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TERT Promoter Mutations Occur Early in Urothelial Neoplasia and are Biomarkers of Early Disease and Disease Recurrence in Urine

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Abstract

Activating mutations occur in the promoter of the telomerase reverse transcriptase (TERT) gene in 66% of muscle-invasive urothelial carcinomas. To explore their role in bladder cancer development, and to assess their utility as urine markers for early detection, we sequenced the TERT promoter in 76 well-characterized papillary and flat noninvasive urothelial carcinomas, including 28 pTa low-grade (pTa LG) transitional cell carcinomas (TCC), 31 pTa high-grade (pTa HG) TCCs, and 17 pTis carcinoma in situ (CIS) lesions. We also evaluated the sequence of the TERT promoter in a separate series of 14 early bladder neoplasms and matched follow-up urine samples to determine if urine TERT status was an indicator of disease recurrence. A high rate of TERT promoter mutation was observed in both papillary and flat lesions, as well as in low- and high-grade noninvasive urothelial neoplasms (mean: 74%). Additionally, among patients whose tumors harbored TERT promoter mutations, the same mutations were present in follow-up urines in seven of eight patients that recurred but in none of 6 patients that did not recur ($P < 0.001$). TERT promoter mutations occur in both papillary and flat lesions, are the most frequent genetic alterations identified to date in noninvasive precursor lesions of the bladder, are detectable in urine, and appear to be strongly associated with bladder cancer recurrence. These provocative results suggest that TERT promoter mutations may offer a useful urinary biomarker, for both early detection and monitoring of bladder neoplasia.

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Disclosures

K.W.K., L.D., N.P., and B.V. are founders of Personal Genome Diagnostics, Inc., a company focused on the identification of genetic alterations in human cancer for diagnostic and therapeutic purposes. They are also members of the Scientific Advisory Board of Inostics, a company that is developing technologies for the molecular diagnosis of cancer. These companies and others have licensed patent applications from Johns Hopkins relevant to the current study.

The terms of these arrangements are being managed by the university in accordance with its conflict of interest policies.

Keywords

TERT promoter; mutation; urine; diagnostics; sequencing

Introduction

Urothelial carcinoma of the bladder is the most common malignancy of the urinary tract with 73,000 new cases and 15,000 deaths expected in 2013 in the US alone (1). These invasive carcinomas arise from histologically well-defined papillary and flat precursor lesions, providing a potential opportunity for early detection and treatment (2). Although urine cytology enjoys a reasonable sensitivity and specificity for detecting high-grade neoplasms, its performance in detecting low-grade tumors is poor, with a sensitivity and specificity of 4% and 30%, respectively (3).

A number of urine-based markers have been developed to improve the accuracy of noninvasive screening and surveillance in bladder cancer. Among Food and Drug Administration (FDA) approved tests, the Immunocy test (Scimedx Corp, Danville, NJ), nuclear matrix protein 22 (NMP22) immunoassay test (Matritech, Cambridge, MA) and multitarget fluorescence in situ hybridization (FISH) (UroVysion; Abbott Park, IL) (4) have demonstrated an overall sensitivity of 70% and a specificity range of up to 89%. Performance inconsistencies, as a result of variability in pre-analytical and analytical specimen factors, have impeded their wide-spread clinical use.

Activating mutations in the promoter of the *telomerase reverse transcriptase* (*TERT*) gene lead to increased telomerase expression and, in doing so, allow some neoplasms to overcome the end-replication problem and avoid senescence. *TERT* promoter mutations were initially described in melanoma (5, 6) and have subsequently been described in a discrete spectrum of cancer types, including 66% of muscle-invasive urothelial carcinomas of the bladder (5, 7). *TERT* is therefore the most frequently mutated gene in advanced forms of this disease, and the localization of these mutations to a small gene region in the *TERT* promoter provides an extraordinary opportunity for biomarker development (7).

For *TERT* promoter mutations to be a useful marker of early, curable disease, these mutations should be present in pre-invasive bladder tumors and shed into the urine. To this end, we have in this study evaluated the sequence of the *TERT* promoter in a large number of curable precursor neoplasms of the urinary bladder. We also determined the sequence of the *TERT* promoter in a separate group of superficial bladder cancers and corresponding follow-up urine samples to establish the feasibility of detecting *TERT* mutations in urine and their potential utility in predicting recurrence.

