

1 **Genomic characterization of upper urinary tract urothelial carcinoma and**
2 **clonal evolution of intravesical recurrences**

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46
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55 **Abstract**

56

57 Background and Objective:

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

64 Methods:

65 We performed targeted next-generation DNA-sequencing of 571 genes in a cohort of
66 276 retrospectively and 138 prospectively enrolled UTUC patients who received
67 radical surgery. Clonality and evolution were assessed in 79 paired UTUC-IVR
68 cases.

69 Key Findings and Limitations:

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

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

80 Conclusions and Clinical Implications:

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

85

86

87 **Summary**

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

93

94 **Introduction**

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

105 Between 22-47% of patients undergoing radical nephroureterectomy (RNU), the
106 recommended treatment for nonmetastatic high-risk UTUC, develops intravesical
107 recurrence (IVR) within two years after surgery [11]. UTUC patients with a history of
108 UCB, which accounts for 19-34% of the patients, are at the highest risk for IVR after
109 RNU [12, 13]. Other clinicopathologic risk factors of IVR include tumor location in the
110 distal ureter, multifocality, and high-grade disease [11, 14]. Based on clinical
111 observations, the seeding hypothesis has been proposed, suggesting that cancer
112 cells from the upper urinary tract spread to the bladder, giving rise to IVRs [15].
113 Genomic studies have reported that most UTUC and IVR share a common ancestor,
114 supporting the seeding hypothesis [16, 17]. However, these studies relied on small
115 cohorts of ≤16 patients, stressing the need for validation in larger cohorts.
116 There is a clear knowledge gap on the molecular biology of UTUC and increased IVR
117 risk after RNU. Following surgery, UTUC patients undergo close surveillance of the

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

126

127 **Patients and methods**

128 The Supplementary material provides full details of the methods.

129

130 **Samples and patient cohort characteristics**

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

142 months) than in the retrospective cohorts (41.2 months, Q1-Q3 = 11.9-83.5 months;
143 p < 0.001, two-sided Wilcoxon rank-sum test). Of 414 UTUC patients, 47 (11.4%)
144 had a history of UCB (UCB-UTUC) and 299 (72.2%) had primary UTUC, of whom 87
145 (21.0%) developed an IVR (UTUC-IVR) after primary UTUC. In 68 cases (16.4%),
146 IVR or history of UCB were unknown. The follow-up time in the UTUC-IVR subgroup
147 was 24.6 months (Q1-Q3: 21.6-51.1) and comparable to the rest of the cohort with
148 25.2 months (Q1-Q2: 16.0-66.3). The median time to IVR was 23.2 months (Q1-Q3:
149 12.8-28.0), and 55.4% occurred within two years after surgery. Within UTUCs with
150 known origin, UTUC in the renal pelvis was 52.5% (n = 180) more frequent than in
151 the ureter (n = 118). In 69.6% of cases, UTUC presented high-grade disease, and
152 concomitant carcinoma *in situ* was identified in 29 (7.0%) patients.

153

154 **DNA-sequencing and clonal evolution**

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

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

162

163 **Statistical analysis**

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

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

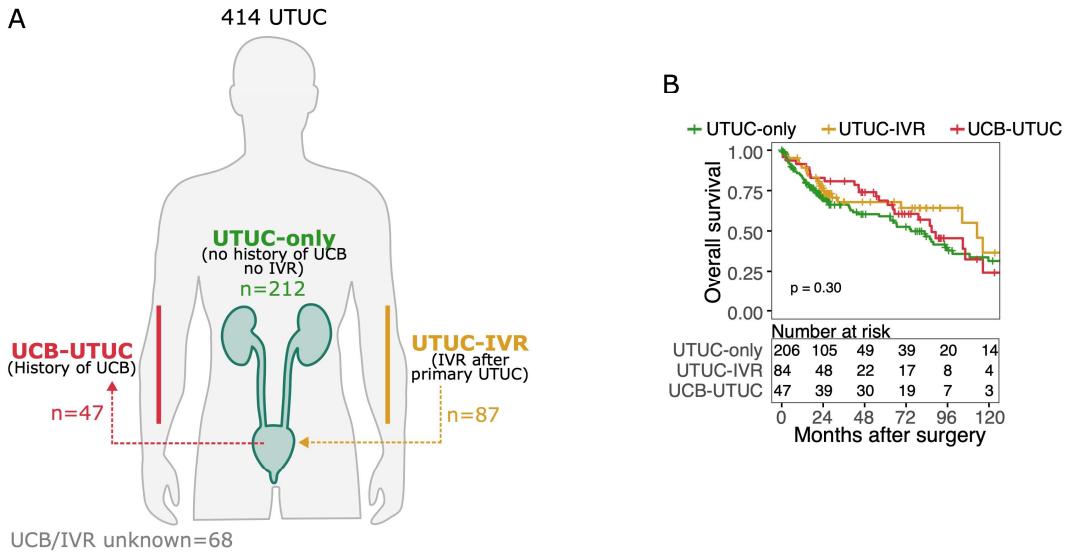
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175 **Results**

