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• GASTRIC CANCER •

Overexpression of cyclin E in Mongolian gerbil with Helicobacter pylori- induced gastric precancerosis

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Abstract

AIM: **To explore dysregulation of cyclin E in malignancies, and to further investigate the role of cyclin E in *Helicobacter pylori (H. pylori)*-induced gastric precancerosis.**

METHODS: **Four-week-old specific pathogen-free male Mongolian gerbils were employed in the study. 0.5mL 1×10^8 cfu·L^{-1} suspension of *H. pylori* NTCC11637 in Brucella broth was inoculated orally into each of 20 Mongolian gerbils, and a further 20 gerbils were inoculated with Brucella broth as controls. 10 of the infected gerbils and 10 of the non-infected control gerbils were sacrificed at 25, 45 wk after infection. The expression of cyclin E was analyzed by RT-PCR and immunohistochemical studies with monoclonal antibody to cyclin E in Mongolian gerbil of *H. pylori*-induced gastric precancerosis.**

RESULTS: ***H. pylori* was constantly detected in all infected animals throughout the study. At 25 wk after infection of *H. pylori*. ulcers were observed in the antral and body of stomach (n=6). Histological examination showed that all animals developed severe inflammation and multifocal lymphoid follicles appeared in the lamina propria and submucosa of gastric antrum. At 45 wk after infection of *H. pylori*, severe atrophic gastritis (n=10). intestinal metaplasia (n=8) and dysplasia (n=6) could be observed. Cyclin E mRNA levels were significantly more at 25 wk after infection of *H. pylori* (1.27±0.26), and at 45 wk after infection of *H. pylori* (1.82±0.39) than control-animals (0.59±0.20, P<0.01); cyclin E mRNA levels were evaluated by 2.2-fold at 25 wk (P<0.01) and 3.1-fold at 45 wk (P<0.01) precancerosis induced by *H. pylori*, when compared with control gastric epithelium of Mongolian gerbil. Immunohistochemical staining revealed exclusive nuclear staining of cyclin E. Furthermore, there was a sequential increase in cyclin E positive cells from normal epithelium to precancerosis.**

CONCLUSION: **Overexpression of cyclin E occurs relatively early in gastric tumorigenesis in this model.**

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INTRODUCTION

Gastric cancer is a major health problem[1-3] and remains the second most common cancer in the world[4-6]. Although epidemiological studies have indicated that *H. pylori* infection plays a crucial role in gastric carcinogenesis in humans[7-22], there is no direct proof that *H. pylori* is actually associated with gastric carcinogenesis[23-26]. The purpose of this study was to elucidate the relationship between *H. pylori* infection and gastric carcinogenesis by using an animal model of long-term *H. pylori* infection, and explore the role played by cyclin E in gastric tumorigenesis[27-29].

Cyclins are positive regulators of cell cycle progression. They function by forming a complex with and activating a class of protein kinases which are essential for cell cycle transitions. As the major regulatory events leading to cell proliferation occur in the G1 phase of the cell cycle, altered expression of cyclins involved in the G1 phase may be an important step in oncogenesis[30-33]. Among G_1 cyclins, an accumulating body of evidence suggests that over expression and rearrangement of cyclin E is associated with malignancy. Over expression of cyclin E has also been found in mouse mammary tumors and is associated iwth tumor development; altered expression of cyclin E can accelerate occurrence and early progression of colorectal cancer, and plays an important role in occurrence of breast cancer[34-37]. But cyclin D_1, over expression in esophageal cancer and breast cancer, has no over expression in gastric cancer, which suggests expression of cyclins has specificity of organs. To gain a further understanding of the role played by cyclin E in gastric tumorigenesis, the study investigates the role of cyclin E in *H. pylori*-induced gastric precancerosis[38-39].

MATERIALS AND METHODS

Animals and preparation

Four-week-old specific pathogen-free male Mongolian gerbils, weighing (20±5)g, were employed in the study. They were housed in individual metabolic cages in a temperature conditioned room (23±2)℃ with a 12 h light-dark cycle, allowed access to standard rat chow (provided by Experimental Animal Center, First Military Medical University) and water ad labium, and acclimatized to the surrounding for 7 d prior to the experiments. *H. pylori*(NTCC11637) was obtained from American Type Culture Collection and cultured on Brucella agar plates containing 70 mL·L^{-1} goat blood in a microaerobic condition (volume fraction; N_2:85%, O_2:5%,CO_2:10%, in aerobic globe box) at 37℃ for 3 d. The strain was identified by morphyology, Gram's stain, urease production and so on.

