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Helicobacter pylori Infection: Augmentation of Telomerase Activity in Cancer and Noncancerous Tissues

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Abstract. Telomerase adds hexameric repeats of 5′-TTAGGG-3′ to the ends of chromosomal DNA (telomere) and has been implicated in cell immortalization and cellular senescence. The aim of this study was to measure quantitatively the telomerase activity and human telomerase RNA component (hTR) content in gastric cancer and to examine the relation between these values and histologic factors including *Helicobacter pylori* as a risk factor for gastric cancer. Telomerase activity was measured by a modified telomeric repeat amplification protocol in cancerous and noncancerous tissues (intestinal metaplasia, chronic gastritis, normal mucosa) from 27 gastric cancer patients; hTR expression was examined by the quantitative reverse transcriptase-polymerase chain reaction using fluorescent probes. Telomerase activity was higher in cancers (total product generated: 33.7) than in noncancerous tissues. Telomerase activity was higher in intestinal metaplasia (16.7) and chronic gastritis (10.6) than in normal mucosa (3.5). In patients with intestinal-type gastric cancer, telomerase activity was higher in intestinal metaplasia with *H. pylori* infection than in that without infection. hTR expression was not correlated with telomerase activity. *H. pylori* infection may influence telomerase activity in cancer and noncancerous tissues.

The ribonucleoprotein enzyme telomerase plays a critical role in the maintenance of telomeres, with effects on cellular mortality and chromosomal protection [1, 2]. Telomerase activity in somatic cells is low and difficult to detect, except in *Tetrahymena,* which has high telomerase activity [3]. Recently, a sensitive polymerase chain reaction (PCR)-based telomerase assay, designated the telomeric repeat amplification protocol (TRAP), has been used to investigate human telomerase activity for cancer and aging research [4]. Telomerase activity appears in 85% of human cancers, including breast, bladder, colon, prostate, and liver, so there may be a use for telomerase as a molecular marker for cancer diagnosis and therapeutic strategies [4–12]. Telomerase activity is also found in precancerous lesions (i.e., gastric intestinal metaplasia and adenomas in the colon mucosa) [13, 14]. Therefore it is thought that activation of telomerase is one step in carcinogenesis [13]. Telomerase activity in cancer has been defined only as positive or negative; it has not been quantified to establish a relation between telomerase activity and the clinical or histologic features of cancer.

Helicobacter pylori infection predisposes to gastric cancer [15] and contributes to the induction of chronic atrophic gastritis and precancerous lesions, such as intestinal metaplasia [16, 17]. It has been reported that *H. pylori* may play an important role in the pathogenesis of the diffuse type of gastric cancer [18, 19]. Little is known about the influence of *H. pylori* infection on genetic alterations and cell immortality in the gastric mucosa. Therefore we quantitatively measured telomerase activity and human telomerase RNA component (hTR) in gastric cancer, intestinal metaplasia, chronic gastritis, and normal mucosa [14, 20].

Materials and Methods

Tissue Samples

Samples of primary tumor and paired noncancerous mucosa exhibiting intestinal metaplasia, chronic gastritis, and a normal appearance in the same resected stomach were obtained from 27 patients with previously untreated gastric cancer following surgery at Sapporo Medical University Hospital between July 1996 and October 1997 (Table 1). After tissue removal, all samples were immediately frozen and fixed in 10% formalin. All histologic factors were evaluated according to the criteria of the Japanese Research Society of Gastric Cancer (Table 1) [21].

Helicobacter pylori *Infection*

Sections were Giemsa-stained, and the rapid urease test (CLO test, Tri-Med Specialties, Lenexa, KS, USA) was performed with fresh samples from the prepyloric antrum, greater curvature of the corpus, and fundus [22]. *H. pylori* infection was defined as positive when *H. pylori* was detected or the CLO test was positive (or both).

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Table 1. Clinical data for gastric cancer cases.

