Mutational Analysis of 472 Urothelial Carcinoma Across Grades and Anatomic Sites

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Abstract

Purpose: The purpose of this study is to characterize the mutational landscape across the spectrum of urothelial carcinoma (UC) to identify mutational features and potential therapeutic targets.

Experimental Design: Using targeted exome sequencing (n = 237 genes), we analyzed the mutation spectra of 82 low-grade nonmuscle-invasive bladder cancers (LG-NMIBC), 126 high-grade (HG) NMIBC, 199 muscle-invasive bladder cancers (MIBC), 10 LG-upper tract urothelial cancers (LG-UTUC), and 55 HG-UTUC.

Results: FGFR3 and KDM6A mutations were significantly more common in LG-NMIBC (72% and 44%, respectively) versus other bladder subtypes. FGFR3 alterations were also enriched in LG-UTUC versus HG-UTUC tumors (80% vs. 16%). In contrast, TP53 and RB1 mutations were significantly more frequent in all 3 HG urothelial carcinoma subtypes than in LG-NMIBC (45%–58% vs. 4%; 9%–22% vs. 0; respectively). Among LG-NMIBC tumors, KDM6A mutations were more common in women than in men (71% vs. 38%). HG-NMIBC and MIBC had higher tumor mutational burden (TMB) than LG-NMIBC (P = 0.001 and P < 0.01, respectively). DNA-damage repair (DDR) alterations were associated with a higher TMB in HG-NMIBC and MIBC tumors, and these two tumor types were also enriched for an APOBEC mutational signature compared with LG-NMIBC and HG-UTUC. Alterations in FGFR3, PIK3CA, and EP300 correlated with worse overall survival in HG-UTUC and occurred concurrently.

Conclusions: Our analysis suggests that a fraction of MIBCs likely arise from precursor lesions other than LG-NMIBC. KDM6A mutations are twice as common in women with LG-NMIBC than those in men. DDR gene mutations and APOBEC mutagenesis drive mutations in HG-NMIBC and MIBC. UTUC has a distinct mutation profile from bladder cancer.

Introduction

The urothelium spans the bladder, ureter, renal pelvis, and a portion of the urethra. Urothelial carcinoma (UC) can arise throughout synchronously and asynchronously predominantly due to toxic exposures and genetic alterations (1). Multiple recent studies have examined the genetic features of muscle-invasive bladder cancer (MIBC), whereas nonmuscle-invasive bladder cancer (NMIBC) and upper tract urothelial cancer (UTUC) are less well characterized (2, 3). The most comprehensive analysis of MIBC was performed by The Cancer Genome Atlas (TCGA) project in which 412 MIBCs were examined, and 58 significantly mutated genes identified, including many potential therapeutic targets (4). However, to date massively parallel sequencing (MPS) efforts focusing on other urothelial carcinoma subtypes such as noninvasive tumors, low-grade disease, and upper tract cancers remain limited. Furthermore, analysis of clinically annotated cohorts with longitudinal follow-up is needed (3, 5, 6).

Many prior studies have characterized genetic events in urothelial carcinoma pathogenesis. Chromosome 9 deletions are among the earliest events (7) and are seen in >50% of NMIBC and MIBC tumors (8). FGFR3-activating mutations are also highly prevalent (80%) in low-grade NMIBC tumors and are associated with favorable outcomes (9). FGFR3 alterations in urothelial carcinoma include chromosomal translocations with several fusion partners which are known to promote tumor development (4, 10). PIK3CA-activating mutations are another common early event (25%) in NMIBC tumors (11, 12) with recent studies showing similar frequency in muscle-invasive disease (4). Alternatively, TP53 alterations are enriched in MIBC disease (48%) and tend to occur in dysplastic lesions with carcinoma in situ (4). Recent reports compared the mutational profiles of UTUC with MIBC. FGFR3 (35%), HRAS (14%), and CDKN2B alterations were more frequent in UTUC highlighting potential site-specific
Sequencing Across the Spectrum of Urothelial Carcinoma

Translational Relevance

Few studies have examined mutation and copy-number alterations across the clinical spectrum of urothelial carcinomas using a single analytic platform. Utilizing our institutional Clinical Laboratory Improvement Amendments–certified next-generation sequencing platform, Oncopanel, we analyzed 472 urothelial carcinomas across tumors grades, stages, and anatomic sites. FGFR3 and KDM6A mutations were enriched in noninvasive, low-grade urothelial carcinoma. In contrast, TP53 and RB1 mutations were more prevalent in more advanced subtypes. PRKDC, a DNA-damage repair gene, was recurrently mutated. HRAS mutations were enriched in upper tract urothelial carcinoma. These observations underscore the differences in genomic alterations across the clinical spectrum of urothelial carcinoma and may inform therapeutic strategies for urothelial carcinoma patients.

