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Brief Correspondence

Comprehensive Molecular Characterization Reveals Genomic and Transcriptomic Subtypes of Metastatic Urothelial Carcinoma

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Abstract

Recent molecular characterization of primary urothelial carcinoma (UC) may guide future clinical decision-making. For metastatic UC (mUC), a comprehensive molecular characterization is still lacking. We analyzed whole-genome DNA and RNA sequencing data for fresh-frozen metastatic tumor biopsies from 116 mUC patients who were scheduled for palliative systemic treatment within the context of a clinical trial (NCT01855477 and NCT02925234). Hierarchical clustering for mutational signatures revealed two major genomic subtypes: GenS1 (67%), which was APOBEC-driven; and GenS2 (24%), which had a high fraction of de novo mutational signatures related to reactive oxygen species and is putatively clock-like. Significantly mutated genes (SMGs) did not differ between the genomic subtypes. Transcriptomic analysis revealed five mUC subtypes: luminal-a and luminal-b (40%), stroma-rich (24%), basal/squamous (23%), and a non-specified subtype (12%). These subtypes differed regarding expression of key genes, SMGs, oncogenic pathway activity, and immune cell infiltration. We integrated the genomic and transcriptomic data to propose potential therapeutic options by transcriptomic subtype and for individual patients. This in-depth analysis of a large cohort of patients

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with mUC may serve as a reference for subtype-oriented and patient-specific research on the etiology of mUC and for novel drug development.

Patient summary: We carried out an in-depth analysis of the molecular and genetic features of metastatic cancer involving the cells that line the urinary tract. We showed that this is a heterogeneous disease with different molecular subtypes and we identified possible targets for therapy for each subtype.

Urothelial cancer (UC) is a molecularly and clinically heterogeneous disease. Comprehensive molecular profiling has been restricted to primary UC [1,2], and a multi-omics characterization of metastatic UC (mUC) is still lacking in the literature. Because of the lethality of mUC, with few therapeutic options available for patients, a multi-omics characterization of mUC could aid in improving patient selection for new and existing therapies. To unravel the molecular landscape of mUC, we conducted a comprehensive genomic and transcriptomic analysis of freshly obtained metastatic biopsies from 116 patients with mUC (Supplementary Tables 1 and 2; see the Supplementary material for methods).

Analysis of whole-genome sequencing (WGS) data was performed on tissue samples from liver, lymph node, bone, and other metastatic sites. A stratification based on the proposed etiology of single-base substitution (SBS) COSMIC signatures [3] (Supplementary Table 3) using unsupervised consensus clustering revealed two major genomic subtypes. GenS1 (67%; Fig. 1) was APOBEC-driven with a large contribution from APOBEC-associated SBS2 and SBS13 signatures (median 54%). GenS2 (24%) predominantly comprised tumors with low *APOBEC* mutagenesis and was characterized by COSMIC signatures of unknown etiology.

To further examine the etiology of these tumors, deconvolution of SBS patterns was performed to identify de novo mutational signatures (Supplementary Fig. 1). This confirmed that GenS1 is APOBEC-driven, whereas GenS2 is dominated by de novo mutational signatures associated with reactive oxygen species (SigF; 0.91 cosine similarity with SBS18) and is putatively clock-like (SigG; 0.90 cosine similarity with SBS5). GenS1 and GenS2 have also previously been identified as the two major genomic subtypes in primary UC [4]. GenS1 was characterized by a higher number of SBS than GenS2, whereas GenS2 had more small insertions and deletions (indels) than GenS1 (Supplementary Fig. 2). Tumors with predicted homologous recombination deficiency were of subtype GenS2 (Fisher's exact test, $p = 0.02$).

The genes most frequently affected by structural variants (SVs) were *CCSER1* (13%) and *AHR* (12%). We identified 71 promoters that were frequently mutated (Supplementary Table 4) of which the promoters of *TERT* (64%), *LEPROTL1* (20%), and *GSTA4* (14%) had the highest mutation rate. *TERT* and *LEPROTL1* were predominantly affected by hotspot mutations. When considering coding and promoter alterations, *TERT* was mutated in 74% of cases. Mutations in the *LEPROTL1* promoter were more frequent in GenS1 than in GenS2 (Fisher's exact test, $p = 0.03$). Significantly mutated genes (SMGs; Supplementary Tables 5 and 6) resembled those reported in primary UC [2] and did not correspond with the genomic subtypes.

