Original Article

Immunohistochemical Evaluation of P53 and Ki67 in Biopsy Samples of Gastritis and Gastric Cancer Patients

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Abstract

Background: Chronic gastritis (CG) is an inflammatory process that can lead to gastric cancer and *Helicobacter Pylori (H. Pylori)*. In this study, immunohistochemical evaluation of P53 and Ki67 in biopsy samples of CG and gastric carcinoma patients with and without *H. Pylori* infection was investigated. **Methods:** From 82 archived paraffin blocks, 42 blocks were selected for CG group and 40 as the gastric cancer group. All CG and gastric cancer cases were subdivided into *H. Pylori* positive and negative subgroups. Monoclonal antibodies specific for Ki67 and P53 were used for immunohistochemical staining. **Results:** The results showed that differences of Ki67 and P53 expressions were statistically significant among patients with CG gastritis and gastric cancer ($P < 0.05$). However, there were not significant differences in Ki67 and P53 expression between *H. Pylori*-positive and *H. Pylori*-negative subgroups of gastritis and gastric cancer ($P > 0.05$). **Conclusions:** The present study proposed that P53 and Ki67 expressions changed in gastric cancer compared to the CG specimens. It seems that overexpression of these biomarkers probably has important roles in the route of carcinogenesis. Our results suggested that these overexpressions were not associated with *H. Pylori* infection. Further studies with larger sample size are needed in this field.

Keywords: *Gastric cancer, gastritis, immunohistochemistry, Ki-67, P53*

Introduction

Chronic gastritis (CG) is an inflammatory disease of the gastric mucosa. It represents a variation of the etiology and host response caused by inflammation.[1] CG in terms of epidemiology and biology has been associated with the development of gastric cancer.[2,3] In developed and developing countries, respectively, cancer is the first and second leading cause of death.[4] Gastric cancer is the fifth most common cancer worldwide. Gastric cancer has an extremely high mortality, and its prognosis is generally poor.[5] It is the third cause of death after the lung and liver cancers.[6]

Gastric cancer was classified as a multifactorial disease that is caused by parallel environmental and genetic factors. Among environmental risk factors for gastric cancer, the critical role of *Helicobacter Pylori* (*H. Pylori*) infection, lifestyle, and nutrition have been noted in some reports. Other important cancer-associated agents are genetic factors that are comprised from gene mutations.[7,8]

Infection of the stomach with *H. Pylori* is the most common cause (almost 90%) of CG worldwide.[9] *H. Pylori* infection is considered as a critical risk factor during the process of gastric carcinogenesis. *H. Pylori*-caused CG is considered as one of the most important precancerous lesions in the stomach.[10-12] Previous studies have also indicated that there was a direct link between *H. Pylori* infection and risk of gastric cancer.[13]

P53, that also is called tumor protein, regulates the cell cycle and it can act as a tumor suppressor.[14-16] The amount of P53 in normal cells is low and steadily is produced and destroyed. P53 binds to genes that regulate DNA repair. P53 controls the process of programmed cell death.[17] In cancer cells, P53 may also be changed and disabled. Previous studies showed that in about 50% of all cancers, *P53* had been changed.[18-20] Geneticists believe that if the P53 was expressed in cancer cells, the growth of these cells could be stopped, and

How to cite this article: Panahi M, Heidari Z, Mahmoudzadeh-Sagheb H, Jahantigh M, Sheibak N. Immunohistochemical evaluation of P53 and Ki67 in biopsy samples of gastritis and gastric cancer patients. Clin Cancer Investig J 2019;8:21-7.