Case	Age (years)	Gender	Tumor sizes[a] (mm)	Histologic type[b]	Tumor invasion depth[b]	Lymph node metastases	Stage[b]	*Helicobacter pylori* infection[c]
1	55	M	11	Diffuse	ss	−	I	+
2	74	M	80	Diffuse	se	+	IV	+
3	77	M	37	Diffuse	mp	−	I	+
4	84	M	20	Diffuse	sm	+	II	+
5	64	F	70	Diffuse	ss	+	III	+
6	77	F	50	Diffuse	ss	+	III	+
7	88	F	36	Diffuse	ss	−	IV	+
8	53	M	40	Diffuse	sm	−	I	−
9	63	M	70	Diffuse	se	+	IV	−
10	75	M	18	Diffuse	mp	−	I	−
11	46	F	25	Diffuse	se	+	IV	−
12	75	F	38	Diffuse	mp	−	I	−
13	78	F	35	Diffuse	sm	+	I	−
14	51	M	15	Intestinal	m	−	I	+
15	51	M	18	Intestinal	sm	−	I	−
16	52	M	24	Intestinal	sm	−	I	+
17	58	M	70	Intestinal	se	+	III	+
18	64	M	70	Intestinal	ss	−	I	+
19	70	M	79	Intestinal	ss	−	I	+
20	71	M	40	Intestinal	ss	+	II	+
21	74	M	110	Intestinal	si	+	III	+
22	75	M	19	Intestinal	m	−	I	+
23	66	M	80	Intestinal	ss	+	III	+
24	55	F	7	Intestinal	m	−	I	−
25	63	M	58	Intestinal	se	+	IV	−
26	66	M	24	Intestinal	ss	−	I	−
27	80	F	25	Intestinal	m	−	I	−

m: mucosa; sm: submucosa; mp: muscularis propria; ss: subserosa; se: invasion to serosa; si: invasion to other organ.

[a]Tumor size was defined as the largest size in extension on the gastric mucosa.

[b]According to the criteria of the Japanese Classification of Gastric Cancer (1995). Intestinal type includes tubular adenocarcinoma, including papillary adenocarcinoma. Diffuse type includes poorly differentiated adenocarcinoma, including signet ring cell carcinoma.

[c]To examine *Helicobacter pylori* infection, a rapid urease test was performed and Giemsa-stained specimens were made. Infection was defined as positive when *H. pylori* was detected, the rapid urease test (CLO test) test was positive, or both.

Telomerase Assay

Cells or homogenized tissues were lysed as described previously [4]. 3[3-Cholaminopropyl diethylammonio]-1-propane sulfonate (CHAPS) extracts were used for the flowing assay. Telomerase activity was measured using a PCR-based TRAP-eze Telomerase Detection Kit (Oncor, Gaithersburg, MD, USA) according to the manufacturer's instructions. The PCR product (15 μl) was electrophoresed in 0.5 × Tris-borate EDTA buffer on 12.5% polyacrylamide nondenaturing gels. The gels were processed for autoradiography on sensitive New A film (Konica, Tokyo, Japan) at −80°C for 16 hours. The signal intensity on the film was measured by a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA, USA). Telomerase activity was defined as [23] (Fig. 1):

$$\text{Total product generated (TPG) units} = \{[(x-x_0)/C]/[(r-r_0)/C_R]\} \times 100.$$

The linearity between signal intensity and protein content is shown in Figure 2.

Quantitative Reverse Transcriptase-PCR for hTR

Total RNA was prepared using a Qiagen RNA/DNA Mini Kit (Qiagen, Hilden, Germany). For the quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay, fluorescent hybridization probes, TaqMan PCR Core Reagents Kit with AmpliTaq Gold (Perkin-Elmer, Foster City, CA, USA), and the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer), were used [24–26]. Primers and probes were designed as described previously [27]. One-step RT-PCR for hTR was performed as described previously [27].

Aliquots of 200 ng of RNA for each sample and 1, 2, 8, 40, and 200 ng of RNA obtained from ASPC cancer cells for a calibration curve were subjected to RT-PCR. The calibration curve as an *xy* (scatter) plot represented the log-input amount (log nanograms of total starting RNA) as *x* and C_T as *y*. The linearity between input RNA (log nanograms) and C_T was obtained. The two formulas for log nanograms of hTR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: hTR, $y = -3.56x + 29.02$ ($r = 0.999$); GAPDH, $y = -3.58x + 28.33$ ($r = 0.998$). When the C_T value of the sample was substituted into the formula for hTR and GAPDH, the level of hTR and GAPDH could be calculated. The normalized level of hTR, an arbitrary number that can be used to compare the relative amounts of hTR in various samples, was determined by dividing the hTR concentration by the GAPDH concentration. The details for this method were described previously [27].

