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Review Article

Development of biomarkers of genitourinary cancer using mass spectrometry-based clinical proteomics

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ABSTRACT

Prostate, bladder and kidney cancer are the three most common types of genitourinary cancer in the world. Of these, prostate and bladder cancers are within the top 10 most common cancers in men. Notably, kidney cancer causes no obvious symptoms in the early stages. To satisfy clinical-management requirements, researchers have developed numerous biomarkers by applying proteomic approaches using clinical serum, urine and tissue specimens, as well as cell and animal models. Through application of biomarker pipeline protocols, including discovery, verification and validation phases, and mass-spectrometric based proteomic platforms coupled with multiplexed quantification assays, these studies have led to recent rapid progress in this area. With improvements in mass-spectrometric based proteomic techniques, numerous promising biomarker candidates and marker panels for various clinical purposes have been proposed. Verification of novel protein biomarker candidates is very resource demanding (e.g. on the clinical and laboratory sides). With the support of national consortia, it is now possible to investigate the future clinical use of such biomarker strategies and assess their cost-effectiveness in personalized medicine.

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1. Introduction

Bladder, kidney and prostate cancers are the three major types of genitourinary cancer in the world, especially among males in developed countries. According to the most recent estimates of the American Cancer Society, there were 79,030, 63,990, and 161,360 new cases of bladder, kidney, and prostate cancer in the United States in 2017. In this same year, estimates of death due to bladder, kidney and prostate cancer were 16,870, 14,400, and 26,730, respectively [1]. Notably, prostate and bladder cancer (BLCA) rank among the top 10 causes of death in males [2]. Given the high morbidity and mortality associated with these cancers, improving early diagnosis and medical treatment is an important issue for achieving better clinical outcomes.

Cancer progression and development are very complicated processes, in part reflecting heterogeneity among patients. Despite considerable effort by the Human Genome Project (HGP) to sequence and map all human genes, for most cancers, cancer progression and drug-treatment strategies still cannot be predicted using only genomic data. The nucleotide sequences of genes are translated into proteins that serve a variety of functions, including catalyzing metabolic reactions, performing DNA replication, and mediating the response of cells to external stimuli, among others. The population of expressed proteins directly controls the physical characteristics of cells, making proteomics technology one of the most powerful tools for cancer research. These techniques allow the profiling of functional proteins and detection of their expression in clinical samples, and are thus invaluable for monitoring changes in the expression of specific proteins during cancer progression and development. Proteomic strategies also offer a compelling approach for uncovering potential biomarker candidates for cancer diagnosis, treatment, and other clinical applications.

In this review, we will introduce key sample-related issues in clinical proteomics, including sample preparation, general clinical sample types and protein purification from clinical samples, as well as the application of mass spectrometry and various protein quantification methods in systems biology. We also discuss the proteomic research pipeline, encompassing the discovery, verification and validation phases. Finally, we highlight potential biomarker candidates for prostate, bladder, and kidney cancer that are worthy of further study.

2. Proteomic techniques

2.1. *Sample preparation and MS-based techniques for proteomic research*

Fig. 1 shows the common workflow for sample preparation and protein identification for proteomic studies in genitourinary cancer using mass spectrometry-based approaches. The protein composition of clinical samples is generally very complex. Moreover, protein concentration usually covers a wide dynamic range in bodily fluids, which are typically used as clinical test samples. Hence, from the standpoint of the feasibility of analysis, it is vitally important to reduce the complexity of the sample during protein enrichment and pre-fractionation for proteomic research. In general, the precipitation of proteins with organic or high-salt buffers using the "salting-out" principle is one of the most common methods of protein enrichment. In practice, the addition of organic or high-salt solvents to a protein solution changes repulsive or attractive electrostatic forces of proteins in the sample, leading to protein precipitation. Trichloroacetic acid, acetone, isopropanol, ethanol, chloroform/methanol and ammonium sulfate are the most common solvents used for this purpose [3,4]. Alternatively, centrifugation is an easy method for protein enrichment, especially when starting with larger volumes of bodily fluids. This approach uses a semipermeable membrane with a molecular weight cut-off in conjunction with centrifugal force to separate proteins from the smaller molecule solvents as a tool for protein fractionation and enrichment. This method is easy to perform, requiring only a few centrifugation steps to concentrate, desalt, and enrich proteins in a sample [5,6]. Given the complex composition of clinical protein samples, noted above, pre-fractionation techniques are important tools for simplifying protein samples for subsequent protein identification and/or profiling. Sample fractionation can reduce ion-suppression effects during ionization of the mass spectrometer, thereby improving the identification of protein categories [7]. Further enrichment techniques, including immobilized metal-affinity and TiO_2-affinity chromatography, are required for detection of post-translational modifications in low-abundance target proteins in human cells [8], such as phosphoproteins [9]. Immuno-affinity chromatography, employing commercial kits, is commonly used to remove high-abundance blood proteins in samples to improve the detection of minor proteins in blood and urine samples [10,11]. However, non-specific or specific binding of non-targeted abundant proteins and the cost of antibody-based products are issues for large-scale studies. Combinatorial hexapeptide ligand libraries have been used to decrease the dynamic range of the proteome and increase the number of detected proteins [10,12]. The advantage of this technique is its applicability to multiple types of clinical samples. However, relative quantitative changes occur in the proteome during processing of combinatorial hexapeptide ligand libraries, potentially preventing determination of the absolute concentration in clinical samples.

After sample preparation, the most common protein identification strategy is bottom-up proteomics. In this approach, proteins are first enzymatically cleaved to peptides for acquisition of liquid chromatography–tandem mass spectrometry (LC–MS/MS) spectra. In-gel and in-solution protein digestion are two typical methods for protein digestion. As the name suggests, in-gel digestions are performed in situ in the gel matrix, and the resulting peptides are extracted for subsequent experiments. This technique is usually applied to samples from SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) or 2-DE (two-dimensional electrophoresis) gels [13]. The drawbacks of in-gel digestion are that it is time consuming, and the efficiency of digestion and peptide extraction are variable. The alternative—in-solution protein digestion—is performed directly

on aqueous protein samples and eliminates the problems of in-gel digestion [14]. Trypsin is the most commonly used enzyme in bottom-up proteomics because of its limited off-site activity, reproducibility of protein cleavage, and appropriate molecular size of tryptic peptides (~700–1500 Da) for LC–MS/MS analysis [9]. Additionally, tryptic or Lys-C peptides can be positively charged at both, the N and C-terminus, which results in more charged peptides that are well suited to peptide sequencing. This enables to read the sequence from both ends and thus, leads to the highest identification rates among all proteases. The most abundant fragment ions in MS/MS spectra are y and b ions when the most common CID or HCD fragmentation methods are applied.

