World Journal of Clinical Urology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.5410/wjcu.v2.i3.20

World J Clin Urol 2013 November 24; 2(3): 20-31
ISSN 2219-2816 (online)

REVIEW

Integrated technologies in the post-genomic era for discovery of bladder cancer urinary markers

Sanaa Eissa, Marwa Matboli

Sanaa Eissa, Marwa Matboli, Oncology Diagnostic Unit, Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Ain Shams University, Abassia, 11566 Cairo, Egypt
Author contributions: Both Eissa S and Matboli M contributed to this paper.
Correspondence to: Sanaa Eissa, Professor, Oncology Diagnostic Unit, Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Ain Shams University, PO Box 11381, Abassia, 11566 Cairo, Egypt. dr_sanaa_eissa@yahoo.com
Telephone: +20-100-1782828 Fax: +20-2-26859928
Received: September 29, 2013 Revised: November 10, 2013
Accepted: November 20, 2013
Published online: November 24, 2013

Abstract

The incidence of bladder cancer (BC) continues to rise with high recurrence and mortality rate, especially in the past three decades. The development of accurate and successful BC treatment relies mainly on early diagnosis. BC is a heterogeneous disease reflected by the presence of many potential biomarkers associated with different disease phenotypes. Nowadays, cystoscopy and urinary cytology are considered the gold standard diagnostic tools for BC. There are many limitations to cystoscopy including being invasive, labor-intensive and carcinoma *in situ* of the bladder may easily be missed. Urinary cytology is still a noninvasive technique with high accuracy in high-grade BC with a median sensitivity of 35%. Furthermore, the need for a sensitive, specific, non invasive, easily accessible BC biomarker is a major clinical need. The field of urinary BC biomarkers discovery is still a rapidly evolving discipline in which more recent technologies are evaluated and often optimized if they are not clinically significant to the urologists. Most of the current strategies for BC urinary biomarker detection depend on integration of information gleaned from the fields of genomics, transcriptomics, proteomics, epigenetics, metabolomics and bionanotechnology. Effort is currently being made to identify the most potentially beneficial urinary biomarkers. The purpose of this review is to summarize and explore the efficacy of gathering the information revealed from the cooperation of different omic strategies that paves the way towards various urinary markers discovery for screening, diagnosis and prognosis of human BC.

Key words: Bladder cancer; Urinary biomarkers; Genomics; Proteomics; Bionanotechnology; Metabolomics; Transcriptomics; Epigenetics

Core tip: Capturing information from in silico data, proteomic data, gene expression data and bionanotechnology data outlines a promising approach to discover significant urinary biomarkers whose activity patterns are discriminative of bladder cancer *vs* control.

Eissa S, Matboli M. Integrated technologies in the post-genomic era for discovery of bladder cancer urinary markers. *World J Clin Urol* 2013; 2(3): 20-31 Available from: URL: http://www.wjgnet.com/2219-2816/full/v2/i3/20.htm DOI: http://dx.doi.org/10.5410/wjcu.v2.i3.20

INTRODUCTION

It is estimated that urinary bladder cancer (BC) is the sixth most common cancer worldwide, with approximately 382660 new cases of BC each year[1,2].

Although the main symptom of BC is hematuria, no symptoms are found in an early stage. About 70% to 80% of patients with newly diagnosed BC present with early stage BC (*i.e.*, stage Ta, Tis, or T1) and low-grade neoplasms that are associated with an excellent prognosis. However, these tumors have a 30% to 70% recur-

rence rate and a strong tendency to progress to invasive cancers in 10% to 30% of patients, with increased risk of metastasis and subsequent mortality. So, early detection of BC is urgently needed to improve prognosis and long-term survival[3].

Nowadays, the standard of care for BC diagnosis and follow-up is through the combination of cystoscopic examination, cytology and histology[4]. However, these methods have a significant financial cost and poor sensitivity for low-grade, well-differentiated lesions. They are also highly subjective investigations and provide little about the molecular characteristics of cancers[5]. Recently, numerous urinary markers have been under study in order to reduce the cost and the frequency of cystoscopies or replace them by non-invasive tests. An ideal test for the detection of bladder tumors should have high sensitivity and specificity; moreover, it is necessary to be objective, accurate, rapid and easy to administer[6].

Urine is an ideal biological fluid representing a gold mine suitable for clinical analysis due to simple, economic and non-invasive collection with large quantities of samples available. Therefore, it has been proposed as a substitute to blood collection as a diagnostic tool or at least as a screening test[7]. Nevertheless, the very low abundance of many candidate targets in urine and the presence of different interfering substances have impeded the development of novel urinary biomarkers that may be clinically useful for BC diagnosis[8].

Integration of different biomolecular signature data set through capturing information from in silico data with multiple omic technologies for genomics, gene expression (transcriptomics) and proteomics is increasingly important to maximize value in biomarker discovery, validation and utilization for early diagnosis or prognosis of cancer[9]. Each one of these technologies provides a snapshot of cell function. However, dynamic understanding of disease processes really needs the integration of all these modalities to the greatest possible extent[10].

