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ORIGINAL ARTICLE

NFKB2 gene expression in patients with peptic ulcer diseases and gastric cancer

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Abstract

Gastric cancer is one of the most common worldwide types of cancer. It is a multifactorial disease and both environmental and genetic factors play an important role in its etiology. Evaluation of the relative expression level of *NFKB2* gene in two groups of patients: peptic ulcer and gastric cancer and its role in the pathomechanism of these diseases was the aim of this study. RNA was isolated from: 79 samples of peptic ulcer, 22 gastric cancer and 11 control tissue. The real-time PCR technique was used to study the expression of *NFKB2* gene. The relative expression level of *NFKB2* gene was a variable in all three studied groups. The relative *NFKB2* gene expression depends on the type of a disease. Peptic ulcer cases showed the increased relative *NFKB2* gene expression to control group ($p = 0.0000$). Cancer cases presented decreased relative *NFKB2* gene expression to normal stomach tissue ($p = 0.0183$). There are statistically important differences in the investigated gene expression between peptic ulcer, where the expression level is higher comparing to gastric cancer and control tissue which confirmed that such an activation is connected with an inflammatory process. The relative expression level of *NFKB2* is decreased in cancer cases as opposed to control tissue and peptic ulcer cases which could suggest that during carcinogenesis of gastric cancer inhibition of NF-kB pathway takes place which could be a promising factor for patients.

Keywords Expression · Peptic ulcer · *NFKB2* · Gastric cancer · Real-time PCR

Introduction

Gastric cancer is the fourth most common cancer incident and the second cause of cancer death, about 738,000 each year [1]. The incidence rates vary wildly between men and women, in men the rates are two- to threefolds higher than in women, among nations are observed more frequently in East Asia, East Europe and South America [1]. Gastric cancer is a multifactorial disease and both environmental and genetic factors play an important role in its etiology, but the mechanism of pathogenesis is still unclear. Some of the risk factors, such as age and sex are not modifiable, whereas smoking and *Helicobacter pylori* infection could be changed [2]. There are still many gaps in our knowledge concerning causes, early detection of gastric cancer and its connection with chronic peptic ulcer disease, that is why dysregulation of several genes and pathways that could play an essential role during gastric carcinogenesis, like Wnt/β-catenin, Hippo and Notch signalling, nuclear factor-kB in peptic ulcer and gastric cancer development should be investigated [3, 4]. NF-kappa-B is a transcription factor present in almost all cell types and is the endpoint of a series of signal transduction events that are initiated by a vast array of stimuli related to many biological processes, such as: inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. NF-kB is a family of bipartite transcription factors that include NFkB1, NFkB2, c-Rel, RelA, and RelB [5]. NF-kappa-B is controlled by various mechanisms connected with post-translational modification, interactions with other factors or inhibitors like NF-kappa-B inhibitor (I-kappa-B) family that kept NF-kappa-B in the cytoplasm as an inactive form [6, 7]. I-kappa-B is phosphorylated by I-kappa-B kinases (IKKs), subsequently degraded, thus releasing the active NF-kappa-B complex which translocates to the nucleus [8, 9]. NF-kB is activated by inflammatory

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factors like like IL-8 also by *H. pylori* infection during the development of peptic ulcer through IkB kinase complex phosphorylates IkB, and then degradation of IkB [10]. Also HuR, a RNA-binding factor that is a direct transcript target of NF-kB is activated in gastric cancer cell lines, its activation has proliferative and anti-apoptotic effects on gastric cancer [11]. The modified expression of NF-kB has anti-apoptotic effects and leads to drug resistance in GC.

The aim of this study was to evaluate the relative expression level of *NFKB2* gene in two separate groups of patients: peptic ulcer and gastric cancer, connection with some clinically important parameters and its role in the pathomechanism of these diseases.

