Original Article

A study on the effect of *Helicobacter pylori* infection on p53 expression in gastric cancer and gastritis tissues

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Abstract

Introduction: *Helicobacter pylori* cause damage to gastric epithelial cells and alterations in the p53 gene that lead to cancer development. This study aimed to determine the correlation of p53 expression with *H. pylori* using immunohistochemistry, RFLP-PCR, and histopathology.
Methodology: Gastric biopsy samples from gastric cancer (GC) (n = 54) and gastritis (n = 31) patients were examined for histopathological changes and expression of p53 protein by immunohistochemistry.
Results: Immunohistochemical analysis of p53 protein expression in *H. pylori*-positive GC sections showed an average of 44.3% positive cells in tumors and 6.9% in normal tissues, as compared to 16.4% and 4.4% in *H. pylori*-negative sections. P53 expression showed significant association with *H. pylori* (*P* = 0.005), invasion depth (*P* = 0.029) and inflammation reaction (*P* = 0.008). In gastritis sections, no difference in the average p53 staining in *H. pylori*-positive or -negative sections was seen. PCR-RFLP results also showed no difference in genotype frequencies of p53 in *H. pylori*-positive or -negative gastritis sections. Histopathology study of *H. pylori*-positive GC sections showed that 97.2% were the intestinal type and 2.8% the diffuse type, while in *H. pylori*-negative sections 35.2% were the intestinal type and 64.8% the diffuse type. Biopsy sections from *H. pylori*-positive gastritis patients revealed more severe inflammation than those of *H. pylori*-negative patients.
Conclusion: Our results show that *H. pylori* infection affects p53 expression in GC. The average p53 expression was significantly higher in tumor than in normal tissues. In gastritis sections p53 expression was significantly associated with *H. pylori*.

Key words: *Helicobacter pylori*; p53; gastric cancer

Introduction

Helicobacter pylori is a type-I carcinogen that plays an important role in gastric cancer (GC) [1]. Genetic alterations in p53 that control the cell cycle might change the susceptibility of tumor cells to trigger apoptosis [2,3,4]. The expression of p53, a tumor suppressor protein, has been found to play a critical role in cell cycle control and apoptosis [5,6].

p53 mutation frequencies were found to increase as gastric mucosa of patients with gastritis progress to intestinal metaplasia (IM), dysplasia and cancer [7]. Earlier reports showed that p53 immunoreactive cells were detected in normal gastric mucosa, in mucosa of patients with gastritis, and in mucosal cells adjacent to tumor tissues [8,9]; However, other investigations found no such cells in normal mucosa or in gastritis tissues [10,11].

In the presence of *H. pylori*, activated leukocytes produce free radicals that exert an effect on the nuclear p53 expression in immunoreactive cells detected in the neck region and the proliferative zone reflecting normal p53 response to DNA damage [12,13]. Such an effect was supported by Satoh *et al*. (2001) who detected significantly higher p53-positive cells during an active *H. pylori* infection than after eradication [14]. In addition, *H. pylori* infection was shown to induce p53 point mutations that were not found in *H. pylori*-negative gastritis patients [15]. Thus *H. pylori* infections and p53 expression might have a synergistic effect on gastric carcinogenesis. Berloco *et al*. [16], however, indicated that *H. pylori* infection in patients from Italy does not affect the p53 pattern in gastric mucosa and that p53 abnormality (overexpression/mutation) showed no association with gastric carcinogenesis in the population studied.

The detection of p53 as a prognostic marker using immunohistochemistry showed results that were in disagreement as far as the prevalence of p53 mutations and tumor type. While some authors found that mutations tend to affect intestinal type tumors, others found that the incidence of mutation is similar in the two main tumor types, suggesting that the p53 gene is a common target in the development of GC in general [17].

Among several polymorphisms described in p53, the codon 72 single nucleotide polymorphism (SNP) on exon 4 is the most common variant associated with cancer development. Three distinct genotype polymorphisms among the p53 codon 72 Arg-Pro (CGC-CCC) were found to exist: a homozygous arginine (Arg-Arg), homozygous proline (Pro-Pro), and heterozygous (Arg-Pro) [6].

