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TOPIC PAPER

Genomic classification and risk stratification of bladder cancer

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Abstract
Bladder cancer is the fourth most common cancer in men and fifth most common overall. The use of next-generation sequencing (NGS) approaches is crucial to precisely characterize the molecular defects of tumors, and this information could be combined with other clinical data, such as tumor histology and TNM staging, with the goal of precise tumor classification. In many settings, targeted NGS is evaluated in patients with first- and second-line metastatic cancer. Yet, in the decade to come we anticipate increased application of precision oncology at all stages of bladder cancer with the aim of customizing cancer treatment. Here, we review the genomic and transcriptomic features associated with risk stratification in bladder cancer and summarize the current efforts for precision oncology in localized urothelial carcinomas.

Keywords Bladder cancer · Genetic mutation · Classifier · Biomarker · Risk-classification

Introduction

Bladder cancer is the fourth most common cancer in men and fifth most common overall [1]. While 80% of patients present with non-muscle invasive bladder cancer (NMIBC), an additional 20% will have locally invasive or metastatic cancer and require both local and systemic therapy [2]. To date, treatment is largely based on clinical parameters, such as TNM staging, with no precision treatments targeting specific tumor vulnerabilities [3]. Yet, the comprehensive genomic profile of both NMIBC and muscle-invasive bladder cancer (MIBC) has identified different molecular features that may help predict risk of aggressiveness and even guide therapy [4]. Towards this goal of precision care, this review will focus on the genomic and transcriptomic features associated with risk stratification and summarize current efforts for precision oncology in localized bladder cancer.

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Non-muscle invasive bladder cancer

Compared to MIBC, NMIBC is nearly fourfold more common with significant heterogeneity between tumors of different stage and grade. Low-grade, non-invasive cancers have a cancer-specific survival of 98% compared to only 84% in high-grade invasive cancers [5–7]. The most comprehensive evaluation of low- and high-grade non-muscle invasive bladder cancer was performed by Hurst et al. in a study that included analysis of genomic copy number (141 tumors), microarray-based gene expression (79 tumors) and whole-exome sequencing (24 tumors) [8]. The authors proposed a classification system discriminating between "GS2 tumors", characterized by the genomic loss of 9q that occurred in 45% of cases and "GS1 tumors". The GS1/GS2 genomic classification accurately predicted pathologic stage, differentiating GS1 and GS2 tumors into low- and high-grade tumors, respectively (28% high-grade GS1 versus 52% GS2; $p = 0.008$). The authors hypothesized that a strong candidate gene lost on 9q was *TSC1,* resulting in higher activity of mTOR signaling, which was confirmed by the identification of the mTOR pathway among the gene sets enriched in the differentially expressed genes. Exome sequencing of 17 GS1 and 7 GS2 tumors found a mean and median tumor mutation burden (TMB) of 1.64 and 2.41 mutations/MB, respectively. Overall, APOBEC $C > T$ mutational patterns were found in 60% of samples and represented 35% of all mutations with a correlation between APOBEC enrichment

and higher TMB. APOBEC-associated mutations, APOBEC RNA expression and TMB were all increased in GS2 compared to GS1 tumors. Since TMB correlated with neoantigen burden, those tumors with a GS2 and high TMB likely have more neoantigens and could potentially benefit from BCG over intravesical chemotherapy. To date, a formal comparison of TMB, APOBEC activity and GS status and response to BCG has not been performed [9]. Thus, non-invasive cancers may be risk stratified by two features: 9q deletion (GS2) and APOBEC signatures. Patients with 9q loss may benefit from targeted mTOR therapy or chemotherapy while those with APOBEC signatures may have a better response to immunotherapy, such as BCG. Evaluation of somatic mutations found that 79% of tumors had an FGFR3 mutation, which were mutually exclusive with mutations in RAS family members. These cancers may be sensitive to targeted therapy for FGFR3-targeted therapies such as erdafintinib, BGJ398 or AZD4547 [10]. To date, two trials have attempted to selectively target FGFR3 in non-muscle invasive bladder cancer (NCT01732107 and NCT02657486). The trial with Dovitinib (HCRN 12-157) resulted in only one CR of 13 patients enrolled, while the BJF398 is open to accrual for patients with FGFR3 mutations [11].