Materials and Methods

Patients and samples

Four hundred and seventy-two urothelial carcinoma patients from Brigham and Women's Hospital (BWH) and/or Dana Farber Cancer Institute (DFCI; Supplementary Table S1.0) had genetic testing of their tumor tissue performed using the Oncopanel assay from Brigham and Women's Hospital (BWH) and/or Dana Farber Cancer Institute (DFCI; Supplementary Table S1.0) had genetic alterations across the clinical spectrum of urothelial carcinoma and may inform therapeutic targets for upper versus lower tract disease, but FGFR3 mutations were not significantly different in frequency when corrected for stage (3).

MPS enables large-scale characterization of genetic changes occurring in all types of urothelial carcinoma. Here, we report the analysis of 472 urothelial carcinoma across the spectrum of clinical disease, using a uniform platform for identification of single nucleotide and indel variants in 237 genes with roles in development of diverse cancer types. This platform also enables analysis of somatic copy-number alterations (CNA), both homozygous deletions and amplification events. We examined the frequency of gene mutations, mutational processes and signatures, TMB, mutation co-occurrence and exclusivity, and CNAs in four urothelial carcinoma subtypes, and assessed the association between genomic alterations and clinical outcome.

Variant assessment

Oncopanel analysis was performed on tumor samples only, so we excluded variants that were likely to be germline rather than somatic, including those seen at an allele frequency >0.1% in the Exome Aggregation Consortium database (19). All nonsense mutations, frameshift insertions or deletions, and splice site alterations affecting consensus nucleotides were considered deleterious. The functional impact of missense mutations was determined using Sorting Intolerant From Tolerant (SIFT) (20) and Polyphen-2 (21). Missense mutations classified as “damaging” in SIFT and/or “probably damaging” in Polyphen2 were considered to be deleterious. We also focused in particular on the DNA-damage repair (DDR) pathway in urothelial carcinoma, because there are many recent reports implicating alterations in this pathway as a driver of tumor phenotype (22). Oncopanel includes 30 genes involved in DDR (ref. 23; Supplementary Table S1.2). Because many of these genes are large and likely to have random variants (both germline and somatic) without functional effect, we used more stringent

Therapeutic targets for upper versus lower tract disease, but FGFR3 mutations were not significantly different in frequency when corrected for stage (3). MPS enables large-scale characterization of genetic changes occurring in all types of urothelial carcinoma. Here, we report the analysis of 472 urothelial carcinoma across the spectrum of clinical disease, using a uniform platform for identification of single nucleotide and indel variants in 237 genes with roles in development of diverse cancer types. This platform also enables analysis of somatic copy-number alterations (CNA), both homozygous deletions and amplification events. We examined the frequency of gene mutations, mutational processes and signatures, TMB, mutation co-occurrence and exclusivity, and CNAs in four urothelial carcinoma subtypes, and assessed the association between genomic alterations and clinical outcome.

Four hundred and seventy-two urothelial carcinoma patients from Brigham and Women's Hospital (BWH) and/or Dana Farber Cancer Institute (DFCI; Supplementary Table S1.0) had genetic testing of their tumor tissue performed using the Oncopanel assay (see below). Low-grade NMIBC (LG-NMIBC), high-grade NMIBC (HG-NMIBC), MIBC, low-grade UTUC (LG-UTUC), and high-grade UTUC (HG-UTUC) tumors were included. Staging and grading of tumors were performed by board-certified pathologists following the criteria of the tumor–node–metastasis classification of the World Health Organization (13, 14). Low-grade TaG1 and TaG2 tumors were considered to be LG-NMIBC; high-grade Tis, TaG2, TaG3, T1G2, and T1G3 tumors were considered HG-NMIBC; and tumors ≥T2 or with lymph node involvement were considered MIBC. LG-UTUC included low-grade TaG1 and TaG2 tumors, whereas HG-UTUC comprised high-grade urothelial carcinoma of all stages (Ta–T4) found in the ureter or renal pelvis; 52 of 55 were ≥T2.

Tissue collection and DNA extraction

Tumor specimen and clinicopathologic information were collected with Institutional Review Board (IRB) approval at DFCI (DFCI IRB protocol 11–104). The study was conducted in accordance with the U.S. Common Rule, and all participants and/or their parent or legal guardian provided written informed consent. DNA was isolated using the QIAamp DNA formalin-fixed, paraffin-embedded Tissue Kit (Qiagen) according to the manufacturer's instructions. DNA was quantified by Nanodrop and pico-Green assays.

