Chapter

Molecular Classification of Bladder Cancer

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Abstract

Bladder cancer is a biologically and clinically heterogeneous disease. Traditional classification systems, based on pathologic grade, stage and clinical prognosis fail to fully explain how tumors with similar pathology exhibit diverse biological behavior. The introduction of transcriptomics technology has allowed us to catalog all of the mRNA expression patterns and DNA alterations in a given tumor thus expanding our understanding of human cancers. Molecular subtype profiling was attempted only recently in bladder cancer, with the earliest attempts dating back to 2010. Several different molecular classification systems have emerged since. Some of these systems address early bladder cancer, while others focus exclusively on the life-threatening muscle invasive tumors. These molecular subtypes have distinct morphological and clinical characteristics with different therapeutic and prognostic implications, particularly in the era of targeted therapies and immunotherapy. However, molecular subtyping is not without its limitations. Despite the rapidly expanding evidence for important clinical implications, much work is still needed to establish the utility (or lack thereof) of molecular subtyping, and its application in daily practice.

Keywords: molecular classification, muscle invasive bladder cancer, taxonomy, transcriptomics, targeted therapy

1. Introduction

Bladder cancer is the most common malignant tumor involving the urinary tract. Histologically, bladder cancers can be urothelial carcinomas, squamous cell carcinomas and adenocarcinomas, out of which urothelial carcinomas constitute over 90% [1]. Urothelial carcinoma is derived from the specialized epithelia of the bladder wall. Using a well-established differentiation program, basal stem cells at the stromal interface self-renew and generate intermediate and superficial urothelial cells to maintain and regenerate the urothelium in response to daily wear and tear. Bladder cancer results from the deregulation of this program. Conventionally, bladder cancer can be divided into non-muscle invasive bladder cancer (NMIBCs) and muscle invasive bladder cancers (MIBCs), based on the invasion of the muscularis propria. The low grade superficial NMIBCs and high grade MIBCs (HG MIBCs) develop along divergent molecular pathways of tumorigenesis and also show diverse biological behavior and molecular profile. Low grade NMIBCs constitute the majority of newly diagnosed bladder cancer cases, typically have a long protracted clinical course characterized by multiple recurrences, and require life-long monitoring, significantly contributing to bladder cancer morbidity. On the

other hand, a large proportion of the MIBCs eventually metastasize, contributing to the bulk of bladder cancer mortality [2, 3].

Low grade papillary urothelial carcinomas occur due to fibroblast growth factor receptor 3 (FGFR3)/RAS/RAF pathway alterations, while the HG MIBCs develop along the TP53/RB1 mutation pathway [4, 5]. Chromosome 9 deletion occurs in the early phase of bladder cancer tumorigenesis. FGFR3/HRAS mutations frequently occur during the development of hyperplasia and low grade (Ta) carcinoma. Hyperplasia develops into high grade urothelial carcinoma (Ta) through the acquisition of CDKN2A alterations, which may progress to become T1 carcinoma after additional TP53/RB1 inactivation. TP53 mutations frequently occur during the development of urothelial dysplasia. These may develop into carcinoma in situ (Tis) after RB1 inactivation, which then progresses through non-muscle invasive infiltrating urothelial carcinomas (T1) to muscle invasive (T2) carcinoma.

Traditional classifications for bladder cancer are mainly based on pathological features and tumor stage. However, even with similar pathological staging and grading, recurrence and progression of bladder cancer shows marked heterogeneity, and directly affects optimal monitoring and treatment response. Only a proportion of cases of bladder cancer of a given grade and stage will progress to a higher stage. Thus the same treatment, such as, transurethral resection alone, or the administration of BCG or neoadjuvant chemotherapy may not be adequate for others. Some tumors are less likely to metastasise and need only local resection, while others are highly invasive and need radical cystectomy and/or other treatments. Currently, there is still no effective means to distinguish between the two. The pathological features of the tumor cannot fully reflect the "intrinsic characteristics" of bladder cancer.

