tumor metabolism through balancing oxidative phosphorylation and glycolysis [58, 59]. Knockdown of SAMMSON resulted in decreased cell viability and apoptosis. SAMMSON localizes to the mitochondria, so its presence in serum may be limited. However, further investigations should be done to confirm its potential as a biomarker.

5.8. SLNCR1

SLNCR1 is a lncRNA that promotes melanoma invasion and is associated with survival outcome. The brain-specific homeobox protein 3a (Brn3a) and androgen receptor (AR) binds to SLNCR1 to increase melanoma invasion by activating the transcription of MMP9, required for the degradation of extracellular matrix during EMT and invasion [60]. Interestingly, the higher incidence of melanoma metastases in males compared to females may be explained by ARs being binding partners for SLNCR1 [60]. Again, there is presently no research on the presence of SLNCR1 in plasma and thus its viability as a melanoma biomarker.

5.9. SnoRNA host gene 5 (SNHG5)

SnoRNA host gene 5 (SNHG5) is a 524 bp lncRNA whose levels were found to be significantly higher in the serum of patients with melanoma compared to normal subjects as well as patients with squamous cell carcinoma [61]. This suggests that this lncRNA plays a role in

melanoma formation and/or metastasis. Its presence in serum is already documented, making it a promising biomarker. However, much more research needs to be done as its mechanism in melanoma has yet to be characterized and the only study looked at just 24 patients with malignant melanoma [61].

5.10. SPRY4-IT1

SPRY4-IT1 is a lncRNA located within the intron of the Sprouty 4 (SPRY4) gene. It was first identified as upregulated in melanoma cells compared to melanocyte and keratinocyte controls [62]. SPRY4-IT1 promotes melanoma cell growth and invasion and also blocks apoptosis [62]. It acts by binding to and inactivating lipin 2, an enzyme involved in fatty acid metabolism [63]. Knockdown of SPRY4-IT1 induces an increase of lipids in the cell and can lead to apoptosis due to lipotoxicity [63]. Based on plasma samples of healthy individuals (N = 79) and malignant melanoma patients (N = 70), SPRY4-IT1 expression was significantly higher in malignant melanoma patients. Additionally, elevated SPRY4-IT1 significantly reduced patient survival rate and is strongly associated with more advanced tumor stage [64]. These data suggest that SPRY4-IT1 may be used as a marker for both diagnosis and staging. SPRY4-IT1 was also found to be present in the plasma of esophageal squamous cell carcinoma patients, and associated with poor prognosis [65]. Limitations of its efficacy as a melanoma biomarker include specificity, as it is also found to play roles in prostate cancer [66], glioma [67], and gastric cancer [68], among others.

5.11. Urothelial carcinoma-associated 1 (UCA-1)

Urothelial carcinoma-associated 1 (UCA-1) is a 1.4 kb lncRNA implicated in several cancers including breast, gastric, and pancreatic [69–71]. A recent study by Wei et. al. showed that UCA-1 is upregulated in melanoma as well [72]. It acts by inactivating miR-507, which in turn leads to the upregulation of transcription factor FOXM1 mRNA and invasion and metastasis [72]. Studies have shown that urine UCA-1 is a possible diagnostic biomarker in bladder cancer [73], though it has not yet been tested as a marker for melanoma.

6. Research and clinical methods

One common method of identifying novel lncRNAs is by RNA-seq expression profiles between cancer cells and normal cells expressing relevant oncogenes [41]. BANCR was discovered through this method. Previously, Sanger sequencing of cDNA libraries and tiling arrays were the preferred method for identifying lncRNA, but they have since been replaced by RNA-seq and other next generation sequencing technologies [74].

To characterize the mechanism of lncRNA action, it is important to determine subcellular localization and binding partners. To determine the subcellular localization of a particular, fluorescence in situ hybridization (FISH) may be used. To find molecular interactions

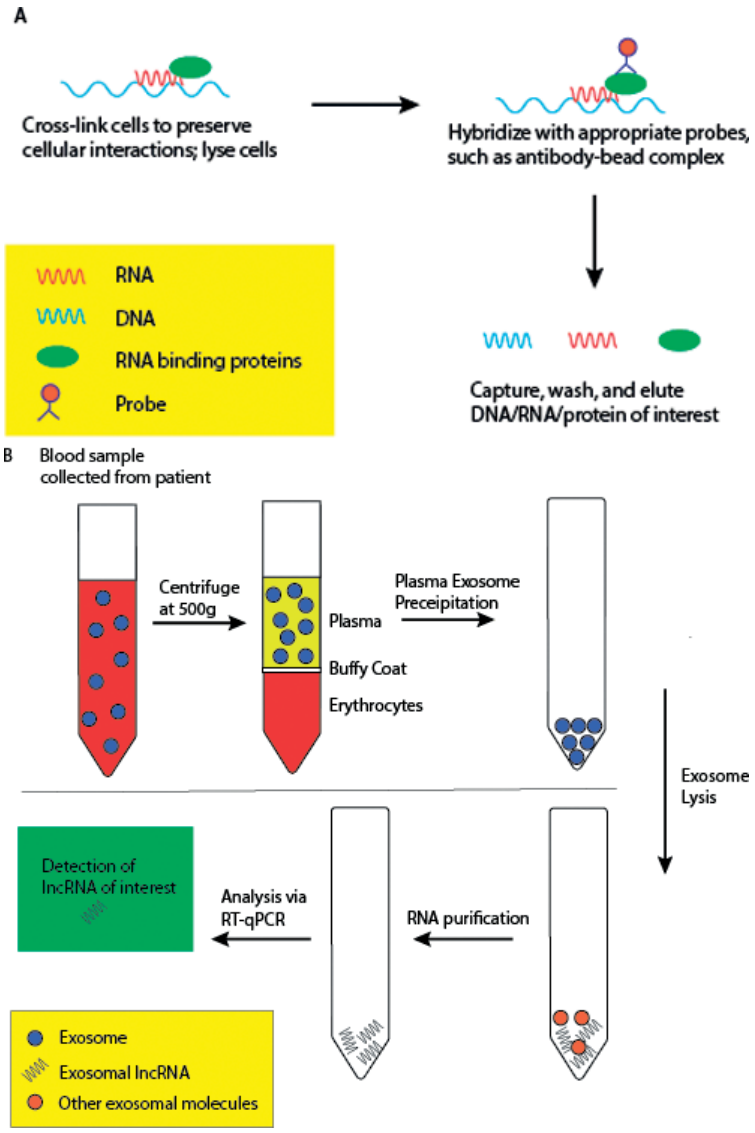

Figure 2. Experimental techniques. (A) General workflow for immunoprecipitations. Many of these pull-down experiments to find RNA binding partners follow a similar protocol, including ChIRP, RAP RNA, and RIP. RNA-DNA: ChIRP, RAP, CHART. RNA-RNA: RAP RNA, CLASH. RNA-protein: RIP, CLIP. (B) Clinical detection of circulating lncRNA. lncRNA are found in serum exosomes. First, a blood sample collected from a patient is centrifuged to separate the plasma. Then, exosomes are precipitated from the plasma, lysed, and RNA collected and purified. This purified RNA can then be analyzed via RT-qPCR to identify any lncRNAs of interest.

between the lncRNA and DNA, RNA, and proteins, a variety of pull-down experiments are performed (**Figure 2A**). To determine RNA-DNA interactions, chromatin isolation by RNA purification (ChiRP) is most commonly used. Other methods include RNA antisense purification (RAP), which uses RNA antisense probes to map RNA interactions with chromatin

[75], and capture hybridization analysis of RNA targets (CHART), which uses capture oligonucleotides that hybridize to the RNA of interest, which is then enriched alongside its targets [76]. To determine RNA-RNA interactions, RAP RNA or cross-linking, ligation, and sequencing of hybrids (CLASH), may be used. RAP RNA uses differential cross-linking with psoralen for RNA-RNA interactions and formaldehyde for protein-mediated RNA-RNA interactions [77]. CLASH, a less labor-intensive method, uses UV cross-linking and sequencing of ligated RNA-RNA hybrids, whereupon chimeric reads are identified using bioinformatics [78]. To determine RNA-protein interactions, RNA immunoprecipitation (RIP) can be used to analyze RNA associations with proteins at specific time points [79], while cross-linking immunoprecipitation (CLIP) maps RNA-protein interactions through in vivo UV cross-linking of cells [80].

Clinically, the detection of circulation lncRNA for use in diagnostics and monitoring would be simple (**Figure 2B**). First, plasma exosomes would need be isolated from a patient's blood sample. Centrifugation of the blood sample first separates the plasma from the cellular components, and the exosomes can then be precipitated from the plasma using one of several techniques: ultracentrifugation, size-exclusion chromatography/ultrafiltration, immunoaffinity capture-based technique, or use of a commercial, exosome precipitation kit [81]. Ultracentrifugation involves spinning the sample at forces up to 1,000,000 g, and is considered the most effective and most used method [81]. Second, RNA extraction for lncRNA analysis would need to be done. The exosomes are lysed, RNA extracted and purified, and then analyzed using qRT-PCR.

7. Conclusion: the future of lncRNAs in melanoma as biomarkers and targets for therapy

Overall, lncRNAs serve as promising biomarkers for melanoma, though much more research needs to be done on them before they can be used clinically. The presence of lncRNA in blood and urine make them particularly valuable to the field of cancer diagnostics as presently, there is a dearth of early diagnostic measures for melanoma. Currently, potential melanomas must be detected by a patient or physician. The major shortcoming of this is that sometimes malignant melanomas do not appear obvious until it is at a late stage, and patients themselves often cannot identify harmful lesions at early stages. Additionally, it is difficult to keep track of potentially malignant nevus in certain areas of the body. Once a potentially tumorigenic nevus or lesion is clinically observed, the first line of diagnostics is the histopathology of biopsies, which are both invasive and expensive.

Diagnoses using circulating lncRNA could serve as an improvement to these biopsies. Not only are they minimally invasive, they are also less expensive and can be conducted at regular intervals for high-risk patients (those with a melanoma in the past 5 years, certain genes, phenotypic red hair, Irish-Scottish ancestry, high mole count, frequent sun exposure, etc.). Moreover, many lncRNAs can also provide valuable prognostic information, including progression, staging, and size to tumor.

Some concerns for the use of lncRNA as biomarkers do exist. The lncRNA must be present in sufficient quantity for it to be able to be detected and analyzed using standard methods. Additionally, as discussed previously, many of these lncRNAs are also upregulated in other cancers, lowering its specificity as a melanoma biomarker. However, this may not be a bad thing, as other malignancies may be able to be "accidentally" detected.

Certain lncRNA can also be used as targets for novel therapies. LncRNAs like BANCR and MALAT-1 are responsible for cell migration and metastases. Targeting or knocking down these lncRNA in vivo may prevent further progression and invasion of early stage melanomas and limit the metastatic activity of late stage melanomas.

In conclusion, lncRNAs are likely to be suitable melanoma biomarkers for a variety of reasons: (1) They are secreted into the bloodstream and easily accessible for analysis using non-invasive and inexpensive methods. Because they are secreted within exosomes, they are also protected from various RNases within the bloodstream. (2) Various lncRNAs are secreted at different time-points of disease progression. Those secreted early on have valuable diagnostic potential while others may be useful in determining disease development and prognosis. (3) LncRNAs are generally highly specific for melanoma, a shortcoming of current protein biomarkers. (4) Noncoding RNAs are responsible for a variety of cellular functions and implicated in many important pathways, making them valuable prognosticators of disease. (5) LncRNA biology is still a relatively novel field, which holds a lot potential as more research is being conducted.

Acknowledgements

Publication made possible in part by support from the Berkeley Research Impact Initiative (BRII) sponsored by the UC Berkeley Library.

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Chapter 3

The Multiple Roles of Tyrosinase-Related Protein-2/L-Dopachrome Tautomerase in Melanoma: Biomarker, Therapeutic Target, and Molecular Driver in Tumor Progression

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70513

Abstract

Cutaneous malignant melanoma (CMM), which is ranked as the 8th most common cancers in the US, makes 4–7% of skin cancers but it causes approximately 80% of skin cancer deaths. CMM is characterized by insidious and fast progression, heterogenic evolution, and significant resistance to numerous therapeutic strategies. CMM is the result of the uncontrolled proliferation of melanocytes, the cells which reside in the basal layer of the epidermis. The most efficient therapy is the surgical removal if the lesion is in an early stage. For metastatic melanomas, there are different strategies, extremely rarely leading to total cure. Tyrosinase-related protein-2 (TRP2) or L-Dopachrome tautomerase (L-DCT) is a member of Tyrosinase-related protein family known for many years for its enzymatic activity in the distal steps of melanogenesis. The modern DCT image is focusing more on processes and mechanisms related to cell development and response to environmental and therapeutic stressors in normal and transformed cell phenotypes. This chapter provides an extended, updated biological status of TRP2/L-DCT encompassing the structural and functional particularities within melanoma molecularity, in the attempt to get new insights into the complex mechanisms of this neoplasm and raise the interest for DCT unexplored yet potential in melanoma diagnosis/prognosis and therapy.

Keywords: tyrosinase-related protein-2, L-Dopachrome tautomerase, melanoma biomarker, structural molecular model, melanoma therapy, melanoma progression, caveolin-1, melanoma signaling pathways

1. Introduction

Cutaneous malignant melanoma (CMM) is a neoplasm generated through the malignant transformation of epidermal melanocytes, the cells which normally reside in the basal layer of the epidermis and produce the skin pigment melanin (**Figure 1A–C**). Noncutaneous melanomas can also develop at other sites populated by melanocytes such as choroidal layer of the eye, respiratory, gastrointestinal, and genitourinary mucosal surfaces, or the meninges. The main incriminating agent for causing CMM remains the UV radiation in interaction with host characteristics (**Figure 1D**). However, CMM may appear in skin areas that are not directly exposed to sun such as palms, soles, or under the nails, which demonstrates a pathogenesis more related to the noncutaneous melanomas. The incidence of CMM has been rising for the last 30 years around the world. Key statistics on CMM released by The American Cancer Society estimate that during 2017, in the US, about 87,110 new melanomas will be diagnosed (about 52,170 in men and 34,940 in women) and about 9730 people are expected to die of melanoma (about 6380 men and 3350 women) [1]. Although CMM makes only 4–7% of skin cancers, this neoplasm causes approximately 80% of skin cancer deaths. CMM is characterized by insidious and fast progression, heterogenic evolution among patients, and significant resistance to diverse therapeutic strategies. CMM is thought to develop in a stepwise manner being initiated with a benign nevus containing cell populations with intense proliferative capacities. Some of these lesions overcome the senescence-inducing signals, exhibit dysplasia (dysplastic nevus), and can progress further toward the malignant stages. The radial growth phase (RGP) is limited to epidermis and has a low invasive potential. In a more advanced stage, the melanoma cells migrate vertically up into epidermis and down into papillary dermis entering a new stage, the vertical growth phase (VGP). In metastatic stage, the tumor cells invade through blood or lymph vessels the distal organs (liver, brain, and lung) where they proliferate, eventually, causing death (**Figure 1D**). The activity of tumor cells is modulated by the complex and dynamic tumor microenvironment that can be extremely heterogenous among tumors of different patients. The multistep process of CMM progression is defined by a plethora of molecular events that are continuously explored, revised, and updated [2, 3].

The only cure for melanoma is the surgical removal of early-stage tumors. For metastatic patients having the median overall survival less than a year, there are different strategies, including combined chemo-/radio- and vaccine therapies, extremely rarely leading to total cure and whose success depends very much on the staging accuracy. Major improvements in the metastatic treatment have been achieved due to advances in understanding the molecularity of this neoplasm. The modern alternative for melanoma evaluation and management is the analysis based on key genes or biomarker(s), pathways, diagnostic technologies, and potentially relevant therapeutics. These tend to replace current limited histological and microscopical evaluation introducing concepts such as “molecular melanoma subtypes” [4], “melanoma disease model (MDM)” [5], or “molecular diagnostic of melanoma” [6], aiming to bring together clinicians, researchers, and pharma for more efficient diagnostic, prognostic, and therapeutic strategies [7, 8]. Tyrosinase-related protein-2 (TRP2, TYRP2) or L-Dopachrome tautomerase (L-DCT) is a member of tyrosinase-related protein (TRP) family known for many years only for its enzymatic activity in the distal steps of melanogenesis. Studies emerging from different

http://dx.doi.org/10.5772/intechopen.70513

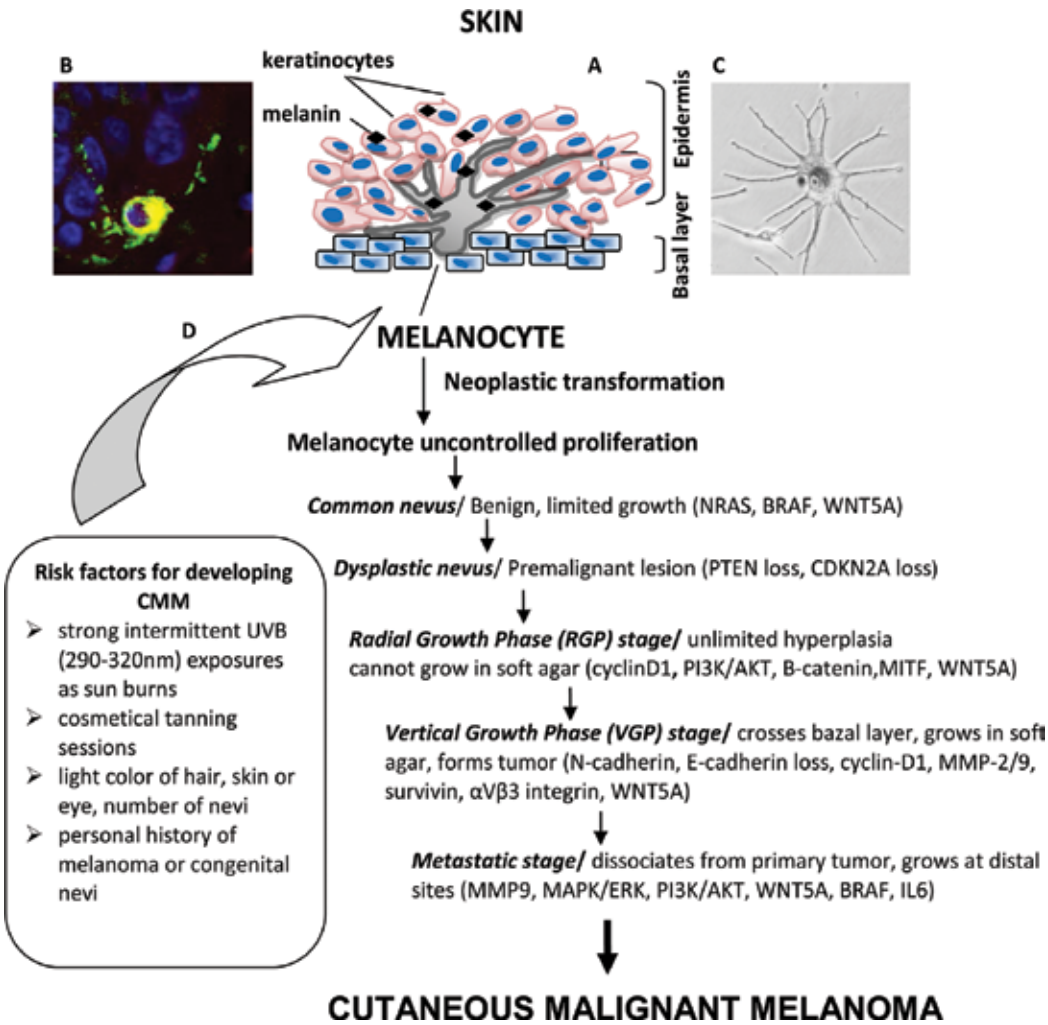

Figure 1. Cutaneous malignant melanoma. (A) Schematic representation of epidermal melanocytes with melanosomes (black dots) exported to the surrounding keratinocytes. One melanocyte and 30–40 keratinocytes form the "epidermal melanin unit". (B) The image of a human melanocyte obtained by confocal fluorescence microscopy of a human skin specimen immunostained for TYR and DCT. The common TYR-DCT staining is in the perinuclear region, whereas TYR staining is visible in dendritic tips too. (C) The image of a human epidermal melanocyte (HEM) in culture, obtained by bright field microscopy. (D) The risk factors for developing cutaneous malignant melanoma and the steps of neoplastic transformation and malignant progression of epidermal melanocytes culminating with the metastatic stage. Several molecular markers and processes emblematic for each tumor stage are indicated.

groups identified TRP2/L-DCT in relation to processes distinct from melanin synthesis (cell protection from environmental and therapeutic stress), melanoma diagnostic (potential biomarker), and therapy (immunotherapeutic target). TRP2/L-DCT is also expressed in precursors of peripheral nervous system associated with developmental processes and in glioma, a brain cancer similar to melanoma in terms of aggressiveness and therapeutic resistance and more recently, unexpectedly, in nonmelanocytic or nonneuronal cellular phenotypes.

This chapter aims to provide an updated status of TRP2/L-DCT in order to demonstrate its multiple implications in melanoma molecularity and therapeutic potential as well as to open up new perspectives for a better understanding of other molecular processes and pathologies. For simplicity, we will further refer to TRP2/L-DCT as DCT.

2. Dopachrome tautomerase: a distinct member of tyrosinase-related protein family

2.1. Structural determinants of DCT

TRPs are type I transmembrane N-glycoproteins. Their polypeptides share significant amino-acid sequence homology and similar patterns of polypeptide chain organization, an amino-terminal signal sequence (residues 1–23 in human DCT) followed by a lumenal domain (aa 24–439), a transmembrane (TM) hydrophobic region (aa 473–493) that inserts the protein into subcellular membranous structures and a carboxi-terminal cytoplasmic (CYT) tail (aa 494–519) interacting with the elements of the sorting and traffic machinery. The lumenal domain encompasses the enzymatic active site shaped by two highly conserved metal-binding regions (MeB1 and MeB2) molded at the core of a four-helical bundle. Interspersed with these two metal-binding regions are two Cys-rich regions (Cys1 and Cys2). Cys1 precedes MeB1 and contains 10 Cys residues conserved only in the human TRPs, and Cys2 located between MeB1 and MeB2 contains six Cys residues of which five are conserved in the human TRPs. Unfortunately, none of the human TRPs have been crystallized, but models of human tyrosinase have previously been developed [9]. Using a similar protocol and based on the high degree of sequence homology among TRPs (about 60% on the entire sequence and 66% in the lumenal domain only), we built a structural model for the lumenal domain of human DCT using as templates the available X-ray structures of tyrosinase proteins from *Bacillus megaterium* [10] (PDB code 3NM8, 3NPY; 2Å resolution) and from *Streptomyces castaneoglobisporus* [11] (PDB code 3AX0; 1.4Å resolution). Alignment between human TRPs and templates sequences (**Figure 2**) was initially generated using CLUSTALW and MULTALIN and further refined by incorporating information on secondary structure elements identified by consensus prediction by several methods, in the case of DCT, and by DSSP assignment in the case of templates.

Despite this high degree of sequence homology between DCT and other human TRPs, distinctive DCT features regarding overall hydrophobicity and charge profiles, active site stereochemistry and composition, N-glycosylation, or phosphorylation patterns generate significant differences in protein function, interaction partners, and sorting/trafficking pathways.

Although the two metal-binding regions in the lumenal domain represent a highly conserved feature of TRP family, DCT has a unique preference for zinc instead of copper, as is in the case of TYR. Purified DCT contains two Zn atoms per protein molecule as measured by atomic absorption spectroscopy and Zn^{2+} chelation experiments. Zn^{2+} is the crucial element that accounts for the tautomerization of L-Dopachrome tautomerase [12]. The enzyme DCT reconstituted with

http://dx.doi.org/10.5772/intechopen.70513

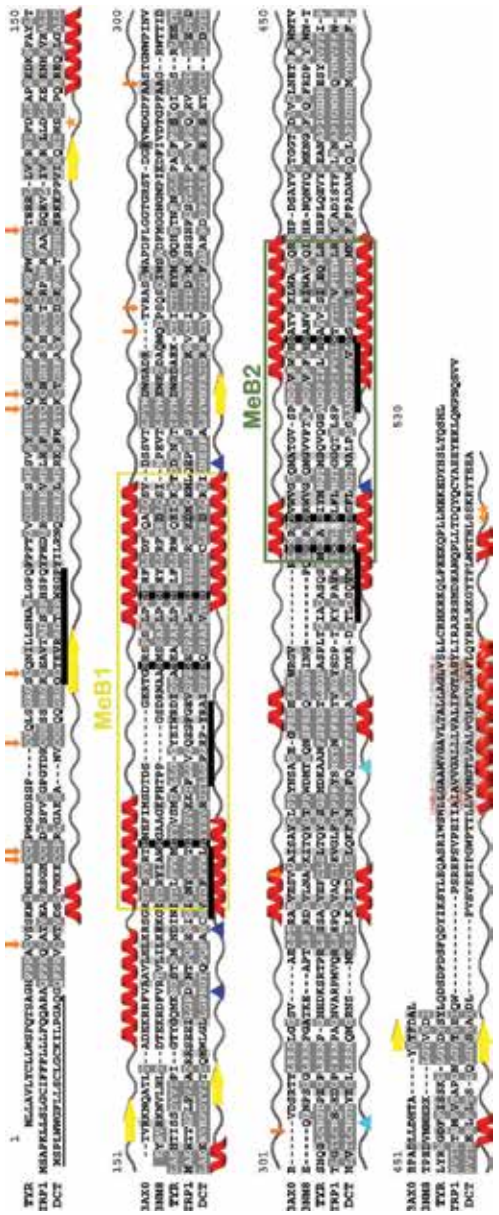

Figure 2. Sequence alignment of human TRPs (TYR, TRP1, and DCT) with the X-ray templates used for modeling DCT (PDB codes 3AX0 and 3NM8). Identical/similar residues between DCT and other sequences are highlighted dark/light gray, metal binding His residues are highlighted black. Assigned/predicted secondary structure elements for templates/DCT are shown above and below the alignment. Membrane pictogram indicates location of (predicted) transmembrane region in all proteins. The rectangles indicate the two Metal-binding regions (MeB1, MeB2). Symbols indicate various functionally relevant residues: stars = phosphorylated residues; diamond = methylated R409 residue; dark triangles = putative N-glycosylation sites, light triangles = experimentally confirmed occupied sites in DCT; arrows = Cys residues. Signal sequence in DCT is thin underlined. The DCT-derived peptides 60–74 [18], 180–188 [19], 197–206 [20], 360–368 [21], 387–395 [22] recognized by CD4+ or CD8+ T-lymphocytes are indicated by thick lines.

Cu^{2+}, which is the cofactor for TYR, or with Fe^{2+}, is inactive, whereas with Co^{2+} is partially active. Unlike the native DCT, which shows a very strict specificity for L-Dopachrome and for which neither dopaminochrome nor D-Dopachrome are suitable substrates, the reconstituted enzyme is stereospecific as well but is also able to rearrange D-Dopachrome into DHI [13]. At this point, it is important to specify that there is also a D-Dopachrome tautomerase (D-DCT, or D-DT) which is decarboxylating D-Dopachrome to DHI. There is no structural or functional relation between L-DCT and D-DT, which is a circulating cytokine, member of macrophage migration inhibitory factor (MIF) protein superfamily with an overlapping functional spectrum with MIF. Within lumenal domain of human DCT, there are 16 cysteine (Cys) residues, clustered into three regions, the first two located N-terminal to MeA and the third between MeA and MeB. In addition to these clustered Cys residues, single Cys residues may be found in the C-terminus cytoplasmic tails of TYR and TRP1 but not of DCT, which indicates a TYR-TRP1 interaction via intermolecular disulfides without DCT participation [14]. This finding is in agreement with our experimental data, showing that DCT does not share common subcellular structures with TYR or TRP1 (see Section 2.3.1.2) and does not support the early theory that all TRPs are possibly interconnected via intermolecular disulfides. Despite the fact that the number of N-glycosylation sites is almost the same in human TYR (seven sites) and DCT (six sites) and they are all located in the lumenal domain, glycosylation pattern is significantly different between TYR and DCT. In the case of human TYR, occupancy of six of the seven sites was demonstrated by site-directed mutagenesis [9], while in the case of DCT, only two sites (N300 and N342) have been experimentally confirmed to be occupied [15] by MALDI/TOF of a truncated version of protein expressed in insect cells. Both N-glycosylated sites in DCT are located in close vicinity (on opposite sides) of the metal containing active site, possibly influencing ligand access within, but only N300 is conserved in all human TRPs while equivalent of N342 is found only in TRP1 not in TYR. The first two N-sites of TYR, which are required for TYR entry in the CNX cycle [16] are not present in DCT, which further supports the idea that TYR and DCT take different intracellular processing pathways. Indeed, our experimental data confirmed that folding pathways, which in all TRPs are dependent on the step of N-glycan processing, are differently regulated within the same cell phenotype and have further distinct impact on their trafficking and stability (see Section 2.3.1.2). Additional unique characteristics of DCT post-translational modifications refer to the methylated residues. A recent large-scale mass spectrometry analysis of arginine-methylated peptides in human T cells [17] demonstrated methylation of R409 in DCT (indicated by a diamond in the alignment in **Figure 2**), located at the end of the second metal-binding region. Structurally, this positively charged residue is positioned in the luminal domain and oriented toward the melanosomal membrane (**Figure 3**), thus likely to interact with the negatively charged head groups of membrane phospholipids. Addition of a methyl group to R409 would shield the positive charge and decrease probability of luminal domain interacting with membrane. Surprisingly, although this residue is conserved in all human TRPs, the same study could not identify similar modification of corresponding residues in the other members of the family. This post-translational modification of DCT could have an impact on interactions between DCT and sorting/traffic machinery and subsequently on DCT intracellular routes. The same study [17] demonstrates that changes in arginine methylation stoichiometry during cellular stimulation in a subset of proteins are critical to T cell differentiation. DCT is a tumor antigen, and several peptides

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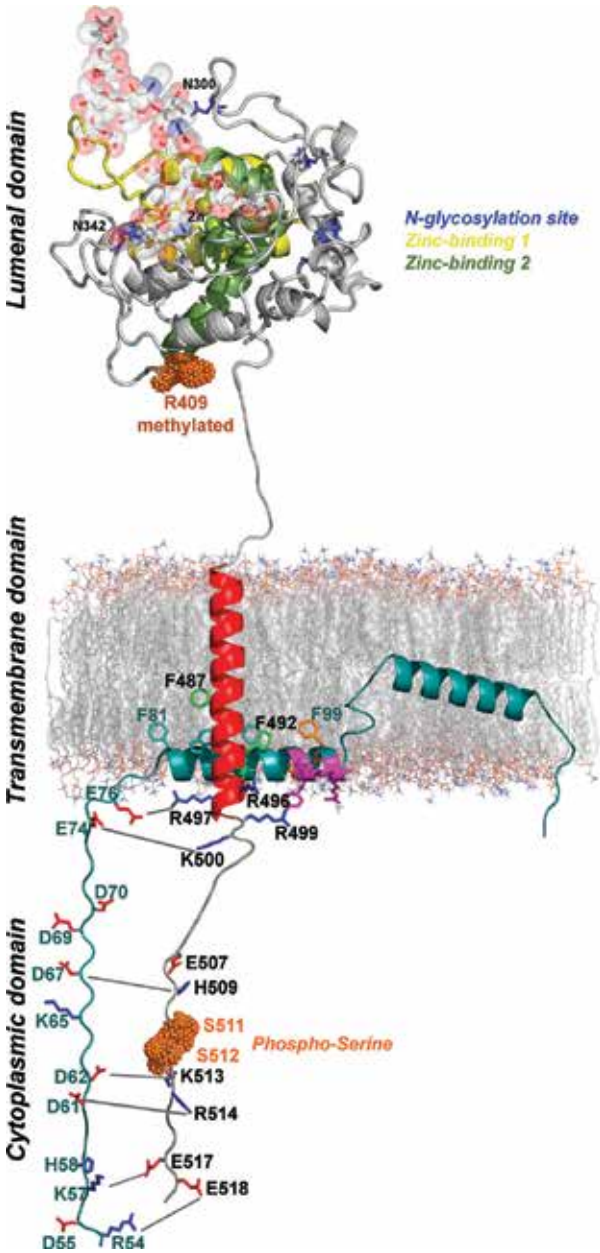

Figure 3. Structural model of DCT protein (cartoon representation) interacting with membrane bilayer and caveolin-1 (Cav1). In the lumenal domain (above membrane) the helical segments indicate the two metal-binding regions, containing two Zn^{2+} ions (shown as opaque spheres). Putative N-glycosylation sites are depicted using thick sticks. Representative structural models of N-glycans (shown as transparent spheres) are attached to glycosylation sites experimentally shown to be occupied (N300 and N342). Methylated R409 (within lumenal domain) and phosphorylated S511, S512 (within cytosolic membrane) are shown as dotted spheres. Within DCT transmembrane region, aromatic residues F487, F492 (thick sticks) and Y495 (behind helical structure) form the Cav1-binding motif. Charged residues in the cytosolic regions of DCT and Cav1 are labeled and shown as sticks, and putative salt bridges are depicted by thin gray lines connecting oppositely charged residues.

derived from it were identified as targets of CD4+ or CD8+ T-lymphocytes, and their position within DCT sequence is presented in **Figure 2** [18–22]. Whether DCT-methylated peptides could be a part of the peptide-methylated pool involved in triggering T-cell differentiation in melanoma would represent a subject worthwhile to be further investigated. Other distinctive features of DCT TM domain are the presence of cholesterol (CRAC) and caveolin-binding motifs, which supports the idea of an interaction with these membrane components. Our detailed computational analysis using various sequence bioinformatics, structural modeling, and molecular simulation approaches allowed us to generate the first complete structural model of DCT in interaction with caveolin-1. This model revealed DCT-specific structural determinants involved in interaction with membranes having specific compositions and possibly regulating its enzymatic activity and intracellular trafficking, as well as its participation in complex processes as signaling pathways [23] (**Figure 3**). The overall model advocates for an interaction between Cav1 and DCT mediated by two distinct regions, one within the membrane (hydrophobicity-driven interaction) and the second cytosolic (electrostatics-driven interaction). The CYT DCT domain is predicted to adopt an extended, possibly disordered conformation and has a net positive charge (7 basic and 3 acidic residues out of 26) whose distribution is complementary to that of Cav1 cytosolic region carrying a negative formal charge, which strongly supports the electrostatic interaction between these regions, facilitated by salt bridges (**Figure 3**, thin lines). Interestingly, the DCT charge distribution in the CYT domain may be modified by the phosphorylation state of two adjacent serine residues (S511, S512 pointed by stars in **Figure 2** and indicated by dotted van der Waals spheres in **Figure 3**) whose phosphorylation was experimentally confirmed by mass spectrometry [24]. We can speculate that phosphorylation of these unique sites may represent a control mechanism for modulating DCT interaction with Cav1 or with other molecules involved in trafficking/sorting/signaling pathways. However, the presence of these interactors would need to be confirmed by additional experimental approaches.

To understand more deeply the specific behavior of TRPs in interaction with cholesterol-rich membranes, we performed molecular dynamics simulations (60 ns) of TYR and DCT TM segments embedded in 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) lipid bilayers in the presence and absence of cholesterol. The 3D structures of TM domains were modeled ab initio as α-helices whose length was based on sequence hydrophobicity and helix propensity profiles which indicated that TYR TM is slightly longer (~4 residues, one helical turn) than DCT TM. Although the two TM domains had identical initial positions and orientations in the membrane bilayer, and the overall helical structure is maintained throughout the entire 60 ns simulations, the TYR TM adopted a more tilted inclination (measured by the angle between α-helix central axis and axis normal to bilayer plane) compared to DCT (upper panels in **Figure 4A**). The magnitude of this tilting effect is likely correlated with the length of the hydrophobic helix segment that needs to fit within the membrane thickness; therefore, the orientation of shorter DCT helix is closer to normal axis while TYR is more tilted (see plot in **Figure 4B**). As expected, tilting is less pronounced in cholesterol-containing membrane due to its increased thickness (lower panels in **Figure 4A**). Surprisingly, cholesterol affects helix translation within membrane in a different manner: while in the cholesterol-free membrane both proteins experience similar levels of helix translation, in cholesterol-containing membrane, DCT translation

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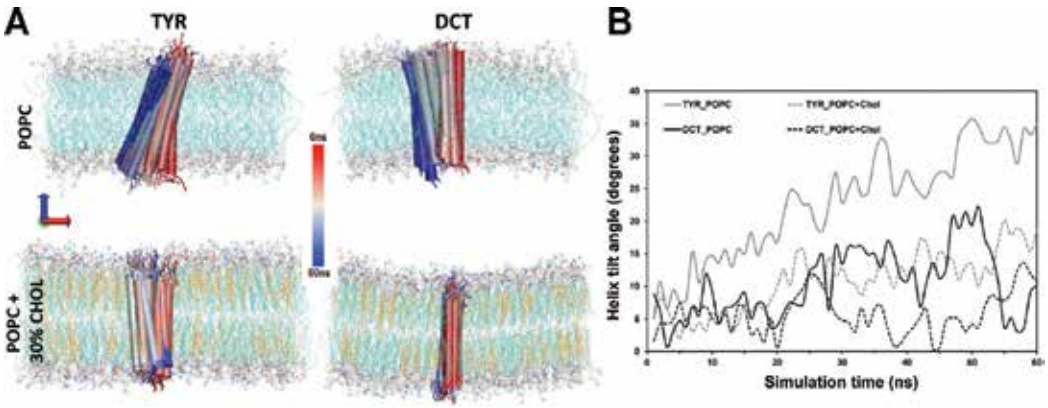

Figure 4. Molecular dynamics simulations (60ns) of transmembrane segments of human TYR/DCT embedded in pure POPC bilayer compared to cholesterol-containing membranes. (A) Structural representation of the transmembrane helix (shown as cylinder) every 2ns in the membrane bilayer; and (B) plot of the helix tilt angle variation during simulation, indicating higher tilt for TYR compared to DCT.

is highly restricted while TYR translation is only slightly affected, suggesting that cholesterol interacts more tightly with DCT, possibly due to the presence of CRAC signature. This would explain the preferential DCT sorting into CRD domains and distinct trafficking along the secretory pathway (see Section 2.3.1.2). This study, presented here for the first time, is one of the few simulation studies on the importance of cholesterol for TM type I protein stability and trafficking. The DCT structural determinants account for its distinct intracellular processing and biological functions.

