

# Genomic characterization of upper urinary tract urothelial carcinoma and clonal evolution of intravesical recurrences

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## Abstract

### Background and Objective:

Patients with upper urinary tract urothelial carcinoma (UTUC) undergoing radical surgery are at high risk of developing intravesical recurrences (IVR). The biology of IVR after surgery for UTUC is poorly understood, and urine markers to replace cystoscopic surveillance of the bladder are lacking. Here, we characterized the genomic landscape of UTUC and paired IVR to discover therapeutic targets and identify diagnostic markers for IVR.

### Methods:

We performed targeted next-generation DNA-sequencing of 571 genes in a cohort of 276 retrospectively and 138 prospectively enrolled UTUC patients who received radical surgery. Clonality and evolution were assessed in 79 paired UTUC-IVR cases.

### Key Findings and Limitations:

Mutations in *TERT* (72%) and *FGFR3* (50%) were highly prevalent in UTUC, while mutations in *KMT2C* were associated with reduced risk of IVR. The mutually exclusive mutational profile of UTUC revealed five genomic subtypes with distinct clinicopathological and molecular characteristics, but none were associated with elevated IVR risk. Clonal evolution of paired UTUC-IVR occurred in 92% of cases via four evolutionary paths, with *FGFR3* as a key driver in the largest path (36%). Additionally, hotspot mutations in the *TERT* promoter, and *FGFR3* and *HRAS* genes were identified as potential markers for noninvasive surveillance by urine testing.

Limitations include cohort heterogeneity and the selected gene-targeted sequencing approach.

## Conclusions and Clinical Implications:

The high *FGFR3* mutation rate in UTUC and its association with IVR development support anti-FGFR targeted therapy to reduce IVR risk. The clonal relationship between UTUC and IVR underscores the potential for patient-friendly noninvasive urine tests for surveillance after radical surgery.

## Summary

Upper urinary tract urothelial carcinoma (UTUC) is a rare cancer with a high recurrence rate after surgery. We found that the *FGFR3* gene is a potential therapeutic target to reduce the risk of recurrence, while recurrent mutations in *TERT*, *FGFR3* and *HRAS* could serve as potential markers for noninvasive surveillance by urine testing after surgery for UTUC.

## Introduction

Urothelial carcinoma (UC), with over 600,000 new diagnoses every year, is among the most common cancers worldwide [1, 2]. Upper tract UC (UTUC) is less common than UC of the bladder (UCB), accounting for only 5-10% of all diagnosed UCs [3]. Due to the relatively low incidence rate, the biology of UTUC remains poorly understood, and treatment approaches are often extrapolated from UCB, resulting in different clinical outcomes [4-6]. Furthermore, up to 60% of UTUC patients have invasive disease at diagnosis *versus* only 25% in UCB, impacting survival probabilities for these patients [7]. At the genomic level, UTUC is associated with Lynch syndrome, whereas UCB is not, and *FGFR3* and *HRAS* somatic mutations are more frequent than in UCB [8-10].

Between 22-47% of patients undergoing radical nephroureterectomy (RNU), the recommended treatment for nonmetastatic high-risk UTUC, develops intravesical recurrence (IVR) within two years after surgery [11]. UTUC patients with a history of UCB, which accounts for 19-34% of the patients, are at the highest risk for IVR after RNU [12, 13]. Other clinicopathologic risk factors of IVR include tumor location in the distal ureter, multifocality, and high-grade disease [11, 14]. Based on clinical observations, the seeding hypothesis has been proposed, suggesting that cancer cells from the upper urinary tract spread to the bladder, giving rise to IVRs [15]. Genomic studies have reported that most UTUC and IVR share a common ancestor, supporting the seeding hypothesis [16, 17]. However, these studies relied on small cohorts of  $\leq 16$  patients, stressing the need for validation in larger cohorts.

There is a clear knowledge gap on the molecular biology of UTUC and increased IVR risk after RNU. Following surgery, UTUC patients undergo close surveillance of the

bladder by invasive cystoscopy, which is uncomfortable and a psychological burden to patients; it is costly and it requires scarce healthcare resources. Therefore, it is crucial to clarify the clonal seeding hypothesis as the main mechanism of IVR following UTUC to identify molecular markers for noninvasive and possible remote surveillance by urine assays. To address these needs and the lack of therapeutic targets to reduce the risk of IVR after surgery for UTUC, we performed a genomic analysis in 414 UTUC patients and sought to clarify the clonality relationship between UTUC and IVR, as well as molecular correlates and evolution of IVR.

## Patients and methods

The Supplementary material provides full details of the methods.

