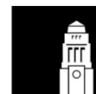

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Report from the International Society of Urological Pathology (ISUP) Consultation Conference On Molecular Pathology Of Urogenital Cancers. II. Molecular Pathology of Bladder Cancer: Progress and Challenges

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Abstract

During the 2019 International Society of Urological Pathology Consultation Conference on Molecular Pathology of urogenital cancer, the Working Group on Bladder Cancer presented the current status and made recommendations on the diagnostic use of molecular pathology, incorporating a pre-meeting survey. Bladder cancers are biologically diverse and can be separated into “molecular subtypes,” based on expression profiling. These subtypes associate with clinical behavior, histology, and molecular alterations, though their clinical utility has not been demonstrated at present and use in bladder cancer is not recommended. Mutations in the *TERT* promoter are present in the majority of bladder cancers, including the non-invasive stage of tumor evolution, but not in reactive conditions. Mutational anlaysis of *TERT* promoter thus distinguishes histologically deceptive cancers from their benign mimics in some cases. A minority of pathologists employ this test. *FGFR3* mutations are common in bladder cancer, and metastatic urothelial carcinoma with such mutations frequently responds to erdafitinib, an *FGFR* inhibitor. Testing for *FGFR3* alterations is required prior to using this drug. Metastatic urothelial carcinoma responds to immune oncology agents in 20% of cases. These are approved as first and second line treatments in metastatic urothelial carcinoma. Several biologic parameters associate with response to immune oncology agents, including tumor mutational burden, molecular subtype, and infiltration by PD-L1-positive lymphocytes, detected by immunohistochemistry. PD-L1 immunohistochemistry is mandatory prior to administering immune oncology agents in the first line setting. In

conclusion, much has been learned about the biology of bladder cancer, and this understanding has improved care of patients with the disease.

Introduction

There are over 500,000 new cases of bladder cancer diagnosed each year worldwide(1). The vast majority are urothelial carcinoma (UC)(2). From a treatment standpoint, bladder cancer is divided into either “non-muscle invasive” or “muscle-invasive” carcinoma. Non-muscle invasive carcinoma accounts for over 70% of new bladder cancer diagnoses(2). This category includes non-invasive papillary UC, flat carcinoma in situ (CIS), and invasive cancer that appears limited to the lamina propria. Treatment is conservative for most cases, with complete resection of cytoscopically visible tumor, often followed by intravesical Bacillus Calmette-Guerin (BCG) if the tumor has high-risk features, such as high-grade histology or lamina propria invasion(3). Muscle-invasive carcinoma includes all carcinomas with evidence of involvement of muscularis propria. It is treated definitively in most cases, typically cystectomy preceded by neoadjuvant chemotherapy (NAC), or chemoradiation(3).

Invasive bladder cancer most commonly has the histomorphology of conventional UC, though a minority are histologic variants. Conventional UC and histologic variants are often present in different areas within the same tumor, with variants arising from conventional UC in most cases. Several histologic variants are uniquely aggressive, and treatment differs from conventional UC. Important variants

include plasmacytoid, squamous, glandular, micropapillary, sarcomatoid, and small cell carcinoma(4).

The purpose of this paper is to review the molecular pathology of bladder cancer and provide recommendations regarding the use of molecular diagnostics based on the pre-meeting survey (**Table 1**) and discussions during the 2019 ISUP consultation conference on molecular pathology in Washington DC. It should be noted recommendations in this manuscript are the result of survey responses and opinions of member in the Working Group, and do not reflect fresh scientific data.

Molecular subtypes of muscle-invasive bladder cancer – biological features

Muscle-invasive bladder cancer may be divided into molecular subtypes based on gene expression, and these molecular subtypes associate with clinical behavior, histology, and treatment response(5-8) (**Figure 1**). Several different molecular classification systems have been developed, with four standing out, developed by The Cancer Genome Atlas (TCGA) consortium(7), Lund University(9, 10), MD Anderson Cancer Center(5), and University of North Carolina(6). A system that combines these molecular subtypes into a unified schema has also been proposed (11). Molecular subtypes within these systems are largely similar, though they differ in clinically and biologically meaningful ways (**Figure 2**).

In these molecular classification systems, over 90% of muscle-invasive bladder cancers classify as either luminal or basal-squamous subtypes, though terminology differs, particularly within the classification system developed at Lund University (5-8). Luminal tumors express high levels of genes associated with urothelial differentiation,

such as GATA3 and uroplakins, and low levels of genes associated with basal or squamous differentiation, such as high molecular weight keratins and p63. Basal-squamous tumors have the opposite expression pattern. The luminal subtype is enriched in tumors with concurrent noninvasive papillary UC, and tends to harbor copy number losses of *CDKN2A* and mutations in *FGFR3*(7). Basal-squamous tumors have a disproportionately high frequency of *TP53* mutation(7).

A small subset of muscle-invasive bladder cancers lacks expression of luminal and basal-squamous genes, and expresses high levels of neuroendocrine genes, such as SOX2 and TUBB2B. These are referred to as “neuronal” or “small cell/neuronal-like,” depending on the classification system(7, 9, 10). Many of these have a histomorphology of conventional UC.

Luminal tumors can be further subtyped by other biological features. For example, the TCGA system classifies luminal tumors into three subgroups: luminal-infiltrated, luminal (without a modifier), and luminal-papillary(7). Luminal-infiltrated tumors express strong stromal and inflammatory signatures. Luminal tumors (without a modifier) have a stromal signature, but a minimal inflammatory signature. Luminal-papillary tumors lack both stromal and inflammatory signatures(7). These subtypes differ in clinical behavior, with superior overall survival seen in luminal-papillary tumors. The system developed at Lund University subdivides luminal tumors based on cell cycle alterations, into urothelial-like and genomically unstable subtypes(8-10). Urothelial-like tumors express genes active in the early part of the cell cycle, while genomically unstable tumors express genes active in the late part of the cell cycle(9). These differences produce distinct profiles by immunohistochemistry. Urothelial-like tumors are

positive for cyclin D1 and RB1, but negative for p16, while genomically unstable tumors are positive for p16, but negative for cyclin D1 and RB1(10). Urothelial-like tumors tend to express FGFR3, while genomically-unstable tumors do not (10). Genomically unstable tumors have greater karyotypic complexity and higher mutational burden than urothelial-like tumors(9).

Basal-squamous cancers can also be further subdivided, dependent on the classification system. The University of North Carolina system recognizes a “claudin-low” subtype, defined by low expression of claudins, remarkably high expression of immune signatures, and enrichment in *EGFR* amplification (12). The Lund system subgroups basal-squamous tumors into basal-squamous-infiltrated and basal-squamous (without a modifier), distinguished by intensity of inflammatory signature(9). That is, the large majority of basal-squamous tumors express a strong immune signature, but this is particularly strong in the basal-squamous-infiltrated subtype. The Lund system also classifies a small subgroup of basal-squamous tumors as “mesenchymal-like,” defined by high expression of epithelial to mesenchymal transition genes, such as *ZEB2*(8-10).

While molecular subtypes appear discreet based on these molecular classification systems, there is a considerable degree of complexity and overlap among subtypes. The most recent iteration of the Lund system attempts to account for this by creating multiple smaller groups within their main subtypes. For example, the urothelial-like subtype is split into three groups: UroA, UroB, and UroC(9). UroA is the prototypical urothelial-like cancer, expressing high levels of luminal genes and low levels of basal genes, as well as high FGFR3, cyclin D1, and RB1, and low p16(9). UroB expresses

relatively high levels of basal-squamous genes, and many would be considered basal-squamous in other classification systems(9). UroC lacks FGFR3 expression, and has features in common with genomically unstable cancers(9).

Some classification systems recognize tumors that are heavily infiltrated by stromal cells. These include the “p53-like” subtype in the MD Anderson Cancer Center system, and the “scar-like” subtype identified in a recent multi-institutional collaboration (5, 13).

Specific histologic variants tend to have specific molecular subtypes. Invasive bladder cancer with squamous histology tends to have a basal-squamous molecular subtype(7). Micropapillary carcinoma tends to be luminal(14), and may be either genomically unstable or urothelial-like in the Lund system(15). Nested, plasmacytoid, and non-enteric glandular carcinoma also appear to be predominantly luminal(15, 16), though these histologic variants are less well-studied. Sarcomatoid carcinoma may be of the mesenchymal-like subtype in the Lund scheme, but is more often of another subtype, and may be urothelial-like, genomically unstable, or basal-squamous(15, 17). Examples of histologic variants and markers of molecular subtype are shown in **Figure 3**.

