Expression of the *P65* gene in gastric cancer and in tissues with or without *Helicobacter pylori* infection*

E. BALCERCZAK[1], T. JANKOWSKI[2], A. BECHT[1], M. BALCERCZAK[1,2], R. JANIUK[2], D. JESIONEK-KUPNICKA[3], J. SZTOMPKA[2], M. MIROWSKI[1**]

[1]Department of Pharmaceutical Biochemistry, e-mail: mirowski@ich.pharm.am.lodz.pl, Molecular Biology Laboratory, 90-151 Lodz, Poland; [2]Department of Surgery Hospital, Leczyca, Poland; [3]Department of Tumor Pathology, Medical University, Lodz, Poland

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A 65-kDa tumor-associated protein (P65) is a potential non-specific tumor marker expressed by many types of tumor cells. Our recent studies indicate that *P65* gene expression is connected with poor prognosis for the patients with colorectal cancer. In the present study *P65* gene expression was determined by means of RT-PCR in the group of 22 gastric cancer and adjacent normal gastric mucosa. Its presence was correlated with some parameters of clinical staging. *P65* gene expression was also determined in 102 tissue antral gastric endoscopic biopsy specimens from the patients suspected of *H. pylori* infection. The presence of *H. pylori* infection was determined by urease test. We found that in the group of gastric cancers, similarly to colorectal cancer, *P65* gene expression was connected with poor clinicopathological parameters as T3, lymph nodes and distant metastases. There was no dependence between *P65* gene expression and *H. pylori* infection. However, more often *P65* gene expression was detected in the group of infected men than women. There was also a statistically significant dependence between age and *P65* gene expression in the group of people above 60 years old. It could be then postulated that *P65* gene expression is connected with poor prognosis for the patients suffering from gastric cancer and that this expression does not depend on *H. pylori* infection.

Key words: P65 gene expression, Helicobacter pylori, gastric cancer

The 65 kDa tumor-associated protein (P65) was described as a potential non-specific tumor marker which is expressed by many types of tumor cells. It was employed to monitor a carcinogenic process in rodent models of liver and skin [7, 10] as well as mammary gland adenocarcinoma [11] and in immunohistochemical analysis of paraffin-embedded tissue slides from human infiltrating ductal breast cancers [9, 12]. It was proved that *P65* antigen may be useful in identification of precancerous changes and may help in the screening examination of women who have a high risk for cancer development.

We have determined the *P65* gene transcript by means of the reverse transcriptase polymerase chain reaction (RT-PCR) in various types of leukemia [2], breast [3], prostate [4] and colorectal cancers [5]. In colorectal tumors the presence *of P65* gene expression was connected with lymph nodes involvement and distant metastases. The detection of *P65* gene transcript in primary tumors might thus serve as an indicator of a poor prognosis [5].

It is already known that *Helicobacter pylori* causes a chronic gastritis and long term infection may lead to increased cell proliferation and may induce DNA injury with irreversible genetic lesions what in consequence induces gastric cancer. In 1994 the International Association for Research on Cancer classify *H. pylori* as a group I carcinogen in human. However, the mechanism underlying the link between *H. pylori* infection and gastric cancer remains still to be elucidated [1].

In this study the *P65* gene expression in gastric cancers was compared with some parameters of clinical staging and the dependence between *H. pylori* infection and the *P65* gene expression was checked.

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**Corresponding author

Material and methods

Tissues. Paired tissue samples from 22 gastric cancers and adjacent normal gastric mucosa obtained from patients of the Oncological Centre in Lodz (Poland) were frozen in liquid nitrogen immediately after surgical resection and stored at –80 °C until processed.

One hundred and two tissue antral gastric endoscopic biopsy specimens from the patients suspected of *H. pylori* infection were obtained from the Hospital in Leczyca (Poland). One half of the tissue was taken for urease test analysis and the second was put directly to the plastic tube containing reagent for RNA isolation (phenozol with inhibitors of endogenous RNA-ases) and stored at 4 °C until isolation procedure.

