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Genitourinary Cancer

TERT Promoter Mutations and TERT mRNA but Not *FGFR3* Mutations Are Urinary Biomarkers in Han Chinese Patients With Urothelial Bladder Cancer

KUN WANG,[a,e,*] TIANTIAN LIU,[c,*] CHENG LIU,[a,*] YAN MENG,[d,*] XIAOTIAN YUAN,[e] LI LIU,[b] NAN GE,[d] JIKAI LIU,[a] CHANG WANG,[e] HONGBO REN,[a] KEQIANG YAN,[a] SANYUAN HU,[b] ZHONGHUA XU,[a] YIDONG FAN,[a] DAWEI XU[d,e]

Departments of [a]Urology and [b]General Surgery, Qilu Hospital, [c]Department of Pathology, School of Medicine, and [d]Department of Urology, Second Hospital, Shandong University, Jinan, People's Republic of China; [e]Department of Medicine, Division of Hematology and Center for Molecular Medicine, Karolinska University Hospital Solna and Karolinska Institutet, Stockholm, Sweden

*Contributed equally.

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Key Words. FGFR3 • Telomerase • TERT • Urinary markers • Urothelial bladder cancer

ABSTRACT

The TERT promoter and *FGFR3* gene mutations are two of the most common genetic events in urothelial bladder cancer (UBC), and these mutation assays in patient urine have been shown to be promising biomarkers for UBC diagnosis and surveillance. These results were obtained mainly from studies of patients with UBC in Western countries, and little is known about such information in Han Chinese patients with UBC. In the present study, we addressed this issue by analyzing tumors from 182 Han Chinese patients with UBC and urine samples from 102 patients for mutations in the TERT promoter and *FGFR3* and TERT mRNA expression in tumors and/or urine. TERT promoter and *FGFR3* mutations were identified in 87 of 182 (47.8%) and 7 of 102 (6.7%) UBC cases, respectively. In 46 urine samples from patients with TERT promoter mutation-carrying tumors, the mutant promoter was detected in 24 (52%) prior to operation and disappeared in most examined urine samples (80%) taken 1 week after operation. TERT mRNA was detected in urine derived from 46 of 49 patients (94%) that was analyzed before operation independently of the presence of TERT promoter mutations. Collectively, *FGFR3* mutations occur at a very low rate in Han Chinese UBC and cannot serve as diagnostic markers for Chinese patients. Han Chinese patients with UBC have relatively low TERT promoter mutation frequency compared with patients in Western countries, and simultaneous detection of both mutant TERT promoter and TERT mRNA improves sensitivity and specificity of urine-based diagnosis. ***The Oncologist*** 2015;20:263–269

Implications for Practice: The TERT promoter and *FGFR3* gene mutations are two of the most common genetic events in urothelial bladder cancer (UBC), and these mutation assays in patient urine have been shown to be promising biomarkers for UBC diagnosis and surveillance, based on the study of patients from Western countries. Surprisingly, we found that the frequency of *FGFR3* mutations was very low in Han Chinese patients with UBC and was unlikely to be a diagnostic marker for them. These patients also had a relatively low rate of TERT promoter mutations, and simultaneous detection of both mutant TERT promoter and TERT mRNA improves sensitivity and specificity of urine-based diagnosis.

INTRODUCTION

Urothelial bladder cancer (UBC), derived from the urothelium, is one of the most common urological malignancies in both Eastern and Western countries, with a global annual incidence rate of 350,000 [1]. Because the pathogenesis of UBC is a multistep process that involves multiple genetic changes including loss of tumor suppressor genes and activation of oncogenes, the molecular dissection of UBC has been one of the most intensively studied areas. The identification of new molecular markers not only allows more complete characterization of UBC but also provides potential tools for UBC diagnosis, surveillance, monitoring of treatment response, and prognosis.

