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Expression of *TERT* in precancerous gastric lesions compared to gastric cancer

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Abstract

The objective of this study was to determine the levels of *TERT* mRNA and TERT protein expression in stomach precancerous lesions such as intestinal metaplasia (IM) and gastric ulcer (GU) and compare them to gastric cancer (GC). Real-time PCR was performed to detect *TERT* mRNA expression levels in 35 biopsies of IM, 30 of GU, and 22 of GC and their respective normal mucosas. TERT protein was detected by immunohistochemistry in 68 samples, 34 of IM, 23 of GU, and 11 of GC. Increased *TERT* mRNA expression levels were observed in a significant number of cases, i.e., 46% of IM, 50% of GU, and 79% of GC. The relative mean level of *TERT* mRNA after normalization with the β-*actin* reference gene and comparison with the respective adjacent normal mucosa was slightly increased in the IM and GU groups, 2.008 ± 2.605 and 2.730 ± 4.120, respectively, but high *TERT* mRNA expression was observed in the GC group (17.271 ± 33.852). However, there were no statistically significant differences between the three groups. TERT protein-positive immunostaining was observed in 38% of IM, 39% of GU, and 55% of GC. No association of *TERT* mRNA and protein expression with *Helicobacter pylori* infection or other clinicopathological variables was demonstrable, except for the incomplete type *vs* the complete type of IM. This study confirms previous data of the high expression of both *TERT* mRNA and protein in gastric cancer and also demonstrates this type of changed expression in IM and GU, thus suggesting that *TERT* expression may be deregulated in precursor lesions that participate in the early stages of gastric carcinogenesis.

Key words: Gastric ulcer; Intestinal metaplasia; Gastric cancer; *TERT*; Gene expression; Protein expression

Introduction

Gastric precancerous lesions such as intestinal metaplasia have been associated with the multistep process of well-differentiated gastric or intestinal-type adenocarcinoma, which develops from active gastritis, frequently associated with *Helicobacter pylori* infection, to gastric atrophy, intestinal metaplasia, dysplasia, and finally to gastric cancer (1). Intestinal metaplasia is characterized by the transformation of the gastric epithelium and glands from secretory to absorptive cells, which closely resemble the mature intestinal epithelium (2). Intestinal metaplasia is a well-established premalignant condition of the stomach and can produce a 10-fold increase in the risk of this neoplasia (3). Another pathway of gastric carcinogenesis includes peptic ulcer, which increases the gastric cancer risk 1.8 times (4). Genetic studies of precancerous gastric lesions are limited and, therefore, a better understanding of the mechanisms involved in gene expression in the premalignant steps, which lead to the development of cancer, is necessary. Among the genes with changed expression in gastric carcinogenesis, *TERT* plays an important role, and can also be overexpressed in premalignant lesions, thus participating in the early progression of disease (5-10).

The *TERT* (telomerase reverse transcriptase) gene encodes the catalytic subunit of telomerase, which elongates the telomere ends using the RNA subunit TERC as a template (9). The stabilization of telomere size is a prerequisite for malignant cells to erase the senescence checkpoint and acquire the capacity to proliferate unlimitedly. Thus, telomerase reactivation is an obligatory event in carcinogenesis and, in fact, increased telomerase activity or *TERT* mRNA expression has been detected in up to 90% of human cancers (11) including gastric cancer (8,10).

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The aim of the present study was to determine the levels of expression of *TERT* mRNA and protein in intestinal metaplasia and gastric ulcer and to compare them to those of normal mucosa and gastric cancer. Additionally, we investigated if there is a correlation of *TERT* levels with *H. pylori* infection and other clinicopathological variables.

Material and Methods

Samples

A total of 87 specimens were evaluated, obtained from 30 patients with gastric ulcer (GU; mean age: 54.90 ± 12.37 years), 35 patients with intestinal metaplasia (IM; mean age: 61.05 ± 12.45 years), all of them gastric cancer free, and 22 patients with gastric adenocarcinoma (GC; mean age: 62.45 ± 14.07 years). Three biopsies from the lesion area and three biopsies from the normal gastric mucosa adjacent to the lesion were collected from each patient during endoscopic evaluation, between March 2006 and March 2008, at the Base Hospital of São José do Rio Preto, SP, Brazil. The biopsies for PCR and immunohistochemical analysis were collected mainly from the antrum and corpus regions of the stomach. Pre-pyloric and non-steroid anti-inflammatory drug-induced ulcers were not included in this study. The biopsies were immediately stored in RNAlater reagent (Ambion) at -20°C until RNA extraction. All specimens were histopathologically diagnosed. Of the 87 specimens evaluated, 68 archival paraffin-embedded, formalin-fixed tissues were used for immunohistochemical staining of TERT protein. Immunohistochemical analysis was performed on a smaller number of samples because the paraffin-embedded tissue blocks were not available for all of them. Information about age, gender, smoking and drinking status, and written informed consent were obtained from all patients, and the study was approved by the Research Ethics Committee (Comitê de Ética em Pesquisa-CEP) of the IBILCE/UNESP.