176 **Clinicopathological correlates with intravesical recurrences**

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

181 Excluding unknowns, the UTUC-only subgroup (no UCB/IVR; $n = 212$) represented
182 61.3% of patients (**Fig. 1A**) versus 13.6% UCB-UTUC and 25.1% UTUC-IVR.
183 Multifocality and $\geq T1$ tumors were overrepresented in the UCB-UTUC subgroup ($p <$
184 0.001, χ^2 test), while age, sex, N stage, carcinoma *in situ*, surgical margins, necrosis
185 and survival status were similar across UTUC subgroups (**Table 1**). UTUC-only was
186 characterized by a higher proportion of lymphovascular invasion and tumors from the
187 renal pelvis ($p = 0.016$, χ^2 test). Overall survival was comparable among the three
188 subgroups, including the UTUC-IVR subgroup (**Fig. 1B**).



189

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

197

198 Table 1. Clinicopathological characteristics of 414 patients with upper tract urothelial carcinoma who
199 received radical surgery stratified by a history of urothelial carcinoma of the bladder or subsequent
200 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)	
ERA*	138 (33.3)	0 (0)	42 (48.3)	96 (45.3)	<0.0001
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)	

201 UTUC: Upper tract urothelial carcinoma.

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

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

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

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

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

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

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

209 CIS: carcinoma *in situ*.

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

211 * Prospective cohort.

212 P values were calculated using the χ^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.

213 *** p value excluding multifocal UTUC.

214

215

216 217 218 The mutational landscape of UTUC

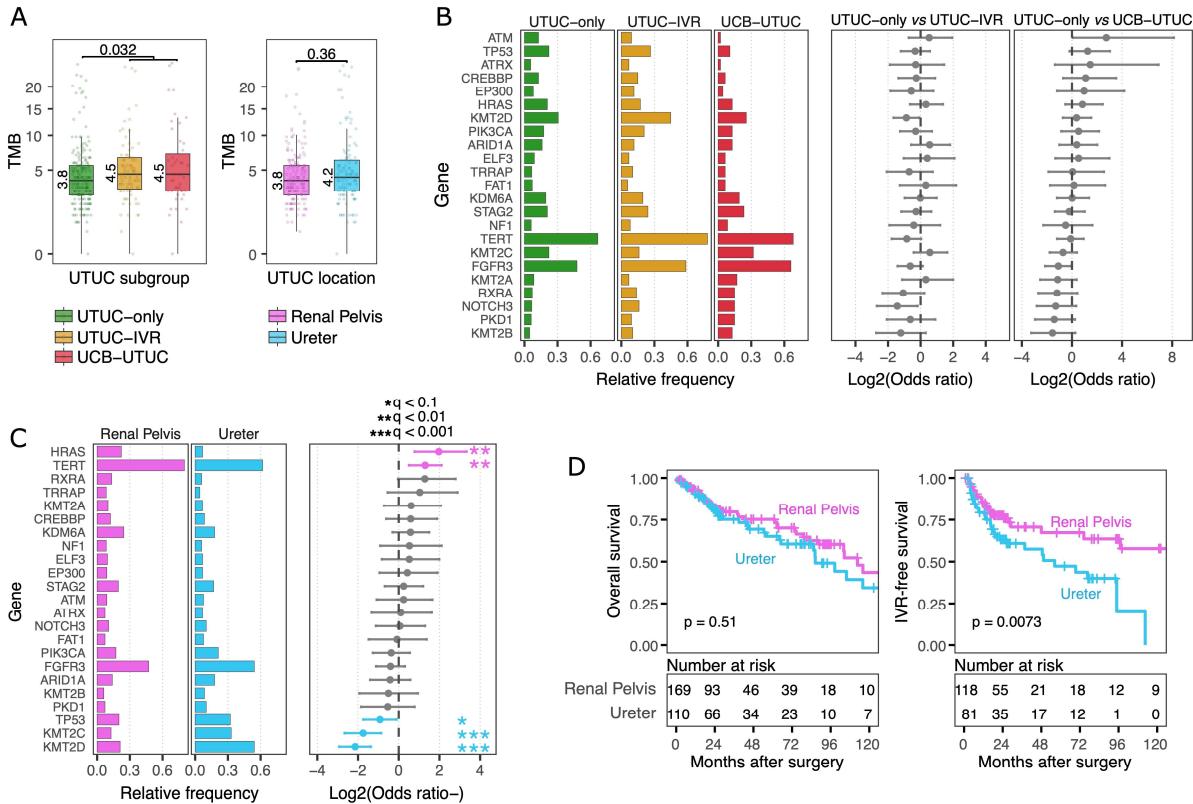
219 The median tumor mutational burden (TMB) of UTUC was 4.18 (Q1-Q3 = 2.79-6.62)

