Telomerase Activity in Patients with Transitional Cell Carcinoma

A Preliminary Study

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BACKGROUND. Telomerase activity is not detectable in normal cells, and their telomers shorten until the chromosome is unable to replicate. Immortal cells have short but stable chromosomes and increased telomerase activity. Transitional cell carcinoma (TCC) has only a few useful markers of diagnostic or prognostic importance. The objective of this study was to determine whether there was a correlation between telomerase activity and the grade or stage of TCC, and whether the enzyme’s activity could serve as a biochemical marker of this tumor.

METHODS. The study included 29 patients with TCC. From each patient, samples of urine cells were obtained, and a cup biopsy was taken from an apparently normal area as well as from a part of the bladder tumor resected transurethrally. Control uroepithelial biopsies were taken from normal transitional cell sites from non-TCC patients. Biopsies or cells were subjected to either histologic examination or telomerase activity determination.

RESULTS. Twenty-six of 29 (90%) of the tumor biopsies exhibited telomerase activity. Most of the cup biopsies were categorized as metaplastic or dysplastic, and 20 of 29 (69%) of these exhibited telomerase activity. Telomerase activity was found in 17 of 21 (81%) of the urine cells but in only 3 of 14 (21%) of control urine cells. All (10 of 10) of the uroepithelial biopsies taken from non-TCC patients did not show any telomerase activity.

CONCLUSIONS. In this study, almost all tumor biopsies exhibited telomerase activity. The high incidence of telomerase activity found in cup biopsies of the malignant field uroepithelial cells from cup biopsies of TCC patients may suggest that telomerase could be activated early in carcinogenesis. A high incidence of telomerase activity was found in voided uroepithelial cells of TCC patients; however, no correlation between this activity and the histologic determination of grading and staging of the tumor was found. Cancer 1999;85:919–24.

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Telomerase activity is essential for the immortalization of tumors; indeed, it is present in germ line cells, in most of the cancerous tumors from different tissues, and in immortal cell lines. However, telomerase activity has not been detected in normal somatic cells or in benign tumors, probably because it is tightly regulated and repressed. These data suggest that telomerase is needed to immortalize cells and contributes to the advancement of the growth toward malignancy. According to a recently proposed model, telomers of normal cells grow shorter and shorter with each cell division until they reach a critical size, which causes cell cycle arrest. This crisis, known as M1, can be circumvented by oncogenic transformation. After such transformation, the cell will continue to divide in a limited way until it reaches the next crisis, known as M2, which is caused by further shortening of the chromosome. At this point, most cells will die except for few cells in which telomerase is activated. These cells retain a very short telomer, which is sufficient to maintain the integrity of the chromosome.

Therefore, activation of telomerase is considered one of the possible mechanisms to immortalize the tumor and probably to promote metastases. Recently, several reports have claimed that the average length of telomers is significantly reduced in malignant cells compared with in normal cell or cells taken from benign growths.

Bladder carcinoma is considered a common malignancy. It is considered the fourth most common cause of death from cancer in men, and over 50,000 new cases of bladder tumor are diagnosed each year in the U.S. Occupational exposure and cigarette smoking are considered major risk factors. Bladder tumors most often present with macrohematuria, are diagnosed by cystoscopy, and are treated by endoscopic resection of the tumor. More than 90% of bladder tumors are transitional cell carcinomas. These lesions may be papillary or solid, single or multiple, superficial or invasive. The degree of the invasion (the stage) and the microscopic arrangement of the tumor (the grade) determine the prognosis of these malignant tumors. Patients with Grade 1–2 tumors and with tumors confined to the mucosa do frequently recur (more than 50% during the first year). Some of these require intravesical therapy with topical agents to reduce frequent recurrences. Follow-up of these patients consists of repeated cystoscopies to identify the recurrences as early as possible, when they are as small as possible. On the other hand, progression into invasive disease or metastases occurs only in 15% of cases. Tumor diagnosed with invasion beyond the lamina propria or bladder muscle requires radical surgical treatment. Adjuvant or neoadjuvant chemotherapy has been reported to improve results of treatment somewhat. Constant monitoring of TCC patients is therefore extremely important. However, current means of follow-up, such as contrast urography, ultrasound, and urinary cytology, lack sensitivity, and some even lack specificity. Furthermore, a definitive diagnosis may sometimes be difficult due to the small size of the lesion, its inaccessibility, or because of the delicate urothelium that crumbles and renders the biopsy inadequate for histopathologic determination. Therefore, a new biochemical marker could prove highly beneficial in the early detection and follow-up of TCC patients.