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Access this article online

Website: www.ccij-online.org

DOI: 10.4103/ccij.ccij_109_18

Quick Response Code:

this situation could provide the opportunity for the immune system to destroy cancer cells.[21-24]

Ki67 as a biomarker of cell proliferation could be expressed and detected in many cancers by immunohistochemistry (IHC).[25] It is believed that the number of cells reacts with Ki67 represents the cell population number or the growth fraction of a cell cycle. It seems that Ki67-positive cells are signs of cellular proliferative activities. Reports are showing that staining with monoclonal Ki67 antibody is the best method to measure cell proliferation in routine experiments.[26-28]

There is some evidence that the expressions of P53 and Ki67 alter in the lesions of gastric cancer. According to previous studies, disorders in proliferation and apoptosis of gastric cells could occur in association with *H. Pylori* infection. However, there are controversial findings in this field.[13,29]

On the other hand, the findings have shown that the expressions of P53 and Ki67 were significantly associated with each other. However, these markers alone had no diagnostic value.[30,31]

Given all the above-mentioned, we proposed that the presence of *H. Pylori* infection in parallel with changes in P53 and Ki67 expression in gastric tissue might lead to progression of gastritis to cancer. The purpose of this study was an immunohistochemical evaluation of P53 and Ki67 in biopsy samples of CG and gastric carcinoma with *H. Pylori* and without *H. Pylori* infection.

Methods

This study approved by the Ethics Committee of Zahedan University of Medical Sciences (ZAUMS) (IR. ZAUMS. REC. 1393.7181). All samples were selected from the archive files of the Clinical Pathology and Cytology Department, Ali-ebne Abitaleb central and referral Hospital of ZAUMS, Zahedan, Iran. All Patients had signed a written consent for using their tissue specimens for research purposes.

Biopsy specimens

From all CG and gastric cancer, endoscopic biopsies blocks that had been collected from July 2010 to December 2015, 82 suitable cases were selected for this study. The inclusion criteria for the selection of archival histological specimens were suitable formalin-fixed, paraffin-embedded tissues with a complete clinicopathological data. Tissue samples from patients with immune disorders, recent recipient of steroids and anti-*H. Pylori* drugs and all specimens with autolysis, inadequate biopsy samples, and accompanied by other gastrointestinal tract malignancies were excluded from the study. Finally, forty-two blocks were selected as indefinite for CG group and forty blocks as gastric cancers. All gastric cancer samples were considered in a single group. Due to limited sample size, tumor stage and its subtypes were not inspected in this phase of our study. Further studies with greater sample sizes regarding cancer stages and subtypes could be done in future. Furthermore, all CG and gastric cancer cases were subdivided into *H. Pylori* positive and negative subgroups that were extracted from their information records. Subgrouping was confirmed with Giemsa staining.

Immunohistochemistry

All IHC procedures were performed using the staining protocol of Novolink polymer detection kit (RE7140-K, United Kingdom). All steps were done at room temperature (25°C) in a humidified chamber. Three microscopic tissue slides were obtained from each block by cutting 3-μm sections with a microtome (Leica RM2255 Fully Automated Rotary Microtome, Germany) and were mounted on slides that were coated with a suitable tissue adhesive (Histogrip CL00-8050, Cedarlane-Canada). One slide was used for P53, the second for Ki67, and the third was served as a negative control. Sections were allowed to air dry at room temperature overnight. Then, the slides were deparaffinized and rehydrated and finally were washed in distilled water. Before staining, the slides underwent a high-temperature, antigen-retrieval technique. The retrieval technique using 0.01 M sodium citrate buffer (pH 6.0) was placed in a laboratory autoclave (1 cycle, 120˚C for 10 min). To neutralize endogenous peroxidase activity, sections were blocked with a peroxidase-blocking reagent (Novacastra peroxidase-United Kingdom) for 10 min and were washed in a bath of Tris-buffer (0.05 M Tris-HCl, pH 7.0–7.6). Then, the slides were incubated with Protein-Block (Novacastra-United Kingdom) for 5 min at room temperature, followed by overnight incubation with the anti-P53 (the Novocastra DO-7 clone recognizes both wild-type and mutant proteins) and anti-Ki67 primary antibodies (mouse monoclonal, Novocastra-United Kingdom) at 4°C. Sections were incubated with Novolink postprimary for 30 min and then with Novolink polymer for 30 min at room temperature. Peroxidase activity was developed with a 3,3′-Diaminobenzidine (DAB) working solution (100 μl of DAB Chromogen added to 1 ml of Novolink DAB substrate buffer) for 30 min.