220 mutations per megabase-pair (**Fig. 2A**), with the UTUC-only subgroup showing the

221 lowest TMB ($p = 0.032$, two-sided Wilcoxon rank-sum test). Although UTUC location

222 was correlated to IVR risk (**Fig. S1; Table S2**), no difference in TMB was observed

223 between renal pelvis and ureter (**Fig. 2A**).



224

225 **Figure 2. Genomic overview and differences between tumor location and between subgroups**
 226 **of upper urinary tract urothelial carcinoma. A)** Tumor mutational burden (TMB) of somatic
 227 mutations, excluding hypermutated tumors, stratified by upper urinary tract urothelial carcinoma
 228 (UTUC) with a history of urothelial carcinoma of the bladder (UCB; UCB-UTUC; n = 47), UTUC with
 229 intravesical recurrence (IVR; UTUC-IVR; n = 83) and UTUC with neither UCB nor IVR (UTUC-only; n =
 230 206) or by UTU location: renal pelvis (n = 174) and ureter (n = 115). Box plots show the median, inter-
 231 quartile range (IQR: Q1–Q3) and whiskers (1.5xIQR from Q3 to the largest value within this range or
 232 1.5xIQR from Q1 to the lowest value within this range). The two-sided Wilcoxon rank-sum test was
 233 performed for differences between UTUC-only and UCB-UTUC + UTUC-IVR, and between UTUC
 234 from the renal pelvis and ureter. Distribution of the most frequently mutated genes ($\geq 7\%$) in the cohort
 235 across **B**) UTUC subgroups and **C**) UTUC location. The odds ratio between pairs of tumor groups and
 236 Benjamini-Hochberg corrected p-values (q) for two-sided Fisher's exact test are displayed and colored
 237 accordingly. **D**) Kaplan–Meier curves for estimates of overall and IVR-free survival by UTUC location.
 238 The log-rank test was used to compare the Kaplan–Meier survival curves.

