MULTIPLEX URINARY TESTS FOR BLADDER CANCER DIAGNOSIS

Virginia Urquidi,[1] Charles J. Rosser,[2] Steve Goodison[3]

1. Associate Professor, Cancer Research Institute, MD Anderson Cancer Center, University of Central Florida College of Medicine, USA
2. Professor, Department of Urology, University of Central Florida College of Medicine, USA
3. Professor, Cancer Research Institute, MD Anderson Cancer Center, University of Central Florida College of Medicine, USA

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ABSTRACT

The development of accurate and reliable molecular assays that could diagnose bladder cancer would be of significant benefit to both patients and the healthcare system. Non-invasive assays that have utility not only for diagnosis, but also for monitoring disease recurrence and response to treatment, are needed. Current urinary tests lack sufficient sensitivity or specificity, often because of a reliance on single biomarkers, but high-throughput technologies are enabling the derivation of more accurate panels of biomarkers. In this article, we review some of the promising investigational studies that are revealing multiplex biomarker signatures that may augment current bladder cancer detection strategies.

Keywords: bladder cancer, biomarkers, non-invasive, urinalysis, diagnosis.

INTRODUCTION

Bladder cancer (BCa) is one of the most prevalent cancers worldwide and there are over 70,000 new cases of BCa each year in the United States alone.[1] If detected early, the five-year survival rate for BCa is >90%, thus timely intervention can dramatically increase the probability of patient survival. Radical surgery is required for muscle invasive lesions, but more prevalent non-muscle invasive BCas can be treated through transurethral resection of the tumour. Unfortunately, more than 70% of patients with non-muscle invasive BCa will have disease recurrence within two years of treatment. Thus, extensive long-term surveillance and repeated surgical intervention are needed to prevent progression of early-stage tumours to the more lethal invasive disease.

The gold standard for BCa diagnosis remains cystoscopic examination of the bladder coupled with voided urine cytology (VUC), the cytologic examination of cellular material present in the urine.[2-4] Cystoscopy is an uncomfortable and costly invasive procedure, which may require anesthetisation of the patient. Evaluation by VUC relies on the microscopic visualisation of shed cancer cells in voided urine. The technique performs well with high-grade and high-stage tumours (T2-T4), but the sensitivity for detecting low-stage tumours is low, ranging from only 20% to 40%.[4-6] The development of accurate and reliable urinary assays that can reduce the need for cystoscopy would be of tremendous benefit to both patients and the healthcare system.

A number of commercial molecular tests have been FDA-approved for specific scenarios. These tests include the measurement of soluble proteins such as: bladder tumour antigen (BTA), nuclear matrix protein 22 (NMP22), proteins detected on fixed urothelial cells (ImmunoCyt™), and chromosomal aberrations detected by fluorescent in situ hybridisation (UroVysion™). Unfortunately, to date, none of these tests have achieved combined sensitivity and specificity values to replace the established cystoscopy and VUC clinical evaluations. This may be due to the reliance of these tests on monitoring single biomarkers. No one biomarker is going to achieve accuracy across the breadth of clinical presentation seen at the urology clinic as not all BCas will harbour any single molecular change.[7] This is supported by the finding that when these tests are combined in one cohort, improvement over single tests is observed,[8-10] however, proprietary issues mean that such combinations are not currently feasible. What is needed are multiplex biomarker assays that can be developed into risk scores and nomograms such that an assay can be applicable over a broad range of disease states. Below, we describe some of the advances in multiplex biomarker discovery for the potential non-invasive diagnosis and monitoring of BCa. In this short review, we focus on

protein and RNA-based studies. Numerous studies that describe alterations in DNA sequence, DNA methylation, and metabolomic signatures associated with BCa have been described elsewhere.[11]

