Genomic distinctions between metastatic lower and upper tract urothelial carcinoma revealed through rapid autopsy

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Abstract

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Conclusions: UTUC demonstrated a lower overall mutational burden but greater structural variability compared to LTUC. Our findings suggest that metastatic UTUC displays a greater
spectrum of copy number divergence from LTUC. Importantly, we identified druggable lesions shared across metastatic samples, which demonstrate a level of targetable homogeneity within individual patients.

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Introduction

Urothelial carcinoma (UC) can occur anywhere along the urinary system. The vast majority of these arise from the lower tract (LTUC) involving the bladder, where up to 30% present with muscle invasion. Standard of care locoregional treatment options include either radical cystectomy and pelvic lymph node dissection or chemoradiation therapy (1-3). Neoadjuvant cisplatin-based chemotherapy achieves a complete response in up to 40% of patients (4-8) yet the overall 5-year survival benefit is only 5-10% (9). Once patients progress, metastatic bladder cancer has a median survival time of 14-15 months (10). For these patients, treatment options may include chemotherapy and immune-modulating approaches (3). Although most patients may achieve initial response, metastatic bladder cancer remains a lethal condition with significant morbidity and mortality worldwide.

Upper tract urothelial carcinoma (UTUC) is a less common entity, accounting for 5-10% of all urothelial tumors, and involves the ureter or renal pelvis (11). UTUC patients often present at an older age, with higher stage disease, which can be associated with worse survival (12-14). Standard of care therapy includes radical nephroureterectomy with consideration for neoadjuvant or adjuvant cisplatin-based chemotherapy in select patients. Platinum based chemotherapy has shown efficacy in a proportion of patients (15, 16) but the overall survival of metastatic UTUC is poor (17, 18).

Given the disease burden and morbidity of both UTUC and LTUC, a better understanding of the pathophysiology of disease progression is required to identify therapeutic targets and predictive
biomarkers to improve outcomes. Genomic sequencing of localized LTUC has led to new insights into the genetic determinants of bladder cancer and potential therapeutic opportunities (19-26). However, less is known about the genomic landscape of UTUC (27-31). For example, recent work has shown that both cancer types share similar mutations; however, UTUC exhibits more alterations in fibroblast growth factor receptors (FGFRs), and higher incidence of microsatellite instability, while LTUC demonstrates more frequent mutations in TP53 (27-30). Clinical studies also suggest that UTUC and LTUC exhibit differential responses to specific systemic treatment regimens raising the question of discordant biology (32-34).

While prior studies of localized urothelial cancers have improved the understanding of disease physiology with potential treatment implications, exceedingly little is known about the genomic architecture of metastatic urothelial carcinoma. Only a singular report conducted genomic analyses across multiple metastatic specimens from the same patients and described early branching evolution in LTUC (35). However, no studies to date have employed contemporary genomic sequencing and analysis to compare primary and metastatic LTUC and UTUC tumors. Furthermore, the extent to which intra-patient heterogeneity exists in individuals with disseminated UTUC is unknown. In this study, we have evaluated a unique set of patient tumors acquired through the University of Washington Urothelial Cancer Rapid Autopsy Program between 2015-2017. In particular, we performed whole exome sequencing on a total of 37 primary and metastatic tumors from 7 UTUC and LTUC patients. A comparative analysis based on mutational burden, mutational signatures, somatic single nucleotide variants (sSNV), and somatic copy number variations (sCNV) revealed unique distinctions between these topologically divergent disease entities across and within patients. In addition, we delineate the clinical
implications of putative druggable lesions across multiple metastases. Together, the genomic landscape of metastatic UTUC and LTUC reveals new differences between the two disease phenotypes and raises the promise of therapeutic opportunities.

Results

The mutational burden differs between localized and metastatic UTUC and LTUC

We conducted whole exome sequencing across multiple tumor specimens in 7 patients with metastatic LTUC and UTUC (Fig. 1A and Supplementary Tables 1 and 2). Three patients had UTUC originating from the renal pelvis (n = 2) and ureter (n = 1) and the remainder had LTUC of the bladder (n = 4). Mean age at diagnosis was 67.3 (+/−11.6 years). Most patients were Caucasian and men (n = 6 and 5, respectively). Three of the seven patients had a history of smoking while two endorsed a history of chemical exposures. A family history of bladder cancer was present in two patients while nearly all patients (6/7) had a family history of other cancers. All patients had predominant urothelial histology with three patients had small areas of squamous differentiation (Supplementary Table 1).

