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PCNA expression in chronic gastritis due to *Helicobacter pylori**

Helicobacter pylori kronik gastritinde PCNA ekspresyonu

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Background/aims: *It has been reported that cases of Helicobacter pylori (H. pylori) gastritis, those with epithelial hyperproliferation, have potential for malignant transformation. This study was planned to determine the effects of H. pylori on gastritis and the gastric epithelial cell proliferation.* ***Methods:*** *In this study, we re-evaluated biopsy samples obtained from 331 cases of chronic gastritis according to Sydney criteria, retrospectively. We examined the gastric epithelial cell proliferation with proliferating cell nuclear antigen in the proliferating zone, in the whole mucosa of intestinal metaplasia and in areas of regenerative atypia, separately.* ***Results:*** *The relationship between H. pylori colonization density and chronic active gastritis was statistically significant. We did not see any relationship between the presence and density of H. pylori in gastric mucosa via proliferating cell nuclear antigen indices. In the presence of lymphoid follicles, we noted a significant expression of proliferating cell nuclear antigen in the areas of whole gastric mucosa and regenerative atypia.* ***Conclusions:*** *In the presence of lymphoid follicles, proliferating cell nuclear antigen expression was observed in the areas of whole gastric mucosa and regenerative atypia, but we did not see any relationship between the presence and density of H. pylori in gastric mucosa via proliferating cell nuclear antigen indices. This observation suggests the role of chronic inflammatory cells in gastric epithelial cell proliferation rather than the bacterium itself.*

Key words: *Helicobacter pylori*, gastric proliferation, PCNA, gastric carcinogenesis

Amaç: *Helicobacter pylori'nin mide epitelinde hiperproliferasyona yol açarak malign değişikliklere neden olabileceği bildirilmektedir. Bu çalışma Helicobacter pylori'nin gastrit bulguları ile ilişkisini ve mide epiteli proliferasyonundaki rolünü araştırmak için planlandı.* ***Yöntem:*** *Histopatolojik olarak kronik gastrit tanısı almış 331 olgudan elde edilen endoskopik biyopsi materyalleri çalışmaya alındı ve Sydney kriterlerine göre tekrar değerlendirildi. Gastrik hücre proliferasyonunu değerlendirmek için normal proliferatif zon, intestinal metaplazi ve reaktif atipi gösteren alanlarda PCNA ekspresyonu araştırıldı.* ***Bulgular:*** *Helicobacter pylori kolonizasyon yoğunluğu ile kronik aktif gastrit arasında anlamlı bir ilişki saptandı. Helicobacter pylori kolonizasyon yoğunluğu ve varlığı ile proliferasyon indeksleri karşılaştırıldığında anlamlı bir ilişki gözlenmedi. Ayrıca lenfoid folikül izlenen olgularda PCNA tüm katlarda ve rejeneratif atipinin izlendiği alanlarda anlamlı pozitiflik gösteriyordu.* ***Sonuç:*** *Lenfoid foliküllerin varlığında, tüm katlarda ve rejeneratif atipinin izlendiği alanlarda PCNA ekspresyonu gözlendi. Ancak Helicobacter pylori'nin varlığı ve yoğunluğu ile gastrik epiteliyal proliferasyon arasında anlamlı bir ilişki saptanmadı. Bu bulgular gastrik epiteliyal proliferasyon üzerinde Helicobacter pylori'nin kendisinden ziyade kronik inflamatuar hücrelerin gastrik hücre proliferasyonunu uyardığını düşündürmüştür.*

Anahtar kelimeler: *Helicobacter pylori*, gastrik proliferasyon, PCNA, gastrik karsinogenez

INTRODUCTION

Helicobacter pylori (*H. pylori*) is the first bacterial infection recognized as a human carcinogen, essentially on the basis of clinical and epidemiological studies (1, 2). It is not clear if the carcinogenic influences of the infection are due to bacterial, host or a combination of both factors. Many recent reports have demonstrated that *H. pylori* infection of the gastric mucosa has been associated with an increase in gastric epithelial cell proliferation (3–7). However, the exact mechanism of the hyperproliferation is controversial. Hyperproliferation may occur as a direct result of the factors produced by *H. pylori* or may be associated with epithelial damage by inflammatory cells induced by *H. pylori*, which may lead to compensatory hyperproliferation. The aim of our study was to

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determine the effects of *H. pylori* on the gastritis and the relationship with gastric epithelial cell proliferation. Proliferating cell nuclear antigen (PCNA) labeling index was used as an indicator of gastric epithelial cell proliferation.

