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Review

Urinary biomarkers in prostate cancer detection and monitoring progression

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ABSTRACT

Prostate cancer (CaP) is the most common cancer in men and the second leading cause of cancer deaths in males in Australia. Although serum prostate-specific antigen (PSA) has been the most widely used biomarker in CaP detection for decades, PSA screening has limitations such as low specificity and potential association with overdiagnosis. Current biomarkers used in the clinic are not useful for the early detection of CaP, or monitoring its progression, and have limited value in predicting response to treatment. Urine is an ideal body fluid for the detection of protein markers of CaP and is emerging as a potential source for biomarker discovery. Gene-based biomarkers in urine such as prostate cancer antigen-3 (PCA3), and genes for transmembrane protease serine-2 (*TMPRSS2*), and glutathione S-transferase P (*GSTP1*) have been developed and evaluated in the past decades. Among these biomarkers, urinary PCA3 is the only one approved by the FDA in the USA for clinical use. The study of urine microRNAs (miRNAs) is another burgeoning area for investigating biomarkers to achieve a pre-biopsy prediction of CaP to contribute to early detection. The development of mass spectrometry (MS)-based proteomic techniques has sparked new searches for novel protein markers for many diseases including CaP.

Urinary biomarkers for CaP represent a promising alternative or an addition to traditional biomarkers. Future success in biomarker discovery will rely on collaboration between clinics and laboratories. In addition, research efforts need to be moved from biomarker discovery to validation in a large cohort or separate population of patients and translation of these findings to clinical practice. In this review, we discuss urine as a potential source for CaP biomarker discovery, summarise important genetic urine biomarkers in CaP and focus on MS-based proteomic approaches as well as other recent developments in quantitative techniques for CaP urine biomarker discovery.

1. Introduction

Prostate cancer (CaP) is the most common cancer diagnosed in men, with an estimated incidence of 16,665 new cases in Australia in 2017 (AIWH, 2017) and 161,360 new cases in the USA in 2017 (Siegel et al., 2017). In 2017, CaP is the second leading cause of male cancer death in Australia with more than 3000 men dying every year (AIWH, 2017). There were an estimated 26,120 deaths from CaP in the USA during 2016 and approximately 12.9% of men will be diagnosed with CaP during their lifetime, based on 2011–2013 data (Insitute, 2015; Siegel et al., 2016). The incidence of CaP increases with age and the risk of a male being diagnosed with CaP by their 85th birthday is 1 in 5 (AIWH, 2014).

There may be no symptoms in the early stages of CaP. In the later stages, symptoms include frequent urination, particularly at night (nocturia), pain on urination (dysuria), blood in the urine (hematuria) or a weak stream and pain in the lower back, upper thighs or hips. More widespread disease often spreads to the bones and gives pain or unexplained weight loss and fatigue. Early detection and treatment can significantly improve CaP survival (Obirieze et al., 2015). The traditional tests used to aid early detection of CaP are digital rectal examination (DRE) and the blood test for prostate specific antigen (PSA).

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A transrectal ultrasound (TRUS) guided biopsy is used to determine its aggressiveness by histopathology.

The serum PSA (PSA) test has been widely used as a screening test for CaP diagnosis for several decades. However, elevated PSA is not specific to CaP as PSA levels often increase in benign prostatic hyperplasia (BPH) and prostatitis, and the false-positive rate (negative prostate biopsy in patients with PSA > 4 ng/mL) of the PSA test is very high (Ferro et al., 1987; Gann et al., 1995; Stamey et al., 1987; Vickers et al., 2008). Furthermore, men with low PSA levels can also develop CaP (Thompson et al., 2004). There are no tests available with sufficient accuracy to screen populations of men for early signs of CaP. Also, a normal DRE result does not rule out CaP (Catalona et al., 1994). Neither test used separately or in tandem, is accurate enough to distinguish potentially fatal cancers from benign tumours (Catalona et al., 1994; Gann et al., 1995). The introduction of PSA testing and DRE leads to a significant increase in the discovery of the disease and has been criticised for contributing to over-diagnosis of CaP (Sandhu and Andriole, 2012). Such over-diagnosis can lead to unnecessary treatments and associated adverse effects such as sexual impotence, urinary incontinence and bowel problems (Gann et al., 2010; Guessous et al., 2016; Ilic et al., 2013; Moyer, 2012). A biopsy is the only way in which a definitive diagnosis of CaP can be made.

Current diagnostic approaches used in the clinic are not useful for the early detection of CaP, or monitoring its progression, and have limited value in predicting response to treatment. Thus, novel non-invasive CaP-specific biomarkers that can assist in the accurate detection of CaP and progression monitoring are in need. In this review, we discuss urine as a potential source for CaP biomarker discovery, summarise several important urine biomarkers identified in CaP and focus on mass spectrometry (MS)-based proteomic approaches for CaP urinary biomarker detection and future exploration.

2. Urine as a potential source for biomarker discovery

Urine is a liquid waste produced from the kidneys, containing inorganic and organic compounds (proteins, hormones and metabolites). The urethra runs through the prostate gland and merges with ejaculatory ducts through which prostate fluid is propelled into the urethra. Studies on urine provide an opportunity to evaluate the well-being of the prostate, and potentially allow early diagnosis of CaP. Urine's anatomic proximity to the prostate gland and the presence of tumour cells in the urine sediment (Dijkstra et al., 2014; Fujita et al., 2009), particularly enriched after a slight prostate massage (Haese et al., 2008), make it possible to develop potential non-invasive diagnoses of CaP using urine based markers.

Urine has become one of the most attractive bio-fluids in clinical proteomics. Compared with other clinical biological specimens, urine has many advantages for determination of both diagnostic and prognostic biomarkers (Fernandez-Serra et al., 2015). It is easy to collect, non-invasive and harmless to the human body. Urine can be obtained in large quantities and there is no significant proteolytic degradation compared with other bio-fluids (Thomas et al., 2010). In addition, urine has a less complex composition compared to serum or plasma, which reduces interferences in isolation and facilitates the evaluation of new biomarkers. A workflow from urine sample collection to biomarker discovery is shown in Fig. 1. The potential urinary biomarkers of CaP, outlined in Fig. 1, will be discussed in the following sections.

3. Genetic biomarkers identified in CaP urine

CaP specific biomarkers can be identified through a urine diagnostic test based on the fact that prostate cells can be detected in urine (Fujita et al., 2009). With the development of molecular biology, massive profiling studies of genes associated with CaP have recently been made possible. The most promising genetic and epigenetic biomarkers including specifically overexpressed genes in CaP cells were identified. These important urine biomarkers in CaP include long non-coding RNA (lncRNA) biomarkers such as prostate cancer antigen-3 (PCA3), CaP-specific fusion gene biomarkers such as transmembrane protease serine-2 (*TMPRSS2*), and CaP specific methylation biomarkers such as glutathione S-transferase P (*GSTP1*). The study of urine microRNAs (miRNAs) is another burgeoning area for investigating biomarkers to achieve a pre-biopsy prediction of CaP to contribute to early detection. The genetic biomarkers identified in CaP urine are shown in Table 1.