RNA extraction and reverse transcriptase

Total cellular RNA was extracted with Trizol reagent (Invitrogen, USA) according to the manufacturer protocol. RNA concentration was determined with a NanoDrop® ND1000 spectrophotometer, and its integrity was confirmed by electrophoresis on 1% agarose gel. RNA samples were stored at -80°C and used for reverse transcription. cDNA was synthesized from 5 µg total RNA using random primers and a High Capacity cDNA Archive Kit (Applied Biosystems, USA), according to manufacturer instructions. The integrity of all cDNA preparations was tested by PCR assay of the β-*actin* gene and visualized on 2% agarose gel after electrophoresis.

Quantitative real-time PCR

The expression of *TERT* mRNA was measured by real-time PCR based on the SYBR Green methodology, using an ABI Prism® 7300 genetic analyzer (Applied Biosystems). The primer sequences to *TERT* and β-*actin* genes were obtained using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), as follow: F - 5' CGG AAGAGTGTCTGGAGCAA 3' and R - 5' GGATGAAGCGG AGTCTGGA 3' for *TERT* and F - 5' TGCCCTGAGGCACT CTTC 3' and R - 5' CGGATGTCCACGTCACAC 3' for β-*actin*. The real-time PCR assays were performed in 10 µL SYBR™ Green Master Mix (Applied Biosystems), 25 ng cDNA and 0.9 µM *TERT* primers. Thermal cycling conditions were: 2 min at 50°C and 10 min at 95°C for initial denaturation, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min, and a dissociation step at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. The samples were assayed in triplicate in each run. β-*actin,* α-*tubulin* and β2-*microglobulin* housekeeping genes were evaluated in lesion and control samples to determine which contained the lowest range of amplification. Next, the β-*actin* gene was used as reference because it showed the lowest variation. Gene relative quantification (RQ) of *TERT* was calculated as described by Livak and Schmittgen (12) and normalized with the β-*actin* control reference gene and corresponding normal gastric mucosa. The transcript levels were considered to be up-regulated when RQ ≥1.5.

Immunohistochemistry

Immunohistochemical analysis was performed in 34 samples with IM, 23 with GU, and 11 with GC. Tissue sections of 4 µm thickness were cut from paraffin-embedded tissue blocks and mounted on glass slides pretreated with 3-aminopropyl-triethoxysilane/acetone (5 mL:250 mL) and dried overnight at 60°C. After deparaffinization and rehydration, antigen retrieval was performed in 10 mM citrate buffer, pH 6.0, for 15 min at 120°C, followed by treatment with 3% H_2O_2 for 20 min to block endogenous peroxidase. The sections were then incubated for 1 h at room temperature with specific mouse monoclonal antibody for TERT (clone 2D8, ABR - Affinity BioReagents, 1:100). After rinsing with Tris-HCl buffer, pH 7.6, the slides were incubated with biotinylated secondary antibody and incubated with streptavidin-biotin peroxidase according to manufacturer instructions (Histostain Bulk Kit, Zymed). The immunostain was visualized with 3,3'-diaminobenzidine tetrahydrochoride (DAB) and counterstained with Mayer's hematoxylin. Negative controls were constructed by replacing the primary antibody with buffer. Tonsil tissue was used as a positive control for TERT antibody. A single pathologist examined all specimens. All analyses were done under a light microscope (400X magnification), and the entire biopsy area of all samples was examined. Immunostain for TERT protein (brown nuclear staining) was graded according to staining intensity as negative (-) (absent brown staining) or positive: weakly stained (+1), moderately stained (+2), and strongly stained (+3), as observed in at least 10% of cells.

Statistical analysis

mRNA relative expression levels are reported using the mean as a point estimator and the range of values. The nonparametric Mann-Whitney U-test was used for comparison of mRNA expression level and clinicopathological variables between groups. The Fisher exact test was used to compare the protein levels between groups and the relationship between protein levels and clinicopathological variables. All statistical tests were performed using the GraphPad Instat Software. The level of significance was set at $P < 0.05$.

The association of demographic and clinical variables such as age, gender, smoking, *H. pylori* infection or the histological type of intestinal metaplasia and gastric cancer with *TERT* mRNA and protein expression was evaluated by the Fisher exact test, but no association was found, with the exception of histological type of intestinal metaplasia (data not shown). Relative *TERT* mRNA expression was increased in the incomplete type of intestinal metaplasia compared to the complete type (3.7 *vs* 1.7, P = 0.0328).