With the rapid development of sequencing, mass spectrometry and other techniques, studies based on the '-omics' technology has transformed our understanding of human cancers. The basic method involves cataloging the entire mRNA expression pattern and DNA alteration profile by sequencing, microarray and other technologies and then performing a cluster analysis of the different genes based on gene expression levels and genes involved in a given biological process. After performing hierarchical cluster analysis on mRNA expression profiling data, clusters were validated by DNA PCR and/or immunohistochemistry (IHC), DNA methylation profiling, miRNA or lncRNA analysis. Among the earliest applications of this approach was to define intrinsic molecular subtypes in human breast cancer by Perou et al., [6]. Subsequently, over the last decade, molecular subtypes with distinct clinical behavior, histology and response to treatment were identified in other malignancies e.g. colon cancer, gliomas, acute leukemias and so on. Molecular subtype profiling was applied only recently in bladder cancer, with the earliest attempts dating back to 2010. Several different molecular classification systems have emerged since, with four standing out in the MIBCs, developed by Lund University group [7–10], The Cancer Genome Atlas (TCGA) consortium [11, 12], MD Anderson Cancer Centre [13] and the University of North Carolina [14]. Several other studies have followed up, in an attempt to unify [15] and reach a consensus [16] between the classification systems. Although each group defended the existence of a different numbers of subtypes (n = 2–6), there was remarkable overall concordance among the groups (**Table 1**). At the highest level, all classification systems recognized the existence of intrinsic luminal and non-luminal (basal) subtypes, which resembled normal luminal/intermediate and basal urothelial cells in gene expression profile.

For the two major types of bladder cancer, NMIBC and MIBC, molecular subtyping of bladder cancer can be divided into early subtyping (which included both NMIBC and MIBC), NMIBC subtyping and MIBC subtyping (**Table 2**).

TCGA [12]	Lund [8]	MDACC [13]	UNC [14]	Properties
Cluster I	UroA, GU	Luminal	Luminal	FGFR mutations, papillary histology
Cluster II	Infiltrated	p53-like	Luminal	CAFs, immune cells
Cluster III	SCC-like, UroB	Basal	Basal	Stem cell markers, squamous differentiation
Cluster IV	Infiltrated	p53-like	Claudin-low	EMTs, CAFs, immune cells

Table 1.
Table showing overlap between the subtypes in the initial classification systems and the cardinal properties of these subtypes, adapted from McConkey et al. [17], CAF-cancer associated fibroblasts, EMT- epithelial-mesenchymal transition.

Subtyping systems	Abbreviation	Number of cases	Subtypes
Early BC subtyping system	Lund 2012 [8] 5 subtypes	n = 308	Urobasal A, genomically unstable, infiltrated, urobasal B and squamous cell carcinoma-like
	Baylor tumor differentiation classification 2012 [19] 3 subtypes	n = 492	Basal, intermediate, and differentiated
NMIBC subtyping	UROMOL 2016 [20] 3 subtypes	n = 400	Class1, 2 and 3
	Van Kessel 2018 [21] 3 subtypes	n = 1239	EAU high risk NMIBCs classified into good, moderate and poor prognostic subtypes
MIBC subtyping	UNC 2014 [14] 2 subtypes	n = 262	Basal-like (including claudin-low), and luminal
	MDACC 2014 [13] 2 subtypes	n = 83	Luminal, p53-like and basal
	TCGA 2014 [12] 4 subtypes	n = 129	Clusters I, II, III and IV
	TCGA 2017 [11] 5 subtypes	n = 412	Luminal-papillary, luminal (not-specified), luminal-infiltrated, basal-squamous, neural
	Consensus classification 2019 [16] 6 subtypes	n = 1750	Luminal-papillary, luminal not specified, luminal unstable, stroma-rich, basal/squamous, and neuroendocrine-like
BC subtyping with both NMIBCs and MIBCs	BOLD 2018 [22] 6 subtypes	n = 2411	Neural-like, luminal-like, papillary-like, HER2-like, squamous cell carcinoma-like and mesenchymal-like

Table 2.
Summary of major molecular subtyping systems, adapted from Zhu et al., 2020 [18].

