Biomarkers in Cancer Therapy

Liquid Biopsy Comes of Age

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Preface

Despite improvements in surgical techniques, chemotherapy and/or radiotherapy, many patients with advanced cancers still suffer rapid recurrence of the disease and a poor outcome. Serum cancer biomarkers are useful tools to evaluate the biological features of cancer cells to predict recurrence, treatment response and patients' survival. Based on the technical development of molecular analyses, various cancer biomarkers, molecular biomarkers and liquid biopsy have been applied for practice. In this book, recent developments in the field of cancer biomarkers and liquid biopsy are concisely reviewed. Clinical significance of serum extracellular vesicles, cell-free DNA, autoantibodies, angiogenic factors and cancer stem cells has been reviewed on various types of malignant tumors. These serum biomarkers have been developed for improving the quality of early diagnosis, monitoring the tumors, predicting treatment response and predicting patients' survival. Thus, several serum biomarkers have potentially become the new molecular targets in cancer treatment.

Tokyo, Japan — Hideaki Shimada

Contents

Part I

Various Strategies to Detect Cancer

Liquid Biopsy Diagnostics Using Extracellular Vesicles

1

Makoto Sumazaki and Koji Ueda

Abstract

Extracellular vesicles (EVs) are membrane-surrounded structures secreted by cells, which involve exosomes, microvesicles, apoptotic bodies, and many others. Recent studies indicated that cancer-associated EVs play pivotal roles in constructing favorable microenvironments for cancer cells through communication with various surrounding or remote cells. In fact, they induce immunosuppression, angiogenesis, and epithelial-mesenchymal transition (EMT) via transport of functional nucleic acids, proteins, and metabolites. *They also transport tumor-associated antigens to antigen-presenting cells*. Since the cargoes of cancer-derived EVs retain the molecular properties of their sources and cancer cells actively release EVs into bodily fluids that are easy to access, EVs are considered to be attractive resources for cancer biomarker development. In the following chapter, we describe the biology of EVs, as well as methods and issues relevant for purifying EVs and measuring EV biomarkers. We also provide an overview of reported EV biomarker molecules and discuss the feasibility of EV-based cancer liquid biopsy.

Keywords

Extracellular vesicles · Exosome · Cancer · Liquid biopsy · Liquid biopsy Protein Tumor-associated antigen · Proteome

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1.1 Introduction

Each year, approximately 14 million people are diagnosed with cancer worldwide. The development of cancer biomarkers as diagnostic tools for the screening or management of cancers may play a crucial role in reducing cancer-related mortality. Indeed, early detection is one of the most effective ways to reduce cancer death rates. Early detection may be accomplished by screening the general population using blood-based biomarkers. Liquid biopsy using blood-based biomarkers may also enable patient-specific targeted therapy or the monitoring of drug resistance acquisition by tumor cells [1]. Circulating tumor cells (CTCs) and cell-free DNA (cfDNA) are promising materials for liquid biopsy; however, several issues remain to be solved, particularly regarding sensitivity [2].

EVs are membranous vesicles released by any types of cells in our body. The functions of EVs include maintaining cellular homeostasis and presenting intracellular antigens [3, 4]. Multiple kinds of stresses, such as hypoxia, pH alteration, oxidative stress, shear stress, or radiation, trigger secretion of EVs [5]. Cancer cells actively secrete EVs even during the early phase of the disease [6]. EVs contain biomolecular cargoes that are protected from degradative enzymes in body fluids [7, 8]. For example, cfDNAs are intensively degraded in blood, which are typically detected as short fragments with less than 100 bp [8]. In contrast, DNA fragments in EVs range from 100 bp to 17 kb in size, indicating that wider range of genetic information would be accessible in EV-DNAs [9]. Protection of RNA cargoes from RNase in biofluids also significantly enhances the depth of transcriptomic analysis by next-generation sequencers [10]. In addition, EV cargoes directly reflect the characteristics of their sources. Indeed, KRAS, TP53, NOTCH1, and BRCA2 mutations were detected in biofluid EVs from patients with pancreatic cancer and ampullary cancer [10]. Cancer-associated EVs also contain cancer-specific protein cargoes. NYO-ESO-1 and TP53 were detected in EVs from cancer patients' sera, which could be effective targets for cancer liquid biopsy [11].

Recent studies of EVs as cancer biomarkers have mainly focused on nucleic acids and proteins. Reports indicated that the diagnostic performance of nucleic acids is excellent, but several major problems remain unresolved. These issues fundamentally come from unfixed EV purification methods. On the other hand, reports on EV proteins are accumulating at a steady rate. These EV proteins are now cataloged in public databases and can be utilized in meta-analyses [12]. For detection technologies, enzyme-linked immunosorbent assay (ELISA), EV microarray, and ExoScreen are widely used in measurements of EV protein biomarkers [13, 14]. These methods have a significant advantage in clinical application of EV protein biomarkers because EV protein concentrations can be directly measured without EV purification, allowing quick, low-cost, and reproducible tests. In this chapter, the reported EV protein biomarkers (Table 1.1) are overviewed, followed by consideration of clinical values for EV-based liquid biopsy (Table 1.2).

Table 1.1 Reported EV protein biomarkers for cancer diagnosis

Type of cancer	Author, year	Number of samples	Type of assay	Target
NSCLC	Wang, 2018	153	ELISA	Lipopolysaccharide-binding proteins
	Sandfeld, 2016	276	EV array	NY-ESO-1
	Ueda, 2014	178	ELISA	CD91
	Yamashita, 2013	9	ELISA	EGFR
Breast cancer	Moon, 2016	169	ELISA	DEL-1
	Lee, 2018	111		
	Toth, 2008	66	FCM	CD45+ LMP
	Salma, 2014	50	ELISA and WB	Survivin, survivin-2B
	Kibria, 2016	120	Micro FCM	CD47
Colorectal cancer	Yoshioka, 2014	385	ExoScreen	CD147
Renal cell carcinoma	Jingushi, 2017	29	LC/MS	AZU1
Melanoma	Peinado, 2012	36	WB	TYRP2

NSCLC non-small cell lung cancer, *EGFR* epidermal growth factor receptor, *DEL-1* developmental endothelial locus-1, *FCM* flow cytometry, *LMP* leukocyte-derived microparticles, *WB* western blotting, *LC/MS* liquid chromatography-mass spectrometry, *AZU1* azurocidin, *TYRP2* tyrosinase-related protein 2

Table 1.2 Studies about EVs liquid biopsy

Type of cancer	Author, year	Target	Utility of liquid biopsy
NSCLC	Lino, 2018	BRAF, EGFR, and KRAS mutations in exoNA	NGS of plasma exoNA for common BRAF, KRAS, and EGFR mutations has high sensitivity compared with clinical testing of tumor and plasma cfDNA
	Elena, 2018	EGFR T790M mutation	The combination of exoRNA/DNA and cfDNA for T790M detection has higher sensitivity and specificity compared with historical cohorts using cfDNA alone
Breast cancer	Yang, 2017	GSTP1 mRNA expression	GSTP1 mRNA containing exosomes predicted clinical outcome of breast cancer with anthracycline/taxane-based chemotherapy
	Wang, 2017	Exosome-carrying TRPC5	Exo-TRPC5 level predicted acquired chemoresistance to anthracycline/taxane-based chemotherapy
	Fang, 2017	Exosomal HER2	Exosomal HER2 expression levels were almost consistent with that in tumor tissue expression
Pancreatic cancer	Allenson, 2017	Mutant KRAS in exoDNA	Mutant KRAS exoDNA was detected in 43.6% of early-stage PDAC patients
Prostate cancer	Kharaziha, 2015	MDR-1, MDR-3, endophilin-A2, and PABP4	Comparative proteomics analysis of exosomes secreted from cell lines showed the candidates of biomarkers for response to docetaxel therapy

NSCLC non-small cell lung cancer, *NGS* next-generation sequences, *EGFR* epidermal growth factor receptor, *BRAF* v-raf murine sarcoma viral oncogene homolog B, *KRAS* Kirsten rat sarcoma viral oncogene, *exoNA* exosomal nucleic acids, *cfDNA* cell-free DNA, *GSTP1* glutathione S-transferase P, *TRPC5* short transient receptor potential channel 5, *HER2* human epidermal growth factor receptor 2, *MDR* multidrug resistance, *PABP4* poly(A)-binding protein

1.2 General Remarks on EVs as Biomarkers

1.2.1 Molecular Characteristics of EVs

EVs are classified into three main groups according to biogenesis processes and particle sizes. (i) Exosomes are 30–200 nm in size and formed as intraluminal vesicles (ILVs) within the lumen of multivesicular bodies (MVBs). MVBs fuse with the plasma membrane to release ILVs. Exosomes from cancer cells contain tumor-associated antigens, and these antigens can transform dendritic cells [15]. (ii) Microvesicles, which are 100–1000 nm in diameter, are formed when cell membranes are partially pinched off and released directly from the originating cells. (iii) Apoptotic bodies are relatively large particles (500–2000 nm) and formed during the late stage of apoptosis. Smaller types of apoptotic bodies (approximately 500 nm in diameter) also contain intracellular antigens [16]. EV subtypes have different cargo profiles, suggesting that their roles may also differ [17].

Members of tetraspanin family (CD9, CD63, and CD81) are generally used as exosome markers. However, these factors are also expressed in apoptotic bodies and microvesicles. These proteins are also expressed on the cell surface and are thus expressed in other types of EVs that are generated by direct budding from the plasma membrane. Different reports have shown that CD9, CD63, and CD81 are abundant not only in exosomes but also in microvesicles or apoptotic bodies; thus, additional factors are needed to discriminate among these different types of EVs [18].

1.2.2 Purification and Detection Methods for EV Biomarkers

Measurement of nucleic acids, such as RNA or DNA, in EVs needs to be accompanied with appropriate EV purification steps. An issue that arises is that the obtained EV population differs according to the used purification methods [19]. The available EV purification methods, such as ultracentrifugation, chromatography, antibody-based capture, and the microfluidic system have advantages and disadvantages. Thus, it is necessary to optimize the method that yields the highest purity of the target population [18]. Proteome analysis plays a vital role in the identification of biomarkers among protein targets. A new analytical strategy has been developed for proteome analysis. This technique uses EVs directly secreted from surgically resected fresh tissues as the subject rather than EVs obtained from patient blood samples or cultured cell lines. This method enabled to deal with high-purity organ-specific EVs and allowed to in-depth OMICS-wide analysis of EVs [19]. For measurement of protein targets expressed on EVs, immunoassays are often employed using a pair of antibodies. After capturing EVs by an anti-tetraspanin antibody, such as an anti-CD9, anti-CD63, or anti-CD81 antibody, a target-specific antibody can detect the targeted EV surface biomarker protein (EV sandwich ELISA). This method enables detection of biomarkers expressed on EVs directly from body fluid samples in a high-throughput manner without any purification processes. This point is a great advantage for clinical application [20].

1.2.3 EV Protein Biomarkers and Liquid Biopsy for Cancer Diagnosis

EV biomarker studies have been accumulated for lung cancer. Ueda et al. [21] isolated exosomes from the serum of lung cancer patients, conducted proteome analysis, and identified 1369 proteins. The investigators successfully distinguished between lung adenocarcinoma patients and controls (healthy donors and benign lung diseases) using Exo-CD91 antigen. Jakobsen et al. [22] and Sandfeld-Paulsen et al. [11] analyzed protein biomarkers in plasma samples from lung cancer patients using EV microarrays and reported an association between NY-ESO-1 and prognosis. As a liquid biopsy of lung cancer, Mohrmann et al. screened nucleic acids in exosomes (exoNA) and detected driver mutations of BRAFV600, KRASG12/G13, and EGFRexon19del/L858R. These mutations were detected more sensitively in exoNA than in cfDNA [23]. Additionally, Castellanos-Rizaldos et al. reported successful detection of EGFR T790M mutation in exoNA, which is responsible for EGFR TKI drug resistance [24].

DEL-1, which is identified via LC-MS/MS analysis of serum from breast cancer patients, was confirmed to have clinical value in a retrospective study using ELISA. The detection of DEL-1 on circulating EVs facilitated early-stage breast cancer diagnosis and discrimination of breast cancer from benign breast disease [25]. Salma et al. suggested that EV-survivin may be useful in breast cancer diagnosis [26]. Kibria et al. suggested that EV-CD47 may be a possible breast cancer biomarker [27]. Toth et al. compared blood samples from breast cancer patients and healthy subjects using fluorescence-activated cell sorting (FACS). They reported that CD45+ leukocyte-derived microparticles had levels of sensitivity and specificity similar to those of the existing biomarker, CA15-3 [28]. GSTP1 and TRPC5 may be useful as negative predictive factors for anthracycline/taxane-based chemotherapy regimens in breast cancer patients [29, 30]. Fang et al. reported that HER2-positive exosomes in plasma from breast cancer patients correlate positively with HER2 expression in breast cancer tissue [31].

Yoshioka et al. reported the utility of ExoScreen and CD147 as EV protein biomarkers for colorectal cancer [14]. Allenson et al. reported the detection of mutated KRAS in exoDNA from early-stage pancreatic cancer patients [32].

Jingushi et al. identified AZU1 as a biomarker candidate via proteome analysis of renal cell carcinoma tissue-exudative EVs [33]. Mass spectrometric analysis of tumor-derived exosomes identified TYRP2, VLA-4, and HSP70 as biomarker candidates [34]. Kharaziha et al. conducted proteome analysis of prostate cancer tumor cell-derived exosomes to identify predictive factors for docetaxel therapy. Their results suggested that MDR-1, MDR-3, endophilin-A2, and PABP4 may be biomarker candidates [35].

1.3 Summary

EVs are attractive resources for cancer liquid biopsies. Some reports already showed favorable results in development of EV-based cancer diagnosis technologies. However, there are still great challenges for clinical application including

standardization of methods for EV purification and an incomplete understanding of the characteristics and molecular composition of EVs. With overcoming these issues, screening the general population with EV biomarker may achieve early detection of cancer and reduce cancer death rates.

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Cell-Free DNA

2

Hiroyuki Yamamoto, Yoshiyuki Watanabe, and Fumio Itoh

Abstract

Cell-free DNA (cfDNA) found in the plasma or serum is derived from multiple sources, including tumor cells. The fraction of cfDNA derived from tumor cells is termed circulating tumor DNA (ctDNA). Despite the low levels of ctDNA in normal cfDNA, advances in digital polymerase chain reaction (PCR) and next-generation sequencing (NGS) technologies have led to significant improvements in the sensitivity and specificity of variant detection. As a result, the literature regarding ctDNA assays has been rapidly increasing. The genomic profiles of ctDNA have been shown to closely match those of the corresponding tumors, which has important implications for both molecular pathology and clinical oncology. Noninvasive diagnostic techniques using ctDNA have the potential to innovate new prognostic factors and direct treatment intervention. Analyses of ctDNA, commonly referred to as "liquid biopsies," can be used for treatment selection, treatment outcome monitoring, residual disease detection, cancer screening in asymptomatic individuals, and tumor dynamics monitoring. In this chapter, we summarize how different ctDNA assays can be used to guide patient care and should be integrated into the clinical practice.

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Keywords
Circulating tumor DNA · Liquid biopsies · Next-generation sequencing · Tumor dynamics · Clonal hematopoiesis

2.1 Structure of cfDNA

Tumor-derived somatic alterations in DNA are detectable in the plasma of patients with cancer in the form of cell-free DNA (cfDNA; Fig. 2.1) [1–5]. This circulating tumor-derived DNA (ctDNA) may be shed from tumors via apoptosis or necrosis. Using quantitative PCR analysis, it has been shown that the fragmentation and concentration of ctDNA are positively correlated with tumor weight [6]. Moreover, patients with metastatic colorectal cancer (CRC) have fivefold higher mean ctDNA fragmentation than healthy individuals.

The short half-life of ctDNA may provide an opportunity for noninvasive detection and diagnosis of solid tumors, evaluation of response to therapy, and monitoring recurrence (Fig. 2.2) [7]. Because ctDNA is reliably shorter than normal cfDNA, size selection for shorter cfDNA fragments can effectively increase the fraction of ctDNA.

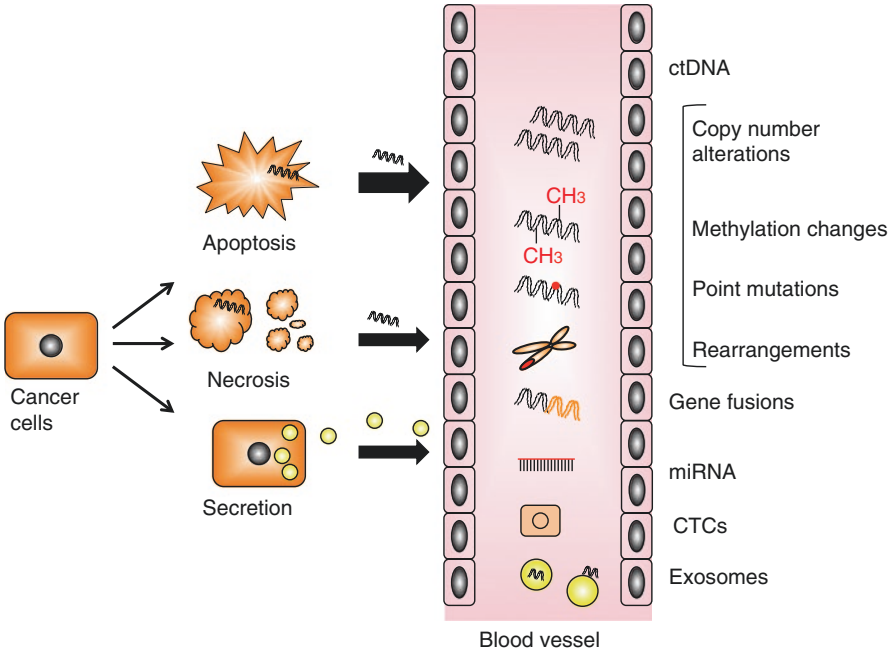

Fig. 2.1 Origins and range of alterations in liquid biopsies. *miRNA* microRNA, *CTCs* circulating tumor cells

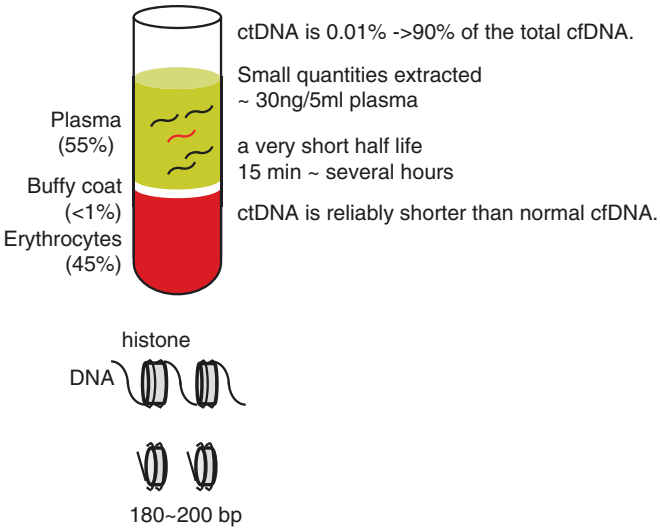

Fig. 2.2 Characteristics of cfDNA and ctDNA. Bottom, cfDNA predominantly consists of nucleosome-protected DNA shed into the bloodstream by cells undergoing apoptosis

2.2 Identification of the Origin of ctDNA

Plasma contains DNA released from various tissues within the body. Using genome-wide bisulfite sequencing of plasma DNA, Sun et al. [8] analyzed the major tissue contributors to the cfDNA pool. In most cases, white blood cells were found to be the predominant contributors; however, in patients with hepatocellular carcinoma, contributions from the liver were found to be increased. In these patients, comparison of methylation results using genomic regions with a different copy number status was then used to determine the tissue type responsible for the aberration.

Lehmann-Werma et al. [9] developed a method to detect tissue-specific cell death based on tissue-specific methylation patterns in cfDNA. They quantified cfDNA carrying the methylation markers of the cell type of interest, e.g., exocrine pancreatic DNA was identified in patients with pancreatic cancer or pancreatitis. These tissue-specific methylation patterns were then used to determine the origin of cfDNA.

The tissue origins of cfDNA can also be determined by nucleosome occupancies because nucleosome positioning varies between cell types. By deep sequencing cfDNA, Snyder et al. [10] generated genome-wide maps of in vivo nucleosome occupancy and found that short cfDNA fragments harbor footprints of transcription factors. Because cfDNA nucleosome occupancies correlate well with the nuclear architecture and gene structure and expression, nucleosome occupancies can be used to identify the cell type of the origin of cfDNA.

cfDNA predominantly consists of nucleosome-protected DNA shed into the bloodstream by cells undergoing apoptosis [11]. Using whole-genome sequencing

of plasma DNA, Ulz et al. identified two discrete regions at transcription start sites at which nucleosome occupancy leads to different read depth coverage patterns for expressed versus silent genes. Thus, nucleosome occupancies can be used to classify expressed cancer driver genes in regions with somatic copy number gains, providing functional information about the cells releasing their DNA into circulation.

2.3 cfDNA as Mobile Genetic Elements

It has been reported that cfDNA can induce DNA damage and mutagenesis by integrating into host cell genomes, thereby acting as mobile genetic elements (Fig. 2.3) [12]. Cell-free chromatin (cfCh) is consistently more active than cfDNA, whereas cfDNA originating from patients with cancer is significantly more active than that originating from healthy volunteers.

cfCh released from dying cancer cells is reportedly a key mediator of both DNA damage and inflammation in adjacent normal cells [13]. It can transform cells in the

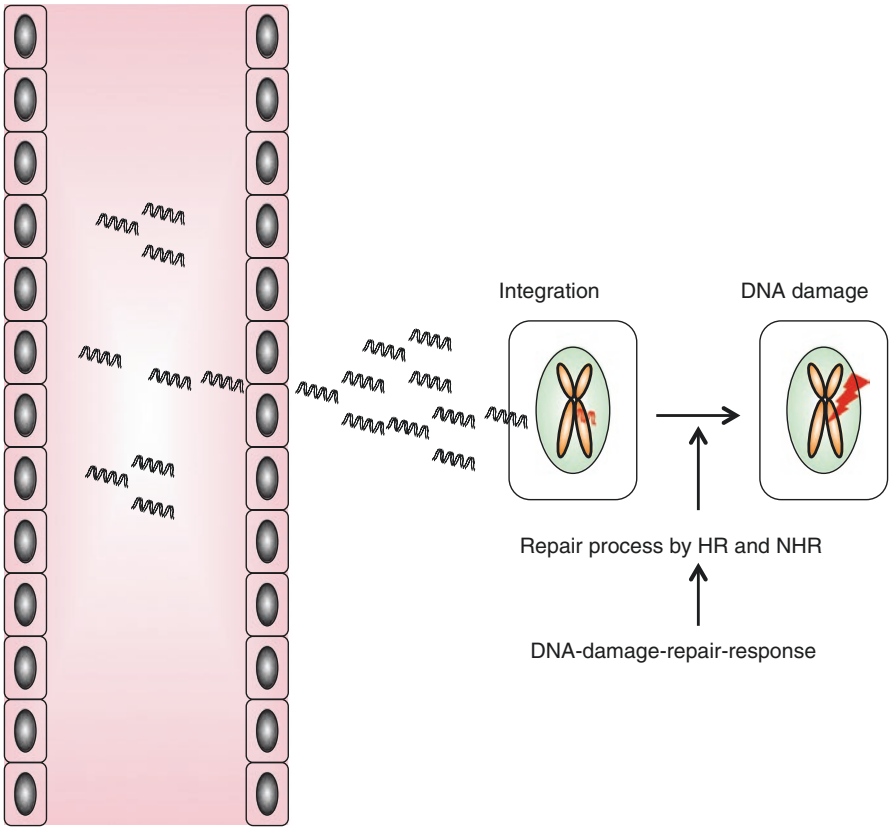

Fig. 2.3 Proposed model of DNA mutagenesis by cfDNA

local microenvironment and those in distant organs, providing a novel mechanism of tumor invasion and metastasis. Importantly, cfCh activity can be abolished by concomitant treatment with chromatin neutralizing/degrading agents, highlighting the therapeutic potential of such agents.

2.4 Cancer Genome Diversity and Cancer Clonal Evolution

Intratumor genetic and functional heterogeneity is acknowledged as a driver of cancer progression [14]. Thus, early detection of relapse following primary cancer therapy or the classification of scattered subclonal metastases may provide novel therapeutic approaches for suppressing tumor recurrence [15]. The potential to use ctDNA for noninvasively tracking tumor evolutionary dynamics in early-stage lung cancer has already been demonstrated [15]. By tracking the subclonal nature of lung cancer relapse and metastases, phylogenetic ctDNA profiling provides a new approach for ctDNA-driven therapeutic intervention.

2.5 ctDNA Assays in the Clinical Setting

Analysis of ctDNA in plasma is a rapidly advancing clinical technique (Fig. 2.4) [11]. ctDNA assays can be used for treatment selection, treatment effect monitoring, residual disease detection, cancer screening in asymptomatic individuals, and tumor dynamics monitoring (Fig. 2.5).

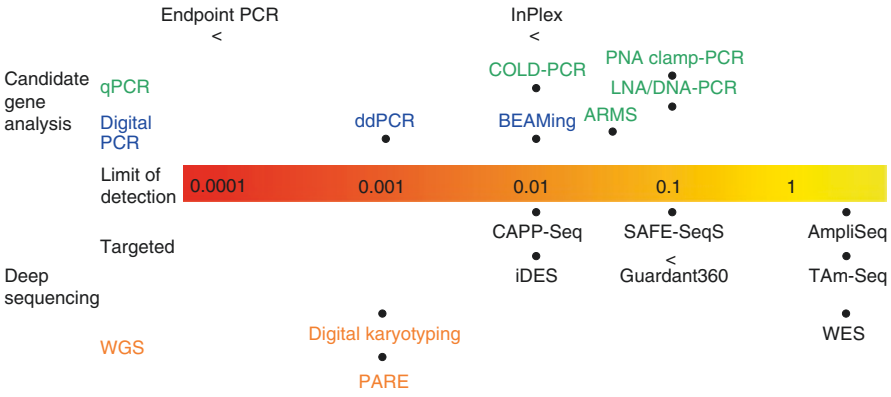

Fig. 2.4 Technologies for ctDNA analysis. *ARMS* amplification refractory mutation system, *BEAMing* beads, emulsion, amplification, magnetics, *CAPP-Seq* cancer personalized profiling by deep sequencing, *COLD-PCR* complete enrichment coamplification at lower denaturation temperature PCR, *ddPCR* droplet digital PCR, *EMA* European Medicines Agency, *FDA* US Food and Drug Administration, *iDES* integrated digital error suppression, *LNA/DNA-PCR* locked nucleic acids/DNA chimera PCR, *PARE* parallel analysis of RNA ends, *PNA clamp-PCR* peptide nucleic acids clamp PCR, *qPCR* quantitative PCR, *SAFE-SeqS* safe-sequencing system, *TAm-Seq* tagged-amplicon deep sequencing, *WES* whole-exome sequencing, *WGS* whole-genome sequencing

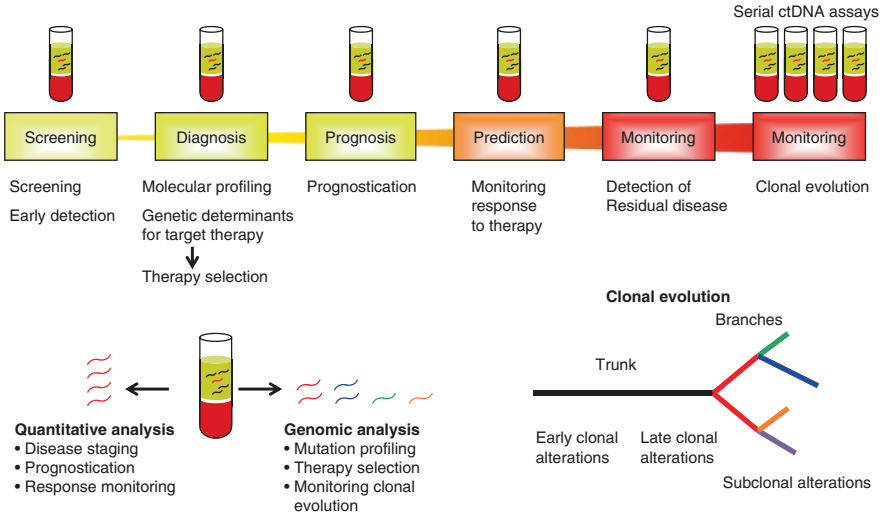

Fig. 2.5 Applications of ctDNA analysis during the course of disease management. Left bottom, the information obtained by ctDNA analysis can be classified as quantitative information relating to tumor burden or genomic information

2.5.1 ctDNA Assays for Cancer Treatment Selection

The clinical validity of ctDNA assays has been demonstrated for the epidermal growth factor receptor (*EGFR*) in non-small cell lung cancer (NSCLC) [16, 17] and *KRAS* in CRC [18, 19]. Clinical validity analysis for next-generation sequencing (NGS)-based panel ctDNA assays reported similar overall concordance with tissue-based genotyping using polymerase chain reaction (PCR)-based assays, although concordance may be reduced for variants at low variant allele fractions (VAFs) (<1%) [20]. Furthermore, ctDNA analysis detected alterations in most patients (82%) with NSCLC, with potentially actionable alterations (61% of which were approved by US Food and Drug Administration) found at the expected frequencies. Therapy matched to ctDNA alterations has shown considerable efficiency, further supporting the clinical utility of ctDNA assays.

2.5.2 ctDNA Assays for Noninvasive Monitoring of Treatment Effects

ctDNA-based methods can be used to monitor the response to cancer treatment, e.g., through quantitative measurement of ctDNA over time. However, validating the ability of ctDNA assay to quantify the tumor burden is technically challenging. The somatic VAF, ratio of somatic variants to nonvariant ctDNA, and detected somatic variant events per unit of plasma have all been used to quantify the tumor burden [21, 22]. Correlations between changes in ctDNA levels and tumor responses/

outcomes have been reported for various cancer types, including lung cancer [23–25], CRC [26, 27], breast cancer [28, 29], lymphoma [30, 31], and melanoma [32]. Studies of multiple cancer types have indicated that ctDNA analysis can identify resistant mutations months before standard radiological methods can [27, 33, 34], thus providing an opportunity to verify whether changing treatment before clinical progression could improve prognosis [35]. However, there is currently no definitive evidence supporting a change in treatment at the time of ctDNA progression.

2.5.3 ctDNA Assays for Detecting Residual Disease

Because ctDNA is generally detected at a lower rate in early-stage cancer than in advanced cancer [36–38], ctDNA assays may be used to detect and monitor residual tumors following curative therapy. Persistent detection of ctDNA after local therapy (surgery or radical radiotherapy) has been shown to predict a higher risk of relapse in CRC [21, 22, 39], breast cancer [40, 41], pancreatic cancer [42], and lung cancer [15].

