Immunohistochemical Detection of Human Telomerase Reverse Transcriptase in Normal Mucosa and Precancerous Lesions of the Stomach

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Telomerase activity confers cell immortality through stabilization of the chromosome, participating in the development of a majority of human cancers. Human telomerase reverse transcriptase (TERT) has been identified as a catalytic subunit of telomerase, and is overexpressed in most gastric carcinomas. We immunohistochemically examined the expression of TERT in normal gastric mucosa and candidate precancerous lesions such as intestinal metaplasia and adenoma. In non-neoplastic gastric mucosa including intestinal metaplasia and normal fundic mucosa, weak but significant expression of TERT was detected in nuclei of epithelial cells located in the lower two-thirds of the glands (wider than the proliferative zone). The telomerase activity was found in a half of gastric adenomas, whose levels of the activity were about 10% of those in gastric carcinomas. TERT protein was expressed in the nuclei of the adenoma cells at moderate levels, that were not necessarily comparable with the telomerase activities. These findings overall suggest that TERT expression may be one of the prerequisites for telomerase activation in an early stage of stomach carcinogenesis.

Key words: Telomerase reverse transcriptase — Gastric mucosa — Intestinal metaplasia — Gastric adenoma — Immunohistochemistry

Telomerase is a key enzyme that catalyzes the synthesis of telomere DNA participating in cell immortalization through stabilization of chromosomal structure.[1–3] Telomerase is expressed in germ tissues as well as the majority of human tumors, including gastrointestinal carcinomas.[4–8] Telomerase activation is associated with an early stage of stomach carcinogenesis. Most gastric carcinomas express strong telomerase activity regardless of histological type and tumor staging, while some intestinal metaplasia and gastric adenomas, candidate precancerous lesions, also express it at various levels.[4, 5, 9, 10] Human telomerase reverse transcriptase (hTERT) has been identified as a putative catalytic subunit of human telomerase.[11, 12] We have reported that 90% of gastric carcinomas express increased levels of TERT mRNA.[7] We also succeeded in immunohistochemical detection of TERT protein in gastric carcinomas.[7] In our previous study, strong expression of TERT was localized in the nuclei of gastric carcinoma cells, while TERT expression in non-neoplastic mucosa, as well as stromal elements except lymphocytes, was weak or negative.[7] *In situ* hybridization study has recently revealed that TERT mRNA is expressed in chief cells in the fundic glands and rarely in surface mucous cells of the stomach.[13] Most recently, we have found immunohistochemically that TERT protein is expressed in the colon, though more weakly than in carcinomas, in non-neoplastic crypt epithelia except those at the tip.[8] These findings prompted us to examine in detail the expression of TERT in gastric non-neoplastic and precancerous tissues by increasing the sensitivity of immunohistochemistry. We report here immunohistochemical detection of TERT protein in normal mucosa, intestinal metaplasia and adenoma of the stomach. The relationship between TERT expression and telomerase activity in gastric adenomas is also shown.

Tissues of non-neoplastic mucosa and adenoma of the stomach were obtained by endoscopic mucosal resection or surgery at Hiroshima University Hospital and related facilities. For immunohistochemistry, tissues were fixed in 10% buffered formalin and embedded in paraffin. To study telomerase activity, tissue samples were frozen in liquid nitrogen immediately after removal and stored at −80°C. HSC-39 cell line, derived from signet ring cell carcinoma of the stomach was kindly provided by Dr. K. Yanagihara (National Cancer Center Research Institute, Tokyo).[14] Polyclonal antibody against TERT protein (EST1.0) was raised in rabbit by injecting glutathione *S*-transferase-fused recombinant protein with amino acid sequences cor-

responding to nucleotides 1687–2042 of hTERT cDNA.[8] The anti-TERT antibodies were purified through affinity chromatography. Immunohistochemical staining was performed as described, with slight modifications.[7, 8] Deparaffinized sections were consecutively subjected to methanol-0.03% hydrogen peroxide treatment to block the endogenous peroxidase activity, microwave pretreatment in citrate buffer to retrieve the antigenicity, and incubation with blocking solution to block non-specific binding sites. The sections were then treated at room temperature with anti-TERT antibody (6 μg/ml) for 120 min or Ki-67 antibody (MIB-1; Medical and Biological Laboratories,