Targeted sequencing

Targeted sequencing was performed using an institutional analytic platform, Oncopanel, that is certified for clinical use and patient reporting under the Clinical Laboratory Improvement Amendments (CLIA) Act (15). Fifty to 200 ng of genomic DNA from each sample was subjected to targeted exon capture and sequencing using 1 of 3 versions of the Oncopanel assay (V1–V3) in the Department of Pathology at BWH. The Oncopanel gene panel includes capture probes for 275 to 447 cancer-associated genes, as well as intronic portions of 60 genes for rearrangement detection (15). Targeted capture was performed using a solution-phase Agilent SureSelect hybrid capture kit and custom bait sets. Sequencing libraries were prepared from captured DNA as described in detail elsewhere (15). Paired-end sequencing was performed on an Illumina HiSeq 2500 sequencer. Reads were demultiplexed using Picard tools (http://picard.sourceforge.net) and aligned to human reference genome b37 using the Burrows–Wheeler Aligner (16; http://bio-bwa.sourceforge.net/bwa.shtml). Low-quality reads and duplicates were filtered out and eliminated using Picard. We focused on our mutational and CNA analysis on 237 genes which were common to all versions of Oncopanel (Supplementary Table S1.1). Single-nucleotide variants (SNV) and small indels were analyzed using MuTect v.1.0.27200 (https://confluence.broadinstitute.org/display/CGATools/MuTect; accessed May 2013) and the Genome Analysis Toolkit (GATK, version 1.6.5-g557da77), respectively. SNVs and small indels were then annotated by Oncotator (http://www.broadinstitute.org/oncotator; accessed May 2013). CNAs were identified using an R-based tool (VisCap-Cancer) that identifies both homozygous deletions and amplifications, the latter defined as >6 copies (17, 18). The mean depth of read coverage for the targeted genes was 275X. The mean, median, and range of percentage of target bases with read depth >30x were 98%, 99%, and 78%–100%, respectively.
criteria to identify deleterious DDR gene alterations. We required that variants be reported recurrently (≥3 times) in the Catalog of Somatic Mutations in Cancer (COSMIC; ref. 24) database to be considered deleterious.

Biallelic alterations in KDM6A were determined based on (1) identification of two distinct inactivating mutations; (2) one inactivating mutation and single copy loss; or (3) two copy loss.

Mutation signature analysis

Excluding LG-UTUC due to limited sample number, SNVs in the remaining 462 samples were classified into 96 base substitution types within the trinucleotide sequence context that includes the bases immediately 5′ and 3′ to each altered base (4). Mutation signature analysis was performed to resolve the SNVs for each sample into a set of characteristic patterns (signatures) to infer the contributions of each signature across samples (25). All SNVs for each sample were projected onto the 30 previously described COSMIC signatures (25) to infer the mutational signature pattern of each sample. However, we observed that broad-spectrum signatures (especially the ERCC2 signature; ref. 26) were under-represented (Supplementary Fig. S1A) compared with signatures with distinct motifs. Therefore, we repeated this projection procedure onto six mutation signatures only (Supplementary Fig. S1B). These six consisted of four identified from de novo analysis of the TCGA MIBC data set (4): APOBEC-a, APOBEC-b, C>T transitions at CpG dinucleotides, ERCC2 (26); and two signatures for which there was at least one sample matching the COSMIC consensus signature for >15 SNVs: Altatoxin-B1 (27) and mismatch repair (28). This projection procedure generated probabilities of mutation signature type for each individual SNV. These probabilities were then summed for all mutations per sample to give a count of SNVs specific to each mutation signature type (Supplementary Table S1.5).

Statistical analysis

Statistical analyses were conducted using R and Python software. Statistical tests included \( \chi^2 \) or Fisher exact tests for categorical variables and the Wilcoxon Rank-Sum test (two-group comparisons) or the Kruskal–Wallis exact test (three-group comparisons) for continuous variables. Patterns of mutation co-occurrence and mutual exclusivity were analyzed using the Fisher exact test applied to 2 × 2 contingency tables for all gene pairs analyzed. The resulting \( P \) values were adjusted with Benjamini–Hochberg correction. A \( P \) value of <0.1 was considered statistically significant. For survival analyses, Kaplan–Meier curves were compared using the log-rank test. Overall survival (OS) was calculated from the date of diagnosis to the date of death or last follow-up. Death from any cause was considered for survival analysis. Patients alive at last follow-up were censored for OS. Progression-free survival (PFS) was calculated from the date of diagnosis to the date of progression, death, or last follow-up. Progression was defined as a transition from NMIBC to MIBC or the development of metastatic tumors for individuals initially diagnosed with localized MIBC. Patients still alive and without progression were censored for PFS. Recurrence-free survival was also assessed in the HG-NMIBC cohort. In patients with HG-NMIBC, recurrence was defined as the reappearance of NMIBC following at least one negative cystoscopy.

Results

Patient demographic and clinical data

To characterize the genomic landscape across the full clinical spectrum of urothelial carcinoma, we identified all patients with urothelial cancer at our institutions (DFCI and BWI) whose tumors had been evaluated for mutations on the same analytic platform, Oncopanel, between the years 2013 and 2017. Of 503 urothelial samples that had been analyzed using Oncopanel, 31 were excluded due to non-UUC histology (tumors predominantly urothelial carcinoma but including up to 30% variant histology were included) or urethral primary sites (Supplementary Fig. S2). Note that 472 urothelial carcinoma samples remained which were comprised of LG-NMIBC (n = 82), HG-NMIBC (n = 126), MIBC (n = 199), LG-UTUC (n = 10), and HG-UTUC (n = 55). Clinical characteristics matched those expected for urothelial carcinoma, with median age at diagnosis of 68 years, male:female ratio of 3.4:1, and 76% current or past smokers (Supplementary Table S1.0). Nearly all patients were Caucasian (95%, Table 1).

