

## Telomerase Is Upregulated in Irreversible Preneoplastic Lesions during Bladder Carcinogenesis in Rats

Toru Shimazui,<sup>1,4</sup> Yoshihiro Ami,<sup>2</sup> Naoto Miyanaga,<sup>1</sup> Yukitaka Ideyama,<sup>3</sup> Takahito Nakahara<sup>3</sup> and Hideyuki Akaza<sup>1</sup>

<sup>1</sup>Department of Urology, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, <sup>2</sup>Department of Urology, Tsukuba Gakuen Hospital, 2573-1 Kamiyokoba, Tsukuba 305-0854 and <sup>3</sup>Pharmacology Laboratories, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585

Multiple occurrence or recurrence after transurethral resection is an important characteristic of superficial bladder tumors. To study bladder carcinogenesis, we focused on detection of telomerase activation, which was investigated in several human cancers, including bladder tumors. We experimentally examined the telomerase activity during bladder carcinogenesis, especially in precancerous lesions, induced by N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) in rats. Male Wistar rats were given 0.05% BBN in water from the age of 8 weeks to 24 weeks. Subgroups were euthanized at 4, 8, 10, 12, 18, and 24 weeks after BBN administration. Using the stretch PCR method, telomerase activity was semiquantified in exfoliated bladder epithelial cells. In addition, telomere length in each subgroup was measured by southern hybridization for the terminal restriction fragment using a (TTAGGG)<sub>4</sub> probe. Statistical analyses were performed using analysis of variance and Fisher's PLSD test. Epithelial cells of normal bladder in the control groups and those of diffuse hyperplasia, which was a reversible change at 4 weeks, expressed no telomerase activity. In contrast, telomerase activity significantly increased in the stage after nodular hyperplasia, an irreversible change at 8 weeks, then elevated with carcinogenesis. However, telomere length was still preserved by the 12th week, and was shortened at 18 and 24 weeks. These results suggest that telomerase activation is probably induced independent of telomere shortening during bladder carcinogenesis in the rat, and might be a biological tumor marker of irreversible preneoplastic lesions, which evolve into bladder tumors in the rat.

Key words: Bladder carcinogenesis — Telomerase — Telomere — BBN — Hyperplasia

Telomerase is a ribonucleoprotein complex that synthesizes telomeric DNA and contributes to the maintenance of telomeres, which are composed of TTAGGG repeats located at the end of the chromosome and play a critical role in chromosome structure and function.<sup>1)</sup> A certain length of telomere is important for cell division; normal somatic cells express very low or undetectable levels of telomerase activity, and progressively lose their telomeric sequence via cell division. Therefore, the activation of this enzyme has been proposed to be a critical event in the immortalization of the cell, and is characteristic of most cancer cells. It has been reported that 80 to 90% of bladder tumors express telomerase activity,<sup>2)</sup> and that the detection of telomerase activity in bladder tumor tissues and/or in exfoliated tumor cells in urine is useful as a new diagnostic tool.<sup>3, 4)</sup>

To examine the critical point at which telomerase activation occurs in the course of bladder carcinogenesis, we measured telomerase activity in bladder epithelium during N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) carcinogenesis in the rat. BBN bladder carcinogenesis is thought to

be a model for superficial bladder tumor. It has been well described that during bladder cancer development, diffuse hyperplasia at 4 weeks after BBN administration is a reversible change, whereas nodular hyperplasia at 8 weeks is an irreversible change toward cancer.<sup>5)</sup> In this study, we aimed in particular to semi-quantify telomerase activity in reversible and irreversible preneoplastic epithelia, in order to detect the critical point of telomerase activation during bladder carcinogenesis. Furthermore, we re-examined whether detection of telomerase activity in exfoliated cells in a urine sample is useful as an early diagnosis for bladder tumors.

### MATERIALS AND METHODS

**Bladder carcinogenesis induced by BBN in rats** A total of 150 male Wistar rats were given 0.05% BBN in water continuously from the age of 8 weeks. Subgroups, each of which consisted of 25 rats, were euthanized at 4, 8, 10, 12, 18, and 24 experimental weeks after BBN administration. Twenty-five rats in each subgroup of the control group (a total 175 rats comprised the control group) were euthanized under anesthesia at 0 experimental weeks and at the same weeks as the BBN subgroups (Fig. 1A). To obtain a

<sup>4</sup> To whom correspondence should be addressed.  
E-mail: torushim@md.tsukuba.ac.jp

sufficient number of urothelial cells, samples from 5 to 10 rats were mixed together then analyzed.

