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Updates on the Genetics and Molecular Subtypes of Urothelial Carcinoma and Select Variants

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SYNOPSIS

Urothelial carcinoma is a morphologically and genomically heterogeneous disease that exhibits a wide spectrum of morphologic features and molecular alterations and subtypes. Classic urothelial carcinoma (not otherwise specified-NOS) is the most common tumor type that develops in the urinary bladder but many, well documented, variant histologies are commonly encountered in approximately one third of invasive urothelial carcinoma, including squamous, glandular, micropapillary, sarcomatoid, small cell/neuroendocrine, clear cell, lymphoepithelioma-like, and plasmacytoid types, among others. In this review we will provide an update on the molecular advances in urothelial carcinoma and some of its variant histologies.

Keywords

Urothelial carcinoma; divergent differentiation; variant histology; squamous; small cell; neuroendocrine; plasmacytoid; micropapillary; molecular subtypes; TCGA

INTRODUCTION

Urothelial carcinoma is the most common cancer that develops in the bladder and is mostly of classical or usual subtype (or NOS) but can invariably exhibit a wide spectrum of variant histologies. This classification has, historically, primarily relied on defined morphological features, but recent technological advances has increased our knowledge of the genomic landscape of urothelial carcinoma and enhanced our understanding of the molecular features associated with this disease and some of its variant histologies. The purpose of this review is

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DISCLOSURE STATEMENT

Nothing to disclose

to highlight the diagnostic and molecular features of the major subtypes of bladder cancer and a subset of its variants whose molecular underpinnings have been recently investigated.

UPDATES ON THE GENOMICS OF INVASIVE UROTHELIAL CARCINOMA (THE CANCER GENOME ATLAS EXPERIENCE)

Recently, the Cancer Genome Atlas (TCGA) bladder cancer group published a comprehensive genomic landscape of 412 muscle-invasive urothelial carcinomas of the bladder.[1] This comprehensive multi-platform analysis led to several findings. It identified a high mutation burden in this cohort which is one of the highest among all cancer types, with a median of 5.8 per Mb and a mean of 7.9 per Mb in coding regions. Whole exome sequencing data analysis revealed that the main mutation signature within these tumors was derived from APOBEC mutagenesis, attributed to a family of enzymes known to contribute to cancer mutagenesis and the development of hypermutation phenotypes. The APOBEC family of enzymes catalyzes typical base changes within a trinucleotide context and two APOBEC-mediated mutation signatures are associated with 66% of the single nucleotide variants (SNVs) within muscle-invasive bladder cancer (MIBC).[1–3] It was shown that the presence of APOBEC3-associated mutation signature is associated with better prognosis and improved 5-year overall survival in patients with MIBC in the updated TCGA cohort. A second mutational signature associated with *ERCC2* mutations is thought to cause approximately 20% of all SNVs. *ERCC2* encodes a DNA helicase that has a central role in the nucleotide-excision repair pathway, a highly conserved DNA repair pathway. Mutations in *ERCC2*, as well as other genes involved in DNA damage response and repair, were shown to be associated with improved response to cisplatin based chemotherapy as well as immune checkpoint blockade and radiation therapy for advanced bladder cancer.[4–8] It was additionally shown that this mutational signature is associated with smoking independent of *ERCC2* mutation status.[9] A third signature in the TCGA analysis was likely related to 5-methylcytosine deamination is associated with 8% of SNVs. From a mutation signature activity standpoint, the TCGA cohort could be grouped into four mutational signature clusters (MSig1 through 4) with distinct mutation burdens and overall survival.[1]

Fifty-two significantly mutated genes representing several pathways involved in cell signaling and other canonical functions (cell cycle and chromatin regulation, receptor tyrosine kinase signaling, transcription, DNA repair, and others) were identified by analysis of TCGA. Examples include mutations in *TP53* (48%), the most commonly altered gene, which were mutually exclusive with *MDM2* amplifications (7%), both events resulting in dysregulation of the cell cycle. *RB1* inactivating alterations were identified in 18% of tumors while *CDKN2A* deletions were observed in 24% of specimens. Both genes are involved in regulation of cell division. Oncogenic alterations within genes involved in cell signaling were noted, including hotspot activating *FGFR3* and *PIK3CA* point mutations as well as *FGFR3* fusions.

