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## Detection of *TERT* promoter mutations in primary adenocarcinoma of the urinary bladder<sup>☆,☆☆</sup>

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### Summary

*TERT* promoter mutations (*TERT-mut*) have been detected in 60% to 80% of urothelial carcinomas. A molecular urine-based screening assay for the detection of *TERT-mut* is currently being pursued by our group and others. A small but significant number of bladder carcinomas are adenocarcinoma. The current study assesses the incidence of *TERT-mut* in primary adenocarcinomas of urinary bladder. A retrospective search of our institutional pathology records identified 23 cystectomy specimens with a diagnosis of adenocarcinoma (2000–2014). All slides were reviewed by a senior urologic pathologist to confirm tumor type and select a representative formalin-fixed, paraffin-embedded block for mutational analysis. Adequate material for DNA testing was available in 14 cases (7 enteric type and 7 not otherwise specified). *TERT-mut* sequencing analysis was performed using previously described SafeSeq technique. Overall, 28.5% of primary adenocarcinoma harbored *TERT-mut*. Interestingly, 57% of nonenteric adenocarcinomas were mutation positive, whereas none of the enteric-type tumors harbored mutations. Similar to urothelial carcinoma, we found a relatively higher rate of *TERT-mut* among

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nonenteric-type adenocarcinomas further supporting the potential utility of *TERT-mut* urine-based screening assay for bladder cancer.

## Keywords

Adenocarcinoma; Telomerase reverse transcriptase; *TERT*; Mutation; Bladder carcinoma; Telomerase

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## 1. Introduction

Telomeres must be maintained to assure cell survival and continued replication [1]. One mechanism by which proliferating cells maintain telomeres is through the action of a complex that includes the product of the *telomerase reverse transcriptase (TERT)* gene adding telomeric repeats to the end of chromosomes [1]. High rates of activating mutations in the upstream promoter of the *TERT* gene have been reported in numerous human cancers [2–5]. Mutations tend to occur in certain “hot spots,” most notably g.1295228C>T and g.1295250C>T. These mutations generate a CCGGAA/T or GGAA/T motif thereby altering the binding site for Ets transcription factor, subsequently leading to increased *TERT* promoter activity [2,6,7].

Our group and others have demonstrated *TERT* promoter mutations (*TERT-mut*) in a large subset (60%–80%) of urothelial carcinomas and some of their histologic variants [8–14]. These mutations have been found in both noninvasive and invasive urothelial carcinomas and have been detected in urine specimens from patients with urothelial carcinoma, making them attractive candidates for early detection and disease surveillance [8,12,15]. In the current study, we sought to determine the rate of *TERT-mut* in primary adenocarcinoma of the urinary bladder.

## 2. Materials and methods

A retrospective search of our institutional archives was conducted for all cases of adenocarcinoma primary to the urinary bladder between the years 2000 and 2014. Focal glandular differentiation can be encountered in association with urothelial carcinomas and other variants. Therefore, to ensure the inclusion of only “pure” adenocarcinoma cases that would satisfy the World Health Organization/International Society of Urologic Pathology 2004 [16] definition of the entity, we limited our study to cystectomy and cystoprostatectomy specimens. Cases with clinical evidence to indicate a secondary involvement of the bladder by an extravesical primary (eg, colorectal) were also excluded. All histologic slides were reviewed to confirm the diagnosis and select a representative tumor section for mutation analysis. A total of 14 cases were included in the study based on the availability of formalin-fixed, paraffin-embedded (FFPE) tissue blocks with sufficient tumor for sampling. The tumor areas were cored with a sterile 16-gauge needle, and the fraction of neoplastic cells was estimated from adjacent sections. The cores were placed in 1.5-mL sterile tubes for DNA purification.

Two sets of negative control samples were also analyzed. Ninety-four peripheral blood samples from healthy population were tested for *TERT-mut* as negative polymerase chain reaction (PCR) procedure controls. Eight FFPE benign transurethral bladder biopsy samples were also used as negative tissue controls.