**Histological examination** Five rats in each subgroup were killed for histological examination. At 4 and 8 weeks after BBN administration, diffuse and nodular hyperplasia were observed without exception, respectively (Fig. 1, B and C). At 12 weeks, nodular hyperplasia was dominant, but transitional cell carcinoma (TCC) had not developed. At 18 weeks, small TCCs were already observed, and at 24 weeks, large tumors were observed in all rats (Fig. 1D).

**Isolation of bladder epithelial cells** To obtain transitional epithelial cells from the rat urinary bladder, we modified the technique used by Kakizoe *et al.*<sup>6)</sup> We previously analyzed several combinations of trypsin and EDTA to determine the optimal conditions for isolating urothelial cells, without contaminating submucosal connective tissue, by histological evaluation (data not shown). Resected bladders were everted and incubated in RPMI1640 with 0.05% trypsin and 0.53 mM EDTA for 10 min at 37°C. Trypsinization was stopped with 10% fetal calf serum (final concentration), and the bladders were then sonicated at 28 kHz twice for 15 s in ice water. Exfoliated cells were collected by centrifugation at 1500 rpm for 10 min, then counted. Cells were washed with PBS, and cell pellets were frozen in liquid N<sub>2</sub> until use.

**Telomerase assay** Bladder epithelial cells (10<sup>5</sup>) from a mixture of samples from five to ten rats were resolved in 100 μl of 3-[(3-cholamidopropyl)dimethylammonio-1-propanesulfonate] (CHAPS) detergent buffer at 4°C, and 20 μl aliquots of the supernatants, which contained telomerase, were used for the telomerase assay using Telochaser (TOYOBO, Tokyo) according to the manufacturer's instructions. Stretch reaction using the extract was performed with an oligonucleotide up-stream primer, 5'-GTA

AAA CGA CGG CCA GTT TGG GGT TGG GGT TGG GGT TG-3', at 30°C for 60 min. Before PCR reaction, phenol/chloroform extraction and ethanol precipitation were performed to remove contaminants. PCR was then performed with the downstream primer 5'-CAG GAA ACA GCT ATG ACC CCT AAC CCT AAC CCT AAC CCT-3' for 30 cycles of 94°C for 45", 60°C for 30", and 72°C for 45". PCR products were detected by 6% polyacrylamide gel electrophoresis followed by Vistra Green (Amersham, Little Chalfont, UK) staining using a BAS 5000 Image analyzer (Fuji Film, Tokyo). Semi-quantification of telomerase activity was done by comparison with the standard telomerase activity in serial dilutions of T24 human bladder tumor cell line, based on measurement of the density of the ladder bands using NIH image ver 1.61.

**mRNA detection of rat telomerase catalytic subunit (rTERT) using RT-PCR** Total RNA was extracted from 10<sup>6</sup> exfoliated bladder cells, which were cumulatively collected from 5 rats by using TRizol (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions. cDNA was generated by reverse transcriptase using 1 μg of the extracted RNA. Expression of rTERT was investigated using PCR with up-primer 5'-GAC ATG GAG AAC AAG CTG TTT GC-3' and down-primer 5'-ACA GGG AAG TTC ACC ACT GTC-3'.<sup>7)</sup> Single 185 bp PCR products were detected on 2% agarose gel electrophoresis with ethidium bromide staining. Expression of rTERT was evaluated by comparison of the results with those of normal controls at each experimental stage.