Material and methods

Patients

The investigated group consisted of two independent groups of patients. The first group comprised 79 RNA samples (43 females; 36 males; the median age of the group was 55 years) isolated from biopsies specimens of gastric mucosa taken during a routine gastroscopy test from patients with peptic ulcer diagnosed at the Department of Surgery, District Hospital, Leczyca Poland. The presence of *Helicobacter pylori* infection and its status was stated by rapid urease test, at the time of gastroduodenoscopy. The second group included 22 cases (8 females; 14 males; the median age of the group was 66 years) RNA samples isolated from tissue specimens taken during operations from patients with gastric cancer diagnosed at the Department of Pathology, Medical University of Lodz, Poland. 11 out of 22 normal tissue samples from patients were collected beyond the margin of cancer tissue. These 11 samples were considered macroscopically as healthy (control group). The investigation was in accordance with the principles of the Declaration of Helsinki and was approved by the Ethical Committee of the Medical University of Lodz (RNN/195/13/KE). All individuals included in the study gave their informed consent.

Rapid urease test

Diagnosis of *Helicobacter pylori* infection, only in the group of patients with peptic ulcer, was performed by rapid urease test (Institute of Food and Nutrition, Poland) at the time of gastroscopy. Mucosa collected from the antrum of the stomach during gastroscopy was placed into medium containing urea and an indicator such as phenol. The test uses the ability of *H. pylori* to secrete urease enzyme which breaks urea down to ammonium and bicarbonate. Ammonium raises the pH of test medium and changes the color of the specimen from yellow (negative) to red (positive).

RNA isolation

RNA was isolated by Total RNA Prep Plus Minicolumn Kit (A&A Biotechnology, Poland) based on RNA isolation method developed earlier. The purity and concentration of RNA samples were assessed nanospectrophotometrically. Until analysis, RNA samples were stored at − 76 °C.

Reverse transcription

cDNA was transcribed from RNA according to High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; USA). For real-time PCR normalization UV absorbance was used to determine the amount of RNA added to a cDNA reaction. PCRs were then set up using cDNA derived from the same amount of input RNA. The final concentration of RNA in reaction mixture was 0.005 µg/µL. As a reference gene, the *GAPDH,* encoding glyceraldehyde-3-phosphate dehydrogenase, was used. Before the quantitative analysis of gene expression during the real-time PCR reaction, parameters were checked using qualitative PCR. PCR reaction mixture for PCR amplification consisted of a cDNA template, with/adding 0.5 µM of each primer, 5 µL of 2 × PCR Super MasterMix (Biotool.com; USA) water to a final volume of 20 µL. Negative control was included in each experiment (sample without a cDNA template).

Real-time PCR

Real-time PCR reactions were done using StrataGene, according to SYBR® Green JumpStart™ *Taq* ReadyMix™ protocol (Sigma Aldrich, Germany). The reaction mixture for both genes consisted of 0.5 µL of each primer (*NFKB2* F 5′-CCA TGA CAG CAA ATC TCC-3′; R 5′-TAA ACT TCA TCT CCA CCC C-3′; *GAPDH* F 5′-TGG TAT CGT GGA AGG ACT CAT-3′, R 5′-ATG CCA GTG AGC TTC CCG TTC AGC-3′), 7.5 µL SYBR-Green ReadyMix, 1 µL of cDNA and distilled water up to the final volume of 16 µL. Samples were tested in triplicates and means of obtained Ct values for both genes were calculated. The reactions for *NFKB2* and *GAPDH* genes were carried out in separate tubes. In each experiment the negative control, also tested in triplicates, was included. The thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 60 s, elongation at 72 °C for 60 s and a final extension step at 72 °C for 3 min. To calculate the relative changes in gene expression, the $\Delta\Delta C_q$ method was used [12].

Statistical analysis

Statistical analysis was performed using the STATISTICA version 12. (StatSoft, Inc., Tulsa, OK, USA) software package. To determine the validity between the R-value and age, gender or clinical and pathological factors the *U* Mann–Whitney test was used. A p-value < 0.05 was assumed as significant in all tests.

Results

The relative expression level of *NFKB2* gene was successfully investigated in 79 samples from peptic ulcer, 22 samples of tissues from gastric cancer and 11 control samples of tissue macroscopically examined as healthy collected beyond margin of cancer tissue from gastric cancer patients.

Expression of *NFKB2* mRNA in peptic ulcer patients cohort

The next part of the research pertained the determination of the relative level of *NFKB2* gene expression in relation to the *GAPDH* gene in the peptic ulcer patients cohort. In this group of patients, the relative expression level of *NFKB2* among all cases was highly variable. The obtained results ranged from 0.1736 to 17.2328. All results are presented in Table 1.