Median follow-up for the cohorts ranged from 20 to 44 months; as of May 2018, 356 were alive and 116 had died. Ninety-four of 472 (20%) tumors had some degree of variant histology (maximum 30%), including 33 squamous, 19 micropapillary, 16 mixed, 10 glandular, 7 sarcomatoid, 4 plasmacytoid, 3 clear cell, and 2 neuroendocrine. Seventy-seven of 472 (17%) had received prior Bacille Calmette Guerin (BCG) intravesical treatment; the remainder had no prior treatment for urothelial carcinoma.

Tumor mutational burden

Two hundred and thirty-seven genes common to all versions of Oncopanel were studied for mutation and CNA events. Tumor mutational burden (TMB) was calculated as the number of non-synonymous protein-coding variants divided by the total sequenced genome length. Excluding LG-UTUC tumors due to small sample size (n = 10), 6,685 somatic mutations were identified in 462 tumors for a mean TMB of 14.03 mutations/megabase (Mb) and a median of 10.9Mb (Supplementary Table S1.3). Both HG-NMIBC and MIBC had a higher TMB than LG-NMIBC (median = 12.1 and 11.4, vs. 9.3, \( P = 0.001 \) and \( P < 0.01 \), respectively; Fig. 1A). HG-NMIBC also had higher TMB levels than HG-UTUC (median for HG-UTUC = 10.9, \( P < 0.05 \); Fig. 1A). Note that 3,471 SNVs and indels (of 6,685, 52%) were judged to be deleterious based on analysis of effects of missense variants (see Materials and Methods for detail; Supplementary Table S1.4).

Mutation signature analysis

In the TCGA MIBC cohort of 412 patients, 5 mutation signatures were identified, reflective of different mutagenic mechanisms operative in bladder cancer (4). Two of these were different variants of APOBEC mutagenesis (C>G or T at TCW) which together accounted for 67% of all SNVs identified and was strongly associated with TMB (4). We used projection mapping to map all somatic mutations identified here onto six mutation signatures. These were the 4 signatures seen in >1% TCGA MIBC samples: two closely related APOBEC signatures, the aging signature (C>T at CpG dinucleotides), and the mutation signature associated with ERCC2 mutations; and two additional signatures seen in this cohort but not in the TCGA MIBC set: the mismatch repair signature and the altatoxin B1 signature (COSMIC...
Table 1. Clinicopathologic characteristics of 472 urothelial carcinoma patients

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signatures 6 and 24, respectively; refs. 27, 28; Supplementary Table S1.5). The combined APOBEC mutation signature was the predominant mutation signature seen in all cohorts. However, APOBEC mutations were significantly more frequent in the MIBC and HG-NMIBC cohorts than the other two cohorts (Fig. 1B and HG-NMIBC cohorts than the other two cohorts (P < 0.01 for each comparison; Fig. 1D). C> at CpG dinucleotide mutations and ERCC2 signature mutations were seen at lower frequency in all cohorts and were of similar frequency among the four (Fig. 1B and C). Three MIBC tumors showed a predominance of C>A transversions, a pattern that is characteristic of the Afatoxin-B1 mutation signature (27). All 3 patients were never-smoking Caucasians without known exposure history. Two patients had high numbers of mutations matching the mismatch repair signature (28). One HG-NMIBC patient had two frameshift mutations in MSH2, with 7 indel mutations in 5 genes, and a TMB of 65.3 mutations/Mb. A second HG-NMIBC patient had frameshift deletions in both PMS2 and MSH2, and a TMB of 74 mutations/Mb. In aggregate, these observations suggest that APOBEC mutagenesis is the predominant mechanism of mutagenesis occurring in all stages and sites of urothelial carcinoma, and that an Afatoxin-like mutation process and defective mismatch repair contribute to a small fraction of urothelial carcinoma patients (each <1%).

### DDR gene alterations

Recent studies have suggested that there is an association between DDR gene alterations and sensitivity to cisplatin-based regimens (29, 30) and to immunotherapy treatment for urothelial carcinoma (6, 31). Thirty DDR genes were assessed in this study. Using conventional criteria (see Materials and Methods), deleterious DDR gene alterations were identified in 253 patients (54%). They were seen at higher frequency in the MIBC (111/199, 56%) and HG-NMIBC (84/126, 67%) compared with LG-NMIBC (37/82, 45%) and HG-UTUC (21/55, 38%; P = 0.0009), similar to overall TMB. Furthermore, DDR gene alterations were associated with higher TMB in both the HG-NMIBC and MIBC tumors (P < 0.0001 and P = 0.0001, respectively; Supplementary Fig. S3).

