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Review

Mass spectrometry-assisted gel-based proteomics in cancer biomarker discovery: approaches and application

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Abstract

There is a critical need for the discovery of novel biomarkers for early detection and targeted therapy of cancer, a major cause of deaths worldwide. In this respect, proteomic technologies, such as mass spectrometry (MS), enable the identification of pathologically significant proteins in various types of samples. MS is capable of high-throughput profiling of complex biological samples including blood, tissues, urine, milk, and cells. MS-assisted proteomics has contributed to the development of cancer biomarkers that may form the foundation for new clinical tests. It can also aid in elucidating the molecular mechanisms underlying cancer. In this review, we discuss MS principles and instrumentation as well as approaches in MS-based proteomics, which have been employed in the development of potential biomarkers. Furthermore, the challenges in validation of MS biomarkers for their use in clinical practice are also reviewed.

Key words: mass spectrometry, proteomics, cancer biomarkers

Introduction

Cancer remains a major life-threatening disease with about 14.1 million new cases and 8.2 million cancer-associated mortalities reported in 2012 [1]. The global demographic and epidemiologic transitions signal an ever-increasing cancer burden over the next decades [2]. Cancer is a multigene disease and each tumor is composed of a variety of cell populations with distinct morphologies and behaviors [3]. Biomarkers such as proteins or biomolecular chemical modifications are quantifiable indicators of a specific biological state. In this respect, cancer-associated biomarkers are useful for studying disease, identifying patients at different clinical stages, and developing adaptive therapies [4]. For example, recent studies have demonstrated that long noncoding RNAs, circular RNAs [5], circulating tumor DNAs [6], and non-essential amino acids that support numerous metabolic processes crucial for the growth and survival of proliferating cells [7] can serve as biomarkers for cancers. Also, epidermal growth factor receptor, which is associated with the development of certain types of cancers [8], is regarded as a useful tool for cancer detection (Figure 1).

Cancer biomarkers can be classified into two categories including disease-related biomarkers and drug related biomarkers [9]. A biomarker should be (i) a mediator of the disease pathology, (ii) present at low and stable expression levels in healthy individuals and higher expression levels in patients, and (iii) simple and quick to evaluate [10]. Such a biomarker can be assayed and linked to cancer using a defined mechanism [11].

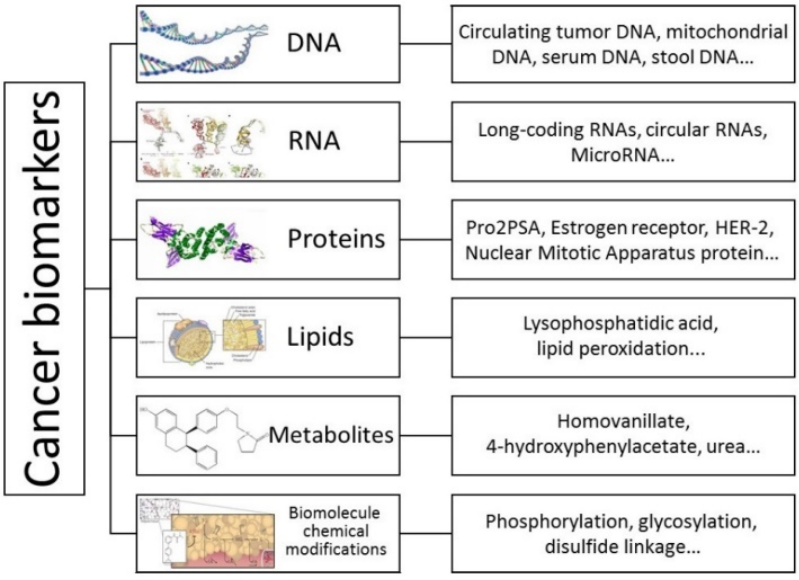

Figure 1. Schematic illustration of biomarkers for various types of cancers. Biomarkers are quantitative indicators of a specific biological state; therefore, cancer-associated biomarkers are useful for understanding the molecular basis of disease, early detection, identifying patients at different clinical stages, and developing a personal therapy.

