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Liquid Biopsy

Edited by Ilze Strumfa and Janis Gardovskis

LIQUID BIOPSY

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Meet the editors

Professor Ilze Strumfa, MD, PhD, is an outstanding medical lecturer, actively involved in research in pathology. She graduated from the Medical Academy of Latvia with a distinction (1998), underwent board certification in pathology (2001), and received her PhD degree in 2005. Currently, she holds the positions of professor and head of the Department of Pathology in the Riga Stradins University (RSU), Latvia. Her 10 years of teaching experience have culminated with the Annual Award (2018) of RSU *for* the most distinguished teacher: *"Lecturer of the Year.*. She is a member of the European Society of Pathology, and an author/coauthor of more than 80 peer-reviewed journal articles and 14 chapters in scientific monographs and medical textbooks. She has supervised nine PhD students creating a team of dedicated young pathologists. Professor Strumfa has been the leading expert in several European and national research projects devoted to the development of diagnostic technologies, neuroendocrine and endocrine tumors, breast cancer, laboratory training in research, and the tumor microenvironment. Her main research interests include morphological and molecular diagnostics and prognostic assessment of malignant tumors, development and standardization of new technologies, as well as digital pathology and other innovations in pathology.

Janis Gardovskis is a professor of surgery, habilitated medical doctor, and practicing surgeon. He is also Head of the Department of Surgery at Riga Stradiņš University and Head of the Department of Surgery in Pauls Stradins Clinical University Hospital. He is an actual member of the Latvian Academy of Sciences and a member of the Latvian Association of Surgeons. Has has fellowships in surgery in Denmark, Lithuania, Finland, Sweden, and Germany. Professor Gardovskis' areas of interest include videosurgery, pancreatic surgery, oncologic surgery, transplantation, hereditary oncologic diseases, and life quality in surgery. He has participated in many international and local scientific projects and clinical trials. He has also participated in the publication of at least five monographs and about 44 scientific publications in international citation databases (Scopus, PubMed), as well as in numerous other papers. About 18 doctoral dissertations were defended under the supervision of Professor Gardovskis.

Contents

Preface

Reliable diagnosis is the cornerstone, starting point, and prerequisite of successful treatment. Therefore, research devoted to innovative diagnostic technologies continues to advance at a rapid pace. The field of liquid biopsy is a bright example of such progress.

Liquid biopsy is a new minimally invasive laboratory evaluation concept that can be used for diagnostic, prognostic, and predictive testing, as well as dynamic monitoring of treatment efficacy or disease course. To achieve these goals, a multitude of specific, targeted tests can be performed to detect free nucleic acids, exosomes, microRNAs, tumor-educated platelets, and whole cells of tumor or fetal origin in different biological fluids, including blood, urine, cerebrospinal fluid, and others.

Although tissue biopsy has long been considered the gold standard of diagnostics, especially regarding malignant tumors, liquid biopsy has the advantages of a non-invasive approach and thus low risk of complications. It is technically feasible even in serious general status or if tumors or metastases are not easily accessible using conventional tissue biopsy. The testing is fast, exact, and can be repeated to ensure real-time follow-up during treatment or surveillance. In contrast to classic tumor markers, e.g., prostate-specific antigen, liquid biopsy is distinguished by high specificity at genomic, proteomic, and cellular levels. It is expected to equal and exceed the diagnostic value of tissue biopsy. The field of liquid biopsies is developing rapidly regarding the selection of targets, technological improvements, and quality assessment. Further research is clearly needed, and a summary of the existing knowledge in this book will support it.

The chapters in this book provide state-of-the-art reviews on the current knowledge and advances in the technologies, software, and diagnosis-based research regarding the field of liquid biopsy. This book is written by a global team of recognized medical experts and researchers. We would like to thank all the authors for their excellence, cooperation, and work input.

We are particularly grateful to Lucija Tomicic-Dromgool, Marina Dusevic, and their colleagues at IntechOpen, one of the world's leading multidisciplinary scientific publishers of open access books. The IntechOpen team was remarkable for their expertise and continuous, kind support in bringing this edition to completion.

Finally, we would like to thank our colleagues and students in Riga Stradins University for the inspiring atmosphere and genuine love of science in general and medical research in particular. The future starts with the bright eyes of students and relevant questions. We hope that the book will provide answers to some of those questions and inspiration for further study, culminating in future research, technologies, and practical implementation of liquid biopsy in clinical medicine.

Ilze Strumfa and Janis Gardovskis
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Introduction

Chapter 1

Introductory Chapter: Liquid Biopsy — A Promising Technology of the Future

Ilze Strumfa and Janis Gardovskis

Additional information is available at the end of the chapter

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1. Introduction

Liquid biopsy represents a spectrum of technologies for the molecular analysis of blood and other biological liquids (e.g. urine, saliva or others) in order to detect cell-free/tumour nucleic acids, exosomes, microRNAs, tumour-educated platelets and circulating or free-floating tumour or foetal cells, depending on the clinical context. In contrast to classic tumour markers, e.g. prostate-specific antigen (PSA) or carcinoembryonic antigen (CEA), liquid biopsy is distinguished by high specificity as it provides genomic, proteomic and cellular characteristics of the disease. The anticipated outstanding reliability of these tests has been reflected in the term itself: "liquid biopsy" is expected to be at least as informative as tissue biopsy, used as the gold standard in certain diagnostic fields, especially in oncology.

The advantages of liquid biopsy include non-invasive approach which is patient-friendly, associated with remarkably low possibility of complications and technically feasible even in patients who are in serious general status or affected by tumour or metastases that are not easily accessible by conventional tissue biopsy. The clinically simple application allows the repeated use of liquid biopsy resulting in real-time follow-up for the disease course. The testing is fast and exact as definite molecular markers are sought for. Generally, liquid biopsy is a much awaited tool to overcome the limitations set by tumour heterogeneity upon conventional tissue biopsy representing only a small part of the whole tumour [1]. However, some technological modifications of liquid biopsy in certain patients can be subjected to the same restrictions regarding heterogeneity. A characteristic example would be the assessment of circulating tumour cells (CTCs) in patients with low burden of malignant cells in the blood. For instance, the diagnostic threshold of

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two CTCs per 7.5 mL of blood ensured high sensitivity and specificity for gastric cancer diagnosis, reaching 85.3 and 90.3%, respectively [2]. Thus, the diagnostic value of liquid biopsy in such setting has proven to be high, but the few malignant cells per sample cannot represent the full scope of cancer heterogeneity, by the number being inferior to tissue biopsy. Similarly, to characterize the tumour exactly, the circulating tumour DNA must represent at least 10% of the whole blood burden of cell-free DNA—a threshold that is not always reached [1].

Liquid biopsies are increasingly applied in the evaluation of oncological patients (**Figure 1**) due to the previously listed benefits over conventional tissue biopsies. However, this approach is not limited to the diagnostics of malignant tumours [3, 4].

In addition to the increasing use in oncology, liquid biopsy can be used in obstetrics and gynaecology to evaluate the molecular characteristics of foetus [3, 4]; in transplantology for the early detection of graft rejection; in haemodialysis and critical care medicine to identify tissue damage; and in rheumatology, e.g. to assess systemic lupus erythematosus [4].

Currently, liquid biopsy is not a routine laboratory test in clinical practice [5], and even its clinical efficacy has been seriously questioned by the American Society of Clinical Oncology and the College of American Pathologists [6], but certain tests are approved for use or reaching the clinical life (e.g. *EGFR* gene mutation testing, approved by the Food and Drug Administration (FDA) on 2016; tumour mutation burden in blood, confirmed in 2018 as an effective tool to predict the efficacy of immunological checkpoint inhibitors), and the related field is rapidly expanding [7, 8]. The development is seen as the progress in technologies, software and quality control systems as well as diagnosis-based research to substantiate many promising clinical applications (**Figure 2**).

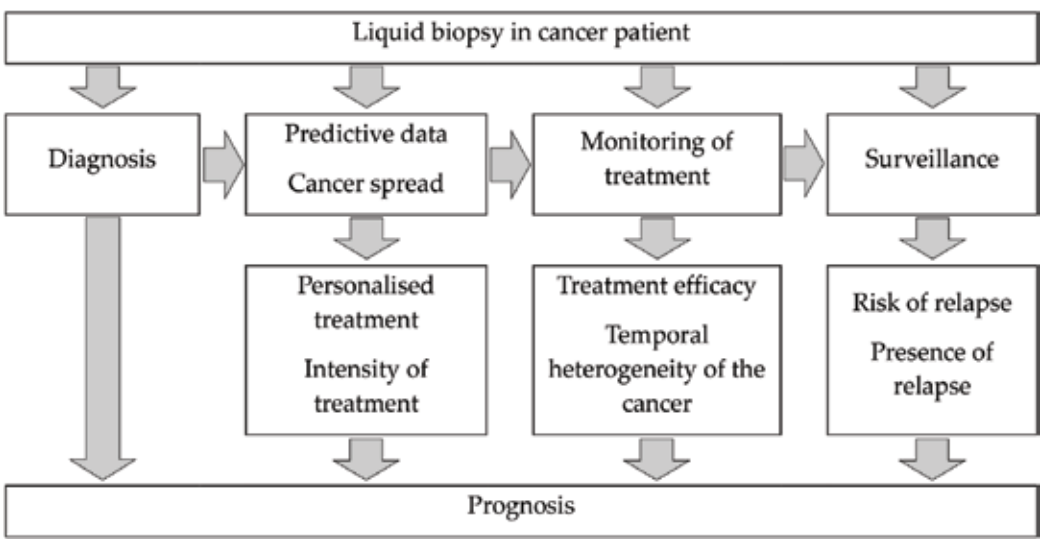

Figure 1. Role of liquid biopsy in the stepwise evaluation of cancer patient.

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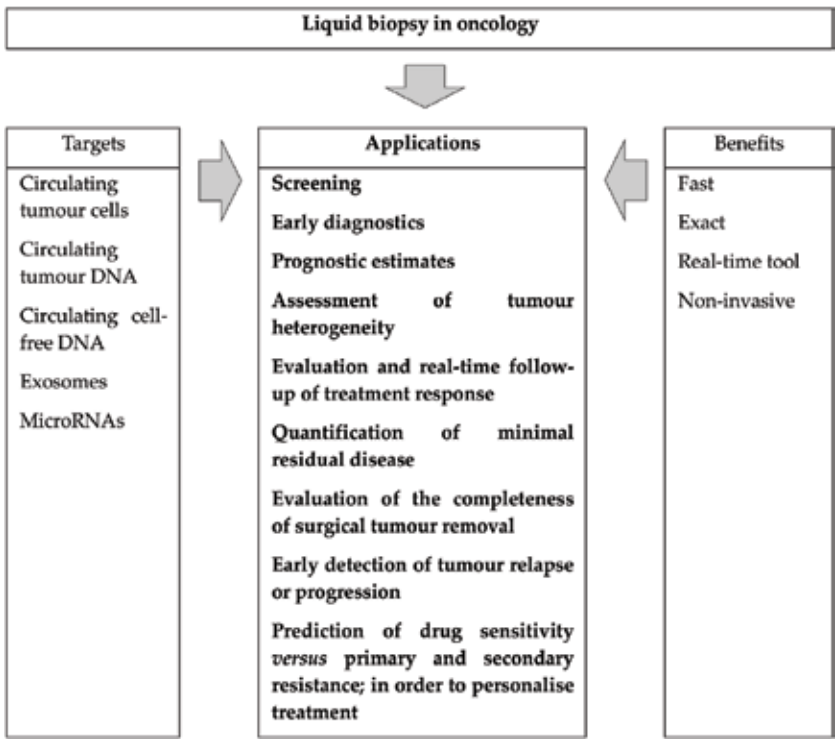

Figure 2. The potential applications of liquid biopsy in oncology.

2. Circulating tumour cells and related issues

Invasion is a typical feature of malignant tumours. An epithelial malignancy, namely, carcinoma, starts its invasive growth from intraepithelial carcinoma in situ, destroys the basement membrane, infiltrates connective tissues and reaches smaller or larger blood and lymphatic vessels, entering the circulation. Thus, at some point of cancer pathogenesis, malignant cells appear in the bloodstream. This is a crucial step of carcinogenesis, leading to the metastatic spread—the hallmark of malignant process. Circulating tumour cells (CTCs) have been described by Thomas Ashworth as long ago as in 1869 [9]. Similarly, circulating foetal cells have been identified in peripheral maternal blood [3]. However, major technological advances were necessary to develop reliable tests to identify the circulating non-haematological cells.

CTCs are generally recognised as non-leukocytic nucleated cells in the bloodstream. They can be recognised by their physical properties including large size, mechanical plasticity and dielectric mobility. Physical filters, density gradient, dielectric, microfluidic or photoacoustic methods are used for physical separation. Immunophenotype by expression of different antigens also can be applied to identify CTCs; the relevant methods include cytometric high-throughput imaging and immunomagnetic and adhesion-based separation as well as negative depletion of leukocytes and CTC identification by tumour markers [5].

Considering the pathogenesis of malignant tumours, the burden of CTCs should appear and increase in parallel with advancing cancer course. Indeed, higher numbers of circulating tumour cells were observed in patients having tumours with higher pT or pN or higher pTNM stage [10]. However, CTCs were found in more than 80% of patients presenting with pT1 and/or pN0 gastric cancer [2]—an interesting finding that has a practical value regarding the possibilities of early diagnostics but also an impact on the theoretical considerations of carcinogenesis.

Thus, CTC detection has manifold roles in oncology. A high number of CTCs is an adverse prognostic factor, shown in breast, prostatic, colorectal, gastric, pancreatic and neuroendocrine carcinomas and sarcomas as well as non-small cell lung carcinoma [5]. However, a diagnostic role has also been confirmed, e.g. in the case of breast, prostate and colorectal carcinoma [5]. Early diagnostics by CTCs has been verified in non-small cell lung carcinoma [5] and gastric cancer [2]. CTCs can provide the information on key genetic features of cancer cells and on the epigenetic changes. Hence, prediction of the treatment response by CTC has been demonstrated in breast, prostate and colorectal cancer as well as in melanoma and non-small cell lung carcinoma. Further, changes of CTC count during treatment dynamically reflects the response to treatment paralleling the residual tumour burden [5].

3. Circulating cell-free and tumour DNA

Circulating cell-free DNA (cfDNA) is present in the blood even in healthy individuals although higher levels are observed in patients diagnosed with autoimmune diseases and especially malignant tumours. Nevertheless, cfDNA seems to represent an essential biological regulatory mechanism. cfDNA is either released passively from dying—apoptotic or necrotic—cells or secreted actively from viable cells. cfDNA can be destroyed by DNAse, but at least part of cfDNA poll follows another way of further biological turnover entering healthy cells. The subsequent genomic integration hypothetically can have a myriad of significant outcomes in health and disease ranging from senescence to autoimmune diseases or transfer of the cfDNA to germ cells.

In cancer patients, a fraction of circulating cell-free DNA burden is attributable to the tumour and consequently is designated as circulating tumour DNA (ctDNA). These DNA fragments are released from neoplastic cells and therefore can reflect the tumour-specific events in DNA, including somatic mutations, methylation patterns and degree of microsatellite instability. Thus, the presence of a malignant tumour can manifest by multiple quantitative and qualitative changes in circulating DNA. First, the concentration and features, e.g. fragment length of cfDNA in cancer patients, differ from healthy individuals. Second, the ctDNA reflects the specific dynamic genetic landscape of the cancer.

Evaluation of circulating DNA in certain situations can be diagnostically useful, e.g. to disclose an occult tumour. However, pitfalls exist, e.g. mutations can be present in cfDNA of healthy volunteers who do not develop cancer at least during the follow-up period. Thus, overdiagnosis of cancer by liquid biopsy must be avoided, especially when screening asymptomatic individuals. In contrast, qualitative or quantitative dynamic changes in the ctDNA of a known oncological patient bring reliable, biologically justified information. In patients with

already confirmed cancer, ctDNA can identify either minimal residual disease after surgery with curative intent or tumour relapse. Similarly, the response to treatment can be monitored. Molecular alterations can be assessed to select personalised treatment.

In addition to the diagnostic, prognostic and predictive role in oncology, cfDNA analysis might be useful in other medical situations. Thus, circulating cell-free DNA can be derived from transplanted organs or from the foetus during pregnancy, serving as an early manifestation of graft rejection or reflecting genetic features on the foetus, respectively. In addition, cfDNA levels can be valuable also as a nonspecific biomarker of tissue damage in critical care medicine and related clinical situations, e.g. sepsis, haemodialysis and others [4].

4. Circulating microRNAs

MicroRNAs (miRNAs) are small, evolutionary conserved, single-stranded, non-coding RNA molecules (approximately 22 nucleotides in length) that bind target mRNA to regulate gene expression [11, 12] at the posttranscriptional level [13]. These molecules act as large-scale molecular switches. MicroRNAs are involved in different physiological and pathological events, including apoptosis, cell proliferation and differentiation; therefore, it is not surprising to see miRNAs participating in carcinogenesis as either tumour suppressors [14, 15] or oncogenes [16]. The cardinal tumour features include cell proliferation, invasion and metastasis as well as activated angiogenesis. miRNAs regulate all the steps. In addition, up- or down regulation of certain miRNAs is associated with the biological potential of cancer, e.g. proliferation, invasivity and epithelial-mesenchymal transition or grade. miRNAs can be assessed either in tissues or in biological liquids, in the last case becoming a target for liquid biopsy.

Again, the diagnostic and regulatory roles of miRNAs are not limited to oncology. Women who develop pre-eclampsia and spontaneous preterm birth are characterised by specific exosomal miRNA profile at early gestation. Considering the interplay between exosomal secretion, oxygen tension and endothelial proliferation, aberrant exosomal signalling by placental cells is suggested to have a pathogenetic role in pregnancy complications [17].

5. Exosomes

Exosomes represent a class of extracellular vesicles mediating intercellular communication. They consist of lipid bilayer, transmembrane and non-membrane proteins and single- and/or double-stranded DNA and RNAs, including microRNAs. A fraction of exosomal proteins are ubiquitous, while others are characteristic for specific cells or tissues.

Exosomes are collected for liquid biopsy analysis by different technological approaches, including methods targeting physical (size, density, sedimentation) or antigenic properties. After the tumour-derived exosomes have been isolated and identified, exosomal protein expression and genetic profile by exosomal RNAs or DNAs or exosomal miRNA signature can be tested. In addition to cancer-produced exosomes, those originating from immune cells also can be detected and evaluated [5].

6. Tumour-educated platelets

Platelets are known to participate not only in blood clotting but also in inflammatory and immune processes, e.g. communicating with lymphocytes or regulating cellular transmigration through blood vessel's wall. In malignant tumours, platelets are involved in neoangiogenesis, induction of epithelial-mesenchymal transformation and metastatic spread of tumour cells, protecting them from the immune system. Contacting with tumour cells, platelets can uptake clinically relevant, tumour-specific biomarkers, as demonstrated in the models of glioblastoma, non-small cell lung cancer or prostate cancer. Such platelets are described as tumour-educated platelets, potentially representing diagnostic or therapeutic targets, as well as a clue to further theoretical investigations of carcinogenesis [18].

7. Conclusions

In conclusion, liquid biopsy represents molecular analysis of biological liquids in order to detect cell-free/tumour nucleic acids, exosomes and/or microRNAs, tumour-educated platelets and circulating or free-floating tumour or foetal cells. These technologies have several advantages including high specifity, non-invasive approach and possibility of repeated use to ensure real-time follow-up of the patient. However, liquid biopsy currently is not a routine laboratory test in clinical practice yet. Major developments are expected in the nearest years, including progress in technologies, software and quality control systems as well as diagnosis-based research to substantiate many promising clinical applications.

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Conflict of interest

Editors have no conflicts of interest.

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Section 2

Liquid Biopsy: Technological Breakthrough

Chapter 2

Profiling Circulating Tumour Cells for Clinical Applications

Kah Yee Goh and Wan-Teck Lim

Additional information is available at the end of the chapter

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Abstract

Circulating tumour cells (CTCs) refer to cells found in the peripheral blood, which are derived from the primary or secondary tumour. They serve as an alternative to study the biology of the primary tumour especially when tissue biopsy is not available. However, major challenges in CTC analysis are the rarity of these cells and the purity of the isolated population. The advancement in technologies allows detection and enrichment of sufficiently pure CTCs at the single-cell level, facilitating downstream molecular characterisation. Single CTC analysis allows detection of key mutations that may be critical to disease management and helps to address the intercellular differences among tumour cells. In this chapter, we discuss the technologies for CTC isolation and the use of CTCs in achieving early detection and prognosis of cancer, real-time monitoring of cancer therapy and tailoring of personalised treatments.

Keywords: cancer, CTC, single-cell analysis, liquid biopsy, personalised treatment

1. Introduction

Cancer is a leading cause of death in many countries [1]. According to World Health Organisation, approximately one in six deaths is attributable to cancer. The development of cancer is a multistage process. Briefly, normal cells undergo transformation into tumour cells, which are defined by various hallmarks including the ability to sustain proliferative signals, evade growth suppressors, promote replicative immortality, avoid cell death and immune destruction, induce angiogenesis and activate invasion and metastasis [2]. This cellular transformation results in

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uncontrolled proliferation and enables tumour cells to migrate from their primary organ to a distant organ, a process known as metastasis. In 90% of cancer patients, death occurs because of metastasis [3, 4].

According to Cancer Research UK, 46% of patients in England are diagnosed in the advance stages of disease, leading to less effective treatment outcomes. To reduce the number of deaths caused by cancer, detection at an early stage of disease development is critical so that clinical intervention can come in place to improve the chances of survival for cancer patients. The diagnosis of cancer involves multiple tests including tissue biopsy, liquid biopsy, imaging scans, genetic tests and an examination of medical history. Tissue biopsy is regarded as the gold standard for the clinical diagnosis of cancer [5, 6], despite the invasiveness and inconvenience of collecting the biopsy sample.

In recent years, liquid biopsy is increasing being used for the detection of cancer because it only requires a routine draw of blood and is less invasive compared to tissue biopsy, which may also not be repeatedly done safely or feasibly [7]. Liquid biopsy includes the analysis of circulating tumour cells (CTCs), circulating cell-free DNA (cfDNA) or exosomes present in the patient's blood [8]. CTCs, in particular, have garnered much attention for its potential clinical utility. CTCs are cells disseminated from the primary or secondary tumour into the peripheral blood and are associated with the development of metastasis. They are precursors of secondary tumour formation and may carry key information relating to the mechanism of metastasis. They are approximately 12–25 μm [9] and present in extremely low numbers (typically 1–10 CTCs per 10 ml of blood or ~1–100 CTCs per 10^9 blood cells) [10–12]. The number of CTCs found in the blood varies with the type and stage of cancer and the treatment provided [13]. Typically, patients in the advanced stages of cancers have higher number of CTCs [14]. CTCs of different cellular morphology may exist in the blood and these include the epithelial CTCs, epithelial-to-mesenchymal (EMT) CTCs and mesenchymal CTCs. Moreover, the mutations found in CTCs are often concordant with the primary tumour [15–17], suggesting that the genetic composition of CTCs is similar to the primary tumour. Thus, CTCs has the potential to be used as a 'surrogate' to study the biology of cancer cells.

In cancer treatment, drug resistance is a major concern. The failure of chemotherapeutic drugs to work in patients lies in the heterogeneity and complexity of cancer cells [18, 19]. Cancer stem cells are resistant to chemotherapy and contribute to the intra-tumoural heterogeneity [20]. Therefore, there is a need for molecular profiling of tumour cells at the single-cell level to better address the intra- and inter-cellular differences in cancer cells and enable clinicians to have a better picture of the disease complexity. While paired tumour/normal tissues is the gold standard for molecular analyses of tumour [21], CTCs may provide information on the dynamic changes in tumour cells when blood is extracted at different times, which cannot be achieved in tissue biopsy. In addition, where tissue is not easily accessible, CTCs may provide a diagnostic window.

However, the main challenges of CTC research are that these cells are extremely rare and the population of isolated CTCs may not be pure due to contamination with white blood cells (WBCs). Therefore, highly sensitive and specific technologies are required to isolate CTCs efficiently. Over the past decade, microfluidics technology has greatly advanced the enrichment and isolation of CTCs from whole blood containing red blood cells (RBCs) and WBCs [22]. Microfluidics deals with the behaviour of fluid passing through the microchannels [23, 24]. It makes use of the laminar flow of fluid in the microchannels to manipulate the fluid to achieve

cell separation. Furthermore, because of the small space and short flow distance in the microdevice, microfluidics-based technologies consume small amounts of reagents and greatly increase the speed and throughput of blood sample processing, allowing clinical adoption. The use of microfluidics facilitates the integration of downstream molecular characterisation of CTCs, which will enhance our understanding on the complexity of cancer development and enable clinicians to develop better therapeutic strategies to eradicate cancer cells and improve the overall survival of patients. In this chapter, we discuss the technologies for CTC isolation and the use of single-cell analysis in achieving early detection and prognosis of cancer, real-time monitoring of cancer therapy and tailoring of personalised treatments.

2. Technologies for enrichment and isolation of CTCs

Many technologies have been developed to enrich and isolate CTCs from the peripheral blood. In most methods, CTCs are separated from the blood cells based on their biological properties and/or physical properties such as size, deformability, density and electric charge. Conventional CTC enrichment systems such as fluorescence activated cell sorters (FACs) have been used to separate CTCs from whole blood based on the expression of cell surface protein markers [25]. Technologies that isolate CTCs based on physical properties also exist. For instance, ISET and ScreenCell use a filtration system to separate the slightly larger CTCs (12–25 μm) from the smaller WBCs (7–15 μm) and RBCs (8 μm) [9]. CTCs and mononuclear cells have a density (<1.077 g/ml) lower than other blood cells (>1.077 g/ml), allowing layered separation of CTCs [6]. CTCs and blood cells exhibit differences in deformability, allowing them to be separated [26]. The dielectric properties of CTCs are different from normal blood cells, allowing separation of CTCs when the cells are subjected to a non-uniform electric field [6, 27]. However, these conventional CTC enrichment methods suffer from limited ability to process large volumes of blood, limited detection sensitivity, inherent losses and poor recovery of viable CTCs, low throughput and insufficient purity due to contamination with WBCs [6].

2.1. Use of microfluidics in CTC enrichment

To overcome these limitations, microfluidics technology offers an alternative platform for isolating CTCs with improved detection sensitivity, high recovery rate, high efficiency and throughput. Each microfluidics platform has its advantages and limitations (**Table 1**). In 2004, US Food and Drug Administration approved the clinical use of CellSearch system (Veridex) for CTC detection in epithelial cancer types such as breast [28], colorectal [29] and prostate cancer [30] for purposes of prognostication. This system uses immunomagnetic and fluorescence imaging technology to enrich and enumerate CTCs from 7.5 ml of whole blood based on the expression of specific proteins in CTCs [31, 32]. CTCs are first separated from other blood cells using magnetic iron nanoparticles coated with antibodies targeted against EpCAM (an epithelial cell adhesion molecule present on the cell surface of CTCs). Subsequently, cells are stained with antibodies targeted against cytokeratin (CK; a protein found in the cytoplasm of CTCs) and CD45 (a cell surface protein found exclusively on WBCs) to differentiate CTCs from contaminating WBCs. DAPI (4′,6-diamidino-2-phenylindole) is also used to stain the nuclei of CTCs and WBCs. Finally, a magnetic field is applied to collect the CTCs, which are identified by positive expression of EpCAM and CK and negative expression of CD45.