In order to delineate differences between localized and metastatic UTUC and LTUC, we analyzed the exomes of 13 metastatic tumor specimens from UTUC patients and 17 metastatic tumor specimens from LTUC patients (Fig. 1A). Primary cancer specimens and germline tissues were also analyzed. In the two patients with a known family history of bladder cancer (UTUC1, UTUC2), no germline pathogenic single nucleotide variants were identified. Interestingly, several
LTUC patients without clear family histories possessed germline loss of function (LOF) mutations including LTUC1 (ALDH1B1; NM00692.5:c.346C>T; g.chr9:38396091-38396091; p.Arg116*), LTUC2 (ATM; NM_000051.3:c.7630-2A>C; g.chr11:108202604-108202604; p.Leu2544Splice), and LTUC4 (ALDH16A1; NM_001145396.1:c.47C>A; g.chr19:49956635-49956635; p.Ser16*). We observed that the average LTUC mutational burden was significantly higher compared to UTUC (average Mut/Mb per patient 6.50 Mut/Mb vs. 3.71 Mut/Mb, \( p = 0.0001 \), Mann-Whitney-U test) (Fig. 1B, Supplementary Table 3). When stratified by primary or metastatic samples, UTUC versus LTUC tumors were also significantly different (6.08 Mut/Mb vs. 2.85 Mut/Mb in primaries; 6.57 Mut/Mb vs. 3.90 in metastases, \( p = 0.002 \), Mann-Whitney-U test, Supplementary Fig. 1). These findings are slightly lower than the mutational burden of primary LTUC observed in the bladder cancer TCGA (8.2 Mut/Mb) (20). Consistent with a recent report in primary UTUC (28), our results demonstrated a lower mutational burden in UTUC than LTUC in both primary and metastatic tumors.

**Mutational signatures remain consistent from primary to metastatic disease, but differ between UTUC and LTUC**

Next, we sought to determine whether our tumor samples comprised specific mutational signatures using the COSMIC signatures of mutational processes in human cancer. Mutational signatures were largely conserved between primary and metastatic tumors in both UTUC and LTUC (Fig. 1C). Overall, we observed mutational signatures for tobacco use, homologous recombination deficiency (HRD), and mitotic clock. Interestingly, the HRD signature was not associated with any pathogenic SNVs to HRD associated genes or biallelic loss of BRCA1/2 (Supplementary...
Tables 4 and 5, Supplementary Fig. 2). However, mono-allelic losses of HRD associated genes have also been described to contribute to defects in homologous recombination (36-38). Interestingly, our patients with the HRD mutational signature (UTUC1-3 and LTUC2 and LTUC4) all possessed multiple mono-allelic losses of genes implicated in homologous DNA recombination (Supplementary Tables 5 and 6).

In addition to the HRD signature, a major fraction of LTUC mutations was enriched for the APOBEC (apolipoprotein B mRNA editing catalytic polypeptide-like) signature, which is consistent with previous reports in lower tract disease (Fig. 1C) (20, 35). The APOBEC family of enzymes function as cytosine deaminases and can edit RNA or ssDNA (39). In tumor cells they are thought to be responsible for hypermutation at cytosine bases in exposed ssDNA and can promote cancer phenotypes (40). We observed that these signatures were maintained between primary tumors and metastatic LTUC tumors and do not appear to change with subsequent therapies (Fig. 1C). Together, these findings suggest that APOBEC mutagenesis is likely a common event in the development of LTUC which is preserved from primary to metastatic disease.

While the APOBEC signature is detected in LTUC tumors, it was observed at high levels in only one of the UTUC patients. Patient UTUC2 was more similar to our LTUC patients with a predominant APOBEC signature (Fig. 1C) while UTUC1 and UTUC3 had mutations enriched for the mitotic clock signature commonly seen in cancer. Notably, UTUC1 and UTUC3 had the lowest somatic mutation burden out of all of our patients as well (mean 2.6 [range 2.3 – 3.0 mut/Mb] vs. 6.4 [range 5.4 – 8.4 mut/Mb] in the remaining patients, p<0.001, two tailed T-test). Interestingly, the APOBEC mutations account for nearly all the differences in mutation rate between UTUC2
and LTUC1-4, and UTUC1 and UTUC3 (Supplementary Fig. 3). Together, these findings suggest that APOBEC-mediated mechanisms may account for the higher mutational load of UTUC2 and LTUC1-4 since the nucleotide editing activity of APOBEC is known to drive somatic DNA mutagenesis in cancer (40). Furthermore, the consistency of APOBEC predominance shared across all tumors samples within individual patients suggest this may represent a driving force in progression.