After DNA purification, samples were analyzed with Safe-SeqS, a sequencing error reduction technology described previously [17], which better discriminates genuine *TERT-mut* from artefactual sequencing variants introduced during the sequencing process. Safe-SeqS amplification primers were designed to amplify segments containing the region of the *TERT* promoter previously shown to harbor mutations in melanomas and other tumors [12] (Fig. 1). The forward and reverse amplification primers contained the *TERT*-specific sequences at their 3' ends and a universal priming site at their 5' end. The reverse primer additionally contained a 14-base unique identifier composed of 14 degenerate N bases (equal likelihood of being an A, C, T, or G) between the universal priming site and gene-specific sequences. The sequences of the forward and reverse primers were either 5'-CACACAG-GAAACAGCTATGACCATGGGCCGCGGAAAGGAAG and 5'-CGACGTAAAACGACGGCCAGTNNNNNNNNN NNNNNCGTCCTGCCCTTCACC or 5'-CACACAGGAA ACAGCTATGACCATGGGCCGAAAGGAAAGGGAG and 5'-CGACGTAAAACGACGGCCAGTNNNNNNNNNNN NNCCGTCCCACCCCTC. PCR products were purified with AMPure and sequenced on a MiSeq instrument. Data were analyzed as previously described [17]. 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 scored as positive were confirmed in at least 1 additional, independent PCR and sequencing experiment.

### 3. Results

#### 3.1. Patient demographics and tumor morphology

The patient demographics, including age, sex, race, TNM stage at time of diagnosis, and outcomes, are reported in Table 1. The mean age of the analyzed patients was 58.3 years (range, 28–74 years). The tumors were primarily located either in the bladder wall or dome (Table 1). Of the 14 patients, 4 (28.5%) died of disease (mean survival from time of surgery, 297.5 days). An additional 5 patients are known to have died; information about the cause of death was not available for these patients.

After morphologic review, 7 of the 14 cases of adenocarcinoma had histologic features of enteric differentiation. These tumors were composed of glands lined by columnar cells with tall eosinophilic cytoplasm and elongated, hyperchromatic nuclei. Typical areas of central (“dirty”) necrosis were identifiable. Variable degree of mucin production was found [18,19]. Four of these tumors with enteric morphology were located in the bladder dome and were consistent with urachal origin. Two of the remaining 3 enteric-type adenocarcinomas involved the trigone region and urethra. The remaining case was centered in the bladder wall. The second subset of cases (7/14) was characterized as nonenteric. This designation encompasses tumors with variable histology, including signet ring, mucinous, and not otherwise specified (NOS). Of the 7 nonenteric cases, 3 had at least a focal poorly differentiated component with intracytoplasmic mucin thereby acquiring signet ring features,

and an additional 3 had areas of extracellular mucinous pools. The final case had nonspecific glandular features and was categorized as NOS (Fig. 2).

### 3.2. *TERT-mut* detection

Overall, *TERT-mut* were identified in 4 (28.5%) of 14 cases of primary bladder adenocarcinoma with all showing an identical mutation type (g.1295228C>T) (Table 2). In the 2 morphologic subsets (enteric versus NOS), *TERT-mut* were only present in the tumors with nonenteric morphology (4/7, 57%) and not found in any of the 7 tumors with enteric differentiation, including those of urachal origin.

All of the blood and FFPE samples used as negative controls tested negative for *TERT-mut*.

## 4. Discussion

Bladder carcinoma is the most common malignancy of the urinary tract and the fourth most common carcinoma in men in the Western world. Among bladder cancers, urothelial carcinoma is by far the most prevalent histologic type, with squamous cell carcinoma and adenocarcinoma making up less than 5% of cases [20]. Per patient, the cost of bladder cancer management is the highest among all tumors, due to the need for long-term monitoring with regular cystoscopy, imaging, and urine cytology [21–23].

*TERT-mut* were first reported in melanoma [2]. Subsequently, our group and others demonstrated the presence of similar mutations in a wide spectrum of solid cancers, including urothelial carcinoma, gliomas, and hepatocellular carcinoma, among others [3–5]. High rates of *TERT-mut* have been conspicuously absent in colorectal and lung carcinomas [3,24].

