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Proteomics in Biomarker Discovery for Prostate Cancer*

Abstract

The use of prostate specific antigen (PSA)-based screening of prostate cancer (PCa) results with over-diagnosis of the disease, unnecessary biopsies and high medical cost treatments. The discovery of new biomarkers in blood, urine or tissue that will permit early detection and treatment of patients with aggressive disease and, concomitantly, avoid overtreatment for low risk cases are urgently needed.

Proteomic technologies are providing the tools needed to discover and identify disease associated biomarkers. The application of these technologies to search for potential diagnostic/prognostic biomarkers associated with PCa has shown constant growth in the last 15 years. The main clinical focus in PCa research nowadays is the discovery of biomarker(s) for diagnosis and distinction between aggressive and indolent cancers, followed by prognostic and response to treatment biomarker(s). Different proteomic technologies and various biological samples have been analyzed with the aim of identifying diagnostic and prognostic biomarkers and developing a deeper understanding of the disease at the molecular level.

Here we will overview the current status in PCa diagnosis nowadays, new emerging biomarkers for PCa, different proteomics technologies applied in the study of PCa and explored sources for biomarker discovery. Emphasis will be given on proteomics research that has been conducted in our lab in the last few years with brief overview of the major findings and putative clinical application.

Keywords: Prostate cancer, benign prostate hyperplasia, diagnostics biomarkers, comparative proteomics.

1. Overview of prostate cancer diagnosis

Prostate cancer (PCa) is the second most frequently diagnosed malignancy in men worldwide [1]. The current FDA guidelines for PCa diagnosis are based on prostate specific antigen (PSA) detection in blood together with digital rectal examination (DRE) for men over 50 years of age. The introduction of PSA in 1994 as FDA approved screening tool for PCa has transformed the management of this disease by increasing the number of men diagnosed with PCa and dramatical decrease of the proportion of men with metastatic prostate cancer at the time of diagnosis. On the other hand, despite the increase in the detection of PCa, the majority of patients with increase in serum PSA have had benign conditions such as inflammation or benign prostate hyperplasia (BPH) or clinically indolent disease. The lack of specificity of the PSA blood test has been recognized especially in patients with total serum PSA levels in range of 2 - 10 ng/ml and it has caused over-diagnosis of PCa ranging from 20-42% [2], leading to unnecessary biopsies, high medical cost treatments and psychological distress of patients.

* Ursprünglich war vorgesehen, dass unser Mitglied Momir Polenakovic am 10. März in der Klasse für Naturwissenschaften und Technikwissenschaften einen Vortrag zu einem Thema der individualisierten Medizin hält. Sein Krankenhausaufenthalt hat dies leider verhindert. Wir veröffentlichen hier den von ihm und seiner Koautorin formulierten Beitrag. *Die Redaktion*

The primary goal in PCa research is to find a biomarker(s) that could detect PCa with high specificity and sensitivity, preferably non-invasively. In addition to diagnostics biomarkers, there is a need for biomarkers that could distinguish between aggressive and indolent cancers and predict the response to therapy. With the advance of -omics technologies in the recent years, a number of new potential biomarkers for screening and diagnosis of PCa have been discovered [3]. Two FDA approved tests (Prostate health index (phi) by Beckman Coulter and PCA3 assay by Progensa) and several clinical laboratory improvement amendments-based laboratory developed tests (Oncotype DX Prostate Cancer Assay, Prolaris score, Prostarix DRE urine test, TMPRSS2-ERG fusion test, ConfirmMDx and Prostate Core Mitomic Test) became available in 2012 and 2013. Although some of these tests substantially improve the detection of early stage prostate cancer and reduce negative biopsies, the ideal PCa screening, diagnostics and prognostic tests are still a subject of intense research.

2. Proteomic technologies applied to prostate cancer studies

Expression proteomics is a branch of proteomics that aims to unravel biological processes based on qualitative and quantitative comparison of proteomes as a function of condition or stimulation (disease, time, drug, etc.). The power of the comparative proteomics studies is based on the identification of proteome changes without prior biological knowledge that subsequently may reveal candidate biomarkers for the conditions of interest. The typical workflow of a comparative proteomics study is presented in **Figure 1**.