2. Early bladder cancer subtyping systems

The earliest representative studies on the molecular classification of bladder cancer were conducted by Sjödahl et al. at the University of Lund, initially proposed in 2010 and finalized in 2012 and the tumor differentiation classification

by Chan et al., of the Baylor University group, 2012 [19]. The Baylor classification focused on tumor biology. They proposed a KRT14/Thy-1/CD44 expressing cancer stem cells as the bladder cancer precursor. This cancer stem cell evolved into a partially differentiated KRT5/KRT17/CD44-positive progeny, which in turn acquired KRT8/18 expression and eventually differentiated into luminal cells expressing uroplakins and KRT20. Tumors were classified into basal, intermediate and differentiated classes based on their resemblance to normal urothelial differentiation. Based on the above classification, the KRT14 group (basal subtype) showed the poorest prognosis and were also found to be resistant to neoadjuvant cisplatin-based chemotherapy.

The Lund University group initially defined two intrinsic molecular subtypes named as MS1 and MS2 based on gene expression, genomic, and gene mutation levels by whole genome comparative genomic hybridization and mutation studies [7]. By combining molecular and pathologic data, it was possible to divide tumors into grade 1 or 2 (MS1) and grade 3 (MS2) (WHO1999), and into Ta (MS1) and ≥ T2 (MS2) stages based on the MS1 and MS2 subtypes.

Subsequently, an extensive biological interpretation of gene expression data identified that biological themes including immune, late cell cycle, keratin, receptor tyrosine kinases and FGFR3 signatures determined their data structure. Based on tumor histopathology, gene signatures, and status of FGFR3, PIK3CA, and TP53 mutations, three major subtypes of UC were defined: urobasal (Uro) (further subdivided into MS1a, MS1b, and MS2b2.1), genomically unstable (GU) (MS2a1 and MS2a2), and SCC-like (SCCL) (MS2b2.2) [8]. Subsequent studies also identified an "infiltrated" group in which the stromal inflammatory transcripts were prominently expressed. Among the urobasal tumors (MS2b2.1), a subset showed "progressed phenotype" with aberrant expression of basal keratins in suprabasal cell layers and upregulation of late cell cycle activity. These tumors were mostly large and invasive, and were named urobasal B to distinguish it from urobasal A tumors that were non-muscle invasive in almost all cases. The molecular subtypes thus defined, transcended pathological staging and all four subtypes (UroA, UroB, GU, and SCCL) were detected among T1 tumors. The initial Lund taxonomic studies included both NMIBC and MIBC and subsequent studies focussed predominantly on MIBC classification.

3. Molecular subtyping of early-stage bladder cancer

Variability in terminology has created a challenge in the molecular classification of early-stage bladder cancer. Treating clinicians emphasize the dichotomous division of BCs into NMIBC and MIBC, and often lump all NMIBCs together when planning molecular studies. In contrast pathologists tend to see a stark difference between non-invasive tumors and invasive tumors limited to the lamina propria and classify accordingly.

Molecular diversity in non-invasive BC differs from that of MIBC. Non-invasive BC histologically includes papillary UC and flat CIS, although both may co-exist in the same patient. As we have discussed earlier, low-grade non-invasive papillary UC has a high frequency of FGFR3 mutation. LGUC progresses to HGUC and invasive carcinoma through the acquisition of TP53 mutations and 9p21 loss involving the gene encoding CDKN2A. In contrast, most CIS lesions have TP53 mutations early in evolution and do not acquire FGFR3 mutations [4, 5].

In the Lund system [8], majority of non-invasive UCs are urothelial-like, while CIS may be either urothelial-like or genomically unstable. They identified a "CIS signature" by utilizing a 16-gene classifier which was specifically expressed in flat

CIS, as well as in early-stage invasive carcinoma with associated CIS, and a large proportion of MIBCs of the basal-squamous subtype. In addition, MIBCs with concurrent CIS had greater genomic instability compared with those without it. Although majority of non-invasive papillary UCs were of luminal subtype, there is substantial molecular diversity among cases. The most clinically relevant diversity was related to cell cycle regulatory genes. Tumors with greater activation of the cell cycle had higher rates of recurrence and progression to MIBCs.

