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CYTOPATHOLOGY

Expression of the RNA Component of Human Telemorase (hTR) in ThinPrep® Preparations from Bladder Washings

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BACKGROUND. The enzyme telomerase is associated with cellular immortality and is expressed in the vast majority of human neoplasms. The expression of the RNA component of human telomerase (hTR) shows excellent concordance with enzyme activity.

METHODS. In this study, hTR expression was analyzed in a series of 18 perioperative bladder washings and compared with histologic diagnoses from material obtained in the same setting. The hTR expression analysis used an ^{35}S-based in-situ hybridization assay. ThinPrep® preparations fixed in PreservCyt® solution (Cytyc Corporation, Boxborough, MA) were hybridized with sense and antisense hTR probes. A 1–4+ grading scheme was used, with appropriate positive and negative controls.

RESULTS. Five of six (83%) lesions with benign histology had hTR expression that was 2+ or less in the exfoliated urothelial cells. In contrast, 11 of 12 (93%) lesions with malignant histology had an hTR expression that was focally 3+ or more, with 7 of 12 (58%) lesions having 4+ hTR expression in at least some urothelial clusters. Although increased hTR expression was present in smears with malignant urothelial cells, a similar trend was not seen with muscularis propria invasion or higher grades of TCC on subsequent histology.

CONCLUSIONS. The use of in situ hybridization technique bypasses the need for stringent specimen processing and allows identification of the specific cell type that expresses telomerase. ***Cancer (Cancer Cytopathol)*** **2001;93:73–79.**

KEYWORDS: hTR, human telomerase RNA component, telomerase repeat amplification protocol (TRAP).

Bladder cancer is a common malignancy. With approximately 55,000 new cases diagnosed in 1999 in the United States, it is the fifth leading cause of cancer after lung, breast, prostate, and colon cancers.[1] Nearly 12,500 patients die from the effects of this cancer each year in this country. The vast majority of these lesions are transitional cell carcinomas (TCCs) that arise from neoplastic transformation of the native urothelium. Approximately 75% of bladder cancers present as "superficial" lesions and are likely to recur in as many as 50–75% of instances.[2] Unfortunately, of the remaining 25% of patients who present with "invasive" lesions, at least 50% already have occult metastases and will die of their disease.[3] Undoubtedly, the physical, emotional, and economic costs of both superficial and invasive bladder cancer are substantial, and early diagnosis and treatment are of paramount importance.

The pathologist has a pivotal role to play in the diagnosis of TCCs. Although the sine qua non of diagnosis rests on the demonstration of

tumor in biopsied or resected surgical specimens, noninvasive techniques, such as the cytologic demonstration of exfoliated tumor cells, have become an important adjunct in diagnosis and follow up for TCCs. Both voided urine samples and bladder washes have been used for this purpose, although the latter is more sensitive for detection of tumor.[4,5] Whereas urinary cytology can detect almost all high-grade urothelial neoplasms, its sensitivity for diagnosing low-grade papillary tumors is low and requires considerable expertise. For example, Grade I TCCs were identified correctly in cytologic preparations in 62% of cases in one study series[6] and in only 16.7% of cases in another series.[7]

To circumvent these shortcomings of conventional cytology, a variety of operator-independent adjunctive markers have been developed to aid in the diagnosis of TCCs. Among these, the two that have shown most promise include the bladder tumor antigen (BTA) test and its derivatives,[8] and the nuclear matrix protein 22 (NMP22) assay.[9] Although the BTA assays seem to have a better sensitivity than exfoliative cytology in diagnosing TCC, they are much less specific and are affected substantially by the presence of inflammation, stones, and instrumentation.[10] Similarly, NMP22 assays have a higher sensitivity than urine cytology alone, but 25–30% of low-grade tumors are not detected by this assay.[11] Previous reports have not been able to demonstrate a significant benefit in combining cytology with the NMP22 test for diagnosing bladder cancer in voided urine.[10]