A more comprehensive evaluation of the genomic landscape of NMIBC was described in a study from the Dyrskjøt group, in which 460 tumors were profiled by transcriptomic analysis, including 345 Ta, 112 T1 and 3 CIS cancers [12]. Tumors were clustered into three groups (clusters 1–3). Cluster 2 tumors were the most likely to be high grade, had concomitant CIS and progressed to MIBC. Compared to the other subtyping taxonomy, Class 1 and Class 3 tumors aligned to Urobasal A (Lund) tumors, with Class 3 more similar to basal tumors (Base47) and Class 2 a mixture of infiltrated (37%) or genomically unstable (57%) from Lund [13]. Class 1 and 2 tumors had the highest expression of uroplakin markers, while Class 3 tumors over-expressed KRT5 and 14 with CD44 stem cell marker. EMT markers were enriched in class 2 tumors. Using RNA-Seq to guide mutation signature analysis, class 2 tumors were over-represented with *APOBEC*-associated mutation signatures. The authors developed a 117-gene signature to help classify tumors to the three clusters and validated their clustering in bladder cancer cell lines, NMIBCs and MIBCs. This 117-gene classifier was also applied by Knowles to the non-invasive tumors and confirmed that GS1 tumors were approximately 50% class 1 with the remaining half being cluster 2 and 3, while the majority of GS2 tumors were cluster 2 [8].

Few studies have focused on biomarkers of risk stratification of T1 bladder cancer. T1 cancers are challenging because they have invasion and share many genetic features of T2 cancers [12, 14]. A focused evaluation of 167 patients with T1 bladder cancer was performed by the Lund group [15]. Using a combination of immunohistochemical and gene expression markers, tumors were grouped into Uro (urobasal), GU (genomically unstable) and SCCL (squamous). GU and SCCL were found to have similar rates of progression, but were more aggressive than Uro tumors. Interestingly, grouped GU and SCCL tumors could be distinguished by the expression of CD3, a T cell marker whose overexpression was associated with worse outcomes. While CD3+ has often been associated with improved response in MIBC, NMIBC tumors with higher CD3 had significantly higher rates of progression (HR 2.33, 95% CI 09–5.01). A microarray-based study of 80 T1 patients identified a 24 gene signature that predicted response to BCG and many of these genes are involved in the immune response [16].

We performed targeted exome sequencing of high-risk NMIBC, which included 22 T1HG cancers [14]. High-risk NMIBCs (22 T1s and 3 TaHG + CIS) at initial presentation were divided into two cohorts: (1) those that recurred and progressed and (2) those that did not recur or progress. A third cohort of MIBC and metastatic bladder cancer from the same institution was evaluated for comparison of genetic mutations on the same platform. While more mutations were found in tumors at the time of recurrence, the cohort was too small to identify a common genetic event that was a driver of progression in multiple samples. Interestingly, most tumors already had a *TP53* mutation, but only tumors at progression had both a *TP53* and *CDKN2A* loss, suggesting that loss of both checkpoints may be necessary for progression. Loss of CDKN2A has previously been described as a key event in the evolution of NMIBC [17]. A formal comparison of total mutation burden (TMB) was made on the Foundation assay comparing all three cohorts. Compared to non-progressors (15 mutations/MB), tumors that progressed had lower TMB (12.8 mutations/MB), which decreased further at progression (10.1 mutations/MB) and was significantly higher than MIBC and metastatic cancers (5.1 mutations/MB). One hypothesis to explain this trend in TMB suggests that contraction of TMB at recurrence is a decrease in neoantigen load, as most patients had received BCG immunotherapy. This contraction of TMB, wherein mutations and neoantigens are decreased as a mechanism to decrease immune recognition, has been demonstrated in patients with metastatic melanoma that progressed after checkpoint immunotherapy [18]. An important next step will be to determine how clonality of each mutation changes in response to immunotherapy.

