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High prevalence of *TERT* promoter mutations in micropapillary urothelial carcinoma

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Abstract

Somatic activating mutations in the promoter of the *telomerase reverse transcriptase (TERT)* gene are the most common genetic alterations in urothelial carcinoma (UC) of the bladder and upper urinary tract. Little is known, however, about *TERT*-mutation status in the relatively uncommon but clinically aggressive micropapillary (MPC) variant. We evaluated the presence of *TERT* promoter mutations in MPC of the bladder and upper urinary tract. A retrospective search of our archives for MPC and UC with micropapillary features (2005–2014) was performed. All slides were reviewed to confirm the histologic diagnosis. Thirty-three specimens from 31 patients had FFPE blocks available for DNA analysis and were included in the study. Intratumoral areas of non-micropapillary histology were also evaluated when present. Samples were analyzed with Safe-SeqS, a sequencing error reduction technology, and sequenced using the Illumina MiSeq

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Compliance with ethical standards

Disclosure/conflict of interest KWK, NP, and BV are founders of Personal Genome Diagnostics, Inc. and PapGene Inc. and advise Sysmex-Inostics. These companies and others have licensed technologies from Johns Hopkins, of which BV, KWK, and NP are inventors and receive royalties from these licenses. The terms of these arrangements are being managed by the university in accordance with its conflict of interest policies.

platform. *TERT* promoter mutations were detected in all specimens with pure MPC (18 of 18) and UC with focal micropapillary features (15 of 15). Similar to conventional UC, the predominant mutations identified occurred at positions −124 (C228T) (85 %) and −146 (C250T) (12 %) bp upstream of the *TERT* ATG start site. In heterogeneous tumors with focal variant histology, intratumoral concordant mutations were found in variant (MPC and non-MPC) and corresponding conventional UC. We found *TERT* promoter mutations, commonly found in conventional UC, to be frequently present in MPC. Our finding of concordant intratumoral mutational alterations in cases with focal variant histology lends support to the common oncogenesis origin of UC and its variant histology.

Keywords

Micropapillary; Urothelial carcinoma; Telomerase reverse transcriptase; TERT; Mutation

Introduction

Somatic activating mutations in the promoter of the *telomerase reverse transcriptase (TERT)* gene, originally discovered in the majority of melanomas [8], have been found to be the most common genetic mutations in urothelial (transitional cell) carcinoma (UC) of either the bladder or upper urinary tract [12, 14]. In a study by Kinde et al. [14], 66 % of muscle invasive (i.e., invading muscularis propria) and 74 % of non-muscle invasive bladder lesions were shown to harbor these alterations (UC).

Greater than 90 % of bladder carcinomas are urothelial type; however, a small subset of tumors are recognized as distinctive histologic variants (e.g., squamous, glandular, microcystic, micropapillary, plasmacytoid, sarcomatoid) [20], raising the question of whether *TERT* promoter mutations are retained in UC with divergent morphologies. Recently, we investigated small cell carcinomas of the urinary bladder and identified *TERT* C228T mutations in 11 of 11 cases [29]. Subsequent studies have also identified *TERT* C228T mutations in the majority of nested and “large nested” variants of UC [30] and UC of the renal pelvis and the ureter [26].

The micropapillary variant of UC, first described in 1994 by Amin et al. [2], is a relatively uncommon but important morphologic subtype. In most cases, it is also associated with conventional UC or mixed with other variant histologies [2, 16]. Several studies have suggested that the presence of invasive micropapillary features confers an adverse prognosis with a higher rate of locally advanced disease and an increased risk of mortality [5, 10, 19]. These differences in biology and response to therapy suggest that alternate molecular pathways may be involved in the divergent development of specific variant histology within UC.

In this study, we sought to address the presence of *TERT* promoter mutations in the micropapillary variant of UC and the implications and clinical relevance of such mutations in aggressive variants of UC.

Material and methods

Patient samples

We searched our electronic pathology database system for the key word “micropapillary” with “urothelial cancer,” “bladder cancer” or combinations of “urothelial,” “bladder,” and “carcinoma.” The search included cases from the years 2005 to 2014. Forty-eight specimens of invasive urothelial carcinoma with micropapillary features were identified. From these, 33 specimens from 31 patients had sufficient tissue for DNA analysis and were included in the study.

