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Coordinate increase of telomerase activity and c-Myc expression in *Helicobacter pylori*-associated gastric diseases

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Abstract

AIM: To detect the telomerase activity and c-Myc expression in gastric diseases and to examine the relation between these values and *Helicobacter pylori* (*H pylori*) as a risk factor for gastric cancer.

METHODS: One hundred and seventy-one gastric samples were studied to detect telomerase activity using a telomerase polymerase chain reaction enzyme linked immunosorbent assay (PCR-ELISA), and c-Myc expression using immunohistochemistry.

RESULTS: The telomerase activity and c-Myc expression were higher in cancers (87.69% and 61.54%) than in noncancerous tissues. They were higher in chronic atrophic gastritis with severe intestinal metaplasia (52.38% and 47.62%) than in chronic atrophic gastritis with mild intestinal metaplasia (13.33% and 16.67%). In chronic atrophic gastritis with severe intestinal metaplasia, the telomerase activity and c-Myc expression were higher in cases with *H pylori* infection (67.86% and 67.86%) than in those without infection (21.43% and 7.14%). c-Myc expression was higher in gastric cancer with *H pylori* infection (77.27%) than in that without infection (28.57%). The telomerase activity and c-Myc expression were coordinately up-regulated in *H pylori* infected gastric cancer and chronic atrophic gastritis with severe intestinal metaplasia.

CONCLUSION: *H pylori* infection may influence both telomerase activity and c-Myc expression in gastric diseases, especially in chronic atrophic gastritis.

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INTRODUCTION

Gastric cancer is one of the most common malignant tumors in the world. Gastric carcinogenesis is a multi-step process progressing from chronic gastritis to glandular atrophy, metaplasia, and dysplasia[1-3]. Genetic analyses of gastric cancers suggest alterations are involved in the structures and functions of several oncogenes and tumor suppressor genes as well as genetic instability[3,4]. However, in addition to these genetic changes, cell immortality is important for the sustained growth properties of gastric cancer cells[4,5]. The ribonucleoprotein enzyme telomerase that synthesizes the G-rich strand of telomeric DNA in germline tissues and in immortal tumor cells plays a critical role in the maintenance of telomeres[6]. By using a highly sensitive PCR-based TRAP assay, telomerase activity has been observed in a wide range of human cancers, including cancers of breast, bladder, stomach, colon, prostate, and liver, so telomerase may be regarded as a molecular marker for cancer diagnosis and therapeutic strategies[7-13]. Telomerase activity is also positive in gastric preneoplastic lesions[14]. Therefore it is thought that reactivation of telomerase may occur at an early stage of carcinogenesis[14].

Myc network proteins are known to be oncoproteins, which are involved in the control of cell porliferation, differentiation and apoptosis[15,16]. The c-Myc oncogene is implicated in the transformation and progression of mutated cells[17-19]. Deregulation of Myc genes is usually caused by chromosomal translocation involving the c-myc gene as well as by gene amplification. Overexpression of Myc is frequently observed in a wide variety of tumor types, and affects both the development and progression of hyperproliferations[20,21].

It is well known that *Helicobacter pylori* (*H pylori*), the main cause of chronic gastritis, is a class I gastric carcinogen[22]. It contributes to the induction of chronic gastritis to cancer through precursor lesions, such as atrophy, metaplasia and dysplasia[23]. Precancerous phenotypic expression is generally associated with acquired genomic instability and activation of telomerase activity[24]. *H pylori* inoculation into Mongolian gerbils has been reported to induce chronic gastritis and intestinal metaplasia. Chronic infection with *H pylori* induces activations of telomerase in gastric mucosa exhibiting intestinal metaplasia[25]. In patients with intestinal-type gastric cancer, telomerase activity was higher in intestinal metaplasia with *H pylori* infection than in that without infection[25,26]. These data 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. To our knowledge, little is known about whether *H pylori* infection induces both genetic alterations and cell immortality in gastric mucosa. Recent evidence suggested that Myc protein was implicated in the regulation of hTERT[27-29]. Therefore we measured telomerase activity and c-Myc expression in *H pylori* infected gastric cancer, chronic atrophic gastritis and chronic superficial gastritis. The results indicate that *H pylori* infection may induce telomerase activity and c-Myc protein expression in the process of carcinogenesis.

MATERIALS AND METHODS

Tissue samples

Samples were obtained from upper gastrointestinal endoscopy or surgical operation during the period of September 2000 to May 2001. It included 20 of chronic superficial gastritis(CSG),

30 of chronic atrophic gastritis(CAG) with mild intestinal metaplasia(IM), 42 of chronic atrophic gastritis with severe intestinal metaplasia, 14 of dysplasia(Dys) and 65 of gastric cancer(GC). Haematoxylin and eosin stains were used for the histopathological diagnosis. Degree of inflammatory reaction and glandular atrophy, intestinal metaplasia, and cellular dysplasia were evaluated according to the criteria of the updated Sydney system. The diagnosis of each sample was based on agreement between two pathologists.