Muscle invasive bladder cancer

Evaluation of locally advanced bladder cancer has been the focus of multiple groups and was performed most extensively by the Cancer Genome Atlas [19]. Originally released

in 2014 with the comprehensive profiling of 131 tumors, the MIBC bladder TCGA was updated in 2017 to reach 432 tumors [20]. Through gene expression and mutation analysis, multiple clustering systems were applied. In the following sections, we review the mutation and gene expression signatures found in MIBC and we discuss about the opportunities of using these information to stratify patient survival.

Genomic mutations are hypothesized to be the origin of most cancers and MIBC has one of the highest total mutation burden [20]. Collectively, single-nucleotide mutations can be analyzed together with the nucleotide context where they occur, and this led to the identification of tri-nucleotide mutation signature [21]. The true role of mutation signatures remains unknown, but the extraction of different signatures can identify a footprint of a how a tumor develops, even the potential initiating cause. Mutational signatures were initially detected in human cancer by the Stratton group and documented in the Catalogue of Somatic Mutations in Cancer (COSMIC) [21]. Multiple groups have then used similar bioinformatics approaches to extract these signatures from TCGA and other cancer datasets. Our group has developed a package to extract signatures from tumors using an R framework [22] and applied it to the study of multiple bladder cancer datasets [23]. The most common signature in the bladder cancer is the two APOBEC signatures, signature 2 and 13, found in 67% of bladder cancers [19, 24]. Tumors enriched with *APOBEC* signatures were associated with higher total mutation burden (TMB) and APOBEC gene expression. While signatures are descriptive, high *APOBEC* tumors were associated with increased neoantigen load and prognostic of a two-fold improved survival of patients (38.2 months compared to 18.5 months, $p = 0.005$) with a 75% five-year survival probability. Our group validated these signatures in a cohort from the Beijing Genomics Institute (BGI) composed of both MIBC and NMIBC [24]. We confirmed the same association of *APOBEC* enrichment with improved survival but found that those patients of Asian race had a lower frequency of *APOBEC* signatures and *APOBEC* gene expression. In those tumors without *APOBEC* enrichment, we found increased frequency of oncogenic drivers *KRAS* and *FGFR3* that were inversely related APOBEC signatures, suggesting that despite the absence of *APOBEC,* these oncogenes may have been the driver of progression. Patients with MutSig2 from the TCGA had the lowest mutation rate and a 5-year probability of death of only 22%. Just as APOBEC mutations are associated with improved survival, signatures related to smoking (COSMIC Signature 5, *ERCC2*, *ERCC2**, MutSig4) were identified in 13.8% of bladder cancer patients. An analysis of mutated genes found in ERCC2 tumors identified an increased association with loss of function mutations of *ERCC2* and nucleotide excision repair. In their multi-variate analysis of features associated with survival, patients who had the best survival on multivariate Cox-regression analysis had the *ERCC2*/MutSig4 signature [19]. In a study comparing humans to mice, our group identified the same signature 5 as the only mutational signature directly induced by carcinogen exposure in the mouse BBN model. This opens the potential for comparison of different therapeutic strategies in a pre-clinical murine model [25].

MIBC has the third-highest rate of total mutations after melanoma and lung cancers, and the total mutation burden may be more prognostic than any one individual mutation since oncogenic drivers are rare. The most frequent mutations occur in the *TP53* and *RB1* genes (48% and 17%, respectively), but tumors with either mutation have no difference in the survival. The second group of frequently mutated genes encodes for COMPASS proteins (*KMT2C*: 19%, *KMT2D*: 28% and *KDM6A*: 26%). Interestingly, KMT2D and KDM6A had mutually exclusive alterations (5.1%, $p = 0.036$), but *KMT2D and KMT2C* mutations occur concurrently (8.7%, $p < 0.001$) [19]. While the role of KMT2D mutations has not been identified, mechanistic investigation of *KMT2C* and *KDM6A* may be critical for H3K27me3 demethylation, involving BAP1 [26]. This interaction may have therapeutic implications by treatment with inhibitors of EZH2 that are currently in trials.