Nagoya; diluted 1:100) for 90 min. To visualize the immune-complex, a modification of the immunoglobulin enzyme bridge technique (ABC method) was employed using SensiTek HRP kit (ScyTek Laboratories, Logan, UT). Since 3,3′-diaminobenzidine tetrahydrochloride (DAB) was used as a substrate, positive reaction was detected as a brown color. The sections were weakly counterstained with 0.1% hematoxylin. The specificity of immunostaining was determined by 1) using irrelevant primary antibodies, 2) omitting the primary antibody, and 3) pre-absorption of the antibody with an excess of specific antigen (recombinant TERT protein). We also confirmed
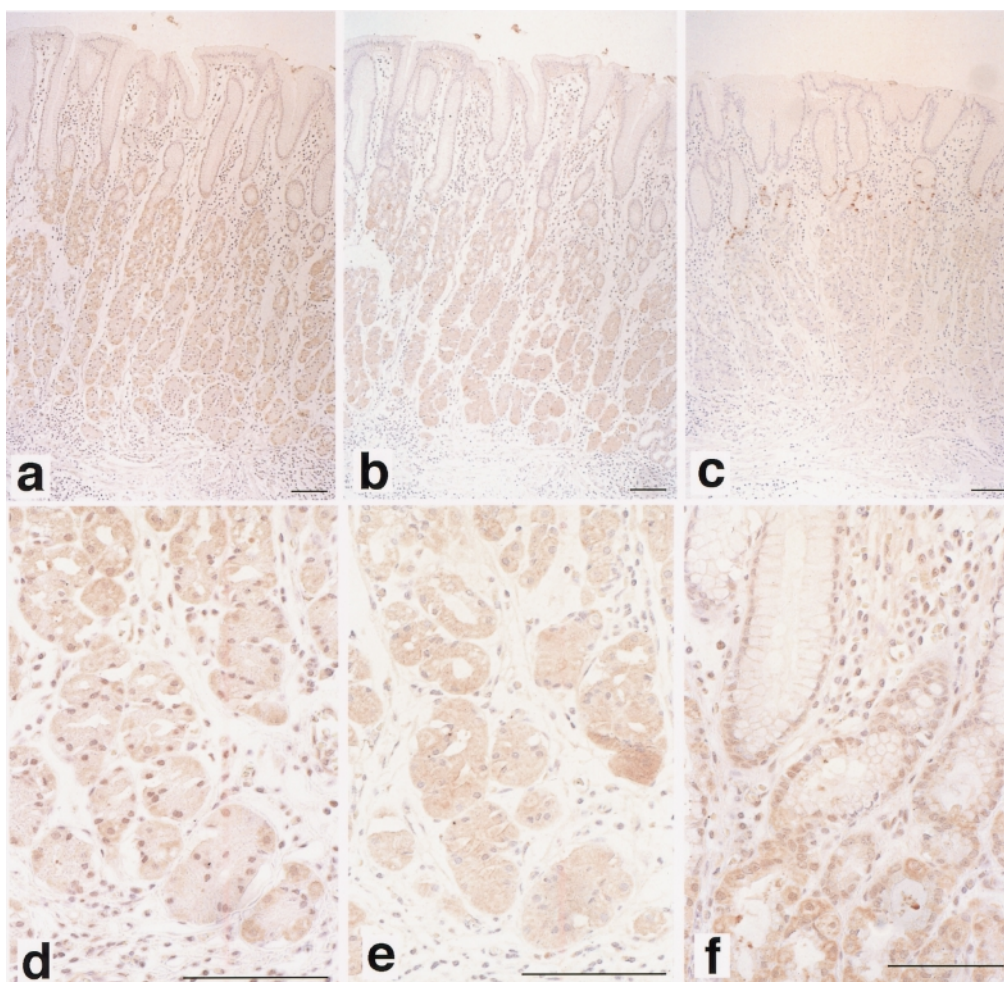

