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ORIGINAL ARTICLE

Quantitative changes of gastric mucosa during carcinogenesis using stereological methods

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Summary

Purpose: *To investigate the effects of helicobacter pylori (H. pylori) infection on the quantitative changes of gastric mucosa in cancerous and precancerous lesions using stereological methods.*

Methods: *One hundred and twenty-two gastric samples were studied. After determination of H. pylori infection, 42 gastric tissues of intestinal metaplasia (IM) patients, 38 specimens from dysplasia (DYS), and 42 specimens from gastric cancer (GC) types were selected from the pathology archive of Imam Ali Hospital, Zahedan, Iran. Gastric tissues were sectioned and stained with p53 and Ki-67 immunohistochemical (IHC) method and haematoxylin/eosin (H&E). Then, the numerical density (NV) of p53-positive, Ki-67-positive cells, eosinophil and polymorphonuclear (PMN) cells were estimated using dissector counting technique in IM, DYS and GC lesions.*

Results: *In IM, DYS and GC specimens, the NV of p53-positive cells in the presence of H. pylori infection (H. pylori+) was significantly higher than in H. pylori absence (H. pylori-). The NV of Ki-67- positive cells only in DYS specimens, showed significant difference between H. pylori+ and H. pylori- groups. The NV of eosinophil cells in DYS and GC specimens in H. pylori+ groups were significantly higher than in H. pylori- groups and the NV of polymorphonuclear cells in IM specimens showed significant difference between H. pylori+ and H. pylori- groups.*

Conclusion: *The results showed that H. pylori infection could cause significant quantitative changes in the cellular structure of gastric mucosa that might be influential on gastric carcinogenesis.*

Key words: *eosinophils, helicobacter pylori, gastric carcinogenesis, numerical density, polymorphonuclear cells, stereology*

Introduction

Gastric cancer (GC) is one of the most common and most malignant tumors [1]. Although the prevalence and mortality from GC decreased over the past 15 years GC is still known as the fourth most common cancer and the second leading cause of cancer-related deaths worldwide [2-5], with approximately 700 000 people dying of this disease each year [6,7]. About 70% of cancer deaths occur in developing countries [8]. GC is the most common malignancy in Iran. In South-East Iran GC is the second intestinal-gastrointestinal malignancy [9]. Gastric carcinogenesis seems to be a multistep process including gastric inflammation, gastric mucosal atrophy, IM, DYS and eventually cancer [10]. The prevalence of GC is influenced by genetic, environmental and infectious factors [9,11]. It has been shown that if genetic factors such as tumor suppressor gene p53 and nuclear proliferation antigen Ki-67 are disturbed or changed, along with environmental and infectious factors, could cause an increased risk for inducing GC. Tumor suppressor gene p53 plays an important role in cell cycle arrest and apoptosis in response to DNA damage. Mutation of this gene is common in many types of

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human cancers. Inactivation of p53 gene by mutation can allow a cell with damaged DNA to escape from normal into an uncontrolled growth. This uncontrolled growth leads to tumor formation and cancer development [12-15]. Ki-67 nuclear protein is associated with cell proliferation that can cause acceleration and progression of tumor growth. Ki-67 nuclear antigen is expressed in growth phases (G1, S, G2 and M), but not in resting phase (G0) of the cell cycle. Differential expression of Ki-67 in different phases of the cell cycle provides information about the active cells and growth rate in the cell cycle. Ki-67- positive cells can be used as a marker of proliferative activity [1,16].

Normal gastric mucosa may contain only a few inflammatory cells. Given that H. pylori can be the most common cause of gastritis, and is considered as a risk factor for gastric carcinogenesis. So H. pylori could cause increased number of inflammatory cells in the inflamed gastric mucosa. It is believed that H. pylori can induce proliferation of gastric epithelial cells. Accumulation of ammonia production on gastric mucous layer by H. pylori can lead to toxic effects and development of gastritis, peptic ulcer and eventually GC [17-19]. Cell counting is a powerful tool for the assessment of the number of cells and other constituents in tissue sections [20]. Zhang et al. in their study compared PMN cells infiltration in H. pylori groups. Their results showed that PMN infiltration in H. pylori+ group was higher than in H. pylori- group and the intensity of inflammation was related with the grade of the PMN cells infiltration and the grade of H. pylori infection [21].