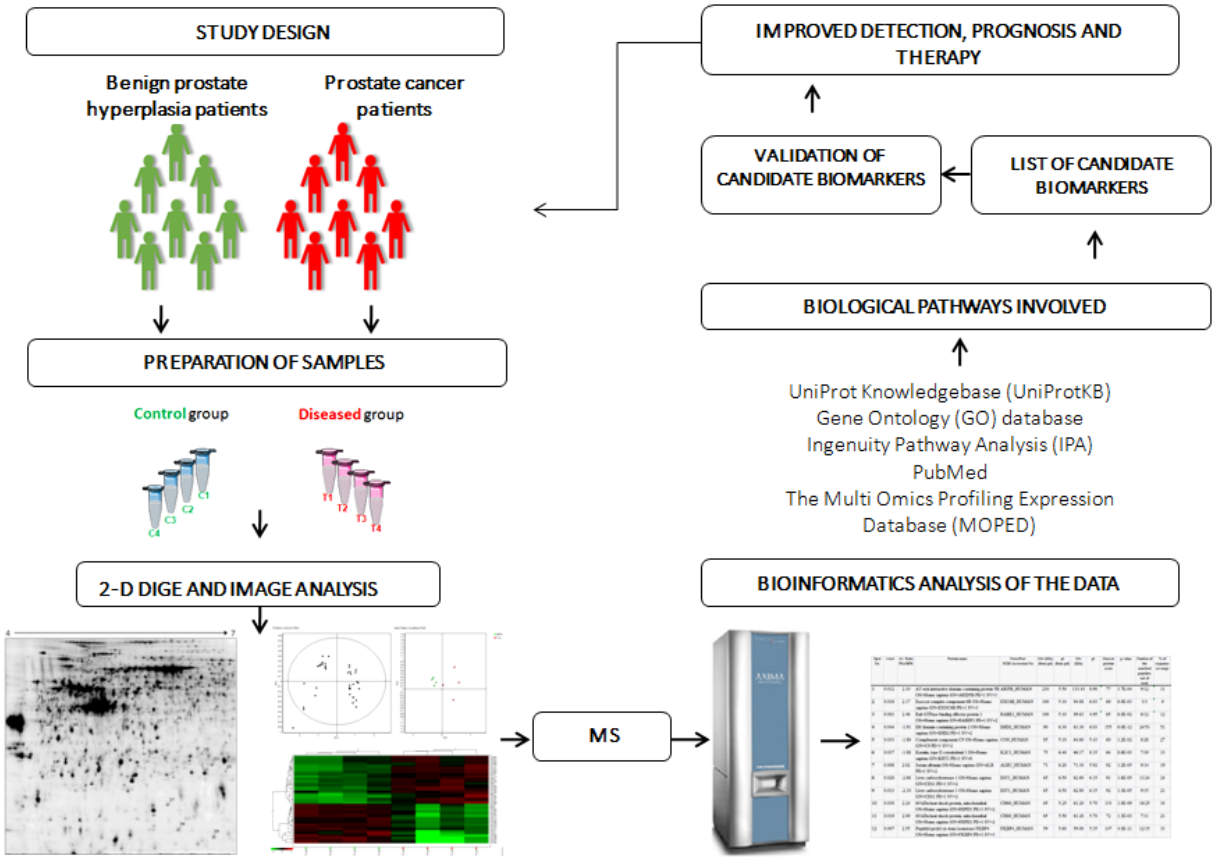

Figure 1. *Typical workflow of the comparative proteomics study*

The recent advances in proteomics are producing powerful platforms that are able to detect and quantify proteins with altered abundance in tissue and many different body fluids (urine, blood, seminal fluid, saliva, sweet and others). These proteomics platforms allow identification of biomarker candidates by simultaneous measurement of hundreds or thousands of molecules and comparison of their abundances between the conditions of interest (ex. disease vs. healthy) in non-hypothesis driven comparative studies.