Fig. 1. Immunohistochemical detection of TERT protein in normal gastric mucosa. Tissue sections were immuno-stained with anti-TERT antibody (a, d, f), with anti-TERT antibody pre-absorbed with TERT protein-antigen (b, e) and with anti-Ki-67 antibody (c). Scale bars indicate 100 μm. In normal fundic mucosa, weak TERT-immunoreactivity was detected in epithelial cells in the lower two-thirds of the glands, while foveolar epithelial cells at the surface were negative (a, b, whole view; d, e, bottom of the glands; f, neck of the glands). Ki-67-positive cells were located at the neck of the fundic glands (c). Positive reaction in the nuclei was not detected by pre-absorbed antibody (b, e). Since brown color in the cytoplasm was not abolished by pre-absorbing with the antigenic peptides, the cytoplasmic staining was non-specific (e).
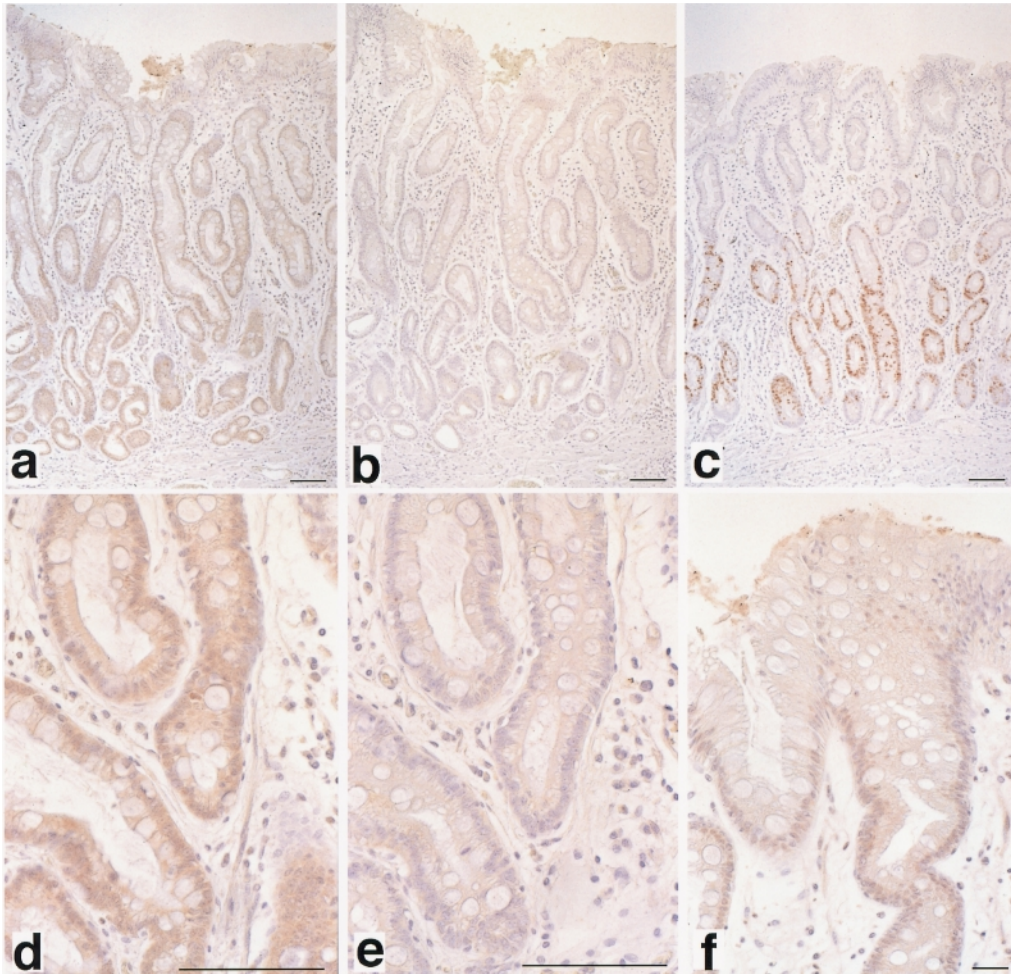
Fig. 2. Immunohistochemical detection of TERT protein in intestinal metaplasia of the stomach. Tissue sections were immuno-stained with anti-TERT antibody (a, d, f), with anti-TERT antibody pre-absorbed with TERT peptide-antigen (b, e) and with anti-Ki-67 antibody (c). TERT-immunoreactive cells were located in the lower two-thirds of the metaplastic glands, while surface epithelia were negative (a, b, whole view; d, e, bottom of the glands; f, surface of the glands). Ki-67-positive cells were located in the lower one-third of the glands (c). Positive reaction in the nuclei was not detected by pre-absorbed antibody (b, e).