Human telomerase is a complex reverse transcriptase enzyme containing both protein and RNA components.[12,13] It binds to TTAGGG telomeric DNA and functions to synthesize telomeric repeats. Telomerase is overexpressed in most cancers and immortal cell lines but is inactive in normal somatic cells, except for male germ cells, and is expressed at lower levels in stem cells of regenerating tissues.[14–16] In tumors, the RNA component of human telomerase (hTR) typically is expressed concordantly with telomerase activity,[17–19] although in some instances, hTR may be expressed independently.[20] In our experience, there is excellent correlation between telomerase activity assessed in cell lines or frozen tissues and hTR expression in the corresponding cell blocks or histologic sections.[17–19] The detection of bladder cancer by identifying telomerase activity in voided urine has been emerging as a promising adjunct in diagnosis.[3,10] Although several published reports now have shown the value of increased telomerase levels in the context of cancer diagnosis, considerable controversy still exists in literature regarding the reliability of the methods used for detecting telomerase in urine samples.

We have developed a ^{35}S-radiolabeled, in situ, hybridization assay to probe for the RNA template component of human telomerase.[21] The in situ method for detecting telomerase RNA has some obvious advantages when compared with an assay that detects holoenzyme activity or an assay based on reverse transcription polymerase chain reaction (RT-PCR). First, it bypasses the need for stringent collection, transport, and storage requirements. Second, because telomerase (and thus telomerase RNA) is expressed in some normal tissue components, such as inflammatory cells and in proliferating stem cells, an in situ method identifies the precise cellular origin of expression. Third, an in situ technique is not affected by the presence of polymerase chain reaction (PCR) inhibitors such as proteases and acidic pH, which commonly are found in urine and interfere with enzyme assays. In this report, we describe for the first time our application of this in situ hybridization analysis to detect hTR expression in exfoliated urothelial cells prepared from bladder washings in 18 patients who were undergoing diagnostic or curative surgery for TCC. We have compared the results of hTR expression with conventional cytology and histology from material collected in the same setting.

MATERIALS AND METHODS

Selection of Archival Material

Bladder washings were collected perioperatively from 18 consecutive patients who were undergoing diagnostic cytoscopic biopsies ($n = 12$), transurethral resection of bladder tumors (TURBT) ($n = 2$), or radical cystectomies ($n = 4$) for previously diagnosed bladder cancer.

ThinPrep® Preparation (Cytyc Corporation, Boxborough, MA)

Three sets of paired slides were made in each case and used as follows: 1) two slides were used for routine cytologic diagnosis and for archival purposes; 2) two slides were used for in situ hybridization with hTR (sense and antisense); and 3) two slides were used for in situ hybridization with a housekeeping gene. In each case, the histologic diagnosis on the concurrently performed biopsy/surgery was retrieved from the surgical pathology archives of our institution. Pathologists not on this study had diagnosed the surgical specimens, and we reviewed the slides only to confirm their diagnoses. Similarly, the pair of ThinPrep® slides that were prepared for diagnostic and archival purposes were reviewed without the in situ material. Although there were patients who had known bladder cancer, the two pathologists scoring the in situ results (A.M. and R.A.) did not have access to existing clinical

or pathologic information on any case until after the grading had been completed.