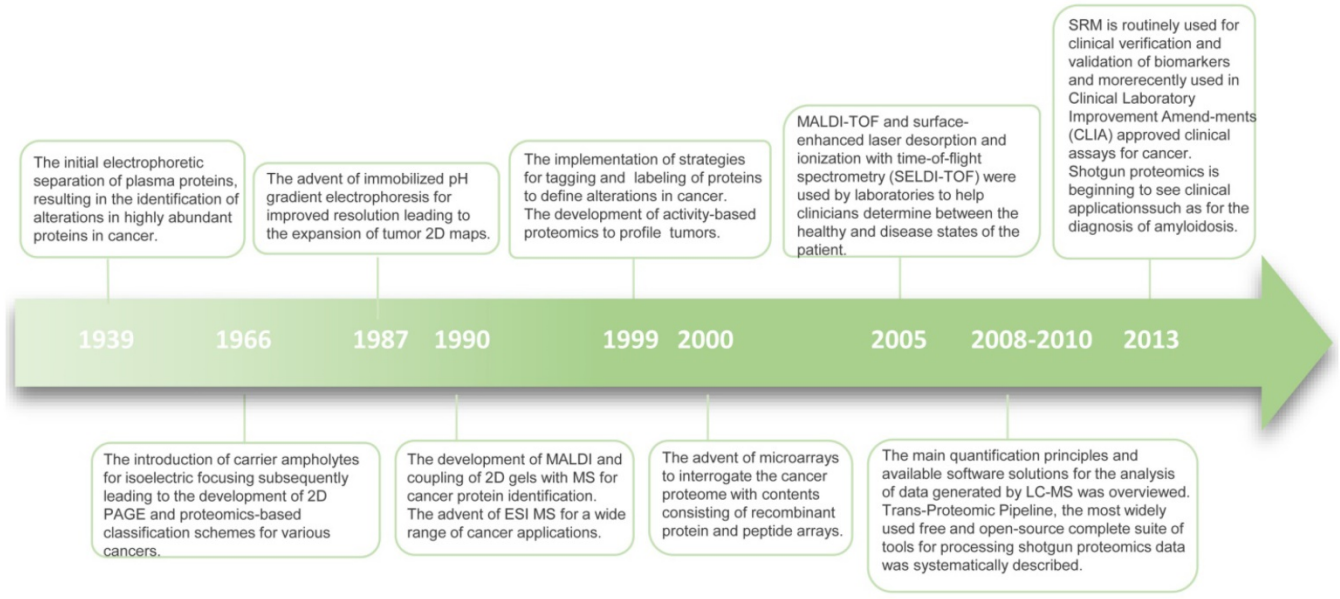

Figure 2. Timeline of progress in proteomics.

Recently, advanced molecular methods have been used in clinical diagnostic laboratories. Most novel techniques are based on transcriptional profiling and DNA methylation. However, compared with the genome and transcriptome, the proteome is more complex and dynamic [12]. The term "proteome" was first used in 1994 to indicate all time- and condition-specific proteins that are simultaneously produced by a cell or a tissue [12]. Proteins are often subject to proteolytic cleavage or post-translational modifications. Although genomics and transcriptomics can provide valuable information, they do not always reflect the variation of encoded proteins. Also, the association between mRNAs and protein expression levels is low compared with that of cell surface proteins [13]. Since proteins are the functional molecules in an organism and may be most ubiquitously affected in disease, therapy response, and recovery, proteomics holds special promise in detecting pathological conditions, predicting the efficacy of treatment, and tailoring personalized medicine (Figure 2) [14].

In a typical clinical proteomic study for diagnostic biomarker discovery, measurement of a large number of proteins in various samples is the first step. The initial protein candidates are proteins

that are differentially expressed in patient and control samples [15]. By confirmation of differential protein abundance in clinically useful samples, candidates can be progressively credentialed to yield a few specific proteins [15]. Candidate biomarker verification should be included in the biomarker development pipeline (Figure 3) to provide reproducible and sensitive quantitative assays [16].

Because of the limited availability and accessibility of suitable reagents, most proteins in a species cannot be detected and quantified by affinity-based assays [17]. Therefore, almost all currently available proteomic procedures and strategies use mass spectrometry (MS) techniques, which are capable of high-throughput profiling of complex samples. Nowadays, non-targeted MS methods have emerged as suitable tools to perform relative quantitation of a large number of proteins to discover novel protein biomarker candidates while targeted MS mode are applied to identify peptides of interest [18, 19]. A variety of MS-based proteomic methods have been developed to identify and quantify proteins in biological and clinical samples [20-23] to obtain biomarker candidates. The present study describes various currently used MS-based proteomic approaches and their applications. Also, the challenges of biomarker validation for their use in clinical practice are discussed.

Principles and instrumentation

MS analysis utilizes electromagnetic fields in a vacuum, where the molecular mass of the charged particle is determined [3]. MS is used to evaluate the molecular mass of a polypeptide or to determine additional structural features [17]. Tandem MS/MS is performed in the latter case to determine detailed structural features of peptides. Moreover, MS-based proteomic methods can also be applied to characterize protein complexes [22]. For example, protein conformation in solution and structural characterization of therapeutic proteins can be studied by hydrogen/deuterium exchange mass spectrometry (HDX-MS) [23].

MS instrumentation

In general, during MS analysis, the analyte is ionized in the gas phase, and the ions are subsequently separated according to their mass-to-charge ratio (m/z). Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are two methods widely used to perform the protein ionization. Both techniques hold great potential for the characterization of biomolecules.

A mass analyzer is an instrument that determines the m/z of ions and the number of ions corresponding to a particular m/z is recorded by a detector. Quadrupole (QD), ion trap (IT), time-of-flight (TOF), orbitrap, and Fourier transform ion cyclotron resonance (FTICR) are common types of mass analyzers. Numerous mass analyzers are often combined to achieve maximum performance [24]. For example, Muntel *et al*. used a quadrupole orbitrap instrument for urine protein biomarker discovery [25]. Moreover, the workflow of a MALDI imaging mass spectrometer (MALDI IMS) enables the histology-directed analysis of the mass spectra using tissues [26, 27]. In addition, optical density mass analyzers, known for their tolerance of high pressure, are particularly suited to the pulsed nature of ESI.