Clinical significance of molecular subtypes of invasive bladder cancer

Clinical outcomes differ among molecular subtypes of bladder cancer in several scenarios. First, molecular subtype associates with overall survival in patients undergoing cystectomy without neoadjuvant chemotherapy (NAC). In the TCGA system, luminal-papillary tumors have the best overall survival, 60% at 5 years, while neuronal-

like have the worst overall survival, 15% at 5 years(7). In the Lund system, UroA tumors have the best overall survival, 60% at 5 years, while UroB and small cell/neuroendocrine-like tumors have the worst overall survival, both ~20% at 5 years(9). Second, in patients with invasive bladder cancer limited to the lamina propria (stage T1), molecular subtype may gauge risk of progressing to muscle-invasive disease. A study by the Lund group showed genomically unstable and basal-squamous T1 cancers have considerably higher rates of progression compared with urothelial-like tumors, 50-60% vs 80% at 5 years(18). Third, molecular subtype appears to associate with benefit from cisplatin-based NAC. A multi-institutional study demonstrated this using a four-group classification, which classified tumors as luminal, luminal-infiltrated, basal, or claudin-low(19). It reported that NAC confers greatest benefit to patients with basal tumors. Unexpectedly, the clinical benefit was independent of pathologic response in this subtype. Though patients with basal tumors appeared to benefit most from NAC, a subset of patients with tumors of other molecular subtypes benefitted from NAC, seen as pathologic response in these other subtypes. The findings thus indicate this test cannot identify which patients will fail to respond to NAC. Notably, an early study showed tumors with a "P53-like subtype" are resistant to NAC(5), though this finding has not been reproduced to our knowledge. Since molecular subtyping cannot identify the patients who do not benefit from NAC, the ISUP working group on bladder cancer does not recommend molecular subtyping to guide treatment with NAC in the routine setting. Likewise, only a minority of pathologists reported ordering tests to guide use of NAC in the ISUP survey (**Table 1**).

Molecular subtypes may differ in response to immune-oncology (IO) agents –this is addressed in detail later in this review.

Intratumoral heterogeneity of molecular subtype in invasive bladder cancer

Several lines of evidence indicate that muscle-invasive bladder cancer is often heterogeneous in molecular subtype, particularly in cases with co-occuring conventional urothelial carcinoma and histologic variant (15, 20, 21). A recent study reported a series of muscle-invasive bladder cancers with spatially distinct regions of conventional UC and histologic variant, which were separately subtyped using the Lund system(15). This showed that tumors of the basal-squamous subtype often co-occur with either urothelial-like or genomically-unstable urothelial carcinoma(15). In contrast, it found no co-occurrence of urothelial-like and genomically unstable carcinoma, suggesting the cell cycle alterations that underlie these subtypes occur early in tumor evolution. Another study showed urothelial carcinomas, particularly of the basal-squamous subtype, often have lymph node metastases with a different molecular subtype(21). These findings provide insight into the role of molecular subtypes in tumor evolution and plasticity, and raise concern for sampling error in laboratory tests that use molecular subtypes to guide therapy.

Molecular classification of early stage bladder cancer

Varible nomenclature has created challenges in the molecular classification of early stage bladder cancer. Treating physicians emphasize the dichotomoy of muscle-invasive vs non-muscle-invasive disease, given treatment implications, and often lump all non-muscle-invasive tumors together when designing molecular studies. In contrast,

pathologists tend to see a stark difference between non-invasive carcinoma and invasive carcinoma limited to the lamina propria, and approach classification accordingly. This may create confusion, particularly if “non-invasive” is taken as shorthand for “non-muscle-invasive.” To prevent confusion in this portion of the review, we use the term “non-invasive” to include only stage Ta non-invasive papillary UC and CIS. We use “non-muscle-invasive” to also include invasive carcinoma limited to the lamina propria.

Molecular diversity in non-invasive bladder cancer differs considerably from that seen in muscle-invasive bladder cancer. Noninvasive UC is classified histologically into non-invasive papillary UC or flat carcinoma *in situ* (CIS), though both may coexist in the same patient. Classically, low-grade, non-invasive papillary UC has a high frequency of *FGFR3* mutation(22) . Evidence suggests these progress to high-grade and invasive carcinoma through mutations in *TP53* and chromosomal losses of 9p21, the locus that includes *CDKN2A*(23-25). In contrast, most CIS lesions develop *TP53* mutations early in evolution, and do not acquire *FGFR3* mutations(23). Regarding molecular subtypes, over 95% of non-invasive papillary urothelial carcinoma and CIS strongly express markers of urothelial differentiation, and weakly express markers of basal-squamous differentiation(15, 26). In the Lund system, the large majority of non-invasive papillary carcinoma are urothelial-like, and CIS may be either urothelial-like or genomically unstable(15).

CIS typically has a “CIS signature,” a 16-gene classifier that is expressed in flat CIS, early stage invasive carcinoma with associated CIS, and a large fraction of muscle-invasive bladder cancers(27), particularly muscle-invasive cancers with a basal-

squamous subtype(7). This signature is usually absent in invasive UC without associated CIS(27). The CIS signature incorporates a diverse set of genes, including BIRC2, an inhibitor of apoptosis, and SDCBP, a linker of syndecan signaling to the cytoskeleton. Separately, greater genomic instability has been reported in muscle-invasive bladder cancers with concurrent CIS compared to those without associated CIS(28).

While the vast majority of non-invasive papillary carcinomas have a luminal subtype, there is substantially molecular diversity among cases(29). The most clinically relevant diversity relates to genes operative in the cell cycle. Tumors with greater activation of the cell cycle have higher rates of recurrence and progression to muscle-invasion(29). This has been shown with various methods, including complex expression signatures(29), immunohistochemistry for cell cycle genes such as cyclin D1(30, 31), Ki-67 labelling index(31), and mitotic index(32). Non-invasive papillary carcinomas are also somewhat diverse in expression of luminal markers, with some tumors being more “luminal” than others, but this does not appear to affect clinical behavior(29, 33).

In the largest series to date that evaluated expression profiles of non-muscle-invasive bladder cancer, Hedegaard *et al*. reported RNA sequencing in 460 patients with non-muscle invasive bladder cancer. (29). The study combined non-invasive papillary UC and invasive UC limited to the lamina propra (stage T1) in the analysis (a few cases of CIS were also included, with a small group of muscle-invasive cancers for comparison). Tumors were classified into three subtypes based on activity of the cell cycle (early vs late cell cycle activation) and relative expression of luminal and basal-squamous genes. Subtypes were named Type 1 (early cell cycle active, higher luminal

expression), Type 2 (late cell cycle active), and Type 3 (early cell cycle active, lower luminal expression). In keeping with prior findings, Type 2 tumors, which included the highest proportion of T1 samples, had greater propensity to progress to muscle invasion, while expression of luminal genes was not associated with outcome (i.e. there was no difference in progression in Type 1 vs Type 3 tumors)(29). This study has greatly expanded our understanding of early stage bladder cancer, but is limited by combining non-invasive papillary carcinoma with T1 carcinomas in the analysis. Details of this study are shown in **Figure 4**.

Non-invasive papillary UC are also diverse in chromosomal instability(34). Broadly separating tumors into chromosomally stable and unstable groups, the unstable group is enriched in tumors with higher proliferation, greater mutational burden, and high-grade histology(34). Unstable tumors may have lower recurrence free survival, though the study describing this did not find a statistically significant association(34).

Because molecular subtyping of non-muscle invasive bladder cancer has not demonstrated clear value in clinical decision making, the ISUP working group on molecular pathology of bladder cancer does currently recommend it on a routine basis, keeping with practice patterns identified in the ISUP survey (**Table 1**).

