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In situ mRNA Hybridization Technique for Analysis of Human Telomerase RNA in Gastric Precancerous and Cancerous Lesions

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Telomerase, the ribonucleoprotein enzyme that elongates telomeres, is repressed in normal somatic cells but is reactivated during tumor progression. The purpose of this study was to investigate the localization of human telomerase RNA (hTR) expression in human gastric precancerous and cancerous lesions by using *in situ* mRNA hybridization (ISH) with avidin-biotin staining. We also examined telomerase activity in these lesions by using hybridization protection assay connected with a telomeric repeat amplification protocol (TRAP/HPA). Analyzed tissue samples were as follows; 132 cases of chronic atrophic gastritis without intestinal metaplasia, 115 incomplete-type intestinal metaplasias, 40 complete-type intestinal metaplasias, 23 hyperplastic polyps, 23 tubular adenomas and 26 adenocarcinomas. In ISH analysis, high levels of hTR expression were observed preferentially in the nuclei at the single-cell level. hTR-expressing cells in carcinomas and adenomas were significantly more frequent than those of the other lesions ($P<0.001$). The expression pattern of hTR in carcinoma and adenoma tissues was heterogeneous and similar intratumor heterogeneity was detected in Ki-67 immunoreactivity. Infiltrating lymphocytes in tissues also exhibited high levels of hTR expression. In TRAP/HPA analysis, carcinomas had significantly more frequent positivity for telomerase activity and a higher level of telomerase activity than the other lesions ($P<0.05$). However, the amount of telomerase activity did not parallel the expression level of hTR. Our data suggest that hTR expression increases in the early stages of stomach carcinogenesis and that sufficient synthesis of hTR is a prerequisite for telomerase reactivation in tumorigenesis.

Key words: Human telomerase RNA — *In situ* mRNA hybridization — Gastric cancer — Gastric precancerous lesion — Telomerase activity

Gastric carcinomas involve genetic alterations in multiple tumor suppressor genes and oncogenes, as well as microsatellite instability.[1–4] The pattern of multiple genetic and epigenetic alterations in gastric carcinomas differs depending on two histological types, well differentiated or intestinal type and poorly differentiated or diffuse type, indicating that they have different genetic involvements.[5] However, reactivation of telomerase, which is responsible for cell immortality, is the most common and fundamental event in gastric carcinomas.[6] In our previous study, telomerase activity was detected in 85% of gastric carcinomas, irrespective of histological type and tumor staging.[7,8] We have also detected telomerase activity in 23% of intestinal metaplasias and 50% of adenomas of the stomach, suggesting that reactivation of telomerase plays a crucial role in the early stage of stomach carcinogenesis.[7]

Recently, the RNA component of human telomerase (hTR) was cloned.[9] In transgenic mouse models, differential regulation of telomerase activity and telomerase RNA during tumorigenesis was reported, i.e., the telomerase RNA (mTR) level was up-regulated in the early preneoplastic stages, although telomerase activity was detected only in the late-stage tumors, and mTR levels did not parallel the amount of telomerase activity.[10] It was also reported that hTR expression did not parallel the increase in telomerase activity in human tumors.[11] We have reported that 81% cases expressed hTR at higher levels in gastric carcinomas than in the corresponding mucosae, although all tumor specimens and the corresponding mucosa expressed various levels of hTR as determined by northern blot analysis.[12] To establish the localization of hTR expression in tissues, *in situ* mRNA hybridization (ISH) is a useful tool, because the technique can detect specific mRNA transcripts in formalin-fixed, paraffin-embedded specimens.[13,14] The purpose of this study was to investigate the localization of hTR expression in gastric precancerous and cancerous lesions by using ISH. Moreover, we examined telomerase activity and Ki-67 immunoreactivity in these lesions.

