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Featured Review Article

Biomarkers for bladder cancer management: present and future

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Abstract: Accurate and sensitive detection of bladder cancer is critical to diagnose this deadly disease at an early stage, estimate prognosis, predict response to treatment, and monitor recurrence. In past years, laboratory diagnosis and surveillance of urinary bladder cancer have improved significantly. Although urine cytology remains the gold standard test, many new urinary biomarkers have been identified. Furthermore, recent advances in genomic studies of bladder cancer have helped to refine our understanding of the pathogenesis of the disease, the biological basis for outcome disparities, and to inform more efficient treatment and surveillance strategies. In this article, the established diagnostic tests, newly identified biomarkers and genomic landscape of bladder cancer will be reviewed.

Keywords: Bladder cancer, biomarkers, disparities

Introduction

Worldwide, an estimated 430,000 patients are diagnosed with bladder cancer annually, and more than 165,000 will succumb to the disease [1]. In the United States there were 72,570 new cases of bladder cancer and 15,210 deaths reported in 2013 [2]. There are estimated to be more than 560,000 people currently living with bladder cancer in the United States. In the past two decades the incidence and mortality of bladder cancer has remained relatively stable, with age-adjusted incidence rates at approximately 21 new cases and 4.4 deaths per 100,000 men and women per year [3]. Because of the growing number of elderly people, however, the number of incidence cases and deaths continues to increase. The rate of bladder cancer in men is about twice that of women, and increases sharply with age, with 9 out of 10 people diagnosed with the disease at an age ≥65. Whites are about twice as likely to develop bladder cancer as African Americans, Hispanics, or Asian/Pacific Islander patients. Yet, African Americans experience significantly worse five-year disease-specific survival when compared to other ethnicities, and such disparities persist even when stratified by age and stage and diagnosis [4].

The single most significant risk factor for bladder cancer is cigarette smoking which, according to the National Institutes of Health-AARP Diet and Health Study Cohort, increases bladder cancer risk by 3.89 and 4.65, in men and women, respectively [5]. Other established risk factors for bladder cancer include various industrial exposures used in a number of occupational settings, and are believed to account for up to 5% of all bladder cancer cases [6].

At initial presentation, about 75% of patients have non-muscle invasive (carcinoma in situ, Ta and T1) disease while 25% of patients have disease that invades the muscularis propria [7]. Non-muscle invasive tumors, are characterized by a high recurrence rate (50%-70%) within 5 years and a relatively low progression rate (approximately 15%), which results in relatively long survival [8]. For this reason, patients with non-muscle invasive bladder cancer are regularly monitored for tumor recurrence and progression with cystoscopy and urine cytology. Given the need for lifelong surveillance and

treatment of patients with non-muscle invasive disease, bladder cancer is the most expensive malignancy per patient to care for from diagnosis to death [9].

Tumors that do invade the muscularis propria require much more aggressive treatments and have a far worse prognosis. Standard treatment for patients with muscle-invasive tumors includes surgical removal of the bladder or concurrent chemotherapy and radiation. In a large series of patients treated with cystectomy, five-year recurrence-free survival rate was only 69% in all patients, and 39% in patients with regional lymph node metastasis [10].

While the TNM staging of bladder cancer guides both treatment and prognosis, there remains substantial heterogeneity among similarly staged patients, with respect to treatment response and overall outcomes. Therefore, there is a critical need for the identification of biomarkers to diagnose bladder cancer at an early stage, monitor recurrence, refine prognostic estimates, and predict response to treatment in patients with bladder cancer. Further, identification of such biomarkers are critical to refining our understanding of the pathogenesis of the disease, the biological basis for outcome disparities, and to informing more efficient treatment and surveillance strategies.

Biomarkers for bladder cancer diagnosis and surveillance

In patients with signs suspicious for bladder cancer (e.g., hematuria), direct cystoscopic visualization of the bladder is the gold standard diagnostic assessment. Newer technologies including optical coherence tomography and confocal laser endomicroscopy may improve the sensitivity and specificity of identifying bladder tumors while also providing pathologic information [11]. These methods, although having a high detection rate, are expensive, time-consuming, invasive and uncomfortable. Bladder cancer screening in the asymptomatic adults has not been widely implemented due to the fact that no screening test has been shown to lower the risk of dying from bladder cancer in people who are at average risk. In 2011, the United States Preventive Services Task Force (USPSTF) concluded that the evidence is insufficient to determine the balance of benefits and harms of screening for bladder cancer (e.g., with urinalysis for microscopic hematuria, urine cytology, or tests for urine biomarkers) in asymptomatic adults [12].

