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Research paper

Urinary *TERT* promoter mutations as non-invasive biomarkers for the comprehensive detection of urothelial cancer

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A B S T R A C T

Background: Recurrent mutations in the promoter of the telomerase reverse transcriptase (*TERT*) gene (C228T and C250T) detected in tumours and cells shed into urine of urothelial cancer (UC) patients are putative biomarkers for UC detection and monitoring. However, the possibility of detecting these mutations in cell-free circulating DNA (cfDNA) in blood and urine, or DNA from urinary exfoliated cells (cellDNA) with a single-gene sensitive assay has never been tested in a case-control setting.
Methods: We developed a single-plex assay (UroMuTERT) for the detection of low-abundance *TERT* promoter mutations. We tested 93 primary and recurrent UC cases and 94 controls recruited in France (blood, urine samples and tumours for the cases), and 50 primary UC cases and 50 controls recruited in Portugal (urinary exfoliated cell samples). We compared our assay with urine cytology.
Findings: In the French series, C228T or C250T were detected in urinary cfDNA or cellDNA in 81 cases (87·1%; 95% CI 78·6–93·2), and five controls (Specificity 94·7%; 95%CI 88·0–98·3), with 98·6% (95% CI 92·5–99·96) concordance in matched tumours. Detection rate in plasma cfDNA among cases was 7·1%. The UroMuTERT sensitivity was (i) highest for urinary cfDNA and cellDNA combined, (ii) consistent across primary and recurrent cases, tumour stages and grades, (iii) higher for low-risk non-muscle invasive UC (86·1%) than urine cytology (23·0%) ($P < 0·0001$) and (iv) 93·9% when combined with cytology. In the Portuguese series – the sensitivity and specificity for detection of UC with urinary cellDNA was 68·0% (95% CI 53·3–80·5) and 98·0% (95% CI 89·3–100·0).
Interpretation: *TERT* promoter mutations detected by the UroMuTERT assay in urinary DNA (cfDNA or cellDNA) show excellent sensitivity and specificity for the detection of UC, significantly outperforming that of urine cytology notably for detection of low-grade early stages UC.
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1. Introduction

Bladder cancer (BC), accounting for 90% of urothelial cancer (UC), has become a common cancer globally [1]. While 70–80% of BCs are non-muscle-invasive carcinoma [2], high rates of recurrence (50–70%) and progression to the muscle (10–20%) require close monitoring after first-line treatment. Upper tract urothelial cell carcinoma

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Research in context

Evidence before the study

In concept, the identification of urinary biomarkers for the detection and monitoring of urothelial cancer (UC; carcinomas of the bladder and of the upper urinary tract) should be enhanced by the vicinity of urine to the tumour. However, FDA-approved urinary biomarkers do not have adequate performance to be clinically useful, so UC detection and management still largely rely on a combination of invasive cystoscopy and urine cytology. While cytology is non-invasive, it fails to identify most low-grade lesions. The discovery in 2013 of two highly recurrent mutations (C228T and C250T) in the promoter of the Telomerase Reverse Transcriptase gene (TERT), in various human cancers and particularly in UCs where it is seen in 60–85% of cases including in early stages lesions reinvigorated the research field of UC biomarkers by providing an excellent opportunity for a simple non-invasive assay for the detection and monitoring of UCs. We searched PubMed with terms "TERT promoter mutations", "urine", "blood", "cell-free DNA", "exfoliated cells", "liquid biopsy", "Bladder cancer", "urothelial cancer" for studies published between Feb. 22, 2013 (initial studies reporting *TERT* promoter mutations in melanoma) and April 1, 2015 (writing of the protocol). A few studies had reported that the mutations could be detected in DNA from exfoliated cells (cellDNA) in urine collected at diagnosis and during follow-ups. They were however based on limited sample size preventing accurate assessment of clinical sensitivity and specificity of the putative biomarkers and the different sources of DNA in urine and blood pairs had not been systematically tested in a case-control setting.

Added value of this study

We evaluated the analytical and clinical validity of *TERT* promoter mutations in body fluids samples for the non-invasive detection of UCs. The novelty of our study is evidenced by: (i) the development of a simple, clinically implementable single-plex assay (UroMuTERT) with unprecedented detection threshold for the detection of low-allelic fraction *TERT* promoter mutations and (ii) the systematic analysis of cell-free circulating DNA (cfDNA) in blood and urine, or DNA from urinary exfoliated cells (cellDNA) in prospectively collected fit-for-purpose samples of UC cases and controls. We compared the sensitivity and specificity in an independent case-control series using retrospectively collected urinary exfoliated cells (cellDNA). *TERT* promoter mutations detected by the UroMuTERT assay in urinary DNA (cfDNA or cellDNA) show excellent sensitivity and specificity for the detection of all forms of UC, significantly outperforming that of urine cytology notably for detection of low-grade early stages UC.

Implications of all the available evidence

Our results suggest that a single-gene assay quantifying tumour-derived *TERT* promoter mutation load in urine cellDNA or cell-free DNA has the required clinical performance to enter the subsequent validation phases with the potential to profoundly change the way urothelial cancer will be detected or clinically managed. We lay out a strategy integrating urinary *TERT* promoter mutations as a primary diagnostic tool to individuals at high-risk or under surveillance for UC recurrence, with the intent to provide urological societies with rationale how it could impact current medical standards for UC detection. Potential health benefits of the biomarkers measured by randomized trials would include (i) improved detection of low-grade early stage UC and therefore timely surgical resection and better survival as well as (ii) reduced numbers of unnecessary cystoscopy of patients with TERT negative test, avoiding discomfort and risk of complications associated with invasive procedures and (iii) reduced high cost of clinical management of suspected UCs and patient non-adherence to screening or surveillance.

