Editorial

Advances in molecular genetics of early-stage urothelial carcinoma

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Bladder cancer is the most common malignancy involving the urinary system. Urothelial (previously known as transitional cell) carcinoma is the predominant histologic type of bladder cancer especially in developed countries (1). Taking into account both histopathological and molecular features, this 'two-pathway' model proposes that papillary non-muscle-invasive bladder cancer (NMIBC) develops via epithelial hyperplasia and recruitment of a branching vasculature, while MIBC derives through flat dysplasia and carcinoma in situ (CIS). Metastasis of NMIBC is rare. Recent genomic and transcriptomic experiments showed that low-grade NMIBC and CIS/high-grade NMIBC/MIBC have distinct mutation and gene expression profiles; even within NMIBCs, considerable heterogeneity exist [reviewed in (2)]. NMIBCs commonly have near-diploid karyotype and few genomic rearrangements. By contrast, MIBCs are commonly aneuploidy with many alterations, including chromothripsis (3). Approximately 75% of newly diagnosed urothelial bladder cancers are early-stage and noninvasive, i.e., NMIBC. However, the incidence and high relapsing characteristics of NMIBC poses a considerable economic burden (4). Treatment of NMIBC involves transurethral resection which may be followed by instillation of a chemotherapy agent, e.g., mitomycin C to delay disease recurrence. Occurrence and mortality rates of urothelial bladder carcinoma including NMIBCs vary across the countries due to differences in risk factors, detection and diagnostic practices, and availability of treatments. Risk factors can be classified into external exposures to carcinogens and genetic predispositions (5). While accumulating evidence, such as the risk is 2-fold higher in first-degree relatives of patients with urothelial bladder carcinoma, suggests that genetic factors are closely associated with its prevalence.

To improve diagnosis and treatment require detailed understanding of pathogenesis and molecular biology in urothelial bladder carcinomas. The main genetic alterations underlying MIBCs involve tumor suppressor genes encoding proteins that regulate cell cycle and apoptosis pathways, including *TP53*, *CDKN2A* (also known as *p16*), *CCND1*, *CDKN1B* (also known as *p27*) and *RB1* [reviewed in (2)]. In NMIBCs, based on clinical outcome endpoints, efforts were undertaken to identify genes with differential expression in bladder cancers by microarray technologies, and have delineated many prognostic biomarkers (6).

Nevertheless, mutations of specific genes and pathways might provide more profound implications. A t(4;14)(p16.3;q32.3) chromosome translocation with breakpoints on 4p16 located 50–100 kb centromeric to the *FGFR3* gene was found in 20–25% of multiple myelomas and

was associated with overexpression of the FGFR3 protein (7). Further studies on solid tumors identified frequent activating mutations of *FGFR3* in human bladder carcinomas (8). Single-strand conformation polymorphism and sequencing analysis in several exons of the *FGFR3* gene, verified that activating point mutations were found in up to 80% of low-grade and low-stage urothelial bladder carcinomas (9,10), triggering downstream signaling pathways including RAS/MAPK, PLCG1, PI3K and STAT (11).

Meanwhile, loss of heterozygosity (LOH), array comparative genomic hybridization and mutation analyses identified genomic heterogeneity at both the chromosome and nucleotide levels in NMIBCs. Deletions of the long arm of chromosome 9 (9q) are frequent detected in NMIBCs at initial diagnosis as well as MIBCs (12). LOH of 9q, homozygous deletion of *CDKN2A* and loss of its expression in NMIBC are predictors of reduced recurrence-free interval. Gene mutations in *FGFR3* and overrepresentation of 8q were uncovered to be mutually exclusive in all NMIBCs and T1/grade 3 tumors (13,14). Recurrent mutation of *STAG2*, which encodes a component of the cohesion complex, has higher mutated rate in NMIBC than in MIBC (15). Activating mutations of *PIK3CA* were found in ~25% of NMIBCs and infrequent in MIBCs (16). Additionally, several studies revealed that a series of gene mutations in NMIBCs involved in PI3K and MAPK signaling pathways [reviewed in (2)]. At the epigenetic level, hypomethylation of non-CpG islands in NMIBCs and extensive hypermethylation of CpG islands in MIBCs were found (17). However, the knowledge regarding NMIBCs does not expand rapidly enough for the application of precision medicine.