that the same concentrations of unrelated recombinant protein as well as bovine serum albumin did not inhibit the reactivity of anti-TERT antibody, and that the recombinant TERT protein did not affect the reactivity of anti-Ki-67 antibody. In order to enhance the sensitivity of TERT staining, an increased concentration of anti-TERT antibody was used and incubation with the antibody and reaction with DAB were performed for longer, as compared with the previous conditions.[7]

Telomerase activity was assayed by the TRAP (telomeric repeat amplification protocol) method with some modification, using extract of tissue protein (0.1 μg) as described.[6] The PCR (polymerase chain reaction) products were analyzed by electrophoresis on a 12% polyacryl-

amide gel, stained with SYBR Green I and visualized. The telomerase activities (TRAP signals) were quantified using a fluorescence image analyzer (FUJI FLA-2000, Fuji Photo Film Co., Ltd., Tokyo), normalized to the internal telomerase assay standard (ITAS) signal.[6] The TRAP signals were confirmed to be sensitive to RNase pretreatment.

We first examined the expression and localization of TERT protein in non-neoplastic gastric mucosas. Normal epithelial cells of the fundic glands were positive to TERT although the immunoreactivity was much weaker than that in carcinoma cells as reported previously (Fig. 1).[7] TERT protein was observed in most epithelial cells in the lower two-thirds of the glands, including parietal cells, chief cells, and mucous neck cells, while foveolar epithelial
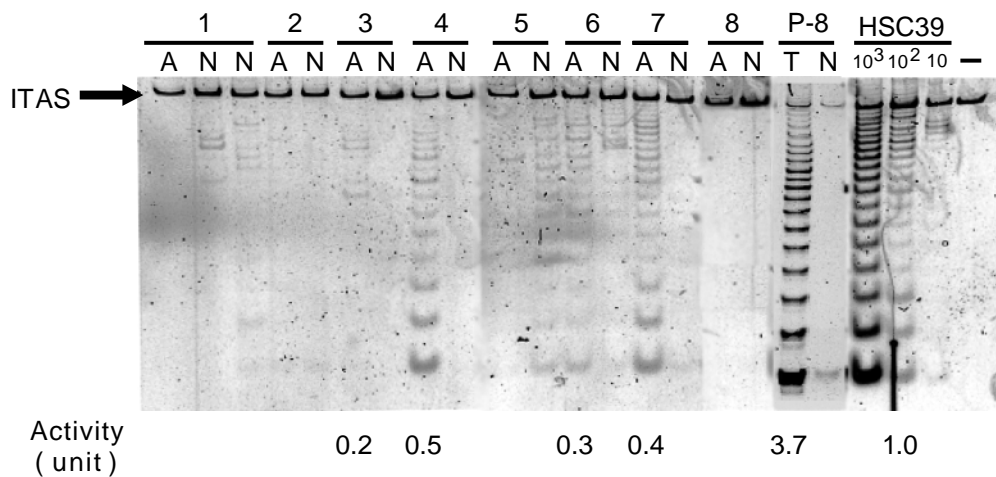
Fig. 3. Telomerase activity in gastric adenomas. Adenoma tissues (A) and corresponding non-neoplastic mucosa from the same patients (N) were assayed for telomerase activity by the TRAP method. The numbers at the top represent the sample number. P-8 is a gastric carcinoma case shown for reference (T, tumor tissue). HSC-39 cells (1000 cells, 100 cells or 10 cells) were used as a positive control. —, buffer containing no sample extract assayed as a negative control. TRAP signals were quantified using an image analyzer, and normalized to the ITAS signal. Arbitrary units, calculated based on the activity in 100 cells of HSC39 taken as 1.0, were used.