Clinical characteristics such as sex, primary cancer subtype, and pretreatment status did not differ between the subtypes. Response to treatment was better among patients with GenS1 in comparison to those with GenS2 tumors (Supplementary Fig. 3). The less prevalent genomic subtypes (9%) were related to the platinum treatment signature (GenS3), the defective DNA mismatch repair signature and microsatellite instability (GenS4), and the reactive oxygen species signature (GenS5).

In conclusion, WGS analyses identified two major genomic subtypes of mUC that correlated with response to treatment. These subtypes resembled different mutagenic processes leading to the development of mUC, although both subtypes showed similar SMG profiles.

A consensus transcriptomic classifier was recently developed for primary UC [5]. However, this classifier does not consider transcriptomic differences inherited from the metastatic site for mUC samples (Fig. 2A) and therefore cannot be applied directly to the present metastatic cohort [6]. Furthermore, transcriptomic subtyping of mUC has not been reported thus far. To identify mUC transcriptomic subtypes, we performed de novo subtyping of RNA sequencing (RNA-seq) data. Hierarchical consensus clustering was applied to organ-corrected paired RNA-seq data for 90 of the 116 mUC patients (Supplementary Table 7) and revealed five transcriptomic subtypes (Fig. 2B and Supplementary Table 8). The phenotypes of the five subtypes were established according to phenotypic signature scores (Supplementary Fig. 4A).

We identified two luminal subtypes (40%) that exhibited high expression of the genes *PPARG*, *GATA3*, and *FGFR3* (Supplementary Fig. 4). The luminal-a subtype had high expression of *PPARGC1B* and *MYCN,* low tumor purity, and a high fraction of natural killer (NK) cells. *NECTIN4* was amplified in 61% of these tumors (Fisher's exact test, $p < 0.001$) and expression of *NECTIN4* was high (Supplementary Fig. 4). The luminal-b subtype had high tumor purity, a low number of SVs, a low fraction of NK cells, high expression of *MYC*, high Myc and RTK-RAS pathway activity (Supplementary Figs. 4 and 5), and a higher proportion of *ELF3* (56%) and *FGFR3* (50%) DNA alterations (Fisher's exact test, $p = 0.002$ and $p = 0.005$) than the other subtypes.

Stroma-rich tumors (24%) showed high expression of *DDR2*, *PDGFRA*, collagens (Supplementary Table 9), and genes associated with stromal content and cancer-associated fibroblasts (*THBS4*, *CNTN1*, *CXCL14* and *BOC*)