Experimental protocol

Suspension 0.5 ML 1×10^8 cfu·L^{-1} of *H. pylori* NTCC11637 in Brucella broth was inoculated orally into each of 20 Mongolian gerbils which had been fasted overnight, for 14 d continuously. A further 20 gerbils were inoculated with Brucella broth as controls. 10 of the infected gerbils and 10 of the non-infected control gerbils were sacrificed at 25, 45 wk after infection. The stomach of each animal was removed and opened for macroscopic observation. For half of each gastric antrum mucosa was dissected for RNA isolation. The remainders of the stomach samples were used for istological examination, which were fixed with neutral-buffered 100 mL·L^{-1}

formalin and processed by standard methods that embedded in paraffin, sectioned and stained with haematoxylin for analyzing histological changes, Giemsa stain for detecting for *H. pylori* and Alcian blue (AB)/PAS stain for examining intestinal metaplasia.

RNA isolation and RT-PCR analysis

Using Tripure isolation reagent (Boehringer Mannheim, Germany), total cellular RNA was isolated from previously frozen tissues according to the manufacturer's instruction. All RNA samples were analyzed for integrity of 18s and 28s rRNA by ethiding bromide staining of 0.5μg RNA resolved by electrophoresis on 12g·L^{-1} agarose-formaldehyde gels. RT-PCR analysis was performed as follows. RNA was incubated at 60℃ for 10 main and chilled to 4℃ immediately before being reverse transcribed. Reverse transcription of 1μg total RNA using antisense primers was performed in a volume of 20μL for 40 min at 50℃, containing 200 U MMLV reverse transcriptase, 1×buffer RT, 1 MU·L^{-1} Rnasin, 0.5mmol·L^{-1} dATP, dGTP, dCTP and dTTP respectively and 0.2μmol·L^{-1} antisense primers including cyclin E and β-actin respectively. The samples were heated to 99℃ for 5 min to terminate the reverse transcription reaction. By using a Perkin-Elmer DNA Thermocycler 4800 (Perkin-Elmer, Norwalk, CT), 5μL cDNA mixture obtained from the reverse transcription reaction was then amplified for cyclin E and β-actin. β-actin was used as the housekeeping gene and amplified with cyclin E as contrast. The amplification reaction mixture consisted of 10×buffer 5μL, 0.2 mmol·L^{-1} dATP, dGTP, dCTP and dTTP respectively, 2.5 U Taq DNA polymerase, and 0.2μmol·L^{-1} each of sense and antisense primers in a final volume of 50μL. The reaction mixture was first heated at 94℃ for 2 min and amplification was carried out for 29 cycles at 94℃ for 0.5 min, 53℃ for 1 min, 70℃ for 1.5 min, followed by an incubation for 7 min at 70℃. The number of amplification cycles was previously determined to keep amplification in the linear range to avoid the "plateau effect" associated with increased numbers of PCR cycles. The PCR primers used were: cyclin E, sense 5'-TAT GGC GAC ACA AGA AAA TG-3'and antisense 5'-GCA AGA GAA GAC AGA CAA CG-3'; β-actin, sense 5'-CCA AGG CCA ACC GCG AGA AGA TGA C-3' and antisense 5'-AGG GTA CAT GGT GGT GCC GCC AGA C-3'. The length of PCR products for cyclin E and β-actin was 770 bp and 587 bp. PCR products were run on a 15g·L^{-1} agarose gel in 0.5×TBE buffer and then analyzed by gel image analysis system. The level of cyclin E was reflected with the ratio of cyclin E/β-actin.

Immunohistochemical staining

Four micrometers paraffin-embedded tisue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was ablated with 10 mL·L^{-1} hydrogenperoxide in methanol. The immunostaining for cyclin E was conduced using the StreptAvidin-Biotin-enzyme Complex kit (Boster, Wuhan). Immunostaining by replacing primary antibody with PBS was also conducted as a negative control. The staining was evaluated semiquantitatively on the basis of the percentage of positive cells, and classified as follows[40]: diffusely positive (+++) when positive cells accounted for more than 70% of the total cells, partially positive (++) when positive cells were 35%-70%, partially positive (+) when positive cells accounted for 5%-35%, and negative (-) when positive cells accounted for less than 5%.

Statistical Analysis

Experimental results were analyzed with Chi-square Tests and K Related Samples Test by SPSS software. Statistical significance was determined at $P<0.05$.