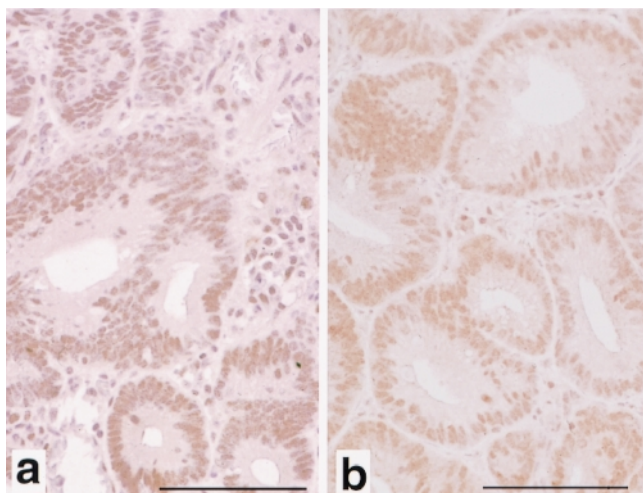

Fig. 4. Immunohistochemical detection of TERT protein in gastric adenomas. Tissue sections were immuno-stained with anti-TERT antibody. Many adenoma cells were positive to TERT protein in their nuclei with moderate intensity in both telomerase-positive (a, case 4 in Fig. 3) and telomerase-negative (b, case 5 in Fig. 3) tumors.

cells at the surface were negative. The distribution of TERT-positive cells was wider than that of proliferating Ki-67-positive cells, which were located at the neck of the fundic glands (Fig. 1, a and c). Since nuclear staining, but not cytoplasmic brown color was abolished by pre-absorbing the antibody with the antigenic peptides, cytoplasmic staining was found to be non-specific (Fig. 1, a, b, d and e). Similarly, epithelial cells at the lower two-thirds of the intestinal metaplastic glands were also weakly positive to TERT, while the surface epithelia were negative (Fig. 2). The immunoreactivity to TERT in the cytoplasm seemed to be slightly reduced by pre-absorbing the antibody with the antigen (Fig. 2, d and e), though we could not clarify the meaning of this phenomenon. As Ki-67-positive cells were located at the lower one-third of the glands, TERT-positive cells were more widely distributed than proliferating cells.

Our previous studies have revealed that a half of gastric adenomas expressed telomerase activity.[4, 10] We then quantitatively examined telomerase activities in gastric adenomas. As shown in Fig. 3, 4 (50%) of 8 adenomas showed telomerase activities at low to moderate levels, while weak signals were detected in non-neoplastic samples of two cases (cases 1 and 5). TRAP signals were quantified using an image analyzer, and normalized to the ITAS signal. They were expressed in arbitrary units calculated based on the activity in 100 cells of HSC39, taken as 1.0. The activity in 4 telomerase-positive adenomas was 0.35±0.01 (average±SD). According to our published data,[7] the activities in gastric carcinomas and non-neoplastic mucosa were 3.32±3.87 and 0.05±0.01, respectively. Therefore, telomerase activity in gastric adenomas was found to be about one-tenth of that in gastric carcinomas, while it was 7-fold higher than that in non-neoplastic gastric mucosa.