**UTUC primary tumors share fewer deleterious mutations with metastatic counterparts compared to LTUC**

In order to determine how sSNVs impact gene function, we adopted an algorithm for calling predicted deleterious/functional driver mutations (Supplementary Fig. 4). We found that UTUC tumors had fewer predicted deleterious mutations compared to LTUC (Supplementary Fig. 2, Supplementary Table 4). Mean and median burden of predicted deleterious mutations were significantly higher in LTUC compared with UTUC (Mean genes mutated in LTUC 59 +/-15 vs. UTUC 33 +/-14; Median LTUC 55 [IQR 49 – 68] vs. UTUC 28 [IQR 24 – 38], two tailed T-test, p<0.001) (Supplementary table 7). These findings are consistent with the overall higher mutational burden (Mut/Mb) of LTUC patients compared to UTUC patients (Fig. 1B). In addition, we sought to determine the extent to which different LTUC and UTUC patients share functional mutations. We observed in our small cohort that there were very few shared mutations between patients (highlighted by the blue areas, Fig. 2A). However, when we compared mutations from different tumors of the same patient, we observed a greater amount of homogeneity (Fig. 2A). When the mutated genes were further stratified into shared (present within every patient tumor), semi-shared
(present in two or more tumors), or private (found in one only tumor of patient), we found that LTUC patients have a greater proportion of shared mutations across primary and metastatic samples compared to UTUC patients (27% vs. 13%, respectively, p < 0.001, Chi-square test) (Figs. 2A-B). Reciprocally, UTUC tumors had a significantly higher proportion of private mutations across tumors (45% vs. 27%, respectively, p < 0.001, Chi-square test). This relationship was maintained when the primary specimens were excluded from the analysis (48% vs. 22%, p < 0.001, Chi-square test) (Fig. 2B). Interestingly, amongst these driver mutations, LTUC patients appear to possess relatively more shared deleterious mutations compared to UTUC patients. These findings suggest increased clonal genomic diversity between metastases within UTUC patients compared to LTUC patients. Interestingly, a recent publication analyzing LTUC and UTUC in patients with a history of both tumors identified clonal relatedness but different mutational patterns, indicating the importance of intra-individual mutagenesis (30).

**UC metastases can arise from non-dominant primary tumor populations**

Next, we sought to determine how specific deleterious mutations differ within and between patients with metastatic LTUC and UTUC. We did not observe any significant enrichment of specific mutations unique to UTUC or LTUC based on an unsupervised analysis of our small patient cohort despite having adequate power to detect shared mutations across individual patients (Fig. 2C and Supplementary Figs. 2, 5). However, we performed focused analysis and identified mutations reported in the urothelial carcinoma TCGA including **ERBB2** (S310F), **KDM6A** (Q555*; novel G407fs), **KMT2C** (novel D348N), **PIK3CA** (E545K), and **TSC1** (novel G147fs) (Fig. 2C and Supplementary Fig. 2). We further conducted gene set enrichment analysis of our functional
mutations identified in both LTUC and UTUC and found that a series of cancer associated pathways were altered. These include the tumor suppressive p53 pathway (FDR = 6.09E-4) and the oncogenic MAPK signaling pathway (FDR = 8.98E-6) both of which are deregulated in bladder cancer (Fig. 2C) (20). In addition, we were powered to observe that both UTUC and LTUC patients harbored a number of loss of function mutations to genes associated with chromatin remodeling including ARID1A, EP300, KDM6A, KMT2D, and KMT2E (Fig. 2C and Supplementary Fig. 2).

UTUC intra-patient tumor analysis uncovered mutations present in all metastases but not present at high levels in the primary tumor. An example of this can be seen with the SWI/SNF complex member ARID1A (AT-rich interaction domain 1A) which is commonly deregulated in urothelial carcinoma (20, 41-43). ARID1A encodes a component of the SWI/SNF protein complex which regulates transcription by altering chromatin structure. In patient UTUC1, we found that the primary tumor harbored a loss of function ARID1A frameshift mutation (2 base-pair deletion) at position Chr1:27107094. However, this was not found in any of the metastatic specimens despite >20x coverage at that position. Instead, all the metastatic sites shared another ARID1A loss of function mutation at position Chr1:27087900 (17 base-pair deletion). Interestingly, upon further analysis of Chr1: 27087900 in the primary specimen, we found that the 17-base deletion was indeed present, but at very low read depth (Supplementary Fig. 6). Interestingly, this finding was not isolated to ARID1A, and a similar phenomenon was observed after baseline filtering for ADAM29, SCNN1A, CFHR4, and MAGI1 in patient UTUC1 (Supplementary Table 8). Importantly, the decrease in the mutational load of these genes in the primary specimen was not secondary to a lower tumor cellularity (Supplementary Table 8). Together, these findings raise the important point that UTUC metastases can arise from non-dominate clonal populations. As this
observation was made within a single patient, it requires validation is a larger cohort and it remains to be determined if this also occurs in LTUC.

