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Morphological and pathologic changes of experimental chronic atrophic gastritis (CAG) and the regulating mechanism of protein expression in rats*

WANG Liang-jing[†§1], CHEN Shu-jie[2], CHEN Zhe[§2], CAI Jian-ting[1], SI Jian-min[†‡2]

([1]*Department of Gastroenterology, the Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*)

([2]*Lab of Digestive Disease, Clinical Institute, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China*)

[†]E-mail: sijm@tom.com

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Abstract: Objective: To study the pathologic change and molecular regulation in cell proliferation and apoptosis of gastric mucosa in rats with chronic atrophic gastritis (CAG), and evaluate the possible mechanisms. Methods: Rats were administered with 60% alcohol or 2% salicylate sodium, 20 mmol/L deoxycholate sodium and 0.1% ammonia water to establish chronic atrophic gastritis (CAG) models. The gastric specimens were prepared for microscopic view with hematoxylin and eosin (H-E) and alcian blue (A-B) stain. The number of infiltrated inflammatory cells, the thickness of the mucosa gland layer (μm) and the number of gastric glands were calculated. The damage of barrier in mucosa with erosion or ulceration, and the thickness of mucin were examined by scanned electron microscope (SEM). The levels of PGE_2, EGF (epiderminal growth factor) and gastrin in the serum were measured with radioimmunoassay or ELISA method. The immunohistochemistry method was used to observe the number of G cells, the expression of protein of EGFR (EGF receptor), C-erbB-2, p53, p16 and bcl-2 in gastric tissue. Results: Under SEM observation, the gastric mucosa was diffused erosion or ulceration and the thickness of mucin was decreased. Compared with normal rats, the grade of inflammatory cell infiltration in CAG rats was elevated, whereas the thickness and number of gastric gland were significantly lower (P<0.05). Compared with normal level of (0.61±0.28) μg/L, EGF in CAG (2.24±0.83) μg/L was significantly higher (P<0.05). The levels of PGE_2 and gastrin in serum were significantly lower in CAG rats than that in normal rats (P<0.05). Immunohistochemistry detection showed that the number of G cell in antrum was lower in CAG group (P<0.05). Immuno-stain showed EGFR protein expression in the basal and bilateral membrane, and the cytoplasma in atrophic gastric gland, while negative expression was observed in normal gastric epithelial cells. Positive staining of p53 and p16 protein was localized in the nucleus of epithelial cells. The former was higher positively expressed in atrophic gland, while the later was higher positively stained in normal gastric tissue. bcl-2 protein was positively stained in the cytoplasma in atrophic gastric gland, while very weakly stained in normal gastric tissue. Conclusion: The pathological findings in gastric gland accorded with the Houston diagnostic criteria of antrum-predominant CAG. CAG in rats was related with the damage of barrier in gastric mucosa and the misbalance of cell proliferation and apoptosis. There was high protein expression of oncogene, while inhibitor of suppressor gene in CAG rats indicated high trend of carcinogenesis.

Key words: Chronic atrophic gastritis (CAG), Rat, Protein expression
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INTRODUCTION

In 1998 gastrointestinal pathologists reached a consensus on the definition of chronic atrophic gastritis (CAG), which was described as programmed loss of gastric gland and/or replacement by intestinal glands in gastric mucosa. CAG was recognized to be closely related with development of gastric cancer and listed as precancerous lesions in this meeting (Genta, 1998). According to Correa (1992)'s cascade of gas-

‡ Corresponding author
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§ The two authors contributed equally to this work