System	Separation principle	Strengths	Weaknesses	References
Antibody-based capture				
CellSearch	Positive selection for EpCAM, CK8/CK18/CK19 and negative selection for CD45	• Food and Drug administration (FDA)-approved for clinical use	• Unable to capture tumour cells that lack EpCAM expression • Only applicable to cancers with epithelial origin • Cells are not viable after isolation	[28–30]
CTC-chip	EpCAM-based	• Sample does not require pre-processing • Cells remain intact and viable after isolation	• Unable to capture tumour cells that lack EpCAM expression • Only applicable to cancers with epithelial origin • Unable to recover tumour cells with 100% purity	[33]
CTC-ichip	Size-based separation followed by negative depletion of white blood cells with CD45 and CD66b magnetic beads	• Fast processing time (8 ml/h) • Has the potential to capture CTCs from any cancer type. • Cells remain intact and viable after isolation	• Unable to capture tumour cells that are smaller or similar in size to blood cells. • Unable to recover tumour cells with 100% purity	[51, 52]
IsoFlux	EpCAM-based	• Higher sensitivity of detecting CTCs than CellSearch system	• Unable to capture tumour cells that lack EpCAM expression • Only applicable to cancers with epithelial origin	[53]
Magnetic Sifter	EpCAM-based	• Allows rapid imaging of captured cells on a small area • Cells remain intact and viable after isolation • Reduces sample losses with minimal pre-processing	• Unable to capture tumour cells that lack EpCAM expression • Only applicable to cancers with epithelial origin	[54]
GEDI microdevice	Prostate-specific membrane antigen (PSMA)/HER2-based	• Higher sensitivity of detecting CTCs than CellSearch system • Device geometry reduces capture of WBCs	• Only applicable to prostate cancer, breast cancer, gastric cancer • Unable to recover tumour cells with 100% purity	[43, 55, 56]

System	Separation principle	Strengths	Weaknesses	References
Label-free capture				
ClearCell FX (spiral chip)	Size-based	• Fast processing time (3 ml/h) • Cells remain intact and viable after isolation • Cost-effective • Has the potential to capture CTCs from various cancer types.	• Unable to capture tumour cells that are smaller or similar in size to blood cells. • Unable to recover tumour cells with 100% purity	[11, 40]
Microfluidic biochip	Size-based	• Fast processing time (7.5 ml of blood in 3 h) • Allows single-cell isolation • Able to isolate viable CTCs with 100% purity • Has the potential to capture CTCs from various cancer types.	• Limited number of cell chambers for imaging • Unable to capture tumour cells that are smaller or similar in size to blood cells.	[16, 17]
Vortex	Size-based	• Fast processing time (7.5 ml of blood in 20 min) • Cells remain intact and viable after isolation • Has the potential to capture CTCs from various cancer types.	• Unable to recover tumour cells with 100% purity • Low CTC capture efficiency • Unable to capture tumour cells that are smaller or similar in size to blood cells.	[57]
Microfluidic device for deformability-based cell classification	Size and deformability-based	• Cells remain intact and viable after isolation • Cost-effective • Has the potential to capture CTCs from various cancer types.	• Unable to recover tumour cells with 100% purity	[35]
DEPArray	Electric charge-based	• Allows single-cell isolation • Able to isolate viable CTCs with 100% purity • Has the potential to capture CTCs from various cancer types.	• Limited throughput • Large amount of sample loses	[58, 59]

Abbreviations used are: CTC, circulating tumour cell; EpCAM, epithelial cell adhesion molecule; CK, cytokeratin proteins.

Table 1. Comparison of selected microfluidics systems used for enriching CTCs.

In 2007, a microfluidics chip developed for CTC enrichment and isolation, known as CTC-chip, was introduced [33]. In this system, CTCs are captured as blood flows through the microchannel containing EpCAM antibody-coated microposts [33]. CTCs are captured with ~50% purity and sample processing takes 1–2 ml/h. Following CTC-chip, a broad range of microfluidic devices were generated to isolate CTCs based on physical size, density, deformability [34–41] or antibody-mediated CTC capture in surface functionalised microchannels [33, 42–47].

Although the affinity binding methods (e.g. CellSearch, CTC-chip) may isolate CTCs of better purity than the physical methods, the strong antibody-antigen interaction in the microdevice may affect the recovery of viable CTCs [11, 48] and hinder subsequent downstream analysis. Additionally, affinity binding methods will lose out on the subpopulation of CTCs that have down-regulated expression of epithelial markers (e.g. EpCAM) such as the mesenchymal CTCs, resulting in an underrepresentation of the actual CTC count in the blood [11, 49]. Therefore, the limitations imposed by affinity binding methods prompt the development of label-free microfluidic devices that can isolate CTCs with increased purity and viability.

To efficiently separate CTCs from the large pool of RBCs and WBCs, a spiral microfluidics chip was introduced in 2013 [11, 50]. The spiral microchannel (500 μm width × 160 μm height) consists of two inlets and two outlets over a length of ~10 cm. Blood (diluted 2–2.5×) is pumped into the outer inlet while sheath fluid is pumped through the inner inlet. The additional sheath fluid in the spiral chip facilitates the Dean migration of large volume of RBCs in a well-controlled manner, thus allowing high haematocrit samples (20–25%) to be processed. In spiral microchannel, CTCs and other blood cells experience Dean drag forces in addition to inertial lift forces and the combined effects cause hydrodynamic focusing of the cells to specific region of the microchannel. The larger CTCs are focused near the inner wall of the channel while smaller WBCs and RBCs are focused along the outer wall, leading to efficient size-based isolation of CTCs [50]. Furthermore, the spiral chip is able to recover >85% spiked cancer cells and deplete almost 100% of WBCs from the blood sample [11, 50]. Trypan blue staining showed that most of the recovered cells (>98%) are viable [11]. Therefore, the spiral chip allows continuous isolation of CTCs in a single step with high sensitivity, recovery and throughput (3 ml of blood can be processed in an hour) [11]. The spiral chip is also designed to have large microchannel dimensions and high flow rate to prevent non-specific binding of CTCs to the walls and eliminate any potential clogging issues [11].

2.2. Microfluidics and single CTC isolation

Most microfluidics systems isolate CTCs in bulk rather than individually. The bulk analysis of CTCs may mask the presence of key mutations that are critical to disease progression, indicating the need for single-cell analysis. To isolate and study CTCs at the single-cell level, approaches such as micropipette aspiration [60, 61] and laser microdissection [62] have been used to manually select the individual CTCs. However, these methods are laborious and suffer from low throughput, making them less suitable for clinical use. Commercial platforms such as DEPArray (Silicon Biosystems), which rely on the dielectric properties of CTC for single-cell isolation, suffer from large amount of sample losses [58].

To address these limitations, Yeo et al. developed a microfluidics device capable of performing high throughput, selective isolation of individual viable CTCs with 100% purity amidst a

large population of WBCs [17]. The separation efficiency is high because as few as 1 CTC in 20,000 WBCs can be recovered. Prior to starting the run, the cell suspension is stained with specific fluorophore-conjugated antibodies such as CK or CD45 to facilitate the differentiation of CTCs from WBCs. The device works on the principle of hydrodynamic focusing to restrict cells to flow in a single stream and hold them passively in active control cell chambers that are positioned along the outer curvature of the channel. CTCs and other blood cells flowing through the channel will experience a slight centrifugal force that facilitates their entry into the cell chambers. Because the microfluidics biochip is integrated into a microscope, the cell sitting in the chambers can be observed under the microscope to determine whether it is a CTC or WBC based on size or staining outcome. Each chamber holds 1 cell at a time and each is connected to a control line that can be activated to eject the selected cell back into the main channel and into the collection well. In this manner, the cells isolated in the chambers can be ejected sequentially so that each cell can be recovered individually. To maximise the recovery of rare CTCs, the effluent of each run is recycled back into the device for three times. The device is able to process 7.5–8 ml of blood in 3 h [16], facilitating the enumeration of CTCs in clinical blood samples in a short period of time. This method also allows the collection of single CTCs for downstream molecular profiling.

3. Clinical applications of CTCs

Given the convenience and ease of obtaining blood samples from cancer patients, CTCs are currently being adopted for clinical practice. This is especially the case when tissue biopsy samples are not readily available. With current technologies, CTCs can be isolated in bulk or individually for studies on disease progression and therapeutic treatment. Single-cell analysis offers several advantages over pooled cell analysis. First, single-cell analysis is able to detect critical driver mutations for drug response that are present in low frequency, which may be masked in pooled cell analysis since the mutations may only be present in a subset of clones. For instance, in stage IV non-small cell lung cancer (NSCLC) patients who developed resistance after tyrosine kinase inhibitor (TKI) treatment, sequencing of single CTC revealed the presence of epidermal growth factor receptor (EGFR) mutation T790 M that confers resistance in a subset of CTCs isolated from each patient (0–3 CTCs carry the mutation among <10 CTCs isolated per patient) [17]. Determining the mutation status of EGFR in NSCLC patients is important for patient stratification and treatment (see Section 3.4). Second, single-cell analysis reveals the heterogeneity in gene mutations and chromosomal copy number aberrations (CNA) among tumour cells while bulk cell analysis would have average out the signals. In metastatic breast cancer, a sequencing analysis of 40 single CTCs from five patients demonstrated heterogeneous mutations in four genes, phosphatidylinositol 3-kinase catalytic subunit alpha (*PIK3CA*), tumour protein P53 (*TP53*), oestrogen receptor 1 (*ESR1*) and *KRAS* [63]. Within the same metastatic breast cancer patient, not all CTCs harbour the particular mutation and different patients harbour different gene mutations [63]. In small cell lung cancer (SCLC) patients, chromosomal CNA profiling of individual CTCs obtained at pre-treatment can predict whether the patient is sensitive or refractory to subsequent chemotherapy at an accuracy of 83.3% [64], facilitating clinical decision-making. Third, single-cell analysis facilitates the study of clonal diversity and mutation evolution over the course of chemotherapy, which is difficult to achieve in bulk cell

analysis [65, 66]. In triple negative breast cancer (TNBC) patients, single-cell DNA sequencing revealed that chemoresistant patients carry pre-existing mutations and CNA that were adaptively selected in response to chemotherapy [65]. Single-cell RNA sequencing also found that chemotherapy induces transcriptional reprogramming to favour the resistant phenotype in TNBC patients who develop chemoresistance [65]. Thus, single-cell analysis provides a better resolution of the tumour profile than pooled sample analysis. More importantly, single-cell analysis of CTC can address the heterogeneous profile of cancer cells at the DNA, RNA and protein level and provide insights in the mechanism of metastasis and drug resistance. Here, we discuss the applications of CTCs that aid in the management of cancer and how single CTC analysis can contribute to achieving personalised medicine in the future.

3.1. Biomarker for early detection of cancer

The presence of CTCs in the peripheral blood acts as a biomarker for the early detection of cancer. Thus, the enumeration of CTC is important for cancer screening, especially in patients who at a higher risk of developing cancer due to genetic predisposition or disease state. The effective use of CTC enumeration in the detection of early-stage cancer has been demonstrated in patients with chronic obstructive pulmonary disease (COPD), who are at a high risk of developing lung cancer [5, 67]. Because of the risk, COPD patients are monitored annually for the development of lung cancer using computed tomography (CT) scan. In a subset of COPD patients without cancer diagnosis, CTCs were found in the peripheral blood. After 1–4 years of CTC detection, lung nodules were indeed detected by CT scan, leading to the diagnosis of early-stage lung cancer. Large-scale clinical studies are being done to further validate the use of CTCs as a diagnostic tool for the early detection of cancer [5]. Therefore, CTC can serve as a potential biomarker for the early detection of cancer so that prompt treatment intervention can come in place to improve the overall health of patients.

3.2. Prognostic marker for overall survival and metastasis

The number of CTCs found in the blood can indicate the state of the disease. Patients in later stages of cancer (e.g. metastatic cancer patients) have higher number of CTCs as compared to patients in the early stages of cancer [14, 68, 69]. Furthermore, the number of CTCs can vary with chemotherapy treatment, allowing clinicians to use CTC counts to determine the treatment efficacy and estimate the overall survival and risk of metastatic relapse. A study on early breast cancer patients [70] measured the number of CTCs before and after adjuvant chemotherapy demonstrated that the persistent presence of CTCs after chemotherapy is associated with poor disease-free survival and overall survival. Moreover, prognosis was worst in patients with >5 CTCs per 30 ml of blood [70]. Similarly, studies in lung cancer patients also showed that higher number of CTCs was significantly correlated to shorter survival [32, 71–73]. Specifically, the presence of >8 CTCs per 7.5 ml of blood after treatment strongly correlated with worse survival in small cell lung cancer patients [74]. In prostate cancer, therapeutic treatment resulting in CTC level dropping from >5 to <5 in 7.5 ml of blood is indicative of better overall survival [55]. Thus, the prognostic cut-off value for CTC is dependent on the type of cancer.

Additionally, CTC enumeration can be used for predicting the risk of metastasis. In non-metastatic colorectal cancer patients, those with >5 CTCs per 2 ml of blood were more likely

to develop distant metastasis than those with <5 CTCs [69]. The strong correlation between CTC count and metastasis relapse have been shown in other types of cancers as well including bladder cancer [75, 76], liver cancer [77] and oesophageal cancer [78]. Thus, these patients may benefit more from early treatment.

3.3. Monitoring treatment response and disease progression

Sequential tracking of CTC number during treatment may inform on treatment response and disease progression, providing important information to clinicians on whether the treatment is suitable for the cancer patient. In advanced NSCLC patients, a significant decrease in CTC count after the second cycle of chemotherapy strongly correlated with better overall survival and progression-free survival [32, 79, 80]. However, a lack of decline in the number of CTCs after chemotherapy may suggest that the patient has developed resistance against the specific drug. Therefore, alternative treatment strategies have to be adopted to curb disease progression and improve patient survival. Another potential application may be the detection of early relapse with regular monitoring of the CTC count in post-surgical cancer patients. This may allow early detection and timely clinical intervention to treat the disease when the disease burden is less.

3.4. Identification of therapeutic targets and drug resistance

Given the continuous improvement in microfluidics technology, Khoo et al. demonstrated that patient-derived CTCs could be cultured into CTC clusters *in vitro* using a microfluidic culture device [81]. Prior enrichment of CTCs and supplements of growth factors are not required, thereby shortening the processing time. Using blood samples from the same patient, CTCs are co-cultured with immune cells in specially formulated microwells to promote the formation of CTC cluster within 2 weeks. The success rate of CTC cluster formation is approximately 50%. With the development of this platform, drug screening can be readily conducted and this can facilitate the discovery and testing of novel drugs that are more efficacious in the treatment of cancer. Furthermore, drug responses can be monitored with varying doses of drug to determine the optimal dose for individual patient.

With the advent of next-generation sequencing, the molecular profile of single CTCs can be obtained at the DNA, RNA and protein level. The genomic profile of single CTCs can be compared to normal or non-malignant cells to identify genes that are differentially expressed, which may mediate the process of metastasis and become potential therapeutic targets. The whole genome sequencing of individual CTCs can reveal genetic alterations such as mutations, copy number variations and single nucleotide polymorphisms that may confer selective advantage to tumour cells [82]. The presence of specific gene mutations in CTCs confers drug resistance and determines the type of treatment to be given to patients. This information is particularly useful when the mutation profile of CTC is concordant with the primary tumour. For example, the CTCs of NSCLC patients harbouring the EGFR T790 M mutation confer resistance to TKI treatment (e.g. gefitinib) [83]. Tracking changes in the CTC count and mutation frequency over the course of treatment allows real-time monitoring of treatment sensitivity and resistance. The early detection of these mutations may provide alternative treatment strategies for NSCLC patients and optimise disease management, leading to improved clinical outcomes [17]. Thus, determining the mutation status of EGFR is crucial since it allows

clinicians to select patients who will benefit from TKI treatment. Furthermore, understanding the key mutations behind drug resistance may help to decipher the mechanism and signalling pathways involved in resistance.

The transcriptome of single CTCs also provides information on the identification of therapeutic targets and drug resistance. In prostate cancer, analysis of the mRNA profile in CTCs is required to determine drug sensitivity or resistance. Specifically, the expression of Arv7 mRNA, a truncated form of androgen receptor that remains constitutively active, in CTCs is predictive of anti-androgen therapy failure with enzalutamide and abiraterone [84, 85]. In prostate cancer patients with Arv7 expression, alternative drugs such as taxanes are used for treatment [86–89]. Additionally, RNA-seq of CTCs can reveal miRNAs that are dysregulated in cancers, making these miRNAs potential targets for cancer therapy.

The expression profile of proteins in single CTCs also plays a role in determining the anti-cancer treatment. For example, oestrogen receptor (ER) is a primary target in the treatment of breast cancer patients. Thus, primary tumours of breast cancer patients are stratified as ER+ or ER- and hormonal therapy is given based on the status of ER expression in the primary tumour. However, breast cancer patients with ER+ primary tumours can harbour ER- CTCs in the blood, which may escape the hormonal therapy [90]. Similarly, metastatic breast cancer patients with HER2+ primary tumours can carry HER2- CTCs in the blood [91–93]. Because of this discordance, HER2-targeted therapies may only be effective against the primary tumour but not the CTCs. Thus, cancer cells will not be fully eradicated and this may lead to metastatic cancer relapse. In this situation, additional treatment strategies have to be adopted to target the CTCs, on top of the primary tumour. Therefore, the monitoring of genetic aberrations is important in identifying acquired mutations that confer resistance to drug therapy.

The expression status of programmed death ligand 1 (PD-L1) in CTCs aids in identifying the groups of patients who are likely to benefit from the immunotherapy as well as predicting the response to the immunotherapy. Tumour cells express PD-L1 that binds to PD-1 receptor found on the surface of activated T cells and B cells to induce an immunosuppressive effect by reducing cytokine production and immune cells proliferation [94, 95]. It was previously shown that metastatic tumour cells have higher PD-L1 expression than primary tumour cells [96]. In breast cancer, the detection of CTCs expressing PD-L1 indicates that patients carry metastatic cells that have the potential to evade immune destruction [94]. Breast cancer patients with a high frequency of PD-L1(+) CTCs are more likely to benefit from anti-PD-L1 immunotherapy than patients with PD-L1(−) CTCs [94]. In a study on NSCLC patients, PD-L1 expression on CTCs was monitored throughout the course of immunotherapy [97]. After 6 months of therapy, patients with PD-L1 expression in CTCs had poor prognosis while patients without PD-L1 expression in CTCs benefitted from the therapy [97].

Given the intra-tumoural and inter-tumoural heterogeneity and dynamic nature of cancer, single-cell analysis of CTC in circulation may provide information on the evolution of tumour and how they evade drug therapy and immune response (**Figure 1**). This enables clinicians to have a more holistic view of the disease complexity and more efficient targeting of cancer cells, moving towards the development of personalised therapy for individual patients.

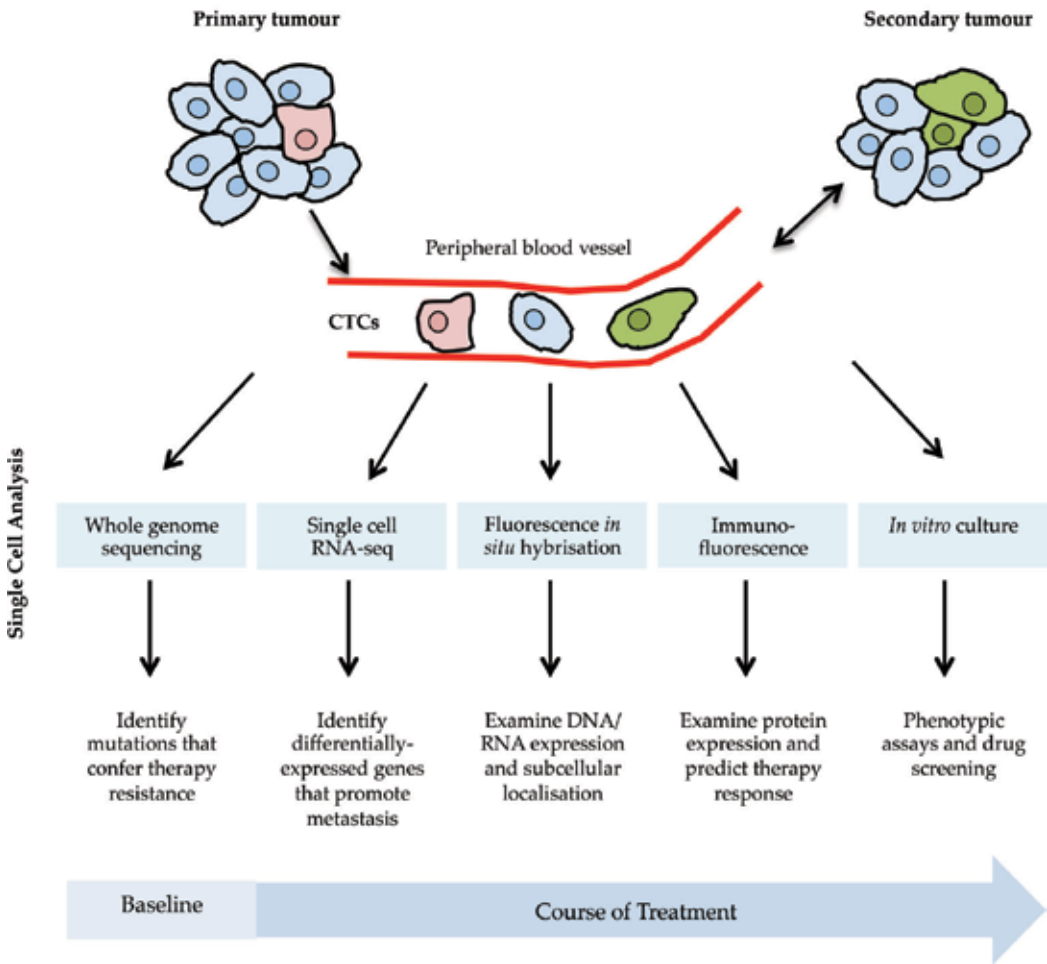

Figure 1. A proposed scheme of how single-cell analysis of CTCs may address tumour heterogeneity. Heterogeneous tumour cells are colour-coded. Heterogeneity stems from genetic or epigenetic changes that confer selective advantage to the tumour cells. Several strategies of single-cell analysis can be adopted to dissect the heterogeneity of tumour cells. Sequential analyses of individual CTCs aids in the monitoring of therapeutic response, tumour evolution and detection of treatment-resistant cells.

3.5. Comparing CTC with cell-free DNA and exosome

Apart from CTCs, other liquid biopsy markers that confer diagnostic and prognostic relevance include the cell-free DNA (cfDNA) and exosomes. Each marker has its own strengths and weaknesses (**Table 2**). cfDNA refers to DNA released from necrotic and apoptotic cells and they can be found in blood plasma. Cancer patients usually have higher concentration of cfDNA compared to healthy individuals [98, 99]. Most cfDNAs in the blood plasma are around 70–200 bp long [100, 101]. cfDNA includes the circulating tumour cell DNA (ctDNA), which are released from CTCs. The fraction of ctDNA contributing to cfDNA is small although usually higher in late stage cancer patients (>5–10%) than patients in the early stages of cancer

Liquid biopsy	Strengths	Weaknesses
Circulating tumour cell (CTC)	• Allows downstream molecular analysis and *in vivo* or *in vitro* functional studies • Able to evaluate the DNA, RNA and protein profile of tumour cells • Able to study the cellular phenotype, morphology and protein localisation	• Extremely rare and challenging to isolate • Heterogeneous population of CTCs may lead to false positive and false negative results
Cell-free DNA (cfDNA)	• High sensitivity in detecting genetic aberrations	• Challenging to isolate pure population of cfDNAs • Molecular characterisation is limited to genomic DNA and unable to evaluate the RNA and protein profile • Unable to perform phenotypic and functional studies
Exosome	• Abundant in the plasma • Allows downstream molecular analysis and functional studies • Able to evaluate the DNA, RNA and protein profile of tumour cells • Allows analysis of inflammatory, stromal and other systemic changes	• Challenging to isolate pure populations of tumour-derived exosomes • Unable to perform phenotypic studies

Table 2. Comparison of the liquid biopsy markers.

(<1%) [98, 102, 103]. A large fraction of cfDNA comes from non-malignant cells that contain wild type DNA. Because of this issue, highly specific and sensitive technologies are required to isolate cfDNA that originates from tumour cells.

The isolated cfDNAs are subjected to sequencing analysis using next-generation sequencing platforms to identify tumour-associated genetic mutations or epigenetic changes. For example, in a large-scale study of NSCLC patients on gefitinib treatment, cfDNA was used as a surrogate of tissue biopsy to identify the EGFR mutations, demonstrating its clinical utility [100, 104]. A comparison on the frequency of mutation detection between cfDNA and CTC revealed that cfDNA showed a higher frequency of the mutation from the same patient [100, 102], suggesting that cfDNA is more effective in detecting these genetic changes. Another advantage that cfDNAs have over CTCs is that cfDNAs can be obtained from bio-banked fluids such as frozen plasma whereas CTCs can only be obtained from peripheral blood [100]. However, the use of cfDNAs as a liquid biopsy marker also poses several limitations. First, because cfDNA can originate from any cell type including normal cells and tumour cells, there will be a high background of wild type DNA and thus isolating a pure population of the rare tumour-derived cfDNA is technically challenging. The abundance of wild type DNA may mask the detection of low copy genetic mutations that could be important for early detection of cancer or drug resistance. Second, molecular profiling of cfDNAs is restricted to the DNA level as the characterisation of the transcriptome and proteome is not possible [100].

Exosomes are membrane-bound microvesicles derived from multivesicular bodies (MVBs) and secreted into the extracellular environment through fusion of MVB to the plasma membrane [8, 100]. They mediate cell-cell communication by transferring biomolecules such as DNA, RNA, proteins and lipids from the donor cell to the recipient cell [105]. Exosomes can originate from many cell types including tumour cells, epithelial cells, fibroblasts, neuronal cells, haematopoietic cells and adipocytes [8, 106]. Most exosomes are around 30–200 nm and are present in large quantities in biological fluids including serum, plasma, urine and saliva [8, 100]. Tumour cells release tens of thousands of exosomes in a day, resulting in hundreds of billions of exosomes per ml of plasma [100, 107]. Since exosomes are abundant, they are easier to isolate compared to CTCs and cfDNAs. However, there is a lack of efficient tumour exosome enrichment method [108] because the isolation of exosomes is largely based on exosome-specific surface markers [100, 109], which do not distinguish between exosomes derived from tumour cells and normal cells.

Because exosomes carry the DNA, RNA and protein content from tumour cells (i.e. cell of origin), they are useful diagnostic and prognostic tools of cancer. Similar to CTCs and cfDNAs, exosomes can be subjected to DNA analysis to determine the genetic aberrations and mutational landscape of tumour cells. Additionally, the RNA and protein profiles of tumour exosomes can be characterised to provide insights into the biology of tumour cells. For example, analysis of exosomal mRNA and proteins allows real-time monitoring of therapeutic response and drug resistance in glioblastoma patients [110, 111]. The up-regulated expression of a panel of serum-derived exosomal miRNAs (miR-1246, miR-4644, miR-3976, miR-4306) serves as a biomarker for the diagnosis of pancreatic cancer [112]. The down-regulated expression of miR-92a is associated with high risk of cancer relapse in hepatocellular carcinoma patients [113]. Furthermore, previous reports showed that tumour exosomes play a role in suppressing immune response, promoting tumour cell growth, angiogenesis and metastasis [100, 114–116]. Similar to CTCs, molecular profiling of exosomes provides insights into the mechanism of metastasis and drug resistance.