Different proteomics technologies have been used so far to study cancer-induced proteomics alterations in prostate tissue and body fluids (**Figure 2**). The first available platform for differential expression-based proteomic was ***two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)***, and latter, an improved version of this technique named difference-in-gel electrophoresis (DIGE). The strength of 2-D PAGE/DIGE platform coupled with mass spectrometry identification lies in separation of intact proteins, visualization and detection of post-translational modifications and cost-effectiveness of the procedure. A major limitation is the analysis of hydrophobic (membrane) proteins, high molecular weight proteins (Mw > 100 kDa), highly acidic (pI < 3) or basic proteins (pI > 9) which cannot be separated and visualized using this method.

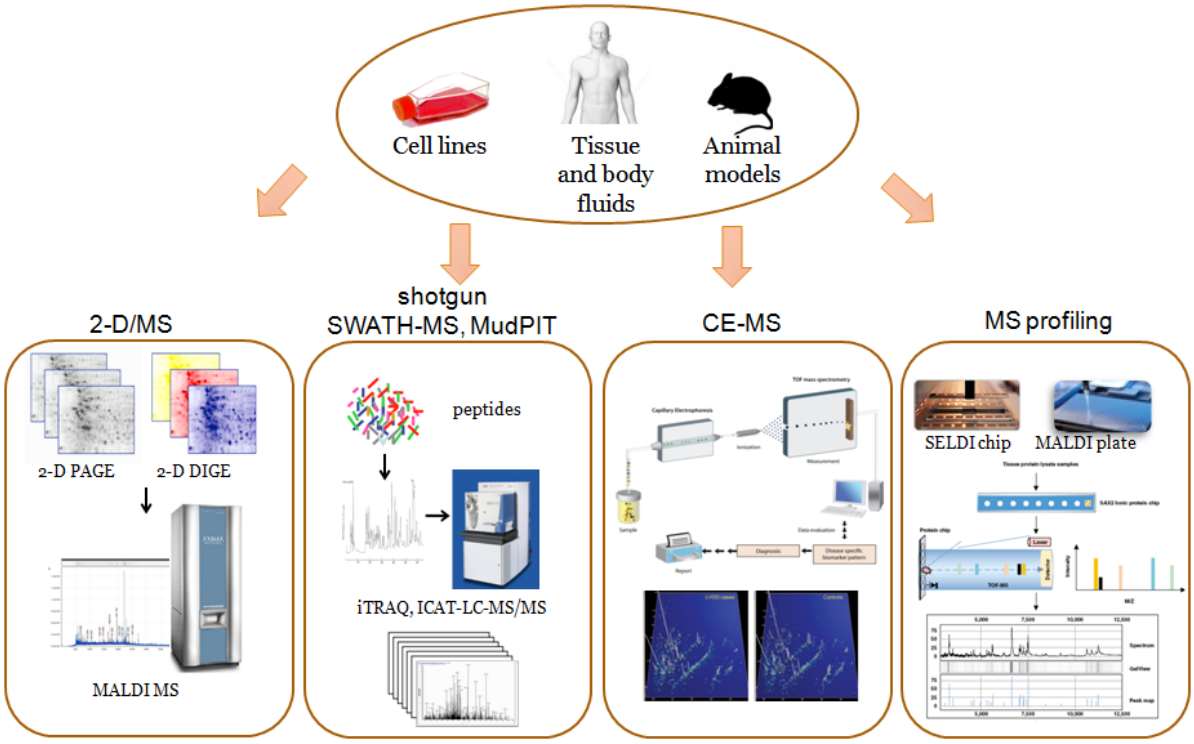

Figure 2. *Proteomic technologies applied to prostate cancer studies.*

The development of non-gel-based, ***"shotgun" proteomic*** techniques has provided powerful tools for studying large-scale protein expression and characterization in complex biological systems. The strength of the shotgun approach are experimental simplicity, increased proteomic coverage compared with the gel-based platforms and accurate quantification while its weaknesses are technical reproducibility, limited dynamic range and informatics challenges related to the enormous complexity of the generated peptide samples. Moreover, this approach cannot identify proteins with multiple modifications because the connection between the peptides that are analyzed in the mass spectrometer and the protein(s) from which the peptides originate is lost during proteolysis.