Since the identification of the association between homologous recombination and sensitivity to chemotherapy, loss of function of genes and pathways involved in DDR has become a predictive biomarker for response to chemotherapy [27]. Approximately, 38% of patients with MIBC have mutations in genes involved in the DNA damage repair pathway. Multiple studies have identified mutations in the nucleotide excision repair gene, *ERCC2*, as predictive of response to cisplatin chemotherapy. Loss of function mutations in *ERCC2* were identified in 9 patients (out of 25) that were responders, compared to no ERCC2 mutations in non-responders [28]. Interestingly, the tumors with *ERCC2* mutations had a significantly higher total mutation burden (15.5 mutations/MB) compared to tumors without ERCC2 mutations ($p = 0.01$) suggesting correlation of ERCC2 with increased DNA damage. Evaluation of further cohorts with both a discovery ($n = 34$) and validation ($n = 24$) subcohorts identified mutations in Fanconi Anemia C (*FANCC*), *RB1* and *ATM* cisplatin susceptibility genes with significantly better pathologic response and overall survival in both cohorts when these mutations were present [29]. In a validation cohort of 45 patients, 8/20 responders had ERCC2 mutations compared to 2/28 non-responders [30]. These data were the basis for an Alliance Trial (NCT03609216), (A031701), "a phase II study of dose-dense gemcitabine plus cisplatin (DDGC) in patients with muscle-invasive bladder cancer with bladder preservation for those patients whose tumors harbor deleterious DNA damage response."

Based on these findings in patients with MIBC, an analysis of 48 tumors treated with chemoradiotherapy was

analyzed for mutations associated with response to treatment [27]. Of the 26 tumors with any DDR alteration, 12 had deleterious DDR mutations, and 6 with ERCC2 mutations. Only 1 of 8 patients with an alteration of in *ERCC2, BRCA1* or *PALB2* had a recurrence and ERCC2 mutations were associated with decreased metastatic recurrence (0 vs 43%, $p=0.04$).

Mutations in DDR-associated genes have also been associated with response to radiotherapy and immunotherapy [31]. Similar to chemotherapy and radiotherapy, a retrospective study of patients with metastatic bladder cancer treated with checkpoint immunotherapy identified an enhanced response when a DDR mutation was identified [32]. These loss of function mutations were associated with increased response (67%) compared to no DDR alteration (18%) and a predicted deleterious DDR mutation had an even greater response rate (80%). The median TMB (calculated by the MSK-IMPACT assay) was greater in deleterious DDR (19.4), compared to DDR mutations (10.2), and those with wild-type DDR (5.72). The increased DDR likely increases the neoantigens of the tumor, to enhance the immune response.

