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Review article

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URINARY AND TISSUE PROTEOMICS OF UROTHELIAL CANCER

SUMMARY

Current explosion of „omics“ technologies within functional genomics and proteomics promises to take a central place in the understanding of pathogenesis, diagnosis, monitoring and treatment of (pre)cancers of many different sites. Urothelial cancer is a common malignant disease characterized by multiple localisations and frequent recurrences. Proteomics is expected to play the key role in early diagnosis and distinction of biological potential among the low grade urothelial cancers, with the long-term goal of predicting their evolution in terms of outcome, avoiding invasive diagnostic procedures. This review covers a selection of advances of proteomics application and its promise for transitional cell carcinoma research. Proteomics offers an attractive approach to biomarker discovery. Protein profiling of urine could provide us with low-cost and noninvasive diagnostic approach for transitional cell carcinoma. Tissue protein profiling is far more complicated than the analysis of fluids, but it could provide more accurate information of healthy and malignant urothelium.

Key words: **urothelial cancer, biomarkers, urinary proteomics, tissue proteomics, diagnosis**

INTRODUCTION

The sequencing of the human genome is only the beginning of the quest to understand the functionality of cells, tissues, and organs, both in health and disease. Compared with the human genome, the human proteome is even wider universe of proteins and their isoforms that are assembled with alternatively spliced messenger RNAs, as well as myriad of other posttranslational modifications. However, our phenotype is a manifestation of the proteome, the full complement of gene products, which are direct executors of the biological processes of the cell. Current explosion of „omics“ technologies within proteomics and functional genomics promises to take a central place in the understanding, diagnosis, monitoring and treatment of (pre) cancers of many different sites. Cancer, being a complex disease that affects a significant fraction of the population, is a prime target for these new technologies. Changes in genes and proteome expression profile occur differently as tumors develop and progress. In addition, the expression levels of many other related genes and proteins are also altered, owing to the functional changes of the different cell populations and/or regulation within tumors. Thus, proteomics technologies are expected to play a key role in the study and treatment of cancer, as they provide valuable information to define and characterize regulatory and functional networks, to investigate the precise molecular defect in diseased tissues and biological fluids, and for developing specific reagents to precisely pinpoint a particular disease or stage of a disease. Applications are still

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limited, but the evidence so far is exciting (1). For drug discovery, proteomics assist with powerful tools for identifying new clinically relevant drug targets, and provide functional insight for drug development (2).

Transitional cell carcinoma (TCC) is typically superficial papillary lesion (stage pTa/pT1) at first presentation and often multifocal. These tumors exhibit a high frequency of recurrence, and many of them will progress to life-threatening malignancies over a long period of time (3). Urothelial cancer belongs to these tumors where a better prediction would have strong impact on the further successfull treatment of patients. Various histopathological and clinical parameters are known to have prognostic significance, but they are not reliable enough to assess with certainty the biological behavior of these tumors. The „gold standard“ includes invasive and expensive procedures. Better and noninvasive prognosticators are urgently needed.

Interest in the proteomics approaches in the past decade has been resuscitated by the development and application of techniques such as protein arrays, two-dimensional gel electrophoresis, and mass spectrometry. Proteomics technologies are expected to play a key role in the study and treatment of urothelial cancer. These techniques provide valuable information for precise definition and characterization of the malignant cell and enables functional insight for diagnostic approach and drug development.

There are important barriers that must be overcame in biomarker discovery for urothelial cancer (2,3). For example, the poor specificity of biomarkers may lead to unnecessary, invasive, potentially harmful interventions; while poor sensitivity can result in misdiagnosis. The eventual “gold standard” for early diagnosis of urothelial cancer would probably consist of multiple assays for analysis of different biomarkers and would contain the adequate algorithm for interpretation of the obtained results. The novel technologies, such as protein arrays, two-dimensional gel electrophoresis, and mass spectrometry could have a greater application in the study and diagnosis of cancer. These technologies are more and more used for the analysis of the clinically relevant samples (biopsy specimens, blood and urine) in order to facilitate the process of the early diagnosis. Studies show that the examination of the proteomics pattern in the urothelial cancer could have the same value as the pathohistological examination of the biopsy tissue specimens (4). The application of the proteomics technology has been used in just a few long-term programs on TCC patients. The usage of these technologies could have not only the diagnostic, but also the profilactic value. The main goal is to identify reliable predictive biomarkers assay using the power of the new “omics” technologies.

