Advances in Brief

Telomerase Activity in Human Bladder Cancer

Yi Lin, Hiroshi Miyamoto, Kiyoshi Fujinami, Hiroji Uemura, Masahiko Hosaka, Yoshiko Iwasaki, and Yoshinobu Kubota

Department of Urology, Yokohama City University, School of Medicine, 3-9, Fukaura, Kanazawa-ku, Yokohama 236 [Y. L., H. M., K. F., H. U., M. H., Y. K.], and Department of Oral Pathology, Kanagawa Dental College, 82 Inaoka-cho, Yokosuka, Kanagawa 238 [Y. I.], Japan

Abstract

Telomerase can synthesize telomeric DNA repeats onto chromosome ends. Telomere length and telomerase activity have recently been implicated in the control of the proliferative capacity of normal and malignant cells. The expression of telomerase activity is concomitant with the attainment of immortality in tumor tissues and cells. Thus, enzyme activity may indicate a prevalent or even ubiquitous tumor producer. In this report, telomerase activity was analyzed in 40 human bladder cancers, 7 normal tissues, and 2 bladder epithelia with dysplasia using a PCR-based telomeric repeat amplification protocol assay. Telomerase activity was detected in almost all bladder tumors (97.5%); only one sample, which was in an early stage, did not express telomerase activity. None of the normal tissues displayed telomerase activity. One of the two bladder epithelia with dysplasia expressed low telomerase activity. The expression of telomerase activity has a clear association with the pathological grade and clinical stage. Most of the tumors with high telomerase activity were in an advanced grade and had deep invasion. Thus, telomerase activity might be suggested to represent an additional required event in the multigenetic process of tumorigenesis in human bladder cancer.

Introduction

Telomeres are the specialized structures at the ends of all eukaryotic chromosomes that are thought to have important functions in protecting genomic DNA from degradation and deleterious recombination events (1, 2). Telomeric DNA may be lost at chromosome ends unless the termini are specifically extended by telomerase (3). In vertebrates, telomerase is a specialized ribonucleoprotein polymerase that consists of hundreds to thousands of tandem repeats of the sequence (TTAGGG)_n and associated protein (4, 5).

The deregulation of telomerase has been suggested to participate in cellular immortalization and oncogenesis. Germline cells, almost all cancer tissues, and cell lines express telomerase activity and maintain telomere length through an indefinite number of cell divisions (6–8). In contrast, normal human somatic cells express low or undetectable telomerase activity and progressively lose their telomeric sequences with replicative senescence in vitro or with normal, in vivo aging (9, 10). Short telomeres, in the absence of telomerase, have been proposed to be the mitotic clock by which cells count their divisions (11). Although the reactivation of telomerase itself may be insufficient for cells to proliferate indefinitely, the expression of telomerase appears to be concomitant with the attainment of immortality in tumor cells (12, 13). Telomerase activity in tumor tissue was first demonstrated in ovarian carcinoma (13) and has now been found in approximately 90% of more than 100 primary tumor biopsies from more than a dozen different tumor types (14), including breast tumor, colon carcinoma, lung cancer, and bladder cancer. In addition, in cultured cells of 18 different tissues, 98 of 100 immortal and none of 22 mortal populations expressed telomerase activity (14). Thus, telomerase activation is supposed to be a critical step in cell immortalization.

We have been interested in telomerase activity in human bladder cancer. However, only three bladder cancer tissues were analyzed in a previous study, although 100% of the bladder tumors (3 of 3) expressed telomerase activity (14). Thus, we studied here the telomerase activity in bladder cancers to confirm whether the expression of telomerase is strongly associated with the malignant potential of bladder cancer and to determine whether or not the association of the telomerase activity was with the differentiation and clinical invasion status of the bladder cancer. In this report, we describe the results of the expression of the telomerase activity in human bladder cancer using the PCR-based TRAP assay. As expected, high levels of telomerase activity were found in almost all human bladder cancers, and its activity was closely associated with the grade and stage of the tumors.

Materials and Methods

Tissue Sample. Tissue samples were obtained from 40 patients with primary bladder cancers by cystectomy or transurethral resection at Yokohama City University Hospital and affiliated hospitals. Detailed clinical and pathological data for each patient were evaluated at the same institutions according to the General Rule for Clinical and Pathological Studies on Bladder Cancer (15), using the TNM classification system of malignant tumors. Normal bladder epithelia were obtained by open surgery in patients with benign prostatic hyperplasia (seven individuals) and in patients with dysplasia (two individuals) by cold cup biopsy. All of the samples were rapidly frozen in liquid nitrogen and stored at −80°C until used for the TRAP assay.

