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Short Communication

Evaluation of TERT promoter mutations in urinary cell-free DNA and sediment DNA for detection of bladder cancer

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ABSTRACT

Background: Cell-free DNA (cfDNA) is proposed to be a valuable source of biomarkers in liquid biopsies for various diseases as it is supposed to partially originate from tumor cells. However, data about the diagnostic implications of cfDNA in urine for the detection of bladder cancer (BCa) is sparse.
Methods: We evaluated the usability of urinary cfDNA for diagnostic purposes compared to urine sediment DNA (sDNA) in 53 BCa patients and 36 control subjects by analyzing two abundant point-mutations (C228T/C250T) in the *TERT* promoter using Next-Generation Sequencing.
Results: Mutations were detected in 77% of the urinary sDNA compared to 63% of the cfDNA samples. Moreover, the *TERT* mutation allele frequencies (MAF) were highly correlated in cfDNA and sDNA. In comparison, the accuracy of the *TERT* assay was higher in sDNA (84%) compared to cfDNA or voided urine cytology (both 77%). Interestingly, MAFs from leukocyte-rich urines were higher in cfDNA than in sDNA, indicating a diagnostic advantage of cfDNA in such urines.
Conclusions: Urine-based mutation detection has the ability to augment and surpass voided urine cytology as the current gold-standard for the non-invasive detection and surveillance of BCa. The analysis of cell-free DNA provides no general diagnostic advantage compared to urine sediment DNA.

1. Introduction

The goal of many urine-based tests for the diagnosis of primary and recurrent bladder cancer (BCa) is the substitution of flexible cystoscopy as an invasive and costly standard diagnostic procedure with < 100% accuracy [1]. Diagnostic tests for the mutation assessment in urine are typically based on urine sediment DNA (sDNA). Advancements in DNA isolation and novel methods such as next-generation sequencing (NGS) also allow the sensitive detection of mutations in cell-free DNA (cfDNA) isolated from urine supernatant. Initial findings supported the hypothesis that urinary cfDNA is enriched with circulating tumor DNA (ctDNA) in urological cancer patients and may hence improve the sensitivity of current urine-based assays [2,3]. In BCa, *TERT* promoter mutations have been identified as the most common recurrent alteration with significance for patient survival and disease recurrence [4]. The detection of *TERT* mutations in BCa patients was already demonstrated in both sDNA [5,6] and cfDNA extracted from urine [7,8]. In order to better understand factors associated with the usability of urinary cfDNA and sDNA as substrates for the detection of BCa, we systematically evaluated these DNA-sources based on the detection of *TERT* promoter mutations, related to clinical and histopathological parameters such as tumor stage and mass, multifocality, as well as on numbers of leukocytes and bacterial load in urine.

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2. Material and methods

2.1. Patients

A total of 89 patients and control subjects were enrolled between February 2016 and September 2017. All participants provided written informed consent prior to study entry. The study was approved by the institutional review board of the Medical Faculty of the Technische Universität Dresden (EK194092004 & EK96032012). 71 patients were admitted for cystoscopy to the Department of Urology of the University Hospital Dresden to rule out BCa due to the occurrence of hematuria and/or suspicions cystoscopy or imaging findings. Of these, 53 patients were histopathologically positive for BCa and 18 patients were negative (noBCa) and allocated to the control group. Furthermore, a second control group comprising 18 patients with urolithiasis was included to increase statistical validity for sDNA and cfDNA analysis. Urolithiasis can be accompanied by hematuria similar to BCa wherefore this condition might serve as suitable control. Demographic characteristics and pathological findings of patients and control subjects are shown in Supplementary Table 1.

2.2. Processing of urine and biopsy samples

Patients with suspicion of BCa underwent standard urological evaluation including cystoscopy and voided urine cytology (VUC). Detailed descriptions of all methods for processing of urine and biopsy samples are shown in Supplementary Data 1. Briefly, spontaneous urine samples were collected before any intervention. The presence of erythrocytes, leukocytes and bacteria was assessed by urine test strip analysis and by routine microscopic analysis of the sediment. A VUC specimen was prepared according to standard procedures. After centrifugation of the remaining urine, the supernatant and the cellular pellet were stored at −80 °C until processing. From all eligible formalin-fixed paraffin-embedded (FFPE) transurethral resections of the bladder (TUR-B) samples ($n = 50$) a fresh hematoxylin and eosin-stained reference section was re-reviewed by an experienced pathologist (US). The stratification of tumor grades and stages was done according to the WHO classification from 2004 [9] and the TNM classification from 2011 [10]. Tumor stage, grade and cell content of all 50 eligible tumor samples and 8 additional histopathologically tumor-free samples are provided in Supplementary Table 3.