In Situ Hybridization (ISH) Assay for hTR

In situ hybridization (ISH) for hTR was performed on uncoverslipped slides from ThinPrep® preparations by a method we have described previously.[21] The plasmid pGEM-5Zf(+) (Promega Corporation, Madison, WI) containing a human telomerase RNA (hTR) complementary DNA (559 nucleotides), obtained from the Geron Corporation, Menlo Park, CA, was used as a template to generate radioactive sense and antisense probes. Briefly, slides were hybridized overnight at 52 °C in 50% deionized formamide, 0.3 M NaC1, 20 mM Tris-HC1 (pH 7.5), 5 mM EDTA, 10 mM NaH_2PO_4 (pH 8.0), 10% dextra sulfate, 1× Denhardt's solution, 500 μg/mL total yeast RNA, 10 mM dithiothreitol, and 50,000 cpm/μl ^{35}S-labeled probe. The slides were washed stringently before treatment with 20 μg/mL of Rnase A at 37 °C for 30 minutes, followed by dipping in Kodak (Eastman Kodak Corporation, Rochester, NY) NTB-2 nuclear track emulsion and exposure for 3 weeks in light boxes with desiccant at 4 °C. The slides were developed in Kodak Dektol developer (31/2 minutes), washed in water (20 seconds), fixed in Kodak fixer (7 minutes), and counter-stained with hematoxylin.

To confirm the presence of intact RNA in hTR negative slides, a second set of ThinPrep® slides from each sample used for hTR expression were also tested for expression of a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The Zba/HindIII fragment from the GAPDH cDNA in pBR322 obtained from the American Type Culture Collection, Rockville, MD, was subcloned into pBluescript and used as previously described.[21]

The in situ results were scored concurrently by two of the authors (R.A. and A.M.), who used a two-headed microscope, followed by review with the others. The investigators were blinded to the routine cytologic and histologic diagnosis in each case, which was made available after the ThinPrep® slides had been scored. A manual scoring system was used because of the presence of scattered background noise, which makes automated image analysis not feasible. Based on our previous experience,[22] we scored in situ hybridization slides from 1 to 4+, with each tier corresponding to a defined and concurrently run positive or negative control. A score of 4+ (strong) was comparable to expression in tissue from an adult colon cancer; a score of 3+ (moderate) was comparable to expression in germinal centers of a lymph node; a 2+ (weak) score was comparable to expression in dividing stem cells of regenerative tissues (either normal respiratory or gastrointestinal tract epithelium); and a 1+ (baseline) score was comparable to expression in sense control for each case. In some cases, there were discernible variations of expression between discrete cell clusters on the same slide, and, in such cases, both subpopulations were graded independently.

TABLE 1
hTR Expression and Cytohistologic Correlation in 18 Patients with Perioperative Bladder Washings

Case	hTR	Cytology	Histology
1	1–2+	Reactive	Cystitis
2	2+	Reactive	Cystitis, focal atypia
3	2+	Degeneration, metaplasia	Cystitis, metaplasia
4	2+	Few atypical urothelial clusters	Focal mild dysplasia
5	2+	Few atypical cells, inconclusive	Cystitis, metaplasia
6	4+	TCC, high grade	Acute, chronic inflammation
7	1+	Degenerated cells	Noninvasive TCC low-grade
8	2–3+	No malignant cells seen	Noninvasive TCC low-grade
9	2–3+	TCC	Noninvasive TCC high-grade
10	2–3+	TCC	High-grade CIS
11	3+	TCC	Invasive TCC high-grade
12	3–4+	TCC	Noninvasive TCC low-grade
13	3–4+	TCC	TCC with focal lamina propria invasion low-grade
14	3–4+	TCC	Invasive TCC high-grade
15	4+	TCC	Noninvasive TCC low-grade
16	4+	TCC	Noninvasive TCC low-grade
17	4+	TCC	TCC with focal lamina propria invasion high-grade
18	4+	TCC	Invasive TCC high-grade

RESULTS

RNA Status in ThinPrep® Smears

GAPDH hybridization signals were positive and similar in slides from all smears examined in this study. In a random subset of cases, hybridization using the sense (control) probe for GAPDH was negative as expected. Thus, no significant RNA degradation had occurred during specimen collection, processing, and fixation.