LITERATURE SELECTION

The published studies that discussed BC biomarkers were identified by searching PubMed for studies that were published between January 2000 and December 2013. The search terms that were used were "bladder", "carcinoma" or "cancer" and "biomarkers" or "bioinformatics" and "genomic", "proteomic" or "epigenetic", or "nanoparticles" without restrictions. In addition, the reference lists of retrieved papers and recent reviews were also examined.

STUDY SELECTION

Any study that matched the following criteria was included: (1) a case-control study design; (2) an association between BC and biomarkers in humans; and (3) BC confirmed by the accepted diagnostic criteria. To evaluate the eligibility of all the studies retrieved from the databases on the basis of the predetermined selection criteria, two independent investigators were used. Disagreements were resolved by discussion.

BIOINFORMATICS AND BC URINARY BIOMARKERS

BC subtypes and biomarkers have been identified using technologies that combine clustering algorithms and visualization tools into web-based bioinformatic databases and those that analyze high-throughput gene expression data[11]. Common analytical tools include the following: Atlas of Genetics and Cytogenetics in Oncology and Hematology (http://atlasgeneticsoncology.org/) is a database that deals with chromosome abnormalities in cancer and genes involved in cancer. This database is provided by experts in cytogenetics, molecular biology with clinicians in oncology and in hematology, and pathologists[12]; Catalogue of Somatic Mutations in Cancer COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) stores and supplies information about somatic mutations in cancer. This database collects information about publications, mutations and samples; Human protein atlas database (http://www.proteinatlas.org/) displays protein expression profiles based on immunohistochemistry for a large number of human tissues, cancers and cell lines, subcellular localization in three cell lines and transcript expression levels in three cell lines; OmniBiomarker (http://omnibiomarker.bme.gatech.edu/) is a web-based bioinformatics tool for developing biomarkers in oncology to anticipate the clinical outcome of promising biomolecules as a biomarker; The NCI's Cancer Biomedical Informatics Grid® (caBIG®) initiative is the most widely used tool at every stage of cancer that facilitates biomarker discovery beginning from selection of target groups until clinical validation step. At the same time, caBIG® also provides information related to basic research free of charge; Gene Expression Profile Analysis Suite or GEPAS (http://www.gepas.org) for microarray analysis; Array Express (http://www.ebi.ac.uk/microarray-as/ae/ca) and the Cancer Biomedical Informatics Grid (caBIG) (https://cabig.nci.nih.gov) are used for storage and management of expression data; Biomedical knowledge discovery server, BioGraph (http://www.biograph.be/about/welcome), is a data integration platform for the purpose and discovery of biomedical information[13]. The database offers prioritizations of supposed disease genes, supported by functional hypotheses. BioGraph can retrospectively validate recently discovered disease genes and identify susceptible genes, surpassing recent technologies, without requiring previous domain knowledge. Briefly, such computational methods integrating multi-omics data will be very precious to select molecular targets, biomarker candidates and to translate them into biologically meaningful hypotheses.

GENOMICS IN BC URINARY BIOMARKERS

Genomics is a discipline that applies recombinant DNA,

DNA sequencing methods and bioinformatics to analyze the function and structure of the whole set of DNA within the cell of an organism, allowing increase of the width of the field with the number of newer markers identification[14,15]. Applying technologies such as gene microarray that can analyze huge number of DNA sequences from many patients very quickly, the field of genomics has identified thousands of genetic duplications and aberrations that may take part in bladder carcinogenesis[16]. BC, with tumor cells being bathed in urine, perhaps provides the best potential use of DNA markers. However, such markers will not become clinically significant until easier detection methods are found, marker standardization occurs and more clear and specific applications for primary diagnosis compared to recurrent disease are performed[17]. Some of the common genetic markers including *FGFR-3* mutations, *p53* and retinoblastoma genes have elucidated several molecular pathways in BC development[18,19].

Larré *et al*[20] designed a comparative genome hybridization (CGH) chip, including loci proposed to be associated with BC for the assessment of bladder tissues. The CGH data were used to develop a diagnostic test that could be performed on urothelial cell pellets. This test had an overall diagnostic accuracy of 91% in 44 samples. The detection of specific urothelial gene mutations is also applicable to disease evaluation[20].

Kucukgergin *et al*[21] assessed stromal cell derived factor 1 (*SDF-1*) 3'A, monocyte chemoattractant protein-1 (*MCP-1*) A2518G, and chemokine receptors *CCR2A*, *CCR5* Δ32 and *CXCR4* gene polymorphisms by PCR and PCR-restriction fragment length polymorphism (RFLP) methods in 142 histologically confirmed BC patients and 197 controls in a Turkish population. Their results suggest that the genetic variants of *SDF-1* 3'A, *CCR2A* V64I and *CCR5* Δ32 gene may contribute to muscle invasive BC in a Turkish population[21].