## Samples and patient cohort characteristics

A multi-institutional international cohort of 414 UTUC patients (31% females) who underwent radical surgery between 2002 and 2020 was investigated. UTUC samples plus 104 IVR samples from 79 patients (16 had multiple IVRs) were collected retrospectively (276 patients; central pathologically reviewed) or in the context of a prospective clinical trial (138 patients; REBACARE trial, METC 2017-227, NL60919.078.17) [18]. IVR was defined as a histologically proven urothelial carcinoma of the bladder. This study was reviewed by the medical ethics board of the Erasmus University Medical Center and approved by all participating institutes. The median age and follow-up were 72 years (quartile1-3 (Q1-Q3): 65-77) and 24.8 months (Q1-Q3: 16.0-65.1), respectively. Among other differences (**Table S1**), the median follow-up was shorter in the prospective (23.6 months, Q1-Q3 = 21.2-25.4

months) than in the retrospective cohorts (41.2 months, Q1-Q3 = 11.9-83.5 months;  $p < 0.001$ , two-sided Wilcoxon rank-sum test). Of 414 UTUC patients, 47 (11.4%) had a history of UCB (UCB-UTUC) and 299 (72.2%) had primary UTUC, of whom 87 (21.0%) developed an IVR (UTUC-IVR) after primary UTUC. In 68 cases (16.4%), IVR or history of UCB were unknown. The follow-up time in the UTUC-IVR subgroup was 24.6 months (Q1-Q3: 21.6-51.1) and comparable to the rest of the cohort with 25.2 months (Q1-Q2: 16.0-66.3). The median time to IVR was 23.2 months (Q1-Q3: 12.8-28.0), and 55.4% occurred within two years after surgery. Within UTUCs with known origin, UTUC in the renal pelvis was 52.5% ( $n = 180$ ) more frequent than in the ureter ( $n = 118$ ). In 69.6% of cases, UTUC presented high-grade disease, and concomitant carcinoma *in situ* was identified in 29 (7.0%) patients.

## DNA-sequencing and clonal evolution

DNA from the tumor and matched normal were sequenced using a panel of 571 genes [19]. Somatic mutations and copy number alterations were assessed as previously described [19, 20].

Clonality assessment was performed on UTUC and matched IVR [21, 22]. For clonally related samples, changes in mutation allele frequency were used as a *proxy* for selection (selection score). Evolutionary paths were interrogated by hierarchical clustering of the selection scores with ConsensusClusterPlus [23].

## Statistical analysis

Analyses were performed using R v4.4.1 [24]. The clonality test was applied with Clonality v1.47.0, which is based on the Neyman-Pearson Lemma test and the

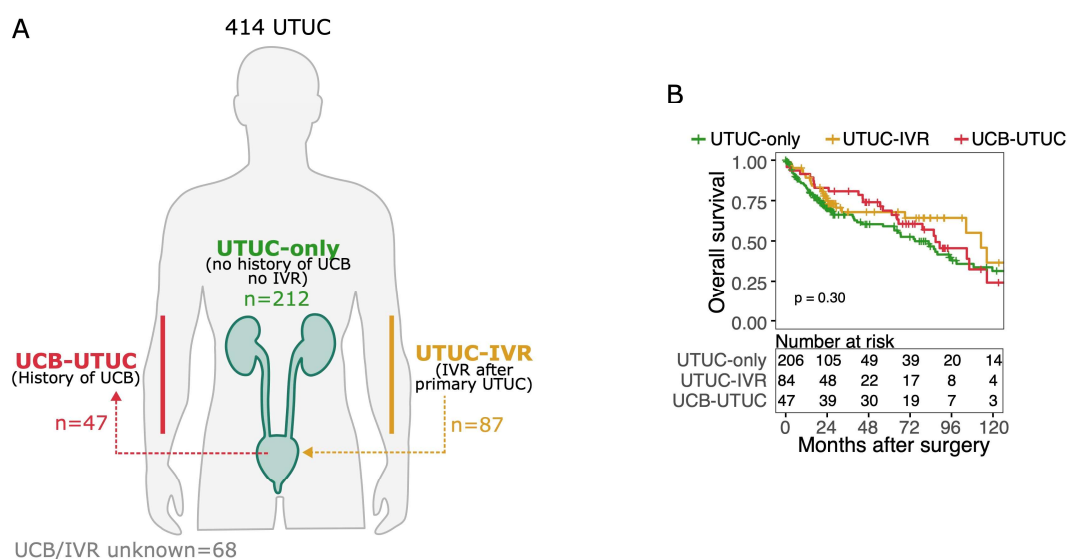
conditional maximum likelihood estimate. The  $\chi^2$  and Fisher's exact tests were used for comparison of categorical values between groups. The Wilcoxon rank-sum test, signed-rank test, and the Kruskal-Wallis test by ranks were used to compare groups with continuous variables. Time from surgery to IVR and death was compared between groups by Kaplan-Meier estimates with the log-rank test. Patients who were lost to follow-up were censored at the date of the last cystoscopy. The Cox proportional hazards regression analysis was applied using the likelihood ratio test. *P-values* were adjusted for multiple testing using the Benjamini–Hochberg method.