H pylori infection

H pylori infection was detected by rapid urease test and bacteria culture. Infection was defined as positive when *H pylori* was detected and/or urease test was positive.

Immunohistochemistry

For each case, 4-μm thick serial sections were cut from paraffin wax blocks, mounted on acid-cleaned glass slides, and heated at 55 ℃ for 60 min. Slides were dewaxed and rehydrated, then endogenous peroxidase activity was inhibited by incubation with 30 mL/L H_2O_2 in methanol for 20 min at room temperature. To reduce non-specific background staining, slides were incubated with 50 mL/L goat serum for 15 min at room temperature. To enhance immunostaining, sections were treated with an antigen retrieval solution (10 mmol/L citric acid monohydrate, pH 6.0, adjusted with 2 mol/L NaOH) and heated three times in a microwave oven at high power for five min. Finally, slides were incubated with appropriate primary antiserum in a moist chamber overnight at 4 ℃. The monoclonal primary antibody, anti-c-Myc, was purchased from Maxim Biotech, Inc. (USA). S-P kit was purchased from Fujian Maixin Ltd (China). Samples were detected according to manufacturer's instructions. The degree of immunopositivity was evaluated semi-quantitatively. A total of 300 cells were counted in random fields from representative areas of the lesions, and the immunoreactive cells were roughly assessed and expressed as percentages. The scoring system for the antibody tested was: 0-5%(negative), 5-25%(low positivity), 25-50%(moderate positivity), >50%(high positivity).

Telomerase assay

Telomerase activity was measured by a modified version of the standard TRAP method, using PCR-ELISA detection kit (Roche Molecular Biochemicals), according to its manufacturer's instructions. Five 10-μm thick cryostat sections of were transferred into a sterile reaction tube containing 200 μL ice-cold lysis reagent. The lysates were homogenized and incubated on ice for 30 min. After centrifuged at 16 000 *g* for twenty minutes at 4 ℃, 175 μL of the supernatant was collected and transferred to a fresh tube. Three micro-litter of tissue extract was incubated with 25 μL of reaction mixture including a biotin labeled P1-TS primer and P2 primer, telomerase substrate, and Taq polymerase for 20 min at 25 ℃. After further incubation at 94 ℃ for 5 min for telomerase inactivation, the resulting mixture was subjected to PCR for 30 cycles at 94 ℃ for 30 s, at 50 ℃ for 30 s, and at 72 ℃ for 90 s, the reaction was held at 72 ℃ for 10 min.

PCR products were denatured and hybridized with a digoxigenin labeled, telomeric repeat specific detection probe. The resulting product was immobilized through the biotin labeled TS primer to a streptavidin coated microtitre plate and detected with antidigoxigenin antibody conjugated with peroxidase. Absorbance values were measured using a microtitre (ELISA) reader at 450 nm with a reference wavelength of approximately 690 nm within 30 min after addition of the stop reagent. Heat-treatment of the cell extract for 10 min at 65 ℃ prior to the TRAP reaction was used to inactivate telomerase protein for producing negative controls. Positive controls were the 293 cells that expressed the telomerase activity. Samples were regarded as telomerase-positive if the difference in absorbance (△A) was higher than 0.2 (A 450 nm–A 690 nm units).

Statistical analyses

The correlations between qualitative data were studied with the χ^2 and Fisher tests. A probability *P* value <0.05 was considered statistically significant.

RESULTS

H pylori infection

One hundred and twelve of 171 cases were *H pylori* positive. The *H pylori* infection rate was 65%(13/20) in CSG, 63.89%(46/72) in CAG, 64.29%(9/14) in Dys, and 67.69%(44/65) in GC. It had no significant difference among these groups.

Telomerase activity

Telomerase activity was measured by a telomerase polymerase chain reaction (PCR) enzyme linked immunosorbent assay, which does not require radioactive PCR amplification and yields a semiquantitative measurement. Telomerase activity was detected in 57(87.69%) of 65 gastric carcinomas examined, 46(63.89%) of 72 CAG and 9(64.29%)of 14 Dys. No telomerase activity was detected in all the 20 CSG. The mean telomerase activity was higher in gastric cancer than in CAG, CSG and Dys (*P*<0.01 or *P*<0.05). Based on intestinal metaplasia, CAG was classified into two groups, severe IM and mild IM. The telomerase activity was higher in CAG with severe IM than that in mild IM (*P*<0.01).

Twenty-nine gastric cancers with complete clinical data were classified by histologic parameters such as gender, tumor location, tumor size and microscopic morphology. The telomerase activity among these groups had no significant difference (data not shown).