In the urinary bladder, *TERT-mut* has also been detected at a high rate in small cell carcinoma, urothelial carcinomas with focal squamous differentiation, and nested variants of urothelial carcinoma [13,24,25]. However, to our knowledge, this study is the first to describe *TERT-mut* in primary bladder adenocarcinoma. Furthermore, the cases evaluated here are intentionally devoid of histologic features of urothelial differentiation (“pure” adenocarcinoma). Here, we demonstrate, for the first time, the presence of *TERT-mut* in a significant subset (57%) of primary adenocarcinomas of the urinary bladder with nonenteric morphology. All of the adenocarcinomas with *TERT-mut* had the same C to T mutation (g. 1295228C>T). Evaluation of prognostic significance of *TERT-mut* was not feasible in this limited study.

Bladder adenocarcinomas may be thought of as morphologically dichotomous, appearing as either enteric-type tumors or adenocarcinoma NOS [18]. The latter encompasses multiple types, including the signet ring and mucinous types. Enteric-type adenocarcinomas of the bladder are morphologically indistinguishable from secondary involvement from colorectal carcinoma; clinical correlation is usually required to establish the diagnosis [26]. A difference in *TERT* promoter mutation rates emerged between enteric adenocarcinoma and adenocarcinoma NOS (0% versus 57%), although the numbers of cases were small and the difference not statistically significant. Intriguingly, *TERT-mut* have not been detected in

colorectal adenocarcinoma [3]. If confirmed, the shared similarities between enteric-type bladder adenocarcinoma and colorectal cancers appear to include the absence of *TERT-mut* in addition to morphologic and immunophenotypic features.

A subset of tumors with enteric-type morphology arise in the dome of the bladder from embryologic remnants of the urachus and are referred to as urachal adenocarcinomas [27,28]. Four of our enteric-type adenocarcinomas were located in the dome of the bladder and were consistent with urachal adenocarcinomas. Interestingly, none of the urachal tumors harbored *TERT-mut* in agreement with their phenotypic enteric differentiation.

Surprisingly, when all cases are taken into account, *TERT-mut* was detected in only 4 (26.7%) of 14 of bladder adenocarcinomas in this study. This figure appears relatively low, considering previous reports have shown that nearly 80% of urothelial carcinomas harbor *TERT-mut*, and it might be expected that all bladder carcinomas would harbor *TERT-mut* at some similar rate [3,8,11,12,15]. This low rate of *TERT-mut* in this study may be in part due to the fact that *TERT-mut* and the presence of enteric differentiation (including urachal adenocarcinomas) appear so far mutually exclusive.

A recent study by Vail et al [14] addressed the potential diagnostic utility of *TERT-mut* in differentiating benign from malignant glandular lesions of the bladder including primary adenocarcinoma of bladder. The study failed to detect *TERT-mut* in 10 cases of primary adenocarcinoma [14]. However, the authors did not further classify the type (enteric versus NOS) of adenocarcinoma analyzed, making it difficult to compare their findings to the current study.

In summary, the present study illustrates the presence of *TERT-mut* in a significant proportion of primary bladder adenocarcinomas of nonenteric morphology, albeit at a slightly lower rate compared to that reported for urothelial carcinoma. In contrast, the enteric-type adenocarcinomas, including urachal carcinomas, lack *TERT-mut*. Our group has previously demonstrated the potential role of urine-based assay for *TERT-mut* as a molecular screening method for the detection of urothelial carcinoma [8,12,15]. Briefly, *TERT-mut* were consistently detected in the urine of 74% of patients with non-invasive urothelial carcinomas and, on follow-up, were predictive of disease recurrence [17]. These results suggest *TERT-mut* as a potential biomarker for development of screening and/or diagnostic tests based on analysis of tumor DNA in urine. One potential limitation of such a molecular urine screening assay for bladder cancer would be the ability to detect other bladder carcinomas beyond urothelial carcinoma, such as adenocarcinoma. The present findings of a high rate of *TERT-mut* (57%) in nonenteric-type adenocarcinoma demonstrate that these tumors may harbor the same biomarker and thus further broaden the clinical utility of a potential assay. Future studies will be needed to address the ability to detect adenocarcinoma in urine samples from patients and determine performance characteristics of such an assay.