Statistical Analyses

The statistical significance of difference was determined by analysis of variance (ANOVA) or the Mann-Whitney U-test. A p value <0.05 was considered significant.

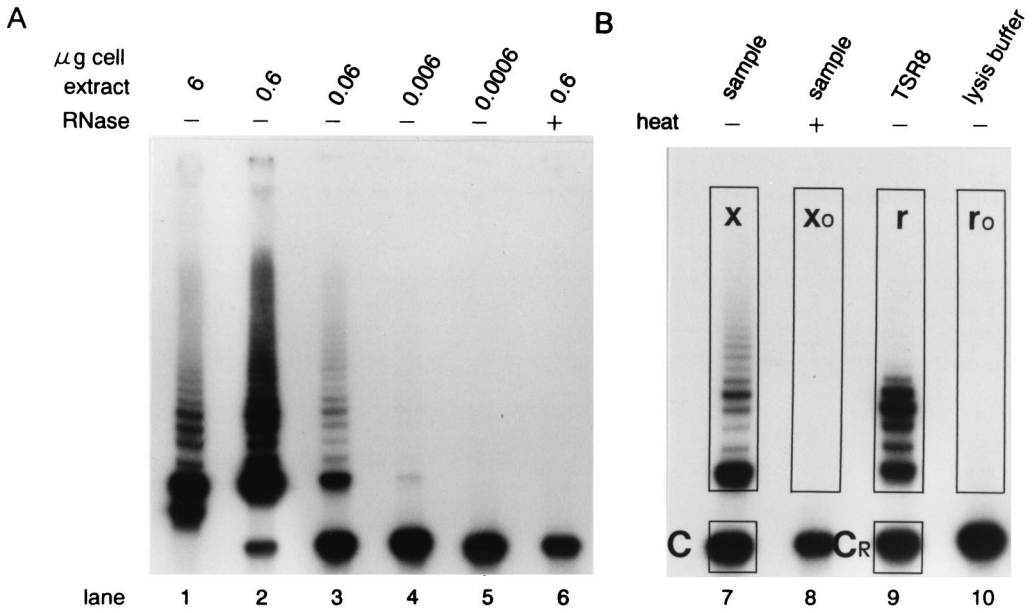

Fig. 1. Quantification of telomerase activity. **A.** Telomerase activity was measured in 6 μg to 6×10^{-4} μg cell extracts from MKN-28 cells (lanes 1–5). The observed telomerase activity was heat-sensitive to 80°C for 20 minutes and was inactivated completely by RNase pretreatment of the cell extracts (lane 6). **B.** Boxed areas show integration of radioactive counts from telomerase products from the non-heat-treated sample extract *(x),* telomerase products from the heat-treated sample extract (x_0), internal control of the non-heat-treated sample extract (C), telomerase products TSR8 quantification control (r), telomerase products from lysis buffer only (r_0), and internal control of the TSR8 quantification control (C_R). These values were used to quantify the level of telomerase activity using the following formula: TPG (units) = $\{[(x - x_0)/C]/[(r - r_0)/C_R]\} \times 100$, where TPG is the total product generated.