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MATERIALS AND METHODS

Tissue samples Surgically or endoscopically removed tissues of the stomach were obtained from Hiroshima University Hospital or its affiliated hospitals from December 1995 to December 1996. Analyzed tissue samples were as follows; 132 cases of chronic atrophic gastritis without intestinal metaplasia, 115 incomplete-type intestinal metaplasias, 40 complete-type intestinal metaplasias, 23 hyperplastic polyps, 23 tubular adenomas and 26 adenocarcinomas. After surgical or endoscopic tissue removal, the samples were immediately frozen in liquid nitrogen and stored at −80°C until use. The definitions of histological classification of gastric carcinomas were made according to the criteria of the Japanese Research Society for Gastric Carcinoma.[15]

Oligonucleotide probe A specific antisense oligonucleotide DNA probe was designed complementary to the hTR transcript.[10] The sequence of the antisense probe for hTR used in this study was 5′-CAC′GGC′GCC′TAC′GCC′CTT′CTC′AGT′TAG-3′. First, the specificity of the oligonucleotide sequence was determined by Genome Data Base search using the Oligo program (National Biosciences, Plymouth, MN); the oligonucleotide showed 100% homology with the hTR sequence and minimal homology with nonspecific mammalian gene sequences. Second, the specificity of the oligonucleotide sequence was confirmed by northern blot analysis. A $d(T)_{20}$ oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. The DNA probe was synthesized with six biotin molecules at the 3′ end (Research Genetics, Huntsville, AL).[16] The lyophilized probe was reconstituted to a stock solution at 1 μg/μl in 10 m*M* Tris (pH 7.6) and 1 m*M* EDTA. The stock solution was diluted with probe diluent (Research Genetics) before use.

Preparation of samples for *in situ* mRNA hybridization Four micrometer thick, formalin-fixed, paraffin-embedded tissue slices were placed on silane-treated ProbeOn Plus slides. The slides were dewaxed and dehydrated with Autodewaxer and Autoalcohol (Research Genetics) followed by digestion with pepsin[17] and then hybridized as described below.

***In situ* mRNA hybridization** ISH was performed as described previously.[18–21] ISH was carried out by using the MicroProbe manual staining system (Fisher Scientific).[22] Hybridization of the probe was carried out for 45 min at 45°C and then the slides were washed three times for 2 min each time with 2× standard saline citrate at 45°C. The slides were incubated with alkaline phosphatase-labelled avidin for 30 min at 45°C, rinsed with alkaline phosphatase enhancer for 1 min and incubated with a chromogen substrate for 15 min at 45°C. A positive reaction in this assay was seen as a red stain. Controls for endogenous alkaline phosphatase included treatment of the samples in the absence of the biotinylated probe and use of chromogen alone.

To check the specificity of the hybridization signal, the following controls were run: (i) RNase pretreatment of tissue sections, (ii) substitution of the antisense probe with a biotin-labelled sense probe, (iii) competition assay with unlabelled antisense probe, and (iv) no probe. All four control treatments showed no signals or markedly decreased signals.

The integrity of the mRNA in the samples was verified by using a poly $d(T)_{20}$ probe. Samples in which RNA was degraded were eliminated.

The expression levels of hTR in tissues were indicated as percentage values of the ratio of positively stained cells in 1,000 tumor cells (adenomas or carcinomas) or 1,000 epithelial cells (chronic gastritis, intestinal metaplasias or hyperplastic polyps) seen in representative areas by high-power field observation.

Assay of telomerase Telomerase activity was assayed by means of hybridization protection assay coupled with a telomeric repeat amplification protocol (TRAP/HPA).[23] Briefly, extracts from cells or tissues were prepared according to the protocol of Kim *et al*.[24] by using the detergent CHAPS. The supernatants were divided into aliquots and quickly frozen at −80°C. Protein concentrations of the tissue supernatants were determined by Coomassie brilliant blue assay. Six micrograms of protein was used for the TRAP assay. The reaction mixture was subjected to polymerase chain reaction (31 cycles at 94°C for 40 s, 50°C for 40 s and 72°C for 60 s). A 5 μl sample of telomerase reaction product was heat-denatured for 5 min at 95°C. Then, 100 μl of acridinium-ester-labelled probe (5′-CCC′TAA′CCC′TAA′CCC′TAA′CTC′TGC′TCG′AC-3′), 3×10^7 relative light units (rlu), in hybridization buffer (0.1 *M* lithium succinate buffer, pH 4.7, containing 20% dodecyl sulfate, 1.2 *M* lithium chloride, 20 m*M* EDTA and 20 m*M* EGTA) was added to each reaction tube and incubation was continued for 20 min at 60°C. A 300 μl aliquot of selection buffer (0.6 *M* sodium tetraborate buffer, pH 8.5, containing 5% Triton X-100) was added to differentially hydrolyze unhybridized probe during incubation for 10 min at 60°C. After the differential hydrolysis, 50 μl of 0.05% phenol red (Gibco BRL, Grand Island, NY) was mixed to quench the chemiluminescence. Quenching by phenol red of acridinium-ester chemiluminescence was required to achieve wide-range linearity, because the labelled probe gave signals that were too high. Chemiluminescence (rlu) was measured in 2 s/tube by a luminometer (Leader I, Gen-Probe Inc., San Diego, CA). To express telomerase activity in tissues we defined the activity equivalent to that in one cell of MKN-1 gastric carcinoma cell line as 1 unit. Telomerase activity of more than 10 units was regarded as positive.

Ki-67 immunohistochemistry Ki-67 immunohistochemistry was performed as described previously.[25] Anti-Ki-67 monoclonal antibody (MIB-1) was obtained from Medical and Biological Laboratories (Nagoya).

Statistical analyses Student's *t* test was used in statistical analyses. The χ^2 test was also used for statistical analysis of the frequency of positive telomerase activity among each histological type. The criterion of significance was set at $P<0.05$.

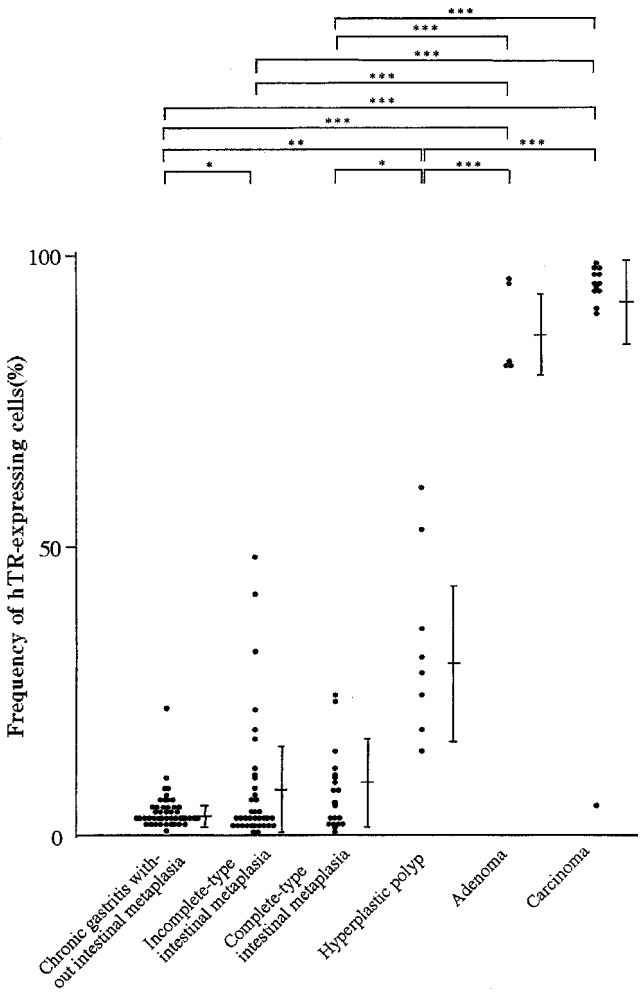

Fig. 1. Summary of hTR expression evaluated by ISH analysis in gastric lesions. hTR-expressing cells in 1,000 tumor cells (adenomas or carcinomas) or 1,000 epithelial cells (chronic gastritis, intestinal metaplasias or hyperplastic polyps) in representative areas were counted and values are indicated as percentages. Hatched bars show the ranges of mean±SD. Student's *t* test was performed for statistical analysis. *, $P<0.05$; **, $P<0.005$; ***, $P<0.001$.

RESULTS

Integrity of mRNA in archival specimens The integrity of the mRNA in each formalin-fixed, paraffin-embedded

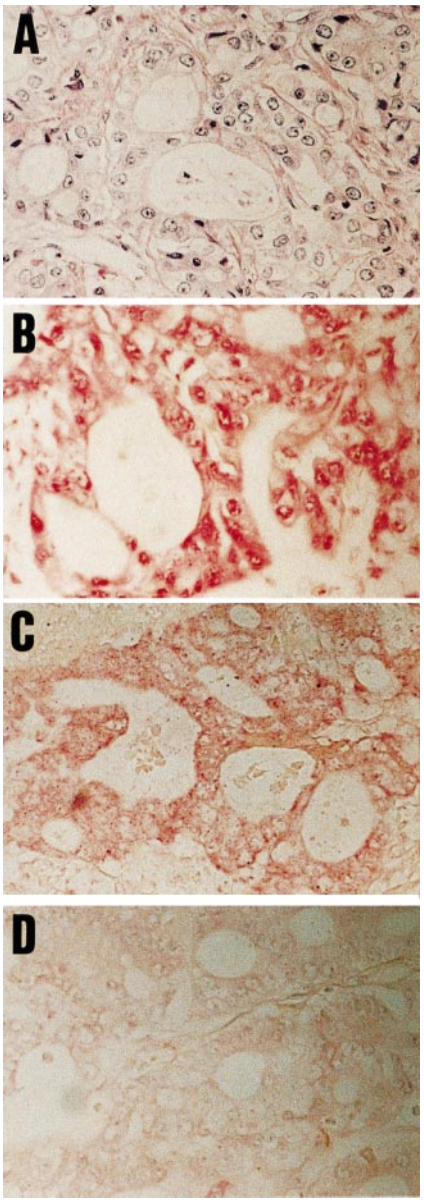

Fig. 2. ISH for hTR expression in a gastric carcinoma. A. Microscopic view showing moderately differentiated adenocarcinoma (HE, original magnification, ×400). B. ISH with a hyperbiotinylated $d(T)_{20}$ oligonucleotide probe, confirming mRNA integrity and lack of degradation. C. ISH with the hyperbiotinylated antisense hTR oligonucleotide probe, showing strong staining in most carcinoma cells. D. ISH with the hyperbiotinylated sense hTR oligonucleotide probe, showing rather weak staining.

tissue was verified by using a poly $d(T)_{20}$ probe. There were 144 (39%) samples that had an intense histochemical reaction, indicating that the mRNA was not degraded. The specimens consisted of samples from 48 cases of chronic atrophic gastritis without intestinal metaplasia, 47 incomplete-type intestinal metaplasias, 22 complete-type intestinal metaplasias, 9 hyperplastic polyps, 5 tubular adenomas and 13 adenocarcinomas.

Expression levels of hTR Next, we examined the expression levels of hTR in gastric precancerous and cancerous lesions by using ISH. High levels of hTR expression were found preferentially in the nuclei of most carcinoma and adenoma cells and some non-cancerous cells, including infiltrating lymphocytes, at the single-cell level. To analyze the expression levels of hTR semiquantatively, hTR-expressing cells in 1,000 tumor cells (adenomas or carcinomas) or 1,000 epithelial cells (chronic gastritis, intestinal metaplasias or hyperplastic polyps) in representative areas were counted and the results are given as percentages. The expression levels of hTR in the present study are summarized in Fig. 1. hTR-expressing cells amounted to 4.3±1.8 (mean±SD) % in chronic gastritis without intestinal metaplasia, 7.9±7.2% in incomplete-type intestinal metaplasias, 11.0±8.7% in complete-type intestinal metaplasias, 30.2±12.7% in hyperplastic polyps, 86.4±7.3% in adenomas and 87.7±12.7% in carcinomas (Figs. 2 and 3). Within each histological type, carcinomas and adenomas possessed hTR-expressing cells significantly more frequently than chronic gastritis with/without intestinal metaplasia, or hyperplastic polyps ($P<0.001$). Intestinal metaplasias and hyperplastic polyps exhibited hTR-expressing cells significantly more fre-

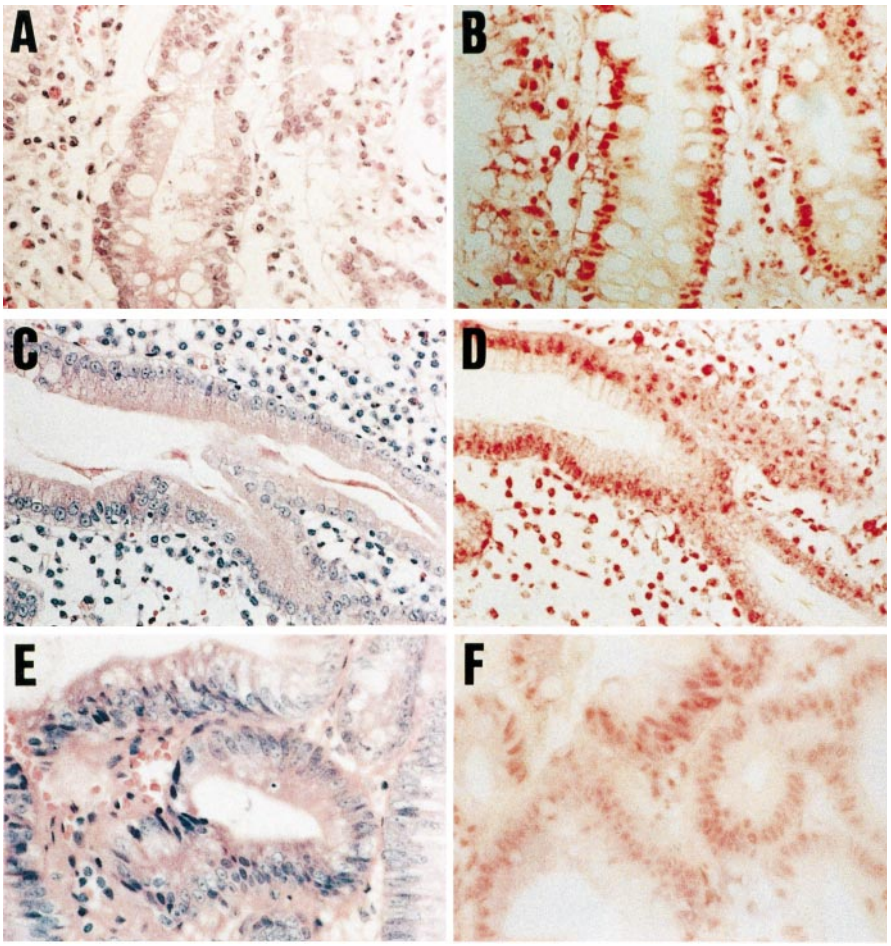

Fig. 3. ISH for hTR expression in gastric precancerous lesions. A, C, E. Microscopic view of complete-type intestinal metaplasia, hyperplastic polyp and adenoma, respectively (HE, original magnification, ×400). B, D, F. High levels of hTR expression were detected in complete-type intestinal metaplasia, hyperplastic polyp and adenoma, respectively.

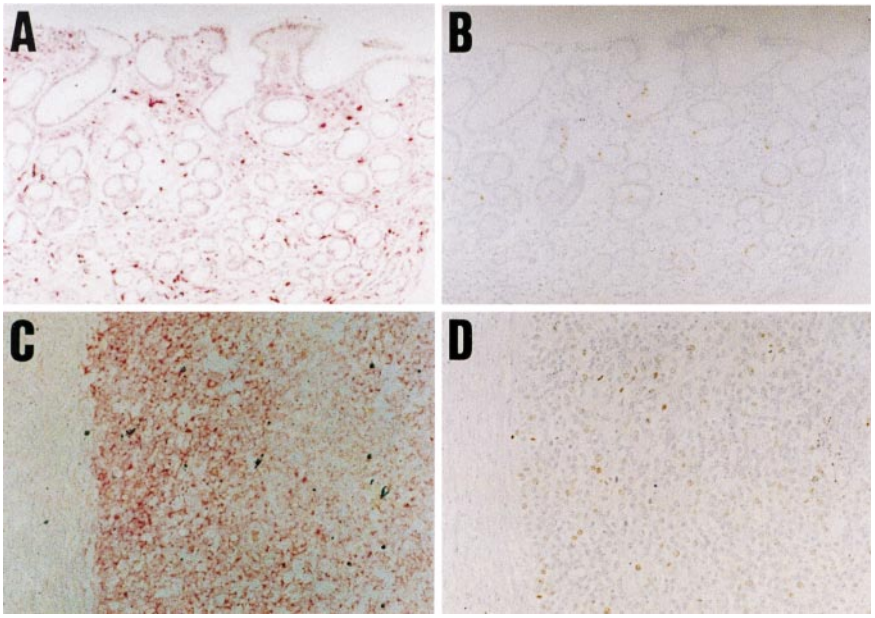

Fig. 4. ISH for hTR expression and Ki-67 immunoreactivity. A, C. ISH for hTR expression in normal mucosa and in poorly differentiated adenocarcinoma, respectively (original magnification, ×100). B, D. Ki-67 immunoreactivity in normal mucosa and in poorly differentiated adenocarcinoma, respectively (original magnification, ×100). The areas of increased cell proliferation defined by Ki-67 immunohistochemistry exhibited high levels of hTR expression in both normal mucosa and cancerous tissues.

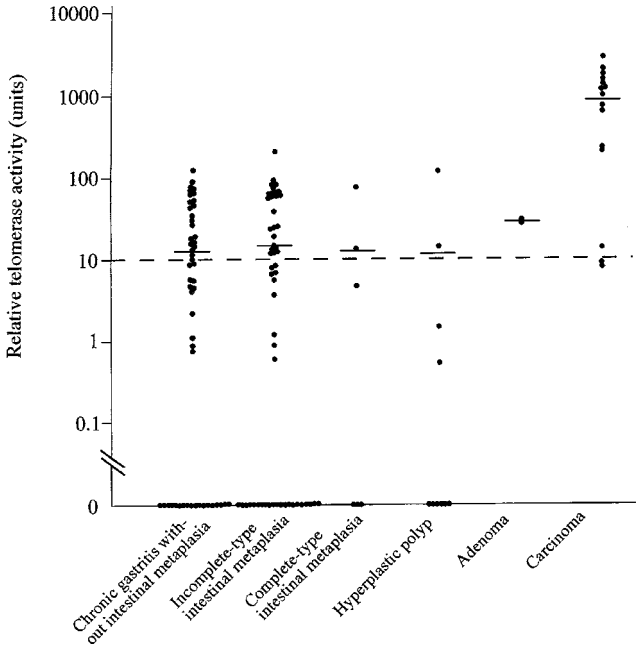

Fig. 5. Summary of telomerase activity evaluated by TRAP/HPA analysis in gastric lesions. The activity equivalent to that in one cell of MKN-1 gastric carcinoma cell line was defined as 1 unit. Telomerase activity of more than 10 units was regarded as positive. Horizontal bars indicate average scores of telomerase activity in each histological type.