2.3. DNA isolation and quantification

Isolation of cfDNA from frozen urine supernatants was carried out with the QIAamp viral RNA Mini kit (Qiagen, Germany). Corresponding sDNA and FFPE-DNA from tumor tissues were isolated using standard procedures (Supplementary Data 1). Resulting DNA was quantified by a β-globin-specific quantitative PCR.

2.4. PCR amplification and sequencing of the TERT promoter region

The *TERT* promoter was analyzed for two C > T transition mutations (chr.5 1,295,228/C228T and chr.5 1,295,250/C250T) according to optimized conditions for the NGS-based detection of low-level point mutations as described previously [11] and in Supplementary Data 1. Briefly, a nested PCR procedure was used to amplify the proximal *TERT* promoter covering nucleotide positions chr5:1295228C > T and 1,295,250C > T (hg19) referred to as C228T and C250T. All primer sequences and PCR conditions are given in Supplementary Data 1. After purification and quantification, barcoded PCR products were sequenced unidirectionally on an Ion Torrent PGM semiconductor-based device (Life Technologies). Data were analyzed using the Torrent Suite Software version 3.2 and the Torrent Variant Caller plugin and alignment to the hg19 human reference genome (http://genome.ucsc.edu/). According to false-positive rates, NGS-based ctDNA detection was conducted with a defined cut-off of 0.4% for the C250T and 0.75% for the C228T mutations. Mutation allele frequencies (MAFs) below the predefined thresholds were considered as wild-type. To assess differences in cfDNA concentrations and MAFs, the non-parametric two-tailed Mann Whitney *U* test was applied. All calculations were performed with Prism 5 (GraphPad, San Diego, USA) and SPSS Statistics 25 (IBM, New York, USA).

3. Results and discussion

Urinary cfDNA could be extracted from samples with median cfDNA yields of 3.6, 2.8 and 14.7 ng cfDNA per ml urine supernatant for the noBCa, urolithiasis and BCa groups, respectively. Compared to all control groups cfDNA amounts were significantly enriched in BCa samples with tumor stage pT1 and ≥pT2 ($p < 0{,}001$), but not with tumor stage pTa (Supplementary Fig. 1/Supplementary Table 2). In total, 83 of 89 (93%) urine samples (52/53 BCa and 31/36 control samples) allowed cfDNA yields above 1 ng DNA and could be analyzed. From the respective 50 eligible FFPE tumor tissues, 43 were positive for one of the two typical *TERT* promoter mutations (86%) (Supplementary Table 3). This is in the range of frequencies previously reported for *TERT* promoter mutations in BCa (65–86%) [4,12]. Of the corresponding 52 urine samples, the two mutations were detected in 40 sDNA (77%) and 33 cfDNA samples (63%), respectively. The comparison of the detected MAFs in urine and tissue samples showed high concordance rates of the FFPE tumor tissue with urinary sDNA (88%) and cfDNA (78%), as well as between urinary cfDNA and sDNA (82%), pointing to a good overall concordance of the DNA profiles in the different substrates (Supplementary Fig. 3). In the control group one of the 15 urolithiasis samples was C228T-positive in sDNA, but not in cfDNA (Fig. 1). Additionally, the C228T mutation was detectable in the FFPE-DNA of three noBCa samples, but not in urine. It could not be clarified, whether these patients developed BCa later. Likewise, *TERT* mutations were already detected in urine-DNA of patients without clinical evidence of BCa [1,12]. The role of *TERT* mutations as early event in urothelial neoplasia and their potential relevance as biomarkers for early disease detection was reported previously [4]. Generally, detection of *TERT* mutations in cfDNA and sDNA was consistent ($r_s = 0.675$; $p < 0.001$), confirming recent reports on a positive correlation between tumor content in the two types of DNA [8]. The resulting MAFs of samples positive for one of the *TERT* mutations were applied as a measure for the relative proportion of tumor-derived cfDNA and sDNA. The MAFs of the combined *TERT* mutations ranged between 2.2% and 71.3% (median = 23.4%) in cfDNA and between 1.5% and 77.3% (median = 24.7%) in sDNA. Initial studies indicated that urinary cfDNA may represent an improved resource for genomic profiling, due to a higher tumor genome burden and key genomic biomarkers, respectively, compared to cellular sDNA [2,3]. Our data did not indicate a significant enrichment of ctDNA in cfDNA compared to sDNA in urine samples (Fig. 1/Supplementary Figs. 2 & 3). However, in line with previous findings [8,13], the proportion of ctDNA showed a slight, but insignificant trend to increase with tumor grade and tumor stage (Fig. 1), but it did not correlate with tumor mass or multifocality (Supplementary Fig. 4). Interestingly, in contrast to the general trend, DNA extracted from leukocyte-rich urines (> 50 cells/µl) showed higher median MAFs in cfDNA than in sDNA, indicating a potential diagnostic advantage of cfDNA in such urine samples due to dilution effects within the cellular fraction (Fig. 1). However, differences were not statistically significant due to small number of patients ($n = 8$) in this subgroup and confirmation in a larger cohort is clearly needed. Overall diagnostic sensitivity of our *TERT* assay was 0.77 in sDNA (Table 1), which is comparable (0.80) [5] or superior (0.55 and 0.62) [6,12] to sensitivities recently reported for the detection of *TERT* mutations in urine sediment. In contrast, sensitivity was lower using cfDNA and VUC. Specificity of the *TERT* assay was very high with 0.97 and 1.00 in sDNA and cfDNA, respectively, compared to 0.93 for VUC.