Sections were counterstained with Mayer's hematoxylin for 5 min and were rinsed with fresh distilled water. Sections were dehydrated and mounted with Entellan (Merck, Germany). Positive controls (colon carcinoma) and negative controls (omission of primary antibody) were run for each batch of slides.

Evaluation of the P53 and Ki67 immunohistochemistry -stained sections

The slides were evaluated independently by two expert observers who were "blinded" to the histological status of the slides. Samples were rated according to a score,

which was calculated by adding the intensity of the stain to the area of the stain. The staining intensity was arbitrarily graded on a scale of four grades: 0, no staining of cancer cells or negative; 1 for weak or mild (yellow or light-brown nuclear stain); 2, moderate staining (dark-yellow, medium-brown stain or a mixture of light and dark stain); and 3, strong staining (dark-brown stain in most of the nuclei).

The area of staining was evaluated using the following scale: 0, 0%–5% >staining of the tumor cells in any field; 1, 5%–25% of tumor cells stain positive; 2, 26%–50% stain positive; 3, 51%–75% stain positive; 4, >75% stain positive. Theoretically, the overall scores could range from 0 to 12. The specimens with a score of >4 were regarded as positive expression and those with a score ≤4 as a negative expression.

Statistical analyses

The nonparametric Mann–Whitney U-test and Kruskal–Wallis test were used to compare the expression scores. $P < 0.05$ was considered as statistically significant. All statistical computations were performed using SPSS for Windows (version 21, Chicago, IL, USA).

Results

Clinical characteristics of patients were presented in Table 1. Age and sex were matched between groups.

Helicobacter Pylori

H. Pylori status was positive in 34 (41.50%) and negative in 48 (58.50%) of total cases. In addition, tissue sections were *H. Pylori* positive in 19 (44.20%) of 43 CG and *H. Pylori* negative in 24 (55.80%) cases. In gastric cancer, 15 (38.50%) of 39 samples were *H. Pylori* positive and 24 (61.50%) samples were *H. Pylori* negative.

Immunohistochemistry

Immunohistochemical staining was done in 82 of the samples. None of the negative controls showed nuclear staining for either of the two markers.

P53 expression

P53 immunohistochemical staining was positive in 41.50% (34 of 82) and negative in 58.50% (48 of 82) of patients. P53 expression difference between patients with gastritis and gastric cancer was statistically significant ($P < 0.05$). P53 expression between *H. Pylori*-positive and *H. Pylori*-negative samples of gastritis and gastric cancer was not statistically significant ($P > 0.05$). In addition, P53 expression among the four subgroups (*H. Pylori* + CG, *H. Pylori* − CG, *H. Pylori* + cancer and *H. Pylori* − cancer) was not statistically significant ($P > 0.05$) [Table 2 and Figure 1]. Then, P53 expression was compared between all *H. Pylori*-positive (34 cases) and all *H. Pylori*-negative (48 cases) patients (regardless of the type of disease); the difference was not statistically significant ($P > 0.05$) [Table 2].