MATERIALS AND METHODS

In this study, we re-evaluated gastric endoscopic biopsy specimens previously diagnosed histopathologically and obtained from 331 patients who had undergone gastric endoscopy at Pamukkale University Hospital over a five-year period (April 1996-April 2001). Exclusion criteria were previous gastrectomy for a malignant tumor and presence or suspicion of a tumor in the gastric biopsy specimens.

Clinical information was obtained from patients' records. For routine histological analysis, biopsy specimens were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E). Pathological examination of gastric endoscopy specimens was performed in the same hospital in the Department of Pathology and a single pathologist reviewed all the slides and evaluated them on a retrospective basis using light microscopy. The presence of *H. pylori*, chronic inflammation, activity, lymphoid follicle, intestinal metaplasia, gastric atrophy and dysplasia was recorded.

We confirmed the presence of *H. pylori* by histopathological examination of the H&E- and toluidine blue-stained tissue sections. In H&E sections, the grade and activity of gastritis, as well as density of colonization with *H. pylori* were evaluated in accordance with the Sydney system (8). Density of *H. pylori* infection in toluidine blue-stained sections was graded as follows: Grade 0: no bacteria detected; Grade 1: sporadic bacteria seen; Grade 2: many bacteria seen in most microscopic fields; Grade 3: bacteria seen in clusters in all fields. The other variables assessed were the presence and degree of lymphocyte/mononuclear cell (0–3) and neutrophil polymorphonuclear infiltration (0–3), atrophy (0–3) and intestinal metaplasia (0–3).

Sections were stained with Alcian blue (pH 2.5)-periodic acid-Schiff to diagnose intestinal metaplasia.

PCNA immunohistochemical evaluation was limited to 59 patients for proliferation zone and 26 patients in the whole mucosa due to technical problems. For PCNA immunostaining, paraffin-embedded sections were de-waxed and hydrated using xylene, graded ethanol and distilled water. Then the slides were immersed for 35 min in 3% H2O2 with methanol to block endogenous peroxidase and then taken to PBS (pH 6). A three-step immunoperoxidase staining technique was performed; the monoclonal DQ–7 mouse anti-human PCNA (DAKO LSAB®) was used. Sections were then incubated with diaminobenzidine (DAB), washed with distilled water, counterstained with hematoxylin, dehydrated through graded alcohols to xylene and coverslipped. After histopathological examination, 72 gastric biopsy specimens, including active gastritis, intestinal metaplasia and regenerative atypia, were stained for PCNA immunohistochemically. We evaluated the PCNA expression in the proliferating zone, in the whole mucosa of intestinal metaplasia and in the areas of regenerative atypia, in order to evaluate the state of proliferation associated with *H. pylori*.

PCNA-positive cells were counted only in well-oriented sections with visible entire gastric pits. Biopsies that did not include surface epithelium, and muscularis mucosae were not used for the determination of proliferation indices. Proliferation of gastric epithelial cells was analyzed by performing direct cell counting of the PCNA-stained cells, and approximately 300 cells of the glandular neck region, which corresponds to the area of cell proliferation, were evaluated for each tissue section. The PCNA labeling index represented the percentage of cells with positive nuclear staining (regardless of the staining intensity) in the total number of cells counted. The cytoplasmic staining was not evaluated.

Data are expressed as mean ± SD. All scores were entered into a database and analyzed using SPSS 10.0 packaged programs. Statistical analysis of observed findings of each group was done by chi-square, sample T and Mann-Whitney U test. Statistical significance of differences and relationships was determined by p values of <0.05.

RESULTS

The distribution of various histopathological features of the gastritis studied is shown in Table 1. Gastric biopsies from 331 patients [182 (55%) male, 149 (45%) female, age range: 15 to 95 years] were studied. The mean age of patients was 51.6 years.

Table 1. *H. pylori* relationship with histopathological features of the gastric mucosa according to the Sydney criteria

	H. pylori (-)	*H. pylori* (+)	*H. pylori* (++)	*H. pylori* (+++)	p value
Neutrophilic infiltration					
0	12 (85.7%)	2 (14.3%)	0	0	0.001
1	31 (37.3%)	33 (39.8%)	19 (22.9%)	0	
2	43 (24%)	57 (31.8%)	55 (30.8%)	24 (13.4%)	
3	15 (23%)	8 (12.4%)	24 (36.9%)	18 (27.7%)	
Atrophy					
Positive	17 (27.9%)	21 (34.4%)	15 (24.6%)	8 (13.1%)	
Negative	84 (31.1%)	79 (29.3%)	73 (27%)	34 (12.6%)	
Lymphoid follicle					
Positive	39 (24.8%)	53 (33.8%)	40 (25.5%)	25 (15.9%)	0.03
Negative	62 (35.6%)	47 (27.0%)	48 (27.6%)	17 (9.8%)	

H. pylori was present in 230 (69.5%) biopsies with evidence of gastritis. *H. pylori* prevalence in young patients was higher than in older patients (age >50) and the difference was statistically significant (p: 0.007).