We next examined TERT protein expression in gastric adenomas. All 8 gastric adenomas including those with undetectable telomerase activity expressed TERT protein. Many adenoma cells were positive to TERT protein in their nuclei with moderate intensity in both telomerase-positive (Fig. 4a; case 4 in Fig. 3) and telomerase-negative (Fig. 4b; case 5 in Fig. 3) tumors. mRNA expression for TERT was confirmed by RT (reverse transcription)-PCR (data not shown). Thus, TERT protein expression was not necessarily comparable with the telomerase activities in gastric adenomas. These findings suggest that TERT expression may be prerequisite for telomerase activation in an early stage of stomach carcinogenesis.

In this study, we demonstrated that TERT protein was expressed in normal fundic mucosa of the stomach, though weakly. These epithelial cells are terminally differentiated and non-proliferating. We have recently reported that non-proliferating colon crypt cells express TERT protein weakly.[8] Therefore, low levels of TERT expression might be characteristic of physically regenerating tissues containing stem cells. TERT in these cells might be synthesized by survivors within the proliferative zone. It is also possible that TERT may be synthesized in differentiated non-proliferating cells at low levels because *in situ* hybridization had revealed the expression of TERT mRNA in chief cells in the fundic glands.[13] Therefore, although they are differentiated and non-proliferating, TERT-positive cells might be competent for regeneration if severe mucosal damage occurs. However, other possibilities can not be excluded, e.g., that these cells contain degraded proteins that retain the antigenicity of TERT but lack enzymatic function.

Human TERT gene promoter has been recently characterized.[15, 16] The promoter of TERT is GC-rich, and lacks both TATA and CAAT boxes, but contains potential myc protein binding sites (E-boxes). Furthermore, the promoter contains multiple consensus motifs for transcription factors, Sp1, AP-2 and so on. These ubiquitous transcription factors may maintain the expression of TERT in normal tissues. In fact, normal gastric mucosa expresses Sp1 although at a much lower level than gastric carcinomas.[17] Enhanced expression of TERT in gastric carcinomas may be associated with increased levels of regulatory factors such as Sp1 and c-myc protein.[18]

It is well known that intestinal metaplasia is a possible precancerous lesion progressing to well-differentiated or intestinal type gastric carcinoma.[19] Many genetic abnormalities found in well-differentiated gastric carcinomas, such as K-*ras* mutations, *p53* mutations and genetic instabilities, are involved in intestinal metaplasia, even though at low frequency.[20–22] In this study, epithelial cells in the lower two-thirds of the intestinal metaplastic glands were positive to TERT. We have previously found that increased telomerase activity and human telomerase RNA (hTR)

were associated with advanced grades of intestinal metaplasia.[9] Gastrointestinal stem cells are characterized by proliferating activity as well as telomerase activity. TERT-positive epithelial cells in the lower two-thirds of the metaplastic glands might function as a reservoir of stem cells. Comparative study of TERT expression and telomerase activity at individual cell levels should be carried out to test this possibility.

