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Study of the correlation between the expression of nuclear factor kappa B and proliferation regulatory proteins and chronic superficial gastritis

Ispitivanje korelacije između ekspresije nuklearnog faktora kapa B i proliferacije regulatornih proteina i hroničnog superficijalnog gastritisa

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Abstract

Background/Aim. Cell proliferation and the regulation of protein expression play an important role in gastritis, but in chronic superficial gastritis (CSG), they are rarely reported. The aim of this study was to determine the relationship between the expression of nuclear factor kappa B (NF-κB) and regulatory proteins and the rat CSG. **Methods.** The CSG rat model was established artificially, by chemical agents and irregular diet. The expression of epidermal growth factor receptor (EGFR) and proliferating cell nuclear antigen (PCNA) in the gastric mucosa of CSG rats was measured by immunohistochemistry, while mRNA expression levels of NF-κB p65 were detected by *in situ* hybridization. **Results.** There was more obvious infiltration of inflammatory cells in the gastric mucosa of CSG rats than in that of control rats, and the inflammation score was significantly increased. The expression levels of PCNA, EGFR, and NF-κB p65 mRNA in the gastric mucosal cells of CSG model rats increased significantly. Correlation analysis showed that the inflammation score was positively correlated with the expression levels of NF-κB p65 mRNA and EGFR, while it presented no significant correlation with the expression level of PCNA. In addition, there was a significant positive correlation between NF-κB p65 mRNA and EGFR levels. **Conclusion.** High expression of NF-κB and EGFR plays an important role in the occurrence and progression of CSG, and it is significantly positively correlated with the degree of inflammation in the gastric mucosa. Therefore, changes in NF-κB and EGFR expression may be used as important indicators for the assessment of CSG; changes in their expression levels are helpful to assess the degree of gastric mucosal lesions and progression of CSG.

Key words:
antigens, nuclear; cell proliferation; dna-binding proteins; epidermal factor growth; gastritis; immunohistochemistry; rats.

Apstrakt

Uvod/Cilj. Ćelijska proliferacija i regulacija ekspresije proteina igraju važnu ulogu u gastritisu, ali u hroničnom superficijalnom gastritisu (HSG) su nedovoljno ispitane. Cilj rada bio je da se ispita povezanost između ekspresije nuklearnog faktora kapa B (NF-κB) i regulatornih proteina i HSG kod pacova. **Metode.** Kod pacova je HSG bio izazvan veštački, primenom hemijskih agenasa i neadekvatnom ishranom. U mukozi pacova sa HSG imunohistohemijski je određivana ekspresija receptora epidermalnog faktora rasta (EGFR) i nuklearnog antigena proliferišućih ćelija (PCNA) dok su hibridizacijom *in situ* određivani nivoi ekspresije mRNA NF-κB p65. **Rezultati.** Utvrđena je veća infiltracija inflamatornih ćelija i skor inflamacije u sluznici želuca pacova sa HSG, u poređenju sa kontrolnim pacovima. Nivoi ekspresije PCNA, EGFR i NF-κB p65 mRNA u ćelijama sluznice želuca pacova sa HSG bili su značajno povećani. Korelaciona analiza pokazala je da je skor inflamacije bio u pozitivnoj vezi sa nivoima ekspresije mRNA NF-κB p65 i EGFR, ali nije bilo značajne korelacije sa nivoom ekspresije PCNA. Dodatno, nađena je značajna pozitivna korelacija između mRNA NF-κB p65 i nivoa EGFR. **Zaključak.** Visoka ekspresija NF-κB i EGFR ima značajnu ulogu u pojavi i progresiji HSG i u značajnoj je, pozitivnoj korelaciji, sa stepenom inflamacije u sluznici želuca. Dakle, promene u ekspresiji NF-κB i EGFR mogu se koristiti kao važni indikatori za procenu HSG, tj. promene u nivoima njihove ekspresije korisne su za procenu stepena lezije sluznice želuca i progresije HSG.

Ključne reči:
antigeni, nuklearni; ćelija, proliferacija; proteini, dnk vezujući; faktor rasta, epidermalni; gastritis; imunohistohemija; pacovi.