Recently, Matsumoto *et al.* [18] proposed that *H. pylori* infection caused an aberrant expression of cytidine deaminase, an enzyme that induces p53 mutations in gastric epithelial cells, suggesting a link between *H. pylori* and genetic polymorphism of p53. Mutations of p53 and/or overexpression of mutant p53 protein have been reported in 60% of all cancers, including gastric cancer samples. Several studies have shown that the detection of p53 in the presence of low-grade dysplasia is a risk factor for progression to high-grade dysplasia or cancer. Thus it is expected that p53 mutation status causes the mutant type of p53 expression [19,20]. However, no correlation was found between p53 mutation analysis and immunohistochemical staining in *H. pylori*-associated gastroduodenal diseases including GC.

Our objectives were to investigate the effect of *H. pylori* on p53 expression in GC resected tumor and normal tissues and in gastritis tissues, and to compare the results of p53 mutation detected by PCR-RFLP and immunohistochemistry in gastritis tissues. This study will enhance our understanding of the effect of *H. pylori* infection on pathogenesis and genetic polymorphism of p53 in patients with GC and gastritis.

Methodology

Biopsy specimens

A total of 96 biopsy samples were collected as follows: 52 samples from gastric cancer patients (12 female) of age range 33-82 years (average 61) and 44 samples from gastritis patients (21 female) of age range 15-84 years (average 46.4). Two biopsy samples from the resected stomach cancer tissues (paraffin-embedded sections), one from the normal tissue, and

one from the tumor tissue were collected from each patient. Sections were examined by PCR-RFLP, immunohistochemical analysis, and histopathology. A written consent was obtained for each patient and the study was approved by the hospital ethical committee.

DNA isolation and PCR

DNA was extracted by the QIAamp DNA Mini Kit (Qiagen, Germany), according to the manufacturer's instructions and stored at -20°C until used.

PCR-RFLP (genotyping of p53 codon 72)

Genomic DNA isolated from antral biopsies of gastritis patients was used. The genetic polymorphism of p53 codon 72 on exon 4 was detected by PCR-RFLP method as described previously [19] using the following primers (forward: 5'-TTGCCGTCCCAAGCAATGGATGA-3'), (reverse: 5'-TCTGGGAAGGGACAGAAGATGAC-3') in a thermal cycler (Techne, Staffordshire, United Kingdom). The PCR program included a cycle of 95°C for 10 minutes, then 35 cycles (95°C for 30 seconds and 60°C for 30 seconds, 72°C for 30 seconds), and 72°C for 5 minutes. The PCR products (p53 codon 72) were digested with the restriction enzymes BstUI (Thermo Scientific, MA, USA) and electrophoresed in 3.5% agarose gel [19].

Immunohistochemistry

Immunohistochemical staining of p53 were performed using streptavidin peroxidase labeled monoclonal antibody (clone DO-7, ScyTek, USA). A positive p53 protein was seen as a nuclear stain and scores of strong, moderate, and weak positive immunolabeling were used. More than 10% of strong or moderately stained cells were considered positive.

Histopathology

Sections of tumor and normal tissues obtained from GC patients and antral biopsy sections from gastritis patients were stained with Giemsa and haematoxylin and eosin (HE) stains. *H. pylori* status and density, chronic gastritis (mononuclear cell activity), neutrophil infiltration, invasion depth, inflammation reaction, glandular atrophy, and IM were evaluated and graded from 0-3 according to the updated Sydney system [21]. The type of GC (intestinal or diffuse) was classified according to the Lauren classification [22]. All sections were morphologically evaluated by an experienced histopathologist who was not informed about the sections.