(UTUCC, 10% of UCs), while different in many aspects, including aetiology, prognosis and treatment guidelines, shares many histological features and genetic alterations with BC [3]. UC detection relies on invasive cystoscopy, imaging approaches such as Computed Tomography and non-invasive urine cytology, however the latter lacking sensitivity in detecting low-grade BC [4] and UTUCC [5]. Performance inconsistencies of FDA-approved urine-based biomarkers prevent their routine clinical use [6]. There is therefore a need for reliable non-invasive biomarkers for early detection and improved surveillance of UC.

Mutations in the promoter of the Telomerase Reverse Transcriptase gene (*TERT*) are frequent in various human cancers. In UCs (in both BCs and UTUCCs), they are observed in 60–85% of cases including early stages [7,8]. These mutations were detected in DNA from urinary exfoliated cells collected prior to diagnosis and during post-surgical follow-ups [8–13]. Recently, urinary cell-free DNA (cfDNA) showed higher analytical sensitivity than DNA from exfoliated cells (cellDNA) for the detection of UC tumour-derived alterations [14]. Assessment of these mutations in different sources of DNA in urine and blood pairs has never been made in a case-control setting with a sensitive single-gene assay.

Because a sensitive and specific biomarker of UC might profoundly influence clinical practice, we developed a single-plex assay, UroMuTERT, based on ultra-deep sequencing of the partial *TERT* promoter and an algorithm for the detection of low-allelic fractions mutations [15]. We assessed *TERT* promoter mutations in DNA from various body fluids (cfDNA in blood and urine, and cellDNA) as putative biomarkers for the detection of UC in two series of cases and controls and compared UroMuTERT diagnostic performance to that of urine cytology.

2. Methods

2.1. Study population and clinical specimens

Participants were recruited from two case-control studies.

2.1.1. DIAGURO case-control study

Recruitment was conducted in the urological department of the Protestant Clinic (Lyon, France) during 2016–2017. Clinical cases included patients with post-surgery histological confirmation of primary or recurrent UC (BC or/and UTUCC (ureter or renal pelvis)) at any stage and grade. Controls were patients with urological pathological conditions other than UC or undergoing colonoscopy. Inclusion and exclusion criteria are summarized in the appendix (p1). Clinical and epidemiological data were collected. Institutional review boards of the International Agency for Research on Cancer and the French Ministry of Scientific Research approved the study protocol (IARC Ethics Committee.,

Project No. 15–23 and CCTIRS No 15.514bis). Written informed consent was obtained for all participants.

Prospective sample collection included trios of tumour-urine-blood (collected before surgery) for cases and duos of urine-blood for controls (appendix p 1). Blood and voided urine samples were processed within the 2 h of collection and DNA from plasma, white blood cells (WBC), urine supernatant (US), urine pellet (UP) and frozen tumour tissue were isolated and quantified using standard protocols (appendix p 2). A qualified pathologist performed histological review of the tumour tissues.

2.1.2. IPO-PORTO case-control replicative series

DNA from UP of 50 primary bladder cancer cases and 50 controls (healthy donors, with no history of cancer) were retrospectively selected from the Biobank of the Portuguese Oncology Institute of Porto for UroMuTERT analysis. Clinical data were collected for all participants. Institutional review boards of the Portuguese Oncology Institute of Porto and the International Agency for Research on Cancer approved the study protocol (Ethics Committee Approvals CES-IPOPFG-EPE 019/08 and IARC Project No. 15-23-A1). Written informed consent was obtained for all participants.

Voided urine samples were collected and processed within the 4 h of collection. DNA was isolated and quantified (appendix p 2). The respective UPs were washed twice with phosphate-buffered saline and stored at −80 °C. DNA was isolated and quantified using similar protocol as for the DIAGURO samples.

3. UroMuTERT assay and mutation analysis

A single-plex of 147 bp was designed to be smaller than the 167 bp average fragment size of cfDNA and to cover the genomic positions of the 2 most prevalent *TERT* promoter mutations (C228T and C250T). The primer sequences are provided in the appendix (p 2). PCR amplification of 50 cycles was performed using 5 ng of cfDNA or tumour DNA, 10 ng of cellDNA and 5× custom Buffer, 100 nM forward and reverse primers and 0.04 U/mL of AccuStart HiFi Taq Polymerase (Quanta BioSciences). The buffer composition is available upon request. Barcoded libraries were prepared as previously described [15]. Emulsion amplification was performed on the Ion OneTouch 2 system using 7 μL of 100pM library and the Ion PI Hi-Q OT2 200 Kit (ThermoscienceFisher Scientific). The sequencing reaction was performed on an Ion Proton System using Life Technologies' Ion PI™ Chip Kit v3 and Ion PI™ Hi-Q™ Sequencing 200 Kit (Thermoscience Fisher Scientific). Genomic DNA from the mutated cell-lines HepG2 and A375 harbouring *TERT* C228T and C250T respectively were serially diluted into human wild-type genomic DNA of a lymphoblastoid cell-line in order to assess the accuracy and the detection threshold of the minimum mutant allelic fractions (MAFs). Variant calling was performed using our Needlestack algorithm specifically designed for the detection of low-abundance mutations [15,16]. It includes C228T and C250T and other rare mutations previously reported (C181T, C176G, C228A, CC242-243TT, G245T) [9,11]. Reads with base quality below 13 at the evaluated positions were excluded. A *p*-value for being a variant (outlier from the regression) was calculated for each sample and transformed into q-values to account for multiple testing. A threshold of Phred scale *q*-values QVAL > 20 was used to call variants ($QVAL = -10 \log_{10}(q\text{-value})$).