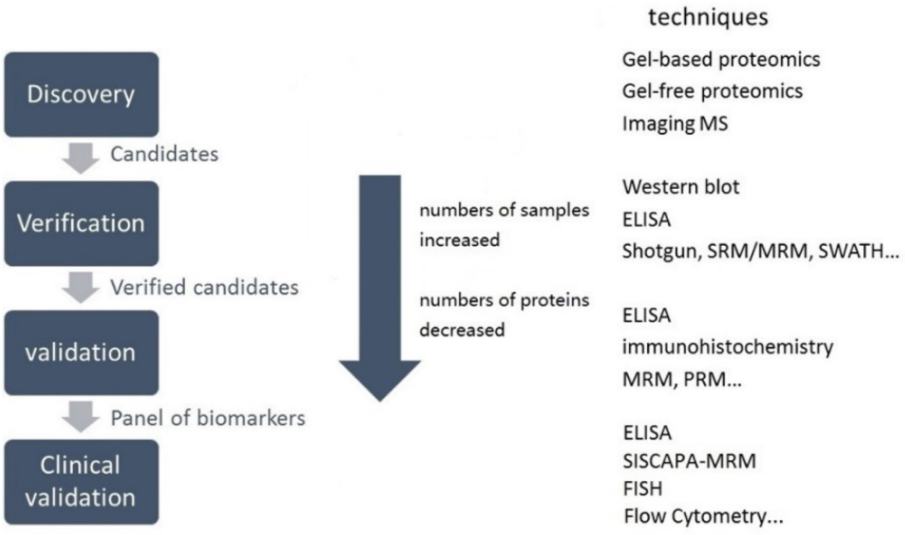

Figure 3. Schematic representation of the various stages in the biomarker pipeline. SISCAPA is the acronym for Stable Isotope Standards and Capture by Antipeptide Antibodies. FISH is short for fluorescent in situ hybridization.

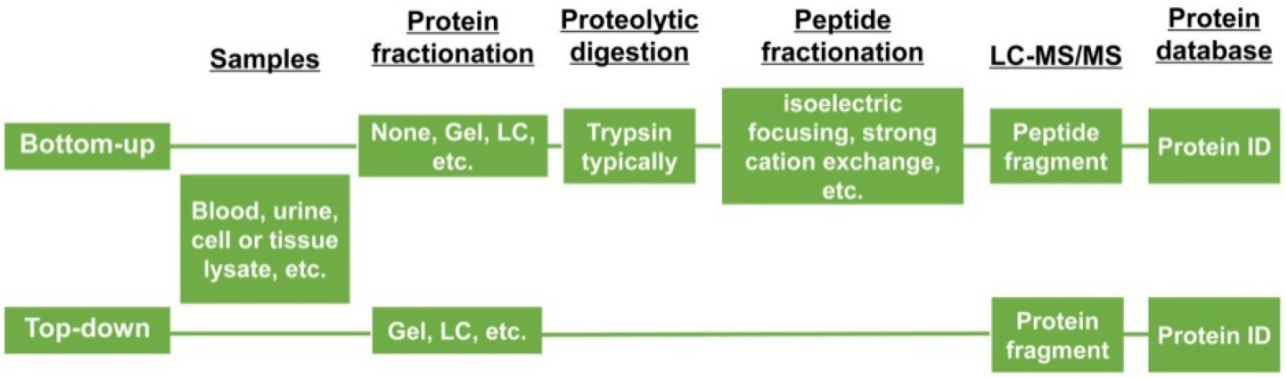

Figure 4. Two categories of proteomic experiments.

MS methodologies

Two-dimensional electrophoresis (2-DE) and chromatography-based proteomics

There are two main approaches to identify proteins applying gel-based proteomics, including bottom-up and top-down proteomics. In the former approach, proteins separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or in some instances, such as shot-gun proteomics wherein the fractionation step is left out, are digested in gel and then analyzed by MS [28, 29]. Which means the proteins are digested using chemicals or enzymes before introducing them into MS. Needless to say, this strategy may have several problems including the occurrence of modifications on disparate peptides. while the top-down approach, on the other hand, both the intact proteins and fragment ions masses can be measured [30] (Figure 4).

2-DE has been applied in proteomic research since its introduction in 1975. For example, Klein *et al*. used the 2DE-MS approach to analyze the nuclear proteome of human gastric cancer cell lines with and without inactivation of hypoxia-inducible factor 1 [31, 32]. The shortcomings of this strategy include a limited dynamic range and low-throughput analysis [3]. Although 2-D gel is still a powerful technique in proteomic analyses [33, 34], such as alternative detection for modification of specific proteins [35], attempts have been made to alleviate these drawbacks by using other techniques such as three-dimensional gel electrophoresis [36].

Shotgun based proteomics

Shotgun proteomics, also referred to as discovery proteomics, is a successfully used method [37]. It is based on employing a liquid chromatography-tandem MS (LC-MS/MS) for data-dependent acquisition (DDA) or in some certain occasions data-independent acquisition (DIA) mode. In DDA mode, peptide fragmentation is guided by the abundance of peptide ions detected in a survey scan. The recorded information of specific ions is searched against a protein database to determine the peptide sequence and protein identity [38]. In addition to its exquisite specificity, DDA-based proteomics has numerous other advantages, including unbiased and free-from hypotheses [39]. DIA offers advantages over conventional DDA methods as it overcomes the stochastic, intensity-based selection of peptide precursors [40].

One of the applications of the shotgun approach is to generate spectral libraries for mass spectrometric reference maps [41, 42]. It has also been used for the analysis of unique types of samples with biological and clinical importance including serum [43] and plasma [44, 45]. In a previous study, shotgun proteomics was applied to detect changes in protein profiles related to lung cancer [46].