2.2. DCT cellular expression

DCT is expressed preponderantly in melanocytes, which originate from neural crest cells (NCC) and migrate during embryonic development to different regions (**Figure 5**). There are also melanocytes in retinal pigmented epithelium (RPE) that originate from the forebrain neuroepithelium and in which DCT expression has also been confirmed [25]. DCT is detected in melanoblast, the progenitor of melanocyte, at embryonic day (E) E9.5, in a SOX10-melanoblast/glial bipotent progenitor, together with microphthalmia-associated transcription factor (MITF) and KIT, whereas TYR or TRP1 are expressed later in the development [26]. In hair follicle, DCT expression has been associated with a pool of melanocytes having stem cell traits of self-renewal and multipotency within the lower permanent proliferation portion of this tisssue [27]. In the precursors of peripheral nervous system which derive also from NCC, the spatial and temporal profiles of DCT expression correlate with neurogenesis during embryonic development and enhance the proliferation of cortical neural progenitor cells and neuroblast migration [28]. A unique cell population called melanocyte-like cells, found within murine and human hearts, that is distributed to the pulmonary veins, atria, and atrioventricular canal, also expresses DCT but has transcriptional profiles distinct from dermal melanocytes. The presence of these DCT-positive cells has been connected with the clinical syndrome of atrial ectopy initiating atrial fibrillation, autonomic dysregulation, and oxidative

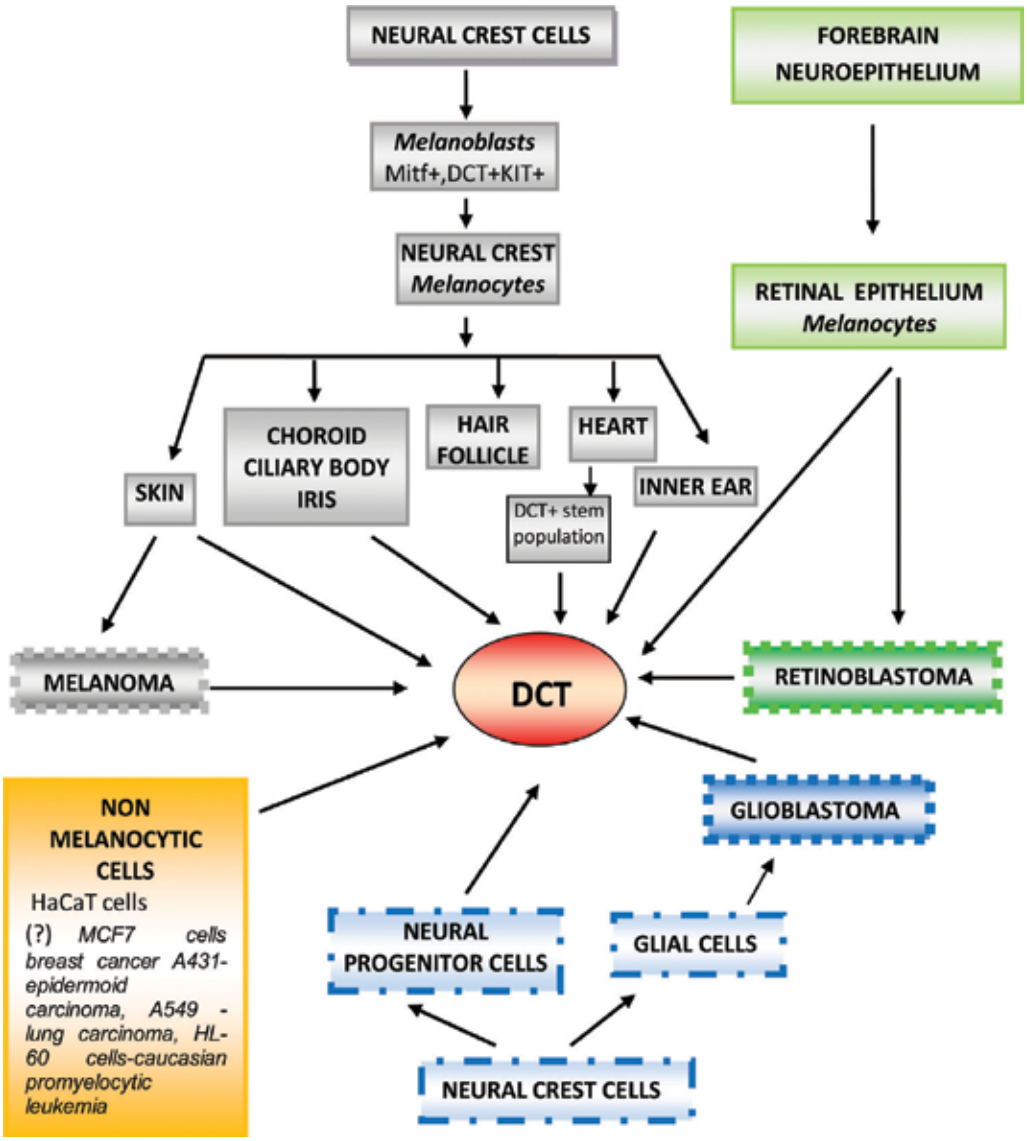

Figure 5. DCT cellular and tissular expression. DCT is primarly expressed by melanocytic (continous line) and neuronal (interrupted line) cells and by their malignant counterparts (dotted line). DCT possible expression in cells of nonmelanocytic origin is also indicated.

stress. It seems that DCT-cardiac melanocytes are involved in maintaining the normal balance of oxidative species in the myocardium [29]. The DCT expression is also retained in the malignat counterparts derived from melanocytes and neuronal cells as melanoma retinoblastoma [30], glioma [31], and glioblastoma [32]. Moreover, the neoplastic cells express different DCT transcripts and in higher amounts compared with the normal cells. For example, in patients with glioma, the DCT mRNA transcripts are in excces of 100,000-fold over that in healthy brain [33]. In amelanotic melanoma cells, in which TYR and TRP1 are downregulated

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or enzymatically inactive, DCT is well expressed [34] and during melanoma malignant progression, DCT expression, unlike TYR or TRP1, remains constant [35]. A recent study presents that DCT is endogenously expressed in HaCaT cells (basal keratinocytes) [36] which has an electrophoretic pattern comparable with DCT in RPE lysate, but distinct from the 68/80 kDa DCT doublet expressed by melanoma cell lines [37, 38]. A significant number of commercially available anti-DCT antibodies include in their technical data sheets, as positive controls for endogenous DCT, cell lysates, or histopathological specimens from cell lines or neoplasms in which DCT is not expected to be expressed such as A431-epidermoid carcinoma (Sigma-Atlas); NBT-II-Nara bladder tumor cells, WEHI-231 B cell line, CTLL-2-cytotoxic lymphocyte (Santa Cruz), human liver cancer tissue lysate, K562 (leukemia) lysate, K-562-chronic myelogenic leukemia, A549-lung carcinoma, HeLa-cervical cancer (Abcam); MCF7 cells-breast cancer, HL-60 cells-caucasian promyelocytic leukemia (Proteintech Group); human cervical cancer tissue (OriGene). Most of them show in WB analysis bands of approximately 50 kDa or/and 30 kDa. Two hypotheses can explain these data: (1) the 50/30 kDa bands are not DCT but possibly contaminants detected due to antibodies cross-reactivity. This would be very unlikely because these antibodies have been raised against different DCT sequences, by different technologies, in different laboratories. However, as many of these antibodies do not show data on these cells having "DCT gene" downregulated or amplified (with specific primers for DCT mRNA), their specificity is still questionable and may induce false-positive results with severe consequences especially in clinic; (2) the 50/30 kDa in nonmelanocytic/-neuronal cells or tissues are indeed derived from DCT (possibly isoforms or degradation products). DCT is expressed in neural crest progenitors that generate multiple cell lineages during development. The demonstrated DCT involvement in anti-apoptotic and stress-resistance pathways (Section 2.4) would qualify it for activated expression in cellular niches of different normal or transformed phenotypes where it would be requested to sustain specific processes. For example, osteopontin, primarily expressed in bone cells (osteoblasts) has become a well-known marker for various neoplasms, including melanoma, where its expression is associated with tumor progression [39]. HaCaT is an immortalized keratinocyte cell line with a high capacity to differentiate and proliferate in which endogenous DCT has detoxification biological activities similar to those already described in melanocytic lineage [36]. These new data consolidate the theory that DCT expression may encompass, indeed, multiple cell phenotypes where it accomplishes, very likely, functions related to cell protection. How is DCT expression activated and modulated in nonmelanocytic/-neuronal cells are questions whose clarification require additional studies. Morevoer, the DCT expression in nonmelanocytic lineages would raise the question whether DCT can still be considered a specific biomarker for the diagnosis of melanocytic lesions.

2.3. Regulation of DCT expression and intracellular processing in melanoma

2.3.1. Intracellular regulation

2.3.1.1. Transcriptional level

The human DCT gene (h-DCT) has 55-kb and was mapped to the chromosomal region 13q31-q32 with a coding region of eight exons all encompassing the open reading frame of the protein [40].

The h-DCT is controlled by the two separate regulatory regions: the 32-bp element and the proximal region [41]. The 32-bp element is a composite enhancer having potential binding sites for transcription factors that contain a basic helix-loop-helix structure (including Microphthalmia-associated transcription factor—MITF), a high-mobility-group (HMG) domain (the TCF/LEF-1 or SOX family), or an Ets domain [42]. MITF is a master regulator of pigmentary system [43], and there is a selective requirement for MITF-M isoform for melanocyte development. The promoter region of MITF-M contains CREB, SOX10, PAX3, and LEF-1 binding sites. The presence within DCT promotor of the 32-bp element containing a CAATTG motif do not produce significant transactivation by MITF, as in case of the other TRPs, suggesting that the mechanism for melanocyte-specific transcription of the DCT gene is different from that of the other TRPs [44]. In addition to MITF, DCT is regulated by SOX10, which is a high-mobility-group transcription factor that plays a critical role in many processes in neural crest cells, including multipotency, proliferation, apoptosis, survival, and commitment to defined neural crest-derived lineages. SOX10 transiently regulates DCT expression during early melanocyte development, independently of MITF function [45] and synergistically with MITF that enhances SOX10-dependent activation of the DCT promoter [46]. Another member of the SOX family, SOX5, inhibits the SOX10-stimulated activity of the DCT promoter in melanocytes [47]. A synergistic transactivation of DCT gene promotor results also from cooperation between TLEF-1 and MITF or between TLEF-1 and TFE3, a MITF-related protein [48]. The TCF/LEF-1 family regulates target gene transcription in response to Wnt signals. The transcriptional regulation of DCT involves also PAX3, a member of a highly conserved family of transcription factors essential to the development of many tissue types throughout embryogenesis and vital to the maintenance of several stem cell niches. Unlike MITF which is an activator of DCT expression, PAX3 inhibits both DCT expression and the ability of MITF to bind to the DCT promoter. PAX3 forms a repressor complex with LEF1 and GRG4 on the DCT enhancer sequence and actively blocks MITF binding. In the presence of beta-catenin, LEF1 forms a complex with MITF and beta catenin and displaces PAX3 from DCT enhancer [49]. Oppositely, SOX10 does not cooperate with PAX3 to activate DCT in combination with PAX3 [50]. OTX2 is a transcription factor that regulates the specific expression of DCT gene in REP. OTX2 binds to the DCT gene promoter *in vivo*, whereas repression of endogenous OTX2 expression results in the decrease of DCT protein content [25]. Our most recent data introduces Cav1 as the newest regulator of the DCT [23] (detailed in Section 5). Several DCT isoforms resulted from translation of introns of DCT are reported. One sequence contains exons 1–4 with retention of intron 2 and part of intron 4 (DCT/TRP-2-INT2) [51], another is from the same sequence except for an extended 3'-untranslated region originating by alternative polyadenylation (Tyrosinase-protein-2 long tail), and the third isoform results from the 3'-untranslated region containing the alternatively spliced last DCT exon (Tyrosinase-protein-2-8b) [52]. Importantly, unlike the fully spliced DCT mRNA expressed in normal skin melanocytes, retina, and melanomas, the DCT/TRP-2-INT2 mRNA is detected only in melanomas, whereas the Tyrosinase-protein-2 long tail and Tyrosinase-protein-2-8b mRNAs are expressed in both melanocytes and melanomas. The DCT/TRP-2-INTL isoform is recognized by a CTL clone and has potent therapeutic value due to its specific and elevated expression in melanoma. Another sequence that containes two novel exons alternatively spliced from the sixth intron between exons 6 and 7 of TRP-2/DCT generates a novel TRP/DCT-2-6b mRNA. The TRP2/DCT-6b isoform is also recognized by a TIL clone and may play a role in tumor regression [53].

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2.3.1.2. Post-translational level

TRPs follow the general secretory pathway: TRP-polypeptide synthesis and folding in endoplasmic reticulum (ER), the N-glycan maturation along the Golgi complex and transport to the steady-state destination, the melanosomes, the site of melanin synthesis and storage. In parallel with our early research on TRP1 intracellular processing in murine melanoma cells [54], studies of other groups were presenting a specific drug-and UV-resistance mediated by TRP2/DCT in melanoma [55–57]. In this context, we considered that deciphering the intracellular processing pathways of DCT would bring fundamental knowledge and possible exploitable information into melanoma development and therapy. The immunofluorescence microscopy images and ultracentrifugation data reveal a unique pattern of DCT subcellular distribution. Unexpectedly, DCT is detected in high amounts in a perinuclear position, co-localizing with the TGN marker, syntaxin 6, and in substructures at plasma membrane (PM), showing weak overlapping with late melanosome markers TRP1 and Rab27a. The maturation kinetics and traffic along the secretory pathway show that ER DCT 68 kDa precursor containing high-mannose N-glycans moves along the Golgi where it acquires complex structures, gradually turning into the DCT 80 kDa mature protein, within approximately 3 h [37] compared to 45 min in which TRP1 becomes a fully glycosylated 75 kDa protein [54]. Similar to TYR and TRP1, DCT interacts with the ER lectin chaperone calnexin that assists normal polypeptide folding of all TRPs [37]. In N-glycoproteins, the glycan procesing in ER interferes with polypeptide folding. The step of N-glycan trimming by glucosidase I and II results in the formation of a monoglucosylated precursor that interacts with the ER lectin chaperones, calnexin, or calreticulin, which assist the polypeptide folding. The inhibition of glucosidase I and II with N-butyldeoxynojirimycin (NBDNJ) perturbs N-glycosylation, resulting in a triglucosylated precursor unable to interact with calnexin. In NBDNJ-treated cells, TRP1 folds in the absence of interaction with calnexin, being rescued by another ER chaperone BiP, leaves ER, and moves along Golgi [54], whereas in the same cells, TRP2/DCT conformation is severely altered, and the misfolded protein is targeted to proteasomal degradation [37]. A more recent study reports that the treatment of Melan-a cells, with the chemical compound, A3B5, results also in proteasomal degradation of DCT but not of TYR [58]. Whether DCT from A3B5-treated cells is targeted to proteasome from the ER, via the well-known retrotranslocation pathway or from a post-ER compartment remains to be further investigated. In any case, this is an additional proof that, indeed, DCT fate in melanoma is distinctly regulated from the other TRPs. Additional information about the DCT biosynthetic pathway came from our investigations of the two human amelanotic melanoma cell lines, MelJuSo (MJS) and SKMel28 (SK28) [23]. In SK28, as in other amelanotic cell lines, pH homeostasis is altered, and TYR is retained in the secretory pathway and prematurely, proteasomally degraded [59]. Importantly, in both MJS and SK28, amelanotic cell phenotypes DCT appears at steady state as a mix of the fully processed protein and the partially glycosylated precursor. This pattern indicates that a significant DCT amount is able to overcome the pH-induced blockade being sorted from the early steps of its biosynthetic pathway in a different cargo than TYR. Our experimental data demonstrate that DCT maturation between ER and Golgi is interrupted or pertured in the presence of nystatin [23] or monensin [60], two pharmacological agents that disrupt CRDs or insert in Golgi CRDs, respectively. A significant amount of DCT is detected by co-localization

and co-immunoprecipitation experiments in complexes with Cav1, an abundant component of CRDs. The association of DCT with Cav1 and cholesterol is supported by our structural analysis (detailed in Section 2.1). Cav1 downregulation has a profound regulatory impact on DCT and subsequently on its entire biosynthetic pathway [23] (detailed in Section 5.3). Our theory is that a significant fraction of DCT is sorted in the early secretory pathway, possibly from ER, in CRDs with Cav1, in a cargo without TYR and trafficked on a route less sensitive to amelanotic acidic pH. Our data is supporting the concept of the selective ER exit sites and ER-Golgi transport [61] and that production of specific lipids might have a regulatory role in cargo recruitment and export from ER [62]. Another cellular parameter regulating DCT processing, between ER and Golgi is the intravesicular pH. The treatment of B16F1 pigmented melanoma cells with bafilomycin (Baf), a specific inhibitor of v-ATPases and pH corrector, slightly increases the amount of DCT mature complex protein [60]. This demonstrates that pH of the secretory pathway is altered in pigmented phenotypes as well, but to a less extent than in amelanotic cells and that only a DCT fraction is trafficked on a route sensitive to pH alterations too. We also found that DCT maturation between ER and Golgi is interrupted by microtubule depolymerization agent nocodazole (NCZ) when DCT is prevented to reach medial Golgi and remains in the form of the 68 kDa precursor [unpublished data]. Post-Golgi, the membrane composition and the interaction of the sorting and trafic machinery with the CYT tail of TRPs decide their destination [63, 64]. The di-Leu motif (QPLLMD) present in both cytoplasmic tails of TYR and TRP-1 and specifically requested for the interaction with the AP-3/AP-1 sorting elements in post-Golgi compartments is absent from DCT CYT domain which has Tyr-like motif (YRRL). The detection of DCT in TGN area and at PM in both murine and human melanoma cell lines with two distinct antibodies and the low amounts in mature melanosomes [23, 37] support the theory that post-Golgi DCT is trafficked on a distinct route than TYR or TRP-1, possibly being recycled from PM via a recycling endosomal (RE) compartment. Interestingly, in GL261 mouse glioma cell line DCT is also detected at PM, which may indicate a post-Golgi common route for DCT in different tumor cells [65]. We discovered an unexpected effect of the lysosomotropic agent chloroquine (CQ) on DCT stability, from both murine and human cell lines. CQ, a well-known pharmacologic agent that accumulates within acidic compartments, usually recommended as inhibitor of lysosomal enzymatic machinery [66] was expected to block DCT constitutive degradation. Instead, we found that DCT amount synthetized within 30 min (pulse), after 3 h (chase), in the presence of added CQ is diverted to a premature degradation pathway, whereas TRP1 stability is not affected in the same cell line. This is not an artifact, given that DCT degradation can be prevented in CQ-treated cells if Baf is present in the system. It is worth mentioning that DCT degradation is significantly decreased if CQ is added at 6 h chase, when probably DCT is in a more protected compartment. The effects of CQ in living systems are pleiotropic, and many of its action mechanisms or targets are still unknown. CQ interferes with the trafficking [67] and recycling processes from PM [68] or with the fusion vesicular processes, by enhancing the rate of the phagolysosomal fusion [69]. Our theory about CQ impact on DCT fate is that in our experimental conditions (mild CQ concentration, 50 μM and short time period treatment of 2 h), CQ potentiates the fusion between a DCT-positive post-Golgi endosomal compartment with a still proteolytically active one, most likely the lysosomes. It will be also interesting to identify which other proteins share the DCT fate in CQ- treated melanoma cells or if the effect of CQ is similar in other cells phenotypes expressing endogenous DCT.

2.3.2. Extracellular regulation

One of the early events in neoplastic transformation of melanocytes is the uncontrolled proliferation. During this step, tumor cells secrete numerous cytokines and growth factors, which can regulate back the tumor cells activities, by binding to self-receptors (autocrine stimulation) or receptors of neighboring cells (paracrine stimulation) and self-sustaining tumor growth signals. In addition, the nutrient deprivation and numerous homotypic cell-cell contacts, established as a result of the alterations that occurred in cell adhesion molecule repertoire, result in activation of multiple signaling cascades. A similar situation to autocrine/paracrine stimulation is simulated in an *in vitro* experimental approach when we cultured three different melanoma cell lines, MNT-1 (pigmented, metastatic stage), MJS (amelanotic, VGP stage), and SK28 (amelanotic, metastatic stage), for various time periods representing subconfluent (48 h), semi-confluent (72 h), and confluent (96 h) stages and when the dramatic increase of DCT mRNA and protein are registered [23] (see also Section 5). Surprisingly, in MNT-1 cells that express all TRPs, only DCT is increasing, whereas neither TYR nor TRP-1 expressions are changed. The process was further dissected in MJS phenotype when subconfluent cells grown in exhausted medium resulted from a confluent culture as well as in nutrient deprivation conditions (2% instead of 10% fetal calf serum) showed also the DCT overexpression [23]. All these data demonstrate that only DCT is the target of autocrine/paracrine stimulation. The DCT increase is more abrupt in MJS than in MNT-1 or SK28 and may be a process which is distinctly controlled in VGP stage phenotypes. The VGP cells express a variety of growth factors for autocrine and paracrine stimulation that enable them with survival and proliferation capacities in growth-factor free medium and with increased invasiveness potential through basement membranes [70]. It appears that DCT remains under the control of extracellular factors even in advanced stages of tumor progression as its increasing expression still persists in the two metastatic cell lines. The identification of the cytokines and growth factors, secreted during intense proliferative step, to which DCT overexpression is activated would be a further important step in elucidating how the expression of this antigen is modulated. Altogether these data demonstrate that DCT fate in melanoma is controlled by multiple and specific factors that do not act in the biosynthetic pathways of the other TRPs. There are several checkpoints in DCT life-cycle: (1) in ER, the DCT stability, controlled by early step of N-glycan processing and polypetide folding assisted by calnexin; (2) between ER and Golgi, the DCT precursor trafficking and maturation, controlled by Cav1 expression, integrity of CRDs and microtubules and pH of the secretory pathway; (3) beyond Golgi, the DCT stability, controlled by CQ; and (4) at transcriptional level, the DCT mRNA and protein, controlled by Cav1 expression, nutrient deprivation and secreted growth factors and cytokines during proliferation step (**Figure 6**).

2.4. DCT: a regulator of melanogenesis, cellular detoxification, and stress-resistance pathways

Melanins represent a group of polymers produced by both normal and transformed melanocytes. The skin melanins are synthetized and deposited within melanocyte-specialized cellular organelles called melanosomes that are finally transferred into epidermal keratinocytes ensuring not only skin pigmentation but also UV light absorption and scattering, free radical

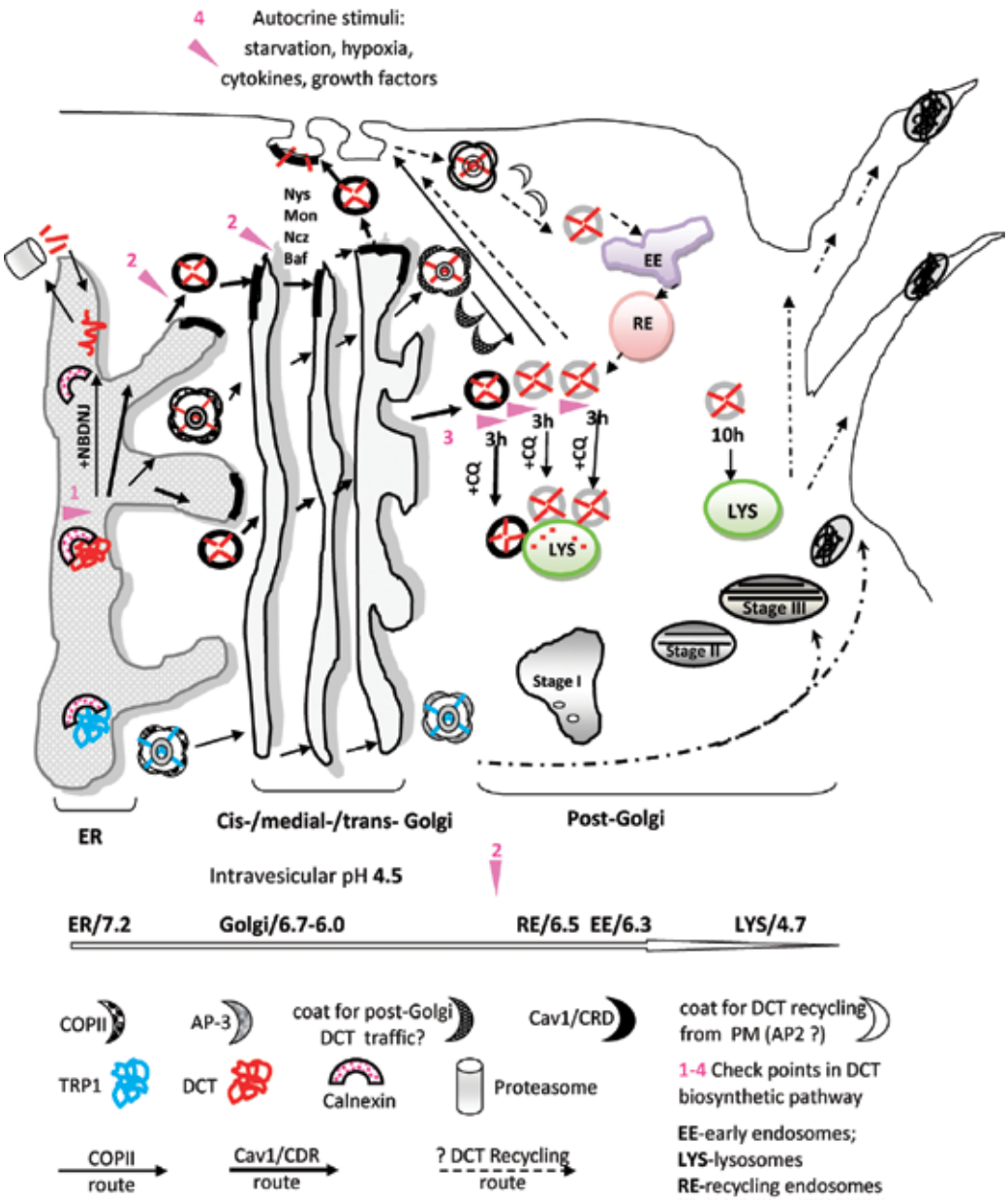

Figure 6. The intracellular journey of DCT in melanoma cells. The DCT biosynthetic pathway within a melanoma cell is schematically presented. All checkpoints along this route are indicated by triangle symbol. The DCT polypeptide is synthetized and folded in ER assisted by lectin chaperone calnexin (Clx). The interruption of N-glycan processing in ER with NBDNJ prevents interaction with Clx. TRP1 is further processed beyond the ER, whereas DCT is targeted to proteasomal degradation (1st checkpoint). Between ER and Golgi, DCT maturation is blocked by disrupting agents of cholesterol-rich domains (CRD) (nystatin-Nys, monensin-Mon) and microtubules (nocodazole-NCZ), intravesicular pH (bafilomycin—Baf) and caveolin-1 (Cav1) downregulation (2nd checkpoint). Post-Golgi, DCT, unlike TRP1, is diverted into a premature degradation pathway induced by CQ treatment (3rd checkpoint). Nutrient deprivation, secreted factors during proliferation and Cav1 gene down regulation are activators of DCT, not of TYR or TRP1, expression (4th checkpoint). Possible DCT recycling route from PM is presented as segmented line. TRP1 post-Golgi route to melanosomes stage III/IV is shown as intrerupted line.

scavenging, coupled oxidation-reduction reactions, and ion storage [71]. TRPs are the main regulators of principal steps of melanin polymer formation (**Figure 7**). TYR is the key-enzyme of melanogenesis that catalyzes the hydroxylation of L-Tyrosine to L-3,4-dihydroxyphenyl alanine (L-DOPA). L-DOPA is rapidly oxidized to DOPAquinone that spontaneously undergoes cyclization to Dopachrome. In the absence of any enzymatic activity, Dopachrome loses carboxylic acid generating 5,6-dihydroxyindole (DHI). TRP2 or L-Dopachrome tautomerase (DCT) acts downstream of TYR by rearranging Dopachrome into DHI-2-carboxylic acid (DHICA) that is further oxidized to the corresponding quinone by the activity of TRP1 in mouse or by TYR in humans. In 1992, Jackson and colab reported the cloning and sequencing of mouse cDNA corresponding to the region of the mice coat color mutation slaty. The gene product was named tyrosinase-related protein-2 (TRP-2) due to its high degree of amino acid identity with the other TRPs [72] or Dopachrome tautomerase (DCT) due to enzymatic activity on Dopachrome [73]. DCT is now well acknowledged as the modulator of melanin qualities. L-Dopachrome is the second branch point which under the unique L-DCT action is transformed into DHICA (**Figure 7**). Melanin derived from oxidation and polymerization of DHI, formed in the absence of DCT are black and insoluble, whereas the DHICA-enriched melanins that contain a higher proportion of carboxylated versus noncarboxylated indolic monomers are brown and more soluble [74]. Despite of numerous mutations identified in other melanosomal proteins, with consequences on pigmentation, no mutations have been described in human DCT, suggesting this is a conserved protein. However, in mouse, mutant alleles of DCT are associated with pigment dilution, producing the slaty (R194Q substitution in the MeA binding domain) and slaty light (G486R substitution in the TM domain) phenotypes. DCT mutations increase pheomelanin and reduce eumelanin produced by melanocytes in culture showing that the enzymatic activity of DCT play a role in determining whether pheo-or eu-melanin pathway is preferred [75]. The intermediates generated during melanogenesis have genotoxic [71] and immunosuppressive properties [76]. DHI is a cytotoxic melanin precursor [77], whereas DHICA is an antioxidant molecule [78], a diffusible chemical messenger [79], and DHICA unlike DHI melanins exhibit potent hydroxyl radical-scavenging activity (**Figure 7**). Moreover, eumelanins bind calcium with an affinity similar to calmodulin and thus interfere with the intracellular calcium regulation [80]. DCT, as a specific limiting factor of DHI concentration and DHICA-eumelanins formation becomes thus a modulator of different processes in melanocyte in which DHICA and DHICA-melanins are involved. To establish the general impact of DCT on a living organism, the DCT gene was targeted during mouse embryonic development [81]. The DCT-KO mice are viable, have a diluted coat color phenotype, due to reduced melanin content in hair but do not show any decrease in melanocyte numbers. However, under chronic UVA-induced oxidative stress in skin of DCT-KO mice compared with wild-type, the level of reactive oxygen species (ROS) and the numbers of apoptotic cells are increased, whereas the amount of eumelanin is decreased [82]. This demonstrates that, in melanocytes, DCT is involved in regulating a protective pathway in response to environmental stressful conditions. The DCT protective effect seems not to be exerted only via its enzymatic activity. The extremely low growth rate for the DCT-slaty and DCT-slatylight melanocytes could not be abgrogated in the presence of catalase, added to culture medium to overcome effects of H_2O_2 resulted from DHI excess due to inactivity of mutated DCT [83]. In transformed melanocytes, DCT is a tumor protector as well. In pigmented melanoma, as in melanocytes, DCT generates DHICA and further

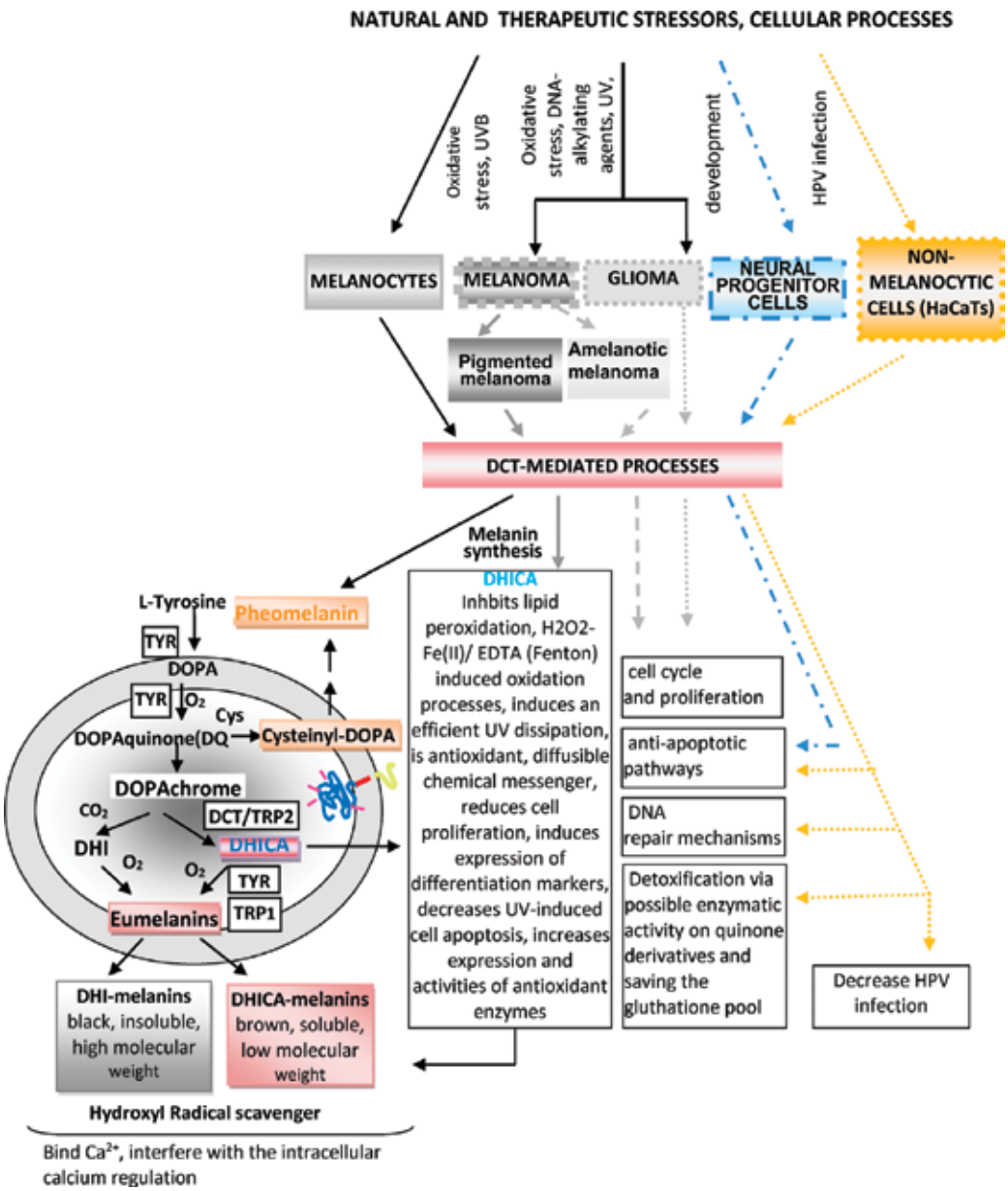

Figure 7. The processes mediated by DCT in different normal and malignant cell phenotypes.

DHICA-eumelanins, both exerting the antioxidant properties (**Figure 7**). However, DCT protective activity is independent of melanin pathway, and this is in good-agreement with finding that DCT is well-expressed in amelanotic cell lines and tumors [34, 35]. In a process of identification of genes associated with cis-diamminedichloroplatinum (II)(CDDP)-and X-ray resistance in the amelanotic melanoma cell line WM35, Bed-David's group found that DCT expression was upregulated in both CDDP- and X-ray resistant mutants compared with the

parental line [84]. On the other hand, DCT ectopic overexpression in melanoma cells abrogates UVB-induced apoptosis [57]. DCT-drug resistance-mediated pathway is related to antitumorals that interferes with DNA replication as CDDP, carboplatin, or methotrexate and is not effective to the ones acting on microtubule formation as paclitaxel. In correlation with our data about DCT intracellular processing, we can speculate that DCT-mediated tumor resistance to the microtubule depolymerizing agents, unlike the one to DNA-alkylating agents, requires mature DCT and not DCT precursor which is the only DCT glycoform in cells treated with microtubule depolymerization agents (Section 2.3.1.2). DCT-radiation resistance is addressed to both X- and UVB-radiation that act on DNA by creating DNA strands and causes the formation of pyrimidine dimers, respectively, and are independent of TYR or TRP1 expression or melanin content [85]. DCT protective effect may be explained by either interference with DNA repair mechanisms or the regulation of anti-apoptotic pathways. DCT anti-apoptotic activity has also been reported in AJS sensory neurons in *C. elegans* [86]. A possible DCT mechanism suggested by the authors would be the activation of the ERK/MAPK stress pathway in response to high DHICA content produced as result of DCT overexpression and enzymatic activity after radiation [84]. However, this will not explain the DCT protective effect in WM35 amelanotic cell line used in these experiments or in other amelanotic phenotypes where melanogenic pathway is interrupted and Dopachrome, DCT natural substrate, is not produced due to TYR inactivity. Specific melanoma protective DCT-mediated effects, independently to melanogenesis, have been demonstrated also in amelanotic melanoma cell line WM35, expressing inducible DCT and subjected to oxidative stress conditions [87]. DCT endogenous expression increases cell viability and intracellular glutathione (GSH)—a key factor of ROS detoxification, whereas ectopic DCT expression decreases nonmelanocytic cell sensitivity to quinone compounds [88]. The DCT detoxification action is dependent of the tautomerase enzymatic activity, is lineage-specific, and is in conjunction with specific metabolites that naturally appear in melanocytes, neural/glial cells, and in melanoma and glioma. A possible mechanism by which DCT prevents quinone toxicity could be explained by the shared homology between quinones derivatives with DCT natural substrate, L-Dopachrome, and to the fact that DCT may have a possible oxidoreductase activity. This, however, is not supported by the presence of Zn^{2+} in DCT catalytic site, as Zn^{2+} has no redox properties, and is unable to catalyze oxidative reactions [12] but possible if the presence of the ferrous-iron would be accepted in the DCT catalytic site [89]. Such a DCT protective mechanism could function in glioma, in which, after targeting DCT by immunotherapy, the tumor cells become more sensitive to chemotherapy [90].

Although the object of this chapter is DCT in melanoma, we consider that it is of importance to discuss the role of DCT in other cell lineages. We have argued about DCT expression in HaCaT cells (basal keratinocytes) [36] (Section 2.2). The effects of DCT downregulation in HaCaTs are similar to the ones reported so far in melanocytic cells, namely increased ROS levels, DNA damage, and altered cell cycle, which furthermore compromise the infection of these cells with HPV. There are several common processes, mainly related to cell protection, with which DCT interferes, regardless the cell phenotypes in which it is expressed. However, these processes are involved in cell-specific responses to different aggressors (e.g., therapeutic stressors in melanoma and viral infection in basal keratinocytes).

