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Telomerase Reverse Transcriptase Promoter Mutations in Bladder Cancer: High Frequency Across Stages, Detection in Urine, and Lack of Association with Outcome

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Article info

Abstract

Background: Hotspot mutations in the promoter of the gene coding for telomerase reverse transcriptase (TERT) have been described and proposed to activate gene expression.

Objectives: To investigate *TERT* mutation frequency, spectrum, association with expression and clinical outcome, and potential for detection of recurrences in urine in patients with urothelial bladder cancer (UBC).

Design, setting, and participants: A set of 111 UBCs of different stages was used to assess *TERT* promoter mutations by Sanger sequencing and TERT messenger RNA (mRNA) expression by reverse transcription-quantitative polymerase chain reaction. The two most frequent mutations were investigated, using a SNaPshot assay, in an independent set of 184 non–muscle-invasive and 173 muscle-invasive UBC (median follow-up: 53 mo and 21 mo, respectively). Voided urine from patients with suspicion of incident UBC ($n = 174$), or under surveillance after diagnosis of non–muscle-invasive UBC ($n = 194$), was tested using a SNaPshot assay.

Outcome measurements and statistical analysis: Association of mutation status with age, sex, tobacco, stage, grade, fibroblast growth factor receptor 3 (*FGFR3*) mutation, progression-free survival, disease-specific survival, and overall survival.

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Results and limitations: In the two series, 78 of 111 (70%) and 283 of 357 (79%) tumors harbored *TERT* mutations, C228T being the most frequent substitution (83% for both series). *TERT* mutations were not associated with clinical or pathologic parameters, but were more frequent among *FGFR3* mutant tumors (p = 0.0002). There was no association between *TERT* mutations and mRNA expression (p = 0.3). Mutations were not associated with clinical outcome. In urine, *TERT* mutations had 90% specificity in subjects with hematuria but no bladder tumor, and 73% in recurrence-free UBC patients. The sensitivity was 62% in incident and 42% in recurrent UBC. A limitation of the study is its retrospective nature.
Conclusions: Somatic *TERT* promoter mutations are an early, highly prevalent genetic event in UBC and are not associated with TERT mRNA levels or disease outcomes. A SNaPshot assay in urine may help to detect UBC recurrences.

1. Introduction

Urothelial bladder cancer (UBC) is heterogeneous at the clinical, pathologic, and genetic levels. Approximately 75% of newly diagnosed tumors are non–muscle invasive (Ta, Tis, and T1); most of them recur and 15–20% progress to invade muscle. The remaining 25% of patients present with muscle-invasive tumors and have a 5-yr survival of <50% [1–3]. Fibroblast growth factor receptor 3 (*FGFR3*) is the most commonly mutated gene in UBC, with an overall frequency of 60% [4–6]. Mutations in Harvey rat sarcoma viral oncogene homolog (*HRAS*), neuroblastoma RAS viral (v-ras) oncogene homolog (*NRAS*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*) are less frequent [6,7]. *FGFR3* mutations are associated with stage and grade and are very common in pTa and grade 1/2 tumors. This makes *FGFR3* mutation analysis in voided urine a useful tool for diagnosing recurrent disease after a primary non–muscle-invasive bladder cancer (NMIBC) [8,9].

Telomerase increases telomere length at chromosome ends. This activity is crucial for proliferating cells: Without telomerase, chromosomes are shortened every cell-division cycle, leading to replicative senescence and genomic instability [10,11]. Telomerase is active in stem cells but becomes downregulated in differentiated cells in somatic tissues. By contrast, it becomes reactivated in many tumors. Somatic hotspot mutations in the promoter of the gene coding for telomerase reverse transcriptase (TERT) catalytic subunit have recently been described in 71% of sporadic melanomas; germline mutations have also been reported in a melanoma kindred [12,13]. The somatic mutations were mutually exclusive C-to-T transitions at nucleotides 1,295,228 (C228T) and 1,295,250 (C250T). These are the first hotspot somatic mutations reported in a gene promoter in human cancers and have been proposed to enhance TERT expression through the creation of novel binding sites for E-twenty-six (ETS)/ELK transcription factors [12,13]. Nevertheless, direct proof for this mechanism is absent. Interestingly, Huang et al. reported the C228T mutation in three of three UBC cell lines [12] and, in a survey of different tumor types, *TERT* promoter mutations were also found in UBC, hepatocarcinoma, glioblastoma, oligodendroglioma, and myxoid liposarcomas, but not in breast, colorectal, and prostate cancers [14]. However, the number of bladder tumors analyzed was small and clinical or pathologic information was not available [14].

