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Expression of Human Telomerase RNA Is an Early Event of Stomach Carcinogenesis

Hiroki Kuniyasu,[1] Tamiko Domen,[1] Takahiro Hamamoto,[1] Hiroshi Yokozaki,[1] Wataru Yasui,[1] Hidetoshi Tahara[2] and Eiichi Tahara[1,3]

[1]*First Department of Pathology and* [2]*Department of Cellular and Molecular Biology, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734*

Expression of human telomerase RNA (hTR) and telomerase activity in gastric cancer and corresponding non-cancerous mucosa were studied. Telomerase activity was detected in 23 (88%) of 26 carcinoma tissues. Although all tumor specimens and non-cancerous mucosa expressed various levels of hTR, 21 (81%) of 26 cases expressed hTR at a higher level in the tumor than that in the corresponding mucosa. All 8 gastric carcinoma cell lines also expressed hTR at high levels. Nine (35%) of 26 non-cancerous mucosa showed telomerase activity and all of them contained intestinal metaplasia. The incidence of telomerase-positive mucosa in grade 2 intestinal metaplasia was significantly higher than that in grade 0 or grade 1 intestinal metaplasia, whereas hTR overexpression was found in grade 0 or grade 1 intestinal metaplasia as well as grade 2 intestinal metaplasia. The degree of *Helicobacter pylori* infection increased in parallel with the level of hTR expression and telomerase positivity. These results overall suggest that *Helicobacter pylori* infection may be a strong trigger for hTR overexpression in intestinal metaplasia, and this may lead to telomerase reactivation.

Key words: Stomach cancer — Intestinal metaplasia — *Helicobacter pylori* — Human telomerase RNA

Gastrointestinal cancers exhibit genetic alterations in multiple suppressor genes and multiple oncogenes, as well as multiple microsatellite instabilities.[1] However, activation of telomerase, which is responsible for cell immortality, is the most common fundamental event in gastrointestinal cancer.[2–5] as well as other human cancers,[6–10] regardless of tumor staging and histological type. Genetic changes in precancerous lesions, including intestinal metaplasia and adenoma of the stomach, indicate that the carcinogenic process leading to well differentiated or intestinal-type gastric cancers involves multiple steps. For example, some gastric cancer cases show identical patterns of *APC* mutation and microsatellite alteration in both intestinal metaplasia and cancer, suggesting single clonal origin of the lesions.[1] Furthermore, we have detected telomerase activity in intestinal metaplasia and adenoma of the stomach,[3] suggesting that reactivation of telomerase plays a pivotal role in the early stage of stomach carcinogenesis.

hTR is cloned as a template RNA of telomerase, a ribonucleoprotein enzyme that synthesizes telomere array, which has 11 nucleotides complimentary to the telomere sequence.[11] hTR is expressed in pre-crisis cell lines and non-neoplastic tissues, as well as in immortalized cell lines or tumor specimens and the expression level is not correlated with the level of telomerase activity.[12] Blasco *et al.* reported that expression of telomerase RNA increased in preneoplastic and early-stage tumors, whereas telomerase activity was evident only in late-stage tumors in two transgenic mice models.[13] In the present study, we have examined whether expression of hTR increases in precancerous and cancerous lesions of the stomach and whether *Helicobacter pylori* infection is implicated in hTR expression and telomerase activity in gastric intestinal metaplasia.

A total of 26 gastric carcinomas and paired non-neoplastic mucosa of the stomach were analyzed for hTR expression and telomerase activity. Non-neoplastic mucosa was confirmed not to contain tumor cells by examination of frozen sections. The degree of intestinal metaplasia juxtaposition to tissues examined for hTR expression or telomerase activity was classified as follows: grade 0, mucosal tissue containing no intestinal metaplasia; grade 1, intestinal metaplasia occupied less than 30% of mucosa; grade 2, intestinal metaplasia occupied less than 70% of mucosa; and grade 3, intestinal metaplasia occupied 70% or more of mucosa. The definitions of stage grouping, histological classification, depth of tumor invasion, and lymphatic metastasis were made according to the criteria of the Japanese Classification of Gastric Cancer.[14] To examine *Helicobacter pylori* infection,

[3] To whom requests for reprints should be addressed.

Abbreviations used are: TRAP, telomeric repeat amplification protocol; hTR, human telomerase RNA; G3PDH, glucose-3-phosphate dehydrogenase.