3. DCT value in the assessment of melanocytic lesions

The diagnostic and prognostic of CMM is in general evaluated histopathologicaly. In particular cases, when it is difficult to discriminate between melanocytic lesions and other resembling tumors as sarcomas, lymphomas, or neuroendocrine tumors, the expression of melanocytic biomarkers is requested, and they are commonly assessed by immunohistochemistry. For patients with unambiguous tumor histologic features, the CMM prognostication relied on Breslow's index, the level of invasion in skin layers (Clark's level), growth pattern (nodular, superficial spreading, etc.), dimensions, and presence/absence of ulceration information proves to be statistically significant in very large clinical cohorts [91]. The panel of melanoma markers is continuously revised and improved in accordance with the new discoveries related to the molecular mechanisms and pathways in melanoma progression [92]. One of the most challenging is the thin melanoma subset, defined by Breslow depth, 1.0 mm representing patients with early-stage disease. Despite that most are thought to have an excellent clinical outcome (85% survival during a 10-year period) and can be treated effectively, 15% of melanoma deaths result from metastases of thin lesions. Furthermore, the clinical outcome of patients with melanoma of intermediate thickness (2.0–4.0 mm in Breslow depth) is less predictable. Clearly, identifying a high-risk population with thin melanomas remains a challenge, and new markers to assist this patient population are expected in order to establish more accurate risk groups with subsequent more aggressive therapeutic approach and tighter follow-up [93]. Our group assessed for the first time, the expression of DCT comparatively with the one of TYR in a panel of formalin-fixed, paraffin wax-embedded benign and malignant melanocytic lesions. The DCT and TYR proteins were analyzed by immunohistofluorescence microscopy in human specimens by simultaneous triple staining, with anti-DCT/-TYR antibodies, followed by secondary antibodies AlexaFluor-labelled and with DAPI for nuclei [38]. This technique allows to follow DCT and TYR expressions in identical cells within different tumor components. In tumor progression, the expressions of melanoma antigens are often lowered [94], and their immunodetection in histological specimens may be enhanced using antibody populations that recognize more than one epitope. In this study, the DCT expression was assessed with a novel anti-DCT antibody raised in our laboratory against the luminal domain of human DCT and in which the bioinformatic analysis identified multiple potential antigenic sites [38]. There is a heterogeneity in the expressions of the two antigens in benign tumors represented by junctional (JNs), compound (CNs), or dysplastic nevi (DNs) and malignant melanomas represented by superficial spreading (SSMs), nodular (NMs), achromic (ACMs), acralentiginous (ALMs) melanomas. Specimens expressing both antigens, only one and negative for both, were present in different numbers in each melanoma subgroup that was analyzed. The melanocyte neoplastic transformation and malignant progression is well correlated with the dissociation of DCT and TYR expression in distinct cell populations. In **Figure 8A** is presented an example of DCT and TYR dissociated expression in distinct tumor cells in a specimen representing a nodular melanoma. Within the double-positive category, we have identified in some specimens a subtype named by us "DCT-phenotype" in which DCT and TYR expressions specifically distributed within cell populations of tumor components create a tumor-specific architecture, with cells Tyr+/DCT- in the subepidermal layer, whereas DCT+/Tyr- cells segregate into deep dermis. The DCT-phenotype was found

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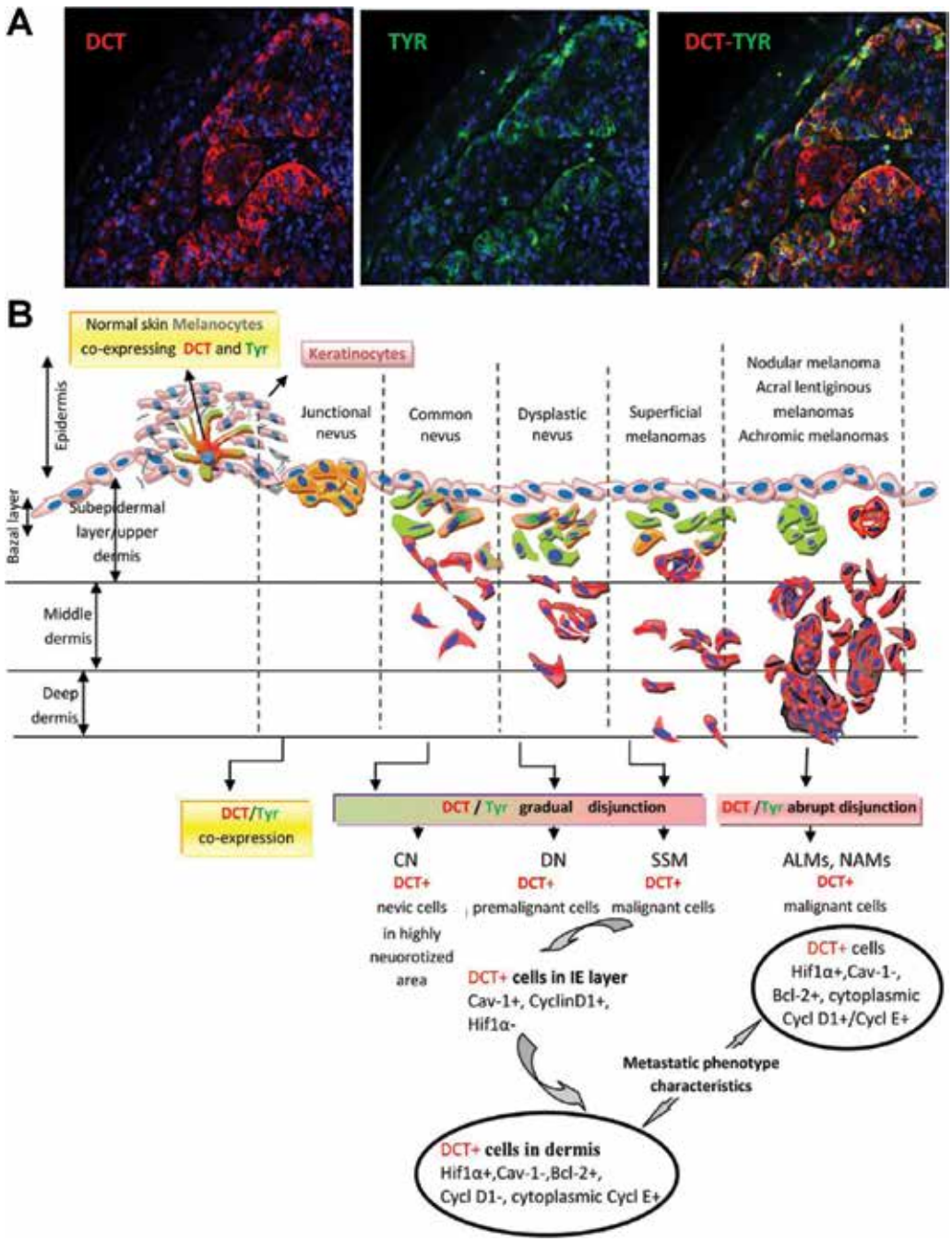

Figure 8. DCT in melanocytic lesions. (A) A nodular melanoma specimen immunostained for DCT and TYR expressions and analyzed by fluorescence microscopy. Tumor cells co-expressing both markers and cells DCT+/TYR- or DCT-Tyr+ can be observed. Unlike TYR, DCT is well expressed in numerous cells. (B) Schematic representation of DCT and TYR dissociation in melanocyte transformation and melanoma progression and molecular anatomy of DCT-phenotype. The switch in molecular repertoire of markers of tumor progression and bad prognosis in DCT+ cells in intraepidermal (IE) layer is indicated. DCT+ cells in deep dermis acquire molecular parameters of metastatic phenotypes [38].

in benign specimens with high neurotization and also in some early malignant ones having low Breslow/Clark indexes but with ulceration. Our theory is that DCT-phenotype is emblematic for a long-lasting, "die-hard" phenotype. The DCT-intense expression is observed in large areas of compound neurotized nevi contributing probably to the well-acknowledged enhanced stability and low proliferation rate of these nevus cells [95] and may not represent a life-threatening problem in benign tumors. However, the superficial malignant melanomas, with low indexes Clark or Breslow but having DCT-phenotype could be a warning signal for considering those specimens as ones of high risk with a possible unfavorable prognostic. The DCT-clones selected in inner dermis of early malignant lesions acquire the expression and subcellular distribution of molecular markers reported to be associated with different types of neoplasms, including melanoma, with extended migratory capacities (caveolin-1-), survival in stressful conditions (cytoplasmic Hif-1α+), activated anti-apoptotic mechanisms (cytoplasmic cyclin D+ and Bcl-1+), angiogenic, and metastatic potential (cytoplasmic cyclin E+) (**Figure 8B**). Several ALMs or ACMs advanced melanomas diagnosed by anatomopathological analysis with bad prognostic detected DCT as the unique melanosomal antigen. The ALMs distinguish themselves from other melanoma types in terms of a worse prognosis, enhanced aggressiveness, and by a more advanced stage at diagnosis [96], whereas some ACMs are characterized by a peculiar and aggressive evolution [97]. It is very possible that DCT expression in ALMs and ACMs mediates tumor stress resistance pathways and contributes to the malignant characteristics of these melanoma categories. DCT could be an useful adjunct marker increasing sensitivity of tumor cell detection in specimens having downregulated other melanoma antigens, and the DCT-phenotype could represent a parameter associated with high-risk for bad disease outcome.

4. DCT as target in melanoma therapy

4.1. Anti-melanoma therapies

The surgical removal is the only cure for melanoma with the condition that the excised lesion be in an early stage. However, the micrometastases cannot be addressed exclusively by the surgery and therefore, combinatorial therapeutical strategies are applied in the attempt to extend survival rates. The treatment options in melanoma are continuously revised, and there are several excellent reviews about this topic [98–100]. The schematic representation of the treatment of metastatic melanoma including different approaches is shown in **Figure 9**.

4.2. Anti-melanoma immunotherapeutic strategies involving DCT

The identification of different T-cell clones in melanoma patients recognizing peptides derived from DCT (**Figure 2**) raised the interest for this antigen in the development of anti-melanoma immunotherapeutical strategies. The cellular vaccine engineered to co-express a DCT epitope, with IFN-γ in the same gene by replacing the IFN-γ signal peptide with a DCT epitope-expressing signal peptide, resulted in decreased B16 tumorigenicity and enhanced immunogenicity after gene transfer. More importantly, irradiated transiently, TRP-2 epitope-expressing, IFN-c

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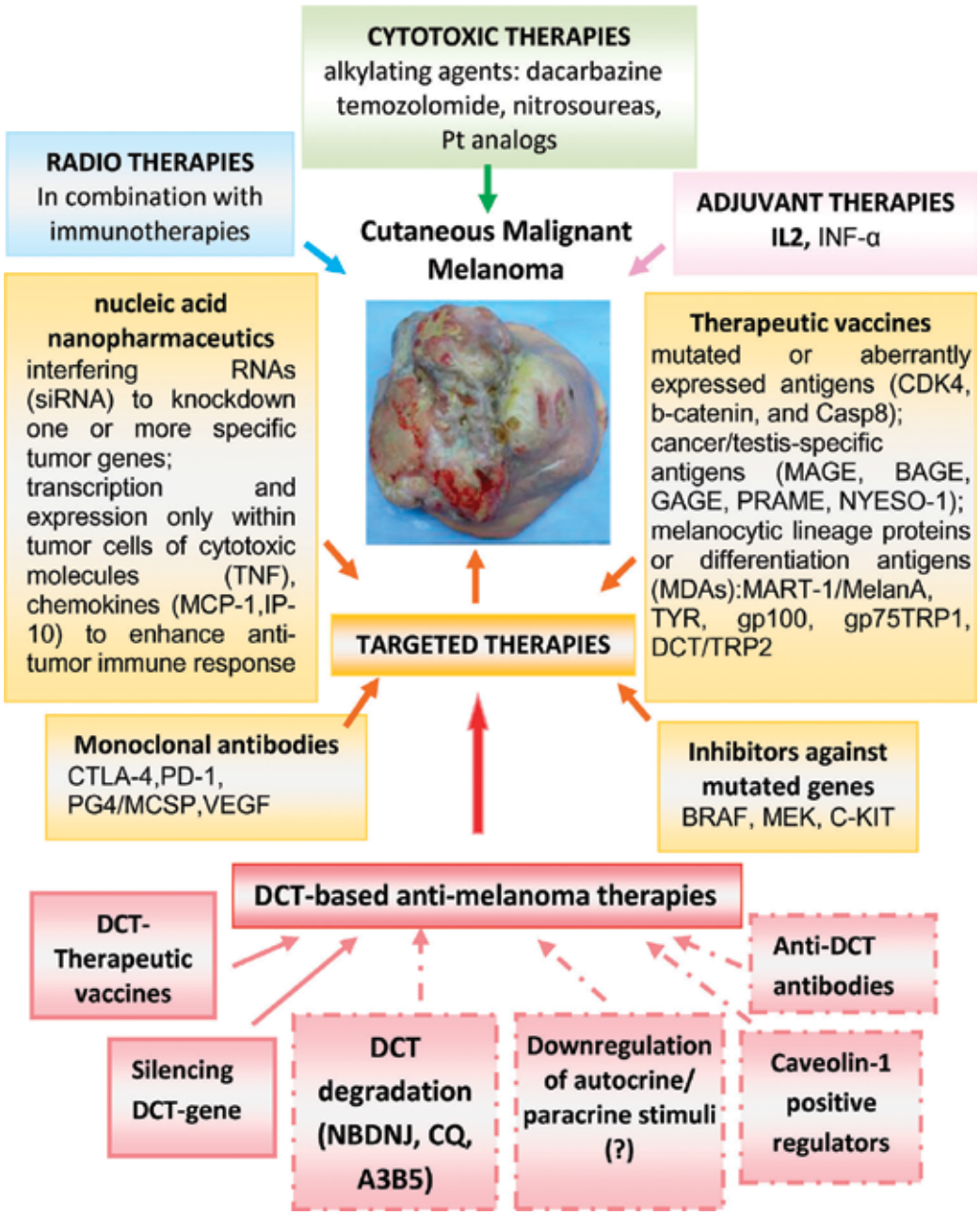

Figure 9. The therapies in melanoma. The different anti-melanoma treatment strategies are presented. DCT-based therapies are integrated part of the targeted therapies. The solid lines indicate the already existing therapies, whereas the dotted lines are proposed as possible adjuvant therapies based on the molecular studies about DCT intracellular processing and stability in melanoma cells. The melanoma specimen is an ulcerated nodular melanoma of a 26-year-old man, from lumbar region (by courtesy of Dr. S. Zurac, Department of Pathology, Colentina University Hospital, Bucharest, Romania).

gene-modified B16 cells worked efficiently as a cellular vaccine to protect animals from parental wild-type tumor challenge [101]. The VacciMax® (VM), a liposome-based antigen delivery platform, has been used to deliver DCT 181–188 in combination with p53-derived peptides. A single administration of VM was capable of inducing an effective CTL response to multiple tumor-associated antigens. The responses generated were able to reject 6-day old B16-F10 tumors [102]. Another plasmide liposome DNA vaccine targeting the DCT in combination with chemokine CCL2 as an adjuvant used xenogeneic (human) DCT in a mouse model and resulted in induction of strong anti-DCT cell-mediated immunity after two vaccinations [103]. A novel vaccine system designed from a long TRP2/DCT peptide with a CD8 epitope (TRP2/DCT 180-88) and a CD4 epitope (TRP2/DCT 88-102) together with α-galactosyl ceramide, a lipid antigen representing a new class of promissing vaccine adjuvants into cationic liposomes tested on mice tumors resulted in the enhanced production of IFN-Υ and increased cytotoxic T-cell responses [104]. Importantly, the antitumor immune activity involving MDAs as immunotherapeutic targets may have as side effects the damage (depigmentation) of the normal tissues that also express the MDAs [105]. However, in a patient receiving infusion with TIL586 (recognizing the DCT 109–205 peptide), tumor regression was observed, but not depigmentation [20], which demonstrates that immunotherapy directed against some DCT epitopes is specific and does not affect normal tissues. In another study, the inoculation of plasmid DNA encoding murine DCT elicited antigen-specific CTLs that recognized the B16 mouse melanoma and protected the mice from challenge with tumor cells. Moreover, mice that rejected the tumor did not develop generalized vitiligo, indicating that autoimmunity is not automatically triggered by administrating therapeutic MDA-based vaccines [106]. The vaccination with bone marrow-derived dendritic cells loaded with DCT peptide resulted in activation of high avidity CTLs mediating protective antitumor immunity *in vivo* without the development of adverse autoimmunity [107]. In a murine therapeutical model, four of seven mice with preestablished tumor remained tumor-free for 80 days after therapeutic vaccination with mouse DCT gene-modified dendritic cells, using a HIV-1-based lentiviral vector demonstrating again that DCT gene transfer to dendritic cells is a potent therapeutic strategy in melanoma [108]. A very important aspect is DCT immune-based therapy in glioma. DCT is expressed in glioma cells naturally, and DCT-specific CTLs have been detected in patients' peripheral blood mononuclear cells [109]. On the other hand, DCT overexpression is associated with tumor cell resistance to chemo- or radio-therapeutic treatments. The theory that DCT is a key player in the synergy between chemotherapy and immunotherapy was demonstrated in a clinical study in which tumor cells escaped from vaccination against DCT were more sensitive to chemotherapy with DNA-damaging drugs.

4.3. Anti-melanoma therapies targeting DCT gene or protein: current status and perspectives

Despite the already acknowledged DCT involvement in melanoma drug-resistance, there are no reports so far, to our knowledge, about melanoma therapies targeting directly the DCT gene or protein. There is, however, a patent claiming the treatment of melanoma cells *in vitro* with antisense nucleotides targeting DCT mRNA in conjunction with DNA-alklylating anticancer drugs [110].

Our data about the intracellular processing and the main checkpoints in DCT fate in tumor cells (Section 2.3) indicate that pharmacological agents that impact DCT stability could represent also potential adjuvants in melanoma therapy. For example, NBDNJ or A3B5 produce specific DCT proteasomal degradation possibly sensitizing tumor cells to therapeutic stress and could also generate DCT-peptides suitable for MHCI presentation and immune response. The selective premature DCT degradation induced in melanoma cells following CQ treatment is another possible way to decrease tumor cell resistance to therapies. CQ has been found to strongly potentiate the inhibitory effect of radiation on tumor cell proliferation [111], to be effective in eliminating chemotherapy-resistant cancer cells and to significantly improve the median survival in glioblastomamultiformis patients [112]. Moreover, the DCT detection at PM by us in melanoma cells [23] and by others in glioma cells [65] introduces DCT as a suitable molecule for targeting tumor cells with specific antibodies. If studies will confirm that DCT is internalized from the PM, this will open interesting perspectives of coupling anti-DCT antibodies with nanocarriers loaded with various antitumor agents. And finally by downregulating DCT (by siRNA or CRISPR/Cas9 system), it can be targeted the Cav1 stability and architecture and possibly some Cav1-mediated pathways including ones involved in tumor progression. The DCT-mediated therapeutic strategies are presented as integrated part of anti-melanoma treatments in **Figure 9**.

5. DCT: a novel molecular driver in melanoma progression

Our most recent studies in two distinct amelanotic melanoma cell lines representing different tumor phenotypes, MJS and SK28, demonstrate a molecular crosstalk, between DCT and caveolin-1 (Cav1), with structural and functional implications [23].

5.1. DCT is associated with Cav1 membranes

DCT and Cav1 are present in common structures in cytoplasm or decorating segments of PM (**Figure 10A**). Both Cav1 monomers/oligomers and DCT-precursor/mature forms have the same distribution along a density gradient in an ultracentrifugation experiment. Moreover, Cav1 has been identified in western blot and mass spectrometry analysis of the immunoprecipitates obtained with anti-DCT antibody from MJS cell lysates [23]. These experimental data are strongly supported by the structural analysis of DCT and Cav1 and by DCT-Cav1 structural model presented in Section 2.1.

5.2. DCT regulates Cav1 assembly and stability and possibly Cav1-mediated cellular processes

The transient downregulation of DCT expression (si-DCT) in MJS and SK28 cells increased the amount of Cav1 protein by its redistribution into more stable, insoluble membrane aggregates with altered morphologies [23] (**Figure 10A**). This is the first report about a melanosomal protein that regulates Cav1 assembly. We postulate that DCT may regulate Cav1- and/or lipid raft structures by competing either with different signaling molecules for Cav1

binding or with Cav1 monomers for Cav1 oligomerization domain or for cholesterol binding. Both caveolae and Cav1-scaffolds are associated with lipid rafts, which are membrane domains with a very dynamic structure abundant in cholesterol, sphingolipids recruiting different molecular players of signaling platforms, and controlling numerous and diverse cellular processes [113]. Either directly or indirectly, DCT as a major regulator of Cav1- or cholesterol-membrane architecture is thus expected to impact also different cellular events mediated by Cav1 (**Figure 10C**). For example, the interaction of membrane/lipid rafts, with the cytoskeleton, has impact on trafficking and sorting mechanisms, formation of platforms for cell anchorage to ECM, transduction of signaling cascades across the PM, cell growth and migration, entry of microorganisms (viruses/bacteria), and toxins or nanoparticles [114]. Indeed, we also observed that in MJS cells having downregulated DCT expression, there was an increase in cell volume, a significant redistribution of actin filaments in cell periphery, and a dramatic decrease in cell proliferation by 20 at 48, 60 at 72, and 75% at 96 h coupled with the cell cycle arrest in G1 [unpublished data]. Interestingly, these effects were less prominent in SK28 phenotype that indicates that DCT-mediated processes are tumor phenotype specific. Importantly, our mass spectrometry analysis of immunoprecipitates obtained from MJS cell lysates with anti-DCT antibodies against N- or C-terminus epitopes has identified as potential DCT interactors, regulators of small GTPases (Arf, Rho and Ras) and numerous proteins involved in anti-apoptotic, proliferative, migration, and invasion mechanisms and pathways [unpublished data]. The structural analysis pointed also the possibility that two Ser residues within DCT CYT subdomain to be phosphorylated (Section 2.1). Our theory based on all these data and preliminary information is that DCT, placed in a molecular environment with Cav1, is a key-molecular player acting on one or more signaling pathways involved in tumor cell survival and morphology, either by itself, as a potential target of the phosphorylation cascades, or as modulator of Cav1 or other participants in regulatory processes (**Figure 10C**). The numerous potential interactors present DCT as a possible new molecular scaffold. Further experimental studies are required to validate these interactions and place DCT in the exact pathway(s) where it operates.

5.3. Cav1 controls DCT gene expression, protein processing, and subcellular distribution

The Cav1 downregulation (si-Cav1) has a dramatic impact on DCT in MJS cells. There is a 20-fold increase over 96 h of Cav1 silencing on DCT mRNA level. Accordingly, there is also a protein increase detected by western blot, and the deglycosylation experiments showed that DCT synthetized in si-Cav1 cells is mainly DCT-precursor. The imagistic studies of confocal immunofluorescence microscopy and Tissue FAXS cytometry quantitative analysis revealed a 7-fold increase in a DCT-population with intense cytoplasmic, but no PM, DCT staining, the "DCT-high clones" (**Figure 10B**). This is the first report about a melanosomal protein/melanoma antigen-regulated by Cav1 and a novel target gene for Cav1. Cav1 is a regulator of several genes as CyclinD or folate receptor promoters [115] or for survivin, a member of the Inhibitor Apoptosis Protein-family [116]. In melanoma, Cav1 function is still ambiguous. In some studies, Cav1 is associated with tumorigenicity [117], whereas others present Cav1 as a tumor suppressor by inhibiting Wnt-β-catenin-TCF/LEF [118], Src/FAK [119] pathways, or attenuating tumor cell motility by disrupting glycosphingolipid GD3-mediated malignant signaling [120]. In the context of DCT-mediating pro-survival and resistance pathways and

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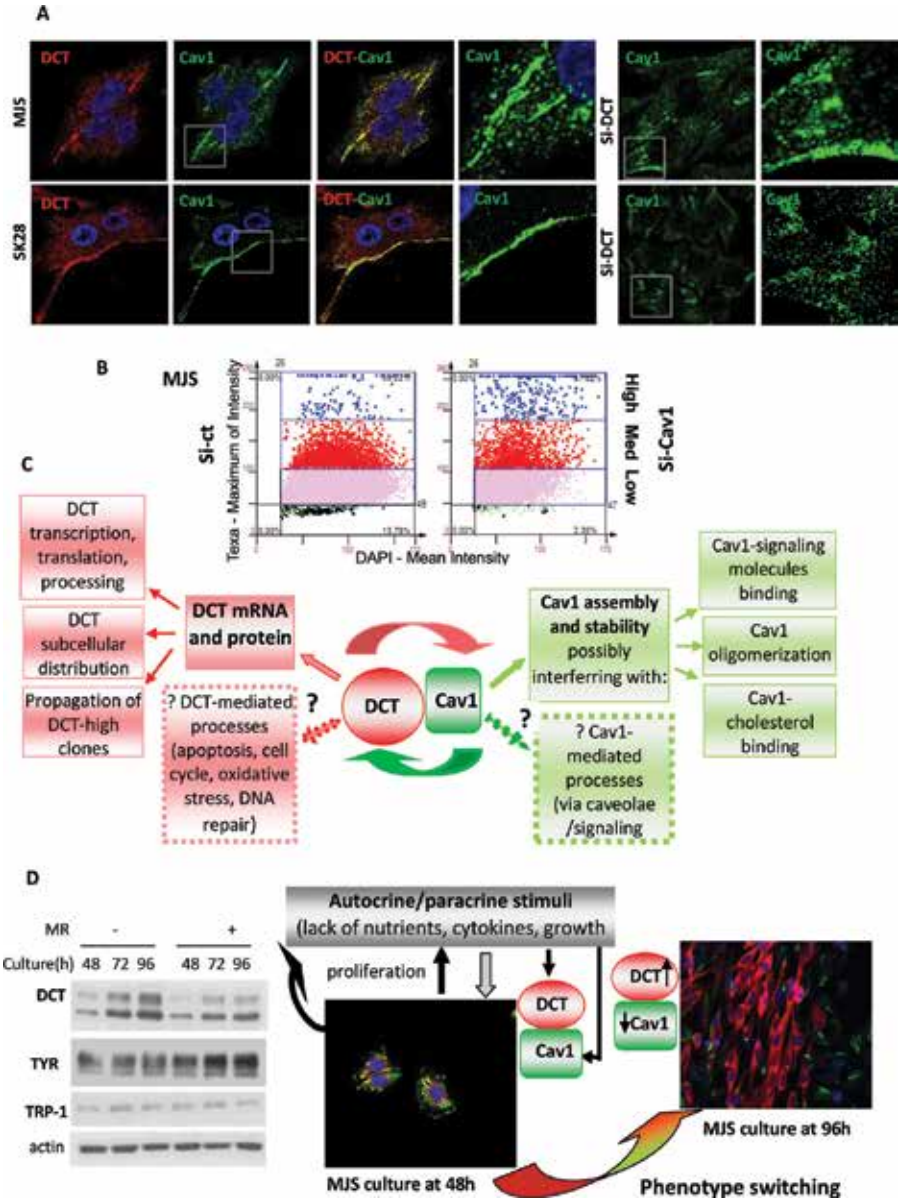

Figure 10. The structural and functional relationship between DCT and Cav-1. (A) MJS and SK28 amelanotic melanoma cells immunostained for DCT and Cav1 and analyzed by confocal fluorescence microscopy demonstrate DCT and Cav1 in cytoplasmic and PM common structures; in DCT downregulated cells, the morphologies of Cav1 positive structures are severely altered. The fourth and the sixth panels represent the enlarged details of the indicated insets; (B) the DCT-high clones in MJS having downregulated Cav1 expression analyzed by tissue FAXS. In the upper part of quadrant are shown the cells with high DCT expression; (C) the crosstalk between DCT and Cav1. The impact of si-DCT on Cav1 and of si-Cav1 on DCT is indicated. Possible processes mediated by either DCT or Cav1 are indicated in dotted boxes; (D) DCT, unlike TYR or TRP1 is overexpressed during transition from subconfluent (48 h) to semi-confluent (72 h) and confluent (96 h). Medium was not replenished for 96 h (MR−) or replenished every 24 h (MR+). Autocrine/paracrine stimulation (starvation, secreted factors by proliferative MJS tumor cells within 48 h) decrease Cav1, increase DCT expressions, and change the cell morphology. The cells at 48 h are polygonal with visible contacts between adjacent cells, whereas cells at 96 h are elongated with no cell-cell contacts and form large clusters.

the upregulation of DCT in si-Cav1 cells, we consider that Cav1 acts as a tumor suppressor gene, at least in this early malignant phenotype. The exact mechanism of how Cav1 controls DCT gene expression and how this intersects DCT-mediated processes (**Figure 10C**) needs to be deciphered and validated in one or more melanoma cell line(s) in addition to MJS.

5.4. DCT and melanoma phenotype switching

The oncogenic epithelial-mesenchymal transition (EMT) is a multistep process by which epithelial cells acquire invasive mesenchymal phenotype characteristics essential in metastatic spread [121]. EMT is regulated and characterized by molecular mechanisms involving specific transcription factors, signaling pathways, and biomarkers. In melanoma cells which do not have epithelial origin, there is a phenotype switching, with similitudes between the EMT program from development, and this EMT-like switch is a major determinant in tumor metastasis [122]. The role of Cav1 in the oncogenic EMT process is significant but controversial and depends on the type of cancer. In bladder cancer cells, Cav1 promotes invasive phenotypes by inducing EMT [123] in A431 human epidermoid carcinoma cells, the Cav1 downregulation by EGF (an EMT inducer) results in E-cadherin loss, and increased tumor cell invasion [124], whereas in primary tumors of head and neck, squamous cell carcinoma increases EMT and prometastatic properties [125]. During transition from subconfluent (48 h) to confluent (96 h) cultures in MJS, SK28, or MNT-1 cell lines, there is an increase in DCT expression, not observed for either TYR or TRP1 and more abrupt in MJS (VGP) than in MNT or SK28 (metastatic) cells (**Figure 10D**). Oppositely, in the same MJS culture, Cav1 was severely downregulated, in the same cells highly expressing DCT. The most stimulating agent for DCT overexpression is the culture medium exhausted in nutrients but rich in cytokines and growth factors secreted by the tumor cells during 96 h proliferation, whereas changing medium every 24 h has a lower impact on DCT increase (**Figure 10D**). EMT can result from multiple extracellular stimuli; for instance, a synergistic effect on EMT has been observed with combined stimulation of EGF and TGF-β [126]. Interestingly, the cell morphology of MJS, but not SK28 cells was dramatically changed during transition from subconfluent to confluent stage from a polygonal, low-expressing DCT/high-Cav1 to an elongated phenotype high-DCT/low- or negative Cav1 (**Figure 10D**). The same phenotype switching has been observed in si-Cav1 cells highly expressing cytoplasmic DCT. Oppositely, si-DCT cells adopt a wider morphology. We consider that, in MJS phenotype, the DCT and Cav1 crosstalk is a possible part of the EMT program. In subconfluent MJS culture (48 h), groups of 2–4 polygonal cells are interconnected via fine filaments and express low DCT and high Cav1. In confluent culture (96 h), the environmental signals trigger probably, independently, the DCT increase and Cav1 decrease. Furthermore, Cav1 downregulation itself sustains even more the DCT increase. The dynamic analysis of tumor cell populations with Tissue FAXS system demonstrates the perpetuation of a subset of DCT-high/Cav1-low, elongated fibroblast-like cells with long extensions, and forming large clusters (**Figure 10D**). This metamorphosis is an *in vitro* recapitulation of an *in vivo* situation encountered during our analysis of the molecular signature of the DCT+ cells in tumor components of human specimens [38]. The tumor cells in subepidermal layer are DCT+/Cav1+, whereas the ones in deep dermis, a more hostile environment, are DCT+/Cav1- (**Figure 8**). In DCT-phenotype, TYR was always in cells from superficial tumor components,

whereas DCT was in the deep dermis ones. This is in good correlation with data showing that in MNT-1 cells expressing all TRPs, during autocrine stimulation only DCT expression is increased [23]. The cross talk between DCT and Cav1, DCT as gene target of autocrine/paracrine stimulation as well as the impact of DCT expression on tumor cell-phenotype proliferation and morphology introduce DCT in the complex signaling pathways and networks regulating tumor progression.

6. Conclusions, open questions, and perspectives

TRP2/L-DCT is, undoubtedly, a benefit for the cell expressing it. In melanocytes, the detoxification processes involve the conversion of DCT natural substrate, DHICA into less toxic products. In nonmelanocytic cells, exogenous DCT is able to decrease the effects of oxidative stress acting on substrate analogs. In melanoma, the "preservation" of the expression of certain melanosomal antigens able to ensure tumor cell viability prevails over that of the key-enzymes for pigment production, and TRP2/L-DCT qualifies for this selection. For this prosurvival molecule, the tumor cells reserve complex transcriptional and post-translational mechanisms distinct from the other TRPs. DCT functions as a sensor in case of the autocrine stimulation/stressful conditions when its expression is highly increased, no matter whether the melanogenic pathway is active or not. There is a molecular crosstalk between DCT and Cav1, a master regulator of numerous cellular processes. The members of signaling platforms identified by mass-spectrometry analysis as potential DCT interactors, as well as the impact of DCT expression on cell proliferation, morphology, and cytoskeleton remodeling are strong proofs that DCT is a key player in cellular processes, acting, in our opinion, as a molecular scaffold within one or more signaling hubs. The recent findings about DCT expression pattern in the tumor architecture in correlation with a stable, longlasting/"die-hard" phenotype in benign lesions and with bad prognostic parameters in malignant lesions advocate for considering DCT as a warning indicative of possibly tumor unfavorable outcome.

On the other hand, TRP2/L-DCT has its own vulnerabilities in terms of stability that can be exploited for therapeutic purposes.

In spite of all these information, the role of DCT in melanoma is far from being elucidated or fully exploited and several issues still need clarification: the molecularity behind DCT regulation by Cav1 and DCT impact on Cav1 structural organization; the decipherment of the signaling pathways in which DCT activates, in amelanotic versus pigmented phenotypes in different stages of tumor progression; how are the DCT structural subdomains involved in DCT tumor cell regulatory mechanisms; the DCT role in tumor cell phenotype switching process; the value of DCT phenotype as prognostic indicative; the efficiency of NBDNJ, CQ, as possible adjuvants in melanoma therapeutic strategies; the clarification of DCT expression in nonmelanocytic/nonneuronal cell lines or tumors.

In melanoma, DCT is a double-edged sword, a lethal weapon for cancer cells serving the tumor progression or an exploitable molecular tool for scientists and clinicians to eradicate the malignant cells.

Acknowledgements

This work was supported by Grant Application 156, Exploratory Research Projects PN-II-ID-PCE-2011-3-0492-1, funded by Ministerul Educației și Cercetării Științifice and by the Academia Română Project 1/2011 of the Institute of Biochemistry. Molecular simulations were performed using the high-performance computational capabilities of the HPC Linux cluster at IBAR and the High-Performance Computing Infrastructure for South East Europe's Research Communities (HP-SEE), a project cofunded by the European Commission (under contract number 261499) through the Seventh Framework Programme. Gabriela Negroiu acknowledges Dr. Sabina Zurac, Department of Pathology, Colentina University Hospital, Bucharest, Romania for providing the image of the specimen in **Figure 9** and for sharing her valuable expertise in melanoma pathology during our collaborative research. Adina Milac is grateful to Dr. Andriy Anishkin, Department of Biology, University of Maryland, College Park, MD, USA for advice and discussions on molecular simulations of cholesterol-containing membranes.

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Section 2

Melanoma: Targets and Treatments

Chapter 4

The Modern Approach to Targeting Melanoma

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73489

Abstract

Melanoma treatment depends largely on the clinical stage of the disease. The preferred treatment is surgical resection of the disease. The surgical margins depend on the depth of the disease. Sentinel lymph node biopsy is generally advised for all lesions greater than 1 mm depth. Complete lymphadenopathy of surrounding lymph nodes is recommended in the presence of nodal disease. There are some controversies surrounding the timing and benefit of complete lymphadenopathy in clinically occult disease. There is evidence to support the role of adjuvant therapies in the form of immunotherapy in regionally advanced disease, and there has been a significant improvement in medical therapies for advanced melanoma. BRAF inhibitors have become mainstay treatment for patients with a BRAF mutation. Immunotherapy is another cornerstone of therapy for advanced melanoma. There is ongoing research to define the optimal therapeutic regimen. Future guidelines will likely incorporate this recent research. Chemotherapy has been relegated to second-line therapy in melanoma.

Keywords: melanoma, staging, therapeutic advances, immunotherapy, chemotherapy

1. Introduction

Melanoma is the deadliest of all the skin cancers. The incidence has been increasing in recent decades. There has been a significant development of therapies for melanoma. For the purpose of chapter, the staging of melanoma will be reviewed. There will be a brief overview of the current recommendations and ongoing research into the different therapeutic approaches.

2. Determining the appropriate therapy

An important determinant of the appropriate treatment strategy is the clinical stage of the primary melanoma. Melanoma is staged by the TNM system. T refers to the primary tumour, N refers to the nodal status and M refers to the metastatic status. The AJCC released updated guidelines for the staging of melanoma in 2016. T1a tumours are less than 0.8 mm deep without ulceration, and T1b tumours are either less than 0.8 mm deep with ulceration or 0.8 mm to 1 mm deep with or without ulceration. T2a and T2b tumours are 1.0 mm to 2.0 mm without and with ulceration, respectively. T3a and T3b tumours are 2.0 mm to 4.0 mm without and with ulceration, respectively. Finally, T4a and T4b tumours are greater than 4.0 mm without and with ulceration, respectively. N1 refers to one-tumour involved or any number of in-transit satellite and/or microsatellite metastases with no tumour-involved lymph node. N1a and 1b refer to one clinically occult (detected by SLNB) and one clinically detected. N2 refers to two or three tumour-involved nodes or any number of in-transit, satellite, and/or microsatellite metastases with one tumour-involved node. N2a and N2b refer to two or three clinically occult and at least one clinically and one clinically occult/clinically detected, respectively. N3 refers to four or more tumour-involved nodes or any number of in-transit, satellite, and/or microsatellite metastases with two or more tumour-involved nodes or any number of matted noes without or with in-transit, satellite and/or microsatellite metastases. N3a, N3b and N3c refer to four or more clinically occult, four or more, at least one of which was clinically detected or the presence of any number or matted nodes or two or more clinically occult or clinically detected and/or the presence of any number of matted nodes, respectively. Satellite metastases are clinically apparent cutaneous and/or subcutaneous metastases within 2 cm of the tumour. Microsatellites are microscopic cutaneous and/or subcutaneous metastases next to or below the melanoma on histological examination. In-transit metastases are clinically apparent cutaneous and/or subcutaneous metastases found greater than 2 cm from the primary melanoma. M0 refers to no distant disease, and M1 refers to distant disease. M1a refers to distant metastases in the skin, soft tissue including muscle, M1b refers to distant metastasis to lung, M1c refers to distant metastasis to non-CNS visceral sites and M1d refers to distant metastasis to the CNS. M can be further risk stratified based on the LDH level [1].

Stage 1 and 2 are defined by tumour size. Stage 3 is defined by positive nodal disease. Stage 4 is defined by the presence of metastases. Based on the TNM staging, stage 0 refers to T1sNoMo, stage 1a refers to T1a/N0/M0, stage 1b refers to T1b or T2a/N0/M0, stage 2A refers to T2b or T3a/N0/M0, stage 2B refers to T3B or T4a/N0/M0, stage 2C refers to T4b/N0/M0, stage 3 refers to any T/N1 or higher/M0 and stage 4 refers to any T/any N/M1 [1].

2.1. Stages 0, 1 and 2

The ESMO guidelines recommend surgical therapy as the primary management strategy in localised melanoma. The guideline recommends against performing routine lymphadenectomy or irradiation to the surrounding lymph nodes. The guideline recommends radiotherapy for local disease control where there are positive margins in lentigo maligna melanoma, in cases of metastases resection where there are positive histological margins or after the removal of bulky disease [2].

http://dx.doi.org/10.5772/intechopen.73489

2.1.1. Surgical

Surgical excision forms the cornerstone of treatment in primary cutaneous melanoma. Following the initial histological diagnosis and microstaging of the tumour, a wider and often deeper excision of the melanoma is performed. The surgical management of melanoma depends primarily on the Breslow thickness, the presence or absence of ulceration, and positivity of sentinel lymph node biopsy [3]. The ultimate aim of surgical therapy irrespective of depth is to obtain clear margins. The extent of the surgical margins is determined by three factors: (1) Wide margins result in a reduced risk of local recurrence. (2) There is no benefit to excising margins beyond 1 cm in thin melanoma. (3) There is no demonstrated benefit to excising margins beyond 2 cm in any thickness melanoma. The guidelines suggest a margin between 1 cm and 2 cm for primary cutaneous melanoma. Furthermore, the guidelines state that an excision should be performed to the level of muscle fascia or depending on tumour location at least to the level of the deep adipose tissue. However, in the case of stage 0 melanoma, an excision margin between 0.5 and 1.0 cm margin is acceptable. In the case of stage 0 lentigo maligna, margins may need to be extended to >0.5 cm, due to characteristically widespread subclinical extension. Permanent section total peripheral margin control and Moh's micrographic surgery have been used to achieve histological control of the margins. However, there is a paucity of evidence to support their use [4].