In this paper, we investigate the frequency of *TERT* promoter mutations in two large series of UBC. In the first one, lacking outcome information, we assessed the relationship between *TERT* mutations and TERT messenger RNA (mRNA) expression; in the second, with patient follow-up, we assessed the relationship between mutations and outcome. Finally, we explored whether mutation detection in urine may be used to identify tumor recurrence.

2. Materials and methods

2.1. Patient characteristics

A set of 111 patients with associated frozen tumor samples came from the Spanish Bladder Cancer/EPICURO Study [15,16] and from the Integrated Study of Bladder Cancer (ISBLAC). Clinical and sociodemographic information was retrieved from hospital records through a structured questionnaire. Subjects were defined as former smokers if they quit smoking ≥1 yr before the date of interview. A second set of 357 patients, with associated formalin-fixed paraffin embedded (FFPE) tumor samples and follow-up information, came from the Erasmus Medical Center and other hospitals in the Netherlands. This cohort comprises 184 patients with primary NMIBC selected to contain a 50:50 distribution of cases with or without progression; the latter was defined by the development of MIBC. It also comprises 173 patients who have undergone a cystectomy for primary MIBC, stage pT2-4. Cause of death for patients in the MIBC cohort was determined from hospital records and/or direct contact with general practitioners. Staging and grading were performed according to the TNM 2002 classification and the three-grade 1973 World Health Organization classification. To confirm staging/grading and ensure uniformity of classification criteria, expert pathologists reviewed diagnostic slides from all tumor blocks. Table 1 shows the characteristics of patients whose tumor samples were studied.

For the urine study, samples were obtained from a consecutive, prospectively sampled collection of urine specimens from two centers (Hospital del Mar, Barcelona, Spain; and Erasmus MC, Rotterdam, Netherlands) participating in the European Community Seventh Framework program–funded "Prediction of bladder cancer disease course using risk scores that combine molecular and clinical risk factors" (UROMOL) study (EU-7FP UROMOL #201663). Samples were obtained prior to cystoscopy at the time when a primary UBC was diagnosed (n = 135), or from patients lacking a prior history of UBC who presented with hematuria for diagnostic work-up and lacked visible bladder tumors at cystoscopy (n = 39). Additional samples (n = 395) were obtained during surveillance for relapsed disease (recurrence or progression) in 194 patients with NMIBC. Primary tumor or recurrence was defined by the presence of a

Table 1 – Characteristics of patients whose tumor samples were studied

Characteristic	Variable	Spain (n = 111), no. (%)	The Netherlands, NMIBC (n = 184), no. (%)	The Netherlands, MIBC (n = 173), no. (%)
Age, yr, median		76	70	71
Sex	Female	13 (12)	46 (25)	28 (16)
	Male	98 (88)	138 (75)	145 (84)
Stage	pTa + pTis	63 (57)	107 (58)	–
	pT1	28 (25)	77 (42)	–
	pT2	13 (12)	–	72 (42)
	pT3	3 (3)	–	75 (43)
	pT4	3 (3)	–	26 (15)
Grade	G1	27 (24)	14 (8)	1 (1)
	G2	28 (25)	101 (55)	13 (7.5)
	G3	56 (51)	69 (37)	159 (92)
Follow-up, mo, median		–	53	21
End point	Progression to MIBC	–	90 (49)	–
	Local or distant progression to MIBC	–	–	78 (45)
	Died of disease	–	–	65 (37)
	Died of other causes	–	–	36 (21)
	Alive at follow-up	–	–	37 (21)
	Lost to follow-up	–	–	35 (20)
TERT status	Mut	78 (70)	147 (80)	136 (79)
	WT	33 (30)	37 (20)	37 (21)
TERT mutations[*]	C228T	65 (83)	121 (82)	113 (83)
	C228A	1 (1.5)	3 (2)	3 (2)
	CC242/243TT	2 (2.5)	NA	NA
	C250T	10 (13)	23 (16)	20 (15)

Mut = mutant; NMIBC = non–muscle-invasive bladder cancer; *TERT* = telomerase reverse transcriptase; WT = wild type.
[*] The C242T mutation was not assessable with the SNaPshot assay.