Urinary proteomics

Urinary proteins include a myriad of proteins and peptides: large scale proteomics profiling of normal human urine samples has revealed the presence of at least 1000 different protein gene products and many more peptide fragments of larger proteins (5). They are all derived from a variety of sources, including glomerular filtration, apoptosis, proteolytic cleavage of cell surface. The majority of urine proteins appear as cleavage products that are found not only as free solutes but also in secreted exosomes (1,5,6). Protein profiling of urine could provide us with low-cost and noninvasive diagnostic approach. There are many of hypothesis-targeted investigations of individual proteins as well as proteome-wide searches for urinary biomarkers of various diseases and their progression. Several recent examples of important diagnostic findings using urine proteomics are described. Some thoughts on the most challenging step are shared: integration of seemingly unrelated findings of gold standard, various protein fragments into a rational pathogenetic pathway(s) (7).

There are already several clinically useful diagnostic tests for bladder cancer which detect the presence of specific tumor products in the urine samples, such as the original bladder tumor antigen (BTA) test, immunoCyt, Telomerase test, Lewis X antigen test; the nuclear matrix protein 22 (NMP22), fibrin/fibrinogen degradation products, vascular endothelial growth factor, hyaluronic acid, hyaluronidase, cytokeratins 18, 19, and 20, survivin, BLCA-4, CD44 and mucin 7 detection (7, 8). In general, these assays are not sufficiently specific to be used to screen the general population for bladder cancer. The problem of almost all urine-based tests is low sensitivity for early detection of carcinoma. For example, BTA and NMP22 could not be reliable for urine samples from patients with gross hematuria, urolithiasis, and genitourinary cancers. NMP22 does not exist in red blood cells, but hematuria significantly increases the urinary NMP22 concentration (7).

Calreticulin (CRT) is a ubiquitous protein that was identified in the sarcoplasmic reticulum of the striated skeletal muscle cell as a Ca^{2+}-binding protein, but also in the endoplasmic reticulum membrane of the nonmuscle cells. CRT plays important role in Ca^{2+} storage and signaling, lectin-like chaperoning, regulation of gene expression, cell adhesion, and autoimmunity, and is also included in the oxidative stress mechanism. The increased production of CRT is found in bladder cancer tissue, in comparison to healthy population. The presence of

this protein was detected also in the urine samples. There are evidences that viable cancer cells could be able to secrete CRT directly into urine (7). The presence of CRT is not specific to bladder cancer only. Future studies should be directed toward determining its presence in various tumors (7).

The initiation of DNA replication represents a potentially attractive target for diagnostic analysis. Proteins of the minichromosome maintenance (Mcm) family (Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7) play a regulatory role in the initiation of DNA replication. These findings were used for detection of cervical cancer. MCM proteins distinguish proliferating from nonproliferating cells and their expression is more sensitive marker of epithelial carcinogenesis than the expression of conventional proliferation markers. The Mcm5 test was significantly more sensitive than the cytological examination of urine. The fraction of Mcm5-expressing cells in urothelial cancer is associated with the pathological grade of transitional cell carcinoma: 78% for poorly differentiated G3 tumors, 70% for moderately differentiated G2 tumors, and 45% for well-differentiated G1 tumors (9).

Besides the explicit identification of specific urinary proteins, there is also a strong need for their quantification. Most biomarker discovery studies use a qualitative approach, looking for proteins or peptides that are either present or not in the diseased urine samples. Assessment of changes in amounts of specific proteins could provide us with useful diagnostic algorithm, for example to predict the biological potential of neoplastic lesions (1,2,10).

