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The Participation of p53 and bcl-2 Proteins in Gastric Carcinomas Associated with *Helicobacter pylori* and/or Epstein-Barr Virus (EBV)

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Abstract

In the presented studies p53 and bcl-2 proteins expression were evaluated in samples of gastric carcinomas in patients with *Helicobacter pylori* or EBV or without *H. pylori*/EBV infection. The studies were conducted on 64 adult patients with gastric adenocarcinomas: 16 patients with *H. pylori* (*cagA*+)-positivity (group 1), 14 with EBV-positive tumours (group 2), 12 with *H. pylori*/EBV-positive tumours (group 3) and 22 patients with *H. pylori*/EBV-negative tumours (group 4). *H. pylori* presence in gastric tumour specimens was detected using Giemsa staining and bacterial culture technique. Moreover, *cagA* gene was detected using PCR. EBV infection was detected based on EBER presence in the tissue by RNA *in situ* hybridization. Expressions of p53 and bcl-2 proteins were analysed using immunohistochemistry. Expression of p53 was noted in 14 (84%) patients from group 1, 8 (57%) patients from group 2, 7 (58%) patients from group 3, and 19 (86%) patients from group 4, whereas expression of bcl-2 was noted in 12 (75%) patients from group 1, in 10 (71%) patients from group 2, 9 (75%) patients from group 3, and 6 (27%) patients from group 4. The obtained results allow the conclusion, that *H. pylori* (*cagA*+)-associated development of the gastric adenocarcinoma is determined by abnormalities in the p53 *protein* function and overexpression of anti-apoptotic bcl-2 protein, whereas EBV-associated adenocarcinomas seem to be related with apoptosis resistance associated with bcl-2 overexpression.

Key words: *Helicobacter pylori*, bcl-2, Epstein-Barr virus, gastric adenocarcinoma, p53

Introduction

It has been well documented that pathogenesis of the gastric cancer is a complex and multi-stage process, with progression in lesions of the mucous membrane from chronic gastritis to chronic atrophic gastritis, intestinal metaplasia, dysplasia and finally gastric carcinoma (Konturek *et al*., 2003; Carcas, 2014). A significant role of infection with *Helicobacter pylori* (*H. pylori*) in the carcinogenesis process was also well established, defining this bacterium as a class I carcinogen for gastric cancer (IARC, 1994). Moreover, involvement of infection with the Epstein-Barr virus (EBV) in etiopathogenesis of gastric cancer was also documented (Czopek *et al*., 2003; Iizasa *et al*., 2012; Chen *et al*., 2012). It is estimated that gastric carcinomas are in approximately 80% cases associated with *H. pylori* infection, whereas in approximately 10% they are associated with EBV infection (Wu *et al*., 2000; HCCG, 2001; Czopek *et al*., 2003; Palli *et al*., 2007). In 40 to 100% of *H. pylori* strains isolated from patients with gastric carcinomas the *cagA* (cytotoxin-associated gene A) (Palli *et al*., 2007) was detected. Crucial role in the carcinogenesis process is played by p53 protein (tumour protein *p53*) and bcl-2 protein (B-cell leukemia/lymphoma-2) (Ozaki and Nakagawara, 2011; Moldoveanu *et al*., 2014; Czabotar *et al*., 2014). p53 has a suppressive effect on the cancerous transformation through induction of apoptosis in cells with defected genome (Vermeulen *et al*., 2003). As for bcl-2, it is the strongest apoptosis inhibitor among all known cell proteins (Mérino and Bouillet, 2009). Nevertheless, it remains unclear whether in gastric carcinoma *p53* abnormalities and bcl-2 expression are dependent on presence of *H. pylori* or EBV infection. Therefore, in this study we investigated p53 and bcl-2 expression in gastric carcinomas in patients with *H. pylori*/EBV or without *H. pylori*/EBV infection.