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Introduction

Chronic gastritis is a common disease in human beings. It is estimated that several hundreds of millions of people worldwide may have chronic gastritis in one form or another [1]. Chronic gastritis is also a common disorder in China and is often underestimated in clinical practice and in real life. Chronic superficial gastritis (CSG) is a clinically common and frequently-occurring disease. If the condition is not resolved in the long-term, it may develop into chronic atrophic gastritis (CAG), with a risk of occurrence of malignant transformation of approximately 2.5–5% [2]. Cell proliferation and the regulation of protein expression play an extremely important role during this period. There have been many reports on the proliferation of gastric mucosal cells in CAG and its effect on the disease progression [3], but in CSG, the proliferation of gastric mucosal cells and the regulation of their protein expression are rarely reported. Therefore, it is important to determine the essence, development, and recovery of CSG and drug targets to study the relationship between the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and regulatory proteins related to the proliferation of gastric mucosal cells and chronic superficial gastritis in CSG rats.

Methods

Spraque-Dawley rats (180–220 g), half male and half female, are purchased from Slack Jingda Laboratory Animal Co. Ltd, Changsha, China, [Certificate No: SCXK (Xiang) 2009-0001]. The rats were fed under the condition of controlled temperature (21 ± 2 °C), relative humidity（about 60 ± 10%), 12-h light/dark cycle, and automatic ventilation 8–15 times every hour. All experiments were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals.

Anhydrous ethanol was purchased from Hunan Hui Hong Reagent Co. Ltd (Changsha, China); 28% ammonia was obtained from Hunan Chemical Industry Research Institute (batch No.20100317; Zhuzhou, China). Sodium deoxycholate was a product of Sigma company (St Louis, USA). Mouse polyclonal antibody against epidermal growth factor (EGF) receptor – EGFR, proliferating cell nuclear antigen (PCNA), and NF-κB/p65 mRNA, *in situ* hybridization Kit and SABC kit were purchased from Wuhan Boster company (Wuhan, China).

CSG rat model was established as follows: rats were administered 2 mL of 60% alcohol once every Tuesday and Friday on an empty stomach; 2 mL of 20 mmoL/L sodium deoxycholate orally once daily; 0.05% of ammonia during weeks 1–6 and 0.1% of ammonia during weeks 7–12 in drinking water; an irregular diet involving 2 days of sufficient feeding and 1 day of fasting was performed [4]. The entire experimental period lasted 12 weeks (there were two deaths due to incorrect administration during the modeling process).

The stomachs were rapidly removed from the abdominal cavity, washed with distilled water, fixed with 4% formaldehyde solution, embedded in paraffin, and cut into slices. The slices were stained with hematoxylin-eosin (HE), and morphology was observed with a microscope. To measure the degree of inflammation of the gastric mucosa, a scoring system ranging from a low score of zero to a high score of 5 was used.

After the experiment, the rats were sacrificed, and blood samples were withdrawn from the celiac artery. The blood samples were centrifuged at 3,000 rpm for 10 min to obtain serum samples. Serum levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured by enzyme-linked immunosorbent assay (ELISA) kits.

Analysis of EGFR and PCNA was performed following the operating instructions, and slices were subjected to immunohistochemistry and image analysis. Under an optical microscope at 400x magnification, 5 images were randomly selected, photos were taken of each section, and each picture was scanned on a computer with an HPIAS-1000 pathological image analysis system. The average optical density and the percentage of positive cells (the area of positive cells/total area of statistical field) were determined.

The tissue sections were treated with 3% H_2O_2 at room temperature for 10 min and washed twice in distilled water. Proteinase was added to the sections at 37 °C for 20 min. The sections were then washed three times with 0.5 mol/L phosphate-buffered salines (PBS), five mins each time. The slides were treated with 20 μL of pre-hybridization liquid at 37 °C for 4 hrs, the excess liquid was absorbed, and each slide was treated with 20 μL of NF-κB p65 oligonucleotide probe hybridization liquid and covered with a special slice overnight at 4 °C. Then, the slides were washed twice in 2% saline-sodium citrate (SSC) for five min each and once in 0.5% SSC and in 0.2% SSC for fifteen min each. Afterward, the slides were treated with digoxygenin for antibody visualization with the addition of pre-biotinylated anti-digoxigenin antibody SABC and biotinylated peroxidase successively. The slides were then incubated at 37 °C for twenty min and washed three times in 0.5 mol/L PBS for five min each. The slides were stained with 3,3'-diaminobenzidine (DAB) and hematoxylin and washed in water. Finally, the slides were covered with neutral gum, and after images were taken, the optical density value and rate of the positive area were detected by an HPIAS-1000 pathological image-text analysis system.

