Human Pathology (2019) **85**, 1–9

ELSEVIER

Human PATHOLOGY

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Original contribution

Targeted sequencing of plasmacytoid urothelial carcinoma reveals frequent *TERT* promoter mutations☆,☆☆

Doreen N. Palsgrove MD[a], Diana Taheri MD[a,b], Simeon U. Springer PhD[c,d], Morgan Cowan MD[a], Gunes Guner MD[a], Maria A. Mendoza Rodriguez MD[a], Maria Del Carmen Rodriguez Pena MD[a,e], Yuxuan Wang BS[d], Isaac Kinde MD, PhD[d], Bernardo F.P. Ricardo MD[a], Isabela Cunha MD, PhD[f], Kazutoshi Fujita MD, PhD[g], Dilek Ertoy MD[h], Kenneth W. Kinzler PhD[c,d], Trinity J. Bivalacqua MD, PhD[i], Nickolas Papadopoulos PhD[c,d], Bert Vogelstein MD[c,d], George J. Netto MD[a,e,*]

[a]*Department of Pathology, Johns Hopkins University, Baltimore, MD 21287, USA*
[b]*Department of Pathology, Isfahan University of Medical Sciences, Isfahan Kidney Diseases Research Center, Isfahan 81746 73461, Iran*
[c]*Department of Oncology, Johns Hopkins University, Baltimore, MD 21287, USA*
[d]*The Ludwig Center for Cancer Genetics and Therapeutics and Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD 21287, USA*
[e]*Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35249, USA*
[f]*Department of Pathology, Rede D'OR São Luiz, São Paulo 03313-000, Brazil*
[g]*Department of Urology, Osaka University, Osaka 565-0871, Japan*
[h]*Department of Pathology, Koç University, İstanbul 34450, Turkey*
[i]*Department of Urology, Johns Hopkins University, Baltimore, MD 21287, USA*

Received 4 September 2018; revised 25 October 2018; accepted 31 October 2018

Keywords:
Plasmacytoid;
Urothelial carcinoma;
PUC;
Telomerase reverse transcriptase;
TERT;
Mutation

Summary 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. Although the *cadherin 1* (*CDH1*) gene is commonly mutated in the clinically aggressive plasmacytoid variant of urothelial carcinoma (PUC), little is known about their *TERT* promoter mutation status. A retrospective search of our archives for PUC and UC with plasmacytoid and/or signet ring cell features (2007-2014) was performed. Ten specimens from 10 patients had archived material available for DNA analysis and were included in the study. Intratumoral areas of nonplasmacytoid histology were also evaluated when present. Samples were analyzed for *TERT* promoter mutations with Safe-SeqS, a sequencing error-reduction technology, and sequenced using a targeted panel of the 10 most commonly mutated genes in bladder cancer on the

☆ Competing interests: K. W. K., N. P., and B. V. 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 B. V., K. W. K., and N. P. 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.

☆☆ Funding/Support: This study was supported by grants from The Johns Hopkins Greenberg Bladder Cancer Institute, The Virginia and D.K. Ludwig Fund for Cancer Research, The Commonwealth Fund, The Conrad R. Hilton Foundation, The Sol Goldman Sequencing Facility at Johns Hopkins, and the National Institutes of Health (grant 5T32CA193145-02 [D. N. P.]).

* Corresponding author at: Department of Pathology, University of Alabama at Birmingham, WP Building, Suite P230, 619 South 19th St, Birmingham, AL 35249-7331.
E-mail address: gnetto@uabmc.edu (G. J. Netto).

https://doi.org/10.1016/j.humpath.2018.10.033

Illumina MiSeq platform. *TERT* promoter mutations were detected in specimens with pure and focal plasmacytoid features (6/10). Similar to conventional UC, the predominant mutation identified was g.1295228C>T. In heterogeneous tumors with focal variant histology, concordant mutations were found in plasmacytoid and corresponding conventional, glandular, or sarcomatoid areas. Co-occurring mutations in *tumor protein p53* (*TP53*, 2 cases) and *kirsten rat sarcoma* (*KRAS*) *viral proto-oncogene* (1 case) were also detected. *TERT* promoter mutations are frequently present in PUC, which provides further evidence that *TERT* promoter mutations are common events in bladder cancer, regardless of histologic subtype, and supports their inclusion in any liquid biopsy assay for bladder cancer.