tric carcinogenesis, gastric cancer was believed to develop from a multistep progression from CAG, intestinal metaplasia (IM), dysplasia (DYS) and subsequently to intestinal-type adenocarcinoma, though the definite mechanisms have not been completely identified. The imbalance in the cellular proliferation and apoptosis of gastric epithelial cells are considered to be attributable to the change of genetic events in CAG. Inhibition of apoptosis or over-proliferation could lead to mutant cells accumulation and the development of gastric neoplasm. Multiple factors are involved in this progression, including change of human environment, gene inherity and medical intervention pathways. Though the helicobacter pylori infection has been revealed to have relation with the progression from CAG to gastric carcinogenesis, there was no convincing evidence to prove this process in clinical studies (Parsonnet *et al*., 1991; Eslick *et al*., 1999). Animal model was available to help to provide information explore the possible mechanisms of gastric carcigenensis and pathological factors in this progression. Our previous studies successfully established stable antrum-predominant CAG in rats by simulating the factors inducing gastric atrophy with helicobacter pylori infection, bile refluxes, alcohol, and nonsteroid anti-inflammatory drugs. We aimed to further explore the regulating method in the cellular cycle by detecting the change of level of regulator growth factors and investigating the expression of series regulator proteins.

MATERIALS AND METHODS

Establishment of chronic atrophic gastritis in rats

CAG models (CAG 1 and CAG 2) in rats were established according to our previous published methods (Si *et al*., 2001; 2004). CAG 1 male Sprague-Dawley (SD) rats (200~220 g) were administered 60% ethanol and 20 mmol/L deoxycholate sodium (1 ml/100 g i.g.) for 24 weeks, and deprived of water by replacement with 0.1% ammonia water. In CAG 2 group, male Wistar rats (130~150 g) were administered 2% salicylate sodium (2 ml i.g.) daily for 6 weeks, and deprived of water which was replaced by 20 mmol/L deoxycholate sodium, placed in stainless cages with 5 animals in each group, at temperature (22±2) °C, humidity 55%~65%, with 12 h dark and light cycles.

Tissue and specimen preparation

After establishing the rat CAG model, the animals were deprived of food for 24 h, and anesthetized with 2% pentobarbital sodium. Five millilitres blood was withdrawn from the carotid artery by catheter. Serum was separated and stored at −40 °C. Gastric specimens were cut along the lesser curvature and greater curvature, and the glossy appearance including color, plica, elasticity and the thickness of mucin in gastric mucosa were observed. Then the specimens were immediately immersed in 10% buffered formalin and embedded in paraffin. Paraffin sections (5 μm) were routinely stained with hematoxylin and eosin (H-E) and alcian blue (A-B) stain. In CAG 2 rats 5 mm×5 mm gastric specimens were taken along with antrum and oxyntic mucosa, and soaked in 2.5% glutaraldehydum and 2% osmium tetraoside, then dehydrated with concentrated alcohol. The microscopic view including erosion, ulceration in mucosa and the thickness of mucin were examined by scanning electron microscope (SEM, Philips XL 30E).

Microscopic view of gastric antrum

Histological change in gastric antrum was assessed by the diagnostic criteria of gastritis in Houston in 1996 (Dixon *et al*., 1996). The mean number of infiltrated inflammatory cells was calculated in each of 10 microscopic fields of antrum mucosa and ranked into 7 scales (0, 0.5, 1, 1.5, 2, 2.5 and 3 scales respectively). The thickness of the mucosa gland layer (μm) and the number of gastric glands per 1 mm were analyzed in each of the 5 microscopic fields.

Hormone detection in serum

The level of serum epiderminal growth factor (EGF) and gastrin (GAS) in CAG 1 rats were measured by radioimmunoassay and ELISA according to the kit's instructions (EGF, Atomic Energy Research Institute, China; Gastrin, Lifkey Company, USA). The serum level of PGE_2 in rats was detected with radioimmunoassay according to the kit's instructions (PGE_2, Shuzhou College of Medicine, China).

Immunohistochemistry detection

Expression of G cell, EGF receptor (EGFR), C-erbB-2, p21, p53, p16 and bcl-2 in gastric tissue