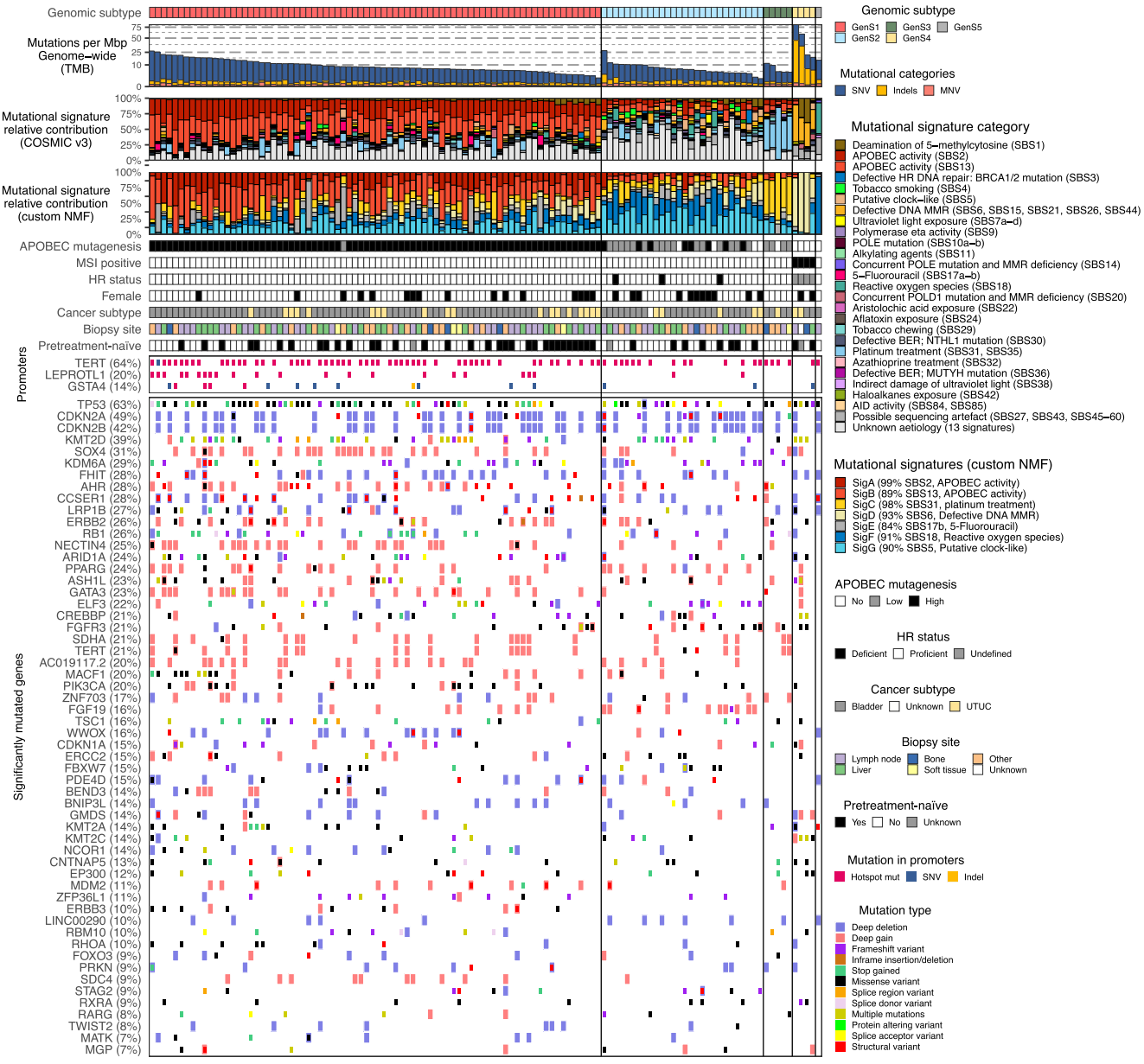

Fig. 1 – Genomic landscape for 116 metastatic urothelial carcinomas stratified by genomic subtype. The analysis was performed on whole-genome DNA sequencing data for freshly obtained biopsy samples from metastatic sites that were centrally reviewed to confirm the diagnosis of metastatic urothelial carcinoma. Tumor samples were classified into genomic subtypes via hierarchical consensus clustering of the relative contribution of COSMIC v3 mutational signatures [3] grouped by etiology. The genomic features are displayed from top to bottom as follows: genomic subtype (GenS1–5); genome-wide tumor mutational burden (TMB) as mutations per Mbp; mutational signatures grouped by etiology (MMR = mismatch repair); relative contribution of seven de novo (custom) mutational signatures via deconvolution of single-nucleotide variants (SNVs) in the 96 trinucleotide context with non-negative matrix factorization (NMF); APOBEC enrichment analysis showing tumors with no, low, and high APOBEC mutagenesis; tumors with microsatellite instability (MSI); homologous recombination (HR) status; female patients; site of origin of the primary tumor (UTUC = upper tract urothelial carcinoma); metastatic site from which a biopsy was obtained; systemic treatment-naïve patients; mutations in the promoter of genes present in >10% of samples; and overview of significantly mutated genes.

[7–9]. Furthermore, these tumors had low tumor purity, a high signature score for epithelial-to-mesenchymal transition, high TGF-β pathway activity (Supplementary Figs. 4 and 5), and a higher rate of *TSC1* DNA alterations than other subtypes (45%; Fisher's exact test, $p < 0.001$).