Although many MS-based proteomic studies were performed using shotgun proteomics, the stochastic sampling of this technique markedly affects reproducible detection [47]. Furthermore, in traditional shotgun proteomics experiments, a large number of MS/MS spectra are collected. Peptide sequences are assigned using database searching algorithms, such as Sequest and PepExplorer, which use rigorous pattern recognition to assemble a list of homologous proteins [48]. However, not all spectra acquired are matched to peptides. To investigate this problem, Chick *et al*. identified unassigned peptides and demonstrated that at least one-third of unmatched spectra arise from peptides with substoichiometric modifications [49].

SRM-based proteomics

The adaptation of targeted data acquisition in the form of selected reaction monitoring (SRM), approximately a decade ago, was initially motivated by the requirement for robust and sensitive

quantification of proteins [50]. Numerous LC-MS workflows employ shotgun LC-MS; however, many others require a significantly higher reproducibility, sensitivity, accuracy, and precision of SRM [51]. SRM, also known as multiple reaction monitoring, uses triple Quadrupole (QD) (Figure 5), where molecular ions are selected in Q1, collision-activated dissociation fragmentation is performed in Q2, and unique fragments ions are evaluated in Q3 [52]. SRM is an attractive choice for sample analysis due to its sensitivity [53].

Advances in SRM have led to the discovery of numerous allergens in food complexes and cancer-related proteins [54, 55, 56]. Recently, by adding an isotopically labeled protein (^{15}N-α-S1-casein), accuracy of SRM analysis was increased [57]. In addition, absolute quantitation (AQUA), which has benefits of linearity over four orders of magnitude [58] and inter-laboratory comparability, also demands its use in allergen quantitation [59].

SRM has also been applied in biological fields [60], metabolic processes [61], signaling pathways [62], and validation of potentially interesting proteins [63]. As protein-protein interaction networks are significantly important in biological processes, it is essential to develop a computational method to predict protein-protein interactions. For example, Huang *et al*. proposed an efficient strategy that used a weighted sparse representation-based classifier model and novel feature extraction to sequence proteins for construction of protein-protein networks. [64]. Since investigation of phosphorylation events may serve an important role in biological research, Angeleri *et al.* developed an efficient strategy to obtain information regarding the phosphorylated sites [65].

Targeted data acquisition by SRM has been successful; however, the technique has intrinsic limitations. For example, the sensitivity of SRM currently cannot achieve the entire space of all organisms. Furthermore, the isolation width of Q1 can lead to false positive identifications [66]. Recent improvements, including time-scheduled SRM or intelligent SRM, have increased the scale and improved the quality of SRM evaluations [67]. In addition, parallel reaction monitoring has been developed markedly in instrumentation and software [68, 69].

Sequential window acquisition of all theoretical mass spectra (SWATH)-based proteomics

SWATH, a recently developed methodology [70, 71, 72] that relies on peptide spectral libraries, can be established by shotgun or obtained from community data repositories. Therefore, in contrast to SRM, SWATH-MS can quantify unlimited number of peptides that are included in spectral libraries.

SWATH-MS can be used in quantitative interaction proteomics [73, 74, 75]. For example, Ortea *et al.* provided evidence that LC-MS/MS combined pre-treatment and SWATH-MS was effective to identify lung cancer biomarker candidates [76]. SWATH-MS is also useful for the identification of candidate biomarkers, which will be further discussed in the following section [77, 78].

Additionally, there have been attempts to optimize the SWATH-MS workflow. The generation of a reference assay library is one of the key challenges and limitations of this approach [79]. It has been demonstrated that combined assay libraries can be used for SWATH data extraction [78], and certain software tools have been proposed for creating combined assay libraries [80, 81]. The parameters of MS detection were also optimized to increase the size of the library and decrease systematic errors [82]. These developments have broadened the application of SWATH.

Multiplexed MS/MS

In SWATH and other DIA approaches, peptides and their modified forms are difficult to distinguish because of the width of the window used for the isolated precursor. Egertson *et al*. introduced and improved the DIA framework, multiplexed MS/MS, to overcome the constraint on the scanning speed of the instrument [83]. The authors also suggested that this method may exploit other strengths of DIA [84].

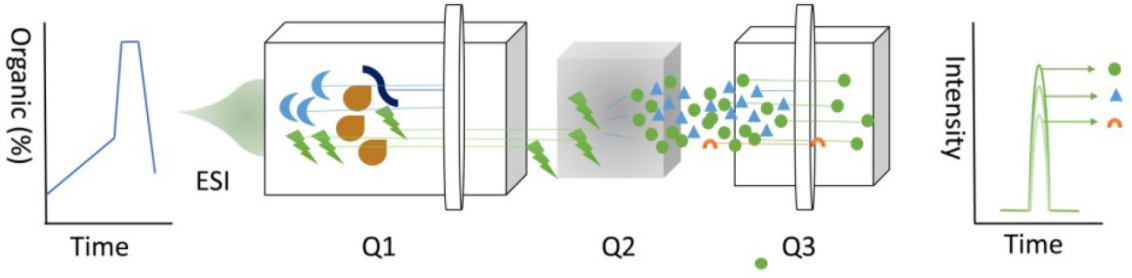

Figure 5. SRM technique.

Multiplexed MS/MS has certain disadvantages. It is more suitable for complex samples rather than simple mixtures due to its likely effect on the detection of low abundance peptides. Furthermore, the de-multiplexing and reconstruction of multiplexed MS/MS data may be a time-consuming process [85].