## Results

### Clinicopathological correlates with intravesical recurrences

By multivariate Cox regression analysis, we identified a history of UCB (hazard ratio (HR) = 3.51 (95% confidence interval (CI): 1.61-7.65),  $p = 0.002$ ) and UTUC in the ureter (HR = 1.85 (95% CI: 1.08-3.15),  $p=0.025$ ) as clinicopathological risk factors for IVR (**Fig. S1**).

Excluding unknowns, the UTUC-only subgroup (no UCB/IVR;  $n = 212$ ) represented 61.3% of patients (**Fig. 1A**) versus 13.6% UCB-UTUC and 25.1% UTUC-IVR. Multifocality and  $\geq T1$  tumors were overrepresented in the UCB-UTUC subgroup ( $p < 0.001$ ,  $\chi^2$  test), while age, sex, N stage, carcinoma *in situ*, surgical margins, necrosis and survival status were similar across UTUC subgroups (**Table 1**). UTUC-only was characterized by a higher proportion of lymphovascular invasion and tumors from the renal pelvis ( $p = 0.016$ ,  $\chi^2$  test). Overall survival was comparable among the three subgroups, including the UTUC-IVR subgroup (**Fig. 1B**).



**Figure 1. Cohort overview of 414 patients with upper urinary tract urothelial carcinoma stratified according to history of urothelial carcinoma of the bladder or intravesical recurrence. A)** Schematic representation of all upper urinary tract urothelial carcinoma (UTUC) samples grouped according to a history of urothelial carcinoma of the bladder (UCB; UCB-UTUC), intravesical recurrence (IVR; UTUC-IVR) or a history without UCB nor IVR (UTUC-only). **B)** Kaplan–Meier curves for estimates of overall survival among UTUC subgroups. The log-rank test was used to compare the Kaplan–Meier survival curves.

**Table 1. Clinicopathological characteristics of 414 patients with upper tract urothelial carcinoma who received radical surgery stratified by a history of urothelial carcinoma of the bladder or subsequent intravesical recurrence.**

Cohort characteristics	Total cohort (N=414)	UTUC subgroups (N=346)			P
		UCB-UTUC (N=47)	UTUC-IVR (N=87)	UTUC-only (N=212)	
Patient Institute, n (%)					
ERL	161 (38.9)	0 (0)	21 (24.1)	74 (34.9)	<0.0001
ERA*	138 (33.3)	0 (0)	42 (48.3)	96 (45.3)	
MAL	96 (23.2)	28 (59.6)	24 (27.6)	42 (19.9)	
EME	19 (4.6)	19 (40.4)	0 (0)	0 (0)	
Age					
Median, years (Q1-Q3)	72 (65-77)	73 (61-78)	71 (66-75)	72 (65-77)	0.90**
Unknown, n (%)	4 (1.0)	0 (0)	0 (0)	0 (0)	
Sex, n (%)					
Female	125 (30.2)	9 (19.1)	29 (33.3)	65 (30.7)	0.18