3.6. Barriers to adoption of CTC as a clinical test

Although CTCs have numerous potential clinical applications, the incorporation of CTCs into routine clinical practice still faces several challenges. First, there is a lack of reproducibility in CTC enumeration when different measurements were taken from the same patient [117]. This variation is likely caused by the extremely low frequency of CTCs in peripheral blood, where >90% of patients with localised diseases and up to 30–40% of patients with metastatic disease do not have >5 CTCs per 7.5 ml of blood [117, 118]. A difference of 1 CTC may lead to different stratification and prognostic outcome [117]. A possible solution to improve the CTC yield is to process larger volume of blood sample from cancer patients [117, 119]. Second, there is a lack of standardisation across the myriad of CTC detection platforms in defining and isolating CTCs [120, 121], resulting in variability of CTC count. Studies comparing EpCAM-dependent and EpCAM-independent CTC enrichment methods using blood samples from the same patient showed that EpCAM-independent methods generate a higher CTC count compared to the CellSearch system [117]. Thus, a universal quality control system is required for detecting and isolating CTCs across the various platforms so as to benchmark the reliability of these methods [117, 120]. Cross-validation studies on CTC enumeration from different laboratories

will also minimise the inter-observer variation [117]. Lastly, the current CTC isolation technologies have limited sensitivity and are not applicable to all types of cancer [117]. Therefore, before CTCs can be fully adopted for routine clinical use, well-designed appropriately powered validation studies are required.

4. Conclusion

Late clinical diagnosis and chemotherapy resistance are the main factors leading to reduced chances of survival for cancer patients. To combat cancer, CTCs provide invaluable information on the status of the disease and the likely outcome of chemotherapeutic treatment. The enumeration of CTCs allows early detection of cancer, prognosis and real-time monitoring of chemotherapy treatment. The single-cell analysis of CTCs provides a wealth of genetic information that enables better understanding of the disease complexity for individual patients and provides the opportunity for the development of personalised treatment. To aid in delivering better therapeutic medicine, current technologies allow CTCs to be cultured *in vitro* for the identification of novel therapeutic targets and optimal drug dosage for individual patients. Therefore, the molecular characterisation of CTCs is important for improving the clinical outcomes in cancer patients.

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Chapter 3

Circulating Cell-Free DNA

Yingli Sun, Ke An and Caiyun Yang

Additional information is available at the end of the chapter

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Abstract

Circulating cell-free DNA (cfDNA) refers to extracellular DNA present in body fluid that may be derived from both normal and diseased cells. The concentration, integrity, genetic, and epigenetic alternations in the cfDNA may suggest pathological conditions of the body, such as inflammation, autoimmune diseases, stress, or even malignancies. cfDNA from patients with malignancies contains variants as those in the tumor tissue cells, thus allowing noninvasive assessment of tumor in real time. The clinical detection of cfDNA is one major application of liquid biopsy and has great application value in the early diagnosis of clinical tumors, real-time progression monitoring, curative effect observation and evaluation, prognosis assessment, and metastasis risk analysis. This chapter summarizes the origin of cell-free DNA and its important clinical applications as a noninvasive biomarker.

Keywords: liquid biopsy, circulating cell-free DNA, cancer, biomarker

1. Introduction

Liquid biopsy, a term relative to tissue biopsy, is a technical way to analyze the nonsolid biological tissue by detection of cells and free DNA that enter body fluids. Liquid biopsy refers to the real-time monitoring of the dynamic alterations of disease by detecting circulating tumor cells (CTCs), circulating cell-free DNA (cfDNA), exosomes and so on. This technique has great application value as a tool for disease early diagnosis, real-time progression monitoring, curative effect observation and evaluation, prognosis assessment, and metastasis risk analysis, with the added benefit of being noninvasive and flexible for repeat tumor sampling [1–3].

Circulating cell-free DNA (cfDNA) is released as single-stranded DNA and double-stranded DNA into body fluids, including the blood [4], sputum [5], urine [6], cerebrospinal fluid [7], or ascites [8] from apoptotic and necrotic cells [9]. cfDNA was first identified by Mandel and Metais

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in the human blood in 1948 [10]. In 1977, Leon discovered that circulating cell-free DNA was also existed in cancer patients [11]. In 1997, Lo et al. found the presence of a small percentage of cfDNA originating from the fetus in the maternal plasma and serum. Then cfDNA was first used for noninvasive prenatal testing, including fetal sex assessment which can identify sex for fetus [12], RhD blood group genotyping, detection of chromosomal aneuploidy, and fetal-related diseases. These diseases include systemic lupus erythematosus (SLE), an autoimmune disease involving multiple systems, multiple organs, and multiple autoantibodies [13], and monogenic diseases, such as *β-globin* gene and *HBB* gene [14]. At present, as an important aspect of liquid biopsy, the detection of circulating cell-free DNA displays its irreplaceable advantages in clinic, including simpleness and accessibility. Compared with the solid biopsy, the liquid biopsy by detection of cfDNA is noninvasive and easily repeated. The detection of cfDNA as a clinic marker has amounts of advantages. The intra-abnormalities can be detected in cfDNA at an earlier time, thus enabling early diagnosis of disease. And the detection of cfDNA makes repeated sampling possible for the monitoring of disease progression, drug response, and prognostic tracking.

2. Clinical applications of circulating cell-free DNA

The concentration, integrity, genetic, and epigenetic alternations in the cfDNA may suggest pathological conditions of the body, such as inflammation, autoimmune diseases, stress, or even malignancies. Different disease-associated molecular characteristics can be detected as the indicators of pathological conditions in the plasma of patients, including the total level and fragment, copy-number aberrations [15–18], methylation changes [19–21], single-nucleotide mutations [16, 22–25], cancer-derived viral sequences [26, 27], and chromosomal rearrangements [28, 29]. cfDNA from patients with malignancies (cell-free tumor DNA, ctDNA) contains variants as those in the tumor tissue cells, thus allowing noninvasive assessment of tumor in real time. ctDNA is a very promising tumor biomarker for cancer diagnosis and monitoring, prognosis assessment, and personalized medication guidance compared with conventional serum markers.

2.1. The size of cfDNA

The length of cfDNA from patients differs from that of healthy groups, which may suggest some kinds of physiological or pathological conditions, including pregnancy, cancer, liver/bone marrow transplantation, SLE, and many other clinical scenarios such as stroke, autoimmune disorders, and myocardial infarction [30–37]. The length of cfDNA was previously identified by gel electrophoresis and electron microscopy (EM) in 1998. Giacona et al. found that most abundant cfDNA fragments from pancreatic cancer patients displayed stronger ladder patterns compared with that from healthy controls, which was equivalent to whole-number multiples (1–5×) of nucleosomal DNA (185–200 bp). The average strand length distributions of DNA (DNA-SL) in pancreatic cancer patients were also obviously shorter (231 nm; median, 185 nm) than average plasma DNA-SL in controls (311 nm; median, 273 nm). There were more excess of short DNA at approximately 63, approximately 126, approximately 189, approximately 252, and approximately 315 nm, corresponding to small multiples of lengths

associated with nucleosomes, in the pancreatic cancer patient plasma than in the plasma of healthy control [38]. The molecular size-distribution profiles of plasma DNA in systemic lupus erythematosus (SLE) patients exhibited a significantly increased proportion of short DNA fragments [22]. Jiang et al. found that the plasma DNA molecules from hepatocellular carcinoma patients were aberrantly short or long through massively parallel sequencing and the aberrantly short ones preferentially carried tumor-associated copy-number aberrations [23]. The study now confirms that the overall size of cfDNA was approximately 166 or 143 bp or even shorter with a periodicity of 10 bp [37]. The size distributions of cfDNA prominent peak were focused in 166 bp for hepatocellular carcinoma (HCC) patients and hepatitis B virus (HBV) carriers [39]. The size of cfDNA fragment was different from the systemic lupus erythematosus patients that the height of the 166 bp peak was reduced and has smaller peaks and healthy individuals [40]. These abundant cfDNA molecules were most likely generated from apoptosis cells accompanied with certain enzymatic cleavage processes shaped by nucleosome-associated DNA packing [34, 40–43]. With the technology development and refinement for the determination of cfDNA fragment size, cfDNA fragment size and its distribution provide important information associated with pathological conditions and display to be a promising indicator for clinical diagnosis.

2.2. cfDNA concentration

The concentration or level of cfDNA could change with different physiological conditions. The study described the concentration of cfDNA in patients with non-small cell lung cancer (NSCLC) was higher than healthy controls, and the average level was 95.67 and 59.60 ng/μl, respectively [44]. The concentrations of overall cfDNA in cancer patients have a significant increase with a wide range (hundreds to thousands ng/ml in the blood) compared with in the healthy controls (a relative level of 30 ng/ml) [45–48]. The level of cfDNA in cancer patients, such as in ovarian cancer, colorectal cancer, and pancreatic cancer, is significantly associated with the cancer-specific survival and can be used as an independent predictor for death [22, 46, 47]. The study found preoperative cell-free DNA levels are significantly elevated in patients with epithelial ovarian carcinoma (EOC), and the cell-free DNA level is a potential predictor for clinical outcome in patients with ovarian cancer. For colorectal cancer patients, the cfDNA level is correlated with a shorter survival and may be a biomarker for survival when it is above 1000 ng/ml [47]. The level of cfDNA is the highest in pancreatic ductal adenocarcinoma compared with pancreatic neuroendocrine tumor and chronic pancreatitis using Alu repeat amplicon [1]. The cfDNA level also can be used as a marker of cellular trauma and inflammation from anesthesia and surgery in clinic. The concentration of cfDNA displayed significant differences and fluctuation pattern during serial perioperative process in donors and recipients undergoing living donor liver transplantation (LDLT). The cfDNA concentration is higher in recipients than in donor undergoing living donor liver transplantation and is an indicative marker for liver injury. The cfDNA level fluctuated from a baseline 37.62 ng/ml to a relative high level of 94.72 ng/ml in recipient who developed postoperative sepsis [49]. In the study of lung cancer, the patients with high baseline cfDNA concentration had a significantly worse disease-free and overall survival than those with lower concentrations [50].

2.3. cfDNA genetic variations

Cell-free DNA generates from apoptosis or necrosis cells and contains the same genetic variations with intra-tissues. cfDNA is widely used as a genetic biomarker for disease diagnosis and monitoring by detection of the copy-number variations, SNPs, and mutation occurred in cfDNA.

2.3.1. Copy-number variations of cfDNA

Copy-number variations (CNV) are always associated with the occurrence of complex disorders. The CNV of urine cfDNA in advanced prostate cancer patients is significantly associated with tumor burden, and the CNV change after stage-specific therapies reflected disease progression status and overall survival [51]. Copy-number variations of HLA-DRB5 in 135 systemic lupus erythematosus (SLE) patients were higher than that in 219 healthy controls and were associated with the risk of SLE. The copy-number at 6p21.32 is aberrant in the majority of SLE patients [52]. In the plasma of neuroblastoma patients, the copy-number alterations of cfDNA displayed concordant high patterns and can be used as a cost-effective, noninvasive, rapid, robust, and sensitive biomarker for neuroblastoma prognosis [53].

2.3.2. Mutation of cfDNA

Mutation is a widespread phenomenon in biology, the effect of which is permanent alteration of nucleotide sequence. Mutations play a vital role in both normal and abnormal biological processes, such as evolution and cancer. Many studies display that the mutation detection in cfDNA will enable noninvasive tumor diagnosis and monitoring with higher sensitivity and specificity in advance [54, 55]. Many patients with advanced lung cancers that are resistant to AZD9291 therapy carried *EGFR C797S* mutation in cfDNA [56]. EGFR mutations in cfDNA were significantly associated with overall survival (OS), progression-free survival (PFS), and response to therapy in the EURTAC trial. The EGFR L858R mutations in cfDNA proved to be a novel prognostic marker [57]. In melanoma, BRAF mutation in cfDNA can be detected earlier than primary lesion [58]. KRAS mutation in cfDNA for pancreatic ductal adenocarcinoma (PDAC) provided a new diagnostic marker and could optimize therapeutic strategies for patients [59].

2.3.3. SNP of cfDNA

Single-nucleotide polymorphism is a variation in a single nucleotide occurring in the genome at a specific position. Detection of SNP in circulating cell-free DNA has been widely used in prenatal screening. The detection of SNP located in SRY gene or TSPY gene in Y chromosome proved to be a highly accurate and clinically applicable noninvasive prenatal diagnosis (NIPD) marker for fetal gender determination [60–62]. The study reported first trimester contingent screening used nuchal translucency and cell-free DNA, the latter has higher detection rate that is up to 98% for trisomy 21, but noninvasive prenatal testing will not be cost-effective associated with traditional [63, 64].

2.4. cfDNA methylation as epigenetic biomarker

Epigenetic modifications are heritable molecular events that affect gene expression without changing DNA sequences, including DNA methylation, histone modification, and so on.

They are stable through cell division. DNA methylation refers to the addition of methyl group to cytosine residues in DNA sequence, and it is the best-studied epigenetic event [65, 66]. The quantitative DNA methylation analysis of tumor-derived cell lines was conducted in 1999 for the first time, and the possibility of using them as noninvasive biomarkers for cancer was examined [21, 67, 68]. Various studies were performed to detect cfDNA to assess the performance of cfDNA methylation as a biomarker [64].

The level of cfDNA methylation for GSTP1 and APC in the castration-resistant prostate cancer patients could be used as a marker reflecting treatment response and prognosis [69]. The BRMS1 promotor in cfDNA could provide prognostic information from the plasma of NSCLC and highly methylated from advanced NSCLC patients [70]. cfDNA epigenetic pattern can be used as an early diagnostic marker for breast cancer [71].

3. Conclusion

At present, cfDNA has been used as an independent marker for prenatal screening and also has great applicable value in the disease prognosis and monitoring, particularly in cancer. When the concentration of cfDNA is above a baseline 30 ng/ml and is closed to hundreds or even thousands ng/ml, or/and when the size of cfDNA is obviously short and displays ladder pattern, or/and when vital genetic and epigenetic mutations are reported in cfDNA, patients should be recommended for further examination. The appearance of cfDNA conforms to the current trend of precision medicine in the disease and achieves accurate diagnosis and precise treatment. However, there are many challenges in the real clinic applications. Firstly, the detected method is not uniform and the standardization process is lacking [72]. Secondly, the level of cfDNA can be too low, so the detection technology needs to be improved to increase the sensitivity and specificity [73, 74].

The study of cfDNA is still in its infancy, and a lot of in-depth research is needed to further confirm its clinical application value.

Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 4

A Review on Machine Learning and Deep Learning Techniques Applied to Liquid Biopsy

Arets Paeglis, Boriss Strumfs, Dzeina Mezale and Ilze Fridrihsone

Additional information is available at the end of the chapter

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Abstract

For more than a decade, machine learning (ML) and deep learning (DL) techniques have been a mainstay in the toolset for the analysis of large amounts of weakly correlated or high-dimensional data. As new technologies for detecting and measuring biochemical markers from bodily fluid samples (e.g., microfluidics and labs-on-a-chip) revolutionise the industry of diagnostics and precision medicine, the heterogeneity and complexity of the acquired data present a growing challenge to their interpretation and usage. In this chapter, we attempt to review the state of ML and DL fields as applied to the analysis of liquid biopsy data and summarise the available corpus of techniques and methodologies.

Keywords: machine learning, deep learning, data analysis, biomarker detection, automated discovery, literature review

1. Introduction

Biological and medical sciences are becoming increasingly data-rich and information-intensive. This tendency, along with the growing availability of such data, provides a better understanding of important questions regarding functions of organisms, causes of diseases, etc. However, both the inherently massive complexity of biological systems and the high dimensionality and noisiness of data thus acquired can make it remarkably difficult to correctly infer such mechanisms. Machine learning (ML) and deep learning (DL) techniques are quickly becoming highly useful tools for solving difficult problems in biology and medicine by providing mathematical apparatus for analysing vast amounts of information that would

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otherwise be difficult to process and interpret. Additionally, these fields themselves provide new challenges for machine learning that can ultimately advance existing ML techniques and give rise to new ones.

The mutual history of machine learning and biological and medical disciplines is both long and complex. An early ML technique, the perceptron, was made in attempt to model the behaviour of biological neurons [1] and was used early on to define the start sites of translation initiation sequences in *E. coli* [2], and can be considered the starting point of the entire field of machine learning. In the last few decades, the power, flexibility, and accessibility of ML and DL techniques have grown considerably, and it can be expected that they will provide significant assistance in the discovery and understanding of the mounting volume of biological and medical data.

In this chapter, we first provide an overview of the commonly used ML and DL techniques and strategies and outline their broad areas of applicability with regard to processing and analysis of biological and medical data. Next, we attempt to summarise the available corpus of research and development concerning the application of ML and DL techniques to the process of analysis and interpretation of biomedical data, focusing on liquid biopsy analysis, outline several of the main avenues of such research, and predict the potential improvements and changes in this highly dynamic and quickly developing field. Expertise in ML is not a prerequisite for this chapter, although we assume basic overall familiarity with the most well-known ML and DL models, techniques, and methodologies.

2. Machine learning strategies

This overview is limited to classical software-based tools and techniques for brevity's sake. Several hardware-based approaches are mentioned in the Future Prospects section.

The ML ecosystem is both extensive and complex [3–5], with many possible ways to subdivide or classify its members. One frequently used classification scheme outlines two broad groups of ML algorithms: supervised learning, where the model is presented with both a set of labelled example inputs and desired outputs (called the training dataset), with the goal to learn a mapping from inputs to outputs, and unsupervised learning, where no labels are given to the model, leaving it to learn the input-output mapping in unstructured data. A notable specific case of supervised learning is reinforcement learning (RL), where training data consists only of positive ("reward") and negative ("punishment") feedback, given according to the model's performance in the training environment.

Another informative approach to classifying ML algorithms is based on the desired type of output of the given model, such as classification (division of the input data into two (binary classification) or more (multi-label classification) predetermined groups), clustering (similar to classification but with the groups not known beforehand), dimensionality reduction (simplification of high-dimensional input data by mapping them into a lower-dimensional space), search, etc. Of these, clustering is particularly notable due to its broad and general applicability

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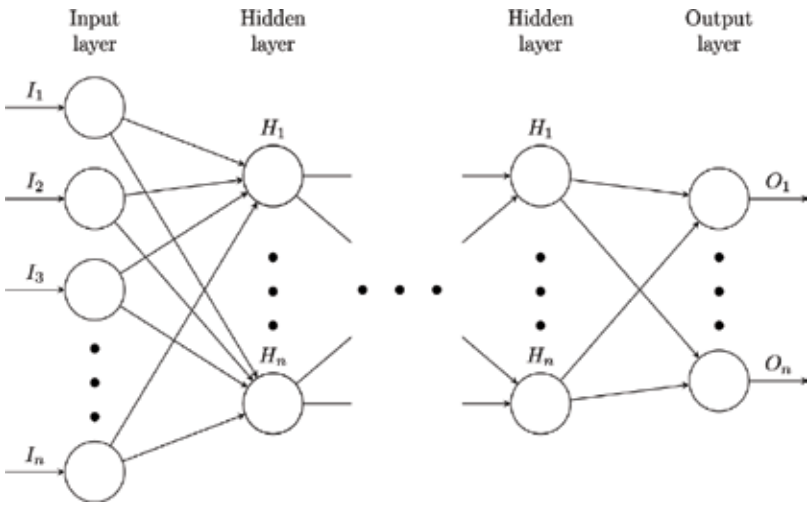

Figure 1. The structure of a typical feed-forward deep neural network, with a fully connected input layer *I*, an unspecified number of hidden processing layers *H*, and an output layer *O*.

and the wide range of models, methods, and algorithms [6–8] that can be employed to carry out cluster analysis.

The notion of "cluster" is often not precisely defined and tends to serve as an umbrella term for various types of data objects, typically groups of data points with small distances (appropriately defined) between group members, higher-density areas of some parameter space, particular statistical distributions, etc. The desired clustering algorithm, therefore, depends on both the given data set and the intended application of the returned results. Due to these complications, clustering, like many other data analysis methods, is typically not fully automated even within the domain of machine learning but instead tends to partially rely on preprocessing and initial parameter selection, based on the specifications of the task at hand.

Deep learning is a subclass of machine learning problems, the distinction being based on the training data representations instead of specific algorithms. Similar to ML in general, deep learning can be both supervised and unsupervised [9]. Deep learning models tend to be vaguely similar to information processing patterns in biological brains (and are therefore often called artificial neural networks), in that they use multiple layers [10] of non-linear processing units (frequently called "neurons", even though their similarity to biological neurons is usually limited) for pattern recognition and transformation, with each successive layer using as inputs the output from a previous layer, forming a hierarchy of representations and levels of abstraction. The number of hidden layers of an artificial neural network broadly determines the "computational power" of the network [11] (**Figure 1**).

Machine learning models have been applied to a wide variety of fields and problem classes, including computer vision, natural language processing, machine translation, bioinformatics and biochemistry [12], with results often similar or superior [13] to those produced by human domain experts.

3. Using machine learning techniques in blood tests

3.1. Classifying blood cells with deep convolutional neural networks

An important part of the data acquired by blood tests is the number of white blood cells (WBCs) or leukocytes, usually differentiated into total and differential WBC count, where the latter describes the absolute and relative numbers of WBC subtypes (neutrophils, lymphocytes, basophils, eosinophils, and monocytes) in the sample. The amount of WBCs in the sample provides information on the state of the patient's innate and adaptive immune system, e.g., a significant changes in the WBC count relative to the patient's baseline is evidence that their body is being affected by an antigen, whereas variations in the specific WBC subtypes can correlate with specific types of antigens or different pathways of immune and inflammatory reaction. Therefore, detailed measurement and understanding of the WBC counts is an important part of the quantitative picture of health and the organism's general condition.

Traditional methods of estimating the WBC count generally fall into one of two categories—manual and automated. The historical manual inspection of the blood sample involved counting the number of cells in a blood sample under a microscope and extrapolating under the assumption of uniform cell distribution across the entire bloodstream. Automated methods involve specialised equipment such as Coulter counters [14] or laser flow cytometers [15] which can provide accurate results and good performance [16] but are generally expensive and require specialised training to operate.

In this light, the ML-based approach provides a potential improvement over the aforementioned techniques due to several reasons. First, it requires far less expensive equipment due to being built around simple imaging solutions. Furthermore, unlike earlier methods, it is able to provide almost instantaneous results after the initial training stage. Finally, its performance can be expected to improve over time, in proportion to growing dataset sizes and, being mostly software-based, it can be expanded and advanced continually and "over the air", without requiring extensive changes in the underlying infrastructure.

We illustrate this approach using an example problem provided by *Athelas* team [17], namely, binary classification of a stained image of a WBC as either polymorphonuclear or mononuclear.[1] The training dataset consisted of hand-labelled images of stained WBCs of all given types in various proportions. Before the dataset could be used, several preprocessing steps were taken, including removing images with multiple cells and using transformations such as flips and rotations in order to increase the size and variability of the training dataset. By using transformed versions of the images, the training dataset size was increased from approximately 350 to 10^4.

For the ML model, *Athelas* team used the LeNet-5 [18] convolutional neural network (CNN) [3, 4, 9] due to its simplicity and availability. The model was tested against a test dataset of 71 images (20% of the original training set and 0.7% of the training set after transformations), with

[1] Eosinophils, basophils, and neutrophils are polymorphonuclear, while lymphocytes and monocytes are mononuclear.

the high accuracy of 98.6%. While the presently used model performs less well (accuracy of 86%) when classifying WBCs into multiple individual type categories as opposed to binary classification, given the high performance and simplicity of this purely software-based approach, *Athelas* team plans to extend it to more complex problems, including datasets containing other cell types, which could enable faster improvement cycles, increased accessibility, and better patient outcomes, compared to previously used methods of cell count analysis.

3.2. Using deep neural networks for detection of ageing-related biomarkers

During the last decade, human ageing research has received an increasing amount of mainstream interdisciplinary attention [19, 20], with an emerging tendency to approach various aspects of the natural ageing process as potentially treatable conditions.

Insilico team developed a DL system designed to predict human chronological age from biochemical data obtained from a basic blood test [21], narrowing an extensive set of potential ageing-related biomarkers to a limited subset of the most salient ones. A dataset of $> 6 \times 10^4$ records was used, with each record consisting of a patient's age, sex, and 46 blood biochemical markers. The dataset was preprocessed, normalising all blood marker values to 0–1 range, and then split into training and test datasets with ratio 90:10.

An ensemble of 21 feed-forward deep neural networks (DNNs) was created as the ML model, with a range of values assigned to DNN parameters such as the number of hidden layers, the number of processing units per layer, activation function, and optimization and regularisation methods. The permutation feature importance method [22] was used to evaluate the relative importance of the various biochemical markers with regard to ensemble accuracy. Batch normalisation [23] was used to reduce the effects of overfitting and increase the stability of convergence of the models.

The best results were obtained from a DNN with five hidden layers, using regularised mean squared error (MSE) function as the loss function, parametric rectified linear unit (PReLU) [24] activation function in each layer, and AdaGrad [25] optimiser of the loss function. The highest-scoring DNN performed with 82% ε-prediction accuracy at $\varepsilon = 10$ (i.e., considering the sample as correctly recognised if the predicted age is ± 10 years of the true age), out-performing several classes of competing ML models. Multiple models for combining individual DNNs into an ensemble (stacking) were evaluated, with the best being the elastic net model [26]. The most important blood markers were discovered to be albumin, glucose, alkaline phosphatase, urea, and erythrocyte count.

Insilico team created an online service (http://www.aging.ai) to make the DNN ensemble available to the general public, allowing patients to use their blood test data to evaluate the age prediction system and serving as a proof of concept for estimating ageing-related variables using readily available biochemical data. Additional data sources, including transcriptomic and metabolomic markers from liquid and individual organ biopsies, as well as imaging data, are being considered. *Insilico* team suggests that similar systems could also be developed for model organisms in order to perform cross-species analysis of individual biological markers and their importance in predicting both chronological and biological age.

3.3. Machine learning-based approach to Alzheimer disease biomarker discovery

In their study, *Smalheiser* team has developed [27] a ML-based model for predicting Alzheimer disease (AD) status of individual samples with high accuracy, using miRNAs and other small RNAs extracted from circulating exosomes obtained from liquid biopsy (blood plasma) samples.

A sample set of $N = 70$ was used to construct the training dataset consisting of normalised miRNA expression data across 465 loci. Cross-validation was used in feature selection to evaluate the impact of values from specific loci as features. The samples were randomly divided into 7 partitions of 5 positive and 5 negative samples each and cross-validation was performed on these partitions, using 6 partitions for training and 1 for evaluation. The random partitioning was repeated 10 times in order to acquire 70 estimate points of the performance measures of interest, one for each sample in the set. These values were averaged and their relative performance was assessed using area under the curve of the receiver operating curve (ROC), Matthews correlation coefficient (MCC) [28], and F1 score.

Smalheiser team evaluated three different ML classifier algorithms—C4.5 decision trees [29] (using the J48 implementation), support vector machines (SVMs) [30], and adaptive boosting (AdaBoost) [31]. After selecting 50 most significant features, as per Mann-Whitney *U* test [32], the C4.5 classifier produced the best results, based on which it was selected as the feature selection method. The feature significance was measured by the number of times the given miRNA locus was used as a node in the decision tree over the 70 runs. The 18 highest-scoring features were selected to move on to the next step. AdaBoost algorithm was used for the final feature selection from the set of 18 features, producing an optimised set of 7 features which were then used with all 70 data samples to produce the final dataset.

The best model used by *Smalheiser* team was able to correctly classify, on average, 29 out of 35 samples from the AD group and 31 out of 35 samples from the control group, yielding accuracy in the range of 83–89%. *Smalheiser* team concluded that ML-based classifiers are able to produce highly accurate predictions of AD occurrence, using a dataset of only 7 miRNAs and that integrating exosome miRNA data with other data is likely to further increase performance of these models.

3.4. Detection and classification of circulating tumour cells using machine learning methods

The presence of circulating tumour cells (CTCs) in blood samples indicates the tumour response to chemotherapeutic drugs and contributes to the mechanism for subsequent growth of derived tumours (metastasisation) in distant tissues. Evaluation of CTCs can yield the diagnosis or help to follow the tumour response to chemotherapeutic drugs.

Mao team designed a deep (six layers) CNN for image-based circulating tumour cell detection with automatically learned network parameters [33]. They used a dataset of 45 phase contrast microscopy [34, 35] images, of which 35 randomly selected images were used for training and the remaining 10 for testing the network. The experiment was repeated 5 times in order to minimise network bias.