1. Introduction

Greater than 90% of bladder carcinomas are urothelial type; however, a small subset of tumors are recognized as distinctive histologic variants (eg, squamous, glandular, microcystic, micropapillary, plasmacytoid, sarcomatoid, etc) [1].

The plasmacytoid variant of urothelial carcinoma (PUC), first described in 1991 by Zuckerberg and colleagues [2] and included in the *World Health Organization Classification of Tumors of the Urinary System* since 2004, is a relatively uncommon but important morphologic subtype. Most cases present at an advanced stage with diffuse and deep involvement of the bladder and perivesical tissue. Outcomes are generally poor with higher rates of recurrence and death than those associated with usual urothelial carcinoma (UC) and a median survival of around 1 year upon development of metastatic disease [3,4].

It is now known that the *cadherin 1* (*CDH1)* gene is frequently altered in PUC [5]. Mutations are most often truncating somatic variants that result in a nonfunctional E-cadherin protein, which prevents tumor suppression and cell adhesion. However, *CDH1* promoter hypermethylation has also been reported in a subset of cases [5]. Beyond *CDH1*, the next most commonly altered gene in PUCs is *tumor protein p53* (*TP53*), followed by *retinoblastoma* (*RB*) *transcriptional corepressor 1* (*RB1*), *AT-rich interaction domain 1A* (*ARID1A*), *cyclin-dependent kinase inhibitor 1A* (*CDKN1A*), and *erb-b2 receptor tyrosine kinase 2* (*ERBB2*) [5].

Somatic activating mutations in the promoter of the *telomerase reverse transcriptase* (*TERT*) gene, originally discovered in most melanomas [6], have been found to be the most common genetic mutations overall in UC of either the bladder or the upper urinary tract [7,8]. In a study by Kinde et al [7], 66% of muscle-invasive and 74% of non–muscle-invasive bladder lesions were shown to harbor these alterations. Recently, we and others have also identified *TERT* promoter mutations in specific lesional subtypes including papillary urothelial neoplasm of unknown malignant potential [9], small cell carcinomas of the urinary bladder [10], micropapillary UC [11], primary squamous cell carcinoma of the bladder [12], primary adenocarcinoma of the bladder [13], nested and "large nested" variants of UC [14], sarcomatoid UC of the upper urinary tract (6/17 cases) [15], and UC of the renal pelvis and the ureter [16]. Little is known, however, about the role of *TERT* promoter mutations in PUCs.

In this study, we sought to address the presence of *TERT* promoter mutations in PUC, as well as mutations in 10 other genes commonly mutated in bladder cancer, and review the clinical relevance of such mutations in this aggressive variant of UC.

2. Materials and methods

2.1. Institutional review board approval

This study was approved by the institutional review board of the Johns Hopkins Hospital and other participating institutions. Clinical information was abstracted from retrospective chart review.

2.2. Patient samples

We searched our electronic Pathology Database System for the key word "plasmacytoid" with "urothelial cancer," "bladder cancer," or combinations of "urothelial," "bladder," and "carcinoma." The search included in-house cases from the years 2005 to 2014. Sixteen specimens of invasive UC with plasmacytoid features were identified. From these, 12 specimens from 12 patients had available material for inclusion in the study. Two cases were excluded because of insufficient tissue for DNA analysis.

Specimen types included bladder biopsies (4 cases), transurethral bladder tumor resections (2 cases), cystoprostatectomies (3 cases), and a transverse colon resection for metastatic tumor (1 case).

All sections were reviewed by a senior genitourinary pathologist (G. J. N.) to confirm the original diagnoses according to the updated 2016 *World Health Organization Classification of Tumours of the Urinary System and Male Genital Organs* [1]. The diagnosis of plasmacytoid variant histology was based on the unifying features of infiltrating, loosely cohesive, homogeneous cells with distinct cell borders resembling plasma cells or lobular carcinoma of the breast. Although, most cases had bland cytologic features, cases with rhabdoid (eccentric nuclei and eosinophilic cytoplasmic inclusions) and/or signet ring-cell–like features (cytoplasmic vacuoles) were also included. Percentage of tumor composed of plasmacytoid features was

not estimated owing to the variability in tumor sampling between specimens. Only specimens with definitive evidence of an invasive plasmacytoid component were included.