**Intra- and inter-patient copy number heterogeneity is prevalent in UTUC**

To determine intra- and inter-patient variability on gene structure, we conducted genome wide copy number analysis. Metastatic tumors exhibited more similarities with other metastases within the same patient than with the primary tumor (Fig. 3A). We found no consistent large copy number amplifications or losses that were uniform across both LTUC and UTUC. However, we observed 8p heterozygous loss across all LTUC tumors, which has been described in urothelial carcinoma (44). Next, we generated per nucleotide Euclidean distances between tumor samples to delineate how similar or different each tumor was within a patient and between patients at the copy number level. In Figure 3B, each patient specimen is represented as a single point, and the distance between each point underscores its relationship to all other tumors. Our analysis revealed that UTUC tumors exhibit substantial differences at the copy number level within patients compared to LTUC tumors. Moreover, as a group, LTUC patients were more similar to each other at the copy number level compared to UTUC patients (Fig. 3B). These findings suggest that UTUC tumors undergo a wider variety of copy number alterations compared to LTUC both within and between patients. This is further supported by hierarchical clustering of gene-restricted copy number profiles, which revealed a greater genomic difference between primary tumors and metastases in UTUC, which was less pronounced in LTUC (Fig. 3B, inset).
Next, we analyzed how copy number alterations impact specific genes and oncogenic pathways (Fig. 3C). We observed that a number of cell cycle genes were significantly altered in both UTUC and LTUC patients through gene set enrichment analysis (FDR = 6.76E-13). These included amplification of cyclin D1 (CCND1) and deletions of the cell cycle inhibitors CDKN2A and CDKN2B (Fig. 3C). MDM2 was amplified exclusively in UTUC patients across every tumor, while we observed no amplification in LTUC patients (Fig. 3C) in our patient population. Interestingly, while we did not observe sSNV mutations to the FGF receptors (Supplementary Fig. 2), their ligands including FGF3, FGF4, FGF19, and the FGFR adaptor FRS2 were amplified in our UTUC but not in our LTUC cohort. These gene specific findings suggest that copy number alterations may drive oncogenic processes through different mechanisms in LTUC and UTUC.

Analysis of putative druggable genomic alterations reveals a remarkable level of similarity between metastases

Given the multiplicity of tumor specimens we had for each patient, we next asked how different tumors within the same patient would be predicted to respond to targeted therapeutics. To do so, we evaluated mutations and structural alterations identified in each tumor for putative druggable target using the Drug Gene Interaction Database (DGIdb), OncoKB, and literature review. Potential therapeutic vulnerabilities included loss of function mutations of ARID1A, an activating PIK3CA E545K mutation, and a loss of function TSC1 mutation. We also observed changes at the gene structure level that may predict for drug sensitivity including amplification of cyclin D1, homozygous deletion of the CDK inhibitor CDKN2A, and genomic gains of FGFR1, FGFR3, and PIK3CA (Fig. 4). Interestingly, when considering only metastatic tissues, ~70% (15/22) of
mutations were present in all samples from the same patient. This finding demonstrates that putative druggable lesions can be homogenous within a metastatic urothelial cancer patient and highlights the potential utility of using a biopsy from a single metastatic site to guide treatment decision making.
**Discussion**

We report the first genomic analysis of primary and metastatic UTUC and LTUC tumors across multiple sites within the same patient and observe several differences between UTUC and LTUC. First, we observed that both primary and metastatic UTUC tumors had on average lower Mut/Mb than LTUC tumors, consistent with previous reports (20, 28). However, a recent study demonstrated that microsatellite instability (MSI) and Lynch Syndrome in UTUC patients is associated with higher mutational burden (30). Interestingly in our study, UTUC patients had a lower mutational burden than LTUC patients. None of our UTUC patients had evidence of perturbations to Lynch Syndrome associated genes such as *MSH2*, *MSH6*, and *MLH1*, or microsatellite instability (Supplementary Tables 4 and 9, Supplementary Fig. 7), which may explain the lower mutation rate amongst the UTUC patients we analyzed.

A major question with regard to the intrinsic differences between UTUC and LTUC has been what are the genomic idiosyncrasies of the two urothelial cancer phenotypes? UTUC more frequently harbor pathologic mutations to *FGFR3*, *HRAS*, *CDKN2B*, *KMT2D*, *KDM6A*, and *NOTCH*, while LTUCs enrich for *TP53*, *RB1*, *ATM*, and *ERBB2* mutations (27-30). In addition to these findings, our landscape view of copy number alterations across metastatic specimens from the same patients revealed that UTUC tumors exhibit more substantial differences at the copy number level compared to LTUC. This is evident both within and between patients and suggests that the fidelity of genome architecture in UTUC may be more disparately affected during tumor initiation and progression than LTUC. However, at the gene-specific level, our population revealed distinct oncogenic pathways consistently mutated in UTUC. For example, we observed the amplification
of MDM2 in all of our primary and metastatic UTUC patient samples (Fig. 3C). This finding suggests that there may be a selective pressure to preserve MDM2 amplification from primary disease to metastatic disease. This is consistent with MDM2’s role as an E3 ubiquitin ligase involved in proteasomal degradation of the tumor suppressor p53. Recently, it has been shown that MDM2 amplification is associated with increased stage and risk of distant recurrence in UTUC (45). Notably, MDM2 amplification was not observed in our LTUC cohort, yet is described to be present in 6% of LTUC (20), which may underlie differences in our small heavily treated patient population.