The UROMOL study of 2016 [20] evaluated expression profiles of NMIBCs including non-invasive papillary UC and invasive UC limited to the lamina propria (stage T1). A few cases of CIS and a small group of MIBCs were also included for comparison. Tumors were classified into 3 subtypes based on relative expression of luminal and basal-squamous markers and cell cycle activity. Three subtypes were proposed, named as Type 1 (early cell cycle activation and higher luminal gene expression), Type 3 (early cell cycle activation with lower luminal gene expression) and Type 2 (late cell cycle activation). Type 2 tumors, which included the highest proportion of T1 samples, had the greatest propensity to progress to muscle invasion. On the other hand, expression of luminal genes did not significantly affect patient outcome. In addition, non-invasive papillary tumors also varied in the degree of chromosomal instability. The unstable group had tumors with higher proliferation, greater mutational burden, and high-grade histology.

Von Kessel et al., in 2018 [21], determined that methylation status of GATA2, TBX2, TBX3 and ZIC4 and mutations in FGFR3, TERT, PIK3CA, and RAS correlated with progression rates of NMIBC. Wild-type FGFR3 and GATA2 and TBX3 methylation were significantly correlated with NMIBC progression. Thus, high risk NMIBC group was reclassified into good, moderate and poor prognostic classes with low, medium and high risk of progression.

However, because molecular subtyping of non–muscle-invasive bladder cancer has not demonstrated clear value in clinical decision making, it is not currently recommended to incorporate it on a routine basis, as per the ISUP recommendations published in 2020.

4. Molecular subtyping of MIBC

Much of the work on molecular subtyping of bladder cancer has been undertaken with MIBCs in consideration and an increasing number of classification systems have emerged with four of them standing out (**Figure 1**). Although the subtypes within these systems are largely similar, they differ in clinically and biologically meaningful ways.

The UNC classification proposed by Damrauer et al. [14], used K2 consensus clustering, to divide tumors into basal (KRT5/6 and CD44) vs. luminal (PPARG, GATA3, KRT20, and UPK2) subtypes utilizing a 47 gene classifier, BASE47. The basal subtype showed similarities with the basal subtype of breast cancers, as demonstrated by applying the PAM50 signature to their dataset. In addition, like in breast cancers, a claudin-low subgroup was identified among basal tumors. The claudin-low subgroup had outcomes similar to basal tumors and was rich in epithelial-mesenchymal transition (EMT) signatures and tumor initiating cell markers. A significant enrichment in genes related to inflammatory cell infiltration and immune checkpoint was also seen in the basal subgroup and, more specifically, among the claudin-low tumors. There was no significant difference in TP53 pathway alterations in the subtypes. The basal subtype, which was more frequent among females, had a high rate of RB pathway gene alterations, while the luminal subtype was rich in FGFR3 and TSC1mutations.

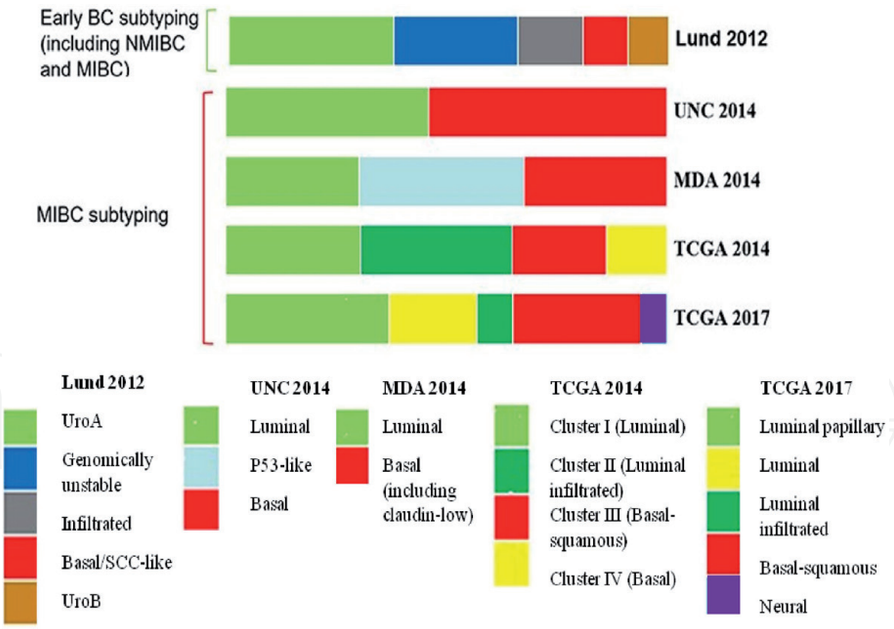

Figure 1.
Figure showing the similarities between the molecular subtypes in the different classification systems. Luminal and basal subtypes exist in all classification systems (figure adapted from Zhu et al. [18]).