Histopathology

Histopathology remains the gold standard for bladder cancer diagnosis and is the most important prognostic factor to predict clinical behavior. In 2004, the World Health Organization revised the classification system, which divided bladder tumors into muscle-invasive urothelial carcinoma and non-muscle invasive urothelial neoplasia. This latter category includes urothelial carcinoma in situ, low and high grade non-muscle invasive papillary urothelial carcinoma, non-muscle invasive papillary urothelial neoplasm of low malignant potential, and urothelial papilloma [13]. Ninety percent of bladder cancer cases are classified as urothelial carcinomas (UC), while the remaining 10% are predominantly squamous cell carcinomas or adenocarcinomas. Protein markers employed as immunohistochemical staining can be helpful to differentiate benign changes from neoplastic processes, especially papillary urothelial neoplasms with low malignant potential (PUNLMP) and low grade Ta bladder cancer. For example, loss of cytoplasmic CD44 expression and increased cytokeratin 20 expression in deeper layers of the urothelium as well as diffused nuclear expression of p53 and high proliferative index (determined by Ki-67 immunostaining) are useful markers for diagnosing urothelial neoplasia [14]. Other protein markers such as Gata3, p63 and MIB-1 have also been used in this setting [15].

Cytology

Urine cytology is a non-invasive method for detecting bladder cancer by identifying abnormal urothelial cells in the voided urine or bladder washes. Urine cytology has high specificity but relatively low sensitivity, particularly in well-differentiated low grade bladder tumors (**Table 1**) [16]. For example, Turco *et al.* [17] evaluated the accuracy of urinary cytology for primary bladder cancer using available population. Cancer registry matching of 2,594 tests revealed 130 incident bladder cancers, of which 97 occurred within 12 months of cytology and were included in this study. Sensitivity for bladder cancer (including categories of atypical, suspicious and positive) ranged between

Table 1. Summary of urine biomarkers for bladder cancer diagnosis and surveillance

Test	Markers	Sensitivity*	Specificity*
Cytology	Urothelial cells	30-92%	93-97%
ImmunoCyt	Urothelial cell and immunostain	55-90%	33-87%
Urovysion	FISH	51-92%	55-95%
BTA stat (point-of-care) and BTA-TRAK	Bladder Tumor Antigen	36-91%	50-90%
BladderCheck (point-of-care) and Bladder Cancer Test	NMP22	34-95%	55-85%
ACCU-DX (point-of-care)	FDP	60-83%	80-86%

*Vary between low grade and high grade urothelial carcinoma.

40.2-42.3%, and specificity was 93.7-94.1%. The positive predictive value of the categories of atypical, suspicious or positive were 11.7, 39.2, and 66.6%, respectively. High tumor grade was associated with significantly higher sensitivity compared to low and intermediate grades combined (p=0.02).

Cytology combined with immunostain

In order to improve diagnostic sensitivity of urine cytology, immunostaining of urothelial cells was developed to be applied to urine specimens (**Table 1**). ImmunoCyt (DiagnoCure, Inc., Quebec, Canada) is a multiplex immunocytofluorescence bladder cancer detection assay that combines fluorescently labeled monoclonal antibodies for M344, LDQ10, 19A211, and a glycosylated form of the carcinoembryonic antigen (CEA). A minimum evaluation of 500 epithelial cells is required, and presence of one fluorescent cell is considered as positive. In a large validation study, Comploj *et al.* [18] evaluated 7,422 samples from 2,217 patients and reported a sensitivity of 34.5% for cytology alone, 68.1% for ImmunoCyt, and 72.8% for the two tests combined. Specificity was found to be 97.9% for cytology, 72.3% for ImmunoCyt, and 71.9% for the combination. Cytology and the ImmunoCyt test together had an overall sensitivity of 72.8%, with 59% for grade 1, 77% for grade 2, and 90% for grade 3 tumors (according to the previous 1973 WHO grading classification system). However, the presence of microhematuria, cystitis and benign prostatic hyperplasia can lead to false-positive results.