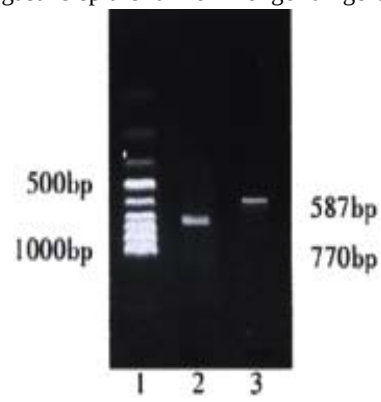
RESULTS

Histophathological findings

H. pylori was detected in gastric antrum and gastric body of all infected animals throughout the study, and more in gastric antrum than gastric body. By the 25th wk after infection of *H. pylori*, ulcers were observed in the antral and body of stomach (n=6). Histological examination showed that all animals developed severe inflammation in the area close to ulcers; multifocal lymphoid follicles appeared in the lamina propria and submucosa; and there were mild atrophic gastritis in all infected animals. By the 45th wk after infection of *H. pylori*, severe atrophic gastritis (n=10), intestinal metaplasia (n=8) and dysplasia (n=6) could be observed. Those metaplastic glands appeared more atypical than the surrounding nonmetaplastic and hyperplastic glands. Severe atrophic gastritis, intestinal metaplasia and dysplasia were gastric precancerosis. In the uninfected animals, there were no significant changes throughout the study.

RT-PCR analysis of cyclin E mRNA expression

There were cyclin E mRNA expression in gastric anturm mucosa of control-animals. cyclin E mRNA levels were significantly more at 25 wk after infection of *H. pylori*(1.27±0.26), and at 45 wk after infection of *H. pylori*(1.82±0.39) than control-animals (0.59±0.20,$P<0.01$); Cyclin E mRNA levels were evaluated by 2.2-fold at 25 wk ($P<0.01$) and 3.1-fold at 45 wk ($P<0.01$) precancerosis induced by *H. pylori*, when compared with control gastric epithelium of Mongolian gerbil (Figure 1-3).



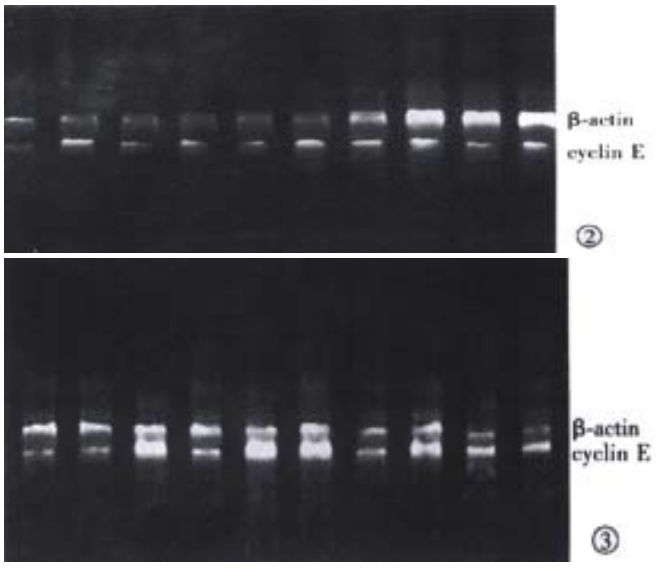


Figure 1 1:cyclin E; 2:PCR marker; 3:β-actin

Figure 2 RT-PCR analysis of cyclin E mRNA levels using β-actin as internal control. Total RNA was first reverse transcribed into cDNA and then amplified by PCR in control.

Figure 3 In 25 wk after infection of *H. pylori*.

Immunohistochemical analysis of cyclin E protein expression

To examine whether increased cyclin E mRNA expression were accompanied by increased expression of cyclin E protein, imunohistochemical analysis was performed. cyclin E protein expression lied in nuclei and cytoplasm. Cyclin E protein expressions were evaluated significantly at 25 wk(P<0.01) and at 45 wk (P<0.01) precancerosis induced by *H. pylori*, when compared with control gastric epithelium of Mongolian gerbil (Figure 4 and Table 1).

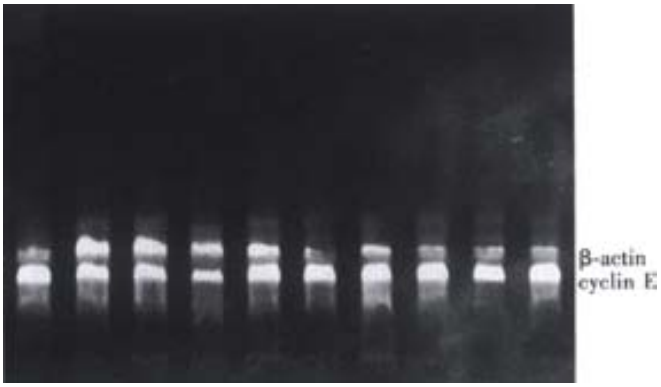


Figure 4 In 45 wk after infection of *H. pylori*.