4. Statistical analyses

Mann-Whitney or Kruskal-Wallis tests were used for comparisons of quantitative variables between groups of patients. Pearson χ^2 tests and two-tailed Fisher exact tests were used for categorical variables. Sensitivities, specificities and accuracy of the putative biomarkers were calculated for the different sources of DNA with confidence intervals computed with the use of Clopper-Pearson method. Positive and negative predictive values (PPV and NPV) were calculated for patients at high-risk of BC, estimated at 30% for patients with haematuria or, patients with lower urinary tract symptoms (LUTS) or others according to Springer and colleagues [12]. Confidence intervals for the predictive values are the standard logit confidence intervals given by Mercaldo et al. 2007 [17]. Analyses were conducted using IBM SPSS Statistics 20.

Table 1
Patient's baseline characteristics.

	DIAGURO cohort (N = 187)		PORTO Cohort (N = 100)	
Characteristics	UC patients (N = 93)	Controls (N = 94)	UC patients (N = 50)	Controls (N = 50)
Median age (range)- yr	72 (42–95)	70 (34–93)	68 (37–91.4)	46 (38–62)
Sex - no. (%)				
Female	17 (18·3)	23 (24·5)	5 (10·0)	26 (52·0)
Male	76 (81.7)	71 (75.5)	45 (90·0)	24 (48·0)
Smoking status - no. (%)				
Never	23 (24·7)	39 (41·5)	–	–
Former	44 (47·3)	44 (46·8)	–	–
Current	21 (22·6)	11 (11·7)	–	–
Missing	5 (5·4)	–	–	–
Alcohol status - no. (%)				
Never	23 (24·7)	22 (23·4)	–	–
Ex-drinker	13 (14·0)	7 (7·4)	–	–
Current drinker	52 (55·9)	64 (68·1)	–	–
Missing	5 (5·4)	1 (1·1)		
Cancer history - no. (%)				
No	68 (73·1)	82 (87·2)	–	–
Yes	18 (19·4)	12 (12·8)	–	–
Missing	7 (7·5)	–	–	–
Diabetes - no. (%)				
No	69 (74·2)	82 (87·2)	–	–
Yes	18 (19·4)	12 (12·8)	–	–
Missing	6 (6·4)	–	–	–
Disease status - no. (%)				
Primary	45 (48·4)	–	50 (100·0)	–
Recurrence	48 (51·6)	–	0 (0.0)	–
Tumour stage - no. (%)				
CIS[a]	12 (12·9)	–	–	–
pTa	51 (54·8)	–	18 (36·0)	–
pTa–CIS	5 (5·4)	–	–	–
pT1	6 (6·4)	–	14 (28·0)	–
pT1–CIS	10 (10·8)	–	–	–
> pT1	6 (6·5)	–	18 (36·0)	
> pT1–CIS	3 (3·2)	–	–	–
Tumour grade - no. (%)				
Low	38 (40·9)	–	12 (24·0)	–
High	55 (59·1)	–	38 (76·0)	–
Tumour risk score - no. (%)				
Low-risk NMIUC[b]	36 (38·7)	–	12 (24·0)	
High-risk NMIUC[c]	48 (51·6)	–	20 (40·0)	
MIUC[d]	9 (9·7)	–	18 (36·0)	
Urine cytology - no. (%)				
Negative	29 (31·2)	–	8 (16·0)	–
Atypical	6 (6·5)	–	–	–
Low grade	3 (3·2)	–	–	–
High grade	28 (30·1)	–	8 (16·0)	–
Missing	27 (29·0)	–	34 (68·0)	–
Median DNA yield (range) - ng/ml				
US cfDNA[e]	5·0 (0·3–808·9)	6·2 (0·1–1073·9)	–	–
UP cellDNA[f]	55·8 (1·1–460·5)	30·93 (1·9–389·8)	–	–
Plasma cfDNA	20·4 (9·3–8833·3)	20·7 (9·3–4466·7)	–	–

[a] Carcinoma In Situ
[b] Low-risk Non Muscle Invasive Urothelial Carcinoma (pTa/pT1, low grade)
[c] High-risk Non Muscle Invasive Urothelial Carcinoma (pTa/pT1, high grade with any stage associated with CIS)
[d] Muscle Invasive Urothelial Carcinoma
[e] Urine Supernatant cell-free DNA
[f] Urine Pellet cellular DNA

Table 2
Performance of body fluid-based *TERT* promoter mutations in detecting UC.