Areas with the highest neoplastic cellularity, as determined from hematoxylin and eosin (H&E)–stained sections of the tumors, were chosen for analysis. Multiple tumor foci were isolated and analyzed separately in specimens that contained more than 1 variant morphology and/or additional foci of conventional UC.

Tumor was cored from formalin-fixed, paraffin-embedded 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. DNA was purified using an All Prep Kit (Qiagen, Hilden, Germany; catalog no. 80204).

Two sets of negative control samples were also analyzed. Eight benign transurethral bladder biopsy formalin-fixed, paraffin-embedded samples were used as negative tissue controls. In addition, DNA from the 94 peripheral blood samples of healthy individuals was used as a control to identify potential false-positive mutations.

2.3. Mutation analysis

We used Safe-SeqS, a sequencing error-reduction technology described previously [17] to discriminate genuine *TERT* promoter mutations from artifactual sequencing variants introduced during the sequencing process. Amplification primers were designed to amplify a 126-bp segment (chromosome 5: 1295100-1295260) containing the region of the *TERT* promoter previously shown to harbor mutations in melanomas and other tumors [6,8]. The forward and reverse amplification primers for the *TERT* promoter region contained gene-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) comprising 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′-CACACAGGAAACAGCTATGACCATGGGCCGCGGAAAGGAAG and 5′-CGACGTAAAACGACGGCCAGTNNNNNNNNNNNNNNCGTCCTGCCCCTTCACC, or CACACAGGAAACAGCTATGACCATGGCGGAAAGGAAAGGGAG and 5′-CGACGTAAAACGACGGCCAGTNNNNNNNNNNNNNNCCGTCCCGACCCCTC (UPS sequences underlined).

DNA was amplified in 25 μL polymerase chain reaction (PCR) reactions using 1× Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific, Waltham, MA USA; catalog no. F-548L) containing 0.5 μM forward and reverse primers (described above). After incubation at 98°C for 120 seconds, 10 cycles of PCR were performed in the following manner: 98°C for 10 seconds, 63°C for 120 seconds, and 72°C for 120 seconds. Reactions were purified with AMPure XP beads (Beckman Coulter) and eluted in 100 μL of Buffer EB (Qiagen, Brea, CA USA; catalog no. 19086). For the second stage of amplification, 5 μL of purified PCR products was 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 [17]. 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 seconds, 17 cycles of PCR were performed in the following manner: 98°C for 10 seconds, 63°C for 120 seconds, and 72°C for 120 seconds. The PCR products were purified with AMPure (Beckman Coulter, Brea, CA USA) beads and sequenced on an Illumina (San Diego, CA USA) MiSeq instrument.

Data were analyzed as previously described [17]. 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 5 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 found in control DNA templates). All sequencing assays

Table 1 Demographic and clinicopathological characteristics

Case no.	Age (y)	Sex	Race	Specimen type	Pathologic stage	Prior surgery and/or treatment	Follow-up (mo)	Outcome
Case 2	73	M	C	Omental nodule excision, transverse colon resection	pM1	Biopsy	8	DOD
Case 3	77	M	C	Cystoprostatectomy	pT3bN0	TURBT	24	DOD
Case 4	59	M	A	TURBT	pT2	TURBT	36	DOD
Case 5	63	M	A	Biopsy	pT1	TURBT	97	AWD
Case 6	51	F	A	Biopsy	pM1	None	2	DOD
Case 7	*64*	M	C	Biopsy	pT2aN0	TURBT, BCG	32	LTF
Case 8	66	M	C	Cystoprostatectomy	pT3aN2	Biopsies	5	LTF
Case 9	65	M	O	TURBT	Unk	Neoadjuvant chemoradiation	7	LTF
Case 10	80	M	C	Biopsy	pT2bN1	Biopsies, BCG	63	LTF
Case 12	62	M	C	Cystoprostatectomy	pT3bN0	TURBT, BCG	30	DOD

Abbreviations: A, African ancestry; AWD, alive with disease; BCG, bacillus Calmette-Guerin; C, Caucasian; DOD, died of disease; LTF, lost to follow-up; O, other; TURBT, transurethral resection of bladder tumor; Unk, unknown; BCG, Bacillus Calmette-Guerin; TURBT, transurethral resection of bladder tumor; AWD, alive with disease; DOD, died of disease; LTF, lost to follow-up;.