**Telomere length assay** To analyze telomere length, we employed southern hybridization for terminal restriction fragments (TRFs) using a (TTAGGG)<sub>4</sub> probe. Genomic DNA was extracted from a mixture of exfoliated urothelial cells from ten rats. Five micrograms of DNA was digested

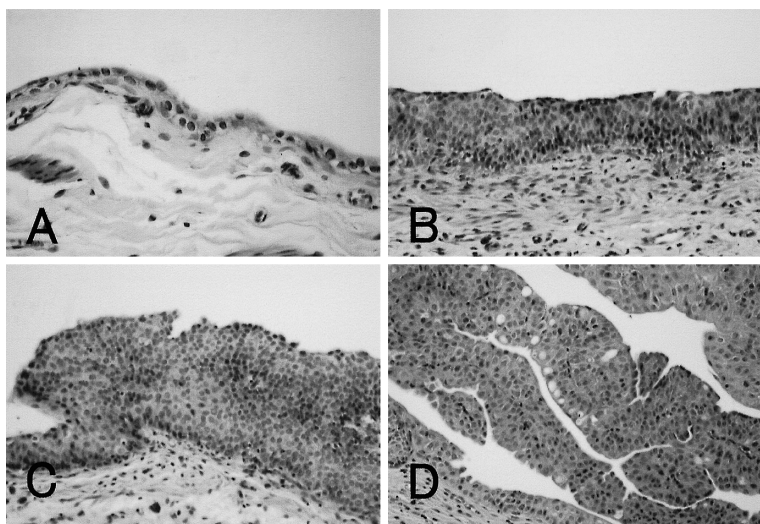


Fig. 1. Histological examination of normal rat urinary bladder shows 2 to 3 layers of transitional cell epithelium without nuclear atypia (A). At 4 weeks after BBN administration, bladder epithelium homogeneously thickens (diffuse hyperplasia) (B). In nodular hyperplasia at the 8th week following BBN administration, nodular hyperplastic lesions are observed in bladder epithelium (C). At 24 weeks, superficial bladder tumors without stromal invasion are microscopically observed in most rats (D).

using *RsaI/HinfI* enzyme mixture at 37°C for 12 h. TRF was resolved in 1% agarose gel by pulse field gel electrophoresis (PFGE) using a CHEF Mapper TM XA (Biorad, Hercules, CA) and transferred onto hybond N+ membrane. Hybridization was performed using a specific probe for the telomere sequence at 42°C overnight. Chemiluminescent detection was carried out using an ECL kit (Amersham) according to the manufacturer's instructions. The range of telomere length was estimated by comparison with a PFGE DNA marker using densitometry.

**Statistical analyses** Differences in semi-quantified telomerase activity between the tumor groups and control groups were analyzed using analyses of variance and Fisher's PLSD test.

## RESULTS

### Histological findings during BBN carcinogenesis in rats

Control rat bladder mucosa consisted of 2 to 3 layers of transitional cell epithelium (Fig. 1A). As stated in "Materials and Methods," at 4 and 8 weeks after BBN administration, diffuse hyperplasia (Fig. 1B) and nodular hyperplasia (Fig. 1C), respectively, were histologically observed in rat bladder epithelia. After 18 weeks, superficial bladder tumor was histologically observed in most of the rats. At 24 weeks, all rats had macroscopic bladder tumors, and these tumors were histologically confirmed as transitional cell carcinoma (Fig. 1D).

### Semi-quantification of telomerase activation during BBN bladder carcinogenesis

Telomerase activity of exfoliated bladder epithelial cells was detected at the 8th week following BBN administration. At the 4th week, no telomerase activity of bladder epithelia was detected on the gel (Fig. 2). No telomerase activity was detected until 4 weeks after BBN administration, whereas at 8 weeks, telomerase activity clearly increased in the epithelial cells showing irreversible nodular hyperplasia. The telomerase activities were semi-quantified by measuring the density of the ladder in comparison with that derived from single cells of T24 human bladder tumor cell line. When the telomerase activity of single T24 cells was set as 1.0, the relative telomerase activities (mean±SD) of single epithelial cells or single tumor cells of rat bladder were 0.023±0.007, 0.059±0.029, 0.176±0.030, 0.203±0.087, 0.270±0.047, 0.326±0.038, and 0.366±0.179 at 0, 4, 8, 10, 12, 18, and 24 weeks after BBN exposure, respectively. Semi-quantification of telomerase activity revealed a significant difference between telomerase activity at 4 weeks and that at 8 weeks ( $P=0.0284$ ), and no difference between that at 0 weeks and 4 weeks ( $P=0.484$ ). After this stage, telomerase activity increased with bladder carcinogenesis (Fig. 3).