Evidence of varying grades of neutrophil activity was found in 234 (70.7%) biopsies. A statistically significant positive correlation (p: 0.001) was found between increasing grade of *H. pylori* colonization density and activity.

Sex and age were not correlated with the degree of cell proliferation (PCNA indices) and presence of activity. Degree of neutrophil infiltration (p: 0.02) and intestinal metaplasia (p: 0.04) increased with increasing age.

One or more lymphoid follicles were observed in 157 (47.4%) biopsies (Figure 1). Presence of lymphoid follicles was significantly correlated with the presence of *H. pylori* (p: 0.03), but no correlation was observed between the density of *H. pylori* colonization and lymphoid follicles.

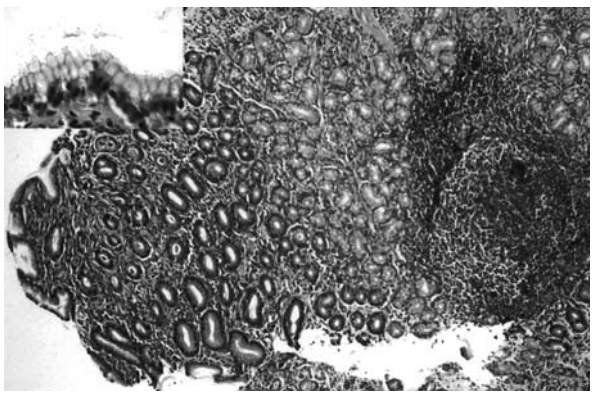

Figure 1. Follicular gastritis that is invariably associated with *H. pylori* infection and *H. pylori* microorganisms on the surface epithelium (H&E, x10)

Atrophy in gastric glands was detected in 61 cases (18.4%) and was correlated with increasing age (p: 0.006). There were statistically significant differences among the two groups in terms of atrophy and presence and degree of intestinal metaplasia (p: 0.004).

Reactive nuclear atypia was detected in 55 cases (16.6%) and it was correlated with the density of *H. pylori* colonization (p: 0.001), not with the presence of *H. pylori*.

PCNA Indices

The distribution of various PCNA indices in different areas of the gastric mucosa is shown in Table 2. We did not see any relationship between the presence and density of *H. pylori* in gastric mucosa via PCNA proliferation indices.

Table 2. PCNA indices in different areas of the gastric mucosa

	N	PCNA indices	Standard Deviation
Proliferative zone	73	27.23	± 9.45
Intestinal metaplasia	39	29.15	± 7.74
Whole gastric mucosa	54	22.88	± 14.62
Regenerative atypia	45	30.26	± 12.83

In the presence of lymphoid follicles, we noted PCNA staining in the areas of whole gastric mucosa (p: 0.018) and regenerative atypia (p: 0.009; Mann-Whitney U test) (Figures 2, 3).

In the presence of chronic active gastritis, we noted high PCNA indices in the areas of intestinal metaplasia, while we observed no differences in PCNA expression in the areas of regenerative atypia and normal proliferating zone. There was no statistically significant correlation with gastric atrophy and PCNA indices.

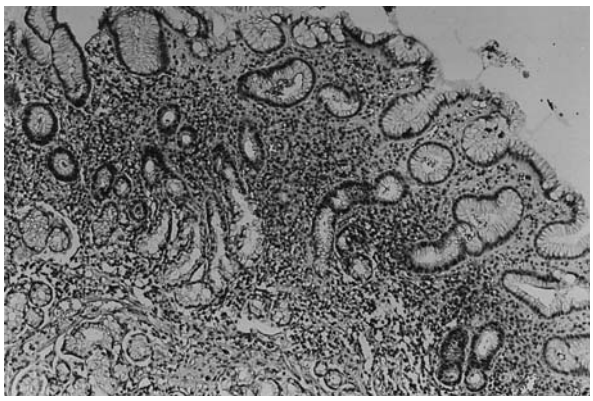

Figure 2. PCNA staining in the proliferating zone (PCNA, x10)