	DIAGURO Cohort				PORTO cohort
	US cfDNA or UP cellDNA	US cfDNA	UP cellDNA	Plasma cfDNA	UP cellDNA
	(N = 187)	*(N = 176)*	*(N = 184)*	*(N = 148)*	*(N = 100)*
C228T or C250T					
True positive - no	81	72	76	5	33
True negative - no	89	86	88	77	50
False positive - no	5	2	5	1	0
False negative - no	12	16	15	65	17
No data - no	0	11	3	39	0
Sensitivity (95% CI) - %	87·1 (78·6–93·2)	81·8 (72·2–89·2)	83·5 (74·3–90·5)	7·1 (2·4–16·0)	66·0 (51·2–78·8)
Specificity (95% CI) - %	94·7 (88·0–98·3)	97·7 (92·0–99·7)	94·6 (87·9–98·2)	98·7 (93·1–100·0)	100·0 (92·9–100·0)
Positive likelihood ratio (95% CI) - %	16·4 (7·0–38·6)	36·0 (9·1–142·2)	15·5 (6·6–36·6)	5·6 (0·67–46·5)	–
Negative likelihood ratio (95% CI) - %	0·1 (0·1–0·2)	0·2 (0·1–0·3)	0·2 (0·1–0·3)	0·9 (0·9–1·0)	0·34 (0·2–0·5)
Positive predictive value[a] (95% CI) - %	87·6 (83·7–90·6)	93·9 (90·4–96·1)	86·6 (82·8–90·0)	70·0 (56·0–83·4)	100
Negative predictive value[a] (95% CI) - %	94·4 (92·7–95·8)	92·6 (90·7–94·1)	93·1 (91·3–94·6)	71·2 (70·6–72·0)	87·3 (85·4–89·0)
Accuracy[a] (95% CI) - %	92·4 (90·6–94·0)	92·9 (91·1–94·4)	91·3 (89·4–93·0)	71·2 (68·3–74·0)	89·8 (87·8–91·6)
All *TERT* mutations					
True positive- no	81	72	77	5	34
True negative- no	88	85	88	77	49
False positive- no	6	3	5	1	1
False negative- no	12	16	14	65	16
No data - no	0	11	3	39	0
Sensitivity (95% CI) - %	87·1 (78·6–93·2)	80·7 (70·9–88·3)	84·6 (75·5–91·3)	7·1 (2·4–16·0)	68·0 (53·3–80·5)
Specificity (95% CI) - %	93·6 (86·6–97·6)	96·6 (90·4–99·3)	94·6 (87·9–98·2)	98·7 (93·1–100·0)	98·0 (89·3–100·0)
Positive likelihood ratio (95% CI) - %	13·7 (6·3–29·7)	24·0 (7·9–73·3)	15·7 (6·7–37·1)	5·6 (0·67–46·5)	34·0 (4·8–238·9)
Negative likelihood ratio (95% CI) - %	0·1 (0·1–0·2)	0·2 (0·1–0·3)	0·2 (0·1–0·3)	0·9 (0·9–1·0)	0·3 (0·2–0·5)
Positive predictive value[a] (95% CI) - %	85·3 (81·3–88·6)	91·1 (87·3–93·8)	87·0 (83·0–90·1)	70·0 (56·0–83·4)	97.1 (82.9–99.6)
Negative predictive value[a] (95% CI) - %	94·4 (92·6–95·8)	92·5 (90·6–94·0)	93·5 (91·7–95·0)	71·2 (70·6–72·0)	75.4 (67.1–82.1)
Accuracy[a] (95% CI) - %	91·6 (89·7–93·2)	92·1 (90·2–93·7)	91·6 (89·8–93·2)	71·2 (68·3–74·0)	83·0 (74·2–89·8)

US cfDNA: Urine Supernatant cell-free DNA.
UP cellDNA: Urine Pellet cellular DNA.
No data denotes samples that were run with the UroMuTERT assay at least twice with two independent amplification reactions and for which no sequencing reads were obtained.
[a] Positive and negative predictive values were calculated for patients at high risk of developing bladder cancer, estimated at 30% for patients with hematuria or, patients with lower urinary tract symptoms or others according to Springer and colleagues [12].

5. Results

5.1. Characteristics of the Diaguro cohort

The cohort included 94 controls and 93 UC cases, of whom 93·5% had BC; 4·3% had mixture of BC and other urogenital tumours and 2·2% had UTUCC. 90·3% of cases were non-muscle-invasive UC (NMIUC), 40·9% were classified as low-grade tumours and 48·4% diagnosed with primary UC. Overall, cases and controls were balanced with respect to baseline characteristics (Table 1). CellDNA yields in urothelial cells (UP) were higher than cfDNA yields in corresponding urine supernatant (US) ($P < 0·0001$, appendix p 3) and was associated, in cases, with high-risk NMIUC (pTa/pT1 high grade and any stage associated with CIS) ($P = 0·0001$) [18] and with invasiveness ($P = 0·001$) (appendix p 4).

5.2. Performance of urinary UroMuTERT in detecting UC (DIAGURO cohort)

Technical validation showed that UroMuTERT could detect C228T and C250T mutations down to respectively 0.8% and 0.5% Mutant Allelic Fractions (MAFs) at sequencing read depth > 10,000× (appendix pp. 5,6). Sequencing results were available for a total of 594 samples corresponding to US cfDNA ($n = 176$), UP cellDNA ($n = 187$), plasma cfDNA ($n = 148$), and tumour DNA ($n = 83$) at a mean depth of 9092×. The sensitivity of C228T and/or C250T was 81·8% (95% CI 72·2–89·2) for US and 83·5% (95% CI 74·3–90·5) for UP (Table 2). While the false positive rate was lower for the US analysis (2·3%) compared to the UP (5·4%), the US assay performance was hampered by the number of samples with no sequencing data ($N = 11$; 5·8%) which was more frequent than for UP ($N = 3$; 1·6%). US and UP median MAF was 19·2%, (range 0·6%–68·8%) and 23·7% (range 1·0%–75·2%) with 34·7% and 22·1% of cases with MAF < 10% respectively (appendix p 6). In both US and UP, MAFs correlated with the tumour risk-score, with significantly higher mutational load in high-risk (pTa/pT1 high grade and any stage associated with CIS) compared with low-risk NMIUC (Low-grade pTa or pT1 tumours) (appendix p 7). Combined urinary cfDNA and urine pellet DNA analysis outperforms either US or UP considered individually; overall sensitivity of 87·1% (95% CI 78·6–93·2) and specificity of 94·7% (95% CI 88·0–98·3), with no missing data reported (Table 2). However, the differences were not statistically significant. Mutational status in US and UP was concordant in 79 of the 86 cases with sequencing data in both sample types (91·9%), of which 79·1% had *TERT* positive results (Fig. 1 and appendix pp. 8–13 for the list of cases and controls with *TERT* variants). Five cases with mutations in UP were negative in US and two cases with a positive test in the US were negative in the UP, indicating that analysis of multiple sources of urine DNA might increase sensitivity. We noted comparable performance in detecting UC when rare but concomitant mutations to the predominant C228T/C250T detected in ten urinary DNAs of cases (C228A, CC242-243TT and a newly discovered G238A) were considered (Table 2, and appendix p 8–11). There was no indication that detection ability of our urinary *TERT* mutations assay is modified by the primary or recurrent status of UC (appendix p14), neither by the tumour grade, risk score or muscle invasiveness (appendix p 15) and the mutational pattern was equally distributed among those categories (appendix p 16). We assessed the analytical sensitivity on the 83/93 available matched tumours and identified urinary *TERT* promoter mutation(s) in US or UP in 71 of the 72 *TERT* mutated tumours (analytical sensitivity of 98·6%; 95% CI 92·5–99·96). Of the 11 tumours without *TERT* promoter mutations, eight were also negative in urine samples, two were positive for C228T in both US and UP (MAF ranged from 1·1 to 7·0%), highlighting the superiority of the urine in 2·4% of cases to detect tumour-derived mutations that are not necessarily captured by a unique piece of tumour tissue due to clonal heterogeneity. One case showed