Table 2 – Characteristics of tumors and patients used for urine diagnostic assays

Characteristic	Variable	The Netherlands no. (%)	Spain no. (%)
Patients with primary tumor diagnosis	Patients, no.	45	90
Stage	pTa	39 (87)	53 (59)
	pTis		3 (3)
	pT1	5 (11)	21 (23)
	pT2-4	1 (2)	13 (15)
Grade	G1	17 (38)	27 (30)
	G2	26 (58)	17 (19)
	G3	2 (4)	46 (51)
Patients under surveillance after NMIBC diagnosis[*]	Patients, no.	118	76
With relapsing tumors			
Stage	pTa	78 (90)	40 (71)
	pTis		2 (4)
	pT1	2 (2)	7 (12)
	pT2-4	5 (6)	4 (6)
	pTx	2 (2)	3 (7)
Grade	G1	25 (29)	23 (35)
	G2	52 (60)	13 (23)
	G3	8 (9)	19 (32)
	Gx	2 (2)	1 (10)
Recurrence-free	Patients, no./urine samples, no.	65/232	20/20

NMIBC = non–muscle-invasive bladder cancer.
[*] Relapsing tumors include both recurrences and progression detected in patients with initial diagnosis of NMIBC.

histologically proven tumor. Table 2 shows the characteristics of patients included in the urine study.

All patients provided written informed consent. The ethics committees of the participating institutions approved all studies. Samples from the Netherlands were obtained according to the Federation of Dutch Medical Scientific Societies' Code for Proper Secondary Use of Human Tissues in the Netherlands (http://www.federa.org/).

2.2. Cell lines

UBC cell lines (Supplemental Table 1) were cultured under standard conditions and used for DNA extraction when at 70–80% confluence. All cultures were *Mycoplasma* free; their identity was tested by analysis of known genetic mutations.