There are several trials performed to determine the optimal extent of the margins when excising melanoma. In thin melanomas, an international, randomised prospective study examined 1 cm margins in the context of primary cutaneous melanomas less than 2 mm depth. A total of 612 patients were included in the trial, with 305 randomised to 1 cm margins and 307 randomised to wide margins less than 3 cm. The disease-free and overall survival was similar in the two groups [5]. In the case of intermediate-thickness tumours (1 to 4 mm depth), a large, multicentre randomised trial demonstrated that margins of 2 cm were acceptable with respect to 5-year survival. Reducing the margins from 4 to 2 cm led to a significant reduction in skin grafting and total length of hospital stay [6]. In another multicentre trial, they investigated the optimal excision margins in high-risk melanoma. High-risk melanoma was defined as localised melanoma 2 mm or greater in thickness on the trunk or limbs. Elective lymph-node dissection, sentinel biopsy and adjuvant therapies were not permitted. Patients were either randomised to 1 cm or 3 cm margins. The trial demonstrated that a 1 cm margin in high-risk melanoma is associated with a significant increase in regional recurrence vs. a 3 cm margin, but both patient populations had a similar overall survival rate [3]. A Cochrane review examined the different excision margins in melanoma. A narrow margin was defined as 1–2 cm, and a wide margin was defined as 3–5 cm. The systematic review included data from randomised trials for 1633 participants in the narrow excision margin group and 1664 in the wide excision margin group. There was no statistically significant difference in terms of overall survival and recurrence free survival between wide and narrow margins. However, there was a trend toward improved overall survival and recurrence free survival. The review concluded that there was inadequate evidence to determine the optimal excision margins for primary cutaneous melanoma [7].

Sentinel lymph node biopsy should be considered in patients with a primary cutaneous melanoma of 1 mm or greater depth. Sentinel lymph node biopsy is generally not advised in

patients with stage 0 or stage 1a melanoma. The use of sentinel lymph node biopsy is only recommended in stage 1B in the presence of adverse prognostic indictors [4]. The likelihood of a positive sentinel lymph node biopsy increases with the Breslow's depth, with 2% in 1 mm, 7% in 1–1.99 mm, 13% in 1–1.99 mm, and 31% in 3 mm. In 710 cases of sentinel lymph node biopsy, 638 (88.5%) were alive without evidence of disease [8].

Lymph node dissection plays an important role in the surgical management of melanoma. The MSLT-1 was a multicentre phase 3 trial, which randomised two groups of patients with localised melanoma more than 1 mm deep to receive either wide excision with lymphatic mapping and sentinel lymph node biopsy with immediate complete lymphadenectomy for sentinel node metastases or wide excision plus postoperative observation with a deferral of the complete lymphadenectomy until clinically evident disease became apparent. The trial comprised of 1270 patients with intermediate-thickness melanoma, 290 with thick melanoma, and 232 with thin melanoma. There was no difference between the immediate and delayed complete lymphadenectomy group in the absence of nodal disease in either the intermediate or thick melanomas group with respect to 10-year melanoma-specific survival rates. The survival rate was much improved in the presence of nodal disease in the biopsy vs. observation group in the intermediate thickness group. However, a similar benefit was not observed in the thick melanoma group. The patients with a positive sentinel lymph node biopsy had a worse prognosis vs. the patients with negative sentinel lymph node biopsy. Thus, there is a clear benefit to immediately complete lymphadenectomy following the identification of clinically occult disease in intermediate-thickness melanoma with respect to nodal recurrence, distant metastases and melanoma-specific survival. The timing of the complete lymphadenectomy does not appear to play an important role in thick melanomas. There is no benefit to immediate complete lymphadenectomy in the absence of clinically occult disease. Finally, the trial demonstrates that sentinel lymph node biopsy serves as an important prognostic tool [9].

Another important trial which assessed the role of complete lymph node dissection was the phase 3, multicentre, DeCOG-SLT trial. They examined whether complete lymph node dissection results in a better overall survival vs. conservative management in patients with positive sentinel lymph node biopsies. The patients had cutaneous melanoma of at least 1 mm depth and positive sentinel lymph node biopsies. The trial randomised 483 patients to either complete lymph node dissection or observation. There was no significant difference in terms of distant-metastasis free survival in the treatment or observation arm (74.9% vs. 77.0%). Furthermore, there was no significant difference in terms of 3 year overall survival between the treatment and observation arm (81.2% vs. 81.7%). There was only a small improvement in disease control in the treatment vs. the observation group 8% vs. 15%). It is important to note that the majority of patients in that study had a low tumour burden [10].

2.1.2. Non-surgical

Surgical management is the treatment of choice for primary cutaneous melanoma. Hence, the non-surgical options should only be advised in specific cases, where surgery is not possible. The options include topical imiquimod, radiation therapy, cryosurgery and observation [4].

2.2. Stage 3

The ESMO guidelines suggest surgical excision and removal of the surrounding lymph nodes. They note that it is not sufficient to merely remove the disease-containing nodes. These guidelines define high-risk situations as the presence of multiple bulky lymph node metastases. The surgical management of stage III melanoma follows the same principles as above. The guidelines suggest the consideration of localised radiation therapy to the surrounding area in the case of high-risk disease. In the presence of inoperable, regionally advanced disease, the guidelines suggest therapies such as isolated limb perfusion, radiation therapy, electrochemotherapy or intralesional therapy [2].

In the presence of high-risk disease, systemic therapy should be considered. A recent phase 3 trial investigated the role of adjuvant immunotherapy in high risk regionally advanced to prevent recurrence. The trial entitled EORTC 18071 enrolled participants who underwent a surgical excision of cutaneous melanoma with clear margins. A total of 951 patients were randomly assigned to the treatment with either placebo or ipilimumab. The median recurrence survival was significantly improved in the ipilimumab treatment arm (26.1 months vs. 17.1 months). There were a large number of patients who discontinued ipilimumab due to adverse events (245/471). These adverse events were most commonly GI (75/472), hepatic (50/472), and endocrine (40/471). There were five treatment-related deaths in the ipilimumab treatment arm (three due to colitis, one due to myocarditis and one due to Guillain-Barré syndrome) [11]. The overall 5-year survival in the ipilimumab treatment arm at 5 years was 65.4% vs. 54.4% in the placebo arm [12].

However, the role of ipilimumab as the optimal adjuvant therapy has recently been challenged with the publication of a phase 3 trial, comparing adjuvant nivolumab vs. ipilimumab in resected stage 3 or 4 melanoma. A total of 906 patients were randomly assigned to either receive treatment with ipilimumab or nivolumab. The patients were followed up for at least 18 months. When the 12-month recurrence-free survival was compared in both groups, it was significantly higher in the nivolumab group (70.5% vs. 60.8% respectively). Nivolumab appeared to have a better overall side-effect profile, with 14.4% reporting grade 3 or 4 adverse treatment effects vs. 45.9% in the ipilimumab-treatment arm. Furthermore, while there were two deaths reported in the ipilimumab arm, there were no deaths recorded in the nivolumab arm [13].

A recent phase 3 trial published in 2017 suggests that the BRAF inhibitor dabrafenib plus the MEK inhibitor trametinib prove the optimal treatment option in stage III melanoma patients with the BRAF V600 mutations. A more detailed discussion regarding the mechanism of these drugs will be discussed in the stage IV section. They randomised patients with either a BRAF V600E or V600 K mutation to receive either a combination of dabrafenib and trametinib or placebo. They recruited 870 patients with adequately resected stage 3 melanoma. The overall 3-year survival was improved in the treatment arm vs. placebo (86% vs. 77%). The 3-year relapse free survival was 58% vs. 39% in the treatment vs. placebo arm, respectively. This trial suggests a role for combination BRAF/MEK therapy in stage 3 melanoma [14].

In conclusion, surgery with removal of surrounding lymph nodes remains the mainstay therapy for stage III disease. Several recent publications suggest a benefit to systemic therapy with checkpoint inhibitors or BRAF inhibitors if applicable in high-risk disease. Future guidelines will likely incorporate this recent research into their treatment strategies for regionally advanced disease.

2.3. Stage 4

The most significant breakthrough in melanoma in recent years has included the therapies designed for metastatic malignant melanoma. The main determinant of treatment strategy is the presence of resectable or unresectable disease. In the presence of resectable disease, the disease may be managed as the above. In the case of unresectable disease, the ESMO guidelines note that the optimal 1st-line therapy in melanoma remains under considerable debate. They suggest either anti-PD1 therapies or BRAFi/MEKi for BRAF-mutated melanomas. Chemotherapy has been relegated to 2nd-line therapy in the guidelines. However, in the case of aggressive metastatic disease, the guidelines note some benefit to polypharmacy, containing paclitaxel and carboplatin/cisplatin, vindesine, and dacarbazine [2]. Furthermore, radiation therapy is recommended in the presence of symptomatic brain metastases or painful bony metastases. However, the guidelines will likely evolve dramatically following the development of further therapeutic strategies.

2.3.1. MAPK pathway inhibitors

Dysregulation of the RAS/RAF/MEK/ERK MAPK pathway plays a pivotal role in the development of MM. In healthy cells, this pathway regulates several physiological cellular processes. The MAPK pathway is activated by growth factors which bind to the extracellular kinase receptor. This receptor-ligand complex leads to autophosphorylation of intracellular domains, which in turn results in phosphorylation and activation of the membrane-bound guanosine triphosphatase RAS. There is dimerisation of the serine/threonine kinases RAF. RAF is encoded by three different isoforms: ARAF, BRAF, and CRAF. The BRAF isoform encodes for the most powerful activator of the MAPK pathway. Activation of RAF causes a phosphorylation cascade, with the eventual activation of ERK. ERK is then free to translocate to the nucleus, where it activates several transcription factors that induce the expression of genes implicated in normal cell turnover and survival [15, 16].

In 2002, there was an exciting discovery that activating mutations in MAPK pathways play an essential role in most MM. A dysregulated MAPK pathway is present in ~40–50% of MM cases. The most common mutation resulting in dysregulation of the MAPK pathway is present on exon 15 and results in the switching of glutamate for valine at codon 600 (V600E). This mutation is located within the activating segment of the kinase domain. The mutant form of BRAF is more potent than the wild type variant. The mutant form results in constitutive activation of the MAPK pathway and increased ERK. The cellular endpoint is increased turnover and survival. The presence of BRAF mutations seems to be dictated by age; with 80% of patients less than 30 years old harbouring a mutation, while only 20% of patients over

80 years old harbouring the mutation. Furthermore, older patients are less likely to have the V600E mutation. Finally, patients with the mutant BRAF had historically worse outcomes than the wild-type BRAF [16].

As a result of this new understanding of the underpinning genetic events that give rise to MM, there have been several drugs developed known as targeted therapy of the MAPK pathway. Initially, the broad spectrum tyrosine kinase, sorafenib, was trialled in melanoma patients [17]. The clinical trial results proved disappointing. There are two targeted therapies subsequently developed and currently licenced, which inhibit mutated BRAF: vemurafenib (formerly known as PLX4032) and dabrafenib. Vemurafenib was the first selective tyrosine kinase inhibitor licenced by the FDA in 2011. Dabrafenib is a potent and selective inhibitor of BRAF V600E kinase. Inhibition of the pathway may also be achieved by MEK inhibitors. Trametinib is a potent and selective inhibitor of MEK 1 and 2. These three MAPK targeted therapies are licenced by the US Food and Drink Authority for single-agent therapy against non-resectable or metastatic cutaneous MM. Combining MAPK pathway inhibitors is an important therapeutic strategy to minimise the development of drug resistance. There is an additional MEK inhibitor, known as cobimetinib licenced for combination therapy with vemurafenib. Similarly, dabrafenib and trametinib are licenced for combination therapy [18, 19]. BVD-523 (ulixertinib), an ERK1/2 inhibitor with high potency and selectivity, is currently under investigation. Preclinical investigations in vivo and in vitro appear promising, and clinical trials are underway. This may prove an important combination therapy or refractory in future clinical practice [20].

An important therapeutic limitation is the development of resistance to the MAPK pathway inhibitors. The current literature suggests that progression-free survival for patients receiving BRAF/MEK inhibitor combinations ranges from 9 to 11 months [21–23]. The exact mechanism of resistance has not been fully elucidated, and there is ongoing research into the development of resistance. Several different mechanisms of resistance have been posed in the literature.

3. Immunotherapy

As seen in the previous section, melanoma is a highly mutated cancer. Similarly, it is extremely an immunogenic cancer. Attenuating the immune system has proved an important therapeutic strategy. Immunotherapy targets 4 broad areas: (1) Checkpoint inhibitors. These agents negatively regulate inhibitors of pre-existing anti-tumour immune response (effectively augmenting the response of the immune system to the tumour cells, e.g., Anti-CTLA-4 inhibitors (ipilimumab), anti-PD-1/PD-L1 antibodies (nivolumab/pembrolizumab/atezolizumab) and IDO1 Inhibitors (Epacadostat); (2) Increasing the anti-tumour T cell response by administration of autologous ex-vivo augmented tumour infiltrating lymphocytes; (3) Administering oncolytic viruses into the metastatic cells to break-up the cells and increase the immune response and (4) Targeting dendritic cells to start and/or increase tumour antigen-specific immune responses [24].

In order to appreciate the mechanism of the immunotherapies, it is necessary to briefly examine the normal physiology of the immune system. T cells are a subgroup of lymphocytes mainly produced in the thymus, which express antigen-recognising T-cell receptor (TCR). Every T-cell has a unique TCR, which recognises a specific antigen. Antigen is presented to the T-cell by the MHC complexes. This causes clonal expansion of T-cells. All progeny of a T-cell express the same TCR, and this expanded pool confers antigen-specific immunity. There are 4 main reasons why the T-cells are such a desirable target for immunotherapy: (1) The response to T-cell is specific, and differentiates between healthy and neoplastic cells; (2) T-cell responses are amplified, resulting in a 1000-fold increase in the response after activation; (3) T-cells travel to the specific area containing the antigen, enabling them to fight distant metastases; and (4) T-cells can remain quiescent, conferring immunity for many years after the initial exposure to antigen [25]. T cells may either be classed as effector T cells or T regulatory cells. Effector T-cells enable immunity and destroy cells with the particular antigen. T regulatory cells are essential for maintaining immunological unresponsiveness to self-antigen and preventing excessive immune responses harmful to the host. There are several different theories about how regulatory T-cells can attenuate the immune response: 1. Secretion of immunosuppressive cytokines by regulatory T cells, cell-contact dependent inhibition, and modification/destruction of APC cells [26–28].

The key molecules required for the activation of both T cell types are similar. T cell activation is mediated by activation of the T-cell receptor and a co-stimulatory molecule, the CD28 receptor on the T cells by MHC peptides, and APC. Both are necessary for generation of an adequate immune response. Antigen is presented to the TCR, and a T-cell receptor complex including CD3, CD2, CD4/CD8/LFA1/CD45R is formed. This activation of TCR generates signal 1. CD28 on T cells is activated by B7.1 and B7.2 on antigen-presenting cells, generating signal 2. B7.1 and B7.2 are generally only expressed on specialised antigen presenting cells, e.g., dendritic and Langerhans. Cytotoxic T-cell associated antigen-4 (CTLA4) and glucocorticoid-induced TNF receptor (GITR) are expressed on T cells. CTLA4 combines with B7.1 and B7.2 and blocks activation of the CD28 molecules. CTLA4 has a greater affinity for B7.1 and B7.2 than CD28. CTLA4 sends a negative signal, downregulating expression of B7.1 and B7.2 [26–28].

3.1. Checkpoint inhibitors

Checkpoint inhibitors are based on the fact that T lymphocytes are essential for the antitumour immunity. Furthermore, an antigen-specific TCR must be activated in the presence of co-stimulatory activation in order to activate the T-lymphocytes [29]. Several inhibitory receptors and ligands present on T cells and tumour cells have been identified as potential targets for cancer immunotherapy. They are essential mediators of immune suppression in the tumour microenvironment [30]. They are different from monoclonal antibodies, which bind and destroy the tumour cells. Checkpoint inhibitors are immunomodulatory antibodies which either stimulate or inhibit the function of cell surface signalling molecules on the patient's own immune cells. This can lead to either upregulation or downregulation of the patient's immune cells [31]. Different negative co-stimulatory molecules exist. Targeting negative co-stimulatory molecules, such as CTLA4 and PD-1, is the basis of checkpoint inhibitors. The advantage of checkpoint inhibitors is that they function irrespective of the patients' BRAF status [32].

3.1.1. CTLA-4 inhibitors

CTLA-4 blocking antibodies prevent CTLA-4 from binding to its ligands B7-1 and B7-2 on APCs. This "unleashes the breaks" on the immune system. Experimental evidence suggests that there is an increase in the absolute number of effector and regulatory T cells in the lymph nodes. However, there is an increase in the effector T cell to regulatory T cell ratio in the tumour microenvironment. Destruction of the regulatory T cells increases the ratio and directly correlated with rejection of the tumour. Another important mechanistic aspect of CTLA4 inhibitors is their effect on FcyR. FcyRs are responsible for the selective depletion of the regulatory T cells. FcyRs are key regulators of the immune response. FcyR is broadly expressed on cells of haemopoetic lineage, including B cells, macrophages, mast cells, NK cell and neutrophils. They can be both activating and inhibitory. Depletion of the T cells results from antibody-dependent cellular cytotoxicity, dependent on tumour-infiltrating CD11b-positive macrophages expressing activating FcyRIV. CTLA4 stimulates activating FcyRIV, inducing the antibody-dependent cellular cytotoxicity. The depletion of regulatory T cells occurs preferentially in the tumour cells for several reasons. Macrophages are a lot more plentiful in the tumour microenvironment (>50 times). Furthermore, there is a greater consumption of the T regulatory cells due to the higher expression of CTLA4 on T regulatory cells vs. T effector cells. This ultimately leads to a higher effector T cell to regulatory T cell ratio in the tumour [31, 33].

In 2010, the CTLA4 inhibitor, ipilimumab, proved to improve overall survival in the groundbreaking phase III clinical trials in patients with advanced melanoma. A total of 676 patients were included in the study with either stage III or IV melanoma. They randomised patients to receive ipilimumab, ipilimumab plus gp100, or gp100 alone (control group). The overall survival of patients who received ipilimumab vs. the control group was 10.0 months vs. 6.4 months. There was no survival difference in overall survival in patients receiving ipilimumab vs. ipilimumab plus gp100. Ipilimumab alone resulted in the best overall response (10.9%) and disease control rate (28.5%). A total of 60% (n = 9/15) of patients receiving ipilimumab alone had a long-term response lasting more than 2 years. It is important to note that 10–15% of participants receiving ipilimumab suffered from grade 3 or 4 immune-related adverse events, most commonly relating to the skin and gastrointestinal system [34]. In clinical trials, greater than 80% of participants experienced adverse events related to therapy with ipilimumab [35]. The most frequent severe immune-mediated adverse effects are enterocolitis, hepatitis, dermatitis (including TEN), neuropathy, and endocrinopathy. These adverse effects generally occur during treatment; however, they may occur in the weeks to months after ipilimumab discontinuation [35]. A pooled analysis was undertaken on the long-term survival data from phase II and III clinical trials, in patients with unresectable melanoma. The data included 1861 patients from 10 prospective and 2 retrospective trials. The median overall survival for patients receiving ipilimumab was 11.4 months. There were 254 patients with 3-year survival follow-up. The 3-year survival rate was 22% [36].

When ipilimumab was combined with dacarbazine, the median duration of the best overall response was 19.3 months vs. 8.1 months in dacarbazine alone at long-term follow-up [30].

EORTC 18071 was a multicentre phase 3 clinical trial with 951 patients comparing adjuvant ipilimumab (dose of 10 mg/kg) with placebo in resected, high-risk stage III melanoma. The overall survival rate at 5 years was significantly higher in the ipilimumab group vs. placebo (65.4% vs. 54.4%). The rate of distant metastasis-free survival at 5 years was increased in the ipilimumab vs. placebo (48.3% vs. 38.9%). 98.5% (n = 465/471) of patients receiving ipilimumab experienced an adverse event of any grade, and 26.2% experienced a grade 3 or 4 adverse event, with 41.6% of patients experiencing grade 3 or 4 immune-related adverse events in the ipilimumab group. The most common immune-related adverse events were GI, hepatic, and endocrine. Five patients (1.1%) died due to adverse events related to ipilimumab [12].

3.1.2. PD-1 inhibitors

The programmed death-1 (PD-1) is a regulatory molecule which is expressed on T cells and operates during the effector phase of T-cell activation. In contrast, CTLA-4 is operational during early activation of T cells in lymphatic tissues. PD-1 interacts predominantly with its two ligands, B7-H1 and B7-DC (PD-L1 and PD-L2) in peripheral tissues, and causes apoptosis and downregulation of T-cell effector function. The function of PD-1/PD-L1 interaction is to minimise the risk of surrounding tissue damage by T-cells. PD-1/PDL-1 therapies are considered "tumour site immune modulation therapy". PD-L1 appears to be upregulated in the tumour microenvironment [30]. In order to extravasate peripheral tissues and organs, the T-cells must have encountered their antigen already in the lymphoid organs. T-cells primed with its antigen develop an immunologic memory and acquire a particular set of adhesion molecules that allow extravasation to the peripheral tissues, including the tumour. Once this T-cell encounters the antigen in the peripheral environment, PD-1 interacts with its ligands and thereby decreases the extent of the immune response. Therefore, PD-1 inhibitors target T-cells already engaged in the ongoing effector T-cell response and hence have a more restricted spectrum of T-cell activation compared with CTLA-3 blocking. This is probably the reason why there is a decreased rate of immune adverse events with anti-PD-1 or anti-PDL-1 antibodies [30].

In 2015, a phase 3 multicentre clinical trial investigated 518 patients with BRAF-negative advanced stage III or IV melanoma randomised to either nivolumab (dose 3 mg/kg) or dacarbazine. At 1 year, the overall survival rate was 72.9% in the nivolumab group vs. 42.1% in the dacarbazine group. The median progression-free survival was 5.1 months in the nivolumab group vs. 2.2 months in the dacarbazine group. The objective response rate was 40% in the nivolumab group vs. 13.9% in the dacarbazine group. Therapy-related adverse effects occurred in 11.7% of the nivolumab group vs. 17.6% in the dacarbazine group. The most common effects included fatigue, pruritus, and nausea [37]. In another clinical trial, an analysis was performed on the safety data relating to nivolumab in both melanoma and other solid tumour groups (n = 306). Some patients were followed-up for safety monitoring over 2 years. The majority of adverse events occurred in the first 6 months of treatment. There were no cumulative toxicities with extended treatment periods [38].

In CHECKMATE 037, a phase-3 multicentre clinical trial, 631 patients with advanced melanoma who progressed after CTLA-4 inhibitor were randomised to receive nivolumab or

chemotherapy as second-line or later-line therapy. Confirmed objective responses were recorded in 31.7% (n = 38/120) in the nivolumab group vs. 10.6% of the investigators' choice chemotherapy group (n = 5/47). Grade 3 and 4 drug-related serious events occurred in 5% (n = 12) of nivolumab-treated patients vs. 9% (n = 9). The grade 3 and 4 toxicities of nivolumab included deranged alanine aminotransferase, anaemia, and fatigue. There were no treatment-related deaths in this group [39].

Pembrolizumab is another PD-1 inhibitor used in clinical practice. KEYNOTE-006, a multicentre phase 3 clinical trial, including 834 participants compared pembrolizumab vs. ipilimumab. They excluded participants who received prior checkpoint inhibitor therapy. The 6-month progression-free survival rates were 47.3% for pembrolizumab every 2 weeks, 46.4% for pembrolizumab every 3 weeks, and 26.5% for ipilimumab. Twelve-month survival rates were 74.1, 68.4 and 58.2%, respectively. The response rate was higher with pembrolizumab, every 2-weeks (33.7%) and 3-weeks (32.9%) vs. ipilimumab (11.9%); 89.4, 96.7 and 87.9% had a sustained response, with a median follow-up of 7.9 months. There was a reduced rate of treatment-related adverse effects of grade 3–5 severity in the pembrolizumab group (13.3 and 10.1%) vs. the ipilimumab group (19.9%). Thus, the efficacy in both pembrolizumab groups was significantly higher than ipilimumab, with reduced treatment-related adverse events [40]. Follow-up of KEYNOTE-006 in 2017 showed overall superiority and progression-free survival of pembrolizumab vs. ipilimumab. The median follow-up was 22.9 months. The 24-month overall survival rate was 55% in the 2-week group, 55% in the 3-week group, and 43% in the ipilimumab group. Most immune-related events occurred within the first 6 months of therapy. Colitis was more common in the ipilimumab group, whereas hepatitis and endocrinopathies were more common in the pembrolizumab group [41]. About 19% (n = 38) treated with pembrolizumab for at least a year developed grade 3–4 treatment-related adverse events. No patients died because of the therapy-related toxicity.

There is ongoing work investigating the role of atezolizumab in advanced melanoma. In a phase 1b trial, atezolizumab was combined with vemurafenib in BRAFV600 metastatic melanoma in 17 patients. It produced an overall response rate of 76%, with three complete responses and 10 partial responses. About 41% experienced grade 3 treatment-related adverse effect and no participants experienced a grade 4 adverse effect or death. Further work is necessary to elucidate the role of atezolizumab in melanoma [42].

A recent analysis was performed on the safety data from 48 trials (n = 6938), including 26 CTLA4, 17 PD-1, 2 PD-L1 trials and 3 CTLA4 and PD1. There were more grade 3/4 immune-related adverse events with CTLA4 inhibitors vs. PD-1 (31% vs. 10%). Colitis, hypophysitis and rash were more common with CTLA4 inhibitors, whereas pneumonitis, hypothyroidism, arthralgia, and vitiligo were more common with PD1 inhibitors. Melanoma patients specifically have a higher incidence of gastrointestinal and skin immune-related adverse effects and a reduced incidence of pneumonitis. The discontinuation rate to immune-related adverse effects was between 3 and 12% in anti-PD-1 trials and 3 and 25% in anti-CTLA4 trials. The most frequent cause of discontinuation was diarrhoea/colitis. Death was an extremely uncommon event for anti-PD1 agents (pembrolizumab 0.1%, nivolumab 0.3%) and mostly occurred

due to pneumonitis. Death occurred in 29 patients receiving CTLA-4 inhibitors and was more often due to gastrointestinal events such as diarrhoea, colitis, and colonic perforation [43].

3.1.3. IDO1-inhibitors

Indoleamine 2,3-dioxygenase (IDO-1) is a significant immunoregulatory enzyme that facilitates immunosuppression, tolerance, and tumour evasion by tryptophan catabolism [44]. It is a molecule which causes oxidative cleavage of tryptophan, an amino acid which is essential for cell proliferation and survival. IDO1 induction triggers dendritic cell apoptosis and inhibits T-cell response. In multiple cancer types, the IDO1 pathway is activated. In vitro, inhibition of IDO1 causes an increase in T and natural killer cells, increase in IFN-production, and reduced switch to regulatory T-cells [45].

Epacadostat is the first IDO-1 inhibitor in its class. Experimental evidence suggests that T-cells stimulated with dendritic cells treated with epacadostat produce a greater number of inflammatory mediators. Furthermore, there appears to be a decrease in the number of regulatory T cells [44]. Echo-202/KEYNOTE-037 was a phase 1 clinical trial, which enrolled 62 patients with advanced melanoma. Patients treated previously with checkpoint inhibitors were excluded. The patients received epacadostat plus pembrolizumab. Grade 3 or higher treatment-related adverse effects occurred in 18%, with the most common being rash, followed by increased lipase. No treatment-related deaths occurred. There were four complete responders, seven partial responders and three stable disease noted [46]. There were phase I/II trials with 40 advanced melanoma patients investigating the tolerability and efficacy of epacadostat plus ipilimumab. Grade 3 or higher immune-related treatment effects occurred in 23% of participants, rash, pruritus, diarrhoea, deranged transaminases, and hypothyroidism were the most commonly reported. Looking specifically at the immunotherapy-naive group, overall response rate was 27–30% (depending on the criteria used). The complete response in both criteria was 10% [47]. There are ongoing phase 3 studies (KEYNOTE-252/ECHO-301) investigating pembrolizumab and epacadostat in advanced melanoma. These trials will likely have a significant impact on the treatment algorithms for advanced melanoma.

3.1.4. Combination therapy

CTLA4 inhibitors and anti-PD1 inhibitors have been recently combined to determine if combination therapy offers improved efficacy vs. monotherapy, with clinically acceptable safety outcomes. Checkmate 067 was a phase 3, multicentre trial which included patients with previously untreated stage III (unresectable)/IV melanoma with known BRAFV600 status. A total of 945 patients underwent randomisation to receive either nivolumab/ipilimumab or ipilimumab alone. All living patients had a minimum follow-up of 36 months, with a median follow-up of 38 months. The overall survival rate at 3 years was 58% in the nivolumab/ipilimumab vs. 34% in the ipilimumab group. Treatment-related adverse events of grade 3/4 were reported in 59% participants in the nivolumab/ipilimumab group, in 21% of the nivolumab group and 28% of the ipilimumab group. 32%, 46%, and 63% of patients received subsequent systemic therapy in the nivolumab/ipilimumab, nivolumab and ipilimumab group, respectively.

There were two deaths related to a study drug within 100 days and two deaths related to a study drug more than 100 days [48].

3.2. Autologous ex-vivo augmented tumour infiltrating lymphocytes

The immune response can be increased by either in vivo vaccination or proliferation of the antigen-specific effectors in vitro followed by transfer to the patient. The APCs used for generating effector responses are critical for determining the specificity and type of immune response. However, the response and essentially the outcome of the T-cells differ hugely whether it is in vivo or in vitro [49].

Adoptive cell therapy, unlike checkpoint inhibitors, creates an immune response, rather than simply "taking the breaks" off the immune system. It involves harvesting the T cells from the patient's serum or tumour and then encouraging them to proliferate in a culture medium in vitro [25]. Adoptive cell therapy involves lymphodepletion prior to infusing autologous tumour infiltrating lymphocytes. In three clinical trials involving 93 patients with refractory melanoma, the response rate varied from 48 and 72%, depending on the chemoradiation strategy employed for the lymphodepletion technique. The median follow-up varied between 10 and 45 months for the trials. The 2-year survival rates ranged from 30 to 42%. There was one treatment-related death [50]. This treatment strategy only experimental and has not been approved by the US FDA for the treatment of melanoma.

3.3. Oncolytic viruses

An oncolytic virus is a non-pathogenic virus which destroys cancer cells, while leaving the normal cells unaffected [51]. The use of oncolytic viruses offers an attractive treatment strategy. The oncolytic viruses result in cytotoxic effects by directly infecting the cancer cells. Furthermore, the viral genome can be manipulated to maximise the beneficial therapeutic effects and to minimise harmful effects. Oncolytic viruses involve the administration of either native or genetically modified viruses, which then enter the tumour cell selectively, proliferate and lyse these cells [52]. The endogenous defence mechanisms against viral-mediated infection are suboptimal in tumour cells. This results in a high turnover of the virus in the tumour cell. The cancer-specific replication is achieved by either selecting a non-virulent virus in humans or by manipulating the genome of the virus [53]. The viruses succeed in destroying the tumour cells through several mechanisms: primary lysis of the cancer cells, powerful bystander effects on healthy cells, provocation of local endogenous antiviral mechanisms and systemic antitumor immunity that can cause regression of the cancer at distant, uninfected cells. There are several types of oncolytic viruses developed: oncolytic poxvirus, oncolytic herpes simplex virus, oncolytic Coxsackie virus and oncolytic reovirus [52].

There are several barriers to treatment with oncolytic viruses: pathogenic potential, suboptimal ability to selectively target cancer cells, degradation by the immune system, and suboptimal ability to trigger T-cell response to neoplasm. Despite these limitations, the first oncolytic therapy known as laherparepvec was approved by the FDA for melanoma patients in 2015. It was indicated for patients with injectable lesions in the skin and lymph nodes that were not amenable to surgical resection [51].

Laherparepvec is a genetically engineered oncolytic virus. It is a genetically modified HSV-1 laherparepvec is used for the treatment of melanoma. It works in two different ways: it replicates more actively in the tumour cells. This causes lysis of the tumour cells. Viral particles and tumour-associated antigens are released from the cells. The viral cells can preferentially target more tumour cells. The tumour antigen can induce an immune response, which is potentiated by the expression of GM-CSF in the laherparepvec. Laherparepvec functions by exploiting the protein kinase R (PKR) pathway. This pathway suppresses viral replication in healthy cells. The usual defence mechanism infected susceptible cell protein 34.5 is usually responsible for overcoming the PKR pathway in HSV. However, laherparepvec is genetically modified to delete the infected susceptible cell protein 34.5 in HSV-1, leaving the cells vulnerable to degradation by PKR. In healthy cells, the PKR pathway is active and causes inactivation of the laherparepvec pathway. However, in the case of tumour cells, PKR is inactive. This leads to the virus actively replicating selectively in the tumour cells. Furthermore, there is a downregulation of type 1 IFN pathway in tumour cells. This leads to a further susceptibility of tumour cells to laherparepvec [54]. The OPTiM trial randomised patients to either intralesional laherparepvec or subcutaneous GM-CSF in patients with stage 3 and 4 melanomas. The trial showed monotherapy with laherparepvec significantly increases the durable response rate vs. therapy with GM-CSF alone (25.2% vs. 1.2%, respectively). It also improved the overall response rate (40.5% vs. 2.3%, respectively). The toxicity profile was similar in both treatment arms, with the majority of toxicities including grade 1 and 2 toxicities [55]. There are ongoing phase 3 trials examining the efficacy of combination therapy with laherparepvec and pembrolizumab in stage 3 and 4 melanomas. Earlier phase 2 trials appear promising.

3.4. Dendritic cells

Dendritic cells are a form of immune cell, which is the more powerful antigen-presenting cell. The cells circulate in their inactive state in the body circulation. When they are exposed to a danger signal, they become activated antigen-presenting cells. They facilitate immune responses in the lymphoid tissue, causing the naïve T-cells to differentiate into effector T cells. DC cells facilitate activation of tumour immunity. They activate antigen-specific T cell responses in melanoma patients. Dendritic cell vaccines are activated dendritic cells containing tumour antigens. Dendritic cells are not advisable as monotherapy in the treatment of advanced melanoma. However, there are promising results when DC viruses are combined with ipilimumab. It is postulated that the immune system is more potent in stage 3 vs. stage 4 melanoma. Dendritic cells show some promise in stage III melanoma. However, phase 3 trials are pending. The safety profile of DC vaccines is favourable when compared with checkpoint inhibitors [56].

4. Chemotherapy

In the advent of targeted therapies, chemotherapy is no longer deemed a first-line therapy for metastatic cutaneous melanoma in the latest ESMO guidelines [2]. However, in the recent past, chemotherapy was an important therapeutic strategy for palliation. Examples of chemotherapeutic agents employed in melanoma include dacarbazine, temozolomide, nab-paclitaxel, paclitaxel, cisplatin, carboplatin, and vinblastine. The only chemotherapy agent approved by the FDA

is dacarbazine. Dacarbazine and temozolomide (an analogue drug) are alkylating agents that damage DNA, leading to cell apoptosis. Multiple phase 3 studies have failed to demonstrate an overall survival benefit for any chemotherapy regimen. Specifically, only 10–20% will respond to dacarbazine. The progression-free rate is 3–6 months with dacarbazine. The adverse effects of dacarbazine include bone marrow suppression and nausea/vomiting. Former combination regiments included BOLD (bleomycin, vincristine, lomustine and dacarbazine), CVD (cisplatin, vinblastine and dacarbazine) and the Dartmouth regimen (dacarbazine, cisplatin, carmustine and tamoxifen). However, the studies failed to demonstrate a benefit to combination chemotherapy vs. monotherapy. Furthermore, the toxicity profiles of the combination therapy were worse than monotherapy. Combining immunotherapy and chemotherapy similarly failed to demonstrate any significant benefit. It also led to worse outcomes in terms of toxicity profiles [57].

5. Conclusion

In conclusion, immunotherapy and targeted therapy in the form of BRAF/MEPK inhibitors form the backbone of therapy for metastatic melanoma. The optimal agents remain under considerable debate. Chemotherapy has been relegated to second-line therapy. Future guidelines will likely reflect this new research.

In conclusion, the mainstay treatment for managing melanoma remains surgery if feasible. There are several adjuvant therapies such as anti-PD1 therapies, CTLA4 inhibitors and BRAF/MEK inhibitors that may play a useful role as adjuvant therapies in high-risk, stage 3 disease. The treatment strategies for advanced melanoma are evolving rapidly. Targeted therapies such as anti-PD1 therapies, CTLA4 inhibitors and BRAF/MEK inhibitors have become mainstay treatment. Further research must be carried out to determine the best regimen. Chemotherapy now only plays a role in rescue therapy.

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Chapter 5

Possibilities for the Therapy of Melanoma: Current Knowledge and Future Directions

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70368

Abstract

This chapter presents an overview of possibilities for the therapy of melanoma, current knowledge and future direction. Skin cancer is one of the most frequent types of cancers. Melanoma is much less common than basal cell and squamous cell skin cancers, but it is far more dangerous. Detailed knowledge of melanoma at the molecular level allows to develop new treatment alternatives and to design effective new drugs. There are two approaches in therapy of melanoma in the present based on immunotherapy and targeted therapy or their combination. Immunotherapy includes immune checkpoint blockades, whereas targeted therapy is represented by protein kinase inhibitors, such as BRAF inhibitors, MEK inhibitors, and NRAS inhibitors. Detailed knowledge of protein structure and the understanding of its role in key signaling pathways in melanoma development lead to the designation of new protein kinase inhibitors in targeted therapy.

Keywords: melanoma, chemotherapy, immunotherapy, targeted therapy, protein kinase inhibitors

1. Introduction

The incidence of melanoma is increasing worldwide. Melanomas represent 3% of all skin cancers but 65% of skin cancer deaths [1]. Melanoma is currently the fifth and sixth most common solid malignancy diagnosed in men and women, respectively [2]. The rates of melanoma have been rising for at least 30 years [3]. Although melanoma is no longer considered just 'one disease', pathologists will continue to have important role in identifying and describing tumor subtypes [4]. More detailed understanding of melanoma allows the development

of new specific treatment alternatives, which are targeted at specific receptors or the genes of tumor cells. In 2011, new molecules were discovered and designed on the basis of new knowledge in the molecular biology of melanoma. These new facts have resulted in the existence of two new approaches to therapy: immunotherapy and targeted therapy of melanoma.