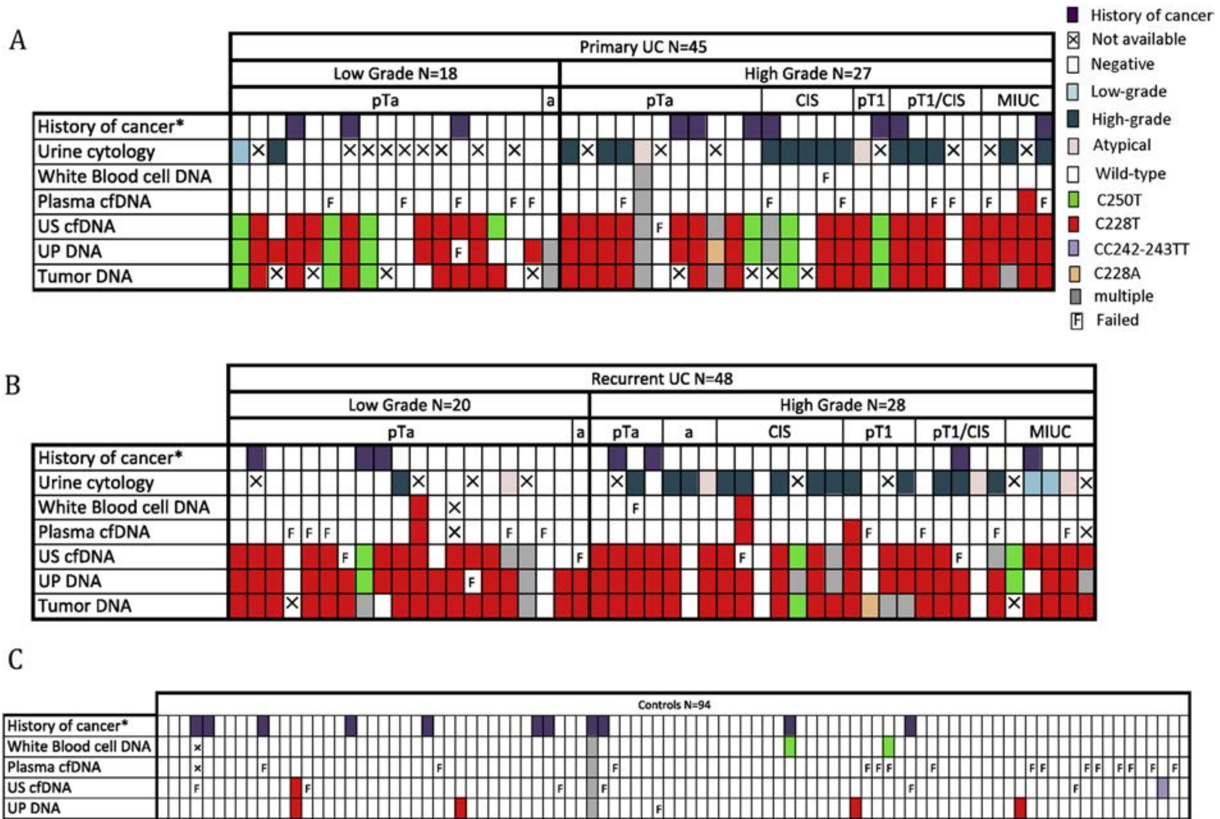

Fig. 1. Overview of the detection of *TERT* promoter mutations by the UroMuTERT assay applied to body fluids and tumours of DIAGURO primary and recurrent UC cases and body fluids of controls. UC denotes Urothelial Carcinoma; US cfDNA denotes Urine Supernatant cell-free DNA; UP DNA denotes Urine Pellet DNA; CIS denotes Carcinoma in situ; MIUC denotes Muscle-Invasive urothelial carcinoma and a stands for pTa/CIS. *other than UC.

discordant results with a mutation C228A detected only in the tumour at 0·4%, likely reflecting a minor tumoral clone undetectable in US or UP.

One of six controls positive in US or UP had a history of prostate cancer. None of mutated controls had however incidental detection of prostate cancer after prostate resection at inclusion ($N = 7$) (Fig. 1). As there was no difference in sensitivity for the detection of primary and recurrent cases (appendix p14), extrapolated PPV and NPV for patients at a hypothetical 30% UC risk, e.g. with haematuria, LUTS or others [12] were calculated on the overall set of data. They were best for US (PPV: 93·9% and NPV: 92·6%) (Table 2) but did not consider missing data ($N = 10$). The combined UP/US analysis overcame these limitations with PPV and NPV of 87·6% and 94·4% respectively.