2.3. DNA isolation and mutation analyses

Tumor cell-containing regions were macrodissected from frozen blocks or FFPE sections after examination of hematoxylin and eosin–stained sections. For MIBC cases, DNA was isolated from tumor obtained at the time of cystectomy or, in case of pT0, of incident transurethral resection. Tumor DNA and leukocyte DNA were extracted using the Qiagen kit for blood and tissue, according to manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Sanger sequencing of polymerase chain reaction (PCR) products was used to identify all possible mutations in the first set of patients (Fig. 1A). A 163-base pair (bp) PCR product encompassing the proximal *TERT* promoter was amplified (primers: CAGCGCTGCCT-GAAACTC [forward] and GTCCTGCCCCTTCACCTT [reverse]); for some samples, a broader 488-bp region of the promoter was amplified (primers: AGCACCTCGCGGTAGTGG [forward], GGCCGATTCGACCTCTCT [reverse]).
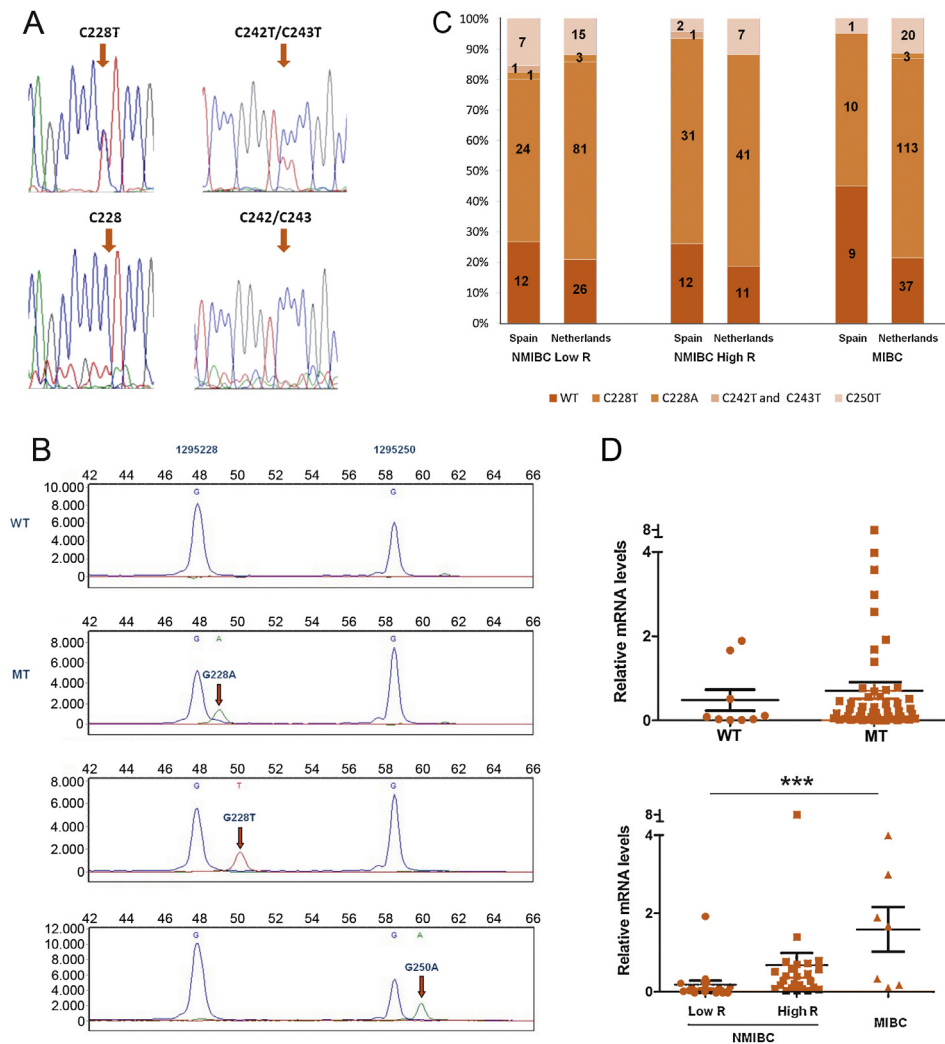
Fig. 1 – Somatic telomerase reverse transcriptase (*TERT*) promoter mutations in urothelial bladder cancer (UBC). (A) The C228T and C242T/C243T mutations are detected in tumor DNA but not in leukocyte DNA (Sanger sequencing). (B) Mutation assay for the indicated nucleotides in the promoter of the *TERT* gene. Upper panel: Control sample with wild-type peaks for positions C228T and C250T (from left to right). The lower panels show mutant samples and the nature of the mutation has been indicated. Note that the assay detects the mutations from the complementary strand, hence the mutations are indicated as G228A, G228T, and G250A. (C) Distribution of *TERT* promoter mutations in UBC according to T and G. Tumors are categorized in three groups: low-risk non–muscle-invasive bladder cancer (NMIBC) (TaG1/G2) (Low R), high-risk NMIBC (TaG3, T1G2/G3) (High R), and MIBC (>T2) for Spain (Sanger sequencing) and the Netherlands (SNaPshot; Applied Biosystems, Foster City, CA, USA). Absolute numbers of cases are given for each mutation and group. (D) TERT messenger RNA levels in bladder tumors assessed by reverse transcriptase-quantitative polymerase chain reaction (*n* = 60); results show the comparison of wild type versus mutant tumors and the comparison of the three tumor groups according to T and G. *** $p \leq 0.001$. WT = wild type; MT = mutant type.

PCR conditions can be provided on request to the authors. *FGFR3* mutation assays were performed as described [8].

For the *TERT* mutational analysis in the second patient group, we designed a SNaPshot assay (Invitrogen Corp, Carlsbad, CA, USA) through a PCR (155 bp) covering the two most frequent mutations identified (primers: AGCGCTGCCTGAAACTCG [forward], CCCTTCACCTTCCAGCTC [reverse]), similar to the previously designed *FGFR3* mutation assay [8]. The PCR was followed by a single-nucleotide extension using probes annealing to the amplicon adjacent to the mutation site. Probe sequences were: T_{23}GGCTGGGAGGGCCCGGA for mutation 1,295,228 (C228T/A) and T_{39}CTGGGCCGGGGACCCGG for 1,295,250 (C250T) at 1 μM and 1.5 μM, respectively (ABI PRISM SNaPshot Multiplex Kit; Applied Biosystems, Foster City, CA, USA). The products were analyzed (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) with the label indicating the presence or absence of a mutation. Genescan Analysis Software v.3.7 (Applied Biosystems, Foster City, CA, USA) was used for data analysis (Fig. 1B).