Matrix assisted laser desorption ionization (MALDI) MS profiling of the proteomics content of the sample such as MALDI-TOF and SELDI-TOF have also been heavily used in PCa research. These

approaches rely on differences in the profile spectrum between two or more groups. SELDI-TOF technology generated a great deal of initial excitement because of the apparent ability to simultaneously detect multiple protein changes in a rapid high-throughput process but is rarely used nowadays due to severe reproducibility problems and low resolution.

Capillary electrophoresis coupled to mass spectrometry (CE-MS) is another approach resolving low molecular mass proteome (peptidome) used in PCa research. The separation in this method is done in a capillary filled with electrolyte where peptides/proteins separate due to differences in the charge-to size-ratio. The approach is suitable for the analysis of peptides and proteins with a broad range of size and hydrophobicity. The weaknesses of this method are precipitation of larger polypeptides and proteins in the CE capillary at the low pH used and the need of suitable software since software solutions provided by the manufacturers of mass spectrometers are inadequate to analyze the pattern of numerous complex samples.

3. Proteomics analysis of prostate cancer

Proteomics research in prostate cancer has shown a steady rise in the last decade and has been reviewed extensively. In our recent paper [4] we made an assessment of the overall expression proteomics studies of PCa investigating all types of human samples in the search for diagnostics biomarkers. Emphasis was put on explored sources for PCa biomarkers and the possible future clinical application of those candidate biomarkers in PCa screening and diagnosis. Here we will give a brief overview of our research which has been focused on the analysis of prostate tissue and urine.

Prostate tissue has advantage over other biomaterials that in addition of being a rich source of potential PCa biomarkers, offers the possibility to clarify the mechanisms of transformation of a prostate normal cell to a tumor cell and subsequent progression to a metastatic state. The analysis of tissue material (as a complex mixture of prostate cells, immune and inflammatory cells, blood vessel cells, fibroblasts) allows detection of the tumor proteome and/or in vivo secretome alterations created by host-tumor cell interactions. A number of comparative proteomic studies investigating prostate tissue have been carried out in the last 15 years with the main objective to find specific diagnostic biomarkers able to distinguish PCa from BPH as well as indolent from aggressive cancers [4].

Our group also carried out a comparative proteomics study of 5 BPH and 5 radical prostatectomy PCa samples by 2-D DIGE/MS and validation of the results on additional 14 PCa and 28 BPH samples using Western blot [5]. Twenty three out of 28 differentially expressed proteins in our study were found associated with cancer in humans or expressed in cancer cell lines according to the Ingenuity Pathway Analysis (IPA) (**Figure 3**). The differentially expressed proteins were involved in 3 possible networks of protein interactions within MAPK, ERK, TGFB1 and ubiquitin pathways. The alterations in these pathways have been previously associated with tumorigenesis, leading to conclusion that they might have impact on the pathogenesis of prostate cancer as well.