5.3. Blood-based detection of TERT promoter mutations

In contrast to the urine, a much lower performance was observed for plasma cfDNA (sensitivity of 7·1%; $P < 0·0001$). Importantly, the five cases with mutations in plasma cfDNA scored positive also for US or UP. The detection of concomitant C228T/C228A in plasma cfDNA, US, and UP at consistent levels (mean of 17·3%/0·4% respectively) in a control prompted us to screen WBC to determine the origin of the multiple TERT positivity. WBC tested positive for C228T/C228A at similar levels, which is suggestive of mosaicism or clonal haematopoiesis associated with haematuria. Two additional controls scored C250T positive (0·5% and 5·5% MAF) but negative in UP and US. Three cases tested positive in WBC DNA (Fig. 1) and plasma cfDNA at similar AFs, and in US, UP and tumour DNA, one of which with C228T levels consistent with a germline or non-clonal mosaicism (MAF range 32·6%–45·8%). In the 2 other cases MAFs were higher in urine (4- and 6-fold) and tumours (2- and 14-fold) than in WBC and plasma, suggesting a dual contribution of clonal or non-clonal mosaicism and tumorigenesis to the urinary mutational load (appendix pp. 8–11).

5.4. Urinary TERT promoter mutations detection in primary UC (IPO-PORTO cohort)

The reproducibility of urinary UroMuTERT was assessed in an independent cohort of 50 primary UC cases and 50 healthy controls, where only urine cellDNA was available (Table 1). 76% of the tumours were classified as high-grade and 64% categorized as NMIUC. The overall sensitivity was 68·0% (95% CI 53·3–80·5) with specificity of 98.0% (95% CI, 89·3–100·0; Table 2). Of the 16 negative cases, 7 were low-grade and 9 were high-grade tumours (appendix pp. 17–19). While no difference in sensitivity of detecting primary or recurrent UC was observed in the DIAGURO cohort (86·7% and 87·5%, appendix p 14), the 68% estimate observed in the PORTO cohort was compared to the sensitivity obtained in the same conditions, e.g. for DIAGURO primary UC detected with cellDNA only (84·1%; 95% CI, 70·0–93·4) and not to the best estimate obtained with the combined urinary cfDNA/cellDNA (87·1%). A borderline non-significant 16·1% difference in detecting primary UC with cellDNA between the two cohorts was observed ($P = 0·07$).

5.5. Comparison of the UroMuTERT performance with urine cytology

Urine cytology data were available in 66/93 (71·0%) and in only 16/50 (32·0%) of UC DIAGURO and IPO-PORTO cases respectively and is therefore discussed in the former case. Urine cytology was positive or atypical in 37/66 cases (56·1%). We found no association between *TERT* positivity in urine and cytology classification (appendix p 15, $P = 0·636$). Sensitivity of UroMuTERT (combined urinary cfDNA/cellDNA) in detecting low-risk NMIUC was significantly higher (86·1%) than that of urine cytology (23·0%, $P < 0·0001$, Fig. 2), whereas

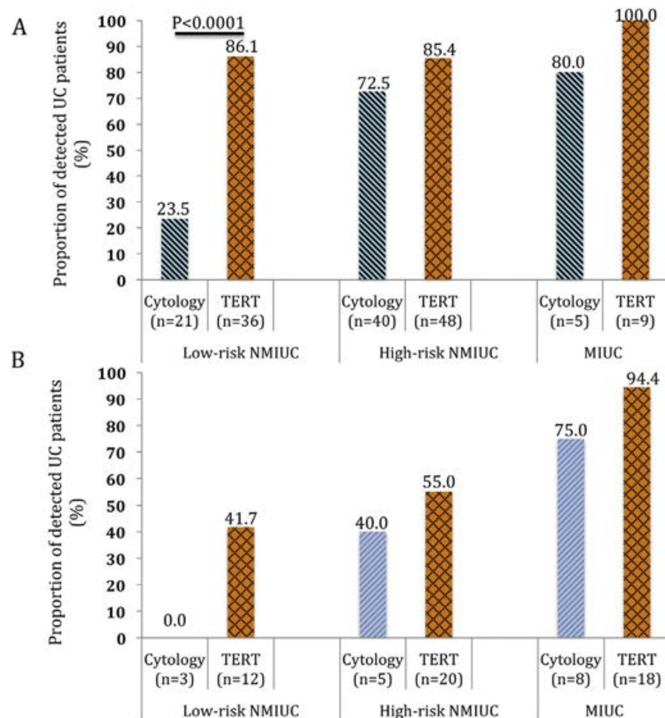

Fig. 2. Performance of cytology and urinary *TERT* promoter mutations in detecting various risk categories of UC in the DIAGURO (A) and the PORTO (B) cohorts. Tumours are categorized in three groups: low-risk non–muscle- invasive urothelial cancer (NMIUC) (pTa/pT1, low grade), high-risk NMIUC (pTa/pT1 high grade and any stage associated with CIS), and muscle- invasive urothelial MIUC (pT2, pT3 or pT4). Risk classification of NMIUC was adapted from Millan-Rodriguez and colleagues [18]. A patient was considered mutated when C228T and/or C250T was found in Urine supernatant cfDNA (US) or in urine pellet cellDNA (UP).

no difference was observed in detecting high-risk NMIUC or MIUC. In the DIAGURO cohort, combined UroMuTERT and urine cytology enabled the detection of 62/66 cases compared to the UroMuTERT only where 59 patients had urine positive test(s) (sensitivity: 93·9%; 95% CI 85·2–98·3 versus 89·4%; 95% CI 79·4–95·6 respectively).