Fluorescent in situ hybridization

Chromosomal alterations are quite common in bladder cancer which can be used for detection via fluorescent in situ hybridization (FISH) assays (**Table 1**). The UroVysion test (Abbott Molecular, Inc., Des Plaines, IL, USA) is a 4-color FISH assay designed to detect aneuploidy of chromosome 3, 7 and 17, as well as loss of the 9p21 locus, using urine specimens from patients with hematuria. The criteria for detecting bladder cancer by UroVysion are: >4 urothelial cells with a gain of >2 chromosome 3, 7, or 17 or >12 cells with loss of the 9p21 locus. In addition, >10 urothelial cells showing gain for a single chromosome 3, 7, or 17; or >10 cells with tetrasomy or near tetrasomy for chromosomes 3, 7 and 17 are also considered abnormal [19, 20]. Dimashkieh *et al.* [21] reviewed 1,835 urine specimens from which 1,045 were from patients undergoing surveillance for recurrent urothelial carcinoma and 790 were included for hematuria. The overall sensitivity, specificity, positive predictive value, and negative predictive value in detecting urothelial carcinoma were 61.9%, 89.7%, 53.9%, and 92.4%, respectively for FISH and 29.1%, 96.9%, 64.4%, and 87.5%, respectively for cytology. The performance of both FISH and cytology was superior in the surveillance population and in samples with high-grade UC. Furthermore, the FISH assay is especially helpful in identifying carcinoma in situ and occult tumors not initially visible on cystoscopy. In patients with history of bladder cancer, a positive FISH results with a negative cystoscopy still predicts a significantly decreased time to recurrence over those patients with a negative FISH and a negative cystoscopy.

Urine protein markers

Many soluble protein markers in voided urine have been explored for bladder cancer diagnosis and screening. These markers include blood group antigens, tumor associated antigens, proliferating antigens, oncogenes, peptide growth factors and their receptors, cell adhesion molecules, tumor angiogenesis and angio-

genesis inhibitors, and cell cycle regulator proteins [22]. Several of these markers, including bladder tumor antigen (BTA-stat, BTA-TRAK), nuclear matrix protein-22 (BladderCheck and Bladder Cancer Test) and fibrinogen degradation products (ACCU-DX), have been approved by the FDA for clinical use (**Table 1**). NMP22 is a structural component of the nucleus that determines nuclear morphology, organizes DNA 3-dimensionally and is implicated in replication and gene expression. Fibronectin is an extracellular matrix component widely distributed on cells and involved in the mechanism of human bladder cancer cell invasion. The bladder tumor antigen (BTA) stat test (Polymedco Inc., NY, USA) is a qualitative, point-of-care test capable of detecting human complement factor H-related protein, known to be produced by several human bladder cell lines but not by other epithelial cell lines. Cytokeratins 8 and 18 are frequently overexpressed in tumor cells and excreted as fragmented urinary proteins. Such proteins can be readily detected in urine by immunoassay and can be used as an aid to the diagnosis and monitoring of bladder cancer patients in conjunction with cystoscopy. Eissa *et al.* [23] measured NMP22, fibronectin, and BTA in voided urine in 168 patients and 47 healthy donors. Overall sensitivity and specificity were 85.0% and 91.3% for NMP22, 83.0% and 82.6% for fibronectin, 67.0% and 80.8% for BTA, and 44.0% and 100% for voided urine cytology. The combined use of all 3 markers increased the sensitivity of cytology from 44.0% to 95.3%. NMP22 may be also useful to improve detection of low grade and non-muscle invasive tumors [24]. The sensitivity for low grade tumors was higher (83.9%) than for high grade tumors (62.5%). Sensitivity of NMP22 for non-muscle invasive vs. muscle invasive disease was also higher (81.8% vs. 57.1%, respectively). However, these tests have high false positivity rates due to presence of inflammatory cells and other contaminated cells.

The ultimate goals of biomarkers for bladder cancer diagnosis and surveillance would be to provide sufficient negative predictive value to allow patients to forgo more invasive tests such as cystoscopy or to risk stratify patients with indolent versus aggressive disease. Currently clinically available tests such as described above have their limitations including complexity, cost, sensitivity and specificity. Therefore, identification of better biomarkers to improve the diagnostic sensitivity and specificity is a top priority.

Genomic landscape and biomarker discovery

The cancer genomics era is developing rapidly, fueled by the emergence of many advanced technologies, including array CGH, DNA microarray, next-generation sequencing, etc. The completion of the cancer genomic landscape not only helps our understanding of the mechanistic basis underpinning particular disease subtypes but also provides opportunities for discovery of new biomarkers for diagnosis, prognosis, and prediction of response. Furthermore, such work facilitates the identification of novel therapeutic targets.