Our study also highlights the potential for putative druggable targets across tumor sites within an individual patient. For example, we found that out of 22 mutations observed within metastatic specimens with therapeutic potential, 15 (~70%) were present in all metastatic tumor specimens in a given individual (Fig. 4). This suggests that specific mutations may be clonally represented in the majority of tumors within a patient. Thus, a biopsy of a single metastatic site may be used in the setting of advanced stage bladder cancer to help guide treatment decisions. This has also been observed in an integrative genomic analyses of metastatic prostate cancer patient specimens through rapid autopsy. Indeed, Kumar et al. found limited genetic diversity within patients compared to substantial differences between patients (46). However, these findings are tempered by the fact that approximately 30% of mutation targets in our cohort were only present in a subset of metastases. For example, patient UTUC1 had only one PIK3CA mutation in a singular lung metastasis. Three other distinct metastases from UTUC1 did not exhibit the PIK3CA E545K mutations despite >33x coverage of the genomic region, >80% power to detect the mutation, and similar tumor cellularity (Fig. 4, Supplementary Figs. 2, 5). Furthermore, we observed that patient
UTUC1 had a TSC1 loss of function mutation which was only present in a primary tumor, but not present in any metastatic tumors (Fig. 4) (47). The emerging technology of circulating tumor DNA (ctDNA) sequencing may represent a potential solution to complement tumor tissue sampling. For example, ctDNA testing of patients with advanced LTUC can detect various germline and somatic genomic alterations including MDM2 amplification, which has the potential to guide therapies and monitor treatment response in a serial manner (42, 48). Future experiments comparing tumor DNA from multiple LTUC and UTUC metastases within a patient and matched ctDNA are needed to further assess its clinical utility.

We observed that metastatic UTUC may not arise from the dominant clonal population in a primary tumor. In particular, patient UTUC1 had an ARID1A frameshift at Chr1:27107094 which was not present in any metastatic specimens. Instead, the metastatic lesions shared an ARID1A frameshift at Chr1: 27087900 (Supplementary Fig. 6). This observation points to the potential importance of ARID1A loss in bladder cancer pathogenesis, which is supported by large genomic studies in bladder cancer and in vivo studies in other cancer models (49, 50). Importantly, we found that the primary tumor specimen did in fact contain the mutation found in the metastatic tissues, but it was well below our read count cut offs. This was observed for a series of other genes as well including ADAM29, SCNN1A, CFHR4, and MAGI1 after baseline filtering and adjusting for tumor cellularity (Supplementary Table 8). As such, it is possible that the metastases in UTUC1 were seeded by a non-dominant clone present in the patient’s primary tumor. This may be due to sampling bias; however, our primary tumor DNA specimens were derived from multiple punches across a tumor block. Moreover, this type of observation has been made in other cancers. For example, it has been shown in prostate cancer that a small tumor clone characterized by PTEN,
SPOP, and TP53 mutations, which was present in a patient’s primary tumor, seeded all the eventual metastases (51). These findings demonstrate the importance of obtaining genomic data in a dynamic manner from patient’s tumors since archival tissues may not adequately represent the diversity or even the clonal make up of metastatic lesions.

Our study has some of inherent limitations. The number of patients analyzed is limited and while we evaluated multiple tumors within patients, they received varying numbers of systemic treatments which may influence the observed differences. All patients in this cohort came from a single center and agreed to participate in a rapid autopsy program, and thus may not broadly represent all UTUC and LTUC. Moreover, our analysis was focused on DNA sequencing, while RNA and protein-based analyses may also identify important differences between UTUC and LTUC biology as well. Despite the limitations, this clinical work provides a much-needed genomic landscape view of metastatic LTUC and UTUC, which will lay the groundwork for future larger studies.