The basal/squamous subtype (23%) had high expression of basal and squamous markers (*DSG3*, *KRT5*, *KRT6A*, and *S100A7*), was enriched among females (52%; Fisher's exact test, $p = 0.004$), had a large fraction of M1 macrophages, and was associated with the poorest outcomes (Supplementary Fig. 3). TGF-β and Myc pathway activity and expression levels of *TGFBR1, MYC, CD274* (PD-L1), and *MSLN*—a tumor-associated antigen—were high. Amplification of *NECTIN4* was absent (Fisher's exact test, $p = 0.001$) and *NECTIN4* expression was low.

The nonspecified subtype (12%) did not clearly overexpress any of the phenotypic markers associated with a basal, squamous, luminal, stromal, or neuroendocrine phenotype, but had a high score for claudin markers, high numbers of indels and SVs, high expression of *APOBEC3B,* high

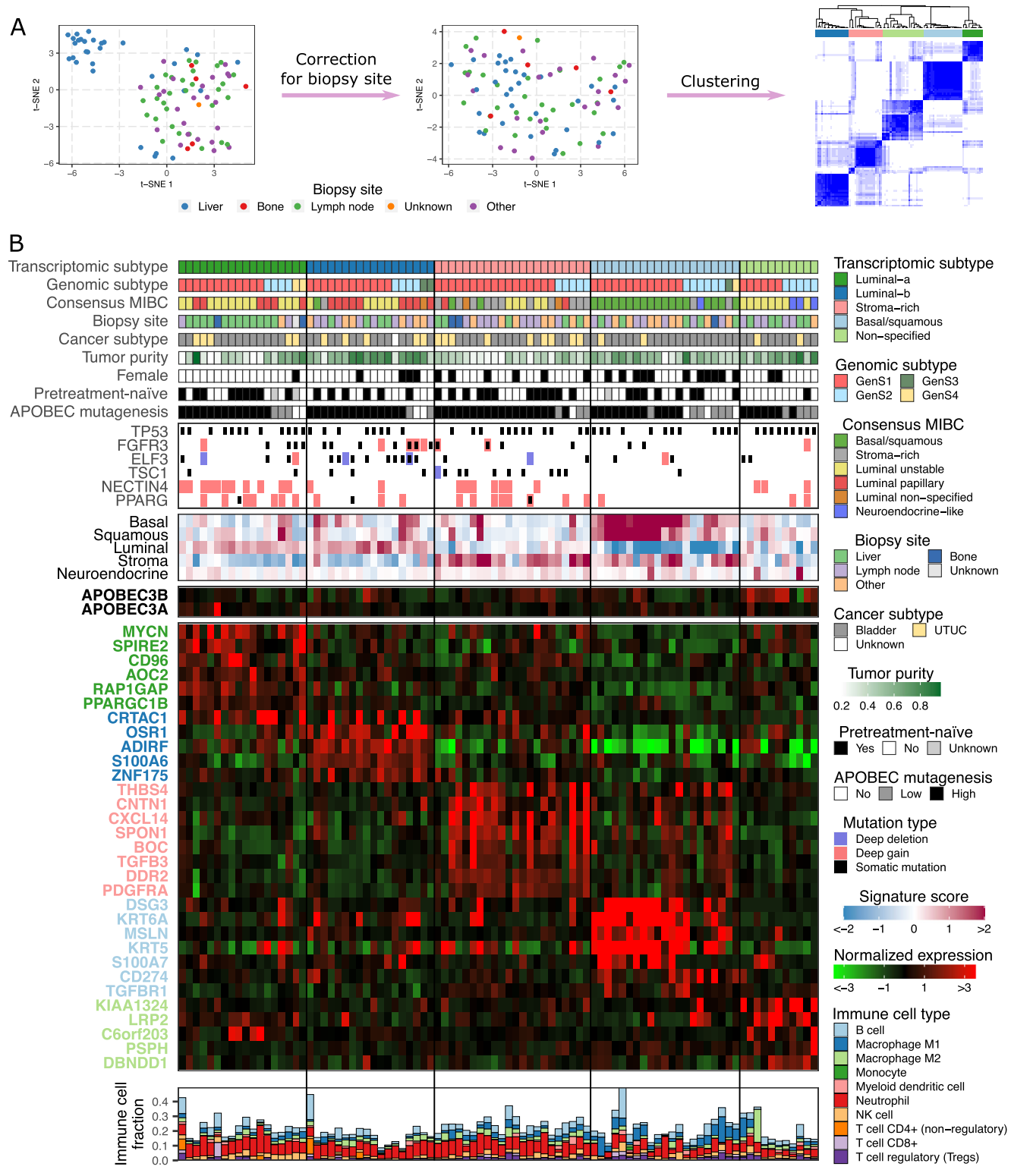

Fig. 2 – Genomic and transcriptomic characteristics of metastatic urothelial carcinoma stratified by transcriptomic subtype. (A) Strategy in the present study to identify transcriptomic subtypes from tissue samples derived from different metastatic biopsy sites for 90 patients with metastatic urothelial carcinoma. Hierarchical consensus clustering was applied on transcriptomic profiles corrected for biopsy site (see the Supplementary material for details). (B) Five transcriptomic subtypes were identified: luminal-a, luminal-b, stroma, basal/squamous, and nonspecified. Features per sample are displayed from top to bottom as follows: transcriptomic subtype; genomic subtype (GenS1–4); transcriptional subtype according to the consensus classifier for primary muscle-invasive bladder cancer (MIBC) [5]; metastatic site from which a biopsy was obtained; site of origin of the primary tumor (UTUC = upper tract urothelial carcinoma); estimated tumor cell percentage; female patients; systemic pretreatment-naïve patients; APOBEC enrichment analysis showing tumors with no, low, and high APOBEC mutagenesis; tumors with genomic alterations in selected genes; signature score (mean expression of genes related to each phenotype) for basal, squamous, luminal, stroma, and neuroendocrine markers; *APOBEC3B* and *APOBEC3A* expression; top overexpressed genes; and immune cell fractions.

cell-cycle pathway activity, and low p53 pathway activity (Supplementary Figs. 4 and 5). This subtype was enriched among patients who were previously treated with chemotherapy (Fisher's exact test, $p = 0.023$).