Somatic mutation

Prior research has shown that different genetic defects generally underlie the two pathways of urothelial tumorigenesis [25]. Low-grade papillary non-muscle invasive tumors are generally characterized by constitutive activation of the receptor tyrosine kinase-Ras pathway, such as activating mutations in the HRAS and fibroblast growth factor receptor 3 (FGFR3) genes. In contrast, high-grade muscle-invasive tumors are characterized by alterations in the tumor suppressor protein p53 (TP53) and retinoblastoma 1 (RB1) pathways. While these initial studies served as a critical foundation for understanding the mechanisms underpinning the different clinical phenotypes of urothelial cancer, the era of high throughput genomics platforms have revealed that urothelial cancer is much more complex and heterogeneous than appreciated with the prior somewhat “simplistic” genomic subsets.

Two recent studies reported genome-wide analysis of bladder cancer by both whole-genome and whole-exome sequencing. One consisted of 99 urothelial tumors from an Asian population [26]. The other consisted of 131 chemotherapy-naive, muscle-invasive, high grade urothelial tumors as part of The Cancer Genome Atlas (TCGA) bladder cancer project [27]. The two studies identified 37 and 32 significantly mutated genes, respectively. Despite obvious differences between the two studies (e.g., the former contains the low grade non-muscle invasive tumors while the latter does not) the two

reported gene lists share a lot in common. For example, among the 19 significantly mutated genes with mutation frequency >5% in the TCGA dataset, 12 are also identified as significantly mutated by the Asian study. Many of them have not previously been reported as statistically significantly mutated in bladder cancer. Pathway analysis showed that somatic mutations are enriched in p53/Rb pathway, RTK/RAS/PI(3)K pathway and histone modification genes. Several of these alterations, particularly those involving the PI(3)K/AKT/mTOR, CDKN2A/CDK4/CCND1 and RTK/RAS pathways, are amenable in principle to therapeutic targeting. The frequent mutations of chromatin remodeling genes, such as ARID1A and EP300, were confirmed by both studies, and were also reported by another independent study of smaller scale from one of the above two groups [28]. The nature of the mutations in these chromatin modification genes indicates many of them are tumor suppressors. The observation that 76% of the tumors analyzed by TCGA had an inactivating mutation in one or more of the chromatin regulatory genes, suggests new possibilities for bladder cancer treatment. Besides these well-known cancer relevant pathways, new genes and pathways were also identified. For example, the Guo 2013 study [26] also revealed frequent genomic alterations in genes involved in the process of sister chromatid cohesion and segregation (SCCS), including STAG2, ESPL1 and NIPBL. Future studies are necessary to elucidate their exact roles and their potential as therapeutic targets.

Copy number variation

Genome-wide analysis of copy number aberration and LOH using array-based comparative genomic hybridization (CGH) or single-nucleotide polymorphism array profiling have revealed regions of alteration. In general, fewer copy number alterations are found in low stage and low grade tumors, and more complex patterns are characteristic of muscle-invasive tumors [29]. Copy number alterations (CNA) have been associated with stage, grade, recurrence, and overall survival.

Both of the two aforementioned studies [26, 27] used GISTIC (genomic identification of significant targets in cancer) to identify recurrent focal somatic CNAs. Guo et al identified 84 regions of focal amplification and 80 regions of focal deletion. The TCGA identified 27 amplified and 30 deleted recurrent focal CNAs. The amplified focal regions detected by both studies encompassed genes such as E2F3, CCND1, MDM2, ERBB2, CCNE1, MYC and FGFR3, most of which have previously reported to be altered in bladder cancer. The deleted regions contain genes such as CDKN2A, RB1 and CREBBP. The most common recurrent focal deletion contained CDKN2A, seen in 50% and 47% samples in the two studies, respectively. In the TCGA bladder cancer study, patients were grouped into 3 clusters based on their somatic mutation and focal CNAs: cluster A was enriched in focal somatic CNAs in several genes and mutations in MLL2; cluster B was characterized by deletion of CDKN2A region and mutation in FGFR3 and papillary morphology; cluster C showed TP53 mutations as well as enrichment with RB1 mutations and amplifications of E2F3 and CCNE1.

mRNA expression

Multiple groups have produced urothelial cancer gene signatures predicting a range of tumor characteristics and outcomes, including stage, molecular subtype, likelihood of recurrence and progression and survival [30]. Markus et al [31] pooled microarray data from 93 patients with similar data from 6 independent published studies, for a total of 578 bladder cancer cases. Comparing non-muscle invasive to muscle-invasive samples, they found significant overlap among the 7 independent studies. They found that fibronectin 1 (FN1) and other members of the integrin signaling pathways were upregulated in muscle-invasive tumors, while members of the TGF-beta signaling pathway were overexpressed in non-muscle invasive tumors, such as SMAD3, SMAD6 and BMP7. However, when comparing the multiple published survival signatures, they observed overfitting in that most signatures achieved significance only in the datasets used to identify them. They then derived a new gene expression signature using machine learning algorithms over the assembled dataset, and showed that this signature combined with a clinical nomogram, can improve the prediction of survival in patients with muscle-invasive bladder cancer treated with cystectomy.