3.1. Long non-coding RNA biomarkers

PCA3, also known as DD3, a prostate-specific lncRNA, was first identified in 1999 by Bussemakers et al. and the gene is located on chromosome 9q21-22 and consists of 4 exons (Bussemakers et al., 1999). The *PCA3* gene is dramatically overexpressed in human CaP tissue relative to normal prostate tissue (Bussemakers et al., 1999; de Kok et al., 2002; Hessels et al., 2003) as the total RNA level ($p < 0.0001$) and as a PCA3/PSA ratio ($p < 0.0001$) (Kulda et al., 2016). However, significant differences in the expression of PSA mRNA in tumour tissue relative to normal prostate tissue were not found (Kulda et al., 2016). De Kok et al. found PCA3 in urine and prostate fluid from CaP patients and suggested using it as a possible urinary biomarker (de Kok et al., 2002). Based on the quantitative real time polymerase chain reaction (qRT-PCR) analysis, the PCA3 test received European Conformity in 2006 and obtained approval by the FDA in the USA in 2012 for clinical use. The PCA3 score is calculated as the ratio of PCA3 to PSA mRNA (PCA3 mRNA/PSA mRNA x 1000) (Luo et al., 2014) in a post-DRE urine sample and was found to be associated with the probability of diagnosing CaP in the prostate biopsy (Crawford et al., 2012).

PCA3 was more accurate in predicting clinically significant CaP than the widely used PSA, and could be used as a basis to decide upon the repetition frequency of biopsy in patients with a previous negative result, to improve the accuracy of CaP detection. However, the definition of the best discriminating value is controversial (Leyten et al., 2014). A recent meta-analysis of 11 clinical studies with 3373 CaP patients found that a cut-off PCA3 of 20 (sensitivity 72%, specificity 53%) was preferable to cut-off of 35 (sensitivity 49%, specificity 74%) (Luo et al., 2014), as unnecessary biopsies can be reduced by more than half at a PCA3 cut-off score of 20. These findings are consistent with a prior study that concluded that a PCA3-based nomogram was more accurate than clinical models without PCA3 and up to 55% of men would avoid biopsy by setting the PCA3 cut-off at 21 and only a few cases of high-grade CaP ($\leq$2%) would be missed (Hansen et al., 2013). In another study, a PCA3 cut-off score of 20 was also suggested in ruling out a repeat prostate biopsy (Wei et al., 2014). In this trial from 11 centres, urine specimens were collected after an attentive DRE and before undergoing prostate biopsy in 859 cases of CaP. Forty-six percent of the men with PCA3 less than 20 would have avoided a biopsy, however, 12% would have had undiagnosed CaP and 3% would have had undiagnosed high-grade CaP. A discriminating value of 35 was suggested in the 2015 clinical guide of the National Comprehensive Cancer Network to decide on a repeat biopsy in patients with a previous negative result (Carroll et al., 2016). However, PCA3's prognostic value is most likely limited as PCA3 was not correlated with biopsy Gleason score and clinical tumour stage (Leyten et al., 2014). It has not found wide use in the medical community due to these factors.

In 2 recent studies, other urinary lncRNAs such as metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1) (Wang et al., 2014), a multiple cancer-associated lncRNA, and FR0348383 (Zhang et al., 2015), a CaP-associated lncRNA, also demonstrated significant correlations with CaP, especially in the "diagnostic grey zone" (PSA 4–10 ng/mL). By setting the MALAT-1 threshold at 25%, or the FR0348383 threshold at 30%, 30–47% or 52% biopsies could be avoided respectively without missing any high-grade CaP (Wang et al., 2014; Zhang et al., 2015). MALAT-1 or FR0348383 have great potential

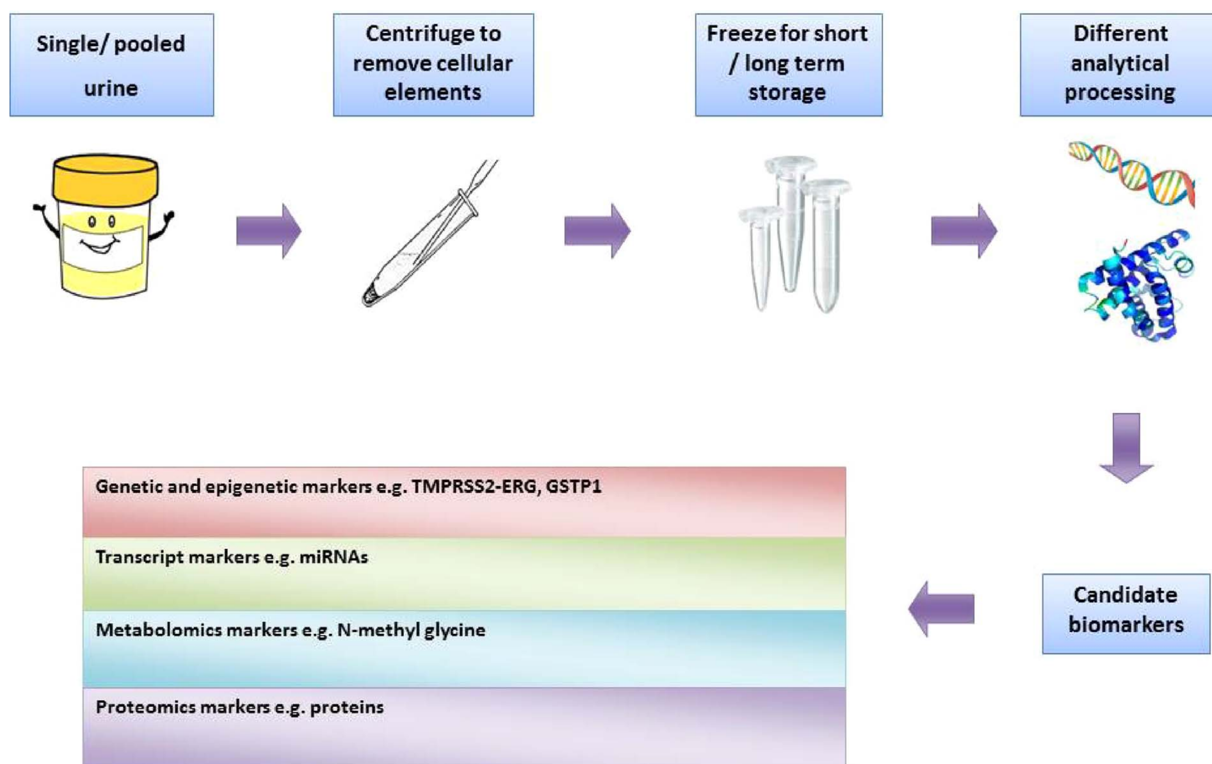

Fig. 1. A workflow showing the summary of urine as a potential source for biomarker discovery.

as independent predictors of CaP.

Besides urinary lncRNAs, clinical utility of a 3 protein-coding gene panel (HOXC6, TDRD1, and DLX1) in urine has been validated in a large multi-centre study (Leyten et al., 2015). Interestingly, the three-gene panel had higher accuracy (AUC, 0.77) compared with urinary PCA3 (AUC, 0.68) or PSA (AUC, 0.72) in predicting aggressive CaP (Gleason score ≥ 7) in biopsies, and integration of three-gene panel with PSA further improved the predictive accuracy (AUC, 0.81). Hamid et al. developed a urinary HOXC6 mRNA detection assay used in early detection of CaP, monitoring progression and response to therapy (Hamid et al., 2015), indicating that HOXC6 up-regulation was specific to CaP, and urinary HOXC6 level was significantly higher in CaP patients compared to men with negative CaP biopsies. The findings suggest urinary HOXC6 is a promising biomarker for CaP detection.