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The CNN received normalised 40 × 40 pixel images as input. They were passed to a layer of 6 convolutional filters with the size of 5 × 5, followed by a max-pooling layer in order to extract the local signal in every 2 × 2 pixel region, defined by the max-pooling function,

$$z^i_{p,q} = \max_{0 \le m,\, n \le 2} \left\{ y^i_{2\times p+m,\, 2\times q+n} \right\}, \tag{1}$$

where (p, q)—pixel coordinates, y—input map, z—output map. This layer was followed by another convolutional filter layer, consisting of 12 filters, and, subsequently, by another max-pooling layer. The last layer was fully connected to the output layer by way of dot product between the weight and input vectors, passed to the sigmoid function which maps the values to the $[-1, 1]$ range. The filter parameters, network bias terms, and weight matrices were automatically adjusted by backpropagation with learning rate set to 0.1.

Mao team compared their CNN-based classifier to a simpler, SVM-based method that depended on hand-crafted feature sets. Using the F-score (harmonic mean of precision and recall scores) as the comparison metric, they found that, after two rounds of five iterations, the F-score of the CNN-based classifier was 0.97, by 18.6 points exceeding the F-score (0.784) of the SVM-based classifier and hand-crafted feature set. They concluded that the CNN-based classifier presents a promising development towards automated CTC detection in images taken from blood samples, and that the technique could be adapted for use with microfluidics-based liquid biopsy platforms for early diagnosis and monitoring.

4. Cancer detection and monitoring using neural network-based methods

4.1. Using artificial neural networks for lung cancer detection and diagnosis

Goryński team describes [36] an artificial neural network (ANN)-based model class used for early detection and diagnosis of lung cancer. In their study, a dataset consisting of a wide range of biochemical parameters obtained from blood samples, as well as results from medical interviews (48 values in total) from 193 patients of mixed age and sex was used to train a family of 10 multilayer perceptron network (MLP) [3, 4] architectures, using a range of activation functions (linear, logistic, and tanh) for both hidden and output layers, as well as varying number of processing units ("neurons") in the hidden layer and different training algorithms (gradient descent, Broyden-Fletcher-Goldfarb-Shanno (BFGS) [37], and scaled conjugate gradient (SCG)) [38].

Goryński team found that two of the trained models, named MLP 48–9-2[2] (trained using BFGS algorithm and using linear and tanh activation functions for hidden and output layers, respectively) and MLP 48–15-2 (SCG algorithm, logistic and tanh activation functions) gave highly

[2]The naming scheme represents the number of "neurons" in the input, hidden, and output layers of the MLP model, respectively.

accurate results in terms of inferring the presence or absence of lung cancer from the given set of variables, with ROC value reaching 99.83%.

Goryński team concluded that these, relatively simple, ANN solutions, while not viable as a full substitute of expert opinion, are nonetheless efficient in early diagnosis and risk prognosis of lung cancer and therefore are promising as potential improvements over and additions to the existing inventory of diagnostic and prognostic methods.

4.2. Mutation prediction and early lung cancer detection in liquid biopsy using convolutional neural networks

The proliferation of cancer cells is driven by specific somatic mutations in the cancer genome [39]. To fulfil the high expectations associated with liquid biopsy, such as comprehensive characteristics of the whole tumour in contrast to limited sampling in the traditional tissue biopsy, or dynamic assessment during treatment, the somatic mutations must be detected with high sensitivity and accuracy; limited coverage depth is not sufficient. *Kothen-Hill* team has demonstrated a CNN-based classifier system named "Kittyhawk" [40] that enables the detection of cancer-related mutations even in extremely low variant allele frequencies (VAFs), more than 2 orders of magnitude lower than is possible with the currently available methods.

For training dataset, whole genome sequencing (WGS) data from 4 non-small cell lung cancer (NSCLC) patients and 3 melanoma patients were used, with $> 1.2 \times 10^7$ reads in total. To ensure adequate genetic context regardless of variants appearing at the end of the read, additional bases were added to both ends of the read. Additional bases were also added to ensure equal read length in cases where a read is shorter than 150 bp.

Kothen-Hill team chose an 8-layer CNN with a single fully connected output layer, similar to the VGG[3] architecture [41], with a perceptive field of size 3 used to convolve the features, based on results of [42] who showed that the tri-nucleotide context contains distinct mutagenesis-related signatures. After 2 successive convolutional layers, downsampling by max-pooling with a receptive field of 2 and a stride of 2 was applied, forcing the model to retain only the highest-importance features, as per [43]. The output of the last convolutional layer was directly connected to a fully connected sigmoid output layer for final classification. A logistic regression layer was used to retain the features associated with the position of the read.

The model was trained using minibatch stochastic gradient decent (SGD) with batch size of 256, initial learning rate of 0.1, and momentum of 0.9, with batch normalisation [23] and a rectified linear unit (RLU) [44] applied after each convolutional layer.

Kothen-Hill team presents the Kittyhawk architecture as a first of its specific kind, being able to avoid the information loss associated with similar earlier architectures. To evaluate the performance of the model, a test dataset consisting of $> 2 \times 10^5$ reads that were split off the training set of reads from the 4 NSCLC patients was used. *Kothen-Hill* team found that the model achieves F1 accuracy of 0.961 when using this test dataset, and 0.92 when using data from an

[3] A CNN architecture developed by the Visual Geometry Group at University of Oxford.

additional independent NSCLC case. When further tested against data from a melanoma case, F1 accuracy of 0.71 was achieved, indicating that the model had learned specific mutation patterns associated with NSCLC, as well as a more general pattern associated with both NSCLC and melanoma.

Kothen-Hill team presents the Kittyhawk CNN model as the first ML architecture designed specifically for detecting cancer-related mutations in a low allele frequency environment, such as liquid biopsy and might serve as the foundation for novel early stage cancer detection techniques that could be used for both screening and prognosis.

4.3. Machine learning and nanofluidics in pancreatic cancer diagnosis

Issadore team has developed a ML-based platform [45] for isolating exosomes from liquid biopsy samples and, using the RNA inside these exosomes to diagnose pancreatic cancer in human and murine cohorts.

Using the Exosome Track-Etched Magnetic Nanopore (ExoTENPO) nanofluidics chip developed as part of the study, *Issadore* team successfully isolated exosomes from cell cultures, as well as human and mouse liquid biopsy (blood plasma) samples. Exosomal mRNA was subsequently extracted and used to develop a predictive panel for pancreatic cancer biomarkers.

Training datasets of 15 mouse and 10 patient profiles, respectively, were created. Linear discriminant analysis (LDA) [46] was used to identify combinations of mRNA profile that discriminated between healthy and tumour-bearing samples. The prediction algorithm was generated by running LDA on the training set, which produced a vector that was used to calculate a weighted sum such that it maximally separates the control group from the sample group with tumours. Two independent blinded test sets, mouse ($N = 18$) and patient ($N = 34$), respectively, were used to evaluate the performance of the LDA classifier. Fisher's exact test was used to quantify the predictive value of the classifier, yielding $P < 0.001$.

Although in their study *Issadore* team focused primarily on the development and evaluation of the ExoTENPO nanofluidics platform, they conclude that even very simple ML algorithms such as LDA can produce good quality predictive models for classifying biochemical and genetic markers and note that more advanced ML solutions could be used in future research in order to further improve performance.

4.4. Machine learning-based RNA sequencing for multi-class cancer diagnostics

Wurdinger team demonstrated a ML-based approach to sequencing and analysis of mRNAs obtained from tumour-educated platelets (TEPs) [47] as a tool for accurate tumour diagnosis, both within a single class and across six different tumour classes.

The initial dataset consisted of blood platelet samples from healthy donors ($N = 55$) and both treated and untreated patients with six different tumour types (NSCLC, colorectal cancer, glioblastoma, pancreatic cancer, hepatobiliary cancer, and breast cancer) in various stages of advancement and metastasis ($N = 228$). After the mRNA extraction, amplification, and sequencing, a set of approximately 5000 different mRNAs was selected for further analysis.

The accuracy of TEP-based multi-class cancer classification in the training dataset ($N = 175$) was estimated, using an SVM algorithm. To cross-validate the SVM for the entire sample set, leave-one-out cross-validation (LOOCV) method was applied. The percentage of correct predictions was reported as the accuracy score. The algorithm was performed 175 times, in order to classify and cross-validate the entire dataset. To determine specific input gene lists for the algorithm, *Wurdinger* team performed ANOVA testing. They selected a set of 1072 mRNAs to use with the training dataset, yielding final accuracy of 96% and ROC value of 0.986. From the patient cohort, all 39 patients with localised tumours and 33 of the 39 patients with primary tumours in the CNS were classified as cancer patients.

Wurdinger team concluded that using the SVM classifier with TEP-based data produces high-accuracy, high-specificity models for liquid biopsy-based diagnostics for several common cancer types. They expect that using more advanced ML algorithms capable of self-learning could further improve the performance of these diagnostic models. They also suggest evaluating systemic factors such as inflammatory diseases and other non-cancerous diseases as potential factors that can influence the mRNA profile.

5. Using machine learning to accelerate DNA sequencing and biomarker development

5.1. A supervised machine learning-based approach to DNA sequence analysis

DNA sequencing and sequence analysis is an important task in many scientific and medical fields that is well-known for being both data-rich and computationally intensive. *Memeti* & *Pllana* describe a ML-based solution for optimised DNA sequence analysis [48, 49]. Their algorithm leverages the increased performance and parallelisation capabilities of heterogeneous (a host central processor (CPU) in combination with a 61-core Intel Xeon Phi co-processor) multi-core computing platform.

Memeti & *Pllana* used the widely known Aho-Corasick (AC) algorithm [50] as the basis for their work, since DNA analysis is a specific case of a string matching problem, where the input text is the given DNA sequence and the alphabet consists of characters corresponding to the four nucleotide bases. AC uses finite automata (FA), a simple type of formal machine in the form of a prefix tree with additional links between internal nodes. These links allow for fast failure transitions (also known as ε-transitions) between branches of the tree that share a common prefix, thus avoiding backtracking. A known drawback of the AC algorithm is its being non-deterministic. *Memeti* & *Pllana* solved the non-determinism issue by modifying the AC finite automaton so that it computes the correct transition for each state, thus eliminating failure transitions and guaranteeing that every character always has the same number of operations associated with it.

A boosted decision tree regression-based predictor [51] was used to estimate the execution time of DNA sequence analysis for both the host CPU and the Intel Xeon Phi co-processor. The predictor's output was used to partition the DNA sequence based on the S-factor,

$$S = \frac{T_{host}}{T_{device}}, \tag{2}$$

where T_{host} and T_{device} are execution times for the host CPU and the co-processor, respectively, and using the partitioning scheme

$$I_{host} = I - I_{device} \tag{3}$$

$$I_{device} = \frac{I}{S+1}, \tag{4}$$

where I is the original DNA sequence, I_{host} is the part of I analysed by the host CPU, and I_{device} is the part of I analysed by the co-processor.

Memeti & *Pllana* used the "single instruction, multiple data" (SIMD) parallelism [52] of both the host CPU and the Xeon Phi co-processor to achieve teraFLOP (10^{12} floating point operations per second) performance. For experimental evaluation of their deterministic finite automata (DFA) algorithm, *Memeti* & *Pllana* used reference genomes of human and 11 different animals from the GenBank sequence database of the National Center for Biological Information, with the average dataset size of 2043 MB. In total, data from approximately 4000 experiments was used to train the performance predictor and to evaluate the DFA performance. The DFA performance was evaluated using different thread affinity modes (*compact*, *balanced*, and *scatter*) and numbers of threads for each of the DNA sequences. The *balanced* thread affinity mode evenly distributes the threads among the computing cores, *compact* mode completely fills a single core with threads before assigning the remaining threads to the next core, while the *scatter* mode distributes threads among the cores in a round-robin sequence.

Memeti & *Pllana* discovered that the balanced thread affinity mode is overall fastest for all of the tested DNA sequences, with second best being the scatter mode. The evaluation of DFA with regard to varying thread counts showed that the algorithm scales well up to approximately 120 threads, whereas in the 180–240 thread range the performance improvement becomes modest due to overhead from thread management operations. Performance-wise, *Memeti* & *Pllana* found that the parallel version of DFA running on a heterogeneous platform has a speed-up from 35.6× up to 206.6×, compared to a sequential (single-thread) version running on the host CPU, with the exact speed-up degree depending on the given host CPU. *Memeti* & *Pllana*. intend to use this work to study and develop highly parallel DNA analysis solutions on more powerful hardware in the future.

6. Future prospects

While the ML models currently used in liquid biopsy analysis in particular and biological and medical research in general (typically different classes of neural networks and linear classifiers) appear to both produce accurate results and show generally high performance, they represent only a narrow subset of machine learning and artificial intelligence solutions [5]. For instance,

a potentially valuable research direction might be in the form of highly advanced probabilistic graphical models [53] augmented with functionality such as one-shot learning [54] and probabilistic program synthesis [55], which could potentially allow researchers to reduce the size of the commonly massive training datasets required for creating ANN- or DL-based models.

Furthermore, with a single exception, all of the studies reviewed here have been focused on the performance and accuracy of software ML models, which is currently the predominant class of machine learning solutions. However, recent advances in general purpose computation using both graphics processing units (GPUs) and specialised application-specific integrated circuits (ASICs) tailor-made for machine learning [56] provide a strong case for the exploration and exploitation of hardware or hybrid ML solutions, as evidenced by, e.g., the results from the AlphaGo experiments and public performance [57].

7. Conclusions

Liquid biopsy-based approaches open many so far little explored and promising opportunities for studying and measuring biological and biochemical markers with broad applications for the monitoring, diagnosis, and prognosis of a large class of diseases and processes. Machine learning, with its advanced pattern recognition capabilities, will likely play an increasingly important role in these fields, as the amount and complexity of data produced by scientific and medical sources already by far exceeds the capacity of unaided human experts and is rapidly increasing with no foreseeable slowdown.

In addition, machine learning tools form a natural synergy with distributed, highly parallel, or cloud-based computation solutions, thus easily yielding to collaboration among researchers and medical professionals from distant locations and involving amounts of data storage and processing power previously available only on dedicated high performance computing (HPC) platforms and supercomputers. It is likely that in the near future the importance of decentralised collaboration will continue to grow, increasing the demand for powerful and easy to use toolset for analysis and processing of biological data.

Based on these trends, we expect that the next generation of liquid biopsy technologies will include many types of machine learning as an integral part of their operation and that this trend could have a significant positive impact on both diagnosis and treatment of patients.

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Conflict of interest

The authors declare that the chapter was written in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Section 3

Liquid Biopsy in the Diagnostics of Malignant Tumors

Chapter 5

Liquid Biopsies in Multiple Myeloma

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Additional information is available at the end of the chapter

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Abstract

Multiple myeloma is the second most common hematological malignancy. It is a heterogeneous disease characterized by focal lesions of malignant plasma cells in the bone marrow. Since bone marrow biopsy is a single-site procedure, its potential is limited in discovering the many clones present in each patient. Thus, a new approach, liquid biopsy, seems to be more relevant in today's world. Liquid biopsy can analyze circulating tumor cells or various circulating molecules (cell-free DNA, microRNA, long non-coding RNA and many others) that originated from the various tumor sites and thus will represent many different subclones. This review summarized current situation in research of liquid biopsies in multiple myeloma.

Keywords: multiple myeloma, liquid biopsy, non-coding RNA, circulating microRNA, cell-free DNA, circulating plasma cells

1. Introduction

Monoclonal gammopathies (MG) are a group of diseases characterized by proliferation of clonal plasma cells (PC). Physiological PC are terminally differentiated B cells, which secrete various types of antibodies used for neutralization of pathogens [1]. This essential function of PC is disrupted in MM patients, and abnormal cells overproduce monoclonal immunoglobulin (M-Ig) [2]. Multiple myeloma (MM) and its precursor disease monoclonal gammopathy of undetermined significance (MGUS) are two most common MG. The less frequent MG include Waldenström macroglobulinemia, solitary plasmacytoma, light chains amyloidosis and plasma cell leukemia [3].

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MM is the second most common hematological malignancy [4]. It is caused by malignant transformation of PC, which infiltrate the bone marrow (BM) disrupting normal hematopoiesis. Moreover, they produce M-Ig that is found in serum and/or urine of MM patients. MM is characterized by a set of clinical features known as CRAB features (hypercalcemia, renal failure, anemia and bone lesions) [5, 6].

MM represents about 13% of all hematological and about 1% of all malignancies. The incidence in the Czech Republic has been reported at 4.8/100000 per year [7], while in Europe it is slightly higher, 6/100000 per year [8]. Interestingly, MM is quite common in North America, Europe and Australia, while it is rare in the Middle East and Asia [9].

In 2003, the International Myeloma Working Group (IMWG) published diagnostic criteria for MM: infiltration of BM by malignant PC >10%, CRAB features and presence of M-Ig in serum and/or urine [2]. Thus, MM was treated only when CRAB feature(s) were fully developed. At that time, most treatment options were quite toxic, which is why treatment was postponed [10]. The past 10 years, however, have brought unprecedented new treatment options (immunomodulatory drugs, proteasome inhibitors, monoclonal antibodies) that improved survival rates as well as quality of life of MM patients. This led to revision of diagnostic criteria in 2014 from diagnostics based on clinical symptoms to diagnostics based on biomarkers allowing earlier treatment of the disease [6].

Since the new drugs have been introduced into clinics, detection of minimal residual disease (MRD) has become even more important as MRD negativity is a prognostic factor in MM. Moreover, sensitivity of the detection method is becoming an issue since new drugs induce deep response, and MRD needs to be measured with sensitivity up to 10^{-6}. The two most common methods used for MRD detection are multiparametric flow cytometry and ASO-PCR (allele-specific PCR) [11–13]. ASO-PCR is based on the detection of patient-specific V(D)J rearrangement in bone marrow plasma cells (BMPC) [14]. Nowadays, next-generation sequencing (NGS) is gaining more attention as a more precise and modern method of detection. However, NGS needs to be standardized and more accessible before it can be used more broadly.

The genome of MM cells is highly unstable and harbors various cytogenetic abnormalities, including translocations, deletions or duplications. From the cytogenetic point of view, MM may be divided into two groups: hyperdiploid and non-hyperdiploid. Hyperdiploid genome is mostly characterized by trisomies of odd chromosomes (3,5,7,9,11,15,19,21) and is connected to better prognosis [5], while non-hyperdiploid genome is characterized by monosomies of chromosomes 8,13,14,16,17 and 22 and recurrent chromosomal translocations involving the immunoglobulin heavy chain (IgH) locus at 14q32. In MM, the most frequent chromosomal translocations are t(11;14)(q13;q32) (15–20% of MM patients) and t(4;14)(p16;q32) (12–15% of MM patients). Other translocations are less frequent, found only in less than 5% of patients (t(14;16)(q32; q23), t(14;20)(q32;q11) and t(6;14) (p21;q32)). In MM cells with t(11;14)(q13;q32), cyclin D1 gene is translocated under the control of immunoglobulin heavy chain enhancer. Similarly, cyclin D3 gene at 6p21 is overexpressed in MM cells carrying t(6;14)(p21;q32) [14].

In addition to MM being a genetically heterogeneous disease, it is also characterized by multifocal tumor deposits throughout the BM and focal lesions elsewhere. Malignant PC in these lesions carry various cytogenetic aberrations with varying level of prognostic value [15].

Diagnosis and monitoring of MM are routinely performed using BM aspiration and/or BM biopsy [6]. However, discrepant results were described from analyses of different biopsy sites within the same patient [16, 17]. Diagnostic biopsies of the BM are obtained only from a single site in the BM what creates a sampling bias and provides only a limited molecular profile as all subpopulations of PC, so-called subclones, are not present in the BM [18].

Liquid biopsies represent one of the possible solutions for more comprehensive analysis of MM patients. Various targets, which can be analyzed in MM samples, include circulating tumor cells [19], cell-free DNA (cfDNA) [20], microRNA (miRNA) [21] and long non-coding RNA (lncRNA) molecules [22]. This review summarizes current knowledge of all aspects of liquid biopsies in MM.

2. Circulating plasma cells

In some cases, PC may migrate out of the BM into peripheral blood (PB), then they are called circulating PC (cPC) [23, 24]. While the reason for the migration is unclear, it is clear that these cPC lose (in some cases only temporarily) their dependence on the BM microenvironment due to the loss of adhesion molecules, increased proliferation, increased number of chromosomal aberrations and increased angiogenesis [25, 26]. The presence of cPC in newly diagnosed MM patients has been associated with shorter survival of patients, and it is an independent negative prognostic factor [24]. It is also possible that it is the first feature of extramedullary relapse, which is characterized by infiltration of PC into soft tissues and bad prognosis for patients [27]. In case that the number of cPC increases to over 20% in PB, it may turn into progression to secondary plasma cell leukemia [28].

cPC can be detected in a small fraction of newly diagnosed MM patients (15%) by conventional morphology [29]. However, this frequency increases up to 50–70% once more sensitive techniques, such as flow cytometry, are used [30]. Interestingly, the presence of cPC has been associated with an increased risk of malignant transformation to symptomatic MM in MGUS patients as well as with an inferior survival among symptomatic newly diagnosed and relapse/refractory MM [31]. In a study by Paiva et al., cPC were analyzed by multiparametric flow cytometry, FISH and cell cycle analysis; cPC were compared to paired PC samples from the BM. Their results showed that cPC are a unique subpopulation of malignant PC in MM; cPC are characterized by decreased expression of integrins (CD11a/CD11c/CD29/CD49d/CD49e) and adhesion molecules (CD33/CD56/CD117/CD138). cPC were also mostly quiescent with higher clonogenic potential than BMPC [31].

Mishima et al. investigated genomic characterization of MM patients using cPC and wondered if mutational profile of cPC is in concordance with mutational profile of PC from the BM. This study showed that both populations have similar mutations. Interestingly, 100% of clonal mutations found in PC from BM were also detected in cPC. Moreover, 99% of clonal mutations of cPC were also found in BMPC. Whole genome sequencing did not find any major differences between these two groups of MM cells, suggesting that the change in biological behavior of cPC could be based on the changes of expression of ncRNA molecules [32].

3. Cell-free DNA

Cell-free DNA are short fragments of DNA not associated with a cell and found in PB and other body fluids, such as urine, saliva, breast milk and others [33–35]. The term cfDNA is a general term that includes circulating DNA of both healthy and tumor origin. As circulating tumor DNA (ctDNA) fragments represent only a fraction of total cfDNA, it is necessary to distinguish the origin of fragments during analysis. Physiological levels of cfDNA in PB of healthy individuals are generally low (10–100 ng/ml). This changes, however, in case of various pathological events. Elevated levels of cfDNA were described in patients with inflammation, trauma, sepsis, stroke or heart attack [36–38], but the highest levels of cfDNA were found in cancer patients, where they reached up to 1000 ng/ml [39, 40]. These findings suggest a correlation between levels of cfDNA and tumor burden. Nevertheless, cfDNA levels were not found to be cancer-specific. Stability of cfDNA is variable, ranging from 15 minutes to 2.5 hours; therefore, the amount of cfDNA cannot be used as a diagnostic marker [41].

Cells can release cfDNA either actively, using exosomes [42], or passively by apoptosis and necrosis (**Figure 1**) [43, 44]. In 2016, however, a study by Bronkhorst et al. proposed that apoptosis and necrosis are not the source of cfDNA and that active secretion is primarily used for cfDNA release [45]. This proposal will require further investigation as it suggests a more active role of cfDNA in cell-to-cell communication. Since cfDNA is released directly from cells, circulating fragments contain the same genetic information as the original cell. In case of cancer cells, this allows detection of cancer-specific genetic and epigenetic aberrations, such as mutations, microsatellite alterations, changes in DNA methylation and others [41].

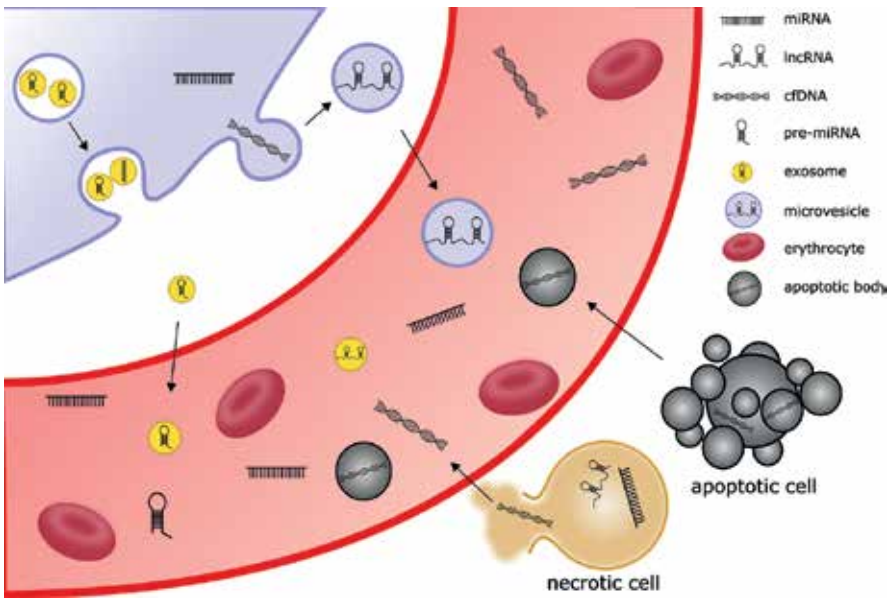

Figure 1. Schematic structure of release of circulating molecules and vesicles into bloodstream. miRNA – Micro RNA, lncRNA – Long non-coding RNA, cfDNA – Cell-free DNA and pre-miRNA – Precursor miRNA.

In the field of MM research, only a small number of cfDNA studies have been published so far. The first pilot study was published by Sata et al. In 2015 [23] they compared ASO-PCR data from peripheral blood mononuclear cells (PBMC), BM mononuclear cells (BMMC), CD20 + CD38− B-cell population in BM and serum cfDNA. Even though the study was quite small and only 20 patients (out of 30 enrolled) were quantifiable, it provided interesting results suggesting further studies and validation are needed. A strong correlation between BMMC and PBMC was found, suggesting circulation of clonogenic PC in PB; PBMC also negatively correlated with treatment as ASO-PCR data from those cells always decreased after treatment. These results suggest a possibility to use PBMC instead of BMMC in monitoring of MRD in MM patients. In addition, DNA sequences found in cfDNA were identical to those found in BM cells in 18/20 cases at diagnosis and 16/20 cases of follow-up samples, while levels of cfDNA remained mostly stable during the course of therapy. Based on these results, the authors assumed that detection of tumor V(D)J rearrangement in cfDNA can reflect presence and persistence of MM clones in patients. However, because of insufficient number of patients who reached complete remission (CR), the potential of cfDNA analysis for MRD monitoring remained unclear [46].

In 2017, three important studies on this topic were published [20, 47, 48]. The first by Kis et al. compared cfDNA analysis to BM analysis regarding molecular profiling of disease. This study screened 64 cfDNA samples from 53 MM patients for sequences of all protein-coding exons of *KRAS*, *NRAS*, *BRAF*, *EGFR* and *PIK3CA* genes. This method allowed for detection of tumor-related fragment of cfDNA at significantly low allele frequencies (0.25%). In 48 cfDNA samples, matching BM data were available. The analysis detected 49/51 (96%) of somatic mutations in cfDNA that were also found in BM; importantly, four additional mutations not detected in BM samples were found in cfDNA (>98% specificity). There were two mutations missed by sequencing of cfDNA samples that were detected during validation by ddPCR in BM samples but not in cfDNA. These outcomes emphasize the potential of cfDNA analysis not only for complex molecular profiling but also for detection of subclones not detected in BM aspirates [20].