Comparison of hTR Expression in Bladder Washings With Routine Cytology and Histology

Table 1 lists the individual cytologic and histologic diagnoses in the 18 study cases, with the corresponding hTR expression. The cases are organized so that the first 6 cases represent cases where a benign histologic diagnosis was rendered, and the last 12 were cases with a malignant diagnosis in the concurrent histologic specimen. The grade of TCC and the pres-

TABLE 2
Summary of hTR Expression in Bladder Washings with Concurrent Histologic Specimens in 18 Patients

hTR	Benign	Malignant
2+ or less	5/6	1/12
Any 3+ or more	1/6[a]	11/12

[a] Patient subsequently detected to have high-grade TCC on repeat cytology and biopsy.

ence or absence of invasion is also tabulated for 12 cases. In five of six cases (83%) with benign histologic diagnoses, there were 2+ or less hTR expression in all exfoliated cells (Figs. 1A,B; Table 2). The cytologic diagnoses in these five cases did not suggest an unequivocal evidence of malignancy either, although in two ThinPrep® smears, there were few atypical urothelial clusters. In one case, there was outright discordance between both hTR and cytology and the corresponding histology (Case 6). While cytology detected high- grade TCC with strong (4+) hTR expression, the histology showed only acute and chronic inflammation. A subsequent biopsy on this patient showed evidence of high-grade TCC, which was confirmed on exfoliative cytology as well.

Of the 12 cases that had malignant histologic diagnoses, 11 cases (93%) had 3+ (moderate) or more hTR expression in the exfoliated cells (Figs. 1C,D; Table 2). In three cases (Cases 8–10), the 3+ hTR-expressing urothelial clusters were focal and distinct from a background of cells that weakly expressed hTR. In the remaining eight cases, the exfoliated cells were either outright 3+ positive or showed intense wall-to-wall 4+ hTR expression. In 1 of 12 cases (Case 7), there was discordance between hTR and histology, with the in situ failing to detect exfoliated neoplastic cells that were present in the histologic resection. When routine cytologic diagnoses were compared with histology in the same 12 cases, 10 of 12 smears were detected to have unequivocal TCC. Of the two cases with a cytohistologic discordance, one smear (Case 7) showed only degenerated cells. This was the same case in which there was a discordance between hTR and histology as well. The second case (Case 8) had been