Conclusions

In this rapid autopsy series, we demonstrate that metastatic UTUC patients exhibited lower mutational burden, but more private deleterious mutations within patients. At the DNA copy number level, we observed greater intra- and inter-individual divergence in UTUC patients. Taken together, these findings may explain differences in clinical behavior and treatment response between UTUC and LTUC. Importantly, despite overall mutational differences, common
druggable targets were identified across metastases underlining opportunities for therapeutic targeting in metastatic urothelial carcinoma.
Materials and Methods

Clinical cohort

Metastases were obtained within 4 hours of death as part of the rapid autopsy program described previously (52). For the logistical framework of the rapid autopsy program, see Supplementary Fig. 8. Specimens were obtained from 7 patients including histopathologically normal tissue (n = 7, fresh frozen), primary tumor samples (n = 7; 5 FFPE, 2 fresh-frozen), and 4-5 metastases per patient (n = 30, fresh frozen) (Supplementary Tables 1 and 2). All patients within this cohort had metastatic urothelial carcinoma, 3 derived from upper tract (UTUC) and 4 derived from lower tract urothelial carcinoma (LTUC) (See Supplementary Table 1 for clinical details); all patients had visceral metastasis, while all but one had liver metastases. Primary or metastatic specimens selected for sequencing had >80% tumor cellularity by genitourinary pathology review (FVL).

Whole exome sequencing

FFPE and frozen total DNA were extracted using the Puregene DNA isolation kit (Qiagen) (See Supplementary Table 2 for listing of samples). Sequencing libraries were prepared from 1 µg of intact genomic DNA quantified on an Invitrogen Qubit 2.0 Fluorometer (Life Technologies-Invitrogen) and Trinean DropSense96 spectrophotometer (Caliper Life Sciences). DNA was fragmented using a Covaris LE220 Focused-ultrasonicator (Covaris) using factory settings for an average size of 200bp. Sequencing Libraries were prepared using the KAPA Hyper prep DNA Library Preparation kit (Kapa Biosoftware) following end repair and A tailing in a single tube
protocol. Library size distributions were validated using an Agilent 2200 TapeStation (Agilent Technologies) and quantified using the Qubit 2.0 Fluorometer. Individually indexed KAPA Hyper libraries were hybrid captured to NimbleGen SeqCap EZ Exome V3+UTR probes (Roche) according to the manufacture’s protocol on a Sciclone NGSx Workstation (PerkinElmer) utilizing a one capture per library strategy. Post capture library size distributions were validated using Agilent 2200 TapeStation and quantified using a Trinean DropSense96 spectrophotometer. Additional quality control during blending of pooled indexed libraries, and cluster optimization was performed using the Qubit® 2.0 Fluorometer. Libraries from frozen tissues were clustered at 4-plex and FFPE libraries at 3-plex per lane on an Illumina v4 flow cell using an Illumina cBot (Illumina, Inc.). Sequencing was performed using an Illumina HiSeq 2500 in high-output 100bp pair end mode using v4 reagents (PE100). Image analysis and base calling were performed using Illumina's Real Time Analysis v 1.18.66.3 software, followed by demultiplexing of indexed reads and generation of FASTQ files, using Illumina's bcl2fastq Conversion Software v1.8.4 (http://support.illumina.com). The raw sequencing data has been submitted to the Database of Genotypes and Phenotypes (dbGaP accession phs001797.v1.p1).

**Sequencing data analysis**

All sequencing reads were aligned to human genome reference Hg19 using Bowtie (53). GATK good practices were followed for pre-processing of all bam files (54). Average read depth was 51x with an average of 93% of nucleotides with >10x coverage (Supplementary Table 10) (55). IntersectBed from Bedtools (56) was used to calculate the on-target aligned read percentage. MuTect (version1) (57) and Strelka (58) was used to call somatic mutations and short indels. We
considered loci for variant calls covered by at least 14 normal reads with variant allele loci with a minimum of 7 alternate alleles and >q20 phred score. Tumor variants were restricted to ≥10% variant allelic frequency to exclude machine errors, sample preservation and Covaris fragmentation technique-associated low frequency false positive calls (59). Oncotator (60) and Annovar (61) were subsequently used for annotation of all called variants. Presumed deleterious non-synonymous single nucleotide variants (sSNVs) were predicted through a consensus call of the majority of 11 mutation functional impact assessor tools (>6/11, 55%; Supplementary Table 11) (60, 61). Additionally, all frameshift and non-frameshift insertions or deletions, splicing, and stop-gain mutations were included in the analysis (Supplementary Fig. 2, Supplementary Table 4). As a quality control measure, we evaluated the sSNVs from a single tumor which was bisected. Half was formalin-fixed paraffin embedded and half of was fresh-frozen. This revealed 80% concordance at the sSNV level demonstrating only a limited impact of the different preservation techniques on our analysis (Supplementary Fig. 9A).