Sjodahl et al [32] applied unsupervised clustering to gene expression profiles from 308 blad-

der tumors (the largest single mRNA expression dataset so far), and defined five major urothelial carcinoma molecular subtypes: urobasal A, genomically unstable, urobasal B, squamous cell carcinoma-like and an infiltrated class of tumors. The five molecular subtypes differ with respect to expression of immune genes, cell-cycle genes, receptor tyrosine kinases, cytokeratin signature genes and cell adhesion genes, as well as mutation frequency of FGFR3, PIK3CA and TP53. They show distinct survival outcomes, with the subtypes of urobasal B and squamous cell carcinoma-like having the worse prognosis. They also showed that the molecular subtypes are independent of pathologic classification. Even though Ta tumors are dominated by the urobasal A subtype, T1 tumors are composed of urobasal A and genomically unstable cases, and the muscle-invasive tumors may be of any subtype. Low grade tumors are predominantly of the urobasal A subtype, whereas high grade tumors can be of any subtype. Their molecular subtypes are consistent in general with the subtypes defined by mRNA gene expression data in the TCGA bladder cancer study.

Volkmer et al [33] classified bladder cancer into three subtypes, on the basis of their differentiation states: basal, intermediate and differentiated. They used a biologically supervised computational approach to define molecular markers of cellular differentiation. They found that keratin 14 (KRT14) designates the most primitive differentiation, and is consistently associated with worse prognosis. Notably, the basal subtype bears similarity to the squamous cell carcinoma-like subtype in Sjodahl 2012 study since KRT14 was overexpressed in both subtypes. In fact, KRT 14 was also overexpressed in one of the four clusters reported by (TCGA 2014) based on RNA-seq data. In addition, the TCGA study reported the signature of this cluster ("basal/squamous-like") is similar to that of basal-like breast cancers, as well as squamous cell cancers of the head and neck and lung. These findings are reminiscent of another result from the study by Markus et al [31]: when they investigated 49 previously published signatures from multiple cancer types, a breast cancer progression signature showed the highest association with overall survival in the MS2 group of their bladder cancer patient cohort. Therefore, there are some consistent discoveries among these recent mRNA expression studies in bladder cancer. Understanding the functional and phenotypic properties of bladder cancer stem cells may be necessary to better understand and harmonize these datasets [34].

microRNA

A growing body of evidence suggests that microRNAs (miRNAs) contribute to bladder cancer development, progression and metastasis. Genome-wide miRNA expression signatures have been used to rapidly and precisely identify aberrant miRNA expression in bladder cancer. Examination of the differential expression of miRNAs between bladder cancer and normal bladder tissue has led to the elucidation of 11 bladder-cancer-specific miRNA expression signature sets [35]. Among the 11 signature sets, the authors reported 15 miRNAs were down-regulated and 7 were miRNAs up-regulated in bladder cancer that have been isolated in three or more expression studies. They also reported 15 miRNAs associated with bladder cancer diagnosis and prognosis. In a parallel review of the current scientific reports that link differences in miRNA expression with the pathogenesis of bladder cancer [36], the authors created the first comprehensive database of miRNA with biased expression profiles in bladder cancer. They identified in total 95 differentially expressed miRNAs, 48 up-regulated in bladder cancer, 35 down-regulated, and 12 contradictory. The molecular targets of these miRNAs have been shown to be involved in crucial cell mechanisms, such as apoptosis, cell cycle progression and epithelial-mesenchymal transition (EMT). A few of these microRNAs and their target mRNAs have been utilized in the TCGA study to cluster bladder cancer patients. For instance, one cluster shows significantly lower expression of miR-99a and miR-100, and up-regulation of their target gene FGFR3. Similarly, two clusters show lower expression of members of the miR-200 family of miRNAs (which target multiple regulators of EMT) and consistent down-regulation of the epithelial marker E-cadherin.