2. Genesis of melanoma

Melanoma is derived from melanocytes—normal pigment cells of the skin. Most commonly, melanoma arises from epidermal skin melanocytes, but primary tumors can also be found lining the choroidal layer of the eye (uveal melanoma) or the mucosal surfaces of the respiratory, genitourinary, and gastrointestinal surfaces [5]. Melanoma is much less common than basal cell and squamous cell skin cancers, but it is far more dangerous. Like basal cell and squamous cell cancers, melanoma is almost always curable in its early stages. However, it is much more likely to spread to other parts of the body than basal or squamous cell cancer if not caught early. Melanocytes that produce melanin are usually uniformly localized at the interface of the dermis and epidermis of the skin. If the melanocytes are found in denser groups, they create different forms of birthmarks—nevus. Later, they may be the cause of developing benign skin tumors—dysplastic nevus [6].

The term "dysplastic nevus" implies that this nevus exists as a distinct and defined entity of potential detriment to its host. Rosendahl et al. examine the current data, which suggest that this entity exists as histologically and possibly genetically different from common nevus, with some overlapping features. Studies show that a melanoma associated with a nevus is just as likely to arise in a common nevus as in dysplastic nevus [7].

Human nevi are benign tumors of melanocytes that are frequently associated with oncogenic mutations predominantly in BRAF V600E. However, nevi typically remain in a growth-arrested state for decades and only rarely progress into malignant melanoma. Very important features of nevus include oncogene-induced senescence [8] and oncogene-induced trans-lineage differentiation [9], which prevent benign nevi from malignant transformation.

In recent years, researchers have learned a great deal about how certain changes in DNA can make normal cells become cancerous. Cancers can be caused by DNA changes that turn on oncogenes or turn off tumor suppressor genes. Changes in several different genes are usually needed for a cell to become cancerous. Damage of DNA may be in the form of inherited genetic mutation, but in most cases it occurs gradually over the life due to the influence of environmental factors, such as UV rays from the sun [10–12].

There are two melanin pigments synthesized in the melanocytes: eumelanin, a dark brown-black insoluble polymer, and pheomelanin, light red-yellow sulfur containing soluble polymer [13]. Pheomelanin has a weak shielding capacity against ultraviolet radiation compared to eumelanin, and has been shown to amplify ultraviolet-A-induced reactive oxygen species. Mitra et al. suggested that the pheomelanin pigment pathway produces ultraviolet-radiation independent carcinogenic contributions to melanomagenesis by a mechanism of oxidative damage [14].

http://dx.doi.org/10.5772/intechopen.70368

The incidence of melanoma is increasing at one of the highest rates of any form of cancer in fair-skinned populations around the world. The exposure to sunlight during the past 50 years is an important factor for the increasing incidence of melanoma. Mortality rates of melanoma show stabilization in Australia, in North America, and also in European countries. Prevention campaigns aim on reducing incidence and achieving earlier diagnosis, which resulted in an ongoing trend toward thin melanoma since the last two decades. However, the impact of primary prevention measures on incidence rates of melanoma is unlikely to be seen in the near future; rather, increasing incidence rates to 40–50/100,000 inhabitants/year should be expected in Europe in the next decades [15].

3. The possible signs and symptoms of melanoma

The possible signs and symptoms of melanoma are new moles or spots on the surface of skin that are changing in size, shape and color. Another important sign is a spot that looks different from all of the other spots on skin. There are the **ABCDE** criteria for these signs, which guide to the usual signs of melanoma:

A – Asymmetry; one half of a nevi or birthmark does not match the other.

B – Border; the edges are irregular, jagged, or blurred.

C – Color; the color is not the same all over and may include shades of brown or black, or sometimes with patches of pink, red, white, or blue.

D – Diameter; the spot is larger than 6 mm across, although melanomas can sometimes be smaller than this.

E – Evolving; the nevi are changing in size, shape, or color.

4. Melanoma classification and staging

The classification schemes **Breslow's thickness (depth)** and **Clark's level** have been developed based on either the vertical thickness of the lesion in millimeters or the anatomic level of invasion of the layers of skin. Breslow's depth is considered significant factor in predicting the progression of the melanoma. Increased tumor thickness is correlated with metastasis and poorer prognosis. Tumors are classified into four categories based on the depth: thickness of 0.75 mm or less, thickness of 0.76–1.5 mm, thickness of 1.51–4 mm and thickness greater than 4 mm.

Clark's level of invasion has far less importance and is used only in the staging of thin melanomas (<1 mm). Tumors are classified into five levels:

Level I – melanoma involves only epidermis (melanoma *in situ*);

Level II – melanoma invades papillary dermis but not papillary-reticular dermal interface;

Level III – melanoma invades and expands papillary dermis up to the interface with, but not into, reticular dermis;

Level IV – melanoma invades reticular dermis but not into subcutaneous tissue;

Level V – penetration of melanoma into the subcutaneous tissue.

Cancer staging system, called the TNM (Tumor, Node, Metastasis) system by the American Joint Committee on Cancer (AJCC) is used for clinical staging [16]. The stage of melanoma refers to the thickness, depth of penetration, and the degree to which the melanoma has spread. The staging is used to determine treatment. There are five stages of melanoma: stage 0 and stages I–IV.

Stage 0 refers to melanoma *in situ*, which means melanoma cells are found only in the outer layer of skin or epidermis. This stage of melanoma is very unlikely to spread to other sites of the body. **Stage I** the primary melanoma is still only in the skin and is very thin. Stage I is divided into stages IA and IB, depending on the thickness of the melanoma and the mitotic rate. **Stage II** melanoma is thicker than stage I melanoma, extending through the epidermis and further into the dermis, the dense inner layer of the skin. It has a higher chance of spreading. Stage II is divided into IIA, IIB and IIC depending on thickness the melanoma and ulceration. **Stage III** melanoma has spread through the lymphatic system, either to a regional lymph node located near where the cancer started or to a skin site on the way to a lymph node. Stage III is also divided into IIIA, IIIB and IIIC depending on the size and number of lymph nodes involved with melanoma and whether the primary tumor appears ulcerated under a microscope. In **stage IV**, melanoma has spread through the bloodstream to other places of the body, such as lung, liver, brain, bone, soft tissue, or gastrointestinal tract. Stage IV is further divided into M1a, it means the cancer has only spread to distant skin and/or soft tissue sites; M1b involves metastasis to the lung; and M1c describes distant metastasis at any other location or an elevated serum lactate dehydrogenase [17].

5. Current possibilities for the therapy of melanoma

Similar to other tumors the progressive stage of melanoma is predictive for therapeutic success. Early stage melanomas (thin tumors) result in a 97% 5-year survival rate of the patients, after surgical removal [18].

6. Surgery and chemotherapy

The treatment of cutaneous melanoma has historically been essentially surgical. Much progress has been made in this area, and the resection margins have been established based on tumor depth. Candidates are also identified for lymphadenectomy, avoiding the morbidity of the procedure in patients who do not require it.

http://dx.doi.org/10.5772/intechopen.70368

Topical formulations are examined and, where available, skin penetration properties of the various drugs are detailed. New strategies for targeted drug delivery to skin cancers are considered with an emphasis on studies conducted *in vitro* with porcine or human tissue, or in patients.

Imiquimod cream may be used to stimulate the local immune response in early stage melanoma patients (**Figure 1**) [19, 20].

The decision to treat melanoma by adjuvant therapy has the opposing arguments: the risk of recurrence, progression and high toxicity, and price of treatment.

The risk of recurrence and death after complete surgical resection of clinically detectable primary cutaneous melanoma ranges from low, intermediate to high risk depending on the stage of disease at diagnosis. This is determined by the depth, ulceration status and mitotic rate of the primary tumor, the presence of regional nodal disease or distant metastasis. For high-risk melanoma, adjuvant therapy is aimed at eradicating melanoma micrometastases in the patients that carry an unacceptable risk of mortality from melanoma recurrence. The ultimate goal of adjuvant therapy is to provide a potential cure before progression of melanoma into advanced inoperable stages [21].

A little progress has been made in systemic treatment since the 1970s when the use of dacarbazine was introduced for the treatment of patients with tumor progression or distant metastasis, with disappointing results.

Dacarbazine and *temozolomide* (**Figure 1**) belong to the group of alkylating agents. These triazene compounds have excellent pharmacokinetic properties and limited toxicity. The active moiety of these drugs is represented by the triazenyl group of three adjacent nitrogen atoms, which are responsible for the physico-chemical and antitumor properties of the molecule. Mechanism of action of both compounds is mainly related to the methylation of O^6-guanine, mediated by methyldiazonium ion, a highly reactive derivative. O^6-methylguanine is responsible for incorrect base pairing and damaging of DNA [22]. Dacarbazine is a prodrug structurally related to purines activated by liver microsomes. This chemotherapeutic agent was approved by the Food and Drug Administration (FDA) for the treatment of melanoma, and often regarded as the standard treatment for advanced melanoma. However, therapy with

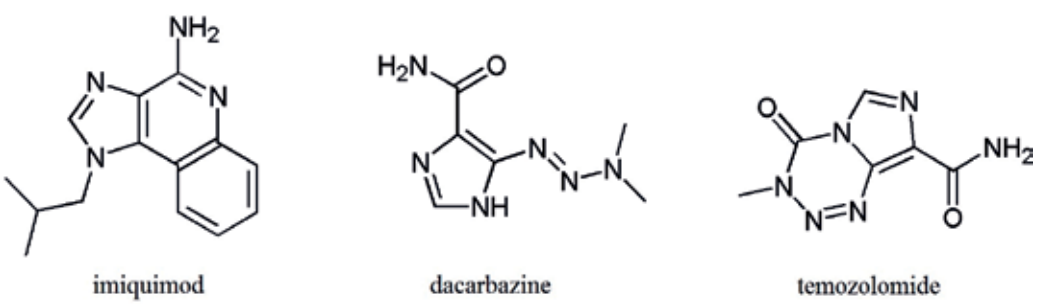

Figure 1. Chemical structures of imiquimod, dacarbazine, and temozolomide.

dacarbazine is characterized with low overall response rates (approximately 10–15%) and there is no valid evidence of survival benefit [23]. Temozolomide is a monofunctional alkylating agent of the imidotetrazine class. It is stable at the acid pH of the stomach and administered orally with 100% bioavailability [22].

7. New approaches

For years, the cornerstones of cancer treatment have been surgery, chemotherapy, and radiation therapy. Significant changes occurred in antitumor therapy for disseminated melanoma during the last decade. Detailed knowledge in the molecular biology of melanoma and immune response lead to the two directions: immunotherapy and targeted therapy. Before 2011, two approved drugs were used to treat patients with metastatic melanoma in the USA: dacarbazine and recombinant human interleukin-2 (IL-2) [24]. The treatment landscape for advanced stage melanoma was revolutionized in 2011 with the approval of ipilimumab and vemurafenib, both of which improved overall survival in phase III clinical trials. More recently, the targeted inhibitors dabrafenib and trametinib have demonstrated similar therapeutic profiles [25]. The latest approved (PD-1)-blocking antibody pembrolizumab is indicated for the treatment of patients with unresectable or metastatic melanoma [26].

8. Immunotherapy

The immune system recruitment may represent a powerful and innovative strategy in cancer therapy. Genetic mutations and alterations in regulatory processes of cancer cells lead to expression of various tumor-related antigens that can be presented to cytotoxic T-lymphocytes by antigen-presenting cells. A major understanding of immune activation, especially T-lymphocyte activation, has identified multiple co-stimulatory and co-inhibitory pathways regulating this process. The two most important targets of immunotherapy are co-inhibitory receptors, such as CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4) and programmed cell death-1 (PD-1) receptor, expressed on the T-lymphocyte surface [26].

A molecule of IL-2 was first approved by the US FDA for immunotherapy of melanoma. It is of limited use due to the serious toxic side effect of this treatment [27]. The first approved checkpoint blocking antibody was ipilimumab.

Ipilimumab (Yervoy®) is a human monoclonal IgG1 antibody that binds the human antigen CTLA-4 located on the surface of T-lymphocytes and blocks its interaction with molecule on the surface of antigen presenting cells. CTLA-4 is a key negative regulator of adaptive immune response and works as a brake on the immune response. Blocking immune response to anticancer leads to a longer and stronger activation of T-lymphocytes and, ideally, to an attack and destruction of the tumor tissue, resulting in long term remission for 15–20% of patients [28]. Although its effectiveness is tested with many carcinomas, the best results were achieved just in the treatment of melanoma [29]. Randomized clinical studies show that the treatment

with ipilimumab leads to a significant extension of the survival of patients with metastatic melanoma. Side effects of ipilimumab are related to the mechanism of its action. Typical side effects are accompanied by diarrhea, skin rash, pruritus, enteritis, vitiligo, endocrinopathies and hepatotoxicity. Ipilimumab is approved in the USA for the treatment of patients with advanced melanoma and in Europe for patients with previously treated advanced melanoma.

Tremelimumab, another drug of this group, is human therapeutic monoclonal antibody IgG2, with the same mechanism of action as ipilimumab. This antibody is currently in progress in phase II/III clinical study [30].

Ipilimumab, in combination with high dose IL-2, and tremelimumab, in combination with interferon alfa provide increased overall response rate, progression-free survival, or higher percentage of complete responses. *Interferon alfa* is FDA approved in adjuvant treatment for patients with high-risk melanoma and it has significant immunomodulatory effects [31–33]. Interferon alfa monotherapy has limited utility in the treatment of stage IV melanoma; therefore, its antitumor activity has led to profound investigation of its use in combination with other therapies [34].

Cancer immunotherapy can be achieved by inhibition of the PD-1/PD-Ll axes, which affect the overall survival in an important fraction of patients. PD-1 is an inhibitory receptor that is upregulated on activated lymphocytes. PD-1 has two known ligands, PD-L1 and PD-L2, which can be expressed on tumor and stromal cells; PD-L1 expression can be induced by cytokines produced by tumor-infiltrating lymphocytes [35].

Pembrolizumab (Keytruda®, Merck & Co) is the first anti-PD-1 immunotherapeutic agent approved by FDA. Keytruda® was granted FDA approval on September 4, 2014 for the treatment of patients with unresectable or metastatic melanoma. This molecule is a potent and highly selective humanized monoclonal antibody of IgG4-kappa isotype, designed to directly block the interaction between PD-1 receptor, expressed on T-cells, and its ligands, PD-L1 and PD-L2, without antibody-dependent cell-mediated or complement-dependent cytotoxicity. In practice, blocking PD-1 activity is believed to prevent inhibition of T-cell immune surveillance of tumors and, in some models, has resulted in decreased tumor growth [26]. The recommended dose of pembrolizumab is 2 mg/kg administered as an intravenous infusion over 30 min every 3 weeks until disease progression or unacceptable toxicity. Most common adverse reactions (reported in ≥20% of patients) included fatigue, cough, nausea, pruritus, rash, decreased appetite, constipation, arthralgia, and diarrhea [36].

Another approach, which has already been tested, is to combine anti-PD-1 and anti-CTLA-4 treatment and is represented by *nivolumab* (Opdivo®, Bristol-Meyers Squibb). Nivolumab is used alone or in combination with ipilimumab [37, 38]. Combination therapy with anti-CTLA-4 and anti-PD-1 monoclonal antibodies has recently led to remarkable antitumor effects, long-term survival and potential cures [39].

BRAF-mutant and BRAF-wild type patients, who progressed after ipilimumab therapy, were included into the Phase III study. One group of patients received nivolumab (3 mg/kg every 2 weeks), and in comparator group patients were treated with chemotherapy. Patients treated with nivolumab demonstrated higher response rate compared to the chemotherapy group—32%

vs. 11% [40]. There was no statistical difference in two contemporary oncology median overall survivals between the study arm with nivolumab, 15.7 months, vs. the comparator group with chemotherapy, 14.4 months. The limitation of the clinical benefit of nivolumab could be related to the fact that control group patients (40%) received pembrolizumab, when progressed during chemotherapy. Furthermore, the number of patients with elevated lactate dehydrogenase levels and brain metastases was imbalanced, favoring the chemotherapy [41]. Adverse events are less frequent in patients treated with nivolumab than in those treated with ipilimumab or chemotherapy [4, 10]. The most frequently observed adverse events included fatigue, pruritus, diarrhea, rash, and nausea. The most commonly observed immune-related adverse events were pruritus, rash, diarrhea, vitiligo, hypothyroidism, and elevated aminotransferase activities [42].

In the study of Pyo and Kang the effects of various immunotherapeutic agents and chemotherapy for unresected or metastatic melanomas were compared. They performed a network meta-analysis using a Bayesian statistical model to compare objective response rate of various immunotherapies from 12 randomized controlled studies. The estimated overall response rates of immunotherapy and chemotherapy were 0.224 and 0.108, respectively. The overall response rates of immunotherapy in untreated and pretreated patients were 0.279 and 0.176, respectively. In network meta-analysis, the odds ratios for overall response rate of nivolumab (1 mg/kg)/ipilmumab (3 mg/kg), pembrolizumab (10 mg/kg) and nivolumab (3 mg/kg) were 8.54, 5.39 and 4.35, respectively, compared with chemotherapy alone. Their results showed that various immunotherapies had higher overall response rates rather than chemotherapy alone [43].

Except immunological checkpoint blockades the approach of *adoptive T cell therapy* seems to be a highly promising in use against cancer including melanoma. The ability of T cells to specifically lyse tumor cells and secrete cytokines to recruit and support immunity against cancer make them an attractive proposition for therapy. Since the first idea in 1989 to genetically redirect T cells, a lot of experiments have been performed. Recent methods of generating tumor-specific T cells include the genetic modification of patient's lymphocytes with receptors to endow them with tumor specificity. These T cells are then expanded *in vitro* followed by infusion of the patient in adoptive cell transfer protocols. Genes used to modify T cells include those encoding T-cell receptors and chimeric antigen receptors. Several trials with gene-modified T cells are ongoing and some remarkable responses have been reported [44]. In fact, current adoptive T cell therapy response rates are 80–90% for hematological malignancies and 30% for metastatic melanoma refractory to multiple lines of therapy. Although these results are encouraging, there is still much to be done to fulfill potential of adoptive T cell therapy, specifically with regard to improving clinical efficacy, expanding clinical indications and reducing toxicity [45].

9. Cancer vaccines

Melanoma vaccines have the goal to induce long lasting immunity against melanoma to prevent the development of metastases. However, melanoma cells express many different tumor-associated antigens. Ideally, vaccines need to contain all these different tumor-associated antigens for antigen-presenting-cells (APC) to induce an adequate immune response [46].

Vaccines specific for cancer antigens exert antitumor effects by inducing cytotoxic T lymphocytes (CTLs) that recognize and attack antigenic cancer-derived peptides comprising 8–10 amino acid residues presented on major histocompatibility complex molecules on the cancer cell surface. Peptides, proteins, mRNA, DNA, and viral vectors can be used as cancer vaccines. Various peptides derived from Wilms' tumor gene-1, glycoprotein 100 (gp100), and melanoma-associated antigen 3 (MAGE-A3) have been used as vaccines against melanoma [47].

Tumor antigen encoded by genes of the MAGE-A family, MAGE-A3 is of importance because they are expressed in a wide array of malignancies including melanoma, brain, breast, lung and ovarian cancer. Its ability to elicit spontaneous humoral and cellular immune responses has been shown in cancer patients. As antigen-specific immune responses can be stimulated by immunization with MAGE-A3, several clinical trials have used MAGE-A3 vaccines to observe clinical responses. The frequent expressions of this antigen in various tumors and its immunogenicity in cancer patients have led to application of this antigen in cancer immunotherapy. Indeed, the initial trials performed with MAGE-A3 peptides showed no significant toxicity [48]. Vaccination with a tumor-specific MAGE-A3 peptide, even without adjuvant, has been shown to induce a CTL response in a melanoma patient followed by reduction in tumor mass. MAGE-A3 protein produced by recombinant technology is more popular in clinical trials as a result of its potential to activate a wide range of T-cell responses as well as its potential application in a larger population of patients with MAGE-A3 expressing tumors [49]. As MAGE-A3 specific therapies have not reached their final goals to cause a significant improvement in the survival of patients. For future perspective of MAGE-A3 therapy, studies are needed to find the most effective vaccine formulations, the most immunogenic adjuvants as well as the most applicable criteria for selection of patients.

10. Oncolytic virus therapy

Over the past several years, oncolytic viruses for treating various cancers have been investigated. Oncolytic viruses play a role in cancer vaccination because antigen-specific immunity is effectively evoked against components released by destroyed cancer cells due to virus-induced production of type I interferon. The effects of oncolytic virus therapy are mediated not only by direct cell disruption, but also by indirect induction of cancer-specific immune responses [48].

Oncolytic viruses selectively replicate within and lyse cancer cells without damaging normal cells. On October 27, the FDA approved the first oncolytic virus therapy, *talimogene laherparepvec (Imlygic™, T-VEC)*. The agency approved T-VEC for the treatment of some patients with unresectable cutaneous, subcutaneous, and nodal lesions in melanoma recurrent after initial surgery [50]. T-VEC is a modified herpes simplex virus, type 1 (HSV-1) that has undergone genetic modifications to promote selective tumor cell replication, while reducing viral pathogenicity and promoting immunogenicity. T-VEC improves overall response rate and durable response rate as a single agent, shows promise in combination therapy with immunotherapy, and is well tolerated. Ongoing trials will determine if T-VEC has a role in early

treatment or in combination therapy for melanoma or other malignancies, such as hepatocellular carcinoma, metastatic liver tumors, advanced non-central nervous system tumors, breast cancer, pancreatic cancer, Merkel cell carcinoma, head and neck cancer, sarcoma, lymphomas. In a Phase I study, T-VEC has been combined with ipilimumab or pembrolizumab, with promising results without overlapping toxicities. The results of larger studies are awaited to further delineate T-VEC's place with combination therapy [51].

11. Targeted therapy

Selected somatic changes such as BRAF mutations have been described, and then applied to the targeted treatments. BRAF gene is located in chromosomal region 7q34; it consists of 18 exons and transcribed mRNA length was 2478 bp. Targeted therapy is based on the knowledge of the molecular biology of the gene encoding the BRAF kinase, belonging to the RAF kinase family. It is a serine/threonine kinase that takes part in the Mitogen Activated

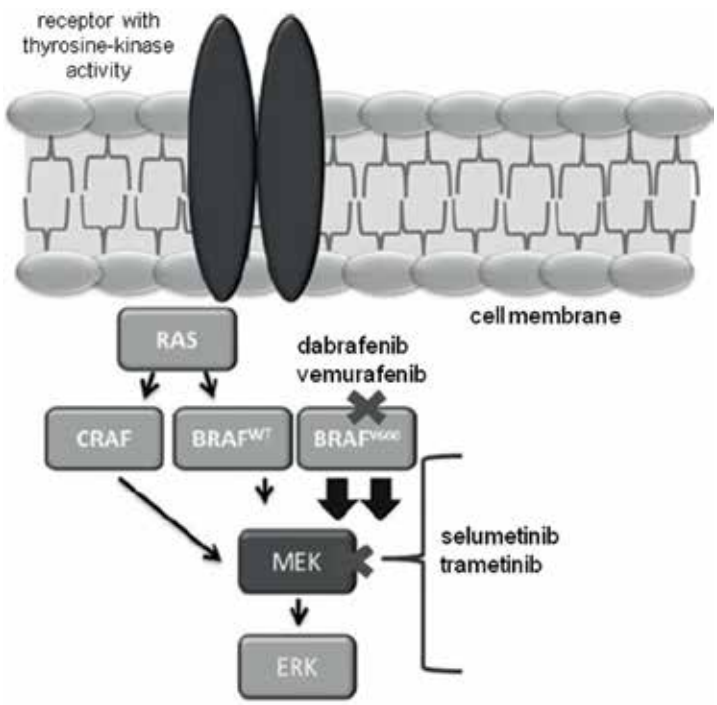

Figure 2. Mechanism of action of kinase inhibitors. This figure shows a schema of signaling pathways triggered by binding of growth factors to tyrosine kinase receptor that triggers RAS, RAF, MEK and ERK pathways leading to cell growth and proliferation. Mutations in BRAF (V600E) can lead to accelerated cell growth and cancer formation of melanoma cells. Inhibition of mutant BRAF by dabrafenib, vemurafenib in the melanoma cells shuts down the signaling pathway causing tumor regression following cell apoptosis, tumor antigen expression and decreased release of cytokines and VEGF. MEK is a member of the MAPK signaling cascade that is activated in melanoma. Inhibition of MEK by selumetinib, trametinib blocks cell proliferation and induces apoptosis (controlled cell death). MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; VEGF, vascular endothelial growth factor.

Protein Kinase (MAPK) cascade, which modulates cell growth and proliferation. This pathway is activated by binding of the extracellular physiological growth factor to its receptor. Conformational change of the receptor leads to the activation of RAS protein (GTP-binding), which activates RAF protein, which activates other kinases MEK and ERK. This pathway may be activated by mutation of specific proteins, including BRAF [52]. It is reported that 40–60% of melanomas have a mutation of the gene leading to the pathological-activated signaling pathways and to uncontrolled growth of malignant transformed cells [53]. The most common gene mutations are V600E or V600K known as an amino acid substitution at position 600 in BRAF, from a valine (V) to a glutamic acid (E) or to a lysine (K), respectively. In the structure of protein kinases there is a DFG motif, which is a highly specific site for interaction with kinase inhibitors. It contains Asp (D), Phe (F) and Gly (G) and exists in a conformational active or inactive state. Just the knowledge in this field has led to the development and screening of new selective inhibitors of BRAF and MEK (**Figure 2**) [54]. Targeted therapy is associated with improved clinical benefit; however, the mechanism of resistance often varies and includes activation of alternative signaling pathways [55].

12. BRAF inhibitors

Vemurafenib (Zelboraf® tablets, Roche) is the first selective inhibitor of BRAF developed by Plexxikon and approved by FDA in 2011 (**Figure 3**). It leads to a rapid, and sometimes the complete remission of the disease in patients with a mutated BRAF V600E. A clinical study on 675 respondents treated with vemurafenib, 960 mg twice daily, demonstrated survival of 6 months in 84% of patients versus 64% of patients treated with dacarbazine. Despite significant benefit in the treatment, there were new challenges identified – the development of resistance to reactivation of MAPK signaling and growth of keratoacanthomas and squamous cells. The most common adverse events were headache, joint pain, fatigue, skin hyperkeratosis and 6% of the patients experienced a squamous cell carcinoma [56].

Dabrafenib (Tafinlar® capsules), developed by GlaxoSmithKline (**Figure 3**), selectively inhibits BRAF ValGlu [57]. It is a thiazole derivative, which binds to the ATP binding site of BRAF kinase. It has a shorter half-life than vemurafenib (5.2 h versus 50 h). In 2009, first clinical studies in

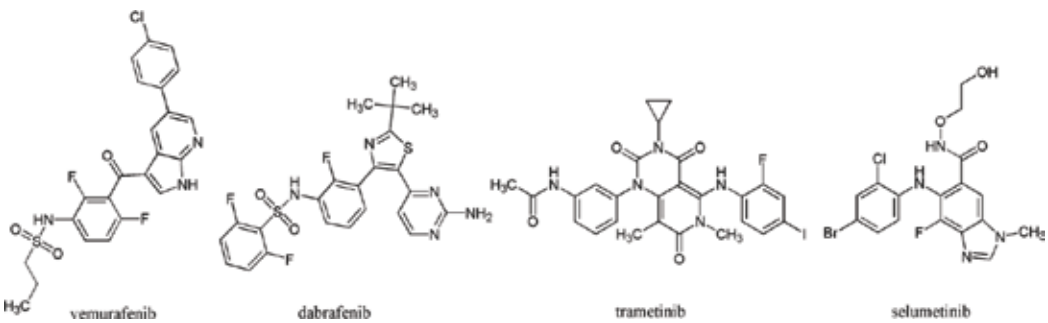

Figure 3. Chemical structures of vemurafenib, dabrafenib, trametinib, and selumetinib.

Phase I/II began. In Phase III clinical trials, the dosing regimen was 150 mg of dabrafenib twice daily, which significantly extended the survival to 5.1 months versus 2.7 months with dacarbazine. Hyperkeratosis, headache and joint pain, fatigue, heartburn have been reported as adverse events [24].

13. MEK inhibitors

Trametinib (Mekinist® tablets, GlaxoSmithKline) is the first selective allosteric inhibitor of MEK1 and MEK2 (**Figure 3**). In May 2013, it was approved by the FDA as a single agent for the treatment of patients with V600E mutated metastatic melanoma [58]. The recommended daily dose of trametinib is 2 mg orally daily. It has a long half-life, i.e. 4 days at the previously mentioned dosing. In Phase III clinical study, trametinib was well tolerated by patients who most commonly experienced side effects such as diarrhea, asthenia, rash, nausea and vomiting [59]. Development of squamous cell carcinoma as a side effect did not occur at all unlike in treatment with BRAF inhibitors [24].

Selumetinib, licensed by Array BioPharma Inc. to AstraZeneca in 2003, inhibits the MEK enzyme in the RAS/RAF/MEK/ERK pathway in cancer cells to prevent the tumor from growing (**Figure 3**). In April 2015, selumetinib was granted Orphan Drug Designation by the U.S. FDA in recognition of the need for new, safe and effective therapies for the uveal melanoma [60]. Uveal melanoma is a rare disease in which cancer cells form in the tissues of the eye. It is the most common primary intraocular malignancy in adults and comprises 5% of all melanomas [61]. In July 2015, AstraZeneca announced that the Phase III clinical SUMIT study of selumetinib in combination with dacarbazine for the treatment of patients with metastatic uveal melanoma did not meet its primary endpoint of progression-free survival. This combination therapy showed an adverse event profile generally consistent with current knowledge of the safety profiles of dacarbazine and selumetinib [62].

Currently, there are being conducted ongoing studies in the elimination of resistance of the MAPK cascade by concomitant administration of inhibitors of MEK and BRAF [52]. This combination of BRAF and MEK inhibitors may prolong progression-free survival, and consequently increase the overall survival of patients. Therapy reactions or responses in patients may be different; the anti-CTLA-4 immunotherapy may lead to long-term response, but not in all patients, whereas targeted drugs may cause responses in most patients, though almost all of them eventually experience relapses due to pre-existing or acquired resistance.

A wide range of mutations are known to prevent effective treatment with chemotherapeutic drugs. Hence, approaches with biopharmaceuticals including proteins, like antibodies or cytokines, are applied [5]. Modern therapeutic approaches in melanoma provide profound and long lasting effects and can even cure some patients. Rational consecutive and combined application of current methods, proper diagnostic and management of related adverse events can prolong life span of patients and meaningfully increase their quality of life [63].

Cobimetinib (Cotellic™) was granted FDA approval on November 10, 2015 in combination with vemurafenib (BRAF inhibitor) for the treatment of patients with metastatic melanoma. The approval was based on the effectiveness of cobimetinib plus vemurafenib in a randomized Phase III clinical trial of 495 patients whose tumors had specific mutations in the BRAF gene and who were not candidates for surgery. Patients who received vemurafenib plus cobimetinib had a median progression-free of 12.3 months, compared with 7.2 months in patients who received vemurafenib plus placebo. At 17 months after initiating treatment, about 65% of patients who received the two-drug combination were still alive, compared with 50% of those who received vemurafenib alone [64].

14. NRAS inhibitors

NRAS-mutant melanoma is a common subtype of this disease with a poor prognosis. NRAS is a low-molecular plasma-membrane–associated GTP-binding protein that constitutively activates intracellular signaling through a variety of pathways, most notably the RAS–RAF-MAPK and PI3K-AKT pathways. NRAS mutations activate MAPK signaling to a similar degree as BRAF mutations and rarely co-occur with mutations in the PI3K-AKT pathways, suggesting that mutant NRAS drives this pathway, too. NRAS mutation occurs in approximately 15–20% of melanomas, and it is the second most common oncogenic mutation in this disease [65].

Farnesyltransferase inhibitors (FTIs) showed the most promising therapy targeting the NRAS mutant. FTIs alter post-translational NRAS modification to prevent insertion into the plasma membrane. A Phase II study of FTI R115777 was performed in advanced melanoma and its results showed no evidence of clinical activity despite potent inhibition of FTIs in tumor tissue. The effect of these inhibitors has resulted in a stage of toxicity involving myelosuppression, nausea/vomiting and anorexia. Gajewski et al. concluded that FTIs were originally developed as RAS inhibitors and they affect several signaling pathways with potential outcomes for out-of-target toxicity. Multiple farnesylated proteins are involved in signal transduction in cancer. FTIs have been developed as a strategy to inhibit the function of these proteins. FTIs inhibit proliferation of melanoma cell lines. Farnesylated proteins are also important for T cell activation and measurement of effects on T cell function was also pursued [66].

Alternative strategies for a directly targeted NRAS mutant include the use of either antisense oligonucleotides or small interfering RNAs (siRNAs, small interfering RNAs) on the mutant NRAS gene. However, this has proved to be technically very demanding and requires advances in siRNA technology before considering use under clinical conditions. Therefore, NRAS mutant melanomas are currently being treated by MEK inhibitors or by newer types of immunotherapy that are not found in the presence of oncogenic mutation.

The first agent to show robust activity specifically in NRAS-mutant melanoma has been an allosteric inhibitor of MEK1 and MEK2 *binimetinib* **(MEK162, ARRY-162)** developed by Array BioPharma. As part of a combined study of BRAF and NRAS mutant melanoma patients, 30 metastatic melanoma patients whose tumors harbored a NRAS mutation were enrolled

and treated with binimetinib. The objective response rate was 21% and the progression-free survival was 3.7 months. Further study in this patient population will be necessary to confirm its clinical activity in comparison to other standard therapies. While prospective data with trametinib in NRAS mutant melanoma patients is not available, early retrospective data from ongoing clinical studies suggests that trametinib may have activity in a subset of NRAS mutant melanoma patients [67].

15. BRAF plus MEK inhibitors

Therapy with a MEK inhibitor in combination with a BRAF inhibitor is more effective and less toxic than treatment with a BRAF inhibitor alone, and has become the standard of care for patients with BRAF-mutated melanoma. Trametinib, the first MEK inhibitor was approved for the treatment of BRAF-mutated metastatic melanoma not previously treated with BRAF inhibitors, and is also approved in combination with the BRAF inhibitor dabrafenib [68].

The clinical study about combination dabrafenib and trametinib versus dabrafenib monotherapy in BRAF V600E/K–mutant metastatic melanoma demonstrated improved progression-free survival and overall survival. Phase III clinical study enrolled previously untreated patients with BRAF V600E/K–mutant unresectable stage IIIC or stage IV melanoma. Patients were randomized to receive dabrafenib, 150 mg twice daily, plus trametinib, 2 mg once daily, or dabrafenib plus placebo. The primary endpoint was progression-free survival; secondary endpoints were overall response, duration of response, pharmacokinetics and safety. Results showed that 423 of 947 screened patients were randomly assigned to receive dabrafenib plus trametinib (n = 211) or dabrafenib monotherapy (n = 212). At data cutoff, outcomes remained superior with the combination: 3-year progression-free was 22% with dabrafenib plus trametinib versus 12% with monotherapy, and 3-year overall response was 44 versus 32%, respectively. Twenty-five patients receiving monotherapy crossed over to combination therapy, with continued follow-up under the monotherapy arm. Of combination-arm patients alive at 3 years, 58% remained on dabrafenib plus trametinib. Three-year overall response with the combination reached 62% in the most favorable subgroup (normal lactate dehydrogenase) versus only 25% in the unfavorable subgroup (elevated lactate dehydrogenase). The dabrafenib plus trametinib safety profile was consistent with previous clinical trial observations, and no new safety signals were detected with long-term use. These data demonstrate that durable survival is achievable with dabrafenib plus trametinib in patients with BRAF V600–mutant metastatic melanoma [69].

The optimal timing and sequence of combination therapy (in particular targeted therapy in combination with immunotherapy) is currently in progress and cannot be precisely predicted for all patients with melanoma. Due to the existence of many potential targets in the immune system many critical questions arise, e.g. which therapy combinations should move forward in development and which patients will benefit from these treatments [70].

http://dx.doi.org/10.5772/intechopen.70368

16. Combination immunotherapy and targeted therapy

Studies about combinations of anti-PD-1/PD-L1 agents with other immunotherapeutic agents are currently conducted in treatment of multiple tumor types. Targeting immune checkpoints such as PD-1, PDL-1 and CTLA-4 has achieved remarkable benefit in multiple cancers by blocking immunoinhibitory signals and enabling patients to produce an effective antitumor response. Inhibitors of CTLA-4, PD-1 or PDL-1 administered as single agents have resulted in durable tumor regression in some patients, and combinations of PD-1 and CTLA-4 inhibitors may even enhance antitumor benefit [70]. The combination of ipilimumab and nivolumab was studied in a phase I trial of 86 patients with pretreated malignant melanoma and demonstrated a 40% objective response rate [71]. In Phase II [72] and III studies [73] of this combination used in the treatment of advanced melanoma response rates were quite impressive, but toxicity was notably increased. Almost 83–89% of patients required either topical or oral immunosuppressive therapy for immune-related adverse events (irAE), which led to treatment discontinuation in 36–47% of all patients [72, 73]. However, almost all of the patients (80–100%) treated with immunosuppressive agents had their irAE completely resolved [74].

Recent study by Kim et al. suggests that the addition of MEK inhibitors to targeted and immunotherapy combinations may be associated with increased toxicity; several patients treated by dabrafenib (BRAF inhibitor), trametinib (MEK inhibitor), and ipilimumab (CTLA-4 inhibitor) developed adverse events related to colonic perforation. This condition found in several patients increases the need to further understand the immunomodulatory effects of trametinib [75].

Promising results have been presented in a Phase I study in BRAF-mutant advanced melanoma patients receiving atezolizumab (anti PD-L1) combined with vemurafenib (BRAF inhibitor) and cobimetinib (MEK inhibitor), with a response rate of 83%; currently a Phase III study is on-going [76].

Atezolizumab (Tecentriq, Genentech Oncology) is PD-L1 blocking antibody that previously received FDA accelerated approval for the treatment of locally advanced or metastatic urothelial carcinoma that has progressed after platinum-containing chemotherapy. Atezolizumab was granted FDA approval on October 18, 2016 for the treatment of patients with metastatic non-small cell lung cancer whose disease progressed during or following platinum-containing chemotherapy. Also the combination of atezolizumab with trametinib in patients with BRAF-wild type melanoma demonstrated encouraging results in an early phase study—a Phase III study is planned [77]. Patients with advanced melanoma and high serum lactate dehydrogenase activity present very poor prognosis, regardless of the systemic treatment used [78]. Current research should be focused on understanding the relationship between high serum lactate dehydrogenase activity and the lack of treatment efficacy with immunotherapy and targeted therapy. Probably novel treatment strategies should be developed in this patient population [42].