**Expression of rTERT mRNA using RT-PCR** Specific primers for rTERT generated a single 185 bp band at a

period later than 8 weeks after BBN administration (Fig. 4). At the 4th week, no PCR product was observed, as was the case in the control group (Fig. 4).

**Telomere-length shortening during BBN bladder carcinogenesis** TRF measurement using southern hybridization revealed that telomere length was shortened at a period later than the 18th week following BBN adminis-

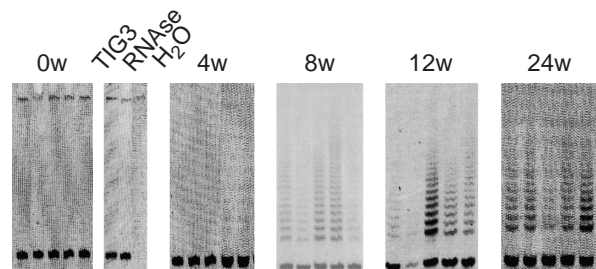


Fig. 2. PCR-based telomerase assay of bladder epithelial cells in rat, in which telomerase activity is detected as the density of the ladder-like PCR products. Telomerase activity is not observed at the 4th experimental week (diffuse hyperplasia), but appears at the 8th week (nodular hyperplasia), and then increases along with carcinogenesis. Negative control assay shows no telomerase activity. TIG3: human fibroblastic cell line. Each lane shows the telomerase activity of bladder epithelial cells from five rats combined at the time of protein extraction.

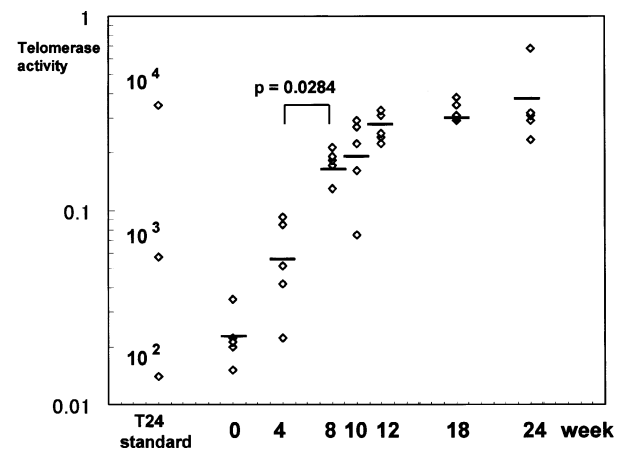


Fig. 3. Semi-quantification of telomerase activity during rat bladder carcinogenesis indicates that a significant difference in telomerase activity is seen between diffuse and nodular (at 8th week) hyperplasia ( $P=0.0284$ ). The telomerase activity in diffuse hyperplasia (at 4th week) is weak. The Y-axis is logarithmic, showing the relative telomerase activity of the samples as compared with that of T24 cell line. The X-axis shows standard telomerase activity using T24 cell line and telomerase activity of the sample at each experimental week. Bars indicate average telomerase activity in each group.

tration (Fig. 5). At the 4th, 8th, and 12th weeks, telomere shortening did not seem to be remarkable in comparison to that in control groups.

**DISCUSSION**

Research on the association of telomerase with human cancer has expanded rapidly in recent years. The presence of telomerase activity has been detected in the vast majority of cancers, including bladder cancer.<sup>4, 8-10</sup> In human bladder cancer, more than 90% of the tumors express telomerase activity, independent of tumor stage.<sup>11, 12</sup> Moreover, this enzyme activity could be detected in exfoliated cells of bladder tumor in urine.<sup>3, 4, 13</sup> However, neither the point at which telomerase is activated during bladder carcinogenesis, nor the specific role of telomerase activation in tumor development has yet been clarified.