Urease test. The biopsy fragments removed from the gastric antrum were placed on a surface of plastic slide covered with immobilized gel containing urea (Pro-Med PPH Gora Kalwaria, Poland) and proceeded according to instruction manual. On the basis of color intensity *H. pylori* infection was determined as high (+++), medium (++) and low (+).

RNA extraction. RNA was isolated by Total RNA Prep Plus Minicolumn Kit (A&A Biotechnology, Gdansk, Poland) based on RNA isolation method developed earlier [6].

Reverse transcriptase reaction (RT). cDNA was obtained by Enhanced Avian RT-PCR (Sigma). Reaction mixture containing RNA in final concentration 0.1 µg/µl, 1 µl oligo(dT) 18 primer (0.5 µg/µl), and 6 µl deionized, nuclease free water was prepared. After mixing and spinning down the mixture was incubated at 70 °C for 10 min, then chilled on ice. After incubation, the following components were added: 2 µl eAMV-RT 10x reaction buffer, 1 µl ribonuclease inhibitor (20 U/µl), 1 µl 10 mM dNTP mix, 1 µl Enhanced Avian Reverse Transcriptase (1 U/µl). Finally, the mixture was incubated at 45 °C for 50 min.

PCR. PCR mixture contained: 1.5 mM $MgCl_2$, mix dNTPs, 0.5 U Taq Polymerase, reaction buffer and 0.5 µM of each primer. DNA was amplified in 35 cycles using the parameters: denaturation (94 °C; 30 sec.), annealing (57 °C; 30 sec.), extension (72 °C; 30 sec.). As a control *ß-actine* gene was amplified. The PCR products were separated by electrophoresis in 1.5% agarose gel.

Statistics. Statistical analysis was done on the basis of exact Fisher test.

Table 1. *P65* gene expression in gastric carcinoma (number of cases).

Clinical stage	TNM	*P65* gene expression		Dependence (exact Fisher test)
		Positive	Negative	
Depth of tumor invasion	T1-T2 T3	4 3	15 0	p=0.0227
Lymph node invasion	N-negative N-positive (N1-N3)	1 6	13 2	p=0.0023
Metastases	M0 M1	4 3	15 0	p=0.0226

TNM classification (T – tumor, N – lymph node metastases, M – distant metastases).

Results

The *P65* gene transcript in 22 paired gastric cancer specimens and adjacent normal gastric mucosa was determined. The expression of *P65* gene was observed in 7 cases of cancers. Unexpectedly, 5 cases of the control mucosa were also positive. For two pairs, expression of *P65* gene was observed in both cancer and normal mucosa cases. The *P65* gene expression was compared with some clinicopathological parameters (TNM classification) such as the depth of tumor invasion (T), lymph node metastases (N), distant metastases (M) (Tab. 1).

Majority of the cases investigated in this study belonged to the group classified as T1–T2. The *P65* gene expression was observed more often in the advanced tumors (T3). There was a statistically significant dependence between the expression of *P65* gene and the depth of tumor penetration (p=0.0227). Another parameter analyzed in this study was the expression of *P65* gene in cases with and without lymph node metastases. This gene expression was detected in 6 from 8 cases with metastases to lymph nodes. These data also revealed significant statistical dependence between the *P65* gene expression and invasion to lymph nodes (p=0.0023). The last investigated parameter concerned distant metastases. In all cancers with distant metastases (3 cases) the *P65* gene expression was observed. There was a statistically significant dependence between *P65* gene and distant metastases (p=0.0226). Example of RT-PCR analysis of the *P65* gene expression in gastric cancers is presented in Figure 1.

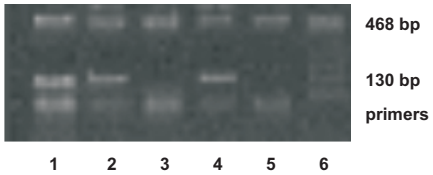

Figure 1. Example of Multiplex RT-PCR analysis of the *P65* and *ß-actine* genes expression in gastric cancer (lane 1 and 2) and in *H. pylori* infected patients (lane 3 to 5). Lane 1,2 and 4 positive *P65* gene expression (130 bp). Lane 6 – molecular size markers.