Al-Kashwan *et al*[22] analyzed TP53 alterations by PCR-single strand conformational polymorphism analysis and DNA sequencing in twenty-nine bladder carcinomas. They found infrequent TP53 mutations, especially insertion A and 196 hotspot codons in 37.9% of the cases, while TP53 overexpression occurred in 58.6% among the Iraqi patients who were exposed to war environmental hazards[22]. Eissa *et al*[23] also evaluated diagnostic efficacy of mutant p53 patients by PCR-SSCP followed by DNA sequencing in urine of 100 patients diagnosed with BC, 93 patients with benign urological disorders and 47 healthy volunteers. The sensitivity and specificity were 59% and 91.4% for cytology, and 37% and 100% for mutant p53, with a significant association observed between disease recurrence and mutant p53, stage and lymph node involvement[23].

Wang *et al*[24] adopted co-amplification at lower denaturation temperature-polymerase chain reaction (COLD-PCR) as a straightforward method with no additional reagents requirements or instruments as a highly sensitive, specific and expedient clinical assay for mutation detection in the *H-ras* gene, including exons 1 and 2, in Chinese patients diagnosed with BC, yielding a 36% improvement in mutation detection compared with conventional PCR. They concluded that silent mutations might be important genomic alterations in BC pathogenesis and recurrence[24].

EPIGENETICS IN BC URINARY BIOMARKERS

DNA methylation

Epigenetics is a field that refers to reversible changes in gene expression caused by mechanisms other than any change in genetic sequence[22]. DNA methylation is the most common epigenetic changes addressed in BC biomarkers. DNA methyltransferase catalyzes the transfer of the methyl group to the cytosine ring of the CpG dinucleotides. When these CpGs present in promoter regions of genes at high density, CpG islands and gene silencing may be caused by their methylation[25]. Hypermethylation of tumor suppressor genes is a common event during tumorigenesis[26,27]. A large number of genes and their methylation state were assessed in their relationship to urothelial cancer. DNA methylation analysis is usually carried out by methylation specific PCR, bisulfate sequencing, methylation sensitive restriction enzymes and methylated DNA immunoprecipitation (MeDIP)[21].

An Egyptian study was conducted on 210 BC patients, 61 patients with benign urological conditions and 49 healthy volunteers. Eissa *et al*[28] evaluated promoter methylation of *RARβ(2)* and *APC* in DNA extracted from exfoliated cells by methylation specific PCR. Methylated *RARβ(2)* and *APC* were significantly higher in BC patients (62.8%, 59.5%) than benign (16.4%, 5%) but not detected in healthy volunteers (0%) at ($P < 0.0001$). Both sensitivities and specificities of the methylated genes for BC detection were superior to urine cytology[28].

DNA methylation status of specific gene promoter regions in bladder tumor cells has been proposed as a marker for primary diagnosis and for detection of recurrence. García-Baquero *et al*[29] conducted evaluation of the methylation of 18 tumor suppressor genes using methylation specific multiplex ligation-dependent probe amplification in 2 prospective, training urine sample sets of 120 preparations and validation set of 128 from patients with BC (170) and controls (78). *HLTF*, *DLC1*, *PRDM2*, *BNIP3 ID4*, *H2AFX*, *CACNA1G*, *TGIF* and *CACNA1A* were methylated in BC. The methylation status of 5 genes (*CCND2*, *SCGB3A1*, *BNIP3*, *ID4* and *RUNX3*) was identified as an epigenetic biomarker for BC and achieved very high accuracy when used as a panel in analysis of urine sediments. ROC analysis revealed significant diagnostic accuracy for RUNX3 and CACNA1A in the training set and for RUNX3 and ID4 in the validation set. CACNA1A methylation correlated with recurrence in the training set, while in the validation set, PRDM2 and BNIP3 were significantly associated

with recurrence respectively[29].

Kandimalla *et al*[30] reported a panel of epigenetic target genes. Genome-wide methylation analysis was performed on 44 bladder tumors using human CpG island microarray, then validation was performed using a next generation sequencer in a retrospective group of 77 independent tumors and urine DNA from four healthy males > 50 years of age was used as reference. They found 4 genes, Zic family member 4 (*ZIC4*), T-box 2 (*TBX2*), T-box 3 (*TBX3*) and GATA binding protein 2 (*GATA2*), that were significantly hypermethylated in tumor samples methylation and associated with progression to muscle-invasive disease in pTa tumors. Individually, methylation of *TBX2* alone showed a sensitivity of 100%, a specificity of 80%, a positive predictive value of 78%, and a negative predictive value of 100%. This panel of methylated gene increased the sensitivity to 91.7% and the specificity to 87.6%. They also declared that the multivariate analysis showed that methylation of *TBX3* and *GATA2* are independent predictors of progression when compared to clinicopathological variables. They further identified and validated 110 CpG islands with differential methylation between tumor cells and control urine. This study was limited by the small number of patients analyzed for testing and validation.

Scher group has reported that the methylation of 3 genes (*BCL2*, *CDKN2A* and *NID2*) detected by nested methylation specific polymerase chain is associated with BC. They were able to differentiate BC from other urogenital malignancies and nonmalignant conditions with a sensitivity of 80.9% and a specificity of 86.4%.