6. Discussion

In this study, we developed UroMuTERT, a simple, non-invasive and sensitive assay with detection thresholds of 0·8% and 0·5% MAFs for C228T and C250T mutations respectively. We evaluated its clinical validity for the detection of UC against urine cytology. Our case-control study shows excellent clinical sensitivity (87·1%), specificity (94·7%) and analytical sensitivity (98·6%) of a single-gene urinary biomarker based on tumour-derived *TERT* promoter mutations for the detection of all forms of UC. The diagnostic performance was best for urinary cfDNA and cellDNA combined. In addition, the ability of UroMuTERT to quantify low-level mutations down to 0·5% enabled the detection of a significant proportion of cases with MAF < 5% (26·4% in US and 13·0% in UP) and is therefore a critical parameter for accurate detection and enhanced sensitivity. Analysing additional rare *TERT* promoter mutations did not improve UroMuTERT performance, as they were concomitant to the prevalent C228T and/or C250T.

In previous studies, sensitivities and specificities of the same markers tested on alternative assays and only in exfoliated urothelial cells (cellDNA) varied from 55% to 62% and from 90% to 99% respectively in patients with incident or early BC and from 42% to 57% and 73% to 90% respectively in patients with recurrent BC [8,9,12,13]. Two studies reported sensitivity of 80% using pre-surgery urine cellDNA but no precision was given on the primary or recurrence status [10,11]. Our UroMuTERT assay demonstrated comparable performance to that of recently developed UroSEEK multiple markers assay (including C228T and C250T) for the detection of primary or early UC (sensitivity of 86·7% versus 83%; Specificity of 94·7% versus 93%) and higher sensitivity for the detection of UC recurrence (87·5% versus 68%) [12].

Previously reported to be mutually exclusive mutations in UC [9,11], we revealed co-occurrence of urinary *TERT* promoters mutations in UC in 10·7% of UC cases, similarly to what has recently been reported in a minority of early-onset bladder tumours [19].

Importantly, our *TERT* mutation biomarkers achieved high specificity against patients with urological pathologies other than UC (including incidental prostate cancer cases) who may benefit from UroMuTERT screening as the symptoms may be similar to the ones observed in UC cases.

Consistent with previous findings [10], the added diagnostic value of the urinary *TERT* promoter mutations as biomarkers was particularly evident for the detection low-risk NMIUC as compared to urine cytology (sensitivity of 86·1% versus 23·5%), where cytology demonstrated poor performance [4]. Combined UroMuTERT and cytology assays improved sensitivity up to 93·9%, allowing detection of 4·5% cases that were negative for UroMuTERT, as similarly observed when combining the UroSEEK assay and cytology for the detection of primary UC (95%) and recurrence (71%) [12].

The UroMuTERT (cfDNA or cellDNA) PPV of 87·6% and NPV of 94·4% extrapolated to at high-risk subjects of developing UC (30% estimated risk [12], see materials and methods) reached 88·4% and 97·4% respectively when combined with cytology and assuming 100% specificity for cytology. Because the risk estimate of 30% may be inflated by the study design, we also considered lower hypothetical risks of 20% and 5% for UC in patients with haematuria [20] and micro-haematuria [21], and obtained PPVs of 81·4% and 48·0% and NPVs of 98·4% and 99·7% respectively, which still demonstrates the superior diagnostic value of combined urinary UroMuTERT and cytology. However, the predictive values of the biomarkers must be accurately assessed in large well-defined high-risk group populations before urological societies may reconsider recommendations for UC screening in such groups [23]. We expect UroMuTERT to change UC detection by complementing urine cytology or replacing urine-based markers which lack performance for clinical utility [22]. Its high accuracy for early-stage UC should improve timely transurethral tumour resections, which in turn will contribute to reduced risk of progression and improved patients' survival. The high NPV in high-risk UC individuals may provide evidence for a reliable substitute to unnecessary cystoscopies to patients with negative tests, avoiding discomfort and risk of complications associated with invasive procedures while reducing high cost of clinical management of suspected UCs and patient non-adherence to screening or surveillance [24,25]. We anticipate that UroMuTERT could target individuals at high-risk or under surveillance for UC recurrence and that only patients with urinary TERT positive tests or with positive cytology results will undergo cystoscopy, such as proposed in the strategy integrating UroMuTERT to current medical standards for UC management (Fig. 3).

The sensitivity of our biomarkers in plasma is poor, reflecting low amounts of tumour-derived mutations in UC patients, which is consistent with recent studies [26]. This clearly indicates the superiority of urine over plasma as the reservoir of UC-derived DNA biomarkers, logically reflecting a prominent concomitant mechanism of urothelial cell carcinoma desquamation and cfDNA release into the urine, which is in close vicinity to the tumour as compared to the release of circulating tumour DNA into the circulating bloodstream. In addition, *TERT* positivity in plasma cfDNA can be confounded with leucocyte-derived mutations. We report for the first time, the rare occurrence of *TERT* promoter mutations in the white blood cells DNA, plasma and urine samples in three UC cases and one control, likely reflecting somatic mosaicism (clonal haematopoiesis or post-zygotic mosaicism) in the control and a probable synergetic mechanism of mosaicism with tumorigenesis in the cases. Its influence on the UC development should be further investigated. In addition, although never reported for C228T but for *TERT*