Protein identification in academic research is usually performed using matrix-assisted laser desorption ionization (MALDI) or electrospray (ESI)–MS. MALDI–TOF (time of flight) MS is usually used in combination with 2-DE [15–19], because of the high resolution of such gels, and suitability for application of TOF-MS for rapid analysis. Although identification of individual proteins or gel-based samples by MALDI–MS is fast, comprehensive protein profiling by MALDI–MS usually fails if sample composition is too complex. The latter ionization method, ESI, is more routinely applied to automatic separation methods, including high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), and substantially diminishes peptide signal suppression. The remainder of this review focuses specifically on LC-based MS techniques for protein quantification.

Quantitative proteomic profiling aims to determine relative amounts of as many proteins as possible and can be performed using label-free methods or differential chemical labeling of peptides in conjunction with LC–MS/MS. In the beginning phase of biomarker discovery, comparative proteomic profiling of clinical specimens uses only limited amounts of protein, and most commonly involves approaches including data-dependent acquisition (DDA) of MS/MS spectra of isotopically labeled peptides. These peptides are generated using isotopic reagents such as isotope-coded affinity tag (ICAT), isobaric tags for relative and absolute quantitation (iTRAQ), tandem mass tags (TMT), dimethylation, or ^{18}O labeling, all of which are difficult to apply for a cell-culture system [6,10,20,21]. Isobaric-tag labeling provides accurate comparisons of multiple samples for protein and peptide quantification. Commercial kits for peptide-level labeling in the discovery phase are available for iTRAQ and TMT labeling [22,23]. Because these methods are compatible with such samples and are suitable for multiplexing, they have been applied in numerous quantitative proteomic studies [6,21,24–26]. Although an additional step is introduced and may not reach 100% labeling yield in an isobaric-tag labeling reaction, the extent of the labeling reaction should be similar for a protein/peptide in treatment and control samples from the same sample matrix. The use of stable isotope-labeled standards can compensate for issues that arise during sample preparation (e.g., sample loss) and proteolysis steps, as well as differences in MS detectors; as a result, they yield more accurate and reproducible quantitative data [27].

The sample size of labeling-based comparative proteomics is limited owing to the relative expense of labeling reagents and limited number of isotopic tags. Label-free proteomics techniques represent an alternative approach. Label-free methods can be divided into two categories: peptide peak intensity-based quantification and spectral counting quantification [28,29], which rely on the number of peptides identified from a given protein [30]. The advantages of label-free quantification include their relative cost-effectiveness and greater dynamic range and proteome coverage compared with label-based methods [28,31,32]. On the negative side, label-free quantification has no multiplexing capability and requires more replicative runs of the instrument to improve the confidence of quantification.

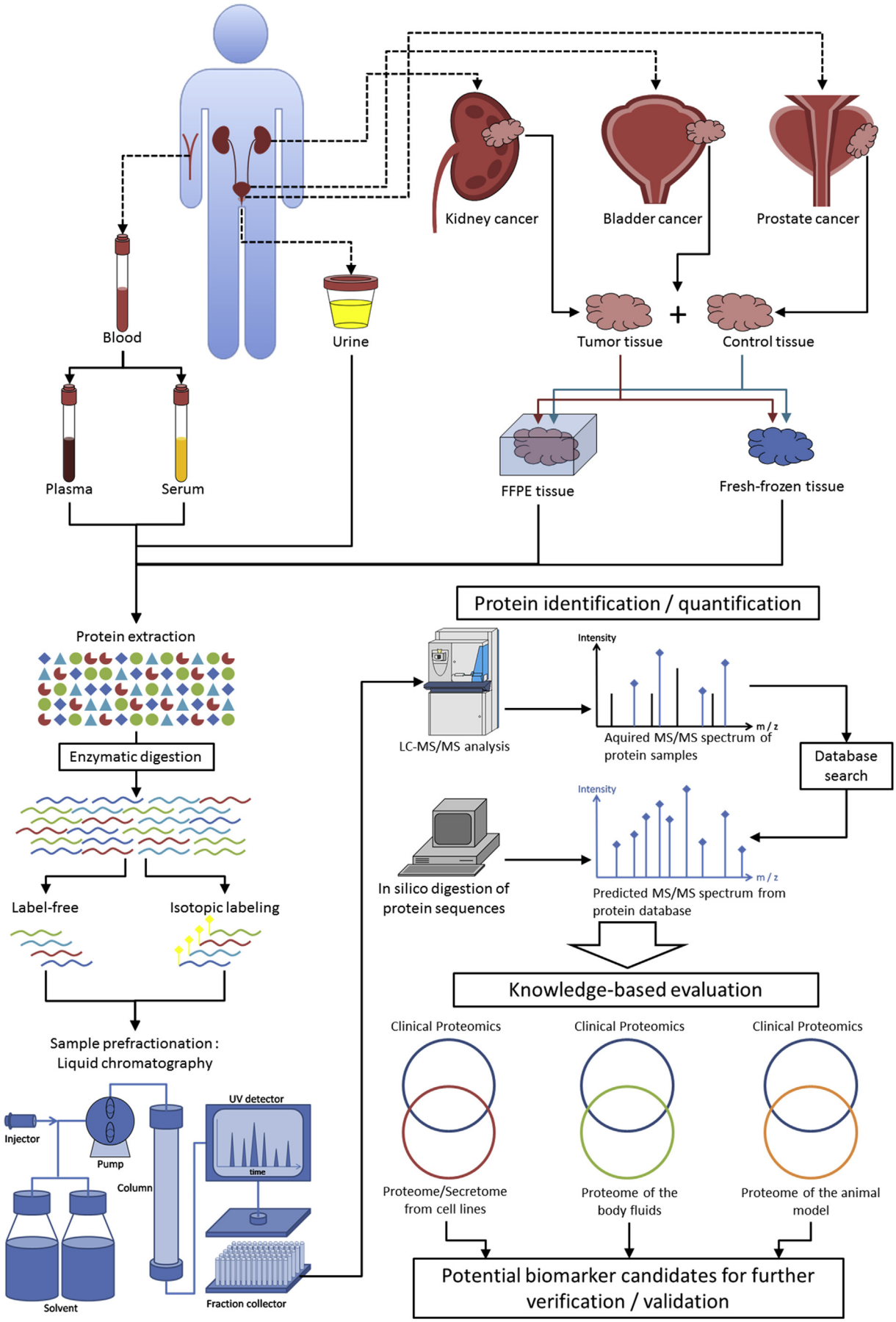
In DDA analysis, the mass spectrometer samples peptides for fragmentation with a selection bias towards those with the strongest signals. The principle underlying DDA thus hinders reproducible detection and/or quantification of minor proteins. As a result, data-independent acquisition (DIA) is gaining increasing attention in proteomic studies [30,33]. The principle of DIA is that all peptides within a wide mass-isolation window ('multiplex fragmentation') are subjected to fragmentation, and the analysis is repeated as the mass spectrometer progresses up the full *m/z* range. Using a computational workflow coupled with a DIA database library, precursor and fragment chromatographic features are detected for peptide sequencing [34]. This results in accurate peptide quantification without limiting profiling to predefined peptides of interest [30]. The acquired MS/MS spectra are matched with predicted mass spectra in sequence databases and are integrated into a comprehensive protein identification base for the selection of biomarker candidates for further evaluation as to clinical relevance. Thus, to summarize technical aspects of proteomic platforms, identification and quantification requirements for comparative clinical proteomics can be achieved using label-free and label-based approaches.