The second important study, although once again lacking a larger patient cohort, was conducted by Oberle et al. and focused on detection of clonotypic V(D)J rearrangement in circulating MM cells and cfDNA. A cohort of 27 MM patients with various treatment regimens based on bortezomib, lenalidomide and panobinostat was examined. NGS was used for identification and tracking of patient-specific V(D)J rearrangements. The identification of rearrangements was successful in only 23 out of 27 patients, and these patients underwent further screening of blood samples before and after initiation of therapy. Baseline screening detected patient-specific V(D)J rearrangement in 71% of cases in circulating MM cells and in 100% of cases in cfDNA. However, these values decreased in follow-up samples to 40% and 34%, respectively. The results also correlated with remission status of patients—91% of non-responders/progressors and 41% of responders to therapy had evidence of persistent MM in circulating cells or cfDNA. Interestingly, positivity in circulating MM cells and cfDNA associated with each other ($P = 0.042$) but disagreed in 30% of cases. This suggests that circulating MM cells are not the only source of MM cfDNA and that cfDNA may reflect tumor burden more comprehensively. All these results indicate that V(D)J analysis from PB samples may be used for evaluation of treatment efficacy and possibly even for MRD prediction [48]. However, validation on a larger cohort is necessary.

The last study was published by Mithraprabhu et al., and the subject of this study was mutational characterization of MM. Paired DNA samples of BM PC and plasma derived cfDNA were analyzed for the presence of activating mutations of four oncogenes—*KRAS*, *NRAS*, *BRAF* and *TP53* by NGS. In total, 48 MM patients (33 relapsed/refractory and 15 newly diagnosed) and 21 healthy donors (HD) enrolled in the study. Overall, 128 different mutations were detected in MM patients (cfDNA = 31, BM = 59 and both = 38), while none were found in HD. Interestingly, almost a quarter of all found mutations were detected only in cfDNA samples. These findings proved spatial heterogeneity of MM and showed that cfDNA molecules are derived from multiple tumor sites within a patient's body. This was supported also by majority of cfDNA-specific mutations found in relapsed/refractory patients (30 mutations) in contrast to newly diagnosed patients (1 mutation) as they are more prone to have multiple focal lesions. Moreover, sequences of cfDNA were evaluated by ddPCR in seven patients throughout their treatment, and changes in fractional abundance were discovered, reflecting progression of disease. This proof-of-concept study confirmed the presence of mutations only in cfDNA and proved that genetic composition of MM is complex and evolves during progression of disease. In addition, it proposed that cfDNA analysis could be used as an adjunct to standard BM biopsy for disease monitoring to enable obtaining more complex results [47].

So far, not many cfDNA studies in MM have been conducted; however, the data are exciting and strongly suggest the future role of cfDNA in MRD monitoring.

4. Non-coding RNA molecules

Protein-coding genes comprise only about 1.5% of the genome. At the same time, it was shown that more than 90% is transcriptionally active [49]. Transcription of this so-called junk DNA leads to creation of thousands of RNA molecules that are not capable of coding proteins. Surprisingly, it was shown that the more complex the organism, the higher number of non-coding RNA (ncRNA) molecules it contains [50]. These molecules have many different functions in the most important cell processes, such as differentiation, proliferation, apoptosis and many others. They are involved in tumorigenesis as well.

Based on their length, ncRNA are divided into two groups: short (sncRNA) and long (lncRNA). SncRNA are smaller than 200 nucleotides (nt), while lncRNA are longer than 200 nt. The first ncRNA molecules that were identified more than 50 years ago were ribosomal RNA (rRNA) and transfer RNA (tRNA) [4, 5, 51]. While there are many classes of ncRNA, the most studied and well known are microRNA (miRNA) and long non-coding RNA (lncRNA).

4.1. Definition and biogenesis of microRNA

MiRNA are short, non-coding, single-stranded RNA molecules about 21–23 nt long. They are involved in regulation of gene expression and influence various cell processes, such as proliferation, differentiation, apoptosis and tumorigenesis. MiRNA genes account for 1–2% of the human genome, and mature miRNA regulate around 50% of protein-coding genes [49].

Based on the canonical model of miRNA biogenesis, miRNA genes are transcribed by RNA polymerase II or III into primary precursors, stem-loop structures (pri-miRNA) that contain 5′ end cap and polyA on the 3′ end. Pri-miRNA are cleaved in the nucleus by RNAse II enzyme Drosha and Pasha leading to pre-miRNA [52]. Pre-miRNA are exported into cytoplasm by transport protein exportin 5 [53]. In the cytoplasm, the pre-miRNA molecule is processed by the RISC complex that contains RNAse III Dicer and protein Argonaute 2 (Ago2). RISC complex cuts the molecule into 20–23 nt long double-stranded miRNA duplex with 2 nt overhang on 3′ ends [54]. One of the strands is the so-called guide strand and is complementary to the mRNA sequence. The other ('passenger' strand) is degraded. Which one of these strands is degraded is based on the stability of pairing on the 5′ end of the miRNA duplex [55]. Based on the level of miRNA/mRNA complementarity, the target mRNA is either silenced translationally in case of non-complete complementarity or degraded in case of 100% complementarity [56, 57].

MiRNA regulate a large spectrum of physiological and pathological processes including oncogenesis; they can act as oncogenes or tumor suppressors. Several mechanisms of miRNA role in tumorigenesis have been described: increased expression levels, amplification, epigenetic silencing or loss of miRNA gene that regulates expression of a tumor suppressor gene [58]. On the other hand, deletion and epigenetic silencing of miRNA gene expression that silences oncogene expression have been described as well [59]. Moreover, mutations in target sequences of mRNA lead to failed translational repression or degradation of target mRNA [60].

In a pilot study of miRNA expression in malignant transformation of PC, increased expression of miR-181a/b, cluster miR-106b-25 (miR-93, miR-106b, miR 25) and miR-21 in MGUS and MM patients in comparison to healthy donors (HD) was found. Interestingly, MM patients showed increased expression of cluster miR-17-92a suggesting a possible role of this cluster in disease progression [61].

4.2. Circulating miRNA

Essentially, all human body fluids (PB, saliva, urine, breast milk, etc.) contain the so-called circulating miRNA [62]. Circulating miRNA are quite stable and resistant to RNases as they are part of protein (Ago2) or lipoprotein (high-density lipoprotein (HDL)) complexes or they are bound inside exosomes—small transport vesicles [63]. It seems that circulating miRNA are involved in cell-to-cell communication as they are exported outside of cells based on biological stimuli. These molecules can also take part in cell processes, such as communication, proliferation, differentiation and in case of tumors also metastases [64]. Specific profiles of circulating miRNA are diagnostic markers differentiating HD from patients, but they also correlate with progression and staging of the tumor [65, 66]. A major advantage of these molecules as potential biomarkers is their simple structure, easy access and measurability by standard laboratory techniques [64].

4.2.1. Circulating microRNA in monoclonal gammopathies

In MM, circulating miRNA were first described in 2012. In a study by Jones et al., PB serum samples of MGUS and MM patients were analyzed in comparison to HD. They found that miR-720, miR-1246 and miR-1308 could serve as potential markers of MG [67].

The success of this study led to other studies, especially in MM. However, different approaches lead to varying results. The main differences were type of samples (serum or plasma of PB), design of experiments (patients vs. HD) and used methods and platforms.

Plasma of PB was reported to have lower levels of miR-92a in newly diagnosed MM patients in comparison to HD. Moreover, the level of miR-92a fluctuated based on progression of disease and treatment response, which would suggest a possible role of this miRNA as a predictive biomarker [68]. Another study showed increased expression of miR-148a, miR-181a, miR-20a, miR-221 and miR-88b in plasma of PB of MM patients in comparison with HD. Expression level of miR-20a and miR-148a was connected to shorter time to relapse of MM; this study suggested that circulating plasma miR-20a could be a marker of worse prognosis of MM [69]. On contrary, another study showed mostly decreased miRNA expression in MM patients compared to HD. MiR-483-5p and miR-20a were shown to have diagnostic and prognostic potential [70].

So far, most studies were performed using serum miRNA. Our own pilot study showed significantly increased levels of miR-29a, miR-660 and miR-142-5p in MM patients in comparison with HD. We showed that circulating serum miR-29a could be a biomarker for MM patients [71]. In our follow-up study, we showed dysregulation of five serum miRNA, miR-744, miR-130a, let-7d, let-7e and miR-34a in MGUS and MM patients in comparison with HD. Multivariate analysis showed that combination of miR-34a and let-7e distinguishes the patient cohorts with good sensitivity and specificity. Moreover, dynamics of serum miRNA with disease progression was shown [72].

In another study, increased expression of miR-181a/b, miR-221, miR-222 and miR-382 was found in relapsed MM patients and MM cell lines [51]. On contrary, lower expression of miR-15a and miR-16 was described; these miRNA have been described in chronic lymphocytic leukemia and seem to be part of pathogenesis of this disease. The genes for these miRNA are coded in the 13q14 locus; this locus is often deleted also in MM [73]. MiR-15a and miR-16 support apoptosis and decrease proliferation of MM cells by AKT and MAP kinase signaling [51].

In a study by Rocci et al., higher levels of miR-25, miR-16 and miR-30a in MM patients correlated with longer overall survival (OS) [74]. Another study showed that miR-19a and miR-4254 distinguish MM and HD. In addition, decreased level of serum miR-19a was positively correlated with international staging system (ISS) stage, presence of del(13q14) and gain 1q21 and shorter progression-free survival (PFS) and OS. Surprisingly, these patients responded to bortezomib better [75].

Serum miRNA were also analyzed at CR after autologous stem cell transplantation (ASCT). MiR-16, miR-17, miR-19b, miR-20a and miR-660 were decreased in diagnostic samples in comparison with CR samples [76]. Patients with lower levels of miR-19b and miR-331 had shorter PFS after ASCT. Level of miR-19b was significantly lower in samples obtained at relapsed than at CR.

The most common clinical manifestation of MM is osteolytic lesions. Increased levels of serum miR-214 and miR-135b were found in MM patients with osteolytic lesions, and their expression correlated with severity of the symptoms [77]. Moreover, increased level of miR-214 associated with shorter PFS and OS.

Using NGS, miRNA (let-7b a miR-18a) from exosomes isolated from serum of MM patients significantly correlated with PFS and OS in univariate analysis and with ISS and cytogenetic abnormalities in multivariate analysis [78]. Moreover, it was shown that levels of exosomal

miR-16-5p, miR-15a-5p, miR-20a-5p and miR-17-5p were significantly decreased in MM patients resistant to bortezomib [79].

As for miRNA in other body fluids, our group performed analysis of circulating miRNA in urine of MM patients in comparison with HD. Unfortunately, we did not find any miRNA significantly dysregulated [13].

While a lot of work was done on circulating miRNA in MG, so far no clear biomarkers of the diseases have been identified. It is possible that MM as a heterogeneous disease will not have a single circulating miRNA as a biomarker. Further studies and standardization of sample processing, types of samples and analytical methods need to be performed.

In our opinion, miRNA have a large potential for diagnostics of monoclonal gammopathies. Most studies were done on diagnostics samples; however, the data are not consistent and more standardization and optimization is needed. The possibility of using miRNA as prognostic or monitoring markers needs to be further validated.

4.3. Long non-coding RNA molecules

LncRNA are an abundant class of RNA between 200 nt and 100 kb long [80, 81]. To date, approximately 16,000 lncRNA have been identified in the human genome (http://www.gencodegenes.org/). On the other hand, the functional characterization of most of them has not been determined yet.

Genes encoding for lncRNA are present in many types of organisms, including animals [82], plants [83], yeast [84], prokaryotic organisms [85] and viruses [86]. LncRNA do not possess protein-coding capacity due to the absence of open reading frames (ORFs) or because of insufficient length of ORFs [87–90]. They can be classified according to their genomic localization into three major groups: long intergenic non-coding RNA (lincRNA), long intronic RNA and long non-coding RNA transcribed from specific genomic regions.

The expression of lncRNA genes is developmental and tissue-specific, and they have been associated with a large spectrum of biological processes, for example, alternative splicing, modulation of protein activity, alternation of protein localization, epigenetic regulation and generally regulation of gene expression. These molecules can be precursors of small RNA and even tools for miRNA silencing [91–96]. LncRNA play an important role both in physiological and pathological processes. The deregulated expression levels of these molecules were identified in a large variety of tumor diseases: breast cancer [97], small-cell lung carcinoma [98] and also in MM [22]. It was shown that alterations in lncRNA can influence regulation of cancer progression [99]. Interestingly, lncRNA seem to have higher tissue specificity even in comparison with protein-coding mRNA and miRNA. Thus, they are even more interesting as new specific biomarkers [88].

Function of lncRNA can also be derived from their localization within the cell. These molecules can be found in the nucleus and in the cytoplasm. LncRNA are preferably located in the cell nucleus, deriving their significant effect on the DNA sequence [88].

The classification of lncRNA can be based on their influence on the DNA sequence. From this perspective, there are two classes of lncRNA: cis-lncRNA (cis-acting lncRNA) and trans-lncRNA (trans-acting lncRNA). Cis-IncRNA can positively or negatively regulate expression

of genes that are located in their genomic proximity [95]. On the other hand, trans-lncRNA regulate expression of distant genes [100]. Many lncRNA are transcribed by RNA polymerase II, just like protein-coding genes. If lncRNA are involved in the regulation of RNA polymerase II, they are transcribed by RNA polymerase III [87, 101–103]. High degree of evolutionary conservation, tissue-specific expression and stability of lncRNA point to significant functional role of these molecules [104].

Dysregulation of lncRNA expression was observed in various human diseases, including cancer. LncRNA may be either oncogenes or tumor suppressors in development as well as progression of tumors [105, 106]. Changes of expression levels of several lncRNA have been reported in several malignancies; other lncRNA seem to be specific for a single tumor, suggesting that these molecules may be good biomarkers for tumor diagnostics as well as prognosis and prediction [107].

Moreover, it was shown that lncRNA are involved in regulation of hematopoiesis, including proliferation, differentiation and apoptosis of hematopoietic stem cells as well as progenitors and precursors of mature blood cells [108, 109]. Dysregulated expression of lncRNA was reported in lymphomas, leukemias and MM. It seems possible that expression profile of these lncRNA could have a potential clinical significance in diagnostics and prognostics of hematologic malignancies.

Current information about the role of lncRNA in pathogenesis of MM is very limited. So far, MALAT1 has been described as a marker of early progression [110]. Expression level of this lncRNA was increased in BM cells of newly diagnosed MM patients and changed during progression of the disease. Patients with lower levels of MALAT1 had a higher risk of early progression.

Handa et al. showed higher expression level of MALAT1 in MM patients in comparison to MGUS and HD [111]. These results are in correlation with another study of Ronchetti et al. who showed dysregulation of 31 lncRNA, including MALAT1, in MM patients [112]. Moreover, this lncRNA may be important in MM pathogenesis through activation of TGF-β, a factor important for osteolytic lesion formation [113].

An earlier study showed decreased expression of MEG3 in MM patients [114]. Decreased expression or loss of this lncRNA seems to be important in various types of human tumors [115]. In a study by Benetatos et al., MEG3 was reported to be lost in more than half of MM patients, and it seemed to have a prognostic significance for MM [116].

Our own study showed that UCA1 might be a marker of MM when HD, MGUS and MM plasma cells were compared (with sensitivity of 85.0% and specificity of 94.7%). UCA1 levels seemed to correlate with albumin and monoclonal immunoglobulin serum levels, cytogenetic aberrations, and survival of MM patients.

4.3.1. Circulating lncRNA

Similar to circulating miRNA, even lncRNA may be detected in body fluids suggesting their possible role as biomarkers for diagnosis, prognosis and prediction. They were found in PB

and urine, but they can also be found within exosomes where they are protected against RNases [117].

Most studies of circulating lncRNA published so far were studies of solid tumors. In prostate cancer, PCA3 specificity was so high that a new test from urine has been approved for usage in Europe; it can be used together with currently used PSA test (prostate-specific antigen) [118–121].

In urinary bladder cancer, increased level of UCA1 was detected not only in the tumor tissue but also in PB and urine of patients [79, 122]. It was shown that based on UCA1 expression, urinary bladder cancer may be distinguished from other urinary bladder diseases with high specificity [28].

Unfortunately, only very few studies were published about circulating lncRNA in MM. In a study of Isin et al. [123], five candidate lncRNA (TUG1, MALAT1, HOTAIR, GAS5, lincRNA-p21) were analyzed in plasma of PB of MM patients in comparison with CLL patients [103]. Plasma of PB of CLL patients contained significantly deregulated levels of lincRNA-p21. On the other hand, MM plasma contained deregulated levels of the other four lncRNA. When compared to HD, MM patients contained only TUG1 deregulated levels. There was a correlation of circulating lncRNA and clinical subgroups of MM, suggesting that TU1 could be a part of MM progression. Another study reported significantly higher levels of PCAT-1 in MM patients in comparison with HD. Its potential as a biomarker was proven by ROC analysis that showed sensitivity of 71.7% and specificity of 93.8%. A possible correlation with MM pathogenesis was suggested by a correlation with β2 microglobulin [123].

While lncRNA molecules are generally described as being more tissue-specific than miRNA, not enough data have been published on circulating lncRNA in MM. Further studies that are more comprehensive are needed to verify their claim as the more specific marker.

5. Conclusion

While not many studies have been published dealing with liquid biopsies of circulating molecules in multiple myeloma, they show a great promise. Liquid biopsies could be used as an adjunct to standard BM biopsy for disease monitoring to enable obtaining more complex results and easier follow-up of patients. While there are many candidate molecules that have been described in this review (cfDNA, miRNA and lncRNA), more studies are needed to validate these findings.

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Chapter 6

Liquid Biopsy in Patients with Thyroid Carcinoma

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Additional information is available at the end of the chapter

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Abstract

Thyroid cancer is a comparatively rare tumor, which affects 1–5% of women and approximately 2% of men, although it is the most common endocrine malignancy worldwide. Furthermore, the incidence of thyroid cancer has been increasing remarkably in the last decades. Currently, diagnosis of thyroid cancer mainly is based on cytological criteria. Although fine needle aspiration is a minimally invasive procedure, complications can occur. Correct diagnosis is mandatory to select patients for surgical intervention and to determine appropriate extent of operation. Overdiagnosis and the associated unnecessary surgery should be avoided as it might also lead to complications. Therefore it is important to practice noninvasive methods not only for early diagnosis of thyroid cancer but also for estimation of prognosis. Liquid biopsy is a promising, noninvasive method that can provide detection of circulating tumor cells (CTCs) as well as circulating nucleic acids such as DNA, mRNA, and microRNA in a blood sample. The aim of the chapter is to highlight the efficacy of liquid biopsy for diagnosis and prognosis of thyroid cancer. The chapter will represent a comprehensive literature review based on recent PubMed publications (mainly 2012–2018).

Keywords: thyroid carcinoma, early diagnostics, molecular diagnostics, liquid biopsy, circulating tumor cells, circulating free DNA, circulating miRNA

1. Introduction

Thyroid cancer is relatively infrequent malignancy, which accounts for about 1–5% of the cancer cases in women and less than 2% in men [1]. Although, it is one of the most common cancer among endocrine malignancies accounting for more than 95% of new cases in the United States [2, 3], according to American Cancer Society, thyroid cancer in the United States in 2019

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is estimated to be about 53,070 new cases (40,260 in men and 37,810 in woman), and more than 2000 people will die from the disease [3]. As well as in the rest of the world, the incidence of thyroid cancer has increased threefold over the past 30 years and is expected to increase by 50–60% between 2010 and 2020 [3–5]. The increase in thyroid cancer incidence rates could be explained by thyroid ultrasound screening with improved technical performance of the equipment, as well as better access to medical examination [1, 6].

Although, there are no significant rise in thyroid cancer mortality rates, growing detection of indolent forms may lead to overtreatment of the patients by performing unnecessary thyroidectomies [7]. General postoperative complications, such as fever, hemorrhages, infection, or cardiopulmonary and thromboembolic events, as well as specific complicationssuch as hypoparathyroidism/hypocalcemia and vocal cord/fold paralysis can be seen. In large population-based study, 27,912 patients were included and analyzed. General postoperative complications were observed in 6.5% of the patients and surgery-specific complications in 12.3% [8]. The diagnostic gold standard for thyroid cancer is the evaluation of histological features, although there still are some differential diagnostic difficulties. To avoid complications, a new, more precise diagnostic tool is necessary to diagnose and manage thyroid cancer patients.

Liquid biopsy is a usual blood sampling method, referred to as a noninvasive procedure to detect components of the tumor which circulates in the bloodstream, for example, circulating tumor cells (CTCs), cell-free nucleic acids, exosomes, or tumor-educated platelets (TEPs); thus it can be a promising method in tumor diagnostics prior to surgery, as well as in monitoring of the disease [9].

2. Detection of specific particles by liquid biopsy

As previously mentioned, in liquid biopsy, circulating tumor cells (CTC), cell-free nucleic acids, exosomes, or tumor-educated platelets (TEPs) can be acquired and studied for different purposes [9, 10].

Circulating tumor cells are cells which detaches from primary tumor and deposits in a patient's blood. The described ability of CTC is particularly important to fully understand metastatic process; therefore a large scientific research field on this topic has evolved. It is believed that CTC can survive in the bloodstream because of the undergoing epithelial-mesenchymal transition (EMT). In this process tumor cells gain plasticity and motility which allows to extravasate from primary tumor into blood and intravasate into distant tissues as well. Detection of CTC can give valuable clinical information about patient's medical status—CTC can be used as a prognostic marker in different carcinomas including malignant thyroid tumors. Analysis of these cells can serve in treatment process as well—response to different pharmaceutical drugs in individual patient can be analyzed [9, 10].

Circulating cell-free nucleic acids (cfDNA, cfRNA, and cfmiRNA) discharge from apoptotic and necrotic tumor cells into the bloodstream. cfDNA originates not only form tumor cells but

also from non-tumor cells after exercise, trauma, or inflammation [10]. Extraction of circulating tumor DNA (ctDNA) alone is a difficult process; therefore mutation in cfDNA particles is being searched and indicates the presence of tumor. A lot of mutation has been analyzed in different types of tumors, for example, epidermal growth factor receptor mutation in non-small cell lung cancer. At present, it is the only liquid biopsy test that has granted and has FDA approval [9, 11].

By liquid biopsy, cfRNA can be analyzed but this particle is not as stable as cfDNA, therefore it is harder to investigate. Another circulating cell-free nucleic acid—microRNAs—is a new and more stable tumor marker in the blood. Exosomes are microvesicles (40–150 nm) that are released in the blood from tumors and normal cells as well. Exosomes contain proteins, DNA, RNA, miRNA, lipids, and metabolites. Tumor-educated platelets (TEPs) are anucleated cell fragments that can be educated by the transfer of tumor-associated particles, mostly RNA [9, 10].

3. Papillary thyroid cancer and liquid biopsy

The large increase in the incidence of thyroid cancer is seen in papillary thyroid cancer (PTC) which represents the major histological type [12]. PTC accounts for 85% of thyroid malignancy [13]. World Health Organization has defined PTC as a malignant epithelial tumor showing follicular cell differentiation and a specific signs of nuclear features which include nuclear enlargement and overlapping, irregular nuclear contours, and nuclear pseudoinclusions or nuclear grooves, as well as optically clear nuclei [14]. PTC measuring 1 cm or smaller in the greatest dimension is defined as papillary thyroid microcarcinoma (PTMC). It is suggested that incidence of PTC increased largely due to an increase in the incidence of PTMC, probably due to thyroid ultrasound screening with improved technical performance of the equipment, as well as better access to medical examination. Foci of PTMC have been reported in up to 22% of surgical thyroid specimens and up to 36% of autopsy series [6, 15].

The main therapeutic method which is used after the diagnosis of PTC is a total of subtotal thyroidectomy with or without radioactive iodine (RAI) and thyroid hormone suppression. Afterward monitorization of disease status is necessary for all the PTC patients which is carried out by measuring levels of serum thyroid-stimulating hormone (TSH) and serum thyroglobulin (Tg) in the blood. Neck ultrasonography is also performed to detect persistent or recurrent PTC nodules in the thyroid gland after treatment [16]. However, if thyroglobulin antibodies (TgAb) are found in the blood, serum thyroglobulin could not be used as a reliable tumor marker because of false negative rate. Furthermore, long time period is needed to observe the changes in the levels of serum TgAb, and this may lead to late diagnosis [17]. Therefore, other biomarkers need to be discovered and used to monitor persistent or recurrent disease.

A new diagnostic tool—liquid biopsy—can be used to analyze circulating tumor cells (CTCs) or circulating epithelial cells (CECs) and circulating cell-free tumor DNA (ctDNA) in the blood

of thyroid cancer patients. This minimally invasive diagnostic method has received a lot of attention over the past years and can also be used to analyze thyroid cancer patients [18]. However, in the case of thyroid carcinoma, only a few studies have been exploring the significance of CTCs in the blood. CTCs are malignant epithelial cells which can separate from primary tumor, invade blood and lymph vessels, and travel through the body to form distant metastases [19]. Salvianti with colleagues proved that quantity of cfDNA with integrity index 180/67 in thyroid cancer patients was higher than in those who had benign thyroid nodules. Therefore, cfDNA could be a suitable marker to diagnose thyroid cancer [20]. On the surface of CTC, epithelial cell adhesion molecule (EpCAM) is found in overexpressed state and therefore can be used for malignant cell visualization from a sample taken by liquid biopsy. Usually EpCAM is overexpressed on tumor cells. The visualization of CTCs can be performed with different antibodies, which allows visualize cells under a fluorescence microscope. In other studies, quantification of various messenger RNAs (mRNAs) is detected in the blood [19].

Not only mRNA but also microRNA could be detected in CTCs [21]. Biochemical alterations of cancer cells are largely supported by noncoding RNA (ncRNA) dysregulation in the tumor site. Noncoding RNAs lack an open reading frame and do not have protein-coding ability. Based on the size of the functional RNA molecule, regulatory ncRNAs are classified as long ncRNAs and small ncRNAs or microRNA (miRNA) [22]. miRNAs are small, evolutionary conserved, single-stranded, noncoding RNA molecules (approximately 22 nucleotides in length) that bind target mRNA to regulate gene expression [23, 24]. MicroRNAs are involved in various physiological and pathological functions, such as apoptosis, cell proliferation, and differentiation, which indicate their functionality in carcinogenesis as tumor suppressor genes or oncogenes [25]. Up- or downregulation of miRNA can influence the tumorigenic outcome depending on the role(s) of the target genes on vital signaling processes [26].

The most often upregulated miRNAs in papillary thyroid cancer (PTC) are miR-146b, miR-222, miR-221, and miR-181b. Overexpressed miR-146b targets retinoic acid receptor beta (RARβ) and causes reduced expression of this gene leading to increased tumor aggressiveness and extrathyroidal invasion. miR-221 and miR-222 target tumor suppressor and cell-cycle regulator p27. Reduced expression of p27 results in increased proliferation of tumor cells. These processes are related to aggressive behavior of tumor, extrathyroidal invasion, and the presence of lymph node invasion [21, 27–29]. miR-181b also is overexpressed in PTC compared to normal thyroid tissue. miR-181b inhibits expression of cylindromatosis (CYLD) gene which acts as tumor suppressor and normally induces cellular apoptosis [28, 30].

The most often downregulated miRNAs in PTC are miR-145, miR-451, miR-613, and miR-137. miR-145 acts a tumor suppressor in thyroid cancer, and downregulation leads to cancer growth through several pathways [28]. miR-451 acts as a tumor suppressor by targeting the PI3/AKT pathway. Downregulation of miR-451a is associated with tumor aggressiveness and the presence of extrathyroidal invasion [27, 28]. miR-613 is involved in PTC cell proliferation and invasion [28, 29]. In PTC, miR-137 expression is downregulated leading to increased cellular proliferation, invasion, and migration [28] (**Table 1**).