We repeated this analysis using a more stringent definition of mutation in DDR genes (see Materials and Methods) and identified DDR gene alterations in 107 patients (23%). ATM, ERCC2, PRKDC, and ATRX were the most frequently mutated genes, with overall mutation rates of 4.3%, 4.1%, 2.4%, and 2.2% (Supplementary Table S1.6). Thirteen of the 23 (57%) ATM variants were truncating mutations, whereas ERCC2 variants were nearly all missense (18 of 21, 86%). As predicted, ERCC2-altered tumors were significantly enriched for the ERCC2 mutational signature across LG-NMIBC, HG-NMIBC, and MIBC tumors (P = 0.0005, P < 0.0001, and P = 0.002, respectively; Supplementary Fig. S4). Throughout the urothelial carcinoma cohort, ever smokers with ERCC2-altered tumors had significantly higher ERCC2 mutation signature activity (median 9.4) than never-smokers with ERCC2-wild-type (WT) tumors (median 2.4) and ever-smokers with ERCC2 WT tumors (median 3.4; <0.0001 for both comparisons). Furthermore, the presence of any DDR variant was strongly associated with higher TMB in both the HG-NMIBC and MIBC cohorts (both P < 0.0001, Fig. 2).

### Focal somatic CNA count increases with bladder cancer invasiveness

Aneuploidy is thought to be a driver of tumor development in many cancer types and has been associated with high-grade tumors and poor outcomes (32). Thus, we examined the distribution of somatic copy number variation (CNV) events across all four urothelial carcinoma cohorts. CNV events were defined as homozygous deletions or amplifications involving any of the 237 genes (Supplementary Table S1.7). MIBC, HG-NMIBC, and HG-UTUC tumors had significantly higher CNV counts than LG-NMIBC (P < 0.0001, P < 0.0001, and P = 0.001, respectively). Sixty-four of 199 (32%) MIBC, 44 of 126 (35%) HG-NMIBC, and 15 of 55 (27%) HG-UTUC had a ≥1 amplification event versus 4 of 82 (5%) LG-NMIBC tumors (each comparison P < 0.0001). Across the entire cohort, 72 of 202 (36%) patients with alterations in TP53 had one or more amplification event versus 55 of 260 (21%)
without alterations in TP53 ($P < 0.001$). No other gene altered in $\geq 10\%$ of our cohort was associated with gene amplification events. This suggests that TP53 alterations in urothelial carcinoma increase genomic instability leading to gene amplifications.

Genomic alteration patterns according to urothelial carcinoma type

A comprehensive view of all mutations and CNA events identified in 472 samples is shown, sorted by urothelial carcinoma subtype, in Fig. 3. Twenty-three of the 237 genes assessed were altered in $\geq 10\%$ of at least one subtype of urothelial carcinoma. CDKN2A was the only gene with homozygous deletions in $\geq 10\%$ for all subtypes of urothelial carcinoma, as has been reported in other studies (3, 4, 33).

The frequency of alterations in each gene was compared among the four predominant urothelial carcinoma subtypes studied here (Supplementary Table S1.8). Five of 237 genes showed a significant difference in alteration frequency (Fig. 4; $q < 0.1$). TP53 alterations were much higher in all 3 HG subtypes in comparison with LG-NMIBC (45%, 58%, and 47%; vs. 4% in LG-NMIBC, $P < 0.0001$, $q = 0.009$). Similarly, RB1 gene variants were also seen more frequently in MIBC, HG-NMIBC, and HG-UTUC than in LG-NMIBC for which no RB1 alterations were identified ($P < 0.0001$, $q = 0.009$). In contrast, both FGFR3 and KDM6A genomic aberrations were seen more commonly in LG-NMIBC (72% and
44%, respectively) than in other urothelial carcinoma subtypes ($P < 0.0001$, $q = 0.009$; $P = 0.0014$, $q = 0.09$, respectively; Fig. 4). HRAS was the only gene to be enriched in the HG-UTUC cohort (7/55, 13%) in comparison with all other urothelial carcinoma subtypes ($P = 0.0017$, $q = 0.09$, Fig. 4).

Sixteen chromatin modifier (CM) genes were assessed for alterations in this cohort, and all showed deleterious alterations in at least one tumor (Supplementary Table S1.9). Note that 340 of 462 (74%) of tumors had alterations in at least one of the CM genes. CM genes were altered in 85% of HG-NMIBC, 77% of LG-NMIBC, 67% of MIBC, and 67% of HG-UTUC ($P = 0.002$). The most commonly altered CM genes were KDM6A (138/462, 30%), ARID1A (118/462, 26%), and CREBBP (72/462, 16%).