Application of MS in cancer biomarker discovery

Gastric, pancreatic, and liver cancers

Gastric cancer has one of the highest mortality rates worldwide [86, 87] urgently requiring its early detection [88, 89]. Studies of gastric cancer biomarkers mainly focus on tissues [90], blood [91], and biological fluids to identify protein, RNA [92], and DNA [93]. MS-based proteomics can aid in the identification of protein biomarkers and help study the mechanisms underlying gastric cancer [94]. Using MALDI-TOF-MS, Yang *et al*. analyzed serum samples obtained from 70 patients with gastric cancer and 72 healthy volunteers and identified two peptides ($P < 0.001$) related to gastric cancer [95]. Quantitative MS-based proteomic approaches include SCI techniques or label-free strategies in gastric cancer research. A variety of sources have been used to identify gastric cancer biomarkers, such as serum, gastric fluid [96, 97], cells obtained from tumor sections [98], cancer stem cells, circulating tumor cells [99], plasma membrane [100], saliva, plasma [101] and cancer tissues [102].

Cancer stem cells (CSCs) have been suggested to be extremely resistant to chemotherapy [103, 104]. Therefore, the identification of CSC markers has become a novel therapeutic perspective. Yashiro *et al*. used CSC-like side population cells to identify novel biomarkers of gastric CSCs [105].

Pancreatic cancer has been described as one of the most lethal tumors [106] with 45220 new cases and 38460 mortalities reported in the US in 2013 [107, 108]. There is a critical need for developing clinically useful biomarkers for pancreatic cancer detection. Carcinoma antigen 19-9 (CA19-9) is a biomarker which has been shown to be significant in the diagnosis, prognosis, and management of pancreatic ductal adenocarcinoma [109]. However, CA19-9 reacts with the sialylated Lewis^{a} blood group antigen present in the glycoprotein serum fraction [110]. 5-10% of the general population has the Lewis^{a-b-} phenotype; therefore, CA19-9 is not an appropriate biomarker for these individuals [111]. To overcome this problem, Yoneyama *et al*. identified insulin-like growth factor-binding protein 2 (IGFBP2) (AUC value of 0.706) and IGFBP3 (AUC value of 0.766) as plasma biomarkers for early detection of invasive ductal adenocarcinoma of pancreas [112]. In another biomarker study, Zhong *et al.* described a 2D-MALDI-TOF-TOF-MS/MS combined strategy for isolating and identifying membrane proteins. Immunohistochemical staining experiment demonstrated that the biomarker candidate they discovered was downregulated in pancreatic cancer tissue ($P < 0.05$) [113]. In another example, Tatsuyuki *et al*. identified novel prognostic markers by applying MS-based proteomic analysis [114].

HCC is the most common primary liver malignant disease [115]. HCC-associated mortality is high due to numerous contributing factors [116]. Therefore, there is an urgent need to develop clinical biomarkers that enable early detection for HCC [117]. Megger *et al*. performed 2-DE and label-free ion intensity-based quantification by applying MS and LC to identify differential protein abundance in HCC and control tissues [118]. Later, the same group combined previous results with label-free analysis [119]. In another study, Wang *et al*. analyzed five HCC subline variants using 2-DE coupled with MALDI-TOF MS [120].

Colorectal cancer (CRC)

CRC is the third most common cancer diagnosed and one of leading causes of cancer-related deaths in the US and [121]. The survival of patients with CRC is primarily associated with the stage of cancer [122]. However, limited number of CRC biomarkers have been developed [123]. Prognostic biomarkers could help the management of CRC [124]. Tomonaga *et al*. used the isobaric tags for relative and absolute quantitation (iTRAQ) shotgun method to discover biomarker candidates, which were subsequently validated by SRM [125]. Bosch *et al*. identified potential cancer markers to improve the diagnostic accuracy of the fecal immunochemical test to detect small traces of the blood protein, hemoglobin [126]. In another study investigating CRC, Peltier *et al.* combined iTRAQ technology [128, 129, 130] with reversed-phase liquid chromatography and MALDI-TOF/TOF to perform quantitative proteomic analysis of adenoma, CRC, and healthy control serum samples [127].

Glycosylation is important in many biological processes, such as immune surveillance for tumors [131, 132, 133]. Protein glycosylation commonly occur with the addition of specific glycan residues to asparagine (N-linked glycosylation) [134]. Sethi *et al*. utilized LC-MS/MS-based N-glycoproteomics to map the N-glycome landscape associated with a panel of colorectal cell lines and described a novel method to identify disease-associated markers [135]. In another

study investigating CRC, a fluorogenic derivatization-LC-MS/MS approach was utilized to perform a differential proteomic analysis of normal and cancer cells [136].

Lung cancer

Lung cancer can be classified into small cell (SCLC) and non-small cell lung cancer (NSCLC) [137, 138]. Numerous previous studies have demonstrated that pleural effusions contain proteins of potential diagnostic value [139, 140]. Recently, the proteome of pleural effusion in patients with NSCLC was investigated using pleural fluid from 20 patients with NSCLC and 10 patients with tuberculosis (Figure 6a) [141].The homodimeric glycoprotein stanniocalcin 2 was reported to serve numerous roles in a variety of cancer subtypes. By applying MS/MS analysis on tissue samples from 53 cancer patients, Na *et al*. revealed that stanniocalcin 2 was upregulated in lung cancer cells [142].