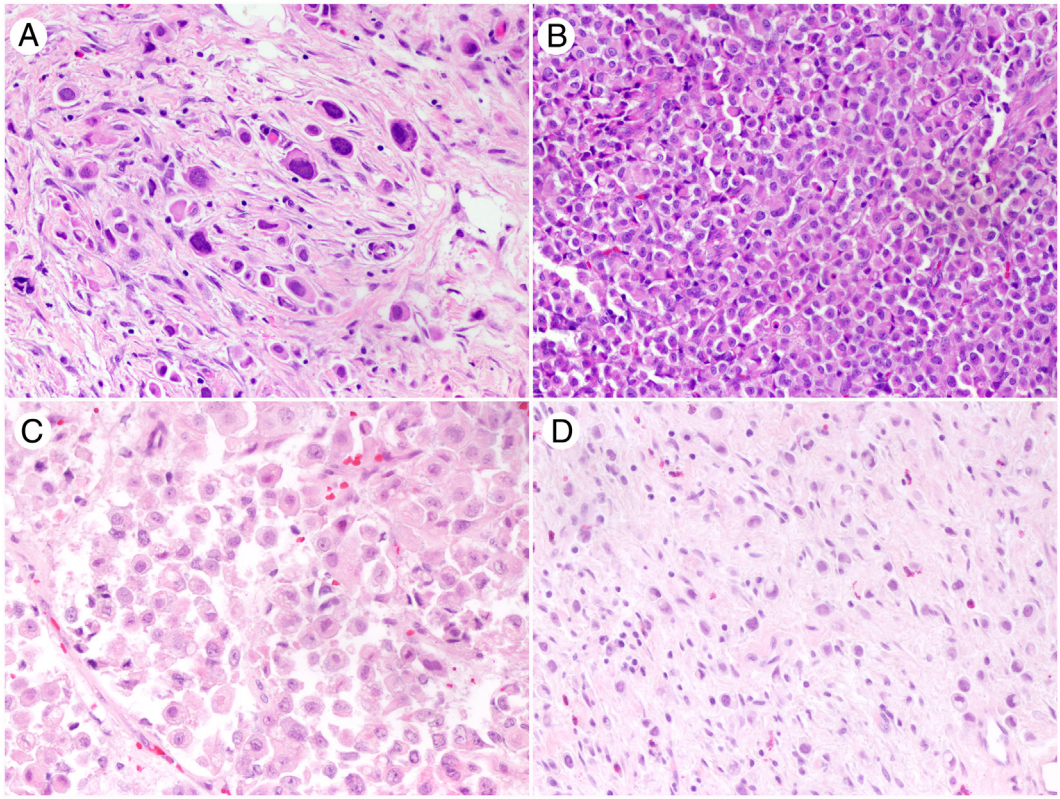

Fig. 1 Examples of PUC with *TERT* promoter mutations (H&E, original magnification ×20): case 2 (A), case 6 (B), case 10 (C), and case 12 (D).

scored as positive were confirmed in at least one additional, independent PCR sequence assay.

Separate multiplex PCR reactions were performed using primer pairs designed to amplify regions of interest from *fibroblast growth factor receptor 3* (*FGFR3*), *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha* (*PIK3CA*), *TP53*, *harvey rat sarcoma* (*HRAS*) *viral proto-oncogene*, *kirsten rat sarcoma* (*KRAS*) *viral proto-oncogene*, *ERBB2*, *cyclin-dependent kinase inhibitor 2A* (*CDKN2A*), *mesenchymal-epithelial transition factor* (*MET*) *proto-oncogene*, *lysine methyltransferase 2A* (*KMT2A*/*MLL*), and *von Hippel-Lindau* (*VHL*) *tumor suppressor gene*. Primer sequences are listed in Supplementary Table. The unusually high guanine-cytosine content of the *TERT* promoter precluded its inclusion in the multiplex PCR design.