3.2. Gene fusion biomarkers

Gene fusion is a result of combining two or more separated genes into one single chimeric gene or transcript, and has been recognised as an important driver of cancer. Genomic rearrangements are suggested to be a major mechanism in driving prostate carcinogenesis (Berger et al., 2011). The *TMPRSS2-ERG* fusion gene is a CaP-specific fusion gene comprising androgen-related transmembrane protease, serine 2 gene (*TMPRSS2*) and ETS-related gene (*ERG*), which results in aberrant expression of the transcription factor ERG. *TMPRSS2-ERG* is the most common gene rearrangement in CaP (Esgueva et al., 2010) and is present in approximately 50% of CaP tissues in Western countries (Cary and Cooperberg, 2013). Additionally, it is not found in BPH samples (Furusato et al., 2011; Jiang et al., 2016). However, the frequencies of TMPRSS2-ERG fusion in men with CaP from Asian countries are lower than those reported from Western countries (Magi-Galluzzi et al., 2011). The fusion rate of the *TMPRSS2-ERG* gene was reported to be 20–28% (Furusato et al., 2011; Miyagi et al., 2010) in Japan, 21% in Korea (Lee et al., 2010) and only 11% in China (Jiang et al., 2016), indicating racial or ethnic disparities in the epidemiology of *TMPRSS2-ERG* fusion.

The mRNA products of the *TMPRSS2-ERG* fusion were first detected in urine by qPCR (Laxman et al., 2006). The urinary TMPRSS2-ERG score was highly correlated to ERG expression in CaP tissue (Young et al., 2012). Integration of the TMPRSS2-ERG in urine with PCA3-score can improve the prediction of CaP. Upon combining TMPRSS2-ERG and PCA3 into a urine test, the diagnostic accuracy was 84%, which was significantly higher than that of the PSA test (Mikhailenko et al., 2014). Incorporation of either urinary TMPRSS2-ERG or PCA3 with PSA can reduce more than half of repeat biopsies with no notable negative consequences in terms of 10-year overall, or 15-year cancer-specific survival (Merdan et al., 2015), suggesting that a large number of men could avoid unnecessary invasive procedures by applying this approach.

Urinary TMPRSS2-ERG and PCA3 have been evaluated to predict CaP (Cornu et al., 2013; Leyten et al., 2014). TMPRSS2-ERG plus PCA3 (cut-off: 25) increased detection sensitivity to 88%. However, TMPRSS2-ERG was not correlated with Gleason score in radical prostatectomy (RP) specimens and its prognostic value needs further evaluation. This was in accord with the results of a previous cohort study of 1180 men treated with RP where TMPRSS2-ERG was associated with tumour stage but not with biochemical recurrence (Lee et al., 2010) or mortality (Pettersson et al., 2012). However, shorter time to recurrence after RP was observed in patients with high expression of TMPRSS2-ERG in CaP tissue and high level of preoperative PSA (Kulda et al., 2016). A combination of high expression of TMPRSS2-ERG in tumour tissue and high preoperative PSA was suggested as a promising biomarker to evaluate the risk of recurrence after RP.

Another gene fusion product, TTTY15-USP9Y, was demonstrated to display significantly higher levels (AUC, 0.828) in post-DRE urine samples in a study of 226 CaP patients in China (Zhu et al., 2015). In this study, the value of PSA level in predicting CaP could be significantly improved when combined with the TTTY15-USP9Y score ($p = 0.001$). In addition, unnecessary biopsies could be reduced by adding the TTTY15-USP9Y score to clinical base models such as PSA level, age, and prostate volume, suggesting the TTTY15-USP9Y gene fusion is a promising biomarker for CaP prediction.

Table 1
Summary of molecular genetic biomarkers identified in CaP urine.

Biomarker	Substrate	Study group	Sample collection	Detection method	Potential application	Year	Reference
PCA3	lncRNA	CaP (n = 802), controls (n = 889)	Post-DRE urine	TMA, HPA	Diagnosis	2012	Crawford et al. (2012)
Metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1)	lncRNA	CaP-positive biopsies patients (n = 85), CaP-negative biopsies patients (n = 133)	Post-DRE urine	qPCR	Diagnosis	2014	Wang et al. (2014)
FR0348383	lncRNA	213 cases included, 72 cases were identified with CaP	Post-DRE urine	qPCR	Diagnosis	2015	Zhang et al., (2015)
Three-gene panel (HOXC6, TDRD1, and DLX1)	DNA	358 cases included, 250 cases were diagnosed with CaP	Post-DRE urine	qPCR	Diagnosis	2015	Leyten et al. (2015)
HOXC6	mRNA	PCa-positive biopsy patients (n ¼ 12) and PCa-negative biopsy patients (n ¼ 13) PCa-positive biopsy patients (n ¼ 12) and PCa-negative biopsy patients (n ¼ 13) PCa-positive biopsy patients (n ¼ 12) and PCa-negative biopsy patients (n ¼ 13) CaP-positive biopsy patients(n = 12), CaP-negative biopsy patients (n = 13)	Post-DRE urine	qPCR	Diagnosis	2015	Hamid et al. (2015)
TMPRSS2-ERG	Gene fusion mRNA	19 CaP patients (11 prebiopsy and 8 pre-radical prostatectomy)	Urine after prostatic massage	qPCR	Diagnosis	2006	Laxman et al. (2006) (Dimitriadis et al. (2013),Tomlins et al., (2016), Mikhailenko et al., (2014),Merdan et al., (2015)
TTTY15-USP9Y	Gene fusion	PCa-positive biopsy patients (n ¼ 12) and PCa-negative biopsy patients (n ¼ 13) PCa-positive biopsy patients (n ¼ 12) and PCa-negative biopsy patients (n ¼ 13) PCa-positive biopsy patients (n ¼ 12) and PCa-negative biopsy patients (n ¼ 13) CaP-positive biopsy patients(n = 75), CaP-negative biopsy patients (n = 151)	Post-DRE urine	qPCR	Diagnosis	2015	Zhu et al. (2015)
Glutathione S-transferase P1 (GSTP1) methylation	Gene methylation	CaP (n = 31), BPH (n = 34)	Urine	MS-PCR	Diagnosis	2016	Minciu et al. (2016), Van Neste et al. (2016)
Panel of four gene methylation (APC, CRIP3, GSTP1, HOXD8)	Gene methylation	CaP patients (n = 153)	Post-DRE urine	qPCR	Diagnosis	2016	Zhao et al. (2017)
miR-375*	miRNAs	CaP (n = 215), BPH (n = 23),controls (n = 62)	Urine	qPCR	Diagnosis	2016	Stuopelyte et al. (2016)
Engrailed-2 (EN2)	Protein	BRCA1 and BRCA2 mutation carriers (n = 276), controls (n = 140)	Urine	ELISA	Diagnosis	2013	Killick et al. (2013)

Notes: BPH, benign prostatic hyperplasia; CaP, prostate cancer; DRE, digital rectal examination; ELISA, enzyme-linked immunosorbent assay; EPS, expressed prostatic secretion; HPA, hybridization protection assays; lncRNA, long non-coding RNA; MS-PCR, methylation-specific polymerase chain reaction; qPCR, quantitative real time polymerase chain reaction; TMA, transcription mediated amplification. * A representation of miRNA.