All data were expressed as the mean ± standard deviation (SD). Statistical analyses were conducted via ANOVA and Bivariate correlation using SPSS Proprietary Software Release 16.0. Data with p values < 0.05 were considered to be statistically significant.

Results

Under the microscope, in the control group, gastric mucosa epithelial cells were in neat rows, with a few inflammatory cells. In the CSG group, the gastric mucosal injury was obvious. There were visible piles of infiltrated inflammatory cells on the surface of the gastric mucosa. The mucosal inflammation score was significantly higher in the

CSG group than in the control group. The mucosal glands in the gastric antrum in the CSG group were arranged in a disorderly manner and irregular, but the glandular layer was obviously not thinner nor thicker (Figure 1).

After 12 weeks of treatment, the levels of TNF-α and IL-6 in the serum of the CSG group were significantly higher than those in the serum of the control rats (Figure 2).

Expression of PCNA was indicated by brown-yellow granules, which were mainly distributed in the nucleus and occasionally in the cytoplasm. In the control group, PCNA-positive cells were rare in the gastric antral mucosa. Compared with that in the control group, PCNA expression was significantly increased and unevenly distributed in the CSG group, and the optical density value and the number of PCNA-positive cells were significantly increased (Figure 3).

Expression of EGFR was indicated by brown-yellow granules, which were mainly distributed in the cytoplasm and cell membrane. Compared with that in the control group, EGFR expression was significantly increased in the CSG group, and the optical density value and the number of EGFR-positive cells were significantly increased ($p < 0.05$) (Figure 4).

Expression of NF-κB mRNA was mainly observed in the cytoplasm, as determined by *in situ* hybridization. In the control group, its expression was very low. Total expression of NF-κB mRNA was evidently increased and unevenly distributed in the gastric mucosal cells of CSG rats compared to those of control rats, and the optical density value and positive area of NF-κB mRNA were increased compared to those in control rats (Figure 5).

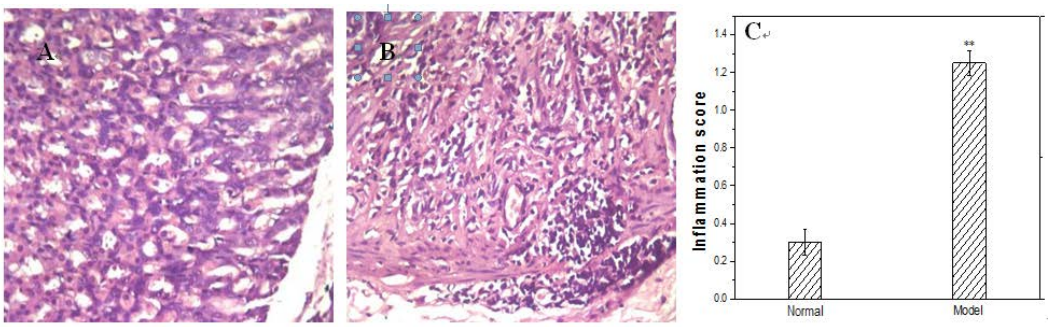

Fig. 1 – Morphologic changes in the gastric mucosa [hematoxylin-eosin staining, ×400]. A) Control group; B) Chronic superficial gastritis (CSG) model group; C) Mucosal inflammation score. [$p < 0.01$, CSG model group *vs* control (normal) group].**

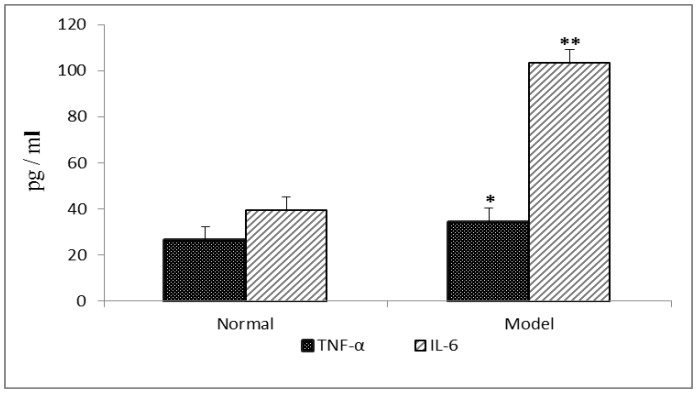

Fig. 2 – The levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the serum [*$p < 0.05$, **$p < 0.01$, chronic superficial gastritis (CSG) model group *vs* control (normal) group after 12 weeks of treatment].

