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Overexpression of Human Telomerase RNA in *Helicobacter pylori*-infected Human Gastric Mucosa

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Telomerase, an enzyme associated with cellular immortality and malignancy, plays an important role in cellular immortalization and tumorigenesis. Furthermore, overexpression of the RNA component of the telomerase, called human telomerase RNA (hTR), has been demonstrated in various human cancers as an early event. The pattern of hTR expression following *Helicobacter pylori* (*H. pylori*) infection in human gastric mucosa was investigated by a radioactive *in situ* hybridization (ISH) assay. Paraffin-embedded sections of 50 biopsy specimens taken from the gastric antrum of individual patients infected to different extents with *H. pylori*, as well as normal gastric mucosa, were studied. In normal gastric mucosa, only weak hTR expression was noted and the expression was limited to basal cells of the gastric glands. However, the degree of hTR expression gradually increased in parallel with the degree of *H. pylori* infection. The mean scores of gastric mucosa with mild, moderate and severe degrees of *H. pylori* infection were 2.3, 2.8, and 3.7 times higher than that of normal gastric mucosa, respectively. The results of this study suggested that up-regulation of hTR expression is a frequent and early event associated with *H. pylori* infection in the gastric mucosa and may play some role in gastric carcinogenesis. Sufficient synthesis of hTR during this early stage may be a prerequisite for telomerase reactivation to occur in gastric cancer.

Key words: Human telomerase RNA — *Helicobacter pylori* — *In situ* hybridization — Gastric cancer

Helicobacter pylori (*H. pylori)* is a gram-negative microaerophilic spiral bacterium that was first isolated in 1982 from a patient with chronic active gastritis.[1)] *H. pylori* is the etiological agent of type B human chronic gastritis, peptic ulcer, and has been documented to be linked with the development of gastric adenocarcinoma and lymphoma.[2, 3)] Gastritis caused by *H. pylori* is often persistent and, if not cured by adequate chemotherapy, can progress sequentially to intestinal metaplasia, dysplasia, and ultimately carcinoma.[2, 3)] Korean people become carriers of *H. pylori* from early childhood and about 80% are carriers after 8 years of age.[4)] Although numerous seroepidemiological studies have consistently proved an association between *H. pylori* infection and gastric cancer, the underlying pathogenesis is still not clear. One well-proven contributing factor is that inflammatory cells associated with *H. pylori* infection release reactive oxygen species and cause DNA damage to the adjacent cells, which can lead to gene modifications that are potentially carcinogenic or mutagenic.[5)]

Tandem repeats of hexanucleotide called telomeres, which are present at the telomeric ends of mammalian chromosomes, are known to stabilize chromosome ends and to protect them from degradation, fusion, or rearrangement.[6, 7)] Progressive shortening of the telomere length may be the major mechanism of cellular senescence[8, 9)] and consequently cell death due to chromosomal instability.[10, 11)] Telomerase is a ribonucleoprotein which contains an RNA template complementary to TTAGGG repeats that permits the *de novo* synthesis of telomeric DNA onto the telomeric ends.[12)] To date, telomerase has not been detected in normal adult somatic tissues except for stem cells of renewal tissues.[13)]

Although not much is known about the exact etiology and pathogenesis of gastric cancer, it is believed that a better understanding of the molecular basis of gastric cancer progression will lead to earlier diagnosis and an improvement of survival rate of gastric cancer. Association of telomerase and cadherin changes with *H. pylori* infection reinforces its etiological role in gastric cancer.[14)] Increased telomerase activity has been found in about 85% of various human cancers, as well as immortal cell lines.[15, 16)] Several studies have shown that increased telomerase activity and up-regulation of TERT expression also occur in the early preneoplastic stage of gastric cancer.[17–19)] However, it is not clear why telomerase activity is increased in precancerous gastric lesions. As Kameshima *et al.* proposed,[20)] our hypothesis was that *H. pylori* infection may be one of the factors that can stimulate the

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expression of the RNA component of the telomerase, called human telomerase RNA (hTR).

Therefore, in this study, to examine whether *H. pylori* infection can induce overexpression of hTR mRNA and to localize hTR mRNA topologically in the gastric mucosa, we investigated the pattern of hTR expression in formalin-fixed, paraffin-embedded normal human gastric mucosa and gastric mucosa having different degrees of *H. pylori* infection using a radioactive *in situ* hybridization (ISH) assay.