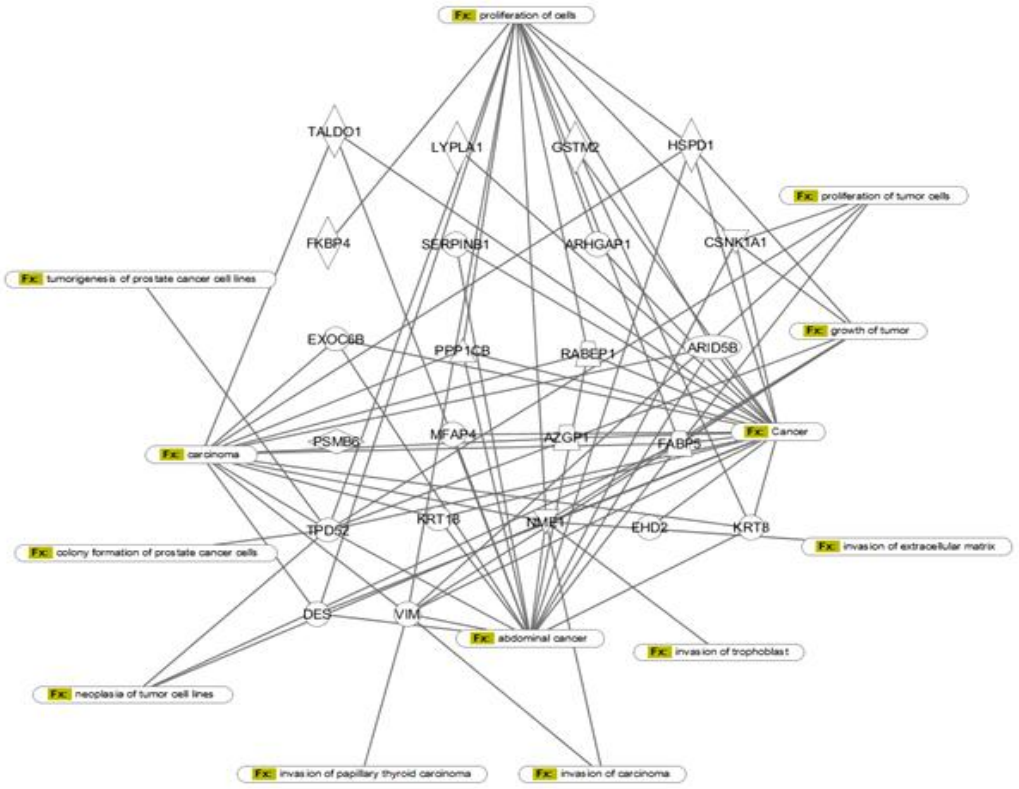

Figure 3. *Association with cancer of the differentially expressed proteins in prostate tissue according to Ingenuity Pathway Analysis (IPA)*

Thirteen of the identified proteins were known cancer markers associated with prostate and other cancers by numerous proteomics, genomics or functional studies. We evidenced for the first time the deregulation of 9 proteins (CSNK1A1, ARID5B, EXOC6B, LYPLA1, PSMB6, RABEP1, TALDO1, UBE2N, PPP1CB and SERPINB1) that may represent novel prostate tumor markers. We selected 3 proteins involved in cell cycle regulation and progression (UBE2N, PSMB6 and PPP1CB) for Western blot validation. Recently, the role of UBE2N in neuroblastoma development was explained by p53 inactivation through formation of monomeric p53 that results in its cytoplasmic translocation and subsequent loss of function. PP1 was associated with PCa in one study where it was nominated as a direct positive regulator of androgen receptor nuclear expression and transcriptional activity. The study of PSMB6 expression in prostate cancer was needed as this protein has been previously associated with the disease in only two studies with contradictory findings. The validation confirmed significantly higher abundances of UBE2N and PSMB6 and significantly lower abundance of PPP1CB in PCa. Our findings are very useful addition to the growing knowledge about the role of these proteins in PCa, providing a starting point for further elucidation of their function in disease initiation and progression.

Urine has become one of the most attractive biofluids in clinical proteomics because it can be obtained in large quantities, can be sampled noninvasively, and does not undergo significant proteolytic degradation compared with other biofluids. It can be viewed as a modified ultrafiltrate of plasma combined with proteins derived from kidney and urinary tract. Proteomic analysis of urine has shown that it contains disease-specific information for various diseases. Up till now, urine has been used as a source of biomarkers for a number of kidney diseases and cancers related to the urogenital system such as bladder and prostate cancer, as well as various systemic diseases [6].