Statistical analysis

A classical regression analysis using the Statistical Package for the Social Sciences (SPSS-19) (IBM SPSS Statistics 19.0, New York, USA) was used for measuring the effect of gender, age, *H. pylori*, IM, atrophy, tumor type, invasion depth, and inflammation reaction on p53 staining in both tumor and normal tissues of GC sections. The regression function is shown below were p53 is the dependent variable and the independent variables were listed above.

$$P53 = \hat{\beta}_0 + \hat{\beta}_1 Gen + \hat{\beta}_2 Age + \hat{\beta}_3 Hp + \hat{\beta}_4 IM + \hat{\beta}_5 Atr + \beta_6 Tt + \hat{\beta}_7 Id + \hat{\beta}_8 Ir$$

The effects of gender, age, *H. pylori*, chronic inflammation, neutrophil infiltration, and IM on p53 staining for gastritis patients were estimated by a regression function given below:

$$P53 = \hat{\beta}_0 + \hat{\beta}_1 Gen + \hat{\beta}_2 Age + \hat{\beta}_3 Hp + \hat{\beta}_4 ChI + \hat{\beta}_5 Nac + \beta_6 IM$$

A *P* value of < 0.05 was considered as statistically significant.

Results
H. pylori

H. pylori in paraffin-embedded tissue sections detected by Giemsa staining and by PCR were positive in 35 (67.0%) of 52 GC samples. *H. pylori* in gastritis biopsies detected either by rapid urease test or PCR were positive in 31 (70.0%) of 44 gastritis samples.

p53 expression

Immunohistochemical analysis of p53 expression in *H. pylori*-positive GC resected tissue sections showed that the average positive cells in tumor and adjacent normal tissues were 44.3% and 6.9% respectively, while in *H. pylori*-negative sections they were 16.4% and 4.4% respectively. The average p53 expression was significantly higher in the tumor tissue in the presence of *H. pylori* than in the absence of *H. pylori*. We have correlated p53 average positive cell ratios with *H. pylori*, gender, age, invasion depth, inflammation reaction, IM, atrophy and tumor type and found a significant association with the presence of *H. pylori* (*P* = 0.005), invasion depth (*P* = 0.029) and inflammation reaction (*P* = 0.008) in tumor tissues, while no such association was observed in normal tissues (Table 1). Figure 1 shows immunohistochemical staining of p53 positive cells in *H. pylori*-positive tumor and normal tissue of intestinal type GC sections. The nuclei of positive cells in the tumor tissue stained more heavily brown in color than those in the normal tissue. Positive cells were commonly found in the neck region of the gastric pits (Figure 2). Figure 3 shows p53 positive cells staining in *H. pylori*-positive tumor tissue (diffuse type) and normal tissue of gastric cancer sections. The nuclei of positive cells were stained brown in color.

Immunohistochemical analysis of p53 expression in antral biopsy sections from gastritis patients showed an average of 29.9% for p53 positive cells in *H. pylori*-positive sections, while in *H. pylori*-negative the average was 21.2%. In these sections p53 expression was significantly associated with the presence of *H. pylori* (*P* = 0.025) and neutrophil infiltration (*P* = 0.002) (Table 2).

p53 genotyping

Arg-Pro, Arg-Arg and Pro-Pro genotypes are among the three common p53 codon 72 polymorphisms. PCR-RFLP results showed that the genotype frequencies of p53 in *H. pylori*-positive gastritis sections were 45% (Arg/Arg), 32% (Arg/Pro), and 23% (Pro/Pro), while in *H. pylori*-negative sections they were 46% (Arg/Arg), 25% (Arg/Pro), and 28% (Pro/Pro).

Histopathology

Histopathology evaluation of the tumor type in GC sections showed that in 35 *H. pylori*-positive GC patients, 34 (97.2%) were of the intestinal type and one was (2.8%) of the diffuse type. Chronic gastritis was detected in all sections and of these 22 (62.0%) had IM and 14 (40%) had glandular atrophy. Of biopsy sections from the other 17 *H. pylori*-negative GC patients, 6 (35.2%) were of the intestinal type and 11 (64.8%) of the diffuse type. All sections showed chronic gastritis and of these 9 (52.9%) had IM. On the other hand, biopsy sections from 44 patients with gastritis revealed that in 31 *H. pylori*-positive patients, 100% showed chronic inflammation, 93.5% showed neutrophil infiltration, and 12.9% showed IM. Biopsy sections from the other 13 patients with no *H. pylori*, 84.6% had chronic inflammation, 38.4% showed neutrophil infiltration, and 7.7% had IM.