There have been extensive efforts in the past decade to identify genetic susceptibility loci for bladder cancer. Recent cancer association studies by candidate gene and genome-wide association study (GWAS) approaches identified at least ten low-penetrance genetic sus-

ceptibility loci for bladder cancer [37]. The ten validated genetic loci include NAT2, GSTM1, 8q24.21 (MYC), 3q28 (TP63), 8q24.3 (PSCA), 5p15.33 (CLPTM1L-TERT), 4p16.3 (TACC3-FGFR3), 22q13.1 (APOBEC3A-CBX6), 19q12 (CCNE1) and 2q37.1 (UGT1A). A recent meta-study of GWAS also identified four more loci that achieved or approached genome-wide statistical significance, but require further studies for confirmation [38].

There have been numerous candidate gene studies reporting positive associations between SNPs and bladder cancer recurrence, progression, and survival. A recent review [39] has summarized them into several carcinogenesis-related processes, including cell cycle and apoptosis (TP53, MDM2, CDKN2A), DNA repair (ERCC6, XPD, XPG, XPF), growth factor signaling (EGFR, TGFBR1), PI3K-AKT (AKT2, PIK3R1, RAPTOR), stem cell signaling (GLI2, SHH, GLI3), inflammation (PPARG, IL-6, NF-kB1), cell adhesion (CDH1) and oxidative stress (HIF1A). However, most of the candidate gene/pathway studies were of limited sample size and had not been validated in independent populations.

Racial disparity in genomic studies

Bladder cancer is the sixth most common cancer and as previously mentioned, it occurs more often in men than in women. Regarding ethnicity, in men, bladder cancer incidence rate in Whites is 39.8 per 100,000 persons about twice comparing to the rates of African Americans and Asians (21.0 and 16.1 per 100,000 persons, respectively). In women, the incidence rate in Whites is 9.5 per 100,000 persons, close to 7.1 per 100,000 persons in African Americans, both much higher than the 3.9 per 100,000 persons in Asians. However, in terms of death rate due to bladder cancer, the difference between White and African American men is smaller, 8.1 versus 5.5 per 100,000 persons. In women, the death rate in African Americans is higher than in Whites, 2.6 versus 2.2 per 100,000 persons, respectively. The worse survival of African American patients compared to Whites can be explained by differences in the pathology of the disease, and in access to effective surveillance and care. There are large genomic studies of bladder cancer focusing on Asian (99 patients in Guo et al, 2013) and White (308 in Sjodahl et al, 2010) populations. However, there are no genomic studies focusing on bladder cancer in African American patients. In the recent TCGA bladder cancer study, there are only 9 samples from African American patients versus >100 samples pertaining to White patients. As discussed above, the genomic alteration pattern of bladder cancer is complex and African American bladder cancer patients are more likely to die due to bladder cancer; thus, there is an urgent need for a systemic genomic study of bladder cancer in African American patients to determine the genomic landscape of the disease and better delineate whether there is a biologic basis for the outcome disparities.

In summary, genomic changes in bladder cancer are complex. There are 4 or 5 molecular subtypes of bladder cancer and different histological types of bladder cancers consist of different proportions of these molecular subtypes. A proper biomarker study should consider heterogeneity of molecular subtypes composition, and may be better to study each molecular subtype separately. With these new insights revealed from recent genomic studies and potential target therapies matching to each molecular subtype, overall survival and treatment outcome is expected to improve in the coming years.

Potential biomarkers for diagnosis, prognosis and prediction

Diagnostic biomarkers

Many investigational tests have be reported in past years. Several of them have showed great potential for clinical applications. However, further studies are needed to demonstrate their diagnostic feasibilities, sensitivities and specificities. Telomerase is a reverse transcriptase responsible for adding tandem repeat sequences (TTAGGG) at the end of the chromosome (telomere). Telomerase activity is increased in many cancers, including bladder cancer, therefore, it can be used as a cancer biomarker. Telomerase consists of three subunits: RNA component (hTR), telomerase-associated protein (TP1), and telomerase reverse transcriptase (hTERT). Telomerase activity can be assessed by a polymerase chain reaction (PCR)-based assay utilizing the telomeric repeat amplification protocol (TRAP). In a prospective case-control series of 218 men, the sensitivity of telomerase with the TRAP assay

was 90% (95% CI, 83-94%) and the specificity was 88% (95% CI, 79-93%). The specificity was increased to 85% for individuals 75 years or younger [40]. However, due to lack of standardization, the clinical application of this biomarker has been limited.