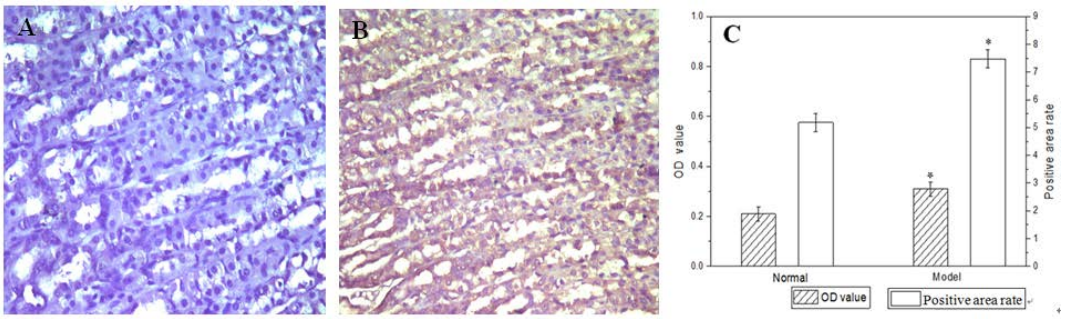

Fig. 3 – Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) expression in rats [hematoxylin-eosin ×400]: A) Control (normal) group; B) Chronic superficial gastritis (CSG) model group; C) Odds ratio (OD) value and positive area rate (*$p < 0.05$, CSG model group *vs* control group).

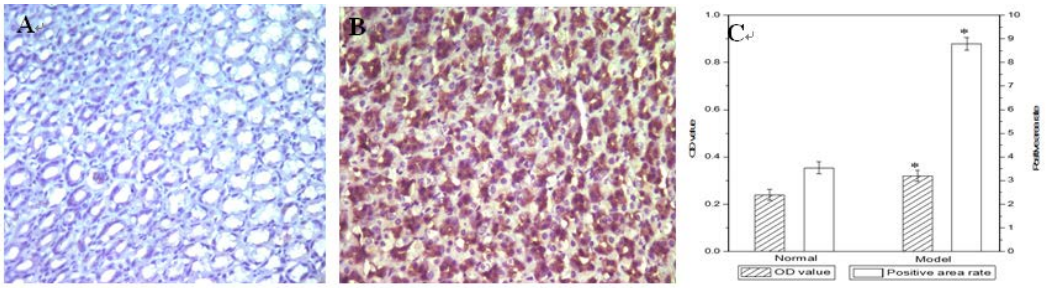

Fig. 4 – Immunohistochemical analysis of epidermal growth factor receptor (EGFR) expression in rats (×400). A) Control (normal) group; B) Chronic superficial gastritis (CSG) model group; C) Odds ratio (OD) value and positive area (*$p < 0.05$, CSG model group *vs* control group).

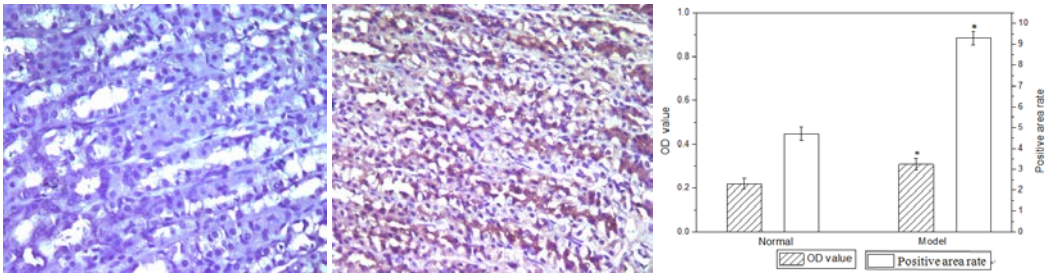

Fig. 5 – *In situ* hybridization analysis of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 mRNA expression (×400): A) Control (normal) group; B) Chronic superficial gastritis (CSG) model group; C) Odds ratio (OD) value and positive area.