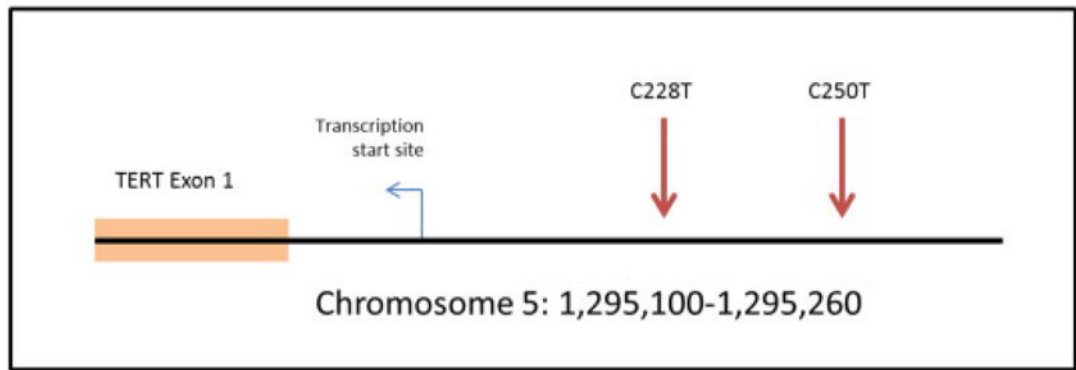
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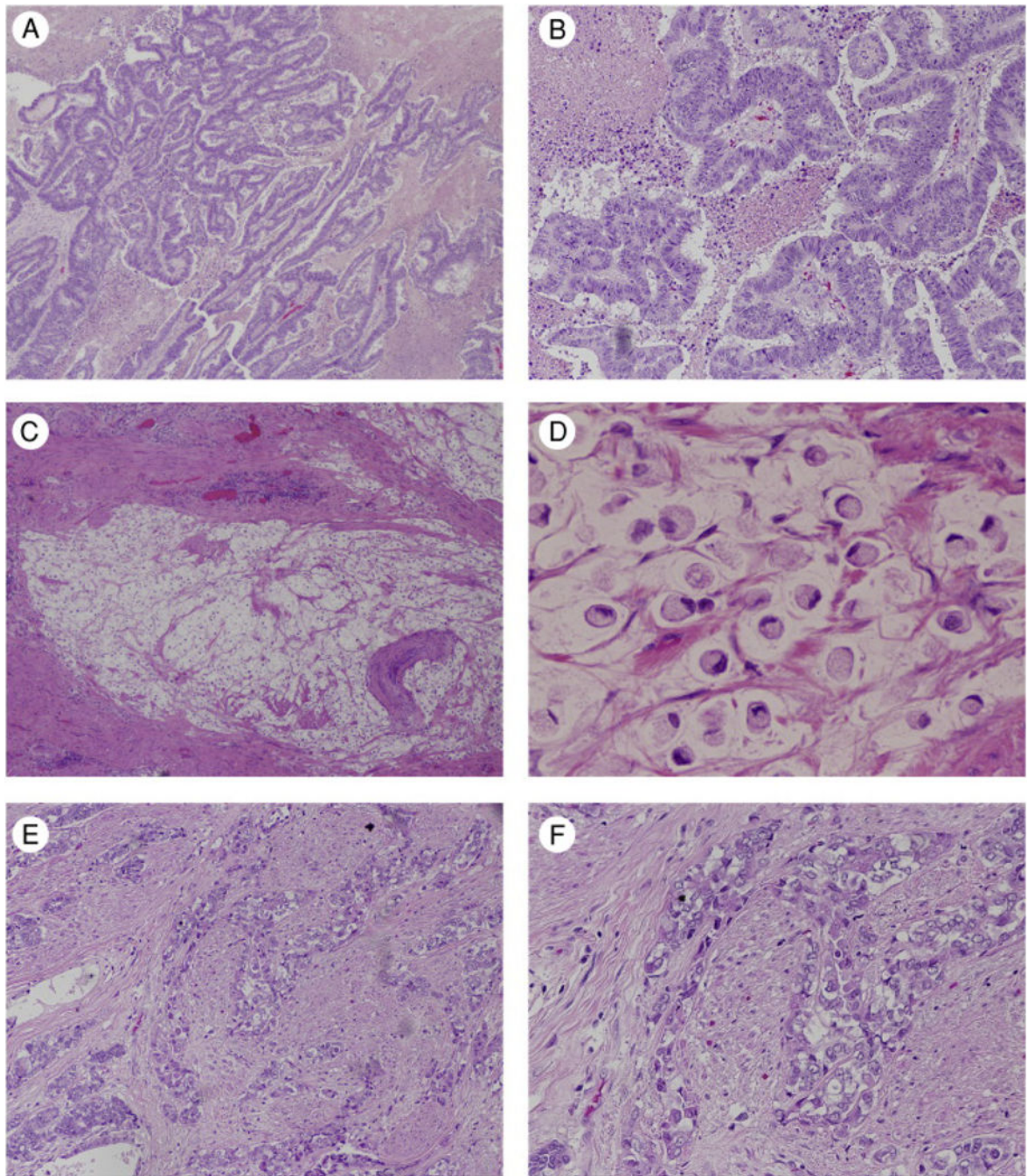
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**Fig. 1.** Two mutational “hotspots” are repeatedly seen in the *TERT* promoter, at position 250 and position 228. Both of the mutations are a C > T base substitution mutation.