MATERIALS AND METHODS

Tissue samples A total of 50 biopsy samples were taken from the gastric antrum of individual patients by the same endoscopist. Two to 3 pieces of the specimens were used for bacterial culture and urease testing. The remaining portions were fixed in 10% phosphate-buffered neutral formalin, routinely processed, and stained with hematoxylin and eosin (H&E) for pathologic diagnosis and with the Warthin-Starry Silver method to detect *H. pylori*. The specimens were regarded as positive for *H. pylori* infection only when the gastric biopsy specimens showed evidence of infection by histopathology and special staining, bacterial culture and urease testing. Histopathologically, *H. pylori*-positive samples were classified as mild, moderate, or severe based on the updated Sydney system.[21] Five normal gastric mucosa taken from patients without *H. pylori* infection were also included and served as negative controls. This study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of Seoul National University Hospital.

ISH for hTR expression ISH for hTR expression was performed using replicate sections of formalin-fixed, paraffin-embedded biopsy specimens mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). Plasmid pGEM-5Z containing a hTR complementary DNA (Geron Corp., Menlo Park, CA) was used as a template to generate both sense and antisense probes. ^{35}S-Labeled single-stranded RNA probes were generated with a commercial kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. To enhance hybridization efficiency, probes were hydrolyzed with alkali to an average length of 200 nucleotides and purified on a Sephadex G-50 column (Boeringer Mannheim, Mannheim, Germany). The specific activity of the radiolabeled probes was about 3×10^7 cpm/μg template DNA.

ISH was performed as previously described.[22, 23] Hybridization reactions were carried out in a water bath overnight at 52°C with hybridization solution containing ^{35}S-labeled RNA probes. Following several thorough washes, the most stringent being 2× SSC (sodium chloride sodium citrate) and 0.1× SSC for 40 min each at 50°C, the slides were dehydrated, dipped in Kodak NTB-2 nuclear track emulsion and exposed for 3 weeks in light-resistant boxes with a desiccant at 4°C. After development, the slides were counter-stained with Gill's 2 hematoxylin and examined microscopically. As a negative control, replicate sections were also hybridized with the sense probe. To confirm the integrity of RNA, the expression of a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was examined concurrently.

The intensity of hTR expression was scored as previously described.[22, 23] A score of 4+ (intense) was comparable to the expression in normal adult testicular tubules, 3+ (strong) was comparable to expression in previously reported carcinomas, 2+ (moderate) was comparable to expression in the germinal centers of lymphoid tissues, and 1+ (weak) was comparable to expression in the basal cells of normal large bronchial epithelium. Signal density of the hTR expression was evaluated by 2 authors (DYK and KH) on a blind basis. To minimize the bias in evaluating the results of ISH, grading of the *H. pylori* infection was performed by JJJ, and ISH of each slide was graded without knowing the results of infection degree.

Statistical analyses Comparisons of the staining intensity were statistically analyzed by one-way non-parametric analyses using SAS programs. Values of $P<0.05$ were considered as statistically significant.

RESULTS

Histopathologically, the study population (n=50) was divided into 4 categories as follows: *H. pylori*-negative gastric mucosa (n=5) and gastric mucosa mildly (n=15), moderately (n=15), and severely (n=15) infected with *H. pylori*.

We used human adult testicular tissues as positive controls which had intense hTR expression (4+), whereas we used slides hybridized with sense probe as negative controls, which gave no signal in any slide tested.

In *H. pylori*-negative gastric mucosa, weak (1+) hTR expression was limited to the proliferating basal stem cells of the gastric glands (Fig. 1B). Lymphocytes in the germinal centers of lymphoid follicles present in the submucosa were weakly to moderately positive.

In gastric mucosa mildly infected with *H. pylori*, the hTR expression was usually weak (Fig. 1D). In contrast, relatively moderate (2+) to strong (3+) hTR expression was detected in the epithelial cells of the gastric glands moderately (Fig. 1F) and severely (Fig. 1H) infected with *H. pylori*. Statistically, the intensity of hTR expression increased gradually as the degree of *H. pylori* infection became more severe (Fig. 2). The mean score of mildly infected gastric mucosa was 2.3-fold higher than that of *H. pylori*-negative gastric mucosa, whereas the mean scores of moderately and severely infected gastric mucosa were 2.8 and 3.7 times higher than that of *H. pylori*-negative