MOLECULAR TAXONOMY OF UROTHELIAL CARCINOMA

While bladder tumors can be grouped based upon significantly mutated genes, and this can also have therapeutic utility through the identification of potentially targetable alterations,

several groups, including TCGA, have also identified RNA expression-based molecular subtypes of bladder cancer that may have both prognostic relevance and prediction for response to a variety of therapies. Over the last several years, different groups independently identified different subtypes based on RNA and/or immunohistochemical expression characteristics. Although the different classifications used different designations to label these different subtypes, significant overlap exists among them.[1, 10–13] Efforts to harmonize and unify the terminology for these subtypes are currently underway to try to classify them into distinct and clinically relevant entities.[14] The most comprehensive of these classifications are the ones proposed by the Lund University group and that of the TCGA.[1, 13] One of the earliest studies by the Lund University group was reported by Lindgren et al, who initially described 2 molecular subtypes of bladder cancer, MS1 and MS2, based upon genome wide gene expression analysis of 144 tumors, including both muscle-invasive and non-muscle-invasive bladder tumors.[15] These two subtypes correlated with stage (MS1 tumors were primarily Ta while MS2 tumors were T2). MS2 tumors were also characterized by high grade as compared to MS1. *FGFR3* mutations were enriched within MS1 tumors (55% vs 7% in MS2, $p<0.05$) and *TP53* mutations were more common in MS2 tumors. Sjödhahl et al subsequently expanded this analysis by performing gene expression profiling and immunohistochemical expression on a larger cohort of tumors and identified 5 discrete molecular subtypes: urobasal A characterized by *KRT5* and *FGFR3* overexpression and a favorable prognosis; genomically unstable, harboring *TP53* mutations and *ERBB2* overexpression and enriched with muscle-invasive high grade tumors; a squamous cell carcinoma-like subtype typified by squamous cell differentiation, overexpression of basal keratins, and a poor prognosis; urobasal B tumors which shared features of the other 3 subtypes; infiltrated tumors with infiltration of immunologic cells and extracellular matrix gene expression.[12, 13, 16] The Lund group continues to refine their classification and modify or add more details to their existing subtypes. For example, they currently include a small-cell/neuroendocrine-like subtype in their classification, which shares features of the genomically unstable subtype but additionally expresses higher levels of neuroendocrine markers such as chromogranin, synaptophysin, neuron specific enolase (NSE, ENO2) and NCAM1 (CD56).[16, 17] They also identified a mesenchymal-like subtype characterized by high expression of mesenchymal markers such as vimentin and ZEB2 and differed from other subtypes by showing low expression of FOXA1, GATA3, KRT5 and KRT14.

The TCGA analysis confirmed the presence of two major luminal and basal subtypes and provided additional discrimination into these two major categories. The basal and luminal subtyping of MIBC was initially proposed following the perceived similarities to the molecular subtypes of breast cancer and was defined based upon distinct expression signatures. Basal tumors express basal type keratins KRT5, KRT14, and KRT6A/B/C; while luminal tumors are characterized by high expression of *FGFR3*, the transcription factors *PPARG*, *GATA3*, *FOXA1*, and *ELF3*, the uroplakin genes found in umbrella cells, and *KRT20*. [10, 11, 18] The TCGA analysis further expanded and refined this classification and identified three subgroups within the luminal subtype along with a basal-squamous subtype as well a distinct neuronal cluster (total of five subtypes). This identified subgroups within the luminal subtype; luminal-papillary, luminal-infiltrated and luminal. The luminal-