Proteomics analysis is good method to use in order to find a new tumor markers in urine sample. For example, prominent biomarker from the diagnostic pattern for urothelial cancer, identified as fibrinopeptide A, is well-known biomarker of ovarian cancer and gastric cancer (10). Discovery and validation of urinary biomarkers can be used in oncology both with regard to cancers of the urinary tract as well as malignancies elsewhere in the body.

Exosomes are the internal vesicles of multivesicular bodies (MVBs). They are delivered to the extracellular fluid by the fusion of the outer membrane of MVB with the plasma membrane. These flattened spheres, bounded by a lipid bilayer, are secreted by the variety of cells. They are significantly smaller than any other membrane vesicles secreted by cells, so that they deserve to be classified as nanoparticles (6,11,12).

Urinary exosomes are secreted from all cell types that face the urinary space including glomerular podocytes, renal tubule cells, and the cells lining the urinary drainage system (6, 12). Exosomes contain both membrane and cytosolic proteins. Because exosomes only account for around 3% of the total protein in normal human urine samples, their isolation can result in a very large enrichment of urinary proteins that can be examined (13). First proteomics analysis of urinary exosomes from normal human subjects identified 295 proteins involved in exosome biogenesis, such as class E vacuolar protein sorting (VPS) proteins: VPS I-II-III, VPS4, and ALIX. These molecules are proved to be one of the most important endosomal sorting complex required for transport (1). The results indicates that exosome isolation is an important step forward in biomarker discovery in urine (1,11). Furthermore, cells of many non-urothelial tumors actively secrete exosomes, and their examination in urothelial cancer proteomics studies could provide us with useful piece of information (14, 15).

It still remains unclear whether renal or urinary drainage tract malignancies are directly associated with excretion of exosomes derived from cancer cells. Considering that exosomes can indirectly stimulate the immune system, they are of enormous interest to both oncologists and immunologists, who are now using them in studies as tumor-antigen bearers in order to trigger the tumor rejection by the body (5, 16).

Tissue proteomics

Proteomics analysis of tissue biopsies is far more complicated than the analysis of fluids, due to the heterogeneous nature of the tissue samples. Although the sensitivity for high-throughput analyses in proteomics has improved, it has not yet been sufficiently resolved, because all tissues, tissue areas and even single cells are heterogeneous on the proteomics level (17). Furthermore, in contrast to serum and other fluids analysis, the analysis of tissues or fractionated cells is more time-consuming because microdissection or cell sorting is necessary. However, it has its advantages, because the chance of finding a reliable tumor marker is much higher (18). Concerning the biomarkers for TCC, the development of genomic markers is still a matter of research, the assays being time-consuming, expensive and difficult to standardise. Identification of proteins present or absent from tumor tissue, compared with tissue from normal or benign disease tissues, may be the easier pathway for the detection of tumor-specific proteins. To date, protein profiling has been successfully applied to multiple cancerous tissues, including human non-small cell lung tumors, gliomas and breast tumors (4, 18, 19).

The specimens that include tumors' and normal mucosal tissues obtained by biopsies and cystectomies have been systematically analyzed exploring the possibility of using proteome

expression profiles of bladder tumors as fingerprints to objectively subclassify histopathological types, and as a staring point for searching for protein markers that may form the basis for diagnosis, prognosis, and treatment. These studies have been complemented with cDNA array analysis, and by genome-wide studies of gene copy numbers, transcripts, and protein levels in pairs of noninvasive and invasive TCCs. Highly deregulated proteins in invasive transitional cell carcinoma were observed by study of (S)-methionine-labeled proteins synthesized by normal urothelium and an invasive TCCs, (Grade 3, pT2-pT4). There were numerous overexpressed proteins, such as heat shock protein 28 (hsp28), proteasome, Proliferating Cell Nuclear Antigen (PCNA), elongation factor-1γ (EF-1γ), specific protein that control microtubule reorganization (MRP14); as well as downregulated proteins, including immunostimulating peptidoglycan monomer (PGM), specific fatty acid binding proteins (PA-FABP, A-FABP), protease inhibitors and tumor suppresors from serpin superfamily (eg. PAI-2, Maspin) versus normal urothelial cells. These studies have revealed several reliable protein markers for TCC progression (2, 20). Results of these studies brought about protein expression databases of TCCs and SCCs, as well as other cell types that are available through the Internet (2,20,21).