Specimen types included bladder biopsies (n = 8), transurethral bladder tumor resections (n = 9), pelvic lymph node dissections (n = 3), cystoprostatectomies with and without pelvic lymph node dissections (n = 9), cystectomy (n = 1), nephroureterectomy (n = 1), pelvic exenteration (n = 1), and small bowel resection for metastatic tumor (n = 1).

All sections were reviewed by a senior genitourinary pathologist to confirm the original diagnoses according to the WHO/ISUP 2004 classification criteria and the updated 2015 International Consultation on Urologic Diseases (ICUD) recommendations [3]. Specimens with surface and/or invasive micropapillary histology in the bladder and upper urinary tract were included in the study. Morphologic criteria used for diagnosis of micropapillary histology included: the presence of epithelial ring forms; multiple nests/balls of cells within the same lacunar space; back-to-back lacunar spaces; reverse polarity; and small branching micropapillae or tufts lacking fibrovascular cores [22]. Specimens with large (>4 cells across in the most narrow focus) tumor nests or otherwise “non-classic” micropapillary features were regarded as conventional UC with micropapillary features [22]. Percentage of tumor composed of micropapillary features was not estimated due to the variability in tumor sampling between specimens and lack of established guidelines in classifying such tumors.

Areas with the highest neoplastic cellularity, as determined from H&E sections of the tumors, were chosen for analysis. Multiple tumor foci were isolated and analyzed separately in the specimens that contained more than one variant morphology and/or additional foci of conventional urothelial carci noma.

Tumor was cored from formalin-fixed paraffin-embedded (FFPE) blocks using sterile 16 gauge needles. One to 4 cores per targeted sample area were removed and placed in 1.5-mL sterile tubes for DNA purification. DNAwas purified using an All Prep Kit (Qiagen, cat. no. 80204).

Two sets of negative control samples were also analyzed for *TERT* mutations. Eight FFPE specimens from benign transurethral bladder biopsy samples were used as negative tissue controls, and 94 peripheral blood samples from a healthy patient population were used as negative PCR procedural controls.

Mutation analysis

We used Safe-SeqS, a sequencing error reduction technology described previously [13, 15], to discriminate genuine *TERT* promoter mutations from artifactual sequencing variants

introduced during the sequencing process. Safe-SeqS amplification primers were designed to amplify a 126-bp segment containing the region of the *TERT* promoter previously shown (Fig. 1) to harbor mutations in melanomas and other tumors [8, 12]. The forward and reverse amplification primers contained the *TERT*-specific sequences at their 3′ ends and a universal priming site (UPS) at their 5′ end. The reverse primer additionally contained a 14-base unique identifier (UID) comprised of 14 degenerate N bases (equal likelihood of being an A, C, T, or G) between the UPS and gene-specific sequences. The sequences of the forward and reverse primers were either 5′-CACACAGGAAACAGCTATGA CCATGGGCCGCGGAAAGGAAG and 5′-CGACGTAAAACGACGGCCAGTNNNNNNNN NNNNNNCGTCCTGCCCCTTCACC, or CACACAGGAAACAGCTATGACCATGGCGGGAAAGGAAAGGGAG and 5′-CGACGTAAAACGACGGCCAGTNNNNNNNNNNNNNNNCCGTCCCGACCCCTC (UPS sequences underlined). These primers were used to amplify DNA in 25 μL PCR reactions in 1× Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, cat. no. F-548L) containing 0.5 μM forward and reverse primers (described above). After incubation at 98 °C for 120 s, 10 cycles of PCR were performed in the following manner: 98 °C for 10 s, 63 °C for 120 s, and 72 °C for 120 s was performed. Reactions were purified with AMPure XP beads (Beckman Coulter) and eluted in 100 μL of Buffer EB (Qiagen, cat. no. 19086). For the second stage of amplification, 5 μL of purified PCR products were amplified in 25 μL reactions containing 1× Phusion Flash High-Fidelity PCR Master Mix and 0.5 μM amplification primers that each contained the first-stage UPS at their 3′ ends and the grafting sequences required to hybridize to the sequencing instrument flow cell at their 5′ ends [13, 15]. The reverse amplification primer additionally included a 6 bp index sequence, unique to each sample, inserted between the UPS and grafting sequences. After incubation at 98 °C for 120 s, 17 cycles of PCR were performed in the following manner: 98 °C for 10 s, 63 °C for 120 s, and 72 °C for 120 s. The PCR products were purified with AMPure and sequenced on a MiSeq instrument.