**sSNV power analysis**

To assess whether each mutant locus had sufficient coverage to reliably determine its mutation status, we calculated the statistical power to call a somatic sSNV given the tumor read depth ($N$), tumor cellularity ($\alpha$), and tumor ploidy ($\phi$). Let $p$ be the expected variant allelic fraction for observing a heterozygous clonal sSNV, where

$$p = \frac{\alpha}{\alpha\phi + 2(1 - \alpha)}$$

For every locus, we computed the theoretical power using the binominal test for observing 3 or more variant reads,
\[ p(X \geq 3) = 1 - [\text{Bin}(0, N, p) + \text{Bin}(1, N, p) + \text{Bin}(2, N, p)] \]

For each sSNV, we extracted the reference and variant read counts and total read depth using the R function `pileLettersAt` from R/Bioconductor package `GenomicAlignments` (v 1.18.1). Indels were excluded from these power calculations. Tumor cellularity and tumor ploidy were estimated by Sequenza. For our analyses, we determined loci to have sufficient coverage for calling somatic mutations if the power estimate is greater than 80%. Overall, we show >80% power to detect mutations across individual patients for approximately 90% of mutations identified (Supplementary Fig. 5).

**Mutational signature analysis**

Mutations per megabase (Mut/Mb) stats included all called variants restricted to defined 104.8 megabase target capture regions (Supplementary Table 3). Mutational signatures were evaluated using the DeconstructSigs (62). In our adopted approach, DeconstructSigs utilizes trinucleotide somatic mutation frequencies, normalized to the exome capture region, followed by weighted reconstruction of the predominant signatures of each sample. A mutation signature analysis was then performed using sSNVs based on 30 predefined COSMIC signatures. Final groups were then clustered based on biological phenotypes (e.g. mitotic clock, APOBEC, homologous recombination deficiency, smoking, etc.)

**Copy number analysis**
Sequenza was used to perform absolute copy number calling and estimation of tumor cellularity and ploidy (63). Exome sequences were aligned using the BWA aligner (64). A Q15 base quality cut-off was used to avoid DNA alignment biases. Furthermore, only high quality (Q40) aligned reads were considered for all downstream copy-number estimation analysis. To avoid genome wide GC content related depth bias, a normalization step was performed using Hg19 GC content data from the UCSC genome browser (63, 65). Initial segmentation calls were made using the circular binary segmentation algorithm from the Bioconductor DNAcopy R package, followed by Sequenza’s probabilistic model-based copy-number estimation. The copy-number estimation utilizes the average depth ratio (tumor versus normal) and B allele frequency (the lesser of the two allelic fractions as measured at germline heterozygous positions) and performs the estimation considering the derived overall tumor ploidy/cellularity, segment-specific copy number and minor allele copy number (Supplementary Table 5). As a quality control measure, we evaluated the sCNV differences from a single tumor half of which was formalin-fixed paraffin embedded and half of which was fresh-frozen. This revealed high concordance in terms of somatic copy number aberration, tumor ploidy and % genome loss estimation, suggesting the differential mode of tissue preservation had no significant effect on estimated sCNV profiles (Supplementary Fig. 9B). Using the genomic segment-specific absolute copy number values, the relative genomic distance between every tumor-pair was calculated as a sum of the per nucleotide Euclidean distances) to derive intra-tumoral and inter-tumoral genomic distances. Mean centering of copy-number variation was used to derive an estimate of percent genome loss and amplification for each tumor tissue. Gene restricted copy number was derived using Gencode v19 annotation references. The recurrently copy number altered genes (altered in >68% of tumors in both LTUC and UTUC) were then checked for pathway enrichment using the GOrilla “GO” term enrichment tool.
**MSI evaluation**

MSIsensor was used to detect replication slippage variants at microsatellite regions for each tumor/normal pair and MSI scores reported (66) (Supplementary Table 9, Supplementary Fig. 7).

**Actionable target analysis**

Actionable mutation prediction was based on called variants only. Both sSNV and sCNV variants were queried using established drug-genotype databases including DGIdb (67) and OncoKB (68) to identify potentially actionable variants. The final list was filtered based on previously published preclinical data and clinical trials in cancer.

**Statistics**

Statistical analyses pertaining to each figure are included within appropriate legends. For direct comparison of continuous variables, two tailed T-test and/or Mann-Whitney-U tests were utilized, while Chi square was employed for categorical variables as appropriate.

**Study approval**

All samples were obtained from patients with signed informed consent under the aegis of the Cancer Donor Program at the University of Washington (University of Washington IRB # 2341).
Authors Contributions

A.C.H., J.L.W., H-M.L., and R.B.M. conceived of and design the project. B.R.W., N.D., S.J., S.A., H.B., L.M., G.H. A.C.H. acquired and analyzed and interpreted the data. F. V-L. conducted all pathological analysis. H.H.C., M.T.S., E.Y.Y., P.G., J.K.L., A.C.H. consented patients for this study. L.K. provided support for the rapid autopsy program. S.K.H. provided statistical support. B.R.W., N.D., and A.C.H. wrote the manuscript. All authors reviewed and approved the manuscript.