2.5.4 ctDNA Assays for Cancer Screening in Asymptomatic Individuals

Because ctDNA has been detected in some patients diagnosed with early-stage cancer, there is intense interest in the use of ctDNA for cancer screening in asymptomatic individuals. However, the application of ctDNA assays for early tumor detection remains challenging [43]. Highly sensitive screening methods for cancer-associated mutations may produce false-positive results because with increasing age, individuals who will not develop cancer will nevertheless develop cancer-associated mutations.

The cfDNA assay for Epstein-Barr virus (EBV) DNA has been shown to detect early-stage nasopharyngeal cancers with a positive predictive value of 11% [44]. The sensitivity and specificity of EBV DNA detection in plasma samples were 97.1% and 98.6%, respectively. Among subjects with nasopharyngeal cancer, screening resulted in earlier detection and better outcomes than those obtained in a historical cohort. While further research is needed, this study highlights the enormous potential of ctDNA analysis for cancer screening [45].

2.5.5 ctDNA Assays for Monitoring Tumor Dynamics

ctDNA analysis can be used to monitor tumor dynamics in patients with cancer who are undergoing surgery or chemotherapy (Figs. 2.5 and 2.6) [21]. For example, ctDNA has been used to genotype CRCs and track clonal evolution during treatment with the EGFR antibody cetuximab or panitumumab [27]. Mutations in various genes, including *KRAS*, were detected in the ctDNA of patients with primary or acquired resistance to the EGFR blockade. Mutated *KRAS* clones

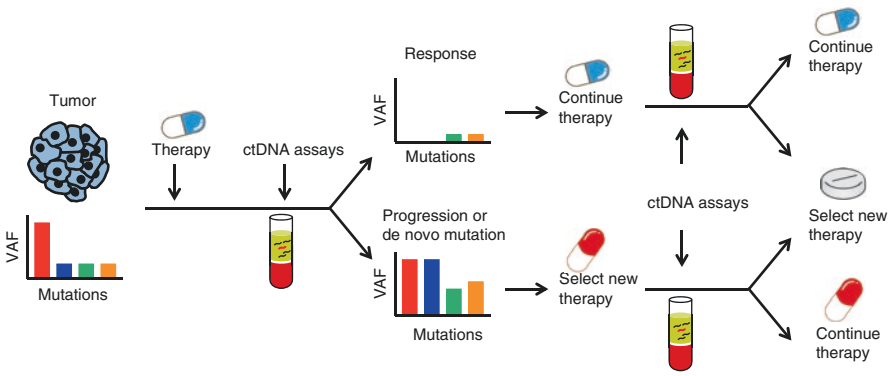

Fig. 2.6 Adaptive monitoring and treatment paradigms using ctDNA assays. The adaptive monitoring and treatment would be facilitated by a fast turnaround time for ctDNA assays, e.g., through the use of point-of-care diagnostics

developing in the blood during EGFR blockade were decreased upon the removal of EGFR antibodies, and the patients regained drug sensitivity. These findings indicate that clonal evolution continues beyond clinical progression and that the CRC genome dynamically adjusts to intermittent drug schedules, providing a molecular explanation for the efficiency of rechallenge therapies based on the EGFR blockade.

Clinical blockade of oncogenes is efficient but only transiently. Therefore, ctDNA-based monitoring of clonal evolution in patients with cancer could be used to develop therapies which will evolve over time, potentially leading to better therapeutic response and survival [46]. In one such application, ctDNA was used to detect *RAS*/*BRAF* mutations in the plasma of 42 patients with metastatic CRC in a phase Ib/II trial of FOLFOX and dasatinib with or without cetuximab [47]. Prior to treatment, sequencing of archival tumor tissue detected mutations in 25 (60%) of 42 patients. However, ctDNA assay detected mutations in 37 (88%) of 42 patients with allele frequencies as low as 0.01%. After treatment, 41 (98%) of 42 patients had *RAS*/*BRAF* mutations detected by the ctDNA assay. These results highlight the importance of obtaining ctDNA-based quantitative data beyond the mere presence/absence of a mutation, regardless of whether patients had preexisting mutations.

In a prospective study, 457 plasma samples were collected from 85 patients with metastatic CRC undergoing chemotherapy [48]. Droplet digital PCR was used to detect *KRAS*-mutated ctDNA in the plasma, and the percentage of *KRAS*-mutated ctDNA in total cfDNA was then calculated. Dynamic changes in *KRAS*-mutated ctDNA were identified as continuous, intermittent, and transient (quick elevation followed by disappearance). Because monitoring *KRAS*-mutated ctDNA during various treatments could detect dynamic changes in *KRAS* status, this method could provide useful information for determining treatment strategy for patients with metastatic CRC.

2.5.6 NGS-Based ctDNA Assays

The Guardant360 is a clinical cfDNA-based NGS platform capable of detecting point mutations, select copy number gains, fusions, insertions, and deletions (Fig. 2.4). It was used to identify potential tumor-related genomic alterations via complete exon sequencing of 73 cancer-related genes in ctDNA extracted from plasma [49]. The Guardant360-based studies on lung cancer and other solid tumors of different tissue origins [49], urothelial cancer [50, 51], breast cancer [52], NSCLC [53, 54], and prostate cancer [55] all support the use of ctDNA profiling as a less invasive approach to monitor cancer progression and select the appropriate drugs during cancer evolution.

2.6 False-Positive Plasma Genotyping Due to Clonal Hematopoiesis

Age-related clonal hematopoiesis (CH), also referred to as clonal hematopoiesis of indeterminate potential, is characterized by recurring somatic variants, which are most commonly associated with hematologic malignancies and mortality, in peripheral blood cells (PBCs) [56–58]. Because most cfDNAs originate from PBCs, CH may give false-positive results during plasma genotyping. The most frequently mutated genes include *DNMT3A*, *TET2*, *ASXL1*, *JAK2*, *TP53*, *GNAS*, *PPM1D*, *BCORL1*, and *SF3B1*. Thus, mutations detected in the plasma, especially in the abovementioned genes, may not represent true tumor genotypes. Although CH is rare in individuals aged <40–50 years, paired PBC genotyping is recommended to avoid misdiagnosis of CH-derived mutations as occult malignancy [59].

2.7 Conclusion

Growing evidence supports that ctDNA assays can serve as less invasive, real-time surrogates for cancer therapeutic tailoring, resistance monitoring, prognosis prediction, and early diagnosis. Furthermore, these assays can reduce biopsy sampling errors related to intra- or intertumor heterogeneity. Because these "liquid biopsies" have demonstrated utility across a range of applications and cancers, their use for clinical benefit has now been realized.

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Autoantibody in Cancer

3

Takaki Hiwasa and Hideaki Shimada

Abstract

An increasing number of autoantibody markers have been reported. These markers are highly sensitive and useful for the diagnosis of early-stage cancer. Anti-p53 autoantibody marker has been applied clinically to cancer diagnosis. Identification of NY-ESO-1 autoantibody in patients with esophageal squamous cell carcinoma (ESCC) has led to cancer immunotherapy. A comparison of the autoantibody markers for ESCC with those for colorectal cancer (CRC) suggests characteristics of each cancer type. Although both of the cancers involve p53 and MYC, ESCC may be driven by the cell cycle progression, whereas CRC appears to depend on intracellular signaling and suppression of apoptosis. An autoantibody analysis has been applied to other diseases, such as atherosclerosis, and has led to identification of some autoantibodies as common markers between cancer and atherosclerotic diseases. Thus, autoantibody analysis is useful not only for the diagnosis of many early-stage diseases but also for the comprehensive interpretation of healthy or disease conditions affecting the whole body.

Keywords

Autoantibody · Biomarker · Esophageal squamous cell carcinoma · Colorectal cancer

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3.1 Autoantibodies Are Sensitive and Stable Biomarkers

To detect the early symptoms of disease, many biomarkers have been studied, including enzymes, antigens, antibodies, DNA, and RNA, among which proteins, such as enzymes, antigens, and antibodies, are stable and the most frequently used. If the target proteins have no enzymatic activity, enzyme biomarkers are not useful. Fine local tissue destruction may cause intracellular proteins to leak out, which then enter systematic circulation [1].When the proteins leak out, antibodies develop. Recent technological progress has revealed that most self-proteins can serve as antigens, against which autoantibodies are developed [2]. Both antigens and antibodies appear at very low concentrations and are rapidly degraded after a single round of tissue destruction. Thus, it may be difficult to detect the levels of these factors. However, repeated tissue destruction and repeated antigen leaking out during the course of disease progression could lead to a tremendous increase in autoantibody levels while maintaining low levels of antigens. Therefore, antibody markers are thought to be much more sensitive than antigen markers.

3.2 SEREX Method to Identify Autoantigens

Sahin et al. introduced an expression cloning method to identify the antigens recognized by serum immunoglobulin (Ig) G from patients with cancer [3]. First, a cDNA library is constructed by using cancer cells and inserted into λZAP II or Uni-ZAP phages. *Escherichia coli* is infected with a phage cDNA library and plated onto agar plates. After the induction of cDNA expression, the expressed protein products are blotted onto nitrocellulose membranes, which are then reacted with serum IgG followed by detection using alkaline phosphatase-conjugated secondary antibodies and a substrate. Each monoclonalized phage clone contains one cDNA only, which can be recombined into plasmids easily. Sequencing of the cDNA insert followed by a BLAST search identified the gene name. Thus, SEREX is a comprehensive and high-throughput method for screening of autoantigens.

Even if the obtained SEREX clone does not contain a full-length cDNA, there is no doubt that the clone contains the epitope site recognized by serum antibodies. Therefore, the SEREX clones are available for the preparation of antigenic recombinant gene products, which can then be used as antigens to examine the antibody levels.

3.3 Method to Examine Serum Antibody Levels

It is necessary to compare the serum autoantibody levels between patients and controls after the selection of candidate autoantigens. Enzyme-linked immunosorbent assay is widely used to examine antibody levels. First, antigenic proteins are

immobilized in the wells of a 96-well microtiter plate through hydrophobic bonding between the proteins and polycarbonate plates. The plate is incubated, washed, and then blocked with blocking reagents, such as albumin and fetal calf serum. The plate is incubated and washed, and sera are added. After washing the plates, the bound IgG antibodies are detected by incubation with horseradish peroxidase-conjugated antihuman IgG antibody followed by addition of a peroxidase substrate. The absorbance of each well is measured by a microplate reader.

Although this is an established method, each individual contains a variety of IgGs, some of which may have more potent hydrophobicity than albumin or fetal calf serum proteins. Such potently hydrophobic IgGs can bind directly to the polycarbonate 96-well plate in spite of blocking and washing and react with the second detection antibody, anti-human IgG. This reaction produces varying background levels depending on individual sera, which interferes with accurate measurement of antibody levels.

The amplified luminescent proximity homogeneous assay-linked immunosorbent assay (AlphaLISA; Perkin Elmer, Waltham, MA) is a recently developed method used to examine antigen and antibody levels. The AlphaLISA kits do not require plate washing. AlphaLISA needs donor beads and acceptor beads, which bind to antigens and serum antibodies, respectively. When the serum antibodies recognize antigens, two beads approach each other to produce luminescent light. The resultant photon counts reflect the amount of antigen–antibody binding. The experimental procedure only requires the mixing of antigens, sera, and two beads; therefore, the background levels are very uniform. Elevated light levels greater than the background level are easily detected. Examples are shown in Fig. 3.1. However, the results can be affected by temperature, air (oxygen), and light. Thus, high levels of experience and skill are needed to produce stable results.

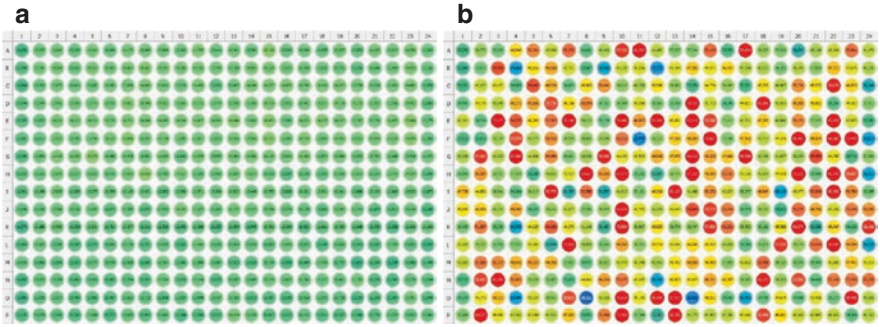

Fig. 3.1 Representative results of AlphaLISA. The antigens, glutathione S-transferase (GST) (**a**) and GST-NBL1 (**b**), were mixed with 384 sera of patients or healthy donors followed by addition of glutathione-conjugated donor beads and antihuman IgG-conjugated acceptor beads. The results of AlphaLISA are shown; higher photon counts are indicated by redder wells

3.4 NY-ESO-1 Is Useful for Diagnosis and Therapeutic Treatment

NY-ESO-1 is one of the SEREX antigens identified as an esophageal squamous cell carcinoma (ESCC) SEREX antigen by Chen et al. [4]. NY-ESO-1 is also named cancer/testis antigen 1B (CTAG1B) [5] and expressed specifically in many types of cancers, such as ESCC, colorectal cancer (CRC), lung cancer, hepatocellular carcinoma, prostate cancer, gastric cancer, breast cancer, and sarcoma [6, 7], with almost no expression in normal tissues. The receptor of NY-ESO-1 has been reported to be calreticulin in dendritic cell surfaces [8]. NY-ESO-1 has been used for cancer immunotherapy, and many cancer vaccines derived from NY-ESO-1 have been developed and have provided promising results [9].

3.5 p53 Antibody in Various Cancers

Among tumor suppressor genes, *p53* has the most potent suppressive activity by inhibiting cell proliferation and by inducing apoptosis throughout most human cancers. Thus, in most tumor cells, p53 activity is kept to a very low level by mutations in the *p53* genes or by inactivating factors. Consequently, *MDM2*, one of the target genes of p53 transactivation, is not expressed abundantly. Because p53 degradation is mainly regulated by MDM2-directed ubiquitination followed by proteasomal degradation, low expression of MDM2 results in high expression of p53 [10]. Tumor tissues are frequently accompanied by tissue degradation, which induces leaking of otherwise intracellular proteins, including p53. Consequently, anti-p53 antibodies are developed. Repeated leaking of p53 protein from tumor tissue increases the antibody levels greatly. Therefore, the diagnosis of cancers by using p53 antibody levels is highly sensitive and specific.

The presence of serum p53 antibody in patients with ESCC was first reported by Shimada et al. [11]. Thereafter, p53 antibodies for other cancers have been reported, for example, gastric cancers, colorectal cancers, and cholangiocarcinoma [12–14]. The presence of serum p53 antibodies has been significantly associated with overexpression of p53 protein in tumor cells [15]. The presence of serum p53 antibodies in patients with ESCC also has been reported to be an independent risk factor for poor overall survival [15–17]. Negative conversion of serum p53 antibodies could be a good indicator of favorable prognosis.

3.6 Autoantibodies in ESCC and CRC

The autoantibodies identified in patients with ESCC and CRC are summarized in Tables 3.1 and 3.2, respectively. There are common autoantibodies between ESCC and CRC, which are p53 tumor suppressor gene product, and include oncogenic proteins, such as MYC, IGFBP2, RALA, and CCND1. Autoantibodies against BIRC5, KRT19, CCNB1, HSPA1A/HSP70, and PUF60/FIR are also observed in

Table 3.1 List of autoantibodies reported for esophageal squamous cell carcinoma (ESCC)

Name	Full name	Function	References
NY-ESO-1/ CTAG1B	Cancer/testis antigen 1B	Cancer/testis antigen, dendritic cell-binding	[4]
NY-ESO-2	U1 sn RNP1 homolog	RNA-binding	[4]
NY-ESO-6/ FUS/TLS	Fused in sarcoma	Oncogene, RNA-binding	[4]
NY-ESO-7/ SNRNP70	Small nuclear ribonucleoprotein, U1 subunit, 70-kD	RNA splicing	[4]
p53/TP53	Tumor protein p53	Tumor suppressor gene	[11, 18]
CIP2A/p90	Cell proliferation-regulating inhibitor of protein phosphatase 2A	Signaling	[19]
TACSTD2/ TROP2	Tumor-associated calcium signal transducer 2	Membrane protein	[20]
SURF1	Surfeit 1	COX assembly	[21]
HOOK2	Hook microtubule-tethering protein 2	Cytoskeleton (microtubule)	[21]
CENPF	Centromeric protein F	Chromosome/ microtubule-binding	[21]
ZIC2	ZIC family, member 2	Transcription	[21]
CCNL2/ hCLA-iso	Cyclin L2	Cell cycle	[21, 22]
HABP4/KI1/57	Hyaluronan-binding protein 4	Protein/RNA-binding	[21]
PDLIM7/ enigma	PDZ and LIM domain protein 7	Cytoskeleton (actin filament)	[21]
HCA25a	Hepatocellular carcinoma-associated antigen HCA25a	Unknown	[21]
SYMPK	Symplekin	Polyadenylation	[21]
SLC2A1/ GLUT1	Solute carrier family 2/facilitated glucose transporter	Membrane transporter	[23]
TRIM-21	Tripartite motif-containing protein 21	DNA-binding	[24]
CDKN2A/p16	Cyclin-dependent kinase inhibitor 2A	Cell cycle	[25]
PRDX4	Peroxiredoxin 4	Redox	[26]
MRLC2	Myosin regulatory light chain	Cytoskeleton (actin filament)	[2]
PIK3R1	Phosphoinositede-3-kinase, regulatory subunit 1 (p85 alpha)	Signaling, enzyme	[2]
MED9	Mediator of RNA polymerase II transcription, subunit 9 homolog	Transcription	[2]
KRT19/ CYFRA21-1	Keratin 19	Cytoskeleton	[2]
STMN1	Stathmin 1/oncoprotein 18	Oncogene, cytoskeleton (microtubule)	[2]

(continued)

Table 3.1 (continued)

Name	Full name	Function	References
PDE4DIP/ myomegalin	Phosphodiesterase 4D-interacting protein	cAMP signaling	[27]
UBE2I	Ubiquitin-conjugating enzyme E2I	Ubiquitination	[28]
HSPA1A/ HSP70	Heat-shock 70-KD protein 1A	Heat-shock protein	[29]
CDC25B	Cell division cycle 25B	Cell cycle	[30]
AISEC	Antigen identified by SEREX for esophageal carcinoma	p53 suppressor	[31]
MKRN1	Makorin 1	Ubiquitination	[32]
ECSA	Esophageal carcinoma SEREX antigen	Unknown	[33]
MMP7	Matrix metalloproteinase 7	Protease	[34]
HMGB1	High mobility group box 1	DNA-binding	[35]
LY6K	Lymphocyte antigen 6 complex locus K	Membrane protein	[36]
ABCC3	ATP-binding cassette C3	Membrane protein	[37]
FOXP3	Forkhead/winged helix transcription factor	Transcription	[38]
HSPH1/ HSP105	Heat-shock 105/110-KD protein	Heat-shock protein	[39]
TPI1	Triosephosphate isomerase 1	Glycolysis	[39]
MDM2	Mouse double minute 2 homolog	Oncogene, ubiquitination	[40]
GSTO1	Glutathione S-transferase omega 1	Detoxification	[41]
BMI1	BMI1 proto-oncogene, polycomb ring finger	Oncogene, ubiquitination	[42]
CCNB1	Cyclin B1	Cell cycle	[43]
MYC	v-MYC avian myelocytomatosis viral Oncogene homolog	Oncogene, transcription	[43]
BIRC5/Survivin	Baculoviral IPA repeat-containing protein 5	Anti-apoptosis	[43]
IGF2BP1/IMP1	Insulin-like growth factor 2 mRNA-binding protein 1	Oncogene, RNA-binding	[43]
IGF2BP2/IMP2	Insulin-like growth factor 2 mRNA-binding protein 2	Oncogene, RNA-binding	[43]
IGF2BP3/IMP3	Insulin-like growth factor 2 mRNA-binding protein 3	Oncogene, RNA-binding	[43]
RALA	v-ral simian leukemia viral oncogene homolog A	Oncogene, signaling	[44]
CCND1	Cyclin D1	Oncogene, cell cycle	[44]
DKK1	Dickkopf WNT signaling pathway inhibitor 1	WNT signaling	[45]
LETMD1/ HCCR	LETM1 domain containing 1	Oncogene, mitochondrial outer membrane protein	[46]

Table 3.1 (continued)

Name	Full name	Function	References
CCNE1	Cyclin E1	Cell cycle	[46]
LGALS1/GAL1	Lectin, galactoside-binding, soluble, 1	Cell proliferation	[47]
L1CAM	L1 cell adhesion molecule	Cell adhesion	[48]
EZR	Ezrin	Cytoskeleton (actin filament)	[49]
STIP1	Stress-induced phosphoprotein 1	Heat-shock protein	[50]
FSCN1	Fascin actin-binding protein	Cytoskeleton (actin filament)	[51]
PUF60/FIR	Poly(U) binding splicing factor 60	RNA splicing	[52]
HS3ST1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	Anticoagulant	[52]
TUBA1B	Tubulin alpha-1B	Cytoskeleton (microtubule)	[52]
AKR1C3	Aldo–keto reductase family 1 member C3	Androgen activation	[52]
BAMBI	BMP and activin membrane-bound inhibitor homolog	BMP inhibitor	[52]
DCAF15	DDB1- and CUL4-associated factor 15	Ubiquitination	[52]
TOP1	Topoisomerase, DNA, I	Transcription	[53]

Table 3.2 List of autoantibodies reported for colorectal cancer (CRC)

Name	Full name	Function	References
CEA/ CEACAM5	Carcinoembryonic antigen	Tumor marker, cell adhesion	[54]
VIL	Villin	Cytoskeleton (actin filament)	[55]
p53	Tumor protein p53	Tumor suppressor gene, transcription	[56]
GRIN1	Glutamate receptor	Membrane receptor	[57]
GAL4	Lectin, galactoside-binding, soluble, 4	Cell adhesion	[57]
NACA	Nascent polypeptide-associated complex subunit alpha	Protein processing	[57]
EEF1D	Eukaryotic translation elongation factor 1 delta	Translation factor	[57]
RBMS1/ MSSP2	RNA-binding motif single stranded interacting protein-1	RNA-binding	[57]
HMG-17	Non-histone chromosomal protein HMG-17	Nuclear protein	[57]
PDXK	Pyridoxal kinase	Circadian oscillation	[57]
SSRP1	High mobility group box (SSRP1)	Chromatin protein	[57]

(continued)

Table 3.2 (continued)

Name	Full name	Function	References
SCAF11/ SRrp129	SR-related CTD associated factor 11	RNA splicing	[57]
LIMS1/PINCH	LIM and senescent cell antigen-like domains 1	Anti-apoptotic, cytoskeleton (actin filament)	[57]
ADSL	Adenylosuccinate lyase	Purine nucleotide metabolism	[57]
MKNK1	MAP kinase interacting serine/ threonine kinase 1	Signaling	[57]
UBE3A/E6-AP	Ubiquitin protein ligase E3A	Ubiquitination	[57]
SNRNP70	Small nuclear ribonucleoprotein, U1 subunit, 70-KD	RNA-binding	[57]
SATB1	Special AT-rich sequence-binding protein 1	Transcription	[57]
KRT19/ CYFRA21-1	Keratin 19	Cytoskeleton, tumor marker	[57]
HSPA1A/ HSP70	Heat-shock 70-KD protein 1A	Heat-shock protein	[57]
TRIP4	Thyroid hormone receptor interactor 4	Transcription	[57]
TBCB/CG22	Tubulin folding cofactor B	Cytoskeleton (microtubule)	[57]
PARP1	Poly(ADP-ribose) polymerase 1	DNA repair, pro-apoptotic	[57]
TPM	Tropomyosin	Cytoskeleton (actin filament)	[58]
UCH-L3	Ubiquitin C-terminal hydrolase isozyme 3	Ubiquitination	[59]
Fas/CD95	FAS cell surface death receptor	Pro-apoptotic	[60]
MUC5AC	Secreted gel-forming mucins	DNA/calcium binding	[61]
NUCB1/ CALNUC	Nucleobindin 1	DNA/calcium binding	[62]
MYC	v-MYC avian myelocytomatosis viral oncogene homolog	Oncogene, transcription	[62]
CCNB1	Cyclin B1	Cell cycle	[62]
CCND1	Cyclin D1	Oncogene, cell cycle	[62]
HSPD1/HSP60	Heat-shock 60-KD protein 1	Heat-shock protein	[63]
NUP62	Nucleoporin, 62-KD	Nuclear pore	[64]
IMPDH1	IMP dehydrogenase 1	Guanine nucleotide synthesis	[64]
PIM1	Oncogene PIM1	Oncogene, anti-apoptotic	[65]
MAPKAPK3	Mitogen-activated protein Kinase-activated protein kinase 3	Signaling	[65]
ACVR2B	Activin a receptor, type IIB	Membrane receptor	[65]
IGF2BP2/ IMP2	Insulin-like growth factor 2 mRNA-binding protein 2	Oncogene, RNA-binding	[66]

Table 3.2 (continued)

Name	Full name	Function	References
BIRC5/ Survivin	Baculoviral IAP repeat-containing protein 5	Anti-apoptotic	[67]
BIRC7/Livin	Baculoviral IAP repeat-containing protein 7	Anti-apoptotic	[67]
XIAP	Inhibitor of apoptosis, X-linked	Anti-apoptotic	[67]
PDIA3	Protein disulfide isomerase A3	Molecular chaperone	[68]
AFP	Alpha-fetoprotein	Plasma protein, tumor marker	[69]
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Oncogene, signaling	[69]
ANXA1	Annexin A1	Anti-inflammation	[69]
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	Oncogene, signaling	[69]
ADAM10	A disintegrin and metalloproteinase domain 10	Protease	[70]
PUF60/FIR	Poly(U) binding splicing factor 60	RNA splicing	[71]
OLFM4	Olfactomedin 4	Retinoic acid signaling	[11]
ITGAM/ CD11b	Integrin, alpha-M	Cell adhesion	[72]
ITGA2	Integrin α2	Cell adhesion	[72]
RALA	v-ral simian leukemia viral oncogene Homolog A	Oncogene, signaling	[73]
LGALS1/ GAL1	Lectin, galactoside-binding, soluble, 1	Cell proliferation	[73]

both ESCC and CRC lists. KRT19 is a frequently used biomarker known as CYFRA21-1 [74]. Other conventional biomarkers, such as CEA and AFP, have also been identified for CRC. Diagnosis using these antigen levels is useful for monitoring the cancer state. Development of autoantibodies can be based on the high expression of antigenic proteins.

In addition to p53, MYC, IGFBP2, RALA, and CCND1, the autoantibody list of ESCC contains other oncogenic proteins such as FUS, STMN1, MDM2, BMI1, IGFBP1, IGFBP3, and LETMD. The autoantibody list of CRC contains PIM1, KRAS, and RAF1. Thus, oncogenesis of ESCC and CRC could be caused by different mechanisms with partly overlapping pathways, including p53, MYC, IGFBP2, RALA, and CCND1.

Cell cycle-related proteins reported were CCNB1, CCND1, CCNE1, CCNL2, CDC25B, and CDKN2A in the ESCC list and only CCNB1 and CCND1 in the CRC list. PUF60/FIR is a c-myc gene transcriptional repressor [74], and its splicing variant directs CCNE overexpression [75]. RALA is involved in the regulation of CCNB-CDK1 kinase [76]. Thus, cyclin-driven cell cycle progression may be more closely involved in ESCC progression. It is well known that cyclins are degraded by the ubiquitination/proteasome system [77]. Ubiquitination-related molecules, such as UBE2I, MKRN1, BMI1, and DCAF15, are listed as autoantibodies for ESCC.

On the other hand, KRAS, RAF1, and their downstream signaling molecules, MKNK1 and MAPKAPK3, are listed as autoantibodies for CRC. The activation of the RAS pathway is frequently accompanied by the induction of apoptosis [78]. Consistently, apoptosis-related molecules, such as PARP1, Fas, LIMS1, BIRC5, BIRC7, XIAP, and ANXA1, are also autoantibodies for CRC. Consequently, activated intracellular signaling-induced growth stimulation with suppression of apoptosis might be the main pathway in CRC carcinogenesis.

Except for KRT19, there are eight cytoskeleton-related proteins in the ESCC autoantibody list but four in the CRC list (Tables 1 and 2). Four of the eight, PDLIM7, MRLC2, EZR, and FSCN1, in the ESCC list, and three, VIL, LIMS1, and TPM, in the CRC list are related to actin filaments. Among microtubule-related molecules, HOOK2, STMN1, CENPF, and TUBA1B have been reported for ESCC, but only TBCB was identified as a CRC antibody. HOOK2 is a microtubule-tethering protein [79]. Upregulation of STMN has been shown to lead to a decreased level of polymerized tubulin [80]. TUBA1B is tubulin alpha-1B, a subunit of microtubules. CENPF connects kinetochores to microtubules [81]. Thus, microtubules are more closely involved in ESCC than in CRC, which may account for the usage of the antimicrotubule anticancer drug, paclitaxel, for the therapy of ESCC but not of CRC [82]. This autoantibody information may be useful for determining treatment strategy.

3.7 Autoantibodies in Other Diseases

Thus far, it is believed that autoantibodies are found in patients with autoimmune disease or cancer. If the development of autoantibodies is the result of inflammation and subsequent tissue destruction, autoantibodies can be induced in other diseases. Recent reports have shown autoantibody biomarkers in metabolic and atherosclerotic diseases, for example, phospholipids [83], apolipoprotein A-1 [84, 85], oxidized low-density lipoproteins [85, 86], heat-shock proteins [85, 87], and NRD1 [88] for cardiovascular disease (CVD); Hsp60 for stroke [89]; insulin [90], glutamic acid decarboxylase (GAD) [91], and protein tyrosine phosphatase IA-2 [92, 93] for diabetes mellitus (DM); RPA2 [94], SOSTDC1 [95], CBX1 [96], and PDCD11 [97] in ischemic stroke; TUBB2C [98], GADD34 [99], and adiponectin [100] in DM; COPE and NBL1 in obstructive sleep apnea [101, 102]; and ATP2B4 [103], BMP-1 [94, 103], DHPS [104], SH3BP5 [105], MMP1 [96], and PRCP [106] in arteriosclerotic diseases.

There is substantial evidence that cancer is related to atherosclerosis-related diseases. For example, DM is also a risk factor for cancers, such as colorectal cancer and esophageal carcinoma [107–109]. Cancer survivors are at higher risk of having modifiable cardiovascular risk factors, such as hypertension and DM [110]. Overweight status and obesity, which are frequently accompanied by atherosclerosis, are associated with increased cancer risk [111]. The anticancer drug paclitaxel has been shown to improve results in diabetics [112]. Among autoantibodies, the atherosclerosis markers, anti-DHPS, anti-ATP2B4, and anti-BMP-1 antibodies, have been

shown to be increased in patients with ESCC [103, 104]. All organs and tissues are interrelated with each other to varying degrees in the human body. Therefore, autoantibody marker analysis can be an effective and excellent approach to developing a comprehensive interpretation of healthy or disease conditions in the whole body.