Table 1 Expression of cyclin E by immunohistochemical staining (n=10)

Groups	Cyclin E				
	-	+	++	+++	Positive %
Control	8	2			20[a]
25 wk after *H. pylori* inf	2	7	1		80[b]
45 wk after *H. pylori* inf	1	6	2	1	90[c]

K related samples test: P=0.002Chi-square test: χ^2=12.344,P=0.002[b] *vs* [a]P=0.007, c *vs* [a]P=0.002

DISCUSSION

H. pylori infection is now know as a major cause of acute and chronic active gastritis, peptic ulcer disease and atrophic gastritis and is also suspected to be involved in the genesis of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma[41-49]. In 1994, the International Agency for Research on Cancer (IRAC), a branch of the World Health Organization (WHO), convened experts from 11 countries to examine the evidence linking a number of infectious agents with human cancer. Although there is no direct proof that *H. pylori* is actually associated with gastric carcinogenesis, epidemiological studies have indicated that *H. pylori* infection plays an important role in gastric carcinogenesis in humans; *H. pylori* was designated as a definite carcinogen (group I) to the human stomach based on prospective case-control studies reported in 1991. Several experiments were conducted in Japan that demonstrated that chronic *H. pylori*-infection models of Mongolian gerbils developed gastric carcinoma. These results will be extremely significant to elucidate the mechanism of gastric carcinogenesis due to *H. pylori* infection[5,50]. Apoptosis, a programmed cell death, was ignored, just like *H. pylori*, only to reappear recently. However, the number of current publications dealing with apoptosis of *H. pylori* has increased exponentially. Although gastric epithelial apoptosis is a programmed physiological event in the superficial aspect of the mucosa and is important for healthy cell turnover, *H. pylori* infection reportedly promotes such a cell death sequence[51-52]. Because apoptosis regulates the cycle of cell turnover in balance with proliferation, dysregulation of apoptosis or proliferation evoked by *H. pylori* colonization would be linked to the gastric carcinogenesis[53-56].

Cyclins are positive regulators of cell cycle progression, initially identified in early cleavage embryos of marine invertebrates as proteins that accumulated during interphase and were degraded at mitosis. Cyclins are now known to be positive regulatory subunits of a class of protein kinase termed cyclin-dependent kinases. These protein kinases have been shown in a number of diverse eukaryotic systems to be the master regulators of major cell cycle transitions[57-59]. Cyclin E is essential for the G1/S phase transitionin the cell cycle. Cyclin E gene amplification and altered expression has been reported in a variety of human cancers[60-61]. Now Cyclin E gene has been defected as oncogene[38]. Cyclin E was synthetized in the mid-term of the cell cycle, expressed the highest level when entering S phase, and degraded through S phase. The level of cyclin-dependent kinase2 kept constant during the cell cycle. Cyclin E, cyclin-dependent kinase2 and cyclin-dependent kinase inhibitor regulated G1/S phase transition by two-way at late stage of G1 phase, Cyclin E accelerated G1/S phase transition by composing and activating cyclin-dependent kinase2.

In the present study, to explore dysregulation of cyclin E in malignancies, and to further investigate the role of cyclin E in *H. pylori*-induced gastric precancerosis, cyclin E mRNA level was measured by quantitative RT-PCR analysis in Mongolian gerbil gastric antrum mucosa. In addition, the expression and localization of protein product was analyzed by immunohistochemistry. Sample size requirement for obtaining sufficient amounts of RNA for RT-PCR analysis did not allowdetection of either cyclin E mRNA level in any specific type of preneoplastic lesions. However, using immunohistochemical technique, cyclin E protein expression in preneoplastic lesions was observed from paraffin-embedded tissue sections. Cyclin E mRNA levels were increased 2.2-fold at 25 wk and 3.1-fold at 45 wk precancerosis induced by *H. pylori*, when compared with normal gastric epithelium of Mongolian gerbil. Immunohistochemical staining revealed exclusive nuclear staining of cyclin E. Furthermore, there was a sequential increase in cyclin E positve cells from normal epithelium to precancerosis. The present study indicated that expression of cyclin E increased from normal epithelium to precancerosis, dysregulation of cyclin E expression occurred relatively early in gastric tumorigenesis in this model and might participate in tumor progression. These findings suggested that *H. pylori*-induced gastric precancerosis was associated with dysregulation of gastric epithelial cell cycle. Further studies were needed to delineate the mechanism of these alterations.

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