The hTERT gene, located on chromosome 5, consists of 16 exons and 15 introns. The core promoter of hTERT including 330 base pairs upstream of the translation start site (ATG) is GC-rich and contains many sites for transcription factors including oncoproteins such as c-Myc, HIF-1, AP2, estrogen receptor and tumor suppressors such as p53, WT1, and Menin [41]. Two highly recurrent and mutually exclusive somatic mutations were found in melanoma and bladder cancer at two residues at -124 (1295228) and -146 (1295250) bp from the ATG start site in the TERT promoter [42]. The C>T (G>A) transition at both sites also resulted in creation of the Ets/TCF binding motifs. In a recent report, Rachakonda *et al.* studied 327 patients with urothelial cell carcinoma of the bladder and found that these somatic mutations occurred in 65.4% of the bladder cancer [43]. Furthermore, these mutations influence both survival and disease recurrence in bladder cancer patients.

Prognostic biomarkers

Although in recent years several markers associated with genetic alterations have been identified both in non-muscle invasive and muscle-invasive bladder cancer, currently there are no validated prognostic molecular markers to guide clinical management of patients affected with this disease [30].

The tumor suppressor gene TP53 is one of the most frequently mutated in bladder cancer. It has been reported to be mutated in 50% of invasive bladder cancer [44] and it confers a significantly decrease survival in those patients [45]. Since mutated TP53 is characterized with a longer half-life than wild-type, immunohistochemistry expression of p53 strongly correlates with gene mutation [46]. Nuclear p53 expression is associated with high grade and high stage bladder cancer, and although it has been shown as an independent prognostic biomarker in several studies mainly centered in non-muscle invasive tumors [47, 48], these results have been disputed by other groups [49, 50]. Mutations of TP53 are significantly associated with RB mutations [51], which have been implicated in progression of high grade muscle-invasive bladder cancer [52]. TP63 is a member of the TP53 family with at least six different isoforms which has also found to have prognostic implications in bladder cancer [53]. Importantly, DeltaNp63 expression has been shown to be associated with a poor prognosis in invasive tumors [54]. Loss of PTEN with the consequent PI3K kinase pathway activation has also been shown in invasive bladder cancer [55], but the clinical significance of this alteration is controversial [56]. Other tumor suppressors genes such as FHIT (Fragile histidine Triad gene) and FEZ1/LZTS1 (Leucine zipper putative tumor suppressor 1) have been also reported to be lost in bladder cancer mainly in high grade tumors and patients whose cancers show this loss present with a poorer prognosis [57-59].

In the past decade, mutation and immunohistochemical expression of several growth factors and receptors such as FGFR3, EGFR, HER2, FGFR1 and FGF2 have been widely studied in bladder cancer as possible prognostic biomarkers. Since their importance resides in the fact that they may be actionable, these factors will be discussed in more detail in the "predictive markers" section below. Mutation rates of H-RAS in bladder cancer varies from 30% to 50% in non-muscle invasive and muscle invasive tumors, respectively. In non-muscle invasive bladder cancer mutations of H-RAS and FGFR3 seem to be mutually exclusive [60] but its clinical significance remains unknown. TSC1 has been shown to be mutated in bladder cancer, more commonly in non-muscle invasive tumors [61] and its prognostic implications are still unclear [62].

Cell cycle regulators such as p21, p16, p15, p27, Cyclin D1 and Cyclin D3 are also important in bladder cancer prognosis, both in non-muscle invasive and muscle-invasive disease, but their clinical prognostic implications have not been widely validated yet. For example, p21 loss is an independent marker of poor survival in muscle invasive tumors [63] and used in combination with p53, p27 and pRB expression it has been shown to be an independent factor for recurrence and progression in non-muscle invasive tumors [64].

In summary, although considerable effort has been put in the last years to find new prognostic biomarkers for bladder cancer (many of

them have not been discussed in this review because they correspond to genes or proteins described in a small series of cases with no further validation), none has been moved to the clinical setting due to their inability to improve results when compared with the current clinicopathological parameters for risk assessment. Nevertheless, combination of several independent and complementary biomarkers and generation of a biomarker nomogram may help further identify patients with a higher recurrence and progression rate in case of non-muscle invasive BC as well as patients with more aggressive invasive tumors.

Predictive biomarkers

High throughput technologies, as discussed above, have greatly expanded our understanding of the molecular classification and pathogenesis of bladder cancer and have also facilitated the identification of potential therapeutic targets and predictive biomarkers. Indeed, given that the vast majority of systemic therapies used to treat human malignancies only confer benefit to a subset of patients, the identification of biomarkers predictive of response to treatment is an area of intense interest. This is particularly true in bladder cancer, a disease for which there have been no new systemic therapies approved for use in the past several decades [65-67]. Only a few studies, however, have prospectively tested putative predictive biomarkers in patients with advanced disease.