Next, we assessed the clinical relevance of genomic alterations and identified potential targetable mutations in 114 of 116 mUC patients, including on- and off-label treatment modalities for UC as well as therapies approved for other tumor types (Supplementary Fig. 6A). In addition, the transcriptomic subtypes may guide the identification of potential therapeutic targets (Supplementary Fig. 6B; the Supplementary material describes the rationale for therapeutic options for mUC patients). Tumors of the luminal-a subtype could benefit from NK cell enhancers and FGFR or PPARγ inhibitors, whereas the luminal-b subtype might be susceptible to FGFR, BET, and RAS pathway inhibitors. The stroma-rich subtype could be sensitive to immune checkpoint inhibitors (ICIs) combined with TGF-β inhibitors. The basal/squamous subtype could benefit from mesothelin-targeted therapy, BET inhibitors, or ICIs plus a TGF-β inhibitor. Individualized targeted therapy should be prioritized in patients with tumors of the nonspecified subtype.

Limitations of the study include the lack of matched primary tumor samples, the heterogeneity of the study population, and the lack of pathology-based data. Despite these limitations, the study defined for the first time the molecular subtypes of mUC on the basis of whole-genome and transcriptome analyses of metastatic biopsies from 116 mUC patients. The findings improve our understanding of the molecular landscape of mUC and may serve as a reference for future drug development in this disease setting.

Author contributions: Joost L. Boormans had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Nakauma-González, Rijnders, van de Werken, Lolkema, Boormans.
Acquisition of data: Lolkema, Boormans, Heijden, Voortman, Cuppen, Mehra, van Wilpe, Oosting, Westgeest, Zwarthoff, de Wit, van der Veldt.
Analysis and interpretation of data: Nakauma-González, Rijnders, van de Werken, Lolkema, Boormans.
Drafting of the manuscript: Nakauma-González, Rijnders, van de Werken, Lolkema, Boormans.
Critical revision of the manuscript for important intellectual content: All authors.
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Data sharing statement: Data are freely available for academic use from https://www.hartwigmedicalfoundation.nl.

Peer Review Summary

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