The epigenetic markers provide a new paradigm in urinary biomarker development for BC[31]. However, the above mentioned markers have been tested in single institutions and with relatively small case control or pilot studies. At the present time, there is no standard method to assess these markers.

microRNA

Hence, it seems to be a good strategy to find cancer-related genes by categorizing methylated genes and microRNA discovery is another major epigenetic event. MicroRNA as a key post-transcriptional regulator of gene expression is small non-coding RNA of 20-22 nucleotides and involved in crucial biological processes, including development, apoptosis and cell division, through improper pairing with target messenger RNA (mRNA)[32]. Array-based profiling, deep-sequencing technologies and qPCR for miRNA analysis are becoming routine technically. They are suitable for the classification of tumors because of aberrant expression of miRNAs in human cancer[33].

Yamada *et al*[34] found the expression level of miR-96 and miR-183 in urine samples was significantly higher in 100 BC than in healthy controls by qPCR. Their results demonstrated that each microRNA has good sensitivity and specificity (miR-96, 71.0% and 89.2%; and miR-183, 74.0% and 77.3%).

Hanke *et al*[35] monitored a number of 157 microRNA species by quantitative reverse transcriptase-polymerase chain reaction in exfoliated urothelial cells in 36 samples. Subsequently, those microRNAs with a higher abundance in urine samples from BC patients were validated in an independent set of urine samples. The study reported that the ratio of miR-126 to miR-182 achieved 72% sensitivity and 82% specificity in 47 samples.

Differential expression of miRNAs was identified by Wszolek *et al*[36] by microarray analysis between noninvasive and invasive BC cell lines and confirmed using (qRT-PCR) within these cell lines. They reported reduced expression of miR-21, miR-30b, miR-31, miR-141, miR-200 and miR-205 in invasive lesions and overexpressed miR-99a in noninvasive BC lesions. Such a diagnostic test, depending on the three most discriminatory miRNAs in this panel (miR-200c, miR-141 and miR-30b), showed a sensitivity of 100% and a specificity of 96.2%.

Tölle *et al*[37] explored the expression of 754 human miRNAs from the Sanger database v14 in the blood and urine samples from 4 controls and from patients suffering from superficial and invasive BC using miRNA microarray. Using the RT-qPCR technique, 6 of the differentially expressed miRNAs were validated in the controls and patients with superficial or invasive tumors. Three blood miRNAs (miR-26b-5p, miR-144-5p, miR-374-5p) were found to be significantly upregulated in invasive bladder tumor patients when compared to the control group. The expression of 2 urinary miRNAs (miR-618, miR-1255b-5p) in patients with invasive tumors was significantly increased in comparison to the control group. The urine miR-1255b-5p had 68% specificity and 85% sensitivity in the diagnosis of invasive bladder tumors.

Pignot *et al*[38] evaluated expression level of miRNAs by quantitative real-time RT-PCR in 11 human normal bladder and 166 bladder tumor samples. The expression level of 804 miRNAs was initially measured and then the differential miRNAs in tumor samples compared to normal bladder tissue were selected for RT-PCR validation in a series of 152 bladder tumors and in six BC cell lines. They reported a panel of 3-miRNA signature (miR-9, miR-182 and miR-200b) was found to be related to bladder tumor aggressiveness and was associated with both recurrence-free and overall survival.

Aberrations in miRNA expression identified between non-muscle invasive BC and muscle-invasive BC provide valuable insight into the molecular mechanisms known to distinguish the unique pathways of bladder carcinogenesis. The limited reproducibility of changes in miRNA expression profiles between studies utilizing in silico miR target-prediction models is due to the heterogeneity of tumor specimens and research methods[39].

TRANSCRIPTOME IN BC URINARY BIOMARKERS

Another component available to be detected in urine is

soluble RNA, including both mRNA and microRNA (miRNA) targets. Quantitative reverse transcription PCR (Q-PCR) and conventional RT-PCR for RNA isolated from exfoliated urothelial cells in urine are the most widely used techniques in novel biomarkers in BC identification and validation[40].

Diverse markers have been discovered but nowadays a very promising mRNA ratio has been assessed[40]. Hanke *et al*[40] isolated RNA from urinary cell pellet and quantified it by reverse transcription quantitative-PCR in 61 patients with BC and 37 healthy donors. The RNA ratio of v-ets erythroblastosis virus E26 oncogene homolog 2 (avian; ETS2) to urokinase plasminogen activator (uPA) enabled the most specific (100%) and sensitive (75.4%) detection of BC from normal urine.

Eissa *et al*[41] evaluated hyaluronidase (HYAL1) and survivin RNA expression by qualitative and semiquantitative reverse transcriptase-polymerase chain reaction in urothelial cells from voided urine in 166 patients with BC, 112 with benign bladder lesions and 100 healthy volunteers. They reported that positivity rates of HYAL1 RNA and survivin RNA on qualitative reverse transcriptase-polymerase chain reaction were significantly different among the study groups. Mean rank using semiquantitative RT-PCR was higher in the malignant compared to the control groups. Using the best cutoffs HYAL1 and survivin RNA sensitivity was 91% and 75%, respectively, with 100% specificity. HYAL1 RNA detected all patients with early stage BC and is more sensitive and specific than urine cytology which is validated in many publications[41-44].