In the present study, telomerase activity was detected at a period beyond the 8th week following BBN administration, at which time nodular hyperplasia could be observed in the bladder, whereas at the stage of diffuse hyperplasia, observed at the 4th week, no telomerase activity was found. Several investigations have reported that precancerous tissues, such as that in dysplasia and atypical hyperplasia, express telomerase activity.<sup>14-17</sup> Shroyer *et al.*<sup>17</sup> reported detection of telomerase activity in 30/30 and 12/13 of moderate and severe dysplasia in the uterine cervix, respectively. In their study, 56% of the reactive hyperplasia also expressed telomerase activity, which was not quantified. Yashima *et al.*<sup>18</sup> described heterogeneous expression of telomerase in carcinoma *in situ* of breast cancer, suggesting that semi-quantification might be useful to evaluate its activity in premalignant lesions. Similarly, in the present study, telomerase activity was observed in hyperplastic nodules of the liver, and it has also been observed in early neoplastic lesions of brain tumors during carcinogenesis in other animal models.<sup>19, 20</sup> Ito<sup>5</sup> reported

that until 4 weeks after BBN administration, epithelial change (e.g., diffuse hyperplasia) was reversible when BBN administration was stopped. In contrast, nodular hyperplasia, which was observed at 8 weeks after administration, was thought to be irreversible because bladder cancer developed even in the absence of BBN administration.<sup>5</sup> Considering the correlation between the significance of premalignant change and the status of telomerase activation, it is suggested that telomerase activation could serve at least as a predictive marker of early neoplastic change, which might evolve into cancer and play a role in malignant progression.

Messenger RNA expression of the catalytic subunit of rat telomerase was also observed after the nodular hyperplasia stage. This corresponds to an elevation of telomerase activity and indicates that detection of rTERT might be useful for early diagnosis of irreversible precancerous lesions. The difference in the level of telomerase activity between precancerous nodular hyperplasia and bladder cancer may be based on either the difference in activity among cells or on heterogeneity in the cellular population which expresses telomerase.

In our study, significant telomere shortening was observed at a phase later than that of telomerase activation. Telomere shortening has been seen in many kinds of cancers simultaneously with telomerase activity,<sup>21</sup> largely because telomere shortening involves or is involved in genetic instability. Harley<sup>22</sup> suggested that telomere loss

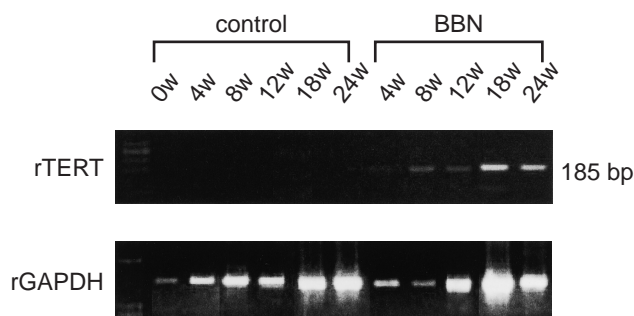


Fig. 4. RT-PCR generates a single 185 bp band using specific primers for rat telomerase catalytic subunit (rTERT). Expression of mRNA of rTERT in the exfoliated cells from bladder is clear at the stage after the 8th experimental week, but is difficult to detect at the 4th week.

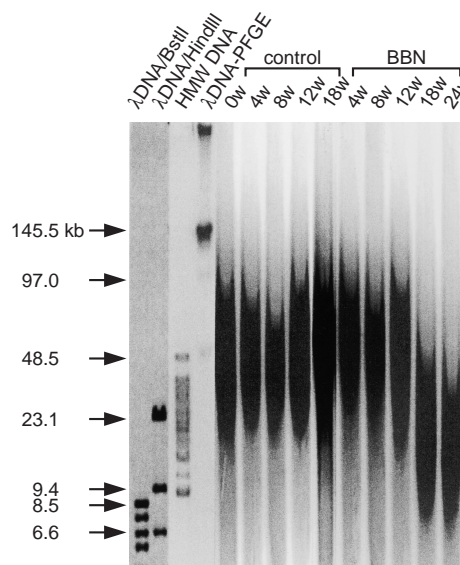


Fig. 5. Telomere length assay shows that length of terminal restriction fragments (TRFs) is preserved until the 12th week following BBN administration. Length of TRFs until the 12th experimental week ranges between 20 and 80 kb, whereas at the 18th to 24th week it ranges between approximately 8 and 40 kb.

