Genomic profile – a possible diagnostic and prognostic marker in upper tract urothelial carcinoma

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Objectives
To investigate gene alterations as diagnostic and prognostic markers in upper tract urothelial carcinoma (UTUC).

Patients and Methods
Patients with UTUC who underwent nephroureterectomy between 2005 and 2012 were followed until November 2020. DNA was extracted from paraffin-embedded tumour tissue. Next-generation sequencing using a 388-gene panel was performed. First a blinded analysis using principal component analysis and hierarchical clustering was used to search for patterns of mutations. Then a comparative analysis using analysis of variance (ANOVA) was used to search for mutations enriched in groups of various grades, stages, and survival. In addition, careful manual annotation was used to identify pathogenic mutations over-represented in tumours of high grade/stage and/or poor survival.

Results
A total of 39 patients were included. All tumour stages and grades were represented in the cohort. The median follow-up was 10.6 years. In all, 11 patients died from UTUC during the follow-up. Tumour mutational burden showed a statistically significant correlation with stage, grade, and stage + grade. Grade 1, Grade 2, and Grade 3 tumours had different mutational patterns. Patients who died from UTUC had pathogenic mutations in specific genes e.g. tumour protein p53 (TP53) and HRas proto-oncogene, GTPase (HRAS). Patients with Ta Grade 1 tumours with a known pathogenic fibroblast growth factor receptor 3 (FGFR3) mutation did not die from UTUC.

Conclusion
The genetic analysis was highly concordant with histopathological features and added prognostic information in some cases. Thus, results from genomic profiling may contribute to the choice of treatment and follow-up regimens in the future.

Keywords
Urothelial carcinoma, Genomic profile, Kidney-sparing surgery, Prognostic marker, UTUC

Introduction
Upper tract urothelial carcinoma (UTUC) is divided into low- and high-risk disease as a basis for the choice of treatment [1]. Nephroureterectomy (NU) is recommended in organ-confined high-risk UTUC, whereas kidney-sparing surgery (KSS) is to be considered for low-risk cases [1]. For low-risk UTUC, KSS has the same oncological outcome as NU [2] without the morbidity associated with more advanced surgery and deteriorated renal function. Unfortunately, the level of evidence for current methods of preoperative risk stratification is weak [1]. Stage and grade are strong prognostic factors [1,3], whereas the prognostic roles of tumour multifocality and tumour size are debated [4,5].
Despite radical surgery, a substantial portion of patients with UTUC develop metastases. The 5-year cancer-specific survival (CSS) is ~50% for high-grade and invasive tumours [1, 5]. Rarely, patients with low-risk tumours die from UTUC [6]. It is unclear why some patients within the same risk group progress while others do not. Assessment of genetic mutations is performed in the clinic for other malignancies [7]. Bagrodia et al. [8] identified differences in tumour mutational patterns related to the prognosis and invasiveness of UTUC. There was a high concordance between mutations found in ureteroscopic tumour biopsies and the corresponding NU specimens [9]. However, with the exception of fibroblast growth factor receptor 3 (FGFR3) and tumour protein p53 (TP53), recent studies of genetic profiles in UTUC have slightly diverging results [10, 11]. A possible reason is that not all genetic variants detected in cancer genes are pathogenic. The type of variant indicates impact: a frameshift mutation is much more likely to have a pathological impact than a synonymous mutation, but a frameshift near the end of the gene might not affect function. In-depth analyses of the likely effects of the detected mutations are scarce in the current literature.

In the present study, we aimed to investigate whether mutational patterns could distinguish aggressive from non-aggressive disease, i.e. to determine the utility of genetic alterations as prognostic markers, over a long follow-up period.

Patients and Methods

In a prospective, consecutive study running from 2005 to 2012, 148 patients with suspected UTUC were included to evaluate diagnostic procedures [12] and for long-term follow-up after treatment. In all, 94 patients had UTUC of which 55 had a NU. A subgroup of 43 patients diagnosed using ureterorenoscopy (URS) was previously included in studies investigating accuracy of ureteroscopic samples [13] and tumour characteristics associated with invasiveness and CSS [5]. The same subgroup was analysed in this pilot study, see Fig. 1. In eight of the 43 cases there was no available tumour tissue left. We aimed to replace these with specimens from the larger cohort. Unfortunately, there were not enough samples with matching histopathological data and a NU specimen available, to replace all eight. Thus, a total of four additional tumours were added, bringing the total to 39 tumours. All patients in this study were treated with NU, which was ‘gold standard’ at the time of inclusion.