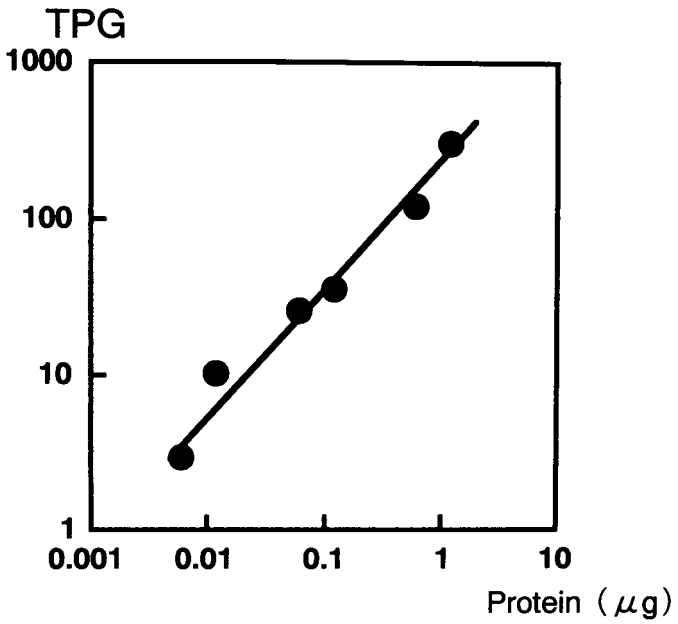

Fig. 2. Relation between protein content and TPG of the human gastric cancer cell line MKN-28. The linearity between signal intensity and protein content is shown.

Results

Telomerase Activity

Telomerase activity was measured by the sensitive PCR-based TRAP assay. High protein content may inhibit Taq DNA polymerase activity in the TRAP assay and cause false-negative results. Therefore to determine the optimal amount of protein, 0.60 and 0.06 μg of protein extracted from tissue samples were tested. Telomerase activity in 0.06 μg of protein was much less than in 0.60 μg of protein (data not shown). Therefore we used 0.60 μg of protein for the following assay. The specificity of the telomerase ladder signal was confirmed by the sensitivity of the cell extracts to heat treatment.

The mean telomerase activity was higher in gastric cancer than in intestinal metaplasia ($p < 0.05$), chronic gastritis ($p < 0.01$), or normal mucosa ($p < 0.01$) (Fig. 3). When intestinal metaplasia was classified as complete or incomplete type, the mean telomerase activity was higher in incomplete metaplasia (TPG 21.5) than in complete metaplasia (12.9), but the difference was not statistically significant.

The relation between clinical and histologic factors and telomerase activity in gastric cancer is shown in Table 2. Telomerase activity was higher in the *H. pylori* infection group than in the noninfection group, but not significantly. When classified by tumor size, mean telomerase activity was higher in $\geq$5 cm tumors than in the <5 cm group, but not significantly. When gastric cancer was classified into intestinal and diffuse types, the mean telomerase activity was higher in intestinal-type gastric cancer than in the diffuse types, but the difference was not statistically significant. There was no significant relation between tumor invasion by depth and telomerase activity in cancer. Telomerase activity was higher in the lymph node-positive group than in the negative group, but not significantly. There was no significant relation between staging and telomerase activity in cancer.

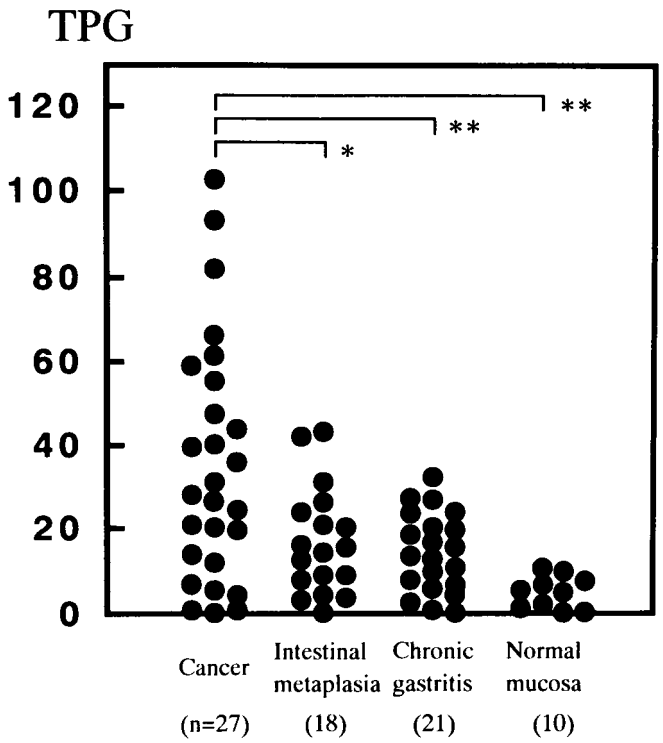

Fig. 3. Telomerase activity of human gastric cancer. Telomerase activity in 0.6 μg protein from samples (cancer, intestinal metaplasia, chronic gastritis, normal mucosa) obtained from patients with gastric cancer. The statistical significance of the difference was determined by analysis of variance (ANOVA) using Scheffe's test. $^{*}p < 0.05$; $^{**}p < 0.01$.