The objective of this study was to determine whether there was a correlation between telomerase activity and the grade or stage of TCC and to establish whether this activity could serve as a new noninvasive biochemical marker for the detection of urothelial carcinoma of the bladder.

MATERIALS AND METHODS

Tissue Samples
Twenty-nine patients with primary or recurrent transitional cell carcinoma were included in the study. If cystoscopy revealed the presence of a tumor in the bladder, the tumor was resected transurethrally using a resectoscope. A cup biopsy was taken from a normal appearing area, remote to the tumor, from each tumor case. In addition, 10 samples of normal urothelium were obtained from patients who underwent surgery for other reasons. Each biopsy was sliced in two: one part was taken for pathologic examination, and the other was immediately frozen in liquid nitrogen to avoid RNA degradation or telomerase denaturation until proteins were extracted.

Urine Samples
Fifty mL of naturally voided urine were collected from 21 patients with confirmed TCC. Fourteen samples of voided urine from control patients or from healthy volunteers were collected in the same manner. The urine samples were centrifuged and washed with phosphate-buffered saline, and the pellet of exfoliated cells was immediately frozen in liquid nitrogen.

Cell Lines
As a positive control for telomerase activity, the cell line 5637 (ATCC HTB-9) derived from human primary bladder carcinoma (the kind gift of Dr. A. Kassel, Carmel Medical Center, Haifa, Israel) was used. The cell line was cultured in RPMI-1640 with 10% fetal calf serum, 1% L-glutamine, and antibiotics (Biological Industries, Kibbutz Beit-haemek, Israel).
Telomerase Activity of Tissue Biopsies

Twenty-six of 29 (90%) of the specimens that were identified as TCC by pathologic examination exhibited telomerase activity. Of these samples, 12 were derived from low grade and stage, superficial bladder carcinoma, and 17 from high grade and stage bladder carcinomas.

Most of the cup biopsies taken from the apparently normal area had histologic reactive changes: 14 were metaplastic and/or dysplastic, 3 cup biopsies were determined to be carcinoma in situ by histology, only 2 were normal, and 5 were inadequate for histologic examination. Twenty of 29 (69%) of these cup biopsies exhibited telomerase activity. These results are presented in detail in Table 1.

All (10 of 10) of the uroepithelial biopsies taken from non-TCC patients did not show any telomerase activity. The absorbance \( A_{450 \text{ nm}} - A_{660 \text{ nm}} \) of each of these samples was below 0.1, with an average value of 0.043.

Telomerase Activity in Urine Samples

Initially, most of the urine samples were negative for telomerase activity (4 of 21). However, after serial dilutions of the protein extracts, resulting in the dilution of Taq polymerase inhibitors, telomerase activity was determined in 17 of 21 (81%) of the exfoliated urine cells. In contrast, urine samples from control patients were mostly negative: only 3 of 14 (21%) of them were positive for telomerase activity.

DISCUSSION

Analysis of the bladder tumor tissue biopsies revealed that almost all (90%) cancerous biopsies exhibited telomerase activity. In the exfoliated urine cells from TCC patients, we showed a very high incidence (81%) of telomerase activity, demonstrating that this noninvasive method is useful as a marker for the detection and follow-up of TCC. These results are particularly encouraging, because the presence of proteases, RNases, and the acidic pH to which exfoliated cells are exposed in the urine might have compromised the efficiency of the assay. Nevertheless, our results show that if the cells are centrifuged and washed immediately after collecting the urine sample, intact cells with telomerase activity are still sufficiently found. These results were obtained after serial dilutions of the protein extracts, which prevented the possible inhibitory effects of macroscopic hematuria on Taq polymerase. Weak telomerase activity can be detected in some inflammatory lesions. However, two patients with a history of TCC were excluded from our study because their specific portions of the biopsies were categorized