Materials and Methods

Patient Samples

This study was approved by the Institutional Review Board of Johns Hopkins University, School of Medicine. Two different sets of samples were analyzed in our study. The first sample set included 76 noninvasive papillary urothelial carcinomas and flat carcinoma *in situ* (CIS) lesions obtained by transurethral bladder resection (TURB) between 2000 and 2012. All specimens were rom the Surgical Pathology archives and were selected only on the basis of specimen availability. Pertinent patient demographics and clinical information were obtained from electronic medical records. All sections were reviewed by three urological pathologists (EM, SFF and GJN) to confirm the original diagnoses. To enrich for neoplastic cells within the tissues, representative formalin-fixed paraffin-embedded (FFPE)

blocks were cored with a sterile 16 gauge needle and tumor areas showing at least 50% neoplastic cellularity were selected microscopically. For eight of the cases, benign adjacent urothelium was macrodissected from FFPE blocks. The cores were placed in a 1.5 mL sterile tube for subsequent DNA purification using an AllPrep DNA/RNA Mini Kit (Qiagen, cat. no. 80204). DNA was purified from peripheral blood buffy coats of 15 patients using the same Qiagen kit.

For the second sample set, we prospectively collected urine samples from 15 separate patients undergoing follow-up cystoscopy for previously diagnosed non-muscle-invasive urothelial carcinoma. We purposely biased this cohort to include patients that recurred within the follow-up period. Immediately prior to follow-up cystoscopy, 25 mL of raw urine was collected and subsequently pelleted by centrifugation at 3,000 *g* for 10 minutes. The pellets were stored at −80° C in 1.5 mL tubes for subsequent DNA extraction. For 14 of these patients, matched FFPE from the original diagnostic TURB was retrieved. These included 13 high-grade urothelial carcinomas (pTa HG and pT1 HG in six and seven cases, respectively), and one low-grade papillary urothelial carcinoma (pTa LG). Twenty 8 μm-thick sections were cut from one representative tissue block in each case and areas containing at least 70% neoplastic cells were microdissected and used for DNA purification using a QIAamp DNA FFPE Tissue Kit (Qiagen, cat no. 56404).

Mutation analysis

Due to their tremendous throughput, massively parallel sequencing instruments are highly cost-effective for DNA mutation analysis. However, sample preparation and sequencing steps introduce artifactual mutations into analyses at a low, but significant frequency. To better discriminate genuine *TERT* promoter mutations from artifactual sequencing variants introduced during the sequencing process, we used Safe-SeqS, a sequencing error-reduction technology described previously (8, 9). As depicted in Fig. 1, Safe-SeqS amplification primers were designed to amplify a 126-bp segment containing the region of the *TERT* promoter previously shown to harbor mutations in melanomas and other tumors (5–7). 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’-CACACAGGAAACAGCTATGACCATGGGCCGCGGAAAGGAAG and 5’-CGACGTAAAACGACGGCCAGTNNNNNNNNNNNNNNCGTCCTGCCCCTTCACC, or CACACAGGAAACAGCTATGACCATGGCGGAAAGGAAAGGGAG and 5’-CGACGTAAAACGACGGCCAGTNNNNNNNNNNNNNNCCGTCCCGACCCCTC (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 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 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 (8, 9). 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 and sequenced on a MiSeq instrument.

Data were analyzed as previously described (8, 9). 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 10× higher than found in control DNA templates from FFPE tissues). Urine samples were considered positive when the frequency of mutation exceeded 0.1% of alleles (a frequency at least 10× higher than found in control DNA templates from urine samples of patients without *TERT* mutations in their primary tumors). All sequencing assays scored as positive were confirmed in at least one additional, independent PCR and sequence assay.

Statistical analysis

The data were analyzed using Stata/SE 12 (StataCorp Inc., College Station, TX). Pearson's chi-squared test was used for analysis of association of categorical variables. A two-tailed probability <0.05 was required for statistical significance.

Results

TERT promoter mutation in papillary and "flat" noninvasive urothelial carcinoma

We used a massively parallel sequencing technology to determine the presence and representation of mutant *TERT* promoter alleles in urothelial cancers. A graphical depiction of the method is shown in Fig. 1 and detailed procedures are provided in the Materials and Methods. In addition to revealing whether mutations are present with a population of DNA templates, this technique provides an accurate determination of the fraction of mutant alleles in the sample. Clinicopathologic characteristics of the 76 noninvasive urothelial carcinomas analyzed in the first phase of this study are summarized in Table 1. They included 59 papillary tumors – 28 low-grade (pTa LG) and 31 high-grade (pTa HG) – plus 17 "flat" urothelial carcinoma *in situ* (CIS). These patients were typical of those with this form of cancer; their average age was 66 years and most (82%) were males (Table 1).