Discussion

H. pylori, a major etiological factor in GC development, acts as an initial triggering mechanism that lasts for years in which chronic gastritis progress to atrophy, IM and cancer [23]. Sixty-three percent of our GC patients were found infected with *H. pylori*

Table 1. Statistical analysis of the association between p53 staining in tumor and normal tissues of gastric cancer sections and gender, age, *H. pylori,* intestinal metaplasia, atrophy, tumor type (intestinal/diffuse), invasion depth and inflammation reaction

	Gastric cancer section							
	Normal tissue				**Tumor tissue**			
	Dependent variable: Sqrt(p53)				**Dependent variable: Sqrt(p53)**			
	Coeff.	**t**	**Sig.**	**VIF**	**Coeff.**	**t**	**Sig.**	**VIF**
Constant	.096	.047	.962		-.864	-.254	.800	
Gender	.098	.145	.885	1.096	.236	.207	.837	1.096
Age	.015	.578	.566	1.129	.009	.202	.841	1.129
H. pylori	.342	.397	.693	2.184	4.324	2.990	.005	2.184
IM	.628	.808	.423	1.949	-2.044	-1.565	.125	1.949
Atr	-.739	-.933	.356	2.024	.636	.478	.635	2.024
Tt	-.331	-.334	.740	2.337	-2.100	-1.261	.214	2.337
Id	.897	1.216	.231	1.216	2.795	2.255	.029	1.216
Ir	-.373	-.534	.596	1.392	3.291	2.804	.008	1.392
N	52				52			
F	.571				2.51			
Sig.	.796				.025			
R	.31				.56			

IM: intestinal metaplasia, Atr: atrophy, Tt: tumor type (intestinal/diffuse), Id: invasion depth, Ir: inflammation reaction.
Coeff: Coefficient
t value
Sig: significance level
VIF: variance inflation factor
R: correlation coefficient

Table 2. Statistical analysis of the association between p53 staining in gastritis tissue sections and gender, age, *H. pylori,* chronic inflammation, neutrophil infiltration and intestinal metaplasia

Gastritis section				
	Dependent variable: p53			
	Coeff.	**t**	**Sig.**	**VIF**
Constant	42.448	4.091	.000	
Gender	.593	.161	.873	1.021
Age	-.241	-1.762	.086	1.335
H. pylori	-12.945	-2.339	.025	1.933
Chi	-16.440	-1.668	.104	1.277
Ni	18.694	3.319	.002	1.689
IM	-5.913	-1.135	.264	1.100
N	44			
F	3.057			
Sig.	.016			
R	.58			