BIOMARKER SIGNATURES FOR NON-INVASIVE BCa DETECTION

Protein Biomarkers

The appropriate use of advanced proteomics technologies has the potential to provide highly efficient biomarkers for BCa detection and monitoring. Capillary electrophoresis-mass spectrometry (CE-MS) was used by Theodorescu et al.[12] to identify urinary biomarkers for BCa in a training set composed of 46 patients with urothelial carcinoma and 33 healthy volunteers. These biomarkers were further refined using CE-MS spectra of another cohort of urine samples from healthy volunteers and patients with malignant and non-malignant genitourinary diseases. A diagnostic biomarker signature of 22 urinary peptides was established using this two-step approach. In a validation study, this signature enabled the correct classification of all urothelial carcinoma patients in a test set containing 31 urothelial carcinoma patients and 138 non-malignant genitourinary disease patients.[12] Another study used an iTRAQ (isobaric tag for relative and absolute quantitation) technique to discover proteins that were differentially expressed between pooled urine samples and non-tumour controls. This strategy identified 55 candidate biomarker proteins.

Conventional techniques confirmed that the level of apolipoprotein A-I (APOA1) was significantly elevated in urine samples from BCa patients.[13] In our own studies, we used a glycoprotein enrichment strategy to profile urine samples from 100 subjects.[14] Combining specific glycoproteins with targets identified in our genomic studies (described below) we subsequently investigated the accuracy of various combinations of protein biomarkers for diagnostic urinalysis in a series of ELISA studies.[15-17] Multivariate analysis identified an 8-protein biomarker panel that achieved 92% sensitivity and 97% specificity in an independent cohort of 64 patients with BCa and 63 controls.[18] The performance of these biomarker panels was far better than current urinalysis tests in the same cohort. Validation of these multiplex biomarker panels in larger, more diverse cohorts is underway. The studies described above show the power of MS-based urinary analysis for the discovery of potential biomarkers. Continuing proteomic technological developments, such as assays for phosphoproteins, glycoproteins or phospho-lipoproteins can achieve reduction of sample complexity for further proteomic analysis of biological fluids, so additional panels of proteins that can be developed into accurate and simple urinalysis assays are likely to be derived in the future.

RNA Markers

Given the advances in RNA/DNA sequencing and hybridisation platforms, one of the most promising sources for the derivation of multiplex diagnostic biomarker signatures is the tumour cell transcriptome. The majority of BCa gene expression profiling studies focused on the analysis of excised solid tumour tissue. These studies have identified gene signatures that are associated with tumour stage,[19-20] disease recurrence and outcome prediction,[19-21] and are most applicable to the development of assays that will aid the histological evaluation of biopsy or excised tumour material. Normal tissue is not readily available for comparison for obvious reasons. Conversely, the analysis of gene expression in naturally shed urothelia (present in all voided urine samples) has several advantages, not least of which is the availability of samples from a range of disease conditions as well as healthy controls. Through polymerase chain reaction (PCR) amplification the analysis can be performed on the minimal cellular material obtained from naturally voided urine, and the detection methods are accurate, quantitative, and economical.

Holyoake et al.[22] used molecular profiling of solid tissues to identify genes over-expressed in tumour stages Ta, T1 or >T1, relative to non-tumour epithelial tissues and inflammatory cells. Using this strategy, transcripts of four genes CDC2, MDK, IGFBP5, and HOXA13 were selected for development of a quantitative RT-PCR urine assay for BCa detection and disease risk stratification of patients. The measurement of the combination of mRNA markers detected BCa at a sensitivity of 85% and a specificity of 80% across all stages, with the best performance with stages >T1 and tumours >1cm in diameter.[22] A recent study by the same investigators compared the performance of assays derived from this biomarker panel in a cohort of 485 patients. The test achieved higher sensitivity (62%) than NMP22 and cytology at a pre-specified 85% specificity, and a modification of the assay detected 82% of BCa cases.[23] Hanke et al.[24] analysed the expression of a selected panel of mRNAs as biomarkers of BCa in whole urine, cell pellets and clarified urine. In a cohort of 98 subjects, they found that the ratio of v-ets erythroblastosis virus E26 oncogene homolog 2 (ETS2) to urokinase plasminogen activator (PLAU) in whole urine facilitated the detection of BCa with a sensitivity of 75% at 100% specificity. Other mRNA-based diagnostic urinalyses have targeted BIRC5 (survivin), HYAL1, KRT20 and MUC7.[25-27] These targets performed similarly at sensitivities between 62-90%, and confirmed that combinations of two to three mRNA markers perform better than single target assays.