were detected with the Envision two-step methods according to the kit's instructions. Gastric tissue specimens in CAG 1 rats were consecutively sectioned into 4 μm thick slices. The paraffin was dehydrated with xylene and concentrated ethyl alcohol. The tissue section was incubated with 3% H_2O_2 for 20 min at room temperature. One percent lemon acid or 0.01 mmol/L citrate buffer fluid was heated at 92 °C. Then the sections were incubated with the primary antibody at room temperature for 30 min. The primary antibody used was EGFR and C-erbB-2 polyclonal human anti-mouse antibody (Santa Cruz and New Markle Company respectively, USA); 13002, D182 and 1302 mouse anti-mouse antibody for p53, p16 and bcl-2 respectively (Zhongshan Biochemical Company, China) and BA0245 rabbit anti-mouse antibodies for GAS (Bo Shide, China). For EGFR and C-erbB-2 staining, the sections were incubated with biotinylated secondary antibody (Envision kit, Dako Company) for 30 min and PBS fluid each for 15 min. The reaction products were invisualized by immersing the sections in diaminobenzidine (DAB) and washing with clear water twice. For GAS, p21, p53, p16 and bcl-2 staining the sections were incubated with anti-rabbit or mouse antibody (Envision HRP, Dako Company) for 10 min. The reaction products were invisualized by immersing the sections in DAB and washing with Tris-NaCl (TBS) for 10 min. Paraffin sections from breast adenocarcinoma with known immunoreactivity to antibodies against EGFR and C-erbB-2 were used as the positive controls. The tissue samples were considered to be positive for expression of p53 and p16, when there was nuclear staining, and bcl-2, EGFR and C-erbB-2 staining in cytoplasma or membrane. Slides were examined under microscope, and positive expression rates were counted in five fields for each specimen and analyzed by computed figure system of Lelca Qwin.

Statistical analysis

All data were put into SPSS 10.0 statistical computer system. Statistical analysis was performed with *t* test or chi-square test between two groups. All *P* values were two-tailed, with statistical significance indicated by a value of $P<0.05$.

RESULTS

Pathological findings of gastric tissue

The glossy gastric mucosa folds were flat or disappeared, with pale appearance and thin mucin in atrophic gastritis rats. The gastric wall elasticity was decreased. Light microscope showed that irregular arrangement and reduction layer of gastric gland, while increasing thickness of musculoris mucosa in atrophic gastritis rats, as shown in Figs.1a and 1b. Massive neutrophil and lymphatic cell infiltration between gastric glands were distributed in CAG 2 rats. H-E and A-B stain showed globlet cell converged in basal mucosa and pits in CAG 2 rats, which indicated intestinal metaplasia, as shown in Figs.1c~1f. Under SEM observation, the gastric mucosa of the rats in CAG 2 was diffused erosion or ulceration and the mucin thickness was decreased (Figs.2a and 2b). The number of inflammatory cells in CAG 2 was more increased than that of control group ($P<0.05$), while the number and thickness of glands in the gastric antrum were significantly decreased ($P<0.01$). Data are showed in Table 1. The scale of inflammation in antrum was 1.21±0.26 in CAG 2 rats, compared with 0.69±0.26 in control group, and was remarkably increasing ($P<0.05$).

Level of gastrointestinal hormone in serum

Compared with normal level of (0.61±0.28) μg/L, EGF in CAG (2.24±0.83) μg/L was significantly higher ($P<0.05$). The level of serum PGE_2 and GAS was more significantly decreased in CAG group than in normal group ($P<0.05$). Data are shown in Table 2.

Table 1 Pathological finding of gastric antrum tissue in rats

Pathological findings	Normal control group	CAG 1 group
Inflammatory scale	0.63±0.36	2.42±0.49*
Thickness of glandular tissue (μm)	13.36±1.87	10.94±1.35#
Number of gland	40.50±1.69	33.17±3.25#

Note: *t* test was performed to compare with the normal control group. * $P<0.05$, # $P<0.01$