MALDI-TOF-MS has been used in numerous cancer studies [143]. It has been shown that endothelial cells (ECs) play an important role in the tumor microenvironment [144, 145] and the properties of tumor-derived ECs are different from normal ECs [146]. Zhuo *et al.* isolated ECs from lung squamous cell carcinoma using magnetic beads (Figure 6b) [147]. Using the same method, Jin *et al*. discovered a protein candidate which was related to the histological presence of lymph node metastasis and neural invasion ($p < 0.01$) [148].

Toxicity and drug resistance remain major challenges facing cancer therapy. Efforts have been made to discover ideal biomarkers to improve the treatment efficiency. Rovithi *et al.* developed a serum peptide algorithm to classify cancer patients with regard to their clinical outcome [149]. To guide the radiotherapeutic method and avoid severe toxicity, Walker *et al*. investigated the alterations in blood during therapy [150].

The collection of saliva is less invasive compared with collection of the blood [151] or tissue making it an attractive biological fluid for diagnosis. Xiao *et al*. used 2D-MS to analyze two pooled samples. The results indicated that saliva analyses might be established for lung cancer detection [152].

Advances in MS help in mapping a large number of mass spectrophotometric peaks to reference libraries [153]. Using LC-SRM, 17 circulating proteins could be identified as potential cancer biomarkers in plasma samples collected from 72 patients [154]. However, despite extensive efforts in lung cancer diagnosis, it remains challenging to move protein candidates in the clinic [155-158].

Melanoma

Melanoma is a skin cancer with a high mortality rate [159]. Besides serum, urine, and cell lines, proteomics can also be used for quantitative analysis on formalin-fixed paraffin-embedded (FFPE) tissues. For example, Byrum *et al*. used label-free quantitative MS to analyze FFPE to identify potential targets for the therapy of melanoma [160]. Qendro *et al.* performed LC-MS/MS to profile five melanoma cell lines, a tissue sample of metastatic melanoma, and a benign melanocyte cell strain [161]. Bioinformatics analysis was performed with each group of proteins to assign over-represented Gene Ontology terms.

Extracellular vesicles including exosomes are one of the mechanisms used for cell-cell communication. Exosomes are initially defined as reticulocyte-secreted vesicles secreted by many cell types [161, 162]. Exosomes play an important role in cancer progression [163]. Previous studies demonstrated that melanoma exosomes may influence disease progression by enhancing immunosuppression [164], angiogenesis, and tumor metastasis [165, 166]. Lazar *et al.* performed proteomic analysis of seven melanoma cell lines and demonstrated that exosomes may be a potential biomarker for melanoma classification [167].

Uveal melanoma (UM) is a primary malignancy of eye the etiology of UM remains poorly understood. According to clinical, histopathological, and genetic features of these tumors, patients with UM can be classified into low-risk and high-risk metastatic groups [168]. Crabb *et al.* performed global quantitative proteomic analysis of UM to increase our understanding of UM metastasis processes and to identify biomarkers of UM metastasis [169]. MS-based proteomics using the untargeted MS method to discover novel protein biomarker candidates and the targeted MS mode to identify peptides of interest, has been a useful tool in melanoma research [170].

Breast cancer

Breast cancer contributes to approximately 14% of the cancer-associated mortality [171]. Although 5-year survival rates have improved, ≥20% of all patients continue to develop metastatic disease with an associated poor outlook [172]. Hormone receptor positive, erb-b2 receptor tyrosine kinase 2 (ErbB2) positive, and hormone (estrogen or progesterone) receptor and ErbB2 negative breast cancers are the four main types of this aggressive disease [173].

Breast milk is an appropriate cancer microenvironment for identifying breast cancer biomarkers. Aslebagh *et al.* used a nanoLC-MS/MS to analyze breast milk samples collected from patients with cancer and controls. The results demonstrated

that sample-specific bands were present between the two groups [174]. Besides milk, serum is also used for identifying breast cancer-specific markers [175-182]. Dowling *et al*. combined metabolomics and proteomics platforms to analyze cancer and non-cancer serum samples [175]. High mobility group protein HMG-I/HMG-Y (HMGA1) abundance level was found to be associated with breast cancer clinicopathological features. Maurizio *et al.* utilized label-free shotgun MS to analyze the proteins extracted from HMGA1-silenced cells and control breast cancer cell line MDA-MB-231 [176]. Ning Qing Liu *et al.* evaluated numerous approaches for global proteome quantification and proteins involved in a signaling pathway in breast cancer tissues were identified (Figure 6c) [177]. Yang *et al*. collected serum samples from 183 breast cancer patients and 64 healthy controls to extract peptides using magnetic beads and analyzed by them MALDI-TOF-MS [178]. Besides serum, urine was also used in proteomic studies to analyze its feasibility as a potential source for breast cancer biomarkers [183].

Ovarian and uterine cervical cancers

Ovarian cancer consists of numerous distinct subtypes [184, 185]. However, the gold-standard biomarker, CA125, only performs well in one of these. A number of novel protein biomarkers relevant to ovarian cancer have been identified using MS-based proteomics [186]. Nepomuceno *et al*. applied LC-MS/MS on tissues obtained from chickens that developed ovarian tumors spontaneously as an emerging experimental model to investigate the ovarian cancer proteome and reported the upregulation of an inhibitor in tumors (p = 0.0005) [187]. Also, Poersch *et al*. performed LC-MS/MS on tumor fluids to identify ovarian cancer-associated protein biomarkers [188].