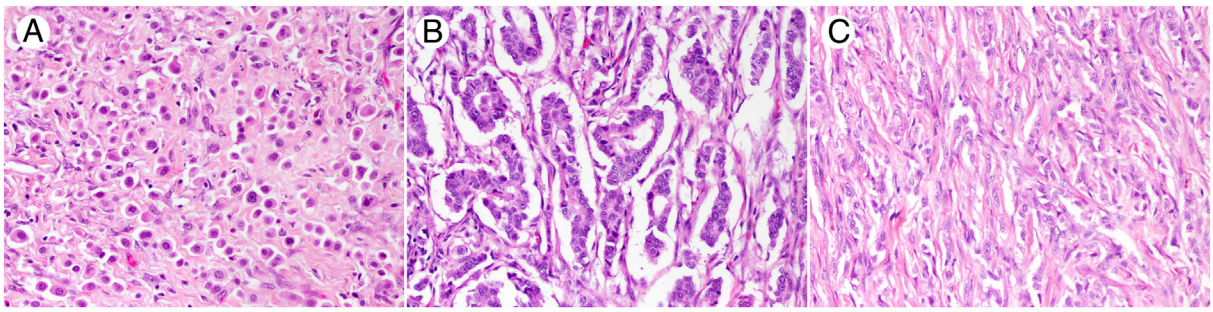

Fig. 2 Case 8: invasive UC composed of areas with plasmacytoid (A), conventional/glandular (B), and sarcomatoid (C) morphology (H&E, original magnification ×20). Each of these areas was isolated and analyzed separately for genetic alterations. The corresponding genetic alterations are shown below each represented morphology.

Table 2 *TERT* promoter mutation findings in PUC

Case no.	Tumor morphologies sampled	UIDs	% Mutant	*TERT* mutation
Case 2	Plasmacytoid	128	75.8	g.1295228C>T
Case 3	Plasmacytoid	1165	0	N/A
Case 4	Plasmacytoid/Conventional	5081	0	N/A
Case 5	Plasmacytoid	1934	0	N/A
Case 6	Plasmacytoid	3383	48.2	g.1295228C>T
Case 7	Plasmacytoid	13 575	0	N/A
Case 8	Plasmacytoid	3949	5.2	g.1295228C>T
	Conventional/glandular	4716	39	g.1295228C>T
	Sarcomatoid	5103	23.2	g.1295228C>T
Case 9	Plasmacytoid with treatment effect	7141	1.2	g.1295228C>T
Case 10	Plasmacytoid/Conventional	8712	65	g.1295228C>T
Case 12	Plasmacytoid	6720	11.9	g.1295228C>T

Abbreviation: TURBT, transurethral resection of bladder tumor.

3. Results

3.1. Patient population

Ten specimens of UC with focal to pure plasmacytoid features from 10 patients (9 men, 1 woman) were analyzed (Table 1). The mean patient age at the time of specimen sampling was 66 years (range, 51-80 years). Five patients (50%) died of their disease, with a median interval from date of diagnosis of 24 months (mean, 20 months; range, 2-36 months). The remaining patients had a median postsurgical follow-up period of 32 months (mean, 40.8 months; range, 5-97 months).

3.2. Tumor morphology

Areas with pure plasmacytoid morphology were isolated in 8 of 10 specimens, whereas 2 specimens demonstrated admixed plasmacytoid and conventional UC. Examples of