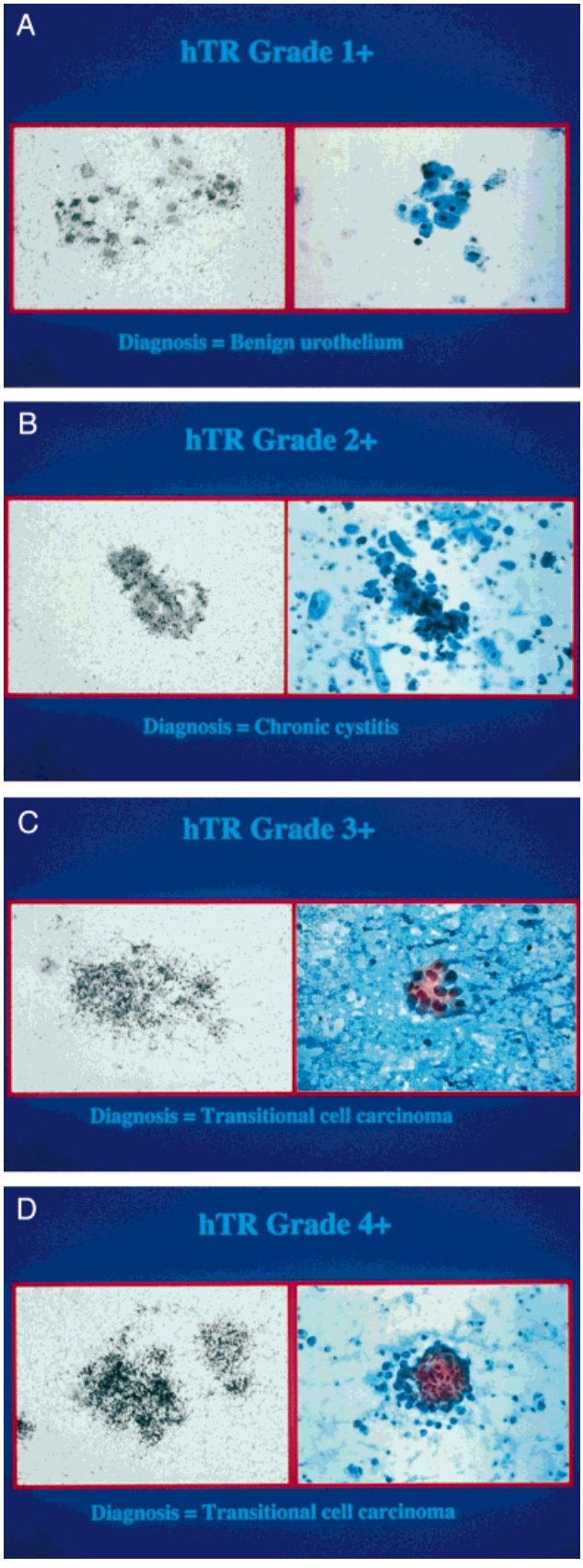

FIGURE 1. Composite figure illustrating hTR expression in exfoliated urothelial clusters (left) with corresponding cytology (right) in ThinPrep® slides from bladder washings. Panels (A) through (D) show increasing intensity of hTR expression from 1 to 4+; (A) and (B) contain benign urothelial clusters; (C) and (D) contain transitional cell carcinoma. The paired hTR and cytology slides in each panel were prepared from the same patient.

rendered a diagnosis of "no malignant cells seen" whereas concurrent histology had shown noninvasive TCC.

We failed to detect a trend of increasing hTR expression with either presence of muscularis invasion or higher grades of TCC. This is illustrated in Table 1, where the last 12 cases composing the malignant histology subset have been listed in order of increasing hTR expression. Thus, Cases 15–18 had nearly equal wall-to-wall strong (4+) hTR expression comprised of two tumors that were invasive and high-grade and two tumors that were noninvasive and low-grade. Similarly, Cases 11–14 had invasive high-grade tumors, one invasive low-grade tumor, and one noninvasive low-grade TCC.

DISCUSSION

There has been a growing interest in measuring telomerase enzyme levels in voided urine for the diagnosis of TCC ever since Lin et al.[23] demonstrated the presence of telomerase activity in 97% of primary tumors using the telomerase repeat amplification protocol (TRAP) assay. They also described an association between high telomerase levels in TCCs and higher tumor grade and stage. Subsequent studies have also found telomerase activity in $>$ 90% of TCCs, and even dysplastic lesions, suggesting that telomerase is upregulated early in neoplastic progression.[24,25]

The use of TRAP assay to assess enzyme activity in urine is difficult. Urine is an aggressive medium, with proteases, RNAses, salts, and urea floating around in an acidic pH environment. Given the instability of the ribonucleoprotein, it is not surprising that investigations conducted to date on voided urine have yielded nonuniform or even contradictory data. Yoshida et al.[24] reported the presence of telomerase activity in 62% of urine samples from TCC patients. It is noteworthy that they reported the absence of detectable telomerase activity in urine from patients who had macroscopic hematuria, suggesting the presence of PCR inhibitors. Similarly, Rahat et al.[25] reported that serial dilutions of voided urine specimens caused a fourfold increase in the percentage of samples that were positive for telomerase activity by the TRAP assay (20–81%), again suggesting the presence of *Taq* polymerase inhibitors. Muller et al.[26] demonstrated the lability of telomerase in urine samples, finding only 7% enzyme activity in voided specimens, whereas RT-PCR for the RNA component was positive in 83% of the same samples. Urine samples had been processed within 15 minutes to prevent degradation or denaturation. Linn et al.[27] compared telomerase activity in archived primary tumors and their matched voided urine samples that had been stored at −80 °C for 6 months to 3 years and found complete loss of enzyme activity in the latter. In contrast, we have been able to perform successful in situ studies for hTR in archival samples that had been stored for up to 10 years previously.[17–19]

We used bladder washes for detection of hTR expression because the sensitivity of bladder washes in detection of urothelial malignancy has been shown to be better than voided urine. Kinoshita et al.[28] compared the telomerase activity in paired voided urine and bladder washings and found a sensitivity of 55% versus 84%, respectively, for detection of TCC. Heine et al.[29] showed an even more dramatic difference in sensitivities for telomerase activity (0% versus 70%) between paired voided urine and bladder washings for detection of TCC. These discrepancies make a strong argument for the use of bladder washings over voided urine samples for detection of telomerase levels, despite the former being an "invasive" test.