The recent breakthrough of RNA sequencing technology (also known as next-generation sequencing) offered greater power, lower costs and new tools to better understanding of the molecular intra- and inter-tumor heterogeneities of NMIBCs, regardless of their histopathological classifications and clinical outcomes. RNA-sequencing technology combines exome and transcriptome sequencing to analyze target samples and compare their sequence variations (isoforms) and mRNA expression levels in one experiment. Using this technology along with whole-genome sequencing, the Cancer Genome Atlas Research Network brought together DNA, RNA, protein and epigenomic profiles to assess tumor and normal samples from 131 individuals with high-grade MIBC. The researchers found dozens of recurrently mutated genes, tracked down potential targets in more than 2/3 of the tumors, and used expression data to define four main subtypes of the disease. Genes involved in chromatin regulation are among those most prone to recurrent mutations in MIBCs (18).

In NMIBCs, using formalin-fixed paraffin-embedded tissue, high concordance with results from matching fresh frozen samples (>0.8 Spearman correlation) was obtained with an average RNA sequencing reads of >100 million per sample using the Illumina HiSeq2000 platform. Low- (n=27) and high-grade (n=22) bladder cancer transcriptomes in 49 tumor samples after transurethral resection of bladder tumor were compared. A total of 947 significantly differentially expressed protein-coding genes were identified. High-grade lesions exhibited distinct inter-tumor transcriptome heterogeneity whereas the transcriptome of low-grade tumors was homogeneous (19). In another experiment, whole-genome, exome and transcriptome sequencings of 38 tumors, including 4 metachronous tumor pairs and 20 superficial tumors, identified the sense strand of *APOBEC* mutational signature in 1/3 of samples, correlating with the mean expression levels. The patient-specific *APOBEC* signature was negatively associated with the expression levels of repair genes but not correlated with clinicopathological parameters. Genetic mutations were related to tumor stages, and the expression levels of chromatin modifiers were associated with survival. Among all mutated genes, *PIK3CA* mutations were identified in the ancestral tumor clones (20).

Given that NMIBC is an extremely heterogeneous disease with widely different outcomes, lately, Hedegaard *et al.* (21) performed a highly comprehensive transcriptional analysis of 460 early stage NMIBCs (345 Ta, 112 T1, 3 CIS) and 16 tumors from patients with MIBC across several clinical centers including Denmark, Sweden, the Netherlands, Spain, Germany and Serbia to dissect major classes of NMIBCs, as well as a unique CIS signature associated with risk of progression to MIBCs. Total RNA-sequencing libraries were prepared for all 476 tumors followed by paired-end sequencing. For each sample, an average of 24.6±0.7 million sequence pairs was generated. Using unsupervised consensus clustering of gene-based expression values with 8,074 genes, NMIBCs can be subgrouped into three major classes regarding to progression-free survival, clinical and histopathological features. The classes were significantly different with respect to progression-free survival, clinical and histopathological features. Besides, 3% of class 1, 81% of class 2 and 16% of class 3 tumors, respectively, progressed to T2+ NMIBC. Compared to class 1 (good prognosis, luminal-like), class 2 (poor

prognosis, luminal-like) and class 3 (intermediate prognosis, basal-like) contained tumors with high stage and grade, concomitant CIS and more frequently progressed to MIBC. Basal-like characteristics have been described in MIBC and some T1 tumors using immunohistochemistry (22). Also, 14 of 16 MIBCs were classified as class 2, similar to the pattern of high-risk NMIBC. Interestingly, in the top 2,000 expressed and most variable non-protein-coding genes, consensus clustering likewise identified three classes of tumors, analogous to the classes obtained using all genes. Compared to previous gene-expression studies, tumors with positive signatures for progression (23) and CIS (24) displayed considerable intersection with those of class 2 tumors.