3.3. Gene methylation biomarkers

Gene methylation, a process by which methyl groups are added to DNA, is essential for normal development. It is associated with numerous key cellular processes, including embryonic development, genomic imprinting, X-chromosome inactivation, repression of repetitive elements, aging and carcinogenesis (Kulis and Esteller, 2010). The glutathione S-transferase P1 (*GSTP1*) gene, located on chromosome 11q13, belongs to the GST family − a group of enzymes involved in Phase II metabolism playing an important role in cell cycle regulation. *GSTP1* methylation, observed in CaP but not in healthy tissue, leads to under-expression of GSTP1 in prostate tissue (Martignano et al., 2016). GSTP1 expression is high in the basal cell layer and luminal cells of benign prostate glands, with progressive loses observed in prostatic intraepithelial neoplasia (PIN) with only the basal cell layer staining, and is not present in CaP glands (Martignano et al., 2016) underlining its involvement in early carcinogenesis. A significant association has been observed between *GSTP1* Ile105Val polymorphism and the risk of CaP among Caucasians rather than Asians or African Americans (Cai et al., 2013; Yu et al., 2013). However, this association was found significant only among Asians in a meta-analysis with a total of 11,762 cases of CaP and 15,150 hospital-based and population based controls from 51 published studies (Zhang et al., 2016a,b).

Zelic et al. (2016) found that *GSTP1* hypermethylation on a negative biopsy was associated with the risk of CaP on a re-biopsy, especially in high-grade CaP. Eighty-seven percent of CaP urine samples contain hypermethylated levels of the *GSTP1* gene (Minciu et al., 2016). In the same study, 12% of BPH also had *GSTP1* hypermethylation and these subjects went on to have a diagnosis of CaP in a repeat biopsy (Minciu et al., 2016). Thus, a test of urinary *GSTP1* hypermethylation for early CaP and discrimination from BPH may be developed in the near future. These findings are in line with a study of DNA-methylation on 7899 prostate core biopsies from 803 CaP patients (Van Neste et al., 2016). DNA-methylation intensities based on *GSTP1*, *RASSF1* and *APC* could help to identify patients harbouring high-grade CaP who were negative upon biopsy histology (Van Neste et al., 2016).

In a meta-analysis of the sensitivity and specificity for CaP detection of *GSTP1* methylation in body fluids (plasma, serum, whole blood, urine, ejaculate, and prostatic secretions), Wu et al. demonstrated that measurement of *GSTP1* promoter methylation in plasma, serum, or urine may complement PSA screening for CaP diagnosis (Wu et al., 2011). The results indicated that the specificity of urinary *GSTP1* promoter methylation was 0.86-0.90 and thus much higher than that of the PSA test (Wu et al., 2011).

Another promising research area involving gene methylation is to examine a panel of DNA methylation markers for the non-invasive diagnosis of CaP. A panel of 4 gene methylation biomarkers (*APC*, *CRIP3*, *GSTP1*, *HOXD8*) from post-DRE urine was able to predict patient reclassification (OR = 2.559; 95% CI = 1.257–5.212) as an independent and superior predictor compared to PSA (Zhao et al., 2017), demonstrating that the combination of several gene methylation markers is a promising method for CaP detection.

3.4. Exosomes and miRNAs biomarkers

Extracellular vesicles (EVs), including exosomes, microvesicles, retrovirus-like particles, and apoptotic bodies, are membrane vesicles released from both normal and cancer cells into the extracellular environment as a means of intercellular communication (Akers et al., 2013). The term exosome was initially coined by Dr. Rose Johnstone (Johnstone et al., 1987) and used for vesicles ranging from 30 to 100 nm that could be isolated from cell culture medium as well as from a variety of bio-fluids such as blood, urine, cerebrospinal fluid, lymphatics, tears, saliva, nasal secretions, ascites, and semen (Lopez-Verrilli and Court, 2013). Exosomes, containing miRNA, mRNA and proteins, were observed in higher numbers in the blood from lung cancer and ovarian cancer patients, suggesting that cancer cells secrete more exosomes than normal cells (Lea et al., 2017; Rosell et al., 2009). Prostate exosomes can be found in CaP urine (Bijnsdorp et al., 2013; Fendler et al., 2016; Nilsson et al., 2009) and enriched after DRE (Dijkstra et al., 2014).

MiRNAs are small non-coding RNA molecules of about 22 nucleotides in length and are involved in almost all biological processes by acting to silence RNA or post-transcriptionally regulate gene expression. Dysregulated expression of miRNAs is related to various diseases, including cancer (Musilova and Mraz, 2015; Pospisilova et al., 2016). Currently, over 2000 human miRNAs have been reported (Griffiths-Jones, 2006) and more than 200 common miRNAs have been evaluated from urine exosomes (Koppers-Lalic et al., 2016). Many studies have proven the usefulness of urinary miRNAs in combination with clinical parameters for enhancing the accuracy of classification of CaP (Bryant et al., 2012; Korzeniewski et al., 2015; Lewis et al., 2014; Sapre et al., 2014; Srivastava et al., 2013). However, the results are divergent because of differences in methodology related to quantitation and normalisation. The urine sediment is used in most studies, while whole urine and urine supernatant are also used, either after a DRE/prostate massage or without DRE. Moreover, according to MacLellan et al., 162 miRNAs were found significantly up-regulated (by ≥3-fold) in haemolysed serum samples (MacLellan et al., 2014), indicating haemolysis should be taken into consideration when testing miRNAs in urine especially after DRE to avoid biasing the results.

Among all the miRNAs reported, miR-141, miR-21, miR-200b, miR-221 and miR-375 have been the most frequently investigated in urine from CaP patients (Bryant et al., 2012; Sapre et al., 2014; Stuopelyte et al., 2016). Stuopelyte et al. found miR-375 in urine was associated with CaP in pT2 stage (Stuopelyte et al., 2016). However, no consensus was found in the study by Koppers-Lalic et al. who observed both miR-21 and miR-375 were decreased in urine in CaP patients (Koppers-Lalic et al., 2016). Although these miRNAs may be involved in cancer pathogenesis (Filella and Foj, 2017), they are not specific for CaP (Huang et al., 2016; Lu et al., 2016; Sekar et al., 2016; Tusong et al., 2017). Currently, the use of urinary miRNAs as predictive biomarkers for CaP is still in its infancy and no firm conclusions can be made regarding the utility of miRNAs in urine. There are many avenues for future studies in this area.

4. CaP urine biomarker identified by proteomics techniques

Proteomics offers a platform for the identification and quantification of novel protein biomarkers. MS-based technologies are one of the most powerful tools for analysing the proteome. Advances in proteomics, especially in MS have led to the identification and quantification of thousands of unique proteins and peptides in complex biological fluids or cell lysates (Yates et al., 2009). In the past few decades, different MS-based proteomics approaches, such as two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), isotope-coded affinity tag (ICAT), isobaric tags for relative and absolute quantitation (iTRAQ), matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF-MS), surface-enhanced laser desorption ionization mass spectroscopy (SELDI-TOF-MS), capillary electrophoresis-MS (CE-MS), liquid chromatography-tandem MS (LC–MS/MS), selected reaction monitoring (SRM)/multiple reaction monitoring (MRM) and parallel reaction monitoring (PRM), have been used in CaP specific urinary biomarker discovery. These techniques allow identification of potential biomarkers and comparison of their abundances between diseased and healthy groups. Table 2 shows urine proteins that have been identified to be differentially expressed in CaP patients compared to healthy controls. Antibody-based methods such as Western blot, immunohistochemistry (IHC) or ELISA were commonly used to validate the potential biomarkers identified by MS-based proteomics.