Data were analyzed as previously described [13, 15]. Briefly, the amplified *TERT* promoter region of reads containing UIDs, where each base of the UID region had instrument-derived quality scores ≥15, was matched to a reference sequence using a custom script. *TERT* promoter sequences with five or fewer mismatches were retained for further analysis. Tumor samples were considered positive if the fraction of mutations exceeded 1 % of alleles (which was a frequency at least 5× higher than that found in control DNA templates). All sequencing assays scored as positive were confirmed in at least one additional, independent sequencing PCR assay.

Results

Patient population

Thirty-three specimens of urothelial carcinoma with focal to pure micropapillary features from 31 patients (24 males, 7 females) were analyzed (Table 1). Two patients had more than one specimen included in the study, which correspond to paired specimen numbers 23–24 and 31–32. The mean patient age at the time of specimen sampling was 70.7 years (range,

52 to 91 years) with a median post-surgical follow-up period of 25 months (range, 1 to 77 months).

Tumor morphology

Pure micropapillary carcinoma (MPC) was isolated in 20 of 33 (61 %) specimens while mixed micropapillary and non-micropapillary components (e.g., glandular, squamous, noninvasive high grade papillary, and conventional urothelial carcinoma) were isolated in 13 of 33 (40 %) specimens. Examples of areas described as “micropa pillary” are shown in Fig. 2b–f. The most common non-micropapillary component was conventional high grade urothelial carcinoma, which was either present alone (five specimens), combined with micropapillary areas (eight specimens), or combined with other non-micropa pillary components.

Twenty-three of 33 (70 %) specimens showed at least 75 % neoplastic cellularity in the areas that were sampled for analysis. The lowest tumor cellularity was 30 % (paired specimen numbers 31–32) with an overall mean of 69 % (range, 30 to >80 %).

TERT promoter analysis

TERT promoter mutations were found in all 33 specimens of primary or metastatic urothelial carcinoma with focal to pure micropapillary features (Table 2). The predominant mutation identified was g.1295228C > T (28 of 33) followed by g.1295250C > T (4 of 33) and g.1295243C > T (1 of 33). Identical genetic abnormalities were detected in paired tumor specimens 23–24 and 31–32. Similarly, all cases with mixed histology showed identical mutations in micropapillary and non-micropapillary areas sampled within the same tumor.

All blood samples and benign urothelial tissue controls tested negative for *TERT* mutations.

Discussion

Micropapillary UC is usually considered an aggressive variant frequently associated with poor clinical outcomes. Any component of micropapillary histology, including surface noninvasive components [7, 23], in UC of the bladder and upper urinary tract [24] is considered to be significant. Studies have shown that as the proportion of the micro papillary component increases, the prognosis worsens [10]. Similar to micropapillary carcinomas in other organs, micropapillary bladder cancer also appears to be less responsive to chemotherapy and intravesical BCG than more common tumors of the same organs [10]. This apparent lack of survival benefit from the addition of neoadjuvant chemotherapy may be related to the frequent presence of lymph-vascular invasion, and occult nodal metastases (27.3 %) [10] observed with micropapillary bladder cancer. Some cases of micropapillary UC, however, do respond to neoadjuvant chemotherapy and are down-staged to ypT0 disease at the time of cystectomy [18].

Our analysis of this tumor cohort expands upon previous reports identifying frequent *TERT* promoter mutations in urothelial carcinomas [1, 14, 25, 29, 30]. In this study, *TERT* promoter mutations were found in all 33 cases of UC with pure to focal micropapillary features. The findings make micropapillary UC the solid tumor type with the highest rate of

TERT promoter mutations. Similar to previous studies on other variants of UC, the predominant mutations identified occurred at positions 124 (C228T) and 146 (C250T) bp upstream of the *TERT* ATG start site [8]. These mutations were also present in areas of non-micropapillary histology in heterogeneous tumors con taining a component of urothelial, glandular, papillary or squamous differentiation in addition to MPC.