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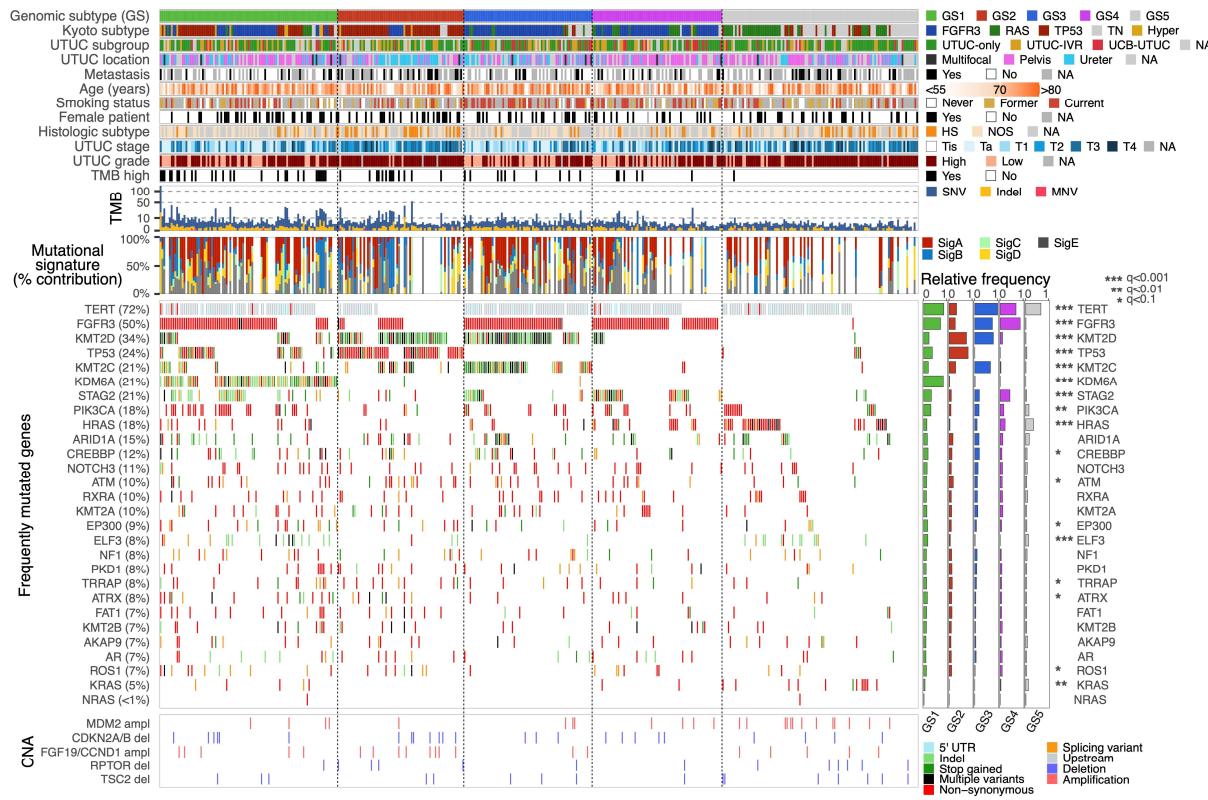
240 Unlike tumor location, we observed limited differentially mutated genes between
241 UTUC subgroups (**Fig. 2B-C**). Mutations in *TERT*, with most occurring in hotspots in
242 the promoter, and *HRAS* were more frequent in UTUC from the renal pelvis, while
243 mutations in *TP53*, *KMT2C* and *KMT2D*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

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

251

252 **Genomic subtypes in UTUC**

253 The distribution of mutually exclusive mutated genes across all 414 UTUC samples
254 revealed five unsupervised hierarchical clusters (**Fig. S3**). These clusters
255 represented distinct genomic subtypes (GS1-5) that partially overlapped with
256 previously defined genomic subtypes of UTUC [25] (**Fig. 3**). GS1 (23.4%), GS3
257 (16.9%) and GS4 (17.1%) were enriched for *FGFR3* mutations, but their differences
258 were defined by the frequency of mutations in *KDM6A*, *KMT2C*, and *KMT2D*. GS2
259 tumors (16.7%) were *TP53* and *KMT2D* mutants with enrichment for wild-type *TERT*.
260 The most prevalent subtype, GS5 (25.8%), had enrichment for *HRAS* and *KRAS*
261 mutations.