Male	287 (69.3)	38 (80.9)	58 (66.7)	147 (69.3)	
Unknown	2 (0.5)	0 (0)	0 (0)	0 (0)	
Follow up					
Median, months (Q1-Q3)	25 (16-65)	64 (43-90)	25 (22-51)	24 (14-45)	<0.0001
Unknown, n (%)	21 (5.1)	0 (0)	2 (2.5)	5 (2.5)	
Location, n (%)					
Renal pelvis	180 (43.5)	19 (40.4)	33 (37.9)	92 (43.4)	<0.0001
Ureter	118 (28.5)	20 (42.6)	32 (36.8)	46 (21.7)	(0.03***)
Multifocal	15 (3.6)	8 (17.0)	1 (1.1)	0 (0)	
Unknown	101 (24.4)	0 (0)	21 (24.1)	74 (34.9)	
N stage, n (%)					
N0	239 (57.7)	14 (29.8)	61 (70.1)	144 (67.9)	0.27***
N1	26 (6.3)	1 (2.1)	4 (4.6)	12 (5.7)	
N2	11 (2.7)	0 (0)	0 (0)	10 (4.7)	
NX or unknown	138 (33.3)	32 (68.1)	22 (25.3)	46 (21.7)	
Surgery, n (%)					
RNU	380 (92.1)	39 (83.0)	76 (87.7)	199 (93.9)	0.030
Partial ureterectomy	32 (7.4)	8 (17.0)	11 (12.3)	13 (6.1)	
Not specified	2 (0.5)	0 (0)	0 (0)	0 (0)	
T stage, n (%)					
pTis	6 (1.4)	2 (4.3)	2 (2.2)	2 (0.9)	
pTa	93 (22.5)	3 (6.4)	26 (29.9)	52 (24.5)	<0.0001
pT1	88 (21.3)	26 (55.3)	19 (21.8)	32 (15.1)	
pT2	66 (15.9)	9 (19.1)	15 (17.2)	32 (15.1)	
pT3	132 (31.9)	6 (12.8)	22 (25.3)	77 (36.3)	
pT4	25 (6.0)	1 (2.1)	3 (3.4)	17 (8.0)	
Unknown	4 (1.0)	0 (0)	0 (0.0)	0 (0)	
Concomitant CIS, n (%)					
Yes	29 (7.0)	4 (8.5)	9 (10.3)	16 (7.5)	0.69
No	220 (53.1)	43 (91.5)	56 (64.4)	121 (57.1)	
Unknown	165 (39.9)	0 (0)	22 (25.3)	75 (35.4)	
Lymphovascular invasion, n (%)					
Yes	55 (13.3)	6 (12.8)	9 (10.3)	40 (18.9)	0.008
No	184 (44.4)	41 (87.2)	53 (60.9)	90 (42.4)	
Unknown	175 (42.3)	0 (0)	25 (28.7)	82 (38.7)	
Necrosis, n (%)					
Yes	15 (3.6)	2 (4.3)	3 (3.4)	10 (4.7)	0.37
No	172 (41.5)	45 (95.7)	44 (50.6)	83 (39.2)	
Unknown	227 (54.8)	0 (0)	40 (46.0)	119 (56.1)	
Surgical margins, n (%)					
Positive	27 (6.5)	7 (14.9)	9 (10.3)	11 (5.2)	0.051
Negative	383 (92.5)	40 (85.1)	78 (89.7)	199 (93.9)	
Unknown	4 (1.0)	0 (0)	0 (0)	2 (0.9)	
IVR, n (%)					
Yes	130 (31.4)	43 (91.5)	87 (100)	0 (0)	

No	216 (52.2)	4 (8.5)	0 (0)	212 (100)	
Unknown	68 (16.4)	0 (0)	0 (0)	0 (0)	
Survival status, n (%)					
Alive	248 (59.9)	21 (44.7)	57 (65.5)	121 (57.1)	0.053
Death	158 (38.2)	26 (55.3)	29 (33.3)	88 (41.5)	
Unknown	8 (1.9)	0 (0)	1 (1.1)	3 (1.4)	

UTUC: Upper tract urothelial carcinoma.

UCB-UTUC: UTUC with a previous history of UCB.

UTUC-IVR: UTUC with subsequent IVR without previous UCB.

UTUC-only: UTUC had neither previous UCB nor IVR.

ERL: patients from the University Hospital Erlangen-Nürnberg, Germany

ERA: patients from the Erasmus University Medical Center Rotterdam, the Netherlands.

MAL: patients from the Virgen de la Victoria University Hospital Malaga, Spain

EME: patients from the University of Malaga and Córdoba Biobank (Reina Sofia University Hospital), funded by the EMERGIA program, Spain.

CIS: carcinoma *in situ*.

Estimates were given as median (quartile 1 (Q1) - quartile 3(Q3)) or frequency (percentage).