As shown by Hedegaard *et al*. (21), there were totally 10,694 significantly differentially expressed protein-coding genes involving in Gene Ontology (GO) cell-cycle (especially, transcriptional factors) and histone/chromatin modifications (especially, histone modifications) processes which were enriched in class 2 and class 3 tumors, respectively. In contrast, among 431 significantly differentially expressed long intergenic non-coding RNAs (lincRNAs), though several were earlier defined to be related to bladder cancer, most remain to be characterized. In order to further molecular stratification of each tumor class, significantly differentially expressed genes involving in cell cycle, cancer stem cells, keratins, uroplakins, epithelial-mesenchymal transition (EMT)/mesenchymal-epithelial transition and differentiation were investigated. Differentiated luminal-like class 1 tumors showed high expression of *UPKs*, *PPARG*, *GRHL3*, *BAMBI* and *SPINK1*. Similarly, *UPKs*, *PPARG*, *KRT20*, *GRHL3*, *BAMBI* and *SPINK1* were significantly upregulated in differentiated luminal-like class 2 tumors. Conversely, *KRT5*, *KRT14*, *KRT15* and *CD44* were significantly high expression in basal-like class 3 tumors. Moreover, EMT transcriptional factors such as *SOX9*, *TWIST1*, *FOXF1*, *ZEB1*, *ZEB2* and *GATA6* were significantly upregulated in class 2 tumors. Both class 1 (*SHH*, *RPSA*, *ALDH1A3*, *ITGA6*) and class 2 (*PROM1*, *ALDH1A1*, *ALDH1A2*, *ALDH1A3*, *NES* and *THY*) tumors highly expressed cancer stem cell markers.

A 117-gene classifier to optimally predict the three classes of NMBICs was furthermore generated and validated in four independent datasets: (I) samples [8 normal specimens and 130 tumors (85 Ta, 37 T1, 8 T2-5) from Aarhus center] which were not included as part of the prospective study, the 117-gene classifier identified three classes of tumors, comparable to the prospective study; (II) analysis of total RNA sequencing from 11 bladder cell lines supported the risk categories detected in tumors; (III) examination on microarray data from 306 tumors (25) using the 117-gene classifier identified class 1 and class 2 tumors, significantly correlated with the Lund taxonomy groups as observed for the discovery cohort; and (IV) the 117-gene classifier was applied to analyze mRNA sequencing data of 408 MIBCs from TCGA and identified class 1 and class 2 tumors when including additional NMIBC class-specific transcripts. Class 1 included mainly tumors with papillary histology, and the two classes showed overlap with TCGA clusters I and II (luminal-like). The molecular characteristics of class 3 tumors were not observed in the TCGA set of MIBC.

Hedegaard *et al*. (21) continued to inspect hotspot mutations using RNA sequencing data and corresponding DNA from 460 NMIBCs and validated 97–99% of mutations in *PIK3CA* (n=419) and *FGFR3* (n=427) identified in RNA sequencing data. Analysis of the most frequently mutated genes (n=168, mutated in ≥10% of tumors) showed a significant enrichment for mutations in genes related to structural and cytoskeletal (GO molecular function) and genes involving in chromatin organization (GO biological process). Pathway analysis demonstrated that mutations in DNA-damage response (52%), *MAPK/ERK* (35%), and *ERBB* family (20%) genes were significantly correlated with class 2 tumors. RNA sequencing data also identified six distinct trinucleotide mutational signatures (signature 1 to 6) and signature 3, *APOBEC*-associated mutational signature (TCT/A, where C is mutated to T or G; C > T or C > G) was predominantly associated with the aggressive class 2 tumors. Expression levels of *APOBEC3A* and *APOBEC3B* were found to be significantly correlated with class 2 tumors and the presence of the *APOBEC*-related mutation signature. Signature 4 (T > C and C > T) was linked to RNA-editing process and significantly correlated with class 3 tumor.

In summary, the presence of stage Ta and T1 tumors with basal-like characteristics (class 3) may resemble a Ta pathway of disease progression, while the high-risk luminal-like tumors (class 2) may resemble the CIS pathway of progression. Class 3 tumors exhibit some of the gene-expression characteristics associated with basal-like MIBC. Progressing tumors showed class shifts from class 3 to class 2 during progress. Three classes represent three developmental pathways of NMIBCs. The identification of subclasses in NMIBCs using the 117-gene classifier may pave the way for optimized surveillance programs and treatment selection. Finally, as pointed out by Hedegaard

et al. (21), evaluation of NMIBCs at the genomic and transcriptomic levels may allow a more effective stratification of patient subclasses, as well as the targeting and optimization of patient-specific therapy (21).

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Footnote

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