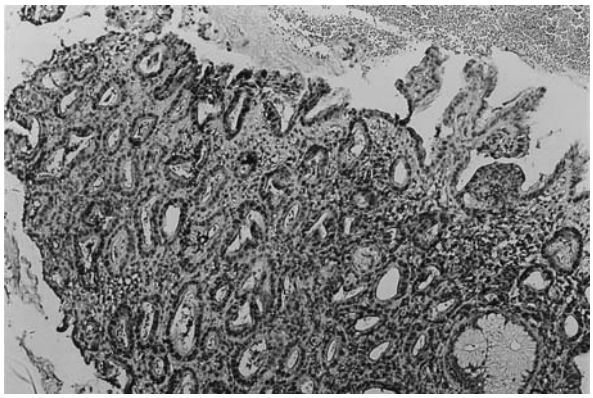

Figure 3. PCNA staining in the areas of whole gastric mucosa (PCNA, x10)

DISCUSSION

H. pylori is associated with chronic atrophic gastritis and an increased risk of gastric carcinoma (1). Gastric carcinoma is a multifactorial disease, involving genetic susceptibility, environmental influences, and infection, generally with *H. pylori*. In the Correa model of gastric carcinogenesis, environmental factors (salt intake, nitrate, a lack of vitamin C and beta-carotene, bile reflux, bacterial overgrowth in atrophic gastritis with nitrosamine formation) contribute to the evolution from normal gastric mucosa through superficial gastritis, multifocal atrophic gastritis, intestinal metaplasia and dysplasia to carcinoma (3).

In most developing countries, *H. pylori* infection is endemic, with as much as 80% of the population infected by age 15. In our study, *H. pylori* was detected in 69.5% of biopsies with evidence of gastritis. *H. pylori* prevalence in young patients was higher than in older patients (age>50), and the difference was statistically significant. This finding may be related to the fact that in developing countries, *H. pylori* is common among children of lower socioeconomic status (9). Also, the incidence of *H. pylori* decreases with progressing preneoplastic lesions, including gastric atrophy, intestinal metaplasia and gastric carcinoma, more prominent in the elderly. Furthermore, gastric epithelial cells may be altered so as to be unfavorable for *H. pylori*. It is obvious that persons infected with *H. pylori* at a young age who generate a marked inflammatory response would be at risk of gastric carcinoma (10).

In our study, sex and age were not correlated with the degree of cell proliferation and presence of active inflammation, similar to the findings of other studies (11, 12). The finding that there is no correlation between gastric epithelial proliferation and a patient's age and sex is not surprising. But, in our study, degree of neutrophil infiltration and intestinal metaplasia increased in conjunction with the patient's age. If active inflammation was present, a higher degree of neutrophils was observed in older patients when compared with the younger patients (age<50). The possible explanation of this result may be the mean age of the patients in our study, 51.6 years, and differences in active inflammation may reflect differences in host susceptibility, in organism virulence, or both. In the literature, there was no specific study about the relationship between active inflammation and age. Why older patients had a higher degree of active inflammation is unclear. The term "active gastritis" refers to the persistence of enzymatically active neutrophils in a gastric mucosa that also contains chronic inflammation. It reflects the continuing destructive activity of neutrophils on the gastric epithelium, and inflammation virtually always coexists with the presence of *H. pylori*. Many more cases need to be evaluated to provide more reliable results about the influence of age on active inflammation.

One or more lymphoid follicles were observed in 47.4% of biopsies. Presence of lymphoid follicles was significantly correlated with the presence of *H. pylori*, but no correlation was observed with the density of colonization. We suggest that lymphoid follicles are virtually pathognomonic for the presence of *H. pylori*, and minimal colonization is enough to result in an immune defense against *H. pylori*. The inflammatory response correlates closely with the density of *H. pylori* colonization, and it was suggested that the presence of neutrophils is a highly specific predictor of *H. pylori* (8, 11, 13).

In our study, a statictically significant positive correlation was found between increasing grade of *H. pylori* colonization density and activity.

Atrophy was found in 18.4% of biopsies, and no association was found in terms of atrophy and PCNA indices. In fact, mucosal atrophy was reported to be associated with increased gastric epithelial proliferation (14). Also, Liu et al. (15) demonstrated that gastric epithelial cell proliferation increases progressively from normal mucosa to atrophic gastritis, dysplasia and gastric cancer. But Peek et al. (16) and Lynch et al. (11) reported that there was no correlation between gastric atrophy and epithelial proliferation. Patchy character and subjective evaluation of the gastric atrophy may be responsible for the controversial results. More studies of other mechanisms appear to be necessary.