Table 1: Demographic features of patients with chronic gastritis and gastric cancer

Groups	Clinical parameters		
	Sex, *n* (%)		Age
	Female	Male	(mean±SEM)
Chronic gastritis	27 (62.80)	16 (37.20)	43.79±2.22
Gastric cancer	21 (53.80)	18 (46.20)	65.12±2.41
Total	48 (58.50)	34 (41.50)	53.93±2.01

SEM: Standard error of mean

Table 2: Immunohistochemical expression of P53 in patients with gastritis and gastric cancer

	n (%)	P53		*P*	
		Positive	Negative		
Gastritis	43 (52.40)	13 (30.20)	30 (69.80)		
H. pylori positive	19 (44.20)	7 (36.80)	12 (63.20)		
H. pylori negative	24 (55.80)	6 (25.00)	18 (75.00)	0.407[a,d]	
Gastric cancer	39 (47.60)	18 (46.20)	21 (53.80)		0.031[a,c]
H. pylori positive	15 (38.50)	8 (53.30)	7 (46.70)		
H. pylori negative	24 (61.50)	13 (54.20)	11 (45.80)	0.966[a,d]	
Total	82 (100.00)	34 (41.50)	48 (58.50)	0.154[b,e]	

Data were presented as number (percentage). a: Mann-Whitney U test, b: Kruskal-Wallis test, c: significant difference between two groups, d: *H. pylori*-positive subgroup compared to *H. pylori* negative, e:between all subgroups

Ki67 expression

Immunohistochemical expression of Ki67 was positive in 61% (50 of 82) and negative in 39% (48 of 82) of all patients. Ki67 expression had significant differences between patients with gastritis and gastric cancer ($P < 0.05$). The expression of Ki67 in *H. Pylori* positive and negative of gastritis and gastric cancer samples was not significant ($P > 0.05$). In addition, Ki67 expression among the four subgroups was not statistically significant ($P > 0.05$) [Table 3 and Figure 2]. Then, the differences of Ki67 expressions in all *H. Pylori*-positive samples were compared with all *H. Pylori*-negative samples (regardless of the type of disease). There were no statistically significant differences between the two groups ($P > 0.05$) [Table 3].

Results of Fisher's exact test confirmed these findings and showed that P53/Ki67 expression was not dependent to *H. Pylori* infection ($P > 0.05$).

We subdivided gastritis samples based on disease severity into mild, moderate, and severe subgroups. Results showed that differences in expression of P53 among gastritis samples with different severity were statistically

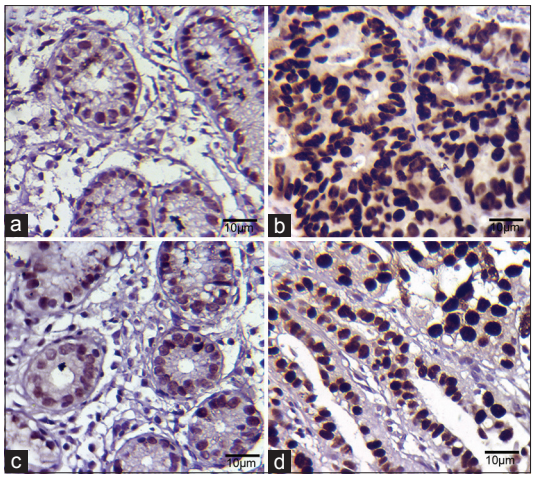

Figure 1: Immunohistochemical staining of P53 in *Helicobacter Pylori*-positive gastritis (a) and *Helicobacter Pylori*-negative gastritis (c) in comparison of *Helicobacter Pylori*-positive gastric cancer (b) and *Helicobacter Pylori*-negative gastric cancer (d), scale bar = 10 μm