TERT promoter mutations were identified in 56/76 (74%) of these urothelial carcinomas (Table 2). In contrast, none of the eight samples of adjacent normal urothelium harbored *TERT* promoter mutations. Additionally, we did not detect *TERT* promoter mutations in 15 samples of peripheral blood from the same patients. Twelve of the blood samples and five of the normal urothelial samples were from patients whose tumors harbored *TERT* promoter mutations. These data demonstrate that the *TERT* promoter mutations in these patients were unequivocally somatic and limited to the neoplastic urothelium in the bladder. The predominant alterations were g.1295228C>T (minus strand of chromosome 5, hg19 assembly) and g.1295250C>T mutations, which accounted for 75% and 20% of the total alterations, respectively. In addition, we identified one g.1295228C>A mutation and two g. 1295242C>T mutations not previously reported (Table S1). The mutations were found in all types and grades of these early cancers: in 76% of papillary lesions and 65% of flat lesions; in 86% of low-grade and in 68% of high-grade lesions (Table 2). None of these differences among subgroups were statistically significant.

The results described above show that *TERT* promoter mutations occur early in bladder cancers and did not correlate with grade or type. Such early mutations would not be likely associated with recurrence or progression, but to evaluate this possibility, our series of

samples included cases both with and without recurrence during follow up. In Tables 3 and 4, the relationship between *TERT* promoter mutation status and tumor recurrence or progression, respectively, are displayed: *TERT* promoter mutation status was not associated with likelihood of recurrence or progression in any subgroup.

TERT promoter mutation in urine samples

We next evaluated whether *TERT* promoter mutations could be identified in cells in the urine. As noted in the Introduction, urine samples are routinely taken at follow-up visits following TURB procedures to help determine whether residual tumor cells are present (via cytology or other methods). We first assessed the tumors obtained from 14 patients undergoing TURB for relatively early (non-muscle invasive) disease. Of these, 11 (79%) harbored *TERT* promoter mutations (Table 5), as expected from the evaluation of the first cohort (Table 2). All of the mutations in the second cohort were at either g.1295228C>T or g.1295250C>T (Table 5).

The 14 patients were monitored for recurrence at subsequent visits. Mutations were assessed in the cell pellets from the urines obtained at the first follow-up visit after TURB in these 14 patients, as described in the Materials and Methods. There was a striking correlation between the presence of a *TERT* promoter mutation in the urine, the presence of the mutation in the original tumor, and recurrence. In the three of 14 patients without a *TERT* promoter mutation in their tumor, no mutation was evident in their urine sample, as expected (Table 5). Of the 11 patients in whom a *TERT* mutation was present in the tumor, seven patients were observed to have a mutation in the DNA isolated from their urine cell pellets; in each case, the mutation was identical to that observed in the primary tumor removed via TURB (Table 5). The bladder cancers in each of these seven patients recurred, either at the first follow-up or thereafter. The proportion of mutant alleles in the cells pelleted from the urine of these patients was often substantial, ranging from 0.17% to 23% with a median of 4.4% (Table 5). We also identified a *TERT* promoter mutation in a urine sample from which no prior tumor was available; this tumor also recurred (Table 5). In contrast, no *TERT* mutations were evident in the urine samples of four patients whose original tumors contained a *TERT* promoter mutation: the tumors of three of these patients never recurred while the fourth developed a recurrence 3.5 months after the urine sample was collected (Table 5). As shown in Table 6, the presence of detectable *TERT* promoter mutations in the urine was strongly associated with recurrence of urothelial carcinoma (P <0.001; Pearson's correlation coefficient =0.87).

Discussion

TERT promoter mutations are detectable in urine, and their presence in urine is strongly associated with bladder cancer recurrence. Muscle-invasive urothelial carcinoma is responsible for the vast majority of bladder cancer related deaths and many of these deaths could be prevented if precursor lesions were detected and surgically excised prior to their invasion into the muscle (10–13). New strategies for the early detection of such lesions are therefore urgently needed (14). Our results show that *TERT* promoter mutations are the most common genetic alteration in noninvasive bladder cancer identified to date, occurring in the majority (74%) of such precursor lesions. They occur in cancers developing through both the papillary and flat routes to tumor progression (15), and occur in low-grade as well as high-grade tumors. We also show that these mutations can be detected in the urine of patients with bladder cancer. Altogether, these results suggest that *TERT* promoter mutations may provide a useful biomarker for the early detection of bladder cancers in the future, and that prospective studies of patients at high risk for this disease are warranted.