Bladder cancer is a heterogeneous disease featuring multiple patterns of gene expression and, hence, tumors can be grouped into molecular subtypes in a manner similar to breast cancer [30]. Molecular clustering is based on gene expression, and the number of subtypes varies depending on the genes that are examined (Fig. 1). For example, the BASE47 classifier groups tumors into only two subtypes, luminal and basal [33]. Luminal tumors were thought to arise as a consequence of expansion of differentiated luminal cells of the bladder, enriched for the expression of uroplakins compared to basal cell compartment located at the base of the lamina propria. These basal cells were hypothesized to be more similar to triple-negative breast cancers expressing high levels of cytokeratin 5 and 14 (KRT5 and KRT14) and stem cell markers (CD44). The two subtype systems evolved into four subtypes (TCGA I-IV) described in the TCGA that included luminal (I), luminal infiltrated (II), basal (III) and squamous (IV) tumors [17]. Further research from the UNC group suggested that a subgroup of basal tumors is more inflamed with immune infiltrates, but also had markers of exhaustion and were similar to claudin-low breast tumors [34]. With expansion of the 2017 publication by the TCGA to over 400 patients, which added NMF clustering methods, five subtypes by $k=5$ clustering were identified [19]. These subtypes were described as luminal papillary (formerly luminal or TCGA I), luminal (derived from the intermediate layer), luminal infiltrated (TCGA II), basal (TCGA III) and neuronal. These subtypes have varying frequencies from 35% (luminal papillary) to neuronal (5%). While clustering has evolved with bioinformatic methodology, the intrinsic biology of subtypes remains an important part of subtyping methodology. Luminal papillary tumors have papillary morphology and have a significantly lower stage compared to the other four subtypes (55% T2 compared to 23% of other subtypes). The molecular driver for luminal papillary is often *FGFR3* with 42/57 tumors from the TCGA with mutations, amplification, fusions or over-expression of *FGFR3*. Luminal tumors express high levels of uroplakins and the umbrella cell markers (KRT20 and SNX31). Luminal infiltrated tumors are distinguished by high rates of myofibroblasts, immune cells with increased expression of immune (PD-L1) markers and EMT markers. Basal tumors express

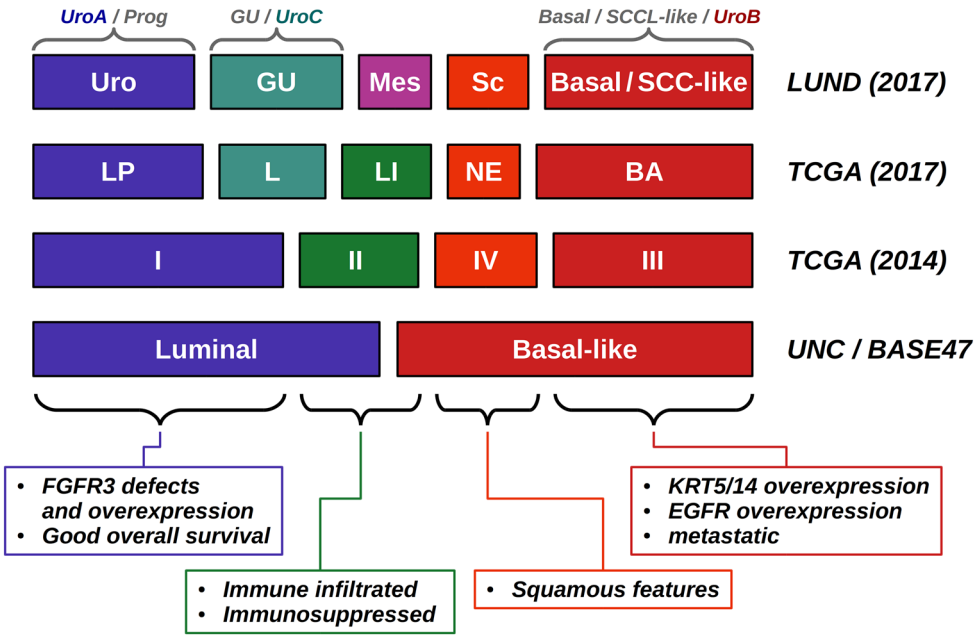

Fig. 1 Subtypes of MIBC according to different systems. Diagram summarizing molecular subtypes of bladder tumors, as identified by RNA-seq data and using different classification approaches. Different subtypes are somewhat overlapping, depending on the gene lists and criteria used for classification. The LUND 2017, TCGA 2017, TCGA 2014, and UNC/BASE47 systems are compared. Molecular and clinical attributes associated with specific tumor groups are indicated

high levels of basal (KRT5) and stem cell markers(CD44), signature 5 genomic mutations, CIS and immune expression. Similar to neuroendocrine tumors, 85% of tumors had *TP53* and *RB1* alterations including loss of *TP53* and *RB1*. Neuronal tumors are not synonymous with neuroendocrine bladder cancer as only 3/17 were histologically classified as NE. Neuronal tumors had the poorest survival and appeared to be the most aggressive subtype of bladder cancer.