Giemsa-stained specimens were made from formalin-fixed paraffin-embedded materials of prepyloric antrum. The bacteria were counted in 50 glands on the Giemsa-stained specimens. Eight gastric cancer cell lines including TMK-1, five human gastric carcinoma cell lines of the MKN series (MKN-1, MKN-7, MKN-28, MKN-45 and MKN-74 [provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima)], KATO-III [provided by Dr. M. Sekiguchi (University of Tokyo, Tokyo)] and HSC-39[15] [provided by Dr. K. Yanagihara (Hiroshima University, Hiroshima)] were also used in this study.

Ten micrograms of total RNA extracted by the conventional guanidium isothiocyanate/cesium chloride method[16] was used for northern blot analysis as described elsewhere.[17] A cDNA probe for hTR in hTR 4.7 plasmid[11] was kindly provided Dr. C. B. Harley (Geron Corporation, CA). G3PDH (Clontech, Palo Alto, CA) was used as an internal control. Signal intensities of transcripts on northern-blot filters were measured by densitometric tracing and corrected based on an internal control. Relative expression levels were represented by using the hTR expression level of TMK-1 cells as a standard (1.0).

The protocol for the extraction of telomerase has been described.[3] Telomerase activity was assayed with 6 μg of extract by the one-tube PCR-based telomerase assay (TRAP method).[2, 18]

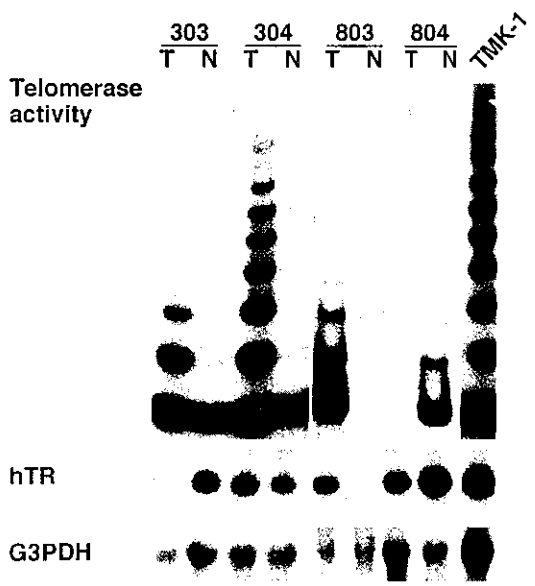

Fig. 1. Telomerase activity and expression of hTR in stomach cancer cases. Telomerase activity was assayed using the TRAP procedure with 6 μg protein of the tissue extracts. hTR expression was detected by northern blot analysis using 10 μg of total RNA. G3PDH was reprobed as an internal control. Labels above sets of lanes: tumor tissue (T) and the corresponding non-neoplastic mucosa (N).

Twenty-three (88%) of 26 tumor specimens showed various levels of telomerase activity (Fig. 1 and Table I). Four (100%) of four well-differentiated type stomach cancer had telomerase activity, and 19 (86.4%) of 22 poorly differentiated type stomach cancer displayed telomerase activity (Table II). There was no correlation between telomerase activity and tumor staging. In 9 (35%) of 26 cases, non-cancerous mucosa exhibited positive telomerase activity.

All tumor specimens and the corresponding mucosa expressed hTR at various levels (Fig. 1, Table I). Of 26 cases, 21 (81%) expressed hTR at a higher level in the tumor than that in the corresponding mucosa. Expression levels in tumor specimens were higher in well-differentiated-type stomach cancer than those in poorly differentiated-type stomach cancer, though the difference was not statistically significant (Table II). Further, no correlation was observed between hTR expression and tumor staging. Although 19 (73%) of 26 cases showed hTR overexpression and positive telomerase activity concurrently, there was no obvious difference in expression levels of hTR between telomerase-positive cases and telomerase-negative cases. To estimate the effect of the difference of material-amounts for TRAP assay and northern blot analysis of hTR, we also examined hTR expression in small amounts of tissue almost equal to those used for TRAP assay and found that the expression levels of hTR were not different from the presented data (data not shown). hTR was expressed in all 8 gastric carcinoma cell lines (data not shown). Compared with telomerase activity previously reported,[3] both hTR expression and telomerase activity were at high levels in all cancer cell lines.