Mutational patterns in HG-Ta and HG-T1 tumors

We then compared the mutational landscapes of both HG-Ta and HG-T1 tumors. None of the 237 genes was significantly altered in one cohort versus the other. In addition, there was no significant difference in the TMB (HG-Ta: 10.9 vs. HG-T1: 14.4, $P = 0.18$) or CNV counts between these two pathologic subsets ($P = 0.59$).

Comparison of LG-UTUC and HG-UTUC

Although limited by the small number of LG-UTUC cases available ($n = 10$), we examined the prevalence of genomic alterations in LG-UTUC in comparison with HG-UTUC (Fig. 3). FGFR3 mutations were seen more frequently in LG-UTUC (80%) than in HG-UTUC (16%, $P = 0.0002$, $q = 0.03$, Supplementary Table S1.10). In contrast, TP53 alterations were seen exclusively in HG-UTUC tumors ([26/55 (47%) vs. 0/10 (0%), $P = 0.004$, $q = 0.15$, Supplementary Table S1.10]. Interestingly, the mutation spectrum of LG-UTUC was quite similar to that of LG-NMIBC tumors with a predominance of FGFR3, KDM6A, and STAG2 alterations in both subtypes.
Genomic alteration patterns according to variant histology

Mutation profiles were compared between 135 pure urothelial carcinoma (including 4 poorly differentiated urothelial carcinomas) and 64 with variant histologies in the MIBC cohort. Among 237 genes, two genes were significantly enriched in the pure urothelial carcinoma cohort versus variant urothelial carcinoma: ARID1A mutations were seen in 47 of 135 (35%) pure urothelial versus 7 of 64 (11%), \( P = 0.0003, q = 0.07; \) STAG2 mutations were seen in 18 of 135 (13%) versus none of 64 (0%), \( P = 0.0009, q = 0.1. \)

Figure 3.
Comutation plot of genes with \( \geq 10\% \) alteration frequency for four subtypes (LG-NMIBC, HG-NMIBC, MIBC, and UTUC) of urothelial carcinoma. Mutation types and clinical features are indicated. For UTUC (lower right), both high-grade and low-grade tumors are included and marked differences in frequency of TP53 and FGFR3 mutations can be appreciated.
KDM6A mutation rate varies according to gender and urothelial carcinoma subtype

The male-to-female ratio for the incidence of bladder cancer is approximately 3:1 in multiple series. The molecular basis for this gender disparity is unknown. Several studies have sought associations between molecular genetic events and gender predisposition (34–36). However, none of the studies have examined the association between gender and molecular alterations in bladder cancer across all grades and stages.

Recently, a significant difference in the frequency of KDM6A mutations according to sex was identified in early stage LG-NMIBC (5). KDM6A is located on Xp11.3 and encodes the histone lysine demethylase UTX (37). In contrast to the majority of genes on the X chromosome, KDM6A escapes X inactivation and often demonstrates two hit mutational inactivation in females in several blood malignancies including T-cell acute lymphoblastic leukemia and B-cell lymphoma (37–39). We examined the prevalence of KDM6A mutations in our cohort according to sex. Although there was no significant difference in KDM6A mutation prevalence according to sex in HG urothelial carcinoma subtypes, women with LG-NMIBC tumors had a significantly higher frequency of KDM6A mutations than men (10 of 14, 71% vs. 26 of 68, 38%, P < 0.05, Supplementary Fig. S5), consistent with the earlier report (5). Furthermore, the majority of women with KDM6A mutations and LG-NMIBC (8 of 10, 80%) showed biallelic inactivation of KDM6A. The frequency of biallelic mutation in KDM6A in women was reduced in HG urothelial carcinoma subtypes, in comparison with LG-NMIBC (10 of 25, 40% vs. 8 of 10, 80%, P = 0.06).

Co-occurrence and mutual exclusion among genetic events

We examined gene mutation co-occurrences in each urothelial carcinoma subtype for every gene altered in ≥10% of patients in that subtype (Supplementary Table S1.1, Fig. 5). Gene mutations in RB1 and TP53 were significantly associated (co-occurred) in both MIBC (P = 0.0002, q = 0.04) and HG-NMIBC (P < 0.0001, q = 0.02; Fig. 5), as noted in previous analyses (4). In contrast, FGFR3 mutations were mutually exclusive with TP53 mutations in MIBC (P = 0.001, q = 0.08, Fig. 5), also similar to prior reports (4, 40). ATM and ARID1A mutations were also found to co-occur in HG-NMIBC (P = 0.0007, q = 0.07, Fig. 5). Power was limited for this analysis in UTUC due to the smaller number of cases, but EP300 and PIK3CA mutations co-occurred in that subtype (P = 0.0003, q = 0.04, Fig. 5), and there was suggestive evidence for several other co-occurrences, including EP300 and FGFR3 (P = 0.006, q = 0.25), and PIK3CA and FGFR3 (P = 0.02, q = 0.48). Neither co-occurrence nor mutual exclusion was seen for any gene pairs in the LG-NMIBC group.