Table 2
Summary of the CaP biomarkers identified from urine by MS-based proteomics.

Biomarker	Potential application	Study group	Discovery method	Validation method	Year	Reference
Fatty acid binding protein 5 (FABP5) ↑, granulin↑, alpha-1-microglobulin/bikunin precursor (AMBP) ↑, charged multivesicular body protein 4a (CHMP4A) ↑, charged multivesicular body protein 4c (CHMP4C) ↑	Diagnosis	Negative biopsy (n = 6), Gleason score 6 CaP (n = 6), Gleason score 8–9 CaP (n = 6); Validation: negative (n = 11), Gleason score 6 CaP (n = 5), Gleason score 7 CaP (n = 7), Gleason score 8–9 CaP (n = 6)	iTRAQ-qLC–MS/MS	SRM/MRM	2017	Fujita et al. (2017)
3 proteins panel (serum PF4V1, PSA, urinary CRISP3)	Diagnosis	BPH (n = 48), high grade prostatic intraepithelial neoplasia (n = 32), localized CaP (n = 47), metastatic CaP (n = 39)	iTRAQ-LC-ESI–MS/MS	ELISA, Western blot	2016	Zhang et al. (2016)
β-2-microglobulin (β–2 M) ↑, pepsinogen 3 group 1 (PGA3) ↑, intestinal mucin (MUC3) ↑	Diagnosis	CaP (n = 90), BPH (n = 83)	iTRAQ-LC/LC/MS/MS	Western blot	2015	Jedinak et al. (2015)
Cyclin-dependent kinase 6, galectin-3-binding protein, L-lactate dehydrogenase C chain	Prognosis	CaP with positive surgical margins (n = 15), CaP with negative surgical margins (n = 15)	2D-MALDI-TOF/MS	N/A	2015	Heger et al. (2015)
Prostatic acid phosphatase (PAP) ↑ secernin-1↑ galectin-3↓	Diagnosis Prognosis	CaP with relapse(n = 7), CaP without relapse(n = 9), NC(n = 7)	MRM-MS	Western blot	2015	Geisler et al. (2015)
α-1-microglobulin/bikunin (AMPB) ↑ transferrin (TF)↓ haptoglobin (HP) ↓	Diagnosis	CaP (n = 8), BPH (n = 16)	2DDIGE/MS	IHC	2015	Davalieva et al. (2015)
221 proteins ↑ 25 proteins ↓ transmembrane protein 256 (TM256) ↑ LAMTOR1↑	Diagnosis	CaP (n = 16), NC (n = 15)	LC–MS/MS	Western blot, ELISA	2015	Overbye et al. (2015)
73 potential biomarkers identified	Diagnosis	Pooled sample:CaP (n = 9), BPH (n = 9), NC (n = 9); Individual sample: CaP (n = 15), BPH (n = 15), NC (n = 15)	LC–MS/MS	N/A	2015	Adeola et al. (2015)
Pyridinoline (PYD) ↑deoxypyridinoline (DPD)↑ osteopontin (SPP1) ↓ prothrombin (F2)↓	Diagnosis	CaP (n = 28), BPH (n = 20), NC (n = 6)	LC–MS/MS	N/A	2015	Li et al., (2015)
β-microseminoprotein (β-MSMB) ↓	Diagnosis	Post-DRE urine samples: CaP (n = 25), BPH (n = 27)	MALDI-TOF-MS	N/A	2014	Flatley et al. (2014)
C-terminal PSA fragment	Diagnosis	Post-DRE urine samples: CaP (n = 50), non-cancer subject (n = 19)	MALDI-DIT-TOF/MS	N/A	2014	Nakayama et al. (2014)
Fibronectin (FN) ↓ TP53INP2↓	Diagnosis	Pooled sample: CaP (n = 8), BPH (n = 12), NC (n = 10)	LC–MS/MS	qPCR	2014	Haj-Ahmad et al. (2014)
Saposin B ↓ Inter-alpha-trypsin inhibitor light chains (ITIL) fragments↓ Inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) fragment ↑	Diagnosis	Early stage CaP (n = 13), BPH (n = 16), NC (n = 15)	2D-MS/MS	Western blot	2013	Jayapalan et al. (2013)
Thy-1 membrane glycoprotein (CD90) ↑ PSA↑	Diagnosis	Pro-operative patient (n = 3), Post- operative patient (n = 3)	ICAT-LC–MS/MS	SRM-MS	2010	True et al. (2010)
Sarcosine	Diagnosis	CaP-positive biopsy patients(n = 59), CaP-negative biopsy patients (n = 51)	LC/GC–MS	N/A	2009	Sreekumar et al. (2009)
49 mass peaks ↑ 23 mass peaks ↓	Diagnosis	CaP (n = 57), NC (n = 56)	SELDI-TOF	N/A	2009	Okamoto et al. (2009)
4 potential biomarkers identified	Diagnosis	CaP (n = 51), NC (n = 35)	CE-MS	N/A	2008	Theodorescu et al. (2008)
Semenogelin I(SEMG1) ↑ Uromodulin (UMOD) ↓	Diagnosis (distinction of CaP and BPH)	Study 1: CaP (n = 89), NC (n = 125) Study 2: CaP (n = 103), NC (n = 38)	MALDI-TOF & LC–MS/MS	N/A	2007	M'Koma et al. (2007)
Calgranulin B/MRP-14	Diagnosis	CaP (n = 6), BPH (n = 6)	2D-MALDI-TOF	IHC	2004	Rehman et al. (2004)

Notes: ↑indicates increased expression. ↓indicates decreased expression. 2D, two-dimensional Gel Electrophoresis; 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; BPH, benign prostatic hyperplasia; CaP, prostate cancer; CE, Capillary electrophoresis; DRE, digital rectal examination; ICAT, isotope-coded affinity tag; IHC, immunohistochemistry; iTRAQ, isobaric tags for relative and absolute quantitation; LC, liquid chromatography; LC/GC–MS, liquid/gas-chromatography coupled to mass spectrometry; LC–MS/MS, liquid chromatography-tandem mass spectrometry; MALDI-TOF-MS, Matrix-assisted laser desorption ionization-time of flight mass spectroscopy; MS, mass spectrometry; MRM, multiple reaction monitoring; NC, normal healthy controls; qPCR, quantitative real time polymerase chain reaction; SRM, selected reaction monitoring.