Tumour Material And Pathological Assessment

We used formalin-fixed paraffin-embedded (FFPE) tumour material from NU specimens. As study inclusion was performed to exclude artefacts and benign germline variants. We filtered using standard quality parameters (see Methods S1 for full information) to remove technical artefacts. We also excluded all variants with a maximum allele frequency of >50%.

DNA Extraction

DNA was extracted using the Promega Maxwell® RCS DNA FFPE Kit and Maxwell® RCS Instrument (Methods S1 for full information). The samples had a mean (range) nucleic acid concentration of 281 (45–779) ng/µL. The samples were delivered for sequencing promptly after DNA extraction.

Genomic Data Processing

Samples were analysed using a custom-designed capture probe panel with 388 genes (Twist Bioscience) with Twist Biosciences enzymatic library preparation and sequencing on a NovaSeq 6000 (Illumina) using a paired-end 150 nucleotide readout, aiming at 30 million read pairs per sample. The FASTQ files were analysed using the bioinformatic analysis pipeline BALSAMIC version 4.0.0 [14], and trimmed reads were mapped to the reference genome hg19. For each sample, single-nucleotide variants (SNVs) and insertions and deletions (INDELs) were called using VarDict version 2019.06.04 [15]. All variants were annotated using Ensembl VEP version 94.5 [16] (for details see Methods S1).

Tumour mutational burden (TMB)

The TMB is the number of somatic coding SNVs per megabase of genome examined. TMB calculations were performed following the method described by Chalmers et al. [17] 2017. To exclude artefacts, we removed all SNVs with a read depth <100 and an alternative allelic depth of <5 from the TMB calculations.

Initial data filtration and bioinformatic analysis

Before the bioinformatic analysis, an initial data filtration was performed to exclude artefacts and benign germline variants.
≥0.5% in any subpopulation in gnomAD version 2.1.1. [18] and rare variants annotated as benign in ClinVar [19] (accessed May–August 2020).

Thereafter, bioinformatic analysis were performed in two stages. A blinded analysis was performed to test whether a clear genetic pattern could be recognised without knowledge of clinical data. Tumours with many and deviant mutations were assumed to represent ≥T2 or Grade 3 any T. This analysis was performed using principal component analysis (PCA) and hierarchical clustering. Next, a comparative analysis was performed to statistically compare patients grouped according to stage, grade, stage + grade and cause of death. The analyses were performed using ANOVA and a custom script, searching for mutations enriched in the various groups (for details see Methods S1).

Manual data filtration to identify pathogenic mutations

The data output after initial filtration was also manually annotated in order to scrutinise the identified mutations and to evaluate whether they were likely to be pathogenic. We excluded gene variants occurring in >60/100000 members of the general population in order to further remove likely germline or benign variants, evaluated the expected impact of the mutation on the function of the protein and whether it was a mutation previously reported in UTUC, bladder cancer or at all in cBioportal [20, 21] (an open-access database for mutations found in various cancer types; see Methods S1 for full information). The rationale for this was that a mutation found in other cohorts of UC was more likely to have a pathological impact, than one never previously reported.
The impact of the identified mutations was estimated from the Variant Effect Predictor’s output [16]:

- High impact: frameshift, splice donor, stop-gained, and splice acceptor variants.
- Moderate impact: missense and in-frame deletions.
- Low impact: synonymous and splice region variants.
- Modifiers: up- or downstream gene variants and intron variants (i.e. non-coding mutations).

All found TP53 mutations were checked in international databases on TP53 mutations (International Agency for Research on Cancer [IARC] version R20, July 2019 [22] and PHANTM [23] accessed 12 January 2021) and were disregarded if labelled functional or wild type.

Microsatellite instability (MSI) analysis

In all, 6% of UCs display MSI [24]. MSI tumours generally show somatic loss of one or two proteins (MutL homologue 1 [MLH1], MutS homologue 2 [MSH2], MutS homologue 6 [MSH6] or PMS1 homologue 2, mismatch repair system component [PMS2]) involved in mismatch repair (MMR) and carry mutations in or hypermethylation of their genes. To estimate MSI, we calculated the percentage of unstable MSI sites in the total number of sites using MSIsensor-pro [25] with the results given as the percentage of analysed sites that demonstrated MSI (see Methods S1 for further information). To confirm MSI status, immunohistochemistry for MMR proteins was performed using standard procedures.