Diagnosis	Type of miRNA	Type of regulation	Outcome	References
Papillary thyroid cancer	miR-146b	Upregulated in cells	Aggressive behavior and extrathyroidal invasion	Rossi et al. [21] Rodriguez-Rodero et al. [27] Boufraqech et al. [28] Chruscik et al. [29]
	miR-222	Upregulated in cells	Aggressive behavior, extrathyroidal invasion, and lymph node metastasis	Rossi et al. [21] Rodriguez-Rodero et al. [27] Boufraqech et al. [28] Chruscik et al. [29]
	miR-221	Upregulated in cells	Aggressive behavior, extrathyroidal invasion, and lymph node metastasis	Rossi et al. [21] Rodriguez-Rodero et al. [27] Boufraqech et al. [28] Chruscik et al. [29]
	miR-181b	Upregulated in cells	Decreased apoptosis of cancer cells	Boufraqech et al. [28]
	miR-145	Downregulated cells	Regulates cancer growth	Boufraqech et al. [28]
	miR-451	Downregulated cells	Tumor aggressiveness	Rodriguez-Rodero et al. [27] Boufraqech et al. [28]
	miR-613	Downregulated cells	Increased cellular proliferation and invasion	Boufraqech et al. [28] Chruscik et al. [29]
	miR-137	Downregulated cells	Increased cellular proliferation, invasion, and migration	Boufraqech et al. [28]

Table 1. Expression of microRNA in PTC.

4. Follicular thyroid cancer and liquid biopsy

Follicular thyroid carcinoma (FTC) is the second most common type of thyroid cancer, comprising 10–15% of all thyroid carcinomas [4, 13, 31]. Although FTC is the second most common type, its incidence has decreased over the past few years [31]. World Health Organization has defined FTC as a malignant epithelial tumor showing follicular cell differentiation in which the diagnostic nuclear features of PTC are absent. Lesions are usually encapsulated and show invasive growth pattern [14]. The diagnosis of FTC is a difficult dilemma in cases when capsular, vascular, or extrathyroidal invasion or metastasizing is not straightforward [32, 33]. A lot of studies have researched biomarkers in liquid biopsy for PTC; however, limited amount of information is found about FTC. One of those studies explored miRNA dysregulation and tried to found miRNA markers for diagnostic purposes in the case of FTC [34]. Dettmer with colleagues found upregulation of miR-182/-183/-221/-222/-125a-3p and a downregulation of miR-542-5p/-574-3p/-455/-199a. They concluded that distinction between FTC and hyperplastic nodules can be done by the use of dysregulated miRNA in the tissues. The miRNAs found

Diagnosis	Type of miRNA	Type of regulation	Outcome	References
Follicular cancer	miR-199a-5p	Downregulated in cells	Increased cellular proliferation	Rodriguez-Rodero et al. [27] Boufraqech et al. [28]
	miR-197	Upregulated in cells	Increased cellular proliferation	Rossi et al. [21] Boufraqech et al. [28]
	miR-346	Upregulated in cells	Increased cellular proliferation	Rossi et al. [21] Boufraqech et al. [28]

Table 2. Expression of microRNA in FTC.

in follicular thyroid cancers (FTC) are also frequently present in other subtypes of thyroid cancer [27]. miR-199a-5p is downregulated, but miR-197 and miR-346 are upregulated in FTC leading to increased cancer cell proliferation [28] (**Table 2**).

5. Medullary thyroid cancer and liquid biopsy

Medullary thyroid cancer (MTC) accounts for only 5% of thyroid cancers; it is responsible for approximately 13% of all thyroid cancer-related deaths [4]. MTC is a malignant thyroid tumor showing evidence of C-cell differentiation [14]. One of the recently studied miRNAs in medullary thyroid cancer is miR-21, which is downregulated especially in the aggressive forms [27]. miR-129-5p also is significantly downregulated in MTC compared to normal tissue leading to increased cellular invasion and migration [28] (**Table 3**).

Diagnosis	Type of miRNA	Type of regulation	Outcome	References
Medullary thyroid cancer	miR-183	Upregulated in tissue	Aggressive behavior and metastatic disease	Rodriguez-Rodero et al. [27] Accardo et al. [35]
	miR-375	Upregulated in tissue	Aggressive behavior and metastatic disease	Rodriguez-Rodero et al. [27] Accardo et al. [35] Boufraqech et al. [28]
	miR-21	Downregulated in tissue	Aggressive behavior and metastatic disease	Rodriguez-Rodero et al. [27] Accardo et al. [35] Boufraqech et al. [28] Pennelli et al. [36]
	miR-129-5p	Downregulated in tissue	Decreased cellular apoptosis and increased cell migration	Rodriguez-Rodero et al. [27] Boufraqech et al. [28]

Table 3. Expression of microRNA in MTC.

6. Anaplastic thyroid cancer and liquid biopsy

Anaplastic thyroid carcinoma (ATC) is aggressive thyroid tumor which consists of undifferentiated follicular thyroid cells [14].

Many miRNAs have been found to be dysregulated in thyroid cancer, but only a few miRNAs are exclusively dysregulated in anaplastic thyroid cancer (ATC) [28]. Loss of miR-200 expression in ATC results in epithelial-mesenchymal transition (EMT) that represses the epithelial features of cancer cells and disrupts the cell-cell adhesion mediated by the loss of E-cadherin. This process enables cells to migrate and invade [26–28].

Overexpression of the miR-17-92 cluster results in downregulation of tumor suppressor PTEN. This process potentiates the activation of AKT/mTOR growth and survival signaling. Another important tumor-suppressive pathway targeted by miR-17-92 is the TGFβ signaling pathway [28]. Increase of miR-17-92 leads to a loss of the tumor inhibitory effect of TGFβ in thyroid cancer cells, which enhances cell proliferation [26–28].

Diagnosis	Type of miRNA	Type of regulation	Outcome	References
Anaplastic thyroid cancer	miR-30 family	Downregulated in cells	Protect cancer cells from apoptosis and autophagy	Sasanakietkul et al. [26] Rodriguez-Rodero et al. [27] Boufraqech et al. [28]
	miR-125a, miR-125b	Downregulated in cells	Promote tumor invasion	Sasanakietkul et al. [26]
	miR-138	Downregulated in cells	Increase with the progression of histological dedifferentiation and malignant behavior	Sasanakietkul et al. [26] Rodriguez-Rodero et al. [27]
	miR-200 family	Downregulated in cells	Increase the invasive potential of tumor	Sasanakietkul et al. [26] Rodriguez-Rodero et al. [27] Boufraqech et al. [28]
	miR-17-92 cluster	Upregulated in cells	Promote tumor growth and invasion	Sasanakietkul et al. [26] Rodriguez-Rodero et al. [27] Boufraqech et al. [28]
	miR-146a, miR-146b	Upregulated in cells	Dysregulated cell differentiation and invasion	Rodriguez-Rodero et al. [27] Sasanakietkul et al. [26]
	miR-221, miR-222	Upregulated in cells	Promote tumor growth and invasion	Rodriguez-Rodero et al. [27] Sasanakietkul et al. [26]
	miR-4295	Upregulated in cells	Increased cell migration and invasion	Rodriguez-Rodero et al. [27] Sasanakietkul et al. [26]

Table 4. Expression of microRNA in ATC.

miR-30 plays a role in thyroid cancer differentiation and progression. Overexpression of miR-30 family members leads to a change in cell morphology and decreased vimentin expression in cancer cells [28]. This is suggesting that miR-30 family members are involved in cancer progression by regulating the EMT process [26–28] (**Table 4**).

7. Poorly differentiated thyroid cancer and liquid biopsy

Poorly differentiated thyroid carcinoma (PDTC) is malignant epithelial cell tumor derived from follicular cells that shows limited characteristics of follicular cell differentiation and is morphologically and behaviorally intermediate between differentiated thyroid carcinomas and anaplastic thyroid carcinoma [14]. miR-23 and miR-150 are downregulated, but miR-146b, miR-221, and miR-222 are upregulated in poorly differentiated thyroid cancer ensuring more aggressive behavior (**Table 5**).

Diagnosis	Type of miRNA	Type of regulation	Outcome	References
Poorly differentiated thyroid cancer	miR-23	Downregulated in tissue	Promote tumor relapse	Sasanakietkul et al. [26] Boufraqech et al. [28]
	miR-150	Downregulated in tissue	Enhance cancer-specific mortality	Sasanakietkul et al. [26] Boufraqech et al. [28]
	miR-146b	Upregulated in tissue	Promote thyroid tumorigenesis	Sasanakietkul et al. [26]
	miR-221, miR-222	Upregulated in tissue	Induce the tumor angiogenesis	Sasanakietkul et al. [26]

Table 5. Expression of microRNA in PDTC.

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Section 4

Other Targets of Liquid Biopsy

Chapter 7

The Diagnostic and Prognostic Application of Heat Shock Proteins and their Post-Translational Modifications from Liquid Biopsies

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Additional information is available at the end of the chapter

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Abstract

Liquid biopsies contain numerous proteins coming from extracellular vesicles (EVs), be it microvesicles or exosomes, released by both normal and tumour cells, as well as the presence of any circulating tumour cells (CTCs). Such proteins can be used as biomarkers for early diagnosis, prognostic assessment, disease progression monitoring, therapy selection and treatment response, particularly in oncology. EVs have been identified as mediators of cell-to-cell communication in both normal and pathological conditions and suggested to play a role in promoting and maintaining cancer dissemination and progression by altering the tumour microenvironment through immune suppression, angiogenesis and metastasis. One class of proteins garnering particular interest are extracellular heat shock proteins (HSPs) (secreted despite no consensus secretory sequence), and their post-translational modifications (PTMs), which are thought to act as key players in intercellular crosstalk and activation of signalling pathways during stress conditions. This review will focus on how characterising and quantifying these proteins can indicate the condition of the physiological system in a variety of pathological contexts.

Keywords: extracellular vesicles, biomarkers, heat shock proteins (HSPs), post-translational modifications (PTMs), intercellular crosstalk

1. Introduction

Liquid biopsies may contain a wide variety of biomolecules including DNA, RNA, proteins and metabolites. When considering the presentation of the numerous proteins within a liquid

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biopsy, these can be free in the plasma, encapsulated within or on the surface of extracellular vesicles (EVs) or still inside cells within the biopsy (such as in the case of circulating tumour cells (CTCs)).

One class of proteins garnering particular interest as part of liquid biopsies are extracellular heat shock proteins (HSPs), and their post-translational modifications (PTMs), mainly because they should not be present in body fluids at the concentrations observed due to their lack of an export sequence and also as a result of the growing evidence supporting the notion that these proteins can mediate intercellular crosstalk and act as messengers that activate signalling pathways during stress conditions.

1.1. Heat shock proteins (HSPs)

HSPs are a class of chaperone proteins ubiquitously expressed in the cells of both prokaryotic and eukaryotic organisms. HSPs have been traditionally named and subdivided into six groups or families based on their molecular weight, namely, the small HSPs (which include HSP27), HSP40, HSP60, HSP70, HSP90 and HSP100 family. However, more recently, a new nomenclature and classification system based on the naming issued by the Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC) has been proposed for classifying human HSPs into the following groups: HSPA (HSP70), HSPB (small HSPs including HSP27), HSPC (HSP90), HSPD/HSPE (HSP60/HSP10), HSPH (HSP110) and DnaJ (HSP40) [1]. Each of these families has members that are constitutively expressed and others that are inducible upon stress.

Under normal physiological conditions, constitutive HSPs fulfil important regulatory roles in a wide range of cellular processes including the synthesis, folding, translocation, assembly and in some cases activation of the proteins they interact with. On the other hand, after an episode of cellular stress, inducible HSPs help to refold and prevent aggregation of misfolded proteins, as well as assist in the proteasomal degradation of misfolded proteins which cannot be recovered. Moreover, HSPs can block apoptotic signalling and increase tolerance to subsequent insults [2].

However, it is now starting to emerge that during stress, the role of HSPs goes beyond what is expected to be their intracellular chaperoning functions for recovery from multiple stress conditions. Despite HSPs acting predominantly intracellularly, they have also been found expressed in the cell plasma membrane and in the extracellular space. Numerous HSPs have been reported to be present in the extracellular space and general circulation, activating a range of signalling pathways depending on the effector cell type or target organ. The role of such extracellular HSPs appears to be that of a systemic warning system of stressful events or chronic conditions, acting by priming the body, of which the immune system is a major effector, in order to prepare for and counteract the spread of the stress insult. Extracellular HSPs thus seem to act as a form of intercellular communication system during stress conditions, particularly those responses linked to oxidative stress, immunity or inflammation [3].

1.2. The presence of HSPs outside cells

When HSPs are present outside cells, they can be found as free proteins in solution or forming part of EVs. EVs can be of various types, with distinct structural and biochemical properties

as well as intracellular site of origin. These include large microvesicles (up to 1500 nm) that are heterogeneous in shape and produced from the plasma membrane, small (50–100 nm) and more uniformly shaped exosomes released from endosomes via the endocytic pathway and apoptotic vesicles produced upon cell death [4, 5].

EVs are released by almost all cell types, both healthy and diseased (including tumour cells). Such vesicles carry a wide range of biologically active molecules including growth factors, cytokines, mRNAs and microRNAs, extracellular matrix constituents and also proteins [6]. The protein fraction consists of cytosolic or plasma membrane components, either inside or on the surface. Their molecular contents have been shown to mediate intercellular communication in a variety of cellular processes, in both normal and pathological conditions, with the transfer of such biomolecules altering the function of the target cells. In the context of cancer, for example, EVs can modulate both the tumour microenvironment and cells and tissues which are located at a distance, affecting the immunity in the area, promoting angiogenesis and bringing about metastasis [7, 8].

EVs are also released by cells in response to being exposed to a stressor or as a result of chronic cellular stress. Such EVs contain particular molecules, including HSPs, whose expression level is directly linked to or induced by the stress insult. Upon reaching their effector cells, and especially when interacting with cells of the immune system, some EV components act as signalling molecules, activating a response in the effector cells which pre-empts the stress insult prior to its spread [3].

Proteomic studies have shown that EVs from serum, saliva, milk or plural effusions contain HSP27, HSP60, HSP70 and HSP90 [9–16] at high concentrations, with the ability to synergise with other encapsulated factors [3]. The delivery of HSPs in EVs provides a much stronger signal to effector cells as exemplified by EVs containing HSP70 producing a 250-fold higher activation of macrophages than an equal concentration of HSP70 in solution [17].

The HSPs encapsulated within or presented on the surface of such EVs, together with changing levels in free HSPs, can thus be valuable disease biomarkers for early detection, diagnosis and therapy selection. However, in order to access them, these proteins need to be purified from the body fluids of patients, characterised, quantified and compared to what is known in the healthy condition.

2. Secretion and uptake of extracellular heat shock proteins

When cells are exposed to a stressor, which includes but is not limited to heat shock, osmotic stress, exposure to heavy metals, hypoxia, ischemia or pathogens, these release signalling molecules in order to alert the rest of the system that a stressful condition is being experienced in some part of the organism and which might potentially lead to a situation of systemic damage. Among the stress signals which can be released by cells in response to an incidence of cellular stress are HSPs and other components of the chaperone (**Figure 1**). It is worth noting that most HSPs lack the consensus signal required for secretion via the classical endoplasmic reticulum (ER)-Golgi pathway [3, 18]. So far, it appears that the secretion of HSPs is achieved via a number of alternative pathways; however, these are still not well defined. Presently, the HSP release mechanisms identified are (but might not be limited to) processes via:

i. Cell lysis—where the process can be the result of a physiologically regulated release of cytokines or necrosis resulting from a pathological condition. Extracellular HSP70 has been suggested to be released into circulation under a variety of pathological conditions which cause widespread cell death as well as the following necrosis of tumour cells [19].

ii. Endolysosomal pathway—where the HSP is translocated into lysosomes and instead of being degraded is translocated out of the cell via endocytosis. HSP27 (dephosphorylated at S15 and S82) [20] and HSP70 [21] have been shown to enter endolysosomes, which are then secreted extracellularly in an ATP-dependent manner, from both tumuor cells and macrophages possibly via some pathway analogous to the ATP-binding cassette (ABC) transport system [21].

iii. Exosomal pathway—where the HSP is contained in secretory vesicles (exosome lumen) which rupture or are lysed once present in the extracellular space. A number of HSPs have been detected within extracellular vesicles including HSP27, HSP70, HSC70, GRP75, GRP78 and HSP90 [22–25].

iv. Inclusion in the exosomal membrane—where the HSP is inserted into the membrane of the secretory vesicles rather than being in the lumen. The isolation of HSP70-containing vesicles, derived from the plasma membrane, indicates that the surface of the vesicle can be used as an export system [17, 26, 27].

v. Secretory-like granules—where the vesicles used to transport the HSP are neither lipid bodies, nor endosomes, or lysosomes. Tumour cells were found to release HSP70 in structures that were only positive for chromogranin A, which is a marker of secretory granules [28].

Once in the extracellular space or general circulation, these HSPs can stimulate a wide range of cell types. However, similar to the secretion mechanisms, the recognition and uptake of HSPs by cells, as well as the role that extracellular HSPs play in cell activation, are poorly understood. HSPs have been reported to bind to a wide variety of receptors on target cells, among which are:

i. Low-density lipoprotein (LDL) receptor-related protein 1 (LRP1; CD91)—a receptor involved in receptor-mediated endocytosis, which is found on numerous cell types including antigen-presenting cells (APCs), known to bind to HSP70, HSP90 and calreticulin [29, 30].

ii. CD40—a member of the tumour necrosis factor (TNF) receptor family that is essential in mediating a broad variety of immune and inflammatory responses and can bind to HSP70 [31, 32].

iii. C-C chemokine receptor type 5 (CCR5; CD195)—a receptor on white blood cells involved in the process by which T cells are attracted to target areas via cytokines, which has also been shown to bind to mycobacterial HSP70 [33].

iv. Toll-like receptors (TLRs)—of the ten TLR receptors found in humans, only TLR2 and TLR4 are so far known to act as HSP receptors. They are known to bind to HSP60, HSP70 and HSP90 [34–37]. It has been suggested that TLR activation by HSP is most likely not the result of a direct binding of HSP70 to these receptors but rather either a low affinity interaction or a secondary activation involving the prior binding of HSP to another receptor [38].

v. CD14—a co-receptor for TLR4 activation, which was found to be also required for HSP70 induction of cytokines [35].

vi. Scavenger receptors (SR)—a family of receptors currently classified into ten subclasses (A–J) based on structure and biological function [39]. At least three SRs bind to and internalise HSPs, namely, lectin-like oxidised LDL receptor 1 (LOX-1), scavenger receptor expressed by endothelial cell 1 (SREC-1) and fasciclin and EGF-like, laminin-type EGF-like and link domain-containing scavenger receptor 1(FEEL-1)/common lymphatic endothelial and vascular endothelial receptor 1 (CLEVER-1), with HSP70 binding to all three, HSP60 binding to LOX-1, HSP90 binding to LOX-1 and SREC-1 and calreticulin binding SREC-1 but not LOX-1 [38, 40–43]. Furthermore, scavenger receptor-A (SR-A) can bind to and internalise HSP90 and calreticulin as well as HSP110 and GRP170 [42, 44]. The sialic acid-binding immunoglobulin-type lectin (Siglec) receptors Siglec-5 and Siglec-14 have also been found to bind to HSP70 [45, 46].

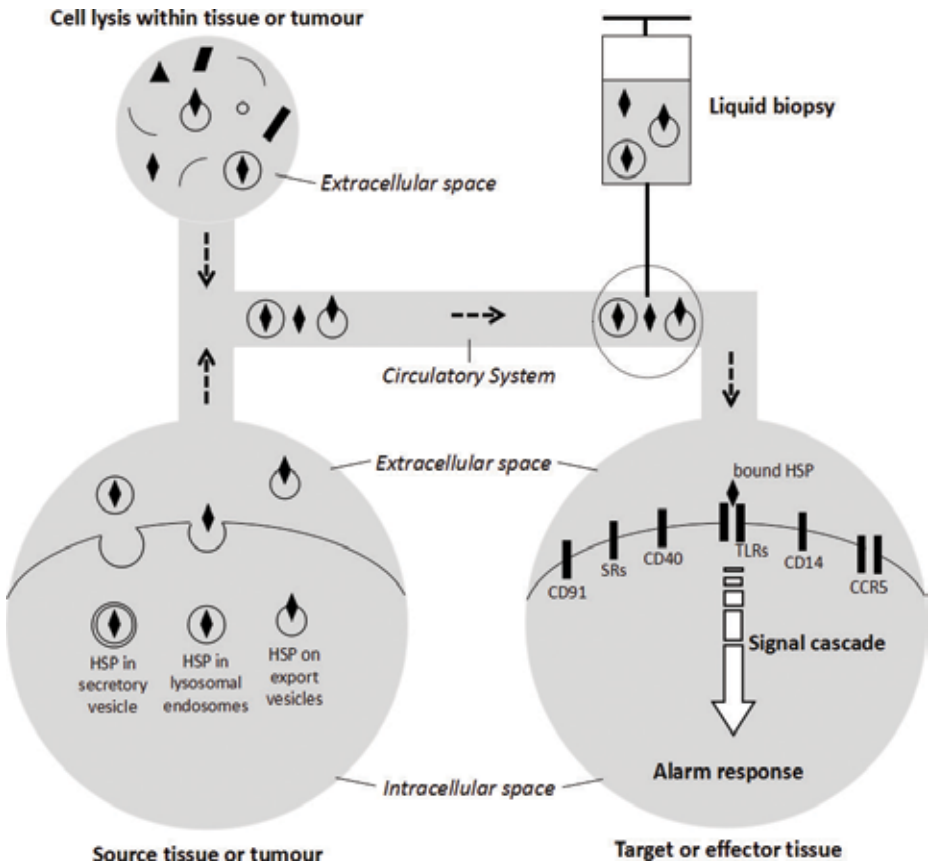

Figure 1. Heat shock proteins (HSPs) are exported into the extracellular space and general circulation via a number of different processes including cell lysis, secretory vesicles, lysosomal endosomes or export vesicles. Once these extracellular HSPs reach the target tissues, they bind to a variety of receptors, which initiate an alarm response. When these extracellular HSPs are collected from patients with chronic diseases and quantified, they can have diagnostic or prognostic value.

3. Biomarker potential of extracellular heat shock proteins

Changes in extracellular HSPs have been detected and implied to be actively involved in many chronic pathological conditions including arthritis, cardiovascular disease, cancer, type 2 diabetes mellitus (T2DM), chronic obstructive pulmonary disease (COPD) and neurodegenerative diseases. However, in order for these extracellular proteins to be used as biomarkers for early diagnosis, prognostic assessment, disease progression monitoring, therapy selection or treatment response, it is essential to characterise their functions and quantify their levels in the selected body fluids for liquid biopsies under both normal physiological conditions and the various pathological contexts.

For example, with respect to cancer, a wide range of studies have linked changes in extracellular HSPs to key mechanisms involved in either the process of malignant transformation or the progression of a tumour via evasion of apoptosis, increased cell proliferation and immortality, invasiveness and metastasis. On the other hand, when it comes to T2DM, because the biochemical mechanisms are not well understood, it is more difficult to link extracellular HSPs to the aetiology of the condition. However, T2DM patients present a two- to fourfold higher risk of developing macrovascular diseases, including coronary artery disease, stroke and peripheral vascular disease, making episodes of cardiovascular complications the major fatality in such patients [47]. Moreover, the sustained hyperglycaemia brings about cellular dysfunction via systematic biochemical changes due to oxidative stress, accumulation of advanced glycated end products (AGEs) and chronic inflammation [48], which are processes highly associated with HSPs.

3.1. HSP27

Extracellular HSP27 has been so far linked to three major functions, immune response modulation, angiogenesis and atheroprotection through a number of mechanisms, which in the contexts of cancer and T2DM can have a significant contribution to the aetiology or progression of the disease.

Immune signalling is activated by extracellular HSP27 via interaction with receptors on the surface of immune or endothelial cells, leading to the differential production and release of cytokines and growth factors, in order to modulate the immune response, cellular migration and proliferation. Extracellular HSP27 interacts with TLR2, TLR3 and TLR4, bringing about NF-κB transcriptional activation and the upregulation of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1), leading to the secretion of TNF-α, IL-6, IL-8, IL-10, IL-1β, IL-12p35 and IL-12p40, colony-stimulating factor 2 (CSF2) and vascular endothelial growth factor (VEGF) [49–52]. The release of IL-10 induced by extracellular HSP27 was found to involve the phosphorylation of p38 and MAPKAPK-2, whilst the upregulation of TNF-α was attributed to the activation of both p38 and ERK1/ERK2 signalling pathways [53]. HSP27 was also found to interact with oestrogen receptor-β (ER-β) [54, 55].

In cancer, extracellular Hsp27 has been reported to exert pro-angiogenic effects via the stimulation of the transcription of the vascular endothelial growth factor (VEGF) gene [50]. Increased

VEGF expression promoted HSP27 phosphorylation through the stress-activated protein kinase 2 (SAPK-2)/p38 pathway, resulting in cytoskeletal rearrangements and endothelial cell migration [56]. Furthermore, HSP27 phosphorylation not only reduced the release of HSP27 in the extracellular space, where the released HSP27 binds to and blocks VEGF [20], but also enhanced intracellular VEGF expression by interacting with the TLR3 on endothelial cells [50].

In the context of diabetes, T2DM patients with cardiovascular disease presented no significant change in serum HSP27 than non-diabetic controls [57]. However, extracellular HSP27 levels were found to be inversely correlated to progression, complexity and instability of plaques found in atherosclerotic human coronary arteries [54, 58], with HSP27 secretion being greatly reduced in atherosclerotic lesions and almost absent in complicated plaques [59]. Lower levels of serum HSP27 were described as being predictive of subsequent heart attacks, strokes or cardiovascular death within the following 5 years [60]. Atheroprotection is thought to be mediated through oestrogen (for the extracellular release of HSP27) as well as via modulation of various processes involved in atherosclerosis, such as cholesterol homeostasis and trafficking, regional inflammation (including mobility of immune cells in plaques and macrophage activation into foam cells) and plaque remodelling by extracellular HSP27 [61]. Extracellular HSP27 seems to be involved in reduced lipid engulfment by macrophages and foam cell formation through the blocking and downregulation of macrophage scavenger receptor A [62, 63], as well as the promotion of cholesterol efflux by enhancing ATP-binding cassette (ABC) transporter activity via the TLR4-induced and NF-κB-mediated release of CSF2 [64]. A similar activation of NF-κB in endothelial cells via TLR2, TLR3 and TLR4 may further worsen the condition [50, 51]. Moreover, patients with T2DM presented accelerated platelet aggregation correlated with the release of phosphorylated HSP27 from platelets induced by thrombin receptor-activating protein (TRAP) activation of Akt and p38 MAP kinase [65, 66].

3.2. HSP60

Till now, extracellular HSP60 has not been linked to any specific function. What has been explored so far is mostly related to its release mechanism. It has been shown that HSP60 is released into the extracellular space via the exosomal pathway, with most of the HSP60 tightly bound to (as opposed to embedded in) the exosomal membrane, rather than housed in the lumen of the exosomes. Moreover, evidence indicates that exosomal HSP60 is at least in part ubiquitinated (but not poly-ubiquitinated, i.e. not marked for degradation), which might act as a signal for the sorting of HSP60 to exosomes [12]. This ties in with its presence in cancer and T2DM, although the significance of its role in the aetiology or disease progression have not been well investigated.

When looking at the cancer context, tumours often tend to present HSP60 in the cell membrane [67] as well as secreted via exosomes [68]. It is hypothesised that cellular stress results in ubiquitination and possibly other post-translational modifications on cytosolic HSP60, which lead to its localisation in the cell membrane and consequently internalisation via lipid rafts, accumulation in multivesicular bodies and release into the extracellular space via the exosomal pathway [12]. Once secreted (either alone or in conjunction with other biomolecules), it then fulfils an as-yet unspecified but probably immunomodulatory extracellular function [69, 70].