The MDA group [13] also classified BC into basal, p53-like and luminal tumors similar to breast cancer. Their luminal and p53-like subtypes had similar mRNA profiles but wild-type p53 gene was significantly activated in the p53-like subtype. All three subtypes had similar frequency of p53 mutations, but the p53-ness contributed by increased wild-type p53 expression was thought to contribute to chemo-resistance in the p53-subtype. The basal subtype was enriched in squamous cell differentiation markers and activated p63 and was more invasive with poorer clinical prognosis.

The TCGA 2014 [12] subtyped MIBCs into four clusters numbered I to IV utilizing an integrated genomic analysis of chemotherapy-naive, invasive UCs by analyzing for somatic mutations, DNA copy number alterations, mRNA and microRNA expression profile, as well as protein analysis, and DNA methylation studies. Cluster I predominantly exhibited papilloma phenotype and was enriched in FGFR3 mutations. Both cluster I and II expressed GATA3, FOXA1, UPK3A transcription factors and uroplakin family of genes and were enriched in RBB2 mutations and ER beta. Cluster III expressed squamoid phenotype and its associated keratin expression. Subsequently in 2017, TCGA expanded their classification [11] into five distinct subtypes, diving luminal tumors into three subtypes, luminal papillary, luminal and luminal infiltrated. They also included a neural subtype in addition to the earlier described basal subtype.

4.1 Intrinsic molecular subtypes and intra-tumor heterogeneity

Intrinsic molecular subtype, a term which first used in breast carcinomas [6] refers to subtypes which reflect an intrinsic property of the tumor. The luminal and basal intrinsic subtypes reflect the property of the tumor cells to show urothelial or basal stem-cell-like differentiation signatures [23].

However, transcriptomic studies identified the entire genetic signature of a tumor, which in the case of invasive malignancies, included variable components of stromal and immune signatures. Thus, in addition to the two intrinsic subtypes, some of the subtypes defined in the various study groups were based on the

characteristics of the tumor-stroma or tumor infiltrating inflammatory cells such as the p53-like'subtype [13], infiltrated subtype [8], luminal-infiltrated subtype [11] as examples. These non-intrinsic subtypes could be recognized as a property of the stroma or inflammatory cells when studies were performed later which localized the gene expression patterns in situ, by immunohistochemistry [10, 23, 24]. Many of these non-intrinsic subtypes could be resolved into luminal and basal subtypes based on tumor phenotype on immunohistochemistry, while others continued to express non-luminal, non-basal phenotype, like the double negative subtype of Dadhania et al., [24].

4.1.1 Intrinsic luminal subtype

At the top of the hierarchical level, MIBCs were divided into luminal-like and nonluminal-like classes based on the presence or absence of bimodally expressed urothelial differentiation signature.

About half of the MIBCs expressed this signature characterized by the expression of KRT20, UPK1–3 (uroplakin 1, 2 and 3), epithelial biomarkers (E-cadherin/CDH1 and members of the miR-200 family), along with transcriptional regulators PPARG (peroxisome proliferator activated receptor-γ), GRHL2–3 (grainyhead like transcription factor), ELF3 and TBX2–3. Luminal MIBCs also displayed active Estrogen Receptor/TRIM24 pathway gene expression and were enriched with FOXA1, GATA3, ERBB2 and ERBB3 expressed on superficial (umbrella cells) and intermediate cells of the normal urothelium [7]. There was increased expression of fibroblast growth factor receptor-3 (FGFR3), with activating FGFR3 mutations in the most differentiated luminal tumors.