Ethical considerations

The study was performed according to the Declaration of Helsinki and was approved by the Regional Ethical Review Board. Informed consent was obtained from all patients.

Results

A total of 39 patients were included and followed until death or 20 November 2020. The median time of follow-up was 10.6 years. At the end of the study, 21/39 patients had died, 11 from UTUC and 10 from other causes, none of the latter with active UTUC at the time of death. Three patients had metastasis of UTUC but were still alive at the end of the study. All tumour grades and stages were represented in the samples, with 31 (79%) being high grade; 12 (31%) were invasive (≥T2), and 24 (62%) tumours were ≥15 mm. For details, see Table 1.

Tumour mutational burden

All samples generated mutational data. The median (range) TMB was 23 (12–116) mutations/mega base pairs (mut/Mbp). One outlier (verified to be an MSI tumour) had a TMB of 116 mut/Mbp. With the outlier disregarded, the range was 12–55 mut/Mbp. In all, 74% of the tumours with a TMB above the median were ≥T2 or Grade 3 any T tumours, compared with 56% in the whole cohort. There was a statistically significant \( P < 0.05 \) difference in TMB between tumours of all stages, Grade 2 vs Grade 3 tumours and Ta–T1 Grade2 vs ≥T2 or Grade 3 any T tumours. There was no correlation between TMB and DNA concentration in the sample or between TMB and age of the specimen, indicating that these parameters did not affect the number of detected mutations before manual filtration.

Bioinformatic analysis

A total of 6540 gene variants were found in the cohort after initial data filtration. In the blinded analysis, tumours were clustered based on these mutations, see Fig. 2. Three groups were obtained from the PCA: one heterogeneous group (black numbers in Fig. 2A) consisting of the majority of individuals and two smaller groups (blue and orange numbers in Fig. 2A) consisting of a total of 13 individuals, each group carrying distinct mutational profiles. Hierarchical clustering (Fig. 2B) identified 14 tumours with a divergent mutational pattern (boxed cluster in Fig. 2B), 13 of which were also found in the smaller groups in the PCA. The number of mutations, i.e. the variant count, differed significantly between the 14 identified tumours and the remaining 25 tumours (Fig. 2C; Mann–Whitney U-test \( P < 0.001 \)). Notably, 12 of these 14 tumours were ≥T2 or Grade 3 any T (Table 1). There was no statistically significant enrichment of lethal or metastatic UTUC in the blinded clustering (the sensitivity of PCA for lethal or metastatic UTUC was 0.43). However, non-parametric re-sampling showed that the blinded selection of suspected ≥T2 or Grade 3 any T samples was superior to random sampling (precision 0.86 [\( P < 0.001 \)] and sensitivity 0.55 [\( P < 0.001 \)].

In the unblinded material, the comparative analysis showed that the ≥T2 or Grade 2 any T tumours showed enrichment of mutations in specific genes, indicating that the same evolutionary pathways were commonly affected in ≥T2 or Grade 3 any T tumours. A comprehensive list of the enriched mutations in the respective groups is listed in Table S1. In all, 10 genes had enriched mutations in both ≥T2, Grade 3 and ≥T2 or Grade 3 any T tumours: rho GTPase activating protein 35 (ARHGAP35), AT-rich interaction domain 1A (ARID1A), B-Raf proto-oncogene, serine/threonine kinase (BRAF), BRCA1 interacting helicase 1 (BRIP1), cAMP response element-binding-protein binding protein (CREBBP), FAT atypical cadherin 1 (FATT), histone-lysine N-methyltransferase 2A (KMT2A), leucine-zipper-like transcription regulator 1 (LZTR1), MAX dimerisation protein MGA (MGA), and SET domain-containing 2, histone lysine methyltransferase (SETD2).
Genes with enriched mutations in patients who died from UTUC were KMT2D, LZTR1 and TP53, all of which are known tumour suppressor genes.

When clustering the unblinded dataset based on genes enriched in ≥T2 or Grade 3 any T tumours, 19/22 ≥T2 or Grade 3 any T tumours were identified (i.e. positioned in clusters containing mostly ≥T2 or Grade 3 any T tumours), yielding a sensitivity of 0.86 and precision of 0.90. This method identified 11/14 lethal or metastatic UTUCs. Two samples with Ta Grade 1 and T1 Grade 1 tumours were positioned among the ≥T2 or Grade 3 any T tumours. One of these patients later died from UTUC. Three samples with ≥T2 or Grade 3 any T tumours did not cluster as such. None of these patients died from UTUC; one died from other causes after 2 years, and the other two were still alive after >11 years of follow-up.