For urine DNA extraction and mutation analysis, cell pellets were washed twice with phosphate-buffered saline, after thawing, by centrifugation for 5 min at 6000 rpm (3000 g). DNA was extracted using the QiAamp Mini and Blood mini kit (Qiagen NV, Venlo, Netherlands) according to the manufacturer's protocol. *TERT* mutational analysis was performed using the SNaPshot assay.

Reverse transcriptase-quantitative PCR (RT-qPCR) was used to assess TERT RNA expression (see Supplement).

2.4. Statistical and bioinformatics analyses

The Predictive Analytics Software v.19 (IBM Corp., Armonk, NY, USA) was used. Statistical differences were considered significant if $p < 0.05$. The chi-square and Fisher exact tests were used to determine the relationships between different variables. Statistical differences between groups were assessed using a nonparametric Wilcoxon test, as appropriate. The log-rank test was used for survival analysis. In the NMIBC group, all outcomes

other than progression appear as censored in the Kaplan-Meier curve. Bioinformatics analysis of the *TERT* promoter sequence is detailed in the Supplement.

3. Results

3.1. TERT promoter mutations in bladder cancer

We first analyzed the occurrence of mutations in UBC cell lines and tumors using Sanger sequencing of PCR products. Of 32 lines analyzed, 4 were wild type and 28 (87%) harbored the C228T (n = 25) or C250T (n = 3) mutations (Supplemental Table 1). The somatic nature of these mutations could not be evaluated due to lack of germline DNA.

Among the 111 UBCs from Spain, covering the full spectrum of the localized disease, 78 were mutated (70%) (Table 1). The most common mutation was C228T (n = 65), followed by C250T (n = 10); two additional rare mutations were C242T/C243T (n = 2) and C228A (n = 1). All mutations were mutually exclusive; their somatic nature was confirmed in 10 cases (Fig. 1A). The C250T mutation was significantly less common in UBC than in melanoma (p = 0.00003); the C228T/C229T mutation reported in melanoma was not found in UBC. *TERT* mutations were not associated with age, sex, or smoking. The frequency of *TERT* mutations was similar in low-risk NMIBC (TaG1 and TaG2; 33 of 45 [73%]), high-risk NMIBC (TaG3, Tis, T1G2, T1G3; 34 of 46 [74%]), and in MIBC (10 of 19 [53%]) (p = 0.192) (Fig. 1C) and in newly diagnosed versus recurrent tumors (p = 0.716). *TERT* mutations were significantly more frequent among *FGFR3* mutant tumors (43 of 49 vs 29 of 54; p = 0.0002).

We analyzed the two most frequent *TERT* mutations in an independent set of tumors from the Netherlands using a specifically designed SNaPshot assay; mutations were found to be mutually exclusive. A high mutation rate was confirmed in both NMIBC (147 of 184 [80%]) and MIBC (136 of 173 [79%]) (Fig. 1C). To assess heterogeneity, we analyzed *TERT* mutations using the SNaPshot assay in multiple (median: 4; range: 2–5) regions of nine NMIBCs. We observed general consistency among the regions, suggesting a low level of intraindividual heterogeneity (Supplemental Table 2). These findings place *TERT* mutations as the

most common genetic alteration in UBC [6,7] and as an early event in urothelial carcinogenesis.

TERT mutations have been proposed to generate novel transcription factor binding sites leading to increased TERT expression [12,13]. Mining the DNA-binding factor database JASPAR, we performed a comparative analysis of the wild-type and mutant sequences and found that the C228A mutation newly reported in this paper was also associated with an increased probability of binding to ELK1 (a member of the ETS oncogene family) and v-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1), as the previously reported mutations. The analysis revealed additional transcription factors that could contribute to TERT overexpression as well some that showed mutation selectivity, such as v-rel avian reticuloendotheliosis viral oncogene homolog (REL) for C242T/C243T or GATA bind protein 2 (GATA2) for C228A (Supplemental Table 3). To assess the relationship between *TERT* mutations and mRNA levels, we analyzed 60 primary UBC samples using RT-qPCR. TERT mRNA levels were higher in *TERT* mutant tumors, but the difference was not statistically significant (Wilcoxon test p = 0.3). TERT mRNA levels were significantly higher in high-risk NMIBC and in MIBC than in low-risk NMIBC (Wilcoxon test p = 0.001) (Fig. 1D).