Finally, we focused on the relationship between intestinal metaplasia and telomerase activity or hTR expression. As shown in Table I, all of 9 telomerase-positive mucosa contained intestinal metaplasia, whereas 7 (41%) of 17 telomerase-negative mucosa showed intestinal metaplasia, the incidence being significantly different ($P < 0.02$). Comparing the incidence of telomerase-positive cases in each grade of intestinal metaplasia and tumor specimens (Table II), its incidence in grade 2 intestinal metaplasia or tumor specimens was significantly higher than that in grade 0 or grade 1 intestinal metaplasia. In particular, all 4 cases of grade 2 intestinal metaplasia exhibited positive telomerase activity. In contrast, hTR was already expressed at constitutive levels in grade 0 or grade 1 intestinal metaplasia. Grade 2 intestinal metaplasia and tumor specimens expressed hTR at higher level than that in grade 0 or grade 1 intestinal metaplasia. Interestingly, more frequent incidence of telomerase activity and higher expression level of hTR

Table I. Expression of hTR and Telomerase Activity in 26 Cases of Human Gastric Carcinomas

Case	Age	Sex[a]	Histological type[b]	Stage[c]	Telomerase activity		hTR expression[d]		Grade of intestinal metaplasia[c]	*Helicobacter pylori* infection[f]
					T	N	T	N		
305	64	M	Poorly	II	Positive	Negative	0.42	0.4	0	—
521	80	M	Poorly	II	Positive	Negative	0.7	1	0	10
220	63	F	Poorly	III	Negative	Negative	1.2	0.6	0	—
218	63	M	Poorly	III	Positive	Negative	1	0.7	0	0
296	57	M	Poorly	III	Positive	Negative	0.3	0.5	0	—
510	57	M	Poorly	III	Positive	Negative	0.12	0.1	0	—
289	79	F	Poorly	IV	Positive	Negative	1	0.7	0	30
304	36	M	Well	IV	Positive	Negative	1.1	0.7	0	—
801	74	M	Poorly	IV	Negative	Negative	0.11	0.09	0	7
506	38	F	Poorly	I	Positive	Positive	0.32	0.3	1	—
803	75	M	Well	II	Positive	Negative	0.7	0.1	1	3
509	63	F	Poorly	II	Positive	Positive	0.2	0.15	1	—
524	57	F	Poorly	II	Positive	Positive	0.7	0.4	1	87
291	62	M	Poorly	III	Positive	Negative	1.6	1	1	4
507	62	F	Poorly	III	Positive	Negative	0.5	0.45	1	4
525	55	M	Poorly	III	Positive	Negative	1.8	0.8	1	65
504	53	M	Well	III	Positive	Positive	0.9	0.4	1	32
802	61	F	Poorly	IV	Positive	Negative	0.12	0.1	1	99
295	59	F	Poorly	IV	Positive	Negative	0.9	0.8	1	25
303	61	M	Poorly	IV	Positive	Negative	0.2	0.8	1	—
519	59	M	Well	IV	Positive	Negative	1.3	0.4	1	—
294	37	F	Poorly	IV	Positive	Positive	1.2	0.5	1	14
527	41	F	Poorly	II	Positive	Positive	0.3	1.1	2	39
523	71	M	Poorly	III	Positive	Positive	1.7	0.5	2	45
514	50	M	Poorly	IV	Positive	Positive	1	0.4	2	159
804	78	M	Poorly	IV	Negative	Positive	0.45	1.4	2	56

a) F, female; M, male.
b, c) According to Japanese Classification of Gastric Cancer (1955). Well, well-differentiated adenocarcinoma, including papillary and tubular adenocarcinoma; poorly, poorly differentiated adenocarcinoma, including signet ring cell carcinoma and mucinous carcinoma.
d, e) Described in the text.
f) Total number of bacteria in 50 antral glands examined; —, not examined.