Table 1 Patient characteristics and results from bioinformatic analysis. The total number of patients was 39.

| WHO 2004 | Stage | Grade | Stage + Grade |
|----------|-------|-------|---------------|
| Low grade, n | Ta-T1 CIS | ≥T2 CIS | CIS only | Grade 1 | Grade 2 | Grade 3 | Ta-T1 Grade 1 | Ta-T1 Grade 2 | ≥T2 or Grade 3 any T |
| High grade, n | 8 | 0 | 0 | 8 | 0 | 0 | 8 | 0 | 0 |
| Total, n | 14 | 12 | 5 | 0 | 11 | 20 | 0 | 9 | 22 |
| Female, n | 6 | 1 | 1 | 1 | 3 | 4 | 1 | 3 | 4 |
| Age at diagnosis, years, mean | 69 | 72 | 72 | 69 | 68 | 73 | 69 | 68 | 72 |
| Year of diagnosis, mean | 2008 | 2009 | 2009 | 2009 | 2009 | 2008 | 2009 | 2008 | 2008 |
| Smoking status at diagnosis, n | Non-smoker | 8 | 5 | 2 | 1 | 5 | 9 | 1 | 4 | 10 |
| Current smoker | 7 | 3 | 0 | 3 | 3 | 4 | 7 | 4 | 2 | 8 |
| Ex-smoker | 7 | 4 | 4 | 4 | 4 | 4 | 7 | 4 | 5 | 8 |
| UTUC tumour size, n | <15 mm | 5 | 2 | 0 | 1 | 3 | 3 | 1 | 3 | 3 |
| ≥15 mm | 15 | 9 | 0 | 6 | 8 | 10 | 6 | 6 | 12 |
| Unknown | 2 | 1 | 0 | 1 | 0 | 2 | 1 | 0 | 2 |
| CIS only | 0 | 0 | 5 | 0 | 0 | 5 | 0 | 0 | 5 |
| Number of tumours | Unilateral | 15 | 7 | 2 | 6 | 7 | 11 | 6 | 7 | 11 |
| Multilobar | 7 | 5 | 0 | 2 | 4 | 6 | 2 | 2 | 8 |
| No visible tumour | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 3 |
| Bladder cancer, n | Primary | 8 | 2 | 4 | 3 | 4 | 7 | 3 | 3 | 8 |
| Secondary | 4 | 1 | 0 | 1 | 1 | 4 | 1 | 1 | 3 |
| Unknown | 0 | 3 | 0 | 0 | 1 | 1 | 0 | 1 | 3 |
| No bladder cancer | 10 | 6 | 1 | 4 | 5 | 8 | 4 | 4 | 8 |
| NU resection margin, n | Negative | 17 | 8 | 4 | 6 | 9 | 14 | 6 | 8 | 15 |
| Positive | 2 | 2 | 1 | 0 | 2 | 3 | 0 | 1 | 4 |
| Unknown | 3 | 2 | 0 | 2 | 0 | 3 | 2 | 0 | 3 |
| Death from UTUC, n (%) | 4 (18) | 6 (50) | 1 (20) | 1 (13) | 2 (18) | 8 (40) | 1 (13) | 2 (22) | 8 (36) |
| Metastasis, n (%) | 4 (18) | 8 (67) | 2 (40) | 1 (13) | 2 (18) | 11 (55) | 1 (13) | 2 (22) | 11 (50) |
| TMB, mut/Mbp, range (median) | 12–37 (22) | 18–116 (30) | 18–55 (38) | 15–37 (23) | 12–30 (18) | 18–116 (30) | 15–37 (23) | 12–30 (18) | 16–116 (27) |
| Bioinformatic analysis, n/N | Blinded analysis | Suspected ≥T2 or Grade 3 any T/total | 4/22 | 7/12 | 3/5 | 2/8 | 0/11 | 12/20 | 2/8 | 0/9 | 12/22 |
| True severe/suspected | 2/4 | 7/7 | 3/3 | 1/2 | n.a. | 12/12 | 1/2 | n.a. | 12/12 |
| Unblinded analysis | Correlation of the mutational profile with histopathological features/total: | 21/22 | 8/12 | 3/5 | 6/8 | 11/11 | 18/20 | 6/8 | 9/9 | 19/22 |