17. Future direction in targeted therapy

Despite extensive new approaches in the treatment of advanced stage melanoma, i.e. chemotherapy, targeted therapy and immunotherapy, response rate is rarely higher than 20%. Especially in the treatment with BRAF inhibitors the drug resistance is very common [79]. Due to this reason there is an urgent need to invent other alternatives and targeted therapies. Preclinical studies looking at least this main drug association strategies seems to be very promising: targeting of either MEK or phosphatidylinositol-3 kinase (PI3K)/mammalian target of rapamycin (mTOR); strategies aimed at blocking anti-apoptotic proteins belonging to B-cell lymphoma (BCL-2) or inhibitors of apoptosis (IAP) families associated with MEK/BRAF/p38 inhibition; co-inhibition of other molecules important for survival (proteasome, histone deacetylase and signal transducers and activators of transcription) [80]. *PI3K-AKT-mammalian target* of rapamycin signaling pathway is important for melanoma initiation and progression so the preclinical investigation of a novel and highly potent PI3K-mTOR dual inhibitor *VS-5584* was realized. VS-5584 induced caspase-dependent apoptotic death in melanoma cells, and its cytotoxicity was alleviated by the caspase inhibitors [81]. Whereas the main aim of inhibiting MAPK signaling pathway is to prevent cancer cell proliferation, apoptosis is controlled by the availability of anti-apoptotic *BCL-2 proteins* (e.g. BCL-2), which reside at the outer mitochondrial membrane. BCL-2 supports neoplastic growth by blocking cell death and this target may be future direction in the treatment of various types of cancers [82]. Development of small molecule inhibitors specific for antiapoptotic BCL-2 proteins is a novel approach not only for therapy of chronic lymphocytic leukemia [83] but is very promising in therapy of advanced melanoma [84]. This new targeted approach could by more successful when the combination with retinoid derivative is used [85]. *Venetoclax (ABT-199)* (**Figure 4**) is the first orally bioavailable selective inhibitor of BCL-2 protein often over-expressed in chronic lymphotic leukemia (CLL) and other types of B-cell related cancers developed by AbbVie in partnership with Roche. It is currently being evaluated in Phase II and Phase III studies for CLL and in Phase I and II studies for several other blood cancers and can be one of the next molecules used in the treatment of melanoma in the near future [86].

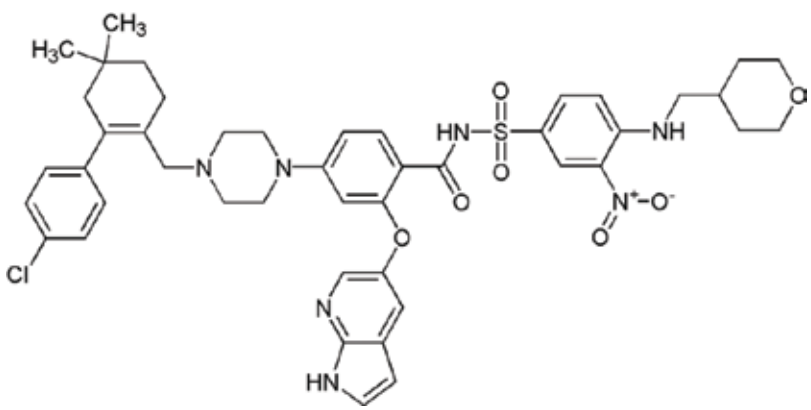

Figure 4. Chemical structure of venetoclax.

In the field of other genetic abnormalities such as *CDKN2A* also known as cyclin-dependent kinase inhibitor 2A, EGF (epidermal growth factor, which plays a role in skin cell growth), *Fas gene*, tumor suppressor gene *PTEN* (phosphatase and tensin homolog), there is a challenge in the research of new therapeutic targets and development of new anti-melanoma drugs in the future that can eventually lead to therapeutic benefit. Recent study by Hodis et al. describes six novel melanoma genes (PPP6C, RAC1, SNX31, TACC1, STK19, and ARID2), three of which RAC1, PPP6C, and STK19 harbored recurrent and potentially targetable mutations [87]. The prevalence of BRAFV600 and KIT mutations were significantly associated with melanoma subtypes and BRAFV600 and TP53 mutations were significantly associated with cutaneous primary tumor location. These results enrich understanding of the patterns and clinical associations of oncogenic mutations in melanoma, which could be the goal of future direction of melanoma therapy [88].

18. Conclusion

The development of drugs in the treatment of melanoma has never been as intense as at present. Single-agent chemotherapy is considered to have rather palliative effect on patients with melanoma; it is usually well tolerated but is associated with lower response rate. Detailed knowledge of protein structures and the understanding of their role in key signaling pathways in melanoma development lead to the designation of new targets for treatment of melanoma. Targeted therapy for patients whose tumors harbor the BRAF mutation achieves high response rates and OS benefit with combination BRAF/MEK inhibition. No other therapy in melanoma has shown a better response rate in late-phase clinical trials than combined BRAF and MEK inhibitors. The rapid kinetics of response to BRAF plus MEK targeted therapies represents the ideal frontline treatment for symptomatic, BRAF-mutant advanced melanoma patients. Although the concept of a combination of immunotherapeutic and targeted agents appears to be crucial in the treatment of melanoma, the synergy between these two approaches in melanoma treatment remains controversial due to the potential increased toxicity. Recently enormous progress in cancer therapy has been achieved by the use of immune checkpoint inhibitors. Activating the body's own immune system has added a novel and powerful therapeutic option for the treatment of melanoma. The potential use of immunotherapy is being extensively explored also in other malignancies. In the future, it is necessary to conduct further clinical trials and collect more data about overall survival, response rates, appropriate timing and sequence of combination therapy to manage the complexity of melanoma treatment.

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Section 3

Non-melanoma Skin Cancers

Chapter 6

Squamous Cell Carcinoma: Biomarkers and Potential Therapeutic Targets

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70767

Abstract

Squamous cell carcinoma (SCC) is the second most frequent non-melanoma skin cancer (NMSC) and carries with it a significant psychosocial and economic burden for both patients and health-care systems. Known risk factors for SCC include chronic ultraviolet (UV) exposure, chronic wounds and inflammation, exposure to certain chemicals and immunosuppression. The considerable risk of SCC recurrence and metastasis has driven the need for the discovery of new molecules that could explain the initiation and biological behavior of this type of NMSC. In this respect, proteomic research techniques have rapidly evolved and adapted in order to connect missing links and single out distinctive skin cancer biosignatures. Proteomic analysis of normal, dysplastic, and malignant keratinocytes appears to be promising in respect to SCC biomarker discovery, with the potential to aid in risk assessment, early detection, disease progression and development of novel targeted therapeutic agents. Identifying changes in the keratinocyte proteome pattern from normal to inflammatory and malignant cells will lead to the discovery of novel SCC biomarkers that could represent valuable tools for patient screening, diagnosis, management and follow-up.

Keywords: squamous cell carcinoma, keratinocytes, carcinogenesis, biomarkers, proteomics, diagnosis, therapy

1. Introduction

Squamous cell carcinoma (SCC) accounts for about 25% of non-melanoma skin cancers (NMSC) and together with basal cell carcinoma (BCC) (75%), it represents the most frequent

skin malignancy worldwide [1, 2]. Particularly, in the last several decades, the risk of developing cutaneous squamous cell carcinoma (cSCC) has been increasing epidemically, reaching approximately 7–11% [3]. Clinically, cSCC shows up red patches, rough or scaly, that can bleed or crust with slow healing. The affected skin is usually the one that is most exposed to sunlight, and body regions such as the head, face, neck and dorsum of the hands can carry a significant risk of developing cSCC. Seldom, it occurs in genital areas. However, it is important to know that cSCC can also be found in scars or skin sores [4]. Although numerous risk factors for developing cSCC have been noted, one of the most significant etiological factors is ultraviolet (UV) light that is responsible for damaging DNA, followed by chemicals, ionizing agents, radiation, chronic skin ulceration, weakened immune system, HPV infection, smoking, light-colored skin, and male gender [4]. In almost 65% of cases, cSCC arises from premalignant conditions such as actinic keratosis [5]. Also, immunosuppression caused by organ transplant or chemotherapy targeting BRAF favors the development of cSCCs with RAS mutations, elevating steadily the incidence of skin cancer by over 65-fold [6].

Even though its mortality rate is relatively low, approximately 2.1%, cSCC has many subtypes that widely vary from harmless to aggressive skin tumors with important metastatic potential, from 2 to 10% [7]. Initially, it invades adjacent tissue, then the regional lymph nodes and ultimately it affects distant organs [8]. The localization of cSCC influences the risk of recurrence and dissemination; cSCC affecting the lips or ears was demonstrated to have a higher risk of invasion (10–25%) [9]. In addition, an up-to-date prospective study established that a primary skin tumor size above 2 cm has a 15% chance of recurrence and a 30% chance of metastasis. Also, histological features such as speed of tumor growth, tumor depth greater than 4 mm, poor differentiation, perineural and subcutaneous invasion is associated with aggressive cSCC, leading to significant morbidity and mortality [8, 10, 11].

Although the vast majority of cSCC usually respond well to conventional treatments including wide surgical excision, chemotherapy, targeted therapy and radiotherapy, none of them can ensure the cure. Hence, approximately 3–5% of cSCCs recur and almost 5% metastasize within 5 years [8]. In addition, approximately 5% of metastatic cSCCs are associated with very poor clinical outcomes. There are no therapies officially approved by the FDA with a specific indication for metastatic cSCC and so the development of new agents has been relatively deliberate, due to a limited knowledge of the molecular basis of this disease. Therefore, there is a high necessity of identifying the complete genomic portrait of cSCC represented by multiple genes with recurrent mutation, amplificatiotn, and deletion including several other alterations which are aimed at developing new biomarker-associated therapeutic targets [12].

2. UV-induced keratinocyte proteome alterations

UV radiation could be considered a “Dr. Jekyll and Mr. Hyde” factor being both beneficial by facilitating vitamin D and endorphins synthesis but also harmful in prolonged exposure of the skin working as a carcinogen [13]. As cellular DNA is the major target for UVB radiation

(290–320 nm), this range has an increased mutagenic and carcinogenic potential by comparison with UVA (320–400 nm), being the most harmful constituent of sunlight that reaches the Earth surface [14]. Chronic and excessive exposure to UV radiation conveys many health risks where, besides photoaging, genomic and proteomic alterations at skin level can lead to immunosuppression favorable to the most common forms of skin cancer, BCC, SCC and melanoma. Genetic factors such as polymorphisms of the melanocortin 1 receptor gene can also influence the skin's sensitivity to UV and enhance cancer risk [15].

The UV-derived effects on skin cells in the proteomic context have not commonly been approached in photobiology and, as a consequence, only a few studies could be retrieved in this domain [16]. Although UVA is about 20-times more abundant than UVB in incident sunlight, its damaging potential on cellular DNA is less dangerous than UVB. The UVA effects are mediated by reactive oxygen species (ROS) that induce oxidative stress affecting the proteome through oxidation of DNA repair proteins, thus inhibiting DNA repair [17].

Almost entirely, published studies refer to UVB effects as triggers of significant alterations in skin cell layers, especially in keratinocytes, the major cell type of the epidermis and the main defensive barrier against external threats. UVB could also raise the increased ROS level responsible for oxidative damage of nucleic acids and proteins. Normal human epithelial keratinocytes isolated from foreskin and subjected to UVB were tested by parallel proteomics approach for assessing the protein expression profile and also for identifying proteins modified through chemical oxidation. In UVB-irradiated keratinocytes, various proteins involved in cellular homeostasis such as cytoskeleton integrity, removal of damaged proteins or heat shock response were differentially regulated (e.g., prohibitin, integrin alpha-3, cytokeratin 5, proteasome subunit alpha type-6) while some specific proteins with roles in cell adhesion, intercellular interaction, and protein folding were carbonylated (e.g., Glucosidase 2 b subunit, GRP 78, actin-related protein 3, annexin 2). These protein alterations driven by UVB exposure could cause cell homeostasis deregulation and eventually trigger cellular senescence or carcinogenesis [18].

Although it was reported that keratinocytes are more resistant to UV than other cell types, recurrent exposures to UVB induce at keratinocyte level, a so-called alternative state of differentiation, noticeable even 64 h after exposure [19]. Thus, a 2D-DIGE proteomic profiling of this specific state revealed a 69 differentially abundant protein patterns belonging to differentiation and survival keratinocyte machinery. Specifically, upon UVB action, an increased expression of a protein called TRI partite Motif Protein 29 (TRIM29) was noticed, further confirmed by Western blot assay. TRIM29 protein protects against UVB exposure damaging effects, as knocking down the TRIM29 expression by RNA interference, the viability of keratinocytes declined. These findings suggest that TRIM29 protein contributes to the survival of differentiating keratinocytes by inducing an alternative differentiation status protecting cells from dying, owing to UVB exposure-related stress [20]. The enhanced expression of TRIM29 as keratinocytes "regenerator" should be associated *in vivo* with the altered expression of other key proteins (heat shock proteins, cytokeratin, and cytoskeletal proteins), inflammation process, epidermis remodeling, and immune response type, as these could be novel mechanisms of keratinocyte survival upon UV damage [16, 21].

3. Chemically induced keratinocyte proteome alterations

Chemically induced tumors in experimental models can mimic all the clinical cancer progress phases being useful in the evaluation of new drugs, studying biological context or in decoding molecular mechanisms responsible for tumor initiation and development. Among chemical carcinogens commonly applied in cancer models are the following: environmental contaminants, N-nitroso compounds, food additives, antineoplastic agents, natural and synthetic substances, etc.

Combining chemically induced cancer models with innovative molecular imaging techniques may help to advance new anticancer diagnostics and therapeutics protocols [22]. By studying skin carcinogenesis, phases of early alterations in the skin layers and of the mechanisms beneath are highlighted. These mouse models share common mechanisms with human skin tumorigenesis, and moreover, there are similarities in terms of genetic milieu caused by carcinogens and pro-inflammatory cytokines and chemokines that favor tumor progression [23].

Different carcinogens imprint different changes on skin cells, including on the keratinocyte proteome pattern. Mancozeb—*ethylene (bis)dithiocarbamate*—is a fungicide and a multipotent carcinogen whose underlying mechanism of action is mostly unknown. By a two-dimensional gel electrophoresis and mass spectrometry analysis, a proteomic profile of mice skin exposed to mancozeb (200 mg/kg body weight) was generated. By comparison with control samples, two significantly upregulated proteins were found, Calcyclin (S100A6) and Calgranulin-B (S100A9); these two proteins are well-known markers of keratinocyte differentiation and proliferation, suggesting their role in neoplastic alterations induced by Mancozeb. The same approach in human keratinocyte carcinogenesis model with HaCaT cells revealed that upregulation of S100A6 and S100A9 confirms the neoplastic potential of Mancozeb. The authors conclude that S100A6 and S100A9 modulate the ERK1/2 signaling pathway underlying in this way the Mancozeb-induced neoplastic potential in human skin [24], and thus, a certain proteome milieu prescribe keratinocyte behavior in a chemically triggered carcinogenesis.

4. Spotting differences between normal and inflammatory keratinocyte proteome pattern

The skin proteome has been the target of intense research in the last years, hence human epidermal keratinocytes, dermal fibroblasts, human epidermis, were characterized regarding their proteome pattern [25–28]. Ong et al. furthered these studies and published the specific proteomic markers in the normal skin and in the one subjected to inflammatory processes. In normal skin, there are several proteins that were reported as having high expression, such as carbonic anhydrase, HSP27, gelsolin, prostate binding protein, MnSOD, a1-antitrypsin, keratin 1 and keratin 10. On the contrary, in keloid scars, there is a low expression or even absence of carbonic anhydrase proving the maintenance of local inflammatory status of the skin. In this manner, the inflamed skin shows intense expression of the proliferative keratin 16 [29]. Other proteomic markers are found to be increased in the inflamed keratinocytes in

comparison to normal skin, such as S100A4 /A8 /A9 /A10 [30]. The over-expression of this protein family was found also in other inflammatory diseases as well as in psoriatic keratinocytes [31, 32]. Over-expression of mast cell proteins was also found in inflamed keratinocytes, namely activation proteins, mast cell b-tryptase, and so on [29]. Mast cell b-tryptase can further induce tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and interleukin-1b (IL-1b) upregulating and also collagen type I and fibronectin expression [33]. Another pro-inflammatory protein found overexpressed in inflammatory keratinocytes, macrophage migration inhibitory factor (MIF), can be involved in the amplification of the inflammatory responses developed during wound healing.

Components of the skin's extracellular matrix (ECM), like the small leucine-rich proteoglycan family members, asporin and decorin, are inhibited by direct binding of the transforming growth factor β (TGF-β) activity [34]. In inflamed keratinocytes, high asporin expression was reported and this overexpression is probably due to the inflammatory response in human dermal wounds [29].

Differences in the proteomic pattern between normal and inflammatory keratinocytes reside in several important classes of overexpressed proteins. These are appending to the inflammation, tumor suppression, and fibrosis processes. The dynamic expression of these proteins can be important in depicting the therapeutic target potential.

5. SCC progression and aggressiveness

Recent studies have compared non-advanced SCC to advanced SCC, in order to identify pathways that are activated in SCC progression. Considering the fact that SCC develops on skin areas exposed to sunlight, UV radiation is found to be responsible for activating signal transduction pathways in the processes of apoptosis, inflammation, proliferation, and differentiation, necessary for SCC development [2].

Molecular marker studies that reflect the initial changes in skin carcinogenesis have shown that the most important gene involved in the ultraviolet radiation effects is the p53 tumor suppressor gene, which plays an important role in apoptosis, cell proliferation, DNA differentiation and repairing process. Mutations of the p53 tumor suppressor gene result in the occurrence of other mutations in cascade with the loss of control of aberrant cell growth, leading to the formation of cancer cells [35, 36].

Also, several biomarkers, such as E-cadherin, Ki-67 and cyclin D1, have been shown to correlate with malignancy in NMSC [37]. Thus, designed to maintain the stability of epithelial tissues, E-cadherin is a Ca(2+) dependent intercellular adhesion molecule, whose downregulation is closely related to the increased potential for tumor invasiveness and metastasis. In SCC, a decrease in E-cadherin expression in the primary lesion is correlated with the development of regional lymph node metastases [38]. Ki-67 is a marker of the cell proliferation and a representative in fast and frequent recurrent aggressive tumors [39]. An important regulator of the cell cycle, cyclin D1 is a proto-oncogene which is essential in the development of skin cancer leading to the organization and abnormal differentiation of tissues [40].

It is known that main factors leading to tumor genesis are mutations in the tumor suppressor genes, such as the APC gene. Thus, mutations at this level lead to the synthesis of a non-functional APC protein that induces β-catenin destruction (the latter being a factor that activates transcription of oncogenes such as MYC and cyclin D1). In addition, 75% of patients diagnosed with cSCC were identified with mutations in NOTCH tumor suppressor genes [41].

Inhibition of squamous cell differentiation has been shown to be one of the most important mechanisms in the development of cSCC. Many therapeutic approaches have been proposed that have had at the forefront this mechanism and the molecules involved. Thus, S100 is a family of Ca-modulated proteins comprising the S100A7-psorasin-protein identified both in the keratinocytes in the psoriatic skin and in the various types of SCC (lung, oral cavity, bladder, skin) with an important role in metastasis and cellular differentiation [42–46]. Furthermore, the gene encoding the information required for the synthesis of this protein is located in the chromosome 1q21 containing the epidermal differentiation complex [47]. Recent studies have highlighted that overexpression of S100A7 is associated with increased cellular survival rates by decreasing cell differentiation, while poor expression of S100A7 correlates with significant cellular differentiation [48].

It has also been found that expression in tumor cells of a member of the signal transducer and activator of phosphorylated pSTAT3 transcription is closely correlated with the depth of tumor invasion and metastasis [49].

A fairly controversial issue has recently been the link between SCC and collagen VII, as a mortality of about 80% has been reported in patients with severe generalized recessive dystrophic epidermolysis bullosa (RDEB) associated with metastatic SCC. Currently, the link between SCC aggressiveness and collagen VII (Col 7) is being debated considering that mortality is high (more than 78%) in patients with severe generalized RDEB with metastatic squamous cell carcinoma. Mutations occurring in COL7AI, the gene encoding information for Col 7 synthesis, cause RDEB [50]. This disease is characterized by the fragility of skin and mucous membranes due to a decrease in Col 7 formation (the main component of the anchored fibrils) leading to formation of blisters and chronic skin trauma (a risk factor for SCC) [50]. There are many studies in progress that try to increase Col 7 synthesis by different methods but it has been observed that high levels of Col 7 are associated with activation of Phosphoinositide 3-kinase pathway which leads to an increase in SCC invasiveness, so there is no formal control of this process [51].

Moreover, tumor clinical factors such as size, anatomical location, tumor thickness, depth of invasion, histopathological subtypes, perineural invasion and inflammation [10, 37] correlate with an increased risk of developing metastatic lesions with significant impact on progression and aggressiveness of SCC. Immunocompromised patients have been shown to have a more aggressive course of SCC. In addition, there is evidence that age and sex can play a role in survival [52]. Although some of these factors provide a perspective on the prognosis and metastatic potential of SCC, they are less used in practice and have not been included in staging schemes [53].

6. Cancer stem cells in SCC

Cancer stem cells (CSC) represent a pluripotent population of tumor cells with self-renewal properties playing an important role in tumor initiation, growth and maintenance [54, 55].

There are many studies, both *in vitro* and *in vivo*, that investigate the involvement of epidermal stem cells in skin carcinogenesis, tumor invasion, metastasis [56, 57] as well as tumor recurrence [58, 59]. Post-initiation, CSCs can generate macroscopic tumors through self-renewal and processes leading to stem cell differentiation generating several cellular variants. Normal epithelial tissue continuously renews and is maintained through the action of proliferating stem cells. When their density decreases, stem cells generate proliferative colonies called holoclones. They bear different characteristics to the abortive colonies of differentiated cells, called paraclones [60]. It is worthwhile highlighting the limited ability of stem cells to renew, making them susceptible to carcinogenesis. This is relevant because it shows the impact of CSC in the development of SCC. It is therefore important to focus on any kind of CSC-related biomarkers that could provide insight into potential therapeutic SCC management schemes.

Cancer stem cells derive either through transformation of normal stem cells (NSCs) or through de-differentiation of tumor cells. Following initial transformations at the level of 17p (TP53) and 3p/9p (p16/FHIT), NSCs give rise to transformed transit-amplifying cells (TACs). These cells first multiply, then expand, and can lead to development of a neoplastic cell field. A further modification of the Rb 13q gene is followed by the inception of the main tumor. Tumor invasion can occur either in a monoclonal or polyclonal cancer inducing way. Thus, tumor CSCs can either spread through lateral migration (CD44h/ALDH1A1h) or they can plant and form a genetically similar tumor, as per the monoclonal model. On the other hand, successive modifications of normal stem cells in the epithelium can lead to the development of independent clones, as per the polyclonal model (**Figure 1**) [61].

Another important biomarker linked to proliferation and differentiation of skin cancers is CD133 [62, 63]. It is a transmembrane hematopoietic stem cell glycoprotein that correlates with an advanced stage of a poorly differentiated tumor, thus having a poor outcome in SCC [64–69]. Several studies have provided evidence that CD133+ CSCs exhibit resistance to apoptosis induced through action on TGF-β, or through tumor necrosis factor. This strengthens the conclusion that new therapeutic agents are required and they need to focus on CD133 being directed at stopping tumor recurrence and metastatic spread [70, 71].

An interesting aspect is that the phenotypic heterogeneity and plasticity of CSC has been associated with epithelial-to-mesenchymal transition (EMT), another important factor linked to both local and remote tumor invasiveness. Although directly responsible for many deaths caused by cancer, its role in SCC is still under debate. EMT is a process involved in embryogenesis and it is designed to create the mesoderm during gastrulation. This is a process through which epithelial cells acquire a migratory mesenchymal phenotype [71]. When the migratory mesenchymal cells mature, they may undergo a reverse process—mesenchymal-epithelial transition, to regain the epithelial phenotype. EMT and non-EMT CSC populations show a strong evidence of CD44 so much that they co-exist transitioning between the two phenotypic states through EMT and reverse mesenchymal-epithelial transition. To note, both cell types are present in oral squamous cell carcinoma (OSCC) generated cells. In addition, recent research that studied the expression of CD44 and epithelial-specific antigen (ESA) clarified that CD44(high)/ESA(low) EMT CSC has a mesenchymal phenotype, while CD44(high)/ESA(high) non-EMT-CSC has epithelial characteristics. To note, EMT CSC requires an ALDH + phenotype (aldehyde dehydrogenase 1) to evolve into non-EMT CSC and to develop metastasis [72].

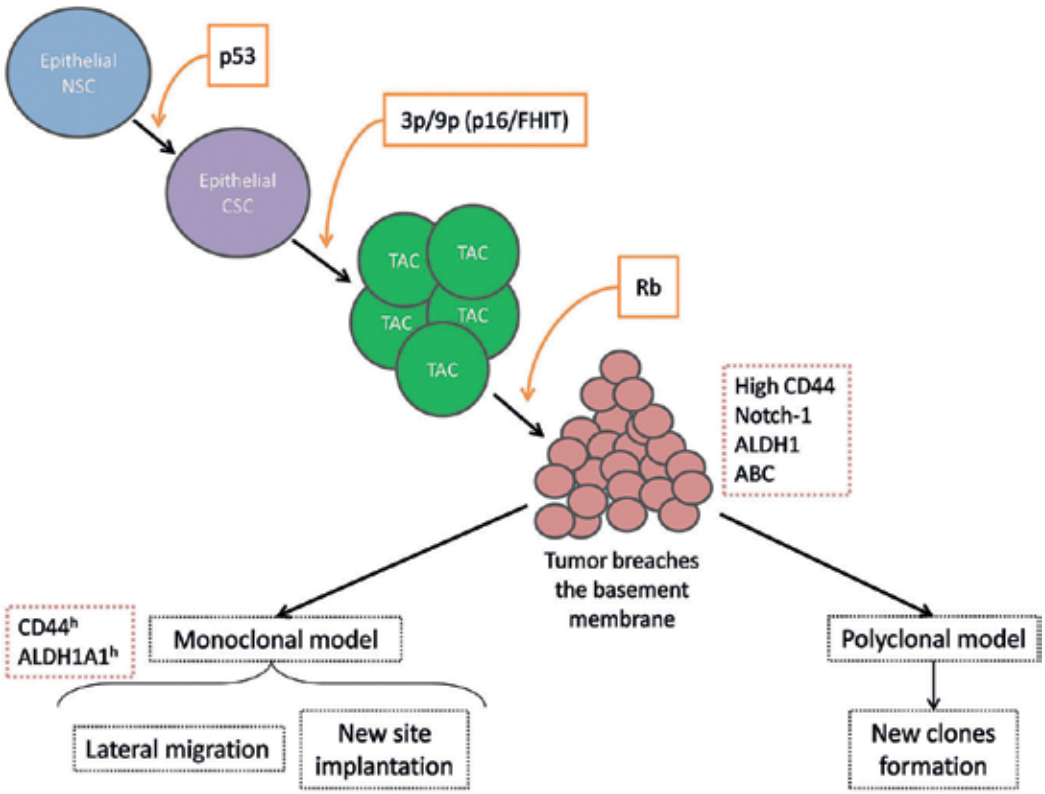

Figure 1. Model for cancer stem cell field onset the process is initiated by a carcinogenic injury producing an alteration (p53/p16/FHIT) in the epithelial normal stem cell (NSC). The cancer stem cell (CSC) will proliferate and form a patch of transit amplifying cells (TAC) which then extends to form a field. At this point, the cells are still in a dysplastic, premalignant stage. It is only after another assault (Rb), one of the field's cells forms the primary tumor. Field cancerization progression takes place through either the monoclonal or polyclonal models. In the monoclonal model, CSCs extend the field by lateral migration (CD44h/ALDH1A1h) or implant at a new site ultimately forming a genetically similar tumor. In the polyclonal model, multiple assaults to epithelial NSCs lead to the evolution of independent clones.

A large number of ALDH1 cells have been detected in lymph node metastases, more than the corresponding primary tumors—indicating the CSC capacity to complete metastatic cascade and to develop metastases.

It has also been shown that a CD44-regulated signaling pathway mediated by the phosphorylation of glycogen synthase kinase 3 (GSK3) can influence CSC phenotypes [73]. Hence, the inhibition of GSK3 reduces the expression of stem cell markers and upregulation of the differentiation markers found in the CD44(high)/ESA(high) cell fraction reversing from EMT and back to the epithelial CSC phenotype [74].

EMT's involvement in several types of cancers such as OSCC [74], breast cancer [75] and others is variable, affecting both tyrosine kinase receptors as well as Wnt signaling pathways [76].

It has been shown that cell lines derived from oral and dermal SCC contain a new population of CSC that influences EMT. It has also been established that EMT is involved not only in

therapeutic resistance but also in tumor recurrence [59, 77, 78] being associated with resistance to epithelial growth factor receptor (EGFR) inhibitors [79]. In epithelial tissues, some stromal signals may induce EMT, leading to downregulation of epithelial processes and upregulation of EMT-inducing transcription factors such as Twist and Snail [80, 81].

7. Current and future molecular therapeutic targets in cutaneous SCC

The high level of gene mutations in UV-exposed skin has hampered the search for novel hints of disease invasiveness and metastatic potential. In SCC, metastasis to regional lymph nodes occurs in roughly 5% of cases and is associated with significant morbidity. Clinical biomarkers of SCC metastasis are currently missing and histological assessment could be unreliable [82]. Cutaneous SCC typically manifests gradually, ranging from a precursor actinic keratosis (AK) to *in situ* SCC, invasive SCC, and finally metastatic SCC. Molecular inquiring into SCC could be done by different experimental models. Although the differences in skin structure amid mice and humans would limit somewhat the correlation with human disease, transgenic mice models have revealed that upregulation of the EGFR/Fyn/Src/Erk pathway acts critical for promoting SCC [83] or UVB-induced cutaneous neoplasia [84].

Latest studies associated omics approaches with humoral immune systems components in SCC involvement; thus recent approaches discern the expression of complement system components in SCC. SCC cell lines and human normal keratinocytes were profiled with an Affymetrix platform and then subjected to quantitative real-time PCR revealing upregulation of complement factor H (CFH) and factor H-like protein-1 (FHL-1) mRNA in cancer cell lines and were proven significantly higher in tumors compared to normal skin. Moreover, immunohistochemistry analysis of CFH and FHL-1 in invasive SCCs, *in situ* SCCs and premalignant lesions (actinic keratoses) showed a specific and stronger expression in SCCs compared with *in situ* carcinoma and actinic keratoses. Not surprisingly, it was found that the level expression of complement factor I (CFI) was higher in the aggressive transformed cell line (RT3) than in less tumorigenic HaCaT cell lines. In addition, by knocking down CFH and FHL-1 expression, proliferation and migration of SCC cells were inhibited, suggesting a role of CFH and FHL-1 in cSCC progression and spotting them as progression markers and potential therapeutic targets in skin SCCs [85, 86].

Huge costs related to skin cancers therapies in general, including SCC became another request for defining reliable biomarkers and better understanding a pathogenesis with significant public health impact. Host immune system influences SCC risk as its incidence is considerably higher in patients with compromised immunity. Very recent studies assign a role for HLA system in SCC risk. Unlike BCC and cutaneous melanoma, SCC often displays partial expression of HLA I proteins, also exhibiting aberrant surface expression of HLA II proteins as a defense mechanism for immune evasion. Analyzing allelic variation and cell-surface protein expression germline of HLA I and II antigens in SCC patients and healthy controls, it was suggested that HLA pattern differs between immunocompetent and immunosuppressed patients regarding the risk for developing SCC. This difference may be owed to some viruses (HIV, HPV) that potentiate tumorigenesis in immunosuppressed patients

[87, 88]. In immunosuppressed HPV-infected patients, it was reported a notable HLA I—SCC connection, probably due to the fact that HLA I processes and presents intracellular peptide antigens, including viral proteins, and thus HPV could be a co-factor of tumorigenesis [87].

Alterations in the composition of basement membrane and dermal extracellular matrix of premalignant lesions are early events in cSCC progression. An influx of inflammatory cells promotes the secretion of proteases, which in turn regulates the availability of growth factors, cytokines, and chemokines and thus influences the growth and invasion of cSCC. Later, the number of inflammatory cells increases with cSCC progression, and the expression of complement factors and inhibitors by tumor cells is induced (CFI, CFH, FHL-1) [89]. A fine interplay between matrix metalloproteinases (MMPs) and their inhibitors could settle the scene for discovering new targets and prognostic or monitoring predictors of the disease. As in cutaneous melanoma, where the role played by MMPs in the phenomenon of regression is an actual approach [90], in SCC, the cellular enzymatic portfolio is a good pool for emerging novel targets coupled to novel biomarkers. For instance, upregulation of MMP-7 expression has also been registered in cSCC, especially in the tumor invasive edge, and moreover activates heparin-binding epidermal growth factor-like growth factor (HB-EGF) promoting cellular proliferation [91] and thus suggesting a future therapeutic effect of HB-EGF antagonists in advanced cSCC [12].

Serine peptidase and their inhibitors (Serpins) are also considered useful for biomarker monitoringing of cSCC progression. Studies performed on serpin family gene expression levels in cSCC cell lines versus normal keratinocytes demonstrate a significantly raised Serpin-A1 expression correlated with the tumorigenic change of keratinocytes [92]. *In vivo* studies correlate Serpin-A1 expression with tumor progression in SCC tumor cells. By using a chemically induced skin carcinogenesis mouse model, as a valuable tool in completing cancer progression profile [23] it was checked the correlation of Serpin-A1 expression with progression of mouse skin SCC [92], suggesting that Serpin-A1 may serve as an useful biomarker for monitoring cSCC progression. Maspin is another member of serpin family—an inhibitor of mammary serine protease—reported as a tumor suppressor in various cancers. Real-time PCR and Western blotting analysis found that Maspin was downregulated in the cSCC tissues compared with the nearby normal tissues. Studies performed on A431 cell line revealed that overexpression of Maspin inhibits growth, cellular proliferation and enhances A431 cells apoptosis by increasing PARP and Bax expression, while decreasing Bcl-2 expression. Therefore, Maspin analysis may provide new insights in the diagnosis and therapy of cSCC [93].

New potential classes of agents for cSCC are also directed to counteract the metastatic feature of this tumor which represents a difficult challenge, knowing that metastatic cSCC has a mortality rate of over 70%. As a comprehensive chemotherapeutic approach in the metastatic form is still lacking, new molecular insights are to be done. Recently, expression of EGFR and nuclear active IκB kinase (IKK) was proved to have a role in metastatic prediction. Thus, a newer and more promising class of agents for metastatic cSCC therapy is represented by EGFR inhibitors. Other advances in finding novel treatments for metastatic cSCC are related to p53 studies, epigenetic approaches such as hypermethylation of specific genes, chromatin remodeling, and the RAS/RTK/PI3K pathway [94]. Molecules with well-established roles in

epithelial adhesion are currently studied regarding their metastatic involvement. Thus, collagen XVII, integrin $\alpha 6\beta 4$ and especially their binding partner laminin 332 are mainly recognized to promote invasion and metastasis in various tumors. By tissue microarray analysis, it was registered that $\gamma 2$ chain of laminin 332 has the highest expression in SCC samples, whereas the expression of collagen XVII and integrin $\beta 4$ greatly differs in SCC and precursors lesions (actinic keratosis and Bowen's disease) [95] and moreover, integrin $\beta 4$ knockdown would reduce the migration of keratinocytes and of malignant cells [96]. All these results suggest the contribution of collagen XVII, integrin $\alpha 6\beta 4$ and laminin 332 to SCC tumorigenesis through their variable expression patterns translated in different migrations and invasion features [97].

Thereby, the tumor microenvironment plays an important role in cSCC progression, offering a genuine reservoir for finding novel targets for both therapeutic purposes and risk assessments in cSCC.

8. Biomarkers of oral SCC

Despite recent advances in diagnosis and therapy, OSCC is still one of the most difficult malignancies to handle due to its great invasive potential both locally and at lymphatic level (in the cervical lymph nodes) [98]. Its occurrence varies across the world as it is closely linked to diet and lifestyle choices (alcohol and cigarettes). OSCC occurs as a result of squamous cells genetic mutations, the new cells developing multiplicative and invasive characteristics [99]. Its genetic heterogeneity can be later highlighted by the fact that many tumors, at a similar stage and location, present significant clinical differences and they can react very differently to treatment. Although the therapeutic strategies are in a permanent development, the survival rate of OSCC patients remains low. It has also been found that predicting treatment outcome using conventional clinical and histopathological parameters carry a low success rate.

It is clear that histopathology remains to this day the benchmark decision-making process as far as diagnosis and treatment are concerned. However, recent molecular studies have made significant progress in understanding and identification of those biomarkers best placed to predict OSCC aggression. Attempts have been made to refine histopathological analysis with immunohistochemistry; this detects gene composition at protein level and brings forward several prognostic tumor biomarkers associated with OSCC's clinical outcome. As such, tumor suppressor genes, oncogenes, angiogenic markers, cell adhesion molecules and cell proliferation markers have been discovered to be potential tools that could help to predict the outcome of OSCC patients [99]. Therapeutic management through molecular inhibition directed at those biomarkers associated with radiotherapy and/or adjuvant chemotherapy are promising treatments for OSCC patients.

EGFR is a transmembrane cell-surface receptor that binds to ligands such as EGF and TGF-α and is one of the most studied OSCC biomarkers. It triggers the activation of the protein-tyrosine kinase system, which acts as a regulator of the signaling process linked to cell multiplication and differentiation [100]. It plays a significant role in OSCC's resilience to radiotherapy.

According to Shiraki et al. [100], cyclin D1 and EGFR together correlate to low survival rates of OSCC patients. It is worth mentioning that despite being an oncogenic gene with a major role to play in tumor invasion, cyclin D1 (independent of EGFR) bears no pathological significance to OSCC.

Recent years have seen a shift toward therapy and prognosis, with a strong emphasis on those molecular biomarkers associated with tumor suppression and apoptosis, especially p53/p63 and Bcl-2 [101]. High levels of Bcl-2 have been proven to correlate to low survival rates of OSCC patients [102–104].

Another important factor in the carcinogenesis of human solid tumors is hypoxia; it is responsible for the adaptive modifications of malignant cells allowing them to survive [105, 106]. Unfortunately, little data is available to help scale its importance within the framework of OSCC prognosis. Antitumor therapy targeting angiogenic biomarkers has been a subject to many recent studies. This is due to the fact that angiogenic processes play a key role in the formation of neo-capillary networks and is essential to cancer growth, progression and metastasis [107]. Thus, the most important angiogenic biomarker involved in carcinogenesis and OSCC tumor dissemination is VEGF which plays a crucial role in the maintenance of tumor vasculature [108, 109].

Tumor invasion is based on several factors, including cellular interaction, requiring both matrix degradation enzymes (MMPs) and cell adhesion proteins (cadherins). MMP is a family of proteases expressed by invasive tumors and adjacent stroma. They were also associated with low survival rates in patients with OSCC without lymph node metastasis [110]. Cadherins are transmembrane glycoproteins with important functions in cell adhesion making them important in tumor invasion and metastasis [111].

9. Biomarkers in genital SCC

Vaginal squamous cell carcinoma (VaSCC) is a tumor with a relatively low occurrence rate of 1–2% of all gynecological malignancies [112], but it can occur in approximately 30% of cervical cancer cases [113, 114]. Despite the low number of studies concerning this type of cancer, epidemiological, virological and clinical-pathological data available show two distinct entities of this genital SCC. They develop through two etiopathogenic pathways: one is linked to HPV infection, while the second is HPV-independent. Available studies do not provide enough information on their significance to the final outcome and they require further investigation. However, it is known that most VaSCCs are closely related to HPV, emphasizing the idea that it shares a common pathway with cervical cancer [114].