Drug resistance is a major challenge for ovarian cancer chemotherapeutic treatments. Therefore, it is essential to discover biomarkers that can distinguish chemosensitive and chemoresistant ovarian cancer patients [189]. Based on the LC-MS/MS results acquired from epithelial ovarian cancer, Chappell *et al*. hypothesized that mitochondrial proteome changes were required to develop chemotherapy drug cisplatin resistance [190]. In another study, Zhang *et al*. analyzed the protein abundance level in chemotherapy drug paclitaxel-resistant ovarian cancer cells and tissues [191].

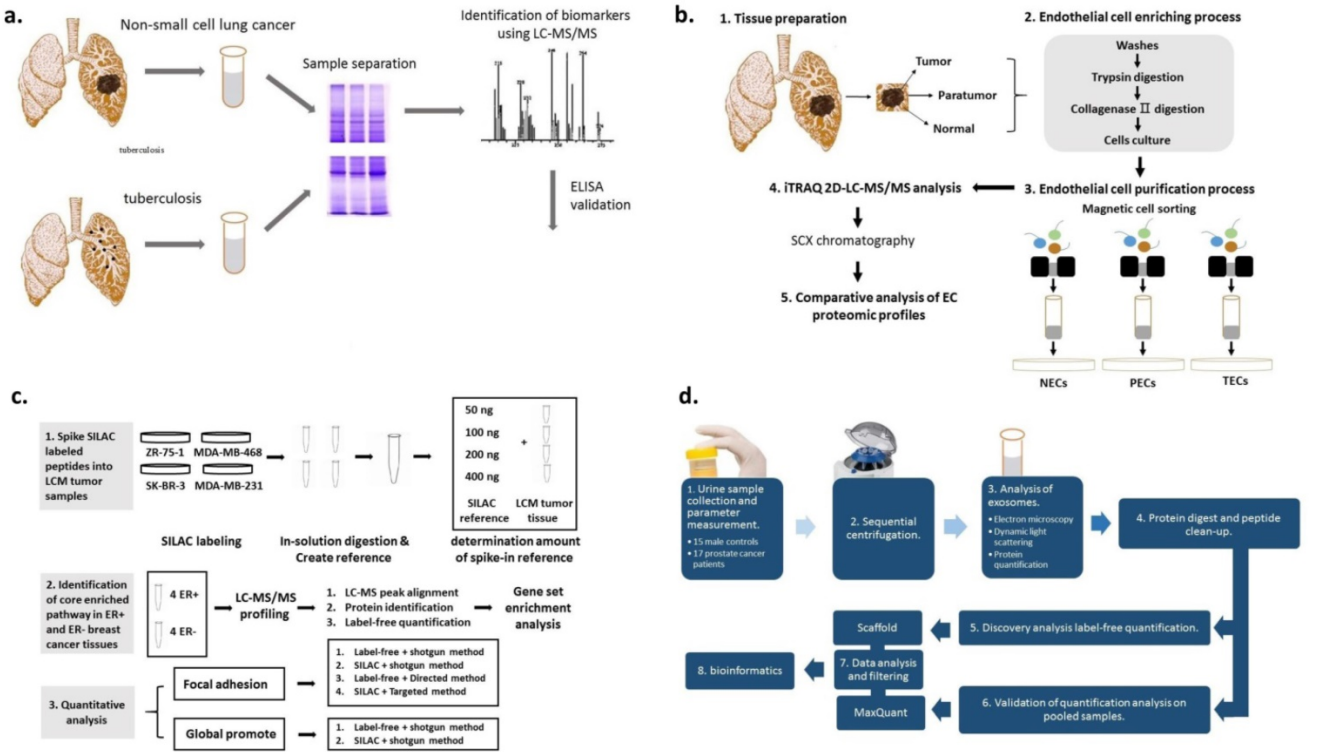

Figure 6. (A) Schematic illustration of proteome screening of pleural effusions to identify biomarkers for NSCLC. 1D SDS–PAGE was performed to separate proteins in pleural fluids. ELISA was used for the validation of protein candidates. (B) Schematic diagram of the experimental design. Normal, para-tumor-, and tumor-derived cluster of differentiation (CD) 105+ endothelial cells (ECs) were isolated, followed by iTRAQ-2DLC-MS/MS-based protein abundance profiling and comparative analysis of profiles. (C) Schematic diagram of the experimental design of systematic comparison between various quantitative methods for quantification of proteins within one pathway. (D) Schematic diagram of the experimental design for the isolation and characterization of urinary exosomes.

Using iTRAQ and LC-MS platform, Shetty *et al.* revealed that major histocompatibility complex class 1 ($p < 0.01$) may be related to ovarian cancer drug resistance [192]. In addition, the mechanism underlying somatic genome effects on the cancer proteome and associations between post-translational modification levels of proteins and clinical outcomes in high-grade serous carcinomas have been investigated [193].

Since Papanicolaou (Pap) test was approved by the US Food and Drug Administration (FDA) in 1996, a vast majority of cervical cancer screening has used liquid-based Pap test [194, 195]. Boylan *et al.* examined the proteins present in residual Pap test fixative samples from females with normal cervical cytology by 2-D-MS/MS and created a "Normal Pap test Core Proteome" [196]. More recently, the same group used iTRAQ to quantify the proteins in Pap test samples from patients with ovarian cancer compared with healthy controls or patients with benign gynecological disease [197]. The labeled samples were analyzed by 2D-LC-MS/MS. The results demonstrated that Pap test samples may be a valuable source for the identification of ovarian cancer biomarkers [197].