Given the high prevalence of *TERT* promoter mutations in early bladder neoplasia, their presence or absence in tumors is of limited prognostic value. However, superficial bladder cancers are currently the most costly solid tumor (per patient) in the US (16, 17). Noninvasive methods to monitor these patients could reduce the cost of caring for these patients as well as the discomfort associated with invasive procedures. Our results are highly encouraging with respect to this potential application. Among patients with *TERT* mutations in their primary tumors, there was a highly significant correlation between the presence of mutations in subsequent urine collections and recurrence (Table 6).

Our results therefore suggest two potential avenues for application of *TERT* promoter mutations in the clinic: early detection in high-risk patients and monitoring of patients with bladder cancer, both through the analysis of urine specimens. It is important to note that both these applications will require further study prior to implementation. For example, we have not yet shown that bladder cancer patients have detectable mutations in urine prior to tumor diagnosis; all of our urine samples were taken at follow-up visits after surgery. Additionally, our study involved only a small number of patients, and we have yet to demonstrate that the analysis of urine for *TERT* mutations improves upon conventional cytology or clinical criteria, nor whether it could partially replace cystoscopy in certain circumstances. Still, our study provides a strong proof-of-principle: *TERT* promoter mutations occur early, are specific for neoplasia, and can be identified in the urine with currently available technologies. Future large-scale studies will be required to determine the clinical utility of this approach for screening or monitoring purposes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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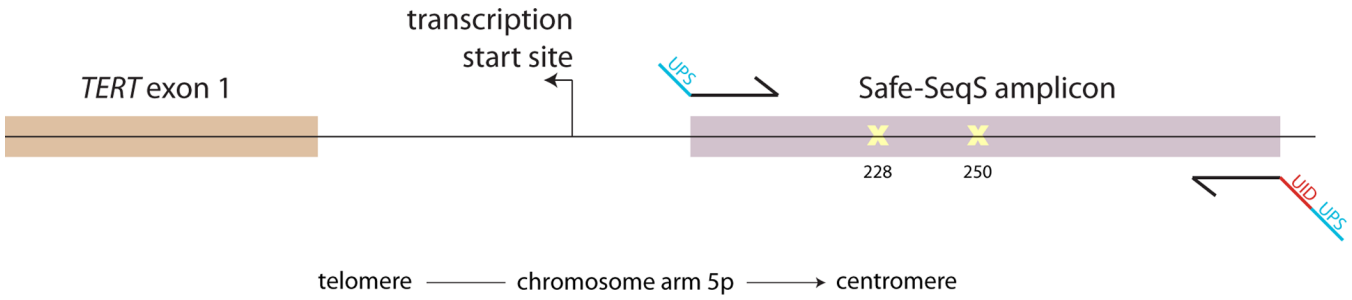

Figure 1.
Schematic of the TERT locus and positioning of the Safe-SeqS amplification primers. The yellow marks indicate the positions (offset by −1,295,000 base pairs) of the most common TERT promoter mutations previously reported and identified in this study. UID, unique identifier; UPS, universal primer binding site.

Table 1

Clinicopathologic characteristics of patients analyzed in this study

	pTa LG (*N* =28)[a]	pTa HG (*N* =31)[a]	CIS (*N* =17)	Total
Age, mean (range)	65.5 (46–84)	67.6 (18–86)	65.6 (54–80)	66.4 (18–86)
Male, percent	73%	84%	94%	82%
Tumor recurred (%)	18/28 (64%)	17/29 (59%)	11/17 (65%)	46/68 (68%)
Tumor progressed (%)	6/28 (21%)	5/29 (17%)	4/17 (24%)	15/68 (22%)
Follow-up months, median (range)	56.5 (2–103)	40 (1–136)	18 (2–43)	38 (1–136)

[a]Recurrence or progression status was not available in two cases. CIS: Carcinoma *in situ*; HG: high-grade noninvasive urothelial carcinoma; LG: low-grade noninvasive urothelial carcinoma; Tumor recurred: tumors recurred within indicated follow-up; Tumor progressed: the recurrent tumor had progressed with respect to stage or grade

Table 2

TERT promoter mutations

***TERT* promoter mutation**	**pTa LG (*N* =28)**	**pTa HG (*N* =31)**	**CIS (*N* =17)**	***P***
Present (%)	24/28 (86%)	21/31 (68%)	11/17 (65%)	0.18

CIS: Carcinoma *in situ*; HG: high-grade noninvasive urothelial carcinoma; LG: low-grade noninvasive urothelial carcinoma.