TRAP Assay. Extracts were prepared from frozen tissues stored at −80°C, followed by the addition of 30–50 μl of

Received 2/2/96; revised 3/20/96; accepted 3/21/96.

1 This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

2 To whom requests for reprints should be addressed. Phone: 81-45-787-2679, Fax: 81-045-786-5775.

The abbreviation used is: TRAP, telomeric repeat amplification protocol.
ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EGTA, 0.5% (3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, and 10% glycerol], depending on the size of the tissue samples. The lysates were on ice for 30 min and then centrifuged at 15,000 rpm for 30 min at 4°C. The supernatants were collected into 500-µl tubes and flash frozen in liquid nitrogen. Protein concentration of the tissue was determined with the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL). Aliquots of extracts containing 6 µg of protein were used for each TRAP assay. T-24 cells (bladder cancer cell line) were used for each TRAP assay. For RNase treatment, 6 µg of extract was incubated with 1 µg RNase Plus (RNase Plus, 5 Prime → 3 Prime Inc., Boulder, CO) for 10 min at 37°C. A volume of 6 µg protein for tissues and 10⁵ cells for T-24 cell lines were incubated with 0.1 µg of TS oligonucleotide (5'-AAATCCGTCGAGCAGAGT-3'), and 0.3 µl of [α-³²P]dCTP (3000 Ci/mmol; Amersham, Buckinghamshire, United Kingdom) for 15 min at room temperature in 50 µl of TRAP reaction mixture, containing 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 50 µM deoxynucleotide triphosphates, 0.5 µM T4 gene 32 protein.

Fig. 1 Telomerase activity in human bladder cancers (T) and normal bladder mucosa (N). In a, telomerase activity signals with (+) or without (−) RNase treatment of the extract are shown. Extracts of a T-24 human bladder cell line (10⁵ cells) having telomerase activity were used as the positive standard. Cases A and B (T₀ and T₁) showed low telomerase activity whereas cases C and D (T₂ and T₃) had higher activity. In b, each extract prepared from a tumor sample was subjected to serial dilutions and analyzed at 6 µg protein (standard condition), at 0.6 µg (10-fold dilution), and at 0.06 µg (100-fold dilution). The dilution series demonstrates at least a 10-fold dilution and perhaps as much as a 100-fold dilution difference in telomerase activity between cases A and B versus cases C and D.
Telomerase activity has been found in a wide variety of malignant tumor samples and in almost cell lines, whereas it was not detected in normal somatic tissues or cell strains (14, 16). It has been suggested that telomerase may play an important role in the growth and development of cancer. Thus, it is worthwhile to examine the expression of telomerase in the development and progression of cancers (17, 18).

Since only three cases of bladder cancers were examined for telomerase activity in earlier work (14), we performed a systematic study of telomerase activity in freshly diagnosed bladder cancers. Our results clearly showed that most of the bladder tumors (97.5%) had telomerase activity, and none of the

### Table 1
Telomerase activity in bladder tumors, normal tissues, and dysplasia

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>Undetectable</th>
<th>Detectable</th>
<th>(High$^a$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder cancer</td>
<td>40</td>
<td>1</td>
<td>39$^b$ (26)</td>
<td>(0)</td>
</tr>
<tr>
<td>Normal</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>(0)</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>(0)</td>
</tr>
</tbody>
</table>

$^a$ Positive, using extracts containing 0.06 μg of protein (100-fold dilution).
$^b$ $P$ was calculated between the bladder tumor and normal tissues ($P < 0.005$).

In addition, all of the 13 bladder cancer samples with low telomerase activity were found to have high telomerase activity (tumors retained a TRAP signal after 100-fold dilution of the extract), and 13 of 39 tumors (33%) were classified to have low telomerase activity (tumors retained a TRAP signal after 10-fold dilution or no dilution of the extract). All of the samples (16 of 16) of cancers with Grade 3 tumors were found to have high telomerase activity, whereas only 20% (2 of 10) of Grade 1 tumors expressed high telomerase activity ($P < 0.005$), and 62% of cancers (8 of 13) with Grade 2 tumors had high telomerase activity, showing significant statistical difference from the Grade 1 groups ($P < 0.05$; Table 2).