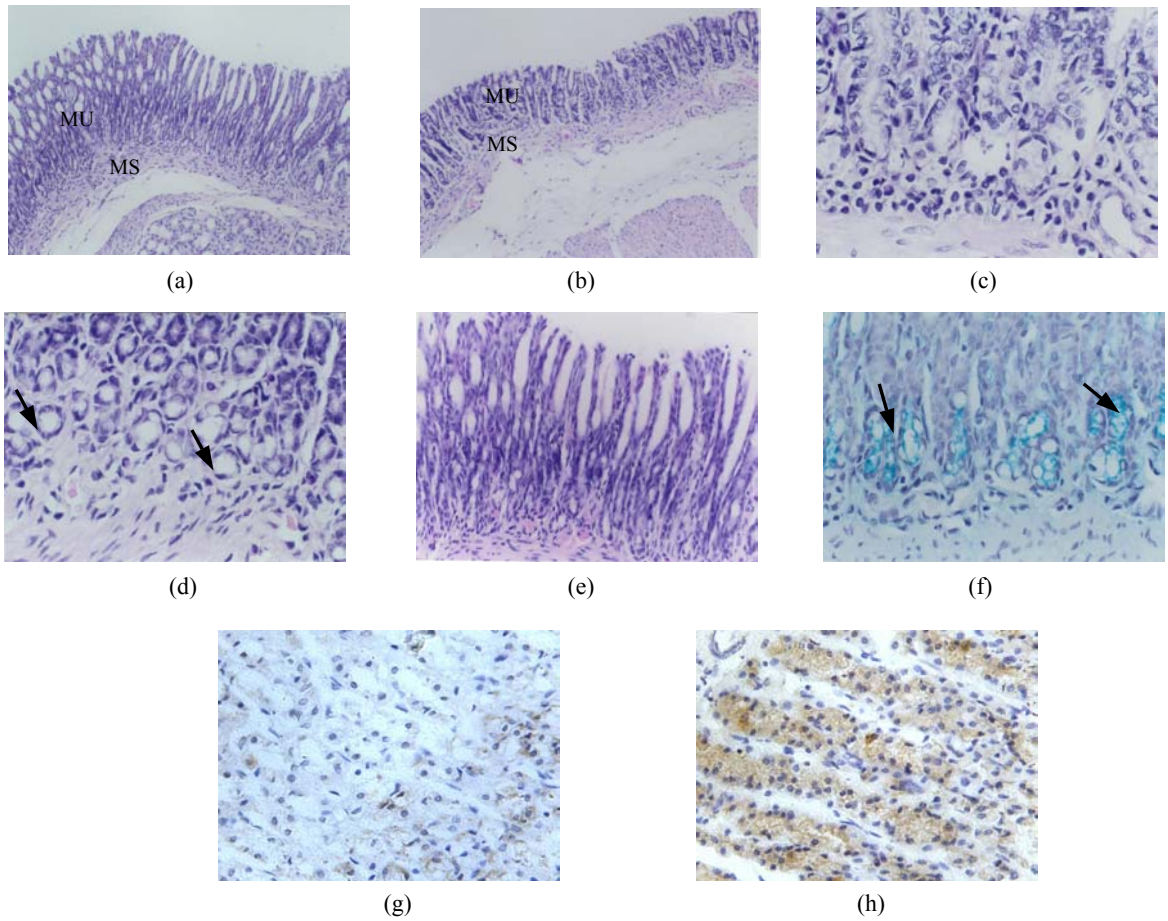

Fig.1 Morphological and pathologic changes of gastric glands and epithelial cells in rats. (a) Regularly arranged gastric epithelial cells, close order of gastric gland and layers in normal rats (H-E×100); (b) Irregularly arranged and reduction layer of gastric gland, thickness of musculoris mucosa in atrophic gastritis rats (H-E×100); (c) Normal H-E stain of gastric tissue; (d) H-E stain shows globlet cells (arrow) converge in basal musoca gland and inflammatory cells infiltration in CAG 2 group (×200); (e) Normal A-B stain of gastric tissue; (f) A-B stain of globlet cells (arrow) in basal musoca gland in CAG 2 group (×200); (g) Negative immuno-stain of G cells; (h) Immumohistochemical stain of accumulation of G cells in the cytoplasm and membrane of antrum gastric gland in line (MU: Muscosa layer; MS: Musculoris mucosa)