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Experimental

Materials and Methods

Patients. The studies were conducted on 64 adult patients with histologically confirmed diagnosis of gastric adenocarcinoma. From patients who underwent routine upper endoscopy antral biopsies were taken. Cancer diagnosis (adenocarcinoma) was verified by analysis of hematoxylin and eosin stained preparations (Shibata *et al.,* 2001; Matsubara *et al*., 2004). Based on presence of infection with *H. pylori* or EBV, three study groups were distinguished. Group 1 included 16 *H. pylori*-positive patients (10 males and 6 females), 59 ± 12.4 years of age. The second group consisted of 14 EBV-positive patients (10 males and 4 females), 61 ± 6.7 years of age. The third group involved 12 *H. pylori*-positive and EBV-positive patients (9 males and 3 females), 62 ± 8.3 years of age. The fourth group included 22 *H. pylori*-negative and EBV-negative patients (17 males and 5 females), 65 ± 7.5 years of age.

H. pylori and *cagA* gene detection. Biopsies of gastric mucosa were plated on Columbia agar (bioMerieux) with 7% sheep blood with antibiotic supplement (*Helicobacter pylori* Selective Supplement (Dent), Oxoid). The incubation was conducted in microaerophilic conditions (Genbox microaer, bioMerieux) for 4 to 6 days at the temperature of 37°C. The isolated *H. pylori* strains were identified based on Gram staining as well as by production of urease, catalase, and oxidase (Szkaradkiewicz *et al*., 2010). For detection of *cagA* gene, the diagnostic kit of PCR-*H. pylori* (DNA Gdańsk) was used. PCR product was subjected to electrophoresis in the 2% agar gel and the result was read after staining with ethidium bromide. Presence of PCR reaction in form of a product of 445 base pairs in size was accepted as the positive test result. Moreover, presence of *H. pylori* was identified in the tissue sections using Giemsa staining (Lee and Kim, 2015).

Detection of EBER (EBER 1 and EBER 2) in tissue material. EBV DNA product in the form of untranslated RNA (EBER 1 and EBER 2) particles was detected in tissue material using *in situ* hybridization (ISH) (Niedobitek and Herbst, 2006; Izadi and Taheri, 2010). The tissue material was fixed in formalin and embedded in paraffin. Five μm thick sections were deparaffinised and digested with proteinase K for 30 min at 37°C, and washed in DEPC. This was followed by inactivation of proteinase K in 0.4% PFD solution for 20 min at 4°C. The hybridisation was performed using a fluorescein-labelled RNA probe of 15 nucleotides in length (PNA Probe/FITC; DakoCytomation) for 15 h at 37°C. After a thorough washing in SWS solution (DakoCytomation) the product was detected using FITC/AP-specific antibodies. BCIP/NBT (PNA ISH Detection kit; DakoCytomation) was used as a substrate.

Immunohistochemical analysis of TP53 protein (p53). Five μm thick sections from the same tumour blocks, used for EBER detection, were immunohistochemically analysed for the presence of p53 protein. Following removal of paraffin, rehydration and blocking of endogenous peroxidase activity with 3% H_2O_2 in distilled water, the tissue was incubated in 10 mM citrate buffer (pH 6.0) in a microwave oven (1000 W) for 15 min. Subsequently, the tissue was rinsed with phosphate-buffered saline (PBS) and treated with the primary antibody, DO-7 mouse anti-human p53 protein (DakoCytomation) in 1:50 dilution, employing 30 min incubation at room temperature. For visualisation of the reaction EnVision+ System-HRP (horseradish peroxidase) kit (DakoCytomation) was used. PBS was substituted for primary antibodies as the negative control. As a positive control, a section of colorectal cancer with high p53 expression was used. In all slides the cells stained per 1000 carcinoma cells were scored. The section was considered p53 positive when at least 10% cell nuclei were stained (Nasierowska-Guttmejer *et al*., 2000).