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Serum Angiogenic Factors as Cancer Biomarkers

4

Hideaki Shimada

Abstract

Expression of angiogenic factors in cancer tissues is frequently associated with aggressive behavior in various cancer types. Although multimodal treatment is applied for such angiogenic tumors, many patients suffer from progressive disease and/or rapid recurrence even after multimodal treatment. Serum angiogenic factors could be useful for predicting malignant potential and monitoring treatment response in such angiogenic tumors. Here, a comprehensive review is provided that focuses mainly on recent advancements in the research on serum angiogenic factors in cancer medicine. We reviewed the clinicopathological significance of vascular endothelial growth factor, thymidine phosphorylase, fibroblast growth factor, midkine, and hepatocyte growth factor. Because these angiogenic factors may be useful biomarkers, serum angiogenic factors may also be useful in the management of cancers.

Keywords

Angiogenic factors · Serum biomarkers · Malignant potential · Prognosis

4.1 Introduction

The aggressive behaviors of "angiogenic cancers" are associated with widespread lymph node involvement, distant metastases, and poor prognosis [1]. Angiogenesis has an essential role in the growth and metastasis of cancers. Vascular endothelial growth factor (VEGF), thymidine phosphorylase (dThdPase/PDECGF), fibroblast

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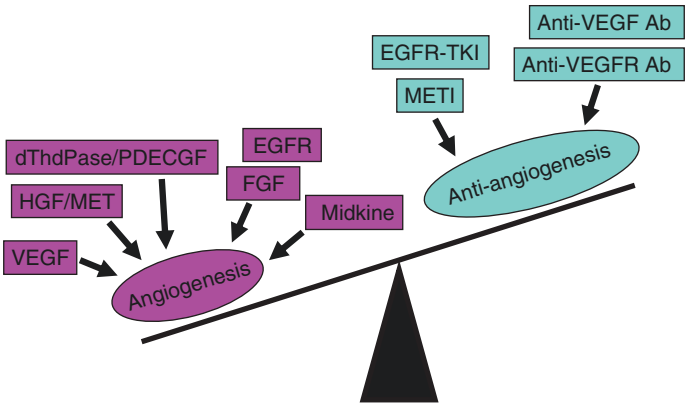

Fig. 4.1 Balance of angiogenesis and anti-angiogenesis in the tumor. *VEGF* vascular endothelial growth factor, *HGF* hepatocyte growth factor, *dThdPase PDECGF* platelet-derived endothelial growth factor, *EGFR* epidermal growth factor receptor, *FGF* fibroblast growth factor

growth factor (FGF), epidermal growth factor receptor (EGFR), midkine (MDK), and hepatocyte growth factor (HGF) have been reported to be key molecules for tumor angiogenesis and aggressive behavior of cancers. They are potentially good candidates for target molecules in cancer treatment (Fig. 4.1). Serum angiogenic factors can be useful to evaluate biological features of tumors to predict recurrence and/or patients' survival [2–4]. In this chapter, we review the predictive aspects of serum angiogenic factors for survival and treatment response.

4.2 Overexpression of Angiogenic Factors and Malignant Potential of Cancer

The VEGF family members have contributing roles in both angiogenesis and carcinogenesis. Seven isoforms of VEGF, VEGF-A, -B, -C, -D, -E, and PLGF-1 have crucial roles through three receptors, VEGF-R1, -R2, and -R3, in positive signaling to induce vasculogenesis, angiogenesis, and lymphangiogenesis. In particular, VEGF expression has been found to be significantly associated with lymph node metastasis, distant metastasis, and survival [2, 4–6]. Therefore, overexpression of VEGF protein is responsible for the malignant potential in carcinoma and is a useful marker for estimating patient prognosis. Hsu JT et al. co-analyzed epidermal growth factor receptor 2 expression and VEGF expression in gastric cancer [7]. They concluded that VEGF overexpression and the presence of vascular invasion were independent poor prognostic factors for HER-2 positive gastric cancer. Polymorphisms in gene-encoding angiogenic factors may alter protein expression [5, 8, 9]. Functional genetic polymorphisms in the VEGF gene have been reported to correlate with VEGF promoter activity, gene expression, protein production, and risk of carcinogenesis, which are modulated by smoking. Thus, germline polymorphisms of genes involved in the tumor angiogenesis pathway independently predict tumor

recurrence and patients' survival (Lurje G). Liu W et al. concluded that the VEGF-634 G > C GG genotype was associated with gastric cancer risk in the overall population, with the VEGF-634 G > C C allele and GG genotype being associated with risk in Caucasians and VEGF+1612G/A with risk in Asians [5].

The other key molecules in the angiogenic pathway are thymidine phosphorylase (dThdPase) and platelet-derived endothelial growth factor [10]. Tabata et al. found that dThdPase-mediated thymidine catabolism could supply the carbon source in the glycolytic pathway and thus contribute to cell survival under conditions of nutrient deprivation. In thymidine phosphorylase dThdPase-expressing cells, thymidine has been found to convert to metabolites, including glucose 6-phosphate, lactate, 5-phospho-α-D-ribose 1-diphosphate, and serine, via the glycolytic pathway both in vitro and in vivo. High dThdPase expression has also been found to be associated with tumor progression and poor prognosis in various cancer types [11–13]. A significant association between dThdPase expression and nodal involvement was observed. Among the various prognostic variables, dThdPase overexpression in cancer tissues was shown to be significantly associated with poor overall survival [14, 15]. High systemic inflammation, an independent risk factor for poor oncological outcome, was significantly associated with dThdPase expression in gastric cancer [16]. dThdPase has become a good target for colorectal cancer of TAS-102, which is an orally administered combination of a thymidine-based nucleic acid analog, trifluridine, and a dThdPase inhibitor, tipiracil hydrochloride [17].

Overexpression of FGF-2 is also associated with tumor recurrence and reduced patient survival [18, 19]. Stromal fibroblasts also contribute to the regulation of extracellular matrix degradation, epithelial cell behavior, inflammation, and cancer progression. FGF receptor (FGFR) 2-positive fibroblasts provide a suitable microenvironment for the development of distant metastases, stimulation of cancer cell proliferation, and induction of angiogenesis. The FGFR pathway has a major role in carcinogenesis by increasing cell proliferation, angiogenesis, and drug resistance. Upregulation of FGFR signaling is a common event in various cancer types. Several FGFR inhibitors are currently under development in clinical trials [20, 21].

4.3 Overexpression of Angiogenic Factors and Treatment Response

The ability to predict treatment response to chemo and/or radiation therapy by analyzing pretreatment biopsy specimens would be valuable for carcinomas. We analyzed the expression of angiogenesis-related factors (p53, dThdPase, and VEGF) by immunohistochemical analysis in patients with esophageal carcinoma prior to treatment [2, 4]. A clinical response was observed in 69% of the patients and was negatively associated with dThdPase and VEGF expressions. Multivariate analysis identified VEGF as a significant independent prognostic factor. These results suggest that expression of angiogenic factors has predictive value for treatment response and outcome in patients with carcinoma.

4.4 Clinical Significance of Serum Levels of Angiogenic Factors

Elevated serum levels of angiogenic factors, caused by increased protein expression, and platelet count were found to be independently associated with poor treatment response and poor prognosis (Table 4.1). Serum angiogenic factors are frequently associated with tissue angiogenic factor overexpression. Thus, increased serum levels of angiogenic factors were found to be associated with poor treatment response and poor prognosis [30–33].

4.4.1 Serum VEGF

Significant differences were observed between serum VEGF categorized by tumor size, tumor depth, lymph node metastasis, and TNM stage. Patients who achieved a partial or complete response to chemo-radiotherapy showed significantly lower serum VEGF levels than those of the non-responder group [3]. Multivariate analysis identified serum VEGF as a significant and independent prognostic factor. Both serum VEGF C and D were associated with both patient survival and tumor progression [31]. Such tendency was observed in various cancer types. Kaplan–Meier analysis showed that CXCL8, VEGF, and pentraxin 3 levels were significantly associated with worse survival in patients with colorectal cancer [34]. Circulating inflammatory mediators efficiently predicted postoperative recurrence after colorectal cancer surgery. On the basis of a randomized phase III study, Spencer SK et al. also reported that low levels of VEGF, VEGF-D, VEGFR-1, and VEGFR-3 correlated with better outcome of the patients with colorectal cancer [35]. Postoperative serum VEGF was also useful for the prediction of colorectal cancer patients' survival. Pascual M et al. analyzed serum VEGF on postoperative day 4 and concluded that the serum level was an independent prognostic factor of decreased disease-free survival and overall survival [36]. On the basis of the monitoring of pro-angiogenic and pro-inflammatory factors during chemotherapy in patients with pancreatic cancer, Arshad A et al. concluded that chemotherapy might reduce concentrations of

Table 4.1 Clinicopathological impact of serum biomarkers in patients with esophageal carcinoma

Serum biomarkers	Reference	Positive rate (%)	Prognostic impact	Treatment resistance	Association with TNM		
					T	N	M
VEGF	[21–24]	37	Positive	Positive	Positive	Positive	BL
dThdPase	[25]	19	Positive	Positive	Positive	BL	Negative
Midkine	[26, 27]	61	Positive	NA	Negative	Negative	Negative
HGF	[10, 12, 28]	NA	Positive	NA	BL	BL	Positive
Platelet count	[29]	21	Positive	NA	Positive	Positive	Positive

BL borderline, *NA* not applicable

Table 4.2 Diagnostic impact of serum biomarkers in tumor staging for esophageal carcinoma

(%)	dThdPase sm<	VEGF N1	VEGF M1	dThdPase M1
Sensitivity	53	59	77	68
Specificity	71	70	64	70
Positive predictive value	91	80	24	24
Negative predictive value	21	46	95	94

sm submucosal tumor, *N1* lymph node metastases, *M1* distant metastases

circulating cytokines and growth factors, which may be associated with improved outcome [37]. On the basis of the monitoring the data of patients with triple-negative breast cancer, Wang et al. reported that serum VEGF could identify patients with favorable or poor responses at an early time point of neoadjuvant chemotherapy.

It was interesting that even 20 years after initial treatment, high serum VEGF was a significant prognostic indicator for overall survival in patients with bladder cancer [24]. VEGF might serve as a complement or alternative to traditional imaging-based response-evaluating methodologies in tailoring systemic treatment strategies for both operable and inoperable advanced cancers (Table 4.2).

4.4.2 Serum Thymidine Phosphorylase (dThdPase)

Serum dThdPase was also measured in patients with esophageal carcinoma [2, 4]. High serum dThdPase levels were associated with tumor size, depth of tumor invasion, and poor treatment response. Platelet count, C-reactive protein, and several inflammatory cytokines were significantly associated with serum levels of angiogenic factors. Interestingly, C-reactive protein, serum dThdPase, and white blood cell count were also significantly associated with thrombocytosis. Even after adjusting for tumor size and TNM factors, multivariate analysis indicated that thrombocytosis was an independent prognostic factor [32]. Torres C et al. assessed the prognostic significance of serum dThdPase and inflammatory cytokines in patients with pancreatic cancer [28]. They found that EG-VEGF/PK1, IL-29, and dThdPase expressions predicted poor prognosis for patients with pancreatic cancer.

4.4.3 Serum HGF

Hepatocyte growth factor (HGF) and its receptor, c-Met, have important roles in esophageal carcinoma development and progression [29, 38]. High Met expression was also reported to be significantly associated with the development of distant metastases and local recurrence in contrast to low Met expression [38]. Grugan KD et al. found that fibroblast secretion of HGF enhanced the ability of transformed esophageal epithelial cells to invade the extracellular matrix [39]. Pretreatment serum HGF levels were found to be significantly higher in patients with esophageal

carcinoma than in control subjects [25]. In a review by Matsumoto et al., changes in serum/plasma HGF, soluble MET, and phospho-MET were noted as having been associated with disease progression, metastasis, therapy response, and survival [40]. Higher serum HGF levels have been associated with treatment resistance, whereas lower HGF levels have been associated with good overall survival after treatment [41].

Rimassa L et al. conducted a randomized study to evaluate the MET inhibitor, tivantinib, in second-line hepatocellular carcinoma patients [42]. A significant interaction between tivantinib and baseline tumor MET in terms of survival was observed. High circulating MET and HGF were significantly correlated with shorter survival. Tumor MET levels were higher in patients treated with sorafenib. Circulating biomarkers such as MET and HGF may be prognostic indicator for the patients with hepatocellular carcinoma.

4.4.4 Serum Midkine

Midkine is a heparin-binding growth factor that has a role in neuronal survival and differentiation. The growth factor is also expressed at higher concentrations in various malignant tumors than in the adjacent normal tissue [22, 43] even in the early stages of disease. Because MDK is a secreted protein, serum midkine concentrations would be expected to increase when tumor tissues express abundant midkine. An enzyme-linked immunosorbent assay was developed to measure serum midkine in cancer patients (Ikematsu), and the overall positive rate in esophageal cancer was found to be 61% [44, 45]. The positive rate increased slightly according to progression of the clinical stages as follows: 53% in stage I, 54% in stage II, 60% in stage III, and 76% in stage IV, respectively [46]. Serum midkine levels in patients with protein positive tumors were significantly higher than those in patients with protein negative tumors. When serum midkine, tumor size, and TNM factors were assessed by multivariate analysis using Cox's proportional hazards model, serum midkine was identified as an independent prognostic factor.

Similar tendency was also reported in patients with head and neck squamous cell carcinoma (Yamashita T). Yamashita et al. reported that the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of serum midkine concentration for detection of head and neck squamous cell carcinoma were 57.3%, 85.3%, 77.6%, 69.2%, and 72.1%, respectively. Serum midkine levels in patients with head and neck squamous cell carcinoma were associated with malignancy, chemosensitivity, and prognosis. As the receiver operating characteristics curve analysis shows, the sensitivity and specificity of the cutoff serum midkine concentration of 400 pg/ml for predicting the presence of NSCLC were 71.2% and 88.1%, respectively. The serum midkine concentration was identified as an independent prognostic factor by multivariate analysis [26]. Serum mesothelin showed moderate sensitivity and high specificity for differentiation of malignant pleural mesothelioma from metastatic malignancy in pleura and from benign pleural diseases. In contrast, midkine was a useful marker for predicting prognosis of patients with mesothelioma [47].

4.5 Conclusions

We reviewed the clinicopathological significance of VEGF, dThdPase, FGF, HGF, and midkine as angiogenic factors. Because of their potential usefulness in differential diagnosis and for prediction of the malignant potential of cancer cells, serum angiogenic factors may be important in the management of cancer treatment.

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Conflict of Interest Disclosure Statement Author Hideaki Shimada owns stock in Cellmid Limited.

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Biomarkers of Cancer Stem Cells in Cancer Therapy

5

Norikatsu Miyoshi, Tsunekazu Mizusima, Yuichiro Doki, and Masaki Mori

Abstract

Cancer therapies, including chemotherapy and radiotherapy, initially achieve good results. Unfortunately, disease recurrence is common. Post-therapy recurrence may depend on a small population of cancer stem cells (CSCs) that self-renew and undergo multipotent differentiation. Tumour CSCs are identified by their expression of surface proteins. CSCs are required for serial transplantation in animal models. However, a specific signature of cell surface proteins that identifies CSCs is unavailable for solid tumours. Here we summarize a new technique for identifying and quantifying CSCs in situ, which may facilitate evaluating therapy. We discuss several preclinical treatments that reprogram CSCs or cause them to be specifically attacked by immune cells. In summary, therapeutics and diagnostics that attack and quantify CSCs, respectively, will be valuable for eradicating cancer.

Keywords

Cancer stem cell · Self-renewal · Heterogeneity · Surface marker

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5.1 Introduction

Cancer, which remains one of the leading causes of death worldwide [1], is a genetic and epigenetic disease that causes uncontrolled cell proliferation. Cancer shows great cellular heterogeneity that is produced by small populations of cancer stem cells (CSCs). CSCs possess self-renewal and multipotent properties [2]; the role of CSCs was first revealed by studies of acute myeloid leukaemia [3, 4]. CSCs are possibly involved in the pathogenesis of several solid tumours [5–9], and recent advances indicate that many cancers possess a differentiation hierarchy that arises from malignant CSCs that undergo uncontrolled proliferation and produce daughter cells with limited proliferative potential [2] (Fig. 5.1).

Many cancers respond to conventional treatments such as chemotherapy and radiotherapy. Nevertheless, certain cancers subsequently recur. Evidence indicates that CSCs cause resistance to conventional treatments [10–12] (Fig. 5.2), and small

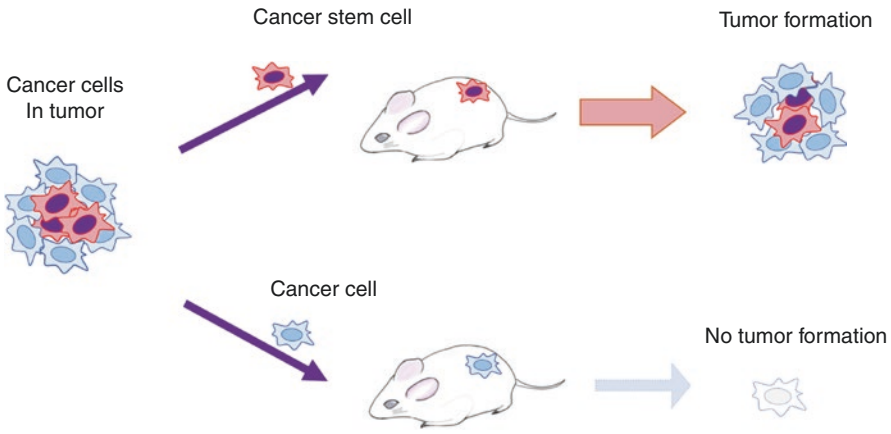

Fig. 5.1 Cancer stem cells in tumours. Cancers producing heterogeneity possess a hierarchy of the differentiation. Transplanted into immunodeficient mice, cancer stem cells can form the tumours

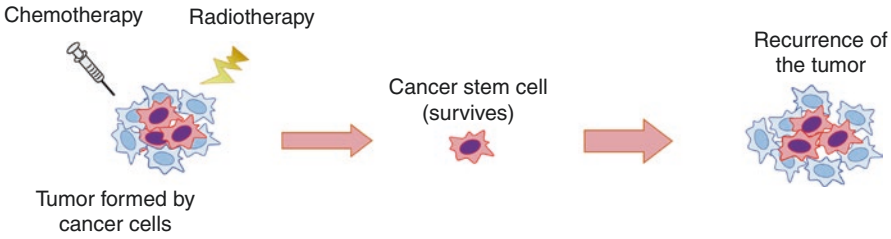

Fig. 5.2 Conventional treatments for cancer producing heterogeneity. The existence of cancer stem cells is the cause of the resistance to the conventional treatments, which results in the tumour recurrence. The small population of the cancer stem cells have the capacity is thought to leads to the resistance to the treatments

populations of CSCs possess persistent proliferative potential, which is detectable using in vitro assays and in vivo animal experiments [13]. Accordingly, malignant tumours are proposed to derive from CSCs with uncontrolled proliferative potential that is associated with dysregulation of differentiation [3, 13].

Here we review recent studies and progress in understanding the properties of CSCs and describe new strategies for targeting CSCs as a component of anticancer therapy.

5.2 Treatment Targeting Surface Markers of CSCs

Numerous published studies devoted to isolating CSCs provide insights into their mechanisms of resistance to treatment [14–17]. The unique properties of CSCs protect them against cytotoxic drugs and adverse responses to DNA damage. For example, analysis of the expression of stem cell markers led to the hypothesis that CSCs are associated with increased recurrence rates and poorer prognosis [18–20]. CSCs in solid tumours usually express organ-specific markers. For example, the surface marker profile related to CSCs in breast cancer is $CD44^{+}CD24^{-/low}Lin^{-}$ [7].

Remarkably, fewer than 100 cells with the CSC phenotype form tumours in mice. In contrast, tens of thousands of cells with alternate phenotypes are not tumourigenic [7]. Furthermore, this tumourigenic subpopulation can be serially passaged in mice, and within each passage, these cells generate new tumours containing tumourigenic $CD44^{+}CD24^{-/low}Lin^{-}$ cells. Similarly, brain tumour stem cells are exclusively isolated from within the cell fraction expressing the neural stem cell surface marker CD133 [6, 21, 22]. Moreover, certain gastrointestinal cancers express CSC surface markers [23–27]. For example, a $CD90^{+}$ hepatocellular carcinoma cell line is tumourigenic [23]. Pancreatic cancer cells expressing CD44, CD24 and epithelial-specific antigen (ESA) possess higher tumourigenic potential [24]. Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) expressed by colorectal cancer stem cells drives self-renewal and the expression of LGR5 is associated with activation of Wnt pathway [20].

Stem cells can be isolated by performing gene expression profile analysis of a side population of cancer cells with low cell turnover. This population extrudes dye, which is a reliable property for isolating stem cells, including CSCs [28, 29]. The two major superfamilies of efflux transporters are the ATP-binding cassette (ABC) transporters and the solute carrier (SLC) transporters. Targeting these efflux transporters as well as CSC-related surface markers in combination with conventional treatments can improve cancer treatment.

Isolating CSCs using flow cytometry is useful for studying cancer cells. However, this procedure stresses the cells and may alter their biology. Therefore, developing a system to visualize CSCs in situ will facilitate analysing authentic tumour cell behaviours within their local microenvironment. Such a technique offers great advantages for cancer research, because it focuses on the characteristics of CSCs, such as their quiescence, low protein turnover rate and decreased 26S proteasome activity [30, 31] (Fig. 5.3). A genetically encoded fusion protein comprising

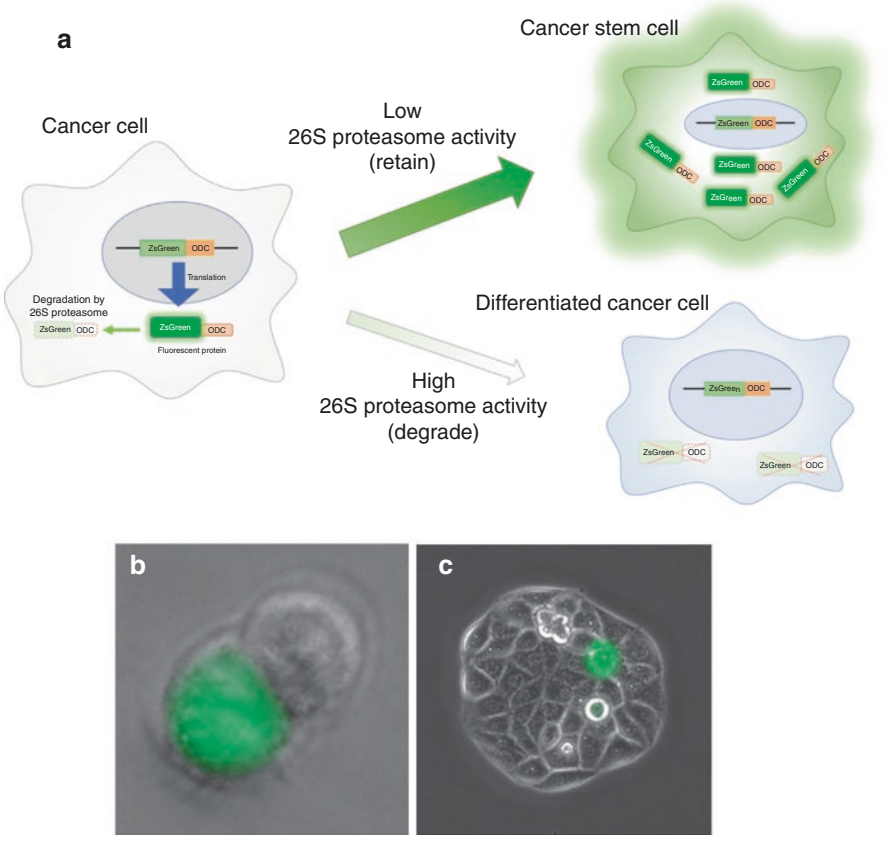

Fig. 5.3 Visualization of CSCs. (**a**) Ornithine decarboxylase (ODC) is normally destroyed by the proteasomes. Based on the decreased activity of 26S proteasome in cancer stem cells, they with low 26S proteasome activity are visualized due to the decreased degradation. They are visualized using a vector coding for a fusion protein of green fluorescence (ZsGreen) and the C-terminal degron of ODC. Cancer stem cells demonstrate asymmetric division (**b**) and finally form the cluster of the cancer cell population (**c**)

green fluorescent protein (ZsGreen) and the C-terminal domain of ornithine decarboxylase (ODC) is retained (green fluorescence-positive) in CSCs with low 26S proteasome activity because of decreased protein degradation. In several solid tumours, the fluorescent cells (ZsGreen-ODC-positive) demonstrate features of stemness, such as tumour formation in xenotransplantation models, and they undergo asymmetric cell division [15–17]. The fluorescent cells are more chemo- and radioresistant compared with nonfluorescent cells [16, 17]. The ZsGreen-ODC system is used to study solid tumours [32, 33], and visualizing CSCs using this system facilitates conducting stem cell research and performing screens to identify novel therapeutic agents.

5.3 Treatments Based on Reprogramming Cancer Cells

We analysed the effects of transcription factors that induce pluripotent stem (iPS) cells as well as cancer-related oncogenes and tumour suppressor genes. These results indicate that the introduction of transcription factors into gastrointestinal cancer cells reprograms the cells to the pluripotent state and sensitizes them to undergoing differentiation [34]. These reprogrammed cells are distinct from parental cells. We hope that the generation of induced pluripotent cancer (iPC) cells will allow us to test previously uncharacterized cancer treatments using differentiation therapy via the induction of drug susceptibility in cancer cells. Reprogramming cancer cells supports the notion that transduction might cause the differentiation of cells to unique cell lineages.

Another goal is to exploit drug discoveries, with the aim of producing therapeutic and diagnostic reagents. For this purpose, the genes encoding the transcription factors OCT3/4, SOX2, KLF4 and MYC were used to transfect cancer cell lines. The cells generated from our study are similar to iPS cells in morphology, embryonic stem cell-like gene expression and epigenetic modifications [35–38]. By controlling culture conditions, the reprogrammed cancer cells can be guided to differentiate into cells of epithelial, mesenchymal, neural or adipose lineages [34]. We demonstrated further that iPC cells undergo multipotent differentiation. We originally hypothesized that iPC cells revert to their original phenotypes. However, we found that these cells lose the ability to form tumours in mouse xenotransplant models. Furthermore, iPC cells become more sensitive to chemotherapy. These findings suggest that reprogramming and epigenetic modifications are promising methods for cancer treatment regardless of the abundance of harboured genetic mutations.

To translate this reprogramming strategy of eradicating CSCs to the clinic, we developed a method for reprogramming murine and human fibroblasts into pluripotent stem cells via a specific combination of microRNAs (miRNAs) [39], which are small noncoding RNAs that silence gene expression to regulate development and differentiation. Moreover, the association of specific miRNAs with pluripotency is established [40–42]. Therefore, we searched for miRNAs that reprogram differentiated cells to pluripotent stem cells. Using somatic cells derived from transgenic mice harbouring a green fluorescent protein inserted downstream of the *Nanog* promoter [43], the identification of miRNAs that reprogram cells to pluripotency was evaluated using fluorescence (a surrogate for NANOG activation). We identified a combination of miRNAs that reprogram mammalian cells to pluripotency through the demonstration that the miRNA-induced reprogrammed cells differentiate into cells of different lineages. The expression of pluripotency markers therefore makes it possible to reprogram cancer cells by administering a combination of miRNAs. Moreover, cancer cell lines reprogrammed with miRNAs exhibit decreased tumour-initiating capacity and became sensitive to chemotherapeutic agents [44]. To confirm the feasibility, safety and effectiveness of administering the miRNAs in vivo, we demonstrated that our combination of miRNAs suppresses tumourigenesis, suggesting that this therapy may be useful for preventing and treating cancer (Fig. 5.4).

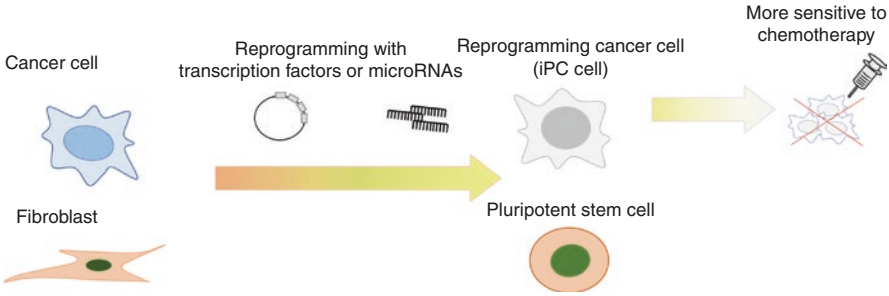

Fig. 5.4 Cancer treatment strategy based on cell reprogramming. As well as normal fibroblasts, cancer cells can be reprogrammed to induced pluripotent stem (iPC) cells by defined transcription factors or microRNAs. The reprogrammed cancer cells (iPC cells) increased the sensitivity for the conventional treatment such as chemotherapy

5.4 Targeting Therapy with the Tumour Microenvironment

Tumour heterogeneity and the microenvironment provide self-protection properties such as the mechanisms enabling dynamic interactions with surrounding epithelial cells, infiltrating immune cells as well as cytokines and chemokines that regulate CSC proliferation and self-renewal. CSCs can be maintained by the tumour microenvironment through the induction of specific features of more highly differentiated tumour cells [45]. Furthermore, the tumour microenvironment plays a key role in regulating the CSC population through direct cell–cell contacts and the secretion of various paracrine factors. These microenvironment factors maintain stemness through self-renewal, which is mediated by the Wnt/β-catenin, Notch and Hedgehog signal transduction pathways.

Wnt activity regulates the self-renewal of CSCs and drives the proliferation and differentiation of transit-amplifying cells [46]. In tumour tissues, Hedgehog signaling, which is important for embryonic development, patterning and differentiation, is associated with the regulation of self-renewal of normal mammary stem cells as well as that of CSCs [47]. Notch signaling controls cell fate during development and aberrant Notch activation contributes to tumourigenesis [48]. Niclosamide, which is a tineacide of the anthelmintic family, is an inhibitor of Wnt/β-catenin and Notch signaling [49]. Thus, niclosamide may be useful for inhibiting CSCs. Mesenchymal stem cells of the tumour-associated stroma affect the behaviours of cancer cells and influence their phenotypes. Prostaglandin E2 (PGE2) secreted by mesenchymal stem cells enables tumour progression via creating a cancer stem cell niche [50]. Targeting the CSC microenvironment may stimulate host antitumour responses, which represents the strategy of blocking tumour-promoting inflammation with a PGE2 receptor antagonist. The best opportunity to demonstrate the effects of targeting CSCs seems to be in the area of combination therapy.