n.a.: not applicable. For grade, CIS only (n = 5) is included in Grade 3. The group ≥T2 or Grade 3 any T includes any stage T2-4 irrespective of grade and Grade 3 tumours irrespective of stage, including any concomitant CIS. Primary bladder cancer was diagnosed before the diagnosis of UTUC, and secondary bladder cancer was diagnosed after UTUC. Metastasis was defined as the presence of UC recurrence in locations other than the bladder. Blinded analysis shows number of suspected ≥T2 or Grade 3 any T/total samples in the group. True severe: fraction of tumours suspected to be ≥T2 or Grade 3 any T in the blinded clustering that were histopathologically classified as ≥T2 or Grade 3 any T, and/or caused metastasis or the patient to die from UTUC. For the unblinded analysis, correlation of mutational profile with histopathological features is shown as fraction of identified samples/total samples in group.

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Enriched mutated genes in the manually filtered data

After manual filtration, 532 mutations in 138 genes occurred at least once. Great care was taken to remove variants that were common germline variants in the normal population, reported to be benign variants, likely technical artefacts, and/or not predicted to have any impact on the protein (Methods S1 for full information). A total of 270 mutations in 71 genes were characterised as likely
pathogenic. In all, 23 genes were mutated in three or more of the 36 informative samples, as shown in Fig. 3. A complete list of gene mutations after manual filtration is presented in Table S2. Three samples had no mutations that fulfilled the quality requirements of the manual filtration. These three tumours had different stages and grades and different sizes. They all had low concentrations of DNA in the sample (<80 ng/μL compared to the cohort mean of 282 ng/μL).

All tumour grades had different mutational patterns. Although all Grade 2 tumours in the study were high grade, they differed from Grade 3 tumours regarding mutational pattern and TMB (Fig. 3). All Ta Grade 1 tumours in the manually filtered data had a FGFR3 mutation, and none had a pathogenic TP53 mutation. The FGFR3 variants occurred in decreasing fractions in Ta Grade 2/T1 Grade 2 and ≥T2 or Grade 3 any T tumours. Known pathogenic TP53 mutations were identified solely in Grade 3 tumours (61% of Grade 3 tumours). One sample (number 10) in the ≥T2 or Grade 3 any T group had a FGFR3 mutation but no TP53 mutation. That tumour was Ta Grade 2 + CIS and the patient was alive after >13 years of follow-up. Erb-B2 receptor tyrosine kinase 2 (ERBB2) mutations occurred in 47% of Grade 3 tumours. One sample with T1 Grade 2 also had an ERBB2 mutation, that patient later died from UTUC.

In total, 11 patients died from UTUC, and three developed metastases but were still alive at the end of the study. Known pathogenic TP53 mutations were found in 45% of the patients that died from UTUC vs 22% in non-metastatic UTUC. An ERBB2 mutation was found in 36% of the samples from patients who died from UTUC vs 18% with non-metastatic UTUC. Three out of four patients with a HRAS mutation in their tumours died from UTUC. Two patients who died from UTUC had tumours with a FGFR3 mutation; one also had a HRAS mutation, and the other had a TP53 variant. One patient with a Grade 1 tumour died from UC after 11 years of follow-up. That tumour lacked a FGFR3 mutation but had a HRAS mutation. In all, 13 of the 14 patients with lethal or metastatic UTUC, had at least one mutation of HRAS, ERBB2 or TP53.

Microsatellite instability analysis

Among the 39 tumours, 37 had MSI scores of 3.08–3.82% (Table S3). Two samples (number 22 and 18) had markedly higher scores: 9.42% and 11.65%. These were classified as MSI-High and confirmed with immunohistochemistry to have loss of MSH2 and MSH6, respectively. Sample 22 had two different mutations in MSH2, sample 18 had two different mutations in MSH6. The tumours were T3 Grade 3 and T3 Grade 3 + CIS and had TMB at the top of the TMB range, 55 and 116 mut/Mbp, respectively. We did not have germline samples or ethical permission to investigate these individuals further.