Bioinformatic analysis of colorectal cancer (CRC) pointed at the HSP60 gene as one of the best indicators for diagnosis [71] and proteomic studies have corroborated this finding [72] giving it diagnostic and prognostic value. Similarly, HSP60 has also been found to be linked to Crohn's disease and ulcerative colitis [73], two conditions with a high risk for CRC development, probably having a pro-inflammatory role in the remodelling of the colonic mucosa via a TLR4-ERK-dependent mechanism [74].

Extracellular HSP60 is also thought to play a role in diabetes, as stresses associated with diabetes result in the expression of HSP60 on the cell surface as well as its extracellular release, such that it has been detected in both the serum and the saliva of T2DM patients [75, 76]. Moreover, T2DM patients with cardiovascular disease were associated with higher levels of circulating HSP60 compared to control subjects without cardiovascular disease [77]. Extracellular HSP60 has been associated with the severity of atherosclerosis and has been proposed as a biomarker for coronary heart disease [78, 79].

3.3. HSP70

Extracellular HSP70 has been shown to have important immunostimulatory properties, activating macrophages, monocytes, dendritic cells (DCs) and natural killer (NK) cells, by acting either as a cross presenter of immunogenic peptides via major histocompatibility complex (MHC) antigens, as a chaperone stimulating both innate and adaptive immunities, or as a stimulator and target for innate immune responses mediated by NK cells [35, 80, 81]. In contrast, some studies have shown that it can also have anti-inflammatory effects by activating both immunosuppressive regulatory T cells (Tregs) and Siglec receptors that block the inflammatory process by interacting with TLRs [82]. Moreover, extracellular HSP70 bound to vesicle membranes has been shown to induce an immunosuppressive effect [27], supporting the notion that HSP70 fulfils different roles depending on the composition, source and effector of the vesicles it is associated with.

Apart from immunity, extracellular HSP70 has been implicated in a wide array of conditions including cancer, diabetes, chronic inflammation, cardiovascular disease, hypertension, preeclampsia, Alzheimer's disease (inhibiting amyloid β aggregation) and ischemia [3, 83, 84].

When it comes to the cancer setting, serum HSP70 levels have been correlated with treatment response and tumour volume [85], making extracellular HSP70 a potential biomarker for cancer [86] both as a candidate biomarker for tumour detection and monitoring clinical outcome of radiotherapy [87], as well as a prognostic marker, such as in CRC, associated with rapid disease progression and poor survival [88]. In some contexts, extracellular HSP70 has even shown potential in discriminating between infection or inflammation and cancer (e.g. chronic hepatitis, liver cirrhosis and hepatocellular carcinoma) [89]. Extracellular HSP70 has been found to increase MMP9 expression by activating NF-κB and AP-1 and that the subsequent increase in pro-MMP9 secretion results in enhanced cell motility and invasiveness [90]. HSP70 was also isolated from the surface of tumour-derived exosomes [26], in which setting it can interact with myeloid-derived suppressor cells, so as to suppress T-cell activation and promote cancer development [27]. Extracellular HSP70 has also been used as a cancer vaccine, such that immunisation of mice with a vaccine made of HSP70-peptide complexes extracted

from fusions between DCs and radiation-enriched tumour cells resulted in a T-cell-mediated immune response against radioresistant tumour cells [91].

In vitro experiments of diabetes have shown that extracellular HSP70 plays a role in diabetic nephropathy in T2DM by promoting inflammation in the proximal tubule cells via a TLR4-NF-κB pathway. HSP70 release induced by the albumin in the proximal tubule cells triggered the overexpression of the inflammatory cytokines monocyte chemoattractant protein' 1 (MCP-1), tumour necrosis factor alpha (TNF-α) and macrophage inflammatory protein 2 (MIP2) [92]. Similar results were obtained in diabetic mice where TLR4 deletion or HSP70 inhibition reduced albuminuria and markers of inflammation and tubular injury [92]. Further supporting these findings, patients with T2DM with albuminuria showed higher serum HSP70 levels [93] as well as an association between urinary HSP70 levels and albuminuria [94]. Serum HSP70 was also found to be higher in patients with diabetic retinopathy, together with HIF-1α compared with subjects without [95] and correlated well with asymmetric dimethylarginine (ADMA) and C-reactive protein (CRP) levels in T2DM patients compared with healthy controls [96].

An inverse association has been reported between levels of HSP70 with the presence and severity of cardiovascular disease [97–100]. Moreover, an inverse correlation was found between HSP70 levels and the risk of future development of atherosclerosis in subjects with established hypertension [101]. Extracellular Hsp70 levels have also been inversely correlated with the risk of cardiovascular disease [97, 101, 102] and the severity and survival after chronic heart failure [103].

3.4. GRP78

Extracellular GRP78 has been documented [104, 105], but it has been studied much more extensively at the cell surface than in the extracellular space or in circulation. GRP78 could be detected in plasma as both full-length and C-terminus fragments [106]. GRP78 is secreted from cells via exosomes, and the release appears to be at least partly controlled by acetylation since the use of histone deacetylase (HDAC) inhibitors could block GRP78 release, causing aggregation in the ER. Suppression of HDAC6 activity leads to GRP78 acetylation, which is then bound to vacuolar protein sorting 34 (VPS34), a class III phosphoinositide-3 kinase, preventing GRP78 from being sorted into multivesicular bodies [107]. Since it has been shown that ER stress can actively promote the expression of GRP78 on the cell surface, and that over-expression of GRP78 can result in similar cell surface localisation, independent of ER stress [102], this might also hold true for extracellular release of GRP78. Once in the plasma membrane GRP78 binds to a wide selection of proteins, which in turn causes signalling cascades through multiple pathways that can result both in cell survival and cell death [108, 109], however the potential interaction or competition of extracellular GRP78 has not been explored. Interestingly, HSP40 (DnaJ) seems to be involved in GRP78 cell surface localisation and silencing of the murine homolog, MTJ-1 abolished cell surface localisation of GRP78 [110], but so far its possible involvement in extracellular release instead has not been investigated.

When looking at cancers, extracellular GRP78 is not commonly investigated; however, some tumours secrete significant levels of GRP78 into the tumour microenvironment [105], and in one study, extracellular GRP78 was identified exclusively in the sera of 28% of gastric cancer

patients but not in healthy controls [111]. It is speculated that ER stress and activation of the unfolded protein response (UPR), an evolutionarily conserved mechanism in which survival or apoptotic pathways are activated in response to ER stress, induce GRP78 in tumour cells leading to increased secretion of GRP78, and by binding to cell surface receptors of endothelial cells, extracellular GRP78 activates ERK and AKT pathways [105].

Useful inferences could be made by looking at cell surface GRP78 which is expressed significantly in human tumours and generally associated with cell proliferation, cell survival, angiogenesis and metastasis [112]. Cell surface GRP78 interacts with α2-macroglobulin, a plasma protease inhibitor, through its amino-terminal domain-activating the PI3K/Akt, ERK1/ERK2 and p38 MAPK pathways, promoting cell proliferation and cell survival via Akt and NF-kB signalling cascades, by inducing the UPR [105, 113, 114]. Moreover, interaction of cell surface GRP78 with teratocarcinoma-derived growth factor 1 (TDGF1; Cripto-1), a small, glycosylphosphatidylinositol (GPI)-anchored protein, modulates activin-A, activin-B, nodal and transforming growth factor-b (TGF-b)-dependent signalling of several ligands via the MAPK/PI3K and Smad2/3 pathway and promotes cell proliferation, downregulates E-cadherin (which decreased cell adhesion) and promotes pro-proliferative responses to activin-A and nodal [115, 116]. Of particular interest is that specifically on the surface of cancer cells but not healthy cells, GRP78 interacts via its amino-terminal domain with extracellular prostate apoptosis response 4 (Par-4), which together with tumour necrosis factor-related apoptosis-inducing ligand or Apo 2 ligand (TRAIL/Apo2L) mediates apoptosis via an extrinsic apoptotic pathway (dependent on ER stress and the Fas-associated death domain (FADD)/caspase-8/caspase-3 pathway) [117]. Similarly, plasminogen kringle 5 (K5), an angiogenesis inhibitor, interacts with cell surface GRP78 via the carboxy-terminal domain, on hypoxic and cytotoxic stressed tumour cells, mediating anti-angiogenic and pro-apoptotic activity following the internalisation of GRP78 by the scavenger receptor low-density lipoprotein receptor-related protein 1 (LRP1) and activation of p38 mitogen-activated protein kinase [118, 119].

The isolation of a tumour-specific variant of GRP78 containing an O-linked carbohydrate moiety with a molecular weight of 82 kDa opens up numerous therapeutic possibilities not only of targeting tumours by specific variants of GRP78 [120] but also of searching for the presence of tumour-specific variants in circulation, as a diagnostic marker.

Once again in the context of diabetes, extracellular GRP78 is poorly investigated. However, data from cell surface expression of GRP78 indicates that the extracellular counterpart might play some role in the cardiovascular complications linked to T2DM. GRP78 has been detected on microparticles shed from activated endothelial cells indicating that GRP78 expression may be involved in regulating thrombosis [121]. Expression of cell surface GRP78 in arterial atherosclerotic lesions negatively regulates the initiation of the tissue factor(TF)-mediated coagulation cascade [122, 123], attenuating procoagulant activity similar to the effect observed from the binding of K5 to cell surface GRP78 on stimulated endothelial cells [119]. Atherosclerotic lesions also present an increase in truncated cadherin (T-cadherin) expression, which interacts with cell surface GRP78, similar to the interaction on vascular endothelial cells [124] and on endothelial cells during tumour angiogenesis [125], promoting cell survival and indicating that this interaction plays a role in vascular tissue remodelling related to stress.

3.5. HSP90

As with most other HSPs, extracellular HSP90 has been mainly studied in relation to inflammation and immunity [126]. However, no specific roles, processes or mechanisms have been elucidated yet.

In the context of cancer, extracellular HSP90 (mainly not only HSP90a but also HSP90b) is known to be involved in tumour cell migration, invasion and metastasis [127–131]. Serum levels of extracellular Hsp90a were significantly higher in the patient groups with tumour burden, with a positive correlation with tumour malignancy and metastasis [132]. The interaction of extracellular Hsp90 with the LRP1 receptor as well as HER-2 activates AKT1/AKT2 (in the phosphatidylinositol-3-kinase (PI3K) signalling pathway) and ERK1/ERK2 signalling cascades giving rise to increased cell migration, supporting growth and survival [128, 133, 134]. AKT activation is sustained by the phosphorylation of the receptor tyrosine kinase ephrin type-A receptor 2 (EPHA2), which is a downstream product of the interaction between LRP1 and extracellular Hsp90 [135]. Also, critical for cell migration is the presence of extracellular HSP90 for the interaction between Src and integrin β1 at focal adhesion points between the cell and ECM [130]. The interaction of extracellular HSP90 with TLR4 also signals through Src, and this transactivates the epithelial growth factor receptor (EGFR), which increases cell migration [136]. It has also been shown that extracellular HSP90 can have a role in ECM remodelling or stabilisation via its direct interaction with fibronectin [137]. Work in colorectal cancer cells showed that extracellular Hsp90 promotes epithelial-to-mesenchymal transition (EMT) via an LRP1-NF-κB pathway [138], whilst exposure of prostate cancer cells to extracellular Hsp90 promoted EMT via a process requiring both matrix metalloprotein 9 (MMP9) and ERK activity [139]. Extracellular Hsp90 was also shown to interact with MMP2 [140]. The activation of ERK by extracellular Hsp90 has also been shown to increase expression of the polycomb repressor complex methyltransferase enhancer of zeste homologue 2 (EZH2), bringing about the epigenetic repression of E-cadherin [141], further supporting the EMT process.

Extracellular HSP90 has not been studied much in the context of diabetes, with the majority of studies investigating HSP90 inhibition in general and thus focusing on intracellular mechanisms whilst not excluding effects by extracellular HSP90. In response to oxidative stress, vascular smooth muscle cells secrete HSP90a, and the stimulation of these cells by HSP90a induces MAPK activity [142]. Similarly, endothelial cells also secrete HSP90 upon activation, and this stimulates angiogenesis [143]. Experiments in diabetic rats have shown that annexin II on endothelial cells interacts with extracellular HSP90a, modulating plasminogen activation to plasmin [144]. Furthermore, HSP90 levels were found to be higher in the serum of patients with atherosclerosis [145]. Exosomes collected from cultured fibrocytes contained HSP90a (among other biomolecules) and enhanced cellular migration and proliferation as well as secretion of type I collagen (COL1) and type III collagen (COL3) and expression of α-smooth muscle actin (α-SMA) [146]. Inhibition of total HSP90 disrupts the IKK complex [147] and JAK2 protein stability [148], blocking the activity of the transcription factors NF-kB [149] and STAT [150], respectively, together with a downregulation in the expression of pro-atherogenic cytokines and chemokines. Dysregulated NF-kB and STAT pathways contribute to diabetic nephropathy [150, 151] and atherosclerosis [152, 153]. The inhibition of HSP90 thus

modulates inflammation and oxidative stress, improving diabetes-associated renal damage and atheroprogression [154], insulin sensitivity [155], high-fat-diet-induced renal failure [156] and diabetic peripheral neuropathy [157].

4. Conclusion

The need to identify biomarkers for complex systemic and chronic diseases is pressing, with an increasing push towards the successful development of therapies aimed at modulating serum levels, blocking receptor binding or inhibiting signalling cascades. HSPs hold great potential as therapeutic targets for those conditions with underlying mechanisms involving accumulation of misfolded or damaged proteins, oxidative stress, altered mitochondrial bioenergetics or dysregulated apoptosis, particularly as a result of their non-chaperoning functions. Studies presented herein suggest that circulating HSP levels may be exploited as biomarkers of such conditions, with cancer and cardiovascular complications linked to T2DM being the contexts used to exemplify.

A major limitation of most studies performed on extracellular HSPs is that their functions and roles in disease have not been elucidated yet. As a result the biochemistry and signalling are investigated very poorly, such as testing for a single downstream product of a complex cascade which can be affected by multiple inputs. Similarly, the PTMs on extracellular HSPs are still in their majority obscure both in abundance and functional significance. Studies conducted retrospectively, on single HSPs in isolation, using small patient groups and without adjustment for confounding effects offer a very poor analysis of the predictive power of HSPs for early diagnosis or prognostic assessment. Thus, in future research, it is important to take into consideration that HSPs do not work in isolation, but act within a network, rather than just detect changes in the total extracellular expression levels of individual HSPs and analyse changes in both total HSP and specific PTMs within groups of chaperone proteins that are functionally relevant to either the development of or resultant from the progression of the condition under investigation. Furthermore, this needs to be performed in large cohorts of well-characterised patients, with prospective validation of promising biomarker panels, if the intent is really their application in a clinical setting.

Conflict of interest

The author declares no conflict of interest.

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Chapter 8

Blood-Brain Barrier Breakdown by Combined Detection of Circulating Tumor and Endothelial Cells in Liquid Biopsy

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Additional information is available at the end of the chapter

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Abstract

Blood-brain barrier breakdown occurring in glioblastoma is a temporary condition often denounced by contrast enhancement upon neurological examination. This condition is useful to increase the intracranial concentration of anti-cancer drugs. The prognosis of glioblastoma and its resistance to conventional therapy has stimulated interest in the search of biomarkers able to unmask and monitor brain barrier breakdown to calibrate the treatment. Despite numerous studies had evidenced the role of circulating tumor and endothelial cells to monitor brain tumor, the mechanism of tumor cells release in the bloodstream and its prognostic significance remain unclear. In this chapter, we want to furnish an update on the relationship between the vascular damage occurring during glioblastoma disease and the reactivity of innate immunity focusing on the cytokines network. Our aim is answer to the question: when and why the liquid biopsy is useful in glioblastoma disease.

Keywords: blood-brain barrier, glioblastoma, liquid biopsy, circulating tumor cells, circulating endothelial cells

1. Introduction

Gliomas are associated with aggressive invasion of the surrounding brain parenchyma. The invasion is due to a combination of transformed cells phenotype changes, innate and acquired immunity and autocrine and paracrine release of growing and permeabilizing chemokines. This intricate scenario develops within the cerebral parenchyma, notoriously protected by an ultraspecialized system. Several experimental models of glioma, have been used in vivo to

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monitor the infiltration of tumor cells in the parenchyma and/or perivascular spaces, even at a single cell level [1–3]. However, the relationship between glioma-induced BBB dysregulation and glioma invasion remains poorly understood. In this chapter we review the natural history of glioblastoma and, on the basis of the scientific evidence published to date, we try to give an explanation and meaning to biomarkers found in the peripheral blood. In particular, we focus our attention on cellular biomarkers, the circulating tumor cells and cellular endothelial progenitors. Our interest is aimed at giving an order in the context of human cell biology of human glioblastoma. This intracranial tumor remains today one of the big killers and represents a major challenge in the field of oncology because it is unresponsive to treatment and able to progress in a way difficult to monitor.

2. Development and structure of the blood-brain barrier

Blood-brain barrier (BBB) separates in a selective manner the nerve tissue of the central nervous system (CNS) from the blood. It is present throughout the CNS, except in the circumventriculars organs, where the capillaries are fenestrated as occurs in choroid plexuses. Indeed, such an anatomical membrane can selectively transport, through the capillary wall, large (>500 Da) or water soluble (hydrophilic) substances, whereas, small, lipid-soluble (hydrophobic) substances can freely pass the endothelium by passive diffusion [4, 5].

Classically, BBB was recognized as the structural base of the so-called *immune privilege* of the brain. Thereby, antigens would be sequestered within the brain and would be invisible to the immune system [6].

This view has been challenged in recent years, due to the discovery of the so-called "glymphatic system" (GS) and of meningeal lymphatics (MLs) of dura mater. GS allow that cerebrospinal fluid flows into brain within periarterial spaces and interstitial fluid and solute clear via perivenous spaces. Furtherly, MLs follow dural blood vessels and cranial nerves and exit the cranium via the foramina together with the venous sinuses, arteries, and cranial nerves in order to join cervical lymph nodes. The relationship of this system with the equilibrium of the blood-brain barrier is unknown and its role in human pathology has yet to be clarified (**Figure 1**) [6].

Tight junctions (TJs) between endothelial cells (ECs) are the main component of BBB. TJs are occluding cell junctions, which act to seal off the intercellular space and consist of transmembrane proteins such as occludins (Oclns) and claudins (Cldns), connected intracellularly to the actin filaments, forming strands in the plasma membrane.

TJs blocks the intercellular pathway and forms a barrier between the arterial blood and the nervous tissue, regulated by the end feet of astrocytes that cover the basement membrane of the capillaries [5].

The development of the BBB begins with angiogenesis, started from invasion of the neuroectoderm by endothelial progenitor cells from pre-existing vessels. The endothelia of these vascular sprouts show many characteristics of mature BBB such as TJs, transcytotic vesicles, nutrient transporters and leukocyte adhesion molecules. Afterwards, contact with CNS cells and pericytes (PCs) allows the full functional maturation of the membrane separating nervous

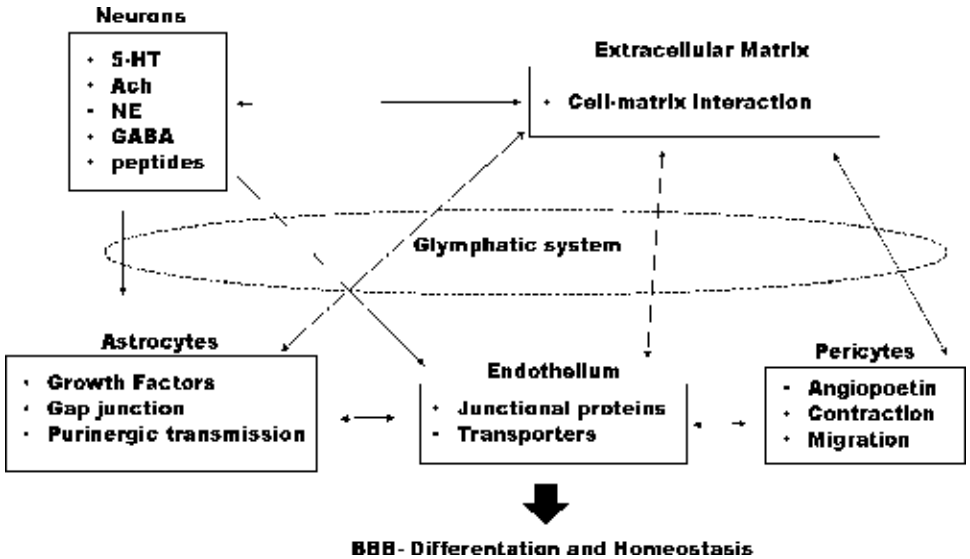

Figure 1. Blood-brain barrier components and their interactions (figure partially modified from Hawkins BT and Davis TP [105]).

tissue from blood. TJs are elaborated and sealed, transcytosis decreases, leukocyte adhesion molecules are downregulated and efflux transporter expression increases [7].

Vascular endothelial growth factor (VEGF), VEGFR-2 and its ligand, play a pivotal role in embryonic angiogenesis and vasculogenesis [8]. In the developing CNS, embryonic brain cells of the subventricular neuroectoderm synthetize VEGF that directs angiogenesis via a concentration gradient through the angiogenic sprouting from vessel networks outside the CNS, in particular, the perineural vascular plexus (PNVP). Within the brain, blood vessels furtherly, then, generate huge networks as the neural tissue grows and concomitantly remodel into a vascular tree with arterial and venous hierarchy [9, 10]. Such a process, when VEGF is reduced or absent, develops in an incorrect way generating decreased blood vessel branching and density in the cortex [11].

Vasculogenesis and angiogenesis have been extensively studied in experimental setting in mouse retina and hindbrain and in zebrafish with the development of the "tip-stalk" model of angiogenic sprouting, which describes the different precursor of endothelial cells within newly formed vascular sprouts:

1. Tip cells drive the sprouting, migrating and extending filopodia that scan the environment for signals that can act as guides for vascular growth.
2. Behind the tip cells, another group of elements, termed stalk cells, proliferate and form the nascent vascular lumen.

Once mature connections and blood flow have been established, the proliferation and the migration of "activate endothelium" ceases. Tip and stalk cells display differential gene expression profiles [12]. The Notch signaling pathway is a very important tool in the regulation

of the tip and stalk cells specification. The activation of Notch signaling inhibits tip cells differentiation and promotes the stalk cell phenotype [13].

Wingless-type mouse mammary tumor virus (MMTV) integration site family (Wnt) pathway plays a pivotal role in angiogenesis both physiological and pathological and in vessels remodeling [14, 15]. Three Wnt signaling pathways are known: the so-called "canonical" Wnt/β-catenin and the two "noncanonical" pathways: the Wnt/calcium (Wnt/Ca2+) and the Wnt/planar cell polarity (Wnt/PCP) [16].

The canonical Wnt/β-catenin signaling pathway targets the regulatory molecule β-catenin. The so-called cytoplasmic destruction complex, consisting of glycogen synthase kinase-3β (GSK3β), Axin, Casein kinase1/2 (CK1/2), Protein phosphatase 2A (PP2A), and adenomatous polyposis coli (APC) leads to a post-translational modification status of β-catenin. In the absence of Wnt signaling, cytosolic β-catenin is phosphorylated by CK1 at Thr41, and GSK3β at Ser33 and Ser37. Phosphorylated β-catenin is then ubiquitinated via the E3 ligase, β-transducing-repeat-containing protein (β-TrCP), and thereby prepared for proteasomal degradation. Conversely, Wnt binding to Frizzled proteins, a Wnt receptors family recruits the co-receptor LRP5/6, that causes an activation of the cytoplasmic phosphoprotein Disheveled. These events lead to the inhibition of GSK3β, thereby promoting the accumulation of unphosphorylated β-catenin and its subsequent translocation to the nucleus, where it binds to a variety of transcription factors, including T-cell factor/lymphocyte enhancing factor (TCF/LEF) and forkhead box (in particular, the FOXO subtypes) family proteins. TCF/LEF represses targets in the absence of signaling, but beta-catenin, when its pathway is activated, enters into the nucleus, binds to TCF on the chromatin and allows the transcription of a number of Wnt target genes involved in cell proliferation, Wnt signal transduction and vascular growth [17, 18].

Tight junctions are organized to form a paracellular seal in transporting epithelia in order to allow the directional transfer of ions and solutes across cell layers.

Tight junctions comprise several trans-membrane proteins (TMPs) which interact with adaptor proteins of the cytoplasmic plaque via their C-terminal domains. TMPs are classified on the basis of their number of transmembrane domains (tetraspan, trispan, and single-span domains) and include the tetraspan Marvel-domain proteins (occludin, tricellulin, and Mar velD3), the claudin family of proteins, the trispan BVES (blood vessel epicardial substance) protein, the single-span JAMs (junctional adhesion molecule-A, -B, and -C), and the polarity determinant Crumbs3. The cytoplasmic plaque is composed of Zona occludens proteins (ZO-1, ZO-2, and ZO-3), multi-PDZ domain protein 1 (MUPP1), cingulin, protein associated with Lin-7 (PALS1), Pals1 associated tight junction (PATJ), protease activated receptor 3 (PAR3) and protease activated receptor 6 (PAR6), which interact in the intracellular space with the cytoskeleton. Moreover, several signaling molecules are associated to the proteins of the cytoplasmic plaque [19].

Oclns and Cldns mainly characterize TJs. The exact role of Ocln is not well known, but it may be a structural component important in in the formation of TJs. Its function is regulated via cytokines, proteases and GTPases [20].

The Wnt/β-catenin pathway, play role in BBB differentiation and may be fundamental in BBB maintenance. Such a pathway is also active in endothelial cells of the adult CNS, providing an essential tool for BBB maintenance [21]. Claudin family member claudin-5 (Cldn5) is important

for TJs formation. Cldn5 is regulated by the Wnt/β-catenin pathway, however activation of VEGF, or other signaling pathways, can oppose the action of Wnt/β-catenin pathway. The Embryonic ablation of Cldn5 in mice induces early postnatal brain edema and death [21]. Claudin-5-deficient mice exhibit an increased leakiness for small-molecular compounds (<800 Da) [22]. BBB leakiness can be tolerated during embryogenesis as long as the placental barrier is functional. A post-natal maturation of brain circulation is a more than likely fact. Indeed, in the mammalian brain, angiogenesis in the cortex well proceeds until 2–3 weeks after birth [23].

Interestingly, in the regions where the corners of three epithelial cells meet, TJs have a specialized structure, the so-called tricellular junctions (tTJs) which contain the tetraspan Marvel-domain protein tricellulin. Such a protein has been detected in the rat and human brain [24, 25]. Tricellular junctions may be critical for the BBB formation. Moreover, lipolysis-stimulated lipoprotein receptor (LSR), a component of paracellular junctions in their three cell membranes meeting points, expression follows CNS angiogenesis and correlates with BBB formation during embryogenesis [26].

PCs are contractile cells surrounding the endothelium of capillaries and postcapillary venules, which are enclosed within the basal lamina of the endothelium along the vessels. They behave as mesenchymal multipotent stem cells giving rise to ECs or smooth muscle cells. PCs play an important role in the angiogenesis and in the BBB integrity. Pericytes show specialized characteristics and roles in different organs such as kidney, liver and brain. Moreover, density of pericytes and vessel coverage vary among tissues [27].

A direct contact between pericytes and ECs is established where the basement membrane is absent via the "peg-and-socket" junctions which are formed by n-cadherin and connexin-43 hemichannels. Adherent junctions between PCs and ECs are also present. Interactions of ECs with PCs and SMCs are pivotal processes in the regularization, remodeling, stabilization and function of vascular wall and BBB, i.e., by the regulation of the transcellular barrier [27–29].

Moreover, the single adhesion receptor CD146 functions on PCs as a co-receptor for Platelet-derived growth factor receptors beta (PDGFR-β) to regulate interactions between ECs and PCs. CD146, shows an initial expression on ECs, during BBB maturation, that slopes down upon PCs recruitment and BBB maturation [30]. Interestingly, astrocytic laminin induces pericyte differentiation from the resting stage to the contractile stage, switching pericyte function from stabilizing the BBB to compromising it [31].