papillary cluster was enriched in tumors with papillary morphology and lower stage and was enriched with FGFR3 overexpression associated with FGFR3 mutations, amplification, and FGFR3-TACC3 fusions. The luminal-infiltrated subtype was distinguished from other luminal subtypes by lower purity that was derived from lymphocytic infiltrates and the presence of smooth muscle and myofibroblast gene signatures. A similar expression signature has been previously reported to be associated with chemoresistance and characterized by a wild-type p53 signature [10] but was also reported to derive most benefit from anti-PDL1 treatment.[19] Tumors included in this subtype had increased expression of several immune markers, including CD274 (PD-L1) and PDCD1 (PD-1). The luminal subtype had the highest expression levels of uroplakins UPK1A and UPK2 as well as genes that are highly expressed in terminally differentiated umbrella cells such as KRT20 (CK20). The basal subtype contained nearly all the tumors that exhibited squamous differentiation by histopathologic review and was thus called basal-squamous. This subtype was associated with high expression of basal and stem-like markers (CD44, KRT5, KRT6A, KRT14) and squamous differentiation markers such as desmocollins (DSC1–3) and desmogleins (DSG1–4), TGM1 (transglutaminase 1) and PI3 (elafin) (Figure 1). It was also enriched in *TP53* mutations, was more common in females and showed a strong immune gene signature expression as well as lymphocytic infiltrates.

The neuronal subtype included 3 cases of neuroendocrine/small cell histology but also 17 additional tumors that had no histopathologic features suggestive of neuroendocrine differentiation. All 20 tumors showed relatively high expression of genes involved in neuronal differentiation and development, as well as typical neuroendocrine and neural differentiation markers such as chromogranin, PEG10, PLEKHG4 and TUBB2B. In only a subset of these 20 tumors were there mutations in both *TP53* and *RBI*, which is the hallmark alteration in small cell/neuroendocrine carcinoma.[20] On the other hand, the majority of tumors in this cluster (85%) had alterations in genes in the p53/cell-cycle pathway and this subtype was associated with the worst clinical outcome.[1]

Applying this molecular classification to other bladder cancer cohorts resulted in patient risk stratification [1, 17] and correlation with responses to chemotherapy or immunotherapy in advanced disease.[10, 19, 21] In other studies using RNA expression or immunohistochemical profiling, some variant histologies of urothelial carcinoma (micropapillary, nested, plasmacytoid) were found to have luminal characteristics whereas urothelial carcinoma with squamous differentiation was found to have basal characteristics. [22, 23]

Despite the important insights provided by these molecular classifications, several questions remain unanswered: are these subtypes impacted by prior therapies for bladder cancer; should subtyping be incorporated into clinical decision-making, and if so, which disease states and/or treatments would be best applicable to such an analysis; how stable these molecular subtypes are within a given tumor and is there variation of subtyping with the same tumor (intratumoral heterogeneity)? These and several other questions are the current focus of several research groups in bladder cancer and should hopefully lead to significant insight into the biology of this disease in the near future, paving the way for novel biomarker-directed therapies.

MOLECULAR UPDATES IN SPECIFIC VARIANTS OF UROTHELIAL CARCINOMA

Plasmacytoid Urothelial Carcinoma

Plasmacytoid urothelial carcinoma is a rare and aggressive variant of bladder cancer characterized by the presence of discohesive, individual cells with eccentrically located nuclei that resemble plasma cells as well cells with intracytoplasmic vacuoles that give the appearance of signet ring cells [24–26]. The aggressive clinical course is characterized by advanced stage at presentation, high mortality rate, high propensity for relapse and, and frequent peritoneal carcinomatosis despite sometimes the apparent initial response to chemotherapy [24–28]. At the molecular level, it has been recently shown that the presence of *CDH1* truncating mutations (or less frequently *CDH1* promoter hypermethylation) is the defining feature of plasmacytoid variant of bladder cancer.[24] Using whole exome and targeted sequencing, truncating somatic alterations in the *CDH1* gene were identified in 84% of plasmacytoid carcinomas and were specific to this histologic variant (Figure 2A). Furthermore, all *CDH1* wild-type plasmacytoid carcinomas were associated with loss of E-cadherin expression, all but one of which were associated with *CDH1* promoter hypermethylation. Aside from *CDH1* mutation, the genomic landscape of plasmacytoid carcinoma was similar to that of urothelial carcinoma, NOS with frequent mutations in chromatin modifying genes, cell cycle regulators and PI3 kinase pathway alterations, suggesting that plasmacytoid and classic urothelial carcinoma likely evolve from a shared cell of origin.[24] This was further supported by performing exon capture and deep sequencing of two adjacent areas of a bladder tumor which contained distinct regions of plasmacytoid and classic urothelial carcinoma (Figure 2B). Both histologic regions shared mutations in *CDKN1A* (A45fs) and *PIK3C2G* (S48R), implying that these were early truncal alterations occurring within a common precursor cell. A *CDH1* Y68fs mutation, along with mutations in other genes was, however, unique to the plasmacytoid component. [24]