Interestingly, all cancer-associated *TERT* promoter mutations discovered thus far generate novel binding sites for the ETS (E26 transformation-specific) family of transcription factors located near the translational start site of *TERT* [8, 9]. The cellular or microenvironmental factors that select for cells with the *TERT* promoter mutations remain unclear. However, it has been suggested that *TERT* promoter mutations preferentially promote tumor progression in differentiated cells which normally have absent to low telomerase and relatively low rates of self-renewal [6, 12]. Chiba and colleagues [6] have proposed that tumor-initiating cells in these specialized tissue types acquire somatic TERT promoter mutations at the point of telomere crisis in the course of cellular transformation in response to chronic injury, thus overcoming this telomere-dependent proliferative barrier early in their progression. Similar findings have been demonstrated in human gastric cancer cells in which bile acids, under acidic conditions, were found to be associated with an increase in *TERT* expression, perhaps through the activation of *c-myc* transcription [27].

The most common *TERT* promoter mutations (C228T and C250T) are believed to result in the creation of novel CCGG AA/T general binding motifs for E26 transformation-specific (ETS)/ternary complex factor (TCF) transcription factors [8, 17]. The somatic mutations at both positions result in a C > T base change and ETS binding sites that differ from pre-existing ETS binding sites (GGAA/T) within the promoter region. Genome wide occupancy studies have shown that multiple ETS factors can occupy any given single ETS binding site within a particular cell type [11]. Non-redundant binding also occurs, but appears to be mediated via protein-protein interactions with partner proteins that stabilize the ETS factors binding to the atypical, low affinity site sequences [11]. While ETS factors are a large family of transcription factors that can recognize these binding sites, a recent study by Bell et al. suggests that the novel ETS binding sites created by *TERT* promoter mutations are specifically and directly bound by GA-binding protein (GABP), a ubiquitously expressed transcription factor has been implicated in the regulation of re-entry into S phase of the cell cycle in quiescent cells [4, 28]. It will be of interest to investigate whether GABP or other *TERT* mutation-targeted therapies can have a role in the treatment of bladder cancer and its more clinically aggressive subtypes.

This study is limited by its relatively small sample size and broad pathologic inclusion criteria, which precluded accurate histologic percentage estimation of micropapillary histology. While micropapillary UC is usually considered to have a more aggressive course than conventional UC, the micropapillary pattern infrequently occurs singly. Our cohort, therefore, included cases with even small foci of micropapillary differentiation, even if they did not satisfy the rigorous demands of pure variant diagnosis. This study also chose to focus on the presence of *TERT* promoter mutations in UC with micropapillary histology compared to conventional UC rather than the prognostic significance of tumors with divergent histologies. In fact, the presence of *TERT* promoter mutations within areas of both focal and

pure micropapillary histology reinforces the morphologic plasticity of UC. Furthermore, the concordant findings of *TERT* promoter mutations in areas of typical UC and divergent differentiation point to a putative stem cell originating urothelial carcinoma capable of generating neoplastic *TERT*-driven populations with different phenotypes [21].

Conclusions

This is the first study to demonstrate the presence of *TERT* promoter mutations in UC with variant micropapillary histology as well as concordant intratumoral mutations in areas with conventional and non-micropapillary divergent morphology. The findings make micropapillary UC the solid tumor type with the highest rate of *TERT* promoter mutations.