As far as the biomarkers linked to genital SCC [23, 115–117] are concerned, their discovery is relevant due to their significant impact on early diagnosis and timely treatment. Numerous studies link p16 expression with a less aggressive form of vulvar SCC and a reduced death rate. On the other hand, patients exhibiting p53 mutation have a worse prognosis, frequent relapses, and greater associated mortality [118]. Other molecular markers with a negative impact on SCC patients are cofilin-1, galectin-7, and wee1 [119]. Moreover, it has been found

that lymphatic invasion and poor tumor differentiation correlate with downregulation of galactin-7 and wee1 [120, 121]. A very important role is played by cofilin which has major implications in carcinogenesis and vulvar SCC invasion [122]. This has turned it into a therapeutic option as it significantly reduces tumor progression. Also, other reports worthy to be considered indicate that downregulation of galectin-7 and high wee1 expressions have been correlated with an increased metastasis risk [120, 121].

Regarding treatment options, surgical resection is associated with a high mortality rate; therefore, attempts are being made to avoid and replace it with radiotherapy associated with chemotherapy [123, 124]. Due to limited options available, there is a real need for new targeted therapies being developed grounded on specific biomarkers.

SCC with penile localization (PSCC) has a relatively low incidence and is associated with poor hygiene, lack of circumcision, HPV infection, and tobacco use [122, 125–127]. Much the same as vulvar SCC, HPV infections play an important role. Starting with the HPV DNA incorporation step into the human genome, E6 and E7 genes deactivate tumor-suppressing genes. Due to the low occurrence rate of PSCC, there are not many studies looking at this type of cancer. These studies have found an increased concentration of Hsp70 [128]. Although not specific to PSCC and present in other types of cancer such as breast, colon, liver, and prostate cancer [129], it is believed to play a protective role for the tumor cells and is thus involved in carcinogenesis. A study looking at families of plaque molecules involved in the binding of filaments, desmosomes, and hemidesmosomes [130] has linked poor expression of plectin (a cytolinker of this family) with rapid cancer progression [131]. Since the diagnosis of inguinal metastases is currently the most important prognostic factor, the discovery of other biomarkers involved in a possible therapeutic management is imperative [128].

10. Conclusions

cSCC is associated with different trigger factors such as UV radiation, especially UVB which induces the alteration of skin layers and therefore the destruction of defensive barrier against external threats, but also the oxidative damage of nucleic acids and proteins through the increased levels of ROS. Therefore, an increased expression of TRIM29 is observed with the survival of differentiating keratinocytes. The chemical factors responsible for inducing SCC are also interfering in the keratinocyte differentiation and proliferation.

The exposure to UV radiation determine mutations of p53 tumor suppressor gene (responsible for apoptosis, cell proliferation, and DNA differentiation) together with the modifications of different biomarkers such as E-cadherin (a decrease in E cadherin expression in the primary lesion is correlated with the development of regional lymph node metastases), Ki-67 (associated with recurrent aggressive tumors) and cyclin D1 (a proto-oncogene which is essential in the development of skin cancer leading to the organization and abnormal differentiation of tissues). Furthermore, the expression of S100A7 which belongs to Ca^{2+}-modulated proteins S100 family is associated with increased survival rate, while its poor expression correlates with significant cell differentiation.

Another important role in carcinogenesis is attributed to cancer stem cells which derive from the transformation of normal cell or through the differentiation of tumor cells migrating through normal tissue. CD 133 is one of the most important biomarkers linked to proliferation and differentiation of skin cancers so that new therapeutic targets are needed to be focused on this transmembrane hematopoietic stem cell glycoprotein.

Regarding the molecular aspects of cutaneous SCC, studies have shown not only the high levels of complement factor H and factor H-like protein 1 mRNA in comparison with normal skin, but also stronger expression in SCC than *in situ* carcinoma or actinic keratosis. In addition, it was demonstrated that knocking down CFH and FHL-1 lead to the inhibition of proliferation and migrations of SCC cells, suggesting their importance as progression markers and potential therapeutic targets in skin SCC.

An important aspect in risk evaluation for SCC is the integrity of immune systems. The high incidence of this malignancy in patients with compromised immune system was observed, pointing out the role of HLA system which varies between immunocompetent and immunosuppressed patients.

Other biomarkers involved in SCC development and progression are MMPs, serine peptidase and their inhibitors (Serpin-A1 being associated with tumorigenic change of keratinocytes and tumor progression). The metastatic prediction is attributed to EGFR and nuclear active IκB kinase (IKK) expression, thus a promising cSCC therapy is represented by EGFR inhibitors.

Oral squamous cell carcinoma is associated with EGFR that not only activates the protein-tyrosine kinase system involved in cell multiplication and differentiation, but also plays an important role in OSCC resilience to radiotherapy. Others biomarkers associated with OSCC are p53/p63 and Bcl-2. Tumor invasion is correlated with both matrix degradation enzymes and cell adhesion proteins.

Genital squamous cell carcinoma is linked with p16 (in less aggressive form of vulvar SCC), p53 (weaker prognosis), cofilin-1, galectin-7 and weel. HPV infection plays an important role in SCC induction and evolution through the deactivation of tumor suppression genes by E6 and E7. Increased Hsp70 is also increased in this type of cancer.

Overall, skin cell carcinoma is one of the most frequent malignancy worldwide that even if it is easily treated and the cure rate is high, there are cases when metastasize can occur. An accurate clinical exam correlated with histological, immunohistochemical and proteomic investigation can establish the biomarkers involved in the development and evolution of this malignancy and reveal the appropriate treatment strategy for each patient.

Due to the fact that SCC is associated with frequent recurrence and sometimes metastasis, it is necessary to realize the study of biological transformation that occurs in these types of cancers. The discovery of various biomarkers can outline the occurrence, evolution and the prognosis of this keratinocyte-derived tumor.

It is important to focus on the analysis of normal, inflammatory and malignant keratinocyte proteome in order to determine novel biomarkers that are associated with the development and progression of SCC and therefore can be used in the early detection, risk assessment, tumor monitoring and also discovery of new therapies for these patients.

Acknowledgements

This paper is partly supported by grants PNII-PT-PCCA-2013-4-1386 (Project 185/2014) and PN-II-PT-PCCA-2013-4-1407 (Project 190/2014) financed by Executive Agency for Higher Education, Research, Development and Innovation.

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Chapter 7

Correlation between Porcine and Human Skin Models by Optical Methods

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.75788

Abstract

Background: Topical photodynamic therapy (PDT) using 5-aminolevulinic acid (ALA) and methyl aminolevulinate (MAL) as precursors of protoporphyrin IX (PPIX) have been used in skin cancer treatment and other skin diseases. To establish new topical PDT, protocols are necessary first to conduct studies *in vivo* using animal skin models. The goal of this study is to evaluate the robust correlation between porcine and human skin models *in vivo* by optical methods to confirm the suitability of porcine skin models to predict drug behavior in the human skin on topical PDT protocols. Methods: The study was performed *in vivo* using porcine and human skin models. In human skin, ALA and MAL cream mixture samples were applied to the inner arm in a circular area of 1 cm^2. In porcine skin, the cream was applied on the back in an area of 4 cm^2, over which an occlusive dressing was placed. PPIX production was monitored for up to 5 h using widefield fluorescence imaging and fluorescence spectroscopy techniques. Results: Human skin models showed similar behavior to porcine skin models, which indicates high similarity between both models and confirms that porcine skin is an adequate model to establish new clinical PDT protocols in human volunteers.

Keywords: 5-ALA, MAL, porcine skin, human skin, widefield fluorescence imaging, fluorescence spectroscopy, photodynamic therapy, skin models

1. Introduction

Photodynamic therapy (PDT) constitutes an alternative therapy in the treatment of cancer and skin diseases. The photodynamic reaction comprises the interaction of a photosensitizer (PS), light (lasers, lamps, and LEDs), and oxygen present in the tissue. The photochemical process occurs when the PS absorbs light in one specific wavelength, interacts with subtracts and oxygen, and produces reactive oxygen species (ROS) and singlet oxygen ($^1O_2$), which are the main causes of PDT damage [1, 2]. Topical PDT using topical medication such as 5-aminolevulinic acid (ALA) and its methyl ester (methyl aminolevulinate [MAL]), has been widely employed to treat skin cancer, skin diseases, and aging skin [2–4]. When methyl, ethers, and other groups are added to ALA, its derivatives become more lipophilic, thereby increasing permeability through the skin [5].

ALA and MAL act as precursors of protoporphyrin IX (PPIX), an endogenous PS produced by mitochondria on cells [2]. While ALA and MAL application on PDT has the advantage of being localized and nonsystemic (transdermal application), it has some limitations as regards penetration through the skin [2, 6, 7].

ALA is a hydrophilic compound, making it difficult to cross the biological barriers of the skin, such as cell membranes. However, it has high efficiency in the production of PPIX. On the other hand, MAL has a lipophilic character allowing it to be transported by nonpolar amino acids via passive diffusion (does not require a driver) facilitating the ability to move across biological barriers reaching higher penetration in the desired tissue, and at a lesser cost than the production of PPIX [8, 9].

It is known that PPIX formation by ALA and MAL application in carcinomas is different to PPIX formation in healthy skin, thus there are few studies comparing ALA and MAL in healthy human skin. Lesar et al. compared the production efficiency of PPIX by the application of ALA and its precursors in various parts of the human body [10]. However, in our study we compared ALA, MAL, and mixtures from both on porcine and human skin models. ALA and MAL as precursors of PPIX were chosen in our study since they are used most in clinical topic PDT [11]. Many types of animal models have been suggested to replace human skin in research on transdermal permeation of molecules [8, 12], including primate, porcine, mouse, rat, guinea porcine, and snake models. Nowadays, the use of primates in research is highly constrained [8]. On the other hand, similarities between porcine skin and human skin models have been discussed in many papers [8, 13].

Animal skin differs morphologically from that of human skin with respect to epidermis and dermis thickness, hair follicles, and other characteristics. Despite their many similarities, porcine and human skin differ regarding structure, immunohistochemistry, and function. Notwithstanding, porcine appears the most suitable animal type to replace human skin in test models [14]. Indeed, porcine constitutes the nonrodent species of choice in the preclinical toxicological testing of pharmaceuticals [13].

The prospect of decreasing the number of human volunteers in studies using *in vitro* and *in vivo* methodologies is an advantage in the development of drugs at pharmaceutical companies

[8, 10]. *In vitro* studies using porcine ear skin as a model for human skin have produced positive results, suggesting a high similarity between both skin models [15].

Research has shown that skin barriers vary among species as regards the amount of free fatty acids and triglycerides and density of hair follicles [8]. Stratum corneum (SC) lipid composition (ceramides, free fatty acids, cholesterol, and cholesterol esters) and organization in biological membranes differ from one species to another. According to Godin, the lack of correlation in transdermal drug permeation among species or different application sites in the same animal model is mainly due to variations in skin thickness, the composition of intercellular SC lipids, and a number of skin shafts [8]. Bearing this in mind, research has shown that porcine ear skin is anatomically similar to human skin regarding lipid composition, which confirms its suitability for use as a new animal model to study adnexal glands. In addition, its anatomic and physiologic characteristics with respect to cardiovascular, urinary, integumentary, and digestive systems are similar to those of human skin [13, 16].

Many authors claim that porcine skin models constitute the most relevant animal model for human skin because porcine skin and human skin have similar histological and biochemical properties [8, 9, 15]. Porcine skin is structurally similar to human skin regarding epidermal thickness and dermal–epidermal thickness ratio; their dermis thickness is approximately 3 mm and their SC and epidermis thicknesses are in the region of 21–26 and 70 μm, respectively [8, 13]. The collagen fiber arrangement in the dermis and the SC proteins (glycosphingolipids and ceramides) present in the porcine skin are also similar to those of human skin [8].

While the vascular anatomy of human skin is superior to that of porcine skin, neonatal porcine skin has the same structure, including sweat glands and hair follicles (730 follicles/cm^2), as opposed to 10 follicles/cm^2 found in adult porcine skin [8, 17]. In this way, in this work we performed the tests in animals of 3–4 months of age.

Nowadays, many scientists consider porcine skin a suitable and readily available model for the human skin barrier and often employ it to test topical and transdermal pharmaceutical formulations both *in vivo* and *in vitro*. Indeed, its application in *in vitro* testing is increasing rapidly. Many studies using porcine skin models have compared its permeability with that of human skin and the results show high similarity [18].

Although several studies indicate similarities between porcine skin and human skin models, predictions about drug behavior in human skin based on results from tests using animal models are still under debate. Some authors believe that animal models constitute useful tools in biomedical research, but remark that effects obtained with animal models are not readily transferable to clinical settings [19].

The purpose of this work is to verify whether there is a robust correlation between porcine and human skin models and, if so, confirm that the porcine skin model is the best alternative to prediction studies with human skin volunteers using optical techniques.

In the previous study [19], porcine skin was studied, and in this chapter we can evaluate the correlation between both models. Seven different samples (ALA, MAL, and mixtures from both) were applied to human and porcine skin and their PPIX production was monitored using widefield fluorescence imaging and fluorescence spectroscopy techniques.

2. Materials and methods

2.1. Chemicals

The PPIX precursors used in this study were ALA and MAL (final concentration of 20%), which were dissolved in different proportions in an oil-in-water (O/W) emulsion. Seven samples (ALA, MAL, and mixtures from both) were prepared in the following proportions: M1 (100% ALA), M2 (80% ALA and 20% MAL), M3 (60% ALA and 40% MAL), M4 (50% ALA and 50% MAL), M5 (40% ALA and 60% MAL), M6 (20% ALA and 80% MAL), and M7 (100% MAL). The emulsion or cream preparation was previously described [19].

Commercial ALA and MAL were obtained from PDT-PHARMA (Cravinhos, São Paulo, Brazil) and were prepared immediately prior to use without previous solubilization because the drug presents elevated solubility in the base cream used.

2.2. Human study approval

This study used a protocol along the lines of the procedures established by Brazil's Human Research Ethics Committee (no. 13556713.8.0000.5504). In addition, a written informed consent was obtained from all participants. Ten female patients aged around 25 years with the clinical diagnosis of normal skin were recruited for this study. To be considered eligible, a patient had to be free of skin disorders on both arms. The volunteers were all women to decrease the variable numbers in the study.

All patients had either Fitzpatrick skin type II (50%) or III (50%). Patients with lesions on the target area or with porphyrin were excluded. Additional exclusion criteria included male volunteers, pregnant or lactating female volunteers, those allergic to ALA and MAL, and volunteers less than 20 and more than 35 years of age. The volunteer protocol followed the standardization previously described [2].

2.3. Topical application of the skin models

The study was performed *in vivo* in human and porcine skin models using ALA, MAL, and mixtures from both cream applications (samples: ALA, M2, M3, M4, M5, M6, and MAL). The cream was applied with a sterile spatula, with a density of 32 mg/cm^2, in tests involving both animals and human volunteers.

In human skin, the cream was applied in seven areas (a circular area of 1 cm^2 each) on each volunteer's right or left inner arm (10 woman human volunteers). Before cream application, the area was cleaned with serum. Skin autofluorescence was used as the control for each cream sample (ALA, M2, M3, M4, M5, M6, and MAL). Data were compared for each treatment group for the same volunteer and among volunteers.

In porcine skin, the cream was applied in a square area of 4 cm^2 (applied on the back). To prepare the cream application it was necessary to remove the hairs from the back. Animal testing involving porcine skin models was performed as described above with seven cream samples (ALA, M2, M3, M4, M5, M6, and MAL) in triplicate (in regard to conditions as well as experiments) [19].

The areas were dressed with an occlusive mask to protect them from light. The cream was removed prior to autofluorescence collection at the skin by fluorescence spectroscopy and widefield fluorescence imaging. After collection, the same amount of cream was applied again under the same conditions at every full hour during 5 h. The experimental procedure was previously described (**Figure 1**) [2, 19].

2.4. Fluorescence collection: widefield fluorescence imaging and fluorescence spectroscopy

A widefield fluorescence imaging system was used for image acquisition. It is a commercial device, produced by MM Optics, Sao Carlos, Brazil, called EVINCE. In brief, the widefield imaging system consists of a lighting device based on LEDs, emitting around 405 nm coupled to a digital camera for image acquisition [2, 19, 20]. The measurements were taken for each sample and different times of PPIX formation. The images obtained by widefield fluorescence were assessed quantitatively using a routine written in Matlab program. The program has defined an array separating the red, green, and blue (RGB) channel colors.

For pixel count analysis, standardization was obtained dividing the red channel by the green channel. This was done to avoid the effects of different shutter speeds and ISO settings for image acquisition. Finally, fluorescence (pixel count) was determined by summing the values of all red channels divided by the sum of all values of the green channel. With these data, it was possible to quantify PPIX production (which shows red fluorescence) in amount and uniformity on the skin extension [2, 19].

For fluorescence spectroscopy analysis, a system with two excitation lasers was used, emitting at 408 nm and 532 nm, respectively. The investigation fiber probe in the Y shape directs the excitation laser to the tissue, while the second arm of the Y shape collects the re-emitted light from the tissue and delivers light to a spectrophotometer. The spectrophotometer used was the USB2000 (Ocean Optics®, USA). A filter was used to remove backscattered light so that only the fluorescence was collected [2, 19, 21].

We recorded the fluorescence spectrum in contact with the tissue at five points in the area where the cream was applied, and equivalent autofluorescence collection was performed. The evaluations from spectral analysis were normalized by total area and subtracted from

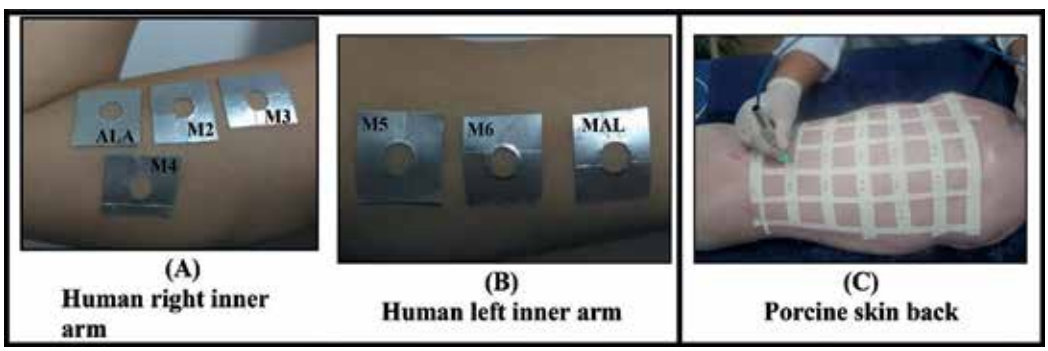

Figure 1. Application of different cream samples (ALA, M2, M3, M4, M5, M6, and MAL) at the skin: (A) human right inner arm; (B) human left inner arm; and (C) porcine skin back.

the autofluorescence. The spectrum evaluations were performed using an Origin 9 program as previously described in our publication [2, 19]. A spectrometer and widefield fluorescence imaging equipment collected skin fluorescence at every full hour during 5 h. At the end of the fluorescence analysis, the cream mixtures were reapplied to the treatment area and covered with an occlusive dressing.

3. Results

Figure 2 shows the PPIX formation after 3 h of cream incubation for human and porcine skin obtained by widefield fluorescence imaging. **Figure 3** shows the quantitative analysis of the PPIX formation by counting pixels of the images and spectroscopy collection acquired by fluorescence techniques. The result of this analysis was acquired by means of the average data collected from volunteers and animals. The results of the porcine skin model were also published previously [19]. The results in **Figure 3** show that the high amount of PPIX production in human and porcine skin occurs for all cream samples (ALA, M2, M3, M4, M5, M6, and MAL) after 3 h of cream application.

Fluorescence imaging shows that PPIX production is heterogeneous for healthy skin in both skin models. Even so, it is possible to verify the differences in PPIX formation to ALA, MAL, and mixtures from both. The results suggest that PPIX formation is greater for ALA than for MAL for both models. In addition, PPIX formation of all sample cream mixtures from ALA and MAL (M2, M3, M4, M5, and M6) was more elevated than MAL and is similar to ALA.

In addition, it is important to mention that for porcine skin preparation it was necessary to shave the back, and for human skin preparation the area was cleaned with physiological serum. This previous skin preparation can interfere with cream sample penetration on the skin as well as PPIX production. This explains the lowest PPIX production for all samples in human skin models when compared to porcine skin models (**Figure 3**).

Figure 4 shows the kinetics study on human skin only, wherein the monitoring of PPIX production was carried out during 5 h, and the measurements were carried out hourly for both

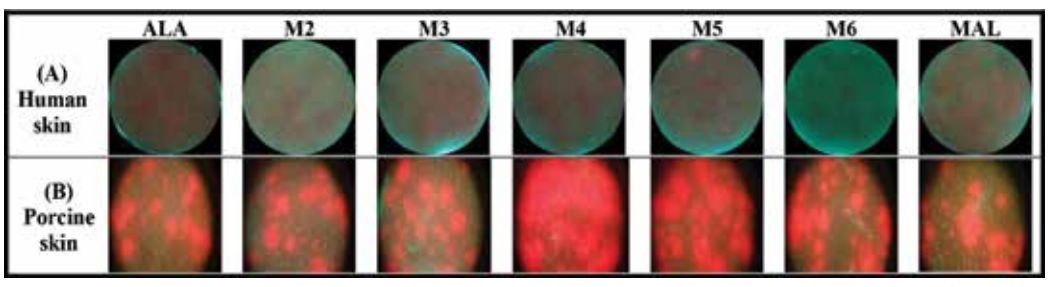

Figure 2. Widefield fluorescence imaging after 3 h of cream sample application (ALA, M2, M3, M4, M5, M6, and MAL): (A) PPIX production in human skin (inner arm) and (B) PPIX production in porcine skin back.

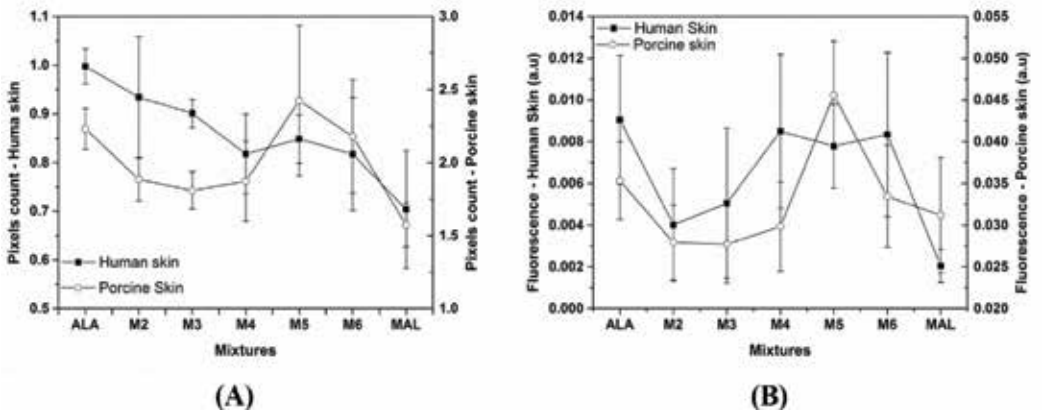

Figure 3. Analysis of PPIX production in human and porcine skin models after 3 h of cream sample application evaluated by (a) widefield fluorescence imaging and (B) spectroscopy fluorescence.

fluorescence techniques. From this study, it was possible to analyze which sample PPIX was produced quickest, and by means of a parameter called the index of fluorescence (IF_{50}) it was possible to quantify the PPIX production in minutes. IF_{50} means 50% of maximum fluorescence value obtained for 5 h.

Although **Figure 4** presents the results of the human skin study, **Table 1** shows the IF_{50} results for the study in human and porcine skin. We have included it here for best comparison. The graphs of the kinetics study in porcine can be observed in previously published work [19].

The results for the kinetics study in the human skin model show that PPIX production is faster with ALA than MAL and cream samples (M3, M4, M5, and M6) for both fluorescence analyses. However, the mixtures M3, M4, and M5 presented better results for PPIX production than MAL in the kinetics study (IF_{50} values). These results suggest that these differences may be due to high variability in human skin experiments. Perhaps these differences can be reduced by using a number of human volunteers.

The results for the kinetics study from **Table 1** show that PPIX production in 5 h (IF_{50}) in human skin models is faster for ALA than MAL, and the opposite occurs for porcine skin models where PPIX production is faster for MAL than ALA. This can be explained by the previous preparation for porcine skin where we can suggest that PPIX production by MAL can be optimized. The other sample creams (M3, M4, M5, and M6) show the same behavior considering the standard deviation.

The values found for IF_{50} through the widefield fluorescence imaging data were closer to human and porcine skin models than the IF_{50} values collected by fluorescence spectroscopy. We believe that this occurred because PPIX production is heterogeneous and the fluorescence spectroscopy measurements are punctual. This punctual fluorescence collection data of PPIX production can suggest false negative or false positive results. On the other hand, by using widefield fluorescence imaging, we can evaluate all PPIX production on the skin surface.

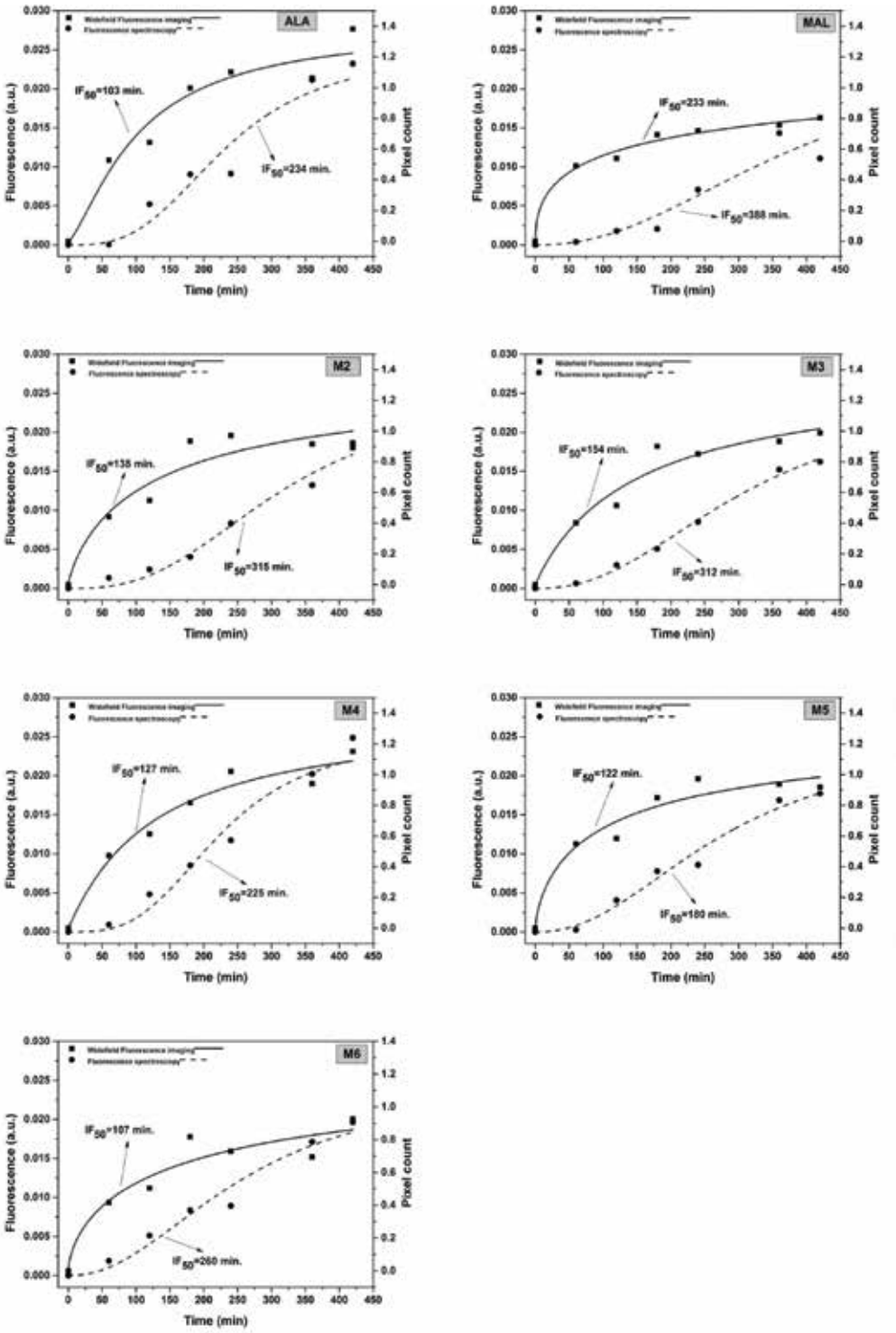

Figure 4. Kinetics of the PPIX production in human skin models by fluorescence spectroscopy and widefield fluorescence imaging evaluations for all cream samples (ALA, M2, M3, M4, M5, M6, and MAL).

Samples	Widefield fluorescence imaging IF_{50} (min)		Fluorescence spectroscopy IF_{50} (min)	
	Human skin	Porcine skin	Human skin	Porcine skin
ALA	103 ± 15	120 ± 10	234 ± 18	230 ± 7
M2	138 ± 27	134 ± 6	315 ± 13	
M3	154 ± 18	40 ± 60	312 ± 6	114 ± 16
M4	127 ± 14	128 ± 8	225 ± 13	17 ± 20
M5	122 ± 21	97 ± 7	280 ± 12	131 ± 17
M6	207 ± 38	120 ± 20	260 ± 15	187 ± 25
MAL	233 ± 18	70 ± 5	388 ± 37	131 ± 9

Table 1. IF_{50} values for widefield fluorescence imaging and fluorescence spectroscopy collected over time (5 h) after sample cream application on human and porcine skin model surfaces.

Figure 5 shows the correlation linear fitting to fluorescence measurements obtained through widefield fluorescence imaging and fluorescence spectroscopy. The fitting in **Figure 5** shows that the red fluorescence signal emitted by PPIX in the porcine and human skin was measured at 3 h following application of ALA and MAL cream mixtures. These results confirm that there is a correlation between both models since the equation line factor obtained was 0.9824, bordering on 1.0, the ideal linear fitting number.

The same linear fitting was performed for fluorescence collected through fluorescence spectroscopy (results not presented here). However, we do not find a correlation between both models (human and porcine skin) by this optical technique due to high variability during fluorescence spectroscopy collection.

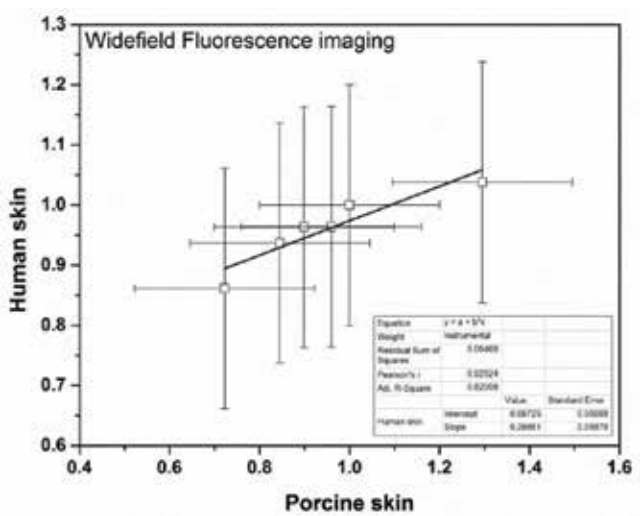

Figure 5. The best correlation analysis between human and porcine skin models by widefield fluorescence imaging.

The fitting results shown in **Figure 5** indicate the best correlation between porcine and human skin models by widefield fluorescence imaging measurements. The possibility of predicting drug behavior on transdermal skin application promotes the success of clinical topical PDT treatment.

4. Discussion

The aim of the study was to evaluate PPIX formation due to ALA, MAL, and cream sample mixtures from both (M2, M3, M4, M5, and M6) application on normal skin models (porcine and human) and then to show that there is a narrow correlation between both models. In this work we use ALA and MAL on topical application as the precursor of PPIX, since these are the most common drugs applied to clinical topical PDT. The fluorescence measurements were collected after 3 h of cream incubation time since this time is also applied to clinical PDT [19, 22].

In our group [22] the clinical PDT studies on skin cancer are done using 20% ALA and MAL cream application in 3 h of incubation time before light irradiation. During this time, PPIX production is elevated since the previous preparation was performed (curettage).

The fluorescence measurements were done using two techniques: fluorescence spectroscopy and widefield fluorescence imaging. With fluorescence spectroscopy using a 532 nm laser (green light) it is possible to evaluate the skin at greater depths (reaching the dermal papillae) when compared with widefield fluorescence imaging using a 405 nm LED (violet light) bringing images from PPIX on the superficial skin [2, 19].

In the study the choice of animal age had great influence; in agreement with the literature the thickness of porcine skin is similar to human skin at around 2 months after birth [8, 13].

PPIX formation on normal skin is not homogeneous and depends on ALA, MAL, and mixtures from both (M3, M4, M5, and M6) penetration through the skin; evaluations using images by widefield fluorescence imaging can be useful and decrease the variability on experiments. Fluorescence spectroscopy evaluation, despite being collected punctually, which can lead to erroneous measurements and high variability, reveals information about PPIX formation on the deeper skin [2, 19] and is important to understand the replacement mechanism of PPIX from deeper layers up to superficial skin layers.

As shown by Valentine et al. [23], there was no difference after increasing the amount of PPIX using ALA and MAL when analyzed by fluorescence spectroscopy using a laser emission at 405 nm (violet light). Fluorescence emission due to 405 nm illumination allows us to measure the output of PPIX on the superficial skin (stratum corneous and superior epidermis). In our work, this superficial skin analysis was performed using widefield fluorescence imaging.

There are few studies concerning the comparison of ALA and MAL in healthy human skin, but Lesar et al. [10] compared the formation efficiency of PPIX from these precursors in various parts of the human body (arm, forearm, back, and legs) with fluorescence (4–29 h) after topical application. They then observed that there were differences in PPIX production, which applied regardless of where the ALA accumulated more PPIX, but the location (back) where they applied the tape striping difference was only after 24 h.

The kinetics study observed that ALA, M4, and M5 indicated the least time of PPIX production (high PPIX production velocity) at the skin. Both studies, human and porcine skin, showed the same behavior. IF_{50} values acquired by widefield fluorescence imaging for both models were very close, with the exception of M3, M6, and MAL. Thereby, it is possible to appreciate the similarity of porcine skin with human skin by first performing clinical tests on porcine skin.

However, it is known that *in vitro* and *in vivo* experiments using the same species show less variability than experiments using human volunteers. The authors suggest that human experiments are done using a greater number of volunteers. The measurement of the correlation coefficient proved that porcine and human skin models have the same behavior with respect to the production of PPIX in quantity as well as in speed of PPIX production through optical methods. The correlation coefficient is a measure of how well the predicted values from a forecast model fit with the real data. We suggest that the best correlation is between porcine and human skin by widefield fluorescence imaging, suggesting this optical method as an important tool to develop new clinical topical PDT protocols.

5. Conclusion

The correlation found between human and porcine skin models measured by widefield fluorescence imaging confirms that porcine skin can be used for establishing human protocols in clinical topical PDT using ALA, MAL, and mixtures from both. The capacity of porcine skin models to predict PDT results in humans can be beneficial to clinical studies optimizing PDT treatment on patients.

Acknowledgements

The authors acknowledge the financial support of the National Council of Technological and Scientific Development (CNPq process 140370/2012-9) and São Paulo Research Foundation (FAPESP/CEPOF). Expressive thanks are extended to Ms. Michelle Requena and all the volunteers for their participation in this study.

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Chapter 8

Molecular Mechanisms and Biomarkers of Skin Photocarcinogenesis

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.70879

Abstract

Skin cancer is the most commonly diagnosed malignancy in the United States and worldwide. While melanoma is the deadliest form of skin cancer, non-melanoma skin cancers, which include basal cell carcinoma and squamous cell carcinoma, are responsible for significant morbidity in millions of Americans each year. While numerous attempts have been made to reduce skin cancer risk factors related to ultraviolet radiation exposure, skin cancer incidence continues to rise. Improved understanding of the molecular pathways underlying skin cancer pathogenesis has led to the investigation of new approaches to skin cancer prevention. In particular, the search for ultraviolet radiation associated biomarkers of skin cancer has become a rapidly expanding and promising area of research. Advances in genetic sequencing have facilitated the discovery of novel biomarkers, which have the potential to profoundly improve patient care. Here we will review the molecular genetics of skin cancer and analyze the existing literature of proposed biomarkers for potential use in skin cancer diagnosis and prevention.

Keywords: ultraviolet radiation, UV biomarkers, basal cell carcinoma, squamous cell carcinoma, actinic keratosis, UV signature

1. Introduction

Skin cancer is the most common type of cancer in the United States and is estimated to affect one in every five Americans [1, 2]. Skin cancer can be classified as either non-melanoma skin cancer (NMSC), which includes basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), or melanoma skin cancer. The costs associated with the treatment of skin cancer are substantial and has placed a significant burden on the healthcare system. In the United States, it is estimated that the combined medical expenditures for the treatment of all skin cancers cost approximately $8.1 billion dollars per year [3].

Unlike many other malignancies, skin cancer is largely preventable in the majority of cases. Ultraviolet radiation (UVR) is strongly associated with cutaneous malignancies and is a primary environmental risk factor for the development of all types of skin cancer [4]. While numerous public health initiatives have increased skin cancer awareness, guidelines developed to mitigate the risks associated with UV exposure are not regularly practiced by many Americans and sunburn rates remain high [5]. Furthermore, recreational and indoor tanning still remains popular among certain groups within the population, which leads to excess UVR exposure [6]. Although most associate sunburns with UV overexposure, few recognize that the increase in melanin production from tanning is triggered by direct UVR damage to skin cells [7]. Repeated exposure to intense UVR in the form of sunburn or tanning can result in cumulative damage within skin cells, leading to cell dysregulation. These sun damaged, cancer-prone cells may exist in the skin for years or even decades before becoming visibly apparent in the form of cancerous or precancerous lesions.

Various strategies to encourage sun protective practices and interventions to modify sun related behaviors have had limited success. Low compliance with UV protection guidelines, particularly among young adults, has been attributed to various factors including inconvenience of sunscreen application and societal attitudes toward tanned skin as a sign of beauty [5, 8, 9]. In addition, the long delay from the time of UV exposure to carcinogenesis lowers risk perception of UVR as being dangerous [10]. Although primary prevention of skin cancer is ideal and remains core to decreasing disease incidence, patient risk stratification following UV exposure is critical for both early detection of skin cancer and prevention of worsening disease.

Biomarker discovery has come to play an increasingly important role in both disease diagnosis and prevention. The application of biomarker-based tests has led to revolutionary changes in medical screening, diagnosis, and targeted therapies for a variety of cancers [11]. While the use of biomarkers has become incorporated into the standard of care for numerous malignancies, the application of biomarker studies within NMSC has not yet been clearly established. By identifying UV biomarkers of NMSC, patients can be risk stratified to ensure routine skin cancer screening and pointed efforts can be made to encourage timely lifestyle changes.