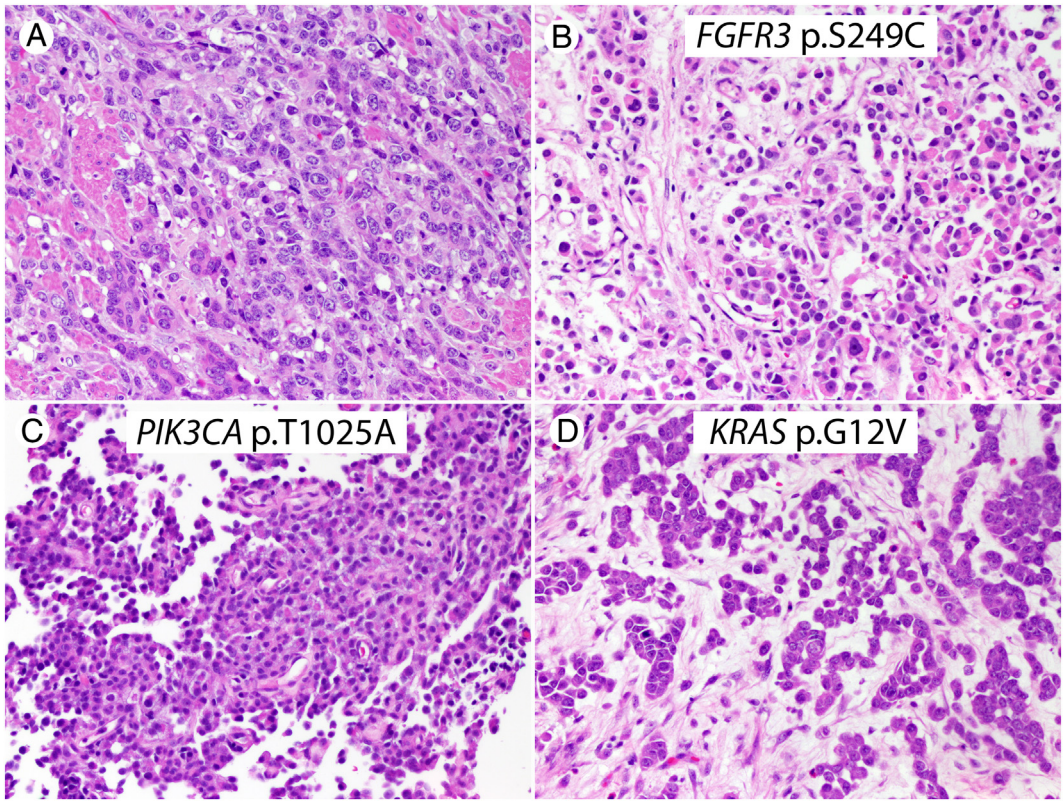

Fig. 3 *TERT* promoter nonmutated cases of PUC: case 3 (A), case 4 (B), case 5 (C), and case 7 (D) (H&E, original magnification ×20). The variants corresponding to each of the represented tumors are shown. Case 3 tested negative for variants in the *TERT* promoter, *FGFR3, PIK3CA, TP53, HRAS, KRAS, ERBB2, CDKN2A, MET, KMT2A/MLL*, and *VHL*.

areas described as "plasmacytoid" are shown in Fig. 1. One specimen (case 8) also demonstrated foci of conventional/glandular and sarcomatoid morphology, which were isolated and analyzed separately (Fig. 2).

Six (60%) specimens showed at least 60% neoplastic cellularity in the areas that were sampled for analysis. The overall mean tumor cellularity was 53% (range, <5% to >80%). One of the samples showed marked pleomorphism in keeping with the patient's history of neoadjuvant chemoradiation.

3.3. *TERT* promoter analysis

TERT promoter mutations were found in 6 of 10 specimens of primary or metastatic UC with plasmacytoid features (Table 2). All mutated cases demonstrated the g.1295228C>T variant. Identical *TERT* promoter mutations were detected in the plasmacytoid, conventional/glandular, and sarcomatoid areas in case 8.

3.4. Targeted analysis of genes commonly mutated in bladder cancer

Additional analysis in selected regions of *FGFR3*, *PIK3CA*, *TP53*, *HRAS*, *KRAS*, *ERBB2*, *CDKN2A*, *MET*, *MLL*, and *VHL* demonstrated alterations in 5 of 10 specimens: *TP53* missense mutations (cases 8 and 12), *KRAS* missense mutations (cases 7 and 8), and *FGFR3* and *PIK3CA* missense mutations (cases 4 and 5, respectively). Three of these cases (cases 4, 5, and 7) were negative for *TERT* promoter mutations (Fig. 3B-D).