The luminal tumors were further sub-stratified into urothelial-like (UroA, UroB and UroC) and genomically unstable (GU) subtypes by the Lund University group [8], luminal papillary, luminal and luminal infiltrated subtypes by TCGA [11] and luminal papillary, luminal non-specified, and luminal unstable subtypes in the Consensus classification [16].

The urothelial-like tumors expressed FGFR3 and CCND1, and frequently showed 9p21 (CDKN2A) loss. On immunohistochemistry, only the urothelial-like tumors retained the basal stratification seen in normal urothelium and express CK5 at least focally, particularly at the tumor-stroma interface [9, 10]. Similar to UroA tumors in the Lund classification, the luminal-papillary subtype in TCGA and Consensus classification were also characterized by FGFR3 mutations; by papillary histology; and by low carcinoma-in-situ scores. Such cancers had a low risk for progression, and while preliminary data suggests a low likelihood of response to cisplatin-based NACT [25], they may respond to tyrosine kinase inhibitors of FGFR3 family [26, 27] or to PPARγ-inhibitors [10] or to Estrogen receptor modulators [28].

Genomically unstable subtype (GU) of luminal tumors expressed FOXM1 and absent to low levels of FGFR3, but not KRT5 [9]. They also frequently showed RB1 loss, and had a high rate of TP53 mutations. Highest ERBB2 expression is also seen in GU subtype. Although they showed urothelial differentiation signature, GU tumors were in fact poorly differentiated and frequently high grade on histology [9, 10]. On immunohistochemistry, they did not express CK5, but expressed late cell cycle makers such as p16 [10]. The luminal unstable subtype of Consensus classification showed similar features to the GU subtype described by Lund University group. These tumors may respond to drugs targeting ERBB2 [23].

In terms of prognosis, luminal papillary tumors or UroA tumors had very good prognosis, while the GU subtype showed an intermediate prognosis compared to urothelial-like and basal/SCC-like tumors [10, 12, 16].

4.1.2 Intrinsic non-luminal subtype: Basal and neuronal

Intrinsic non-luminal MIBC included basal MIBC (Choi et al., 2014) [13] and neuronal or small cell neuroendocrine MIBC. The basal subtype has been renamed basal-squamous in the later classification systems as it is characterized by squamous differentiation [8, 12].

Basal-squamous MIBCs expressed signal biomarkers similar to normal basal cells in the urothelium like high molecular weight cytokeratins KRT1, KRT5, KRT6, KRT14, KRT16, 15 KRT6A, KRT6B, KRT6C and CD44 and CDH3 [8, 11]. However, unlike normal basal urothelial cells which retained urothelial differentiation factors (GATA3 and PPARG), the basal-squamous subtype showed down-regulation of this signature. Interestingly, they had a higher incidence in females unlike all the other subtypes which were male predominant [11].

Basal MIBCs were also characterized by up-regulation of the epidermal growth factor receptor (EGFR) and other ligands of the epidermal differentiation complex such as S100A7 and SPRR1B, similar to basal breast and head and neck squamous cell carcinomas. Cell cycle regulator p63 played a central role in controlling the basal pathway of differentiation, and STAT3, NFκB, and Hypoxia Induced Factor-1α (HIF-1α) were also involved [23].

Without treatment, basal MIBCs had poorer survival [13, 14] but they responded well to neoadjuvant chemotherapy [11]. Because NACT pathological complete response is associated with excellent long-term survival, aggressive early management of basal MIBCs with NACT offers the best chance for improved survival for these patients.

This subtype also had the strongest immune expression signature, including T cell markers, inflammation genes and lymphocytic infiltrates. It is predicted that the basal-squamous subtype may respond to anti-PD-L1, anti-PD-1 and anti-CTLA-4 agents [28, 29]. EGFR-, NFκB, HIF-1α/VEGF, and/or STAT3-targeted agents may also have a role within this subtype [27].

The neuronal subtype showed no histopathological distinction from other types of MIBC in most cases. Nonetheless, they had high levels of TP53 and RB1 mutations, similar to small cell carcinomas in other tissues. It had the worst survival of the mRNA expression subtypes, making it important to recognize [10, 11].