Another study published by this group assessed urinary fibronectin (FN), relative telomerase activity (RTA) and cytokeratin 20 (CK20) mRNA in comparison with voided urine cytology (VUC) in 132 patients with BC, 60 patients with benign bladder diseases, and 48 apparently healthy individuals[45]. Detection of CK20 was carried out by conventional RT-PCR in urothelial cells from voided urine, estimation of fibronectin by ELISA and relative telomerase activity by telomeric repeat amplification protocol (TRAP). The overall sensitivity (89.3%) and specificity (98.4%) were the highest for CK20 mRNA compared to all investigated markers. The efficacy of urinary CK20 mRNA in BC diagnosis was validated in many publications[46-50].

C-X-C chemokine receptor 4 (CXCR4) and CXCR7 were estimated by Yates *et al*[51] in BC cell lines, tissues (normal = 25; BC = 44) and urine specimens (n = 186) by qPCR and/or immunohistochemistry. CXCR7 messenger RNA levels were 5 to 37-fold higher than those for CXCR4. CXCR7 messenger RNA levels and CXCR7 staining scores were significantly higher in BC than in normal tissues. CXCR7 level was elevated in exfoliated urothelial cells from high-grade BC patients (90% sensitivity; 75% specificity) while CXCR4 level was unaltered.

Bongiovanni *et al*[52] performed real-time reverse transcriptase-polymerase chain reaction to evaluate Bradeion/SEPT4 transcript levels in urine samples from 17 healthy controls and 41 patients with BC. Relative quantification analysis of Bradeion transcript showed 92.68% sensitivity and 64.71% specificity. This preliminary study supports the possible usefulness of Bradeion as a urinary marker of BC.

Brems-Eskildsen *et al*[53] measured urinary mRNA levels of PPP1CA, hTERT, MCM5 and SENP1 by q-RT-PCR from 123 prospectively cross-sectional collected urine samples from patients with BC (54 patients with recurrent BC at sampling, 59 patients with previous BC and no tumor at sampling, 10 patients with a primary BC at sampling). The sensitivity and specificity of these mRNA markers were: for hTERT: 86%; SENP1: 71.7%; MCM5: 95.45%; and PPP1CA: 91.3%. Follow-up data resulted in sensitivity and specificity values: for hTERT: 62/84; SENP1: 63%; MCM5: 83.6%; and PPP1CA: 98.5%. The best combination was hTERT and cytology with a sensitivity of 71% and a specificity of 86%, but the combination of hTERT and MCM5 also increased the detection rate.

Rosser *et al*[54] applied cDNA microarray to explore the molecular signatures of BC in urine pellet from 46 individuals. They reported 14 overexpressed and 10 decreased genes in exfoliated tumor cells. Finally, they built a panel of 14 genes as a potential molecular pattern for diagnosing BC with 90% sensitivity and 65% specificity. This study is limited by the small sample size and low specificity but is still significant as it used the exfoliated cells as a source to perform cDNA microarray analysis. This molecular signature motivated another group to validate them in a larger study applying Q-PCR. Holyoake *et al*[55] investigated the expression of 14 different genes by Q-PCR using voided urine from 75 transitional cell carcinoma (TCC) patients and 77 control patients. In their analyses they developed a panel of 4-marker involving CDC-2, HOX-A13, MDK and IGBP-5 mRNAs detected 48%, 90% and 100% of stage Ta, T1, and > T1 TCCs, respectively, at a specificity of 85%.

PROTEOMICS IN BC URINARY BIOMARKERS

Proteomics refers to the large-scale experimental analysis of proteins, mainly their structures and functions using diverse technologies such as 2-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS)[56]. After initial screening, more traditional tests (*e.g.*, ELISA, zymography, western blot) can be carried out to evaluate the clinical efficacy of promising biomarkers[57].

SELDI (surface enhanced laser desorption/ionization) is the best MS-technique used to characterize biomarkers from biological fluids such as urine and blood[58]. Such high throughput technology can analyze only small molecular mass proteins and miss relatively higher molecular mass biomarkers. Several markers have been gleaned from such technology, including TATI (tumor associated trypsin inhibitor), MMPs (matrix metalloproteinase) and CXCL-1[59].

Chen *et al*[60] used 17 biomarkers for BC diagnosis which were already discovered using isobaric tagging absolute and relative quantitation (iTRAQ), then validated by multiple reaction monitoring-based mass spectrometry in urine samples from 57 patients with hernia, 76 BC and 23 urinary tract infection. Prothrombin had the highest sensitivity, 71.1% and 75.0% specificity for discriminating BC from non-cancerous patients. They generated six-peptide panel (apolipoprotein A-II precursor, ceruloplasmin, adiponectin, afamin, complement C4 gamma chain and prothrombin) to differentiate BC subjects from non-cancerous subjects, with a 76.3% positive predictive value and a 77.5% negative predictive value.