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References


Figure 1

A

Patients

<table>
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<th>UTUC2</th>
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<th>LTUC2</th>
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</tbody>
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Treatments

- BCG
- Cisplatin
- Docetaxel
- Check Point Inhibition

Patient Samples

- Germline tissue
- Primary tumor

Location of Metastases

- Lymph node
- Pleural
- Lung
- Intestine
- Liver
- Pancreas
- Omentum

B

Mutational burden of UTUC vs. LTUC

Mutations per Mb

p = 0.0001

UTUC vs. LTUC

C

Mutation signature frequencies

- Others
- Tobacco
- HRD
- APOBEC
- Mitotic clock

UTUC1, UTUC2, UTUC3, LTUC1, LTUC2, LTUC3, LTUC4
Figure 1. Whole exome sequencing of metastatic upper tract urothelial carcinoma (UTUC) and lower tract urothelial carcinoma (LTUC) reveals mutational heterogeneity and distinct mutational signatures.

A) Clinical features of rapid autopsy patients (N=7) including gender, smoking history, treatment history, and location of metastatic lesions. Of note, the ‘intestine metastasis’ (LTUC2) refers to an implant to the small bowel serosa from carcinomatosis.

B) Overall mutational burden of each sequenced tumor stratified by UTUC (N=16) and LTUC (N=21) is shown (box and whiskers plot representing median and interquartile range; p-values calculated using Mann-Whitney-U test).

C) Mutational signature analysis across each primary and metastatic UTUC and LTUC tumor specimen represented as a proportion of all mutations within a given tumor. All mutation analyses were performed following baseline read filtering (see methods). HRD: Homologous recombination deficiency; APOBEC: Apolipoprotein B mRNA, Catalytic Polypeptide.
Figure 2

A

B

Primary and metastatic samples

Metastatic samples only

Proportion of Mutated Genes

Private
Semi-Shared
Shared LTUC
Shared UTUC

p<0.0001

p<0.0001
Figure 2
Figure 2. Metastatic UTUC tumors exhibit more deleterious private mutations compared to metastatic LTUC tumors.

A) Heatmap representing the absolute number of predicted deleterious genes mutated that are shared between all pairs of tumors reveals limited inter-patient homogeneity across both UTUC (N=16) and LTUC (N=21). Here, we constructed a per-gene binary indicator vector for every tumor and calculated the number of impacted genes that are shared between all pairs of tumors which are plotted in the matrix. The numbers of shared mutations are indicated by the color scale. (LN = lymph node).

B) Predicted deleterious mutations across UTUC (blue) and LTUC (yellow) tumors represented as a percentage of all shared (colored), semi-shared (present in >1 sample), and private (only mutated in single samples) mutations. Left panel includes both primary and metastatic specimens while the right panel is limited to metastases only (p-values calculated using the Chi-square test).

C) Representative Bladder Cancer TCGA mutations identified in our patient cohort as well as genes identified by gene set enrichment analysis (tumor suppressive p53 pathway (FDR = 6.09E-4) and the oncogenic MAPK signaling pathway (FDR = 8.98E-6).
Figure 3

B

C

[Image of a figure showing various cancer types and data visualization]
Figure 3. Metastatic UTUC exhibits significant intra- and inter-patient copy number heterogeneity compared to metastatic LTUC.

A) Copy number analysis at the chromosome level normalized for baseline ploidy across each primary and metastatic UTUC (N=16) and LTUC tumor (N=21).

B) Multi-dimensional (2D) scaling (MDS) analysis utilizing estimated genome wide absolute copy number (UTUC: unshaded circles; LTUC: shaded circles). Results are stratified by primary (black border) vs. metastatic samples per patient. Axes represent multi-dimensional inter-sample distances projected to 2 dimensions using classical Multi-dimensional scaling. Inset: hierarchical clustering dendrogram using genome wide, gene-restricted, copy number profiles. The height of dendrogram delineates the cumulative copy number differences between tumor specimens.

C) Representative gene specific copy number analysis reveals altered cell cycle genes (delineated by gene set enrich enrichment analysis, FDR = 2.95E-4) as well as group of FGF family genes.
Figure 4. Significant homogeneity of druggable genomic alterations are present in urothelial carcinoma patients.

All deleterious sSNVs and copy number alterations were screened for potential druggability using DGIdb, OncoKB, and published literature. This analysis reveals that out of 22 mutation targets observed within metastatic specimens with therapeutic potential, 15 (~70%) were present in all metastatic tumor specimens within a given individual. However, out of 20 mutations present in primary tumors, only 11 (55%) were also present in all metastatic tumors. LOF = loss of function mutation, GOF = gain of function mutation, Homo-del = homozygous deletion and Het-loss = heterozygous loss.