Subtyping is truly a tumor segmentation approach defined by gene expression as few mutations are unique to each subtype [19]. In addition to the *FGFR3* mutations, luminal papillary tumors are enriched with *KDM6A* mutations (23%), which were also found more frequently in low-grade luminal tumors. Luminal papillary tumor also had the lowest frequency of *TP53* mutations (32%) and highest frequency of *STAG2* mutations (20%). This can be contrasted with basal tumors that had 60% mutations in *TP53*, 41% deletion of *CDKN2A* and 24% loss of function in *KMT2D* but only 19% mutations in *KDM6A*. This lack of direct co-concordance between mutations and subtypes has led many to speculate that subtypes are more plastic and may change over time or after exposure to different therapies. Targeting tumors based on molecular subtype may be a very challenging task, due to tumor heterogeneity, timing, progression, and location of the tumor sampled. Since subtyping is based on gene expression patterns, the methodology has evolved from microarray (first generation) to fully transcriptomics by RNA-Seq (second generation). We are likely near the third generation of subtyping in which each patient will likely be classified for diagnostic purposes based on a panel of genomic information that will take into account both tumor heterogeneity (for example, using RNA-seq data from multiple sampling locations, or single-cell analysis technology) and evolution (for example, by monitoring gene expression changes with respect to time or recurrence).

Lund molecular taxonomy

An alternative to the TCGA-based classification is the Lund molecular taxonomy [35]. The Lund taxonomy differs from subtyping described in earlier sections by inclusion of both gene expression profiling with immunohistochemical staining. The Lund Urobasal (Uro) subtype class encompasses UroA, UroB and UroC subtypes that are the most urothelial-like of Lund classification, similar to luminal tumors described by TCGA [13]. While many of the urothelial differentiation genes are shared, UroA tumors uniquely express the HoxA and HoxB genes suggestive of active retinoid and PPAR-gamma pathways [36]. UroB tumors express KRT5 and KRT14 of basal urothelial cells with significantly worse prognosis compared to UroA and UroC. UroC has features similar to genomically unstable (GU) tumors but distinct from GU tumors by expression of FGFR3, CCND1 and RB1 with low CDKN2A expression. GU tumors have the highest rate of *TP53* mutations, inactivation of RB1 and high CDKN2A with the highest overall total mutation burden. The Lund basal/SCC-like subtype tumors express high levels of KRT5, KRT14 and low levels of GATA3 and FOXA1 with increased MYC activity. Mesenchymal-like (Mes-like) tumors express high levels of ZEB2 and VIM with no expression of KRT5, KRT14, GATA3 or FOXA1. The neuroendocrine-like (Sc/NE-like) tumors are distinguished by the expression of FOXM1/MYBL2/E2F1/E2F2/MuvB with only *MYCN* activity and no uroplakin expression [34]. Recently, the Lund classification was converted to a single-patient classifier using only gene expression (the LundTax classifier) [37].