The epidermal growth factor family of receptors has been demonstrated to be altered at the genomic and protein level in bladder cancer samples and preclinical studies have demonstrated the activity of targeting EGFR or HER2 [68-71]. However, trials exploring anti-EGFR or anti-HER2 therapies in the clinic have generally not stratified patients based on the presence of the "target" or have used a variety of assays that have not been analytically validated [72, 73]. A small trial that evaluated the activity of the dual EGFR and HER kinase inhibitor, lapatinib, in patients with her-2 amplified tumors (centrally tested) demonstrated no significant antitumor activity in patients with bladder cancer [65]. Recently, HER2 mutations have been demonstrated in bladder cancer in genomic analyses by the TCGA and other and such tumors may be particularly sensitive to HER2 blockade.

Though activating mutations of FGFR3 are present in a large proportion of patients with non-muscle-invasive bladder cancer, such mutations are also present in 10-20% of muscle-invasive specimens. A prospective clinical trial recently evaluated the activity of the FGFR kinase inhibitor, dovitinib, in patients with FGFR3 mutant bladder tumors [74]. Unfortunately, there was no anticancer activity demonstrated and it is unclear if this is related to the particular therapy versus the relevance of FGFR3 as a target in these advanced tumors. Recent work from Knowles et al has demonstrated cross-talk between FGFR3 and EGFR suggesting that inhibiting both may be necessary for therapeutic benefit [75].

Bladder cancer is a relatively chemotherapy sensitive neoplasm. However, only a subset of patients respond to the current armamentarium of cytotoxic agents. There has been an interest in developing predictive biomarkers, not only for novel therapeutics, but also to refine use of existing drugs. DNA-repair genes, or their protein products, have been explored based on the notion that tumors with higher levels of DNA-repair genes may be more resistant to cytotoxic chemotherapy. Excision repair cross complementing 1, and BRCA1 have been evaluated in bladder tumors but have not reliably correlated with response to treatment [76, 77].

The genomic complexity of bladder tumors, and the complexity of DNA damage repair pathways, it is unlikely that a single gene or protein product can serve as a useful predictor of response to conventional cytotoxic chemotherapy. On the other hand, gene signatures may offer a more complete representation of the complexity of these pathways. Theodorescu and colleagues have developed a novel bioinformatic approach known as Coexpression Extrapolation (COXEN) [78, 79] that utilizes publicly available data regarding gene-expression profiling and drug sensitivity from the NCI-60 panel of tumor cell lines as a "Rosetta Stone" to predict chemo-sensitivity of gene-expression profiled bladder cancer samples using a computational algorithm. The ability of this approach to predict response to neoadjuvant chemotherapy in muscle-invasive-bladder cancer will be evaluated in an upcoming national clinical trial.

The promise of whole genome sequencing to identify potential predictive biomarkers was recently demonstrated by Iyer and colleagues [80]. In a study exploring the mTOR inhibitor, everolimus, as second-line therapy for patients with metastatic bladder cancer, a single patient had a durable response to treatment while a few other patients had minor tumor regressions. The investigators used an "outlier" approach to identify the presence of TSC1 mutations that correlated with drug sensitivity.

The genomic landscape of bladder cancers, now well-defined through the work of the TCGA and other groups, sets the stage for the functional validation, and subsequent clinical evaluation, of a large number of potential predictive biomarkers that could change the outlook for patients with this disease.

Future perspectives

With the increasing understanding of genomic alterations in bladder cancer, we anticipate that new genetic and epigenetic biomarkers will be discovered and validated. Furthermore, rapid progress in proteomics and metabolomics would add additional markers to improve the usefulness and accuracy of diagnostic, prognostic and predictive markers. To validate these potential biomarkers requires large cohorts of patients with high-quality biologic samples and complete clinical annotation. Therefore, the formation of multicenter consortia is necessary to accumulate prospectively enough patients with multiple clinical samples and longitudinal follow up. The areas of further development include urine sample collection and preservation, simple, low-volume and low-cost assay format, and improved sensitivity and specificity of individual markers. Finally, it will be necessary to combine using bioinformatics tools different types of urine biomarkers including exfoliated cells, DNAs, RNAs, proteins, metabolites into integrated biomarker signatures of risk, prognosis and prediction, which will achieve better sensitivity and specificity compared to individual markers. Such signatures will eventually lead to the development of panel of highly predictive markers which can be applied in clinical practice through multiplex approaches.

Disclosure of conflict of interest

None.

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