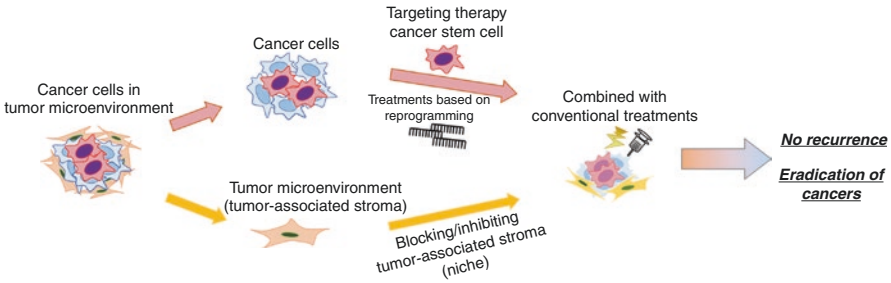

Fig. 5.5 Strategy for the cancer therapy focusing on the cancer stem cells and its tumour microenvironments. In the tumour, the heterogenous cancer population and its microenvironments provide self-protection properties. Using stem cell-specific markers or visualizing system to detect the cancer stem cells, the targeting therapy including reprogramming-based treatments can improve the conventional treatments for the cancer. Targeting its tumour microenvironment also enhanced the treatment strategy. These cancer therapies lead to the reduction of the recurrence and eradiation of the cancer

5.5 Conclusions

It is generally considered that the relative quiescence and resistance to anticancer treatment are associated with a long cellular life span. CSCs, through their self-renewal and drug-resistant capacities, may share properties that are conducive to proliferation and differentiation relevant to anticancer therapy. Unfortunately, a specific set of markers that distinguish CSCs from normal stem cells is not available. It will therefore be important to enhance our knowledge of the characteristics of CSCs and normal stem cells. If specific markers for these stem cells can be identified, it will be possible to isolate, identify and analyse these minor populations within tumours. Elucidating the properties of CSCs properties combined with analysis of the tumour microenvironment may lead to the development of novel and effective anticancer therapies (Fig. 5.5).

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Part II

Biomarkers for Individual Cancers

Specifics 1: Head and Neck Cancer and Esophageal Cancer

6

Shuhei Ito, Kensuke Koike, and Koshi Mimori

Abstract

Head and neck cancer (HNC) and esophageal cancer (EC) are aggressive diseases associated with high morbidity and mortality. The poor prognosis is mainly attributed to the absence of specific symptoms during early-stage cancer and the lack of reliable biomarkers. The identification of biomarkers has the potential to aid early diagnosis and prediction of recurrence and therapeutic efficacy. Biomarkers can also enable the improvement of long-term prognosis through personalized treatment strategies. The discovery of noninvasive methods to detect and monitor tumors remains a major challenge in clinical oncology. In this chapter, we review the development and feasibility of biomarkers, especially via "liquid biopsy," such as circulating tumor cells (CTCs), cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), circulating microRNAs (miRNAs), and autoantibodies against tumor-associated antigens (TAAs). We highlight new insights into the biomarkers of HNC and EC in clinical application and identify promising avenues of research in this emerging field of study, ultimately leading to improved HNC and EC patient care.

Keywords

Esophageal cancer · Head and neck cancer · Liquid biopsy · Circulating tumor cell · Cell-free DNA

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6.1 Head and Neck Cancer

6.1.1 Introduction

Head and neck cancer (HNC) is the sixth most common type of cancer worldwide. The predominant histological type is squamous cell carcinoma (SCC) that mainly occurs in the oral cavity, oropharynx, hypopharynx, and larynx [1]. Environmental factors, such as alcohol and tobacco abuse and human papillomavirus (HPV) infection, are implicated in the development of HNC. Despite advanced surgery and therapeutic strategies, the survival rate of HNC is very poor especially in cases where the disease is locally advanced [2]. Current diagnostic procedures, including clinical examinations, imaging techniques, and biopsies, have certain limitations, such as unsatisfactory resolution, high costs, patient inconvenience, severe invasiveness, and high error rates [3]. However, continued improvement in the understanding of liquid biopsy provides new possibilities to predict therapy response and identify new targets for cancer management [4, 5]. The aim of this review is to summarize current knowledge about liquid biopsy biomarker molecules, such as circulating tumor DNA (ctDNA) and microRNA (miRNA), as well as their potential clinical applications in HNC.

6.1.2 Blood-Based Biomarkers (Liquid Biopsy)

6.1.2.1 Circulating Tumor DNA (ctDNA)

It is believed that dying tumor cells release small fractions of their DNA into the bloodstream. In cancer patients, ctDNA is found within the cell-free segment of blood, representing a variable fraction of the total circulating DNA and can been found together with DNA fragments of normal cells, known as cell-free DNA (cfDNA) [6]. Recent study has shown proof-of-principle for ctDNA as a biomarker in HNC [7]. Some studies have correlated the abundance of ctDNA with tumor size and stage, with as much as 40% of ctDNA levels in metastatic cancers but as low as 0.1–1% in premalignant or early-stage disease [8]. From this point, high sensitivity detection system is required for detection of ctDNA. With the improvement of NGS technologies, it is possible to employ targeted deep-sequencing analysis to detect ctDNA, with high sensitivity (0.1–1%). A study using NGS for the analysis of ctDNA showed that it is possible to search for new acquired mutation after recurrence or treatment, which is considered useful for elucidating the time course of tumor genome and the mechanism of resistance to therapy [9]. Furthermore, some findings demonstrate the importance of examining a combination of circulating mediums (i.e., blood and saliva) or appropriate bodily fluids according to tumor type to achieve the highest sensitivity. For instance, when both plasma and saliva were tested in combination, the overall ctDNA detection rate was 96%, irrespective of tumor location or stage. In contrast, when plasma and saliva are analyzed individually, the detection rate is reported to be between 86 and 100% and 47 and 70%, respectively [1].

6.1.2.2 Circulating MicroRNAs (miRNAs)

In recent years, the biomedical fields have manifested a rapidly expanding interest in a relatively small number of small genes—miRNAs [10]. MicroRNAs are well-recognized, noncoding, regulatory RNA molecules, about 22 nucleotides in length [11]. MicroRNAs are not involved directly in protein coding, but facilitate the expression of more than one-third of the protein-coding genes in the human genome. They have been shown to function as proto-oncogenes or tumor suppressors, and their aberrant expression has been reported in many types of cancer [12]. A number of recent studies have identified specific miRNA alterations in HNC, reporting miR-21, miR-184, miR-133a/133b, miR-137, and miR-193a to be up- or downregulated [11, 13]. For example, miR-21 and miR-184 are well-established oncogenic miRNAs that enhance cell proliferation and suppresses apoptosis [14–17]. On the other hand, several studies have demonstrated the tumor suppressor functions of miR-133a/miR-133b, miR-137, and miR-193a, inhibiting proliferation and inducing apoptosis in HNC cell lines. Furthermore, it has been suggested that both miR-137 and miR-193a are silenced by DNA hypermethylation in HNC [18, 19]. The ectopic transfection of miR-137 or miR-193a into oral squamous cell carcinoma lines lacking their expression significantly reduced cell growth, with downregulation of the translation of cyclin-dependent kinase 6 or E2F transcription factor 6, respectively [19].

A study investigating cancer treatment resistance reported that EMT (epithelial to mesenchymal transition) and low expression of EMT-inhibiting miRNAs, especially miR-203, measured in pretreatment material, causes intrinsic radioresistance of HNC, which could enable identification and treatment modification of radioresistant tumors [20]. Based on these findings, miR-203 may potentially serve as a new diagnostic and therapeutic target for the treatment of HNC [21]. Unique miRNA expression patterns may become specific and sensitive biomarkers for diagnosis and prognosis of HNC. Finally, these miRNAs may also serve as therapeutic targets in HNC prevention [10].

6.2 Esophageal Cancer

6.2.1 Introduction

Esophageal cancer (EC) is a highly lethal malignancy and the eighth most common cancer worldwide and the sixth leading cause of cancer-related death worldwide [22]. The asymptomatic nature of this disease results in late diagnosis and 5-year survival rates of approximately 20–50% for resectable cases, even with preoperative chemotherapy [23, 24]. The poor prognosis of EC highlights the need for precise diagnostic methods to shift diagnosis to the earliest possible stage, especially for individuals with risk of EC. Although endoscopy and tissue examination from biopsy are currently the standard approaches for detection and diagnosis of EC, they are uncomfortable procedures, especially when used Lugol's iodine solution [25], invasive and not cost-effective for screening and surveillance. Therefore, there is a

considerable demand for developing noninvasive (tissue biopsy-free) and economical biomarkers to assist with earlier detection and better clinical management of EC.

The most effective strategy to improve the prognosis of advanced EC is neoadjuvant chemotherapy (NAC), followed by surgery [23, 24]. However, not all EC patients benefit from NAC, and currently, responders cannot be identified before treatment because no predictive markers have been established. Patients who undergo neoadjuvant chemoradiotherapy (NACRT) or definitive chemoradiotherapy (CRT) and achieve pathological complete response (pCR) can be expected to survive longer [26], and previous studies demonstrated that surgery may be unnecessary in patients who respond to CRT [27]. On the other hand, the subgroup of patients who show no response to NACRT are only exposed to adverse events, and they could become inoperable cases. Therefore, it is critical to identify in advance the therapeutic effects of neoadjuvant and definitive CRT to prevent noneffective and potentially harmful therapies to nonresponding patients. Thus, stratification of patients according to biomarker expression will help to guide individualized treatment strategies for EC patients. Although several biomarkers for EC have been detected, such as serum squamous cell carcinoma (SCC) antigen (SCC-Ag), carcinoembryonic antigen (CEA), and serum anti-p53 antibody (p53-Ab) and CYFRA21-1 [28–31], more accurate molecular biomarkers that can be translated to widespread clinical practice are urgently required.

6.2.2 Tissue-Based Biomarkers

Multiple gene alterations are associated with the development and progression of EC; these include mutation, epigenetic alteration, messenger RNA (mRNA) expression, and protein expression [32]. In addition, a variety of changes can occur in cell-cycle regulation, growth factors and their receptors, and DNA repair system [33]. Recently, studies of the mutational landscape of esophageal squamous cell carcinoma (ESCC) patients have identified mutations in genes that regulate the cell cycle (*p53, CCND1, CDKN2A, FBXW7, RB1, NFE2L2*); epigenetic processes (*MLL2, EP300, CREBBP, TET2*); and NOTCH (*NOTCH1, NOTCH3*), WNT (*FAT1, YAP1, AJUBA*), and receptor-tyrosine kinase-phosphoinositide 3-kinase signaling pathways (*PIK3CA, EGFR, ERBB2*) [34–37]. In addition to these alterations, molecular alterations associated with the risk factors: tobacco smoking and drinking for SCC and Barrett's esophagus for adenocarcinoma may also be useful as biomarkers in EC [32]. To predict the response of neoadjuvant therapy, predictive biomarkers using a biopsy specimen have been identified at the level of alteration of genomic DNA, gene expression of mRNA, miRNA and protein expression, as well as the clinical factors such as age, histology, and tumor invasion [38]. Patients with normal *p53* have notable benefit from NAC with cisplatin/fluorouracil compared with those of mutant *p53* status [39]. Additionally, the expression of Rad51, which is a key factor in homologous recombination in DNA double-strand break repair in pretreatment biopsy specimens, was reported to be a predictive factor for response to NACRT [40].

Thus, comprehensive understanding of molecular characteristics of EC and identification of genetic biomarkers are important for diagnosis and evaluation of therapeutic effect. However, one of the main challenges is that the characteristics of the biopsy specimen do not reflect those of whole tumors because of intratumor heterogeneity. In addition, the characteristics of the biopsy or resected specimen from primary lesion are often not similar to those from metastatic (recurrent) lesion in the same patient.

6.2.3 Blood-Based Biomarkers (Liquid Biopsy)

6.2.3.1 Circulating Tumor Cells (CTCs)

Circulating biomarkers for EC represent a new approach for the early detection and monitoring of tumor dynamics [41]. Circulating tumor cells (CTCs), derived from the primary tumor, are thought to be the mechanism of tumor spread. These cells are released into the circulation and may form micrometastasis, which is a key initial step leading to recurrence and distant metastasis. Currently, in cancer research new CTC assays such as CellSearch System [42] are used in addition to conventional techniques such as immunohistochemical assay, cytomorphology with immunocytochemistry, RT-PCR, and nucleic acid sequence-based amplification [43, 44]. CTCs are related to tumor progression, relapse, metastasis, and patients' prognosis in ESCC [45, 46] as well as in patients with HNC, gastric cancer, and colorectal cancer [47–49]. In EC patients, previous meta-analysis demonstrated that the presence of CTCs is associated with poor OS and PFS, especially in Asian and SCC patients [50]. In addition, the monitoring of CTC status may be a promising indicator for evaluating the efficacy of chemotherapy or chemoradiotherapy [45].

6.2.3.2 Cell-Free DNA (cfDNA) and Circulating Tumor DNA (ctDNA)

cfDNA is present in the blood as small fragments emerging from dying cells undergoing apoptosis and necrosis. Therefore, cfDNA, which can be obtained in a noninvasive manner via "liquid biopsy," is a potential source of biomarkers. ctDNA is the tumor-derived cfDNA in the plasma of cancer patients and is often present in cancer patients without detectable CTCs. ctDNA has also become a promising biomarker for early detection, tumor progress monitoring, and resistance mutation identification. This is due to the fact that cancer-associated genetic alterations such as point mutation, copy number variations, chromosomal rearrangements, and methylation patterns can be detected in ctDNA. Since the amount of ctDNA in total cfDNA is small (< 1.0%), the detection and quantification of ctDNA are very challenging [51–53]. Through the use of digital PCR-based technology and deep-sequencing analysis in various cancer types, previous studies have reported a positive correlation between the concentration of ctDNA and tumor stage and volume [54, 55]. In ESCC patients, the feasibility of exome and targeted sequencing of ctDNA to detect somatic mutations and monitor the treatment effect has been demonstrated [56]. In addition, next-generation sequencing (NGS) of cfDNA in plasma of 13 patients with ESCC demonstrated that the original panel including 53 genes enabled the

diagnosis of tumor recurrence with greater accuracy than conventional tumor markers (SCC, p53-Ab) or imaging methods [57]. The low-cost and sensitive target multigene panel for deeper sequencing coverage could promote the clinical use of ctDNA as a noninvasive biomarker.

DNA methylation is a critical epigenetic mechanism that plays a crucial role in pathogenesis of ESCC [58, 59]. As a noninvasive test, blood sample collection is a promising route for clinical application of methylation screening. The methylation of *EPB41L3*, *GPX3*, and *COL14A1* genes of the circulating cfDNA was only found in ESCC patients' plasma, but not in normal individuals, and combined methylation analysis of these genes has the potential to be a valuable diagnostic tool of noninvasive testing (sensitivity, 64.3%; specificity, 100%) [60]. Moreover, hypermethylation of tumor-related genes (*RAR-β, DAPK, CDH1, p16, RASSF1A*) in cfDNA was detected in ESCC patients, and diagnostic accuracy was increased when methylation of these genes was analyzed in combination (sensitivity, 82.2%; specificity, 100%) [61].

6.2.3.3 Circulating MicroRNAs (miRNAs)

miRNAs are single-stranded, ~22-nucleotide-long, well-conserved, and noncoding RNAs that can regulate target gene expression at the posttranscriptional level. Aberrant expressions of miRNAs have been shown to be associated with promotion or suppression of metastasis in various types of cancers. Circulating miRNAs have drawn attention as biomarkers for gastrointestinal cancer [62, 63] because they are highly stable and resistant to degradation. Previous meta-analyses demonstrated that miRNA analysis may serve as novel noninvasive biomarkers for ESCC with excellent diagnostic characteristics [64], where elevated levels of miR-21 and miR-1246 and decreased levels of miR-375 are indicative of poor prognosis of EC [65–67]. Moreover, the profile of seven serum miRNAs (miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a, and miR-127-3p) has potential to serve as a noninvasive biomarker for diagnosing ESCC [68]. In terms of response to chemotherapy, the overexpression of miR-21 in plasma has been shown to contribute to chemoresistance and could be used as a biomarker for predicting chemoresistance in patients with ESCC [69]. The levels of circulating miR-200c can also be useful for predicting the response to NAC and prognosis in EC patients [70].

6.2.3.4 Autoantibodies Against Tumor-Associated Antigens (TAAs)

Autoantibodies, which are produced as a response to TAAs, are rarely observed in healthy individuals, and they can indicate malignant transformation before standard clinical tests and may be useful as early detection biomarkers. Compared with TAAs, the corresponding antibodies are likely to be stable and persistent in serum samples [71] and may serve as new screening markers [72–74].

Serum Anti-p53 Antibody

In a previous systematic review, the most frequently assessed autoantibody was anti-p53 [31]. *p53* is a tumor suppressor gene that plays a role in DNA repair and cell cycle arrest and is the most common mutation in cancers, including ESCC [32,

75]. The frequencies of *p53* gene mutations were between 17 and 84% of ESCC tissue [76] and are also found in dysplastic lesion, indicating an important role of *p53* abnormality in esophageal carcinogenesis. *p53* mutation can cause accumulation of nonfunctional, highly stable mutant protein that has a significantly longer half-life than the native protein. The accumulated p53 protein acts as an antigen, with subsequent production of p53-Ab, which are detected in tissue, blood, and other body fluid of various cancer type patients, including EC [77]. Therefore, p53-Ab in blood may be useful as a biomarker in the diagnosis of EC, since the p53 protein and p53-Ab are absent in normal plasma. Although, in previous meta-analyses, the sensitivity of circulating p53-Ab in serum was low, in spite of its high specificity [31, 78], the prognostic significance [79] and association with pathological tumor response to NAC [80] of serum p53-Ab have been documented in ESCC patients.

Other Autoantibodies

A previous systematic review reported 35 different circulating autoantibodies against TAAs such as p53, NY-ESO-1, HSP70, c-Myc, MMP-7, Prx VI, Bmi-1, p16, p62, Survivin, and CDC25B that could be useful for early detection of EC, and analysis revealed that for single autoantibodies, specificity was very high (median 98.3%), but sensitivity was low (median 26.7%) [31].

The development of multi-marker panels of autoantibodies could be a promising approach for early detection with higher sensitivity and specificity. Based on the high specificity for EC, serum NY-ESO-1 antibodies were reported to be one of the first choices for EC, even in patients with stage I tumors [81], and a clinical trial has shown that it is well tolerated and antibody responses to NY-ESO-1 vaccine were elicited in ESCC patients [82].

6.2.3.5 Serum Tumor Markers

Serum tumor markers are measurable serum molecules that can be useful in the diagnosis, prognosis, and clinical management of patients. A meta-analysis demonstrated that CEA Cyfra21–1, p53-Ab, SCC-Ag, and VEGF-C are highly specific, but not sufficiently sensitive to diagnose EC [83]. To increase the power of EC detection, it is essential to identify patterns of multiple biomarkers for combined detection, such as autoantibodies in EC patients. With regard to treatment response and prognosis, ESCC patients with combined high pre-NACRT serum VEGF-A and TGF-β1 levels measured using proximity ligation assay (PLA) followed by enzyme-linked immunosorbent assay (ELISA) had significantly worse survival rates [84]. In addition, serum profiling of 84 cytokines in ESCC patients with NACRT plus surgery revealed high levels of serum soluble interleukin-6 receptor, and this was correlated with a poor response to preoperative therapy and unfavorable outcome [85], probably because persistent systemic inflammatory host status causes a possible mechanism of resistance to NACRT. Regarding the epigenetic biomarkers, a previous meta-analysis demonstrated that the area under the summary receiver operating characteristic curve (AUC) for diagnosis of EC based on *CDKN2A* methylation were 0.82 in tissue samples and 0.90 in blood samples, respectively, and the pooled

odds ratio (OR) of blood samples (OR = 34.98) was remarkably higher than that of tissue samples (OR = 11.60). Those results suggested that hypermethylated *CDKN2A* may be a useful noninvasive biomarker for blood detection [86].

6.2.3.6 Exosome

Exosomes are small extracellular vesicles that range from 40 nm to 150 nm in size and contain DNA, mRNA, ncRNA (miRNA, lncRNA), proteins, and lipids. Exosomes are thought to have some function, particularly in signal transduction, and play an important role in cancer progression [87, 88]. Although it is difficult to differentiate whether exosomes originate from the tumor itself or as a result of the host response against the tumor, contents of exosomes have been of interest in cancer research, especially as potential diagnostic markers. Exosomal miR-21 expression was upregulated in serum in ESCC patients compared with that in benign control group and was associated with tumor progression and aggressiveness [89]. A previous report investigating healthy controls and Barrett's esophagus demonstrated combined serum exosomal miRNA ratios have the potential to be used as biomarkers for the detection of esophageal adenocarcinoma [90]. Moreover, the amount of exosomes in the plasma, as measured using the acetylcholinesterase (AChE) activity, was an independent prognostic marker, and low levels of exosome showed poor prognosis in ESCC patients [91].

6.2.4 Biomarkers Using Breath and Headspace Vapor (Gas Analysis)

Gene and/or protein alterations in tumor development may lead to peroxidation of the cell membrane species and emission of volatile organic compounds (VOCs). There has been growing interest in the measurement of VOCs in breath as optimal noninvasive marker. Previous report identified approximately 2000 different VOCs in breath, even from normal healthy volunteers [92]. Breath VOCs have already shown clinical usefulness as biomarkers for various cancers including gastric cancer [93], colorectal [94], lung cancer [94, 95], breast cancer [94, 96], and prostate cancer [94]. Moreover, distinct exhaled breath VOC profiles can distinguish patients with esophageal and gastric adenocarcinoma from noncancer controls [97]. Recently, a new methodology using a modified proton-transfer-reaction mass spectrometer (PTR-MS) to evaluate the ionic characteristics of exhaled VOCs was applied and differentiated between the EC patients and the healthy people [98]. In addition, the quantification of VOCs in the headspace vapor of urine samples [99] and gastric contents [100] from gastric or EC using selected ion flow tube mass spectrometry (SIFT-MS) have been reported. In urine samples, ROC analysis using the concentration of six VOCs (acetaldehyde, acetone, acetic acid, hexanoic acid, hydrogen sulfide, and methanol) resulted in AUC of 0.904 [99]. In gastric contents, ROC analysis using the concentration of four VOCs (acetaldehyde, formaldehyde, hydrogen sulfide, and methyl phenol) resulted in AUC of 0.9 [100]. These results demonstrate the potential for VOC profiling as a new screening test in gastric or EC.

6.2.5 Future Directions

Considering the worldwide incidence of EC, new diagnostic and prognostic approaches are needed to improve the clinical management and survival rates.

Present surveillance and diagnostic methods for EC are invasive and expensive. Although there is current development and validation of new less invasive biomarkers for EC, translation to clinical practice has been slow. Since circulating markers in blood via liquid biopsy were discovered, they have offered a promising outlook for the improvement of diagnosis and prognosis of EC. In addition, a combined approach of multi-marker panels using state-of-the-art bioinformatics has potential to improve specificity. It will also be necessary to investigate the biomarker utility of the other liquid biopsy sample mediums such as saliva and urine. Furthermore, although it is critical to understand the mechanisms of production of VOCs in cancer cells, thanks to the development of VOC detection technologies, breath and headspace vapor are potential odorant biomarkers [101]. However, the detailed investigation of biomarker focused on the differentiation between SCC and adenocarcinoma was lacking despite their distinct molecular characteristics. To prevent EC, it is essential to educate the public about the dangers of carcinogens, such as alcohol and cigarette smoke, with respect to SCC [32] and Barrett's esophagus in adenocarcinoma [102], and to detect this disastrous disease at the early stage. The development of novel biomarker assays is urgently required to improve diagnostic and therapeutic approaches of EC.

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Biomarkers in Gastric Cancer

7

K. Yamashita

Abstract

The most major biomarkers of gastric cancer are serum CEA and CA19-9, and they are increased in serum of adenocarcinoma patients. Both markers do not increase during early-stage carcinogenesis, so they are not useful for early diagnosis of gastric cancer. In the outpatient center after gastrectomy, however, simple biomarkers are still convenient, and serum RNA (microRNA and long noncoding RNA) biomarkers are newly emerging, as they exhibit higher sensitivity than the conventional serum markers. Gastric cancer is characterized by unique progression styles such as peritoneal dissemination, lymph node metastasis, and liver metastasis, which are consistent with macroscopic appearance of the tumors. Macroscopic type I/II gastric cancer tended to metastasize to liver, while type III/IV gastric cancer is prone to peritoneal dissemination. Increased trend of type III/IV gastric cancer has been lately recognized in developed countries, which has thus demanded diagnostic markers of peritoneal dissemination in gastric cancer clinics. Cytology test by microscopy is a gold standard for diagnosis of peritoneal remnant disease, but its sensitivity is insufficient because gastric cancer with negative cytology test frequently recurred in the peritoneum, especially in type III/IV gastric cancer. Highly sensitive biomarkers to detect minute peritoneal dissemination should be urgently required. Nevertheless, either CT or even PET-CT is unreliable to diagnose peritoneal recurrence even in combination with serum biomarkers, and the best tool to confirm the peritoneal recurrence is diagnostic laparoscopy at present. DNA markers using promoter DNA methylation of the tumor suppressor genes also have a great potential to detect early recurrence of the peritoneal dissemination on the diagnostic laparoscopy.

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Keywords
Gastric cancer · Serum · RNA · DNA · Peritoneal dissemination

7.1 Introduction

Gastric cancer is the fifth most common malignancy and the third cause of cancer-related deaths worldwide and increasing, accompanied by population aging [1]. Adenocarcinoma is the dominant histology of gastric cancer, and both intestinal-type and diffuse-type gastric cancers are distinct histological entities. The former is caused by chronic inflammation of persistent *Helicobacter pylori* infection in the normal gastric mucosa or precancerous lesions such as metaplasia [2], and the latter is more affected by genetic factors such as *CDH1*/*RohA* abnormalities [3]. Cancer Genome Atlas Network elucidated the four distinct molecular categories in gastric cancer, which are designated as EBV (Epstein-Barr virus) associated, MSI (microsatellite instability) associated, GS (genomic stable), and CIN (chromosomal instable) gastric cancers [4]. EBV associated, MSI associated, and CIN gastric cancers are characterized by *p16* gene silencing together with *PI3K* gene mutation, *MLH1* gene silencing, and *p53* gene mutation, respectively, and prone to intestinal-type gastric cancer, while GS gastric cancer is enriched in disuse type histology.

Gastric cancer is characterized by unique progression styles such as peritoneal dissemination, lymph node metastasis, and liver metastasis, which are consistent with macroscopic appearance of the tumors [5, 6]. Type I/II gastric cancer tended to metastasize to liver like colorectal cancer, while peritoneal dissemination is frequently seen in type III/IV gastric cancer. Intriguingly, type I/II gastric cancer included more intestinal-type histology, while type III/IV gastric cancer is usually diffuse-type histology [7]. The most unique finding is that type IV gastric cancer designated as scirrhous type is inevitably diffuse-type gastric cancer and exhibited the most dismal prognosis among gastric cancer [5].

Diffuse-type gastric cancer is characterized by deeper invasion and emerging peritoneal disease, which could explain its poor prognosis. Reflected on this unique distribution of the prognostic features, intestinal type is occupied half of total gastric cancer, while 70% of the recurrent disease are diffuse-type gastric cancer. Recent increased trend is observed in diffuse-type gastric cancer rather than in intestinal-type gastric cancer [8], which may be explained by prevalent western lifestyle accompanied by increased frequencies of gastroesophageal reflux disease in developed countries, which has highly demanded diagnostic markers of peritoneal dissemination and novel therapeutic strategies of the minimal residual disease in the peritoneum.

7.2 Plasma/Serum Conventional Biomarkers for Gastric Cancers

The most major biomarkers of gastric cancer are serum CEA and CA19-9 [9]. CEA is a glycoprotein designated as carcinoembryonic antigen, and CA19-9 represents sialyl Lewis a (SLA) epitope recognized by mouse monoclonal antibody NS19-9

largely on mucin in blood, and they are increased in serum of adenocarcinoma patients including gastric cancer.

CEA is a large protein with molecular weight of 180–200 kDa. CEA that was administered into blood was proved to be accumulated in the liver, and macrophage cells designated as Kupffer cells in the liver which are treated with CEA increased inflammatory cytokines such as interleukin-6, interleukin-10, and TNF-alpha [10], suggesting that CEA in blood can make the specific niche which can support liver metastasis. Actually, increased serum CEA is consistent with liver metastasis in colorectal cancers. Even in gastric cancer, serum CEA is often increased in gastric cancer with liver metastasis. However, serum CEA is elevated approximately in 10, 20, 25, and 40%, in gastric cancer with stage I, II, III, and IV, respectively [9]. Prognosis of gastric cancer with stage I/II is very excellent, so it is important for serum biomarkers to diagnose gastric cancer with stage II (~80% of survival rate) [11, 12]. Due to its low frequencies (~20%) of gastric cancer with stage II, it is not useful for serum CEA to diagnose early-stage gastric cancer [9].

CA19-9 is different from CEA, because it is not protein itself. It represents sialyl Lewis a (SLA) antigen epitope on the glyco-chain conjugated with Ser/Thr on the mucin (O-glycan, e.g., MUC1 [13]) or proteins (N-glycan, e.g., CD44 [14]). Usually, serum CA19-9 in cancer patients is supposed to represent serum mucins. Mucin is a huge molecule with molecular weight of 1000–10,000 kDa and plays an important role of immunological signal transduction as robust amplifiers in blood. Representative structures of mucins are shown in Fig. 7.1b, where tandem repeats of the O-glycan are considered to multiply the signals [15, 16]. Serum mucins from the cancer patients can bind with Siglec family on inflammatory cells. We previously demonstrated that mucins from the cancer patients showing high serum CA19-9 can bind with immature dendritic cells (DC) through Siglec-9 and suppressed IL-12 production [13]. This finding suggested that serum CA19–9 may represent control immunosurveillance in the host of the cancer patients. Serum CA19-9 can at least partially reflect CA19-9 immunostaining of the tumor cells in gastric cancer and supposed to indicate suppression of the immune status. Serum 19-9 of pancreatic cancer is much higher than other gastrointestinal adenocarcinoma, and CA19-9 immunostaining is recognized in the tubular cells of pancreaticobiliary

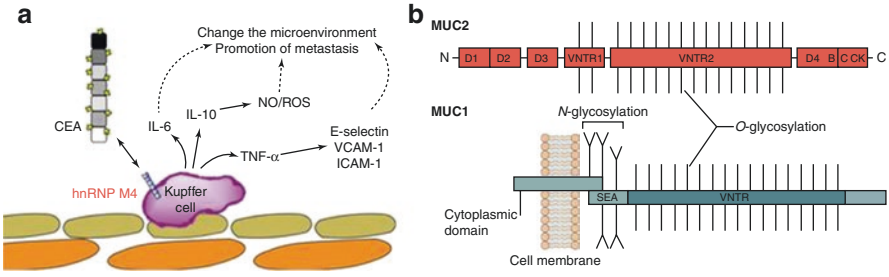

Fig. 7.1 (**a**) CEA can bind hnRNP 4 on Kupffer cells in the liver, and the Kupffer cells induced various cytokines to make microenvironment suitable for metastasis of cancer cells. The figure is modified in reference [10]. (**b**) Molecular structures of the representative mucin. Both MUC1 and MUC2 harbor variable numbers of tandem repeat (VNTR) domains, with multiple O-glycosylations which can amplify the signal to immune cells. This figure is modified in reference [17]

tract, and preoperative treatments for obstructive jaundice can remarkably decrease serum CA19-9 so much, suggesting that serum CA19-9 is not always representing mucins produced only from tumor cells. Serum CA19-9 is detected in gastric cancer with stage I, II, III, and IV in about 10, 20, 30, and 40%, respectively [9]. The frequencies are also similar with serum CEA. Due to its low frequencies of gastric cancer with stage II (~20%), it is not useful for serum CA19-9 to diagnose early-stage gastric cancer, either.