### Table 2
The relationship of telomerase activity and pathological grade and clinical stage in bladder tumors with telomerase activity

<table>
<thead>
<tr>
<th>Tumor</th>
<th>n</th>
<th>Low$^a$</th>
<th>High$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>10</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Grade 2</td>
<td>13</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Grade 3</td>
<td>16</td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>
| $^a$ Positive, using extracts containing 6 μg or 0.6 μg of protein but negative when 100-fold dilution.

#### Results
In this study, telomerase activity in 40 bladder cancer tissues, 7 normal bladder epithelium tissues, and 2 bladder dysplasia tissues were used for the TRAP assay. Telomerase activity was detected in 39 of the 40 bladder cancer tissues (97.5%). In contrast, the enzyme activity was not detected in any of seven histologically normal bladder epithelia (Fig. 1a; Table 1). Because of the variation in the intensity of the signals in the TRAP assay in the cancer specimens, which might reflect differing activities of the enzyme, we estimated the activity of telomerase by using the results of serial dilutions of each extract of 6 μg of protein (standard condition), 0.6 μg (10-fold dilution), and 0.06 μg (100-fold dilution). In 39 bladder cancers with telomerase activity, twenty-six tumors retained the telomerase activity, even with 100-fold dilution (Table 1).

To determine whether or not dysplasia of bladder tissues express telomerase activity as in bladder tumors, we analyzed two bladder mucosa with dysplasia. The results showed that one sample (1 of 2) expressed telomerase activity, although activity was not found in a 10-fold dilution of the sample (Table 1). This result might suggest that telomerase may play an important role in the development of preneoplastic lesions in bladder mucosa.

To understand the relationship of telomerase activity and clinical features in bladder cancer, we divided the 39 tumors with positive telomerase activity into two groups according to the telomerase activity and analyzed the relationship with the grade and stage of the tumors. Twenty-six of the 39 tumors (67%) were classified to have high telomerase activity (tumors retained a TRAP signal after 100-fold dilution of the extract), and 13 of 39 tumors (33%) were classified to have low telomerase activity (tumors retained a TRAP signal after 10-fold dilution or no dilution of the extract). All of the samples (16 of 16) of cancers with Grade 3 tumors were found to have high telomerase activity, whereas only 20% (2 of 10) of Grade 1 tumors expressed high telomerase activity ($P < 0.005$), and 62% of cancers (8 of 13) with Grade 2 tumors had high telomerase activity, showing significant statistical difference from the Grade 1 groups ($P < 0.05$; Table 2).

In addition, all of the 13 bladder cancer samples with low telomerase activity were found in early stage (T₁-T₄) tumors (Table 2), and the only one bladder cancer with undetectable telomerase activity was also found in this early stage (T₁), whereas all of the bladder cancers with muscle invasion, 6 tumors (6 of 6) with stage T₂ and 9 (9 of 9) with stage T₃-T₄ expressed high telomerase activity (Fig. 1b; $P < 0.025$). The T₂-T₄ human bladder tumor cell line, which was used as a positive control, also had expression in telomerase activity, even in 100-fold dilution, as shown in Fig. 1a.

#### Discussion
Telomerase activity has been found in a wide variety of malignant tumor samples and in almost cell lines, whereas it was not detected in normal somatic tissues or cell strains (14, 16). It has been suggested that telomerase may play an important role in the growth and development of cancer. Thus, it is worthwhile to examine the expression of telomerase in the development and progression of cancers (17, 18).
normal bladder epithelia had telomerase activity. The failure of
detection of telomerase activity in only one bladder cancer
sample of 40 might have been due to inactivation of the enzyme
during the assay procedure. The variations of telomerase activity
may have resulted from high protein concentration or Taq
polymerase inhibitors present in the tissue extracts (19). The
extract was reexamined with serial dilutions and mixed with an
extract of telomerase-positive T-24 cell line. The TRAP signals
did not appear after serial dilutions; thus, there was no evidence
of inhibitors (data not shown).

Although the assay itself is nonlinear and at times the nature of
the results is such that it is difficult to make definite statements
about the relative levels of activity (19), we found a correlation
of telomerase activity with the differentiation of cancer cells and
with the clinical stage in bladder cancers. All of the 16 bladder cancers
with high grade (G3) tumors expressed high telomerase activity,
while 80% (8 of 10) low grade tumors (G1) showed low
telomerase activity ($P < 0.005$). In addition, all of the bladder cancers
with muscle invasion ($T_{2}$-$T_{4}$) expressed high telomerase activity ($P <
0.025$). As shown in other types of tumors (18, 20), high telomerase
activities were found in advanced-stage tumors, whereas low
telomerase activities always occurred in early-stage tumors. These
data suggest that malignant progression could be dependent on the
activation of telomerase.