It is already well documented that *H. pylori* causes critical alterations in gastric mucin structure. Long-term bacterial infection is associated with the development of gastritis and peptic ulcer and is presumed to be a risk factor for gastric cancer. In our further studies we would like to answer the question whether there is a dependence between the *P65* gene expression and presence/absence of bacterial infection in biopsy specimens taken from patients with suspicion of *H. pylori* infection.

The antral gastric endoscopic biopsy specimens (n=102) were checked by semi-quantitative urease test for detection of *H. pylori* infection and *P65* gene expression. *H. pylori* positive cases (n=78) were divided into three groups according to the grade of infection. In 18 cases the grade of *H. pylori* infection was determined as high (+++), in 20 as medium (++), in 40 as low (+) and 24 did not reveal *H. pylori* infection. *P65* gene expression was detected in 37 cases, 27 out of them were *H. pylori* positive (Tab. 2). *P65* gene expression was observed mainly in biopsy specimens classified as a low grade (+) of *H. pylori* infection. Example of the RT-PCR analysis of the *P65* gene expression in *H. pylori* infected patients is presented in Figure 1. There was no statistically significant dependence between the *P65* expression and *H. pylori* infection (p=0.5298) as well as infection grade.

The analyzed population consisted of 48 women and 52 men (for 4 patients information about the gender was missing). In the group of men 40 cases were *H. pylori* positive but only in 10 cases the *P65* gene expression was observed (Tab. 3). In the group of men without *H. pylori* infection, in 7 out of 12 cases the *P65* gene expression was detected. There was a statistically significant dependence between *P65* gene expression and *H. pylori* infection in the group of men (p=0.0412). In the group of women 37 were *H. pylori* positive, the *P65* gene expression was observed only in 17 of them. Of 11 cases without *H. pylori* infection only in 3 women the investigated gene expression was detected. The statistical analysis also did not reveal dependence.

The *P65* gene expression was compared with the age of patients. In the whole examined population there was no statistically significant dependence between *P65* gene expression and the age of patients. We divided investigated population into four groups according to the age: first group <20, second 20–40, thrid 40–60 and fourth >60 years (Tab. 4). Only in the last group, people above 60 years, there was a dependence between *P65* gene expression and age of patients (p=0.0466).

Table 2. Grade of *H. pylori* infection and presence/absence of *P65* gene expression in the group of 102 antral gastric endoscopic biopsy specimens

P65 gene expression	Grade of H. pylori infection							
	(+++)		(++)		(+)		(–)	
	N	%	N	%	n	%	n	%
Presence (n=37)	9	24	5	14	13	35	10	27
Absence (n=65)	9	14	15	23	27	42	14	22

p=0.5298 – insignificant dependence

Table 3. Expression of *P65* gene in the group of men with or without *H. pylori* infection

Number of cases (n= 52)	*P65* gene expression	
	Presence	Absence
H. pylori positive	10	30
H. pylori negative	7	5

p=0.0412 – significant dependence

Table 4. Expression of *P65* gene in the group of 98 antral gastric endoscopic biopsy specimens divided according to the age of patients

P65 gene expression	Age of patients							
	<20		20–40		40–60		>60	
	n	%	n	%	n	%	n	%
Presence (n=37)	2	2	9	9	7	7	19	19.5
Absence (n=61)	4	4	11	11	27	28	19	19.5