In addition to these serum markers, several glyco-antigens were potential alternatives like sialyl Tn (STN), CA72-4, and CA125, which showed low sensitivity of gastric cancer, either; serum CA72-4 is a bit higher than either serum CEA or serum CA19-9 [9], although lower specificity (elevation in other diseases) is frequently experienced in the outpatient center. Such alternative serum markers uniquely represent specific situation of various clinical aspects of gastric cancer. For example, STN immunostaining is significantly correlated with lymph node metastasis, and serum STN may represent aggressive lymph node disease [18]. With regard to CA72-4 or CA125, they were reported to be significantly associated with peritoneal dissemination [19]. The CA125 level was significantly correlated with the degree of peritoneal dissemination and the existence of malignant ascites. About half of the peritoneal recurrences are positive for serum CA125, so it could be well referred in such specific clinical situation. Nevertheless, as the best optimized diagnostic tool, serum markers are considered as less powerful as compared to the recent excellent diagnostic modalities such as staging laparoscopy due to its capacity to explore the pathological finding. That is why the conventional serum biomarkers are considered as supplemental tools for diagnosis of gastric cancer.

7.3 Plasma/Serum RNA Biomarkers for Gastric Cancers

In order to increase sensitivity of gastric cancer diagnosis, noncoding RNA is promising. Noncoding RNAs are largely composed of microRNA (miR) and long noncoding RNA (lncRNA). miR is a single-strand RNA length ranged from 21 to 25 base pairs (bp) and processed from primary microRNA transcripts (pri-miRNA), while lncRNA is defined as the size longer than 200 bp. miR can regulate target gene expression by binding to their 3'untranslated region, while lncRNA can antagonize it by its hybridization with miR as a molecular sponge. Interestingly, lncRNA cannot be degraded by RNase, and miR or lncRNA derived from primary cancer can be detected in serum/plasma of the cancer patients.

Recent review articles presented the representative noncoding RNA biomarker candidates such as let-7a, miR-21, miR-93, miR-192a, miR-18a, and miR-10b in plasma/serum of gastric cancer patients, where they are usually onco-miR which is overexpressed in primary cancer tissues [20]. The biomarker candidates showed significantly high AUC of the ROC curve than the conventional serum biomarkers; however large-scale validation is not sufficient at present. On the other hand, there have been a few reports regarding circulating lncRNA of serum/plasma of gastric cancer patients. H19 is the lncRNA located on chromosome 11p15 and a precursor

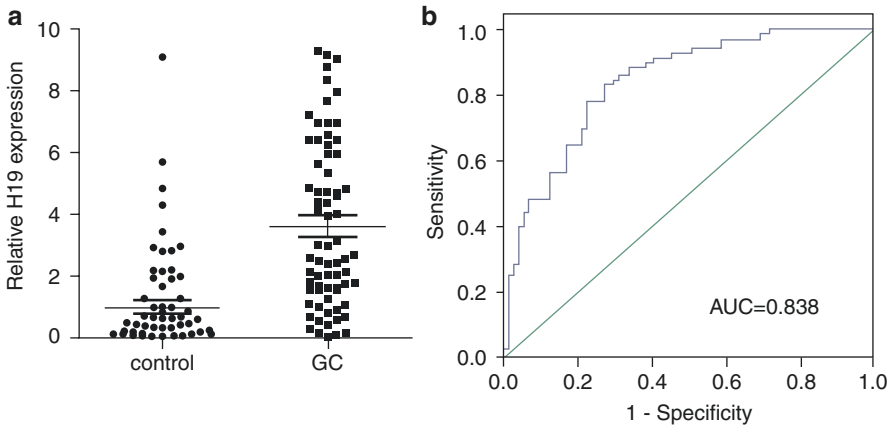

Fig. 7.2 (**a**) The lncRNA H19 in plasma as a novel biomarker for diagnosis of gastric cancer. LncRNA detected by PCR is significantly elevated in plasma of gastric cancer patients as compared to the control patients. (**b**) ROC curve of plasma H19 to diagnose gastric cancer. AUC shows 0.838. This figure is cited by reference [22]

of onco-miR-675 and involved in sponge antagonism for critical tumor suppressor-miR such as let7, miRNA-200, and miRNA-34, targeting to Cbl, Zeb1, Snail1, IGF2BP3, and c-myc [21]. H19 in plasma was recently proved to be elevated in primary gastric cancer by independent research groups [22] (Fig. 7.2) and promising for early detection of gastric cancer even in the outpatient center. H19 can be detected because lncRNA was not degraded by RNase in human blood [22].

7.4 Peritoneal Solution Biomarkers of Gastric Cancers

Pathological cytology test is insufficient diagnostic tool for microscopic peritoneal dissemination, because gastric cancer with negative cytology test often recurred in the peritoneum, especially in type III/IV gastric cancer [5]. Highly sensitive biomarkers to detect minute peritoneal dissemination are thus urgently demanded. As either CT or PET-CT is unreliable to diagnose peritoneal recurrences, either, the best diagnostic tool to confirm the peritoneal recurrence after gastrectomy is the diagnostic laparoscopy at present. On the other hand, in the outpatient center after gastrectomy, simple biomarkers are convenient such as serum markers, and serum CA125 can more accurately represent peritoneal dissemination as compared to other serum markers [19]. Nevertheless, it can never overcome diagnostic capacity by the diagnostic laparoscopy, because it can directly obtain the pathological cytology and/or histological test samples.

DNA markers have emerging potential to detect minimal residual disease even in CY0 patients due to its high sensitivity, hence predicting early recurrence of the peritoneal dissemination even on diagnostic laparoscopy. CEA mRNA is known as such a representative DNA marker [23]. For this assay, mRNA was transcribed to

Table 7.1 Diagnostic outcomes of DNA CY test according to mRNA, nonHRMG, and HRMG

	mRNA	NonHRMG	HRMG
Sample stability	△	○	○
Diagnostic sensitivity	23%	25%	20%
Diagnostic sensitivity in CY0	10%	7% (~pT2)–20% (pT3~)	10%
Diagnostic sensitivity in CY1	69%	75%	100%
Marker	CEA	BNIP3/CHFR/CYP1B1/MINT25/SFRP2/RASSF2	CDO1
References	[23]	[24]	[25]

HRMG highly relevant methylation gene

cDNA for PCR amplification for the peritoneal lavage solution, so investigation of CEA mRNA expression was included among the DNA cytology test. Although it has several weak points with regard to molecular stability (mRNA), sensitivity (in stage IV), or specificity (expression of the non-cancerous mucosa tissues) (Table 7.1), it can actually detect minimal residual disease of the peritoneum much more than the conventional cytology test and predict poor prognosis.

Recently, epigenetic DNA biomarkers are newly emerged [26, 27]. Promoter DNA methylation of the tumor suppressor genes are candidates such as CDO1 in gastric cancer. Diagnostic performance of the promoter DNA methylation can be alternative to that of CEA mRNA in the peritoneum, if several markers were combined. For example, combination of the BNIP3, CHFR, CYP1B1, MINT25, SFRP2, and RASSF2 genes was used for detection of the minimal residual disease in peritoneum, and the diagnostic performance mimics those of the cytology test by using CEA mRNA [24].

On the other hand, rigorous comprehensive exploration for cancer-specific promoter DNA methylation identified eminent DNA biomarkers such as CDO1 [27]. Conveniently, only one DNA marker could override the six DNA biomarker combinations, putatively due to its high potential to diagnose the tumors from the corresponding normal tissues. Such excellent markers had been discovered by pharmacological unmasking microarray for champion genes for several decades. DNA CY1 diagnosed by CDO1 methylation showed twofold higher sensitivity than the conventional cytology test, and all CY1 cases were positive for DNA CY1 test [25] (Table 7.1). Moreover, it can predict future recurrences of the peritoneal disease [25]. Now, the prospective clinical trials using 400 samples are being conducted to demonstrate diagnostic accuracy and prediction (UMIN000026191), and digital PCR will augment the diagnostic accuracy [28]. Finally the weak point of this therapeutic strategies is that prognostic efficacy of the interventional chemotherapy against such minimal residual disease is unknown at present, even though it could be feasible.

7.5 Conclusion

Conventional blood biomarkers such as CEA and CA19-9 are elevated in a portion of gastric cancer patients; however they are not useful to diagnose early-stage disease. On the other hand, the newly emerging blood RNA markers are promising for

cancer surveillance, because they are more frequent than the conventional blood biomarkers. Nevertheless, importantly, diagnosis of peritoneal dissemination of gastric cancer is highly demanded in gastric cancer clinic, as gastric cancer phenotypes (diffuse-type histology representing macroscopic type III/IV gastric cancer) representing peritoneal disease progression are increasing. Biomarkers using promoter DNA methylation of the tumor suppressor gene have a great potential to detect such minimal residual disease in the peritoneum, and digital PCR will augment the diagnostic accuracy in the near future.

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Liquid Biopsy in Hepatocellular Carcinoma

8

Eiichiro Suzuki, Tetsuhiro Chiba, and Naoya Kato

Abstract

Hepatocellular carcinoma is the major cancer-related death worldwide, and the prognosis is grim. Therefore, there is an urgent need of detection at early stage, effective treatment options, and prevention of recurrence. Recently, liquid biopsies, such as circulating tumor cells, circulating tumor DNA, and microRNA, have accumulating evidence that is useful for these needs.

In this review, we summarize the current advance of liquid biopsies and discuss the perspective.

Keywords

Hepatocellular carcinoma · Liquid biopsy · Circulating tumor cell · Circulating tumor DNA · MicroRNA

8.1 Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death, and its incidence has been increasing worldwide [1]. In Japan, HCC is the fifth most common cause of cancer-related death [2]. Due to advances in medical technology, mainly in the field of imaging, the rate of early detection of HCC has been increasing, and the emergence of molecularly targeted therapies has enabled HCC patients to receive systemic chemotherapy [3]. However, in many patients,

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the cancer is still detected in the intermediate or advanced stages, and these patients cannot receive curative treatments such as resection or radio frequency ablation. Even after curative treatments, the recurrence rate is high due to the existing liver damage [4].

For advanced cases, systemic chemotherapies have been the main treatment option. Currently, three molecularly targeted anticancer drugs—sorafenib [5], regorafenib [6], and lenvatinib [7]—are available for advanced HCC. However, due to the lack of a biomarker that can aid decision-making regarding which drug to use, these three drugs are generally used according to the physician's choice in daily practice.

To help physicians choose the appropriate drug, liquid biopsies have already shown promising applications in clinical settings for some cancers, including colorectal cancer [8], breast cancer [9], and lung cancer [10]. These biopsies, which can be performed on blood or body fluids, provide genetic and epigenetic information and can be performed repeatedly with low invasiveness. Also, they provide real-time information about cancer status. In this paper, we review liquid biopsies, focusing particularly on blood-based circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and microRNAs (miRNAs) from blood samples in HCC cases (Fig. 8.1). We also discuss current perspectives on these procedures.

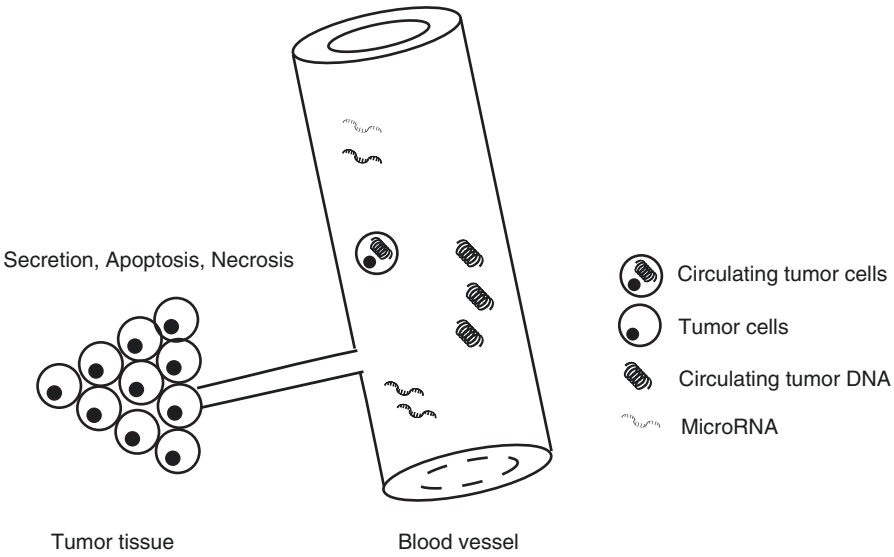

Fig. 8.1 Circulating tumor cell, circulating tumor DNA, and microRNA as liquid biopsy

8.1.1 Circulating Tumor Cells

The path to tumor metastasis mainly begins with tumor cells in systemic circulation [11]. CTCs originate from a primary organ and reach distant metastatic sites without being blocked by a barrier system; the idea for how this occurs was originally based on the "seed and soil" theory [12]. CTCs play an important role in diagnosis, because finding them helps in the early detection of cancer, and CTC analysis offers valuable information including DNA, RNA, and protein levels. This is because CTCs reflect the real status of the cancer, unlike tissue biopsies, which are collected before treatment.

Various techniques for liquid biopsies have been developed, mainly divided into either physical or biological methods. Recently, biological methods using antibody-based enrichment techniques have been developed. In particular, CellSearch, which can detect the epithelial cell adhesion molecule (EpCAM), was approved by the Food and Drug Administration in the USA (Fig. 8.2) [13]. This system consists of a CellPrep system, a CellSearch epithelial cell kit, and a CellSpotter analyzer. It

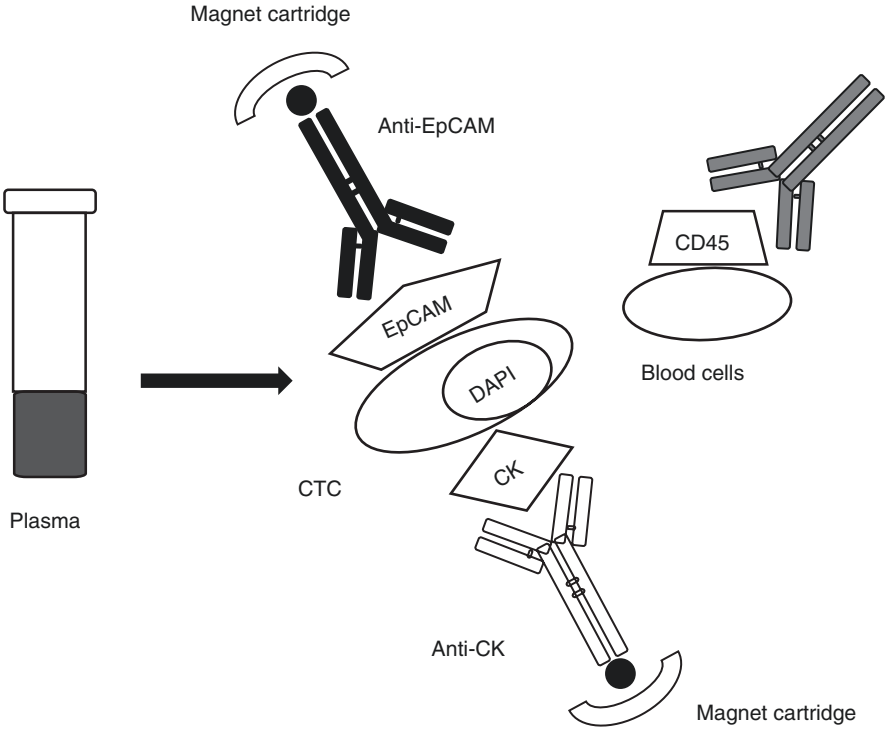

Fig. 8.2 CellSearch system technology. *CTC* circulating tumor cell, *EpCAM* epithelial cell adhesion molecule, *CK* cytokeratin

enumerates CTCs of epithelial origin in whole blood using antibodies that bind to cytokeratins 8, 18, and 19, an antibody to CD45 conjugated to allophycocyanin, and the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI). A meta-analysis with 998 HCC subjects including advanced cases showed a pooled sensitivity of CTC detection of 67% at a pooled specificity of 98% [14].

One limitation of early detection is poor sensitivity; with current techniques, it is difficult to detect a sufficient number of tumor cells with a high sensitivity and specificity, especially in early stages [15]. In addition, the epithelial-mesenchymal transition (EMT) is an important event that occurs when tumors shed into metastasis, and the EMT may disrupt epithelial cells and EpCAM expression [16]. In fact, only 35% of HCC-derived CTCs express EpCAM [17]. To overcome these challenges, new innovations are needed to detect higher numbers of CTCs and greater amounts of specific antibodies in addition to EpCAM.

8.1.2 Circulating Tumor DNA

Circulating tumor DNA (ctDNA) consists of extracellular nucleic acid fragments that flow into the plasma from tumor cells [18]. The mechanism of DNA shedding involves necrosis, apoptosis, and secretion by tumor cells. The concentration of ctDNA in a healthy individual is 1–10 ng/ml, and cancer patients have a higher amount [19]. Tumor-derived DNA fragments are shorter than non-tumor-derived DNA fragments, and after curative treatment, cell-free DNA (tumor- plus non-tumor-derived DNA) integrity increases and returns to normal levels [20]. This suggests that DNA integrity may be a potential predictive marker for the diagnosis, treatment, and monitoring of HCC. Compared to CTCs, the fraction of ctDNA can be more easily detected [21], and ctDNA shows the total heterogeneity of all the tumors and thus reflects the total tumor status.

Recent studies have focused not only on quantitative analysis but also on aberrations and methylation of DNA. The utility of analyzing ctDNA by next-generation sequencing (NGS) has been increasing. In addition, recent whole-genome sequencing showed the presence of somatic mutation of *TERT* promoter, the *WNT/beta-catenin* pathway, the *PI3K-AKT-mTOR* pathway, the *MAPK*-pathway in patients with HCC [22]. Ikeda et al. analyzed 32 ctDNA fragments in 26 HCC patients and detected 100% genetic alteration and serial ctDNA emergence of a new gene alteration [23]. Although the ctDNA demonstrated excellent sensitivity and specificity, the detection rate was strongly affected by the tumor burden and tumor type, namely, a rate of somatic mutation is 15–56% for operable cases; on the other hand, in advanced stages, the ctDNA detection rate rose to 74–88% [24]. Recently, Cai et al. analyzed 574 genes from the ctDNA of 4 HCC patients; 96.9% of these patients' tissue mutations could also be detected in plasma samples [25]. The authors concluded that ctDNA could overcome the issue of tumor heterogeneity and track therapeutic responses in real time.

It is well known that methylation is a common epigenetic regulation mechanism and that the methylation of suppressor genes has been connected with carcinogenesis. Thanks to the development of NGS techniques, methylation can aid in the diagnosis of HCC [26].

Xu et al. have developed HCC-specific methylation marker panels comparing HCC tissue and peripheral blood mononuclear cell methylation as high as 1000 cases [27]. Compared to normal controls, this model achieved 85.7% sensitivity and 94.3% specificity. The main limitation of this method is the low detection rate in early stages, as mentioned above.

8.1.3 MicroRNAs

miRNAs are small, noncoding, interfering RNAs that are 21–30 nucleotides long; they play a crucial role in cellular processes and carcinogenesis [28]. Namely, the upregulation of miRNAs inhibits tumor suppressor genes, and the downregulation of miRNAs inhibits oncogenes. A total of over 2300 miRNAs have been identified. Recent studies have shown that miRNAs are associated with pathogenesis of cancers and not only genetic but also epigenetic modification of carcinogenesis [29, 30].

miRNAs have two ways of expanding into the bloodstream. One is secretion through cell death by apoptosis and necrosis, and the other is release in a package into a small membrane vehicle called an exosome. In the plasma, the miRNA binds to particular proteins such as Argonaute 2 and high-density lipoproteins and escapes RNase [31]. Cancer cells secrete exosomes with miRNAs to assist their invasion or metastasis.

Among miRNA groups, certain specific miRNAs are associated with HCC prognosis. In the liver, some miRNAs are associated with HBV and HCV infection and liver fibrosis. For example, the expression of miR-34 has been shown to be increased in patients with hepatic fibrosis, HCV, alcoholic disease, and NAFLDS, in addition to HCC [32].

Li et al. first demonstrated that miRNA expression profiles could be useful biomarkers for distinguishing between HCC with no HBV infection and HBV-positive HCC [33]. Zhou et al. identified using seven miRNAs and improved the diagnostic accuracy of HCC [34]. More recently, Lin et al. analyzed over 300 HCC and non-HCC patients and identified 7 different miRNAs (miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505) with higher sensitivity than AFP and a similar specificity [35]. Based on these data, in China, this system has been approved for clinical use and is now widely available.

Regarding recurrence, miR122, miR26a, and miR29a may be predictive markers for recurrence [36]. If the quantity of these miRNAs with tumor markers can be monitored, effective treatment can be possible. The limitation of miRNAs is their diagnostic accuracy. The measurement methods used are quantitative RT-PCR, microarray, and NGS, and it is difficult to compare among these methods.

8.2 Perspective

Compared to their use in other malignancies such as lung cancer, breast cancer, and colon cancer, the advance of liquid biopsies in the field of HCC has been slow. One reason is the typical HCC problem of background liver damage. Most HCCs occur in the damaged liver due to HBV, HCV, and nonalcoholic steatohepatitis, and the difference in etiology may affect the results of mutation or the quantitative amount of CTCs, ctDNA, and miRNA. In addition, when comparing HCC data with controls, the controls depend on the background of the liver, and careful attention is needed to interpret the results.

Recently, a blood test called CancerSEEK, which uses protein biomarkers and somatic mutations for the early detection of HCC and seven other cancers, has shown promising results (Fig. 8.3) [37]. Surprisingly, the overall sensitivity and specificity are 98% and 99%, respectively, and the sensitivity for HCC is highest among stage I cancer cases. In addition, the plasma from all HCC cases has been shown to have at least one mutation in 16 genes including *TERT*, *TP53*, and *PIK3CA*. These results suggest that the detection rate of HCC, which is expected to have low sensitivity and specificity of detection due to background liver damage, can be made high by combining a protein marker with genetic alteration. However, although healthy individuals were chosen as the controls, the control panels were affected by inflammation and cirrhosis. The CancerSEEK method is also attractive in terms of its low cost. The research teams have tried to keep the cost below $500 per sample.

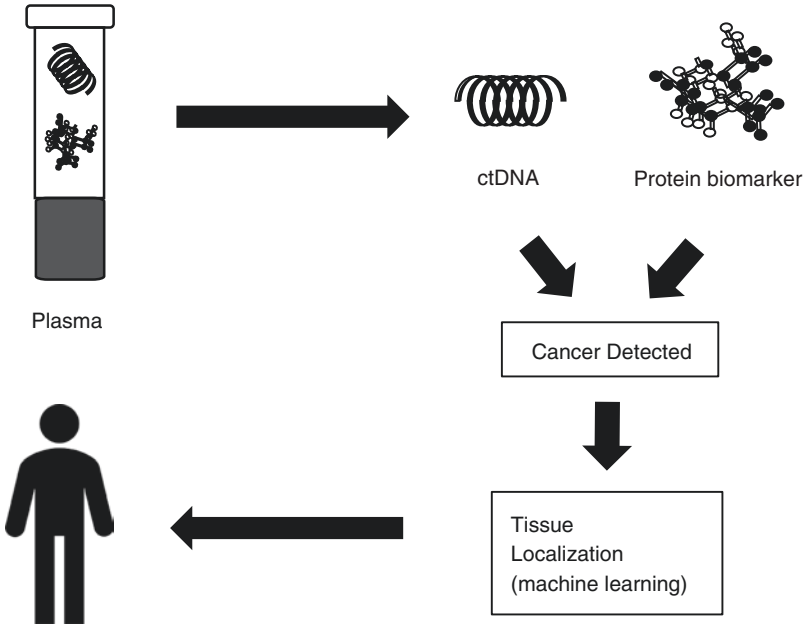

Fig. 8.3 CancerSEEK system

If this is possible, the era of precision medicine, in terms of not only sensitivity and specificity but also low cost, will soon arrive.

For a decade, clinical trials have been conducted on new, effective molecularly targeted drugs. In addition to "all comer" clinical trials, some trials have focused on somatic mutations. If these mutations can be identified by CTCs or ctDNA, then suitable drugs against cancer can be found. For example, a copy number gain for FGF19 and FGF/FGF4 amplification is a predictive marker for sorafenib response [38, 39]. In non-small-cell lung cancer, the use of the *EGFR* mutation in liquid biopsies is now widely available. If such changes can be found before systemic chemotherapy, HCC patients are expected to achieve a good response.

Nowadays, clinical trials using immune checkpoint inhibitors are being widely conducted, including for HCC. In the USA, the PD-1 antibody nivolumab was approved for sorafenib-failed advanced HCC [40]. In non-small-cell lung cancer, PD-1 expression in over 50% of tumor cells was correlated with the improved efficacy of pembrolizumab [41]. Whether the amount of PD-1 expression is also a predictive marker in HCC is controversial; however, the amount of quantitative change in expression may be a predictive marker for efficacy in patients with HCC receiving immune checkpoint blockers.

In conclusion, we review liquid biopsies in patients with HCC. Liquid biopsies are useful as diagnostic, therapeutic, prognostic factors and will play a crucial role of the new era of precision medicine.

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Biomarkers of Pancreatic Cancer

9

Takahiro Kishikawa, Minoru Tada, Motoyuki Otsuka, and Kazuhiko Koike

Abstract

Pancreatic cancer is regarded as one of the most intractable cancers. One reason for its poor survival rate, despite remarkable developments in anticancer therapy, is the lack of useful biomarkers for detecting early-stage disease. Consequently, there is an urgent need to develop biomarkers that enable the detection of early-stage tumors. Emerging evidence suggests that tumor cells release substantial amounts of nucleic acids into the bloodstream. Recent genomics technologies and the innovation of measuring platforms make it possible to identify novel nucleic acid biomarkers at sufficient levels for quantitative analyses. One of the most highly assessed circulating tumor DNAs, *KRAS* gene mutation, is observed in most pancreatic cancers. It is released from tumor cells and can be detected in the bloodstream and pancreatic juice, demonstrating its use in predicting prognosis and treatment response as well as a detection marker. On the other hand, the expression profiles of circulating cell-free RNAs, such as microRNAs and long noncoding RNAs, are drastically altered during oncogenesis and treatment. They are also important functionally as a tool for cell-to-cell communication. Although further technical advances in cost-effectiveness and reproductivity are required for their clinical use, circulating nucleic acids may be able to identify novel biomarkers that will help improve the prognosis of pancreatic cancer.

Keywords

Biomarker · Pancreatic cancer · Circulating tumor DNA · Cell-free RNA

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9.1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most intractable cancers. Its recurrence rate continues to increase, and it has become the third leading cause of cancer death, surpassing breast cancer. The incidence and death rate of pancreatic cancer are high. More than half of pancreatic cancer patients are diagnosed at an advanced stage. Despite remarkable developments of anticancer drugs, such as molecular targeting therapy and immunotherapy, the 5-year survival rate remains below 10%, which is significantly low compared to other cancers [1]. The most important goal for patients with PDAC is to establish a relevant and highly sensitive tool that enables its early detection [2].

Circulating biomarkers are useful for screening because of the ease of sample collection and their relatively noninvasive nature; moreover, repeated collection for monitoring diseases is possible. Several biomarkers are routinely adopted for specific cancer, such as SCC, PSA, and CA125; however, there is only one molecule, CA19-9, that has been approved by the US FDA for monitoring the progression and therapeutic response of PDAC. Although CA19-9 is widely used for monitoring the response to chemotherapy or surgical dissection, its sensitivity and specificity are insufficient as a diagnostic biomarker of early-stage pancreatic cancer [3, 4].

The fields of genomics, proteomics, and molecular imaging have recently matured expeditiously. This progress not only contributes to our understanding of the complicated and heterogenic landscape of cancer but also sheds light on the minor factors and extremely minute targets that had been impossible to detect. Furthermore, innovative technologies improve them to a level where they are acceptable for clinical use in both the aspects of reliability and cost-effectiveness. Several types of emerging biomarkers have been identified, including glycoproteins, circulating tumor cells, small peptides, and metabolites, which make efficient use of comprehensive screening platforms and highly sensitive assessment technologies [5]. Among them, circulating nucleic acids have shown remarkable benefits as a useful and versatile material in the last decade. DNA and RNA are easily amplified and highly applicable to various objects by changing the primer sequence. Moreover, they are relatively resistant to degeneration or modification relative to protein- or carbohydrate-based tumor makers. This chapter focuses on circular nucleic acids as diagnostic and prognostic biomarkers of pancreatic cancer.

9.2 Circulating Tumor DNA (ctDNA)

In 1977, Leon et al. first reported cell-free circulating DNAs in serum using a radioimmunoassay [6]. Notably, the development of polymerase chain reaction (PCR) made it possible to isolate specific tumor-derived mutated sequences (called ctDNA) from serum and plasma of cancer patients. It is commonly accepted that apoptotic cells release DNA into the bloodstream, which is cleaved in approximately 180 base pairs and packaged into nucleosomes [7]. It has been suggested that cancer patients have higher levels of cell-free DNA than healthy individuals. Hyperproliferation and

rapid cellular turnover under stressful circumstances induce increased apoptosis and necrosis in a tumor, which may cause aberrant release of cleaved DNA compared to normal tissues. However, the identification of ctDNA remains challenging, since ctDNA from a tumor may be significantly lower compared with total circulating DNA from the whole body. In the 1990–2000s, the detection of ctDNA with traditional methods, such as Sanger sequencing or standard PCR, was limited to patients in advanced stage due to its insufficient sensitivity. Allele-specific PCR methods have been applied since the mid-2000s for the detection of hotspot mutations in serum and plasma, and some assays are available as kits that are approved for clinical use but still have limited analytical sensitivity. However, there have been several recent advances in measuring platforms, including the digital PCR and targeted next-generation sequencing (NGS). Digital PCR assays on microfluidic platforms are quantitative and highly sensitive and can be used extensively to quantify single cancer-specific mutation. For a larger number of loci, targeted sequencing using PCR amplicons have been used, which can increase the depth of target read and reduce costs compared to entire exome sequencing [8]. These innovative technologies have made it possible to detect ctDNA as low as 0.1% in the blood, which has resulted in an explosive increase in the number of studies on the clinical utility of ctDNA.

The Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) gene has been frequently studied as a pancreatic cancer-specific marker. *KRAS* mutation is commonly observed in various type of cancer, in particular, reaching nearly 100% in pancreatic cancer [9]. Furthermore, most of the detected mutation is concentrated on several hotspots, which is one reason why many researchers are interested in the detection of mutated *KRAS* in plasma. Accumulating evidence suggests that mutant *KRAS* is specifically detected in the bloodstream of pancreatic cancer patients [10]. Although the sensitivity varies, ranging 26–94% depending on the quantitation methods and patient background, the evolution of technologies has improved not only advanced-stage but also early-stage cancer patients. Notably, ctDNA purified from pancreatic juice exhibits higher sensitivity and specificity than plasma samples [11–14]. Although an endoscopic approach is required to obtain pancreatic juice and the procedure includes the risk of complications of acute pancreatitis, it can be used as an additive diagnostic marker combined with cytological assessment [12]. In addition to *KRAS* mutation, combination mutant allele-specific PCR and NGS evaluating other genes, such as *TP53*, *SMAD4*, *CDKN2A*, *PIK3CA*, and *NRAS*, enable us to measure multiple mutations, resulting in increased sensitivity [15–17]. Moreover, epigenetic changes, such as DNA hypermethylation, could be used as biomarkers for cancer screening since epigenetic modulations are also found in ctDNA [18, 19].