2.2. *Clinical specimens and experimental design of clinical proteomic studies of urological cancer*

The availability of human specimens is an important issue for biomarker discovery in both initial studies and in final applications. Therefore, we have focused on a consideration of specimens that are easily accessible and collected in this review.

2.2.1. *Blood*

Blood contains different blood cells and platelets, which are essential factors in the circulatory system. Because it can be conveniently collected in a relatively non-invasive manner, blood is the most frequently used bodily fluid in the clinic. Blood contains a variety of bioactive molecules, including proteins, metabolites, mineral ions, hormones and carbon dioxide, some of which are secreted from cells, tissues, glands or organs. Because blood perfuses all tissues and organs, it is capable of transporting these nutrients throughout the body so as to maintain a physiological balance. Therefore, blood is also representative of the overall physiology in humans [35]. It has been shown that the composition of the human plasma proteome is complex and its protein concentration exhibits a wide dynamic range, e.g. over 10 orders of magnitude. Serum albumin comprises more than 60% of total serum protein [36].


Kidney cancer
Bladder cancer
Prostate cancer
Blood
Urine
Tumor tissue
Control tissue
Plasma
Serum
FFPE tissue
Fresh-frozen tissue
Protein identification / quantification
Protein extraction
Enzymatic digestion
Label-free
Isotopic labeling
Sample prefractionation :
Liquid chromatography
Injector
Pump
Column
UV detector
time
Solvent
Fraction collector
LC-MS/MS analysis
Intensity
m/z
Aquired MS/MS spectrum of protein samples
Database search
In silico digestion of protein sequences
Predicted MS/MS spectrum from protein database
Knowledge-based evaluation
Clinical Proteomics
Clinical Proteomics
Clinical Proteomics
Proteome/Secretome from cell lines
Proteome of the body fluids
Proteome of the animal model
Potential biomarker candidates for further verification / validation

A total of 3509 plasma proteins summarized from 178 individual proteomic experiments recently were reported in the Human Plasma Proteome Draft of 2017 [37]. However, only hundreds of high/medium-abundant proteins were routinely detectable in blood in a single proteomic experiment and indicated the difficulty in routinely detection of minor plasm proteins. Therefore, analyzing the comprehensive proteomic profile of blood by MS is a challenge, but still attracts considerable interest as part of efforts to discover biomarkers from serum, blood, or plasma [38–41].

2.2.2. *Urine*

The principle components of urine, which is produced by the kidney, include water, glucose, salt and proteins [42]. The production of urine by the kidney provides a mechanism for removing waste from the blood, highlighting the importance of this organ in maintaining the physiological homeostasis of the human body. The kidney contains two major functional components: glomeruli and renal tubules. The function of glomeruli is to filter plasma (generating the glomeruli filtrate), whereas the function of renal tubules is to reabsorb most of the glomeruli filtrate. Healthy individuals produce ~1–2 L of urine per day. By comparison, approximately 150–180 L of glomeruli filtrate is produced per day, indicating that most of the glomeruli filtrate is reabsorbed. Because urine is filtered from plasma by glomeruli, it also contains a majority of plasma proteins. Thus, urine also retains some key information from plasma. Urine secreted by the kidney is collected by the bladder, where it is stored before disposal by urination. According to a previous study, ~70% of urinary proteins come from urinary organs and the urinary tract, whereas the remaining 30% represents proteins filtered by the glomerulus [43]. These attributes, together with the advantage of non-invasive sampling, have established urine as a good source for numerous studies of urogenital and systemic diseases [44–46]. The development of ultra-performance liquid chromatography (UPLC) enhances the resolution, sensitivity and efficiency of separation, with recent studies showing that up to 1500–3500 proteins can be identified by LC-–MS/MS in a single analysis of the urinary proteome [47–49].

2.2.3. *Tissue*

Although acquiring tissue samples from the human body is invasive, such samples offer the advantage of directly reflecting the condition of organs or lesions. Fresh-frozen (FFr) and formalin-fixed paraffin-embedded (FFPE) tissue samples are the two major types of samples available in clinical practice. Fresh freezing is the best method for retaining the representative molecular properties of tissues. Freezing of organs or tissue biopsies is performed immediately after surgery without the need for involved processing procedures. Frozen storage can prevent degradation of proteins by endogenous proteolysis and metabolite change [50], and as such is the ideal way to maintain the molecular information from the original tissue. However, the freezing option necessitates storage space and associated costs. Accordingly, FFPE is an alternative, convenient and cost-effective method for preserving tissue samples. In the general FFPE protocol, fresh tissue is fixed with formalin and embedded in paraffin. Formalin fixation causes cross-linking of proteins; therefore, an antigen retrieval step is necessary to break cross-links and allow correct identification of proteins. A previous study that used a targeted approach to compare the two sample preparation types [51] showed that chemical modifications induced by formalin fixation decrease the sensitivity for low-abundance proteins. This problem can be overcome by increasing sample input to making analysis of proteins in FFPE tissue samples by MS workable. For biomarker candidates discovered from tissue specimens, subsequent evaluations of clinical performance are commonly conducted using bodily fluids obtained through non-invasive methods.

3. Workflow of the biomarker development pipeline

The process of developing biomarkers is generally divided into several phases involving increasing patient population sizes and decreasing biomarker candidate numbers [52], as shown as Fig. 2. Biomarker development should start from a clearly defined, unmet clinical need for a biomarker or biomarker panel among a specific population, and should end with the ability to distinguish diseased patients from non-diseased individuals. In protein biomarker discovery projects, the secretome of cell models, bodily fluids and/or tissues are usually used first to generate lists of biomarker candidates (Fig. 1), classically by comparing case and control samples. Longitudinal studies, in which each “early case” subject is its

Fig. 1 – The workflow for sample preparation and protein identification for proteomic studies in genitourinary cancer using mass spectrometry (MS) based approaches. The proteomic profiles of cancer tissue and control tissue are compared to discover dysregulated proteins that are associated with cancer progression. Fresh frozen tissue samples and formalin-fixed paraffin-embedded tissue samples are two major types of tissue samples. Plasma, serum and urine are the materials most suitable for the development of diagnostic biomarkers because they can be obtained through minimally invasive procedures. Blood, the most widely collected and used bodily fluid in clinics, exhibits a very complex composition and wide dynamic range of protein concentrations. Urine is filtered from plasma by glomeruli and, therefore, also contains a majority of plasma proteins as well as proteins secreted from urological organs. Urine is a good sample source for non-invasive diagnosis. Disease model systems are also integrated into the workflow to minimize the impact of the heterogeneity of clinical specimens used for the selection of biomarker candidates. Extracted proteins are prepared by subsequent enzymatic digestion, fractionation and labeling procedures, and protein expression in cancerous and control specimens is compared using proteomics approaches that couple label-free or isotopic labeling with liquid chromatography–tandem mass spectrometry (LC–MS)/MS for the discovery of protein biomarker candidates. The list of protein biomarker candidates for further verification or validation can be improved by integrating results with proteomic changes observed in cell or animal models.