Table II. Relationship between Telomerase Activity and hTR Expression in Gastric Carcinoma and Corresponding Non-neoplastic Gastric Mucosa

		No. of cases	Cases with positive telomerase activity	Relative hTR expression[h]	*H. pylori* infection[i]
Intestinal metaplasia[a]	Grade[b] 0	9	0[d, e, f]	0.53±0.1	11.8±6.4
	1	13	5 (38.5%)[g]	0.48±0.08	37.0±12.5
	2	4	4 (100%)[d]	0.85±0.24	74.8±28.0
Carcinoma	Well[c]	4	4 (100%)[e]	1.00±0.13	—
	Poorly[c]	22	19 (86.4%)[f, g]	0.72±0.11	—

a) Corresponding non-cancerous mucosa, resected from the same patient from whom the tumor specimens were taken.
b) Grading of intestinal metaplasia is described in the text.
c) See footnote to Table I.
d, e) $P<0.01$, by χ^2 test.
f) $P<0.001$, by χ^2 test.
g) $P<0.02$, by χ^2 test.
h) Mean±SE.
i) Number of bacteria per 50 glands, mean±SE.

were found in grade 2 intestinal metaplasia than in poorly differentiated type stomach cancer. However, the difference between them was not statistically significant. We also examined *Helicobacter pylori* infection in normal antral mucosa (Table II). The density of the bacterium in glands increased as the grade of intestinal metaplasia advanced, in parallel with hTR expression level and telomerase positivity. All the intestinal metaplasia examined in this study were of incomplete type morphologically.

The pattern of multiple gene changes in gastric cancer differs depending on the histological type, well-differentiated or intestinal type and poorly differentiated or diffuse type, indicating that they have different genetic involvements.[1, 19] In our previous study, telomerase activity was detected in 85% of stomach cancer tissues, irrespective of histological type and tumor staging, suggesting that telomerase activity is the most common event of stomach cancer. Interestingly, we found 3 telomerase-negative stomach cancers, all of which were poorly differentiated.[3] It has been reported that telomerase-negative immortalized cells have extremely long telomeres.[20] Thus, a telomerase-independent pathway might exist in the oncogenesis of some poorly differentiated stomach cancers with long telomeres.

hTR expression was detected in all non-neoplastic stomach mucosa of various grades of intestinal metaplasia. In stomach, various kinds of inflammation affect the growth of the mucosa. In particular, intestinal metaplasia or chronic atrophic gastritis exhibits accelerated cell growth and active cell cycling.[21, 22] Although factors which up-regulate hTR expression are still unknown, continuous inflammatory and regenerative processes might stimulate hTR expression by affecting stem cells, and subsequently might enhance the activity of telomerase in non-cancerous mucosa of the stomach. Therefore, it is likely that hTR overexpression in non-cancerous mucosa might reflect one of the earliest processes in multistep carcinogenesis of the stomach.

Importantly, 35% of non-neoplastic mucosa which exhibited incomplete-type intestinal metaplasia showed positive telomerase activity. Moreover, higher incidence of telomerase activity was observed in more advanced grades of intestinal metaplasia (Table II). Infection of *Helicobacter pylori* is an important risk factor of gastric cancer,[23] and in the present study, the density of the bacterial infection correlated with the grade of intestinal metaplasia, the level of hTR expression and telomerase positivity. Although we have no direct evidence for the implication of *Helicobacter pylori* in the reactivation of telomerase, continuous inflammatory status caused by *Helicobacter pylori* may play some role in inducing chronic regenerative activity or chronic mitogenesis which could facilitate increased mutagenesis in gastric mucosa. In fact, *Helicobacter pylori* has been reported to induce the production of reactive oxygen species, as well as reactive nitrogen species, which may cause active tissue regeneration as well as genotoxicity, by infiltrating white blood cells.[24] Moreover, in some cases, we observed shortening of telomere lengths in intestinal metaplasia (data not shown), possibly caused by active cell-proliferation of metaplastic mucosa. These observations suggest the existence of "stem-cell hyperplasia" or already immortalized somatic cells in intestinal metaplasia. We have evidence that intestinal metaplasia is an actual precancerous lesion which shares not only epigenetic changes, but also genetic alterations including *APC* mutation and CD44 abnormal transcript.[1] Furthermore, microsatellite instability, as well as *p53* mutation,[25, 26] has been detected in 33% of intestinal metaplasia.[27]

We observed a difference of timing between telomerase reactivation and hTR expression in the development of incomplete-type intestinal metaplasia, which is evidently implicated in well-differentiated-type stomach carcinogenesis. Since hTR is a template component of the enzyme, precedence of hTR expression is compatible with telomerase reactivation. To activate telomerase, the protein component of the enzyme would also be essential. Two protein subunits from the ciliate *Tetrahymena* were purified and the cDNAs were cloned.[28] To elucidate the precise functions of hTR and the mechanism of telomerase reactivation, protein component(s) of the telomerase will require further investigation.

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