4.1.3 Non-intrinsic subtypes: P53-like, luminal-infiltrated, stromal-like, infiltrated, claudin-low

P53-like MIBCs showed some overlap in gene expression with luminal and basal subtypes of the UNC classification but were characterized by the expression of an active wild-type p53-associated gene expression signature [13]. P53-like subtype of bladder cancer responded poorly to neo-adjuvant chemotherapy [13, 28, 30]. Wild-type p53-induced reversible senescence and quiescence had been implicated in causing chemo-resistance. However, even though p53-associated expression signatures were present, *TP53* mutation frequencies were found to be similar in luminal, p53-like and basal subtypes defined by Choi et al., (2014). The p53-ness as measured by mRNA expression was found to be a more accurate predictor of de novo and induced MIBC chemo-resistance than analysis of *TP53* mutational status [23].

The luminal-infiltrated subtype reported by the TCGA was characterized by low tumor purity, with high expression of epithelial-mesenchymal transition (EMT) and myofibroblast markers, and of the miR-200 s. It showed medium expression of PD-L1 and CTLA4 immune markers. This subtype had been reported to respond to immune checkpoint inhibitors like Anti-PD-L1 [29].

Stromal-like subtype from the Consensus classification, the infiltrated subtype from the Lund classification [10] and claudin-low subtype of the MD Anderson Cancer Centre classification [14] all showed similar features of low-tumor purity, high EMT and stromal related transcripts with increased cancer stem cell-like gene expression profile. Claudin-low tumors described by Damrauer et al. [14], in addition, showed increased expression of claudins-1, 3, and 7 and had a similar expression profile to the claudin-low breast cancer subtype. Dadhania et al., [24] in their meta-analysis of the TCGA, Lund and MD Anderson cohorts also identified a subset of tumors with low urothelial and basal expression signatures, which they termed "double negative", which showed similar expression profile to claudin-low tumors.

With tumor progression, alterations are seen both in the intrinsic characteristics of the tumor cell, as well as in the tumor microenvironment (TME). Early MIBC molecular classification systems mainly focused on the molecular classification of tumor cells themselves. With a deeper understanding of BC cells and their TME, molecular subtyping efforts have begun to focus more on intratumor heterogeneity, stromal-extracellular matrix (ECM) interactions and immune cell infiltration, allowing further refinement of the molecular subtypes. Currently, studies on molecular subtyping are mainly based on whole tumor DNA or RNA studies rather than focusing on a single tumor cell. In this method, intratumoral heterogeneity can greatly affect the accuracy of molecular subtyping. Warrick et al. [31] conducted a pathological examination on 309 bladder cancer markers and found that nearly one fourth of them exhibited intratumoral variation in tissue samples. Out of the 83 specimens subtyped by them with the Lund subtyping system, 39% exhibited molecular heterogeneity. Even among the subtypes, the basal-squamous subtype particularly showed the greatest variability; with approximately 78% of these tumors simultaneously exhibiting the genomically unstable or urothelial-like subtype.

Several immunohistochemistry based algorithms have been developed in an attempt to classify bladder cancer into clinically and prognostically significant molecular subtypes [9, 10, 24, 32]. The use of immunohistochemistry as a surrogate to molecular testing shows promise in making molecular subtyping amenable to widespread use. The use of a simple panel comprising of a luminal urothelial markers like GATA3 and basal keratin marker like KRT5 can help identify a GATA3 positive, KRT5 low luminal subtype and a GATA3 negative, KRT5 high basal subtype. Tumors which are negative for GATA3 and have low keratin may be further tested for mesenchymal or neuroendocrine markers. The luminal tumors may also be further subtyped into uro-like tumors which are p16 negative and genomically unstable tumors which show p16 positivity [33]. The subtypes thus identified have demonstrated significant prognostic and predictive value [24, 32].

5. Clinical significance of molecular subtypes of bladder cancer

Not only do clinical outcomes differ among the molecular subtypes, but also therapeutic response. The gold-standard for management of MIBCs for disease confined to the pelvis includes radical cystectomy preceded by platinum based neoadjuvant chemotherapy. Although a significant minority of patients treated this way achieves durable response and improved cancer specific survival, a sizeable fraction does not respond. In fact, a meta-analysis has suggested that there is only 5% absolute survival benefit at 5 years for patients treated in this manner [34]. In addition, concerns regarding delayed surgery and risk of serious morbidity have limited the usage rates of NACT for cystectomy patients at 25% or less [35, 36]. Biomarker tests

that predict chemo-response could address these problems by identifying patients most likely to benefit. In this regard, data suggests that NAC confers the greatest benefit in basal tumors [11, 29], while the "p53-like subtype" has been reported to confer chemo-resistance [13]. However, current ISUP working group guidelines of 2020 do not recommend routine subtyping to guide NACT [37].