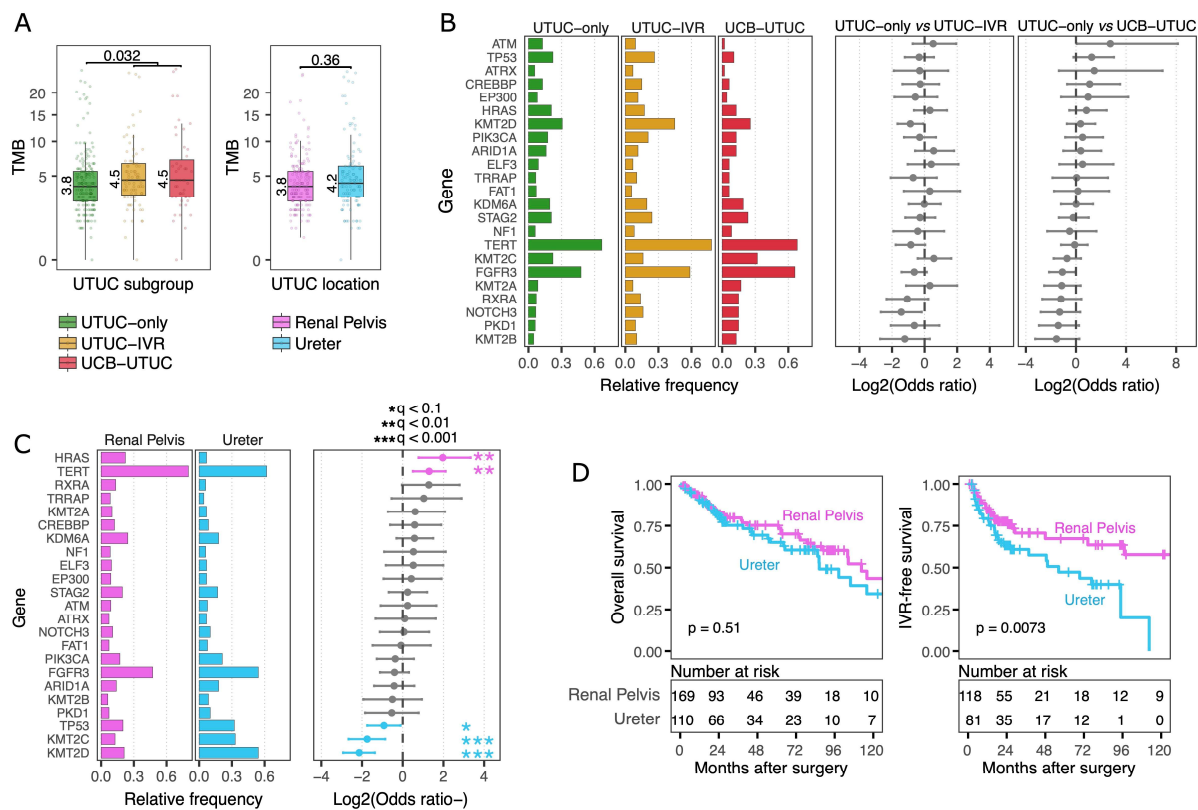
\* Prospective cohort.

P values were calculated using the  $\chi^2$  test (two-sided for pairwise comparisons), except for age in which the \*\*Kruskal-Wallis test for continuous age values was applied. Unknowns were excluded.

\*\*\* p value excluding multifocal UTUC.

## The mutational landscape of UTUC

The median tumor mutational burden (TMB) of UTUC was 4.18 (Q1-Q3 = 2.79-6.62) mutations per megabase-pair (**Fig. 2A**), with the UTUC-only subgroup showing the lowest TMB ( $p = 0.032$ , two-sided Wilcoxon rank-sum test). Although UTUC location was correlated to IVR risk (**Fig. S1; Table S2**), no difference in TMB was observed between renal pelvis and ureter (**Fig. 2A**).



**Figure 2. Genomic overview and differences between tumor location and between subgroups of upper urinary tract urothelial carcinoma.** **A**) Tumor mutational burden (TMB) of somatic mutations, excluding hypermutated tumors, stratified by upper urinary tract urothelial carcinoma (UTUC) with a history of urothelial carcinoma of the bladder (UCB; UCB-UTUC; n = 47), UTUC with intravesical recurrence (IVR; UTUC-IVR; n = 83) and UTUC with neither UCB nor IVR (UTUC-only; n = 206) or by UTU location: renal pelvis (n = 174) and ureter (n = 115). Box plots show the median, inter-quartile range (IQR: Q1–Q3) and whiskers (1.5xIQR from Q3 to the largest value within this range or 1.5xIQR from Q1 to the lowest value within this range). The two-sided Wilcoxon rank-sum test was performed for differences between UTUC-only and UCB-UTUC + UTUC-IVR, and between UTUC from the renal pelvis and ureter. Distribution of the most frequently mutated genes ( $\geq 7\%$ ) in the cohort across **B**) UTUC subgroups and **C**) UTUC location. The odds ratio between pairs of tumor groups and Benjamini-Hochberg corrected p-values (q) for two-sided Fisher's exact test are displayed and colored accordingly. **D**) Kaplan–Meier curves for estimates of overall and IVR-free survival by UTUC location. The log-rank test was used to compare the Kaplan–Meier survival curves.