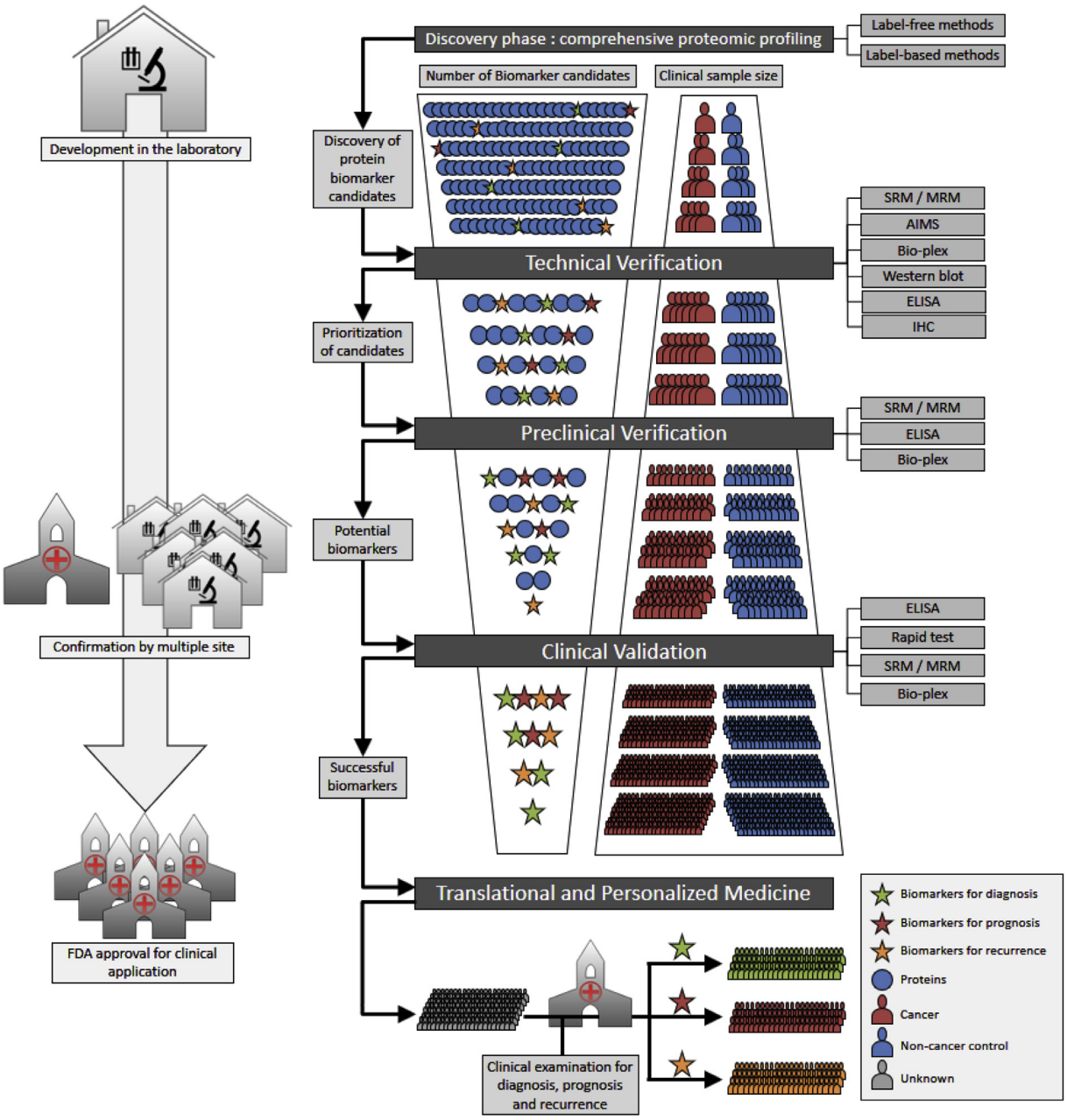


Fig. 2 – Biomarker discovery pipeline for clinical application. The pipeline starts from a clearly defined, unmet clinical need for a biomarker or biomarker panel capable of distinguishing diseased patients from non-diseased individuals among a specific population. In the discovery phase, samples prepared from a small number of individual samples, or pooled samples, are used to profile proteomic changes and generate a list of biomarker candidates. Individual variations may cause uncertainty and add cost to subsequent biomarker verification and validation steps. For selection or prioritization of biomarker candidates, a targeted quantification assay is then used to bridge the gap between the discovery phase in the laboratory and validation in clinics. Progressing through technical and pre-clinical verification, multiplexed targeted or advanced methods are used on an increasing number of samples, ultimately leading to the selection of a single or several promising biomarkers for use in the final development of a high-throughput assay for translational and personalized medicine using a large number of samples from multiple sites or hospitals. Hospital-based clinical studies will be essential for acceptance and use of results from marker discovery studies in a clinical setting.

own control for the later sample, are also used. The list of putative biomarkers is then refined by performing a verification step on an increasing number of plasma samples using targeted approaches. Ultimately, these analytically verified candidates are clinically validated in large cohorts so as to demonstrate the utility, specificity and sensitivity of a biomarker or a panel of biomarkers, and show their clinical relevance as prognostic or diagnostic tools.

The detailed progression of biomarker development can best be described as a multiphase approach linking

biomarker-candidate discovery, technical verification, pre-clinical verification, clinical validation, and translation into clinical practice for personalized medicine.

3.1. *Discovery phase of the biomarker pipeline*

As depicted in Fig. 2, the main aim of the discovery phase is to comprehensively quantify thousands of proteins in individual or pooled samples of human bodily fluid, tissues or cells, or animal models of genitourinary cancers. Because of the enormous instrumental resources required to comprehensively quantify proteins, a relatively smaller number of individual clinical specimens or disease models can be profiled at this stage of the biomarker pipeline. The opportunity to perform first-stage screening in a higher number of samples may help to improve the statistical significance of the discovery findings and reduce the number of pilot assays that need to be developed afterward, which again requires substantial resources. Dysregulated proteins are selected as potential biomarker candidates for further "technical verification" using an alternative analytical tool, for example using an MS-based method with a different quantification principle, or antibody-based methods, including Western blotting or enzyme-linked immunosorbent assay (ELISA), to confirm the quantitative results of MS-based data.