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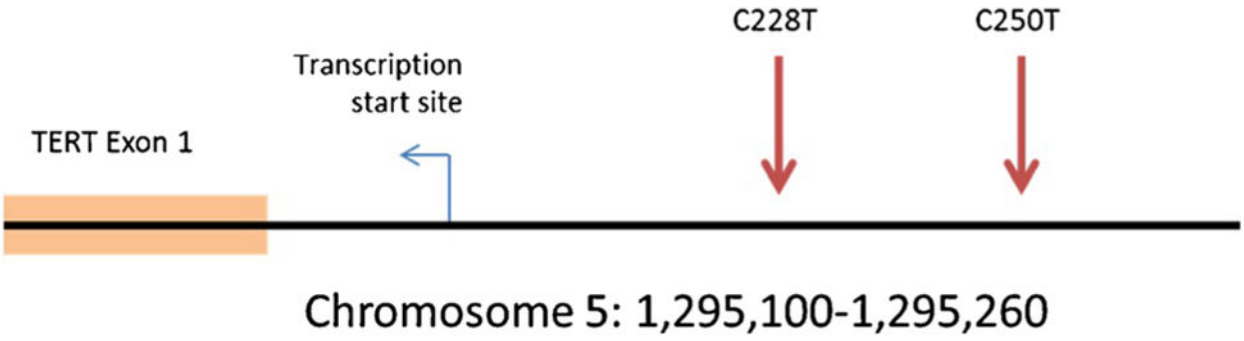

Figure 1.
Two mutational “hotspots” that are repeatedly seen in the *TERT* promoter occur at position 250 and position 228. Both of the mutations are a C > T base substitution mutations.