Stereology is a technique for accurate estimates of the number of cells in biological structures. Stereology makes it possible to elicit three-dimensional (3D) data from two-dimensional (2D) images [22-27]. Dissector technique allows estimating the density of particles by counting small microscopic particles with unbiased manner in a virtual 3D volume, by two physical or optical consecutive sections separated by a distance "t". By using the dissector method we can obtain estimation of any numerical quantity without the factors arising from different causes including particle size, orientation of particles and section thickness [20,24]. As an advantage, the dissector method provides the possibility of generalizing data to the entire structure. Given that there is no precise data concerning structural changes of the stomach during carcinogenesis, and due to the prevalence of GC and changes in the gastric mucosa structure, the stereological changes of gastric mucosa in patients with GC and precancerous lesions were investigated in the present study.

Methods

Study group

Gastric specimens obtained at endoscopic biopsy from patients admitted during 2010-2015 years were used. The specimens were obtained from the pathology archives of Imam Ali hospital, Zahedan, Iran. This project was approved by the ethics committee of Zahedan University of Medical Sciences (IR.ZAUMS.REC.1394.53).

An experienced pathologist reviewed the original sections to confirm the histopathological diagnosis. From each selected formalin-fixed paraffin-embedded tissue block, two 3 µm-thick sections were cut and prepared for the application of physical dissector method. One of these two sections was selected as the "reference section", while the other was the "look-up section". The distance between the surfaces of section pairs was 15 µm. The sections were mounted on HistoGrip (CEDARLANE, Canada)-coated glass slides, then dewaxed and rehydrated in distilled water and finally stained according to the IHC and H&E staining protocols for stereological analysis. Immunostaining was done for the determination of the p53-positive and Ki-67-positive cells, and the sections were boiled at 120°C for 20 min in sodium citrate buffer (PH:6.0). The p53 antigen was stained by applying a monoclonal antibody (RTU-P53-DO7, Novocastra, England), and Ki-67 antigen was stained by applying another monoclonal antibody (RTU-Ki-67-MMI, Novocastra, England). Immunostaining was precisely described in our previous studies [25,26].

To calculate the NV of eosinophils and PMN cells, the H&E staining paired slides were used. The dissector counting technique was used to estimate the NV of cells using the formula:

$$NV = \frac{\sum Q^-}{\sum frame \times vdi\,\mathrm{sec}\,tor}$$

Where *NV* is the estimated numerical density of cells (cell number per unit volume), Σ^{Q-} is the total number of cells counted in the frames, Σ^{frame} is the number of frames in each sample which in this case was 10, and *vdissector* is the volume of each frame that was obtained using the formula:

$$Vdis\,\mathrm{sec}\,tor = Aframe \times H$$

Aframe is the area of each frame with the magnification (×400), *H* is the distance between sections which in this case was 15µm. *Vdissector* was 63375 µm^3 in this study.

Two pathologists examined the slides under a light microscope at high-power magnification (400×). In each slide, 10 fields were selected in systematic random manner by movement of the microscope's stage in X and Y directions with the aid of vernier scale in the reference section and then applying similar movements exactly on the second photomicroscope with look-up section [22] . Total cells were counted in the corresponding area (*Aframe*: 65×65 µm^2), and only nuclei stained unequivocally were considered positive. The fundamental rule of the dissector principle is to count the particle profiles

which are visible on the reference section but not in the look-up section. Since every particle generally has only one tip in a given direction, such an approach gives an unbiased estimate of the particle number in a given dissector volume. It is crucial to distinguish the profiles belonging to different particles clearly for an unbiased estimation [20].

H. pylori in gastric epithelial lesions was determined using a modified Giemsa staining method.

Statistics

Statistical analyses were performed using SPSS 16 software. All data are presented as mean±standard error of the mean (SEM). Statistical differences between independent groups were assessed using the nonparametric statistical tests of Kruskal Wallis and Mann-Whitney U tests. The significant level was set at $p<0.05$.

Results

In this study, 122 tissue blocks were included, 77 from male and 45 from female patients with a range age of 18-92 years (median 65). Gastric lesions based on histopathology and presence of H. pylori were selected as IM (n = 42; 20 H. pylori + and 22 H. pylori-), DYS (n = 38; 19 H. pylori+ and 19 H. pylori-) and GC (n = 42; 19 H. pylori+ and 23 H. pylori-). H. pylori was positive in 51(47.54%), and negative in 64 (52.45%) of all gastric lesions. The specimens selected were similar to our previ ous study [26].