***TERT* promoter mutation**

Mutations in the promoter of the gene *TERT* are present in 60-80% of urothelial carcinomas(35-40). Mutations predominantly occur at two residues, -124 and -146 base pairs from the transcription start site. These are typically C>T transversion mutations. Mutation at -124 is more common. *TERT* promoter mutations are an early event in

bladder cancer evolution, present in the majority of both CIS and non-invasive papillary urothelial carcinomas, including low-grade tumors(36, 38, 39). Papillary urothelial neoplasm of low malignant potential (PUNLMP) also commonly harbors *TERT* promoter mutation, seen in 30-60% of cases(38, 39). Studies differ in the frequency of *TERT* promoter mutations identified in urothelial papillomas, with reported rates up to 46%, and some studies showing 0%(40, 41). *TERT* promoter mutations are rare in inverted urothelial papilloma, with most studies showing inverted papillomas lack these mutations(39, 41-43). Some authors take this, as well as the benign behavior and near ubiquitity of mutations in the MAP kinase/ERK pathway in these lesions, as evidence that inverted papillomas are a distinct type of indolent low-grade urothelial neoplasia that does not progress to carcinoma(44). *TERT* promoter mutations are retained as tumors evolve from non-invasive to invasive carcinoma. Likewise, these mutations are seen in the majority of bladder squamous cell carcinoma(45), UC with glandular differentiation(46), sarcomatoid carcinoma(40), plasmacytoid UC(47), and micropapillary UC(48). TERT promoter mutations are rare in enteric-type bladder adenocarcinomas and urachal carcinomas(46, 49).

TERT promoter mutations do not occur in reactive urothelial proliferations. Thus, they have great diagnostic utility in distinguishing urothelial carcinoma from its benign mimics. Mutational analysis of the *TERT* promoter distinguishes nested urothelial carcinoma from proliferative cystitis(50), and noninvasive papillary neoplasia from polypoid cystitis(43), with high specificity, albeit at only 60-80% sensitivity. The high specificity and presence in low-grade disease make analysis of *TERT* promoter superior to the immunohistochemical panel of CK20/CD44/p53, because these markers are

relatively non-specific and utility is limited to distinguishing CIS from reactive atypia(51). *TERT* promoter mutations are also rare in prostatic adenocarcinoma(52), and may be useful in distinguishing these from urothelial carcinoma, particularly highly evolved variants, such as small cell carcinoma(53, 54). However, immunohistochemistry readily distinguishes prostate cancer from urothelial carcinoma in the large majority of cases, using markers such as NKX3.1 and PSA(55), making *TERT* promoter unnecessary in distinguishing prostate from bladder cancer in most cases.

In contrast to molecular subtype, there is minimal intratumoral heterogeneity in *TERT* promoter mutations in urothelial carcinoma(56), corroborating *TERT* promoter mutation as an early event in bladder cancer evolution. Despite its potential utility, a minority of pathologists use *TERT* promoter analysis, per the ISUP survey (**Table 1**).

FGFR3 alterations

Oncogenic alteration of *FGFR3* is present in approximately 15% of muscle-invasive bladder cancers. The majority are activating point mutations in exon 7 or exon 10, and a minority are activating rearrangements, such as *FGFR3-TACC3* fusions (7). The luminal subtype of bladder cancer is enriched in *FGFR3* mutations and FGFR3 overexpression(7, 9). Muscle-invasive cancers that harbor *FGFR3* alterations tend to have a characteristic histology, seen as a bulky, exophytic papillary neoplasia, composed of tumor cells with irregular, koilocytoid nuclei(57). While a subset of basal-squamous cancer harbor *FGFR3* alterations, probably because they evolved from a noninvasive papillary UC, the majority of basal-squamous tumors lack these alterations and underexpress FGFR3(7). FGFR3 is a target of erdafitinib, a pan-FGFR inhibitor.

Recently, results of a phase II clinical trial showed objective response rates of approximately 30% in patients with metastatic or unresectable urothelial carcinoma who received erdafitinib(58, 59). Only patients with oncogenic *FGFR3* mutation, or *FGFR2* or *FGFR3* fusion (*FGFR2/3* fusion), were included in the trial(59). Superior response was seen in tumors with *FGFR3* mutation compared with *FGFR2*/3 fusion, 49% vs 16% response rates, respectively(59). The United States Food and Drug Administration (FDA) has granted accelerated approval of this drug in patients with advanced or metastatic urothelial carcinoma, with relevant *FGFR* alterations, whose disease has progressed during or following treatment with platinum-based chemotherapy, including in the adjuvant and neoadjuvant settings(58). Testing may be performed using the FDA-approved companion diagnostic (a specific RT-PCR kit (58)), or other methods, including next generation sequencing(60). The ISUP working group does not advocate any specific method for interrogating *FGFR3*. Instead, we emphasize testing must be performed using a validated test in an accredited laboratory, and recommend the pathologist be familiar with technical performance of the assay. The National Comprehensive Cancer Network (NCCN) has recently recommended that molecular/genomic testing be performed on patients with Stage IIIB, IVA, and IVB bladder cancer, preferably at the time of diagnosis. Though there may be benefits to this approach, the ISUP working group does not recommend testing all patients with advanced stage disease at the time of diagnosis. Instead, we recommend FGFR testing be performed more selectively, on patients with advanced disease who have progressed following platinum-based therapy, or who have another indication to perform testing, based on the judgement of treating physicians. This recommendation prevents

unnecessary testing that burdens patients and healthcare systems with avoidable financials costs.

Mutations in *FGFR3* are more common in non-muscle invasive bladder cancer(8), particularly non-invasive papillary urothelial carcinoma, 75% of which have mutation in this gene(22). In patients with non-muscle invasive bladder cancer, mutation in *FGFR3* is association with improved clinical outcomes, specifically lower rates of progression to muscle-invasive disease(61-66). For example, van Kessel et al. showed non-muscle invasive bladder cancers harboring *FGFR3* mutation have lower risk of progression to muscle invasion, though this association was not seen on multivariant analysis including tumor grade(66). Similarly, van Rhijn et al. combined *FGFR3* mutational analysis and MIB-1 index to generate a “molecular grade” in a cohort of patients with non-muscle invasive bladder cancer(65). This molecular grade predicted progression to muscle invasion, including in a multivariate model incoporating tumor grade. Grade was not significant in this model, arguing that the molecular test largely acted as a more informative proxy for tumor grade. The close association between *FGFR3* status and grade in these studies may be telling. That is, *FGFR3* mutation is common in non-invasive papillary UC, including low-grade tumors, but is rare in CIS(22). Thus, studies that pool diverse non-muscle invasive cancers together, and separate them based on *FGFR3* status, likely generate one group heavily enriched in non-invasive papillary UC, including low-grade tumors, and another group highly enriched in CIS and T1 disease associated with flat CIS. The former group would behave more indolently with such a grouping, likely explaining the findings of these studies.

The ISUP working group on molecular pathology of bladder cancer considers it premature to replace histologic grade with a molecular test, or incorporate *FGFR3* mutation analysis into clinical decision making in patients with non-muscle invasive bladder cancer, and thus does not recommend these.

Immuno-oncology

Tumors defend against immune attack by upregulating genes active in “immune checkpoint”, including CTLA-1 and PD-L1, which inhibit immune cells directed against the tumor(67). Drugs that target immune checkponts, so-called immune-oncology (IO) agents, have dramatically changed the treatment of metastatic bladder cancer. Cisplatin is the standard first line therapy for metastatic bladder cancer(3). However, many patients are ineligible for cisplatin because of comorbidities, including chronic kidney disease, peripheral neuropathy, and poor functional status. IO agents are the first successful first line treatment for metastatic bladder cancer in decades, and may be given to cisplatin ineligible patients. At the time of writing, two IOs - pembrolizumab and atezolizumab - have been FDA approved for first line therapy in metastatic UC(68). Five agents – pembrolizumab, atezolizumab, durvolumab, avelumab, and nivolumab - have been FDA approved as second line agents in metastatic UC(69). Response is seen in approximately 20% of patients, many of these durable(70-76).