Telomerase is an RNA-dependent DNA polymerase responsible for synthesizing telomeric DNA at the ends of chromosomes, and telomerase-mediated lengthening of telomeres is required for sustained cellular proliferation [2–4]. Consistent

Correspondence: Yidong Fan, M.D., Department of Urology, Shandong University Qilu Hospital, Jinan, 250012, People's Republic of China. Telephone: 86-531-82166701; E-Mail: fanyd@sdu.edu.cn; or Yan Meng, M.D., Department of Urology, Shandong University Second Hospital, Jinan, 250036, People's Republic of China. Telephone: 86-531-88382223; E-Mail: mengyan2000@126.com Received October 4, 2014; accepted for publication December 8, 2014; first published online in ***The Oncologist*** *Express* on February 5, 2015. http://dx.doi.org/10.1634/theoncologist.2014-0391

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with its biological activity, telomerase is observed to be silent in most normal somatic cells, with a limited life span while activated in up to 90% of human malignancies, acquiring an infinite proliferation potential [2, 4]. Although telomerase is a huge ribonucleoprotein complex, its catalytic component—telomerase reverse transcriptase (TERT)—is the determinant for controlling telomerase activity [2–4]. Consequently, telomerase silence and activation in normal and malignant cells results generally from the repression and derepression, respectively, of the *TERT* gene. Numerous studies have evaluated telomerase activity and TERT as cancer diagnostic markers and therapeutic targets, given their cancer-specific expression or activation [3, 5–7]. Widespread telomerase activation and TERT expression, for example, occur in UBC, and detection of telomerase activity and/or TERT mRNA in voided urine has been successfully applied to urine-based diagnostics for and recurrence surveillance of UBC [8–18]; however, non-tumor-derived TERT expression poses a potential drawback. Inflammatory lymphocytes, for example, may contain substantial amounts of TERT transcripts or telomerase activity and may cause a false-positive result when exfoliated into urine [8]. Consequently, more specific telomerase-based diagnostic markers are required.

Hotspot mutations in the *TERT* core promoter region (C228T and C250T) were recently identified in human malignancies [13, 19–30], and up to 84% of UBC tumors harbor TERT promoter mutations [27, 28]. These novel genetic events create ETS/TCF binding motifs in the *TERT* promoter and stimulate TERT transcription, thereby activating telomerase in UBC cells [20, 23]. More important, because these mutations are absent in normal cells, mutation detection in voided urine from patients with UBC has been tested as a biomarker for urine-based diagnostics and recurrence surveillance [21, 23, 28].

Fibroblast growth factor receptor 3 (FGFR3) is a tyrosine kinase receptor that mediates the effects of fibroblast growth factors and stimulates the RAS-mitogen-activated protein kinase and phosphatidylinositide-3 kinase-AKT pathway, triggering a variety of cellular processes [31]. Earlier experimental studies established a causal link between the presence of FGFR3-activating mutations and tumorigenesis. Indeed, the activating mutation of the *FGFR3* gene is one of the most frequently mutated genes in patients with UBC [31]. In particular, 60%–80% of non-muscle-invasive UBC has been observed to harbor FGFR3 mutations [32–40]. The mutations have also been used as urinary biomarkers for UBC diagnostics and recurrence monitoring. Interestingly, the occurrence of TERT promoter and FGFR3 mutations is highly correlated; moreover, the combined detection of both TERT promoter and FGFR3 mutations in the urine of patients with UBC has been shown to increase sensitivity and specificity of UBC diagnosis [28].

Wu et al. [23] reported a TERT promoter mutation (C228T/A and C250T) rate of 54.2% in a cohort of Han Chinese patients with UBC, indicating relatively fewer mutations compared with Western patients; however, little is known about the FGFR3 mutation in Han Chinese patients with UBC. Because this information is important to the development of mutant TERT promoter- and FGFR3-based UBC diagnostics and disease surveillance, we addressed this issue by sequencing the proximal TERT promoter region and FGFR3 (exons 7, 10, and 15) in UBC tumors derived from 182 Han Chinese patients at diagnosis. Our results revealed that 47% of these patients carried C228T/A or C250T TERT promoter mutations, and surprisingly, only 6.7% had *FGFR3* gene mutations. Given such a low frequency of FGFR3 mutations, their detection is unlikely to be a useful biomarker for Chinese UBC diagnostics. We further explored the combined assay of the mutant TERT promoter and TERT mRNA in voided urine from patients with UBC, and our findings demonstrate the feasibility and accuracy of their simultaneous detection for urine-based UBC diagnostics.