Rosse *et al*[61] evaluated the urinary concentration of eight biomarkers (CA9, APOE, MMP-9, PAI-1, VEGF, IL-8, ANG and MMP-10) by ELISA assay in 102 BC subjects and 206 subjects with different urological disorders. They reported that this 7-biomarker model has a sensitivity of 74% and specificity of 90%. This study was limited by being performed on banked urines and the lack of VUC and UroVysion data on controls.

In another study published by Goodison *et al*[62], the urinary concentration of 14 biomarkers (OPN, MMP-9, MMP-10, APOE, CCL18, A1AT, ANG , VEGF, CD44, CA9, PAI-1, IL-8, PTX3 and SDC1) was measured by ELISA in voided urines from 127 patients (64 tumor bearing subjects). They reported a panel of 8-biomarker achieving the most accurate BC diagnosis (sensitivity 92%, specificity 97%) and highly accurate combination of 3 of the 8 biomarkers (IL-8, VEGF and APOE) (sensitivity 90%, specificity 97%) in comparison with the commercial BTA-Trak ELISA test (sensitivity of 79% and a specificity of 83%) and voided urine cytology (33% sensitivity) in the same subjects.

Li *et al*[63] identified 16 urinary proteins including Gc-globulin (GC) from BC patients and normal controls by two-dimensional fluorescent differential gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF/TOF MS). The urinary GC protein from cases and controls were further assessed by western blotting and ELISA showing 82.61% sensitivity and 88.24% specificity. Another Chinese group used 2-dimensional electrophoresis combined with MALDI-TOF/TOF MS and SWISS-PROT database to explore urinary proteins in patients with BC and in normal controls[64]. They identified 14 proteins, including 2 putative proteins [fatty acid-binding protein adipocyte, myoglobin, beta-2-microglobulin isoform 2 of fibrinogen alpha chain, apoA-I, gelsolin, isoform 1 of gelsolin, prostaglandin D(2) synthase 21 kDa (brain), keratin type II cytoskeletal 1, type II cytoskeletal 8, protein AMBP, transthyretin, putative uncharacterized protein ALB, putative uncharacterized protein MASP2 (fragment)]. apoA-I was confirmed by western blot analysis, concluding that proteomic analysis of urine may be a noninvasive and highly efficient strategy for searching for new bladder tumor biomarkers.

Zoidakis *et al*[65] applied immobilized metal affinity chromatography in urine samples from patients with non invasive and invasive BC and the eluted proteins were analyzed by 1D-SDS-PAGE followed by band excision and liquid chromatography tandem MS. They found that MMP9, fibrinogen forms, clusterin, aminopeptidase N, profilin 1 and myeloblastin were differentially expressed in urine from patients with aggressive compared with non aggressive BC and benign controls, then further validated by western blot or ELISA analysis. This study reported that profilin 1 is strongly associated with BC paving the way for its further assessment in BC diagnostics.

Lindén *et al*[66] screened the urine samples from BC patients by mass spectrometry (MS) and western blot (WB)/dot blot (DB). 29 proteins had a significantly higher abundance in BC samples compared with control urine samples. Then four selected proteins were confirmed with western blot: apolipoprotein E, fibrinogen β chain precursor, leucine-rich α-2-glycoprotein 1 and α-1-antitrypsin. Dot blot analysis of a separate urine sample set pointed out fibrinogen β chain and α-1-antitrypsin as the most significant biomarkers with sensitivity and specificity values in the range of 66%-85%. When the Human Protein Atlas (HPA) was explored, it also revealed that BC tumors are the proposed source of these proteins.

Bryan *et al*[67] explored urine samples from 751 patients with BC and 127 controls using MALDI-TOF-MS. They declared that albumin, total protein and hematuria were elevated in T2+ patients. Hematuria was found in 39% of patients with Ta/T1 disease and in 77% of patients with T2+ disease. Taken together, great consideration should be given when applying omic in searching for urinary biomarkers because blood proteins may give false-positive results.

METABOLOMICS IN BC URINARY BIOMARKERS

Metabolomics is defined as "quantitative measurement of the unique chemical fingerprints that elucidate metabolic response of living systems to pathophysiological stimuli or genetic modification"[68]. It provides information that cannot be obtained directly from the gene expression profiles or even the proteomic fingerprint of an individual. Application of urine-based metabolic profiling is achieved using high pressure liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) which may identify specific biomarker patterns that can aid diagnosis of BC[69].

In a study published by the Pasikanti group, gas chromatography mass spectrometry (GC-MS) was used for urinary metabolic profiling of BC patients and non-BC controls and concluded that urinary metabolomics is highly compliant to the noninvasive diagnosis of BC[70].

Huang *et al*[71] enrolled twenty-seven BC patients and 32 healthy volunteers to perform metabolomic profiling to identify a potential unique biomarker pattern in urine

as a noninvasive strategy for BC detection. They utilized a liquid chromatography-mass spectrometry based method. Carnitine C9:1 and component I were identified as a biomarker panel, with 92.6% sensitivity and 96.9% specificity for all patients and 90.5% and 96.9%, respectively, for low-grade BC patients.

