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ARTICLE

Proliferating Cell Nuclear Antigen (PCNA) Immunostaining in *Helicobacter Pylori* Infection: Impact of Eradication

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Despite the fact that the association of *Helicobacter pylori (H. pylori)* with an increased risk of gastric cancer has been well documented, the exact mechanisms of this association have not been fully elucidated. The aim of the present prospective study was to contribute to the exploration of these mechanisms by studying the relationship between *H. pylori* infection and proliferating cell nuclear antigen (PCNA) immunostaining in endoscopic biopsies in gastric antrum. Furthermore, we examined the impact of *H. pylori* eradication on this relationship. We studied 28 *H. pylori* positive patients and the results were compared with 22 endoscopically and histologically normal *H. pylori* negative patients (control group) who were comparable to the *H. pylori* positive group for age and sex. In addition all *H. pylori* positive patients were examined before and after treatment aiming to eradicate *H. pylori*. In the *H. pylori* (+) patients the median PCNA index was 35 (range 8-58) and this was significantly higher than the respective number in the control group [5.5 (2–14), p<0.001]. In patients studied before and after successful eradication of *H. pylori* (n=10) the corresponding numbers were 35 (8-56) and 7 (4–13) (p<0.01) the latter not being significantly different from the control group of *H. pylori(-)* patients. On the contrary, in patients without successful H. pylori eradication (n=18) the PCNA indices before and after treatment were similar [35.5 (21-58) vs 31.5 (20-56)]. It is concluded that *H. pylori* infection alters the replication cycle of the gastric mucosa inducing hyperproliferation, which return towards normal after successful *H. pylori* eradication. (Pathology Oncology Research Vol 5, No 4, 304–308, 1999)

Keywords: *Helicobacter pylori* infection, *H. pylori* eradication, PCNA immunostaining, gastric proliferation, gastric carcinogenesis

Introduction

Helicobacter pylori (H. pylori) infection has been recognized as the principle cause of type B gastritis and peptic ulcer disease[1-5] and in addition a close association between *H. pylori* and gastric malignancy has been found,[6-13] mainly on the basis of seroepidemiological data. Furthermore *H. pylori* has been classified as type I carcinogen for gastric cancer[14] by the International Agency for Research on Cancer (IARC). However despite the well-documented association of *H. pylori* with an increased risk of gastric cancer, the exact mechanisms by which it affects the gastric mucosa, have not been elucidated. One possible assumption is that *H. pylori* infection alters the replication cycle of gastric mucosa inducing hyperproliferation and possible ploidy abnormalities.[15] However, the evidence in the literature is not conclusive and therefore more information is needed on this important subject of contemporary interest. The aim of our prospective study was to assess whether *H. pylori* infection affects gastric cell proliferation and in addition to examine the impact of eradication of the bacterium on this. To do this the proliferating cell nuclear antigen (PCNA) labeling index was used as an indicator of the alterations induced in the cellular replication cycle by *H. pylori* infection. There is increasing evi-

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dence that PCNA assessment is a useful tool to evaluate cell proliferation and previous studies have shown that PCNA index correlates with the S-phase fraction of tumor cells determined by DNA flow cytometry.[16-18] Furthermore it has been found that PCNA immunostaining correlates with that detected by Ki67,[19] the latter marking cells in G1 and G_2 phases in addition to those in S-phase and also with thymidine labeling index[20] and with bromodeoxyuridine uptake in carcinoma cell lines.[21]

Materials and Methods

Patients and samples

For the purposes of this study we prospectively studied 50 consecutive dyspeptic patients [32 men, 18 women, median age 39.5 (range 18-67)], referred for upper gastrointestinal endoscopy. None of the subjects studied had undergone upper gastrointestinal surgery and none had used antibiotics, bismuth, PPIs or NSAIDs during the previous 8 weeks. During endoscopy antral biopsies were taken from each patient for *H. pylori* detection and histological assessments. Patients, who were found to be *H. pylori* positive, were examined before and after treatment for *H. pylori.* Treatment was attempted with the administration of a double therapy scheme (Omeprazole 20 mg bid + 1 g Amoxycillin bid) received for 14 days. In these patients endoscopies with biopsies were performed before starting therapy and were repeated four to six weeks after the completion of treatment.