Gene mutations associated with progression and/or survival

Metastasis occurred in 40 of 126 (32%) MIBC patients during the follow-up interval (median follow-up 20 months). None of the commonly mutated genes (≥10%) in MIBC showed an association with metastasis.

Progression to MIBC occurred in 16 of 122 (13%) subjects with HG-NMIBC during this study. None of the commonly mutated genes in HG-NMIBC were associated with this event. Recurrence of HG-NMIBC is a common clinical problem necessitating frequent and long-term monitoring. We examined the possibility that local recurrence might correlate with mutation spectrum in the 72 HG-NMIBC whose specimen was analyzed at the time of diagnosis. Recurrence occurred in 22 of 72 (31%) patients and was seen at higher frequency in tumors with CREBBP mutations (9 of 18, 50%) than in those without CREBBP mutations (13 of 54, 24%, P < 0.05, Supplementary Fig. S6A), although there was no difference in time until recurrence according to CREBBP mutation status (Supplementary Fig. S6B). No other frequently-altered gene was associated with recurrence, nor were DDR-gene pathway alterations as a group.

The association between frequently altered genes and OS was assessed for both the MIBC and HG-UTUC cohorts. No gene was significantly associated with OS in MIBC. In contrast, alterations in each of PIK3CA, EP300, and FGFR3 were all associated with poorer OS in UTUC (P < 0.0001, q = 0.0002; P < 0.0001, q = 0.0001; P < 0.05, q = 0.08, respectively, Supplementary Fig. S7). Interestingly, all 4 patients with concomitant alterations in PIK3CA, EP300, and FGFR3 died (median OS of 144 days), whereas a fifth patient with alterations in EP300 and PIK3CA died 350 days after the date of initial diagnosis.

Discussion

Here, we report mutational analysis on one of the largest series of urothelial carcinoma patients ever described, analyzed at a single institution using a uniform MPS panel covering 237 cancer genes. The mutation and CNV event calls were performed using a standardized pipeline for alteration identification based on high read-depth MPS, with adjudication by board-certified molecular pathologists without bias as to the site of origin, grade, or clinical features of these patients. Assessments of missense mutation significance were also performed blinded to urothelial carcinoma subtype and clinical details. These factors provide confidence about the observations made here.

Several genes had markedly different rates of mutation in different subtypes of urothelial carcinoma (Fig. 4). TP53 and RB1 mutations were seen at much higher frequency in all of HG-NMIBC, MIBC, and UTUC than in LG-MIBC, whereas the converse applied to KDM6A and FGFR3, for which mutations were much more common in LG-NMIBC and LG-UTUC. Furthermore, TMB was similar in MIBC and HG-NMIBC, and higher than seen in
either HG-UTUC or LG-NMIBC. These and related observations have at least three important consequences. First, HG-NMIBC is genetically more similar to MIBC than it is to LG-NMIBC, consistent with prior reports (41–43). Our unbiased mutation assessment provides strong confirmation of this important distinction and validates the long-standing practice of differentiating between low- and high-grade noninvasive disease for clinical management.

Second, these observations on HG-NMIBC suggest the potential clinical utility of mutational assessment in these tumors, either broadly, or focused on FGFR3, KDM6A, TP53, and RB1, which may have important value in clinical management strategies. This will require further study in prospective cohorts. However, it is notable that neither TP53 nor RB1 mutations were prognostic for progression in the HG-NMIBC cohort, although this may reflect the limited number of events here despite the large number of patients studied, and/or the limited period of clinical follow-up available (median 30 months).

Third, these observations focus attention on the question of why KDM6A and FGFR3 mutations are not seen at higher

Figure 5.
Correlation between observed and expected co-occurrence of mutations for four urothelial carcinoma subtypes (LG-NMIBC, HG-NMIBC, MIBC, and HG-UTUC) showing only genes with ≥10% alteration rate.
crebbp gene alterations present at diagnosis. Crebbp encodes the transcriptional coactivator and histone acetyltransferase crebbp-binding protein (47). Alterations in crebbp have been seen at relative high frequency in esophageal and small cell lung cancer (48, 49). Our data suggest the possibility of treatments including histone deacetylase inhibitors for crebbp-altered HG-NNIBC.