Several features of bladder cancer associate with response to IOs (**Table 2**). First, tumors that are innately infiltrated by CD8 T cells tend to respond(75). Likewise, greater response is seen in tumors expressing genes associated with effector CD8 cells, such as CXCL9 and CXCL10, and tumors with high interferon gamma signaling, a

biologic process associated with CD8 T cell activation(75, 77). Second, tumors with a greater number of somatic mutations, i.e. tumor mutational burden, tend to show greater response to IOs(75, 76). Third, molecular subtypes of bladder cancer have differential response rates to IOs. For example, infiltrated tumors, such as basal-squamous and luminal-infiltrated cancers, appear more sensitive to IOs than luminal tumors without inflammation(76). Uniquely, genomically unstable tumors appear disproportionately sensitive to IOs, even in cases without significant inflammation(77). This may relate to the high tumor mutational burden in this subtype. Tumor mutational burden provides predictive information beyond molecular subtype, because tumors with higher mutational burden have superior response to IOs within individual subtypes(75). Fourth, tumors are more likely to respond to IOs if they are infiltrated by PD-L1+ tumor infiltrating lymphocytes(70, 71, 73-76, 78). Some data suggest that cancers with PD-L1+ tumor cells are more likely to respond to IOs, but the evidence is comparatively weak (70, 71, 76). Immunohistochemistry for PD-L1 in bladder cancer is shown in **Figure 5.**

Although many biological features associate with response to IOs, a subset of tumors lacking these features do respond, and no biomarker can satisfactorily predict which patients will fail treatment. However, biomarker testing for PD-L1 expression does have an important and emerging role in guiding use of IOs, specifically in patients receiving them in the first line setting. In two ongoing phase 3 clinical trials (Keynote 361 for pembrolizumab, ClinicalTrials.gov Identifier NCT02335424; and IMVigor 130 for atezolizumab, ClinicalTrials.gov Identifier NCT02108652), patients in single-agent IO arms had inferior survival compared to those in standard of care chemotherapy arms, but only if the tumor had low PD-L1 expression by immunohistochemistry(68).

Therefore, patients whose tumors express high PD-L1 may still receive IO monotherapy, but those with tumors that express low PD-L1 should receive cisplatin or carboplatin-based chemotherapy(79, 80). However, if patients are ineligible for platinum-based agents, they should receive IO therapy regardless of the tumor's PD-L1 expression status(80). Immunohistochemistry is thus mandatory in the first line setting for most patients, with FDA approved companion diagnostics for both pembrolizumab and atezolizumab(81, 82).

Keynote 361 and IMVigor 130 used different scoring systems to evaluate PD-L1 expression in tumors, and different antibody clones in their immunohistochemistry assays, creating a challenge in implementing these as diagnostic tests. That is, different antibodies for PD-L1 immunohistochemistry must be used for different IOs, and their evaluation criteria differ. Keynote 361 for pembrolizumab used Dako antibody 22C3, and quantified expression using a "combined positive score," the number of cells expressing PDL1 (including tumor cells, lymphocytes, and macrophages) divided by the total number of tumor cells x 100(82). A score ≥10 is considered positive. In contrast, IMVigor 130 for atezolizumab used Ventana SP142, and quantified as the percent area of the tumor involved by tumor infiltrating immune cells that express PD-L1, with ≥5% considered positive(82). Concordance is good between these two antibodies when using their FDA-approved criteria for evaluation, with kappa values in the range of 0.6-0.8(83, 84). Despite similarity, these antibodies should not be used interchangably. Evaluation of these immunostains may be challenging given their complexity. The ISUP working group thus recommends labs that interpret PD-L1 immunohistochemistry (companion diagnostics) should have protocols for internal quality control, to ensure

consistency in results, though we make no specific recommendation on how those should be constructed at the present time. While no companion diagnostic is required for use of IOs in the second line setting, several “complementary” tests are available, which may help oncologists guide therapy, but these are not required. The evaluation criteria for different IOs are presented in **Table 3** (80-82, 85-89). Comparison of different antibodies for PD-L1 is presented in **Figure 6**.

The ISUP survey revealed that a narrow majority pathologists perform PD-L1 immunohistochemistry in their own laboratory, and typically order at request of treating physicians (**Table 1**). The ISUP working group recommends that immunohistochemistry for PD-L1 be performed routinely on metastatic bladder cancer, with the appropriate antibody for a given drug. Testing may also be performed selectively in patients without metastatic disease, who are not candidates for standard therapy, based on the judgement of treating physicians.

The FDA has also approved pembrolizumab in any solid tumor that is microsatellite instability (MSI)-high or mismatch repair (MMR) deficient, that has progressed following prior treatment and has no satisfactory alternative treatment option(90, 91). MSI can be measured using PCR based methods, and MMR deficiency can be tested with immunohistochemistry for MLH1, PMS2, MSH2, and MSH6, with loss of expression considered MMR deficiency(92). Less than 1% of bladder cancers are MSI-high(93). However, invasive upper tract urothelial carcinomas are MSI-high/MMR-deficient in approximately 20% of cases(94). Assaying for MSI or MMR is low-yield in bladder cancer, but potentially useful to guide treatment in upper tract UC that has metastasized. Upper tract urothelial carcinoma is a characteristic tumor of Lynch

syndrome, an inherited cancer syndrome in which a mismatch repair gene is deleteriously mutated(95). Cancers arising in Lynch syndrome are MMR deficient in the majority of cases, and undiagnosed cases of this syndrome can be detected by screening specific tumor types for MMR deficiency, such as endometrial cancer colorectal carcinoma(96). Given the association between Lynch syndrome and upper tract UC, screening these tumors for MMR deficiency can also detect undiagnosed cases of Lynch syndrome(95). The ISUP working group recommends universal screening of newly diagnosed upper tract UCs with immunohistochemistry for MLH1, PMS2, MSH2, and MSH6.

Urine biomarkers for disease monitoring

In patients with bladder cancer, tumor cells are frequently shed into the urine, making urine a useful specimen to monitor for recurrence, particularly in patients with non-muscle invasive disease. Indeed, cytologic evaluation of urine is standard of care in monitoring for recurrence in this patient population. Urine may also be subject to molecular studies. Fluorescence *in situ* hybridization (FISH) for chromosomal alterations is commonly utilized, usually the Urovysion assay, which interrogates cells shed in the urine for aneuploidy of chromosomes 3, 7, and 17, and losses in 9p21, common events in high-grade bladder cancer(97). Adding this test to standard urine cytology increases sensitivity for detecting recurrence according to most studies(98). Patients with normal cystoscopy and negative urine cytology are at substantially increased risk of recurrence if FISH is positive, a situation termed “anticipatory positive.”(99) However, other than predicting a positive finding later, the value of urine FISH is not firmly established. The

test is not necessary in following up patients with early stage bladder cancer in the opinion of the ISUP working group.

Mutational analysis may also be performed on tumor cell DNA from urine specimens. Described tests typically evaluate combinations of genes altered in bladder cancer, such as *FGFR3* and *TERT* promoter, with focus on mutational hotspotspots(100-104). This may be combined with methylation analysis of specific genes(105). Urine-based mutational tests have higher sensitivity than urine cytology, and can detect low-grade neoplasia, an advantage over FISH. However, as with FISH, a positive mutational analysis may warn of a future recurrence, but is itself insufficient evidence to treat for recurrence. This test may be useful to monitor some patients and triage cystoscopy in the future, but its clinical value in not yet clear. The ISUP working group on molecular pathology of bladder cancer does not recommend it at this time. Most pathologists do not routinely use urine-based molecular tests at present, as shown in the ISUP survey (**Table 1**).

Urine biomarkers for primary diagnosis

Molecular tests in the urine, both FISH and mutational analysis, are often positive in patients with newly diagnosed bladder cancer(104). However, any patient with a positive urine molecular test still needs conventional workup to establish the diagnosis, including cystoscopy with biopsy. Furthermore, it is unclear how one should handle a positive urine molecular test and a negative workup. While molecular tests may have a role in the primary diagnosis of bladder cancer in the future, such as identifying patients

who can safely forgo full work up(106), their use is inappropriate in this setting at the present time.

Circulating tumor cells and circulating tumor DNA

Recently developed technologies can identify tumor cells within a patient's blood, termed circulating tumor cells (CTCs). Currently, the only FDA-approved system for collecting and enumerating CTCs is the CellSens system(107). In short, this is an immunomagnetic system that uses antibodies against EpCAM and pan-cytokeratin to positively identify carcinoma cells, and CD45 to negatively select leukocytes(108). Additional antibodies may be added to select subgroups of CTCs, such as HER2 in breast cancer(109). This system can assess the burden of CTCs, but cannot isolate them for molecular analysis. Increased burden of CTCs associates with aggressive disease in patients with bladder cancer, including higher stage and inferior survival(110). This technology offers promise in the care of patients with advanced stage bladder cancer, such as early detection of relapse or development of therapeutic resistance, but its role is not yet established.