Data published by the TCGA Consortium stated that about 69% of BCs contain potentially actionable therapeutic targets which associate with specific molecular subtypes [11]. Mutations and amplifications of FGFR3 are seen in 50–80% of superficial bladder cancers and up to 20% of MIBCs [19]. Luminal-papillary tumors demonstrate a high rate of these alterations [11]; however, clinical trials have not yet incorporated molecular classification to determine patient eligibility. Similarly PIK3CA is also a frequently mutant gene and therapies targeting PI3K pathway have also shown preclinical BC trials [38]. Other targets such as ERBB2 and TSC1 are also being investigated as therapeutic targets.

Molecular subtyping may also provide a guide to BC immunotherapy. Anti-programmed death 1 (PD-1) and anti-programmed death-ligand 1(PD-L1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) are important second line therapies for MIBC with NAC failure. A few are also approved as first line therapy for cisplatin ineligible cases. Unfortunately, not all patients benefit from immunotherapy [39, 40]. Testing for biomarkers of response involves IHC for PD-1 or PD-L1 or estimation of tumor mutation burden, micro-satellite instability or immune-microenvironment of the tumor. However, none of these biomarkers have shown overwhelming predictive efficacy over others [41–43]. The luminal infiltrated subtype in the TCGA 2017 subtyping system was found to be enriched in PD-L1, CTLA-4 and other immune signatures. In particular, although this subtype did not respond well to NACT, they showed good response to anti-PD-L1 and anti-PD-1 treatment. The basal subtype has also shown response to immunotherapeutic agents in addition to being sensitive to NACT [11, 29]. Molecular subtypes may thus help define patient selection for immunotherapy.

6. Conclusion

Compared to traditional classification of BCs, molecular subtypes provide more information regarding tumor biology, prognosis and treatment. In general, BC can be divided into luminal and non-luminal subtypes based on their degree of urothelial differentiation. The luminal subtype is further subdivided into those with papillary features, which are superficial, predominantly non-invasive. Though they carry good prognosis when compared to other treatment-naïve subtypes, they do not respond well to conventional NAC and may benefit from targeted therapies. The luminal infiltrated type has more inflammatory and stromal signatures. They are more invasive than luminal papillary tumors and may respond well to immunotherapies. Basal/squamous tumors express stem cell and squamous differentiation associated gene expression signatures. They are aggressive untreated, but respond well to NACT as well as immunotherapies but are insensitive to radiotherapy. The neural subtype forms a minority of non-luminal tumors with neuronal or neuroendocrine phenotype and usually carry poor prognosis but respond to NACT. There are an increasing number of molecular subtyping systems being constantly updated. While they carry great potential to reform BC prognostication and therapeutics, they are not entirely without limitations. Accessibility is a key issue in the present times. Molecular subtyping is mainly based on "static" research, especially in NMIBC, and enables a one-time detection and analysis of tumor specimens rather than "dynamic" tracking to over the disease course. It has also mainly focused

on genome and transcriptome research so far but proteomics and immune status of tumors are also closely related to their development. Therefore, the implementation of multiomics is a key necessity in future studies on molecular subtyping. Intra-tumor heterogeneity also provides another challenge with patient outcome being dominated by one subtype more than the other. With the rapid development of single-cell high-throughput sequencing, mass spectrometric analysis, immune cell analysis and other technologies, the accuracy of the molecular subtyping prediction system need further improvement. Compared to the existing classification system, molecular subtyping methods offer a more comprehensive analysis, particularly to guide adjuvant chemotherapy, targeted therapy and immunotherapy. In the future, these classifications will become an important complementary approach to traditional pathological classification.

Conflict of interest

The authors declare no conflict of interest.

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