Immunohistochemical analysis of bcl-2 protein. Five μm thick sections from the same tumour blocks were immunohistochemically analysed for the presence of bcl-2 protein. Following removal of paraffin, rehydration and blocking of endogenous peroxidase activity with 3% H_2O_2 in distilled water, the tissue was incubated in 10 mM citrate buffer (pH 6.0) in a microwave oven (1000 W) for 15 min. Subsequently, the tissue was rinsed with phosphate-buffered saline (PBS) and treated with the primary antibody, FLEX monoclonal mouse anti-human bcl-2 oncoprotein (DakoCytomation) in 1:50 dilution, employing 30 min incubation at room temperature. For visualisation of the reaction EnVision+ System-HRP kit (DakoCytomation) was used. Finally, the site of immunoprecipitate formation was detected by applying diaminobenzidine (DAB; Sigma). PBS was substituted for primary antibodies as the negative control. As a positive control, a section of colorectal cancer with high bcl-2 expression was used. In all slides the cells stained per 1000 carcinoma cells were scored. The section was considered bcl-2 positive when at least 10% cell cytoplasms were stained (Nasierowska-Guttmejer, 2001).

Statistical analysis. Differences in frequencies of p53 and bcl-2 positive results were compared with Fisher's exact test. Relationships with *P*-values higher than 0.05 were considered insignificant.

Results

***H. pylori* and EBV infection.** Among studied patients with gastric carcinoma, were 16 (25%) patients with *H. pylori* infection constituted group 1 (Fig. 1), in which presence of *cagA* gene was detected in 14 iso-

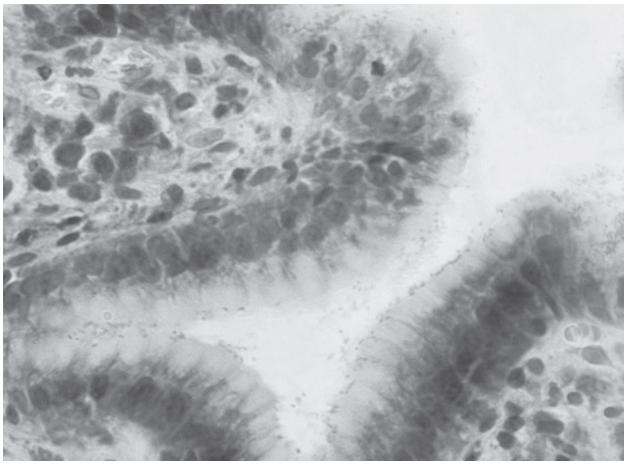

Fig. 1. *H. pylori*-positive gastric adenocarcinoma.

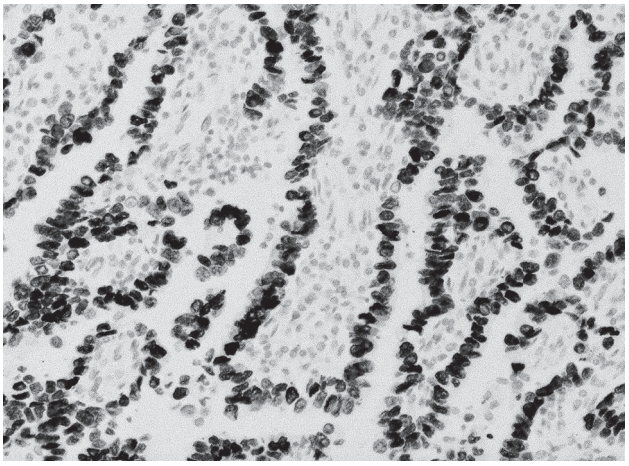

Fig. 3. Expression of p53 in *H. pylori* (*cagA*+)-positive gastric adenocarcinoma.

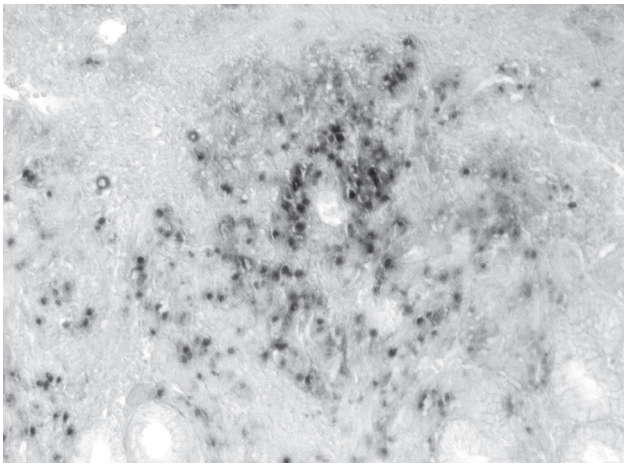

Fig. 2. EBER-positive gastric adenocarcinoma.

lated strains of the bacteria. Group 2 included 14 (22%) patients proved to be EBV-positive (Fig. 2). Group 3 included 12 (19%) patients found to be *H. pylori*-positive and EBV-positive. Group 4 included 22 (34%) patients in whom neither EBV infection nor *H. pylori* infection could be detected.