Tumor DNA from a patient's blood can be sequenced, and the information garnered may inform on prognosis and guide therapy. In bladder cancer, patients with detectable circulating tumor cell DNA (ctcDNA) have higher risk of recurrence post-cystectomy, greater risk of progression in early stage disease that is managed conservatively, and overall increased risk of metastasis(107, 111). This technology is newer than CTCs, and has no clear clinical indication at the moment. However, developments in non-small cell lung cancer offer insight into future uses, as the FDA

recently approved mutational analysis of *EGFR* from ctcDNA to guide use of therapy in this tumor type(112). Mutational analysis, of *FGFR3* for example, may be used similarly in patients with bladder cancer in the near future.

In the opinion of the ISUP working group on molecular pathology of bladder cancer, testing for circulating tumour cells and tumour DNA is investigational at this time, and is not recommended in routine diagnostics.

Histologic variants - molecular associations and diagnostic considerations

Invasive conventional UC expresses markers of luminal and basal-squamous differentiation, though to varying degrees in individual cases. Markers commonly positive in conventional UC are GATA3, p63, and 34BE12(2). Specific histologic variants have unique molecular features, including gene expression, which may present diagnostic challenges.

Plasmacytoid urothelial carcinoma appears histologically as infiltrative single tumor cells, similar to lobular breast cancer and gastric signet ring cell carcinoma. It is a uniquely aggressive variant, with >80% of cases presenting with stage T3 or greater(113, 114). Conservative management is inappropriate for any patient with this variant, including those without definite muscularis propria invasion at TUR(114). Accurate diagnosis is thus imperative, particularly in these T1 cases. Like lobular breast cancer and gastric signet ring cell carcinoma, plasmacytoid urothelial carcinomas lose e-cadherin function, often via mutation(115). Immunohistochemistry for e-cadherin is likewise typically negative in plasmacytoid urothelial carcinomas(116). However, not all plasmacytoid urothelial carcinomas lose e-cadherin expression, and not all urothelial

carcinomas that lose e-cadherin are plasmacytoid(10, 116). Despite these limitations, immunohistochemistry for e-cadherin may be diagnostically useful on occasion, such as distinguishing artifactual discohesion from true plasmacytoid histology, though it is not required to make the diagnosis. Given the histologic similarity of this variant to lobular breast cancer, signet ring cell carcinoma, and even plasma cell neoplasia, these tumors may be considered in the differential diagnosis of plasmacytoid urothelial carcinoma. Signet ring cell carcinomas tend to be negative for GATA3 and uroplakin II, so these are the best stains in this differential diagnosis(117). A considerable minority of plasmacytoid UCs express CDX2 and p-CEA, making these less valuable tests in this differential diagnosis. ER-alpha and mammaglobin tend to be negative in plasmacytoid urothelial carcinoma, but positive in lobular breast cancer, making these useful in this distinction. A considerable minority of plasmacytoid urothelial carcinomas express PR and GCDFP-15, making these less useful in this setting(117). Like plasma cell neoplasms, plasmacytoid urothelial carcinoma frequently expresses CD138(118). Keratins are the most useful test to distinguish this variant from plasma cell neoplasia.

Micropapillary urothelial carcinoma histologically appears as small nests within retraction spaces. This variant is aggressive, though opinions differ on whether presence of this variant alone provides sufficient grounds for early cystectomy in T1 cases(119-121). Micropapillary cancers are enriched in *HER2* amplification and overexpression(95, 96). HER2 expression may be heterogeneous within a given bladder cancer, with overexpression present only in micropapillary areas(122). However, not all micropapillary bladder cancers overexpress HER2, and not all bladder cancers that overexpress HER2 are micropapillary(123, 124). HER2

immunohistochemistry is not required to diagnose micropapillary UC, and its value in the diagnosis in unclear. The ISUP working group recommends against HER2 staining in this setting, in keeping with the practice of most pathologists in the ISUP survey (**Table 1**). Notably, it is also unclear if HER2 overexpression in bladder cancer associates with response to trastuzumab, though one small negative trial has been reported(125).

Small cell bladder cancer is histologically similar to small cell lung cancer. It is aggressive, with 5 year survival rates of less than 30% in the pre-NAC era(126, 127). However, the variant responds uniquely well to NAC followed by cystectomy, with 80% survival at 5 years(128). The NAC regimen differs for small cell carcinoma compared to conventional UC, being cisplatin and etoposide, the same regimen used in small cell lung cancer, instead of gemcitabine and cisplatin or methotrexate, vinblastine, doxorubicin, and cisplatin(3). Also different from conventional UC, patients with stage T1 small cell carcinoma receive NAC with cystectomy, given the aggressiveness of the variant and its good NAC response(4). Accurate diagnosis of this variant is therefore imperative. Small cell bladder cancers have mutations in *RB1*, *TP53*, and the *TERT* promoter in the large majority of cases, though these alterations alone appear insufficient for small cell transformation(126). This variant expresses neuroendocrine markers, including synaptophysin, chromogranin, and CD56, and lacks expression of conventional UC markers, including high-molecular weight keratins and p63(129). CD56 is the most sensitive marker (>90% sensitive) but the least specific, while synaptophysin and chromogranin are the more specific but less sensitive (70% sensitive)(130). It has

recently been shown that a newer marker, INSM1, has superior sensitivity and specificity for neuroendocrine tumors of the thoracic cavity(131). This may prove to be a useful marker in small cell carcinoma of the bladder, though it has not been reported to our knowledge. A subset of urothelial carcinomas have “small cell histology,” but these express typical UC markers and lack expression of neuroendocrine markers(129). The ISUP WG recommends to confirm the morphological diagnosis of small cell carcinoma with immunohistochemistry to exclude this mimic. Most pathologists in the ISUP survey reported confirming all or most small cell bladder cancers with immunohistochemistry, in keeping with this recommendation (**Table 1**). A small subset of histologically conventional UCs have an immunophenotype more keeping with small cell carcinoma, though they lack its histology(7, 8, 10, 132). While these tumors are particularly aggressive, it is unclear if they respond to NAC like small cell bladder cancer. Some bladder cancers may have ambiguous histology, but have immunotypic features like small cell carcinoma. Large cell neuroendocrine carcinoma is rarely seen in the urinary bladder(133), and it is unclear if it responds to NAC like small cell bladder cancer. Current NCCN guidelines recommend treating bladder cancers with neuroendocrine features like small cell bladder cancer(3). The ISUP working group agrees with this recommendation. We specifically recommend that physicians treat similarly to small cell carcinoma both large cell neuroendocrine carcinoma and high-grade carcinoma with ambiguous histology but immunophenotypic features of small cell carcinoma.

Conclusion

In conclusion, there has been substantial progress in understanding the biology of bladder cancer. Much of this has translated to improved patient care, such as *FGFR3* mutation analysis and PD-L1 immunohistochemistry, which vitally guide treatment. However, despite our greater understanding, it has been difficult to translate many findings into clinical practice. Much work remains.

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Figure Legends

Figure 1: Gene expression in muscle-invasive bladder cancer.

Heatmap demonstrating gene expression in muscle-invasive bladder cancer, organized by The Cancer Genome Atlas (TCGA) subtype. The majority of tumors are either luminal or basal-squamous. Luminal tumors subtype into luminal-papillary, luminal, and luminal-infiltrated, differentiated largely by stromal and inflammatory signatures. A small subset of tumors has a neuronal-like subtype. Papillary histology and *FGFR3* alterations are enriched in luminal tumors, while squamous histology and *TP53* mutations are enriched in basal-squamous tumors. This heatmap was generated with publicly available data from from the TCGA study through the Genomic Data Commons by the National Cancer Institute (https://portal.gdc.cancer.gov/).

*FGFR3 alteration refers to *FGFR3* mutation, amplification, or rearrangement.

Figure 2: Biological features underlying different molecular classification systems.

A: The Lund system broadly divides tumors into luminal, basal-squamous, and small cell/neuroendocrine-like subtypes (the schema does not use the term “luminal” but it is used here for illustration). Luminal tumors are subtyped into urothelial-like and genomically unstable subtypes. Urothelial-like subtypes may be further divided based on expression of basal-squamous markers and FGFR3, and the genomically unstable subtype may be further divided based on signatures of inflammation.

B: The Cancer Genome Atlas (TCGA) system divides tumors into luminal, basal-squamous, and neuronal-like subtypes. The luminal group includes three subtypes, which differ in stromal and inflammatory signatures.