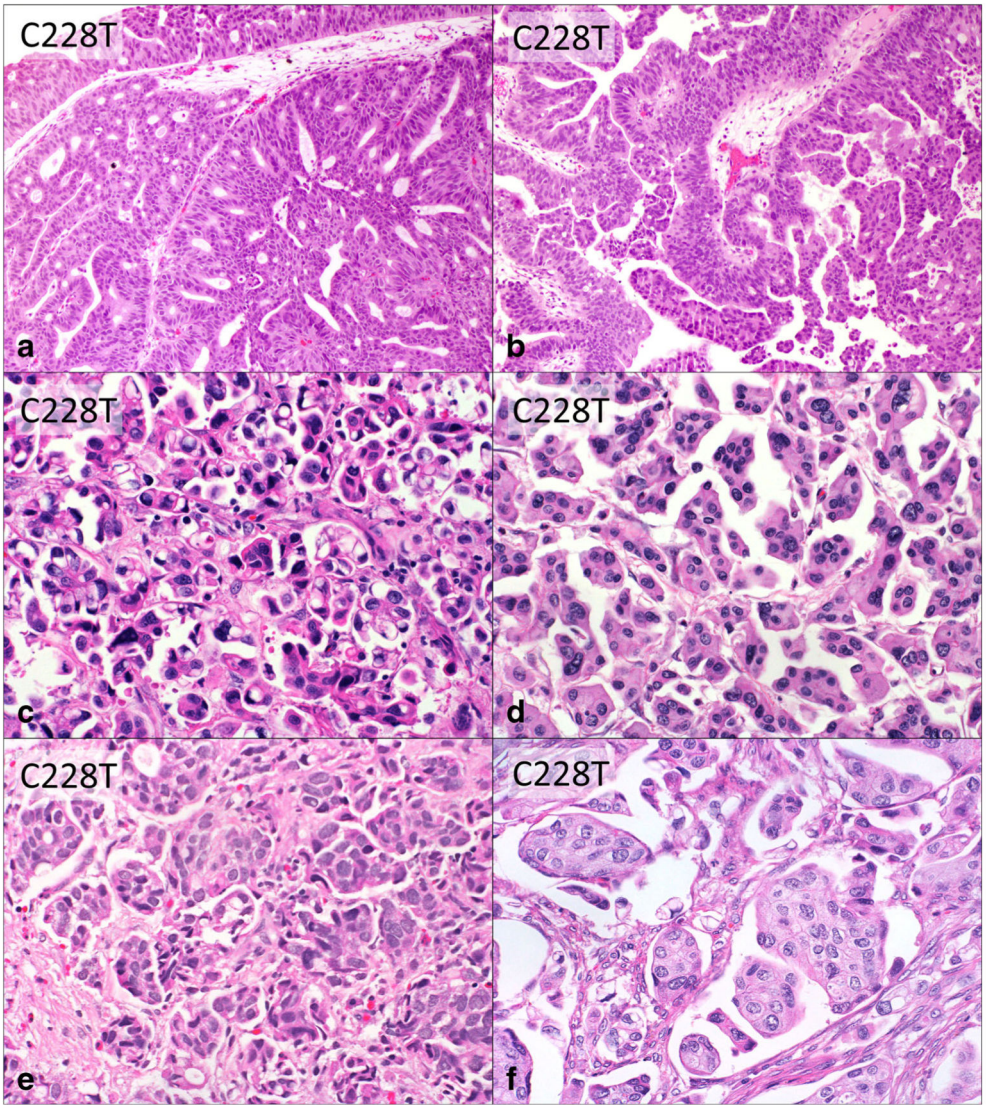

Fig. 2.

(H&E, 10X) Invasive urothelial carcinoma with glandular differentiation (**a**) and separate focus of noninvasive micropapillary carcinoma showing typical tufts of urothelial cells lacking fibrovascular cores (**b**) in the same patient sample. (H&E, 20X) Invasive micropapillary carcinoma composed of multiple uniformly sized nests of urothelial cells occupying stromal spaces (**c**, **d**). (H&E, 20X) Invasive urothelial carcinoma with micropapillary features composed of irregularly sized nests of urothelial cells (>4 cells across in the most narrow focus) occupying stromal spaces (**e**, **f**). The type of TERT promoter mutation corresponding to each of the represented tumors is shown. Note the identical type of mutation shared by areas represented in **a** and **b** of the same patient.

Table 1

Demographic and clinicopathologic characteristics

Specimen	Age	Sex	Race	Pathologic stage	Clinical stage	Prior surgery and/or treatment	Follow- up, months	Outcome at last follow- up
1	78	M	W	Ta	0	Mitomycin C	18	AWD
2	60	M	B	T1	I	BCG	77	DOO
3	80	M	B	TisN2	IV	TURBT	3	AWD
4	68	M	O	T3aN0	III	Biopsy	17	AWD
5	66	M	W	T3N2M1	IV	Cystoprostatectomy, chemo	51	DOD
6	64	M	W	T2a	II	ND	40	DOD
7	81	M	W	T2bN0	II	Biopsy	8	AWD
8	73	F	W	T3bN0	IV	Chemo	68	DOD
9	58	M	W	T3bN0	IV	Chemo	45	AWD
10	69	M	W	T4aN3	IV	Chemo	21	DOD
11	83	F	W	T1	I	ND	28	DOO
12	58	M	W	T2a	II	Biopsy	25	AWD
13	83	F	W	T2aNX	II	Biopsy	45	DOD
14	79	M	W	T3bN2	IV	BCG, mitomycin C	26	AWD
15	70	F	W	T1	I	ND	64	NED
16	76	M	W	T2aN2	IV	Biopsy	2	AWD
17	63	F	W	T2a	II	BCG	24	DOD
18	60	M	W	T3NX	III	ND	50	NED
19	59	M	W	T2aN2	IV	TURBT, chemo	10	AWD
20	77	M	W	T3bN2	IV	TURBT, chemo	12	AWD
21	58	F	W	T1	I	Left nephroureterectomy, BCG	47	NED
22	67	M	B	T2a	II	ND	12	DOD
23[a]	69	M	W	T2a	II	ND	34	NED
24[a]	69	M	W	T2bN0	II	TURBT	34	NED
25	91	M	O	T1	I	ND	32	AWD
26	88	F	W	T2a	II	ND	4	DOD
27	62	M	W	T3bN2	IV	TURBT	8	DOD
28	65	M	W	T1	I	TURBT, BCG	20	NED

Specimen	Age	Sex	Race	Pathologic stage	Clinical stage	Prior surgery and/or treatment	Follow- up, months	Outcome at last follow- up
29	81	M	W	T2a	II	Radiation for prostate cancer	12	DOD
30	75	M	W	T2aN2	IV	TURBT	1	AWD
31[b]	52	M	W	T2a	II	TURBT, BCG, Mitomycin C	62	AWD
32[b]	52	M	W	T2a	II	TURBT, BCG, mitomycin C	62	AWD
33	80	M	B	At least T1	I	ND	11	AWD