Interestingly, 2 additional *TP53* mutations, p.R248W (VAF 1.4%) and p.R342* (1%), were detected in the areas with conventional/glandular morphology but not in the separately isolated areas with plasmacytoid or sarcomatoid variant morphology in case 8 (Table 3).

All blood samples and benign urothelial tissue controls tested negative for *TERT, FGFR3*, *PIK3CA*, *TP53*, *HRAS*, *KRAS*, *ERBB2*, *CDKN2A*, *MET*, *MLL* (*KMT2A*), and *VHL* mutations. One case of PUC (case 3, Fig. 3A) was negative for variants in all gene regions evaluated.

Table 3 Detected alterations in genes other than *TERT* in PUC [a]

Case no.	Gene	Mutation(s) detected	UIDs	% Mutant
Case 4	*FGFR3*	p.S249C	10 284	28.4
Case 5	*PIK3CA*	p.T1025A	3541	37
Case 7	*KRAS*	p.G12 V	3278	21.3
Case 8 [b,c]	*TP53*	p.P278A	2165	24.1
	KRAS	p.G12D	3067	23.4
Case 12 [c]	*TP53*	p.R175H	4194	4.1

Abbreviation: TURBT, transurethral resection of bladder tumor.

[a] Panel included regions of interest from *FGFR3*, *PIK3CA*, *TP53*, *HRAS*, *KRAS*, *ERBB2*, *CDKN2A*, *MET*, *MLL*, *PLEKHS1*, and *VHL*.

[b] The *TP53* and *KRAS* variants in case 8 were detected in all 3 separately isolated areas with variant morphology (plasmacytoid, glandular/conventional, and sarcomatoid); average UIDs and % mutant across all analyzed areas are reported here.

[c] Cases 8 and 12 also demonstrated *TERT* promoter mutations.

4. Discussion

As an aggressive histologic variant, accurate pathologic recognition and early detection of PUC of the urinary bladder is critical to improving patient survival. Unfortunately, the rarity of plasmacytoid carcinoma makes it difficult to define the optimal treatment strategy.

In this study, we attempted to identify genetic aspects of PUC that may assist in developing noninvasive approaches for detection of this rare subtype. Similar to previous reports identifying frequent *TERT* promoter mutations in UCs [18], we found *TERT* promoter mutations to be present in 60% of UC with pure or focal plasmacytoid features. Our additional targeted analysis of genes commonly mutated in bladder cancer (*FGFR3*, *PIK3CA*, *TP53*, *HRAS*, *KRAS*, *ERBB2*, *CDKN2A*, *MET*, *MLL*, and *VHL*) demonstrated alterations in 50% of PUC cases. Three of these were negative for *TERT* promoter mutations. Combining both *TERT* promoter mutations and the additional targeted gene analyses, 9 (90%) of our 10 PUC cases had at least 1 detectable genetic alteration. This finding supports the potential utility of a targeted panel inclusive of all these genes (as the one recently described by our group [19]) in a noninvasive urine based molecular detection assay for all subtypes of bladder cancer, including the aggressive plasmacytoid variant.

The high frequency of *TERT* mutations in UC makes them key constituents of any somatic mutation panel for detecting primary disease and subsequent tumor sequencing for surveillance of residual disease and/or recurrence [20,21]. Although urine is the ideal source of biomarkers for bladder cancer due to its direct contact with the tumor, plasma liquid biopsies may also be used, particularly in the setting of metastatic disease [22,23].