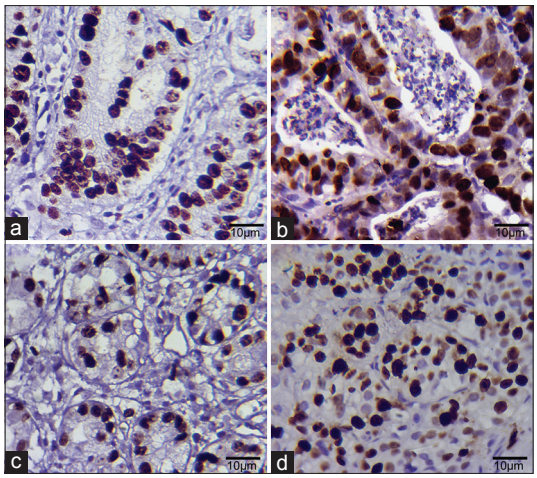

Figure 2: Immunohistochemical staining of Ki67 in *Helicobacter Pylori*-positive gastritis (a) and *Helicobacter Pylori*-negative gastritis (c) in comparison of *Helicobacter Pylori*-positive gastric cancer (b) and *Helicobacter Pylori*-negative gastric cancer (d), scale bar = 10 μm

Table 3: Immunohistochemical expression of Ki67 in patients with gastritis and gastric cancer

	n (%)	Ki67		*P*
		Positive	Negative	
Gastritis	43 (52.40)	21 (48.80)	22 (51.20)	
H. pylori positive	19 (44.20)	8 (42.10)	11 (57.90)	
H. pylori negative	24 (55.80)	13 (54.20)	11 (45.80)	0.437[a,d]
Gastric cancer	39 (47.60)	29 (74.40)	10 (25.60)	0.019[a,c]
H. pylori positive	15 (38.50)	12 (80.00)	3 (20.00)	
H. pylori negative	24 (61.50)	17 (70.80)	7 (29.20)	0.638[a,d]
Total	82 (100.00)	50 (61.00)	32 (39.00)	0.090[b,c]

Data were presented as number (percentage). a: Mann-Whitney U test, b: Kruskal-Wallis test, c: significant difference between two groups, d: *H. pylori*-positive subgroup compared to *H. pylori* negative, e:between all subgroups

significant ($P < 0.05$). Nevertheless, this was not significant about Ki67 expression ($P > 0.05$) [Table 4].

Discussion

H. Pylori infection is impressed about 50% of the world population and could be seen in all age groups. It is able to produce large amounts of urease enzyme. The bacteria can be found in the deep layer of mucus, close to the epithelial mucosa where there is a physiological PH for its surviving. Urease enzyme makes an alkaline environment within the acidic environment of the stomach[32,33] and has a very important role in stomach carcinogens;[34] so, *H. Pylori* is the first line of the gastric carcinogen list. The colonization of *H. Pylori* in the gastric mucosa, resulting in inflammation and immune responses that leading to the destruction of the mucus layer and histological changes such as atrophy, metaplasia, and dysplasia.[10,35-37] Correa *et al*. noted that changes in gastric mucosa lesions caused by *H. Pylori* infection in CG can eventually lead to intestinal types of gastric cancer.[38] Based on Correa's hypothesis, host immune responses to *H. Pylori* infection can cause superficial gastritis. Then, if the inflammation persists, it probably causes atrophic gastritis, metaplasia, and in severe cases lead to dysplasia in the gastric mucosa. These changes have a key role in the development of gastric cancer, and if there are other environmental factors such as high salt intake, smoked food, and low intake of fresh fruits and vegetables, the chance of gastric cancer goes up.[39,40]

Correa has reported that the *H. Pylori* infection is a necessary, but not a sufficient cause for gastric cancer. *H. Pylori* presence is a major etiological cause of gastric cancer. This bacterium triggers a mechanism that probably causes progression of CG to atrophy, intestinal metaplasia, and cancer.[41] Watari *et al*. compared the effects of the presence or absence of infection with *H. Pylori* in patients with gastritis and gastric cancer. Their results showed that eradication of *H. Pylori* caused cells tried to prevent, fixed malignant process, and protected themselves.[42] Some reports demonstrated that P53 had a possible involvement in *H. Pylori*-related gastric cancer.[29] Our findings showed that there was no significant difference in P53 expression between CG samples with *H. Pylori* infection and *H. Pylori* negative. However, the highest percentage of P53 expression was observed in CG patients with *H. Pylori* positive status. One reason is that the effects of *H. Pylori* on P53 expression might be modulated by inflammatory cells and cytokines present in the gastric mucosa colonized by *H. Pylori*.[43-45]