The NV of the p53 positive and Ki-67 positive cells was compared between H. pylori+ and H. pylori- subgroups in different pathological stages (Table 1). p53 and Ki-67 positivity was clearly localized in the nuclei of malignant cells. Our data showed that in IM, DYS and GC, the NV of p53 positive cells in H. pylori+ subgroup were higher compared to H. pylori- subgroup (Table 1, Figure 1). There were significant differences in the NV of p53 positive cells between all H. pylori+ and H. pylori- subgroups of all groups (p=0.001). From the IM to DYS there was a trend of progressive increase in NV of p53-positive cells in H. pylori+ subgroup.

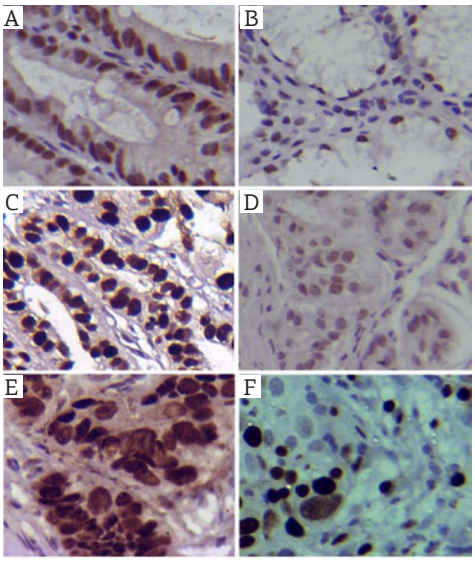

Figure 1. Measuring numerical density of p53 positive cells in gastric mucosa. Intestinal metaplasia in H. pylori positive **(A)** and H. pylori negative cases **(B)**; Dysplasia, in H. pylori positive **(C)** and H. pylori negative cases **(D)**; gastric cancer in H. pylori positive **(E)** and H. pylori negative cases **(F)**. IHC stain× 400.

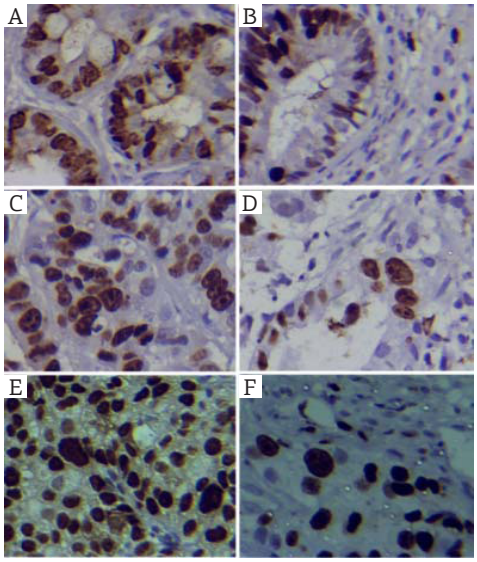

Figure 2. Measuring numerical density of Ki-67 positive cells in gastric mucosa. Intestinal metaplasia, in H. pylori positive **(A)** and H. pylori negative cases **(B)**; Dysplasia in H. pylori positive **(C)** and H. pylori negative cases **(D)**; gastric cancer in H. pylori positive **(E)** and H. pylori negative cases **(F)**. IHC stain× 400.

Table 1. Comparison of numerical density (NV) of p53- positive and Ki-67- positive cells in gastric cancer and precancerous lesions in the presence or absence of Helicobacter pylori.

Group	*H. pylori*	*p53 positive cells (n/mm³)*	*p value*	*Ki-67 positive cells (n/mm³)*	*p value*
IM	+	168204 ± 8142	0.001	103494 ± 11097	0.147
	-	138138 ± 34767		83069 ± 7054	
DYS	+	206456 ± 10314	0.001	181375 ± 4890	0.001
	-	139021 ± 7366		132211 ± 4559	
GC	+	184033 ± 22140	0.005	179715 ± 14236	0.146
	-	134533 ± 8642		143795 ± 11224	

Results are expressed as mean ± SEM. IM: intestinal metaplasia, DYS: dysplasia, GC: gastric cancer, H. pylori: helicobacter pylori