Unlike tumor location, we observed limited differentially mutated genes between UTUC subgroups (**Fig. 2B-C**). Mutations in *TERT*, with most occurring in hotspots in the promoter, and *HRAS* were more frequent in UTUC from the renal pelvis, while mutations in *TP53*, *KMT2C* and *KMT2D* were more frequent in UTUC from the ureter. Despite these differences and distinct risk of IVR, overall survival between the ureter and renal pelvis was comparable (**Fig. 2D**).

A multivariate Cox regression analysis of the TMB and the most frequently mutated genes revealed that no genomic features were associated with increased risk of IVR (**Fig. S2A**). However, a reduced IVR risk was associated only with *KMT2C* mutations (HR = 0.30 (95% CI: 0.13-0.71), p = 0.006), which was an independent genomic predictor when accounting for UTUC location (**Fig. S2B**).

## Genomic subtypes in UTUC

The distribution of mutually exclusive mutated genes across all 414 UTUC samples revealed five unsupervised hierarchical clusters (**Fig. S3**). These clusters represented distinct genomic subtypes (GS1-5) that partially overlapped with previously defined genomic subtypes of UTUC [25] (**Fig. 3**). GS1 (23.4%), GS3 (16.9%) and GS4 (17.1%) were enriched for *FGFR3* mutations, but their differences were defined by the frequency of mutations in *KDM6A*, *KMT2C*, and *KMT2D*. GS2 tumors (16.7%) were *TP53* and *KMT2D* mutants with enrichment for wild-type *TERT*. The most prevalent subtype, GS5 (25.8%), had enrichment for *HRAS* and *KRAS* mutations.



**Figure 3. Genomic landscape of five genomic subtypes of upper urinary tract urothelial carcinoma.**

Tumor and patient characteristics, and genomic features of genomic subtypes of upper urinary tract urothelial carcinoma (UTUC) are displayed from top to bottom as follows: Genomic subtype of mutually exclusive mutated genes; Kyoto genomic subtype; UTUC subgroup; UTUC location; metastasis; patient age; smoking status; female patient, yes or no; histologic subtype; T stage; UTUC grade; tumor mutational burden (TMB) high tumors (>10 mutations per megabase); TMB; de novo mutational signatures (SigA-E) of tumors with  $\geq 10$  mutations; frequently mutated genes in this UTUC cohort, including *KRAS* and *NRAS*, and their frequencies across genomic subtypes applying  $\chi^2$  test with Benjamini-Hochberg corrected p values (q); and copy number alterations (CNA) of selected genes.

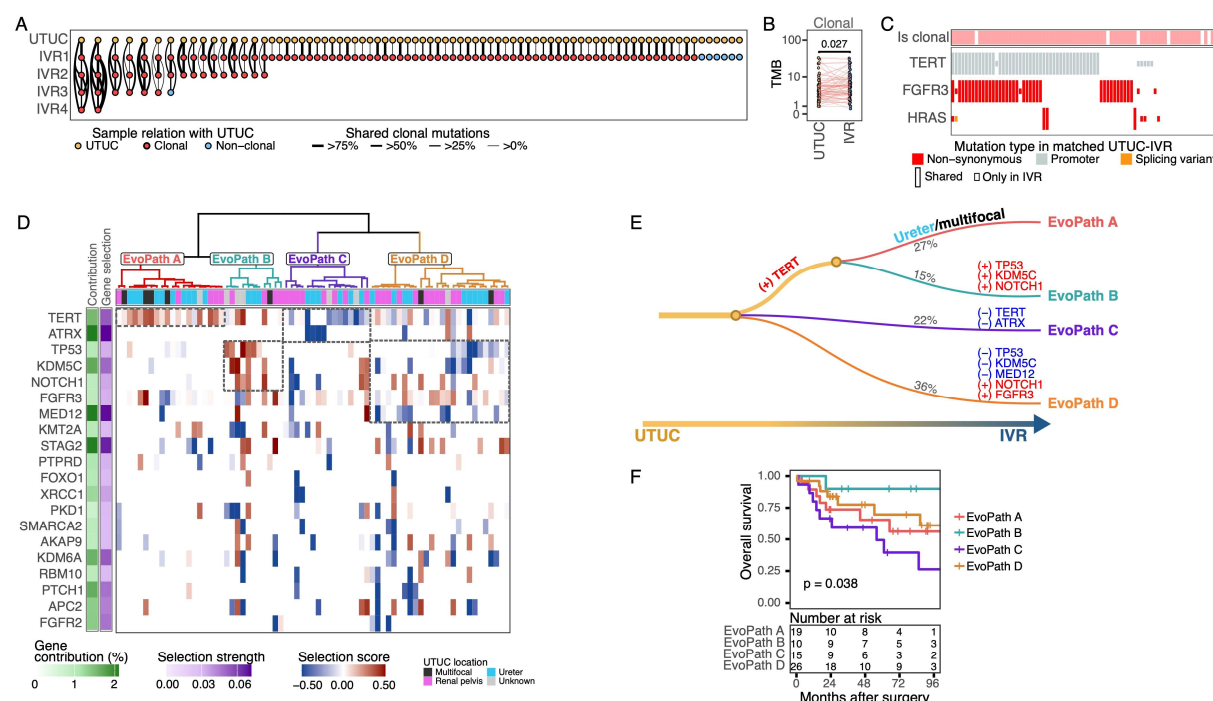
We identified five *de novo* mutational signatures (Fig. S4, Table S3) related to APOBEC activity (sigA), age (sigB), aristolochic acid signature (sigC), defective DNA