Chi: chronic inflammation, Ni: neutrophile infiltration, IM: intestinal metaplasia.
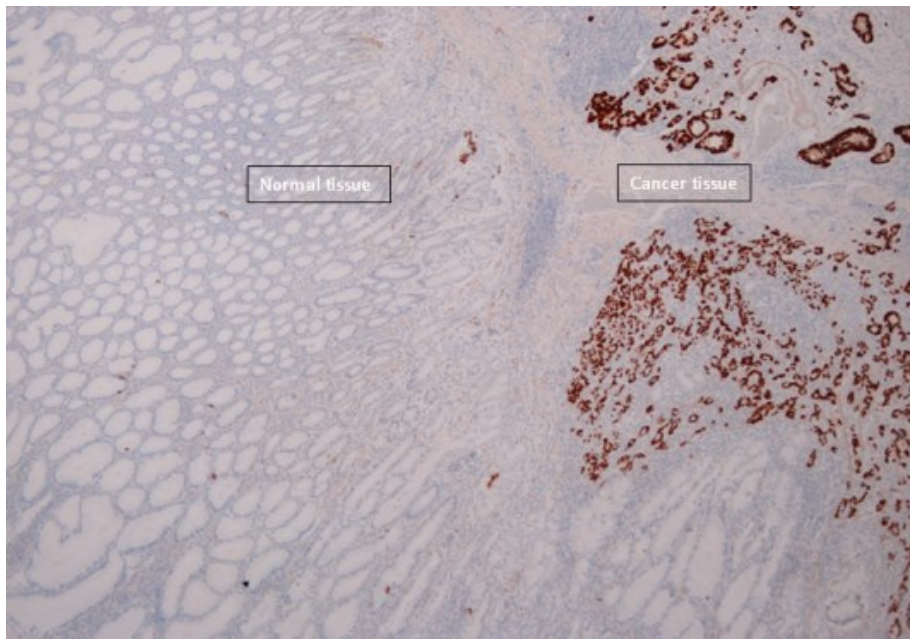Coeff: Coefficient
t value
Sig: significance level
VIF: variance inflation factor
R: correlation coefficient

Figure 1. Immunohistochemical staining for p53 in *H. pylori*-positive tumor tissue (intestinal type) and normal tissue of gastric cancer section
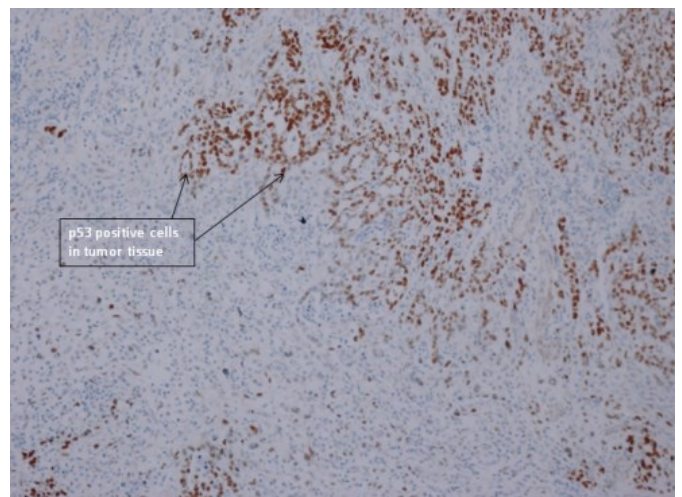

The nuclei of positive cells were stained brown in color. (Magnification: 40X)

Figure 2. Immunohistochemical staining for p53 in *H. pylori*-positive tumor tissue (intestinal type)
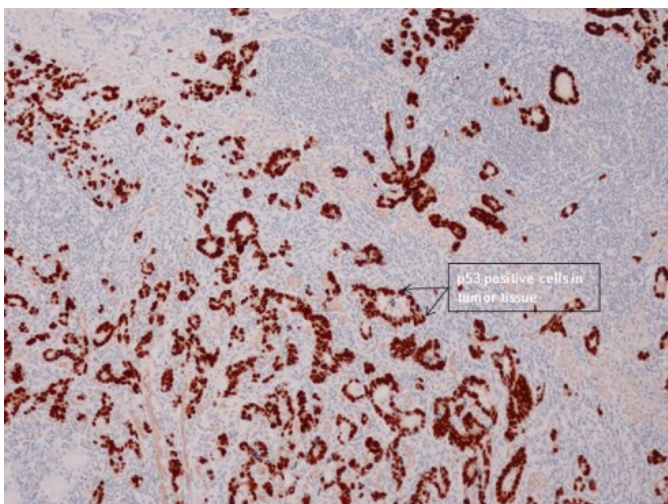
Positive cells were commonly found in the neck region of the gastric pits (arrows). (Magnification: 100X)

Figure 3. Immunohistochemical staining for p53 in *H. pylori*-positive tumor tissue (diffuse type) and normal tissue of gastric cancer section