On the basis of rapid urease test results, a group of patients with peptic ulcer was divided into two subgroups: patients uninfected (N = 47) and infected (N = 32) with *Helicobacter pylori*. There were no statistically significant differences of the *NFKB2* gene relative expression level between the subgroup of patients infected and uninfected with *H. pylori* ($p = 0.7006$) (Fig. 1).

Additionally, the results of rapid urease test allowed to distinguish within the *H. pylori*-infected patients those in whom the severity of the infection was estimated as one, two or three pluses during semi-quantitative rapid urease test. Patients whose *H. pylori* infection severity was assessed as two or three pluses were combined into one subgroup. Next,

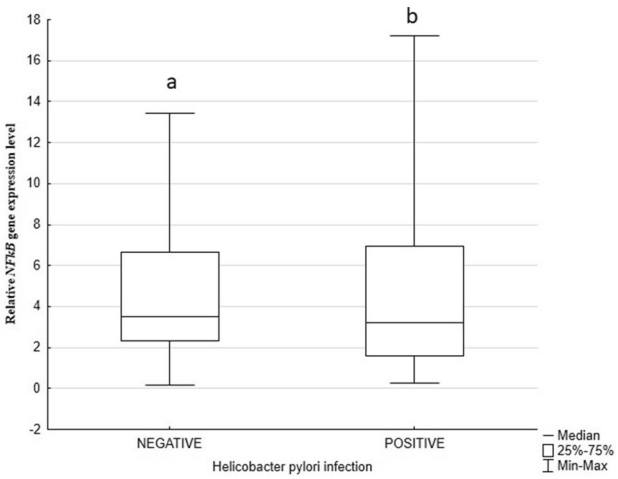

Fig. 1 Relative *NFKB2* gene expression in peptic ulcer cells uninfected and infected with *H. pylori*. Figure shows the level of expression of the *NFKB2* gene depending on the rapid urease test result: **a** negative—no *H. pylori* infection in the group of patients with peptic ulcer patients and **b** positive—current *H. pylori* infection in the peptic ulcer patients. The data plot **a**, **b** for each of the rapid urease test results represents the median value along with the minimum and maximum values and the lower and upper quartiles

the relative expression levels of *NFKB2* gene were evaluated between this combined subgroup and the group of patients in whom the result was rated at one plus. There were no statistically significant differences ($p = 0.8330$).

Then, the relative expression of *NFKB2* mRNA, depending on gender and age in this group of patients was rated. No statistically significant differences were found ($p = 0.7070$ and $p = 0.1715$, respectively).

Expression of *NFKB2* mRNA in gastric cancer patients cohort

In the second part of the study, the relative level of *NFKB2* mRNA was evaluated in the group of patients with gastric cancer. All results are presented in Table 2.

The dependences of the relative expression level of *NFKB2* with clinicopathological parameters were evaluated.

Table 1 Relative expression level of *NFKB2* mRNA in peptic ulcer patients

Relative *NFKB2* mRNA level	N	Median	Min	Max	Lower quartile	Upper quartile
All cases	79	3.4913	0.1736	17.2328	1.6269	6.6518
Men	36	3.3583	0.2571	13.3037	1.5418	6.7849
Women	43	3.6521	0.1736	17.2328	1.9504	6.6518
Uninfected with *H. pylori*	47	3.5278	0.1736	13.4272	2.3194	6.6518
Infected with *H. pylori*	32	3.2240	0.2571	17.2328	1.5874	6.9278
Severity of infection assessed at (+)	20	3.7966	0.2571	13.3037	1.5874	6.9278
Severity of infection assessed at (++) and (+++)	12	2.3912	0.5166	17.2328	1.7430	8.7130

Table 2 Relative expression level of *NFKB2* mRNA in stomach cancer patients

Relative *NFKB2* mRNA level	N	Median	Min	Max	Lower quartile	Upper quartile
All cases	22	0.6558	0.2787	2.4095	0.4502	0.9343
Men	14	0.6298	0.3376	2.4095	0.3887	1.0307
Women	8	0.6774	0.2787	2.0663	0.5013	0.8375
TNM Tis or I	11	0.6879	0.2787	2.0663	0.5047	1.0307
TNM II or III	11	0.6072	0.3376	2.4095	0.3887	0.9343
G1	11	0.6668	0.2787	1.7255	0.3803	0.7407
G2 and G3	11	0.6072	0.3703	2.4095	0.4502	1.2300