Expression of p53 protein. Results of detection of p53 protein are summarized in Table I. In group 1, among 16 patients with *H. pylori*-associated gastric carcinoma nuclear expression of p53 in most tumour cells was demonstrated in 14 (87%) cases (Fig. 3). In the remaining 2 (13%) cases no p53 could be detected. In group 2, among 14 patients with EBV-associated gastric carcinoma in 8 (57%) cases nuclear expression of p53 protein was noted in most tumour cells. In the remaining 6 (43%) cases no p53 could be detected. In group 3, among 12 patients with *H. pylori*-positive and EBV-positive gastric carcinoma in 7 (58%) cases nuclear expression of p53 protein was noted in most tumour cells. In the remaining 5 (42%) cases no p53 could be detected. In the group 4, among 22 patients with EBV-negative and *H. pylori*-negative gastric carcinoma, p53 protein nuclear expression was noted in most tumour cells in 19 (86%) cases. No p53 was detected in the remaining 3 (14%) cases. Expression of p53 in individual groups of patients, 1 to 3, with *H. pylori* and/or EBV infection, was not significantly more frequent than in patients without these infections ($P = 1.0000$, $P = 0.1111$ and $P = 0.0975$, respectively).

Expression of bcl-2 protein. Results of detection of bcl-2 protein are summarized in Table I. In group 1, among 16 patients with *H. pylori*-associated gastric carcinoma expression of bcl-2 was demonstrated in most tumour cells in 12 (75%) cases (Fig. 4). In the remaining 4 (25%) cases no bcl-2 could be detected. In

Table I
Expression of p53 and bcl-2 in gastric carcinoma in patients with or without documented infection with EBV or *H. pylori*.

Groups of patients with gastric carcinoma (n)	Number of patients with expression (%) of:			
	p53		bcl-2	
	(+)	(–)	(+)	(–)
Group 1 *H. pylori*-positive (cagA+) (n = 16)	14 (87%)	2 (13%)	12 (75%)*	4 (25%)
Group 2 EBV-positive (n = 14)	8 (57%)	6 (43%)	10 (71%)*	4 (29%)
Group 3 *H. pylori*-positive, EBV-positive (n = 12)	7 (58%)	5 (42%)	9 (75%)*	3 (25%)
Group 4 *H. pylori*-negative, EBV-negative (n = 22)	19 (86%)	3 (14%)	6 (27%)	16 (73%)

* significantly different as compared to group 4.

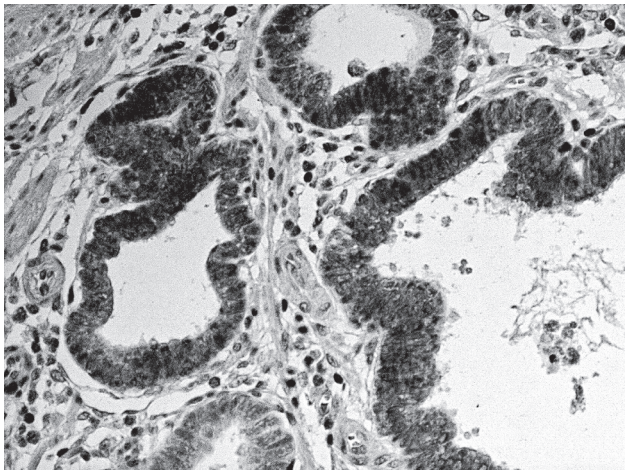

Fig. 4. Expression of bcl-2 in *H. pylori* (*cagA*+)-positive gastric adenocarcinoma.