Pasikanti *et al*[72] conducted a urinary metabotyping in another study in 38 BC patients and 61 non-BC controls using two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC-TOFMS). Urinary metabotyping characterized 46 metabolites which are human specific to BC with 100% specificity and 71% sensitivity in detecting BC *vs* 100% specificity and 46% sensitivity for cytology. They suggested potential roles of kynurenine in the malignancy and therapy of BC. In addition, altered metabolic pathways extracted from urinary metabotyping shed new insights on the mechanism of BC.

BIONANOTECHNOLOGY IN BC URINARY BIOMARKERS

However, the above mentioned BC diagnostic methods are not very powerful methods in detection of very early stages of cancer[73]. Also, some of them are quite costly and not available for many people. Therefore, the development of novel, specific, reliable and easily accessible technology for detecting BC early is of great importance[74-82].

Nanotechnology has been progressing very rapidly during the last few years and with this, properties of nanoparticles that provide an enriched medium for the selective capture and uptake of urine biomarkers due to their unique optical, chemical and physical magnetic properties[74,83]. Many classes of nanoparticles (such as gold nanoparticles, quantum dots, magnetic nanoparticles) have been proposed to be applicable in diagnosis, monitoring and treatment of disease[75,84-86].

The Wang *et al*[87] has reported that human telomerase activity can be visualized by using primer-modified Au nanoparticles. Our research group developed a gold nanoparticle (AuNP) assay for direct detection of unamplified hepatoma upregulated protein (HURP RNA) in urine samples from 50 bladder carcinoma patients, 25 benign bladder lesions and 25 controls[88]. They purified HURP RNA using magnetic nanoparticles functionalized with HURP RNA-specific oligonucleotides and detected by RT-PCR and gold nanoparticles. The developed HURP RNA AuNP assay has sensitivity and a specificity of 88.5% and 94%, respectively, and a detection limit of 2.4 nmol/L. Nossier *et al*[89] developed a simple colorimetric gold nanoparticle (AuNP) assay for rapid and sensitive detection of urinary HAase activity. The assay depends on charge interaction between polyanionic hyaluronic acid (HA) and cationic AuNPs stabilized with cetyltrimethylammonium bromide (CTAB) led to formation of gold aggregates and a red to blue color shift. HAase digests HA into small fragments preventing the aggregation of cationic AuNPs. The AuNP HAse assay has a sensitivity of 82.5% and a specificity of 96.1% and a short turnaround time of 2 h[89].

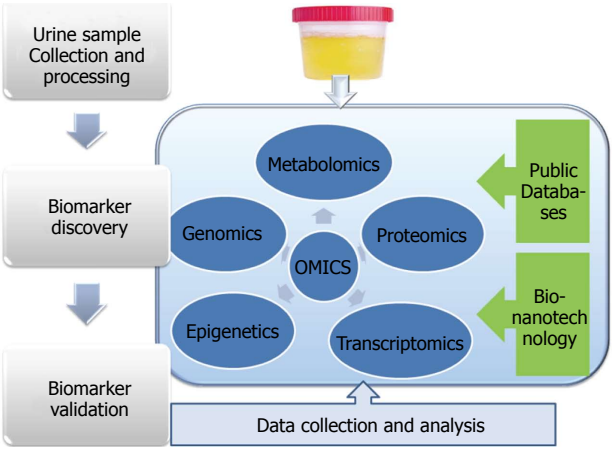

Figure 1 Integration of OMICS strategies for urinary bladder cancer biomarker discovery and validation.

IDENTIFICATION OF WHOLE TUMOR CELLS BY RAMAN SPECTROSCOPY

Raman spectroscopy is a technique based on excitation of vibrational models in the chemical bonds that hold molecules together[90]. Thus, it provides a measure of biologically active molecular groups[91,92]. Many authors applied Raman spectroscopy successfully in discriminating tumor cells from normal cells. Beside accuracy and non invasiveness, Raman spectroscopy is a fast and promising tool for BC screening in high risk populations[93,94]. Shapiro *et al*[95] used a Falcon Raman microscope to diagnose BC from epithelial cells found in urine of 344 patients (116 patients without urothelial cancer, 92 patients with low grade tumors and 132 patients with high grade tumors). They concluded that Raman molecular imaging is a powerful technique for BC diagnosis, with 92% sensitivity and 91% specificity in agreement with many recent studies[96].

Finally, although many policies and guidelines have been developed to evaluate potential BC biomarkers, no proper validation has been achieved until now, except for a few biomarkers[97]. For clinical application, any biomarker should be validated in a large number of samples with different ethnic origin and in different institutes, followed by approval from the FDA[98]. Public and private resources should offer financial support. Collaboration among researchers in universities, clinicians and industrial participants should be encouraged to bring biomarkers from the bench to the clinic[99,100].