H pylori infection and telomerase activity in patients with gastric cancer and CAG are shown in Table 1. The mean telomerase activity was higher in CAG with severe intestinal metaplasia and *H pylori* infection than in that without infection (*P*<0.05), but there was no significant difference between those with and without *H pylori* infection in gastric cancer and CAG with mild intestinal metaplasia.

Table 1 Telomerase activity and c-Myc expression in *H pylori* infected gastric diseases

Diagnosis	Samples number	*H pylori* infection	Telomerase activity + (%)	P	c-Myc expression + (%)	P
GC	65	+(44)	38 (86.36)	>0.05	34 (77.27)	<0.05[a]
		-(21)	19 (90.48)		6 (28.57)	
CAG with MM	30	+(18)	3 (16.67)	>0.05	4 (22.22)	>0.05
		-(12)	1 (8.33)		1 (8.33)	
CAG with SM	42	+(28)	19 (67.86)	<0.01[b]	19 (67.86)	<0.01[d]
		-(14)	3 (21.43)		1 (7.14)	

χ^2 test and Fisher's exact test. [a]*P*<0.05 *vs* c-Myc expression from samples without *H pylori* infection. [b]*P*<0.01 *vs* telomerase activity from samples without *H pylori* infection. [d]*P*<0.01 *vs* c-Myc expression from samples without *H pylori* infection. MM, mild intestinal metaplasia SM, severe intestinal metaplasia.

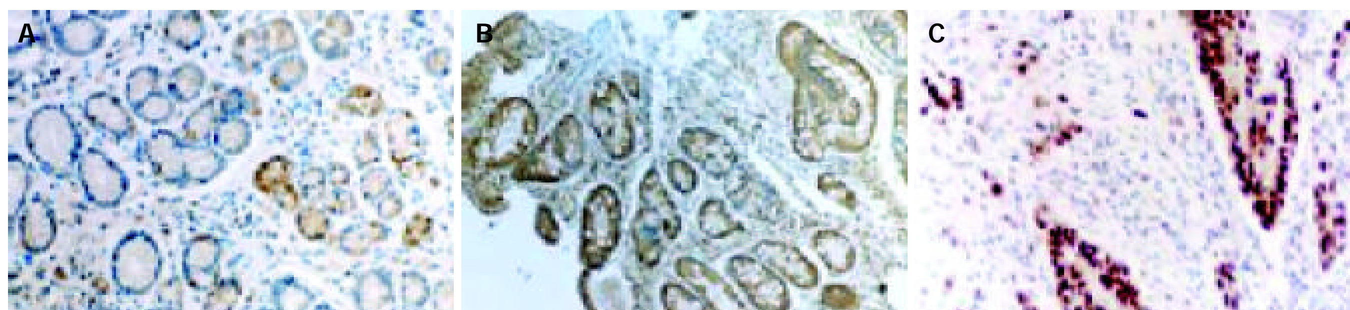

Figure 1 c-Myc protein expression in chronic superficial gastritis, chronic atrophic gastritis and gastric adenocarcinoma. A: c-Myc protein expression in chronic superficial gastritis. B: c-Myc protein expression in chronic atrophic gastritis. C: c-Myc protein expression in gastric adenocarcinoma.

c-Myc expression

c-Myc was expressed in 40 of 65(61.5%) gastric cancers, 20 of 42(47.62%) CAG with severe IM, 5 of 30(16.67%) CAG with mild IM, 8 of 14(57.14%) Dys and 1 of 20(5%) CSG. The expression of c-Myc was highest in gastric cancer, and c-Myc expression in CAG with severe IM was significantly higher than that in CAG with mild IM. The c-Myc immunoreactivity was localized in both cytoplasm and nucleus (Figure 1).

H pylori infection and c-Myc expression in patients with gastric cancer and CAG are shown in Table 1. The expression of c-Myc was higher in *H pylori* infected gastric cancer and CAG with severe IM than in those without infection (P<0.01), but there was no significant difference between those with and without *H pylori* infection in CAG with mild IM.

Coordinate up-regulation of telomerase activity and c-Myc expression in H pylori infected gastric cancer and CAG with severe intestinal metaplasia

The telomerase activity and c-Myc expression were coordinately increased in *H pylori* infected gastric cancer and CAG with severe IM (Figure 2). The rate of co-expression was 89.47%(34/38) and 100%(19/19), respectively.