group 2, among 14 patients with EBV-associated gastric carcinoma in 10 (71%) cases expression of bcl-2 protein was noted in most tumour cells. In the remaining 4 (29%) cases no bcl-2 could be detected. In the group 3, among 12 patients with *H. pylori*-positive and EBV-positive gastric carcinoma, bcl-2 protein expression was noted in most tumour cells in 9 (75%) cases. No bcl-2 was detected in the remaining 3 (25%) cases. In the group 4, among 22 patients with EBV-negative and *H. pylori*-negative gastric carcinoma, bcl-2 protein expression was noted in most tumour cells in 6 (27%) cases. No bcl-2 was detected in the remaining 16 (73%) cases. Expression of bcl-2 was found to be more frequent in the group of patients with *H. pylori* infection ($P = 0.0036$), the group of EBV-associated gastric carcinoma patients ($P = 0.0159$) and the group of *H. pylori*-positive and EBV- positive gastric carcinoma patients ($P = 0.0120$) but no difference in the frequency could be demonstrated in the groups of patients with EBV-negative and *H. pylori*-negative gastric carcinoma.

Discussion

In the present study, expressions of p53 and bcl-2 proteins have been examined in *H. pylori*- and/or EBV-associated and *H. pylori*/EBV-negative gastric carcinomas. Presence of *H. pylori* in the isolated samples of gastric tumour has been directly detected using Giemsa staining method of histological sections (Lee and Kim, 2015), and the bacterial culture from the obtained samples. In turn, infection with EBV has been detected on the basis of EBER presence in tumour cells. It has already been well documented that EBER is expressed in all forms of EBV latency and can always be detected by in situ hybridization (Murray and Young, 2002; Iizasa *et al*., 2012; Chen *et al*., 2012). Thus, the techniques employed in this study provided a reliable and a highly specific diagnosis of *H. pylori*/EBV infection.

The obtained results have shown that in most cases of both *H. pylori* (*cagA*+)-positive (group 1) and *H. pylori*-negative gastric carcinomas (group 4) a disturbed function of *p53* gene is observed. The data may suggest that *H. pylori* (*cagA*+) is not responsible for development of mutation in *p53* gene and the demonstrated overexpression of p53 in cancerous tissues. The suggestions are supported by results of earlier studies conducted by Berloco *et al.* (2003) and Targa *et al*. (2007). On the other hand, however, Suriani *et al.* (2008) demonstrated *H. pylori* CagA-specific antibodies in sera of 82.6% patients with *H. pylori*-negative gastric cancer. This indicates that patients with gastric carcinoma, even if *H. pylori*-negative at the time of this testing, might have been infected by *H. pylori* before onset of the disease and in the meantime the pathogen underwent eradication. Taking the above into account, it is possible that at least a proportion of group 4 patients have earlier been infected with *H. pylori* (*cagA*+). In addition, the percentage of patients manifesting expression of p53 in group 1 as well as in group 4 was high while results of p53 expression in all examined groups manifested no significant differences. In such a context it seems probable that *H. pylori* (*cagA*+) may induce *p53* mutations already at the early phase of carcinogenesis. The conclusion seems to be supported by studies of Kuniyasu *et al*. (2000) and of Kountouras *et al.* (2008) who detected *p53* mutations in at least 30% cases of intestinal metaplasia and in 58% patients with gastric dysplasia. Moreover, recent data indicate that infection with *H. pylori* may lead to an increased expression of *p53* apoptosis inhibitor (iASP), mediating the anti-apoptotic process (Meng *et al*., 2013). Thus, the pathogen may induce mutations of *p53* gene, and also its deregulation, which results in promotion of gastric cell proliferation and cancerous transformation.

However, proportions of patients with p53 expression were lower in the analysed groups 2 and 3. Thus, the obtained results may suggest that abnormalities in *p53* gene in gastric cancer are not EBV-dependent, consistent with results of our earlier study (Szkaradkiewicz *et al*., 2006). Such a suggestion is supported by other studies indicating that *p53* gene mutations are seldom identified or are absent from EBV-associated neoplasias (Petit *et al*., 2001; Wang *et al*., 2005). In addition, expression of p53 has been observed more frequently in group 4 than in groups 2 and 3. The observation corresponds to the earlier published studies of Ishii *et al.* (2004). The authors showed that frequency of p53 overexpression was lower in EBV-positive than in EBV-negative early stage gastric adenocarcinomas. Thus, the data might suggest that EBV interacts with *p53* gene, preventing or reducing expression of p53. In such a context, the suggestion that EBV infection promotes the development of gastric cancer by a *p53* pathway-dependent mechanism seems doubtful.