Although low-graded, TCCs have the different biological potential in the sense of their evolution. So, the distinction of biological potential among low grade tumors is essential. Studies with invasive TCC samples have led to the development of novel strategies for the identification of tumor heterogeneity among low-grade papillary TCCs. These encompasses a blind and systematic studies concerning the profile of the proteome expression in biopsy specimens, from both normal and tumor originated tissue. So far, the most investigated potential biomarkers were keratins 5, 20, and 8 (2, 21), fibroblast growth factor receptor-3 (FGFR3), hamartin, 14-3-3sigma, Aurora-A, and E-cadherin (22).

So far, these studies have identified several types of cancer heterogeneity among low grade tumors that affect either the basal proliferative compartment, the umbrella cells, or the suprabasal layers (2, 20). Observed results clearly illustrate the potential of combining proteomics with other methodology, such as immunohistochemistry to reveal cancer heterogeneity and to correlate it with outcome. Undoubtedly, long-term prospective study involving a larger sample size with regular follow-up is required to assess reliable results applicable in clinical practice.

CONCLUSION

Proteomics complement genomic-based approach in the study of cancer. Concerning the biomarkers for TCC, the development of genomic markers is still a matter of research. So far, designed assays are time consuming, expensive and difficult to standardise. The identification of proteins that are present or absent in tumor tissue and their quantification, compared with normal or benign diseased tissue, may lead to the discovery of urothelial cancer specific proteins. Proteomics technologies are hoped to provide insight into the molecular complexity of the disease process and thus enable the development of tools to help in treatment, as well as in detection and prevention. It is especially valuable in the discovery of biomarkers, because the proteome reflects both the intrinsic genetic program of the cell and the impact of its immediate environment. Protein expression and function are subject to modulation through transcription, as well as through posttranscriptional and translational events. Ideally, molecular profiling would provide a state of the art assessment of urine and tissue analyses for early detection, different preventive and therapeutic approach of TCC. The implementation of discovery-driven translational research will not only require coordination of basic research activities, facilities, and infrastructures, but also the creation of an integrated and multidisciplinary environment with the participation of a dedicated team of clinicians, oncologists, pathologists, immunologists and epidemiologists. Issues related to sample collection, handling, and storage, number of patients, availability of normal controls, tissue banks, quality of the clinical information, follow-up studies, and ethical considerations are critical, and must be carefully considered.

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URINARNA I TKIVNA PROTEOMIKA UROTELIJALNOG KARCINOMA

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SAŽETAK

Skorašnji ekstenzivni razvoj "omics" tehnologija koje ispituju funkcionalni genom i proteomiku sve više zauzima centralno mesto u razumevanju patogenetskih mehanizama, dijagnoze, praćenja i tretmana (pre) kanceroznih lezija različitih tkiva. Urotelijalni karcinom je relativno česta maligna bolest koja se karakteriše multiplom lokalizacijom i čestim recidivima. Proteomika bi mogla da odigra ključnu ulogu u ranoj detekciji karcinoma, diferencijaciji različitih tumora niskog gradusa, sa različitim biološkim potencijalom u cilju blagovremene predikcije, izbegavajući upotrebu invazivnih dijagnostičkih procedura. Ovaj članak prikazuje neke novine u primeni i mogućnostima primene proteomika u istraživanju tranziciocelularnog karcinoma. Urinarna proteomika je atraktivan pristup u istraživanju biomarkera karcinoma. Određivanje proteina u urinu je na putu da postane jeftina i neinvazivna dijagnostička procedura u detekciji tranziciocelularnog karcinoma. Karakterizacija proteina u tkivu je komplikovanija procedura u odnosu na ispitivanja u telesnim tečnostima, ali može obezbediti preciznije informacije o stanju normalnog i maligno transformisanog urotelijuma.

Ključne reči: **urotelijalni karcinom, biomarkeri, urinarna proteomika, tkivna proteomika, dijagnoza**