3.2. *Verification phase of the biomarker pipeline*

There is a critical bridge between the discovery phase—which generates a general list of biomarker candidates—and validation for potential clinical use. The purpose of the bridge between discovery and clinical validation, the so-called verification phase, serves to confirm expression of the protein and prioritize the numerous biomarker candidates from the discovery phase to create a list of the most promising candidates for use in developing higher-throughput tools. The verification phase can be divided into two stages: technical verification and pre-clinical verification. Because many current protein biomarker candidates were discovered using an MS-based shotgun approach, it is advisable to technically verify expression of the protein using a different targeted quantification method, either MS-based or non-MS-based, using a similar sample size. For Western blotting and ELISA, high-quality antibodies are necessary for protein quantification. Unfortunately, high-quality antibodies are not usually available for novel potential targets [53]. In general, pre-clinical verification is performed using targeted approaches and increasing sample sizes, typically on the scale of tens to hundreds of samples. Developing ELISAs for all potential biomarker candidates is difficult and is not a high-throughput strategy; thus, it lacks the economic benefit desirable for the technical verification stage of newly discovered protein biomarker candidates. Therefore, one of the bottlenecks of traditional biomarker pipelines is the inability to quantitatively verify the majority of novel candidates generated in discovery-phase studies—a key step in prioritizing targets for subsequent development [54]. This hurdle can now be overcome using the multiple reaction monitoring (MRM) mode, also called selected-reaction monitoring (SRM) [55,56], or parallel-reaction monitoring (PRM) [57], an MS-based instrumentation model for targeted quantification that was selected as Method of the Year in 2012 [58,59]. ELISA and MRM can both be used to quantify protein levels in large cohorts of samples: ELISAs can quantify a single candidate in many samples at once, whereas MRM assays are capable of quantifying multiple protein candidates in all samples in a single MS run. Verification and validation steps often require the development of assays, which in turn require the investment of time, resources and expertise, and possibly additional upstream development [60]. Although quantitative MRM assays take time and entail investment costs for instrumental resources, they are capable of quantifying proteins without being compromised by antibody availability or quality [60]. Other antibody-based techniques, such as Western blotting, immunohistochemistry, Bio-Plex and Olink Proximity Extension Assay (PEA) technology, are also commonly used in the verification phase to evaluate the correlation of protein expression between tissue and body fluids.

Based on the results of verification and candidate prioritization, a limited number of promising potential biomarker candidates are selected for further evaluation in the clinical validation phase. This phase aims to translate outcomes of preclinical end-point studies performed in an academic setting using state-of-the-art technology to clinics using tools that fulfill clinical requirements of an In Vitro Diagnostic Device (IVD) test [52]. High-throughput data generation and analysis capability is necessary because, typically, hundreds to thousands of specimens from multiple sites, such as hospitals and other laboratories, must be analyzed in this phase to determine the sensitivity, specificity, false-positive and -negative rates of biomarker tests, as well as additional statistical parameters of resulting classifications of subjects into respective groups.

The final phase of the biomarker discovery pipeline aims to accomplish the unmet clinical need defined at the beginning of the study by achieving a biomarker test suitable for personalized medicine. The application of a biomarker (or panel) may be adjusted during the progression of the study because of unexpected factors. Economic issues, including the cost of the biomarker test versus original medical assays, are calculated at this phase, as is the overall effectiveness of disease management in terms of quality of life, mortality and cost to the patients themselves, the community and the government.

4. Potential biomarkers identified using proteomic techniques in genitourinary cancers

As noted above, ~70% of urinary proteins come from urinary organs and the urinary tract [43], making urine a suitable specimen for the proteomic discovery phase in genitourinary cancers. Because of its advantage of non-invasive sampling, urine is used as the clinical specimen of choice for many proteomic studies on bladder, kidney, and prostate cancers as in Tables 1–3. Tables 1–3 also summarize studies on the development of biomarkers for genitourinary cancers using tissue, blood, cyst fluid, and microparticles [18,19,21,47] as sample materials. The sample size of each group in the discovery phase shown in Tables 1–3 ranged from 4 to 425 patients.

4.1. Potential biomarkers for bladder cancer

A number of proteins have been reported as potential biomarkers for various applications in BLCA. For the diagnosis of BLCA, previous studies by independent groups identified apolipoprotein A1 (APOA1) as a potential biomarker, either alone or as part of a biomarker panel [6,17,61]. APOA1 was validated for the diagnosis of BLCA by ELISAs, exhibiting an overall sensitivity and specificity of 94.6% and 92.0%, respectively, and an area under the curve (AUC) of 0.982, an indicator of its ability to detect BLCA For detection of early-stage BLCA, the sensitivity and specificity of APOA1 were 83.8% and 94.0%, respectively, and the AUC was 0.978 [6]. A separate study showed differential expression of APOA1 in urine from BLCA patients and normal controls in 2-DE MS analyses, and validated APOA1 for the diagnosis of BLCA by ELISA, reporting a sensitivity and specificity of 89.2% and 84.6%, respectively [17]. Further combining cytology with APOA1 expression increased the diagnostic sensitivity to 93.7% [17]. Transgelin 2 (TAGLN2) is a novel biomarker candidate recently identified in the BLCA tissue proteome that was shown to be differentially expressed in both bladder tissue and urine specimens [21]. Immunohistochemical analyses showed that TAGLN2 expression was almost 30-fold higher in tumor tissues compared with adjacent non-tumor tissue; this study also showed that TAGLN2 in tissue was capable of detecting BLCA with an AUC of 0.999 [21]. Gc-globulin (Gc) was identified in the urine of BLCA patients by two-dimensional difference gel electrophoresis (2D-DIGE) and MALDI-TOF–MS [16]. In this study, urinary Gc, determined by ELISA, was able to detect BLCA with a sensitivity of 92.3%, specificity of 83.0%, and AUC of 0.964. Urinary Gc was also able to differentiate infiltrating urothelial carcinoma from BLCA with an AUC of 0.889. Similar results were obtained by immunohistochemical and Western blot analysis of bladder tumor tissue [16]. Using ELISAs, Urquidi et al. [62] performed validation studies of C–C motif chemokine 18 (CCL18), plasminogen activator inhibitor 1 (PAI-1) and CD44—selected from previous proteomic profiling research [63]—investigating their diagnostic performance in BLCA. They also compared the performance of CCL18 in diagnosing BLCA with that of a United States Food and Drug Administration (FDA) approved BLCA detection assay (BTA-Trak). These studies, performed on 63 non-cancer and 64 cancer specimens, validated the diagnostic ability of CCL18, revealing a sensitivity of 88.0%, specificity of 86.0%, and AUC of 0.919. By comparison, BTA-Trak showed a detection sensitivity and specificity of 80.0% and 84.0%, respectively, and an AUC of 0.819 [62]. The combination of CCL18, PAI-1, and CD44 achieved a sensitivity of 86%, specificity of 89%, positive predictive value of 89%, negative predictive value of 86%, and AUC of 0.938. Using capillary electrophoresis coupled to mass spectrometry (CE–MS), Frantzi et al. performed a large-scale, multicenter validation study of urine-based peptide biomarker panels for the detection of BLCA [64]. In this study, urine samples from primary BLCA patients (n = 341) and controls (n = 110) as well as recurrent BLCA patients (n = 109) and corresponding controls (n = 316) were used for the biomarker discovery phase. They validated two biomarker panels, a 116-peptide model for primary BLCA and a 106-peptide model for recurrent BLCA, using two independent samples sets, including primary (n = 270) and recurrent (n = 211) validation sets. For detecting primary BLCA, the 116-peptide panel showed a sensitivity of 91%, a specificity of 68%, and an AUC of 0.87. For detecting recurrence, the 106-peptide panel showed a sensitivity of 87%, a specificity of 51%, and an AUC of 0.75. For detecting low-risk recurrence, the combination of the 106-peptide panel with cytology increased the performance, as evidenced by the resulting AUC of 0.90, which outperformed that of either single test alone (cytology, 0.64; peptide panel, 0.79) [64].