9.3 Circulating Cell-Free RNA (cfRNA)

In contrast to ctDNAs, the origin and manner of release of extracellular cancer-associated RNAs are quite heterogeneous. While RNA from dead or dying cells is associated with apoptotic bodies or protein complexes, living cells actively release RNA encapsulated in lipoprotein complexes named exosomes or microvesicles

which function as a shield from RNase digestion in biofluids. They can also be incorporated into remote cells and are believed to play an important role in cell-to-cell communication [20].

The most well-studied cfRNAs is a family of microRNAs (miRs). They are small, single-stranded noncoding RNAs (ncRNA) consisting of 18–22 nucleotides that suppress the translation of various genes [21]. miRs play important roles in cell proliferation, differentiation, and apoptotic induction by targeting the mRNAs, and one single miR affects the expression of a number of coding genes. There have been many reports on the aberrant overexpression or downregulation of miRs in various cancer tissues, and alteration in specific miRs have been associated with cancer development. Without exception, tumor-related miRs play a critical role in tumorigenesis, invasion, metastasis, and chemoresistance in pancreatic cancer that could provide the basis for their development as novel diagnostic, prognostic, and/or therapeutic targets [22].

On the other hand, high-throughput RNA sequencing techniques have revealed that long noncoding RNAs (lncRNAs) are transcribed as frequently as protein-coding mRNAs. Most lncRNAs are supposed to possess biological functions, and increasing numbers of cancer-associated lncRNAs, such as HOTAIR, MALAT-1, ANRIL, H19, PVT1, and PCA3, are upregulated in various cancer cells [23] and may play important roles during oncogenesis, functioning as epigenetic regulators, guides for alternative splicing, decoys of miRNAs, and scaffolds for protein complexes. To date, several reports have identified lncRNAs, such as HITTIP-005 and RP11-567G11.1 [24], Linc-pint [25], HSATII [26], HOTAIR, and PVT1 [27], as novel biomarkers of pancreatic cancer in blood and saliva.

Very recently, circular RNAs (circRNAs), which are closed RNA transcripts generated by back splicing of pre-mRNA, have attracted much attention as a new family of ncRNA. The emergence of NGS has demonstrated that circRNAs are considerably abundant and conserved among various biological systems. Moreover, the expression of circRNAs is tissue- and disease-specific [28]. The expression profiles are also altered in cancerous tissues, including pancreatic cancer [29], and they may be released into the circulation as well as other cfRNAs in cancer patients. Due to their circular structure, circRNAs are considered more resistant to RNase in biofluids than linear transcripts, giving them an advantage as a stable biomarker. At present, there is no circRNA biomarker specific to pancreatic cancer, but some studies have shown that specific circRNA in cancer tissue can be used as a diagnostic or prognostic indicator [30–32]. However, thousands of circRNAs have not been functionally clarified, which may open a new field of biomarkers.

9.4 Approach for Detection of Precancer Lesion

In the pancreas, an intraductal papillary mucinous neoplasms (IPMN) is a pathological state regarded as a high risk for the occurrence of PDAC. According to several retrospective studies, the incidence of PDAC in IPMN patients is 0.68–1.1% per year [33]. Therefore, there is an urgent need to develop a noninvasive biomarker

for IPMN for screening high-risk patients in the clinic. Although emerging technologies such as high-throughput sequencing and digital PCR have altered the criteria for measurable ranges of minute nucleic acid in biofluids, it is still challenging to detect precancerous lesions, which indicate a high risk for cancer development; this is because low levels of ctDNA correlate with tumor burden and tumor-specific mutations, such as *KRAS* and *GNAS*, are also detected in inflammatory tissues at low levels. However, several RNAs, including ncRNAs, begin to be dysregulated from the precancerous stage mainly due to epigenetic alternations. Several studies have indicated tissue specificity and dynamic alternation of ncRNA expression profiles during oncogenesis, including precancerous lesions. Some reports have demonstrated that cfRNA is a useful indicator for detecting IPMN, which is completely undetectable with CA19-9 [26, 34].

9.5 Potential for Prognostic Marker

Another factor that prohibits pancreatic cancer from having a better prognosis is its notorious resistance to chemotherapy and radiotherapy. In addition, although several treatment combinations are currently available to metastatic pancreatic cancer patients, no molecular marker is routinely used in the clinic to predict the response to treatment or to evaluate efficiency [3]. Liquid biopsy has two relevant advantages compared with tissue biopsy: its noninvasiveness and repeatability. In particular, the latter is important when considering longitudinal sampling to monitor recurrence or to evaluate the therapy response. Certainly, changes in DNA mutation patterns in serum samples collected over time can indicate altered pathways or clonal evolution of the disease, and changing of mutant DNA suggests an altered disease burden. During the course of treatment, alternation in circulating DNA mutation patterns can indicate the emergence of resistant clones and prompt changes in treatment [35]. A previous meta-analysis also showed that *KRAS* mutations, rather than CA19-9, in plasma DNA were stronger prognostic factors for survival [36, 37], and some studies have used longitudinal monitoring of ctDNA [38, 39]. Additionally, the levels of several individual miRs, including miR-10b, miR-21, miR-31, miR-126, miR-335, and miR-373, correlate with metastatic outcome in carcinoma patients [40]. In addition, other classes of ncRNAs, such as HOTAIR [41] and HULC [42], have been proposed as putative biomarkers for metastatic potential in tumors. Similar strategies may be applicable for the evaluation of circulating RNAs.

9.6 Conclusion

In addition to the evolution of genomics tools and measurement technologies, circulating nucleic acids have also gained increasing attention as a useful material for liquid biopsy. Both ctDNAs and cfRNAs are highly beneficial to the pancreatic cancer field, representing possible novel diagnostic and/or prognostic markers. Although further improvements in procedures and relevant validation with a larger

cohort are required to validate their clinical use, the discovery of more specific and sensitive markers in the near future could provide hope for improvements with regard to this miserable disease.

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Biomarkers of Lung Cancer: Liquid Biopsy Comes of Age

10

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Abstract

Liquid biopsy provides a new diagnostic dimension for cancer based on molecular information obtainable from fluid samples such as peripheral blood and urine. In comparison with conventional tissue sampling, liquid biopsy is noninvasive, safe, and easily repeatable, and these favorable characteristics have rapidly expanded its range of application to the management of lung cancer patients. Two sources of information can be obtained by liquid biopsy: circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA)/cell-free DNA (cfDNA). As tumors show heterogeneity, it is anticipated that ctDNA may be more representative of the whole tumor cell population than small samples obtained by conventional tissue biopsy. The current applicability of liquid biopsy has been achieved through advances in highly sensitive analytic technologies such as digital PCR and next-generation sequencing. It has been reported that epidermal growth factor receptor (EGFR) gene mutation and anaplastic lymphoma kinase (ALK) gene rearrangement can be detected in CTCs in patients with non-small cell lung cancer (NSCLC) with specific genetic alterations. New methods of detecting and isolating CTCs with higher efficiency and selectivity are now being developed. This review discusses the techniques of liquid biopsy and their clinical application in the context of lung cancer and addresses some of the challenges that may lie ahead.

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Liquid biopsy · Non-small cell lung cancer (NSCLC) · Circulating tumor cell (CTC) · Cell-free DNA (cfDNA) · Circulating tumor DNA (ctDNA) · EGFR mutation · T790M

10.1 Introduction

In recent years, the molecular landscape of lung cancer has been explored extensively by large-scale genome sequencing, and molecular therapeutics targeting mutated/rearranged oncogene products, such as epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and c-ros oncogene 1 (ROS1), have been incorporated into routine clinical practice. It is now indispensable for oncologists to determine the genetic status of newly diagnosed non-small cell lung cancer (NSCLC) prior to the initiation of any treatments. Previously, genetic alterations in individual tumors were tested only once by analysis of tissue biopsy or archive pathology specimens. However, the genetic status of tumors may change during the course of treatment. Liquid biopsy provides a minimally invasive means of repeat testing over time [1]. Such real-time genetic monitoring may thus provide an alternative diagnostic dimension by which precision medicine can be offered to NSCLC patients.

10.2 Materials of Liquid Biopsy

Liquid biopsy is a revolutionary technique that has opened up novel perspectives [2] and offers a promising source of genomic information in patients with NSCLC. Circulating tumor DNA (ctDNA)/cell-free DNA (cfDNA), circulating tumor cells (CTCs), and exosomes secreted by tumor cells are the most studied materials that can be obtained by liquid biopsy (Fig. 10.1) [3]. The exosome is a

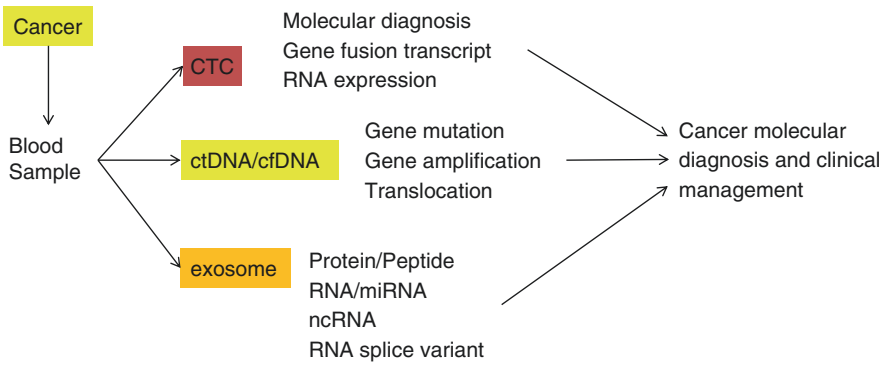

Fig. 10.1 CTC, cfDNA/ctDNA, and exosomes as source materials obtained by liquid biopsy for personalized medicine

type of extracellular vesicle secreted by various cells, including cancer cells. Exosomes retain various types of molecular information about their cells of origin, but their clinical utility in the context of liquid biopsy has not been established and therefore will not be discussed in this review.

The advantages of liquid biopsy include the possibility of repeated sampling, low cost, safety, and the fact that it can yield information on the complete molecular status of the tumor [4]. Biopsy of small tissue samples may provide only a snapshot of the overall tumor heterogeneity and may not represent all of the tumor cell populations present. Thus, blood-based genetic testing may revolutionize the daily clinical practice of cancer therapeutics. Herein, we describe the current techniques and clinical applications of liquid biopsy.

10.2.1 ctDNA

DNA fragments with mutated sequences can be detected in the peripheral blood of cancer patients and are referred to as ctDNA. These short DNA fragments are believed to be shed into the bloodstream from tumor cells during cell death processes, such as apoptosis and necrosis, or by other unknown mechanisms, and their sequence identity confirms that they originate from tumor cells. One of the challenges to analysis of tumor-derived ctDNA stems from its low abundance (<0.01%) in peripheral blood [5]. Several techniques have been developed to detect minute and specific genetic alterations and biomarker molecules that are diagnostically useful in peripheral blood with high sensitivity and specificity. These techniques include real-time quantitative PCR (qPCR), the Scorpion amplification-refractory mutation system (ARMS), digital PCR (dPCR) [beads, emulsion, amplification, and magnetics (BEAMing) and Droplet Digital PCR (ddPCR)], and targeted parallel sequencing [next-generation sequencing (NGS)] (Table 10.1). The Cobas (EGFR Mutation Test v2, Roche) assay, based on the Scorpion ARMS reaction, is able to detect single-base changes or small deletions by the use of allele-specific primers.

Table 10.1 Overview of the various techniques used for cfDNA analysis of cancer patients

Method	Approach	Sensitivity (%)	Mechanism
qPCR	Candidate gene	10	Specific TaqMan probes
Scorpion ARMS	Candidate gene	1	Specific Scorpion primer-probe complex
dPCR	Candidate gene	0.01	Specific TaqMan probes
BEAMing	Candidate gene	0.01	Magnetic beads
NGS	Screening of the genome	≤0.04	Parallel sequencing

cfDNA cell-free DNA, *qPCR* quantitative PCR, *ARMS* amplification-refractory mutation system, *dPCR* digital PCR, *BEAMing* beads, emulsion, amplification, and magnetics, *NGS* next-generation sequencing

ctDNA/cfDNA has also been detected in other body fluids such as urine, saliva, pleural/peritoneal effusion, and cerebrospinal fluid (CSF) and contains useful tumor-derived genetic information. These fluids can potentially be used as sources of genetic information.

10.2.2 Real-Time qPCR

Real-time qPCR is the most commonly used platform for quantification of nucleic acids with specific sequences. Two major techniques are available: the TaqMan-based PNA-LNA (peptide nucleic acid-locked nucleic acid) clamp assay and the Scorpion ARMS assay [6–8]. The ARMS assay is a PCR-based method for detecting single-base mutations and small deletions by amplification of target sequences using sequence-specific primers, detection of a PCR product indicating the presence of the intended target sequence. Because the ARMS assay is relatively insensitive for detection of sequences that are not abundant, it is often combined with the Scorpion assay. The assay consists of a PCR primer covalently linked to a probe. Therefore, the presence of a mutation can be detected by Scorpion ARMS with high sensitivity in a real-time setting.

10.2.3 Digital PCR (dPCR) and BEAMing

Droplet Digital™ PCR (ddPCR™, Bio-Rad and RainDrop™, RainDance Technologies) and BEAMing (beads, emulsion, amplification, magnetics; Sysmex) are two PCR-based techniques based on a similar principle [4, 9]. DNA is diluted down to single DNA molecules that can be physically separated into individual reaction compartments. Basically, the dPCR approach is similar to real-time qPCR. However, the DNA templates are partitioned into thousands of individual, parallel PCR reactions. Detection of the signal indicates the presence of the target sequence. As a result, dPCR allows the detection of mutated ctDNA in a high background of wild-type cfDNA, rendering it highly sensitive. The advantage of digital approaches is their high specificity, high sensitivity, speed, independence from qPCR equipment, ease of use, and comparatively low cost.

The major disadvantage of qPCR and dPCR is that they are restricted to predefined genetic alterations. However, the detection of previously unreported genetic alterations is neither required nor helpful for making therapeutic decisions. If a distinctive mutation is identified, a personalized assay can be individually designed for each patient, being of potential benefit for early detection of recurrence, monitoring of disease progression, and identification of resistance prior to drug administration.

10.2.4 Next-Generation Sequencing (NGS)

Unlike qPCR and dPCR, NGS is a method capable of detecting novel genetic changes without modification of the protocol. NGS is based on the production of

short sequences from single DNA molecules and their comparison to a reference sequence. This parallel short sequencing of millions of DNA templates results in the sequencing of a large portion of the genome. Whole-genome sequencing makes it possible to screen not only point mutations and insertions/deletions but also rearrangements and copy number aberrations, thus facilitating a comprehensive survey of an individual cancer genome [10].

10.2.5 Circulating Tumor Cells (CTCs)

CTCs are shed from either primary or secondary tumor sites, migrate into the circulatory system, and are considered responsible for the development of distant metastases [11]. Intact cancer cells are considered advantageous as a source material for liquid biopsy over cfDNA and exosomes. However, CTCs are extremely sparse, occurring at a frequency as low as 1 CTC per 10^6–10^7 leukocytes, even lower numbers being present in patients with early-stage disease [12]. Various techniques have therefore been tried for isolation of CTCs from the peripheral blood of cancer patients. The CellSearch(®) (Menarini Silicon Biosystems, Firenze, Italy) system, using the antibody-based immunomagnetic technique and image cytometry, is currently the only CTC diagnostic platform approved by the US Food and Drug Administration (FDA) for prognostication of metastatic breast, prostate, and colorectal cancer [13]. Initially assessed as non-leukocytic, nucleated cells of epithelial origin, CTCs do not have well-defined morphological characteristics. The CellSearch system exploits the characteristic expression of cell surface markers (CD45-negative, EpCAM-positive) of epithelial cancer cells.

10.2.6 Clinical Application of CTCs

It has been proposed that an increased CTC count is correlated with poor outcome in lung cancer patients. Several studies have assessed CTCs as prognostic and predictive markers of lung cancer [14, 15]. Patients with SCLC have on average ten times more CTCs than patients with any other tumor type [16]. However, the appearance of CTCs may vary according to cancer type and stage. As the clinical significance of CTC detection and counting in NSCLC patients remains unclear [17], a well-designed prospective trial would be warranted.

The genetic information provided by CTCs can help to clarify the different clinical behavior of individual tumors. It has been reported that EGFR mutation and ALK rearrangement can be detected in CTCs extracted from NSCLC patients with specific genetic alterations [18, 19], indicating that CTCs would be a promising additional source of information for real-time assessment of genetic progression during the course of molecular therapeutics targeting these and other genetic alterations [20]. However, the development of a more sensitive and reproducible method for isolation of CTCs and its standardization would seem to be essential [21].

10.3 Liquid Biopsy for Companion Diagnosis

The Cobas® EGFR Mutation Test v2 (Roche Molecular Systems, Inc.) was approved by the FDA as the first technique for in vitro ctDNA diagnostics in 2016. This is a real-time PCR-based test for detection of predefined mutations of the EGFR gene in NSCLC patients for whom tissue specimens are not available. This assay detects EGFR exon 19 deletion and L858R/T790M point mutation in cfDNA, thus identifying patients who would benefit from EGFR-tyrosine kinase inhibitor (TKI) therapy.

In the phase I AURA clinical trial of osimertinib for EGFR-mutant NSCLC with acquired EGFR-TKI resistance, EGFR genotyping of plasma cfDNA was performed in 237 patients using the BEAMing assay. The cfDNA assay detected T790M mutation with 70% sensitivity and 69% specificity in comparison to tissue genotyping [22]. Patients with T790M mutation in their ctDNA had therapeutic outcomes similar to those of patients whose tumor tissue showed the same mutation. However, the 30% false negativity rate of cfDNA genotyping indicated the need for confirmatory tumor biopsy in patients who were negative in the plasma T790M test.

Matched tumor tissue and plasma samples from patients enrolled in the AURA extension and AURA2 phase II studies were examined by the Cobas EGFR mutation test [23]. As a reference, plasma was also assessed by a next-generation sequencing method [MiSeq (Illumina Inc., San Diego, CA)]. Agreement between the Cobas plasma and tissue tests was 65% (95% CI: 61–69) for T790M mutation. Comparison of the Cobas plasma test with MiSeq demonstrated agreements of 90% or higher [23]. The plasma test, however, did not detect the T790M mutation in approximately 40% of patients who were positive for T790M by tissue biopsy. Therefore, to mitigate the risk of a false-negative plasma test result, it is advised that, whenever possible, any plasma T790M-negative test result should be reconfirmed by contemporaneous tissue biopsy (Fig. 10.2) [24].

10.4 Future Direction of Liquid Biopsy

Another field of interest currently being investigated is the relevance of CTC testing as a companion diagnostic approach for predicting the efficacy of immune checkpoint inhibitors. CTCs from 70% of patients with hormone receptor-positive, human epidermal growth factor receptor 2 (HER2)-negative breast cancer were reportedly positive for programmed cell death-1 ligand-1 (PD-L1) [25]. Similar results have been reported for patients with NSCLC [26, 27]. In a study of stage IV NSCLC patients treated with the anti-PD-1 (programmed cell death 1) therapeutic antibody nivolumab, patients with PD-L1-positive CTCs showed a significantly poorer outcome than patients with PD-L1-negative CTCs [28]. Given the growing importance of immunotherapy in current medical oncology, tissue and CTC PD-L1 expression and their correlation with response to immune checkpoint inhibitors will need to be investigated further.

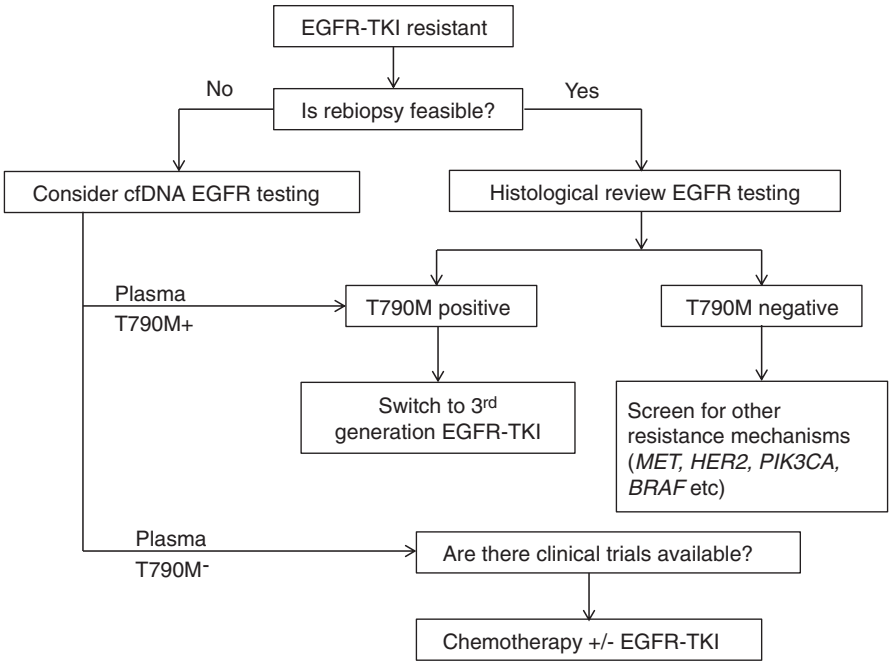

Fig. 10.2 Management of TKI resistance in EGFR-mutated non-small cell lung carcinoma

10.5 Conclusion

Liquid biopsy is now playing an evident role in the tailoring and monitoring of treatments for patients diagnosed with cancer. The recent successful detection of early cancers by the multi-genetic and proteomic tests [29, 30] may further facilitate the possible application of liquid biopsy to cancer screening in the future.

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Biomarkers for Breast Cancer Treatment

11

Tetsu Hayashida and Yuko Kitagawa

Abstract

The current treatment strategy for breast cancer is highly segmented by the difference of intrinsic subtypes, and it is no longer possible to be understood as a single disease. To conduct a tailor-made medicine, biomarker studies to define the molecular and oncological characteristics of breast cancer tissue from each patient are essential. It has been already established by many clinical trials and meta-analysis that ER, PgR, and HER2 are important biomarkers for predicting the effect of endocrine therapy and anti-HER2 therapy. Multigene assay is also turned to be not only a prognostic predictor but also a predictor of the effect of chemotherapy which contributes to clinical decision-making. The aim of this review on biomarker in breast cancer is to discuss the importance to know the biology and the current standards for the clinical application.

Keywords

Breast cancer · Biomarker · ER · PgR · HER2 · Multigene assay · BRCA · CEA · CA15-3

Various translational and clinical studies based on basic research have been intensively conducted to solve a clinical question to use the precise treatment method in appropriate situation and timing, which directly affects clinical judgment. Therefore, the current treatment strategy for breast cancer is highly segmented by the difference of intrinsic subtypes, and it is no longer possible to be understood as a single disease. To conduct such a tailor-made medicine, biomarker studies to

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define the molecular and oncological characteristics of breast cancer tissue from each patient are essential. Historically, it has been recognized that female sex hormones are involved in the proliferation and progression of breast cancer. Therefore, researches on estrogen receptor (ER), one of the major biomarkers, have been advanced from long ago, which contributes to the prosperity of biomarker research in the field of breast cancer.

11.1 Hormonal Receptors

11.1.1 The Research Background of Hormonal Receptors in Breast Cancer

ER is and has always been the most important biomarker for breast cancer treatment. It was identified as a protein binding to estradiol, and its functional role was reported to be involved in breast cancer proliferation from the 1960s to the 1970s [1]. McGuire and his colleagues reported that expression analysis of ER in breast cancer is valuable for predicting the effect of endocrine therapy [2]. Then, tamoxifen was approved as a selective estrogen receptor modulator (SERM) and started to be used for breast cancer treatment. Expression analysis of ER and progesterone receptor (PgR) by immunohistochemistry was established in the 1990s with advances in the quality of antibodies and antigen retrieval methods. Due to its simplicity and high success rate, immunohistochemistry has spread all over the world, and even today it is still practiced daily.

11.1.2 The Structure of Hormonal Receptors

Two types of isoforms, ER-α and ER-β, have been identified; however, the clinicopathological significance of ER-β in breast cancer is still unknown. ER-α exists at 6q 25.1 and consists of six functional domains as a protein structure. Activation function-1 (AF-1) and AF-2, which are present at the N-terminal and C-terminal of the ER protein, respectively, are transcriptional activation domains. It is known that AF-2 is essential for promoting proliferation mainly in mammary gland tissue, and it is activated to ligand dependence such as estradiol. PgR exists at 11 q22–q23. Since PgR is a direct target gene of ER, expression of PgR is also enhanced by stimulation with estradiol. Therefore, depending on the presence or absence of PgR expression, it is possible to predict to some extent whether ER-mediated pathway acts properly. The expression of ER and PgR in breast cancer cells is determined by immunohistochemical staining (IHC) as described above. More than 1% as occupancy threshold is recommended in the ASCO/CAP guideline. The Allred's score looks at what percentage of cells test positive for hormone receptors, along with how well the receptors show up after staining. This information is then combined to score the sample on a scale from 0 to 8 and score > 2 is judged as positive.

11.1.3 Hormonal Receptors as Biomarker for Breast Cancer Treatment

It is established by many clinical trials and meta-analysis that hormone receptors are important biomarkers for predicting the effect of endocrine therapy. In the NSABP-B23 trial, no significant difference was observed both in disease-free survival (DFS) and overall survival (OS) even when tamoxifen was administered to ER-negative and axillary node-negative breast cancer [3]. Early Breast Cancer Trialists' Collaborative Group (EBCTCG) conducted meta-analysis on 21,457 women enrolled in 20 randomized trials of 5-year tamoxifen versus no treatment after surgery. In ER-positive disease (n = 10,645), the recurrence rate reduction averaged 39%, and the breast cancer mortality rate reduction averaged 29% by tamoxifen treatment [4]. Among women whose tumors did not express ER, tamoxifen had no effect on recurrence. Expression of PgR did not influence whether tamoxifen reduced the risk of recurrence, suggesting that the utility of PgR as predictive factor for endocrine treatment was not confirmed in this study. On the other hand, there was a report showing that PgR levels were strongly correlated with time to recurrence for tamoxifen-treated patients and more strongly for anastrozole-treated patients in trans-ATAC study [5]. There is also another meta-analysis by EBCTCG on 37,000 women enrolled in 55 trials. The study showed that for 5 years of adjuvant tamoxifen, the proportional recurrence reductions produced among these 30,000 ER-positive patients were 47% whereas 6% in ER-negative patients [6].

11.2 HER2

In 1985, a tyrosine kinase located on 17q11.2–q21.1 was cloned, and the sequence was shown to be highly homologous to EGFR and v-erbB. It turned out to be the same as the oncogene known as "neu" since the positions of the chromosomes encoded are the same [7]. Japanese researcher group confirmed it as a glycoprotein of 185 kDa [8]. This gene is distinct from EGFR and named c-erbB-2.

It was reported that either gene amplification or protein overexpression of HER2 is observed with poor prognosis in about 15–25% of breast cancer patients [9]. It has also been reported that HER2-positive breast cancer shows resistance to endocrine therapy [10]. Despite these reports of a prognostic and predictive factor, what is the most useful in breast cancer treatment is the role as a predictor in anti-HER2 therapy. Therefore, the HER2 testing is recommended by most of the guidelines for breast cancer treatment. The presence or absence of expression in breast cancer cells is determined by a combination of IHC method and in situ hybridization (ISH).

11.2.1 HER2 as Biomarker for Breast Cancer Treatment

As described above, HER2 positivity in breast cancer cells had been considered as a prognostic factor because of its poor prognosis; however, the significance as a

predictive factor of effect on anti-HER2 treatment is important in current breast cancer treatment. In the meta-analysis involving 7 trials with 1497 patients in 2014, treatment with trastuzumab significantly improved response rate to HER2-positive advanced metastatic breast cancer (HR = 1.58, 95% CI 1.38–1.82). Moreover, the combined HRs for OS and PFS favored the trastuzumab-containing regimens (HR 0.82, 95% CI 0.71–0.94, and HR 0.61, 95% CI 0.54–0.70, respectively) [11]. In the neoadjuvant setting, another meta-analysis showed that the addition of trastuzumab in HER2-positive breast cancer improves the probability of achieving higher pathological complete response (pCR) (RR 1.85, 95% CI: 1.39–2.46) [12]. In addition, the network meta-analysis assessing the efficacy and safety of neoadjuvant therapy for HER2-positive breast cancer also demonstrated that patients in dual targeting arms had statistically significantly more pCR than those in trastuzumab monotherapy (chemotherapy + trastuzumab + pertuzumab vs chemotherapy + trastuzumab, OR = 2.29, 95% CI 1.02–5.02) [13]. Similarly, in adjuvant setting, the meta-analysis involving 11,991 patients with HER2-positive breast cancer revealed that the combined HRs for OS and DFS significantly favored the trastuzumab-containing regimens (HR 0.66; 95% CI 0.57–0.77; and HR 0.60; 95% CI 0.50–0.71, respectively) [14].

11.3 Ki67

The nuclear protein Ki67 is regularly used for distinguishing actually proliferating cells in the cell cycle, since it is expressed in all phases of cell cycle, including G1, S, G2, and M, but is absent in resting cells (G0) and differentiated cells. Therefore, it is considered to be useful as a tumor proliferative marker not only in breast cancer but also in other tumors. However, no universal criteria for judging whether Ki67 is high or low has been established. To solve this problem, it was proposed to set the cutoff value to 14% for determining luminal-A or luminal-B based on the report of Cheang et al. in St. Gallen International Breast Cancer Conference in 2011 [15, 16]. However, the same conference in 2015 concluded that it is clinically useful to measure the high and low of Ki67 value, but it is impossible to determine over universal cutoff value beyond inter-facility disparity [17].

11.3.1 Ki67 as Biomarker for Breast Cancer Treatment

Since Ki67 is widely involved in the sensitivity of chemotherapy and endocrine therapy for breast cancer patients, it was shown to be useful in various clinical situations. As a prognostic factor, de Azambuja et al. conducted the meta-analysis evaluating the impact of Ki67 on DFS and on OS in operable breast cancer. Ki67 positivity is associated with higher probability of relapse in all patients (HR = 1.93, 95% CI 1.74–2.14), in node-negative patients (HR = 2.31, 95% CI 1.83–2.92) and in node-positive patients (HR = 1.59, 95% CI 1.35–1.87) [18]. Furthermore, Ki67 positivity is associated with worse survival in all patients (HR = 1.95, 95% CI

1.70–2.24), node-negative patients (HR = 2.54, 95% CI 1.65–3.91) and node-positive patients (HR = 2.33, 95% CI 1.83–2.95). The utility as a predictive factor for the effect of chemotherapy was also reported. In the study involving 552 cases of invasive ductal carcinoma, high expression of Ki67 was an independent predictor of pCR in neoadjuvant setting (OR = 3.5, 95% CI 1.4–10.1) [19]. Among patients receiving the chemotherapy regimens containing taxane, DFS also improved significantly in cases with high expression of Ki67 in adjuvant setting [20]. On the other hand, several studies were reported saying that Ki67 is not an independent predictor for chemotherapeutic effect [21, 22]. Therefore, it is a matter to be carefully considered to predict by Ki67 if chemotherapy should be added as an adjuvant or neoadjuvant therapy.