Helicobacter pylori detection

In each subject *H. pylori* was sought in two ways, i.e. the rapid urease test[22] and histology. For histological detection slides were stained with Giemsa, modified for *H. pylori* and then the presence of *H. pylori* was microscopically evaluated.[23] For the purposes of this study, patients with one test positive were not included. Thus, patients were considered to be *H. pylori* (+) when the bacterium was identified in both tests and negative when the bacterium was not identified in both tests. In addition, after treatment, *H. pylori* eradication was confirmed with the ^{13}C urea breath test.[24]

Histological assessments

Antral mucosal biopsy specimens were immediately fixed in buffered neutral formalin and embedded in paraffin. Sections were then stained with haematoxylin and eosin for diagnosis and evaluation of gastritis, with modified Giemsa staining for *H. pylori* detection, as developed above and PCNA immunostaining for proliferation assessment. All histological slides were reviewed by the same experienced pathologist.

In haematoxylin-eosin sections the diagnosis and evaluation of gastritis, was based on accepted criteria.[25] For PCNA immunostaining, paraffin-embedded sections were dewaxed and hydrated using graded ethanol and distilled water. Then the slides were immersed for 10 min in 3% H_2O_2 to block endogenous peroxidase and then taken to PBS (pH 7.6). A three-step immunoperoxidase staining technique was performed; the mouse monoclonal anti-PCNA/cyclin antibody (clone PC-10, Dako) was used. A dilution of 1:10 with one-hour incubation was found to be optimal. Very light haematoxylin counterstain was performed before mounting. For evaluation of PCNA immunostaining, the slides were examined using a 10 objective lens and ten fields were randomly selected. PCNA positive cells were counted in the glandular neck region, which corresponds to the area of cell proliferation.[15] The PCNA index represented the percentage of cells with positive nuclear staining (regardless of the staining intensity) in the total number of cells counted.

Statistical analysis

All statistics were computed using GraphPad, PRISM, Version 2.0. The results are represented graphically as boxes and whiskers or individual data points, whereas data in the text are expressed as median values with ranges. As most data showed skewing, comparisons between the multiple groups were performed using non-parametric Kruskal-Wallis analysis of variance. If the result of this was significant, then simple comparisons between pairs of groups were performed with the non-parametric Mann Whitney U test.[26] Comparisons between proportions were made by the Fisher's exact test. A p value of less than 0.05 was considered significant.

Results

Among 50 patients studied, there were 28 (56%) *H. pylori* positive patients (13 duodenal ulcer, 15 non ulcer) and 22 (44%) endoscopically and histologically normal *H. pylori* negative patients (control group). These two groups

Table 1. **Demographic data in *H. pylori* (-) and *H. pylori* (+) patients studied (NS=not significant).**

	H. pylori (-) *(n=22)*	*H. pylori* (+) *(n=28)*	*p*
Age (yrs) median (range)	38.5 (19-66)	39.5 (18-67)	NS
Sex (M/F)	15/7	17/11	NS
Smoking habits	14/22 (63.6%)	17/28 (60.7%)	NS
Daily alcohol consumption	5/22 (22.7%)	7/28 (25%)	NS