might be an obligatory step in the progression and immortalization of most cancers. Miura *et al.*<sup>15)</sup> showed that telomere shortening may eventually lead to the reactivation of telomerase, which may then contribute to malignant conversion, based on clinical analysis of hepatocellular carcinogenesis. Although it has not been clearly established whether premalignant bladder lesions express telomerase activity, clinical investigation has shown that superficial bladder tumors usually exhibit strong telomerase activity and very short telomeres.<sup>23)</sup> However, an *in vitro* study showed that bladder cancer cell lines expressed strong telomerase activity with long telomeres, which may, however, be an *in vitro* artifact.<sup>24)</sup> In addition, prompt telomerase activation was observed in a chemical carcinogenesis model of the rat,<sup>25)</sup> suggesting that telomerase might be activated at a phase earlier than that of telomere shortening. In view of our findings in this study, telomerase activation might be a primary event during BBN bladder carcinogenesis in the rat, and might lead to malignant conversion without frequent genetic alteration. Thus, telomerase activation in this model seemed to be induced directly by BBN. However, it has been reported that p53 alteration of the tumor in this model was more frequent than that in human superficial bladder tumor.<sup>26, 27)</sup> Therefore, the correlation between p53 alteration and telomerase activation remains controversial, as noted previously.<sup>20, 28, 29)</sup> On the other hand, Broccoli *et al.*<sup>28)</sup> reported a lack of detectable telomere shortening during telomerase activation in mouse mammary tumors, which had very long telomeres like those in the rat. Hence, another possible explanation is that significant telomere shortening cannot be detected because of very long telomeres in the rat, the result of which is that genetic instability does not appear in the pre-neoplastic state. Further, contamination with exfoliated normal bladder epithelial cells with long telomeres might prevent detection of telomere shortening.

## REFERENCES

- 1) 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., Le, S., West, M. D., Harley, C. B., Andrews, W. H., Greider, C. W. and Villeponteau, B. The RNA component of human telomerase. *Science*, **269**, 1236–1241 (1995).
- 2) Shay, J. W. and Bacchetti, S. A survey of telomerase activity in human cancer (Review). *Eur. J. Cancer*, **33**, 787–791 (1997).
- 3) Kinoshita, H., Ogawa, O., Kakehi, Y., Mishina, M., Mitsumori, K., Itoh, N., Yamada, H., Terachi, T. and Yoshida, O. Detection of telomerase activity in exfoliated cells in urine from patients with bladder cancer. *J. Natl. Cancer Inst.*, **89**, 724–730 (1997).
- 4) Yoshida, K., Sugino, T., Tahara, H., Woodman, A., Bolodeoku, J., Nargund, V., Fellows, G., Goodison, S., Tahara, E. and Tarin, D. Telomerase activity in bladder carcinoma and its implications for non-invasive diagnosis by detection of exfoliated cancer cells in urine. *Cancer*, **79**, 362–369 (1997).
- 5) Ito, N. Early changes caused by N-butyl-N-(4-hydroxybutyl)nitrosamine in the bladder epithelium of different animal species. *Cancer Res.*, **36**, 2528–2531 (1976).
- 6) Kakizoe, T., Hasegawa, F., Kawachi, T. and Sugimura, T. Isolation of transitional epithelial cells from the rat urinary bladder. *Invest. Urol.*, **15**, 242–244 (1977).
- 7) Nozawa, K., Kurumiya, Y., Yamamoto, A., Isobe, Y., Suzuki, M. and Yoshida, S. Up-regulation of telomerase in

Superficial bladder cancers are usually treated by transurethral resection (TUR), and the urinary bladder can be preserved. However, this tumor has a strong tendency to recur, with multiple occurrences frequently observed. Intravesical recurrence or the multiple occurrence of bladder tumors can be considered to be a result of the seeding of tumor cells derived from a single progenitor cell,<sup>30, 31)</sup> or the expression of independent neoplastic events (i.e., new second recurrence, so-called second primary cancer).<sup>32)</sup> Hinotsu *et al.*<sup>33)</sup> recently reported that the recurrence of superficial bladder tumor was typically observed at the early phase and late phase after TUR. Intravesical chemotherapy could prevent the early-phase recurrence after TUR, but not the late-phase recurrence, suggesting that the late-phase recurrence might be based on the second primary cancer. In the case of the latter type of recurrence, detection of telomerase activity might be a useful tool to predict intravesical recurrence during a longer follow-up period in patients with superficial bladder tumors, because of the expression of telomerase in precancerous lesions. Detection of telomerase reverse transcriptase mRNA on dysplastic or hyperplastic epithelium would be of great significance to the investigation of the early phase of cancerous lesions during bladder carcinogenesis. To determine the tissue localization of telomerase expression, RNA *in situ* hybridization or RT-PCR using extracted RNA from micro-dissected tissue on histological specimens might be useful in future studies.

## ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan (No. 12031015-16).

(Received November 30, 2001/Revised February 13, 2002/  
Accepted February 22, 2002)

- primary cultured rat hepatocytes. *J. Biochem.*, **126**, 361–367 (1999).
- 8) Kamata, S., Kageyama, Y., Yonese, J. and Oshima, H. Significant telomere reduction in human superficial transitional cell carcinoma. *Br. J. Urol.*, **78**, 704–708 (1996).
  - 9) Lin, Y., Miyamoto, H., Fujinami, K., Uemura, H., Hosaka, M., Iwasaki, Y. and Kubota, Y. Telomerase activity in human bladder cancer. *Clin. Cancer Res.*, **2**, 929–932 (1996).
  - 10) Heine, B., Hummel, M., Muller, M., Heicappell, R., Miller, K. and Stein, H. Non-radioactive measurement of telomerase activity in human bladder cancer, bladder washings, and in urine. *J. Pathol.*, **184**, 71–76 (1998).
  - 11) Kyo, S., Kunimi, K., Uchibayashi, T., Namiki, M. and Inoue, M. Telomerase activity in human urothelial tumors. *Am. J. Clin. Pathol.*, **107**, 555–560 (1997).
  - 12) Rahat, M. A., Lahat, N., Gazawi, H., Resnick, M. B., Sova, Y., Ben-Ari, G., Cohen, M. and Stein, A. Telomerase activity in patients with transitional cell carcinoma: a preliminary study. *Cancer*, **85**, 919–924 (1999).
  - 13) Kavalier, E., Landman, J., Chang, Y., Droller, M. J. and Liu, B. C. Detecting human bladder carcinoma cells in voided urine samples by assaying for the presence of telomerase activity. *Cancer*, **82**, 708–714 (1998).
  - 14) Montalto, M. C. and Ray, F. A. Telomerase activation during the linear evolution of human fibroblasts to tumorigenicity in nude mice. *Carcinogenesis*, **17**, 2631–2634 (1996).
  - 15) Miura, N., Horikawa, I., Nishimoto, A., Ohmura, H., Ito, H., Hirohashi, S., Shay, J. W. and Oshimura, M. Progressive telomerase shortening and telomerase reactivation during hepatocellular carcinogenesis. *Cancer Genet. Cytogenet.*, **93**, 56–62 (1997).
  - 16) Hytioglou, P., Kotoula, V., Thung, S. N., Tsokos, M., Fiel, M. I. and Papadimitriou, C. S. Telomerase activity in pre-cancerous hepatic nodules. *Cancer*, **82**, 1831–1838 (1998).
  - 17) Shroyer, K. R., Thompson, L. C., Enomoto, T., Eskens, J. L., Shroyer, A. L. and McGregor, J. A. Telomerase expression in normal epithelium, reactive atypia, squamous cell carcinoma of the uterine cervix. *Am. J. Clin. Pathol.*, **109**, 153–162 (1998).
  - 18) Yashima, K., Milchgrub, S., Gollahon, L. S., Maitra, A., Saboorian, M. H., Shay, J. W. and Gazdar, A. F. Telomerase enzyme activity and RNA expression during the multistage pathogenesis of breast carcinoma. *Clin. Cancer Res.*, **4**, 229–234 (1998).
  - 19) Tsujiuchi, T., Tsutsumi, M., Kido, A., Kobitsu, K., Takahama, M., Majima, T., Denda, A., Nakae, D. and Konishi, Y. Increased telomerase activity in hyperplastic nodules and hepatocellular carcinomas induced by a choline-deficient L-amino acid-defined diet in rats. *Jpn. J. Cancer Res.*, **87**, 1111–1115 (1996).
  - 20) Sakitani, H., Tsujiuchi, T., Kobitsu, K., Kido, A., Iki, K., Takahama, M., Nakamura, M., Sakaki, T., Nakae, D., Konishi, Y. and Tsutsumi, M. Increased telomerase activity and absence of p53 mutation in oligo-astrocytomes induced by N-ethyl-N-nitrosourea in rats. *Cancer Lett.*, **126**, 157–164 (1998).