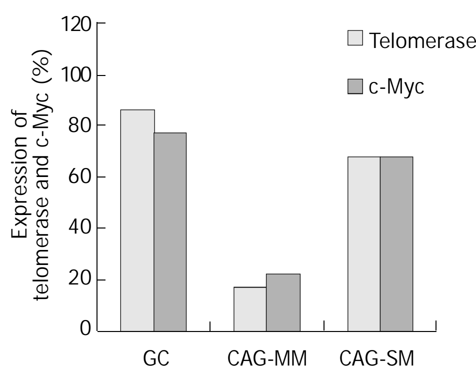

Figure 2 Coordinate increase of telomerase activity and c-Myc expression in *H pylori* infected gastric cancer and CAG with severe intestinal metaplasia. The rate of co-expression was 89.47%(34/38) and 100%(19/19), respectively. (CAG-MM: chronic atrophic gastritis with mild metaplasia; CAG-SM: chronic atrophic gastritis with severe metaplasia.).

DISCUSSION

In the present study, we demonstrated that most gastric tumors displayed telomerase activity, regardless of histological types. Telomerase activity was highest in cancer tissue, followed by chronic atrophic gastritis with complete intestinal metaplasia, dysplasia, chronic atrophic gastritis with mild intestinal metaplasia and chronic superficial gastritis. Telomerase activity was higher in CAG with severe metaplasia and *H pylori* infection than in that without *H pylori* infection (P<0.01). Our results indicate that telomerase activity is one of the most common and fundamental events of gastric cancers. What is more important is that telomerase was activated in precursor lesions, such as chronic atrophic gastritis with intestinal metaplasia and dysplasia. It suggested that there were a certain number of immortal cells existing in these lesions. In addition, *H pylori* infection might induce telomerase activity in chronic atrophic gastritis with intestinal metaplasia.

Recently, it was reported that *H pylori* infection played a role in activation of telomerase. Chronic infection with *H pylori* induced the activation of telomerase in gastric mucosa exhibiting intestinal metaplasia[25]. In patients with intestinal-type gastric cancer, telomerase activity was higher in intestinal metaplasia with *H pylori* infection than in that without infection[26]. We found that telomerase was activated in *H pylori* infected precursor lesions, such as chronic atrophic gastritis and dysplasia, and telomerase activity rose higher during the progression of the lesions. These data indicate that *H pylori* infection may contribute to gastric tumorigenesis through induction of telomerase activity, and that telomerase activation in *H pylori* infected chronic atrophic gastritis with intestinal metaplasia and dysplasia may be correlated with oncogenesis. It remains to be defined how *H pylori* infection activates telomerase. *H pylori* infection might influence the negative regulator of telomerase activity during early stages of stomach carcinogenesis[26]. In addition, inflammatory gastric mucosa caused by *H pylori* infection could produce free radicals and cytokines that may induce telomerase activity[30].

Frequent genetic alterations, such as c-myc proto-oncogene have been found in gastric pre-malignant lesions[31,32]. Amplified c-Myc, which controls cell growth and cellular differentiation[33,34], has been reported in small percent of gastric carcinomas[35,36]. Expression of c-Myc was proved to be more frequent in poorly differentiated gastric cancer cells, and more frequent in gastric adenocarcinoma than in adenoma and has also been proposed as an aid to differentiate between the two conditions[37,38]. In the present study, c-Myc was expressed in 61.54%(40/65) of gastric cancers, 47.62%(20/42) of chronic atrophic gastritis with severe IM, 16.67%(5/30) of CAG with mild IM, and 5%(1/20) of chronic superficial gastritis patients. The expression of c-Myc was highest in gastric cancers, and c-Myc expression in CAG with severe intestinal metaplasia was significantly higher than that in CAG with mild intestinal metaplasia. The expression of c-Myc was higher in *H pylori* infected gastric cancer and CAG with severe intestinal metaplasia than in that without infection (P<0.01). We found that c-Myc expression in both gastric cancer and chronic atrophic gastritis with severe intestinal metaplasia is correlated with *H pylori* status. Previously, a study by Nardone *et al* showed that *H pylori* infection affected the expression of c-Myc in chronic gastritis, but the positive rate was very low (15%). The expression of c-Myc was very high in *H pylori* infected CAG in our study because we classified chronic gastritis as CSG

and CAG, the latter was further classified as CAG with mild intestinal metaplasia and with severe intestinal metaplasia.

Recent evidence suggested that the Myc protein could bind to the promotor of human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase[27-29] . It has been shown that hTERT is the determinant of telomerase activity control. A study by Hiyama *et al*. demonstrated a close association between a high level of telomerase activity and amplification of the myc locus in neuroblastomas. Thus, up-regulation of Myc due to gene alterations leading to subsequent activation of hTERT expression, may be one cause for telomerase activation in tumors. Interestingly, we found that telomerase activity and c-Myc were co-expressed in *H pylori* infected chronic atrophic gastritis. The correlation was not significant in patients without *H pylori* infection or in patients with *H pylori* infection but without gastric atrophy. Therefore, it is suggested that the presence of both gastric atrophy and chronic *H pylori* infection is essential for cell immortality and genomic instability.

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