11.4 Oncotype DX

In 2001, Sorlie et al. analyzed the gene expression profile of intrinsic gene on the microarray platform and reported that breast cancer can be classified into each subtype by its gene expression pattern [23]. The classification by multigene assay was advanced to predict the prognosis and the therapeutic effect of breast cancer, and many practical applications are produced in conjunction with various clinical trials. Oncotype DX, considered to be the most successful on a commercial basis among them, is recommended for suitable breast cancer patient by various guidelines. Oncotype DX calculates recurrence score (RS) by measuring the expression of 16 cancer-related genes and 5 reference genes in mRNA extracted from formalin fixed paraffin-embedded sample. RS defines less than 18 as low risk and medium risk from 18 to 30. Thirty-one or higher is defined as high risk, and it is considered clinically useful in the following aspects.

In NSABP-B14, a clinical trial comparing the group administered with tamoxifen alone and the placebo group, the prognosis of the low RS group was significantly better than that of the high RS group, and the multivariate analysis showed RS is the strongest prognostic factor [24]. Correspondingly, Trans-ATAC trial also showed that RS is a strong prognostic factor even in the group of postmenopausal patients who received aromatase inhibitor [25]. The valuable point of Oncotype DX is not only a prognostic predictor but also a predictor of the effect of chemotherapy which contributes to clinical decision-making. NSABP-B20 trial examined the additional effect of chemotherapy on tamoxifen for lymph node-negative and ER-positive breast cancer. In this trial, the additional effect of chemotherapy was observed only in the high RS group. Similar results were obtained from SWOG8814 sample analysis. Therefore, it is useful for deciding the introduction of chemotherapy as adjuvant therapy after surgery [26, 27]. Moreover, a partial result of the TAILORx trial, which is a prospective clinical trial using Oncotype DX, was already reported. In the low-risk group with RS <11, the 5-year DFS was 93.8% and the 5-year OS was 98.0% when only endocrine therapy was performed as adjuvant therapy [28]. This extremely good prognosis suggests that these patients don't need chemotherapy as adjuvant therapy at all.

11.5 BRCA1/2 Mutation

If the patient has a pathologic germline mutation in the BRCA1 or BRCA2 gene, she is diagnosed with hereditary breast and ovarian cancer syndrome (HBOC). This genetic test is usually performed on breast cancer patients with a rich family history, breast cancer patients with juvenile onset, triple-negative breast cancer patients, and patients with multiple histories of breast cancer morbidity. Then, risk reduction surgery or unusual surveillance corresponding to the medical and social condition is required for HBOC patients. The BRCA1/2 genes are important for the repair of double-strand DNA breaks by the error-free homologous recombinational repair. It was clarified that the DNA repair enzyme does not react, and the cells are induced to synthetic lethal when Poly (ADP-ribose) polymerase 1 (PARP1), a protein that plays a role of repairing single-strand breaks, is inhibited in a breast cancer patient with pathogenic BRCA1/2. The phase 2 clinical trial in 2009 revealed that the drug applying this phenomenon, PARP inhibitors, act safely and effectively against breast, ovarian, and prostate cancer in patients with germline mutations of BRCA 1/2 [29]. Therefore, it was suggested that genetic test for BRCA1/2, "BRACAnalysis," may be a companion diagnostic blood test used to determine if an individual with metastatic breast cancer might benefit from PARP inhibitor, which has been verified by several clinical trials.

The OlympiAd trial is a phase 3 clinical trial involving HER2-negative locally advanced and metastatic breast cancer patients with germline mutations in BRCA 1/2. Patients who had taken up to two previous lines of therapy were randomized 2:1 to PARP inhibitor, olaparib, (205 patients) or a drug of the physician's choice (91 patients). The primary endpoint was PFS. The trial met its primary endpoint and improved median PFS by 2.8 months (7.0 vs 4.2 months) with a hazard ratio of 0.58 (95% CI 0.43 to 0.80) [30]. It is especially promising to see that olaparib was also effective against triple-negative breast cancers that arise in HBOC.

According to the result of the OlympiAd trial, all patients with HER2-negative advanced breast cancer will theoretically be subject to this genetic test. However, it is necessary to establish a system that goes beyond the usual clinical examination, since clinical and social care for HBOC must be taken into consideration for the patient herself and her relatives. For facilities that do not satisfy the criteria, a coordination system with facilities that meet the requirements must be established to provide appropriate medical service such as genetic counseling. Also, careful handling as personal information should be taken into consideration.

11.6 Tumor Marker

CEA and CA15-3 are often measured during breast cancer treatment; however, it can't be asserted that robust evidence was established, since little prospective randomized controlled trial on these tumor markers was reported. ASCO provided guidelines in 2013 on follow-up observation after the initial treatment of breast cancer. The guideline assessed nine systematic reviews on postoperative follow-up

observation and five randomized controlled trials. No studies investigating the effectiveness of tumor markers were found among them. Therefore, they concluded the utility and application to routinely test tumor markers including CEA and CA15-3 for asymptomatic patients were not demonstrated [31]. On the other hand, prospective clinical trials showing that tumor marker surveillance contributed to the improvement of prognosis were reported, although the number of patients was relatively small. Nicolini et al. compared 32 patients who started treatment after confirmation of recurrence by diagnostic imaging and 36 cases who started treatment after only tumor marker elevation in 68 eligible patients. As a result, it was reported that the OS in the tumor marker group improved with a significant difference [32]. Furthermore, another group reported that MRI and/or PET scan in case of tumor marker elevation contributed to the early detection of breast cancer recurrence. In this study, 44 out of 813 patients were observed elevated tumor markers, 36 of whom (79.5%) had the recurrence in the median observation period of 63 months. The median overall survival of these relapsed cases was 41.1 months [33]. Relatively good sensitivity and specificity in the study suggest that tumor markers are useful for asymptomatic recurrent breast cancer detection. However, it is necessary to conduct a randomized controlled trial to confirm if there is a reasonable contribution to the prolongation of survival rate, cost-effectiveness, etc. Studies on the usefulness of CEA and CA15-3 in postoperative surveillance are relatively old. Due to the development of medicine and medical technology, it is necessary to consider that the treatment for advanced breast cancer has dramatically improved to be effective. Therefore, it is necessary to verify the possibility that early detection of asymptomatic recurrent breast cancer contributes to the improvement of patient prognosis.

ASCO guidelines in 2007 presented that monitoring tumor marker is useful for estimating the therapeutic effect of advanced breast cancer. The increase or decrease of these markers may reflect the therapeutic effect when no measurable lesion is found in the diagnostic image, although measurement of CEA or CA15-3 alone is not enough to make a clinical decision [34]. However, tumor marker elevation within 4–6 weeks after a change to new treatment may not always reflect the response of treatment.

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Biomarkers of Prostate Cancer

12

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Abstract

Prostate cancer is one of the most common male malignancies in the Western countries and recently has been dramatically increasing in Asian countries including Japan. Serum prostate-specific antigen (PSA) is the most widely utilized biomarker among solid cancers. During the management of prostate cancer patients, measurement of serum PSA is useful for screening, diagnosis, risk classification, assessment of treatment effect, prediction of outcomes, and detection of recurrence or progression. In addition, several novel biomarkers such as proPSA, neuroendocrine markers, bone turnover makers, and urine PC3 are used for efficient diagnosis or precise evaluation of the disease status. Based on recent advances in technology, liquid biopsy, including circulating tumor cells, cell-free DNA, and microRNA which are taken from blood sample, has been introduced for clinical practice.

Keywords

Prostate cancer · Prostate-specific antigen (PSA) · Screening · Risk classification Biochemical progression · Liquid biopsy · Circulating tumor cells · Androgen receptor

12.1 Introduction

Prostate cancer is one of the most common male malignancies in the Western countries [1]. Although the incidences of prostate cancer in Asian countries including Japan were considered to be low, recently they have been dramatically increasing,

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Table 12.1 Purposes of biomarkers of prostate cancer

Serum markers
• PSA (prostate-specific antigen)
Screening, risk classification, monitoring
• proPSA
Diagnosis
• NSE (neuron-specific enolase)/chromogranin A
Neuroendocrine differentiation
• Bone turnover markers
Evaluation of bone metastasis, osteoporosis due to hormone therapy
Urine markers
• PC3
Diagnosis
Liquid biopsy
• CTC (circulating tumor cells)
Prognostic factor, selection of treatment
• cfDNA (cell-free DNA)/ctDNA (circulating tumor DNA)
Prognostic factor
• miRNA (microRNA)
Prognostic factor

probably due to rapid aging of population, change to the Western lifestyle, and spread of serum prostate-specific antigen (PSA) as a screening tool. Serum PSA is the most widely utilized biomarker among solid cancers. During the management of prostate cancer patients, measurement of serum PSA is useful for screening, diagnosis, risk classification, assessment of treatment effect, prediction of outcomes, and detection of recurrence or progression (Table 12.1). In addition, several novel biomarkers have been proposed to evaluate the disease status more precisely. Based on recent advances in technology, liquid biopsy, including circulating tumor cells, cell-free DNA, and microRNA which are taken from blood sample, has been introduced for clinical practice.

12.2 Serum Markers

12.2.1 PSA (Prostate-Specific Antigen)

PSA is a glycoprotein which consists of 237 amino acids [2]. PSA belongs to human kallikrein family and possesses serine protease activity. The prostatic epithelial cells produce PSA protein and secret it into semen. Because all prostatic epithelial cells including normal, benign hyperplasic, and cancerous cells are expressing PSA, PSA is not prostate cancer-specific but prostate-specific. The androgen-response element is located within upstream of the PSA gene, and thus the expression of PSA is regulated by androgen (androgen-dependent). In the prostatic tissue of cancer or inflammation, or when mechanically stimulated, tissue structure is destroyed, and basement membrane is destructed, resulting in leakage of PSA protein into blood

circulation. Therefore, elevated levels of serum PSA are observed in not only prostate cancer but also prostatitis, needle biopsy, or urethral catheterization.

Screening There is a relationship between serum PSA levels and detection rate of prostate cancer. The cutoff level of serum PSA for prostate cancer is usually set at 4 ng/mL. Sensitivity of serum PSA, digital rectal examination, and transrectal ultrasonography were reported to be 80–82%, 48–62%, and 44–55%, respectively [3–5]. Since serum PSA increase along with age, the age-specific PSA range may be utilized: 0.0–3.0 ng/mL for 64 years old or younger, 0.0–3.5 ng/mL for 65–69 years old, and 0.0–4.0 ng/mL for 70 years old or older [6]. Increased level of serum PSA is also observed in patients with benign prostatic hyperplasia or prostatitis, whereas 5-alpha reductase inhibitors for benign prostatic hyperplasia and male pattern baldness and antiandrogens are known to decrease serum PSA level down to approximately 50% [7]. Serum PSA measurement is the most commonly utilized method of prostate cancer screening. A European randomized controlled trial demonstrated that screening by PSA could achieve 21% decline in prostate cancer mortality after 13 years follow-up [8]. On the other hand, PSA screening might induce overdiagnosis and overtreatment by detecting clinically insignificant prostate cancer. To overcome this dilemma, balance between benefit and harm of PSA screening should be discussed between candidate and his physician to obtain informed decision-making whether to receive PSA test or not. Moreover, active surveillance without therapeutic intervention is one of the choices for small and slow-growing prostate cancer [9]. To improve the efficacy of detection of prostate cancer, some of PSA-related markers have been proposed [10]. PSA density (PSAD) is the value of serum PSA divided by prostate volume; PSA velocity (PSAV) is absolute annual increase in serum PSA (ng/mL/year). Free/total (f/t) ratio of PSA has value in stratifying the risk of prostate cancer in men with total PSA level of 4–10 ng/mL.

Risk Classification Serum PSA level is a significant prognostic factor after commencement of therapies such as active surveillance, surgery, radiotherapy, and hormone therapy. For decision-making of therapeutic strategy of prostate cancer, risk classification is widely used based on T stage, Gleason score, and serum PSA [11]. Although several classification methods have been advocated, PSA level of less than 10 ng/mL, 10–20 ng/mL, and 20 ng/mL or greater is generally defined as low-, intermediate-, and high-risk groups, respectively.

Monitoring To assess the effect of treatment and to detect recurrence or progression of the disease, the concentrations of serum PSA are periodically measured following the treatment. Serum PSA value is declined by the therapeutic effect and thus is a significant predictor of prognosis. Increase in PSA often precedes clinical recurrence and called as PSA recurrence or biochemical recurrence. Because PSA kinetics are different among the therapies, the distinct definition of recurrence is applied according to each therapeutic method. After radical prostatectomy, all

prostate tissue is supposed to be removed, and thus serum PSA level must be undetectable. Therefore serum PSA value of 0.2 or 0.4 ng/mL is used for PSA (biochemical) recurrence after surgery [12]. In case of definitive radiotherapy, since the normal prostatic cells must be remained even if all cancerous cells were excluded, serum PSA of 2.0 ng/mL greater than the nadir level is defined as PSA recurrence [13]. For hormonally treated patients, the definitions of castration-resistant prostate cancer (CRPC) are 25% or more increase above 2.0 ng/mL from the nadir level, which is confirmed 4 weeks or later in Japan [14], and three consecutive rises in PSA 1 week apart resulting in two 50% increases over the nadir and PSA >2.0 ng/mL in the Europe [15]. At the recurrence or progression of prostate cancer, PSA kinetics may be useful for evaluating and predicting outcomes. PSA doubling time (PSA-DT), which measures the exponential increase in serum PSA over time, may have a prognostic role in treating prostate cancer [16] and be utilized for selection of treatment modality.

12.2.2 proPSA

proPSA is an isoform of serum-free PSA, and several lengths of proPSA are exiting in serum. Among [−7]proPSA, [−5]proPSA, [−4]proPSA, and [−2]proPSA, [−2]proPSA isoform (p2PSA) and related index (Prostate Health Index: PHI) [=(p2PSA/free PSA) × $\sqrt{(\text{total PSA})}$] are most useful for differentiating prostate cancer and nonmalignant conditions and reducing the number of unnecessary prostate biopsies in PSA-tested men [17].

12.2.3 NSE (Neuron-Specific Enolase)/Chromogranin A/proGRP (Pro-Gastrin-Releasing Peptide)

In a majority of prostate cancer, the development and growth of tumor is androgen-dependent. For the management of advanced prostate cancer, hormone therapy by means of androgen deprivation therapy (ADT) is utilized as a principle modality of treatment. On the contrary, neuroendocrine cancer or neuroendocrine differentiation may exist initially or arise during hormone therapy. Neuroendocrine cells lack the androgen receptor and are resistant to ADT. NSE, chromogranin A, and proGRP are useful serum or tissue markers for neuroendocrine differentiation [18]. Neuroendocrine differentiation is examined by immunohistochemical staining of the tissue or measurement of serum concentration of specific protein such as NSE, chromogranin A, proGRP, and synaptophysin.

12.2.4 Bone Turnover Markers

Bone is the most common site of metastasis in prostate cancer. And skeletal-related symptoms are often observed in advanced prostate cancer patients, due to bone

metastasis, osteoporosis by ADT, radiotherapy, and increased risk of falls by nocturia, sarcopenia, and anemia. Bone turnover markers, which consist of bone resorption and formation markers, are utilized for evaluation of bone condition and detection and follow-up of bone metastasis [19]. Although osteoplastic metastasis is common in prostate cancer, both of bone resorption and formation markers are often elevated in prostate cancer patients with bone metastasis. Bone resorption markers include deoxypyridinoline (DPD) in urine, type I collagen cross-linked N-telopeptide (NTX) in urine or serum, and pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen (1CTP) in urine or serum. Bone formation markers include bone-specific alkaline phosphatase (BAP) and osteocalcin and procollagen type I carboxy-terminal propeptide (P1CP).

12.3 Urine Markers

12.3.1 PC3 (Prostate Cancer Gene 3)

Prostate cancer gene 3 (PCA3) is a prostate-specific, noncoding mRNA biomarker and is detectable in urine sediments obtained after three strokes of prostatic massage by digital rectal examination. A urine test for PCA3 is useful for detection of prostate cancer in men with elevated PSA [20]. Currently, the main indication for the PC3 test is to determine whether repeat biopsy is needed after an initially negative biopsy.

12.4 Liquid Biopsy

A number of novel agents including abiraterone [21], enzalutamide [22], docetaxel [23], and cabazitaxel [24] have been introduced for the treatment of advanced CRPC. Because the effects of these agents depend on the tumor characteristics, the appropriate selection of sequential therapies became important to accomplish personalized medicine for each patient. To obtain the tumor feature in each stage of disease progression, tissue biopsy of primary and metastatic sites would be required. However, it must be difficult to get enough tissue sample since the most frequent site of metastasis from prostate cancer is bone. Therefore less invasive liquid biopsy has been studied to compensate tissue biopsy.

12.4.1 CTC (Circulating Tumor Cells)

It was reported that the number of circulating tumor cells in blood is a significant prognostic factor [25]. But the method of evaluating CTC has not been established, and CTC is not used for routine clinical practice. In CRPC patients, the androgen receptor in CTC was examined, and it was found that there were several splicing variants lacking some of the exons of androgen receptor gene. The splice variant 7

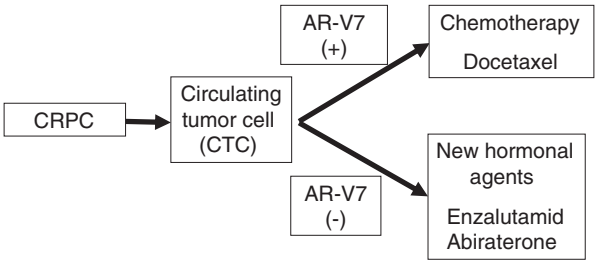

Fig. 12.1 Choice of treatment by splice variant 7 of androgen receptor (AR-V7) in castration-resistant prostate cancer (CRPC)

of androgen receptor (AR-V7) loses the steroid-binding domain of the receptor and is able to activate the androgen-responsive genes in an androgen-independent manner [26]. In this context, the CRPC patients with AR-V7-positive CTC would not respond to the novel hormonal agents such as abiraterone and enzalutamide but respond to chemotherapy such as docetaxel [27]. On the contrary, abiraterone or enzalutamide would have the favorable effect in those without AR-V7. Therefore, examination of AR-V7 in CTC is thought to be a tool for selection of therapeutic agent in CRPC patients (Fig. 12.1).

12.4.2 cfDNA (Cell-Free DNA)/ctDNA (Circulating Tumor DNA)

The concentration or fragmentation of cfDNA from blood has been studied as diagnostic and prognostic indicators. Recently, multi-genome analysis of ctDNA was proposed for precision medicine of prostate cancer [28].

12.4.3 miRNA (MicroRNA)

miRNA is a noncoding RNA of 20–25 base length and is thought to regulate gene expression within cells. Several miRNAs were shown to be overexpressed in prostate cancer cells and may be a biomarker for diagnosis and prediction of outcomes in prostate cancer [29].

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Biomarkers of Gynecological Cancers

13

Tatsuyuki Chiyoda, Ai Dozen, Keiko Saotome, Yoshiko Nanki, and Daisuke Aoki

Abstract

Tumor markers are molecules or substances produced by malignant tumors or the surrounding tissues that enter the circulation in detectable amounts. The majority of biomarkers are tumor-associated rather than tumor-specific and show elevated levels in multiple cancers. Tumor markers thus can be helpful for differential diagnosis but are not themselves diagnostic. In the field of gynecologic malignancies, biomarkers of ovarian cancer have been eagerly investigated owing to the difficulty in screening. Despite large efforts to develop novel biomarkers, cancer antigen 125 (CA125) has been the only biomarker clinically used, and no other marker has been able to outperform CA125. Noncoding RNAs, metabolites, and circulating tumor DNAs have recently emerged as cancer biomarkers and are being applied to clinical practice. Genomic biomarkers with predictive values are now used to select therapeutic drugs, especially molecular-targeted drugs. In this chapter, we describe ovarian cancer biomarkers in detail. In addition, biomarkers of uterine cancer and cervical cancer are summarized.

Keywords

Ovarian cancer · Uterine cancer · Cervical cancer · Biomarker · CA125 · BRCA · Homologous recombination · Microsatellite instability · Noncoding RNA

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13.1 Ovarian, Fallopian Tube, and Peritoneal Cancers

The lifetime risk of developing ovarian, fallopian tube, and peritoneal cancer (OC) is approximately 1–2% in women. Over 239,000 new cases are diagnosed worldwide each year [1]. Seventy to eighty percent of OCs are serous histologic type. Less common types include endometrioid (10%), clear cell (10%), mucinous (3%), transitional (Brenner) (<1%), and undifferentiated carcinomas (<1%) [2]. As OC is a heterogeneous disease, one biomarker may be unlikely to be effective for the diagnosis of each subtype.

13.1.1 Serum Biomarkers

13.1.1.1 CA125

Cancer antigen 125 (CA125) is the only marker used for the clinical diagnosis and management of OC. Despite decades of research to identify better biomarkers than CA125, no single marker superior to CA125 has been found. CA125, first described by Bast et al. in 1981, is a high molecular weight (5 million Dalton) heavily glycosylated transmembrane mucin (*MUC16*) [3, 4]. CA125 is expressed in the epithelium of fallopian tube, endometrium, and endocervix. CA125 expression is also detected in mesothelial cells in the pleura, pericardium, and peritoneum, particularly in areas of inflammation and adhesion. CA125 expression is undetected on the surface epithelium of normal fetal and adult ovaries, except in inclusion cysts, areas of metaplasia, and papillary excrescences [5]. Therefore, CA125 expression is not specific for OC.

The widely adopted cutoff value of 35 U/mL in routine clinical practices is based on the results of a study, wherein only 1% of 888 normal healthy persons had CA125 levels higher than 35 U/mL [6]. However, the level of CA125 tends to be lower in postmenopausal women; hence, a cutoff value of 26 U/mL has been suggested for postmenopausal women [7]. Approximately 85% of patients with OC have CA125 levels of >35 U/mL. CA125 level may be elevated in benign conditions, including ovarian cyst and endometriosis, as well as in other malignancies such as breast, pancreas, lung, gastric, biliary tract, liver, and esophageal cancer and in physiological states such as pregnancy and menstruation (Table 13.1) [8–10].

Screening

The importance of tumor markers and ultrasonography in the screening of OC is yet not established by prospective studies. CA125 is useful for monitoring patients with OC [11]. Elevated CA125 levels have been reported in 50% of patients with stage I and >90% of patients with stage II–IV OC [8, 11, 12]. The specificity of CA125 is suggested to be improved when used in combination with transvaginal ultrasound (TVS) or upon continuous monitoring of CA125 levels over time [13, 14]. However, the false-positive and false-negative results achieved with both CA125 and TVS and the absence of any data confirming the usefulness of the screening method for OC detection at an earlier stage indicate that these tests are

Table 13.1 Elevations of CA125 in women with benign gynecologic disease and gynecologic malignancies

		CA125 > 35 U/mL (%)
Benign gynecologic disease	Ovarian cysts[a]	14
	Germ cell tumors (mature teratoma)[a]	21
	Sex cord stromal tumors (thecoma, fibrothecoma)[a]	52
	Cystadenoma, adenofibroma, cystadenofibroma[a]	20
	Serous epithelial tumors[a]	20
	Mucinous epithelial tumors[a]	18
	Endometriosis/endometriomas[a]	67
	Abscess/hydrosalpinx/PID[a]	37
	Fibroid (leiomyomas)[a]	26
	Benign, other (normal ovaries)[a]	22
Gynecologic malignancies	Ovarian cancer (overall)[b]	80
	Ovarian cancer (serous papillary)[b]	84.4
	Ovarian cancer (mucinous)[b]	68.8
	Ovarian cancer (clear cell)[c]	78.9
	Endometrium/endocervix[b]	50
	Squamous cervical cancer[b]	16.7

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not recommended and should not be routinely used to screen women with high risks for OC [15–17].

As 3.0–4.1% of healthy women have elevated CA125 levels, the use of this marker in a standalone test has several limitations [18], which may be overcome with the use of TVS. TVS as a second-line test in women with elevated CA125 levels in combination with CA125 monitoring may provide high specificity (99.9%) [19]. A statistical algorithm (Risk of Ovarian Cancer Algorithm, ROCA), based on the age-specific risk of the disease and the behavior of CA125 over time in women with OC versus normal controls, has shown significant improvement in the sensitivity and specificity of CA125 interpretation for primary invasive OC [20–22]. Algorithms such as ROCA rely on the behavior of a biomarker from disease onset to clinical presentation and use data accumulated in large trials over many years. The Shizuoka Cohort Study of Ovarian Cancer Screening was a randomized control trial of 82,487 low-risk postmenopausal women who were assigned to a screening group using an annual TVS and CA125 or a control group. During the first screening round, 27 OCs were detected, and 32 OCs were reported in the control group.

The proportion of stage I OCs was higher in the screened group (63%) than in the control group (38%) [23]. However, no statistically significant difference was observed, and the impact of mortality is yet unreported.

In the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), 202,638 postmenopausal women aged 50–74 years were randomized to either control, an annual screening with TVS, or an annual CA125 screening with second-line TVS (multimodal strategy [MMS]) groups in a 2:1:1 manner. The sensitivity, specificity, and positive predictive value (PPV) for all primary OCs were 89.4%, 99.8%, and 43.3% for MMS and 84.9%, 98.2%, and 5.3% for TVS, respectively [24]. The mortality outcome data at a median follow-up of 11.1 years was as follows: OC was diagnosed in 1282 (0.6%) women, including 338 (0.7%) in the MMS group, 314 (0.6%) in the TVS group, and 630 (0.6%) in the no screening group. Of these women, 148 (0.29%) in the MMS group, 154 (0.30%) in the TVS group, and 347 (0.34%) in the no screening group died of OC [25]. The relative mortality reduction was 15% in the MMS group and 11% in the TVS group, but these reductions were not significant (MMS, $p = 0.10$; TVS, $p = 0.21$). Further follow-up is planned until the end of 2018. A total of 14 women in the MMS arm and 50 in the TVS arm per 10,000 screens underwent surgery as a result of positive screen results and were then found to have only benign ovarian lesions or normal ovaries [25].

The US Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial enrolled 78,216 women aged 55–74 years, with 39,105 women randomized to OC screening [26]. Women were screened using serum CA125 level at a cutoff value of 35 U/mL and TVS for 3 years followed by CA125 alone for additional 5 years. At the baseline screening round, a total of 566 surgeries were performed as diagnostic follow-up to positive screening, resulting in a diagnosis of 18 OCs (the ratio of surgeries to invasive cancers, 31 to 1). Of the 18 cancers, 83% were stage III or IV. Over three subsequent annual rounds of screening, 604 additional surgeries following positive screening and 42 additional cases of OC (the ratio of surgeries to cancers of 14 to 1) were detected. Of these OCs, 67% were stage III or above [27]. At a median follow-up of 12.4 years, PLCO showed no mortality benefit with screening, with 118 and 100 deaths in the screening and control arm, respectively (mortality rate ratio [RR], 1.18; 95% confidence interval [CI], 0.82–1.71). From 3285 of 39,105 women with false-positive results, 1080 underwent surgery as a part of the diagnostic workup. Of these 1080 women, 163 women experienced at least one serious complication (15%) [26]. These results show that OC screening increases the risk of complication from false-positive results with no reduction in mortality.

The target population for screening includes women who have a strong family history of OC. Most of this risk is attributed to mutations in the *BRCA1*, *BRCA2*, and DNA mismatch repair (MMR) genes, with cumulative risks by the age of 80 years for OC of 44% in *BRCA1* mutation carriers, 17% in *BRCA2* mutation carriers [28], and 12% in women with mutations in the MMR genes [29]. Mutations in moderate-penetrance genes such as *RAD51C*, *RAD51D* [30], and *BRIP1* [31] are also reported to be associated with lifetime risk of OC. In these women, the primary recommendation is risk-reducing salpingo-oophorectomy (RRSO). Screening with TVS and CA125 starting at the age of 30–35 years is usually offered to those that

refuse to undergo RRSO, although it cannot prevent the diagnosis of advanced OC [32, 33]. An approach based on 3–4 months of screening with CA125, interpreted using ROCA, was evaluated in prospective screening studies in the UK (UK Familial Ovarian Cancer Screening Study [UKFOCSS Phase II]) [34] and in US trials by the Cancer Genetics Network and Gynecological Oncology Group and the US-based Cancer Genetics Network (CGN) [35]. In the former study, 4348 women underwent ROCA screening every 4 months. TVS was performed annually when ROCA results were normal or within 2 months of an abnormal ROCA result. Modeled sensitivity, PPV, and negative predictive value (NPV) for OC detection within 1 year were 94.7%, 10.8%, and 100%, respectively. Seven (36.8%) of the 19 cancers diagnosed within 1 year after prior screen were stage IIIb–IV as compared to 17 (94.4%) of 18 cancers diagnosed more than 1 year after screening ended ($p < 0.001$) [34]. In the latter studies, 3692 women were screened with serum CA125 every 3 months, evaluated using a ROCA, which triggered TVS. Specificity and PPV were compared with levels derived from general population screening. Specificity for TVS referral was 92% versus 90% ($p = 0.0001$), and PPV was 4.6% versus 10% ($p > 0.10$). Three of six invasive cancers were early-stage (I/II; 50% vs 10% historical *BRCA1* controls; $p = 0.016$). ROCA flagged three of six (50%) cases before CA125 exceeded 35 U/mL. These studies show that ROCA-based screening is an option for women at high risk of OC who decline RRSO, given its high sensitivity and significant stage shift. However, it remains unclear whether this strategy would improve survival in screened high-risk women.

Whether the raised CA125 level in asymptomatic postmenopausal women is a predictor of non-gynecologic cancer is yet unclear. A study group of 771 women with elevated CA125 level (≥30 U/mL) and a control group of 771 women with CA125 < 30 U/mL followed up for a mean of 2269 days reported that the elevated CA125 level was not a predictor of non-gynecologic cancer [36]. However, CA125 level was associated with a significantly increased risk of death from all causes in the next 5 years [37]. In the study of 5550 women from Swedish Regional Cancer Registry, breast cancer and lung cancer were overrepresented among women with elevated CA125 values ($p = 0.015$ and $p < 0.001$, respectively) [38].