Few studies have attempted to characterize the molecular landscape of plasmacytoid UC [5,24]. Al-Ahmadie et al [5] recently reported that plasmacytoid UC frequently, if not always, have truncating somatic mutations in *cadherin 1* (*CDH1*), similar to signet ring cell gastric carcinoma and lobular carcinoma of the breast [25,26]. Of the 30 patients sequenced, 10 (30%) demonstrated *TERT* promoter mutations (VAF range, 0.5-0.61), and 1 had a *TERT* missense variant of unknown significance (Fig. 4). *TP53* was frequently comutated in 7 of the 10 *TERT* promoter-mutated samples as was *PIK3CA* (4/10). Other comutations included *ERBB2* and *KMT2A*, 1 case each. Among the cases without *TERT* promoter mutations, *FGFR3* (splice site) was mutated in 1 case, *CDKN2A* was altered in 3 cases (1 amplification, 2 missense mutations), and *ERBB2* was altered in 5 cases (1 amplification, 4 missense mutations). A search of the GENIE Public Cohort database (v3.0.0) [27] reveals 7 cases of plasmacytoid/signet ring cell bladder carcinoma. Like the Al-Ahmadie cohort,

Fig. 4 Tumor samples with sequencing and copy number data from the bladder cancer, plasmacytoid variant MSKCC cohort [5] and the GENIE Public Cohort database (v3.0.0) [27]. Data accessed via cBioPortal v1.13.3-SNAPSHOT [28,29].

CDH1 mutations were frequent (6/7) as were *TP53* and *PIK3CA* mutations. *TERT* promoter mutations were not reported in any of the cases. All cases were submitted from 1 of 2 institutions (DFCI, 1 case; MSKCC, 6 cases) that list *TERT* among the genes targeted by their sequencing panels (DFCI-ONCOPANEL-1 [30], MSK-IMPACT341 [31], and MSK-IMPACT410); however, the MSK-IMPACT panels were the only ones that specifically targeted the *TERT* promoter region in the GENIE cohort. The discrepancy in *TERT* promoter mutation detection in the Al-Ahmadie cohort and the MSK-IMPACT GENIE cases is interesting because the same clinical assay (MSK-IMPACT341) was used to sequence most cases in both groups. The relatively higher detection rate of *TERT* promoter mutations in the current study (60%) may reflect the sensitivity of the method used for detection compared with the large, capture-based targeted sequencing method used in the Al-Ahmadie cohort and the MSK-IMPACT GENIE cases.

Lobular carcinoma of the breast and signet ring cell carcinoma of the stomach should always be considered on the differential of PUC. Although *TERT* promoter mutations are much rarer in tumors of the breast (0.9% in one study [32], including 2 HER2-positive cases and 1 triple-negative cancer case) and stomach (11% in one study [33], including 2 cases of diffuse type and 9 of intestinal type), their presence alone is not enough to differentiate these tumor types by site of origin. One study of gastric cancers, however, which looked at the entire *TERT* promoter region, did note that novel mutations were more frequent than hotspot *TERT* promoter mutations [33].

The most common *TERT* promoter mutations (g.1295228C>T and g.1295250C>T) are believed to result in the creation of novel CCGGAA/T general binding motifs for E26 transformation-specific (ETS)/ternary complex factor transcription factors [6]. The somatic mutations at both positions result in a C>T base change and ETS binding sites that differ from preexisting ETS binding sites (GGAA/T) within the promoter region. Although ETS factors are a large family of transcription factors that can recognize these binding sites, a recent study by Bell et al [34] 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. Currently, novel therapeutic strategies to target the GABP transcription factor complex and/or mutant *TERT* promoter are under active investigation.

We also found concordant *TERT* promoter mutations in areas of nonplasmacytoid histology in heterogeneous tumors containing components of conventional urothelial, glandular, or sarcomatoid morphologies. The presence of *TERT* promoter mutations within areas of both focal and pure plasmacytoid histology reinforces the morphologic plasticity of UC. The

concordant findings of *TERT* promoter mutations in areas of typical UC and divergent differentiation point to a putative stem cell originating UC capable of generating neoplastic populations with different phenotypes [35].

This study is limited by its small sample size due to the rarity of PUC variant. However, the methodology of the mutational analysis, particularly for *TERT* promoter mutations, and the selection of cases from a single institution with strict histopathologic criteria are some of its strengths.

5. Conclusions

We report a high incidence of *TERT* promoter mutations in UC with variant plasmacytoid histology as well as concordant intratumoral mutations in areas with conventional and nonplasmacytoid divergent morphology. The findings provide further evidence that *TERT* promoter mutations are common events in bladder cancer regardless of histologic subtype and should be included in any noninvasive liquid biopsy assay for UC.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2018.10.033.

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