C: The University of North Carolina (UNC) system separates tumors into luminal and basal subtypes (which are equivalent to basal-squamous in the Lund and TCGA schema), and recognizes a group of basal tumors that express low levels of claudins.

D: The MD Anderson system separates tumors into luminal, basal (which are equivalent to basal-squamous in the Lund and TCGA schema), and P53-like, the latter enriched in expression of stromal genes.

Figure 3: Markers of Lund subtypes with histology in muscle-invasive bladder cancer.

A: Invasive conventional urothelial carcinoma with a urothelial-like subtype, expressing FGFR3 diffusely, GATA3 diffusely, and CK5 at the tumor periphery.

B: Invasive squamous carcinoma with a basal-squamous subtype, lacking expression of FGFR3 and GATA3, and diffusely expressing CK5.

C: Micropapillary carcinoma with a urothelial-like subtype, expressing cyclin D1, retaining RB1 expression, and losing p16 expression. Expression of cyclin D1 is patchy but strong (arrow).

D: Micropapillary carcinoma with a genomically unstable subtype, diffusely expressing p16, but lacking expression of RB1 and cyclin D1.

Figure 4: Gene expression in non-muscle invasive bladder cancer.

A: Expression of early and late cell cycle genes in non-invasive papillary urothelial carcinomas, grouped as Type 1, 2, or 3, based on the system developed by Hedegaard et al. The Type 2 group is enriched in tumors that express late cell cycle genes. This figure was generated from publicly available data from the study by Hedegaard et al.

B: Expression of cell cycle genes in T1 bladder cancers shows the large majority of these tumors express late cell cycle genes, and the majority (72%) are classified as Type 2. This figure was generated from publicly available data from the study by Hedegaard et al.

C: Progression free survival (PFS) in patients with non-muscle invasive bladder cancer, generated from the data from the study by Hedegaard et al., including non-invasive papillary and cT1 tumors. Type 2 tumors have inferior PFS. This figure was generated from publicly available data from the study by Hedegaard et al.

Figure 5: PD-L1 immunohistochemistry in muscle-invasive bladder cancer.
(A) Invasive urothelial carcinoma containing a large number of tumor-infiltrating lymphocytes, which (B) express PD-L1 by immunohistochemistry, though the tumor cells lack expression of PD-L1. (C) Invasive urothelial carcinoma containing a large number of tumor-infiltrating lymphocytes. (D) Both tumor cells (large arrow) and tumor infiltrating lymphocytes (small arrow) express PD-L1 in this tumor. The antibody shown here is Ventana SP263.

Figure 6: Comparison of multiple antibodies in PD-L1 immunohistochemistry.

Shown here is PD-L1 immunohistochemistry performed on the same tumor, using four different antibodies, including the companion diagnostics Dako 22c3 and Ventana SP142, and the complimentary diagnostics Dako 28-8 and Ventana SP263 (see **Table 1** for interpretation details). While the antibodies show generally consistent results, intensity and character of staining differs in tumors cells and immune cells in this example. This minor variability translates to good, but not excellent, concordance among antibodies.

Table 1: Survey results, 256 total respondents.	
Do you use mutational analysis of TERT promoter for differential diagnosis such as distinguishing nested variant urothelial carcinoma from proliferative cystitis?	Yes (3%) No (97%)
Do you use immunohistochemistry, such as E-cadherin, to aid in the diagnosis of plasmacytoid urothelial carcinoma?	Yes (38%) No (62%)
Do you use immunohistochemistry, such as HER-2 staining, to aid in the diagnosis of micropapillary variant of urothelial carcinoma?	Yes (13%) No (82%)
Do you perform confirmatory immunohistochemistry (e.g. synaptophysin, chromogranin A) to establish a diagnosis of poorly differentiated neuroendocrine (small cell) carcinoma?	Yes in most or all cases (81%) Only in cases posing diagnostic challenge (14%) Rarely or never (5%)
Do you perform urine-based tests (such as UroVysion FISH) beyond urine cytology?	Yes (24%) No (76%)
Do you use immunohistochemistry or mutational analysis for FGFR3 to provide prognostic information in non-muscle invasive bladder cancer?	Immunohistochemistry (3%) Mutational analysis (4%) Immunohistochemistry and mutational analysis (1%) None (92%)
Does your laboratory perform molecular testing on Liquid Biopsies (that is serum or plasma) in patients with (advanced) urothelial carcinoma?	Yes (8%) No (92%)
Do you regularly perform laboratory tests to assign a molecular subtype to bladder cancer?	Yes (7%) No (93%)

Which of the following tests do you recommend to guide use of neoadjvuant chemotherapy in muscle invasive bladder cancer?	Immunohistochemistry (13%) RNA-based test that assigns molecular subtype (4%) No laboratory test (81%) Other (3%)
Do you perform PDL1 immunohistochemistry on metastatic bladder cancer to guide treatment with checkpoint inhibitors?	Yes (56%) No (44%)
When is immunohistochemistry for PD-L1 performed in your center	Reflex testing (8%) On request (64%) Not done (29%)
Who performs the majority of next generation sequencing of solid tumors for your anatomic pathology group?	Commerical laboratory that specializes in next generation sequencing of tumor tissue, such as Foundation Medicine or Caris (23%) Commerical laboratories that are more generalized, such as Quest or Neogenomics (6%) Laboratories based in academic medical centers, such as ARUP or M-Labs (6%) In-house molecular laboratory (38%) Other (6%) Not applicable (21%)

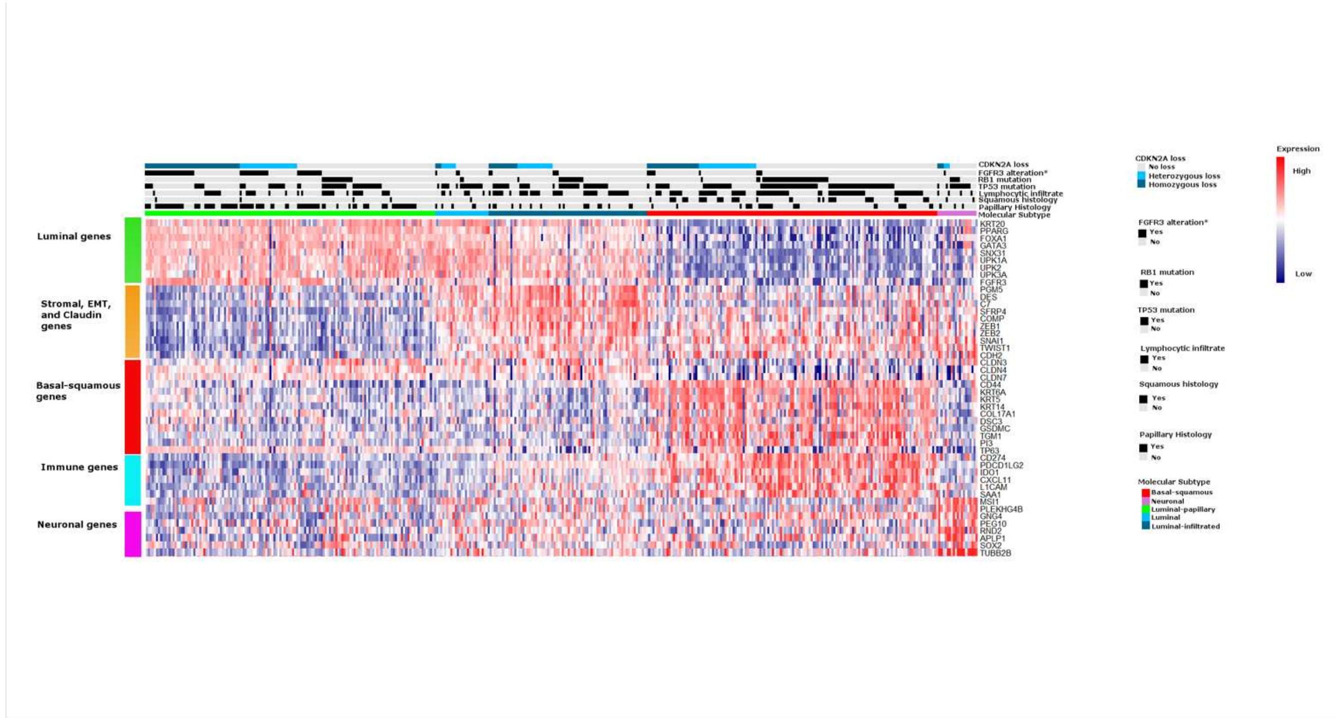
Table 2: Biological features associated with response vs resistance to immune-oncology agents.