BCG bacillus Calmette-Guerin, *chemo* chemotherapy, *TURBT* transurethral resection of bladder tumor, *ND* none documented, *AWD* alive with disease, *DOD* died of disease, *DOO* died of other causes, *LTF* lost to follow-up, *NED* no evidence of disease

[a,b]Samples from same patient

Table 2

TERT promoter mutation findings in micropapillary variant of urothelial carcinoma

Specimen	Sample type	Tumor morphologies sampled	UIDs	% mutant	*TERT* mutation
1	Biopsy	Conventional with micropapillary areas	4467	69.2	g.1295228C > T
2	Biopsy	Micropapillary with focal glandular areas	991	39.4	g.1295228C > T
		Glandular	1632	35.4	g.1295228C > T
3	Cystoprostatectomy, lymph node	Micropapillary	2655	5.5	g.1295228C > T
4	Cystoprostatectomy	Micropapillary	5875	39.0	g.1295228C > T
5	Small bowel resection	Micropapillary	653	53.0	g.1295228C > T
6	Biopsy	Micropapillary with focal glandular areas	4741	29.7	g.1295228C > T
7	Cystoprostatectomy	Conventional	4552	64.0	g.1295228C > T
		Micropapillary	2839	65.4	g.1295228C > T
		Conventional with squamous and micropapillary areas	4646	46.3	g.1295228C > T
8	Pelvic exenteration	Micropapillary	1772	42.4	g.1295228C > T
9	Cystoprostatectomy	Micropapillary	2424	32.1	g.1295228C > T
10	Cystoprostatectomy	Micropapillary	101	49.5	g.1295228C > T
11	Biopsy	Micropapillary	3980	65.0	g.1295250C > T
12	TURBT	Micropapillary	19,051	66.3	g.1295228C > T
13	Cystectomy	Micropapillary	3449	57.6	g.1295228C > T
14	Cystoprostatectomy	Micropapillary	10,266	37.8	g.1295250C > T
15	TURBT	Conventional	12,009	46.3	g.1295228C > T
		Micropapillary with focal glandular areas	8129	32.4	g.1295228C > T
16	Lymph node excision	Micropapillary	6938	39.0	g.1295228C > T
17	TURBT	Conventional with focal glandular features	3384	61.6	g.1295228C > T
		Micropapillary with focal conventional areas	4451	53.2	g.1295228C > T
18	Nephroureterectomy	High grade papillary with micropapillary features	4357	60.5	g.1295243C > T
19	Lymph node excision	Micropapillary	6784	68.5	g.1295228C > T
20	Cystoprostatectomy	Micropapillary	4027	38.6	g.1295228C > T
21	Biopsy	Conventional	3467	42.0	g.1295228C > T
		High grade papillary with micropapillary and glandular features	3008	51.8	g.1295228C > T
22	Biopsy	Micropapillary	20,879	78.3	g.1295228C > T

Specimen	Sample type	Tumor morphologies sampled	UIDs	% mutant	*TERT* mutation
23[a]	TURBT	Conventional with micropapillary areas	8037	71.9	g.1295250C > T
24[a]	Cystoprostatectomy	Micropapillary	5982	56.2	g.1295250C > T
25	Biopsy	Conventional with micropapillary areas	6741	1.1	g.1295228C > T
26	TURBT	Conventional with micropapillary areas	10,338	71.9	g.1295228C > T
27	Cystoprostatectomy	Conventional	3948	89.3	g.1295228C > T
		Micropapillary with focal conventional areas	3371	60.1	g.1295228C > T
28	TURBT	High grade papillary with micropapillary areas	369	24.1	g.1295228C > T
29	Biopsy	Conventional with micropapillary areas	6891	39.4	g.1295228C > T
30	Lymph node dissection	Micropapillary	12,963	10.4	g.1295228C > T
31[b]	TURBT	Micropapillary	6089	35.5	g.1295228C > T
32[b]	TURBT	Micropapillary	9351	24.0	g.1295228C > T
33	TURBT	Conventional	11,119	38.1	g.1295228C > T
		Micropapillary	6986	37.4	g.1295228C > T

UIDs unique sequence reads, *TURBT* transurethral resection of bladder tumor

[a,b]Samples from same patient