CONCLUSION

BC remains an expensive cancer due to life-long surveillance involving upper tract imaging, urinary cytology and cystoscopy. However, as combined cystoscopy with cy-

Table 1 Urinary bladder cancer biomarkers

Biomarker/signature	Technology used	Ref.
MCP-1 A2518G, SDF-1 3'A and chemokine receptors CCR2A V64I, CCR5 Δ32, CCR5 59029 and CXCR4	PCR-restriction fragment length polymorphism	[21]
TP53	PCR-single strand conformational polymorphism analysis, DNA sequencing and immunohistochemical analysis	[22]
H-ras gene mutations	COLD-PCR	[23]
RARβ(2) and *APC* promoter methylation	Methylation specific PCR	[27]
SCGB3A1, *BNIP3*, *ID4* and *RUNX3*	Multiplex ligation-dependent probe amplification	[28]
TBX2, *TBX3*, *GATA2* and *ZIC4*	Genome-wide methylation analysis	[29]
BCL2, *CDKN2A* and *NID2* genes methylation	Nested methylation specific polymerase chain	[30]
miR-96 and miR-183	Q-PCR	[33]
miR-618, miR-1255b-5p	RT-qPCR	[36]
RNA ratio of v-ets erythroblastosis virus E26 oncogene homolog 2 (avian; ETS2) to urokinase plasminogen activator (uPA)	Reverse transcription quantitative-PCR	[41]
HYAL1 and survivin RNA	Qualitative and semiquantitative reverse transcriptase-polymerase chain reaction	[42]
FN, RTA, and CK20	Detection of CK20 by conventional RT-PCR, estimation of fibronectin by ELISA and relative telomerase activity by TRAP	[46]
CXCR4 and CXCR7	qPCR and/or immunohistochemistry	[52]
Bradeion/SEPT4 transcript	Real-time reverse transcriptase-polymerase chain reaction	[53]
hTERT, SENP1, PPP1CA, and MCM5 mRNA	q-RT-PCR	[54]
HOX-A13, IGBP-5, MDK, and CDC-2	cDNA microarray, Q-PCR	[55,56]
Afamin, adiponectin, complement C4 gamma chain, apolipoprotein A-II precursor, ceruloplasmin and prothrombin	iTRAQ	[61]
IL-8, MMP-9, MMP-10, PAI-1, VEGF, ANG, CA9 and APOE	ELISA assay	[62]
IL-8, MMP-9, MMP-10, SDC1, CCL18, PAI-1, CD44, VEGF, ANG, CA9, A1AT, OPN, PTX3 and APOE	ELISA assay	[63]
GC	Two-dimensional fluorescent differential gel electrophoresis and MALDI-TOF/TOF MS	[64]
beta-2-microglobulin, fatty acid-binding protein adipocyte, gelsolin, isoform 1 of gelsolin, myoglobin, isoform 2 of fibrinogen alpha chain, apoA-I, prostaglandin D(2) synthase 21 kDa, protein AMBP, transthyretin, keratin type II cytoskeletal 1, type II cytoskeletal 8, putative uncharacterized protein ALB, putative uncharacterized protein MASP2 (fragment)	2-dimensional electrophoresis combined with MALDI-TOF/TOF MS and SWISS-PROT database	[65]
MMP9, fibrinogen forms, and clusterin, aminopeptidase N, profilin 1 and myeloblastin	1D-SDS-PAGE followed by band excision and liquid chromatography tandem MS	[66]
Fibrinogen α chain precursor, apolipoprotein E, α-1-antitrypsin, and leucine-rich α-2-glycoprotein 1	MS and western blot/dot blot	[67]
Carnitine C9:1 and component I	Liquid chromatography-mass spectrometry based method	[72]
Kynurenine	Two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC-TOFMS)	[73]
HURP RNA	Conventional RT-PCR and AuNP nanoassay	[89]

MCP-1: Monocyte chemoattractant protein-1; SDF-1: Stromal cell derived factor 1; COLD-PCR: Co-amplification at lower denaturation temperature-polymerase chain reaction; ZIC4: Zic family member 4; Q-PCR: Quantitative real time PCR; FN: Fibronectin; TBX2: T-box 2; CXCR4: C-X-C chemokine receptor 4; RTA: Relative telomerase activity; CK20: Cytokeratin 20; GATA2: GATA binding protein 2; HYAL1: Hyaluronidase; TRAP: Telomeric repeat amplification protocol; iTRAQ: Isobaric tagging absolute and relative quantitation; CXCR4: C-X-C chemokine receptor 4; GC: Gc-globulin; MALDI-TOF/TOF MS: Matrix-assisted laser desorption time-of-flight mass spectrometry; MS: Mass spectrometry.

tology is considered the corner stone for BC diagnosis, it is necessary to search for an economical and efficient method to replace these deficient traditional methods. Many of the urinary markers currently available appear to be alternatives to cytology with a lower price and higher sensitivity, especially in detecting low-grade, non-muscle invasive cancers. Modern technologies, including mass spectroscopy, liquid chromatography, next generation sequencing, gene-expression profiling, metabolic profiling, nanoassays and epigenetic markers, are promoting more and more biomarker discoveries each month (Table 1, Figure 1). Finally, these versatile and newer strategies should be integrated to trace which markers may be clinically efficient and refinement of these markers which will help the urologist in critical evaluation of BC. Consequently, further and in-depth studies are required to determine the accuracy and widespread applicability of these modalities in guiding urinary markers discovery in BC.

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