4.1. Gel-based proteomics

In order to be able to analyse complex samples, high-resolution electrophoretic separations of proteins became available in the 1970s, followed by successful reports of high resolution two-dimensional gel electrophoresis (2DGE) in protein separation (MacGillivray and Rickwood, 1974; O'Farrell, 1975). A CaP specific biomarker calgranulin B/MRP-14 was reported in a study of voided urine after prostatic massage using 2DGE following MALDI-TOF-MS (Rehman et al., 2004). In this pilot study, calgranulin B/MRP-14 was present in 4 of 6 urine samples from CaP patients but not in any of patients with BPH. Kiprijanovska et al. created an initial proteomic map from urine of CaP patients by 2D-MS (Kiprijanovska et al., 2014). Although the proteome map showed a limited number of proteins, wherein 125 spots were identified by MS and 45 distinct proteins were revealed, the study provided some leads to understand the molecular bases of CaP. Four urinary proteins were found to be significantly altered in early stage CaP by using a combination of 2DE and MS/MS (Jayapalan et al., 2013). In that study, patients with early CaP presented lower levels of saposin Bandinter-alpha-trypsin inhibitor light chain (ITIL) fragments in their urine, while the level of an inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) fragment increased. 2DGE coupled with MALDI-TOF-MS was also used in the discovery of urinary biomarkers related to surgical margin status after RP, wherein 3 uniquely identified proteins (cyclin dependent kinase 6, galectin-3-binding protein and L-lactate dehydrogenase C chain) were found to be associated with CaP (Heger et al., 2015). The proteins identified in the positive surgical margins (inadequate cancer clearance) group may be helpful for research on the molecular mechanisms of CaP and assist in the assessment of risk of disease progression after surgical treatment. Using 2D-DIGE/MS, Davalieva et al. reported 3 potential CaP biomarkers in urine (Davalieva et al., 2015). The level of urinary α-1-microglobulin/bikunin (AMPB) (50% specificity, 94% sensitivity) was significantly higher in CaP, but levels of transferrin (TF) (94% specificity, 56% sensitivity) and haptoglobin (HP) (56% specificity, 94% sensitivity) were lower. The integration of HP with AMBP yielded higher accuracy (AUC = 0.848) than PSA, which underlines its potential to improve the sensitivity and specificity of CaP detection.

4.2. MS-based isobaric labelling proteomics

Isobaric labelling methods, such as ICAT and iTRAQ, are MS-based strategies used in quantitative proteomics that are able to determine the amount of protein without undertaking a traditional 2D gel approach. In one report, PSA and thy-1 membrane glycoprotein (CD90) were detectable in urine from CaP patients via ICAT/LC–MS/MS and both proteins were present in the pre-operative samples but below the detection limit in the post-operative samples (True et al., 2010). In another report, 3 proteins, β-2-microglobulin (β–2 M), pepsinogen 3 group 1 (PGA3) and intestinal mucin (MUC3), were identified by iTRAQ/LC–MS/MS in the urine of CaP patients. Multivariate logistic regression analysis revealed an AUC of 0.668, 0.625 and 0.618 for each marker alone, demonstrating that β–2 M, PGA3 and MUC3 can, to some extent, sensitively and specifically differentiate between patients with BPH and localised CaP (Jedinak et al., 2015). The diagnostic accuracy increased (AUC, 0.710) when these markers were multiplexed, and further improved when the data were combined with clinically defined categories of PSA (AUC, 0.812). This small panel of biomarkers, especially in conjunction with PSA, may be clinically useful for non-invasively discriminating between BPH and CaP (Jedinak et al., 2015). Another small panel of 3 proteins (serum PF4V1, PSA, and urinary CRISP3) has been identified using iTRAQ as being able to accurately diagnose CaP (Zhang et al., 2016a,b). This panel of markers had higher CaP predictive accuracy (AUC, 0.941) compared with PSA alone (AUC, 0.757). Importantly, a combination of serum PF4V1 and urinary CRISP3 could achieve greater discrimination (AUC, 0.895) even in the grey zone of PSA (4–10 ng/mL) and also had the potential to distinguish CaP from high-grade prostatic intraepithelial neoplasia (HGPIN) (AUC, 0.934). In a recent study on urinary EVs, where proteins were labelled with iTRAQ and analysed by LC–MS/MS, 5 proteins were found to be associated with Gleason Score (GS), including granulin, fatty acid binding protein 5 (FABP5), alpha-1-microglobulin/bikunin precursor (AMBP), charged multivesicular body protein 4a (CHMP4A) and charged multivesicular body protein 4c (CHMP4C) (Fujita et al., 2017). The results indicate FABP5 has potential to predict high GS CaP with higher AUC values of 0.757 (CaP ≥ 6) and 0.856 (GS ≥ 7) compared with AUC values of 0.593 (CaP ≥ 6) and 0.511 (GS ≥ 7) by PSA.

4.3. MALDI-TOF-MS & SELDI-TOF-MS

MALDI is a soft ionization method in MS typically used with TOF-MS for the analysis of biopolymers and large organic molecules, such as DNA, proteins, peptides, polymers and other macromolecules. M'Koma et al. firstly employed MALDI-TOF in profiling the urine proteome to distinguish patients with CaP and BPH (sensitivity 67%, specificity 71%) (M'Koma et al., 2007). In the following study for identifying proteins of interest by LC–MS/MS sequencing, uromodulin (UMOD) and semenogelin I (SEMG1) were identified as 2 biomarkers that could help discriminating CaP from BPH (M'Koma et al., 2007). Using MALDI-MS profiling, another potential biomarker β-microseminoprotein (β-MSMB) was found to be significantly lower in post-DRE urine samples from CaP patients (Flatley et al., 2014). High classification accuracy for CaP could be obtained (sensitivity 96%, specificity 26%) by combining PSA levels with MALDI-MS measured β-MSMB levels. In a recent study, a C-terminal fragment of PSA, a 2331 Da peptide, was discovered in post-DRE urine from 50 CaP patients by using MALDI-TOF-MS (Nakayama et al., 2014). The presence of this peptide in urine provides the opportunity to elucidate intracellular and/or extracellular PSA fragmentation mechanisms.

An extension of MALDI-TOF that is widely used in proteomics is SELDI-TOF. A protein profiling study of post-prostatic massage urine specimens via SELDI-TOF revealed 49 mass peaks that were significantly up-regulated and 23 peaks that were significantly down-regulated in CaP compared with non-cancerous controls (Okamoto et al., 2009). Since only the mass to charge (m/z) values can be obtained, combining urinary analysis of SELDI-TOF and follow-up identification approaches such as LC–MS/MS will most likely help in discovery of urine-based biomarker for CaP.

4.4. CE-MS & LC–MS/MS proteomics

CE is an electrically-driven liquid-based separation where analytes are separated based on their difference in charge and size. Conversely, LC is a pressure-driven liquid-based separation in which analytes are separated according to their adsorption/desorption kinetics. Coupled with MS, these techniques provide high-efficiency separation and identification in a single analysis (Jianyi Cai, 1995). Using CE-MS coupled with MALDI-TOF-MS, Theodorescu et al. revealed several polypeptides from urine that enabled correct diagnosis of CaP with 92% sensitivity and 96% specificity, respectively (Theodorescu et al., 2005). In a study of urine from CaP patients using CE-MS, 4 proteins, including sodium/potassium-transporting ATPase γ, Collagen α-1 (III), Collagen α-1 (I) and Psoriasis susceptibility 1 candidate gene 2 protein (SPR1), were identified as biomarkers for CaP diagnosis (Theodorescu et al., 2008). Using liquid/gas-chromatography (LC/GC)-MS, Sreekumar et al. found that sarcosine, an N-methyl derivative of glycine, was present in urine and its presence was significantly increased during progression from normal through localised to metastatic CaP (Sreekumar et al., 2009). Urinary sarcosine had a higher predictive value than PSA in differentiating CaP patients with PSA levels of 2–10 ng/mL. This finding was further confirmed by an observation via LC–MS from Cao et al. who described that urinary sarcosine level was significantly