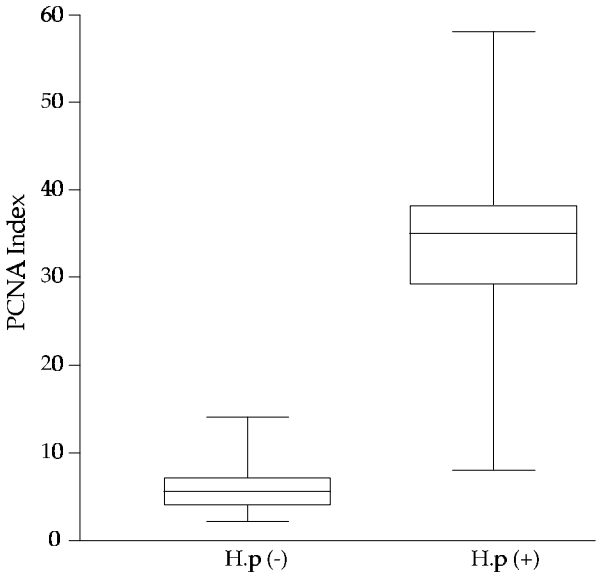

Figure 1. PCNA index in H. pylori (-) and H. pylori (+) patients (p<0.001). Results are expressed as boxes and whiskers. Boxes indicate 25-75% range and central vertical lines indicate median values. Whiskers represent upper and lower extremes.

were comparable for age and sex and other demographic parameters *(Table 1).*

In the *H. pylori* (+) patients the median PCNA index was 35(range 8-58) and this was significantly higher than the respective number in the control group [5.5 (2–14), p<0.001] *(Figure 1).* Treatment was successful in 10/28 (35.7%) *H. pylori* positive patients (i.e. bacterium eradication and normalization of gastric histology). In these patients the PCNA corresponding indices before and after eradication were 35 (8-56) and 7(4–13) (p<0. 01) *(Figure 2)* the latter not being significantly different from the control group of *H. pylori* (-) patients. On the contrary in patients without successful *H. pylori* eradication after treatment (n=18) the PCNA indices were not significant [35.5 (21-58) Vs 31.5 (20-56)] *(Figure 3).*

Discussion

This study addressed the relationship between *H. pylori* infection and PCNA immunostaining in endoscopic biopsies in the gastric antrum, as PCNA has been demonstrated to be a useful tool in evaluating cell proliferation. We showed that *H. pylori* alters the replication cycle of the antral mucosa, inducing hyperproliferation, which returns to normal only in patients with successful *H. pylori* eradication. So far evidence in the literature on the association between *H. pylori* infection and gastric cell proliferation and especially on the impact of *H. pylori* treatment has been conflicting. Thus some researchers[27] have found that the significant reduction in epithelial cell proliferation after *H. pylori* treatment was independent of successful eradication of *H. pylori* and they attributed their results directly to the effect of treatment used. On the contrary, other researchers[15,28] showed that *H. pylori* infection induced gastric epithelial cell proliferation in the antral mucosa, which decreased significantly after *H. pylori* eradication. They did not show a similar reduction in those whose infection was not eradicated. Similar long-term observations in-vitro, were noticed by Lynch et al.[29] From the above it seems that there are four prospective studies; the present study, as well as the three mentioned above[15,28,29] which are in agreement on hyperproliferation in *H. pylori* positive subjects and the beneficial consequences of successful *H. pylori* eradication, whereas there is only one study which does not confirm this notion.[27] It is notable therefore that on this important subject of current interest, the conclusions of four independent studies from different parts of the world are in agreement.

Apart from the above studies, which examined gastric cell proliferation before and after *H. pylori* treatment, the effect of *H. pylori* infection on gastric cell proliferation rates, per se, has been examined in various other studies, using various methodologies. Thus, increased[30,31] or unaltered proliferation rates were found.[32] Although the reason for these conflicting results is not clear, a possible explanation is that among *H. pylori* strains there is heterogeneity in cytotoxin associated gene A (cagA) status. Hence, the cagA (+) and cagA(-) strains may differ in their ability to induce proliferative responses, with the cagA (+) *H.*