Although the effects of *H. pylori* on the microenvironment of the gastric mucosa are not completely understood, several pathways can be identified to explain the association between *H. pylori* and gastric carcinoma. The mechanisms involved in carcinogenesis are probably multiple and include induction of a hyperproliferative state of gastric epithelium, depletion of protective epithelial mucins and DNA damage related to abundant release of reactive oxygen metabolites by leukocytes (17).

Recent reports have proposed that mucosal hyperproliferation represents an early step in carcinogenesis and is an important indicator of increased risk for gastric carcinoma (3–5). It has been clearly shown that *H. pylori* infection increases gastric epithelial cell proliferation, demonstrated by interventional and non-interventional methods for proliferating cell detection (3, 6, 7, 18–20), although the exact mechanisms by which *H. pylori* promotes cell proliferation have not been fully elucidated.

Much effort has been made to identify *H. pylori*-related factors contributing to pathogenesis, including urease, adherence factors for colonization, inflammation and virulence factors that are involved in the induction of alterations in the gastric epithelial cells (21-24). There are also other factors that influence gastric epithelial cell proliferation such as salt intake, ammonia and bile reflux.

The pathogenicity of *H. pylori* may involve not only bacterial virulence factor but also host immunity. Although criteria for causality have not been completely satisfied for *H. pylori* and gastric carcinoma, there are reasonable mechanisms by which chronic inflammation could induce carcinogenesis (25). This hypothesis is strengthened by the documented relationship between cancer and chronic inflammation in the literature, regardless of the etiology. Chronic inflammation, which has been causally linked with malignancies, is thought to induce cancer by stimulating cell proliferation and increasing free-radical formation. Increased gastric epithelial cell proliferation increases the risk for DNA damage by replication error, and replicating single strands of DNA are more susceptible to the genotoxic effects of endogenous inflammation-related mutagens and exogenous dietary mutagens (7, 10). This increased mitogenesis leads to intestinal metaplasia followed by dysplasia and gastric carcinoma (19, 26). Mutagenic bacterial metabolites are also suspected to increase risk for cancer. However, in vivo data on human carcinogenesis by bacterial metabolites are inconsistent. Parsonnet (27) suggested that *H. pylori* infection has been linked to cancer by two mechanisms: induction of chronic inflammation and production of carcinogenic bacterial metabolites. *H. pylori* damages the gastric mucosa, leading to a compensatory hyperproliferation and increasing the replication zone which may expose vulnerable cells to luminal exogenous or endogenous mutagens (7).

Smoot et al. (28) suggested that direct contact by *H. pylori* inhibits gastric cell proliferation, supporting the theory that the hyperproliferation of gastric epithelial cells seen in vivo with *H. pylori* gastritis is a reflex response to cell injury, not directly caused by bacterial contact. It seems more likely that the chronic inflammatory cells, which only slowly regress following eradication of the bacteria, provides the stimulus to gastric epithelial proliferation (11). Although several possible carcinogenic mechanisms can be proposed, many studies support the notion that *H. pylori* and resultant inflammation causes gastric epithelial hyperproliferation (4, 11, 19).

Since successful treatment of *H. pylori*-induced gastritis results in clearance of the bacteria as well as the inflammation, it has been difficult to decide whether *H. pylori* or inflammation accounts for the hyperproliferation of gastric epithelial cells. In our study, we were not able to evaluate the gastric epithelial cell proliferation after *H. pylori* eradication. In both in vitro and in vivo experiments, *H. pylori* infection has been shown not only to induce cell proliferation, but also to provide a source of endogenous mutagens through its

acute and chronic stimulation of inflammatory cells. But the finding that eradication of *H. pylori* does not lead to an immediate decline in epithelial proliferation argues against a direct effect (11). Indeed, recent in vitro studies indicate that *H. pylori* itself has a suppressant effect on epithelial proliferation (29). Our findings indicate that gastric epithelial cell proliferation is related with the histological parameters like lymphoid follicles and intestinal metaplasia, so we concluded that *H. pylori* induces epithelial cell proliferation indirectly by inflammatory cells rather than the bacterium itself, similar to the findings of Lynch and Liu et al. (11, 15).

Only less than 10% of *H. pylori* carriers develop malignancy, and increased epithelial cell proliferation is one of the earliest identifiable abnormalities in the development of gastric carcinoma. From the biological point of view, progression to atrophy, intestinal metaplasia and gastric carcinoma seems to be dependent on other factors, primarily genetic and environmental, and requires further DNA changes. A combination approach that includes *H. pylori* eradication and dietary supplementation may be necessary. Furthermore, new studies must be performed to investigate the role of the bacterium-related metabolites and other factors.

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