Table 3 Relative expression level of *NFKB2* mRNA in morphologically normal tissue taken beyond the tumor margin

Relative *NFKB2* mRNA level	N	Median	Min	Max	Lower quartile	Upper quartile
All cases	12	0.9964	0.4435	2.0618	0.7949	1.2200
Men	8	0.9964	0.4435	2.0615	0.8280	1.3419
Women	4	0.9617	0.7866	1.3198	0.7949	1.2200

According to TNM stage the stomach cancer patients cohort was divided into two subgroups: patients with Tis or I stage and patients with II or III stage. There were no statistically significant differences between these two subgroups of patients ($p=0.3144$). Next, in order to evaluate the differences of *NFKB2* gene expression level depending on histological grade, the group of patients with stomach cancer was divided into two subgroups: patients with low histological malignancy degree (G1) and patients with high histological malignancy degree (G2 or G3). The analysis between these two groups of patients was performed. However, no meaningful difference in the level of *NFKB2* gene expression was found ($p=0.7928$) Following that, the relative expression of *NFKB2* mRNA, depending on gender and age in group of patients with stomach cancer was rated. No statistically significant differences were found ($p=0.3909$ and $p=0.3461$, respectively).

Expression of *NFKB2* mRNA in morphologically normal tissue taken beyond the tumor margin

After that, the relative expression level of *NFKB2* mRNA in the morphologically normal tissue taken beyond the tumor margin was evaluated. The expression level was a variable in this group. Data is presented in Table 3.

Expression of *NFKB* mRNA in samples from peptic ulcer, gastric cancer and control group

Finally, samples were divided into three groups: peptic ulcer, gastric cancer and macroscopically normal gastric tissue which formed the control group. The analysis showed that relative *NFKB2* gene expression level depends on the type of tissue. Peptic ulcer cases showed the increased relative *NFKB2* gene expression to control group ($p=0.0000$) (Figs. 2, 3). Then the difference in relative *NFKB2* gene expression between gastric cancer cases and control group was obtained. The results of this comparison showed that cancer cases present a decreased relative *NFKB2* gene expression as opposed to the control tissue ($p=0.0183$) (Figs. 2, 3). Also the comparison between stomach cancer cells and peptic ulcer cases was performed and showed a statistically significant lower relative *NFKB2* gene expression

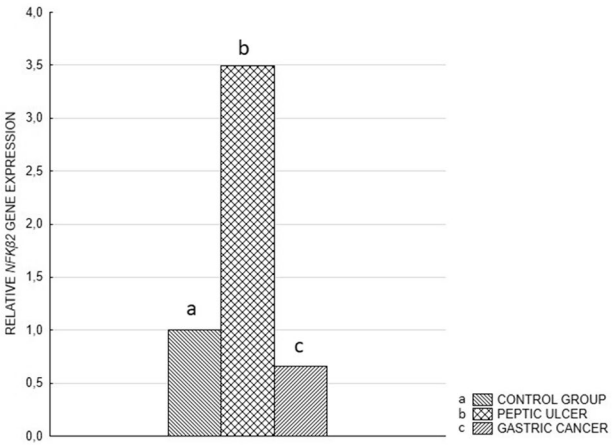

Fig. 2 Median relative *NFKB2* gene expression in peptic ulcer, gastric cases and the control group. Figure shows the dependence of the level of expression of the *NFKB2* gene depending on the type of tissue in which the analysis was performed: **a** macroscopically normal gastric tissue (control group), **b** biopsies of the gastric mucosa (group of patients with peptic ulcer disease); **c** gastric cancer tissue (group of patients with gastric cancer)