Astrocytes (ACs) surround microvessels and capillaries and interact with endothelial cells through the end-feet of their processes. ACs play critical roles in regulating cerebral blood flow in response to neuronal activity by relaying signals and maintain BBB function by inducing barrier properties and the polarization of transporters [28]. Under steady-state conditions ACs promote BBB homeostasis through soluble factors such as Sonic hedgehog (Shh), retinoic acid (RA), glial-derived neurotrophic factor (GDNF) and angiopoietin1 (Ang-1), which interact with receptors on ECs to increase junctional protein expression, raise transendothelial electrical resistance (TEER), and reduce permeability. Furthermore, knocking out a-dystrobrevin (a-DB), a scaffolding protein of the astrocytic endfeet, or astrocyte-secreted laminin a2, leads to down-regulation of junctional proteins and a leaky BBB [32]. Interactions between ACs and ECs are very important not only for formation and maintenance of BBB, but also for astrocytic differentiation [21].

Morphology and function of BBB are linked as shown by microscopic observation. Early ultrastructural studies where performed by administration of silver nitrate in the drinking water of rodents [33]. They showed the presence of very scarce quantity of silver around the capillaries in the cerebral cortex, medulla, and cerebellum. In such brain regions, capillaries are continuous. In other areas, such as neurohypofisis, area postrema, pineal body and intercolumnar tubercle, heavy accumulation of silver was present around fenestrated capillaries [34]. Using lanthanum and horseradish peroxidase as tracers, via intravenous injection, it has been demonstrated that these substances are unable to penetrate between the endothelial cells because of the tight junctions presence (zonulae occludentes). Regarding the contribution of astrocytes to the BBB, it is well known that its end-feet form a relatively complete layer, but the junctions between are gap junctions and not of the occluding kind [35]. Perivascular endfeet of astrocytes do not provide an effective barrier even if substances should pass through the endothelial cells into the brain. Indeed, after intravenous infusion of peroxidase, endothelial cells show micropinocytotic vesicles containing the tracer [36]. Such a transendothelial cell barrier is very selective and based on carrier-mediated transports, but is not furtherly mechanically regulated.

Caveolae are small, bulb-shaped, plasma membrane invaginations. They have been described to have a function in endocytosis and transcytosis and in in maintaining the lipid composition of the membrane, as well as acting as signaling background.

While endothelial cells in peripheral organs, such as the lung and heart, are enriched in caveolae, in BBB only a small number of caveolae are detectable [24]. Mfsd2a (transporter of the major facilitator superfamily domain-containing (Mfsd) family) contributes to the regulation of vesicular traffic in BBB endothelial cells [37] through the transport of the essential omega-3 fatty acid docosahexaenoic acid (DHA). The expression of Mfsd2a becomes upregulated in ECs with the maturation of BBB. Gene ablation of Mfsd2a in mice results in BBB leakiness and increased vesicular traffic in ECs.

Caveolins (Cavs) are thought to play a role in the regulation of BBB function. Cavs, are a family of integral membrane proteins which represent both positive and negative regulators of intracellular signaling as scaffolding proteins that regulate the intracellular distribution of the signaling molecules. Cav-1 overexpression protects the integrity of the BBB mainly by preventing the degradation of TJ proteins in rats [38].

Cav-1 is a marker of caveolae in endothelial cells and is important in the regulation of various functions like endocytosis, transcytosis, signal transduction, and molecular transport. Recent studies on mice indicate that the suppression of the caveolae pathway requires the transport of lipids, notably DHA-containing phospholipids, by Mfsd2a to regulate CNS endothelial cell plasma membrane composition and to inhibit caveolae vesicle formation [39].

Moreover, Cav-1 regulate the angiogenic response by influencing VEGF receptor 2 (VEGFR2) phosphorylation and internalization [40, 41].

In BBB the basement membrane (BM) represents the noncellular component. Astrocytes, PCs and ECs synthetize and secrete molecules which constitute the BM surrounding the external surface of the endothelial cell, composed by type IV collagen, fibronectin, heparan sulfate,

nidogen, osteonectin and laminin. BM functions as a charge and molecular weight barrier and is able to interact with integrins in order to regulate permeability and cellular transport also across the BBB. Dystroglycans and integrins are transmembrane receptors that allow BBB cells to interact with BM. During brain angiogenesis in mice, ECs show α4β1 and α5β1 integrin, whereas in adult animals α4 and α5β1integrins promote stabilization of vessels [42], α4β1 and α5β1 integrin induce cell proliferation through MAPK signaling in human ECs. Moreover, β1 integrin interaction with laminin maintains levels of claudin-5 in TJs [43, 44].

3. Blood-brain barrier damage

The endothelial cells (ECs) compose the wall of vessels and capillaries and represent the primary blood-tissue barrier. The ECs acting as a protective filter are able to regulate the passage of molecules and immune cells and the level of specialization of each blood-barrier is determined by the functions of endothelial-wall. In the brain, there is the higher level of specialization of the endothelial wall. The filter function of BBB is carried out by ECs strictly interconnect with numerous tight junctions.

Pathological conditions within the central nervous system like ischemia, inflammation or tumor growth lead to blood-brain barrier (BBB) dysfunction, emphasizing that the permeability barrier regulation is principally provided by the local microenvironment and its maintenance is a necessary condition in any circumstance. In many brain tumors morphological irregularities of the perivascular space correlate with a breakdown of the BBB [45–47].

Wolburg et al. [48] found that claudin-3, a key component of BBB tight junctions, is lost in glioblastoma. Further evidence on claudin-1 loss in tumor microvessels, as well as downregulation of claudin-5 and occludin in hyperplastic vasculature, result in a phenotypic change in BBB function due to leaky tight junctions and hyperpermeable endothelial cells.

A recent study by Watkins et al. [49] using a mouse model demonstrates that astrocytic endfeet displace from their position alongside endothelial cells with disruption of the communication between the astrocytes and vasculature and that single glioma cells were sufficient to produce local BBB opening.

The structural perturbations of the vascular barrier during tumor progression, in other organs was demonstrated, are successive to the release in the tumor microenvironment of specific cytokines that downregulate the transcription of these structural proteins [50].

The crucial point is the vascular damage that the tumor direct and indirect operas through the release on the next microenvironment of chemical factors able to increase the permeability of the tissue vessels reducing their protective barrier function. This could explain the pathogenesis of the BBB damage occurring during the development of glioblastoma. The BBB damage induced by the glioblastoma represents a strategy employed to control the BBB opening to allow the passage of drugs [51].

The detection of endogenous circulating molecules normally restricted by the BBB, namely albumin, immunoglobulin G, or fibrinogen, in the brain parenchyma, using immunohistochemistry

or immunofluorescence is one of the most straightforward in situ techniques used to assess BBB impairment [52].

Measurement of exogenous tracer extravasation remains a technique of choice in preclinical studies testing for BBB permeability. A variety of detectable exogenous tracers has become available, and methods have been developed to evaluate the kinetics of their extravasation. Due to variations in tightness of the BBB, the extravasation of different BBB permeability tracers across an impaired BBB critically depends on their physicochemical properties and molecular weight.

In recent years, different in vivo imaging techniques have contributed to the understanding of BBB dysfunction in disease [52]. Among these, noteworthy:

- In vivo confocal microscopy, an important tool for the high-resolution dynamic fluorescence imaging in BBB research. This technique has the advantage of time-lapse imaging of the same subject. The apparent disadvantages of this technique are the technical complexity and invasive nature, because it requires opening of a cranial window in experimental animals.
- Noninvasive fluorescence imaging (NFI), image acquisition is fast (in the range of seconds), and the imaging equipment is comparably inexpensive, simple, and easy to use. However, NFI has a limited resolution (1–2 mm) and quantitation of the imaging signal has several pitfalls.
- Nuclear imaging, rarely used for clinical imaging of BBB impairment. Examples of nuclear imaging techniques are single photon emission computed tomography (SPECT) and positron emission tomography (PET).
- Magnetic resonance imaging, MRI upon intravenous injection of a contrast agent, usually gadolinium-diethylene triamine pentaacetic acid (Gd-DTPA; MW 550 Da), is the most commonly used noninvasive imaging technique for detection of BBB impairment in both clinical and preclinical studies.

With the exception of brain tumors, where angiogenic biomarkers have been targeted for molecular imaging [53], cerebrovascular biomarkers have not yet been fully exploited for molecular imaging of brain diseases.

Despite this advance the problem to track BBB opening remains for insufficient selectivity, specificity of the tools provided and for the no sufficiently abundancy [52] of the biologics-based molecular imaging probes today available. In this prospective the liquid biopsy could represent an opportunity to track in real time the BBB opening. The concept is based on the causal release of tumor cells in the systemic circulation through the BBB damage. The structural perturbation of the BBB and the consequent modification of its permeability could induce cell spread independently by the molecular equipment expressed by cancer cell. In this direction, the detection of the circulating cancer cells in glioblastoma cases could acquire a specific significance related to the permeability of the BBB more than a prognostic value related to the dissemination phase of the disease (**Figure 2**).

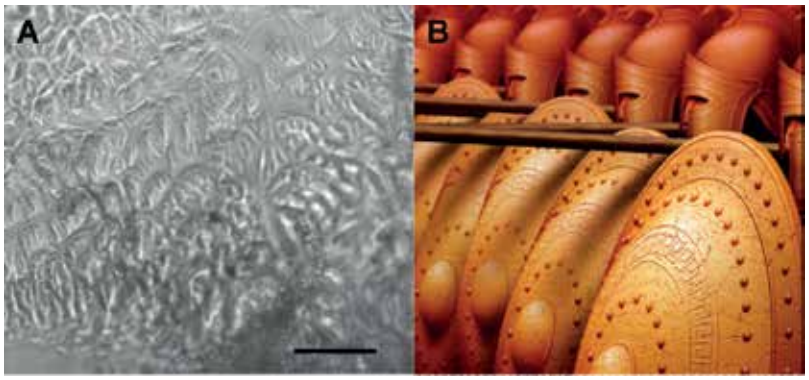

Figure 2. Vessel's arrangement of endothelial cells acting as protective filter (A) mimics an array of warriors (B) placed to protect the tissues. In (A), primary human endothelial culture. Scale bar 100 μm (figure partially modified from Malara et al. IJC Heart & Vasculature [106]).

4. Glioblastoma's angiogenesis

Gliomas are a heterogeneous group of neoplasms derived from glial cells that account for 40–45% of all intracranial tumors. The most malignant type of glioma, Glioblastoma multiforme (GBM), account for approximately 12–15% of all intracranial neoplasms and 50–60% of all astrocytic tumors. In most European and North American countries, incidence is approximately 2–3 new cases per 100,000 people per year. Nearly 260,000 patients worldwide are diagnosed annually with primary malignant brain cancer [54, 55]. The World Health Organization defines GBM as a cancer of grade IV characterized as malignant, mitotically active, and predisposed to cellular necrosis [56].

Untreated patients with glioblastoma multiform uniformly die within 3 months from the diagnosis. Treated patients with a protocol including surgical resection, radiation therapy, and chemotherapy, have a median survival of 14.6 months, with fewer than 25% of patients surviving up to 2 years and fewer than 10% up to 5 years [52, 57, 58]. Moreover, males had a slight preponderance over females, with a male-to-female ratio of 1.6:1. GBM may manifest in persons of any age, but it affects adults preferentially, with a peak incidence at 45–70 years [55]. Glioblastomas are tumors that display extensive morphological and molecular heterogeneity, and thus may reflect their origin from different population of astrocytes, and possibly from oligodendrocytes and ependymal cell lineages. GBM rarely metastasize outside the brain [59]. GBM can be classified into primary type and secondary type, according to whether they are generated de novo or by progression of lower-grade tumors. Histologically, primary and secondary glioblastomas are largely indistinguishable, but they differ in their genetic and epigenetic profiles [60]. GBM is a highly aggressive tumor with distinct histopathological features, including high proliferation, necrosis and considerable neovascularization (i.e., angiogenesis), leading to vessels that exhibit morphological abnormalities and "leakiness" [60]. It is generally accepted that the degree of angiogenesis is correlated to the malignancy of the tumor [58, 61]. GBMs are the most lethal cancer and the most vascularized brain cancer, with

the highest degree of vascular proliferation and endothelial cell hyperplasia [62]. Patients with high tumor microvascular densities exhibit shorter postoperative survival rates than patients with low microvascular densities [63, 64].

Angiogenesis and tumor cell invasion play a critical role in GBM development and growth, even during the earliest phases [65, 66].

Angiogenesis requires three distinct steps: (1) blood vessel breakdown, (2) degradation of the vessel basement membrane and the surrounding extracellular matrix (ECM), and (3) migration of endothelial cells for the formation of new blood vessels. The first step (1) in forming new blood vessels from existing vessels is the dissolution of aspects of native vessels [67].

Tumor angiogenesis results from a balance between pro-angiogenic factors and anti-angiogenic factors with a shift toward angiogenic factors that stimulate uncontrolled and disorganized vascular growth. These molecular factors can be secreted by cancer, endothelial, stromal, and blood cells and by the extracellular matrix [68].

Pro-angiogenic factors include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), acidic fibroblast growth factor, basic fibroblast growth factor (bFGF), placental growth factor, angiopoietin-2 (Ang-2), and interleukins, whereas anti-angiogenic factors include angiostatin, endostatin, thrombospondin 1, and endothelial monocyte-activating polypeptide 2 [69, 70].

The VEGF play a major role in GBM angiogenesis by stimulating capillary sprouting from pre-existing vessels toward VEGF-expressing tumor cells. Tumor VEGF expression and angiogenesis are mainly hypoxia-driven, but can also be promoted by other vascular cytokines and constitutively expressed by genetic tumor mutations [71].

Another mechanism of neo-vascularization includes the recruitment of endothelial progenitor cells (EPCs) that have been proposed to originate from different sources, including bone marrow, the existing vasculature, or adipose tissue [72, 73].

Upon BBB injury, endothelial progenitor cells (EPC) contribute (directly or as a source and carrier of pro-angiogenic factors) to BBB re-endothelialization. EPC are involved to the cerebral angiogenesis associated with physiological conditions (i.e., activity-induced neurogenesis) or pathological conditions (i.e., tumor progression). In adults, there are three origins of EPC:

- **i.** Bone marrow origin (EPC derived from bone marrow multipotent hemangioblasts [VEGFR2(+)VEcadherin(+)CD45(−)
- **ii.** Mesenchymal origin SC) (CD73(+)CD90(+)CD105(+)CD34(−)CD45(−)];
- **iii.** Tissue origin (EPC found at the sites of extensive angiogenesis but demonstrating no signs of hematopoietic origin, being, probably, derived from tissue multipotent cells) [73–75]

In pathological conditions, angiogenesis and vascular remodeling are usually considered as significant components of brain tissue repair program after injury (hypoxic, ischemic, traumatic, inflammatory, toxic, etc.) and the mobilization of EPC from bone marrow correlates to the severity of cerebral alterations [76].

A key challenge in the field of BBB permeability is the current shortage of the knowledge on the most efficient way to cross BBB used by tumor cells. It has been suggested that the brain endothelial cells can actively participate in metastatic progression and stimulate increased BBB permeability.

During vasculogenesis, EPCs are mobilized from the bone marrow by increased concentrations of chemokines, growth factors and other soluble factors in serum, including stromal cell-derived factor-1 (SDF-1), VEGF, and granulocyte-monocyte colony stimulating factor (GM-CSF) [77, 78].

Given the high rate of angiogenesis in glioblastomas and the lack of prognostic markers for anti-VEGF treatments in general, the kinetics and prognostic relevance of circulating endothelial cells was assessed relatively this their potential role. It is clear that molecules with high pro-angiogenic potential provide the recruitment of EPC from bone marrow (VEGF, IL-8, IGF, etc.) [81]. Moreover, EPC provide paracrine signaling to facilitate angiogenesis [79, 80]. It has been suggested that quantification of CECs is useful to identify patients who might benefit from anti-angiogenic treatments [81]. Batchelor et al., in a series of patients with glioblastoma treated with AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, found that viable CEC number increased when tumors escaped treatment [82]. Indeed, CECs, rare in healthy individuals, increase in vascular disorders and tumors due to vascular damage such as the GBM, in which, the VEGF is highly expressed and may mobilize endothelial precursors from the bone marrow [83–85]. During GBM progression, the extracellular matrix (ECM) changes its flexibility and structure (i.e., content of fibrillary proteins as collagen, fibronectin, etc.) and malignant infiltration of glioma may stimulate the development of distinct provisional ECM patterns. Indeed, tumor cells induce changes in ECM by secretion of growth factors and angiogenic factors such as VEGF, bFGF, PDGF, and TNF-α [86]. The combined release of these factors have a synergic effect on the mobilization of EPC [87]. Further studies are needed to assess the role of EPC in liquid biopsy as marker of BBB dysfunction.

5. Microenvironment in glioblastoma

Cytokines play a critical role in contributing to the complexity and lethality of glioblastoma characterized by diffuse invasiveness, immunosuppression, aggressive proliferation, vascularization, and resistance to conventional radiotherapy and chemotherapy. Cytokines consist in small molecules with diverse effects depending on the microenvironment. Cytokines comprise glycoproteins and polypeptides that exert pro-inflammatory, anti-inflammatory or immunosuppressive action. In addition to the tumor cells, immune cells, extracellular matrix, blood vessels, the cytokines are an integral part of the network GBM tumor microenvironment-associated. Glioblastoma arises from astrocytes and their precursors, neural stem cells. The resulting tumor is a heterogeneous cellular population composed of both undifferentiated and differentiated cells and containing subpopulations of tumor cell with different grade of tumorigenic property. Recently, study on GBM progression reveals a feed-forward mechanism with the epidermal growth factor receptor variant III (EGFRvIII) and STAT3 defined by the presence of cytokine receptor OSMR on the surface of non-tumorigenic and tumorigenic brain tumor cells.

In particular, EGFRvIII-OSMR complex signals activates STAT3, and STAT3 signals upregulates OSMR expression. The result is a feed-forward signaling mechanism that drives oncogenesis in GBM. One of principal activator of the JAK-STAT signaling pathway is IL-6. Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates the immune response, but also plays a role in promoting tumor growth and survival [88, 89]. In gliomas, the level of IL-6 gene expression increases with the grade of malignancy. In GBM, the, amplification/overexpression of the IL-6 gene appears to be a common feature [90]. Array studies have reported that the number of IL-6 gene copies was increased in 40–50% of GBM [91, 92]. In ependymoma, with inflammatory phenotype, was described a constitutive activation of the IL6/STAT3 pathway and crosstalk between tumor and immune cells with significant increase in STAT3 and IL8 secretion in tumor microenvironment. Moreover, IL-6 and IL-8, EGF, and other cytokines are involved on the regulation of endothelial function at BBB level, with several potential outcomes: increased permeability, generation of relaying signaling including another cytokine(s) and soluble mediators potentiating the effects of another cytokine(s), modulation of efflux transporters). Even though the BBB within the tumor is considered "permeable," in large parts of gliomas the BBB more closely resembles the intact BBB and prevents efficient passage of cancer therapeutics, such as small molecules and antibodies. Recently, was reported a tumor screening study that combined the detection and analysis of circulating tumor DNA with protein markers comprising some interleukins and the increase of chemokines (IL-6, IL-8) with encouraging results [93]. The big limit in the use of circulating tumor DNA in the screening phase of tumor disease is due to the poor specificity of this biomarker. In fact, mutated circulating DNA may be found in the peripheral blood of healthy subjects, it represents the genetic segments discarded during the normal proliferation of the cells in the body, for example during the hematopoiesis.

6. Circulating biomarkers of glioblastoma

The dissemination of cancer cells in the bloodstream transforms a limited disease in a systemic pathology with remarkable prognostic and therapeutic complications for the patients. The phase of dissemination is a complex plan of action, in which, independently by type of tumor and its location, there are two main actors, the endothelial and the tumor cells. The endothelial cells compose the wall of vessels and capillaries and represent the primary blood-tissue barrier. The adhesion molecules expressed on ECs surface condition the permeability and trans-endothelial resistance to cell crossing [6].

In the brain, the filter function of BBB is carried out by ECs strictly interconnect with numerous tight junctions. The tight junctions give continuity to the endothelial wall eliminating the intercellular space. Astrocyte end-feet, pericytes and microglia interacting with ECs draining fluids in the glymphatic system. Glymphatic system controls the levels of concentration of solutes in the neuronal interstitium and it is connected with the blood circulation [6].

Animal models combined with observations on humans, are still promising tools in order to clarify mechanisms of passage of malignant cells trough the BBB. Indeed, similarly to what is observed in epithelial tumors have been demonstrated by patient-derived GBM xenografts that acquisition of a mesenchymal phenotype is mandatory for malignant cells in order to enter the blood stream and behavior as CTCs [94].

The detection of CTCs in the field of CNS represents a promising noninvasive technique to facilitate early diagnosis and monitoring tumor evolution [95, 96]. The significance of the CTCs detection in intracranial tumors, commonly related to metastatic phase of the cancer disease, was hardly applied for the very few case with extracranial metastases observed although the high malignancy and invasiveness of glioma [97]. More recently, it was reported that disseminating cells of medulloblastoma were able to infiltrate the leptomeninges through hematological way [98]. Garzia et al. described an in vivo experiment consisting in *via surgical* union of two mice by their flanks. The cancer cells of medulloblastoma implanted in one mouse model were able via hematological to give leptomeningeal metastatic disease in the other one mouse. The surgical union shared not only the cells but also the biochemical environment. The biochemical composition of cancer microenvironment consists by a mixture of permeabilizing and inflammatory cytokines. Many types of cytokines like Histamine, TNFa, IL1b and in particular the axis, chemokine CCL2-CCR2 receptor, sustain BBB alterations. Their action plays on BBB by activating cytoskeleton redistribution, downregulating structural proteins and favoring the tight-junction opening [99]. Garzia et al. to obtain leptomeningeal relapsing lesion used an experiment that shared the microenvironment rather than to inject only cancer cells. The tumor cells, although expressing molecular equipment involved in cell movement, were not able, alone, to determine leptomeningeal metastasis. The tumor cytokines mediated BBB-opening and successively favored cellular BBB-crossing.

Moreover, due to the contribution of other studies demonstrating that CTCs can be detected in all pathological subtypes of glioma, irrespective of different malignant degree, now, the reductive misconception of *metastatic CTCs* has been challenged suggesting that CTCs should be considered a common property of glioma lesions [100]. The behavior of the CTCs suggest that the pathogenesis of their release in the blood stream is independent from the grade of malignancy of intracranial tumor, and such as happened in other tumor diseases, this phenomenon happened in each phase of cancer development [101].

The BBB confers to intracranial tumor diseases a different nosology. The presence of CTCs in the bloodstream of patients with intracranial tumors acquires a diverse significance respect to cancers located in extra-cranial sites. Regarding the intracranial cancers could be more appropriate to use CTCs as biomarkers of BBB-damage rather than biomarkers of a preferential/alternative dissemination way. The understanding of this scenario is important to focus the better targeting strategy. The interference on the interaction between cancer-endothelial cells or the reduction of the cytokines levels could be the key targets for pharmacological interventions for the inhibition of cell spread by an intracranial tumor.

Another emerging role in the study of glioblastoma and liquid biopsy are represented by the detection in the peripheral blood of extracellular vesicles (EVs) of tumor origin. EVs are secreted by tumor cells, are mediators of intercellular communication that transfer nucleic acids, proteins and lipids. The liquid biopsy of EVs in patients with GBM has the role to clarify their implications in tumor progression, as a tumor biomarker for tracking GBM progression and as a potential therapeutic target/delivery system [102]. Therapeutic experimental protocols involving EVs delivery of miRNA were performed for GBM. The deletions on chromosome 10 are a common chromosomal alteration found within GBM with leads to a loss of miR-146b normally located at 10q24 [103]. In GBM the loss of miR-146b facilitates migration and invasion. Moreover, in a study using an in vivo model, the exosomes derived

Figure 3. Schematic representations of endothelial-cancer cells interaction. (A) Interstitium space at brain (blood-brain barrier, BBB) and at blood barrier (BB) of other organs. Vessels (red), pericyte (green-cell), astrocyte end-feet (yellow-cell) and cancer cells (blue cells). (B) Cancer cells cross BB through pre-existing inter-endothelial spaces, (C) while at BBB overcome the absence of intercellular spaces realizing permeabilizing cytokines (green balls).

from marrow cells were administered in intracranial rat tumors with consequent reduction of glioma cell invasion, migration, viability and expression of EGFR normally amplified in approximately 40% of all GBM [104]. The possibility of miRNA-based approaches using exosomes have a strong therapeutic potential, which could be achieved through the delivery and transfer agents in those cases of GBM in which the BBB opening is a rare event. In these cases, the liquid biopsy negative for the detection of EPC and CTCs could be useful to select the patients eligible to this type of approach (**Figure 3**).

7. Conclusive remarks

In conclusion, the answers to the question when and why the liquid biopsy is useful in glioblastoma disease, could be different in function of the phase of disease:

1. Diagnostic glioblastoma phase: the liquid biopsy, searching and characterizing the circulating glial cells, could discriminate between glioblastoma and other intracranial lesions, supporting the clinical imaging investigations and in substitution of the stereotactic biopsy procedure when this procedure is at high risk of side effects.

2. Prognostic evaluation and monitoring phase: the prognostic value of the CTCs in glioblastoma patients should be clarified by further studies. Their release in the bloodstream not should be considered as a marker of dissemination and consequently of a bad prognosis. Their detection in the blood is in function of the particular anatomic location of cancer inducing local structural perturbations and only partially dependent by its grade of malignancy. In fact, the release of CTCs in the blood is conditioned by many local factors such as peritumor inflammation reaction, structural changes on the BBB, altered cancer microenvironment, etc. On this way, could be better to analyze the circulating segments of tumor DNA, carrying of hotspot mutations specifically related to glioblastoma, to define prognosis and to monitor during the treatment the emergence of aberrant mutations in advance to predict chemo resistance.

3. Anticancer-treatment design phase: the combining detection of CTCs and EPCs as markers of the opening status of BBB could be useful to individuate the better timing of treatment,

in which therapeutic drugs may be favorite to cross BBB. Moreover, those patients in which the BBB opening is not detectable through liquid biopsy could be considered a different therapeutic approach using EVs.

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Reliable diagnosis is the cornerstone, starting point, and prerequisite of successful treatment. Therefore, development of innovative diagnostic technologies represents a hot topic in medical research.

Liquid biopsy is a novel, minimally invasive laboratory evaluation concept for diagnostic, prognostic, and predictive testing, as well as dynamic monitoring of treatment or disease course. To achieve these goals, a multitude of specific, targeted tests can be performed to detect free nucleic acids, exosomes, microRNAs, tumor-educated platelets, and whole cells of tumor or fetal origin in different biological fluids, including blood, urine, cerebrospinal fluid, and others. Although tissue biopsy has long been considered the gold standard of diagnostics, especially regarding malignant tumors, liquid biopsy has the advantages of a non-invasive approach and thus low risk of complications. It is technically feasible even in serious general status or if tumors or metastases are not easily accessible using conventional tissue biopsy. The testing is fast, exact, and can be repeated to ensure real-time follow-up. In contrast to classic tumor markers, liquid biopsy is distinguished by high specificity at genomic, proteomic, and cellular levels. It is expected to equal and exceed the diagnostic value of tissue biopsy. The field of liquid biopsies is developing rapidly regarding the selection of targets, technological improvements, and quality assessment.

This book, written by a global team of recognized scientists, comprises state-of-the-art reviews on the current knowledge and advances in the technologies and software for liquid biopsy. Examples of practical application of liquid biopsy to evaluate thyroid cancer, multiple myeloma, etc. are discussed as well. The book is intended to serve as a reference for scientists and clinicians interested in the development and practical implementation of liquid biopsy.

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