The nuclei of positive cells were stained brown in color (arrows) (Magnification: 40X).

and the percentage was also high (70.4%) in our gastritis patients.

p53 alterations were shown to have an important role in GC development worldwide. Immunohistochemical analysis of p53 overexpression in our tumor tissue GC sections showed that the average staining was significantly higher in the presence of *H. pylori* than in the absence of *H. pylori* and also higher than that in the normal tissues, excluding *H. pylori.* Also, in our antral biopsy sections from gastritis patients, the average results of p53 expression were significantly associated with the presence of *H. pylori* and neutrophil infiltration. Previously, Polat *et al.* [12] investigated p53 expression in *H. pylori*-positive and -negative Turkish patients with gastritis using histological evaluation and reported a 10.9% overexpression of p53 in the presence of *H. pylori.* Similarly, Sadeghi *et al.* [24] showed that p53 expression was more prevalent in an *H. pylori*-positive group and indicated that this overexpression might contribute to the pathogenesis of gastritis and GC. However, Zhang *et al.* [25] reported no p53 protein expression in normal gastric mucosa but such protein was significantly increased in GC tissues in the presence of *H. pylori*, and they concluded that *H. pylori* infection might strengthen p53 mutation. On the other hand, Berloco *et al.* [16] reported earlier that *H. pylori* infection does not affect p53 expression in gastric mucosa and that p53 mutations did not correlate with *H. pylori* in GC patients in the Italian populations. In another study, no expression of mutant type p53 protein was found in *H. pylori*-positive or -negative gastric mucosa of the control group [19].

The role of *H. pylori* in the expression of p53 was studied earlier by Satoh *et al.* [26], who found that p53 positive cells in *H. pylori*-infected gastric mucosa before treatment decreased significantly one month after *H. pylori* eradication. Andre *et al.* [27] also suggested that *H. pylori* cagA-positive strains contribute significantly to p53 alteration in GC.

Murakami *et al.* [15] reported that *H. pylori* infection can induce p53 point mutations in gastric mucosa of gastritis patients that in turn leads to dysplasia or carcinoma. Similarly, Takeda *et al.* [28] also reported that an overexpression of p53 was associated with gastric mucosal alterations in early tumor tissues. p53 overexpression has been reported in 17% to 90.7% of invasive tumors. The degree of p53 expression correlates with the proliferative rate of the tumors, perhaps explaining the higher incidence of p53 positivity in intestinal versus diffuse GC [29].

Earlier studies have shown that p53 mutations can occur in the early stages of GC development, present even in normal mucosa, and increase in frequency during the progression of the developed cancer. Reports on the frequencies of p53 mutations showed a prevelance rate that varied between 0% and 77% in gastric carcinomas [29]. Since the majority of published reports on the detection of p53 expression varied in terms of the antibodies used, the method of detection, and the different interpretation approaches, it is not surprising to find contradictions in the results presented by those studies.

Determination of the genotype frequencies of p53 in our gastritis sections using PCR-RFLP showed no significant differences in the prevalence of allelic variations in both *H. pylori*-positive and -negative gastritis sections. Fenoglio-Preiser *et al.* [29] also showed no differences in p53 polymorphism in the presence or absence of *H. pylori* infection.

We have also found that p53 was significantly associated with the presence of *H. pylori*, invasion depth, and inflammation reaction in tumor but not normal tissues of GC sections. These observations show that *H. pylori* has a direct effect on tumor cell kinetics. In addition, the majority of tumors detected in our *H. pylori*-positive sections were of the intestinal type, emphasizing the important role of *H. pylori* in this process. In gastritis sections, p53 was significantly associated with the presence of *H. pylori* and neutrophil infiltration.

H. pylori infection affects p53 expression in tumor tissue of GC sections. The average p53 expression was significantly higher in tumor than in normal tissues. In gastritis sections p53 expression was significantly associated with *H. pylori*. Overall, the detection of p53 in tumor tissue of resected GC sections provided additional supportive evidence for the effect of *H. pylori* on its expression.

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