**Fig. 2.** Representative images of the histologic subtypes. A and B, Tumors with enteric differentiation are indistinguishable morphologically from colon adenocarcinoma. Tumors with nonenteric histology include signet ring cell adenocarcinoma (C and D) and adenocarcinoma NOS (E and F). Hematoxylin and eosin, original magnification,  $\times 100$  (A, C, and E) and  $\times 200$  (B, D, and F).

**Table 1**

Patient demographics, outcome, tumor subtype, and pathologic findings in 14 primary urinary bladder adenocarcinomas included in the study

Case	<i>TEXT-mut</i>	Adenocarcinoma subtype	Age	Sex	Location	Race	pTNM stage	Recurrence	Progression	Survival outcome
1	Y	NOS	68	M	Wall	W	T3	NX	N	UNK
2	N	NOS	48	F	Trigone, urethra	B	T4	NX	Y	DOD
3	Y	NOS	64	M	Wall	W	T4	N0	N	UNK
4	N	Enteric	46	M	Wall	B	T2	N0	Y	AWD
5	Y	NOS	67	M	Wall	W	T3b	N2	N	UNK
6	N	Enteric	28	M	Urachus	W	T3	NX	Y	DOD
7	N	NOS	71	M	Wall	W	T3	N0	N	UNK
8	N	Enteric	53	M	Urachus	W	T3a	N0	N	NED
9	N	NOS	52	M	Trigone, ureter	B	T2b	NX	Y	UNK
10	N	Enteric	69	M	Trigone, urethra	B	T4	N2	N	DOD
11	N	Enteric	55	M	Urachus	W	T3	N1	Y	DOD
12	Y	NOS	74	M	Wall	W	T2a	N0	N	NED
13	N	Enteric	51	M	Urachus	W	T4b	N2	Y	AWD
14	N	Enteric	70	M	Trigone, urethra	W	T4	N0	N	NED

NOTE. Patient age reported at time of surgery. Tumor stage reported at diagnosis.

Abbreviations: Y, yes; N, no; M, male; F, female; W, white; B, black; DOD, died of disease; DWD, died with disease; AWD, alive with disease; NED, no evidence of disease; UNK, unknown cause of death.

**Table 2***TERT-mut* status and mutation type by histologic type of adenocarcinoma

Adenocarcinoma histologic type	<i>TERT-mut</i> detected	<u>Mutation type</u>	
		228 T>C	250 C>T
<b>Enteric (n = 7)</b>	0/7 (0%)*	–	–
<b>NOS (n = 7)</b>	4/7 (57%)*	4/4 (100%)	0/4 (0%)
<b>All (n = 14)</b>	4/14 (28.5%)	4/4 (100%)	0 (0%)

\*  $P = .06$  (Fisher exact test) for difference in *TERT-mut* rates in enteric versus NOS subtypes.

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