Results

Real-time PCR analysis was performed for all 87 samples and their corresponding adjacent normal gastric mucosa and the results of mRNA expression are summarized in Table 1. In the GU and GC groups, no *TERT* expression was detected in 8/30 (27%) and 3/22 (14%) samples, respectively. After normalization with the β-*actin* reference gene and comparison with the respective adjacent normal mucosa, the relative expression of *TERT* mRNA in the groups was found to be increased in 16 (46%) of 35 IM samples, in 11 (50%) of 22 GU specimens, and in 15 (79%) of 19 GC samples. Considering all samples, the mean ± SD expression levels of *TERT* mRNA in the IM, GU, and GC groups were 2.008 ± 2.605, 2.730 ± 4.120, and 17.271 ± 33.852, respectively. Due to the high interindividual variability in each group, no significant differences in *TERT* mRNA expression were found between the three groups.

Figure 1 shows representative immunohistochemistry results for TERT protein in *H. pylori*-negative normal gastric mucosa showing negative immunostaining (Figure 1A) and positive immunostaining in the lesions evaluated. Positive nuclear staining for TERT protein (Figure 1B-D) was observed in 13 (38%) of 34 IM specimens, in 9 (39%) of 23 GU samples, and in 6 (55%) of 11 GC samples, ranging from weak (+1) to moderate/strong intensity (+2/+3), which was the most common staining (Table 1). Both GC and GU presented a diffuse staining distribution in most cases, while about 50% of the IM cases displayed a focal staining distribution, i.e., exclusively in the metaplastic glands (goblet and non-goblet cells). There were no significant differences in TERT protein expression between groups.

Table 1. Relative expression of *hTERT* mRNA normalized to β-*actin* and normal gastric mucosa and expression of TERT protein in intestinal metaplasia (IM), gastric ulcer (GU), and gastric cancer (GC) groups.

	IM	GU	GC
TERT mRNA	N = 35	N = 30	N = 22
Up-regulated cases	16/35 (46%)	11/22 (50%)	15/19 (79%)
Mean ± SD	2.008 ± 2.605	2.730 ± 4.120	17.271 ± 33.852
Range	0.001 to 11.484	0.000 to 12.861	0.005 to 129.636
TERT protein	N = 34	N = 23	N = 11
(-)	21 (62%)	14 (61%)	5 (46%)
(+1)	1 (3%)	1 (4%)	0 (0%)
(+2/+3)	12 (35%)	8 (35%)	6 (55%)

N = number of samples. The Mann-Whitney U-test was used to compare mRNA expression levels and the Fisher exact test to compare protein expression between groups. There were no statistical differences between any pairs of groups.

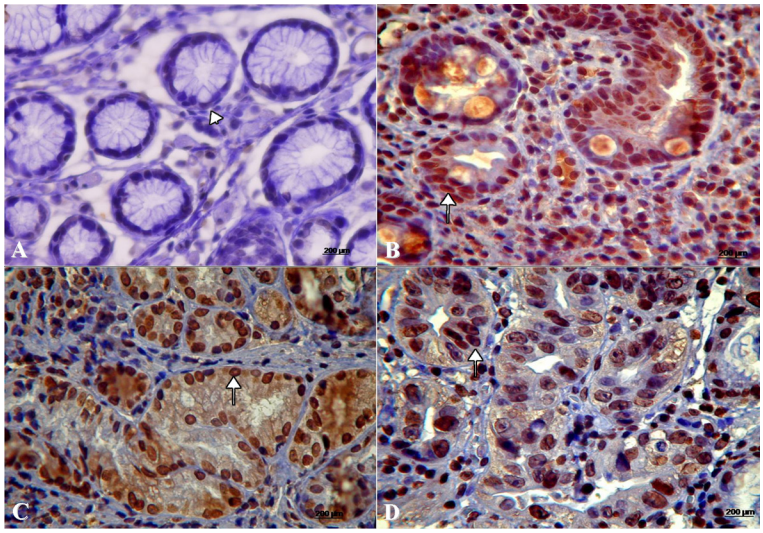

Figure 1. Immunohistochemical analysis of TERT protein in normal gastric mucosa showing negative expression (arrowhead, A), TERT nuclear positive immunostaining in intestinal metaplasia (B), gastric ulcer (C), and intestinal-type gastric cancer (D). Arrows point to immunostaining nucleus. Mayer's hematoxylin stain. Original magnification: 400X.

Discussion

We used real-time PCR and immunohistochemistry to determine the levels of *TERT* mRNA and protein expression in precancerous lesions such as intestinal metaplasia and gastric ulcer and their relationship with expression in gastric cancer. Our findings demonstrated slightly elevated levels of *TERT* mRNA expression (mean 2.0 and 2.7 times, respectively) in about 45-50% of IM and GU specimens compared to 79% of the cases of gastric adenocarcinoma that showed high expression of *TERT* mRNA (~17 times), but without statistically significant difference from other precancerous lesions.