Table 3

Correlation between *TERT* promoter mutation status and tumor recurrence.

	Recurrence on follow-up	Number of patients	*TERT* mutation present (%)	*TERT* mutation absent (%)	*P*
pTa LG (*N* =28)[a]	Yes	17	16/17 (94%)	1/17 (6.0%)	0.21
	No	9	7/9 (78%)	2/9 (22%)	
pTa HG (*N* =31)[a]	Yes	17	13/17 (77%)	4/17 (24%)	0.14
	No	12	6/12 (50%)	6/12 (50%)	
CIS (*N* =17)	Yes	11	7/11 (64%)	4/11 (36%)	0.90
	No	6	4/6 (67%)	2/6 (33%)	

[a]Recurrence status was not available in two cases. CIS: Carcinoma *in situ*; HG: high-grade noninvasive urothelial carcinoma; LG: low-grade noninvasive urothelial carcinoma.

Table 4

Correlation of *TERT* promoter mutation status and tumor progression

	Progression on follow-up	Number of patients	*TERT* mutation present (%)	*TERT* mutation absent (%)	*P*
pTa LG (*N* =28)	Yes	6	6/6 (100%)	0/6 (0%)	0.31
	No	22	17/22 (77%)	3/22 (14%)	
pTa HG (N =31)[a]	Yes	5	4/5 (80%)	1/5 (20%)	0.45
	No	24	15/24 (63%)	9/24 (38%)	
CIS(*N* =17)	Yes	4	3/4 (75%)	1/4 (25%)	0.60
	No	13	8/13 (62%)	5/13 (39%)	

[a]Progression status was not available in two cases. CIS: Carcinoma *in situ*; HG: high-grade noninvasive urothelial carcinoma; LG: low-grade noninvasive urothelial carcinoma; NA: not available.

Table 5

Correlation of *TERT* mutation status in original diagnostic transurethral resection biopsy (TURB) tissue and *TERT* mutation status in urine collected at follow-up.

Patient	Original diagnostic TURB mutation (%)	Follow-up urine mutation (%)	Tumor grade	Recurrence at time of urine collection	Recurrence after urine collection
1	g.1295228C>T (11%)	g.1295228C>T (6.3%)	pTa HG	Yes	NA
2	g.1295250C>T (4.1%)	g.1295250C>T (23%)	pTa HG	Yes	No
3	g.1295228C>T (5.9%)	g.1295228C>T (0.17%)	pT1 HG	Yes	NA
4	Absent	Absent	pT1 HG	No	No
5	Absent	Absent	pT1 HG	No	No
6	g.1295228C>T (6.7%)	g.1295228C>T (0.64%)	pT1 HG	Yes	NA
7	g.1295228C>T (8.7%)	g.1295228C>T (4.6%)	pTa LG	Yes	Yes
8	g.1295228C>T (7.8%)	Absent	pTa HG	No	Yes
9	g.1295228C>T (7.0%)	Absent	pT1 HG	No	No
10	g.1295228C>T (5.1%)	Absent	pTa HG	No	NA
11	g.1295228C>T (4.8%)	Absent	pT1 HG	No	No
12	g.1295228C>T (5.5%)	g.1295228C>T (5.1%)	pT1 HG	Yes	Yes
13	Unknown	g.1295228C>T (6.6%)	Unknown	Yes	Yes
14	Absent	Absent	pTa HG	No	No
15	g.1295250C>T (23%)	g.1295250C>T (0.69%)	pT1 HG	Yes	Yes

Genomic coordinates refer to the minus (−) strand of chromosome 5 (hg19 assembly); HG: high-grade noninvasive urothelial carcinoma; LG: low-grade noninvasive urothelial carcinoma; NA: not applicable.

Table 6

Correlation of *TERT* promoter mutation status in follow-up urine samples with recurrence

***TERT* mutation in follow-up urine**	**Number of patients**	**Recurred**	**Did not recur**	***P***
Present	8	8/8 (100%)	0/8 (0%)	<0.001
Absent	7	1/7 (11%)	6/7 (89%)	(r =0.87)[a]

[a]Pearson coefficient of correlation