Although telomerase activity might always accompany
later events of cancer progression (18), the details remain ob-
scure at present. In our experiments, of 24 early-stage superfi-
cial tumors ($T_{1}$-$T_{2}$), 11 samples (46%) also showed high telom-
erase activity. It should be emphasized that telomerase activity
not only occurred in later events in human bladder cancer
progression but also is responsible for early stages. This indi-
cates that telomerase activity may appear at the same time or in
an earlier event than in the detection of tumors on a clinical level.

Bladder dysplasia are thought to be precarcinoma lesions of
the bladder. Some of these do in fact develop further into carci-
noma. Thus, it is very important to understand or to predict the
malignant potential of dysplasia before they become carcinoma.
We found telomerase activity in one of the two patients with
dysplasia, although the telomerase activity was low. These two
patients underwent transurethral resection for superficial bladder
cancer 3 and 5 years ago and had experienced frequent intravesical
tumor recurrence since then. During this time, we found some
epithelial abnormalities through the cystoscope and took biopsies
of these lesions via cold cup. The pathological diagnosis was
dysplasia. We are now carefully following up on these patients.

In conclusion, the present study demonstrates that the ma-
Jority of the human bladder cancers studied expressed telom-
erase activity, whereas all of the normal samples had no detectable
telomerase activity. The tumors with high telomerase activity
have been accompanied with high grade and advanced stage.
Thus, bladder tumors with high telomerase activity might have
more malignant potential. Furthermore, telomerase activity may
be a useful diagnostic marker as well as a prognostic marker in
human bladder tumors in the future.

References
1. Makarov, V. L., Lejnine, S., Bedoyan, J., and Langmore, J. Nucleo-
somal organization of telomere-specific chromatin in rat. Cell, 73:
775–787, 1993.
3. Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A.,
Chiu, C. P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J. H., Le,
S. Y., West, M. D., Harley, C. B., Andrews, W. H., Greider, C. W.,
and Villepontone, B. The RNA component of human telomerase. Science
4. Moyzis, R. K., Buckingham, J. M., Cram, S., Dani, M., Deaven,
L. L., Jones, M. D., Meyene, J., Ratliff, R. L., and Wu, J. A highly
conserved repetitive DNA sequence (TTAGGG), present at the
6. Counter, C. M., Botelho, F. M., Wang, P., Harley, C. B., and
Bacchetti, S. Stabilization of short telomeres and telomerase activity
accompanied with immortalization of Epstein-Barr virus transformed human
in vivo in human malignant hepatopoietic cells. Oncogene, 9: 3043–
Functional characterization and developmental regulation of mouse
9. Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai,
E. V., Futcher, A. B., Greider, C. W., and Harley, C. B. Telomere length
10. Vaziri, H., Dragowska, W., Allsopp, R. C., Thomas, T. E., Harley,
C. B., and Landorp, P. M. Evidence for a mitotic clock in human
11. Harley, C. B. Telomere loss: mitotic clock or genetic time bomb?
12. Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G.,
Greider, C. W., Harley, C. B., and Bacchetti, S. Telomere shortening
associated with chromosome instability is arrested in immortal cells
M. D., Ho, P. L. C., Coviello, G. M., Wright, W. E., Weinrich, S. L.,
and Shay, J. W. Specific association of human telomerase activity with immor-
15. Japanese Urological Association and the Japanese Society of Pa-
thology. General Rule for Clinical and Pathological Studies on Bladder
16. Hiyama, E., Hiyama, K., Yokoyama, T., Matsuura, Y., Piatyzek,
M. A., and Shay, J. W. Correlating telomerase activity levels with human
17. Chadeneau, C., Hay, K., Hirte, H. W., Gallinger, S., and Bacchetti,
S. Telomerase activity associated with acquisition of malignancy in
18. Hiyama, E., Yokoyama, T., Tatsumoto, N., Hiyama, K., Imamura,
Y., Morakami, Y., Kodama, T., Piatyzek, M. A., Shay, J. W., and
telomeric repeat amplification protocol (TRAP) result in increased reliabil-
20. Tahara, H., Nakashima, T., Kitamoto, M., Nakashio, R., Shay, J. W.,
Tahara, E., Kajiyama, G., and Ide, T. Telomerase activity in human live
tissues: comparison between chronic liver disease and hepatocellular
Telomerase activity in human bladder cancer.

Y Lin, H Miyamoto, K Fujinami, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/2/6/929

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.