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Comparative study of *TERT* gene mutation analysis on voided liquid-based urine cytology and paraffin-embedded tumorous tissue☆,☆☆

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A B S T R A C T

Noninvasive reliable urine-based screening method for detection of urothelial carcinoma (UC) is still highly elusive. Recently, studies have shown the presence of telomerase reverse transcriptase (*TERT*) gene mutation in a high number of UCs. This finding can be used as a marker in screening voided urine samples. The aim of this study was to assess sensitivity of *TERT* mutation in detecting UC between liquid-based cytology (LBC) voided urine and formalin-fixed, paraffin-embedded neoplastic tissue (FFPE). Voided urine of 29 patients was collected before surgery via LBC. Subsequently, neoplastic tissue from transurethrally resected tumors of the same patients was analyzed. Both LBC and paraffin-embedded tissues were analyzed independently for the *TERT* gene mutation using Sanger sequencing and next-generation sequencing. Using Sanger sequencing, *TERT* mutation was detected in 17 of 29 samples of voided urine, whereas 4 cases showed weak positivity. Of 17 patients with *TERT* mutation, 6 had mutation in C250T and 11 in C228T. Using next-generation sequencing, 19 of 28 LBC (1 case was not suitable for analysis) were positive for *TERT* mutation, of which 5 contained C250T mutation and 14 had C228T mutation. Sanger sequencing was performed in all 29 resected UC cases. *TERT* gene mutation was found in 21 cases in FFPE, for which 6 tumors had mutation in C250T, and C228T mutation was found in the remaining 15 tumors. *TERT* promoter mutation is not positive in all UCs, and that negative result in LBC samples does not exclude the possibility of UC. It is evident from our results that there is 100% agreement of results between the material from FFPE and the corresponding LBC material in cases of high-grade UC. In contrary, the agreement rate between results of FFPE and LBC material (analyzed by Sanger sequencing or next-generation sequencing) varied in low-grade lesions. The use of such a test is more clinically relevant for detecting recurrence in the surveillance setting such as known UC patients with associated *TERT* promoter mutation (from routine-processed histologic samples).
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1. Introduction

Voided urine cytology is used for screening patients with hematuria, follow-up in patients with known urothelial carcinoma (UC), and detection of "flat" lesions (ie, UC in situ). Two types of cytologic techniques used for voided urine screening include conventional cytology (CC) and the relatively new technique liquid-based cytology (LBC). The main difference between the 2 methods is in processing of the material. Both methods serve, among others, to detect neoplastic cells in urine, but their predictive value is very limited, especially for detecting low-grade UC. Therefore, cystoscopy still remains the standard method for the detection of early low-grade bladder neoplasms.

Great efforts have been expended on exploring urine-based tests/biomarkers for the noninvasive detection of UC, with particular success being achieved measuring tumor-specific nucleic acid variants. Two of the most frequently mutated genes in bladder cancer with point mutation hotspots are *FGFR3* and *TERT*, which have been assessed as biomarkers for detecting UC in urinary DNA in different studies [1]. The *TERT* promoter is mutated in approximately 65% of bladder tumors regardless of stage and grade and represents the best single biomarker for UC for detecting primary bladder tumors [1].

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We designed a comparative study to assess sensitivity of *TERT* mutation in detecting UC between LBC voided urine and formalin-fixed, paraffin-embedded (FFPE) neoplastic tissue.

2. Materials and methods

Twenty-nine patients who suspected to have either primary or recurrence UC were prospectively enrolled in the study. These patients had abnormal finding during dispensary cystoscopy (patients with recurrent UC) and/or had positive findings on ultrasonography or computed tomographic scan with initial presentation of macroscopic hematuria.

Voided urine was collected before surgery and fixed using LBC technique. Subsequently, transurethral resection of neoplastic tissue was performed and was fixed in 4% formaldehyde and embedded in paraffin using routine procedures. Two-micrometer-thin sections were cut and stained with hematoxylin and eosin. Grading system according to the 1973 World Health Organization (WHO) as well as the most recent 2016 WHO classification was used in all cases [2,3].

Both samples from LBC and FFPE tissues were analyzed independently for the *TERT* gene mutation using Sanger sequencing (SS) and next generation sequencing (NGS).

2.1. DNA extraction and quality control

DNA from FFPE tissue was extracted using a QIAsymphony DNA Mini Kit (Qiagen, Hilden, Germany) on the automated extraction system (QIAsymphony SP; Qiagen) according to the manufacturer's supplementary protocol for FFPE samples (purification of genomic DNA from FFPE tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution). The content of each LBC vial was centrifuged at 600*g* for 10 minutes, the pellet was resuspended in 600 μL of phosphate-buffered saline, and DNA was isolated using the QIAsymphony DNA DSP mini kit. Concentration and purity of isolated DNA were measured using NanoDrop ND-1000 (NanoDrop Technologies Inc, Wilmington, Delaware). DNA integrity was examined by amplification of control genes in a multiplex polymerase chain reaction (PCR) [4].