Here we review the relevant literature regarding skin cancer pathogenesis, molecular genetics, and potential biomarkers for use in NMSC. In addition, we will critically analyze the strengths and limitations of various types of biomarkers and detection methods. Due to the significant differences that exist at the molecular level between the development of melanoma and NMSC, an in-depth discussion of related literature in melanoma research is beyond the scope of this review. As such, this discussion will focus on biomarker discovery and its applicability to NMSC diagnosis and prevention.

2. Non-melanoma skin cancers

Each year in the United States over 5.4 million cases of NMSC are treated in more than 3.3 million people [2]. Actinic keratosis (AK), the most common precancerous lesion, affects

more than 58 million Americans and represents the large number of individuals at risk for the future development of NMSC [12]. While genetic factors such as skin type and family history contribute to an individual's risk of development of skin cancer, UVR exposure and age are the leading risk factors in skin cancer pathogenesis. It is estimated that approximately 90% of NMSCs are associated with excessive exposure to UVR and incidence increases with age [13, 14]. Other general risk factors include chronic arsenic exposure, radiation therapy, photosensitizing drugs, certain genetic disorders, and prolonged immunosuppression [15].

2.1. Basal cell carcinoma

Basal cell carcinoma is the most common cutaneous malignancy and represents approximately 80% of all NMSCs [16]. BCCs arise secondary to malignant transformation of cells in the basal layer of the epidermis and its appendages. While BCCs can be aggressive and destructive to surrounding tissue, it has low metastatic potential: estimated at only 0.0029–0.55% [17]. The majority of BCCs arise sporadically and only a small number of cases are inherited, which typically arise within the setting of syndromic disorders such as nevoid basal cell carcinoma syndrome. It is estimated that approximately 70% of BCCs occur on the face and 35% of patients with one BCC will go on to develop another BCC within 5 years [18]. Based on histologic examination, BCC can be classified as nodular, infiltrative, micronodular, superficial, sclerosing, or morpheaform subtypes. Analysis of the histologic growth pattern and cell differentiation is critical as it provides a means to further categorize the lesion as a high or low risk BCC [19]. Infiltrative, sclerosing, morpheaform, and micronodular BCCs are considered high-risk subtypes given the higher likelihood of subclinical spread and more frequent local recurrence [20]. However, patient treatment and prognostication are frequently complicated by the fact that more than 30% of BCCs have a mixed pathology that combines both less aggressive and more aggressive subtypes within the same lesion [21].

2.2. Squamous cell carcinoma

Cutaneous squamous cell carcinoma arises due to malignant proliferation of epidermal keratinocytes and is the second most common type of skin cancer. In a 2013 meta-analysis, the number of new SCCs (exclusive of SCC in situ) in the United States was estimated to be between 186,157 and 419,543 cases [22]. While a number of genetic disorders are associated with increased risk of both BCC and SCC, many hereditary syndromes are skewed toward development of cutaneous SCC (**Table 1**) [23]. Actinic keratosis, which results from the proliferation of atypical epidermal keratinocytes, is strongly associated with UV exposure and is considered a precancerous lesion of SCC [24]. Although some have suggested that AKs are also precursors of BCCs, this association is less clear [25]. Most AKs will not progress to SCCs and many will either regress or persist as AKs [26]. While the likelihood of an individual AK progressing to SCC is low, the presence of AKs is a marker of chronic sun damage and indicates an increased risk of NMSC [27, 28]. In general, SCC is considered highly curable and metastasizes in less than 1–5% of cases [29, 30]. Whereas specific subtypes of BCC are associated with higher or lower risk, there is less consensus as to which features best characterize the aggressiveness of cutaneous SCC. Acantholytic, adenosquamous, and desmoplastic

Fanconi anemia
Xeroderma pigmentosum
Ferguson-Smith syndrome
Rothmund-Thomson syndrome
Oculocutaneous albinism
Epidermolysis bullosa
Epidermodysplasia verruciformis
Dyskeratosis congenita
Bloom syndrome
Werner syndrome

Table 1. Hereditary syndromes predominantly associated with increased risk of cutaneous SCC.

subtypes are recognized as high risk variants; however, traditionally low risk variants may achieve high risk status based on tumor features including size, location, and lesion depth, which have been independently correlated with SCC risk [31].

3. Role of ultraviolet radiation in the development of NMSC

The link between UVR exposure and NMSC is well established [32]. Sun light exists on the electromagnetic spectrum and is divided into ultraviolet, visible, and infrared light based on wavelength. UVR is further broken down into three subtypes: type A (UVA), type B (UVB), and type C (UVC), which, again, are distinguished from one another by wavelength [32]. Of the UVR that reaches earth, 97.5% is comprised of UVA (315–400 nm) and 2.5% is UVB (280–315 nm) [33]. UVC is entirely absorbed by the atmosphere; however, UVC (200–290 nm) is emitted by man-made sources such as welding torches and mercury lamps. Thus, by and large, UVA and UVB are the most clinically relevant sources of UVR as it pertains to skin cancer [34].

While the daily dosage of UVB is significantly less when compared to UVA, UVB is considered far more dangerous. Based on the optical properties of skin, UVB is strongly absorbed within the stratum corneum and epidermis, therefore exerting a stronger effect on epidermal keratinocytes [34]. UVB is directly absorbed by proteins and nucleic acids and is responsible for the majority of sunburns. Melanin, found in the basal layer of the epidermis, is an important chromophore within the skin and primarily acts to absorb UVR. Upon stimulation by UVR, melanocytes undergo melanogenesis, which results in the increased production of melanin pigments that cause the skin to visibly tan [35]. Due to this property, melanin's photoprotective nature is believed to protect skin cells from UVB radiation damage. Furthermore, this reasoning has been used as evidence to support the observation that the incidence of skin cancer is much lower in individuals with higher levels of melanin at baseline (i.e. darker skin phenotypes) [36]. However, when effects of UVR exposure outpace the synthesis of melanin or its capacity to absorb harmful rays, skin damage will clinically appear as a sunburn.

The wavelength of UVB radiation primarily corresponds to the absorption spectrum of DNA. Upon skin exposure to UVB radiation, electromagnetic energy is absorbed by biologic molecules and transformed into chemical energy [34]. When UV photons are absorbed by DNA molecules, electrons are excited to a high energy state, which can result in formation of photoproducts [37]. The two major DNA lesions induced by UVB radiation are the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4PPs). These lesions are ultimately caused by the misbonding of two pyrimidines, either thymine or cytosine, within the same DNA strand. Both lesions can lead to genetic mutations including C→T and CC→TT transitions; however, CPDs are considered more carcinogenic because they are more prevalent and less efficiently repaired than 6-4PPs [34]. If left unrepaired, these dimers become mutagenic, which highlights the equally important role of the DNA repair system in skin carcinogenesis. Pyrimidine dimers are normally removed by nucleotide excision repair (NER) enzymes. In patients with xeroderma pigmentosum (XP), a disease characterized by defective NER enzymes, individuals exhibit a decreased ability to repair DNA mutations, especially those caused by UVR. In individuals with XP, this clinically manifests as the development of NMSC and melanoma skin cancers at a young age [38]. In building on the knowledge learned from patients with XP, studies have subsequently sought to uncover novel defects in NER enzymes within the general population. It has been suggested that polymorphisms within NER enzymes increase susceptibility to the development of NMSC [39]. While polymorphic variants of DNA repair genes may contribute to an individual's risk of developing NMSC following UV exposure, it is unclear at this time to the extent by which one is affected.

In contrast to UVB, the exact role of UVA in skin carcinogenesis is far more nebulous. Although UVA is partially absorbed by the epidermis, it also penetrates to the dermis where collagen fibers function to scatter light [34]. Until relatively recently, UVA has long been considered to play a minor role in the development of skin cancer as photons of UVA are not within the absorbable wavelength of DNA [34]. However, molecular studies have since illuminated the potentially significant function of UVA in photocarcinogenesis. Recently, research has shown that UVA causes indirect DNA damage via the generation of reactive oxygen species, and DNA-protein crosslinks, as well as direct DNA damage by formation of CPDs or single-strand DNA breaks [40, 41]. While the role of UVA radiation in the formation of skin cancer is not yet clarified, epidemiologic studies also seem to support its harmful effects. Indoor tanning beds, which primarily emit UVA radiation, have been linked to the increased incidence of skin cancer among users [42, 43]. It has been reported that just one indoor tanning session can increase a user's risk of developing SCC by 67% and BCC by 29% [44]. There is thus compelling evidence to elevate the role of UVA in the formation of skin cancer by perhaps alternative pathways that lead to carcinogenesis.

4. Molecular mechanisms underlying skin carcinogenesis

Our understanding of cancer biology and the molecular pathology underlying malignant transformation has grown considerably within the past several decades. In particular, the advent of high-throughput sequencing technologies has enabled the detection of various mutational signatures. Dysregulation of proto-oncogenes and tumor suppressor genes, which

are critical for controlling cellular growth, is the mechanistic basis of cancer development [45]. Gain of function mutations convert proto-oncogenes into oncogenes, which lead to unregulated cell growth [46]. Likewise, mutations in tumor suppressor genes, which normally inhibit cell growth, also lead to uncontrolled cellular proliferation due to loss of negative control [47].

Past studies on UV radiation in skin carcinogenesis have identified at least three pathways involved in skin cancer development (**Figure 1**): genetic mutations, epigenetic changes and alterations in gene expression. While these three pathways can act alone to cause cancer, they often interact with each other to trigger cancer development.

4.1. Gene mutations

UVR is recognized to induce a specific pattern of genetic mutations, namely C→T and CC→TT substitutions. To date, this specific mutation is found to be specifically enriched in skin cancers [48]. Since UVR, in particular UVB, is known to induce these types of mutations, C→T transitions are now widely referred to as 'UV signatures' [49]. Our understanding of these specific mutations as being UV dependent has enabled deduction of UV induced genetic mutations by backward inference and pattern recognition. While there have been many pathways implicated in skin carcinogenesis, there is strong evidence to support the impact of selected genetic mutations as being crucial for malignant transformation and tumorigenesis within clonal populations.

4.1.1. P-53 and skin cancer

Often described as the 'guardian of the genome,' the tumor suppressor protein p53 is arguably one of the most important regulatory proteins for its role in maintaining cellular integrity.

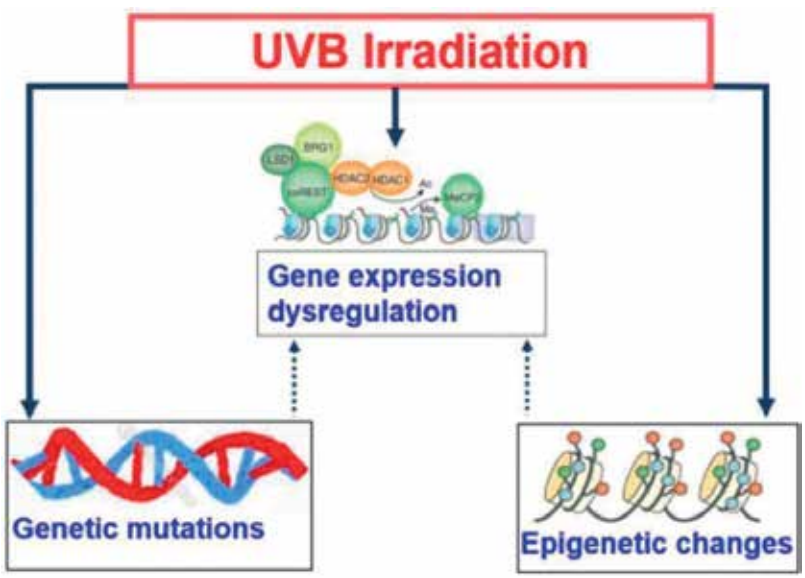

Figure 1. Schematic illustration of major molecular mechanisms underlying UV-induced skin photocarcinogenesis.

p53 is a transcription factor that is responsible for controlling genes involved in cell cycle regulation, apoptosis, and DNA repair [50]. In skin cancer, many mutations in p53 are characterized by the C→T and CC→TT transitions, which are characteristic of the UV mutational signature. It is estimated that mutations in p53 occur in 58% of SCCs and 33% of BCCs [51, 52]. Mutations in p53 are found in many malignancies, which suggest its involvement in aberrant signaling pathways and subsequent DNA damage. However, in skin cancer, each p53 allele often carries different mutations at different locations along the gene. This is opposed to other cancers in which the p53 mutations occur within conserved regions [53]. Although p53 mutations in skin cancer do not consistently occur at the same locus, mutations do not occur randomly. Rather, these UV signature mutations accumulate in 'hot spots,' which, importantly, are different from regions of p53 that are mutated in internal malignancies [54]. This suggests that the proclivity for these mutations to occur within specific loci is perpetuated by a selective advantage. p53 mutations are believed to confer resistance to apoptosis in response to UVR, thereby leading to positive selection of p53 mutant cells and clonal expansion [55].

4.1.2. Basal cell carcinoma and PTCH mutation

Patched (PTCH) is a transmembrane receptor protein that suppresses the hedgehog (HH) signaling pathway. While SCCs are believed to originate in the interfollicular epidermis, histologic evidence suggests that BCCs preferentially arise within stem cells of the hair follicle [56]. HH signaling through PTCH is critical for maintenance of skin stem cell populations, regulation of hair follicle, and sebaceous gland development. Binding of HH protein to the PTCH receptor inhibits the activation of smoothened (SMO) protein to dampen the expression of the HH pathway. Inactivating mutations in the PTCH gene or gain of function mutations in SMO can lead to constitutive expression of the HH pathway [57]. Aberrations in the HH signaling pathway are now recognized as major contributors in BCC tumorigenesis. Mutations in the PTCH gene were initially detected in patients suffering from basal cell nevus syndrome [58]. It was later discovered that a significant proportion of sporadic BCCs and BCCs arising in patients with XP also carried mutations in PTCH [59, 60]. High levels of mutant PTCH transcripts have been found in BCCs but not in normal skin or other types of tumors, making this a relatively specific genetic mutation observed in BCC [61]. The molecular link between PTCH mutations and BCC formation is regarded as a major scientific discovery; however, the exact mechanism by which over expression of the HH pathway leads to unrestricted proliferation of skin basal cells is not known.

4.1.3. Complexity of mutational patterns in squamous cell carcinoma

While mutations in the HH signaling pathway appears to be the most important genetic change leading to BCC oncogenesis, the search for a pathway of similar significance in SCC development is ongoing. Whole exome sequencing has revealed a very high mutational burden in cutaneous SCC with an average of one mutation per 30,000 base pairs [62]. Efforts to identify key driver mutations in SCC have thus been hindered by the high background mutation rate. Furthermore, when compared to BCCs, delineation of the mechanisms underlying SCC formation is somewhat more complex. While BCCs are largely believed to arise 'de novo,' SCCs can arise from clinically apparent precursor lesions, namely actinic keratoses [63].

Histologically characterized by atypical keratinocytes, AKs give rise to approximately 65% of SCCs [25]. In addition to clinical observation, this notion has been further supported by genomic analysis. While AKs and SCCs exhibit similar karyotypes, AKs demonstrate less genotypic complexity, suggestive of an earlier stage of tumor development [64]. While the genetic relationship between AKs and SCCs has not yet been clearly defined, it appears that AKs and SCCs exist on a continuum in which certain mutations drive progression from premalignant to malignant forms [65]. Thus, mutations in both AKs and SCCs have been explored for their potential roles as drivers of carcinogenesis.

Increasing evidence suggests that the underlying pathogenesis of cutaneous SCC involves mutations in several genes and pathways. Besides p53, published research has mainly focused on a handful of key mutations frequently found in cutaneous SCC including NOTCH, RAS, EGFR, TGFB, NF-KB, and most recently, KNSTRN [66]. KNSTRN gene, one of the newest genetic mutations reported in SCC, encodes a kinetochore associated protein that modulates anaphase onset and chromosome segregation during mitosis. Recurrent UV signature point mutations in KNSTRN at codon 24 (p.Ser24Phe) have been observed in 19% of SCCs and 13% of AKs [67]. Functionally, this specific mutation in KNSTRN results in disruption of chromatid cohesion in normal cells, which can lead to aneuploidy and chromosomal aberrations. Since KNSTRN mutations occur rarely in other malignancies, this may represent a previously unrecognized oncogene in skin tumorigenesis; however, studies to clarify its clinical applicability are needed.

4.2. Epigenetic alterations

Apart from the mutagenic effects of UVR on the genome, the role of epigenetic changes induced by UVR in skin cancer remains underappreciated. Epigenetics refers to heritable changes in gene expression that are not due to alterations within the DNA sequence itself. This includes changes in DNA methylation, histone modification, and miRNAs. The human genome encodes a subset of genes that function in epigenetic modifications of the genome and thus regulate the activities of other genes. While the list of such epigenetic regulators continues to grow, DNA methyltransferases (DNMTs) and histone modifying enzymes are among the best studied epigenetic regulators [68]. DNA methylation is a process catalyzed DNMTs by which a methyl group is added to the 5′ carbon of a cytosine ring of DNA. These modified cytosine residues are often adjacent to guanine bases, resulting in the formation of a CpG dinucleotide. These CpGs are often concentrated in short CpG-rich DNA segments, known as CpG islands, the majority of which are found in the promoter region of genes [68]. Methylation events in promoter regions can have profound effects via the secondary effects on transcription.

Both DNA hypomethylation and hypermethylation have been implicated in tumorigenesis of skin cancers. In particular, DNA hypermethylation in CpG islands is an important mechanism by which tumor suppressor genes are silenced. This in turn leads to downstream effects on gene expression that may eventually lead to a neoplastic phenotype [69]. Like other human malignancies, the development of skin cancer involves a complex interplay between environmental factors and alterations in gene expression within skin cells. While the

development of skin cancer is multifactorial, epigenetic alterations are believed to be among the earliest detectable changes in UVR exposed skin [70]. Over exposure of the skin to UVR leads to oxidative stress, inflammation, and DNA damage, factors that are known to significantly alter epigenetic pathways. In particular, chronic inflammation has been shown to accelerate the formation of DNA methylation changes [71]. Thus, environment-induced aberrant expression of epigenetic regulators can trigger widespread epigenetic changes that may subsequently disturb a variety of downstream target genes, potentially increasing the risk of cancer development.

Despite the increasing recognition that abnormal DNA methylation is a crucial factor in skin carcinogenesis, histone modifications serve as another dynamic epigenetic pathway that is frequently altered in cancer. Histone modifying enzymes can be divided into multiple families [72, 73]. Among these, proteins involved in histone acetylation and deacetylation have been shown to play important roles in both normal development and disease states. Limited data suggest that silencing of tumor suppressor genes in UV-induced skin leads to photocarcinogenesis and aberrant epigenetic modifications including alterations in DNA methylation and histone acetylation at specific loci [74, 75]. UV-induced differential gene expression has also been linked to changes in global H3 lysine 27 acetylation (H3K27ac), an epigenetic marker of active promoter and enhancer regions. Using ChIP-seq analysis, one study revealed that UVR induced genome-wide loss of H3K27ac, as well as regional gains in H3K27ac levels. Upon further analysis, UV-induced differential H3K27ac acetylation was functionally correlated with differential gene expression was observed [76]. The genome wide loss of H3K27ac may be attributable to the suppression of histone acetyltransferases activities, whereas the regional gain of H3K27ac may occur secondary to the binding of UV-responsive transcription factors, such as JUN/FOS or TP53, which subsequently recruit HATs to their target regions [76, 77]. Though the epigenetic mechanisms underlying the effects of UVR in promoting skin cancer warrant more extensive studies, the use of histone biomarkers for clinical diagnosis and/or prognosis is an interesting approach that is also being investigated for use in other malignancies [78, 79].

There is a growing body of evidence to suggest that non-coding RNA (ncRNA) is involved in the development of many malignancies, including skin cancer [80]. ncRNAs are RNA transcripts transcribed from DNA that are not translated into protein and classified based on size: small non-coding RNA (<200 nucleotides) and long non-coding RNA (>200 nucleotides). While there are various different subtypes of ncRNA, microRNA (miRNA), a subtype of small non-coding RNA, is perhaps the most widely studied. miRNAs are single stranded ncRNA molecules that modulate gene expression by binding to the 3′ untranslated region of target mRNA. This ultimately causes mRNA instability and eventual degradation, leading to subsequent alterations in cell differentiation, metabolism, apoptosis, and signal transduction [81]. Studies have demonstrated that UV irradiation of human keratinocytes modulates the expression of numerous miRNAs. In addition to a common miRNA response, UVA and UVB were also shown to induce wavelength specific miRNA expression signatures [82]. Although interpretation of changes in UV-responsive miRNA expression is complex, miRNA mediated gene silencing likely has important downstream effects, which contribute to the development of skin cancer.

While less studied than short non-coding RNA, long non-coding RNA (lncRNA) has recently become an area of significant interest within cancer research. It is now known that lncRNA can effect gene expression through interactions with transcription factors and can interact with miRNAs to regulate mRNA stability [83, 84]. Though thousands of lncRNA transcripts have been identified, the majority remain uncharacterized with unknown functions [85]. Nonetheless, a growing number of lncRNAs are being investigated for their role in cancer growth, tumor initiation, and metastasis.

5. Current measures of ultraviolet radiation exposure and skin damage

The current indicator of skin sun damage relies on the use of minimal erythema dose (MED), which refers to the amount of UVR that produces visible skin redness within 24 hours following exposure [86]. As an indicator of UV damage, MED is insensitive and inadequate because UV-induced molecular damage may occur at sub-MED UV doses [87, 88]. Other markers of UV exposure include clinical findings such as solar lentigines and solar elastosis. While these lesions are completely benign, they do have a positive association with NMSC, mainly due to the fact that they arise secondary to photodamage [89]. Although clinical findings of photodamage provide prognostic value, these lesions are neither sensitive nor specific as markers of skin cancer risk as many individuals with solar elastosis and lentigines will never develop skin cancer [89].

While the association between UVR and skin cancer is well established, quantitative assessment of skin UV exposure and its effect on skin cancer development remains unknown. In a small case control study of 58 patients with cutaneous SCC, the risk was greatest in patients who had more than 30,000 hours of cumulative lifetime sun exposure [90]. This is in contrast to BCCs where studies suggest that intense, intermittent sun exposure resulting in sunburns may be more important for the development of BCC [91]. Quantifying the amount of sun exposed hours necessary to induce NMSC is technically challenging and is not practical for implementation as a risk measure at the population level.

6. Biomarkers as a novel tool in monitoring ultraviolet skin damage to improve skin cancer prevention

Biomarkers are defined as measurable cellular, biochemical or molecular alternations in biological media such as blood or tissue. Several FDA-approved multi-gene panel tests are now approved for risk prediction and diagnosis of various cancers; however, no similar biomarker tests exist for patient risk stratification of NMSC [92]. At present, there are no skin cancer screening guidelines in the United States. Despite this, physicians are routinely confronted with the decision of who should receive total body skin exams and at what time interval. The United States Preventive Task Force (USPSTF) has repeatedly stated that in the absence of randomized controlled clinical trials, there is insufficient evidence to recommend skin cancer

screening in the general population [93]. While the majority of the USPSTF skin cancer screening report focuses on melanoma, one of the main arguments made against screening for NMSC is that there is limited evidence regarding the diagnostic accuracy of primary care physicians to correctly identify BCCs and SCCs. Given the lack of rigorous dermatology training in most medical school and primary care residencies, this argument is not necessarily unfounded [94]. Although there are several other arguments to be made against the USPSTF recommendations and reasoning, the lack of clear skin cancer screening guidelines is problematic.

It is unlikely the USPSTF will obtain a satisfactory level of evidence in the near future to recommend skin cancer screening in the United States given the large number of patients required to adequately power a clinical trial [95]. It thus behooves physicians and scientists to search for alternative measures as a means to quantify skin cancer risk. The presence of an objective UV biomarker test could facilitate patient triage by identifying high risk individuals for dermatology referral. Identification of susceptible patients in the primary care setting via the use of an accessible genetic screening test would thus provide physicians with an evidence based method to make informed decisions regarding which patients should receive regular skin cancer screening.

The development of a UV biomarker panel has the potential to have a profound impact on patient care. Breakthroughs in next generation sequencing technology have provided a powerful tool for identifying biomarkers of a given physiological status or exposure [96]. Many studies have attempted to identify biomarkers that correlate UV exposure and skin damage with variable success; however, no consensus UV biomarkers have been established to date. Various types of biomarkers have been investigated including DNA, RNA, and protein.

7. Candidate biomarkers for assessing UV damage: strengths and weaknesses

7.1. DNA markers

Given the potent mutagenic effect of UVR and its role in skin carcinogenesis, skin cancer research in the past several decades has primarily focused on mapping cancer-related mutations. Such efforts have been greatly enhanced by recent advances in whole-genome and whole-exome sequencing studies, which have identified recurrent genomic aberrations that underlie the development of BCC and SCC [97]. As such, these particular gene mutations have been suggested for use as markers of skin cancer risk. Unfortunately, numerous issues have been encountered with this approach.

Based on our current understanding of cancer development, tumor growth is initiated by the presence of driver mutations, which lead to clonal expansion of mutant cells. This increases the total number of cells that are at risk to develop further mutations and malignant transformation [98]. Given the high prevalence of p53 mutations observed in NMSC, this had previously been suggested as a potential genetic marker of patient risk. Upon further investigation, it was found that mutant clones with p53 UV signature mutations were found in high numbers

within clinically normal skin [99]. As another example, both copies of NOTCH1 are frequently inactivated in SCCs via point mutations and copy number alterations. While studies have demonstrated that up to 60% of SCCs have mutations in NOTCH1, approximately 20% of clinically normal skin cells also carry this mutation [100]. These instances suggest that DNA mutation alone is insufficient to drive skin carcinogenesis. Although this may signal that a significant number of keratinocytes are predisposed to developing skin cancer, the specific combination of events leading to malignant transformation is not well understood.

One of the more promising DNA-based UV biomarkers involves the use of mitochondrial DNA (mtDNA) as a biomarker of cumulative UVR exposure and oxidative stress [101]. Given that mitochondria lack classical NER pathways, UV signature mutations that form within mtDNA have limited capacity for repair [102]. Thus, the entirety of UV induced damage can be observed and correlated quantitatively with UV exposure. Since mutations in nuclear DNA (nDNA) are capable of repair via NER enzymes, the UV mutational burden in nDNA is less representative of lifetime UV exposure. Furthermore, individual variability in NER enzyme activity makes the assessment of nDNA mutations more complicated as repair rates are not uniform [39]. The use of mtDNA as a marker of UV damage is appealing; however, the most important question: whether there is a relationship between mtDNA mutations and actual disease: remains to be answered. Additional studies are required to establish a functional correlation between mtDNA mutation and skin cancer development.

7.2. RNA markers

Fundamental to the central dogma of molecular biology, RNA is a necessary intermediary between DNA and protein. The exploration of mRNA-based UV biomarkers is an exciting area of research that has been facilitated by recent advancements in next-generation sequencing technology [96]. While RNA only exists for a finite period of time before being degraded, it is easy to detect and quantify at very low levels [103]. There are currently numerous methods available by which RNA expression can be analyzed in a reliable and reproducible fashion. Compared to DNA biomarkers, RNA biomarkers provide a snapshot of temporal and spatial changes in regulatory pathways, which cannot be observed with singular DNA changes.

The increasing power of high throughput sequencing has allowed for detection of changes in both protein-coding RNA (i.e. mRNA) and non-coding RNA (i.e. small nuclear RNA, micro RNA, small nucleolar RNA, lncRNA) expression with high sensitivity and specificity [104]. Non-protein coding RNAs are well known to play regulatory roles in gene expression via post-transcriptional modification and there is a growing body of evidence that implicates non-coding RNA as key regulators of tumor pathways [105, 106]. Studies have demonstrated that specific UV induced miRNAs are differentially expressed in NMSCs (**Table 2**). While altered miRNA expression has been found within malignant tumors themselves, various cancers also demonstrate specific miRNA aberrations within serum and plasma [107]. Therefore, analysis of circulating miRNA expression levels can be potentially employed for use in a non-invasive biomarker test for NMSC. Plasma profiling for early detection of NMSC has been explored in one recent study, which showed significant changes in expression of miRNA-19a, miRNA-25, miRNA-30a, miRNA-145, miRNA-186 [108]. While these are interesting findings, extensive validation of the clinical utility of these miRNAs as NMSC biomarkers is required.

microRNA	Expression	NMSC association	Reference
miR-203	Downregulated	BCC	[133]
Let-7a	Downregulated	BCC	[124]
miR-21	Upregulated	BCC, SCC	[124–129]
miR-29c	Downregulated	BCC	[130]
miR-130a	Upregulated	BCC	[126, 130]
miR-124	Downregulated	SCC	131]
miR-203	Downregulated	SCC	[132]
miR-184	Upregulated	SCC	[132]
miR-30a	Downregulated	SCC	[126]
miR-387	Downregulated	SCC	[126]
miR-135b	Upregulated	SCC	[126]
miR-424	Upregulated	SCC	[126]
miR-766	Upregulated	SCC	[126]
miR-145	Downregulated	SCC	[126]
miR-140-3p	Downregulated	SCC	[126]
miR-26a	Downregulated	SCC	[126]
miR-31	Upregulated	SCC	[127, 134]
miR-205	Upregulated	SCC	[135]
miR-365	Upregulated	SCC	[136, 137]
miR-1	Downregulated	SCC	[138, 139]
miR-34a	Downregulated	SCC	[140]
miR-124/214	Downregulated	SCC	[131]
miR-125b	Downregulated	SCC	[127]
miR-193b/265a	Downregulated	SCC	[141]
miR-199a	Downregulated	SCC	[142]
miR-361-5p	Downregulated	SCC	[143]
miR-483-3p	Downregulated	SCC	[144]

Table 2. Aberrantly expressed microRNAs in NMSC tumors.

LncRNA is also being explored for its utility as a biomarker for cancer diagnosis and therapy. Following genome wide association studies, it is now known that 88% of trait associated single nucleotide polymorphisms (SNPs) are located in intergenic regions [109]. Since the majority of lncRNAs are transcribed from these regions, it has been suggested that SNPs of lncRNA may represent differential disease risk. While studies are limited, the potential role of circulating lncRNA for use as a biomedical tool is exciting. Dysregulation of lncRNAs has

been reported in a number of malignancies including colon, prostate, breast, and liver cancer where they act as tumor suppressors and oncogenes [110]. Similar to miRNAs, lncRNA can easily be detected by PCR in bodily fluids. Studies demonstrating the detection of lncRNA PCA3 in urine and lncRNA HULC in blood have been suggested as novel modes of cancer screening and diagnosis for prostate cancer and hepatocellular carcinoma respectively [111–113]. With regard to skin cancer, the aberrant expression of lncRNA appears to be functionally important in skin carcinogenesis. In one study, lncRNA lincRNA-p21—a transcriptional target of p53 and HIF-1α—was found to be highly inducible by UVB radiation and crucial for p-53 mediated apoptosis of damaged keratinocytes [114]. The interaction between the vitamin D receptor and lncRNA also appears to play an important role in maintaining cellular homeostasis prevention of skin tumor formation [115]. In another study, differential expression analysis revealed 1516 lncRNAs were upregulated and 2586 lncRNAs were downregulated in cutaneous SCCs when compared to normal controls [116]. A similar study demonstrated analogous findings in BCCs that showed upregulation of 1851 lncRNAs and downregulation of 2165 lncRNAs when compared to normal skin [117]. While the prognostic, diagnostic, and therapeutic application of lncRNAs within skin cancer is not yet developed, their potential role in the molecular pathogenesis of NMSC warrants further analysis.

In addition to non-coding RNA, mRNA has been explored extensively for its use as a biomarker in numerous studies [118–123]. Similar to miRNA, multiple mRNA transcripts have been found to be differentially regulated in response to UV radiation. Despite the large number of differentially expressed genes identified so far, there is no consensus in terms of which genes are the most sensitive and specific markers of NMSC. Due to frequent inter-individual variations in the expression of many genes, it is unlikely that a singular mRNA will be sufficient as a reliable biomarker. There is thus a growing consensus that multi-gene biomarker panels will be required for the development of a robust and reliable screening test.

Analysis of combined transcriptomic data from previous studies has been complicated by large variations in experimental design including cell type, UV exposure, dose, and time points of analysis [118–123, 145, 146] (**Table 3**). Therefore, development of a validated consensus UV biomarker panel has not yet been achieved. However, a recent transcriptomic profiling study has begun to shed light on the viability of UV biomarker panel for clinical application [147]. In this study, rigorous bioinformatics and statistical analyses were performed to identify UV-responsive genes that are conserved among different donors, in response to various UVR doses, and at different time points after UV exposure. Through this comprehensive transcriptomic analysis, 401 conserved UV-responsive genes were identified out of approximately 4000 U-induced differentially expressed genes detected following each specific UVR condition. Through RNA-seq analysis, this study also generated a SCC-specific signature based on differential gene expression analysis of five pairs of human SCC tumor tissue and adjacent normal skin tissue. Moreover, there is a significant similarity between the conserved UV responsive genes and the SCC signatures genes. The conserved UV-responsive gene set has little similarity to gene sets dysregulated in other human cancers, highlighting their specificity for skin cancer gene expression signatures [147]. Additional validation studies will be needed to select which of these conserved UV-responsive genes may be used to develop a consensus UV biomarker panel.

Author	UV type & dose (mJ/cm²)	Transcriptional analysis: time post UVR (hours)	Cell type	Platform
Rieger and Chu [118]	1 (UVC)	4	Lymphoblastoid cell lines	U95A-v2 chips
Dazard et al. [119]	20–80 (UVB)	0, 0.5, 3, 6, 12, 24	NHEK vs. SCC	U95A-v2 chips
Takao et al. [120]	10 (UVB)	6	NHEK	HuGene FL chips (6800)
Dawes et al. [121]	1000 (UVB)—rat 3 MED (UVB)—human	48	Human vs. rat epidermal cells	RNA-Seq
de la Fuente et al. [122]	300 (UVB) + 3700 (UVA)	6	NHM, MHEK, NHDC	Whole genome chips
Yang et al. [123]	25 (UVB)	24	NHM	U133+-v2 chips
Koch-Paiz et al. [145]	5 (UVA) + 12.5 (UVB) + 1.2 (UVC)	6, 12	Differentiated mammary epithelium	7684 probes cDNA array
Enk et al. [146]	150–250 (UVB)	2, 24, 72	NHEK	U95A-v2 chips

Abbreviations: NHEK, normal human epidermal keratinocytes; NHM, normal human melanocytes; NHDC, normal human dendritic cells.

Table 3. Comparison of study design and methods of previous gene expression profiling studies analyzing differential expression of UV induced mRNA.

7.3. Protein markers

The majority of genes function through their protein products. Therefore, it is generally accepted that gene activity and function are positively correlated with their protein expression level. Characteristics of protein expression such as variation in quantity, timing of synthesis, and interaction with other proteins has been studied as a means to correlate molecular mechanisms with clinical pathology. Although there are benefits to directly measure changes in protein expression, the use of protein biomarkers has several significant challenges. The main limitation with the use of protein markers lies within currently available detection methods. Protein biomarker panels can be expensive because each protein requires a specific antibody for detection. Secondly, it remains technically challenging and laborious to standardize the quality and specificity of antibodies in their research and clinical applications. Synthetically produced protein antibodies often exhibit great variability between manufacturers, increasing the likelihood of inconsistent results [148]. Moreover, there is a significant number of proteins whose activity depends on specific forms of posttranslational modifications, thus making it more complex in interpreting their clinical relevance solely based on their expression levels [149]. For these reasons, no studies have focused on the role of aberrantly expressed proteins in NMSC. Unless improvements in technology are able to overcome the aforementioned issues, the use of protein biomarkers for patient risk stratification of NMSC is unlikely to be practical.

8. Conclusions

Skin cancer is the most commonly diagnosed cancer in the U.S. and has become a major and growing public health problem. Despite numerous public health initiatives to promote sun safety, many Americans do not adhere to recommended guidelines to protect themselves from UV exposure. Given the long lag between UV induced skin damage and clinically apparent skin cancer, this reduces the perceived risk of UVR and does not encourage timely behavior modification.

Our understanding of the pathogenesis of skin cancer at the molecular level has dramatically expanded within the past several years. Although there is still much to be learned about the underlying mechanisms of skin cancer pathobiology, advances in genetic sequencing have provided great insight into the ways in which effective tests may be developed for patient risk stratification of NMSC. This has since paved the way for pursuit of novel applications of this information, which have the potential to profoundly improve patient care.

Clinical biomarker discovery has led to revolutionary changes in medical screening, diagnosis, and target based therapies for a variety of cancers. In the era of precision medicine, individualized patient care is becoming increasingly important in all fields of medicine. While UVR has long been known to be a key risk factor for skin cancer development, increasing evidence has demonstrated that its role in carcinogenesis is likely multifactorial and involves multiple biologic pathways. Despite this, identification of cellular dysregulation in key regulatory pathways has provided insight into potential biomarkers of disease.

Various types of biomarkers including DNA, RNA, and protein have been suggested for use in diagnostic and prognostic testing for various malignancies. Identification of individual biomarkers that produce consistent and reliable information on UV damage has posed a significant clinical challenge. We believe that a successful clinical test consisting of a panel of UV signature genes will provide the most sensitive and specific means for patient risk stratification of UV skin damage. Within NMSC research, RNA-based UV biomarkers currently exhibit the most promise for future clinical application given the multiple, reliable, and cost-effective modalities for RNA detection.

The current lack of skin cancer screening guidelines in the United States has resulted in a non-standardized approach to skin cancer screening and physician risk assessment. Thus, a UV biomarker-based screening test could provide an objective and evidence based method to determine which patients should receive regular skin cancer screening facilitate the identification of high risk individuals for dermatology referral and regular skin cancer screening. By encouraging early risk assessment, we believe that a biomarker-based diagnostic test will greatly improve skin cancer prevention and reduce skin cancer incidence.

Furthermore, translation of UV biomarker expression patterns into a risk score would hopefully offer quantitative and convincing evidence to alert susceptible individuals and encourage UV protective behaviors. Finally, development of a reliable UV biomarker panel could be used for other purposes such as testing the UV-protective effects of sunscreens. We believe this area of research deserves continued attention as the development of UV biomarker based tests has the potential to completely transform the preventative paradigm pertaining to skin cancer.

Acknowledgements

We thank the funding support by NIH/NIAMS grant K01AR064315, the Columbia University Herbert Irving Comprehensive Cancer Center (P30 CA013696), and the Center for Environmental Health in Northern Manhattan (P30 ES009089).

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Edited by Miroslav Blumenberg

Human skin cancers, the most common type of tumors, represent a significant health burden. The deadliest is unquestionably melanoma. Half of melanomas have an activating mutation in the *BRAF* gene, prompting development of novel drugs, vemurafenib and dabrafenib, specifically targeting mutated BRAF. Trametinib and cobimetinib, which block MEK, a BRAF effector protein, have been used in combination with BRAF inhibitors. A promising new melanoma treatment is immunotherapy, approach that boosts patient's own immune system to attack cancer. Pembrolizumab and nivolumab inhibit PD-1, whereas Ipilimumab targets CTLA-4, another immunity check point, to boost the immune response. Here we focus on pathways, mechanisms, targets and treatments of human skin cancers, with particular emphasis on the new developments in the research on melanomas.

Published in London, UK

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ISBN 978-1-83881-278-2