Table 2. Correlation between histologic factors and telomerase activity.

Factor	No.	Telomerase activity (TPG, mean ± SD)
Helicobacter pylori infection		
Positive	17	41.2 ± 30.5
Negative	10	21.0 ± 23.8
Tumor size (cm)		
<5	16	32.5 ± 30.1
≥5	11	35.6 ± 30.0
Histology (type)		
Intestinal	14	41.7 ± 33.2
Diffuse	13	25.2 ± 23.1
Tumor invasion by depth		
m, sm	9	43.3 ± 26.6
mp, ss	12	29.2 ± 31.7
se, si	6	28.3 ± 30.5
Lymph node involvement		
Negative	15	32.1 ± 30.9
Positive	12	35.8 ± 28.9
Stage		
I	15	34.9 ± 31.5
II	2	50.8 ± 43.8
III	5	31.2 ± 20.3
IV	5	18.2 ± 28.0

TPG: total product generated.

Helicobacter pylori infection and telomerase activity in patients with intestinal-type gastric cancer are shown in Table 3. The mean telomerase activity was higher in intestinal metaplasia with *H. pylori* infection than in that without infection ($p < 0.01$), but there is no significant difference between those with and without *H. pylori* infection in cancer and chronic gastritis samples.

Table 3. Telomerase activity in patients with intestinal-type gastric cancer.

Diagnosis	*Helicobacter pylori* infection (TPG, mean ± SD) Positive	Negative	p^{*}
Cancer	49.2 ± 33.5 ($n = 10$)	22.8 ± 27.2 ($n = 4$)	NS
Intestinal metaplasia	20.1 ± 11.7 ($n = 7$)	1.3 ± 1.8 ($n = 3$)	$< 0.01^{**}$
Chronic gastritis	8.0 ± 7.4 ($n = 9$)	5.9 ± 5.4 ($n = 3$)	NS

*Mann-Whitney U-test.
$^{**}p < 0.05$ indicates statistical significance.

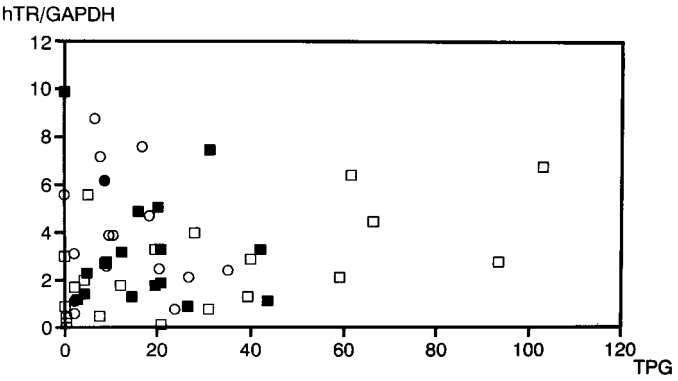

Fig. 4. Quantitative analysis of human telomerase RNA component (hTR) expression in gastric cancer samples. Open squares: gastric cancer; filled squares: intestinal metaplasia; open circles: chronic gastritis; filled circles: normal mucosa; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Human Telomerase RNA Component

Telomerase activity and hTR expression were plotted (Fig. 4). No correlation between telomerase activity and hTR expression could be shown. Moreover, high hTR expression was observed in some samples of normal mucosa and chronic gastritis and intestinal metaplasia.

Discussion

The present study demonstrates that telomerase activity is highest in cancer tissue, followed by intestinal metaplasia, chronic gastritis, and normal mucosa; in patients with intestinal-type gastric cancer, telomerase activity was higher in intestinal metaplasia with *H. pylori* infection than that without infection ($p < 0.01$). Our results indicate that telomerase is activated in precancerous lesions, such as gastric intestinal metaplasia and chronic gastritis, suggesting that small numbers of immortal cells exist in these lesions [13]. In addition, *H. pylori* infection may induce high telomerase activity in intestinal metaplasia.