Functional cell lines studies supported a significant role of *CDH1* loss in promoting cell discohesion and stromal invasion, which could explain the higher incidence of both local recurrence and cancer-specific mortality as well as the higher rate of peritoneal spread than those with pure urothelial carcinoma. By performing Clustered Regularly Interspersed Palindromic Repeat (CRISPR)/Cas9-mediated knockout of *CDH1* in two *CDH1* wild-type urothelial carcinoma cell lines (RT4 and MGHU4), loss of E-cadherin expression resulted in increased invasion and migratory capability of MGHU4 and RT4 cells. It was also shown that loss of E-cadherin expression was observed only in the invasive plasmacytoid carcinoma and was retained within the non-invasive component. This work and that of others reporting common E-cadherin loss by immunohistochemistry in the majority of plasmacytoid carcinomas [25, 29], indicate that E-cadherin loss, typically as a result of *CDH1* mutation and less commonly as a result of *CDH1* promoter methylation, is the defining molecular event of the distinct local invasion and spread patterns observed in patients with plasmacytoid carcinoma. Notably, in contrast to the germline *CDH1* mutations typically seen in patients with diffuse hereditary gastric cancers and a subset of lobular breast cancer, no germline *CDH1* mutations were identified in plasmacytoid urothelial carcinoma.[24]

These results indicate that somatic loss-of-function mutations in *CDH1*, with consequent E-cadherin loss, leads to enhanced cellular migration and invasive properties in plasmacytoid carcinoma, characterized by marked cell discohesion and single cell infiltration.

Micropapillary Urothelial Carcinoma

This is a rare variant of urothelial carcinoma with reported aggressive clinical course. Although it is now increasingly recognized, it still lacks application of strict diagnostic criteria and as a result suffers from high degree of interobserver variability. This becomes even more problematic particularly that many clinicians advise early cystectomy for this disease even in the absence of invasion into the muscularis propria.[30] Morphologically, this tumor is characterized by the presence of small tight clusters of high grade tumor cells lacking true fibrovascular cores and present within lacunar spaces (Figure 3).[31] The basis behind this appearance, in the namesake entity in the breast, is the “reverse orientation or polarization” of the basal and luminal aspects of tumor cells resulting in the lack of cohesion between tumor and stroma.[32, 33]

At the molecular level, higher rates of *ERBB2* amplification are reported in micropapillary urothelial carcinoma than in classic urothelial carcinoma and in some reports this amplification was additionally associated with worse cancer-specific survival following radical cystectomy.[34, 35] In a recent study, we reported high rates of intratumoral heterogeneity of *ERBB2* amplification within tumor containing both micropapillary and classic urothelial components as *ERBB2* amplification was more commonly amplified in the micropapillary than the classic urothelial components.[36]. Moreover, the rate of *ERBB2* in the classic urothelial components in these mixed (micropapillary+urothelial) tumors was much higher than the reported rates in pure classic urothelial carcinoma or those not mixed with micropapillary components[1, 37, 38] (Figure 4), indicating a possible role of *ERBB2* activation in the development of this aggressive variant of urothelial carcinoma. It has been recently reported that mutations in known hotspots in *ERBB2* are common in micropapillary carcinoma of the bladder [39] but it is likely that the frequency of such mutations is not higher in this variant histology than it is in classic urothelial carcinoma. In another recent study employing RNA expression profiling of micropapillary bladder cancer, there was common downregulation of miR-296 and activation of chromatin-remodeling complex RUVBL1, but it remains unclear what role these alterations play and what they contribute to the development of micropapillary urothelial carcinoma.[23]