Table 1

Correlation between gastric mucosal inflammation score and the expression levels of PCNA, EGFR, and NF-κB p65 in the chronic superficial gastritis group

Inflammation	Inflammation score	Optical density of EGFR	Positive area of EGFR	Optical density of PCNA	Positive area of PCNA	Optical density of NF-κB p65 mRNA	Positive area of NF-κB p65 mRNA
Score							
r	1	0.674	0.200	-0.393	-0.260	0.520	0.606
p		0.033*	0.317	0.167	0.267	0.076	0.042*
Optical density of EGFR							
r			-0.203	-0.334	-0.178	0.666	0.599
p			0.315	0.209	0.337	0.036*	0.058
Positive area of EGFR							
r				-0.619	-0.718	0.148	0.475
p				0.050	0.022*	0.364	0.117
Optical density of PCNA							
r					0.325	-0.325	-0.557
p					0.216	0.216	0.078
Positive area of PCNA							
r						-0.465	-0.578
p						0.123	0.067
Optical density of NF-κB p65 mRNA							
r							0.661
p							0.026*
Positive area of NF-κB p65 mRNA							
r							1
p							

***Confidence (one-sided) is 0.05, the correlation is significant.**

PCNA – proliferating cell nuclear antigen; EGFR – epidermal growth factor receptor; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells.

The statistical results, summarised in Table 1, revealed that in the CSG group, there was a significant correlation between inflammation score and NF-κB p65 mRNA and EGFR levels, but the correlation coefficient between PCNA and inflammation score was very small and presented no statistical significance. Conversely, NF-κB p65 mRNA expression was significantly correlated with EGFR expression, and there was a negative correlation between PCNA and EGFR expression (Table 1).

Discussion

Here, we explored the relationship between the expression of nuclear factor kappa B, EGFR, and PCNA in CSG rats. The pathological results showed that in the control group, the epithelial cells of gastric mucosa were orderly arranged, inflammatory cell infiltration was occasionally found on the surface of the mucosa, and the mucosal glands of the gastric antrum were orderly arranged. In the CSG group, the gastric antrum mucosa was obviously eroded, and there was a large number of infiltrating inflammatory cells, indicating that there was an obvious inflammatory reaction in the gastric mucosa. The inflammation and lesions were mainly located in the antropyloric region of the stomach, and the inflammation scores were higher in the model group than in the normal group and were closely related to inflammatory cell infiltration and damage. The arrangement of gastric antrum mucosal glands was disordered, but there was no obvious thinning or thickening, which was significantly different from CAG and the canceration stage of gastritis [5], indicating that the change in gastric mucosal cells was within the physiological range.

The statistical analysis revealed that in the normal group, the correlation coefficient was very small in the 5% level of significance, and there was no significant difference indicating that there was little change in the expression levels of PCNA, EGFR, and NF-κB p65 and there was no obvious correlation. However, in rats with chronic gastritis, the gastric mucosa was markedly inflamed, and there was a significant positive correlation between inflammation score and the expression of EGFR and NF-κB, with correlation coefficients of 0.674 and 0.606, respectively, and the difference was statistically significant. Meanwhile, there was also a significant positive correlation between the expression of EGFR and NF-κB, with a correlation coefficient of 0.666. The correlation between inflammation score and expression of PCNA was very low and negative, and there was no statistical significance. In other words, based on the changes in the contents of nuclear factors and cell proliferation factors related to CSG and morphological changes, during the development of chronic gastritis, the degree of inflammation of the gastric mucosa was positively correlated with the expression of EGFR and NF-κB p65, and there was also a significant positive correlation between NF-κB p65 and EGFR. The result showed that the expression of EGFR and NF-κB p65 played an important role in the occurrence and development of gastritis.