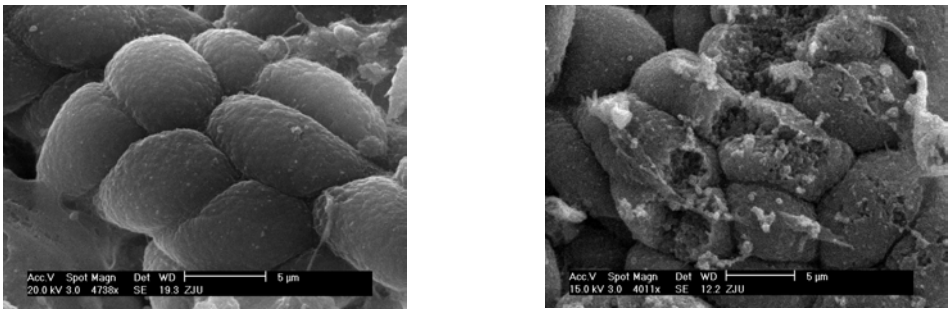

Fig.2 Gastric mucosa under SEM observation. (a) SEM shows regular gastric pit, without erosion, with massive mucin in normal rats (×4738); (b) Gastric pit disorder, diffused ulcer and erosion change of epithelial cells, with thin mucin in experimental gastritis rats (×4011)

Table 2 Level of EGF (μg/L), PGE_2 (μg/L) and GAS (pg/ml) in serum of rats

Serum hormone	Normal group	CAG group
EGF (μg/L)	0.61±0.28	2.24±0.83$^{\#}$
PGE_2 (μg/L)	125.1±41.3	73.7±6.7$^{\#}$
GAS (pg/ml)	139.25±17.32	118.38±13.62*

Note: *t* test was performed to compare with the normal group. $^{*}P<0.05$, $^{\#}P<0.01$

Detection of G cells in antrum

Immunohistochemical stain showed accumulation of G cells localized in the cytoplasma of the middle or lower layer in antrum gland, and were ranged irregularly or in line. As shown in Figs.1g and 1h, the number of G cells was 33.75±7.34 in gastric mucosa in CAG rats, which was significantly lower than that of 68.76±21.75 in normal group ($P<0.05$).

Detection of expression of proteins

Immunohistochemistry detection showed EGFR protein expression in the basal and bilateral membrane, and the cytoplasma in CAG gastric tissue. C-erbB-2 was identified with weak expression in membrane and cytoplasma in atrophic gastric gland. Immuno-stain showed negative expression of EGFR and C-erbB-2 protein in normal gastric epithelial cells (Figs.3a~3d).

The positive staining of p53 and p16 protein was localized in the nuclear body. The former were higher positive expressed in atrophic gland, while the later was higher positive stained in normal gastric tissue (Figs.3e~3h). bcl-2 protein was positively stained in the cytoplasma in atrophic gastric gland, but very weakly stained in normal gastric tissue (Figs.3i and 3j).

DISCUSSION

Administering rats with 60% ethanol or 2% salicylate sodium directly damaged the gastric mucosa. Twenty mmol/L deoxycholate sodium and 0.1% ammonia administration could simulate the damage by bile refluxes and hyperammonia induced by helicobacter pylori infection. This study successfully established atrophic gastritis, which revealed that pathological view accorded with the diagnostic criteria of antrum-dependent atrophic gastritis. The urease-ammonia (NH_4OH) system has been proposed to play a major role in the pathogenesis of the helicobacter pylori-associated gastritis, single application of ammonia was accompanied by a decrease in gastric blood flow (GBF) to approximately 30% of the normal value, and be inhibitor of prostaglandin (Brzozowski *et al*., 1996). When the bile refluxes into the stomach, bile salt may damage the lipoprotein in musoca. When the gastric mucosa was exposed to ethanol, or salicylate sodium (an inhibitor of prostaglandin-cyclooxygenase), the extent of mucosa damage was greatly attenuated. Chronic atrophic gastritis in rats was related with the damage of the gastric mucosa barrier and further inducing of the deep injury in glandular tissues by long term stimulating with inflammatory factors, while the gastric mucosa incompletely regenerating.