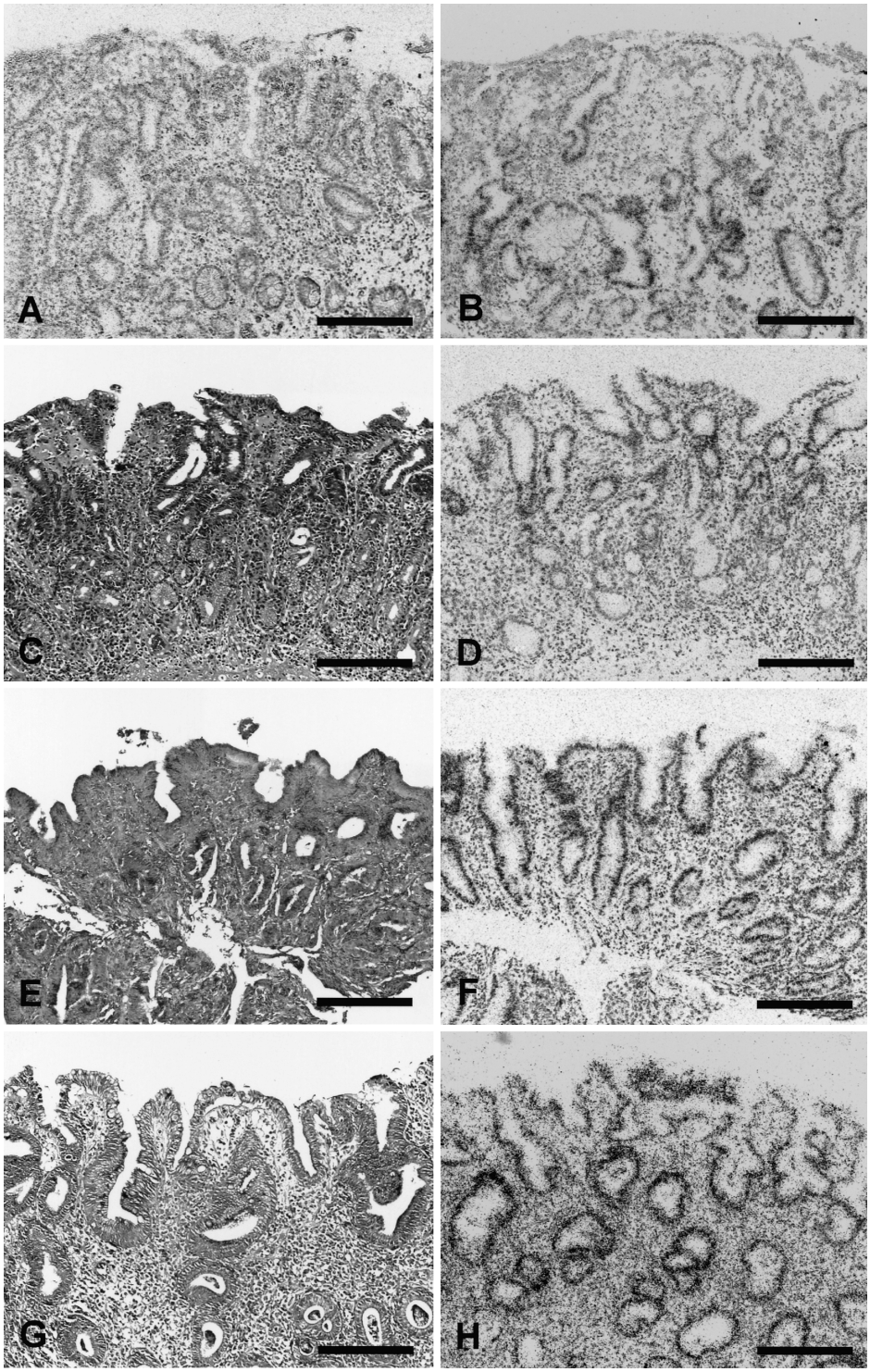

Fig. 1. hTR expression in *H. pylori*-negative gastric mucosa (A, B), and in mildly (C, D), moderately (E, F) and severely (G, H) infected gastric mucosa. Both H&E staining (A, C, E, G) and ISH (B, D, F, H) images are presented. Note the weak (+) hTR expression in the proliferating basal cells of the gastric glands of *H. pylori*-negative gastric mucosa (B) and in the glandular cells of mildly *H. pylori*-infected gastric mucosa (D). Moderate (2+) and strong (3+) levels of hTR expression are noted in the glandular cells of moderately (F) and severely (H) *H. pylori*-infected gastric mucosa, respectively. Bar=200 μm.