262

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

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

274

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

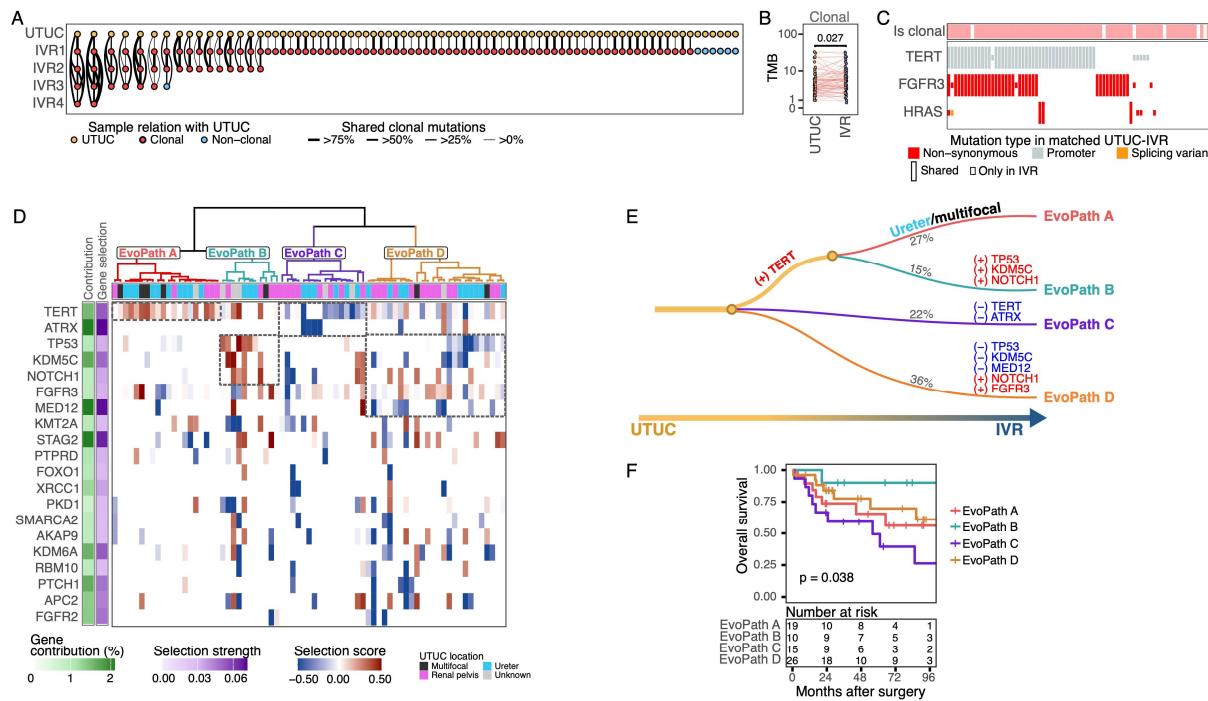
277 mismatch repair (sigD) and background mutations (formalin-fixed paraffin-embedded
278 damage; sigE). These signatures were equally distributed across genomic subtypes
279 (**Fig. S5A**). Similarly, the distribution of UTUC subgroups, patients' sex and age were
280 comparable among the genomic subtypes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

289 A classifier based on a multinomial regression model was developed to assess the
290 genomic subtypes in five external cohorts of UTUC ($n = 384$, **Fig. S6, Table S4**) [10,
291 25-28]. Despite incomplete genomic datasets for some external cohorts (e.g., lack of
292 insertions/deletions or not reported *TERT* promoter mutations), they consistently
293 showed that GS5 had low TMB, validating our observation. We also confirmed that
294 UTUC from the renal pelvis, increased tumor stage and high tumor grade were
295 associated with GS2. Furthermore, we confirmed that UTUC subgroups, patients' sex
296 and age were comparable among the genomic subtypes. In our cohort, we observed
297 no differences in overall survival or IVR-free survival between the genomic subtypes,
298 which was confirmed in the external cohorts. However, metastatic events, which were
299 more common in GS2 in both our cohort and external cohorts, translated into
300 differences in metastatic-free survival.

301

302 Clonal evolution of intravesical recurrences

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



313

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

325

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

340

341 **Discussion**

342 UTUC is a relatively rare form of UC with a high risk of recurrence in the bladder
343 following surgery, for which molecular markers for surveillance of patients are lacking
344 in clinical practice. In this study, we aimed to characterize the genomic landscape of
345 414 UTUC patients treated with radical surgery and identify molecular markers of
346 IVR. In-depth analysis of 79 patients with paired UTUC-IVR further refined our results
347 and delineated the clonal evolution of IVR.

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

354 A clonal relationship between UTUC and paired IVR was observed in 92% of
355 patients, which is in line with other studies reporting 73-100% [16, 17]. This result,
356 together with *TERT* promoter, *FGFR3* and *HRAS* mutations in over two-thirds of
357 cases, underscores the potential of surveillance by urine assays. Assays that include
358 these three genes, among other markers, have shown important diagnostic accuracy
359 [29, 30], which could accelerate their clinical implementation for the surveillance of
360 patients after surgery for UTUC.

361 UTUC develops differently across locations, showing different sets of driver genes [8,
362 25]. These differences are also reflected in the distinct evolutionary paths of UTUC to
363 develop in the bladder. EvoPath D, the preferred evolutionary path for one-third of

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

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

379

380 **Conclusions**

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

386

387

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418

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451

452 **Data availability**

453 The data that support the findings of this study are available from the corresponding
454 author upon reasonable request. Data from external cohorts are freely accessible at
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457 The genomic subtype classifier is available as an R package at
458 <https://github.com/erasmus-ur/UTUCclassifyGS>

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