Our results also showed that there was no significant difference in P53 expression between *H. Pylori*-positive and *H. Pylori*-negative individuals with gastric cancer. Although expression of P53 in *H. Pylori* negative gastric cancers was more than other subgroups. Surprisingly,

Table 4: Immunohistochemical expression of Ki67 and P53 in patients with different severity of gastritis

Gastritis	*n* (%)	Ki67		*P*	P53		*P*
		Positive	Negative		Positive	Negative	
Mild	14 (32.60)	8 (57.10)	6 (42.90)	0.627[b]	5 (35.70)	9 (64.30)	0.020[b,c]
Moderate	13 (30.20)	5 (38.50)	8 (61.50)		7 (53.80)	6 (46.20)	
Severe	16 (37.20)	8 (38.10)	8 (36.40)		1 (6.20)	15 (93.80)	

Data were presented as number (percentage). b: Kruskal-Wallis test between Gastritis three subgroups, c: Significant difference between groups

the highest frequency of P53 expression was related to *H. Pylori*-negative patients with gastric cancer.

There are some reports concerning the mutation in the P53 gene in preneoplastic lesions of the stomach caused by *H. Pylori* infection.[13] Therefore, alerted expression of P53 could be a useful molecular marker in early stages of gastric carcinogenesis in the presence of *H. Pylori*.[29,46] It is shown that modified gastric mucosa in gastric cancer cases might become inhospitable for the continued infection with *H. Pylori* and its colonization. Thus, most of *H. Pylori*-negative cancer cases might have been positive in earlier stages of carcinogenesis process. We discussed it in our previous study.[10] In addition, we compared the expression of P53 between all *H. Pylori*-positive subjects and *H. Pylori*-negative subgroups and found that the difference was not statistically significant between these two subgroups. These results suggested that P53 expression probably is not dependent on the presence of *H. Pylori* infection.

Several independent studies are done on the key proteins that are associated with cell cycle. These studies have demonstrated that how the expression of these proteins can be associated with prognosis and responses to treatment in patients with gastritis. In the present study, higher expression of P53 was observed in gastric cancer compared to CG. We found that there was a significant difference in immunohistochemical expression of P53 between CG and gastric cancer samples. This result suggests that P53 may be one of the essential factors for trigger carcinogenesis in gastric lesions. Seo *et al*. showed that there is an association between P53 expression, tumor invasion, and progress to the later stages of the disease and metastasis of cancer cells. Tumors with higher P53 expression had a worse prognosis compared to other groups.[47] Two other studies about P53 expression in CG samples indicated that P53 mutation could be an early event in the pathogenesis of gastric cancer.[48,49] In other words, these results are proposed that the expression of P53 could be the sign of the onset of a malignancy in the stomach. Li *et al*. have shown the lack of P53 expression in the normal samples and dyspeptic patients. P53-positive immunohistochemical staining was detected in intestinal metaplasia and gastric carcinoma samples. In addition, they reported that there was a higher presence of *H. Pylori* in gastric carcinomas compared to gastritis.[50]

The expression of Ki67 between *H. Pylori*-positive and *H. Pylori*-negative CG samples was not significant. However, positive expression of Ki67 in *H. Pylori*-negative CG sample was more common. We found that Ki67-positive expression had a higher percentage in the gastric cancer samples with positive status of *H. Pylori* infection. However, this difference was not statistically significant. On the other hand, we subdivided total samples into *H. Pylori*-positive and *H. Pylori*-negative subgroups and compared Ki67 expression between these two subgroups. We did not find a significant difference in expression status between them. Therefore, *H. Pylori* infection probably has no significant role in the expression of Ki67. Therefore, gastric cell hyperproliferation might occur independently of *H. Pylori* infection and is not influenced by it. However, the frequency of positive Ki67 expression in gastric cancer samples with positive *H. Pylori* status was more than other groups.