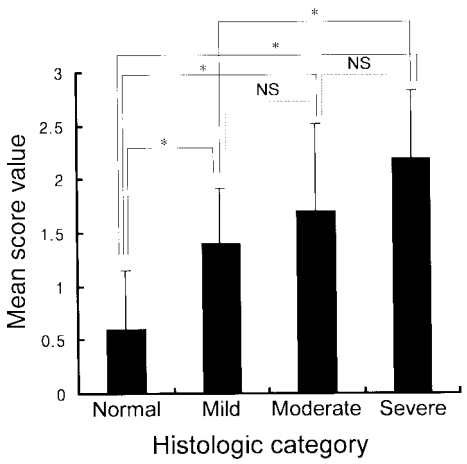

Fig. 2. The relative levels of hTR expression of *H. pylori*-infected gastric mucosa. Note that the degree of hTR expression gradually increases as the degree of *H. pylori* infection gets more severe. *, significant ($P<0.05$). NS, not significant.

gastric mucosa, respectively (Fig. 2). There was a significant increase of hTR expression between mildly and severely *H. pylori*-infected gastric mucosa, whereas there was no significant difference of hTR expression between mildly and moderately and between moderately and severely *H. pylori*-infected gastric mucosa (Fig. 2).

DISCUSSION

Telomerase activation is essential for the stabilization of telomere length, through which immortalization and oncogenesis are achieved. Recent findings suggest that up-regulation of telomerase activity is one of the most common and fundamental steps in cancer development. In addition, overexpression of hTR has been demonstrated in several human cancers as an early event and appears to be a useful indicator of prognosis, or may be useful in the diagnosis of some tumors, including transitional cell carcinoma of the upper urinary tract, prostate cancer, esophageal cancer, and childhood neuroblastic tumors.[24–27] Our previous study has shown that dysregulation of hTR expression is a frequent and early event associated with the progression of gastric cancer.[23] In that study, although some heterogeneity of hTR expression was noted, there was a tendency for intensity of hTR expression to increase gradually as the cancer progressed to a more advanced stage.

Using northern blotting analysis, Kuniyasu *et al.* demonstrated overexpression of hTR in precancerous gastric tissues.[28] They also showed that the level of hTR expression generally increases in parallel with the degree of *H. pylori* infection. However, since inflammatory cells infiltrated in the gastric mucosa following *H. pylori* infection are often telomerase-positive and could be a source of hTR, resulting in false-positives, it must be established that increased hTR expression in *H. pylori*-infected gastric mucosa was indeed contributed by inflamed gastric mucosal tissue, rather than by inflammatory cells themselves. Recently, the ISH method was developed and used to detect the expression of hTR to compensate for several defects of the TRAP assay.[22, 23] Since the ISH technique can use frozen and paraffin-embedded specimens, this method can precisely localize the cells expressing hTR. In this study, gastric mucosa free of *H. pylori* infection expressed only low levels of hTR and the expression was limited to the proliferating basal stem cells of the gastric glands. However, weak to strong hTR expression was detected in the epithelial cells of the gastric glands of *H. pylori*-infected gastric mucosa. Topologically, unlike that of the *H. pylori*-negative gastric mucosa, hTR expression of *H. pylori*-infected gastric mucosa was not limited to the basal stem cells of gastric glands but was diffusely distributed in the epithelial cells of the gastric glands. Moreover, the degree of hTR expression gradually increased in parallel with the degree of *H. pylori* infection.

Our findings confirm and extend those of Kuniyasu *et al*. Overall, *H. pylori* infection is probably a strong trigger for hTR expression in the early stage of gastric carcinogenesis and sufficient synthesis of hTR during this early stage may be a prerequisite for telomerase reactivation in gastric cancer. Although hTR was up-regulated in precancerous gastric tissues, its expression did not always parallel the telomerase activity.[19] Since hTR is the RNA component of telomerase, a certain level of hTR expression might be required for telomerase reactivation, and this might explain the timing difference between telomerase reactivation and hTR expression.

Most gastric cancers express high levels of hTR, which is essential for the survival of cancer cells. Naka *et al.* demonstrated the growth-inhibitory effect of antisense hTR on gastric cancer cell lines, showing that it caused telomere shortening, leading to cell death or cellular senescence.[29] Factors that can induce or be involved in the overexpression of hTR in precancerous gastric tissues are not known. *H. pylori* causes release of reactive oxygen and reactive nitrogen species, which may be a strong trigger for “stem cell” hyperplasia in intestinal metaplasia. It is therefore probable that a series of genetic changes during *H. pylori* infection may allow a small number of cells to undergo additional mutation which may activate or up-regulate hTR in the precancerous stage and then clonal development of immortal cancer cells occurs.

In conclusion, up-regulation of hTR expression is a frequent and early event associated with *H. pylori* infection in the gastric mucosa. Thus, further studies are necessary to investigate the mechanism or factors involved in hTR expression following *H. pylori* infection.

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