p=0.0466 – significant dependence

Discussion

Our recent study assessed clinical utility of the estimation of *P65* gene expression in patients with colorectal cancer [5]. A statistical correlation was found between *P65* gene expression and depth of wall penetration, invasion to lymph nodes, distant metastases and vessel invasion. The incidence of *P65* positive cases increased with the increasing clinical stage of Astler-Coller's classification. Potential prognostic value of *P65* gene transcript determination in colorectal cancer stimulated us to study *P65* gene expression in gastric cancers. The presence of *P65* expression was found only in 1/3 of the analyzed cases which may suggest that *P65* gene is not crucial in gastric neoplastic transformation. Unexpectedly, about 1/4 of control gastric mucosa specimens was also *P65* positive. It may be explained probably by high proliferation potential of gastric mucosa cells and their constant exposure to chemical carcinogens what may increase different mutations rate. Another explanation could be the fact that these control tissue were taken and classified as nonneoplastic during surgical operation on the basis of macroscopic appearance of mucosa. The presence of micrometastases connected with embolisms of cancer cells in lymph vessels in submucosa may be a reason of *P65* gene expression appearence in these theoretically normal mucosa. Till now, we have not been able to detect *P65* gene transcript in control tissue of the breast [3], bone marrow [2] and colorectal mucosa derived from healthy patients [5] but this gene expression has been observed in high percentage in benign prostatic hyperplasia (BPH)

To assess clinical utility of the estimation of *P65* gene expression in gastric cancer we have compared it with several clinicopathological parameters. The data revealed significant statistical dependence between the *P65* gene expression and depth of tumor, lymph nodes invasion as well as distant metastases what may indicate poor prognosis for the patients. Therefore, we suggest that *P65* may participate in growth- and differentiation-related tumor progression events of gastric cancer. Similar tendency has been observed for the patients with colorectal cancer [5].

The association between *Helicobacter pylori* infection and

gastric malignancies has been suggested during the last decade. This bacteria appears to play a prominent role in the very initial steps as causative agent of chronic gastritis which under the influence of different factors usually leads to atrophy, metaplasia, dysplasia and cancer development. Recent work with the use of a Mongolian gerbil treated with chemical carcinogens, has proven that *H. pylori* infection may be considered as a promotor in gastric carcinogenesis [14]. It was also postulated that gastric mutant frequency in Big Blue transgenic male mice (C57B1/6) inoculated with *H. pylori* was 4-fold higher in infected mice [14]. Significant progress in the area of clarification of the exact mechanisms underlying the link between *H. pylori* infection and gastric carcinogenesis has been made by employing cDNA microarray analysis that allows to detect broad patterns of differentially expressed genes induced by *H. pylori* in the gastric cancer cell line (AGS) cocultured with *H. pylori* in comparison to uninfected AGS cells. From 6000 genes present in the array approximately 200 genes showed marked changes. Among them genes involved in the processes which dysregulation may induce neoplastic transformation i.e. *c-jun*, *jun-B*, *c-fos*, *cyclin D1*, *serine threonine kinase prim-1* and *ATF3* [13]. As we have described earlier [10] P65 antigen can be detected in the very early stages of chemical hepato- and skin carcinogenesis. That is why we would like to get an answer whether *P65* gene expression may be connected with *H. pylori* infection. We have analyzed about 100 antral gastric endoscopic biopsy specimens for the presence of *P65* gene transcript. The highest percentage of *P65* gene expression was noticed in biopsy specimens where the grade of *H. pylori* infection was determined as low. There was no statistically significant dependence between them. In the group of men and also in the whole group of patients (women and men) with age over 60 we found statistically significant dependence between *P65* gene expression and *H. pylori* infection. The lack of dependences between *P65* gene expression and medium and high grade of *H. pylori* infection suggests that *H. pylori* is not involved in the process of *P65* gene activation. This hypothesis is confirmed by the fact that the above mentioned dependence was noticed in the patients with age over 60 when the probability of mutation is higher. Furthermore, *H. pylori* has been suggested to be a tumor-promoter in gastric carcinogenesis [15] whereas, it has been proven that anti-P65 antibodies detected only preneoplastic foci that were tumor-promoter independent [7, 8].

In the case of gastric cancers *P65* gene expression, similarly to colorectal cancers [5], is connected with poor prognosis for the patients because its expression is detectable in cases with lymph nodes and distant metastases. *P65* gene expression does not depend on *H. pylori* infection. The final answer about the mechanism of *P65* gene activation and its role in gastric carcinogenesis will be possible after cloning and sequencing the gene and employing microarray analysis containing cDNA sequences typical for genes that are crucial for gastric carcinogenesis, including the *P65* gene.

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