Telomerase Activity of the 5637 Cell Line

In all experiments, telomerase activity of the 5637 cell line produced strong telomerase activity, with an average absorbance \( A_{450 \text{ nm}} - A_{660 \text{ nm}} \) of 1.930. When diluted, telomerase activity persisted, even at a total protein amount of 0.06 pg (data not shown). Thus, we used this cell line as a positive control for telomerase activity.

RESULTS

Telomerase Activity of the 5637 Cell Line

The telomerase activity was determined by using the telomerase polymerase chain reaction–enzyme-linked immunosorbent assay (PCR-ELISA) kit (Boehringer Mannheim, Mannheim Germany) according to the manufacturer’s instructions. This nonradioactive method is based on the recently developed telomeric repeat amplification protocol (TRAP) assay and has been previously used in the determination of telomerase activity in TCC patients and shown to produce similar results to those obtained by the radioactive TRAP assay. Cellular proteins were extracted from the cells, from the urine samples, or from the 5637 cell line by using the lysis buffer provided in the kit. The extracts were flash frozen in liquid nitrogen and stored in aliquots until the biochemical determination of telomerase activity. The quantity of all proteins was determined by Bradford reagent (Bio-Rad Laboratories, Hercules, CA), so that equal amounts of protein were assayed. Taq polymerase inhibitors are often present in protein extracts, and their presence may result in a false-negative result. Serial dilutions of the sample dilute both these inhibitors and telomerase activity. Therefore, we used 10-fold and 100-fold dilutions to provide a wide range in which inhibitors and telomerase were diluted in a ratio that still allowed the detection of telomerase activity, as described by Kyo et al. In some of the cases, telomerase activity decreased gradually after 10-fold and 100-fold dilutions. In other cases, negative telomerase activity was enhanced after a 10-fold dilution, and even more so after a 100-fold dilution. In a third group, negative samples restored telomerase activity after 10-fold dilutions but became negative after 100-fold dilutions. To test which of the negative samples was truly negative for telomerase activity, all negative samples were diluted 10-fold and 100-fold, and the reaction was repeated with the diluted protein extracts. Because of the exponential nature of the PCR reaction, the test is not quantitative, but qualitative only. Hence, all samples that yielded telomerase activity in any of the dilutions were considered positive, whereas all samples that did not yield telomerase activity in all dilutions were considered negative.

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by histologic examination only as chronic and acute inflammation, without traces of tumor. These two biopsies exhibited no telomerase activity, even after serial dilutions, indicating that infiltration of white blood cells did not affect the specificity of the test.

However, no clear correlation between telomerase activity in the urine samples and grade and stage of the tumor as determined by histologic examination was found. This finding is in accord to other studies.\textsuperscript{18–20} In contrast to those studies \textsuperscript{18–20} that found no telomerase activity in normal tissue specimens,\textsuperscript{18–20} we show here a high incidence of telomerase activity in the nonmalignant, normal-looking bladder cup biopsies taken from TCC patients. This difference could result from the distinction we made between normal patients, who had no history of urogenital cancer, and between cup biopsies taken from known TCC patients near the area of the overt tumor. These cup biopsies were nonmalignant on histologic examination; however, most of them were determined to be dysplastic. Hyperplastic, dysplastic, and metaplastic changes of the urothelium are histologic expressions of changes in the bladder microscopic architecture, including an increase in the number of cell layers, and epidermatoid (squamous) or glandular changes of the normal transition cell epithelium. Aspects of these changes are considered precancerous.\textsuperscript{22} Thus, telomerase activity found in this group may represent activation of telomerase in the early stages of the carcinogenesis pathway. The expression of telomerase activity in the cancer field in a high percentage of the cases suggests that it may be useful in determining individuals at risk and help in the follow-up of diagnosed TCC patients, although it is less effective as a diagnostic tool.