In our own studies we performed genome-wide mRNA profiles of urothelia obtained from over 90 urine samples. We reasoned that profiling the actual material that would be subject to diagnostic assay in the clinic would circumvent any confounding factors inherent to tissue profiling. The resulting profiles were analysed using advanced feature selection algorithms[28-30] to reveal an optimal gene signature for BCa association. A 14-gene signature model was derived and monitoring of this signature using quantitative RT-PCR was able to detect BCa with 100% specificity at 90% sensitivity in an independent cohort of 81 cases.[31,32] In comparison, cytological evaluation of this cohort diagnosed only 35% of tumour cases correctly. In a study utilising a similar strategy, a panel of 384 genes identified in tissue-based analyses were subsequently tested in urothelial samples using quantitative RT-PCR.[33] These analyses identified a 12-gene signature that achieved high accuracy (89% sensitivity and 95% specificity) in identifying BCa cases in a cohort of 211 subjects. Despite significant differences between the studies, with respect to the biomarker discovery phase, both groups were able to derive molecular signatures that could accurately classify BCa samples. This demonstrates that a multiplex quantitative RT-PCR test on voided urine sample holds promise as a non-invasive urine-based assay in the evaluation of patients being investigated for BCa. Although a quantitative RT-PCR test has some upfront processing requirements, it has the advantage of being developed into an assay that can be automated and highly standardised for consistency between laboratory sites.

Another urothelial RNA source for potential biomarker discovery is the transcribed, non-protein coding microRNA (miRNAs) component. To date, over 1500 human miRNAs have been identified and characterised to some extent. Each miRNA controls the expression of multiple genes, and so this molecular family may represent an opportunity to identify biomarkers of a higher order. Assay-based profiling and deep-sequencing approaches for miRNA analysis are becoming routine, and studies targeting miRNAs as potential diagnostic biomarkers are increasing accordingly. Tumour tissue profiling studies have identified the expression of single miRNA transcripts as being associated with primary BCa or outcome, and some of the candidate biomarkers have been confirmed in urine samples.[34-39] More recent studies have derived signatures or panels of miRNA biomarkers with good diagnostic performance for urinalysis. Hanke et al.[40] examined the expression of 157 miRNAs in exfoliated urothelial cells using quantitative RT-PCR and reported that the ratio of miR-126 to miR-182 achieved 72% sensitivity and 82% specificity in a cohort of 47 samples. A quantitative PCR study of a panel of 15 miRNAs in 121 urine samples revealed that the combination of 3 miRNAs (135b/15b/1224-3p) detected BCa with high sensitivity (94.1%), but specificity was lower (51%).[41] The monitoring of miR-222 and miR-452 has been reported to be to helpful in tumour stratification and for non-invasive diagnosis,[38] and the expression of miR-96 and miR-183 have been shown to augment cytology and to correlate with advancing tumour grade and stage.[39]

CONCLUSION

The inadequate power of single biomarker assays means that the non-invasive detection of BCa remains a challenge. Advances in molecular techniques, especially profiling approaches, have enabled investigators to derive a new generation of compound molecular diagnostic signatures that may provide assays with the desired clinical utility. Such multiplex biomarker systems for BCa diagnosis are still at an early stage compared with the FDA-approved markers. Promising signatures and panels of markers have been derived and tested on varied cohorts, but require further validation in independent studies. The hope is they will provide informative robust tests across the broad range of clinical presentation. Once optimised, multiplex diagnostic assays may enter the clinical setting to augment, or eventually even replace, cystoscopy and/or cytology for diagnosis, disease recurrence monitoring, and the monitoring of response to treatment. A major advantage of multiple biomarker assays is the results can be input into algorithms to provide a continuous score for prediction of disease status or prognosis. Furthermore, algorithms that incorporate clinical data and molecular risk scores into a nomogram can give physicians the most valuable guidance regarding patient management.[42,43]

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