4.2. Potential biomarkers for kidney cancer

For the diagnosis of kidney cancer, Bosso et al. used an automated sample-preparation system to analyze 29 controls and 39 renal cell carcinoma (RCC) patients using MALDI-TOF–MS and LC-ESI–MS/MS for the discovery of kidney cancer-specific MS signals [65]. In this study, a cluster of three MS signals (m/z = 1827/1914/1968) was able to discriminate patients from controls with a specificity and sensitivity of 100% and 95%, respectively. Another study used a similar strategy to identify kidney cancer-specific proteins in a total of 162 serum samples using a combination of magnetic bead-based weak cation exchange chromatography and MALDI-TOF–MS [66]. Three candidate peaks identified from RNA-binding protein 6 (RBP6), tubulin beta chain (TUBB), and zinc finger protein 3 (ZFP3) were upregulated in the clear cell RCC (ccRCC) group and exhibited a tendency to return to healthy control levels after surgery [66]. In this study, this three-peptide panel identified ccRCC patients with a mean sensitivity of 88.38%, a mean specificity of 91.67%, and an AUC of 0.81–0.83 [66]. For large-scale validation, Frantzi et al. initially verified a panel of 86 RCC-associated peptides using urine samples from 40 RCC patients and 36 controls, demonstrating a sensitivity of 80%, a specificity of 87%, and an AUC of 0.92 [67]. They subsequently evaluated specificity in 1077 control samples, including age-matched normal controls (n = 218) and disease control patients with related cancer types or renal diseases (n = 859). Their results showed that the panel of 86 RCC-associated peptides was able to discriminate RCC from closely related cancer types and non-malignant renal and systemic diseases [67]. Recently, Jeremiah et al. measured urine AQP1 (aquaporin 1) and PLIN2 (perilipin 2) by ELISA and Western blotting, respectively, in urine samples from 720 patients undergoing routine abdominal-computed tomography, 80 healthy controls, and 19 patients with pathologically confirmed RCC. Their results validated the ability of a screening protocol using urinary AQP1 and PLIN2 to diagnose patients with occult RCC [68].

4.3. Potential biomarkers for prostate cancer

The prostate-specific antigen (PSA) test, used to diagnose prostate cancer (PCa), has contributed to the early detection of prostate cancer [69]. Ueda et al. subsequently identified neuropeptide-Y (NPY) in plasma from prostate cancer patients by low-molecular-weight proteome profiling [70]. They developed a unique technology—Quick Enrichment of Small Targets for Mass Spectrometry (QUEST–MS)—for low-molecular-weight proteome profiling [70], and further

Table 1 – Summary of protein biomarker candidates discovered using proteomic platforms in bladder cancer.*

Biomarker	Sample type	Cohort	Method	Result			Reference
				Sensitivity	Specificity	AUC	
Apolipoprotein A-I	Urine	Discovery phase: 14 controls/23 BLCA patients	iTRAQ with LC–MS/MS				[6]
		Validation phase: 50 controls/76 BLCA patients	ELISA	100.00%	92.00%	0.98	
	Urine	Discovery phase: 10 controls/10 BLCA patients	2-DE–MS				[17]
		Validation phase: 40 controls/40 BLCA patients	ELISA	92.50%	80.00%	0.95	
Transgelin-2	Tissue/urine	Discovery phase: 4 pairs BLCA patients and adjacent control	iTRAQ with LC–MS/MS				[21]
		Validation phase: 68 controls/137 BLCA patients	ELISA	53.30%	80.90%	0.70	
Reg-1	Tissue/urine	Discovery phase: 7 cytology negative/7 cytology positive	2D-DIGE with MALDI-TOF–MS				[15]
		Validation phase: 48 controls/32 BLCA patients	ELISA	81.30%	81.20%	0.88	
Gc-globulin	Tissue/urine	Discovery phase: 12 controls/12 BLCA patients	2D-DIGE with MALDI–TOF–MS				[16]
		Validation phase: 40 controls/91 BLCA patients	ELISA	92.31%	83.02%	0.96	
Alpha-1-antitrypsin	Urine	Discovery phase: 46 controls/54 BLCA patients	LC–MS/MS				[91]
		Validation phase: 35 controls/35 BLCA patients	ELISA	74.00%	80.00%	0.82	
C–C motif chemokine 18	Urine	Candidates was selected from other studies					[62]
		Validation phase: 63 controls/64 tumor-bearing subjects	ELISA	88.00%	86.00%	0.91	
Prothrombin	Urine	Candidates was selected from other studies					[56]
		Validation phase: 80 controls/76 BLCA patients	MRM–MS	71.10%	75.00%	0.80	

(*continued on next page*)

Table 1 – (*continued*)

Biomarker	Sample type	Cohort	Method	Result			Reference
				Sensitivity	Specificity	AUC	
6-Marker panel	Urine	Candidates was selected from other studies					[56]
		Validation phase: 80 controls/76 BLCA patients	MRM–MS	76.30%	77.50%	0.81	
TACSTD2	Urinary microparticles	Discovery phase: 9 controls/9 BLCA patients	Dimethy labeling with LC–MS/MS				[47]
		Validation phase: 81 controls/140 BLCA patients	MRM–MS	73.60%	76.50%	0.80	
116 Peptide panel	Urine	Discovery phase: 110 controls/241 primary BLCA patients	CE–MS				[64]
		Validation phase: 102 controls/168 primary BLCA patients	CE–MS	91.00%	68.00%	0.87	
106 Peptide panel	Urine	Discovery phase: 316 controls/109 recurrent BLCA patients	CE–MS				[64]
		Validation phase: 316 controls/109 recurrent BLCA patients	CE–MS	87.00%	51.00%	0.75/0.87 (combining with cytology)	

*AUC—area under the curve; BLCA—bladder carcinoma; iTRAQ—isobaric tags for relative and absolute quantitation; LC–MS/MS—liquid chromatography–tandem mass spectrometry; ELISA—enzyme-linked immunosorbent assay; 2-DE-MS—two-dimensional electrophoresis MS; 2D-DIGE—two-dimensional difference gel electrophoresis; MALDI–TOF—matrix-assisted laser desorption ionization–time of flight; MRM–MS—multiple reaction monitoring MS; CE–MS—capillary electrophoresis coupled to MS.