Small Cell/Neuroendocrine Carcinoma of the Bladder

This is a rare variant of bladder cancer that is morphologically identical to the small cell carcinoma of the lung and other organs, but may be admixed with a classic urothelial component (invasive or in situ) as well as other divergent differentiation including squamous, glandular and sarcomatoid with or without heterologous elements in up to 50% of cases.[40]. The genomic landscape of small cell carcinoma of the bladder is still not fully defined but a few recent studies provided insights into the molecular characteristics of this disease and identified some similarities and differences between small cell and urothelial components within the bladder as well as with small cell carcinoma of the lung.[20, 37, 41, 42] A near universal finding was the presence of co-alterations in *TP53* and *RB1* resulting in

loss of function of both genes. In one study, *TP53* and *RBI* alterations were detected in 90% and 87% of cases, respectively, and 80% of tumors displayed co-alterations of both genes (Figure 5).[20] Further, in some tumors without *RBI* loss of function mutations, there was loss RB expression by immunohistochemistry, suggesting an alternative mechanism, such as epigenetic silencing, that contributed to RB loss. Interestingly and perhaps not surprisingly, unlike lung small cell carcinoma, commonly mutated genes in urothelial carcinoma were also found mutated in the bladder small cell carcinoma cohort, including *TERT* promoter mutations (95%) and truncating alterations within chromatin modifying genes such as *CREBBP*, *EP300*, *ARID1A*, *KMT2D* in approximately 75% of samples.[20, 42]. Notable exceptions include the near absence of *KDM6A* truncating mutations, *CDKN2A* deletion and *CCND1* amplifications in the bladder small cell carcinoma cohort, compared to urothelial, carcinoma where such alterations are common. *E2F3* amplification was found in both small cell and urothelial bladder tumors, while this event was rare in small cell lung cancer.[20]

A high level of chromosomal instability was observed in bladder small cell carcinoma, including whole genome duplication in 72% of tumors that correlated with the presence of *TP53* missense mutations, particularly those associated with biallelic silencing. The APOBEC mutation signature that was identified within muscle-invasive bladder cancer from the TCGA bladder cancer study [43] was observed in 95% of small cell bladder cancer in this cohort; notably, small cell lung cancers are typically characterized by a mutation signature associated with tobacco exposure distinct from the APOBEC signature.[20] in a subset of cases with mixed urothelial and small cell components, sequenced the macrodissected components separately identified share alteration between the two components in addition to private alterations in each of them, further supporting the concept that small cell carcinoma of the develops form a precursor urothelial carcinoma. It still remains unexplained; however, how small cell carcinoma develops and what molecular mechanisms underlie its development from its urothelial precursor beyond the combined *RBI/TP53* alterations which are co-mutated in a subset of classic urothelial carcinoma that clearly does not display small cell/neuroendocrine differentiation.[1, 37, 44] It also remains unclear how true small cell carcinoma, as defined histologically, is related to the neuronal/neuroendocrine subtype of bladder cancer, as defined by the TCGA and Lund group classifications.

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Key features**Bladder Cancer Genomics**

- Bladder cancer is genomically heterogeneous and is associated with high mutation burden
- Mutational signature strongly associated with APOBEC activity
- Different mutational signatures may be associated with different clinical outcomes

Key features**Molecular taxonomy of urothelial carcinoma**

- Urothelial carcinoma can be grouped into distinct molecular subtypes based on expression profiles
- Many classification systems exist for this molecular subtyping, with significant overlap among them
- Some of the molecular subtypes may have predictive or prognostic significance
- The stability of these subtypes within the same tumor and disease states is under investigation

Key Features**Plasmacytoid urothelial carcinoma**

- Rare and aggressive variant of bladder cancer
- Discohesive and infiltrating growth
- Loss of E-cadherin expression due to truncating *CDH1* mutations or promoter hypermethylation is pathognomonic
- Genomic background otherwise similar to urothelial carcinoma
- No association with *CDH1* germline mutations

Key features**Micropapillary Urothelial Carcinoma**

- Rare and aggressive variant of bladder cancer
- Characterized by small tight clusters of high grade tumor cells lacking true fibrovascular cores and present within lacunar spaces
- Strong association with *ERBB2* gene amplification and HER2 overexpression

Key features**Small cell/neuroendocrine Carcinoma of the Bladder**

- Rare and aggressive variant of bladder cancer
- Similar to small cell carcinoma in other organs
- Strong association with *TP53/RB1* co-alterations
- Genetic background similar to bladder cancer and strongly associated with APOBEC signature
- Aside from *TP53/RB1* co-alterations, genetic background is different from lung small cell carcinoma