Inflammation is a crucial factor involved in the pathogenesis of gastric mucosal lesions in CSG. Gastric mucosal damage is accompanied by a substantial increase in the contents of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. The results showed that TNF-α and IL-6 expression levels in the CSG group were significantly higher than those in the control group. In the inflammatory environment, macrophages express a variety of cytokines, such as TNF-α, IL-1β, IL-6, and IL-1. The transgenic expression of inflammatory cytokines in the mouse stomach leads to gastritis and gastric tumor development [6–8], the development of gastritis is also related to cytokine gene polymorphisms [9]. IL-1β and IL-6 are pleiotropic inflammatory cytokines expressed during gastric inflammation, and their overexpression can induce gastric mucosal injury. Proinflammatory cytokines can induce the infiltration of neutrophils, trigger the production of additional inflammatory cytokines and result in an inflammatory response that aggravates gastric tissue damage [10, 11]. Moreover, inflammatory cytokines may trigger oxidative stress pathways and produce reactive oxygen species, which can lead to oxidative damage in gastric mucosal cells. However, the expression of proinflammatory factors is regulated by NF-κB. NF-κB is an important transcription factor expressed in the process of inflammation and immune response. There are functional combination points for NF-κB in many gene promoters and enhancers, which may regulate transcription and expression of many cell factors and inflammatory mediators related to inflammation [12] and mediate both acute and chronic inflammation [13]. After NF-κB is activated, it translocates into the nucleus, where it binds to specific sequences in promoter regions of target genes, further activates target genes, and causes the release of inflammatory factors (IL-1, IL-6, and TNF-α) and inflammatory response by regulating the transcription and expression of genes related to inflammation. Meanwhile, inflammatory factors can also cause further NF-κB activation and induce a cascade response. As a result, inflammation will continue and increase [14]. The experimental results revealed that in control rats, the positive expression of NF-κB p65 in the gastric epithelium was lower than that in CSG rats, and the optical density and the positive area of NF-κB p65 expression were significantly increased, which is consistent with a report by Cui et al. [15], who indicated that NF-κB plays an important role in CSG. Moreover, correlation analysis showed that CSG was closely and positively related to NF-κB p65 expression. Thus, the measurement of its changes could help assess stomach health and the degree of inflammation in the gastric mucosa.

The aberrant activation of NF-κB is invariably associated with inflammation. Activated NF-κB can cause gastritis via the induction of proinflammatory cytokines [16] and reactive oxygen species (ROS), which play an important role in DNA and cell membrane damage in gastric epithelial cells. The inflammation score is an important index used to evaluate the gastric mucosal inflammatory injury. This experiment showed that when the inflammation score was

significantly increased in model rats, high expression of NF-κB was also detected, which was consistent with the changes in the cytokine level and the inflammation score, indicating the degree of gastric mucosal damage. The odd ratio (OD) value of NF-κB was positively correlated with the inflammation score in rats, and there was a correlation between the activity of NF-kB and the inflammatory score, indicating that NF-κB expression correlated well with the severity of gastritis. These results are in agreement with findings by other researchers [17, 18]. The intensity of NF-κB staining correlates with the density of the inflammatory cell infiltrate comprised of neutrophilic and lymphocyte infiltrates but not eosinophilic infiltrates [19], indicating that there were plenty of neutrophilic infiltrates in CSG, which is consistent with the above pathological results. Research has shown that the activation of NF-κB is the primary factor responsible for the initial inflammatory response [18]. The role of NF-κB appears to be predominant in the early stages of the disease when it is responsible for the induction of neutrophilic infiltration. There is no role for NF-κB in the later stages of the disease [19]. Atrophy occurred in the later stages of the disease, and the grade of atrophy had either no or a negative correlation with NF-κB [20]. Atrophic gastritis gradually evolved from superficial gastritis, and superficial gastritis is the early stage of atrophic gastritis. Therefore, high expression of NF-κB also indicates that superficial gastritis at this time is the early stage of gastritis in the rat model.