Prognosis

CA125 was a prognostic factor in patients with stage I or II OC, while it had no prognostic value in patients with advanced stage OC [39]. Patients with stage I OC and preoperative serum CA125 levels <65 U/mL had a significantly longer survival compared to stage I OC patients with preoperative serum CA125 ≥ 65 U/mL (hazard ratio [HR] = 3.4, $p = 0.01$) [40]. An Australian multicenter study of 518 patients with stage I OC revealed the association between CA125 levels more than 30 U/mL and higher grade, substage 1B and 1C, non-mucinous histologic type, and old age. The 5-year overall survival rate was 82% for patients with CA125 levels >30 U/mL, and 95% for patients had CA125 levels of ≤30 U/mL ($p = 0.028$) [41]. Postoperative CA125 levels were also reported as significant prognostic factors [42]. Patients with premaintenance baseline CA125 level of ≤10 U/mL showed superior progression-free survival (PFS) compared with those with higher levels (even in the normal range) [43]. CA125

continues to be of prognostic significance upon recurrence; patients with normal CA125 levels (≤35 U/mL) at relapse had better prognosis than those with elevated levels [44].

Although CA125 levels are widely used to monitor the clinical course of OC, CA125 should not be used as the sole criterion to determine clinical response. Two-third of patients with serum CA125 values of 20–35 U/mL had grossly visible disease at second-look laparotomy [45]. Serial measurements are more informative than cutoff values. A study proposed using at least a 50% decrease to define response and using ≥two times the upper limit of the reference range or the nadir value to define progression, which was incorporated into the RECIST criteria [46].

Detecting Recurrence

In a retrospective analysis of 58 patients, a follow-up based on physical examination and CA125 level could identify 53 out of 54 (98%) patients with recurrence; thus, computed tomography (CT) scan is not recommended during the follow-up period [47]. Whether to treat patient upon recurrence was solely dependent on the level of CA125 or until symptomatic presentation was a subject of debate. The MRC OV05/EORTC55955 trial showed no benefit in CA125 monitoring in the follow-up of patients with OC, as patients randomized to immediate (based on CA125 levels, $n = 265$) or delayed chemotherapy (when signs and symptoms of recurrence were present) failed to demonstrate any difference in survival (early arm, median survival 25.7 months; delayed arm, median survival 27.1 months; HR 0.98; $p = 0.85$) [48]. The European Society of Gynecological Oncology (ESGO) stated that monitoring CA125 level may be beneficial for certain patient groups, especially when the patient is a candidate for secondary cytoreductive surgery, and that CA125 remains as the most important biomarker for OC, excluding tumors of mucinous origin [49].

13.1.1.2 Human Epididymis Protein 4

Despite the efforts to find a marker with performance better than, or comparable to, that of CA125, only one marker developed and clinically used is the human epididymis protein 4 (HE4) [50]. HE4 is a glycoprotein in the epithelial cells of the epididymis, and the increase in HE4 serum levels in OC was first observed in 1991 [51]. HE4 expression level varies between histological subtypes of OC and is deemed positive in 93% of serous, 100% of endometrioid, 50% of clear cell, and 0% in mucinous OC [52].

Screening

In comparison with CA125, HE4 levels are relatively stable in the serum across the menstrual cycle [53]. HE4 levels are lower in premenopausal women than in postmenopausal women. There is no clear evidence whether HE4 may act as a better biomarker than CA125. Urban et al. demonstrated that HE4 outweighs TVS both as a first-line and second-line test but may not outperform CA125 (first-line sensitivity: CA125, 46.4%; TVS, 28.6%; HE4, 35.7%) [54]. In the nested case-control study within PLCO screening trial, none of the five predictive models, each

containing six to eight biomarkers, nor a model derived from all of the 28 markers evaluated in the PLCO set showed improvement over CA125 alone [55].

Differential Diagnosis

The level of HE4 was shown to decrease during pregnancy (median levels of 30.5 pmol/L) and is around 50 and 60 pmol/L in pre- and postmenopausal women, respectively; these values increase with age (median 109.5 pmol/L in women over 80 years) [9, 56]. HE4 has also emerged as a serum biomarker for pulmonary adenocarcinoma, lung cancer, chronic kidney disease, renal failure, and kidney fibrosis.

13.1.1.3 The Risk of Ovarian Malignancy Algorithm

The Risk of Ovarian Malignancy Algorithm (ROMA) is a logistic regression algorithm that was first described by Moore et al. in 2009 and uses serum HE4 and CA125 levels plus menopausal status to assess the risk of OC in women with pelvic masses [57]. In EPIC cohort that compares 810 invasive OC and 1939 controls within 6 months of diagnosis, the median HE4 level was 29.1 pmol/L in OC cases and 18.9 pmol/L in control cases ($p < 0.0001$), although CA125 remains the single best marker for the early detection of OC [58]. The results of EPIC cohort show that HE4 level was higher in older patients and current smokers and inversely associated with oral contraceptive use duration, parity, and old age at menopause [59]. Statistically lower HE4 levels were observed in *BRCA1* gene mutation premenopausal carriers, while a minor increase of HE4 level was observed in occult OC cases [60].

13.1.1.4 Cell-Free DNA

Cell-free DNA has been increasingly investigated as a potential biomarker. Preoperative plasma total cell-free DNA levels are significantly elevated in patients with OC (median preoperative cell-free DNA level of 10,113 GE/mL in patients with OC; 2365 GE/mL in patients with benign ovarian neoplasms [$p < 0.0001$] and 1912 GE/mL in controls [$p < 0.0001$]). Elevated plasma cell-free DNA is an independent predictor for death from disease in OC [61]. Using a method called tagged-amplicon deep sequencing (TAm-Seq), mutations of the tumor suppressor gene *TP53* in circulating DNA of 46 plasma samples of advanced OC were identified at allele frequencies as low as 2% and sensitivity and specificity of >97% [62]. In relapsed treatment courses, a decrease in *TP53* mutant allele fraction (MAF) of ≤60% was associated with poor response and helped in the identification of cases with time to progression (TTP) < 6 months at 71% sensitivity and 88% specificity, indicative of the potential role of circulating tumor DNA as a highly specific early molecular response marker in OC [63].

13.1.1.5 DNA Methylation

DNA hypermethylation at specific sites of a gene, mainly at CpG islands, is a common event in human cancer, resulting in direct effects on gene expression. Changes in DNA methylation patterns are frequently observed in OC, and each histological subtype is characterized with a different methylation motif [64, 65]. Methylation of

ESR1 encoding for estrogen receptor alpha was found in 28.3–48.5% of primary OC tissues and 38.0% of plasma samples [66]. However, DNA methylation profile has not yet been used in clinical practice.

13.1.1.6 Metabolites

Metabolomics is the analysis of small molecular weight metabolites of different biochemical classes in the body. As metabolic fluctuations lie downstream of alterations at the DNA, RNA, and protein levels, metabolomics may be sensitive and comprehensive functional readouts of biological systems. Sixty-six invasive OC and nine borderline tumors of the ovary were analyzed by gas chromatography/time-of-flight mass spectrometry (GC-TOF MS), which revealed significant differences in 51 metabolites between borderline tumors and OC ($p < 0.01$) [67]. Metabolite profiling of plasma from 50 serous OC and 50 serous benign controls identified 34 metabolites with significantly different expression ($p < 0.05$), and a multivariate classification model built with the top four lipid metabolites achieved an estimated area under the curve (AUC) of 0.85 [68].

13.1.1.7 MicroRNAs

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by translational inhibition or mRNA degradation. Initial studies examining tissue profiling of OC and normal ovaries showed the upregulation of miR-200a, miR-200b, miR-200c, and miR-141 and downregulation of miR-199a, miR-140, miR-145, and miR-125b1 [69]. Levels of eight miRNAs (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, and miR-214), previously demonstrated as diagnostic factors, were detected in exosomes isolated from sera specimens of women with various stages of OC. These miRNAs were undetectable in the control group, suggesting that the miRNA profiling of circulating tumor exosomes could potentially be used as a diagnostic strategy [70]. Resnick et al. showed that miR-21, miR-92, miR-93, miR-126, and miR-29a were significantly overexpressed in the serum of patients with OC than in control subjects ($p < 0.01$), and miR-155, miR-127, and miR-99b were significantly downregulated ($p < 0.01$). Among the miRNAs upregulated in OC, miR-21, miR-92, and miR-93 were elevated in three patients with normal preoperative CA125 level [71]. Zhang et al. summarized the clinically relevant miRNAs in OC that showed consistently downregulated expression (Let-7a/b/d/f, miR-22, miR-31, miR-34a/b/c, miR125b, miR-127-3p, miR-152, miR-155, miR-181a-3p, and miR-382) and upregulated expression (miR-15a/16, miR-20a, miR-23a/b, miR-30a/b/c, miR-92, miR-93, miR-106a, miR-146b, miR-182, miR-200, miR-203, miR-205, and miR-223) [72].

13.1.1.8 Long Noncoding RNAs

Long noncoding RNAs (lncRNAs), nonprotein-coding transcripts longer than 200 nucleotides, are known as key regulators of various biological processes. lncRNAs are involved in the development and progression of many cancers. Wang et al. reported decreases in the levels of AC092214.10, CYP3A5, LEMD1, PART1, RNF157-AS1, and RP11-532F12.5 and increases in levels of AC010680.1, ADAMTS9-AS1, ADAMTS9-AS2, AK021537, AK125532,

LOC386758, RP1-78O14.1, RP11-597D13.9, and LEMD1-AS1 in patients with OC than in benign control subjects [73]. Other lncRNAs upregulated (HOTAIR, H19, XIST, HOST2, AB073614, ANRIL, CCAT2, C17 or f91, MALAT1, NR-026689, and TUG1) or downregulated (BC200, GAS5, and HOXA11AS) in OC were summarized elsewhere [74, 75].

13.1.1.9 Carcinoembryonic Antigen

Elevated carcinoembryonic antigen (CEA) levels associated with colon and pancreatic cancer are also related to benign diseases of the liver, gastrointestinal tract, and the lung. Immunohistochemistry analysis of 189 borderline tumors and 571 OC tissues revealed that 18% of borderline tumors and 4% of OCs were positive for CEA expression and a higher proportion of mucinous tumors than other histological subtypes were positive for CEA expression [76].

13.1.1.10 Alpha-Fetoprotein

Alpha-fetoprotein (AFP) is an oncofetal protein produced by the fetal yolk sac, liver, and upper gastrointestinal tract. AFP level is rarely elevated in epithelial OC, but an increase in AFP level is observed in patients with germ cell tumors (yolk sac tumors, immature teratomas or dysgerminomas), especially in younger women [77]. In women with yolk sac tumors, AFP is a reliable marker for monitoring therapeutic response and detection of recurrence [78].

13.1.1.11 Inhibin

Inhibin is a heterodimeric glycoprotein composed of a common alpha-subunit and one of two beta-subunits, producing either inhibin A of inhibin B. Inhibin is elevated in patients with granulosa cell tumors. Inhibin is upregulated in 18% of serous, 84% of mucinous, 54% of endometrioid, and 100% of granulosa OC [79].

13.1.1.12 Cytokines

Serum levels of macrophage colony-stimulating factor (M-CSF) were shown to be elevated in 64% of OC and were associated with OC stages [80]. Interleukins (ILs) have also been studied in OC. Although IL-6 level is elevated in 50% of OCs and associated with worse survival, the combination of IL-6 and CA125 does not improve the sensitivity of CA125 alone [81]. IL-7 was also reported to be elevated in OC, and the combination of IL-7 and CA125 could accurately predict 69% of OC [82].

13.1.1.13 Other Markers

Some of the human kallikrein gene families expressed in OC cells (KLKs 2–11 and 13–15) or dysregulated in serum (KLKs 5–8, 10, and 13) have been reported as OC biomarkers [83]. Osteopontin is a secreted extracellular matrix glycoprotein involved in a number of cellular processes, including wound healing, inflammation, immune response, and tumorigenesis. Despite an encouraging AUC value of 0.85, the use of osteopontin as a biomarker is not recommended due to selection bias [84]. Elevated serum mesothelin levels are associated with poor survival in OC [85]. It is important to note that no single marker or combination of markers has emerged with

evident clinical advantage over CA125 in women with OC, except in specific tumor subtypes such as germ cell tumors and granulosa cell tumors.

Predictive Genomic Biomarkers in Ovarian Cancer

About 22% of high-grade serous OC have either germline or somatic *BRCA1* or *BRCA2* (*BRCA1/2*) mutations, and about 11% loss in BRCA1 expression is related to DNA hypermethylation. About 20% of OC have alterations, including mutations in Fanconi anemia genes and other genes associated with DNA repair (i.e., *ATM*, *ATR*, *CHEK2*, and *RAD51C*), *EMSY* amplification, and loss of *PTEN*. Thus, approximately half of OC have homologous recombination (HR) deficiency (HRD) [86]. HR is a high-fidelity DNA double-strand break repair mechanism that uses sister chromatids as a template.

Poly(ADP-ribose) polymerase (PARP) inhibitors have been reported to be selectively lethal to BRCA1/2-impaired cells [87, 88]. This result is explained by the synthetic lethality caused by HRD and DNA single-strand break repair inhibition in response to PARP inhibitors. In fact, patients with germline mutations of *BRCA1/2* received the greatest benefit and HRD-positive OC with wild-type *BRCA1/2* had prolongation of PFS [89, 90]. Approaches for measuring HRD include (1) sequencing of DNA repair genes; (2) measurement of "genomic scars," including genomic wide losses of heterozygosity (LOH), telomeric allelic imbalance, and large-scale transitions; (3) protein expression analysis of HR genes; and (4) functional assays [90].

13.2 Uterine Cancers

Uterine cancer is generally a disease of postmenopausal women, and most cases are diagnosed in early stages, owing to the clinical symptoms of abnormal vaginal bleeding. At present, there are no serum markers with established role in clinical management of uterine endometrial cancer (EC).

13.2.1 CA125

About 26.2% of uterine ECs had elevated CA125 levels [91]. A cutoff level of 20 U/mL of CA125 could detect myometrial invasion to more than one-half of the myometrium with a sensitivity of 69.0%, specificity of 74.1%, PPV of 58.8%, and NPV of 81.6% [92]. Others reported that the elevated CA125 level (>40 U/mL) significantly correlated with higher stage, higher grade, and increased depth of myometrial invasion as well as lymph node metastases, and presence of lympho-vascular space involvement in EC [93].

13.2.2 HE4

HE4 is also elevated in EC. HE4 was more sensitive than CA125 in detecting advanced stage disease. In addition, a significant correlation was observed between

large primary tumor diameter and deep myometrial invasion [94]. A population-based study demonstrated HE4 as a better predictor of outer-half myometrial invasion (AUC = 0.76) than CA125 (AUC = 0.65), particularly in patients with low-grade endometrioid tumors (AUC 0.77 vs 0.64 for CA125) [95].

13.2.3 Other Markers

miRNAs (miR-93, miR-205, miR-944, let-7, nc886, and miR-145) and lncRNAs (OVAL, HOTAIR, SRA, H19, TUG1, BANCR, NEAT, ASLNC04080, and LINC00958) are upregulated in EC [96].

Predictive Genomic Biomarkers in Uterine Cancer
Approximately 25% of ECs have defects in the DNA MMR system manifested by errors in DNA replication of trinucleotide repeat regions, referred to as microsatellite instability (MSI) [97]. Tumors with MSI have an underlying defect in one of the MMR genes (*MSH2*, *MSH6*, *MLH1*, or *PMS2*) or *EPCAM* deletions, which may be either germline (Lynch syndrome) or somatic. MSI-high (MSI-H) ECs are associated with high neoantigen loads and number of tumor-infiltrating lymphocytes (TILs), along with the overexpression of PD-1 and PD-L1 [98]. Thus, MSI-H EC has become an attractive candidate for immune checkpoint inhibitors. A phase II trial of anti-PD-1 therapy (pembrolizumab) in MSI-H tumors demonstrated a 71% immune-related objective response rate in non-colorectal tumors, including EC [99]. About 5–10% of ECs show very large number of single-nucleotide variations, with a particular preference for C > A transversion mutations attributed to the mutations in the exonuclease domain of *POLE*. *POLE* is the gene encoding for the catalytic subunit of DNA polymerase epsilon, which is involved in DNA replication, proofreading, and repair. *POLE*-mutated ECs have significantly better PFS than other ECs [97].

13.3 Cervical Cancers

The screening strategy for cervical cancers is based on exfoliative cytology and high-risk human papilloma virus (HPV) DNA detection. To date, there has been no serologic marker that is sensitive or specific enough for screening purposes.

13.3.1 Squamous Cell Carcinoma Antigen

Squamous cell carcinoma antigen (SCC-Ag) is a clinically used serum marker for squamous cell cervical carcinoma that was first reported by Kato and Torigoe in 1977 [100]. Abnormal level of SCC-Ag is detected in 28–88% of patients with squamous cell carcinoma [101]. A meta-analysis study showed that the serum SCC-Ag level was consistently associated with recurrence and survival in the newly diagnosed cervical cancer cases [102].

13.3.2 Serum Fragments of Cytokeratin

Serum fragments of cytokeratin (CYFRA) are a measure of the serum concentration of fragments of cytokeratin 19, an acidic subunit of cytokeratin that is expressed in normal epithelium and cervical cancer. CYFRA 21-1 was reported to be positive in the majority of patients and in all patients with advanced cervical cancer, and its expression is well correlated with tumor size and stage [103]. CYFRA 21-1 elevation was observed in 26–63% of patients with cervical cancer [104].

13.3.3 CA125

Elevated CA125 levels were detected in 20–75% of patients with cervical adenocarcinoma [104] as well as in patients with squamous cell cervical cancer. CA125 was elevated in 75% of patients with adenocarcinoma as against only 26% of patients with squamous cell carcinoma [105].

13.3.4 Other Markers

The sensitivity of carcinoma embryonic antigen (CEA) for cervical cancer detection is 32% in squamous cell carcinoma and 38.5% in adenocarcinoma [106]. Immunosuppressive acidic protein (IAP) was reported to be elevated in 53% of squamous cell carcinoma and 40% of adenocarcinoma. High level of serum IAP was significantly related with worse survival [107]. Upregulation in serum levels of vascular endothelial growth factor C (VEGF-C) was thought to be a unique phenomenon for the early diagnosis of cervical cancer metastasis [108].

miR-21, miR-29a, miR-25, miR-200a, and miR-486-5p were reported to be useful markers in serum samples for cervical cancer detection. A panel of five miRNAs constitutes a more sensitive and specific diagnostic test than SCC-Ag and CA125 [109]. Cervical cancer miRNA biomarker is well described elsewhere [110]. lncRNAs such as HOTAIR, MALAT1, CCAT2, SPRY4-IT1, RSU1P2, CCHE1, lncRNA-EBIC, and PVT1 are reported as candidate biomarkers in cervical cancer [111].

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Biomarkers of Malignant Pleural Mesothelioma

14

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Abstract

Malignant pleural mesothelioma (MPM) is a rare tumor with a poor prognosis. Blood biomarkers of MPM would be useful in clinical practice, as they could aid radiological evaluation by reflecting prognostic information and predicting the effects of treatment. The many reports on MPM blood biomarkers have focused on their utility as screening or diagnostic tests. Carcinoembryonic antigen (CEA) and cytokeratin-19 fragments (CYFRA 21-1) have been used to aid in the diagnosis of MPM but are not useful as blood biomarkers. The most frequently studied blood biomarker of MPM is the soluble mesothelin-related peptides (SMRPs). Other potential biomarkers are megakaryocyte potentiating factor (MPF), also called N-ERV/mesothelin, which is formed from the same precursor protein as soluble mesothelin; osteopontin, a glycoprotein that mediates cell–matrix interactions; and fibulin-3, an extracellular glycoprotein. A combination of the best-performing marker and highest-value marker, as determined by ongoing research, will likely improve the accuracy and rapidity of MPM diagnosis in the near future.

Keywords

Malignant pleural mesothelioma · Soluble mesothelin-related peptide · Megakaryocyte potentiating factor · Osteopontin · Fibulin-3

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14.1 Soluble Mesothelin-Related Peptides (SMRPs)

SMRPs are the best-known putative biomarker in the serologic diagnosis of malignant pleural mesothelioma (MPM) [1]. Soluble mesothelin (SM) is a 40-Kd glycosylated protein on the surface of cell membranes and is overexpressed in pancreatic cancer, ovarian cancer, mesothelioma, and other cancers. SM is not a cancer-specific antigen but is thought to be a differentiation antigen expressed in normal cells of the pleura, peritoneum, and pericardium. The SM gene encodes a precursor protein of 69 Kd, and this glycoprotein is cleaved with furin-like proteinase and released to the blood as the 31-Kd megakaryocyte potentiating factor (MPF) at the N-terminal side. The glycoprotein on the C-terminal side binds to the cell membrane as SM. Three SM variants are known, one of which has a carboxyl terminal region and is liberated from the cell membrane by the absence of a glycosylphosphatidylinositol (GPI) anchor (Fig. 14.1) [2].

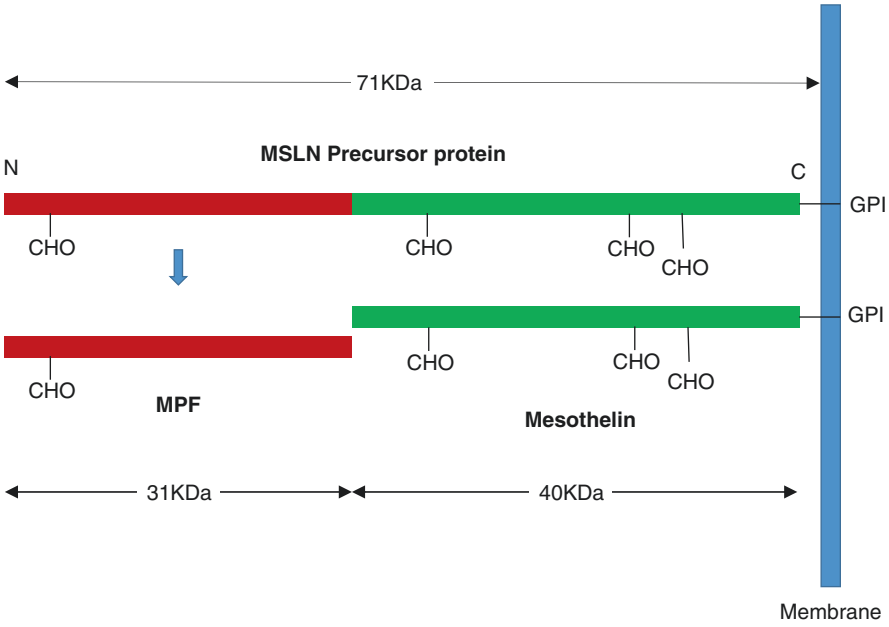

Fig. 14.1 Maturation of the mesothelin protein. The mesothelin precursor protein is synthesized as a 622 amino acid polypeptide and has a calculated molecular weight of 77 kDa. Potential signal peptide and a glycosylphosphatidylinositol anchor signal sequence are predicted at the NH_2 and COOH ends, respectively. The precursor protein has four possible glycosylation sites (CHO) and a furin cleavage site. Cleavage at the furin site produces membrane-bound mesothelin (green) and secreted protein megakaryocyte potentiating factor (red)

Serum/plasma SMRPs are the most studied biomarker of prognostic and therapeutic effects [1]. Early studies focused primarily on differentiating MPM from other malignant or benign lung lesions of SMRPs. In a study of 91 patients with MPM who received various chemotherapy regimens, there was a significant difference in overall survival (OS) (17.1 months vs. 8.4 months) between those with low (<3.5 nmol/L) and high baseline SMRP levels [3]. This association was statistically significant in multivariate analysis (hazard ratio [HR], 1.9; 95% confidence interval [CI], 1.1–3.5; $P = 0.025$) [3]. However, OS and baseline serum SMRPs were not correlated in two other studies, of 96 and 82 patients [4, 5].

Two meta-analyses investigated the sensitivity and specificity of serum SMRPs in the diagnosis of MPM. A systematic review and meta-analysis of 12 studies comprising a total of 717 MPM patients and 2851 control subjects, including healthy controls and non-MPM patients, showed a sensitivity of 64% for diagnosis of MPM (95% CI, 61–68%) and a specificity of 89% (95% CI, 88–90%) [6]. Hollevoet et al. [7] examined 16 studies comprising a total of 4491 controls and 1026 MPM patients. The results showed a sensitivity of 32% (95% CI, 26–40%) and 95% specificity for SM. SMRPs are a useful marker for detecting progression of malignant mesothelioma and assessing tumor response to treatment. However, the low sensitivity of SM (35–50%) limits its value in diagnosis [8].

14.2 Megakaryocyte Potentiating Factor (MPF)

MPF is a 31-kDa secreted cytokine derived from mesothelin cleavage. MPF was assessed by ELISA in serum samples from MPM patients and healthy controls. Serum MPF levels were higher in MPM patients than in the controls, patients with benign asbestos-related disease, and lung cancer patients [9].

Tajima and colleagues [9] measured MPF and osteopontin (OPN) in 14 MPM patients before and after treatment with various chemotherapy regimens. Despite the small sample, the ratio of pre- to posttreatment levels was lower in patients with disease progression, as determined with the Response Evaluation Criteria in Solid Tumors (RECIST), than in those with a partial response ($P < 0.05$). In a larger study, Hollevoet et al. [10] investigated 62 patients who underwent pneumonic total resection ($n = 14$) or received pemetrexed/platinum chemotherapy ($n = 48$). In the surgery group, median MPF after treatment was 76% lower, and median SM was 78% lower in five patients. These results suggest that serum MPF predicts treatment response. Analysis of other covariates showed that baseline serum MPF was correlated with OS ($P = 0.040$). The serum biomarkers MPF, SM, and CA-125 were tested for correlation with treatment response in a phase I dose-escalation study of anti-mesothelin immunotherapy, called SS1P. In 20 patients, all 3 biomarkers were strongly significantly correlated with an outcome of partial response, stable disease, or progressive disease [11].

14.3 OPN

OPN is a secreted glycoprotein that is important in several biological processes, such as cell–matrix interactions, immunological modulation, tumor development, and cell migration. Serum OPN levels are elevated in several cancers, including MPM, colon, lung, and breast cancer. Therefore, serum OPN is a potential biomarker for early detection of MPM [12].

Grigoriu et al. [12] measured baseline serum OPN and SM in a cohort of 96 MPM patients. Baseline serum OPN was significantly associated with OS in multivariate analysis (HR, 3.46; CI, 1.1–10.9; $P = 0.034$). At a cutoff of 350 ng/mL, patients with low serum OPN had a median OS of 15 months, as compared with an OS of 5 months for patients with high serum OPN levels. Hollevoet et al. [10] reported that baseline OPN correlated with OS and progression-free survival and was an independent factor not correlated with other biomarkers or tumor stage. Pass et al. [13] investigated the benefit of adding baseline plasma biomarker levels to mesothelioma European Organization for Research and Treatment of Cancer (EORTC) prognostic indicators and Cancer and Leukemia Group B (CALGB) indicators. Baseline levels of plasma OPN, SM, and fibrin-3 were measured in a discovery cohort of 83 patients, and plasma OPN and SM were independently correlated with OS. Mundt et al. [14] analyzed OPN concentrations in serum and pleural effusion in two separate cohorts and evaluated its role in diagnosis and prognosis at baseline. Serum OPN was correlated with OS (HR, 2.5; CI, 1.4–10.3) when the median of the dataset, 185 ng/mL, was used as the cutoff value.

14.4 Fibulin-3

Fibrin-3 is a secreted glycoprotein containing epidermal growth factor and is involved in regulating MPM cell proliferation and migration. Fibrin-3 is also encoded by the fibulin-like extracellular matrix protein-1 (*EFEMP-1*) gene [15].

No study has investigated whether serum or plasma fibrin-3 is a significant marker of baseline prognosis. Hooper et al. [16] measured serum fibulin-3 after two cycles of pemetrexed/cisplatin and after chemotherapy. Baseline levels were high for epithelial cell subtypes, but there was no correlation with OS when histological subtypes were analyzed separately. In addition, continuous sampling did not predict treatment response or progression-free survival. Creaney et al. [5] focused on diagnostic utility and measured serum and pleural fibulin-3 in a prospective cohort of 82 MPM patients. Fibrin-3 level in pleural effusion (median, 1331 ng/mL) was significantly higher ($P = 0.002$) in patients with biphasic or sarcomatous histology than in those with epithelial subspecies (median, 426 ng/mL). However, serum fibulin-3 was not associated with OS.

14.5 Other Biomarkers

14.5.1 Vascular Endothelial Growth Factor (VEGF)

Serum pan-VEGF levels were analyzed and compared in 51 MPM patients and 42 asbestos-exposed persons without MPM. Serum VEGF level was significantly higher in the MPM population and increased in relation to tumor stage. The median was high for epithelial cell sarcoma tissue (1071 ± 816 pg/mL vs. 580 ± 279 pg/mL), but the difference was not significant. Multivariate analysis showed no significant correlation with OS [17]. Kao et al. [18] randomly tested thalidomide as a chemotherapeutic adjuvant ($n = 34$) or as a single agent. In multivariate analysis, baseline serum VEGF was the only significant biomarker that predicted OS ($P = 0.025$); median survival was longer in persons with a low VEGF level. In addition, median OS was longer in patients with high baseline levels who underwent chemotherapy than in those with lower baseline levels ($P = 0.050$).

14.5.2 High-Mobility Group Box 1 Protein (HMGB1)

HMGB1 is a damage-associated molecular pattern (DAMP) molecule that mediates several biological processes, such as transcription, cell proliferation, DNA repair, and inflammation. Acetylation of HMGB1 prevents nuclear translocation and results in HMGB1 accumulation in cytoplasm [19]. Inflammatory cells such as granulocytes and macrophages can release acetylated HMGB1 from cytosol in the extracellular environment, which increases proinflammatory activity [20]. HMGB1 is also passively released by cells undergoing programmed cell necrosis. MPM cells actively secrete HMGB1 in an autocrine manner, as shown by interference of HMGB1 antagonists against MM proliferation in vitro and in vivo [21]. Serum and plasma HMGB1 levels were reported to be higher in MPM patients than in healthy individuals. A systematic review and meta-analysis showed that HMGB1 is a prognostic marker of MPM [22].

14.5.3 Midkine

Midkine is a heparin-binding growth factor that promotes cell survival, growth, and migration [23]. It is markedly expressed during embryogenesis, especially during pregnancy, but is substantially downregulated in healthy adults. However, midkine overexpression has been observed in pathological conditions, including cancer [24]. In a study of 198 patients in 3 groups—95 patients with malignant mesothelioma, 56 patients with metastatic pleural cancer, and 47 patients with nonmalignant pleural disease (20 with benign asbestos pleurisy and 27 with benign pleural disease)—the cutoff

values were 1.5 nmol/L for mesothelin and 421 pg/mL for midkine. The sensitivity and specificity of midkine were 61.1% and 41.1%, 61.1% and 48.1%, and 61.1% and 75.0%, respectively, to distinguish malignant mesothelioma from metastatic cancers, benign pleural diseases, benign asbestos pleurisy, and pleural diseases other than malignant mesothelioma, respectively. An elevated baseline midkine level was associated with survival, after adjustment for stage, histological subtype, and treatment schedule (HR, 1.84; 95% CI, 1.09–3.09; $P = 0.022$) [25].

14.6 Conclusion

MPM is frequently diagnosed at an advanced stage; thus, earlier diagnosis is important. The author evaluated new and potentially more sensitive and specific MPM biomarkers, including serum SMRP, MPF, OPN, and fibulin-3, which yielded promising results. The combination of the best-performing marker and highest-value marker will enable more accurate and earlier diagnosis of MPM.

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