2.2. Mutation analysis by SS

Mutation analysis of part of *TERT* promoter was performed using PCR and direct sequencing. Polymerase chain reaction was carried out using primers shown in Table 1. The reaction conditions were as follows: 12.5 μL of HotStar Taq PCR Master Mix (Qiagen), 10 pmol of each primer (Table 1), 10 ng of template DNA, and distilled water up to 25 μL. The amplification program consisted of denaturation at 95°C for 15 minutes and then 40 cycles of denaturation at 95°C for 1 minute, annealing at 65°C for 1 minute, and extension at 72°C for 1 minute. The program was finished with 72°C incubation for 7 minutes. The PCR products were checked on 2% agarose gel electrophoresis. Successfully amplified PCR products were purified with magnetic particules Agencourt AMPure (Agencourt Bioscience Corporation, A Beckman Coulter Company, Beverly, Massachusetts). Products were then bidirectionally sequenced using Big Dye Terminator Sequencing kit (Life Technologies, Foster City, California), purified with magnetic particules Agencourt CleanSEQ (Agencourt Bioscience Corporation), all according to the manufacturer's protocol, and run on an automated sequencer ABI Prism 3130xl (Life Technologies) at a constant voltage of 13.2 kV for 11 minutes.

Table 1
Primers used for amplification of hotspot *TERT* mutation [23]

Name	Primer sequence 5′-3′
TERT-F2	CACCCGTCCTGCCCCTTCACCTT
TERT-R2	GGCTTCCCACGTGCGCAGCAGGA

2.3. Mutation analysis by NGS

Targeted sequencing of LBC urine samples was performed on the Ion Torrent PGM platform (Life Technologies) with Hi-Q Sequencing Kit (Life Technologies). Sequencing library was prepared by ligation of adapters to the same PCR products that were used for SS using Ion Plus Fragment Library Kit (Life Technologies).

Sequencing data were processed by the Torrent Suite Software V5.0.2 and analyzed via the Integrative Genomics Viewer. The minimum coverage depth for each sample was set to 500× with the cutoff value for positive samples set to 2.5% [1].

3. Results

Table 2 presents clinicopathologic data on 29 patients enrolled in this study. Thirteen patients had recurrent UC, whereas 16 were newly diagnosed UC cases. Patients' age ranged from 35.2 to 87.4 (median, 69 years; mean, 68.6 years), with 26 being male and 3 female.

Urothelial carcinoma was confirmed histologically in all 29 cases. Pathologic staging included pTa in 19 cases, pT1 in 9 cases, and pT2a in 1 case. Grade 1 (WHO 1973) or low-grade (WHO 2016) was found in 14 cases, grade 2 (WHO 1973) or low-grade (WHO 2016) in 5 cases, and grade 3 (WHO 1973) or high-grade (WHO 2016) in 10 cases [2,3].

Results of molecular genetic analyses are summarized in Table 3. Detection of *TERT* mutation in LBC was performed in all 29 cases—using both SS and NGS methods. Sanger sequencing detected *TERT* mutation in 17 of 29 cases (4 case with weak positivity). Six of 17 mutation C250T, and 11 of 17 had mutation C228T. Analysis with NGS methods was performed in 28 LBC (1 case was not suitable for analysis), which demonstrated positivity in 19 cases. Five of 19 LBC contained C250T mutation and 14 of 19 had C228T mutation. Sanger sequencing of FFPE was performed in all 29 cases and showed *TERT* gene mutation in 21 cases. Six of 21 tumors had mutation in C250T, and C228T mutation was found in 15 of 21 tumors.

Among the low-grade UC cases, *TERT* mutation was detected in 13 of 19 FFPE specimens, with concurrently positive cases in 9 of 19 LBC samples (using SS), and 11/18 LBC specimens (using NGS). Among the high-grade UC patients, positive *TERT* mutation was found in 8 of 10 cases, with similar findings for corresponding LBC materials.

Among the newly diagnosed low-grade UC cases, *TERT* mutation was found in 7 of 12 cases using FFPE tissue. Concurrent positive with *TERT* mutation were identified in 6 of 12 LBC samples using SS and 6 of 11 LBC materials using NGS. For low-grade recurrent UC cases, 6 of 7 patients were found to have *TERT* mutation using FFPE tissue. This corresponds to 3 of 7 LBC materials using SS and 5 of 7 LBC samples using NGS, concurrently. Furthermore, *TERT* mutation was found in 3 of 4 newly detected high-grade UC and in 5 of 6 cases of recurrent high-grade UC via FFPE tissue as well as corresponding LBC materials.

4. Discussion

Urothelial carcinoma is the seventh most common neoplasm worldwide, with an estimated global incidence of 330 380 new cases in 2012. Mortality rate differs by sex, with 2 to 10 deaths per 100 000 males per year and 0.5 to 4 deaths per 100 000 females per year [3]. The high recurrence rate of UC presents constant challenge for urologists and stress for the patients. Standard follow-up procedure comprises voided urine cytology, ultrasonography, and cystoscopy. However, less invasive procedures are highly in demand for years. Conventional cytology provides relatively good results in the diagnosis of high-grade tumors, with a sensitivity of 79% and a much higher specificity up to 100%. Conventional cytology in cases of low-grade UC has a sensitivity of 25% to 45% and a specificity of 98% [5-7]. Despite this, more sensitive and reliable methods, which would be comparable to cystoscopic findings, are desirable.

In recent years, attention has been directed toward *TERT* gene mutation. The mutations of *TERT* gene have been detected in several human

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