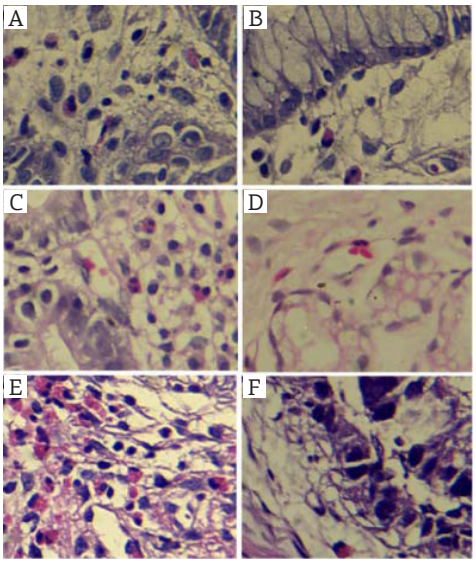

Figure 3. Measuring numerical density of eosinophil cells in gastric mucosa. Intestinal metaplasia in H. pylori positive **(A)** and H. pylori negative cases **(B)**; Dysplasia in H. pylori positive **(C)** and H. pylori negative cases **(D)**; gastric cancer in H. pylori positive **(E)** and H. pylori negative cases **(F)**. H&E stain× 400.

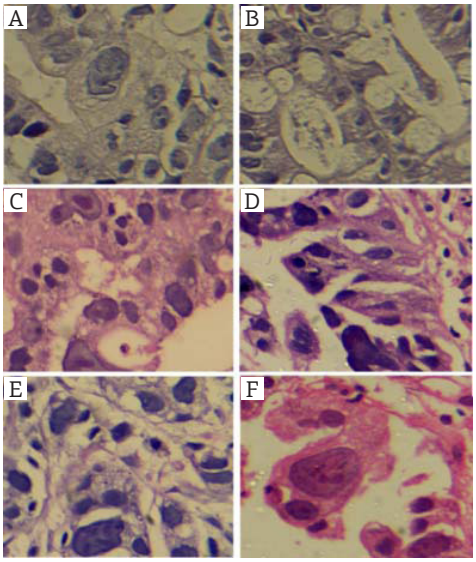

Figure 4. Measuring numerical density of polymorphonuclear cells in gastric mucosa. Intestinal metaplasia in H. pylori positive **(A)** and H. pylori negative cases **(B)**; Dysplasia in H. pylori positive **(C)** and H. pylori negative cases **(D)**; gastric cancer in H. pylori positive **(E)** and H. pylori negative cases **(F)**. H&E stain× 400.

This study showed that in patients with DYS, the NV of Ki-67-positive cells in the presence of H. pylori infection was significantly higher than in the absence of the infection, while in the IM and GC specimens this difference was not statistically significant (Table 1, Figure 2). In addition, there were significant differences of NV of Ki-67 positive cells between all H. pylori+ and H. pylori- subgroups of all groups (p=0.001).

The NV of the eosinophils and PMN cells was compared between H. pylori+ and H. pylori- specimens at different pathological stages (Table 2). In the IM group no significant differences in NV of the eosinophil cells between H. pylori+ and H. pylori- subgroups were detected. In DYS and GC specimens, the NV of the eosinophil cells were higher in the H. pylori+ subgroup compared with the H. pylori- subgroup. There was a trend of progressive increase in NV of the eosinophil cells in H. pylori+ subgroups from IM to GC types of specimens (Table 2, Figure 3). Kruskal-Wallis test showed statistically significant differences in NV of the eosinophil cells between all H. pylori+ and H. pylori- subgroups of all groups (p=0.002).

Only in the IM group the NV of the PMN cells was significantly different between H. pylori+ and H. pylori- subgroups, while in the DYS and GC groups no significant differences were noticed between their H. pylori+ subgroups (Table 2, Figure 4). Kruskal-Wallis test showed no significant differences between all H. pylori+ and H. pylori- subgroups of all groups (p=0.275).