KEY POINTS

- Bladder cancer is morphologically and genomically heterogeneous
- Urothelial carcinoma has high mutation burden associated with variable mutational signatures and variable clinical outcomes
- Many molecular subtypes of urothelial carcinoma are present based on expression profiles
- Some variants of urothelial carcinoma can be characterized by specific genetic aberrations
- Most variant histologies harbor similar genetic alterations to those seen in classic urothelial carcinoma

Figure 1:
Invasive urothelial carcinoma with features of basal subtype by immunohistochemistry.
There is morphologic evidence of squamous differentiation in the tumor. The tumors lacks
GATA3 expression and shows over expression of basal keratins CK5/6 and CK14.

Figure 2:

Comparison of the genomic landscape of plasmacytoid urothelial carcinoma and urothelial carcinoma, NOS. **A)** Heatmap comparing the frequency and distribution of *CDH1* alterations and select co-altered genes within 25 plasmacytoid urothelial carcinoma (PC), a prospective cohort of 62 urothelial carcinomas (including 6 with plasmacytoid histology), and 121 muscle-invasive urothelial carcinoma, NOS samples from the initial TCGA paper. Star: Six *CDH1* mutant plasmacytoid urothelial carcinomas from the prospective clinical cohort. **B)** Phylogenetic tree depicting divergent evolution of plasmacytoid and urothelial NOS components within a tumor with distinct histologic regions. Exon capture and deep sequencing of macrodissected components identified shared *CDKN1A* and *PIK3C2G* mutations in both components, suggesting that these are truncal alterations occurring within a common precursor cell. However, a *CDH1* frameshift mutation was unique to plasmacytoid-variant histology, and the remaining genetic alteration profiles of the two histologic components were distinct. Red and green lines in the phylogenetic tree indicate plasmacytoid and urothelial NOS components, respectively. (From Al-Ahmadie, H.A., et al.,

Frequent somatic CDH1 loss-of-function mutations in plasmacytoid variant bladder cancer.
Nat Genet, 2016. **48**(4): p. 356–8; with permission.)

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Figure 3.

An example of HER2 expression IHC score 3+ in the micropapillary component (MP) with *ERBB2* amplification. There is no HER2 expression in the NOS component (score 0) and no *ERBB2* amplification. A and B represent panoramic view of MP and NOS components. (From Isharwal, S., et al., *Intratumoral Heterogeneity of ERBB2 Amplification and HER2 Expression in Micropapillary Urothelial Carcinoma*. Hum Pathol, 2018 Mar 27 [Epub ahead of print]; with permission.)

Figure 4.

ERBB2 Amplification in classic “not otherwise specified (NOS)” urothelial carcinoma and the NOS and micropapillary components of micropapillary urothelial carcinoma (MPUC) containing both components. The prevalence of *ERBB2* amplification in NOS bladder cancer is derived from TCGA dataset (6.3%). *ERBB2* amplification was significantly more common in MPUC, both within the MP and NOS components, as compared to pure NOS bladder tumors. (From Isharwal, S., et al., *Intratumoral Heterogeneity of ERBB2 Amplification and HER2 Expression in Micropapillary Urothelial Carcinoma*. Hum Pathol, 2018 Mar 27 [Epub ahead of print]; with permission.)

Figure 5. A) a heatmap depicting patterns of mutations in *TP53*, *RB1*, the *TERT* promoter, and other cell-cycle regulators small cell carcinoma of bladder cohort from reference [20] and urothelial carcinoma from the initial TCGA cohort. B) Commonly mutated genes in small cell bladder carcinoma, urothelial carcinomas, and small-cell lung carcinoma (dark blue, light blue, and red, respectively) are grouped on the basis of their alteration frequency (*, nominal *P* value < 0.05; Fisher exact test. Hom., homozygous; LOH, loss of heterozygosity; N/A, not available). (From Chang, M.T., et al., *Small cell carcinomas of the bladder and lung are characterized by a convergent but distinct pathogenesis Clin Cancer Res.*, 2017. 24(8):1965–73; with permission.)