It has been stated that telomere maintenance due to telomerase activation contributes to cancer cell formation by increasing the cell's life span. This allows the accumulation of additional genetic alterations required for cancer development (13). Thus, telomerase activity or *TERT* mRNA expression may play an important role as diagnosis and prognosis markers in different types of neoplasia (10).

Recently, increased telomerase activity or mRNA expression has been demonstrated in cancers of the oral cavity (14), ovaries (15), uterine cervix (16), lung (17), as well as gastric cancer, and there are also some reports about intestinal metaplasia, chronic gastritis, and gastric ulcer (5-10,18). Earlier clinical studies indicated that telomerase activity and *TERT* are valuable biomarkers for discriminating between normal and malignant gastric tissues (8).

In gastric cancer, telomerase re-expression has been demonstrated in 61 to 90% of cases (9,19). Furthermore, some studies have reported increased telomerase activity in 24% of chronic gastritis and in 10 to 79% of intestinal metaplasia cases, albeit lower than that observed in dysplasia or cancer (5,6,9). Gulmann et al. (9) observed that TERT protein expression was similar in IM and normal mucosa adjacent to gastric cancer (~38% of cases), whereas in carcinoma the TERT expression rates were higher (~50% of cases), as also shown by our results with 38% of IM cases *vs* 55% of GC cases. These investigators suggest that IM may harbor molecular abnormalities similar to those in gastric mucosa close to cancer and that early genetic instability requires telomerase re-expression in order to overcome telomere shortening. Another study reported that expression of the *TERT* gene was significantly more frequent in chronic gastritis with IM than in gastritis without IM, suggesting that *TERT* expression is induced at an early stage of gastric carcinogenesis (5).

In the present study, no association of *TERT* mRNA and protein expression with *H. pylori* infection and other clinicopathological variables was found, except for the incomplete type *vs* the complete type of IM. Intestinal metaplasia has been classified into two major categories on the basis of morphology and enzyme histochemistry: type I or complete type of IM and the incomplete type including types II and III (20). Some studies have suggested that high risk of gastric cancer is more common with the incomplete type of IM (3,21). In a prospective trial on 1281 subjects with IM, Filipe et al. (3) reported a 3.8-fold increased risk for gastric cancer in the presence of the incomplete type compared to the complete type of IM. We found higher mean levels of *TERT* mRNA in patients with the incomplete type of IM than patients with the complete type (3.7 *vs* 1.7; $P = 0.0328$). Thus, we may speculate that *TERT* expression in the incomplete type of IM may indicate genetic changes that may facilitate malignant progression.

Peptic ulcer is characterized by a circumscribed loss of tissue that occurs in portions of the gastrointestinal tract exposed to chlorohydropeptic secretion, occurring in 5 to 10% of the population (22). Studies have shown a relationship between increased risks of gastric cancer in patients with a diagnosis of gastric ulcers, but not in patients with duodenal ulcer (4). Furthermore, it is well known that during the process of ulcer healing, complex biological responses occur, including cell proliferation, migration, differentiation, regeneration, active angiogenesis, and extracellular matrix deposition, many of them also participating in the carcinogenic process (23). We found a 2.7-fold increased expression of *TERT* in GU samples compared to normal mucosa. This result can be related to GU healing process. On the other hand, high *TERT* expression is known to play a role in the initial events of carcinogenesis by stimulating deregulated cell proliferation that can lead to cancer.

There are few studies on *TERT* expression in GU and the results are contradictory. While Hu et al. (18) did not detect telomerase activity in any of 14 GU cases evaluated, Yao et al. (24), similarly to our study, showed a positive rate of *TERT* expression in 33% of GU cases and verified high levels of *TERT* in 7 of 8 early-stage gastric cancers (88%), which was remarkably greater than that of the precancerous lesion group (36%). Therefore, these data, taken together, suggest that *TERT* overexpression may be an early event in carcinogenesis of the stomach, although more studies are needed to clarify this issue.

In conclusion, this study underscores the presence of high levels of expression of *TERT* mRNA and protein in gastric cancer and shows that *TERT* may be up-regulated in precursor lesions such as intestinal metaplasia and gastric ulcer in cancer-free patients and can, therefore, act as early events in gastric carcinogenesis. In gastric ulcer, these findings of changed expression may be related to the response of the tissue in the healing process, but we cannot rule out the possibility that these alterations, together with other genetic alterations, may pose a greater risk of malignant progression in a percentage of gastric ulcer cases. Thus, further investigation of precancerous lesions is needed to determine the expression levels of other genes involved in the initial steps of gastric carcinogenesis.

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