Gastric adenoma is an important precancerous lesion, although the idea of an adenoma-carcinoma sequence in stomach carcinogenesis is still under discussion.[23] However, our accumulated evidence suggests that 30–50% of gastric adenomas share not only epigenetic, but also genetic alterations observed in well differentiated gastric cancer.[23] The most interesting feature in this study was that most adenoma cells were positive for TERT protein, although a half of the adenomas showed undetectable telomerase activity. It is unlikely that telomerase activity by mesenchymal components such as leukocytes accounts for the discrepancy between the enzyme activity and TERT expression in adenomas, because the extent of infiltrating lymphocytes in telomerase-positive adenoma was similar to that in telomerase-negative adenoma. Therefore, some other factors than TERT might play a role in the regulation of telomerase activity in gastric adenomas. It has recently been shown that telomerase activity in human development is regulated not only by TERT expression, but also by alternative splicing of TERT transcripts.[24] Although we do not know at present whether alternative splicing occurs in gastric tumors, the anti-TERT antibody used in this study (against protein corresponding to nucleotides 1678–2042 of hTERT cDNA) can detect the reported alternatively spliced transcripts with critical reverse transcriptase motifs deleted (α deletion, 2186–2221; β deletion, 2342–2524).[24] It is also probable that post-translational modification of TERT, such as phosphorylation, may be involved in the regulation of telomerase activity. Other candidates for modifying telomerase activity are telomere-specific DNA binding proteins. The function of human telomeres requires TRF1 (telomeric repeat binding factor-1) and TRF2.[25, 26] TRF1 negatively regulates telomere length maintenance, while TRF2 protects telomeres from end-to-end fusion.[25, 26] TRF1 is believed to act in a *cis* mode by inhibiting telomerase at telomere termini, although it does not control the expression of telomerase. Recently, tankyrase, a protein with homology to the catalytic domain of poly-adenosine diphosphate (ADP)-ribose polymerase, has been shown to bind to TRF1.[27] Further study should be performed to examine the alterations of telomere binding proteins and poly-ADP-ribosylation in the course of multistep carcinogenesis of the stomach.

In conclusion, the findings observed in the present study overall suggest that the presence of TERT is prerequisite

for telomerase activation, and post-translational modification or other mechanisms to activate the enzyme may contribute to the conversion of gastric adenoma to carcinoma.

REFERENCES

1) Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, 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 immortal cells and cancer. *Science*, **266**, 2011–2015 (1994).

2) Blackburn, E. H. Telomerase. *Annu. Rev. Biochem.*, **61**, 113–129 (1992).

3) Shay, J. W. and Bacchetti, S. A survey of telomerase activity in human cancer. *Eur. J. Cancer*, **33**, 787–791 (1997).

4) Tahara, H., Kuniyasu, H., Yokuzaki, H., Yasui, W., Shay, J. W., Ide, T. and Tahara, E. Telomerase activity in preneoplastic and neoplastic gastric and colorectal lesions. *Clin. Cancer Res.*, **1**, 1245–1251 (1995).

5) Chandeneau, C., Hay, K., Hirte, H. W., Gallinger, S. and Bacchetti, S. Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res.*, **55**, 2533–2536 (1995).

6) Nakayama, J., Tahara, H., Tahara, E., Saito, M., Ito, K., Nakamura, H., Nakanishi, T., Tahara, E., Ide, T. and Ishikawa, F. Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. *Nat. Genet.*, **18**, 65–68 (1998).

7) Yasui, W., Tahara, H., Tahara, Ej., Fujimoto, J., Nakayama, J., Ishikawa, F., Ide, T. and Tahara, E. Expression of telomerase catalytic component, telomerase reverse transcriptase, in human gastric carcinomas. *Jpn. J. Cancer Res.*, **89**, 1099–1103 (1998).

8) Tahara, H., Yasui, W., Tahara, Ej., Fujimoto, J., Ito, K., Tamai, K., Nakayama, J., Ishikawa, F., Tahara, E. and Ide, T. Immuno-histochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and no-tumor tissue sections. *Oncogene*, **18**, 1561–1567 (1999).

9) Kuniyasu, H., Domen, T., Hamamoto, T., Yokozaki, H., Yasui, W., Tahara, H. and Tahara, E. Expression of human telomerase RNA is an early event of stomach carcinogenesis. *Jpn. J. Cancer Res.*, **88**, 103–107 (1997).

10) Hiyama, T., Yokozaki, H., Kitadai, Y., Tahara, Ej., Tahara, H., Ide, T., Haruma, K., Yasui, W., Kajiyama, G. and Tahara, E. *In situ* hybridization technique for analysis of human telomerase RNA in gastric precancerous and cancerous lesions. *Jpn. J. Cancer Res.*, **89**, 1187–1194 (1998).

11) Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Lingner, J., Harley, C. B. and Cech, T. R. Telomerase catalytic subunit homologs from fission yeast and human. *Science*, **277**, 955–959 (1997).

12) Meyerson, M., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R. L., Davidoff, M. J., Liu, Q., Bacchetti, S., Haber, D. A. and Weinberg, R. A. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell*, **90**, 785–795 (1997).

13) Kolquist, K. A., Ellisen, L. W., Counter, C. M., Meyerson, M., Tan, L. K., Weinberg, R. A., Haber, D. A. and Gerald, W. L. Expression of *TERT* in early premalignant lesions and a subset of cells in normal tissues. *Nat. Genet.*, **19**, 182–186 (1998).

14) Yanagihara, K., Seyama, T., Tsumuraya, M., Kamada, N. and Yokoro, K. Establishment and characterization of human signet ring cell gastric carcinoma cell lines with amplification of the c-*myc* oncogene. *Cancer Res.*, **51**, 381–386 (1991).

15) Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M. and Inoue, M. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Res.*, **59**, 551–557 (1999).

16) Horikawa, I., Cable, P. L., Afshari, C. and Barrett, J. C. Cloning and characterization of the promoter region of *human telomerase reverse transcriptase* gene. *Cancer Res.*, **59**, 826–830 (1999).

17) Kitadai, Y., Yasui, W., Yokozaki, H., Kuniyasu, H., Haruma, K., Kajiyama, G. and Tahara, E. The level of a transcription factor Sp1 is correlated with the expression of EGF receptor in human gastric carcinomas. *Biochem. Biophys. Res. Commun.*, **189**, 1342–1348 (1992).

18) Wu, K.-J., Grandori, C., Amacker, M., Simon-Vermot, N., Polack, A., Lingner, J. and Dalla-Favera, R. Direct activation of *TERT* transcription by c-MYC. *Nat. Genet.*, **21**, 220–224 (1999).

19) Correa, P. Human gastric carcinogenesis: a multistep and multifactorial process. *Cancer Res.*, **52**, 6735–6740 (1992).

20) Tahara, E., Kuniyasu, H., Yasui, W. and Yokozaki, H. Gene alterations in intestinal metaplasia and gastric cancer. *Eur. J. Gastroenterol. Hepatol.*, **6**, 97–102 (1994).

21) Ochiai, A., Yamauchi, Y. and Hirohashi, S. p53 mutations in the non-neoplastic mucosa of the human stomach showing intestinal metaplasia. *Int. J. Cancer*, **69**, 28–33 (1996).

22) Hamamoto, T., Yokozaki, H., Semba, S., Yasui, W., Yunotani, S., Miyazaki, K. and Tahara, E. Altered micro-

satellites in incomplete-type intestinal metaplasia adjacent to primary gastric cancers. *J. Clin. Pathol.*, **50**, 841–846 (1997).

23) Tahara, E., Yokozaki, H. and Yasui, W. Stomach—genetic and epigenetic alterations of preneoplastic and neoplastic lesions. *In* "Molecular Pathology of Early Cancer," ed. S. Srivastava, D. E. Henson and A. Gazdar, pp. 341–361 (1999). IOS Press, Amsterdam.

24) Ulaner, G. A., Hu, J.-F., Vu, T. H., Giudice, L. C. and Hoffman, A. R. Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. *Cancer Res.*, **58**, 4168–4172 (1998).

25) van Steensel, B. and de Lange, T. Control of telomere length by the human telomeric protein TRF1. *Nature*, **385**, 740–743 (1997).

26) van Steensel, B., Smogorzewska, A. and de Lange, T. TRF2 protects human telomeres from end-to-end fusions. *Cell*, **92**, 401–413 (1998).

27) Smith, S., Giriat, I., Schmitt, A. and de Lange, T. Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science*, **282**, 1484–1487 (1998).