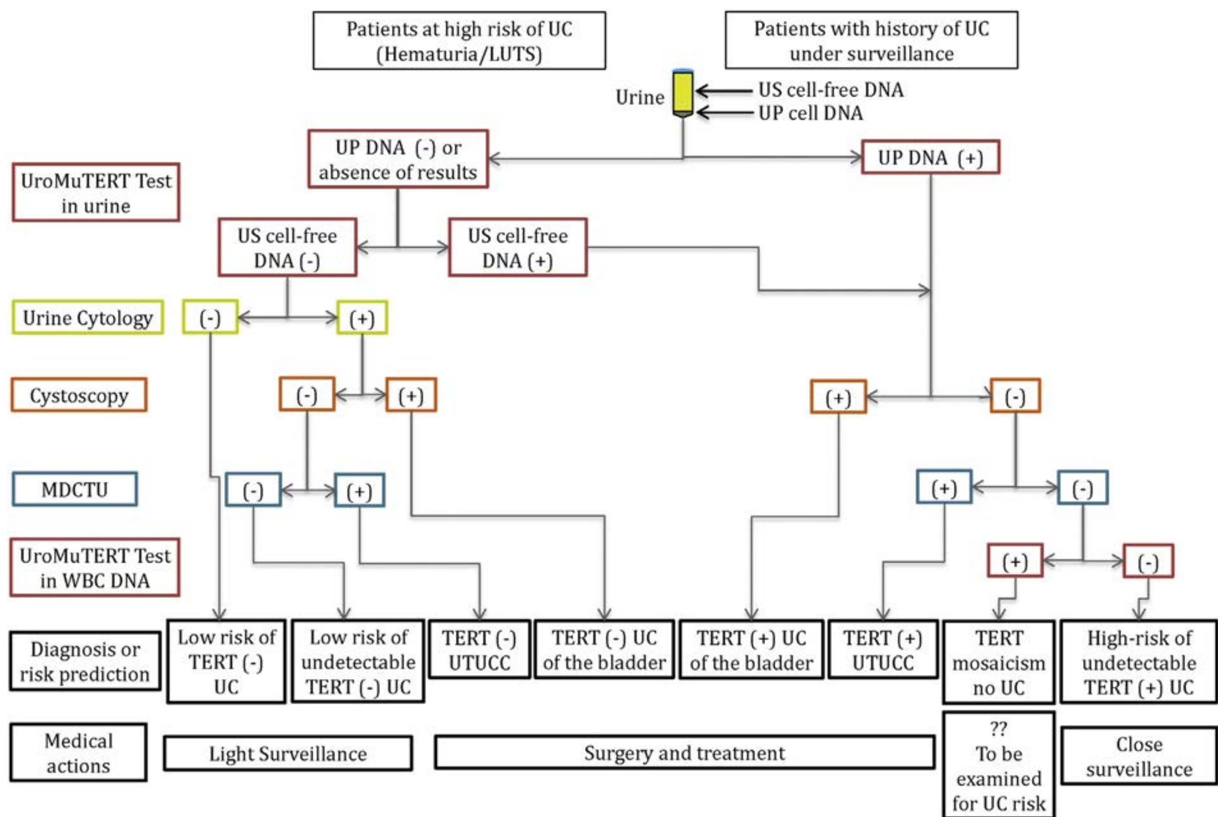

Fig. 3. Proposed strategy integrating urinary *TERT* mutations analysis to current medical standards for the management of UC of the bladder and of the upper urinary tract. UC denotes Urothelial Cancer; UTUCC denotes Upper tract urothelial cell carcinoma; LUTS denotes Lower urinary tract symptoms; US denotes Urine Supernatant; UP denotes Urine Pellet; WBC denotes White Blood Cells; MDCTU denotes Multidetector Computed Tomographic Urography.

c.-57T>G in familial melanoma [27], we cannot exclude that the C228T identified in one case at 35–40% MAF is of germline origin. Blood-based clonal mosaicism has been associated with age and increased risk of solid and haematological cancers [28] and recent evidence showed that blood-based mutations could predict the risk of acute myeloid leukaemia years prior to the disease onset [29]. Rare mosaicism in patients with UC has been observed [30] and may therefore add a layer of complexity in the interpretation of an urinary *TERT* positive test with negative subsequent cystoscopy or urography. In such scenario, screening leucocyte DNAs should infer about patients with *TERT* mosaicism or germline variants, which may, if proven to be at higher risk by future studies, benefit from regular urinary *TERT* mutations testing (Fig. 3).

One limitation of our study is that we could not assess paired urinary cfDNA and cellDNA in the replication Portuguese series. Focusing on urinary cells we found a non-significant lower sensitivity in detecting Portuguese primary UC (66·0–68·0%) than French primary UC (84·0%), raising the question of whether the sensitivity is upward biased in the initial French series or whether there are differences in sampling or in *TERT* promoter mutations frequency between the two cohorts or whether it results of a combined effect. While there is limited evidence that indicates that *TERT* promoter mutations frequency in UC varies according to geographical regions or under specific conditions or exposures, the origin of potential differences that impact the biomarker sensitivity should be addressed in large multi-cohort studies.

In conclusion, our study demonstrates unprecedented performance of a single-gene assay quantifying tumour-derived *TERT* promoter mutation load in urine for the detection of all forms of UC and lays the foundations for large-scale validation and clinical utility studies for implementation into clinical practice. The role of rare *TERT* promoter mutations in leucocytes on UC development and its impact on the clinical use of the biomarkers should be further examined.

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Declaration of interests

we declare no competing interests.

Author contributions

AM and FLCK supervised the project. AM, GB, EW, JM, CJ, GS, FLCK contributed to the study design. AM, EV, SM, NF, BDT, GP, SMR, RH, CJ contributed to recruitment of participants and collection of samples and medical data. GD, NF, OL, PF, CC designed and conducted the experiments. CV, TMD, MF did the bioinformatics data analysis. PHA and GB did the statistical analysis. BAA conducted pathological examinations. PHA, MZ, MIH, JM, CJ, GS AND FLCK interpreted the validation set. PHA and FLCK wrote the manuscript. All authors reviewed the manuscript and approved the final version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.05.004.

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