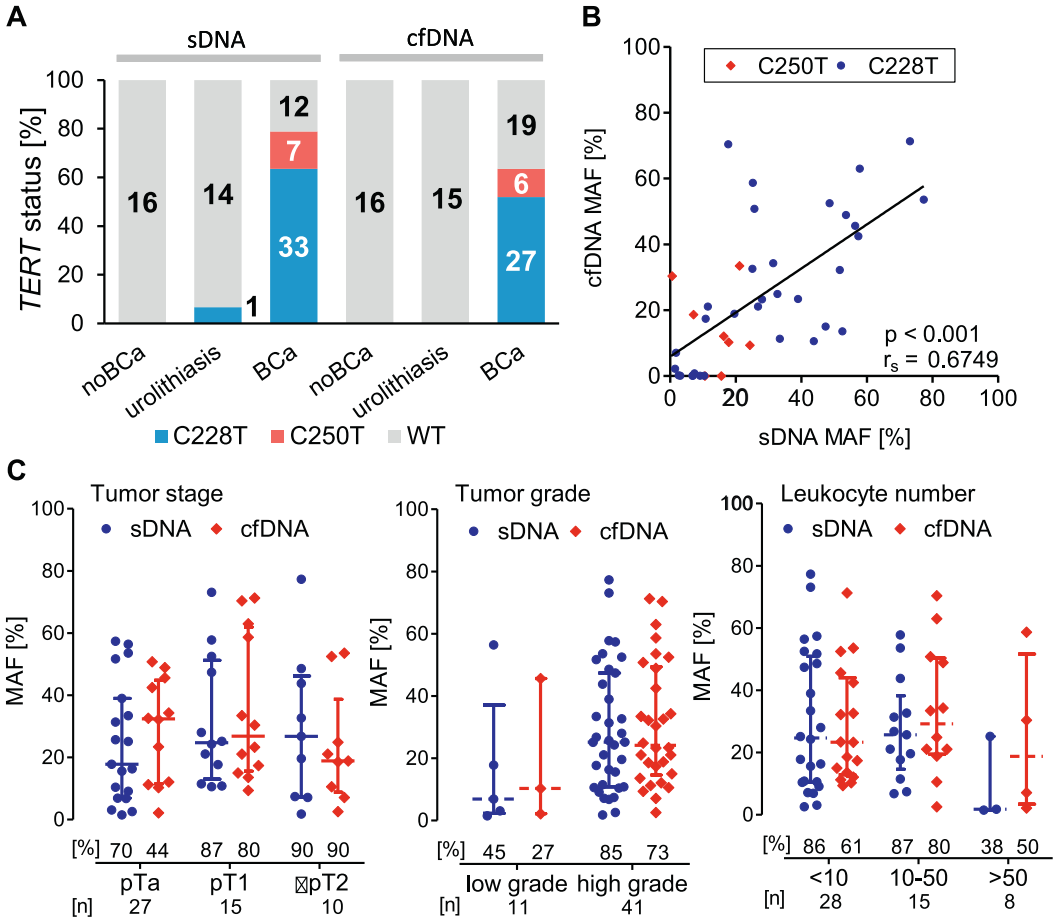

Fig. 1. Detection of the *TERT* mutations C228T and C250T in urinary sDNA and cfDNA.
A) Overview (percentage and total number) of the detected *TERT* promoter mutations C228T and C250T, as well as wildtype (WT) promoters in sDNA and cfDNA samples per group.
noBCa: histopathologically unconfirmed BCa suspicion.
B) Spearman's rank correlation coefficient (rs) between MAFs of the *TERT* promoter mutations C228T (blue) and C250T (red) in urinary sDNA and cfDNA from BCa patients. Samples were sequenced with a median coverage of 25,238 reads (range 10,693–97,893).
C) MAFs of *TERT*-mutated samples in sDNA and cfDNA from BCa patients in dependence on tumor stage, tumor grade and leukocyte number in urine samples. Leukocyte number (in cells/µl) was available for only 51 of the 52 tumor-derived urine samples. MAF distributions are shown in dot plots with median values and interquartile range as horizontal lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Diagnostic performance of the *TERT* assay in cfDNA and sDNA in comparison to VUC.

	***TERT* assay**		**VUC**
	sDNA	**cfDNA**	
Sensitivity	**0.77**	0.63	0.67
Specificity	0.97	**1.00**	0.93
PPV	0.98	**1.00**	0.95
NPV	**0.71**	0.62	0.62
pLR	23.85	NA	10.10
nLR	**0.24**	0.37	0.35
Accuracy	**0.84**	0.77	0.77

Sensitivity, specificity, positive and negative predictive value (PPV, NPV), positive and negative likelihood ratio (pLR, nLR) and accuracy are given for the *TERT assay* for all analyzed urine samples in comparison to voided urine cytology (VUC). The best overall values are marked in bold. Values were calculated according to standard statistical methods.

Finally, the accuracy of the *TERT* assay in sDNA was clearly higher (84%) compared to cfDNA or to voided urine cytology (both 77%) (Table 1). Presumably, the slightly lower diagnostic performance of the mutation assay in cfDNA was partially due to the lower cfDNA extraction yields compared to sDNA. Although median