Table 2 – Summary of protein biomarker candidates discovered using proteomic platforms in kidney cancer.*

Biomarker	Sample type	Cohort	Method	Result			Reference
				Sensitivity	Specificity	AUC	
3-Marker panel	Urine	Discovery phase: 29 controls/39 RCC patients	MALDI-TOF–MS				[65]
		Validation phase: 9 controls/19 RCC patients	MALDI-TOF–MS	100.00%	85.00%	N/A	
Peptide panel	Serum	Discovery phase: 64 controls/58 RCC patients	MALDI-TOF–MS/LC–MS/MS				[66]
		Validation phase: 64 controls/58 RCC patients	MALDI–MS	88.38%	91.67%	N/A	
14 3-3 Protein beta/alpha	Cyst fluid	Discovery phase: 76 controls/89 RCC patients	2D-DIGE with LC–MS/MS				[18]
		Validation phase: 76 controls/89 RCC patients	ELISA	N/A	N/A	0.88	
Peptide panel	Urine	Discovery phase: 104 controls/58 RCC patients	SELDI-TOF–MS				[92]
		Validation phase: 43 controls/28 RCC patients	SELDI-TOF–MS	67.80%	81.40%	N/A	
TSP1/ENO2	Tissue interstitial fluid (TIF)/Serum	Discovery phase: 5 controls/5 RCC patients	LC–MS/MS				[93]
		Validation phase: 4 RCC patients	MRM–MS/ELISA	TSP1 was 14-fold and higher in RCC patients; ENO2 was 4-fold and higher in RCC patients			
Peptide panel	Urine	Discovery phase: 68 controls/40 RCC patients	CE–MS				[67]
		Validation phase: 46 controls/30 RCC patients	CE–MS	80.00%	87.00%	0.92	
AQP1/PLIN2	Urine	Candidates was selected from other studies					[68]
		Validation phase: 80 controls/19 RCC patients	ELISA	95.00%	98.00%	0.99	

*SELDI-TOF MS—surface-enhanced laser desorption/ionization time-of-flight MS; RCC—renal cell carcinoma.

Table 3 – Summary of protein biomarker candidates discovered using proteomic platforms in prostate cancer.*

Biomarker	Sample type	Cohort	Method	Result			Reference
				Sensitivity	Specificity	AUC	
Neuropeptide-Y + PSA	Plasma	Discovery phase: 43 controls/73 PCa patients	QUEST–MS				[70]
		Validation phase: 45 controls/65 PCa patients	MRM–MS	81.50%	82.20%	0.88	
CD14	Urine	Discovery phase: 16 controls/16 PCa patients	LC–MS/MS				[71]
		Validation phase: 16 controls/19 PCa patients	ELISA	81.00%	100.00%	N/A	
Autoantibody of PRDX6 and ANXA11	Tissue/Serum	Discovery phase: 20 controls/24 PCa patients	2-DE with MALDI-TOF–MS				[19]
		Validation phase: 20 controls/20 PCa patients	2-DE immunoblotting	90.00%	100.00%	N/A	
21-Marker panel	Seminal plasma	Discovery phase: 55 controls/70 PCa patients	CE-MS				[94]
		Validation phase: 27 controls/48 PCa patients	CE–MS	83.00%	67.00%	0.75	
Peptide panel	Urine	Discovery phase: 41 controls/21 BPH patients/26 PCa patients	CE–MS				[95]
		Validation phase: 41 controls/21 BPH patients/26 PCa patients	CE–MS	92.00%	96.00%	N/A	
130 Verifiable peptide signals	Urine	Discovery phase: 125 BPH patients/52 HGPIN patients/89 PCa patients	MALDI-TOF–MS				[96]
		Validation phase: 125 BPH patients/89 PCa patients	MALDI-TOF–MS	71.20%	67.40%	N/A	
130 Verifiable peptide signals	Urine	Discovery phase: 125 BPH patients/52 HGPIN patients/89 PCa patients	MALDI-TOF–MS				[96]
		Validation phase: 52 HGPIN patients/89 PCa patients	MALDI-TOF–MS	80.80%	81.00%	N/A	
Peptide-signal panel	Serum	Discovery phase: 45 PCa patients (Gleason score < 7)/54 PCa patients (Gleason ≥ 7)	SELDI-TOF–MS				[73]
		Validation phase: 15 PCa patients (Gleason score < 7)/15 PCa patients (Gleason ≥ 7)	SELDI-TOF–MS	73.3%	60.00%	0.90	
Lamin A	Tissue	Discovery phase: 23 PCa patients (Gleason score < 7)/23 PCa patients (Gleason ≥ 8)	2D-DIGE with MALDI-TOF–MS				[74]
		Validation phase: 23 PCa patients (Gleason score < 7)/23 PCa patients (Gleason ≥ 8)	2D-DIGE with MALDI-TOF–MS	N/A	N/A	0.88	

5-markers panel	Serum/Tissue	Discovery phase: Purified tissue and serum samples from wild-type and prostate-specific Pten -knockout animals Validation phase: 54 PCa patients with Gleason score < 7 and ≧ 7	LTQ-FT—MS MRM–MS	60.90%	67.80%	0.79	[75]
FABP5	Urine/urinary extracellular vesicles	Discovery phase: 6 controls/6 PCa patients (Gleason score < 7)/6 PCa patients (Gleason score ≧ 7) Validation phase: 11 controls/18 PCa patients	iTRAQ with LC–MS/MS MRM–MS	N/A	N/A	0.76	[46]
		Discovery phase: 6 controls/6 PCa patients (Gleason score < 7)/6 PCa patients (Gleason score ≧ 7) Validation phase: 11 controls/5 PCa patients (Gleason score < 7)/13 PCa patients (Gleason score ≧ 7)	iTRAQ with LC–MS/MS MRM–MS	60.00%	100%	0.86	

*QUEST–MS—Quick Enrichment of Small Targets for MS; LTQ-FT–MS—LTQ-Fourier Transform MS.