There are several comparative proteomics studies aiming to find diagnostic PCa biomarkers in urine [4]. Our group also focused on identification of non-invasive biomarkers in urine with higher specificity than PSA. In the first preliminary study we have determined the protein components of urine from PCa patients by conventional 2-D PAGE [7]. The MS identification of the most prominent

125 spots from the urine map revealed 45 distinct proteins. Comparison with other published studies analyzing normal urine proteome pointed out 11 proteins distinctive for PCa, among which E3 ubiquitin-protein ligase rififylin (RFFL), tumor protein D52 (TPD52) and thymidine phosphorylase (TYMP) were associated specifically with cellular growth and proliferation. Although the presented urinary proteome map from patients with PCa has limited number of identified proteins, the information regarding their position, molecular mass, possible posttranslational modifications and presence of different protein fragments contribute to the growing knowledge of prostate cancer pathophysiology. In the next study, we tested urine samples from PCa and BPH patients by 2-D DIGE coupled with MS and bioinformatics analysis [8]. We analyzed 56 urine samples divided into screening set consisting of 8 PCa and 16 BPH samples and validation set consisting of an additional 16 PCa and 16 BPH urine samples. Statistically significant 1.8 fold variation or more in abundance, showed 41 spots, corresponding to 23 proteins. Seventeen of the identified proteins have been associated specifically with PCa in different proteomics studies. Moreover, five of the proteins associated with PCa in previous study [7] such as TYMP, ENDOD1, RFFL, CRYZL1 and ILF2 were also detected with differential abundance in this study. Nine proteins with differential abundances were acute phase response proteins and the expression pattern of 4 differed from the defined expression in the canonical pathway. All of the acute phase response proteins with differential abundances in this study were reported as differentially expressed in other proteomics studies of PCa using tissue or body fluids and some of them in proteomics studies of bladder and renal cancer. Worth mentioning is that among these proteins was inter-alpha-trypsin inhibitor (ITIH4) which already was reported to be up-regulated in the urine of PCa patients in the study of Jayapalan et al., [9]. The urine levels of TF, AMPB and HP were validated and the expression level was confirmed by immunoturbidimetry. The concentration of AMPB in urine was significantly higher while levels of TF and HP were significantly lower in PCa in comparison to BPH. The combination of TF, AMBP and HP increased the individual diagnostic accuracy (AUC=0.723-0.754) and the highest accuracy, greater than PSA was obtained for the combination of HP and AMBP (AUC = 0.848).

Furthermore, we have evaluated the PCa specificity of urinary HP and AMBP in independent set of PCa, BPH, bladder cancer (BC) and renal cancer (RC) patients **(Figure 4)**. The nonparametrics Kruskal-Wallis test comparing AMBP and HP urine concentrations among the 4 groups, showed significantly different distribution between groups for both AMBP (p=0.0004) and HP (p<0.0001). The concentration of HP in the PCa group showed a significantly lower level compared to BPH (p=0.036), BC (p<0.0001) and RC group (p=0.0002).

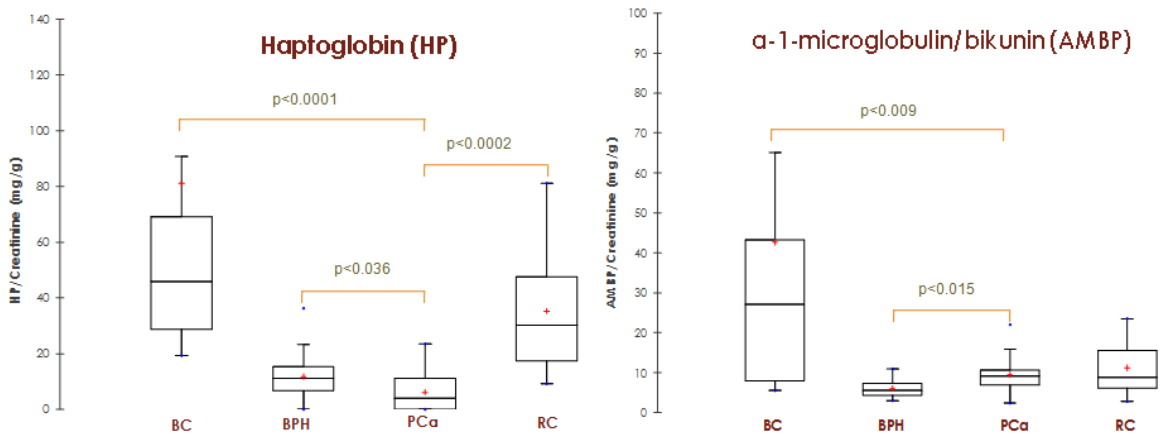

Figure 4. *Validation of urinary HP and AMBP in larger cohort of PCa, BPH, bladder cancer (BC) and renal cancer (RC) patients*