concentrations of sDNA and cfDNA were in the same order of magnitude in urine (42.8 ng sDNA/ml vs 14.68 ng cfDNA/ml for the BCa group), extraction yields of both substrates differed considerably due to different urine volumes used for extraction of cfDNA (median = 280 µl urine supernatant according to vendors recommendations) and sDNA (median = 33 ml of total urine). Accordingly, median extraction yields of sDNA significantly exceeded yields of cfDNA (~10 to 100-fold). Assuming a similar tumor content in both substrates (Fig.1), higher yields of sDNA used for PCR amplification will automatically correlate with higher numbers of mutant molecules that can potentially be detected. Isolation of cfDNA remains challenging and needs further optimization and probably larger urine volumes. In addition, the use of sDNA may be favorable in clinical settings due to advantages with respect to costs and sample processing. The *TERT* assay could play a role in the non-invasive detection and surveillance of BCa, especially if the evidence for a relation between *TERT* promoter mutations and prognosis [7] should be affirmed.

4. Conclusions

The detection of *TERT* mutations is feasible in urinary cfDNA, but it

provides no general advantage for the non-invasive detection of BCa compared to the use of sDNA. However, the analysis of cfDNA might provide advantages in patients with urinary infections or other reasons of leukocytes in the urine. In general, the performance of the *TERT* assay is superior compared to VUC. Further studies with larger cohorts of patients are needed to confirm the diagnostic performance of the *TERT* assay and to determine the potential to surpass voided urine cytology for the non-invasive detection of bladder cancer.

Conflicts of interest

CT is CEO and co-owner of AgenDix GmbH, a company performing molecular diagnostics. The remaining authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2018.11.009.

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