Patients and Methods

Patients and Tumor Specimens

The study was conducted with 182 patients with UBC who underwent surgery at Shandong University Qilu Hospital and Second Hospital in Jinan, People's Republic of China. UBC tumors from 181 patients were transitional cell carcinoma, and 2 were adenocarcinoma. Tumors were also obtained at recurrence from 3 of 182 patients, for a total of 185 tumor specimens. Tumor grading and staging were performed according to the criteria of the World Health Organization and the TNM classification of the International Union Against Cancer (seventh edition). Patient clinical characteristics including: sex, age at diagnosis, tumor size and other histopathological characteristics, and metastases and recurrence are summarized in Table 1 and supplemental online Table 1. The specimens were collected after surgical treatment and kept frozen at −70°C or paraffin-embedded until use. All samples were collected with informed consent and approval by the institutional ethics committee.

Voided Urine Samples From UBC Patients

Urine was collected from 102 patients with UBC before surgical treatment (supplemental online Table 2) and from 10 patients without cancer who were recruited as controls. Fifty mL of urine was centrifuged, and the pellet was kept at −70°C until use.

DNA Extraction and Sequencing

Genomic DNA was extracted from frozen and/or paraffin-embedded tumor tissue samples and urine pellets using Qiagen DNA extraction kits (Qiagen, Venlo, The Netherlands, http://www.qiagen.com). The two mutations defined as C228T and C250T in the TERT core promoter correspond to positions 124 and 146 bp upstream of the ATG site (Fig. 1A). The target region was amplified using polymerase chain reaction (PCR) followed by Sanger sequencing, as described previously [13]. PCR was performed with the following primer pairs: 5′-CAC CCG TCC TGC CCC TTC ACC TT-3′ (forward) and 5′-GGC TTC CCA CGT GCG CAG CAG GA-3′ (reverse). The C228T and C250T mutations were verified by sequencing from both directions. The same set of DNA from these specimens was also analyzed for the alterations of the *FGFR3* gene by Sanger sequencing, and the specific PCR primers are as follows [40–43]: exon 7, 5′-AGT GGC GGT GGT GGT GAG GGA G-3′ (forward) and 5′-AGC ACC GCC GTC TGG TTG GC-3′ (reverse); exon 10, 5′-CAA CGC CCA TGT CTT TGC AG-3′ (forward) and 5′-CAA GAT CTC CCG CTT CCC G-3′ (reverse);

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Table 1. Comparison of TERT promoter mutations with clinical characteristics in patients with urothelial bladder cancer

Parameter	TERT promoter mutation		
	Mutated ($n = 87$)	Wild type ($n = 96$)	p value[a]
Age at diagnosis, years			
Mean	63.93	64.94	.543
Median (range)	65 (39–89)	65.5 (21–87)	
Sex, *n*			.0678
Female	9	20	
Male	78	76	
TNM stage, *n*			.861
<pT2	67	75	
≥pT2	20	21	
Pathology stage, *n*			.109
G1	4	13	
G2	37	35	
G3	45	47	
Tumor size, no.			.153
<3 cm	10	19	
≥3 cm	71	70	
Number of tumors			.364
Multiple	37	34	
Single	50	62	
Lymph metastases, *n*			.174
Yes	2	7	
No	85	89	
Recurrence, *n*			.227
Yes	17	12	
No	70	84	
hTERT mRNA expression, *n*	27	16	.438
Mean±SD	0.5126 ± 2.2769	0.1643 ± 0.2392	

[a]Not statistically significant.
Abbreviation: hTERT, human telomerase reverse transcriptase.

and exon 15, 5′-GAG AGG TGG AGA GGC TTC AG-3′ (forward) and 5′- TCA TGC CAG TAG GAC GCC T-3′ (reverse).