validated screening results in an independent sample set (n = 110) using targeted MRM–MS technology. For prostate cancer diagnosis, the combination of NPY and PSA showed 81.5% sensitivity, 82.2% specificity, and an AUC of 0.88 [70]. Another study showed that the combination of urinary PSA with CD14 could be used to differentiate benign prostatic hyperplasia (BPH) from cancer using an MS-based proteomic profiling approach. In this study, the combination of urinary CD14 and PSA exhibited a sensitivity of 81–94% and a specificity of 84–100% for the differential diagnosis of BPH and cancer [71]. Ummanni et al. identified autoantibodies against peroxiredoxin 6 (PRDX6) and annexin A11 (ANXA11) in the serum of prostate cancer patients as prostate cancer classifiers [19], showing that these two potential prostate cancer-associated autoantibodies could be used to discriminate prostate cancer patients from healthy controls. The sensitivity of each antibody alone varied from 70% to 80%, but the combination of both PRDX6 and ANXA11 antibodies exhibited 90% sensitivity and 100% specificity for the diagnosis of cancer [19]. In prostate cancer, Gleason grading system is used to evaluate prognosis of prostate cancer patients from histologic pattern of prostate biopsy [72]. The performances of novel proteomic biomarkers associated with the Gleason score are included for the prediction of aggressive prostate cancer [46,73–75]. In these studies, Al-Ruwaili et al. performed validation of the peptide-signal panel in serum from low- and high-Gleason score PCa by surface-enhanced laser desorption/ionization time-of-flight MS (SELDI-TOF–MS). This peptide-signal panel can discriminate the high-Gleason score PCa with an AUC of 0.90 [73]. Furthermore, Fujita et al. identified a FABP5 protein in urinary extracellular vesicles from high-Gleason score PCa. FABP5 present a greater diagnostic ability (AUC = 0.86) for discrimination of high-Gleason score PCa from general PCa (AUC = 0.76) [46]. These biomarkers are helpful in the diagnosis of aggressive PCa, and can be applied in the prediction of PCa prognosis.

The major studies describing the development of biomarkers for bladder, kidney, and prostate cancers using proteomic platforms are summarized in Tables 1–3, respectively.

5. Further evaluation of urinary protein biomarkers for translational research and personalized medicine

It has been argued that hematuria and non-cancerous diseases interfere with previously discovered biomarkers, affecting their specificity for use in screening [76,77]. Hematuria—the presence of blood-derived proteins in urine—may be caused by nuclear secretion, bleeding, or angiogenic mechanisms when tumor development. Hematuria is one of the major symptoms of the urological cancer. The presence in urine of blood cells, which contain many major plasma proteins, further adds to the complexity of the urine proteome composition, creating an inherent challenge to discovering highly specific biomarkers. Even in some tissue proteins shown to be novel bladder or kidney cancerous tissue-leakage biomarkers, their urinary concentrations in hematuria are also increased and result in poor organ specificity. One way to address this problem is to exclude all typical plasma proteins

as biomarker candidates; however, in doing so, the most sensitive biomarkers are often lost. These plasma protein biomarkers in urine usually show greater sensitivity in detecting urological cancer, but may exhibit lower selectivity in cancer screens. The combination of sensitive plasma proteins and other specific tissue-leakage proteins in urine is a strategy worth further consideration for different clinical applications. An alternative approach is to increase the cut-off values for plasma protein concentrations in urine. Because these proteins are commonly present at higher concentrations in urine specimens from cancer patients than in those from non-malignant subjects with hematuria [6], malignant and non-malignant urological diseases are still distinguishable using the plasma proteins in urine for non-invasive screening or advanced purposes.

6. Cost-effectiveness of using biomarkers in genitourinary cancer

The final assessment of a biomarker or panel will be its performance in cost–benefit studies in different population-based cancer programs that use biomarkers to solve real-world clinical problems through the use of a decision model designed in advance. The cost-effectiveness of a biomarker strategy will be evaluated based on saved lives and changes in total costs, including the biomarker test itself, infrastructure resources, and avoided or replaced medical events [78]. The economic value is also affected by the sensitivity, specificity, prevalence in the population, and acceptance of the assay by clinicians and patients [79]. Biomarkers are expected to offer the most cost-effective benefit in identifying high-risk patients with a history of disease or in monitoring the recurrence or metastasis of cancer. For screening assays, a relative low-cost biomarker assay is required for targeting individuals with no or only minor illness symptoms [80]. Multiplexed assays may show greater cost-effectiveness by lowering the frequency of measurement and the corresponding cost of population-wide screening, and by refining results originally obtained by less cost-effective methods from a smaller number of subjects [81]. Using BLCA as an example, a diagnostic assay with superior sensitivity and similar specificity to the FDA-approved urinary biomarkers, NMP22 (nuclear matrix protein-22), fluorescence in situ hybridization (FISH), BTA-Trak, fluorescent immunohistochemistry (ImmunoCyt) or cystoscopy, will enable a reduction in the number of required cystoscopy procedures by at least 50% during primary diagnosis of BLCA patients as well as during follow-up monitoring. If the price of a biomarker assay is less than 50% of the cost of cystoscopy, reports indicate that it will be cost-effective for use as a biomarker for BLCA management [46,79,82]. In one case, the clinical effectiveness and cost-effectiveness of photodynamic diagnosis was compared with that of white light cystoscopy and the combination of urine biomarkers and cytology for the detection and follow-up of BLCA [83]. However, the clinical effectiveness, in terms of added life-years, and the extra costs associated with societal acceptance of replacing white light cystoscopy with photodynamic diagnosis, was not clear. A model for estimating cumulative cancer-related costs and efficacy of screening (compared with no screening) in a high-risk BLCA population was created using a urine-based tumor marker over a 5-year period [84]; the authors of this study concluded that the urine-based markers are cost-effective in a high-risk population. Notably, PSA tests, a mainstay of routine clinical monitoring, were recently shown to offer no significant benefit in terms of all-cause mortality, a conclusion that remains a matter of controversy [85–87]. Baseline PSA levels, warning thresholds, and screening intervals must be considered in the context of the target population. The American Urological Association (AUA) recommends shared decision-making for men age 55–69 years who are considering PSA screening and does not recommend routine PSA screening in men over age 70 years or in those who have less than a 10- to 15-year life expectancy [88,89]. Some potential urinary protein biomarkers have been reported in the case of RCC, including NMP22, NGAL (neutrophil gelatinase-associated lipocalin), AQP1, and PLIN2 [90]. Economic evaluations of these biomarkers remain limited to low-mass drug/immunotherapy responses, metastasis or progression, which are not directly related to proteomics.

7. Conclusions and perspectives

With the continuing improvement of MS-based as well as non-MS based proteomics, we would expect more previously discovered biomarker candidates to be verified and validated in blood or urine specimens for non-invasive clinical applications. Despite many novel protein biomarker candidates discovered using proteomics techniques, verification of a biomarker panel is more challenging than a single marker, which usually reports with a clearly-defined cut-off concentration value. With the use of a classification algorithm for deciding biomarker combination as a risk score, verification for the biomarker panel in another large independent set of samples by an alternative lab without establishing the computing algorithms is not feasible. More studies, especially collaborative investigations, are needed to evaluate the comparative effectiveness and health economic aspects of novel biomarkers (or panels), given the currently limited availability of data for genitourinary cancer. The link of the biomarker (or panels) behaviors between tissue and body fluid in individual patients will be also important to reflect the disease heterogeneity for addressing the unmet needs in drug responses and resistance of genitourinary cancer.

Conflicts of interest

The authors declare no conflict of interest.

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