The concentrations of AMPB in the PCa group showed significantly higher level compared to BPH group (p=0.015) and opposite, significantly lower level compared to BC group (p=0.009). There was

no significant differences in AMBP concentrations between PCa and RC group (p=0.258). The expression level of AMBP was also increased in BC and RC group as in PCa group, although the median levels between PCa and BC groups were significantly different. Contrary, the expression level of HP which was decreased in PCa compared to BPH, showed increased expression in BC and RC group. In conclusion, the results from evaluation of HP and AMBP urinary levels in urogenital cancers highlight the urinary HP as a biomarker for PCa. Though maybe HP is not specific protein for PCa, it could become an important check index and improve the sensitivity and specificity for early diagnosis.

4. General conclusions from prostate cancer proteomics studies

What can be concluded from the overall analysis of candidate biomarkers proposed so far by the comparative proteomics studies is that most of the data obtained until now is quite heterogeneous and there is a small percentage of overlap between independent studies [4]. In addition, most of the proteins that overlap between independent studies are discovered by gel-based proteomics methods. While most of the candidate biomarkers in tissue are highly expressed in prostate, the blood and urine biomarkers are mainly transporters and structural proteins not expressed specifically in the prostate and involved in a variety of biological processes among which the most prominent are immune response, protein metabolism and transport.

There is also a low level of transfer of tissue biomarkers into body fluids. A general discrepancy between tissue and body fluids findings can be observed in the cancer biomarker studies [10]. This lack of detection or low transfer of cancer biomarkers into the circulation may be due to the low levels of tumor associated proteins in tissue being released into the body fluids (serum, urine) where they are masked by high abundant serum proteins and therefore undetectable with the present method. Alternatively, proteins may be differentially expressed at the tumor level but the increase or decrease in circulation may be negligible owing to the greater mass of unaffected tissues. Lastly, levels of the proteins in tumor tissue may be unchanged compared to unaffected tissues, but because of altered processing, increased turnover and cell breakdown, the proteins may appear at increased concentrations in the circulation, as in the case of PSA. In our experience, from 28 proteins identified with altered abundance between PCa and BPH tissues [5] we have detected only 4 (ALB, GC, AZGP1 and TPD52) in the urine of PCa patients [7]. Among them, only vitamin D binding protein (GC) was found with differential abundance in urine samples [8] with the same fold change as in the study analyzing tissue.

5. Challenges and future perspectives

Comparative proteomics studies of malignant and benign prostate tissues have identified a large number of candidate biomarkers for PCa. So far, most of the proteomics studies of PCa have been limited to biomarker discovery and just few of them have tried to validate the proteomic data both in larger cohort and in different populations. These studies helped to some extend in the elucidation of the molecular events underlying PCa progression. However, clinical application of most of these biomarkers is still lacking.

Having in mind the overall accomplishment and recognized challenges in the proteomics research of PCa so far, in the future there is a need for:

- Validation of the many candidate biomarkers that have been discovered
- Comparative analysis of well-defined samples using highly sensitive proteomics techniques and combination of proteomics methods to increase sensitivity of detection and minimize method-associated bias
- Novel markers with high sensitivity and specificity for diagnosis and identification of aggressive disease
- Transfer from biomarker discovery to rigorous validation and application of the findings in clinical trials.

Hopefully, lessons learned from proteomics studies of PCa so far, may subsequently speed up the discovery process and lead to reliable and sensitive biomarkers for PCa in near future.

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