RNA Extraction and Quantitative PCR for TERT mRNA Determination

Total cellular RNA in UBC tumors and exfoliated cells from urine was extracted using Trizol (Life Technologies; Thermo Fisher Scientific, Paisley, U.K., http://www.lifetechnologies.com). cDNA was synthesized using random primers (N6) (Amersham; GE Healthcare, Buckinghamshire, U.K., http://www3.gehealthcare.com) and M-MLV reverse transcriptase. Quantitative PCR was carried out in an ABI7700 sequence detector (Applied Biosystems; Thermo Fisher Scientific) using the SYBR Green kit (Applied Biosystems) and the specific primer pair for TERT transcripts, as described previously [13]. β2-microglobulin (β2-M) was PCR-amplified as an internal control. Levels of TERT mRNA were calculated based on the threshold values and normalization of β2-M expression.

Statistical Analyses

Differences in the TERT promoter mutation frequency between tumors with *FGFR3* alteration, sex, clinical stage, and metastasis were determined using Fisher's exact test. Student's *t* test was used to analyze differences in age and tumor size between the TERT promoter mutation-positive and -negative groups. Differences in TERT mRNA levels were examined using the Mann-Whitney rank sum test. All tests were two-tailed and computed using SigmaStat3.1 software (Systat Software, Inc., Richmond, CA, http://www.systat.com). p values of $<.05$ were considered statistically significant.

Results

A Relatively Lower Frequency of TERT Promoter Mutations in Han Chinese Patients With UBC

Sanger sequencing was used to determine TERT promoter mutations in tumor DNA derived from 182 patients with UBC. A total of 87 UBC tumors (87 of 182, 47.8%) were found to carry TERT promoter mutations, 38 in C228T, 15 in C250T, 1 in C228A, and 1 in CC242-243TT (Fig. 1A and B). Four different mutations were mutually exclusive, consistent with published observations [19, 20]. Altogether, C228T, C228A, and C250T comprised 47% of the mutation frequency in 182 Han Chinese UBCs, lower than 65%–84% observed in patients from Western countries (Table 2).

In addition, we analyzed three tumors at recurrence from three patients with C228T-harboring UBC tumors at diagnosis and observed the presence of the same mutation in these consecutive samples.

Because TERT promoter mutations facilitate gene transcription by creating de novo ETS binding motifs [20, 23], we were interested in the relationship between the presence of the mutation and the abundance of TERT transcripts. Total RNA from 46 patients' tumors was available and analyzed for TERT mRNA expression using quantitative PCR. TERT transcripts were detected in 42 of 46 tumors (91%), and 3 of 4 tumors lacking TERT mRNA were TERT promoter mutation negative. The tumors carrying TERT promoter mutations tended to express higher TERT mRNA, but the difference between mutation-positive and -negative tumors was not statistically significant (mutation vs. wild type: 0.555 ± 0.473 vs. 0.148 ± 0.057; $p = .438$) (Table 1).

The Relationship Between TERT Promoter Mutations and Clinical Variables in Patients With UBC

We then determined the potential association of the presence of TERT promoter mutations with clinical variables in this cohort of patients. Male patients tended to have a higher rate of TERT promoter mutations than did female patients, but the difference did not reach statistical significance (51% vs. 23%; $p = .068$) (Table 1). Patient age, tumor size and number, and TNM and pathological stages also were not correlated with the TERT promoter mutation status (supplemental online Table 1; Table 1).