mismatch repair (sigD) and background mutations (formalin-fixed paraffin-embedded damage; sigE). These signatures were equally distributed across genomic subtypes (**Fig. S5A**). Similarly, the distribution of UTUC subgroups, patients' sex and age were comparable among the genomic subtypes (**Fig. S5C-D and S5F**). TMB was lowest in the GS5 subtype, while highest in the GS1 subtype ( $p < 0.0001$ , Kruskal-Wallis test; **Fig. S5B**). UTUC from the ureter was more common in GS2 and GS3, and UTUC from the renal pelvis was enriched in GS4 ( $p < 0.0001$ ,  $\chi^2$  test; **Fig. S5E**). Metastasis and variant histology occurred more frequently in GS2 ( $p = 0.01$  and  $p = 0.001$ , respectively,  $\chi^2$  test; **Fig. S5G and S5I**). GS3 tumors were enriched for current smokers ( $p = 0.003$ ,  $\chi^2$  test), while higher stage and high-grade tumors were frequently found in GS2 and GS5 tumors ( $p < 0.0001$  in both,  $\chi^2$  test; **Fig. S5H and S5J-K**).

A classifier based on a multinomial regression model was developed to assess the genomic subtypes in five external cohorts of UTUC ( $n = 384$ , **Fig. S6, Table S4**) [10, 25-28]. Despite incomplete genomic datasets for some external cohorts (e.g., lack of insertions/deletions or not reported *TERT* promoter mutations), they consistently showed that GS5 had low TMB, validating our observation. We also confirmed that UTUC from the renal pelvis, increased tumor stage and high tumor grade were associated with GS2. Furthermore, we confirmed that UTUC subgroups, patients' sex and age were comparable among the genomic subtypes. In our cohort, we observed no differences in overall survival or IVR-free survival between the genomic subtypes, which was confirmed in the external cohorts. However, metastatic events, which were more common in GS2 in both our cohort and external cohorts, translated into differences in metastatic-free survival.

## Clonal evolution of intravesical recurrences

Clonality assessment of 79 matched UTUC-IVR cases found 92% (73/79) of IVR to be clonally related to UTUC, which was accompanied by an increment in the TMB (Fig. 4A-B;  $p = 0.027$ , two-sided Wilcoxon signed-rank test). In more than half of the 'non-clonal' cases (4/6), clonality assessment was limited due to a low number ( $\leq 2$ ) or lack of mutations in the matched UTUC-IVR (Table S5), suggesting that the proportion of clonality was likely higher than what we report here. In 70% (55/79) of paired UTUC-IVR cases, hotspot mutations in either *TERT*, *FGFR3* or *HRAS* genes may serve as potential urine biomarkers for surveillance after surgery for UTUC [29] (Fig. 4C), which may increase to 78% (62/79) if we consider mutations exclusively appearing in the IVR.



#### Figure 4. The clonal evolution of upper urinary tract urothelial carcinoma and intravesical

**recurrences. A)** Evolutionary trees of all upper urinary tract urothelial carcinomas (UTUC) and their corresponding intravesical recurrences (IVR) per patient. Clonal relations between UTUC and IVRs (IVR1-4) are represented by connecting lines. **B)** Comparison of the tumor mutational burden (TMB) between UTUC and IVR for clonally related cases using the average TMB for multiple IVRs (two-sided Wilcoxon signed-rank test). **C)** Hotspot mutations in the *TERT* promoter, *FGFR3* and *HRAS* in UTUC and IVR. **D)** Hierarchical clustering of the selection score in genes mutated in at least two patients. Only genes with the top 20 strongest selection scores and with the highest contribution to variability across cases are displayed. **E)** Graphical representation of the four clusters from (D) into evolutionary paths (EvoPath A-D) of IVR. **F)** Kaplan-Meier survival curves of EvoPaths. P-value was estimated with the log-rank test.