Two additional samples had single pathogenic mutations in MMR genes: one in MLH1 (sample 20) and one in MSH6.
(sample 38). Both had normal MSI scores. Sample 20 had normal MMR proteins according to immunohistochemistry. Sample 38 showed heterogeneity, with some tumour areas lacking MLH1 and PMS2, possibly due to hypermethylation.

This pattern is not consistent with a defect in MSH6 and therefore a staining artefact cannot be ruled out.

**Discussion**

All tumours in our present cohort were high risk according to European Association of Urology (EAU) guidelines [1]. However, when analysing tumour mutations by stage, grade, and clinical outcome, we identified several interesting patterns:

1. There was a statistically significant difference in TMB between tumours of all stages, Grade 2 vs Grade 3 tumours, and Ta–T1 Grade 2 vs ≥T2 or Grade 3 any T tumours.
2. Patients who died from UTUC had pathogenic mutations in specific genes e.g. TP53 and HRAS. All three patients with Grade 1 and Grade 2 tumours who died from UTUC had superficial tumours (<T2) but had tumour mutations indicating aggressive disease.
3. None of the patients with Ta Grade 1 tumours with a known pathogenic FGFR3 mutation died from UTUC regardless of tumour size and multifocality.
4. Although all the Grade 2 tumours were high grade in our present study, the Grade 2 and Grade 3 tumours had different mutational patterns and statistically significant differences in TMB. They also had different CSSs: 47% of patients with exophytic Grade 3 tumours died from UTUC compared to 18% in the Grade 2 group.
5. Tumours with MSI could be identified using the program MSIsensor [25].

Our present findings suggest that genetic analysis may be used for risk stratification and helping treatment decisions, although verification in larger cohorts is needed.

The strengths of our present study are the prospective collection of data, the long follow-up, and the representation of all tumour grades and stages. The methods used are well established, and the pipeline and gene panel are being implemented in clinical practice, which facilitates validation, reproducibility, and clinical use. Like many studies of UTUC, our present cohort is small, but an advantage of this smaller cohort is that in-depth analysis is feasible. Last, FFPE samples carry more artefacts than freshly frozen tissue, but we have taken several steps to eliminate such artefacts (initial data filtering, Methods S1).

In our present study, higher TMB was seen in more aggressive tumours, consistent with the findings of Nassar et al. [26]. We did not see a statistically significant difference in TMB between Grade 1 and Grade 3 tumours ($P = 0.12$) or Ta–T1 Grade 1 and ≥T2 or Grade 3 any T tumours ($P = 0.066$). A possible explanation might be that the Grade 1 group was small (eight patients) and contained two outliers, which affected our present results regarding Grade 1 tumours. Furthermore, as our panel included only genes relevant to cancer, which are more likely to be mutated in tumour samples, the TMB is likely to be higher in our present results than if we would have used whole-exome sequencing (WES). This matter of methodology does not affect the comparative results within our present study; however, our results cannot be compared directly with others using WES or other gene panels, like the study by Su et al. [11], who found a markedly lower mutational load in UTUC when performing WES.

In the manual filtration, we scrutinised the likely effect of all identified mutations and disregarded reportedly benign gene variants. All Ta Grade 1 tumours in the manually filtered analysis had a known pathogenic FGFR3 mutation, consistent with the findings of Sfakianos et al. [27], who found FGFR3 mutations in 96% of low-grade UTUC tumours, and those of Bagrodia et al. [8], who found them in ≥80% of Ta tumours.Audenet et al. [28] found FGFR3 mutations in 31% of high-grade tumours. We had a similar initial frequency (28%) of variants of FGFR3, but after manual filtration 14% of the high-grade tumours in our present cohort had a known pathogenic FGFR3 mutation. Bagrodia et al. [8] found FGFR3 mutations in 10–20% muscle-invasive tumours, compared to 9% in ≥T2 tumours in our present study. Sfakianos et al. [27] found TP53 mutations exclusively in high-grade tumours, similar to our present results with known pathogenic TP53 mutations solely in Grade 3 tumours.

The presence of a FGFR3 mutation has been linked to favourable prognosis and outcome, and the presence of TP53 mutation has been linked to poor prognosis [8, 27]. Similar to our present results, Sfakianos et al. [27] found a mutually exclusive mutation pattern among FGFR3, HRAS, and TP53 in high-grade tumours. The minor discrepancies between our present results may be due to differences in methodology: Sfakianos et al. [27] only looked at certain mutations in oncogenes and others in tumour suppressors; we included all mutations in all genes and then manually checked in a multitude of databases to determine if the found mutations were likely benign or pathogenic. Eight of our samples had FGFR3 and TP53 or HRAS mutations. After manual filtration, two tumours had combinations of verified known pathogenic FGFR3 and TP53 or HRAS mutations. Both of these patients died from UTUC, suggesting a negative prognostic effect of HRAS and TP53 mutations that overrules the less aggressive effect of a FGFR3 mutation.