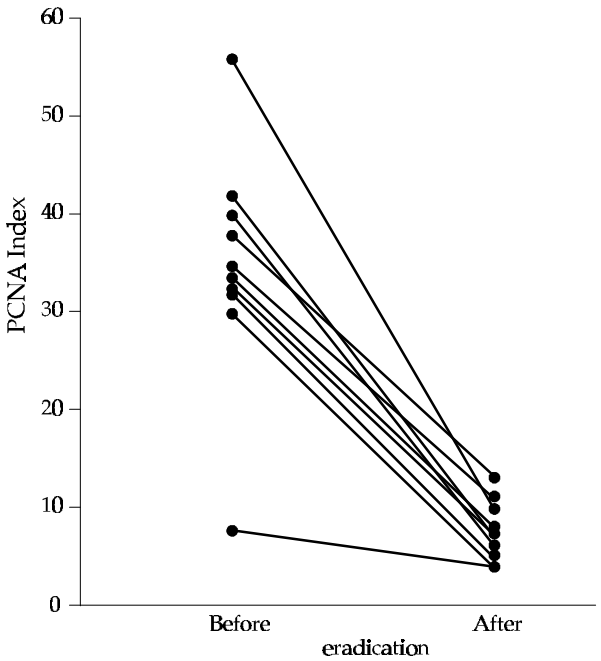

Figure 2. PCNA index in patients (n=10) before and after successful eradication of H. pylori (p<0.001).

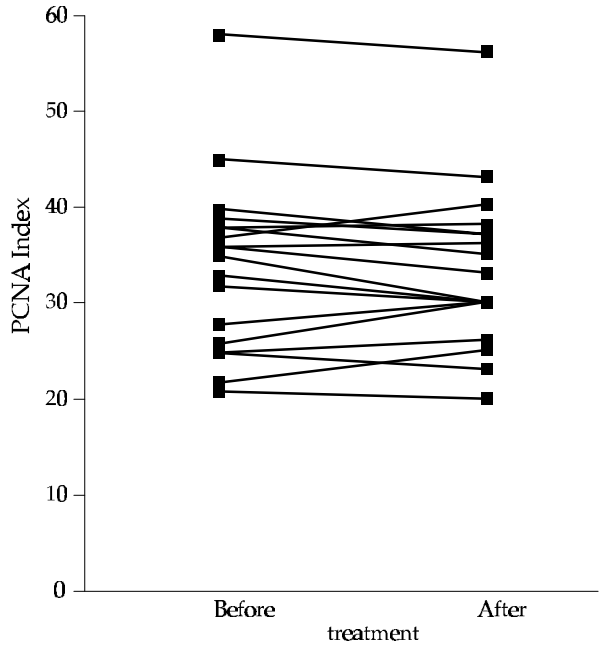

Figure 3. PCNA index in patients (n=18) before and after unsuccessful H. pylori treatment (NS).

pylori strains producing increased proliferation rates in comparison to cagA (-) strains.[33]

The reason why *H. pylori* infection should increase proliferation rates is not known, but there are a number of possible explanations. Thus, Konturec et al[34] studied epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) in antral biopsies of duodenal ulcer and non-ulcer dyspepsia patients and found that in *H. pylori* infection and the resulting antral gastritis, there was increased cell proliferation, which was paralleled by an enhanced expression of both EGF and TGF. Based on these results they raised the possibility that the enhanced expression of both growth factors was responsible for the increased mucosal cell proliferation. However, other, as yet unknown, factors that may stimulate cell proliferation, such as reactive oxygen metabolites produced by polymorphonuclear cells or other bacterial factors may also be involved.

Undoubtedly the implication of the increased proliferation rates on gastric carcinogenesis in *H. pylori* infection is significant. However, other parameters, not examined in this study, which are essential in gastric tissue homeostasis, such as apoptosis, must be taken into account, although the data on this subject are not unanimous in the literature. Thus an increased apoptosis rate has been reported by some studies[35] whereas other studies did not confirmed this.[33] It seems therefore that more data are needed on this important issue.

In conclusion, we found that *H. pylori* infection is associated with increased proliferation rates which return to normal after successful eradication. The former stresses the significance of *H. pylori* infection in gastric carcinogenesis, whereas the latter stresses the possible significance of *H. pylori* eradication in preventing gastric cancer.

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