In turn, in analysis of our results devoted to bcl-2, we have documented expression of the anti-apoptotic protein in most cases of *H. pylori* (*cagA*+)-positive gastric carcinomas (group 1). In parallel, frequency of bcl-2 expression has been higher in patients of groups 1–3 than in patients of group 4. Therefore, it can be concluded that chronically developing infection with *H. pylori* (*cagA*+) determines an increase in bcl-2 expression, which in turn promotes development of gastric cancer. The conclusion finds support in earlier publications (Konturek *et al*., 2003; Lima *et al*., 2008). Moreover, Bartchewsky *et al*. (2010) demonstrated that in *H. pylori* infection, manifested by chronic gastritis, an increased Bax expression initiates cell apoptosis, but in patients with gastric cancer the pathogen induces the anti-apoptotic gene *bcl-2*. In addition, the presented *in vitro* investigations demonstrate that the *H. pylori*-secreted vacuolating cytotoxin – VacA exerts a pro-apoptotic effect on epithelial cells, acting in an opposite manner to anti-apoptotic activity of CagA (Oldani *et al*., 2009; Matsumoto *et al*., 2011). Therefore, the early period of infection with *H. pylori*, due to action of VacA, develops with overexpression of the pro-apoptotic protein while translocation of CagA to epithelial cells mobilizes overexpression of bcl-2 and, as a result, assures cellular survival. Recently presented data indicate also that CagA may activate human epidermal growth factor receptor 2 (HER2) in gastric cells (Shim *et al*., 2014). Activation of the oncogene plays an important role in cell proliferation and tumorigenesis (Gravalos and Jimeno, 2008; Bollig-Fischer *et al*., 2010).

In turn, results presented in this study, related to bcl-2 in patients carrying only EBV-positive (group 2) versus EBV-negative gastric carcinomas (group 4) have demonstrated significant differences in expression of the protein between the two groups. The results contradict studies of Ishii *et al*. (2001) and Lima *et al*. (2008) but are consistent with earlier results of Kume *et al*. (1999), who documented high bcl-2 expression in EBV-positive gastric carcinomas. The obtained by us data allow to conclude that EBV-infection induces bcl-2 expression in gastric epithelial cells. It is already well known that EBV latent membrane protein (LMP)-1 inhibits cell apoptosis, elevating levels of bcl-2 (Zimber-Strobl *et al*., 1996; Thompson and Kurzrock, 2004). Nevertheless, investigations on EBV gene expression in gastric carcinomas documented type I latency pattern, that used to be restricted to EBV nuclear antigen (EBNA)-1, the $EBER_s$, and secretion of BARF0 gene proteins (Thompson and Kurzrock, 2004; Sivachandran *et al*., 2012). At the same time, until now no reports have appeared which would document upregulation of bcl-2 – associated with EBNA-1 and BARF0 in epithelial cell lines (Fu *et al*., 2013). Nevertheless, EBERs may induce up-regulation of bcl-2 in immortalized nasopharyngeal epithelial cells, causing their tumorgenesis (Wong *et al*., 2005). Moreover, it is demonstrated that BARF0 may stimulate expression of HER2 in epithelial cells, which may point to involvement of still another molecular mechanism in pathogenesis of EBV-infected gastric carcinoma cells (Lin *et al*., 2007). Such data, in the context of results presented by us seem to indicate that also the development of EBV-associated gastric carcinomas is dependent on bcl-2 anti-apoptosis activity.

In conclusion, our current studies indicate that the development of *H. pylori* (*cagA*+)- positive gastric adenocarcinomas is linked to abnormalities in function of p53 protein and the overexpression of anti-apoptotic bcl-2. On the other hand, the development of EBV-positive gastric adenocarcinomas seems to be related with apoptosis resistance associated with bcl-2 overexpression.

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