All clonally related UTUC-IVR (n = 73) were analyzed to define the evolution of UTUC towards IVR. Hierarchical clustering of selection scores of mutated genes revealed four evolutionary paths (EvoPaths A-D; **Fig. 4D-E**). EvoPath A (27%) was driven by a positive selection of *TERT* promoter mutations and was favored by multifocal tumors and tumors from the ureter (p = 0.042, one-sided Fisher's exact test). In addition to a positive selection of *TERT*, EvoPath B (15%) was driven by a positive selection of mutant *TP53*, *KMD5C*, and *NOTCH1*. EvoPath C (22%) was characterized by negative selection of *TERT* and *ATRX* mutant genes, and patients harboring these tumors had the poorest overall survival compared to other EvoPaths (**Fig. 4F**). Tumors in EvoPath D (36%) followed a more complex path involving positive and negative selection of several genes, including *TP53*, *KMD5C*, *NOTCH1*, *MED12* and *FGFR3* mutations. The dependency of EvoPath D on the positive selection of *FGFR3* mutations, a driver of IVR, may represent susceptibility to targeted therapy.

## Discussion

UTUC is a relatively rare form of UC with a high risk of recurrence in the bladder following surgery, for which molecular markers for surveillance of patients are lacking in clinical practice. In this study, we aimed to characterize the genomic landscape of 414 UTUC patients treated with radical surgery and identify molecular markers of IVR. In-depth analysis of 79 patients with paired UTUC-IVR further refined our results and delineated the clonal evolution of IVR.

Our analysis identified five genomic subtypes of UTUC based on mutually exclusive mutated genes. Despite clinical differences between the subtypes, no differences were observed in the risk of IVR. These subtypes only partially overlapped with the previously defined genomic subtypes of UTUC [25], offering more granularity for *FGFR3*-mutated tumors, which is the most affected gene by protein-coding mutations.

A clonal relationship between UTUC and paired IVR was observed in 92% of patients, which is in line with other studies reporting 73-100% [16, 17]. This result, together with *TERT* promoter, *FGFR3* and *HRAS* mutations in over two-thirds of cases, underscores the potential of surveillance by urine assays. Assays that include these three genes, among other markers, have shown important diagnostic accuracy [29, 30], which could accelerate their clinical implementation for the surveillance of patients after surgery for UTUC.

UTUC develops differently across locations, showing different sets of driver genes [8, 25]. These differences are also reflected in the distinct evolutionary paths of UTUC to develop in the bladder. EvoPath D, the preferred evolutionary path for one-third of

cases, is driven by positive selection of mutated *FGFR3*, suggesting that blocking this evolutionary path with FGFR inhibitors may result in an overall reduction in the risk of IVR after radical surgery. This could be achieved by identifying *FGFR3*-mutated UTUC in preoperative biopsies obtained through diagnostic ureteroscopies and offering these patients perioperative intravesical instillation with a FGFR3-inhibitor. Together, our data indicate the potential benefit of novel intravesical drug-delivery systems, such as the TAR-210 with erdafitinib that targets *FGFR* mutant UTUC to reduce the risk of IVR [31, 32].

Limitations of our study included the use of targeted DNA-sequencing, restricting our analysis to 571 genes and differences in the proportion of IVR reported between the retrospective and prospective cohorts. The prospective cohort was designed to review each case meticulously and identify IVRs, while excluding previous UCB, leading to a higher proportion of IVRs [18]. This may explain differences in the proportions of IVR and median follow-up time, supporting the impression that IVR might be underreported in the retrospective cohorts.

## Conclusions

This study advances the molecular characterization of the relatively rare UTUC and reinforces the central role that FGFR-directed therapies may play in shaping future treatments for these patients to reduce the risk of IVR. Our results show that UTUC patients undergoing radical surgery could potentially be monitored by non-invasive, patient-friendly, urine-based molecular assays to detect IVR.

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## **Author Contributions**

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### **Competing Interests Statement**

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## Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Data from external cohorts are freely accessible at <https://www.cbioportal.org/> and <https://portal.gdc.cancer.gov/>. Pre-processed data from the Tokyo cohort were kindly provided by Seishi Ogawa and Yoichi Fujii [25].

The genomic subtype classifier is available as an R package at <https://github.com/erasmus-ur/UTUCclassifyGS>

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