Discussion

In this research, the NV of p53, Ki-67, eosinophils and PMN cells in GC and precancerous lesions (IM and DYS) were compared in the presence or absence of H. pylori. The NV of p53 positive cells was significantly higher in H. pylori+ compared with the H. pylori- subgroups in all study groups. It is considered that the increased number of p53-positive cells in the gastric mucosa in these

Table 2. Comparison of numerical density (NV) of eosinophil and polymorphonuclear cells in gastric cancer and precancerous lesions in presence or absence of Helicobacter pylori

Group	*H. pylori*	*eosinophil cells (n/mm³)*	*p value*	*polymorphonuclear cells (n/mm³)*	*p value*
IM	+	19648± 4353	0.138	164338 ± 35820	0.024
	-	11619 ± 2954		66989 ± 2179	
DYS	+	22422 ± 4756	0.04	62119 ± 24674	0.37
	-	9218 ± 2939		106467 ± 33653	
GC	+	34858± 6596	0.002	113027 ± 40889	0.769
	-	10084 ± 2874		91381 ± 29884	

Results are expressed as mean ± SEM. IM: intestinal metaplasia, DYS: dysplasia, GC: gastric cancer, H.Pylori: helicobacter pylori

lesions may result from DNA damage induced by H. pylori infection. These results are in agreement with the results of the expression of p53 in our previous study [26].

Only in DYS the NV of Ki-67-positive cells showed significant difference between H. pylori+ and H. pylori- subgroups.

This investigation was the first to study the NV of p53 and Ki-67-cells in gastric lesions. No quantitative and stereological studies have been carried out yet on microscopic parameters of the gastric mucosa and the number of p53 and Ki-67 positive cells. Based on cell counting method our results suggested that the number of p53 and Ki-67 positive cells can be associated with H. pylori infection, and thus it can be concluded that H. pylori can be a risk factor for gastric carcinogenesis.

In this study, no statistically significant difference was identified in the IM group between the NV of eosinophil cells in H. pylori+ and H. pylori- subgroups, but in DYS and GC groups the differences between their subgroups were statistically significant. Piazuelo et al [27] evaluated the eosinophil cells density in gastric mucosa in two different contains: low-risk and high-risk of a Colombian population. In their study eosinophil density in the mucosal layer was lower in early stages of gastric carcinogenesis, then in advanced disease stages in low-risk areas. The authors suggested that the high density of eosinophils in the early stages of gastric carcinogenesis was due to long-term Th2 response to the presence of H. pylori and proposed that it was a limiting factor for further tissue damage and disease progression towards gastric carcinogenesis. On the other hand, they stated that in high-risk areas eosinophil density in IM, DYS and GC was higher in early stages of gastric carcinogenesis and they noted that eosinophils likely had a poor/weak anti-inflammatory response to the presence of H. pylori in gastric carcinogenesis in the early stages. Delays in the inflammatory response leads to tissue damage and disease progression to cancer . Our study showed that the number of eosinophil cells in advanced stages was increased significantly in the gastric mucosa infected with H. pylori and we pointed out the possible roles for H. pylori infection in the development of gastric cancer. The results of our study are aligned with results from high-risk areas proposed by Piazuelo et al [27].

Our data showed that in the IM group, the NV of PMN cells in H. pylori+ specimens was higher than H. pylori- ones. With regard to DYS group, the NV of PMN cells in H. pylori+ group were lower than H. pylori-, while in the GC group the NV of PMN cells in H. pylori+ samples were slightly higher than in H. pylori- ones, but these differences were not statistically significant.

Similarly, Brenes et al. [28] showed that the number of PMN cells was higher in the early stages of gastric carcinogenesis in the H. pylori infected group, but in the specimens from the patients cleared from H. pylori there was a significant reduction in the number of PMN cells and also other histopathological parameters were improved after H. pylori eradication . In another study van Grieken et al [29]. found that in H. pylori infected patients the numbers of inflammatory cells were higher than in non-infected patients. Satoh et al. [30] found that before eradication, in the early stages of gastric carcinogenesis the number of PMN cells was significantly higher in regions with p53-positive cells, but after eradication the number of PMNs was significantly reduced in these areas.

Our data suggested that the number of cells at the earlier stages of carcinogenesis were affected by H. pylori infection, and H. pylori infection is essential for the onset of disease. However, with disease progressing to cancer no increase in the number of cells was shown in presence or absence of H. pylori. According to the results of this study, it can be concluded that H. pylori infection inflicts some changes in gastric mucosa structures including increase in the number of p53, Ki-67, eosinophils and PMN cells, all of which might eventually lead to gastric carcinogenesis. In summary, the cell counting method is a powerful tool for the assessment of mucosal structure, number of cells and grade of inflammation.

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Conflict of interests

The authors declare no confict of interests.

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