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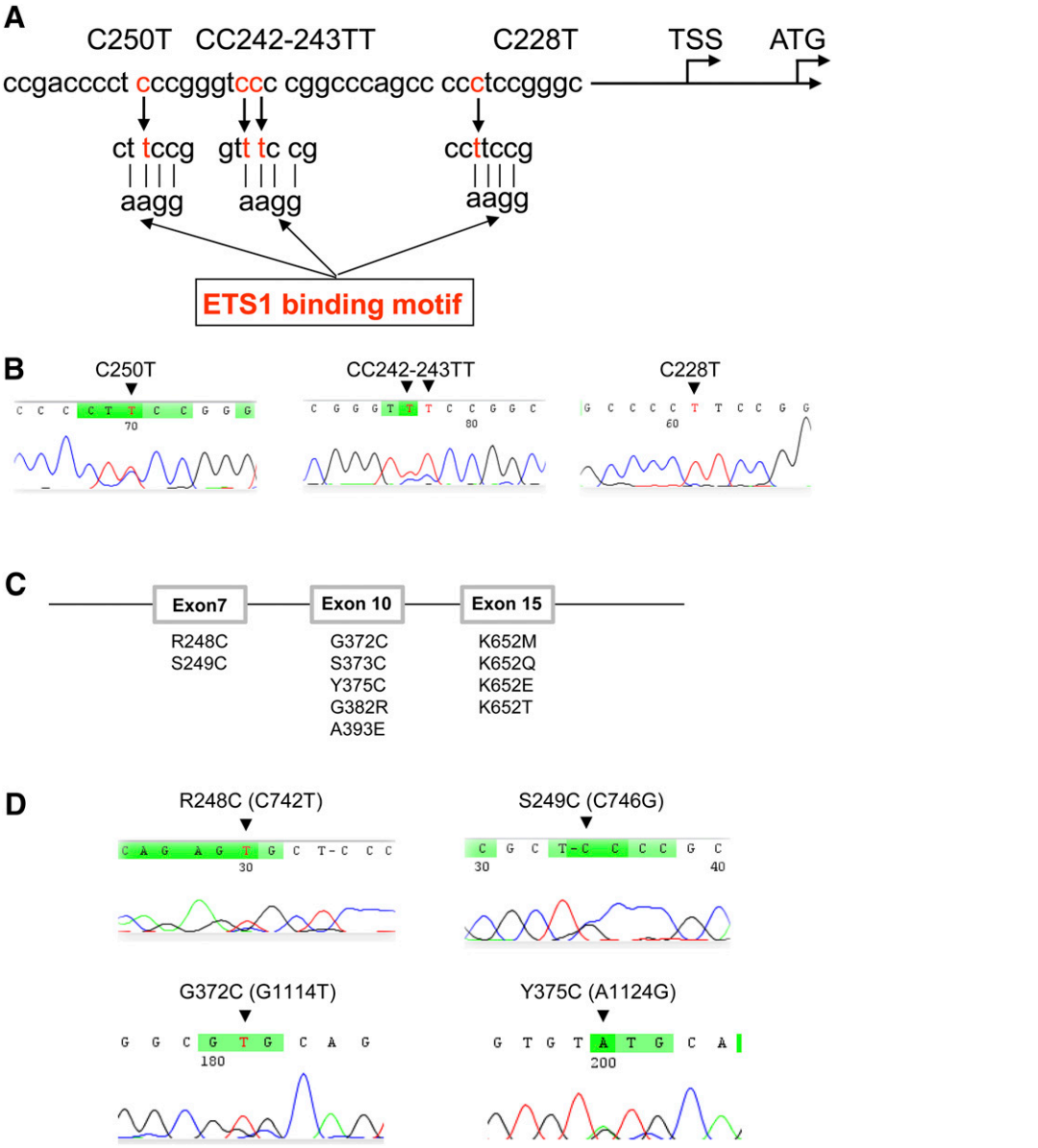

Figure 1. TERT promoter and FGFR3 gene mutations detected in urothelial bladder cancer (UBC) tumors. **(A):** Location of C228T, CC242-243TT, and C250T (in red) in the TERT core promoter. TSS and ATG indicate transcription and translation start sites, respectively. The mutations create de novo binding motifs (GGAA) for the transcription factor ETS1. **(B):** Sequencing chromatographs of the TERT promoter locus in tumor genomic DNA obtained by Sanger sequencing from three patients with UBC. C to T transition at C250, CC242-243TT, and C228 in the *TERT* promoter locus were identified in three tumors, respectively. **(C):** The schematic expression of frequently mutated positions at the *FGFR3* gene. **(D):** Sequencing chromatographs of the FGFR3 locus in tumor genomic DNA obtained by Sanger sequencing from three patients with UBC. Shown are R248C, S249C, G372C, and Y375C mutations identified in four tumors.