3.2. TERT mutations and patient outcome

The set of tumors from the Netherlands was used to assess tumor prognostic value. Of 184 patients with NMIBC, 90 (49%) experienced progression to MIBC (Table 1). There was no relationship between progression-free survival and the presence of mutations (log-rank test p = 0.984) (Supplemental Fig. 1). In the group of 173 patients with MIBC, 78 (45%) patients had local or distant progression and *TERT* mutation status was not associated with progression-free survival (log-rank test p = 0.160). Furthermore, 101 (58%) patients died during follow-up, including 65 (37%) who died of bladder cancer (Table 1). Overall- and cancer-specific survival were not related to *TERT* mutations (log-rank tests p = 0.987 and p = 0.834) (Supplemental Fig. 1). Among patients with MIBC, 25 received adjuvant chemotherapy with curative intent; *TERT* mutations were not predictive for survival in this subgroup or the group without chemotherapy.

Table 3 – Sensitivity and specificity of urine diagnostic assays

	Variable	The Netherlands, no./total (%)	Spain, no./total (%)	Netherlands and Spain, no./total (%)	Netherlands and Spain, p value
Primary tumors, sensitivity	*FGFR3*	17/42 (40)	29/86 (34)	46/128 (36)	–
	TERT	21/37 (57)	52/81 (64)	73/118 (62)	*FGFR3 vs TERT, p < 0.0001*
	TERT + FGFR3	20/28 (71)	53/77 (69)	73/105 (70)	*TERT vs TERT + FGFR3, p = 0.23*
Recurrent tumors, sensitivity	*FGFR3*	15/68 (22)	9/56 (16)	24/124 (19)	–
	TERT	18/63 (29)	30/50 (60)	48/113 (42)	*FGFR3 vs TERT, p = 0.0001*
	TERT + FGFR3	21/53 (40)	31/50 (62)	52/103 (50)	*TERT vs TERT + FGFR3, p = 0.238*
Recurrence-free FU urine samples, specificity	*FGFR3*	208/232 (90)	19/20 (95)	227/252 (90)	–
	TERT	149/200 (75)	11/18 (61)	160/218 (73)	*FGFR3 vs TERT, p < 0.0001*
	TERT + FGFR3	131/183 (72)	11/18 (61)	142/201 (71)	*TERT vs TERT + FGFR3, p = 0.529*

FGFR3 = fibroblast growth factor receptor 3; FU = follow-up; *TERT* = telomerase reverse transcriptase.

3.3. *TERT mutation detection in exfoliated cells in urine*

Patients with NMIBC require continued follow-up due to frequent tumor recurrences. We assessed whether *TERT* mutations can be detected in exfoliated tumor cells in voided urine and compared the findings with *FGFR3* mutations (Table 3). Of 39 urine samples from patients with hematuria and in whom no bladder tumor was visible at cystoscopy (17 of 39 [44%] female; median age: 54 yr), 4 were positive for *TERT* mutations, resulting in a specificity of 90%. None of these four patients developed a UBC or another tumor in the subsequent >3.5 yr. In urine samples obtained before resection of a primary bladder tumor, the sensitivity of incident tumor detection was 36% (46 of 128) for *FGFR3*, 62% (73 of 118) for *TERT*, and 70% (73 of 105) for *TERT* and *FGFR3* combined (*FGFR3* vs *TERT*, $p < 0.0001$). We then tested 395 urine samples prospectively collected from 194 patients under surveillance after a primary NMIBC diagnosis. Sensitivity of detection of relapsing (recurrence or progression) UBC developing after a primary NMIBC was 19% (24 of 124) for *FGFR3*, 42% (48 of 113) for *TERT*, and 50% (52 of 103) for both combined (*FGFR3* vs *TERT*, $p = 0.0001$). In the recurrence-free urine samples, a *TERT* mutation was detected in 58 of 218 cases, corresponding to a specificity of 73%, whereas the specificity for *FGFR3* mutation was 90% (*FGFR3* vs *TERT*, $p < 0.0001$).

4. Discussion

TERT promoter hotspot mutations constitute a new type of somatic genetic alterations in cancer. A recent survey in a wide range of tumors indicated a selective mutational pattern associated with tumor histology and tissue of origin [14]. It has been proposed that mutations occur more commonly in tumors derived from tissues with low proliferation rates [12–14]. Based on the analysis of 21 UBCs, a high mutation rate was proposed, but there was no clinical or pathologic information on these patients.