  - 21) de Lange, T. Telomere dynamics and genome instability in human cancer. In “Telomeres,” ed. E. H. Blackburn and C. W. Greider, pp. 265–293 (1995). Cold Spring Harbor Laboratory Press, New York.
  - 22) Harley, C. B. Telomere loss: mitotic clock or genetic time bomb? *Mutat. Res.*, **256**, 271–281 (1991).
  - 23) Kamata, S., Kageyama, Y., Yonese, J. and Oshima, H. Significant telomere reduction in human superficial transitional cell carcinoma. *Br. J. Urol.*, **78**, 704–708 (1996).
  - 24) Kageyama, Y., Kamata, S., Yonese, J. and Oshima, H. Telomere length and telomerase activity in bladder and prostate cancer cell lines. *Int. J. Urol.*, **4**, 407–410 (1997).
  - 25) Miura, M., Karasaki, Y., Abe, T., Higashi, K., Ikemura, K. and Gotoh, S. Prompt activation of telomerase by chemical carcinogens in rats detected with a modified TRAP assay. *Biochem. Biophys. Res. Commun.*, **246**, 13–19 (1998).
  - 26) Spruck, C. H., 3rd, Ohneseit, P. F., Gonzalez-Zulueta, M., Esrig, D., Miyano, N., Tsai, Y. C., Lerner, S. P., Schmutte, C., Yang, A. S. and Cote, R. Two molecular pathways to transitional cell carcinoma of the bladder. *Cancer Res.*, **54**, 784–788 (1994).
  - 27) Masui, T., Dong, Y., Yamamoto, S., Takada, N., Nakanishi, H., Inada, K., Fukushima, S. and Tatematsu, M. p53 mutations in transitional cell carcinomas of the urinary bladder in rats treated with N-butyl-N-(4-hydroxybutyl)-nitrosamine. *Cancer Lett.*, **105**, 105–112 (1996).
  - 28) Broccoli, D., Godley, L. A., Donehower, L. A., Varmus, H. E. and de Lange, T. Telomerase activation in mouse mammary tumors: lack of detectable telomere shortening and evidence for regulation of telomerase RNA with cell proliferation. *Mol. Cell. Biol.*, **16**, 3765–3772 (1996).
  - 29) Vaziri, H. and Benchimol, S. Alternative pathways for the extension of cellular life span: inactivation of p53/pRb and expression of telomerase. *Oncogene*, **13**, 7676–7680 (1999).
  - 30) Habuchi, T., Takahashi, R., Yamada, H., Kakehi, Y., Sugiyama, T. and Yoshida, O. Metachronous multifocal development of urothelial cancers by intraluminal seeding. *Lancet*, **342**, 1087–1088 (1993).
  - 31) Takahashi, T., Habuchi, T., Kakehi, Y., Mitsumori, K., Akao, T., Terachi, T. and Yoshida, O. Clonal and chronological genetic analysis of multifocal cancers of the bladder and upper urinary tract. *Cancer Res.*, **58**, 5835–5841 (1998).
  - 32) Heney, N. M., Daly, J., Prout, G. R., Jr., Nieh, P. T., Heaney, J. A. and Trebeck, N. E. Biopsy of apparently normal urothelium in patients with bladder carcinoma. *J. Urol.*, **120**, 559–560 (1978).
  - 33) Hinotsu, S., Akaza, H., Ohashi, Y. and Kotake, T. Intravesical chemotherapy for maximum prophylaxis of new early phase superficial bladder carcinoma treated by transurethral resection. A combined analysis of trials by the Japanese Urological Cancer Research Group using smoothed hazard function. *Cancer*, **86**, 1818–1826 (1999).