Chronic inflammation of the gastric mucosa has a positive correlation with EGFR expression. EGFR is the receptor for EGF, and EGF is secreted by the submandibular gland, salivary gland, pancreas, and duodenum [21]. It can restrain the secretion of gastric acid, protect the gastric mucosa, and promote gastric epithelial repair and regeneration [22, 23]. These functions of EGF are achieved when EGF binds to its receptor, and changes in EGFR expression directly affect the function of EGF. The expression of EGFR was rare in the intact gastric mucosa; however, when the gastric mucosal barrier was damaged, its level was elevated. The study showed that there was a low expression of EGFR in the gastric epithelium of control rats and that the expression was higher in model rats, which may be a protective response to inflammatory stimulation in the process of modeling. EGFR expression was high in the mucosal surface layer but low in the deep and muscle layers of the stomach [24], consistent with the pathological location of superficial gastritis. Therefore, the increase in EGFR expression can also imply that there may be inflammation in the gastric mucosa. In addition, the increase in EGFR may also result from overexpression of NF-κB in CSG. Correlation analysis showed that there was a positive correlation between EGFR and NF-κB, with a correlation coefficient of 0.666. NF-κB is a transcription factor that can initiate and regulate the gene transcription and expression of many factors (including EGFR). The appropriate expression of EGFR is beneficial for the repair and regeneration of gastric mucosa [25], but in the case of long-term stimulation, because of repeated inflammatory stimulation, uncontrolled cell proliferation and carcinogenesis will occur [26, 27]. Therefore, it is helpful to assess the degree of mucosal lesions and the progression of CSG to detect changes in EGFR expression, just as PGI and PGII, gastrin-17, and *Helicobacter pylori* antibodies may be used as stomach-specific biomarkers for the noninvasive assessment, diagnosis, and screening of atrophic gastritis [28].

However, increased expression of EGFR has dual effects on gastric mucosal cells; while moderate expression of EGFR can promote mucosal epithelial cell proliferation and is beneficial for mucosal repair, overexpression may be related to canceration [29–31]. The inflammatory microenvironment of the gastric mucosa induces the activation of EGFR signaling. EGFR activation plays a critical role in gastric disease risk. EGFR can combine with EGF and affect the pathway of oncogene expression, disrupting the normal self-regulation of the cell cycle and resulting in the development of gastric cancer. There was high expression of the EGFR protein in the cancer stage of atrophic gastritis in rats, which indicated high trends of carcinogenesis in CAG [32–34]. The expression of EGFR ligands was significantly upregulated in both K19-C2mE mouse gastritis tissues and Gan mouse gastric tumors. These EGFR ligands are induced via a COX-2/PGE2-associated inflammation-dependent mechanism, which leads to the acceleration of tumor cell proliferation. Treatment with an EGFR inhibitor significantly suppressed gastric tumourigenesis [35], and EGFR mutations can modify the responsiveness to EGFR-inhibiting drugs and are associated with acquired resistance to inhibitors [36]. Therefore, the abnormal expression of EGFR is a molecular marker of the malignant growth trend of gastric epithelial cells, which is closely related to the occurrence of carcinogenesis [37].

It has also been shown that the relationship between inflammation of gastric mucosal and expression of PCNA is very small, with no significant difference. PCNA was discovered by Miyachi et al. [38] in the serum of patients with systemic lupus erythematosus in 1978 and was named so because of its presence in proliferative cells (including normal proliferating cells and cancer cells). The expression of PCNA has close relationships with the synthesis of DNA in cells [39], playing an important role in the start of cell proliferation, and is highly involved in cell cycle regulation, replication, repair, and apoptosis [40]. The expression of PCNA occurs mainly in the S and early G2 phases of the cell proliferation cycle, closely reflecting dynamic changes in cell proliferation, and an increase in PCNA expression suggests that cell proliferation is active. Our study found that expression of PCNA was lower in the control group and greater and uneven in the CSG group, as the optical density value and the positive area of PCNA expression increased significantly, indicating that mucosal repair was accelerated. However, the mechanism is not clear and requires further study. Increased expression of PCNA can accelerate mucosal repair and alleviate mucosal inflammation. However, if the stimulating factors exist for a long time, repeated stimulation of the inflammatory microenvironment will lead to its overexpression and then to unregulated mucosal hyperplasia

and the occurrence of gastric carcinoma. There have been many reports [41] regarding the high expression of PCNA in gastric cancer and precancerous lesions.

Conclusion

Our findings show that in the gastric epithelium, the high expression levels of NF-κB and EGFR play important roles in the occurrence and progression of CSG and that there is a strong correlation between NF-kB activation and inflammation score, which indicates the degree of inflammation in the gastric mucosa, suggesting that NF-kB activation is important for neutrophil infiltration and inflammatory factor production. Therefore, changes in NF-κ B and EGFR may be used as important indicators for the assessment of CSG, and the changes in their expression help assess the degree of gastric mucosal lesions and the progression of CSG.

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