Our present results suggest that the mutational profile might have a stronger prognostic impact than some of the prognostic factors used today, i.e. size and multifocality. All patients in our present study were treated with NU. It may be, that patients with Grade 1/low-grade tumours with a known pathogenic FGFR3 mutation, without TP53, HRAS,
ERBB2 and CREBBP mutations, safely can be offered organ-sparing treatment to a greater extent than today. On the other hand, even small unifocal Grade 1/low-grade tumours with mutations consistent with aggressive disease should perhaps not be offered organ-sparing treatment. These hypotheses need to be tried and validated in another prospective and larger cohort. In our present study, all Grade 2 tumours were high grade, but there were notable differences in gene profiles and CSSs between Grade 2 and Grade 3 tumours, suggesting that using both the WHO 1999 and 2004 classifications is of prognostic value when diagnosing UTUC. Our present findings are consistent with the results of van Rhijn et al. [29], who concluded that a combination of the WHO 1999 and 2004 classifications was superior in prognostic evaluation for UC of the bladder compared to either system alone. Gene sequencing in the diagnostic evaluation may enable risk stratification in high-grade tumours in a more nuanced way than can be offered today.

In all, 47% of the patients with exophytic Grade 3 tumours died from UTUC despite receiving curative intent treatment. Although there are studies showing promising results of neoadjuvant chemotherapy there are no published randomised controlled trials. Hence, neoadjuvant chemotherapy is not a recommendation in the EAU guidelines [1]. Regarding adjuvant treatment, the POUT trial (ClinicalTrials.gov Identifier: NCT01993979) by Birtle et al. [30] showed significantly improved disease-free survival in patients with locally advanced UTUC who received adjuvant therapy after surgery. Our present study was initiated long before the results of the POUT study and in our material only four patients received adjuvant chemotherapy: one because of non-radical NU and three because of later metastasis. Two of the patients who received adjuvant chemotherapy were still alive at the end of the study. TMB and MSI are emerging biomarkers for susceptibility to immune checkpoint inhibitors [17]. Additional information obtained by genomic profiling may help identify patients who can benefit from additional treatment in the future.

Our present results add further knowledge of mutational patterns correlated with long-term CSS, and our analysis of known pathogenic mutations suggests a strategy for predicting prognosis in patients with multiple FGFR3, TP53 and HRAS mutations. Further research should include validation of diagnostic samples in a prospective study and a standard procedure for the analysis of mutations.

**Conclusion**

Genetic analysis was highly concordant with histopathological features related to prognostic stratification. In discordant cases, the genetic profile for the combined stage and grade correlated better with clinical outcome than did histopathological features. Genomic profiling at diagnosis may be a valuable tool for deciding treatment strategies in the future.

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**Conflict of Interest**

The authors have no conflict of interest.

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Abbreviations: CIS, carcinoma in situ; CREBBP, cAMP response element-binding-protein binding protein; CSS, cancer-specific survival; EAU, European Association of Urology; ERBB2, Erb-B2 receptor tyrosine kinase 2; FGFR3, fibroblast growth factor receptor 3; HRAS, HRas proto-oncogene, GTPase; KSS, kidney-sparing surgery; LZTR1, leucine-zipper-like transcription regulator 1; MLH1, MutL homologue 1; MMR, mismatch repair; MSH(2)(6), MutS homologue (2) (6); MSI, microsatellite instability; NU, nephroureterectomy; PCA, principal component analysis; PMS2, PMS1 homologue 2, mismatch repair system component; SNV, single-nucleotide variant; TMB, tumour mutational burden; TP53, tumour protein p53; URS, ureterorenoscopy; (UT)UC, (upper tract) urothelial carcinoma; WES, whole-exome sequencing.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Comprehensive list of the enriched mutations in the respective groups.

Table S2. Complete list of gene mutations after manual filtration.

Table S3. MSI scores for the 39 tumours.

Table S4. Genes previously reported to be mutated in UTUC.

Method S1. Supplementary Methods.