It has been reported that infiltrating lymphocytes express telomerase activity in inflammatory tissues such as pancreatitis and

hepatitis [11, 28, 29]. Our preliminary study revealed that telomerase activity of normal T and B cells is <1% of levels in a gastric cancer cell line (MKN-28) (manuscript submitted), implying that infiltrating lymphocytes should not influence the measurement of telomerase activity in chronic gastritis. In addition, we found no correlation between the intensity of telomerase activity and the amount of infiltrating lymphocytes (data not shown). In chronic gastritis mucosa, local production of free radicals and inflammatory cytokines [e.g., tumor necrosis factor-α (TNFα) and interleukins (IL-6, IL-8)] by infiltrating monocytes, lymphocytes, or the gastric mucosa itself has been reported [30–33]. These reports suggest that free radicals and cytokines can induce telomerase activity in inflammatory mucosa. In addition, we cannot rule out the possibility that genetic alterations caused by DNA degeneration due to repeated degradation and regeneration in inflammatory tissues affect telomerase activity [34]. There is evidence that the *TPR-MET* gene rearrangement exists not only in gastric cancer but also in chronic gastritis mucosa [35].

Genetic alterations, such as the *p53* mutation, *APC* gene mutation, replication errors at microsatellite loci (RERs), and genetic instability are found in gastric intestinal metaplasia [36–39]. In addition, it is generally thought that incomplete intestinal metaplasia is more likely to become cancerous than the complete-type metaplasia [40]. Our data demonstrate that telomerase activity of the incomplete type is higher than that of the complete type. If telomerase is activated in gastric intestinal metaplasia harboring the genetic alteration mentioned above, these telomerase-positive cells may grow clonally and subsequently develop into gastric cancer.

Recently, the World Health Organization International Agency for Research on Cancer demonstrated a strong relation between gastric cancer and *H. pylori* infection. *H. pylori* is a definite carcinogen, and *H. pylori* infection has been reported to be a significant risk factor for intestinal metaplasia [15, 16, 41]. However, it is not known if *H. pylori* infection induces genetic alterations and cell immortality in the gastric mucosa. Our present results revealed that in patients with intestinal-type gastric cancer telomerase activity was higher in intestinal metaplasia with *H. pylori* infection than in that without infection ($p < 0.01$). In addition, telomerase activity is higher in intestinal-type gastric cancer than in the diffuse type. The incidence of *H. pylori* infection was higher in intestinal-type gastric cancer (71%) than in diffuse-type gastric cancer (54%). How *H. pylori* infection induces high telomerase activity is not clear. One explanation based on our results is that *H. pylori* infection may influence the negative regulator of telomerase activity during the early stages of stomach carcinogenesis.

We tested whether *H. pylori* infection can induce telomerase activity in gastric mucosa using the Mongolian gerbil model system. We verified that chronic infection with *H. pylori* induces activation of telomerase in gastric mucosa exhibiting intestinal metaplasia [42]. This report on the Mongolian gerbil is in agreement with our present results: that in patients with intestinal-type gastric cancer telomerase activity in intestinal metaplasia was higher with *H. pylori* infection than without the infection ($p < 0.01$).

We measured hTR expression by TaqMan PCR, which is a quantitative RT-PCR using a dual-labeled fluorogenic probe [27]. Our results showed no correlation between hTR expression and telomerase activity in gastric cancer, intestinal metaplasia, chronic gastritis, or normal mucosa. High hTR expression was observed in some samples of normal mucosa, chronic gastritis, and intestinal metaplasia. Thus taken together with previous reports, hTR is expressed in normal, precancerous, and cancer tissues; and its expression levels are variable [14, 43, 44]. Our data suggest that extinction of telomerase activity by transfection of an antisense hTR sequence might be irrelevant to gene therapy for cancer.