Feature	**Associated with response vs resistance**
Tumor infiltrating CD8 T cells	Response
Markers of interferon gamma signaling	Response
Higher mutational burden	Response
Luminal subtype, lacking tumor infiltrating lymphocytes	Resistance
Genomically unstable molecular subtype, even if lacking tumor infiltrating lymphocytes	Response
PD-L1+ tumor infiltrating immune cells	Response

Table 3: Immuno-oncology agents used in bladder cancer, with the associated antibodies used for analysis of PDL1 expression in tumors by immunohistochemistry, evaluative criteria, and clinical utility.

Drug	Antibody used for immunohistochemistry for PDL1	Evaluative Criteria for a positive score	Utility in clinical management
Pembrolizumab	Dako 22C3	Combined positive score (CPS) ≥10 *CPS is the number of cells expressing PDL1 (including tumor cells, lymphocytes, and macrophages) divided by the total number of tumor cells x 100	Mandatory (companion) diagnostic in the first line setting
Atezolizumab	Ventana SP142	PDL1 expression in tumor infiltrating immune cells in ≥5% of tumor area	Mandatory (companion) diagnostic in first line setting
Durvalumab	Ventana SP263	≥25% tumors cells with membranous staining; or Percent of tumor area involved by immune cells ("immune cells present", ICP) >1% and percent of immune cells in tumor positive for PDL1 (IC+) ≥25%; or ICP=1% and IC+=100%	Optional (complementary) diagnostic
Nivolumab	Dako 28-8	≥1% tumor cells with membranous staining	Optional (complementary) diagnostic
Avelumab	None currently available		

Figure 1:



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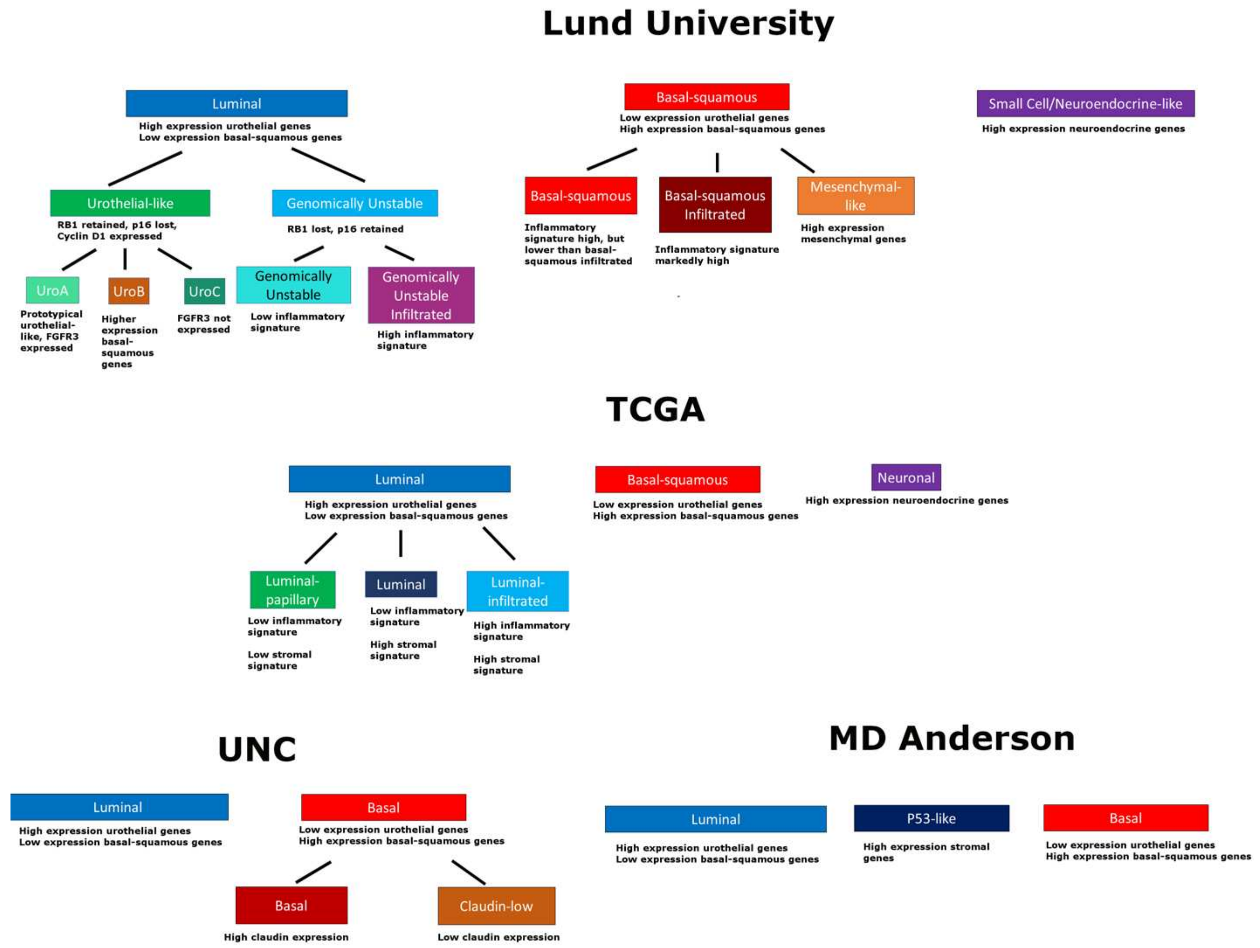
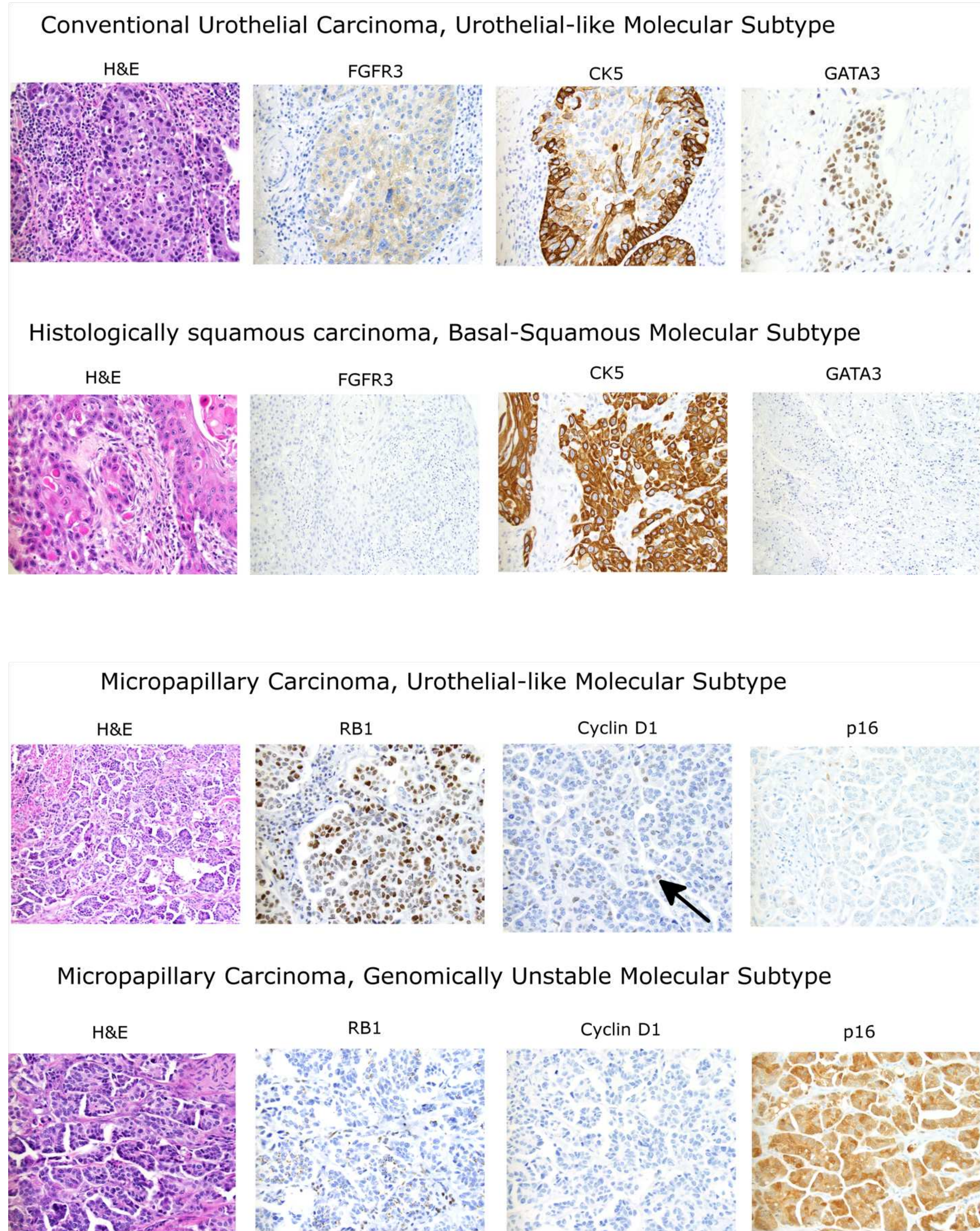