Urinary cancers

Urinary cancers include kidney, bladder, prostate, and testicular cancers [198]. Sensitive and accurate MS quantitative analyses have been introduced for biomarker discovery in these cancers [199]. Zhao *et al.* performed quantitative proteomic analysis on clear cell renal cell carcinoma (RCC) and adjacent kidney tissues using LC-MS/MS [200]. Urine is a rich resource for the investigation of kidney physiology as well as diagnosis of glomerulonephritis, hypertensive nephropathy, and renal cancer [201]. Sandim *et al.* investigated the proteins in urine samples collected from 64 patients with clear cell RCC and compared them with the healthy controls [202], whereas Neely *et al.* combined proteo-transcriptomic analysis and investigated alterations in protein abundance [203].

Prostate cancer is among the most common types of adult malignancies with an estimated 220,000 American males diagnosed with the disease annually [204]. Sensitive biomarkers would improve the efficiency of diagnosis, prognosis, and personalized therapy of prostate cancer. Øverbye *et al.* identified proteins with differential abundance in 16 prostate cancer patients compared to 15 healthy controls by MS-based proteomics (Figure 6d) [205]. Kim *et al.* developed SRM-MS assays in post-digital rectal examination urine samples. The results demonstrated that this strategy may accurately identify non-invasive biomarkers [206].

Urine is also considered to be an attractive source for bladder cancer biomarkers identification [204, 208]. Guo *et al.* proposed a strategy to identify urine proteins associated with bladder cancer [209]. In Europe and North America, a majority of bladder cancers are urothelial carcinomas [210]. Lin *et al.* used MALDI-TOF spectrometry on urinary exosomes for the determination of urothelial biomarkers [211].

MS-based proteomics has also been used to identify testicular cancer biomarkers. Liu *et al.* used the proteomics platform to identify proteins that participate in spermatogenesis and can, therefore, serve as novel targets for the treatment of male infertility and cancer [212]. The proteins they identified may also be used for personalized therapy for patients with testicular cancer.

Challenges in biomarker implementation and future prospects

Cancer progression is a comprehensive event that makes biomarker development a challenging task. Despite rapid advances in academia and industry, not many biomarkers move on to clinical practice [213]. Failure of cancer biomarkers appears to be due to several distinct challenges depicted in Figure 7 [214].

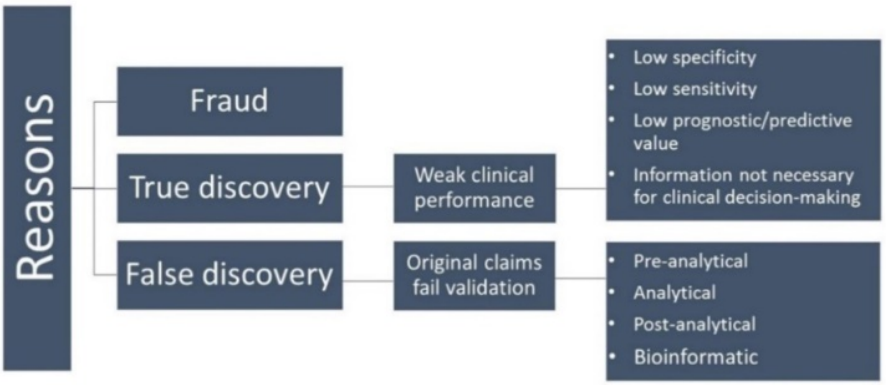

Figure 7. Schematic illustration of potential reasons for failure of biomarkers in clinical practice.

The first category of fraud is quite rare [215]. False discovery is a major reason for failure of biomarkers to reach the clinic. These biomarkers fail at independent reproduction in the validation phase [216, 217]. Small sample size as well as control samples used in the experiments that are not matched for age, sex, and race can lead to deceptive results [218]. Other important issues to be considered include, criteria for selection and inclusion of samples, strict standards for collecting and handling samples, suitability of the methodology, for the analysis of the data obtained, and, most importantly, independent validation of the identified biomarkers [219, 220].

Although few cancer biomarkers have entered clinical use, there are numerous ways to improve the situation. For biomarkers with low specificity and sensitivity that are not suitable for clinical use, it is possible to combine a panel of different biomarkers to identify clinical scenarios [214]. For example, a novel ovarian cancer biomarker, human epididymis protein 4, is not superior to CA125, which is an FDA-approved marker for ovarian cancer [221]. However, by combining human epididymis protein 4 and CA125, diagnosis of malignant versus benign pelvic masses can be improved [222]. For false discovery or artefactual biomarkers, understanding of the biological and molecular heterogeneity of disease states is required to guide the experimental design [223]. In addition, efforts should be taken made for improving the MS technologies to explore proteins with lower abundance [224].

Conclusion

Because of recent advances in MS-based proteomics together with streamlined sample preparation, improved instrumentation, and combination of various analytical platforms, numerous cancer biomarkers have been identified with diagnostic and prognostic values. The challenge is to realize the diagnostic and prognostic potential of these biomarkers in the clinical practice.

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Competing Interests

The authors have declared that no competing interest exists.

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