In a series of 31 UTUC samples, Moss and colleagues (2) identified fgf3, pik3ca, and tp53 mutations in 60%, 27%, and 33%, respectively, of high-grade samples. One patient was microsatellite instability-high. A larger cohort of 60 HG-UTUC (3) showed fgf3 to be altered in 36% of HG-UTUC with HRAS alterations occurring in 14% of the tumors samples, whereas pik3ca and tp53 mutations were seen in 10% and 25%, respectively (3). In our series, tp53 was the most frequently altered gene (47%), with fgf3 and pik3ca mutations occurring only in 16% and 15% of cases. This enrichment for tp53 alterations in our HG-UTUC cohort may stem from the high proportion of invasive tumors (52/55) versus 37 of 59 and 10 of 31 in the Sfakianos (3) and Moss and colleagues (2) cohorts, respectively. A striking finding in our cohort was the association of fgf3, ep300, and pik3ca mutations with poor OS in UTUC, though the relatively small number (n = 55) of tumors analyzed also leads us to emphasize the need for replication of these findings in other UTUC cohorts. Nonetheless, based on our findings, there appear to be two distinct types of HG-UTUC. The first is characterized by co-occurring alterations in pik3ca, ep300, and/or fgf3 and exhibits a median survival of only 5 months. The second type of HG-UTUC had no alterations in any of these 3 genes, and had a median survival of 26 months. There are significant limitations to this study. The panel of genes was not chosen based on knowledge of mutation spectrum in urothelial carcinoma, but rather cancer-associated genes as a whole, and assessed only 24 of 58 genes that were found to be significantly mutated in MBc in the concurrent TCGA analysis (4). Among the genes altered in ≥10% of the TCGA MBc tumor specimens, 13 of 20 genes were part of our sequencing panel. Second, as only tumor samples were analyzed, we could not assess somatic versus germline status with certainty. We used Sift and Polyphen2 to assess the significance of missense variants but recognize that it is very likely that some “significant” variants were excluded by this process, whereas other “non-significant” variants were erroneously included. However, it is notable that even with known germline versus somatic status, it is often difficult to determine functional significance of mutations. Nonetheless, our mutation frequencies in the MBc subset were similar to the mutation frequencies identified in the TCGA based upon tumor-normal comparison, suggesting that our mutation calling approach was effective if not perfect.

Herein, using a standardized CLIA-approved mutation assessment platform, we show that (1) the mutation spectrum and TMB of HG-NNIBC are virtually identical to MBc and distinct from LG-NNIBC. Over half of both HG-NNIBC and MBc is not derived from LG-NNIBC, given the major differences in fgf3 mutation rates in these tumors. (2) KDM6A mutations are much more common in women with LG-NNIBC than men, but this is not seen in other urothelial carcinoma subtypes, suggesting an important difference in pathogenesis for LG-NNIBC. (3) A subtype of HG-UTUC characterized by alterations in any of fgf3, pik3ca, and ep300 is associated with much worse clinical outcome than HG-UTUC without alterations in any of those genes.
Disclosure of Potential Conflicts of Interest

R. Umeton is an employee of and has ownership interests (including patents) at Health Catalyst Inc. L. Harshman is a consultant/advisory board member for Exelixis, Bayer, Genentech, Dendreon, Pfizer, Medivation/ Astellas, Kew Group, Covus, Merck, Novartis, Jounce, OnClive, PER, and reports receiving commercial research grants from Bayer, Soto, Bristol-Myers Squibb, Merck, Dendreon/Valient, Janssen, Medicines/astellas, Genentech and Pfizer. E.M. Van Allen has ownership interests (including patents) in Genome Medical, Syapse, and Tango Therapeutics, reports receiving speakers bureau honoraria from Illumina, is a consultant/advisory board member for Genomic Medicine, Invitae, Tango Therapeutics, and Foresite Capital. M. Preston reports receiving commercial research support from Merck. Institutional Research funding. K.W. Mouw is a consultant/advisory board member for Pfizer and EMD Serono, and reports receiving commercial research funding from Pfizer. T.K. Choueiri is a consultant/advisory board member for AstraZeneca, Alexion, Sanoﬁ/Aventis, Bayer, Bristol-Myers Squibb, Cerulean, Eisa, Foundation Medicine Inc., Exelixis, Genentech, Heron Therapeutics, Roche, GlaxoSmithKline, Merck, Novartis, Peloton, Pfizer, Prometheus Labs, Corvus, Calithera, Analysis Group and Takeda. G. Sonpavde reports receiving speakers bureau honoraria from Physician & Education Resource (PER), and Research to Practice (RTP), is a consultant/advisory board member for Bayer, Sanofi, Pfizer, Novartis, Exelixis, Eisai, Janssen, AstraZeneca, Merck, Genentech, EMD Serono, Astellas, and reports receiving commercial research grants from Sanofi, Bayer, Boehringer-Ingelheim, Merck, Astra-Zeneca, Bristol-Myers Squibb, Pfizer and Celgene. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.H. Nassar, K. Lundgren, L. Harshman, M. Preston, J. Bellmunt, T.K. Choueiri, G. Sonpavde

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.H. Nassar, R. Umeton, J. Kim, E.M. Van Allen, M. Preston, F. Dong, J. Bellmunt, K.W. Mouw, T.K. Choueiri, G. Sonpavde, D.J. Kwiatkowski

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Study supervision: T.K. Choueiri, G. Sonpavde, D.J. Kwiatkowski

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Mutational Analysis of 472 Urothelial Carcinoma Across Grades and Anatomic Sites

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