Very Low Incidence of *FGFR3* Gene Mutations in Han Chinese Patients With UBC

We next analyzed the *FGFR3* gene by sequencing the hot-spot mutation-localized regions including exon 7, 10, and 15 (Fig. 1C). A total of 104 tumors were screened, and 7 were observed to carry mutations (2 with R248C/C742T, 2 with Y375C/A1124G, 2 with G372C/G1114T, and 1 with both S249C/C476G and G372C/G1114T) (supplemental online Table 1; Fig. 1C, 1D). The FGFR3 mutation was not associated with grade, stage, invasiveness, or TERT promoter mutations (supplemental online Table 1; Table 1). The mutation frequency was much lower than that in non-Chinese patients (Table 2).

Detectable Mutant TERT Promoters in Urine Derived From Patients With UBC

Having demonstrated the presence of TERT promoter mutations in UBC tumors from this cohort of patients, we sought to determine whether the mutant sequence was detectable in patients' voided urine, using Sanger sequencing. Urine samples were available from 102 patients prior to surgical treatment. Of these 102 patients, 46 had mutation-carrying tumors, whereas the remaining 56 lacked the mutation in their tumors (supplemental online Table 2; Table 3). The mutant sequence was detected in 24 of 46 urine samples (52%) derived from patients with tumors harboring TERT promoter mutations (supplemental online Table 2; Table 3). There was no association

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Table 2. Reports of TERT promoter and *FGFR3* mutations in urothelial bladder cancer

Authors	Number of cases, mutated/analyzed, (%)	Countries
TERT promoter		
Current study	89/200 (47)	PRC (Han Chinese)
Wu et al. [23]	117/216 (54.2)	PRC (Han Chinese)
Rachakonda et al. [24]	214/327 (65.4)	Europe
Hurst et al. [21]	201/262 (77.9)	U.K.
Allory et al. [28]	359/468 (74)	Europe
Liu et al. [27]	44/52 (84.6)	U.S.
FGFR3		
Current study	7/104 (6.7)	PRC (Han Chinese)
Wallerand et al. [35]	43/110 (39)	France
van Rhijn et al. [38]	172/286 (60)	Netherlands
Hernández et al. [43]	387/764 (50.1)	Spain
Lamy et al. [33]	43/107 (40.2)	France
Jebar et al. [39]	54/98 (55)	U.K.
Lindgren et al. [34]	46/75 (61)	Sweden
Bodoor et al. [46]	39/121 (32.2)	Jordan
Miyake et al. [41]	24/45 (53.3)	Japan
Dodurga et al. [40]	33/56 (59)	Turkey

TERT promoter mutations include only C228T and C250T mutations.
Abbreviation: PRC, People's Republic of China.

between positive urine detection and tumor size or disease stage and grade. In urine specimens from 56 mutation-negative patients with UBC, the mutation was undetectable in the vast majority (53 of 56, 95%); however, 3 of these patients (5%) were C228T or C250T positive, as determined using Sanger sequencing, and thus inconsistent with tumor tissue results (supplemental online Table 2; Table 3). Taken together, in TERT promoter mutation-positive tumors, the sensitivity for urinary assay using Sanger sequencing is 52%, whereas the specificity was 95% (53 of 56) when all analyzed urine samples were counted together.

To determine whether the mutant TERT promoter present in patients' urine would disappear following a surgical treatment, we analyzed consecutive urine specimens from 15 originally urine-positive patients at 1 week after operation (supplemental online Table 2). TERT promoter mutations remained detectable in 3 of 15 urine samples and became undetectable in the rest, indicating that most patients had fast clearance of the urinary mutant promoter once tumors were removed. One patient with a C228T-negative tumor had detectable urinary C228T prior to operation and exhibited a wild-type TERT promoter in his urine specimen after the surgical operation.