Cystoscopy is the primary method for detecting of primary bladder carcinoma and for the follow-up of such patients. However, small tumors or tumor hidden by the mucosa folds may be overlooked during the procedure. Cells that are just beginning the cancerous transformation will most probably not be recognized by this method and will be regarded as normal cells. Detection of telomerase activity in urine samples of TCC patients might offer the opportunity to identify recurrent tumors in their early stages and to treat the patients accordingly.

As previously mentioned, follow-up of bladder TCC patients is involved with frequent cystoscopies, which present a heavy burden on the patient, the urologist, and the health care system. Therefore, the search for markers of bladder tumor diagnosis is

### Table 1

| Biopsies taken from tumor area in TCC patients | Urine samples | Cup biopsies taken from normal-looking area in TCC patients |
|-----------------------------------------------|--------------|-----------------------------------------------------------|
| Low grade and stage TCC (Ta G1–2, T1 G1–2)   |              |                                                          |
| No. of patients                               | Mean age (yrs) | Gender (M:F) | Telomerase activity | Telomerase activity | Histology | Telomerase activity |
| 12                                           | 74            | 8:4          | 91.6% (11/12)     | 75% (6/8)           | Carcinoma in situ | 100% (1/1) |
|                                              |               |              | (4 N.D.)          |                     | Dysplasia and metaplasia | 71.5% (5/7) |
|                                              |               |              |                   |                     | Normal | 0% (0/1) |
|                                              |               |              |                   |                     | SNAH   | 33.3% (1/3) |
|                                              |               |              |                   |                     | Total   | 63.6% (7/12) |

| High grade and stage TCC (T1 G3, T2 G1–3, T3A, T3B) | 17 | 73 | 13:4 | 88.2% (15/17) | 84.6% (11/13) | Carcinoma in situ | 100% (2/2) |
|                                              |    |    |      |              |              | Dysplasia and metaplasia | 75% (9/12) |
|                                              |    |    |      |              |              | Normal | 0% (0/1) |
|                                              |    |    |      |              |              | SNAH   | 100% (2/2) |
|                                              |    |    |      |              |              | Total   | 76% (13/17) |

TCC: transitional cell carcinoma; SNAH: specimen not adequate for histologic examination; N.D.: not done.
constantly ongoing. Urinary cytology lacks sensitivity and misses a great number of low grade tumor recurrences. A few potential markers were recently introduced to improve the diagnosis and prognostic prediction: Lewis X antigen expression has been found in malignantly transformed bladder epithelium. The expression of ABO blood group antigens has been associated with aggressive tumor behavior. Urinary BTA (bladder tumor antigen) test has low sensitivity for the detection of low grade and high grade TCC. This low sensitivity is true for the BTA stat and trak assays and ranges between 26% and 58%. Inflammatory conditions of the bladder and other irritative changes may result in false-positives and reduce the specificity of this assay. Although a careful review of inflammatory conditions of the bladder and other irritative changes may result in false-positives and reduce the specificity of this assay, and thus may weaken the usefulness of this assay as a marker for the detection of bladder carcinoma. Urinary NMP22 has also recently been used for the detection of recurrences of uroepithelial carcinoma, and thus may weaken the usefulness of this marker for the detection of bladder carcinoma. Urinary NMP22 has also recently been used for the detection of recurrences of uroepithelial lesions. A sensitivity of 70% and specificity of 86% have been found. Although a careful review of the threshold value of 10 U/mL would result in a different sensitivity and specificity in a variety of clinical settings. Although the sensitivity of these methods is quite promising, it is obvious that cystoscopy is still the gold standard for TCC detection and follow-up.

In summary, this study showed that telomerase activity was detected in almost all malignant tumors, in a high incidence of cup biopsies, and in a high incidence in urine samples taken from TCC patients. Although no correlation was found between the grade and stage of tumors and telomerase activity, the ability to detect telomerase activity in urine samples with such sensitivity and specificity indicates that this assay is a good marker for the detection of TCC and follow-up of TCC patients.

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