Figure 3



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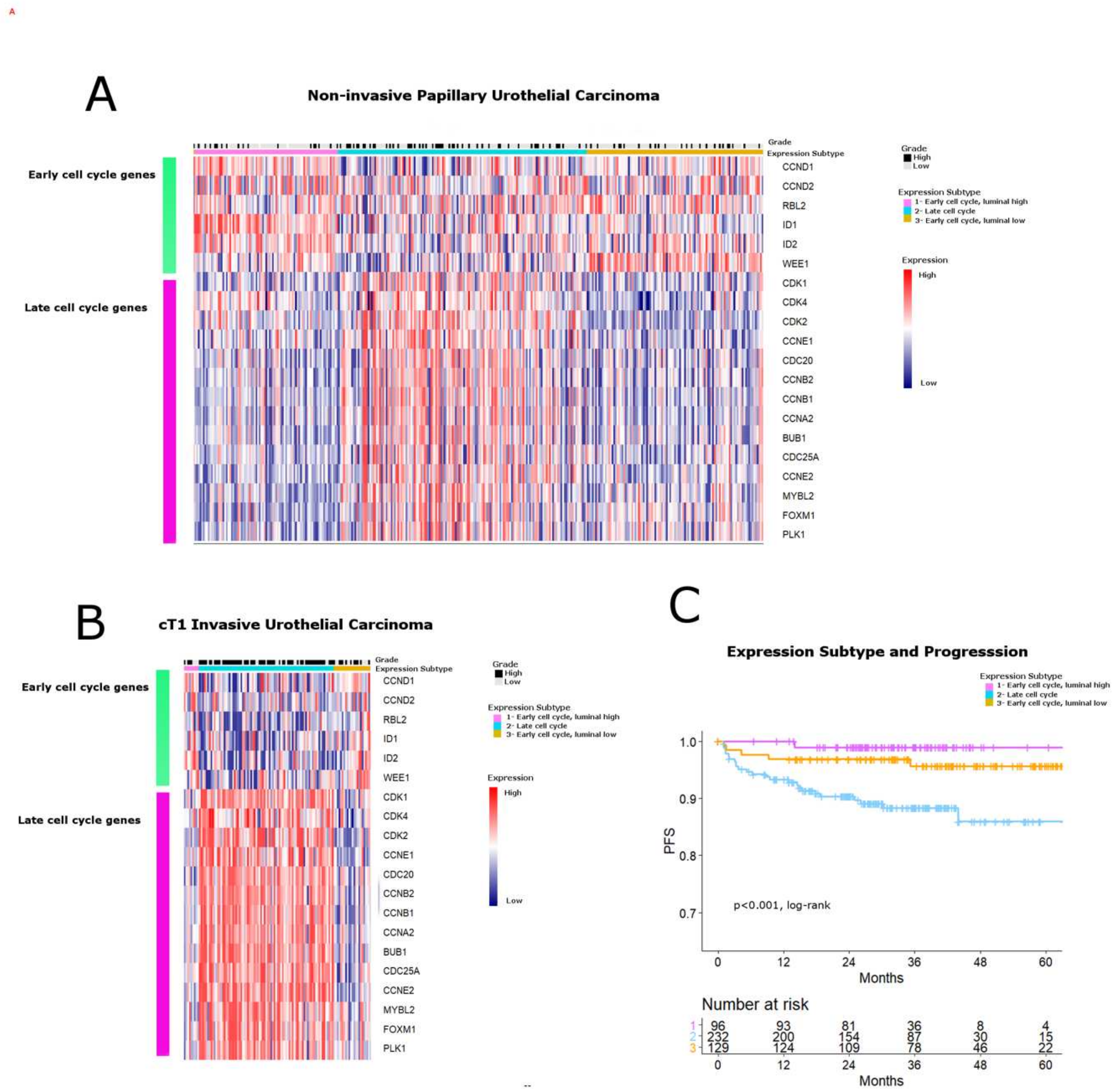

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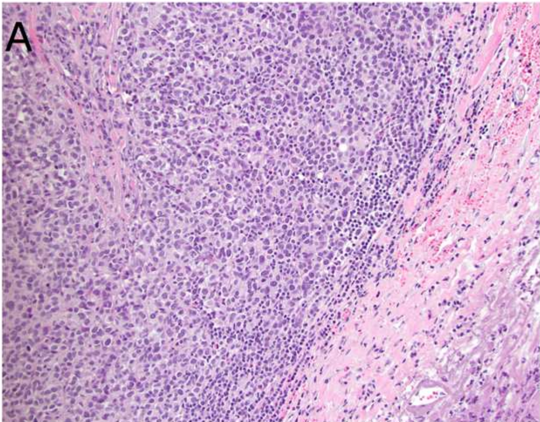

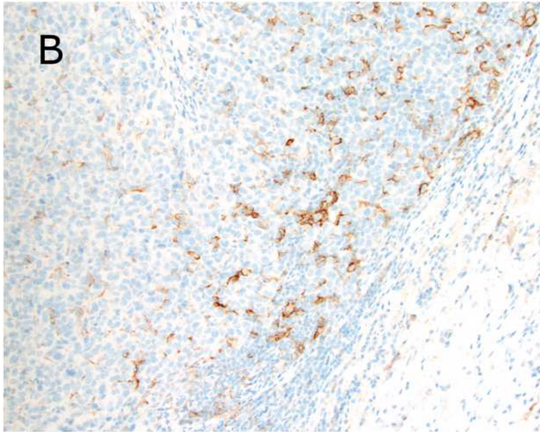

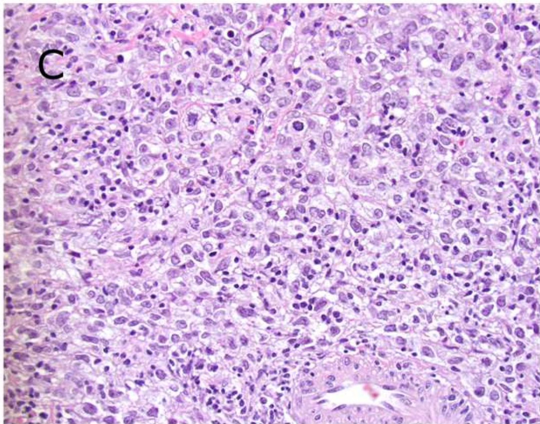

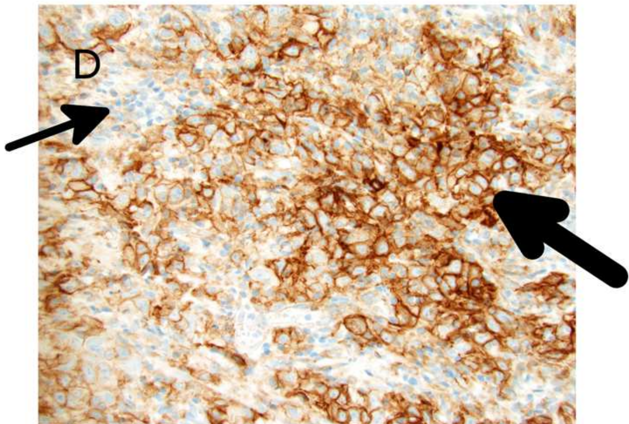

Figure 6: