Expression of Telomerase Catalytic Component, Telomerase Reverse Transcriptase, in Human Gastric Carcinomas

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Telomerase activity is believed to be crucial for cellular immortality, which is considered to participate in the development of a majority of human cancers. Human telomerase reverse transcriptase (TERT) has recently been identified as a catalytic subunit of telomerase. We examined the expression of TERT and other telomerase components such as human telomerase RNA component (hTR, encoded by TERC) and human telomerase-associated protein (TEP1) by reverse transcription-polymerase chain reaction in human gastric carcinomas and non-neoplastic mucosa, in addition to measuring the telomerase activity. Of 20 gastric carcinomas examined, 18 (90%) and 18 (90%) showed increased expression of TERT and higher telomerase activity in comparison with corresponding non-neoplastic mucosa, respectively. Increased expression of hTR/TERC was also observed in 15 (75%) of the gastric carcinomas. Immunohistochemically, strong expression of TERT protein was detected in the nuclei of the tumor cells of all carcinoma tissues, while the expression of TERT in non-neoplastic mucosal cells as well as stromal elements (except lymphocytes) was weak or negative. These findings suggest that increased TERT expression associated with telomerase activity may serve as a novel marker for the diagnosis of stomach cancer.

Key words: Telomerase catalytic component — TERT — Gastric carcinoma

An important step in the pathogenesis of cancer is the acquisition of cellular immortality.[1] The key enzyme responsible for this is telomerase, a reverse transcriptase that catalyzes the synthesis and extension of telomere DNA in germline tissues and in immortal tumor cells.[1–3] This ribonucleoprotein enzyme compensates for the telomere loss that occurs during replication and makes possible the indefinite proliferation of cells.[1] Telomerase activity is expressed in a variety of human tumors such as breast cancer, colon cancer, prostate cancer, hepatoma, leukemia and so on.[4–9] We have reported increased telomerase activity in a majority of gastric carcinomas.[5, 10] We have also found that the expression of the human telomerase RNA component (hTR, encoded by TERC)[11, 12] is increased in gastric carcinomas.[10] However, the precise localization of telomerase-positive cells remains to be elucidated.

Human telomerase reverse transcriptase (TERT) has recently been identified as a putative catalytic subunit of human telomerase.[13, 14] The expression of TERT is closely correlated with telomerase activity *in vitro* and *in vivo*.[13, 14] Introduction of the gene encoding TERT into telomerase-negative normal fibroblasts induces telomerase activity.[15] We have also demonstrated a significant corre-

lation between TERT expression and telomerase activity in hepatocellular carcinomas.[15] In the present study, we searched for telomerase activity and the expression of TERT and other telomerase components such as hTR/TERC and human telomerase-associated protein (TEP1/TLP1)[16, 17] in gastric carcinoma and non-neoplastic mucosa by reverse transcription-polymerase chain reaction (RT-PCR), and studied the expression of TERT protein immunohistochemically using anti-TERT antibody.

Tissues of adenocarcinoma and corresponding non-neoplastic mucosa of the stomach were obtained by surgery at Hiroshima University Hospital and related facilities. Histology, depth of tumor invasion and tumor stage were classified according to the criteria of the Japanese Classification of Gastric Cancer.[18] For molecular analyses, tissue samples obtained at the time of surgery were immediately frozen in liquid nitrogen and stored at −80°C. Histologically, tumor samples consisted mainly of carcinoma tissue and non-neoplastic mucosa did not contain tumor cells. Neither tumor nor non-neoplastic tissues showed significant inflammatory involvement (mild lymphocytic infiltration was sometimes present). For immunohistochemistry, tissues were fixed in 10% buffered formalin and embedded in paraffin. 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).[19]

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The frozen tissues were powdered in liquid nitrogen and divided into two samples, for telomerase assay and RT-PCR, allowing the two methods to be carried out with identical samples. Telomerase activity was assayed by the TRAP (telomeric repeat amplification protocol) method with some modifications, using extract of tissue protein (0.1 μg) as described.[15] The PCR products were analyzed by electrophoresis on a 12% polyacrylamide gel, stained with SYBR Green I and visualized. The TRAP signals were confirmed to be sensitive to RNase pretreatment. To examine the expression of mRNAs for hTR/TERC, TEP1 and TERT, RNA was extracted from the tissues using an RNeasy mini kit (Qiagen, Hilden, Germany) and treated with DNase. RT-PCR was performed with total RNA (0.1 μg) using the GeneAmp EZ r*Th* RNA PCR kit (Perkin-Elmer, Foster City, CA). The thermal cycles were: 94°C for 30 s and 62°C for 45 s (65°C for TERC) for 25 cycles for TERC and G3PDH, and 30 cycles for TERT and TEP1. The amplified products were fractionated on a 6% non-denaturing polyacrylamide gel (hTR/TERC, TEP1) or on a 2% agarose gel (TERT, G3PDH). Gels were stained with SYBR Green I and visualized. The primer sets used were described elsewhere.[15] All samples were assayed simultaneously for a particular set of primers to minimize experimental deviation. The expression of G3PDH was examined as a positive control. The telomerase activity (TRAP signals) and the expression of telomerase components (RT-PCR products) were quantified using a fluorescence image analyzer (Fuji FLA-2000), normalized to the internal telomerase assay standard (ITAS) signal and the G3PDH RT-PCR signal, respectively.[15]

Polyclonal antibody against TERT protein (EST1.0) was raised in rabbit by injecting partial peptides with amino acid sequences from the middle part of TERT and affinity-purified anti-TERT antibody was used as described.[15] 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 (3–5 μg/ml) 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)[20] was employed
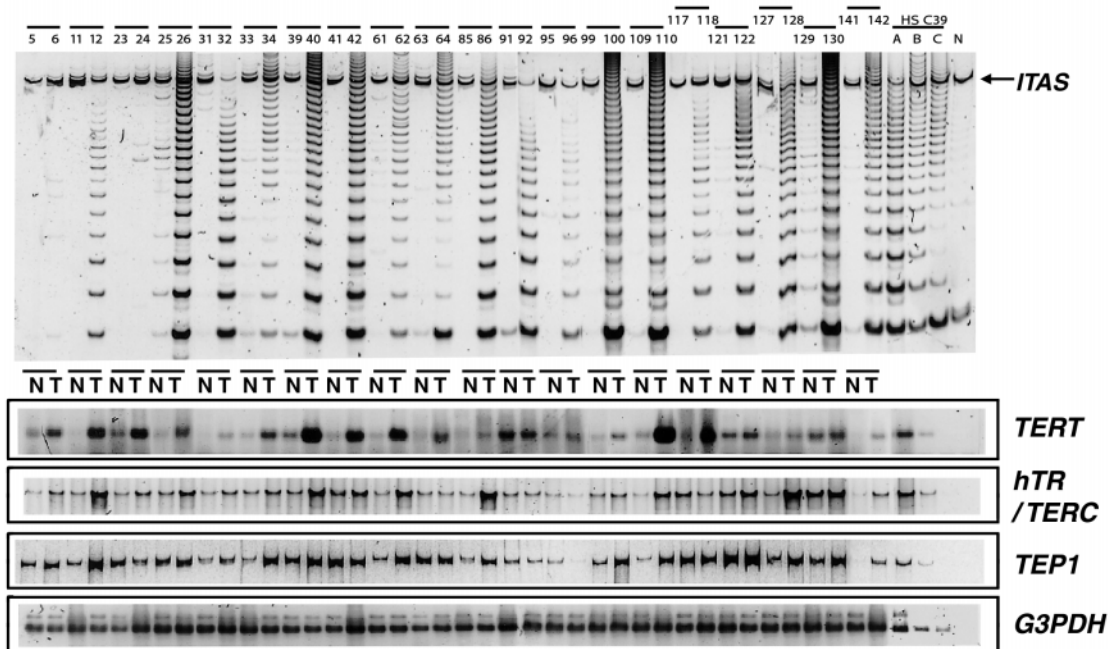

Fig. 1. Telomerase activity and TERT, hTR/TERC and TEP1 expression in human gastric carcinomas and corresponding non-neoplastic mucosa. Tumor tissues (T) and corresponding non-neoplastic mucosa from the same patients (N) were assayed for telomerase activity by the TRAP method (upper panel), and for mRNA expression of TERT, hTR/TERC and TEP1 by RT-PCR (lower panel). The expression of G3PDH was examined as a positive control. The numbers at the top of the upper panel are the sample numbers. Samples containing 0.1 μg protein and 0.1 μg RNA were used for each TRAP and RT-PCR assay, respectively. HSC-39, a human gastric carcinoma cell line, was used as a positive control; A, 1000 cells for TRAP and 0.1 μg of RNA for RT-PCR; B, 100 cells for TRAP and 0.01 μg of RNA for RT-PCR; and C, 10 cells for TRAP and 0.001 μg of RNA for RT-PCR. N: buffer containing no sample extract assayed as a negative control. ITAS: internal telomerase assay standard.

using a SensiTek HRP kit (ScyTek Laboratories, Logan, UT). Since 3,3′-diaminobenzidine tetrahydrochloride was used as 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) preabsorption of the antibody with an excess of specific antigen (TERT peptides).

We first examined the telomerase activity by TRAP assay and the expression of TERT, hTR/TERC and TEP1 by RT-PCR in 20 cases of gastric carcinoma. As shown in Fig. 1 and Table I, telomerase activity was higher in 18 (90%) of the gastric carcinomas than in the corresponding non-neoplastic mucosas. Increased expression of TERT was detected in 18 (90%) of the gastric carcinomas compared with that of the corresponding non-neoplastic mucosas. However, in a few cases, the telomerase activity and the expression level were not well correlated. While samples 91, 92 showed higher telomerase activity in the tumor tissue than in the non-neoplastic mucosa, the levels of TERT expression were almost equal in the tumor and in the nonneoplastic sample. On the other hand, samples 5, 6 and 23,

24 showed significant levels of TERT expression, but negligible telomerase activity. No obvious correlation was found between telomerase activity or TERT expression and clinicopathological parameters of gastric cancer such as histology, depth of tumor invasion and tumor stage (Table I).

Increased expression of hTR/TERC was also observed in 15 (75%) of 20 gastric carcinomas, while the level of TEP1 expression was higher in the tumor tissues than in non-neoplastic mucosas in 12 (60%) cases (Fig. 1 and Table I). Some discrepant results were also detected between telomerase activity and the level of hTR/TERC expression. For example, samples 95, 96 and 129, 130 showed higher telomerase activity in the tumor tissues than in the non-neoplastic mucosa, but the levels of hTR/TERC expression were not consistent with this.

We next immunohistochemically studied the expression and localization of TERT protein in gastric carcinomas and non-neoplastic gastric mucosas. TERT protein was detected in all gastric carcinoma tissues examined. The nuclei of many carcinoma cells showed strong immunoreactivity to TERT, which was abolished by pre-absorbing the antibody with an excess of specific antigen (Fig. 2).

Table I.   Summary of Telomerase Activity and Expression of Telomerase Components in Gastric Carcinomas

Case	Sample	Age	Sex	Hist.	Depth	Stage	Activity		TERT		hTR		TEP1	
							T	N	T	N	T	N	T	N
1	5,6	57	M	por2	ss	3a	0.0	0.0	11.8	3.4	3.4	0.9	15.2	9.9
2	11,12	60	M	tub2	se	3a	0.8	0.0	18.1	0.6	10.7	1.8	24.8	6.4
3	23,24	67	M	tub2	se	4b	0.1	0.0	19.0	8.9	3.8	1.7	8.3	14.5
4	25,26	77	F	por1	ss	1b	3.1	0.2	15.4	2.0	5.9	2.7	14.2	16.5
5	31,32	67	F	tub2	ss	1b	16.9	0.0	3.0	1.0	3.2	1.4	9.8	3.9
6	33,34	76	F	tub1	m	1a	0.6	0.1	12.0	1.3	4.5	2.1	18.8	4.6
7	39,40	69	F	tub2	ss	3a	2.5	0.1	27.8	4.6	9.3	3.4	20.8	21.9
8	41,42	82	M	pap	se	4b	3.7	0.0	21.3	2.0	6.6	5.3	17.0	20.0
9	61,62	69	F	muc	ss	3a	0.6	0.0	19.1	1.6	7.9	1.7	18.5	6.6
10	63,64	57	F	por2	se	4a	1.2	0.1	3.5	13.1	1.7	2.0	15.3	9.7
11	85,86	59	M	tub2	se	4b	3.1	0.0	8.9	3.7	10.2	1.0	12.9	4.6
12	91,92	81	F	por1	ss	1b	5.2	0.1	14.0	16.5	1.9	2.4	3.1	6.0
13	95,96	69	M	pap	ss	1b	0.8	0.0	13.7	8.8	0.3	1.2	0.0	2.0
14	99,100	57	M	tub2	se	4a	6.8	0.0	5.5	1.7	3.1	2.3	19.0	7.6
15	109,110	64	F	tub1	sm	1a	6.9	0.1	30.6	1.7	6.9	0.6	14.8	1.6
16	117,118	74	F	tub2	ss	2	0.7	0.0	23.4	9.5	2.6	5.8	17.0	18.3
17	121,122	75	F	tub1	se	3b	2.0	0.0	12.1	7.1	7.8	5.7	29.5	30.8
18	127,128	58	M	pap	se	3b	3.3	0.1	7.5	3.9	10.8	2.2	19.3	11.8
19	129,130	70	M	por2	se	4b	6.2	0.4	14.4	8.5	10.2	6.5	22.6	11.8
20	141,142	89	M	por2	se	4a	2.3	0.1	3.2	0.3	2.7	0.4	5.5	0.0
					T>N		18/20 (90%)		18/20 (90%)		15/20 (75%)		12/20 (60%)	

Histology (Hist.), depth of tumor invasion (Depth) and tumor stage were classified according to the criteria of the Japanese Classification of Gastric Cancer.[18] The telomerase activity (TRAP signals) and the expression of telomerase components (RT-PCR products) were quantified using a fluorescence image analyzer, normalized to the ITAS signal and the G3PDH RT-PCR signal, respectively. The units are arbitrary, and were calculated based on the telomerase activity and the amount of telomerase components in 100 cells of the HSC39 cell line, taken as 1.0.
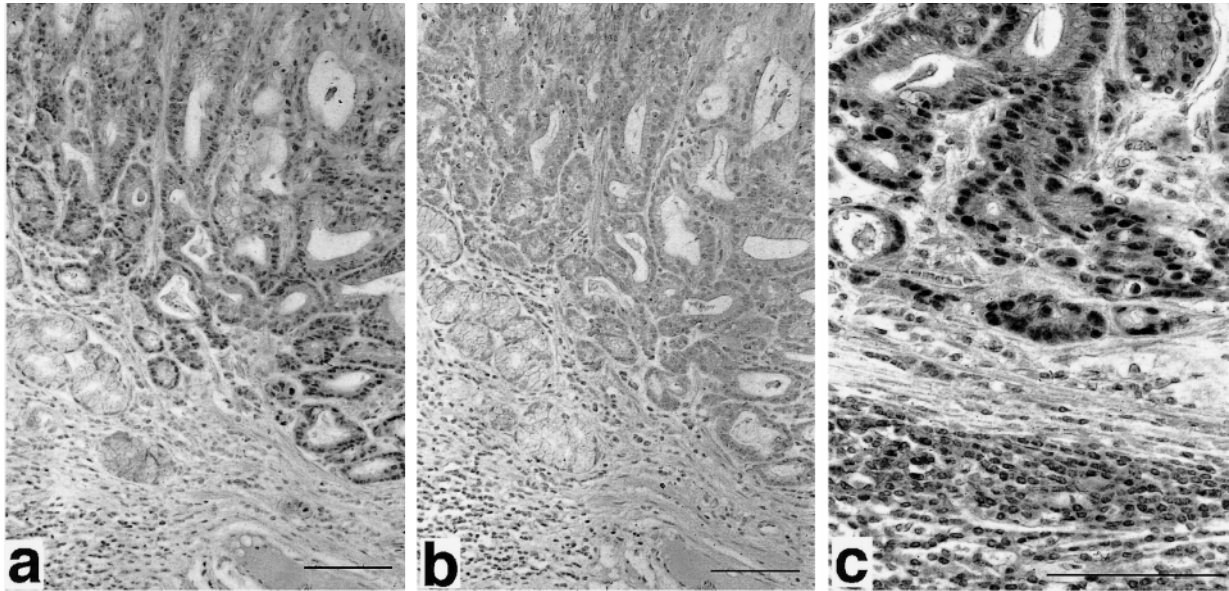
Fig. 2. Immunohistochemical detection of TERT protein in human gastric carcinomas. Tissue sections were immunostained with anti-TERT antibody (a, c), or with anti-TERT antibody pre-absorbed with TERT peptide-antigen (b). Scale bars indicate 100 μm. Most carcinoma cells showed strong immunoreactivity to TERT in their nuclei, while the stromal elements (except lymphocytes) were negative to TERT (a, c). Pre-absorbed antibody (b) gave no positive reaction in the nuclei.

The expression of TERT in carcinoma cells was not directly correlated with proliferating Ki-67-positive cells (data not shown). The stromal elements such as fibroblasts, endothelial cells and smooth muscle cells (except lymphocytes) were negative to TERT. Lymphocytes infiltrating in the stroma as well as forming lymphoid follicles were TERT-positive in their nuclei. Furthermore, only weak expression of TERT was detected in non-neoplastic epithelial cells of the stomach.

The higher telomerase activity and increased hTR expression in most of the gastric carcinomas examined in this study are consistent with our previous results.[5, 10] Here, we also found increased expression of TERT in most of the gastric carcinomas compared with that of the corresponding non-neoplastic mucosas. However, some gastric carcinomas showed a discrepancy between the telomerase activity and the expression level of TERT. Furthermore, the discrepancy between telomerase activity and the level of hTR/TERC expression was more evident than that between the enzyme activity and the level of TERT expression. We have already reported that, in hepatocellular carcinomas, the relative titers of telomerase and TERT expression showed a good correlation when these values were relatively high.[15] Our present results should be free from experimental problems, because we used identical samples for the two methods and in addition, the linearity of the reaction was confirmed in RT-PCR (data not shown) and the signal intensity of ITAS was consistent in TRAP assay. We do not yet know the reason for the discrepancy, but several explanations are possible. RNase, possibly present in gastric tissues, might modulate the TRAP assay, resulting in underestimation of telomerase activity. It is also likely that post-translational modification of TERT such as phosphorylation may be involved in the regulation of catalytic activity. Alternatively, other factors than TERT might play some role in the regulation of telomerase activity in a minor proportion of gastric carcinomas. Nevertheless, the present observations suggest that telomerase activity associated with increased TERT expression may be involved in the development of a majority of gastric carcinomas. Moreover, TERT expression might be more important than hTR/TERC in the regulation of telomerase activity in gastric tumors.

In the present study, we immunohistochemically demonstrated strong expression of TERT in many gastric carcinoma cells. The expression of TERT in non-neoplastic mucosal cells as well as stromal elements (except lymphocytes) was weak or negative. These findings overall suggest that increased TERT expression associated with telomerase activity may play a key role in the development of gastric carcinoma. Importantly, immunohistochemical techniques enable us to differentiate whether telomerase activity is due to epithelial components or lymphocytes. We therefore consider that immunohistochemical detection of TERT is a novel tool for the diagnosis of stomach cancer.

Very recently, an *in situ* hybridization study has revealed that TERT mRNA is expressed in chief cells in

the fundic glands and rarely in surface mucous cells of the stomach.[21] The expression of TERT protein in non-neoplastic mucosal cells should be examined in detail to shed light on stem cell kinetics, cell differentiation and carcinogenesis of the stomach.

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