Combining genetic risk stratifiers

While basic research on risk stratification in bladder cancer is promising, there are few CLIA-grade biomarkers that directly impact treatment. Next-generation sequencing of patients with metastatic cancer, by any commercial platform, may identify drivers that can be targeted in bladder cancer. While these targeted sequencing panels can expose the "long tail" of urothelial carcinoma, they often describe the overall mutation rate or total mutation burden (TMB). From studies of PD-1/PD-L1 inhibitors, a higher TMB has been associated with response to immuno-oncology (IO) agents, and analysis of IMVigor 210 and 211 cohorts has demonstrated greater response to metastatic bladder cancer in patients with high TMB [38]. The identification of DDR mutations may have direct interaction with TMB and both have been shown to directly impact response to IO and systemic chemotherapy. Thus, targeted genomic sequencing with TMB may have direct impact on patient care. We hypothesize that subtyping may predict response to therapy, while the choice of treatment may take into account the rare number of activating mutations in bladder cancer (Fig. 2). Finally, two studies of tumor subtyping by RNA expression analysis of pre-treatment bladder tumors identified basal tumors to have the most significant survival benefit to neoadjuvant chemotherapy [39]. Thus, multiple RNA expression platforms are under development to combine both subtyping and immune scores. Currently, there is one RNA based platform by GenomeDx that mirrors their prostate Decipher [39]. We caution hesitation to utilize this classifier clinically, as it has not been prospectively validated. We anticipate that several trials will test the function of these expression platforms in the near future.

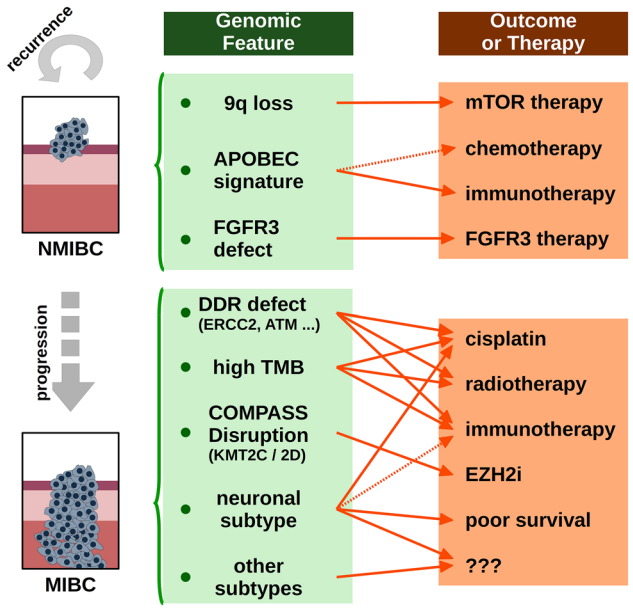

Fig. 2 Leveraging genomic data to maximize treatment safety and efficacy. Based on the molecular alterations detected in a tumor, it may be possible to predict patient trajectories and recommend optimal treatments. The diagram summarizes some of the distinctive molecular alterations commonly identified in NMIBC and MIBC, and links them to the expected patient outcome, as well as to the therapeutics that could be beneficial to the patient, given the current understanding of tumor biology, trial results, and in vitro or in vivo data

Conclusions

Bladder cancer is a heterogeneous disease, comprising many tumor subtypes with differences in histology, genomic aberrations, as well as prognosis and sensitivity to anti-cancer treatments. Incorporating genomic information in the diagnostic process has the potential to transform the way bladder cancer is treated and improve outcomes. In the last 10 years, many genomic determinants of bladder cancer were identified, and specific associations with tumor pathology and patient trajectories were revealed. In the decade to come, we anticipate that NGS-based analyses will be leveraged to customize anti-cancer treatments and procedures, in an effort toward precision oncology. Cancer subtypes that are known to be more likely to recur or progress may be treated using more aggressive treatments closer follow-up recommended. Likewise, patients affected by invasive tumors may benefit from a more comprehensive characterization not just limited to the tumor histology, but expanded information of gene expression, gene mutations, and mutational signatures. Bioinformatic approaches allowing identification of patients with high neo-antigen-load tumors or strong immune-suppression already exist and could be applied in the clinic to maximize drug safety and efficacy. In conclusion, leveraging tumor genomic data is expected to improve classification of bladder cancer patients, revealing risks and predicting efficacy of different therapeutic options and this will be a crucial step towards precision oncology.

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Compliance with ethical standards

Ethical statements All research involving human subjects should refer back to primary studies.

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