Résumé

L'addition répétée de 5′TTAGGG-3′ hexamérque par la télomérase est impliquée dans l'immortalisation et la sénescence cellulaires. Le but de cette étude a été de quantifier l'activité de la télomérase et le contenu du composant ARN dans la télomérase humaine (hTR) dans le cancer gastrique et d'évaluer le rapport entre ces valeurs et les facteurs histologiques y compris la présence *d'hélicobacter pylori* (*H. pylori*) comme facteur de risque du cancer gastrique. On a mesure l'activité de la télomérase par un protocole d'amplification répétée, et l'expression hTR par une réaction en chaîne de la polymérase transcriptase quantitative inversée en utilisant des sondes fluorescentes, dans des tissus cancéreux et non cancéreux (métaplasie intestinale, gastrite chronique et muqueuse normale) provenant de 27 patients ayant un cancer gastrique. L'activité télomérasique dans les cancers (produit total généré (PTG): 33,7) a été plus haut que dans les tissus non cancéreux. L'activité de la télomérase dans la métaplasie intestinale (PTG : 16,7) et dans la gastrite chronique (PTG : 10,6) a été plus élevée que dans la muqueuse normale (PTG : 3,5). Chez les patients ayant un cancer gastrique de type intestinal, l'activité en télomérase dans la métaplasie intestinale a été plus élevée en cas d'infection par *l'H. pylori* qu'en son absence. L'expression hTR n'était pas corrélée à l'activité de la télomérase. L'infection *H. pylori* pourrait influencer l'activité de la télomérase dans les tissus cancéreux ou non cancéreux.

Resumen

La telomerasa, que añade 5′TTAGGG-3′ a las terminaciones del DNA cromosómico (telómera), ha sido implicada tanto en la inmortalización como en la senescencia celulares. El propósito del presente estudio fue determinar cuantitativamente la actividad de la telomerasa en el contenido del componente de telomerasa del RNA (hTR) en el cáncer gástrico y examinar la relación entre estos valores y los factores histológicos, incluyendo al *Helicobacter pylori,* como riesgo de cáncer gástrico. Se midió la actividad de telomerasa en tejidos cancerosos y no cancerosos (metaplasia intestinal, gastritis crónica y mucosa normal) en 27 pacientes con cáncer gástrico, mediante un protocolo modificado de amplificación telomérica repetida, y la expresión de hTR mediante la reacción en cadena de la polimerasa transcriptasa reversa utilizando sondas fluorescentes. La actividad de la telomerasa en el cáncer apareció más alta (producto total generado: 33.7) que en los tejidos no cancerosos. También apareció más alta en la metaplasia intestinal (16.7) y en la gastritis crónica (10.6) que en la mucosa norma (3.5). En los pacientes con cáncer gástrico de tipo intestinal, la actividad de la telomerasa en la metaplasia intestinal con infección con *H. pylori* apareció más alta que en los casos libres de infección. La expresión de hTR no se correlacionó con la actividad de telomerasa. La infección con *H. pylori* puede influir sobre la actividad de telomerasa en tejidos con cáncer y no cancerosos.

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Invited Commentary

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This interesting paper demonstrates the influence of *Helicobacter pylori* infection in telomerase activation. Kameshima et al. used noncancerous mucosa as paired samples with primary tumor from gastric cancer patients for measurement of telomerase activity and the human telomerase RNA component (hTR). In patients with intestinal-type cancer, telomerase activity of the metaplastic lesions with *H. pylori* infection revealed significantly higher levels than in those without infection, although only small numbers of patients were evaluated. These results indicate that *H. pylori* infection may contribute to the precancerous stage through induction of telomerase activity, and that telomerase activation in intestinal metaplasia with *H. pylori* infection may be correlated with oncogenesis, though the mechanism remains to be defined. This paper is important for understanding the immortal status of gastric mucosa as a background for cancer progression. Another paper has revealed that the ectopic expression of a telomerase gene may be essential to convert normal human cells into tumorigenic cells [1] and might support the results by the authors.

As they noted, although hTR is the essential component for the telomerase molecule, the correlation between telomerase activity and hTR expression is controversial [2]. The antisense inhibitor of hTR has been reported to have an inhibitory action for telomerase activity and an anticancer effect [3, 4], and the possibility of its clinical application should be pursued. hTERT, which is another component of the telomerase molecule, has been reported to correlate with telomerase activity [5, 6]. It has been reported that telomerase activation through hTERT plays a crucial role in the carcinogenesis of human cells [1]. The same hypothesis can be applied to gastric cancer with *H. pylori* infection. hTERT would be expected to be a diagnostic marker and a new target for telomerase inhibition.

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