EGF played a critical role in gastric tissue repair and cell regeneration. EGFR (C-erbB-1) and C-erbB-2 were two of the most important factors in the EGFR family. EGF would combine with its receptor (EGFR) in gastric epithelial basal or bilateral membrane and showed its effects. Studies demonstrated that in normal gastric epithelial cell, EGFR was rarely detected because of not being exposed in intact epithelial membrane. When damage of gastric barrier and perfusion rate in the epithelia membrane increased, the level of the expression of EGF/EGFR was elevated. Brzozowski *et al*.(1996) reported that repeated stimulation with ammonia in rats could result in adaptive cytoprotection and cell proliferation. The effects of EGF on gastric mucin biosynthesis were increased, and the EGFR-mRNA expression was high in the surface mucosal layer but low in the deep and muscle layers of the stomach (Ichikawa *et al*., 2000). Abnormal expression of EGFR has been identified as a molecular marker of dysphasia and malignant growth in gastric epithelial cells, and had close relationship with carcigenesis (Wang *et al*., 2002; Kopp *et al*., 2002). Our study showed that there was high expression of EGFR and C-erbB-2 protein in atrophic gastritis in rats, which indicated high trends of carcinogenesis in CAG. EGFR would combine with EGF and took effect in the pathway of the oncogene expression, breaking the normal self-regulation of the cell cycle, and resulting in the development of gastric cancer.

Gastrin secreted by G cell and facilitated gastric mucous cell proliferation, mucous regeneration and