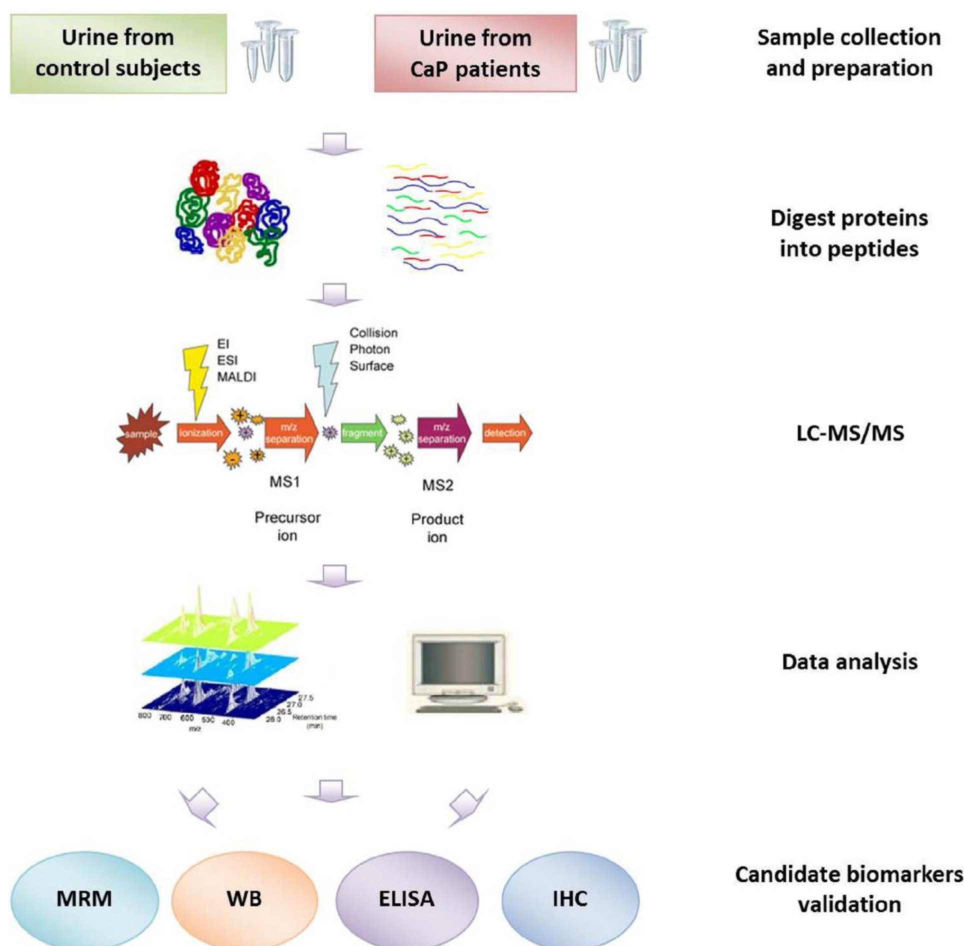

Fig. 2. A diagram showing CaP urine biomarker discovery by LC–MS/MS proteomics and validation of potential biomarkers. Urine samples from CaP patients and control subjects are collected and centrifuged to remove cellular elements. Proteins are harvested by precipitation and digested by trypsin. LC–MS/MS reference and protein profiles are acquired. The LC–MS/MS data can be uploaded for bioinformatics analysis. The potential biomarker candidates identified are validated using multiple reaction monitoring (MRM), Western blot (WB), enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC).

higher in CaP patients than that in normal healthy controls and AUCs were improved when combining with PCA3 or free PSA (Cao et al., 2011).

With the development of chemical analysis, LC–MS/MS has emerged as an innovative analytical technology that provides extra precision and higher sensitivity by measuring the *m/z* of the compound as well as its intermediates (Vogeser and Parhofer, 2007). This technique is becoming more and more important in the detection of cancer biomarkers. The work flow for urinary CaP biomarker discovery and validation using LC–MS/MS proteomics is shown in Fig. 2. LC–MS/MS and CE-MS/MS demonstrated significant complementarity when testing normal human urine where over 30% more peptide sequences were identified with LC–MS/MS compared to CE-MS/MS (Klein et al., 2014). In one LC–MS/MS study of urinary proteins there was significant down-regulation of fibronectin (sensitivity 75%, specificity 50%) and TP53INP2 (sensitivity 75%, specificity 7%), and this improved the chances of accurately predicting CaP (Haj-Ahmad et al., 2014). In another study, LC–MS/MS analysis demonstrated high levels of pyridinoline (PYD) and deoxypyridinoline (DPD), and low levels of osteopontin (SPP1) and prothrombin (F2) in the first voided urine after massage from CaP patients (Li et al., 2015). Adeola et al. found a total of 73 proteins were differentially expressed in pooled and individual urine samples from CaP patients, as compared with BPH and healthy control subjects, using LC–MS/MS (Adeola et al., 2015). Urinary exosomal proteomic profiling by LC–MS/MS has shown that 246 proteins were differentially expressed in CaP compared with healthy subjects, and the majority of them (221) were up-regulated (Overbye et al., 2015). Two promising biomarkers, TM256 (94% sensitivity at 100% specificity) and LAMTOR1 (81% sensitivity at 100% specificity), were identified from this proteomic study (Overbye et al., 2015). Among the 246 proteins, flotillin2, transmembrane protein 256 (TM256), Rab3B and LAMTOR1 were further validated as being at higher levels in independent CaP urine samples by Western blot, while flotillin 2 and PARK7 were further validated in urine by ELISA (Wang et al., 2017). TM256, which located at the plasma membrane and in exosomes, is associated with uncontrolled growth in leukaemia cells (Gu et al., 2010). A combination of urinary TM256 and LAMTOR1 could be promising in predicting CaP with an increased sensitivity of 100% compared to single TM256 (Overbye et al., 2015). Due to the complexity of CaP, finding a panel of biomarkers instead of a single molecule would probably be a viable route to the diagnosis and prognosis of the disease.

Initial proteomic profiles from CaP tissue using iTRAQ and 2D-LC–MS/MS, found that glutathione peroxidase 3 precursor (GPx3), cofilin-1 (CFL1), heat shock protein-90β (HSP 90β), zinc alpha 2-glycoprotein (ZAG) and secreted protein acidic and rich in cysteine (SPARC), were detected in CaP tissue. These potential biomarkers identified were further investigated in urine samples, and ZAG was found to have a discriminative potential for CaP early diagnosis (Katafigioti et al., 2016).