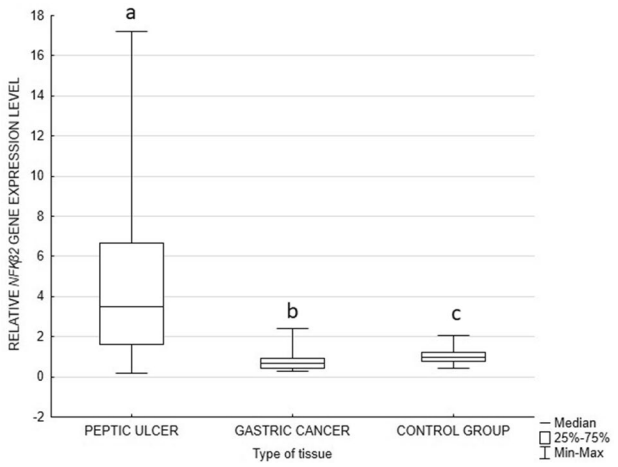

Fig. 3 Relative *NFKB2* gene expression in peptic ulcer gastric cancer cases and the control group. Figure shows the dependence of the level of expression of the *NFKB2* gene depending on the type of tissue in which the analysis was performed: **a** biopsies of the gastric mucosa, **b** gastric cancer tissue, **c** macroscopically normal gastric tissue. The data plot **a**, **b**, **c** for each of the tissues/study groups represents the median value along with the minimum and maximum values and the lower and upper quartiles

in cancer tissue compared to peptic ulcer cells ($p = 0.0000$) (Figs. 2, 3).

Discussion

The correlation between NF-κB, its signals and gastric cancer phenotype remains unclear, as is the association of this activation with peptic ulcer disease development and progression, which could be one of the causes of gastric cancer. The aim of the study was to determine the level of *NFKB2* mRNA in peptic ulcer and gastric cancer and compare the obtained results to control tissue, including other important clinical parameters, like presence of *H. pylori* infection, TNM staging and others.

Inappropriate *NFKB2* activation as a recurrent feature is well documented in MALT lymphomas, PMBL, multiple myeloma, and Hodgkin's lymphoma [13–16] and solid tumours [17, 18]. In our study we showed that the level of mRNA is lower in patients with gastric cancer when compared to the control tissue or patients with peptic ulcer. Doffey et al. [19] observed that blocked Nf-κB function in head and neck squamous cell carcinoma cells could be important for inhibition of growth and metastases and showed that this inhibited xenograft-derived tumour growth. Inhibition of NF-κB in mutated Kras-induced lung cancer and pancreatic cancer greatly reduced tumour initiation and progression [20]. In this study no differences were observed between the gene expression level and some clinicopathological parameters, like clinical staging and histological grading, which could suggest lack of connection with worse prediction or prognosis for the patients, but it could be explained by a small group of investigated cancers. *NFKB2* family consists of transcription factors that form homo- or heterodimerization of the subunits RelA, RelB, c-Rel, NF-κB1, and NF-κB2. They can be activated in two ways: classical and an alternative one, in which pro-inflammatory cytokines are very important [21, 22]. During activation through the alternative pathway, an IKK (IκB kinase) complex consisting of two IKKα subunits phosphorylates p100, which is the primary gene product encoded by *NFKB2,* that plays an essential role in many chronic inflammatory diseases [8, 23, 24]. In our study such an observation was confirmed, the level of *NFKB2* mRNA in peptic ulcer patients was higher than in the control tissue. On the one hand, several factors, such as persistent infections with *H. pylori* the pro-inflammatory microenvironment of the cancer, self-reactive immune receptors as well as genetic lesions altering the function of key signalling effectors, contribute to constitutive NF-κB activity in these malignancies [9, 25]. On the other, chronic inflammatory microenvironment may lead to immunosuppression and cancer development which can be visible as NF-κB protein p50 expression inducing the immunosuppressive microenvironment [26]. In our study no significant differences of the *NFKB2* gene relative expression level between the subgroup of patients infected and uninfected with *H. pylori* were observed also no correlation between severity of *H. pylori* infection and *NFKB2* mRNA level was found.

The relative expression level of *NFKB2* is significantly lower in cancer cases than the control tissue, which could suggest that during carcinogenesis of gastric cancer, inhibition of NF-κB pathways takes place which could be a promising factor for patients. There are differences important statistically in the investigated gene expression between peptic ulcer and gastric cancer cases and also the control tissue, which could confirm that when peptic ulcer cases its activation, it is connected with the inflammatory process.

This article does not contain any studies with animals performed by any of the authors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (The Ethics Committee of the Medical University of Lodz, number (RNN/195/13/KE) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Written informed consent was obtained from the patients prior to their participation in the research.

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