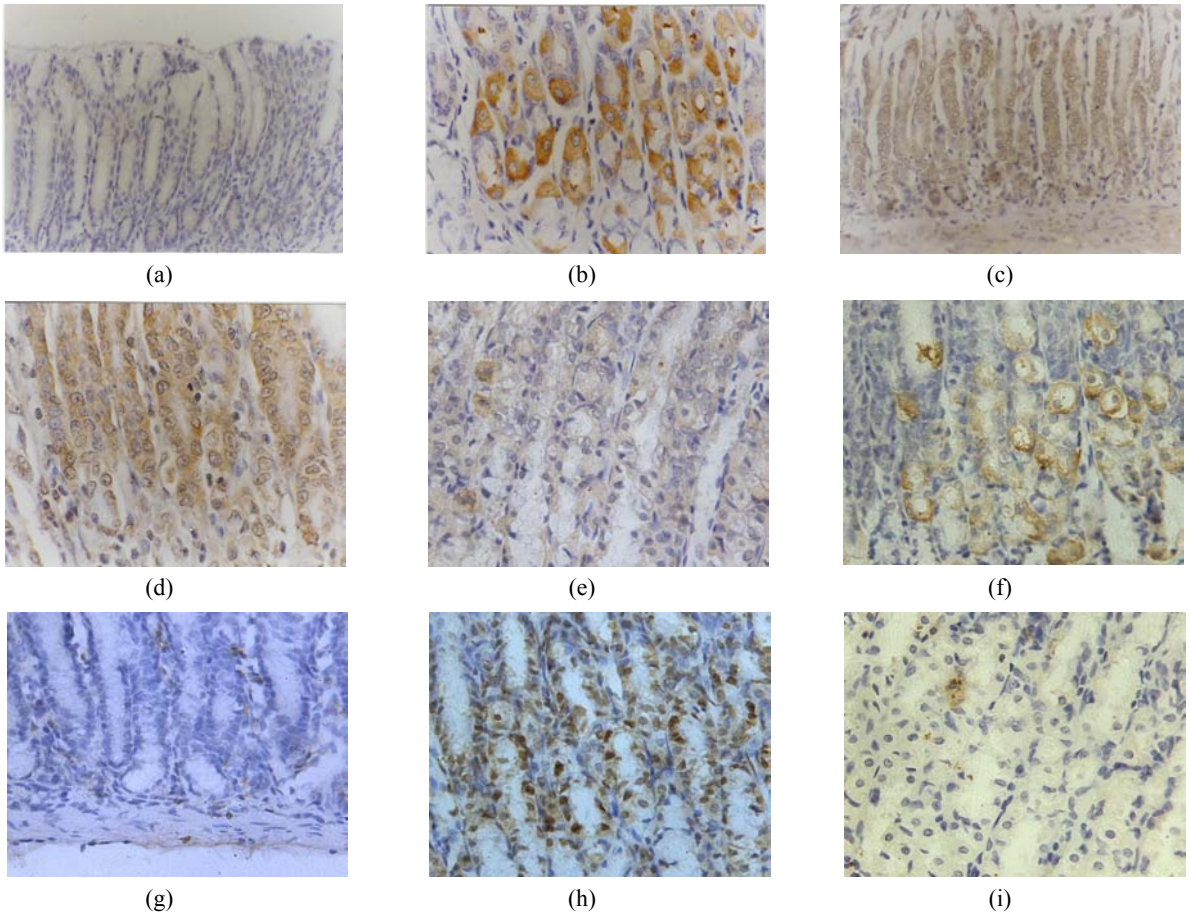

Fig.3 Immuno-stain of series regulator proteins in gastric glands. (a) Negative immuno-stain of EGFR in normal gastric tissue; (b) EGFR protein expression in the basal and bilateral membrane in atrophic gastric gland; (c) Negative immuno-stain of C-erbB-2 in normal gastric tissue; (d) C-erbB-2 expression in the membrane in atrophic gastric epithelial cell; (e) Negative immuno-stain of p53 expression in normal gastric tissue; (f) p53 protein expression in the basal and bilateral membrane in atrophic gastric gland; (g) Negative immuno-stain of p16 protein in atrophic gland; (h) p16 protein positive expression in the basal and bilateral membrane in normal gastric epithelial cell; (i) Negative immuno-stain of p21 expression in normal gastric epithelial cell; (j) bcl-2 protein positive expression in the basal and bilateral membrane in atrophic gastric gland (All figures are shown with immumohistochemical stain×400)

increasing GBF. Gastrin is mostly expressed in the lower layer of gastric tissue in antrum. The level of the gastrin and the number of G cells were decreased in CAG rats accordingly. Studies demonstrated that these changes were more remarkable in moderate or severe grade atrophic gastritis (Larsson and Hougaard, 1993; Lin *et al*., 1996). It could be explained that the injury was deep in low layer of gland tissues, where G cells localize.

Gastric mucosa continuously renews epithelial cells by maintaining the balance of cell proliferation and apoptosis. Apoptotic cells were identified in gastric surface epithelium of normal rats. With the progression of atrophic gastritis, the generative cell zone shifts downwards in the neck region of the mucosal glands, in intestinalized glands, both apoptotic and proliferating cells are present in deeper portions of the glands (Ishida *et al*., 1996). The balance of cell apoptosis and proliferation occurred in a cell cycle-dependent manner. Apoptosis occurs in late G1 or G2 phases. p53, a tumor suppressor gene, is thought to play a critical role in the multistep process of gastric carcinogenesis. p53 regulates the cell cycle checkpoint-related gene p21, whose expression directly

suppresses the cell cycle in the late G1 and may consequently induce apoptosis. The apoptosis induced by wild-type p53 can be blocked by apoptotic inhibitor regulator bcl-2 (Merritt *et al*., 1994; Girinsky *et al*., 1995). It has been shown that in normal gastric mucosa, the expression of bcl-2 and p53 is topographically restricted. bcl-2 and p53 expression is confined to only a few regenerative epithelial cells of the mucous neck region. Such topographic expression is increased in chronic gastritis or intestinal metaplasia. In dysplasia, bcl-2 expression increases and extends up the parabasal and superficial epithelium; weak p53 is expressed throughout the full thickness of the epithelial membrane (Cho and Kim, 1998). p16 gene is also called multiple tumor suppressor, for its role as inhibitor of Rb gene expression, and in suppressing cell proliferation. bcl-2 is important negative regulator of cell apoptosis. Studies showed that the production of p16 was significant lower with the progression from atrophic gastritis, to intestinal metaplasia, subsequently to gastric carcinoma. The inhibitor action of p16 was considered as the early event of carcinogenesis (Zhou *et al*., 2001). Cho and Kim (1998) revealed that overexpression of bcl-2 in patients with CAG and IM. p53 mutation and accumulation occur in the process of transformation from premalignant lesions to gastric carcinoma. We found that the level of protein p16 was significantly decreased in CAG rats, when compared with normal rats. There was overexpression of bcl-2 in CAG rats, whereas no significant change of p53 expression was demonstrated in our animal experiment. The overexpression of bcl-2 may play a critical role in the progression of premalignant lesions, while the p53 mutation happened late in malignant events.

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