Hayama *et al*. have indicated that *H. Pylori* cells were not able for adhering to epithelial cells with proliferative status. On the other hand, the rate of cell proliferation in the presence of *H. Pylori* was elevated without bacterial attachment to the epithelial cells. It could be a reason for the absence of *H. Pylori* in some gastric biopsies with higher levels of Ki67 expression.[51] Furthermore, other factors could modulate the effect of *H. Pylori* in Ki67 expression. Zhang *et al*. have found that there was increased expression of CDX2 (a proliferative factor in tumor cells) in higher levels of *H. Pylori* infection. They also have used the immunohistochemical expression of Ki67 to show that their findings could confirm proliferating activity of cells.[52]

According to our results, the difference of Ki67 expression between gastritis and gastric cancer samples was significant. Positive expression of Ki67 in gastric cancer samples was more than CG samples that were similar to Li's studies.[50] Since the carcinogenesis invariably starts with the cellular hyperproliferation,[53,54] the increased expression of Ki67 in gastric cancer samples probably was due to higher proliferation of tumor cells.

Our results showed that P53 expression among mild, moderate, and severe gastritis cases was statistically significant. The highest expression frequency of P53 was related to moderate gastritis. We found no significant difference in Ki67 expression among mild, moderate, and severe gastritis cases.

It is demonstrated that expression levels of P53 and Ki67 could be influenced by numerous factors, including

the presence of different bacterial strains and different virulence genes such as CagA and VacA. The variations in pro- and anti-inflammatory cytokine genes may influence individual responses to carcinogenic exposures, and grouping of gastric carcinoma into intestinal type and diffuse type. These factors can affect the levels of P53 and Ki67 expressions too.

Geography and ethnic origin of individuals could be another possible reason for different expressions of these factors among various populations and inconsistent results among different studies. Since the earlier reports have shown that there is an ethnic variation in the allele frequencies of P53 gene polymorphisms among different populations,[55] it is expected that the expression of this gene varies among different ethnicities. Investigations in other geographical regions with different ethnicity and larger sample sizes could lead to more findings about the role of P53, Ki67, and *H. Pylori* in the progression of gastritis to cancer.

On the other hand, environmental factors that are responsible for significant different prevalence of gastric cancer in various populations such as differences in the lifestyle, especially nutritional could be able to alter the resistance of individuals to carcinogen agents like *H. Pylori* infection.[56,57]

Our findings suggest that in patients with gastric cancer, *H. Pylori* may not exclusively play the main role in the gastric carcinogenesis. According to the results, we found that the process of carcinogenesis begins from the early stages of CG. In addition, other environmental factors such as individual diet, smoking, and socioeconomic status could be effective as well as *H. Pylori* infection in this process. Since the etiology of gastric cancer is complex and multifactorial,[58] there is a need for more research in this regard. The main limitations of the current study were a small sample size in subgroups. Further studies with larger sample size are needed to investigate the effects of *H. Pylori* along with other environmental factors, host-related elements as well as genetic and epigenetic alterations in the rate of gastric carcinogenesis.

Conclusions

The present study proposed that P53 and Ki67 expressions changed in gastric cancer compared to the CG specimens. It seems that overexpression of these biomarkers probably have important roles in the carcinogenesis progression. In addition, our results suggested that these overexpressions were not associated with *H. Pylori* infection. Further studies with larger sample sizes are needed in this field for precise clarifying these findings.

Acknowledgments

The authors acknowledge Zahedan University of Medical Sciences that funded this project (Grant no. 7181) and B. Moudi and M. Narouei for their helps.

Financial support and sponsorship

This project was funded by Zahedan University of Medical Sciences (Grant no. 7181).

Conflicts of interest

There are no conflicts of interest.

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