4.5. Targeted proteomics by SRM/MRM and PRM

Instead of trying to detect all proteins in a complex mixture, targeted proteomics only monitors specific peptides and focuses on their corresponding fragments during acquisition, allowing high sensitivity and specificity for absolute quantification of the proteins of interest. SRM/MRM is now one of the most widely used forms of MS-based absolute quantitation. Under SRM/MRM, the mass spectrometer is set up to monitor only specific *m/z* values of interest; consequently, the probability of detecting even low levels of a peptide in the presence of a

complex mixture of peptides is much higher. Shi et al. developed a highly sensitive assay based on SRM/MRM for quantification of Anterior gradient 2 (AGR2) in urine and serum of CaP patients (Shi et al., 2014). SRM/MRM and ELISA results showed a good correlation ($R^2 = 0.91$) in measuring AGR2 concentrations in urine and a significant difference ($p = 0.026$) was observed in the urinary AGR2/PSA concentration ratios between non-cancer and cancer subjects (Shi et al., 2014). Three proteins, including secernin-1, prostatic acid phosphatase (PAP), and galectin-3, which were initially found to be associated with CaP by analysing prostate tissue samples using 2D-DIGE combined with MS, were chosen for a proof of principal of SRM/MRM-MS study, using urine from CaP patients with or without relapse and healthy controls (Geisler et al., 2015). PAP showed higher levels in the urine of CaP patients compared to healthy control subjects, but there was no significant difference in the urine between patients with and without relapse while Galectin-3 showed significantly lower levels in urine from CaP patients with relapse compared to those without relapse (Geisler et al., 2015). However, secernin-1 was not detected in patient urine samples using SRM/MRM. Percy et al. recently developed a robust method utilising a bottom-up reversed-phase liquid chromatography (RPLC)/SRM/MRM-MS with stable isotope-labelled standards (SIS) for quantifying a highly multiplexed set of 136 proteins in urine from CaP patients (Percy et al., 2015). This MRM-based method has excellent reproducibility with average CVs of 8.6% in the concentrations of the 136 proteins in urine samples. The average dynamic ranges for the quantifier peptides of the 136 proteins were predominantly in the 100–360 range. These findings suggest MRM-MS is a useful tool for validation of urine biomarkers in CaP.

A novel variant, PRM, analyses the fragment ions with an Orbitrap mass analyser rather than a quadrupole MS. Due to the high resolution and reduced likelihood of being affected by interfering ions, PRM provides high selectivity and sensitivity, and high-throughput quantification with less effort than the traditional SRM/MRM assay on QqQ instruments (Gallien et al., 2012). Adeola et al. have recently developed a penultimate pre-validation workflow to help improve clinical translation of potential biomarkers from discovery proteomics experiments, wherein targeted validation was performed by PRM using previously discovered potential CaP biomarkers (Adeola et al., 2016). PRM revealed a top 12 potential biomarkers that were differentially expressed between CaP and BPH/healthy control samples (Adeola et al., 2016). These targeted proteomics approaches provide a more useful means of validation of a large number of potential protein or peptide biomarkers than Western blot and IHC.

4.6. Data-independent acquisition (DIA)

Although the traditional shotgun proteomics by data-dependent acquisition (DDA) is a powerful technology for delivering large numbers of identifications for MS discovery in complex samples, it has problems such as missing data during acquisition. To address this issue, the use of DIA, based on the construction of reference spectral libraries and special data processing tools is rapidly expanding in discovery proteomics. In contrast to DDA where a fixed number of precursor ions are selected and analysed by tandem MS, DIA acquisition gives complete coverage of all precursor ions within a selected m/z range and analyses all fragment ions in a second stage of tandem MS, allowing unmatched proteome coverage with reproducible and precise quantification (Bruderer et al., 2015). Sequential window acquisition of all theoretical fragment-ion spectra (SWATH)-MS is based on a DIA mode and has been used in biomarker discovery in ovarian cancer using human serum samples, and one novel potential marker- Protein Z was identified and further validated for ovarian cancer early detection (Russell et al., 2016). Martínez-Aguilar et al. conducted SWATH-MS from 32 primary thyroid carcinomas to quantitate over 1600 proteins from 5 μg frozen tissue and made the novel observation that TGFβ-induced protein βig-h3 (TGFBI) was found to be frequently overexpressed in follicular carcinoma compared with follicular adenoma (Martinez-Aguilar et al., 2016), suggesting that this protein could differentiate benign follicular adenoma from follicular carcinoma and papillary carcinomas in thyroid. SWATH-MS has been used for identifying urinary and serum exosome biomarkers for radiation exposure and a total of 47 differentially secreted proteins were identified in urinary and serum exosomes (Kulkarni et al., 2016), indicating its feasibility for use in urine analysis. Combined with 2D-DIGE and iTRAQ, SWATH-MS was used to identify a panel of candidate markers in breast cancer urine, which could discriminate $HER2^+$ breast cancer from healthy controls (Gajbhiye et al., 2016). The DIA strategy shows promise in proteomics and could benefit urinary CaP specific biomarker discovery in the future.

5. Challenges in CaP urine biomarker research and future perspectives

In the past few decades, many potential biomarker candidates have been proposed in CaP research. However, the lack of follow-up validation leaves these studies still at the discovery stage. Although antibody-based Western blot or ELISA can be used for validation, as long as antibodies are available or can be made, none of the candidates are routinely used in clinical practice. Research efforts need to be moved from biomarker discovery to validation in a large cohort or separate population of patients and translation of these findings to clinical practice.

The collection and handling of urine specimen is critical in protein analysis. Whilst urine is a valuable source of candidate biomarkers, the variation in protein levels is high not only from person to person (Crosley et al., 2009), but also within different sample collection method (Thomas et al., 2010). The rapid high throughput proteomics techniques enable identification of valid biomarkers in a large cohort of hundreds to thousands, which cannot be produced in a single laboratory. Thus a standard protocol for sample collection and processing is necessary to address these situations. Several studies reveal exciting avenues for standardization in analytical methodology (Mischak et al., 2010; Thomas et al., 2010). A mid-steam collection of first morning urine followed by low-speed centrifugation to remove cell debris is preferred guidelines in our group (Beretov et al., 2014). One important challenge is to enhance the sensitivity of the technique in discovery proteomics. As low abundance proteins can be easily masked by highly abundant proteins, new techniques are required to enrich low abundance proteins in urine for CaP diagnosis. Another barrier is the compatibility of proteomics data with current databases. For example, there is no available database that can be used to analyse SWATH data, and coordinated efforts are required to improve this situation. Since each proteomics platform has its strengths and drawbacks, combining them together may make the laboratory work more efficient and productive. Improvements in methodology are the subject of future research.

Extending CaP biomarker into panels may be beneficial. Since a single biomarker may not reach the required sensitivity and specificity, a combination of biomarkers from the same or different bio-sources may achieve high accuracy in clinical tests. Ideal CaP biomarkers are produced directly from the tumour epithelial or stromal cells or specifically in response to the presence of the tumour. The candidate biomarkers discovered in tissue provide a promising research orientation for targeted urinary proteomics research, as the proteomic changes in tissue may better indicate pathophysiology and validation of these biomarkers in urine may reduce the time from bench to clinical practice.

6. Conclusions

Since the utility of PSA is limited and controversial, the search for novel CaP-specific biomarkers, especially from non-invasive bio-fluids is an important pursuit. Urinary biomarkers for CaP represent a

promising alternative or an addition to traditional biomarkers. Although none of the potential biomarkers to date have been translated into clinical practice, integration of different urinary and/or serum markers in a multiplex setting (such as gene panel HOXC6-TDRD1-DLX1 combined with urinary PCA3, urinary TMPRSS2-ERG or PCA3 with PSA, urinary TTTY15-USP9Y with PSA, urinary β-2M-PGA3-MUC3 with PSA, serum PF4V1 with urinary CRISP3, urinary β-MSMB with PSA, and urinary TM256 with urinary LAMTOR1) may enhance clinical prediction and provide a strategy to resolve the dilemma of early detection and staging of CaP. Future success in biomarker discovery will rely on collaboration among clinics and laboratories. Standards and guidelines are needed for facilitating discoveries by providing a framework for specimen collection, processing, analysis, and data reduction. In addition, research efforts need to be moved from biomarker discovery to validation in a large cohort or separate population of patients and translation of these findings to clinical practice.

Conflict of interest

None.

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