TERT mRNA Present in Urine From Patients With UBC

TERT mRNA was previously observed to be detected in urine from patients with UBC. To probe whether a relationship exists between TERT promoter mutations in tumors and presence of TERT mRNA in patient urine and whether the combined assay of both TERT promoter mutations and TERT mRNA is capable of increasing the sensitivity and specificity for UBC diagnostics, we determined urinary TERT mRNA in 49 patients. As controls, urine specimens from 10 healthy adults were assessed, and TERT mRNA was undetectable in all of them. Urine specimens from 47 of 49 patients (96%) contained TERT mRNA at different levels (supplemental online Table 2). Urinary TERT mRNA tended to be more abundant in patients with TERT promoter mutation-carrying tumors than in those without the mutation (1.31 ± 0.668 vs. 0.498 ± 0.184), but the difference was not statistically significant ($p = .496$). Of note, the combination of both mutation and mRNA results further increased sensitivity to 98%.

Discussion

Recent evidence has shown that UBC is very heterogeneous clinically, pathologically, and genetically. Because of a high risk of recurrence, life-long surveillance is recommended [1]. Cystoscopy and urinary cytology are useful tools for UBC diagnostics and disease monitoring; however, the former is invasive and expensive, whereas the later may be insufficiently sensitive, particularly to low-grade disease, and prone to interobserver and intraobserver variability [44]. Consequently, accurate, noninvasive tests are needed for initial diagnosis, surveillance, monitoring of treatment response, and prognosis. The identification of prevalent TERT promoter and *FGFR3* gene mutations in UBC, together with their absence in normal tissues and cells, makes them potential urinary markers for disease diagnostics [21, 23, 28, 31].

To determine the sensitivity and specificity of TERT promoter mutations in urine-based tests, it is essential to define the prevalence of these genetic events in UBC tumors. Analyses of European and American patients with UBC demonstrated 65%–84% mutation frequencies (Table 2) [21, 23, 27, 28]; however, a relatively lower mutation rate (54%) was found in a cohort of 216 Chinese patients with UBC [23]. In the present study, we showed an even lower occurrence of the mutation (47%) in Han Chinese patients with UBC. These results indicate that TERT promoter mutations occur less frequently in Han Chinese patients with UBC than in those from Western countries. This information must be taken into consideration when urine examination of the mutant TERT promoter is performed on Han Chinese patients with UBC for diagnostic and monitoring purposes.

It is currently unclear what causes different TERT promoter mutation frequencies between Han Chinese and Western patients with UBC. Potential reasons include differences in ethnicity, in oncogenic factors or pathways, and in carcinogen exposure. We also noted that renal pelvic and ureter carcinomas have very different occurrences of TERT promoter mutations, despite originating from the same urothelium or belonging to transitional cell carcinoma as UBC [25]. The mutation frequency in renal pelvic carcinoma is approximately 50% and is very similar between Chinese and U.S. patients [22, 23, 25]. Based on our analyses of small numbers of ureter carcinoma samples, only 11% of these tumors harbored the mutation [25].

Although a relatively lower frequency of TERT promoter mutation occurs in Han Chinese patients with UBC, the mutation assay may still be a urinary marker for those patients with mutation-carrying tumors. Sanger sequencing is specific, but its sensitivity is not high enough. In the present study, we demonstrated 52% sensitivity for the Sanger sequencing assay in tumors with mutations. A more sensitive approach is

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Table 3. Concordance of TERT promoter mutations between tumor tissues and urine samples in urothelial bladder cancer patients, as determined using Sanger sequencing

	Tumor tissues				
Sanger	**Mutant, *n***	**WT, *n***	**Sensitivity, %**	**Specificity, %**	**Accuracy, %**
Urine samples			52	95	75
Mutant, *n*	24	3			
WT, *n*	22	53			
Total	46	56			

Abbreviation: WT, wild type.

required to reliably detect the mutant promoter present in patients' voided urine. In contrast, despite 95% specificity with Sanger sequencing, it remains unclear where the mutant promoter sequence detected in 3 urine samples (5%) came from, given the lack of TERT promoter mutations in these patients' tumors, or whether they were simply false positive due to contamination. Dissociation between tumor and urine tests was also reported by Allory et al. [28]. Using Sanger sequencing and SNaPshot to examine TERT promoter mutations in bladder cancer, they found that the mutant TERT promoter was present in urine but not in tumors from 4 of their 39 bladder cancer patients [28]. During a 3.5-year follow-up period, these 4 patients did not have recurrent disease.