In this paper, we provide definitive evidence on the occurrence of *TERT* promoter mutations in UBC. First, we show a high mutation rate (70%), which was replicated in an independent series (79%) using different assays, thus making *TERT* the most frequently mutated gene in UBC. Second, we show a similar mutation frequency across stages and grades, strongly suggesting that *TERT* mutations participate in the two major genetic pathways involved in UBC. Third, we show a distinct mutational spectrum in comparison with melanoma with a dominance of the C228T mutation. Finally, we show a lack of association with progression in NMIBC or with the development of local or distant metastases in MIBC.

The high frequency of hotspot mutations renders *TERT* a very attractive target for diagnosis of bladder tumors, both primary and recurrent, using body fluids such as urine or blood. We show that tumor cell detection in urine is feasible, with a sensitivity of 62% at initial diagnosis and 42% at recurrence. For urine diagnostics during surveillance, the patients were not stratified for mutation status of the primary tumor; we expect that when this is performed,

sensitivity of detection of recurrent UBC will increase. Accordingly, *TERT* mutation was assayed concomitantly in tumor tissue and urine in 31 cases: *TERT* was concordant in 26 cases (19 mutated, 7 wild type), one tumor harbored a C242T mutation not assessable by the SNaPshot assay, and four mutations were detected in urine but not in tumor samples. We show that *TERT* mutation detection has a higher sensitivity than *FGFR3* mutation for tumor cell detection in urine, and assays of both *TERT* and *FGFR3* mutations might have improved sensitivity, though the difference was not statistically significant in our study. By contrast, *TERT* mutations may have a lower specificity than *FGFR3* mutations, as four mutations were detected in 39 urine samples from patients without cancer and in 58 of 218 of patients in clinical remission. In this group, we cannot distinguish reduced specificity from higher lead-time bias in detection of tumor relapse. Larger, well-designed, prospective studies are required to establish the clinical usefulness of mutation detection in urine. As previous reports have proposed TERT expression or activity assessment in urine as a tool to detect recurrence, it will be of high interest to compare *TERT* mutation with them and with other candidate biomarkers in future studies [17,18].

Several observations support the notion that *TERT* somatic mutations are an early event in urothelial carcinogenesis, including their occurrence in a small fraction of subjects without UBC, their presence in tumors of both the papillary and invasive pathways, and the low level of intraindividual heterogeneity when multiple tumor regions were analyzed. UBC cell lines are also very frequently mutated, and we noticed that most of the mutant ones lacked the wild-type allele. These observations also suggest that mutant cells may display a growth advantage over wild-type cells.

The mechanisms through which *TERT* promoter mutations contribute to UBC and other tumors remain to be determined. One possibility is that the acquisition of new, putative transcription binding sites leads to altered regulation of gene expression, as previously proposed, and also suggested by our bioinformatics analysis using the DNA-binding factor database JASPAR. However, we failed to find a significant association between *TERT* mutation and expression. This could be explained by alternative mechanisms of TERT upregulation in wild-type tumors, also leading to TERT overexpression, or by the participation of *TERT* mutations in additional biologic processes.

Given its reverse transcriptase activity, telomerase is a potential therapeutic target. Recent developments suggest that azidothymidine (AZT), an inhibitor of HIV reverse transcriptase, also inhibits telomerase. Hence, it is conceivable that AZT or other telomerase inhibitors may suppress the growth of UBC overexpressing the enzyme [19].

One of the limitations of our work is the retrospective nature of the cohorts used for the outcome analysis, even though our results do not make positive claims. Another is the lack of comparison for urine-based diagnostic assays between *TERT* mutation, cytology, and other biomarkers. However, the high frequency of these mutations in urine reported herein provides evidence for future prospective and comparative studies. Overall, the novelty of *TERT* mutation in

UBC is strengthened by the independent analysis that we performed in two different centers.

5. Conclusions

Mutations in the promoter of the *TERT* gene are the most frequent somatic mutations in tumors of the urinary bladder. Mutations occur with similar frequency, regardless of stage or grade, and are not associated with clinical outcome. *TERT* mutation assays may be used for tumor cell detection in urine and TERT may be a therapeutic target.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2013.08.052.

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