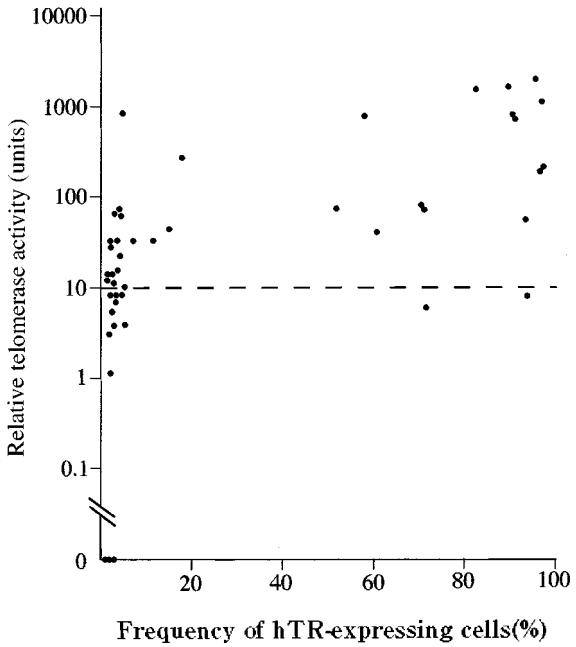

Fig. 6. Relationship between expression level of hTR and telomerase activity in gastric lesions. There is no significant relationship between expression level of hTR and telomerase activity in gastric lesions.

quently than chronic gastritis without intestinal metaplasia ($P<0.05$).

hTR-expressing cells in normal mucosae were mainly located in the neck of the gastric glands, the so-called "proliferation zone." A similar distribution of Ki-67-positive cells was observed in these tissues (Fig. 4, A and B). hTR-expressing cells in normal mucosae may be mainly stem cells. On the other hand, a large proportion of carcinoma and adenoma cells showed high levels of hTR expression. The expression pattern of hTR in these tissues was heterogeneous, and similar intratumor heterogeneity of Ki-67 immunoreactivity was detected (Fig. 4, C and D). Thus, we observed that areas of increased cell proliferation defined by Ki-67 immunohistochemistry exhibited high levels of hTR expression in both non-cancerous and cancerous tissues.

In addition, infiltrating lymphocytes in tissues showed high levels of hTR expression (Fig. 4A). The immunohistochemical distribution of hTR-expressing infiltrating lymphocytes was different from that of positive reaction for UCHL-1 (T-cell marker) or L26 (B-cell marker) (data not shown).

Telomerase activity We then analyzed telomerase activity in gastric samples by TRAP/HPA (Fig. 5). Positive telomerase activity was detected in 12 of 53 (23%) chronic gastritis without intestinal metaplasia, 24 of 54 (44%) incomplete-type intestinal metaplasias, 2 of 7 (29%) complete-type intestinal metaplasias, 2 of 10 (20%) hyperplastic polyps, 2 of 2 (100%) adenomas and 13 of 15 (87%) carcinomas. Carcinomas exhibited positive telomerase activity significantly more frequently than did chronic gastritis with/without intestinal metaplasia, or hyperplastic polyps ($P<0.05$, by χ^2 test). Mean telomerase activity was 16.5 units in chronic gastritis without intestinal metaplasia, 20.2 units in incomplete-type intestinal metaplasias, 18.3 units in complete-type intestinal metaplasias, 13.4 units in hyperplastic polyps, 46.0 units in adenomas and 955.1 units in carcinomas. Carcinomas had significantly greater telomerase activity than the other lesions ($P<0.001$).

Relationship between hTR expression and telomerase activity We analyzed the relationship between the expression level of hTR and the telomerase activity in gastric lesions. Telomerase activity did not parallel the expression level of hTR (Fig. 6).

DISCUSSION

Several researchers have reported high levels of hTR expression in malignant tissues but not in non-malignant tissues by using the ISH technique.[26–30] In these reports, ISH was performed with radioisotope-labelled probes. We have developed an ISH method with avidin-biotin staining to analyze the localization of hTR expression in formalin-fixed, paraffin-embedded tissues. Our study is the first to analyze hTR expression by ISH with avidin-biotin staining. This method is convenient because it avoids the need for radioisotope-labelled reagents.

In this study, high levels of hTR expression were found preferentially in the nuclei of most carcinoma and adenoma cells and some non-cancerous cells, including infiltrating lymphocytes, at the single-cell level. hTR is supposed to be localized mainly in the nuclei, because hTR is a part of telomerase, which synthesizes TTAGGG telomeric DNA repeats onto chromosomal ends *de novo*.[31] Our results on hTR expression detected by ISH do not conflict with the idea that hTR is located in the nuclei.

In previous reports, high levels of hTR expression were noted not only in cancers with telomerase activity, but also in cancers without telomerase activity.[11, 12] Non-malignant tissues also have various levels of hTR expression.[8] In this study, 86.4±7.3% of adenoma cells and 87.7±12.7% of carcinoma cells showed high levels of hTR expression. In addition, 7.9±7.2% of epithelial cells from incomplete-type intestinal metaplasias, 11.0±8.7% of epithelial cells from complete-type intestinal metaplasias, and 30.2±12.7% of epithelial cells from hyperplastic polyps also exhibited high levels of hTR expression. On the other hand, only 4.3±1.8% of epithelial cells from chronic gastritis without intestinal metaplasia showed high levels of hTR expression. These data imply that hTR overexpression may be an early event in carcinogenesis of the stomach.

Furthermore, gastric carcinomas often show intratumor heterogeneity histologically, within the same tumor sample. We also observed intratumor variation in proliferation activity (defined in terms of Ki-67 immunohistochemistry) and the areas of increased cell proliferation exhibited high levels of hTR expression (Fig. 4). Similar results have been reported in human brain astrocytomas.[28] These data suggest that high levels of hTR expression may be related to the proliferation activity defined by Ki-67 immunoreactivity.

Telomerase activity has been detected in a large number of cancers by using highly sensitive polymerase chain reaction (PCR)-based assays.[6, 7, 9, 23] Telomere stabilization through telomerase reactivation is probably crucial for survival and sustained replication of tumor cells. It has also been reported that not only cancer cells, but also some normal adult somatic cells, including male germ cells, intestinal stem cells and peripheral lymphocytes, have telomerase activity.[32, 33] In this study, carcinomas had significantly stronger telomerase activity than the other lesions ($P<0.001$). High levels of hTR expression and strong telomerase activity were concurrently observed in the majority of carcinoma tissues, but not in chronic gastritis and intestinal metaplasias. However, the amount

of telomerase activity did not parallel the expression level of hTR (Fig. 6).

It has been reported that lymphocytes possess telomerase activity,[26, 32, 33] and we found by ISH analysis that infiltrating lymphocytes in the mucosae had high levels of hTR expression. Therefore, we analyzed the relationship between telomerase activity and amount of infiltrating lymphocytes in the mucosae, in chronic gastritis without intestinal metaplasia and intestinal metaplasias. As lymphocytes infiltrated extensively in the mucosae, the telomerase activity tended to increase (data not shown). Thus, the telomerase activity in these noncancerous lesions may partially reflect the amount of infiltrating lymphocytes in the mucosae.

Recently, the human telomerase catalytic subunit gene, *hTERT*/*hEST2*, has been cloned.[34, 35] *hTERT*/*hEST2* is expressed at high levels in telomerase-positive tissues, but is undetectable in telomerase-negative tissues.[36] Moreover, the mRNA is up-regulated concomitantly with the activation of telomerase during the immortalization of cultured cells and is down-regulated during cellular differentiation. It is suggested that the induction of *hTERT*/*hEST2* mRNA expression is required for telomerase reactivation. ISH for *hTERT*/*hEST2* might allow us to estimate the amounts of telomerase activity in non-frozen samples, and could be helpful for clinical diagnosis.

In conclusion, our data suggest that, although hTR is present in normal human tissues such as stem cells or infiltrating lymphocytes, it increases in an early stage of stomach carcinogenesis. Sufficient synthesis of hTR may be a prerequisite for telomerase reactivation in tumorigenesis.

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