Because TERT and telomerase activity is widely present in UBC, its detection in patient urine has been tested as molecular markers for UBC diagnostic and follow-up [8]. The present results demonstrated high sensitivity for urinary detection of TERT transcripts. Despite this, non-tumor-derived TERT expression poses potential drawbacks. Inflammatory lymphocytes may contain substantial amounts of TERT transcripts or telomerase activity and may cause a false-positive result when exfoliated into urine [8]. This scenario may occur more frequently in women [45]. Because TERT promoter mutations are exclusively absent in normal cells and tissues, the combined detection of both TERT transcripts and mutant promoter may solve this problem in TERT promoter mutation-carrying UBCs by increasing detection specificity. Our present results support simultaneous detection of both TERT promoter mutations and TERT mRNA as urinary markers for UBC.

Given the observed prevalence of the *FGFR3* mutation in UBC [32–40], the detection of the mutant FGFR3 has been widely investigated for UBC diagnostic and prognostic purposes. It is surprising to observe an extremely lower frequency of *FGFR3* mutations in our cohort of patients than in Western patients. The analyzed patients were randomly recruited with different stages and grades, and hence the difference is unlikely to be the result of bias in patient selection. Of note, patients with UBC from other Asian countries such as Japan and Jordan exhibit higher frequency of the *FGFR3* mutation [41, 46]. It is unclear what causes such a dramatic difference in the *FGFR3* mutation between patients from China and other countries. More investigation is needed.

Conclusion

We identified frequencies of 47% for TERT promoter mutations and 6.7% for *FGFR3* mutations in Han Chinese patients with UBC, lower than rates reported for patients with UBC from Western countries. This finding should be considered when these mutations are used as biomarkers for urine-based UBC diagnosis and recurrence surveillance. *FGFR3* mutation detection is not suitable for diagnosis and outcome prediction in Han Chinese patients with UBC, whereas urine-based TERT promoter mutation assays are specific to patients with the mutant promoter-carrying UBC, and the combined TERT mRNA and mutant promoter detection may increase sensitivity and specificity for UBC.

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Author Contributions

Conception/Design: Kun Wang, Tiantian Liu, Cheng Liu, Yan Meng, Yidong Fan, Dawei Xu

Provision of study material or patients: Kun Wang, Cheng Liu, Yan Meng, Xiaotian Yuan, Li Liu, Nan Ge, Jikai Liu, Hongbo Ren, Keqiang Yan, Zhonghua Xu

Collection and/or assembly of data: Kun Wang, Cheng Liu, Yan Meng, Xiaotian Yuan, Li Liu, Nan Ge, Jikai Liu, Chang Wang, Hongbo Ren, Keqiang Yan, Sanyuan Hu, Zhonghua Xu, Yidong Fan

Data analysis and interpretation: Kun Wang, Tiantian Liu, Cheng Liu, Yan Meng, Xiaotian Yuan, Li Liu, Nan Ge, Jikai Liu, Chang Wang, Sanyuan Hu, Zhonghua Xu, Yidong Fan, Dawei Xu

Manuscript writing: Kun Wang, Tiantian Liu, Cheng Liu, Yan Yidong Fan, Dawei Xu

Final approval of manuscript: Kun Wang, Tiantian Liu, Cheng Liu, Yan Meng, Xiaotian Yuan, Li Liu, Nan Ge, Jikai Liu, Chang Wang, Hongbo Ren, Keqiang Yan, Sanyuan Hu, Zhonghua Xu, Yidong Fan, Dawei Xu

Disclosures

The authors indicated no financial relationships.

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