The TRAP assay and RT-PCR for hTR often can yield false-positive results because they fail to identify the cell types expressing telomerase. Kavaler et al.[30] reported the presence of enzyme activity in 16 of 47 (34%) patients who had nonmalignant pathologies, almost all of whom had chronic or severe inflammation. Conversely, cytologic evaluation detected only one false-positive patient in this subgroup. Similarly, Muller et al.[26] reported hTR by RT-PCR in 27% of patients with benign urologic conditions such as urinary tract infection. Unfortunately, a significant number of patients with TCC receive intravesicle chemotherapy with resultant acute and chronic cystitis. The presence of false-positive results in inflammatory conditions severely restricts the role of TRAP or RT-PCR among patients being followed for recurrence of TCC.

In contrast to TRAP or RT-PCR, in situ hybridization of exfoliative specimens for hTR expression does not require any special collection, storage, or transport conditions. All the ThinPrep® slides used in this study were prepared from bladder washes fixed routinely in PreservCyt® solution, stored at room temperature for over 6 months. As mentioned, we previously have performed studies on archival tissues more than a decade old. First, unlike PCR assays, in situ hybridization is not hindered by *Taq* polymerase inhibitors present in urine. Second, the use of a methanol-based medium such as PreservCyt® solution, which inactivates RNAses, ensures excellent preservation of RNA for extended periods of time.[31] Last, the grading of hTR expression under direct observation ensures that the precise cell types expressing telomerase are identified and that false positives arising from benign inflammatory conditions are avoided.

In this study, there was a trend toward moderate-

or high-hTR expression in exfoliated cells and a trend toward malignancy in the concurrent histologic sections. Eighty-three percent of lesions with a benign histologic diagnosis had 2+ (weak) or less hTR expression. Similarly, 93% of cases with malignant histology had hTR expression that was at least focally 3+ (moderate) or more (Table 1). We did not perform any statistical analyses on our results because of the small numbers of study cases. Of 18 cases, there were 2 samples that had discordance between hTR expression and histology. In one case, which was benign on histology, the hTR was strongly positive, suggestive of TCC. Subsequent urine cytology as well as rebiopsy showed high-grade TCC. The second case failed to detect more than baseline hTR expression despite the presence of malignant cells on the biopsy. This was also the case where urine cytology revealed only degenerated cells, suggesting that the entire specimen might have been lacking in any malignant cells. We did not repeat the bladder wash in this patient. Although increased hTR expression was present in smears with malignant urothelial cells, a similar trend was not seen with muscularis propria invasion or higher grades of TCC on subsequent histology. Previous reports have also failed to show a relation between telomerase enzyme levels or hTR levels measured by RT-PCR in exfoliated samples and the tumor grade and stage in histologic sections.[25,26,29]

Because of the small numbers of study cases, statistical analyses were not performed, nor were sensitivity and specificity of this technique calculated for the diagnosis of TCC. A larger series is needed to address these issues in the future and to establish whether the trends observed in this study reach statistical significance. This preliminary study demonstrates that an in situ hybridization assay for hTR permits the use of archival cytologic specimens and bypasses the need for stringent collection, transport, and storage conditions. Second, in situ hybridization allows identification of the specific cell type expressing telomerase. The role of hTR as an adjunctive tool to routine cytology in the diagnosis of urothelial neoplasms needs further elucidation in future studies.

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