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NF-κB/p65 expression before and after treatment in rectal cancer patients undergoing neoadjuvant (chemo)radiotherapy and surgery: prognostic marker for disease progression and survival

R. VOBORIL[1,*], V. RYCHTEROVA[2], J. VOBORILOVA[3], M. KUBECOVA[4], J. FANTA[1], J. DVORAK[5]

[1]Department of Surgery, First Faculty of Medicine, Charles University in Prague and Na Bulovce Hospital, Prague, Czech Republic; [2]Department of Pathology, Third Faculty of Medicine, Charles University in Prague and Faculty Hospital Kralovske Vinohrady, Prague, Czech Republic; [3]Department of Biochemistry, Cell and Molecular Biology, Division of Cell and Molecular Biology, Third Faculty of Medicine, Charles University in Prague, Prague, Czech Republic; [4]Department of Radiotherapy and Oncology, Third Faculty of Medicine, Charles University in Prague and Faculty Hospital Kralovske Vinohrady, Prague, Czech Republic; [5]Department of Oncology, First Faculty of Medicine, Charles University in Prague and Thomayer Hospital, Prague, Czech Republic

[*]*Correspondence: rene.voboril@seznam.cz*

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Nuclear factor-kappaB (NF-κB), especially p65 subunit, has been associated with origin and progression of cancer as well as with the resistance to radiotherapy and chemotherapy in experimental models. The aim of the present study was to determine expression of NF-κB/p65 in tumor specimens before and after treatment of rectal cancer patients and to evaluate possible relationship between expression of NF-κB/p65 before and after (chemo)radiotherapy, other tumor characteristics and the clinical outcome. Furthermore, NF-κB/p65 was studied in relationship to pathologic response to preoperative (chemo)radiotherapy. Fifty patients with rectal cancer undergoing neoadjuvant (chemo)radiotherapy and surgery were included in the study. Pre-treatment rectal cancer specimens were obtained from diagnostic colonoscopy. Post-treatment rectal cancer specimens were obtained from surgically removed part of the rectum with the tumor. NF-κB/p65 expression was determined by immunohistochemistry and analysis was performed both in biopsies and in post-treatment tumor samples. Cytoplasmic positivity in tumor cells and nuclear positivity in lymphocytes were detected. High NF-κB/p65 positivity in pre-treatment tumor samples was significantly associated with shortened overall survival (OS). Disease-free survival (DFS) tends to be shortened as well. In post-treatment tumor samples, high NF-κB/p65 positivity was neither associated with shortened OS nor with shortened DFS. In post-treatment samples residual tumor cells deeply infiltrating the wall of the rectum with high NF-κB/p65 expression were found. The cells were linked to significantly worse clinical outcome in terms of shortened OS and DFS. NF-κB/p65 positivity did not correlate with pathologic response to preoperative (chemo) radiotherapy. In conclusion, our data suggest that high level of NF-κB/p65 subunit may be associated with more aggressive features of the tumor, higher metastatic potential, and shortened overall survival, but it does not correlate with resistance to (chemo)radiotherapy. Consequently, the level of NF-κB/p65 may help to select those patients who have poor prognosis and are candidates for more intensive anticancer therapy. For these purposes both pre-treatment and post-treatment tumor samples may be used.

Key words: rectal cancer, chemoradiotherapy, NF-kappaB, p65 subunit, immunohistochemistry, prognostic factor

Colorectal cancer is a significant cause of morbidity and mortality in Western countries. More than 30% of all cases are localized in the rectum, with a less promising outcome after surgery than for colon cancer, as seen in local recurrence rates of 20% to 50% after traditional surgical treatment [1]. Moreover, rectal cancer is characterized by a higher incidence of regional lymph nodes and distant metastases [2, 3].

Radiotherapy administered in pre-operative regimen is used in the treatment of advanced rectal cancer. It may downstage the tumor and may increase the possibility of a sphincter sparing procedure. Furthermore, pre-operative radiotherapy may reduce the rate of local recurrence and distant metastases. Thus, it may improve the chance of survival in patients with resectable rectal carcinoma [4, 5]. However, not every rectal

tumor responds well to radiotherapy. It is documented that some tumors respond well to standard radiotherapy, while others remain non-responsive [6, 7]. It is not known why such differences in response to radiotherapy occur in rectal cancer patients. Some recent studies have shown that patients, who respond to pre-operative radiotherapy with or without chemotherapy as demonstrated by pathologic response, have lower rates of local recurrence [8, 9]. Their survival is also possibly improved when compared to non-responsive patients whose tumors are either partially or totally unresponsive [10, 11].

One of mechanisms involved in cell survival is activation of nuclear factor kappab (NF-κB), an inducible transcription factor. NF-κB has been shown to be associated with origin and progression of cancer by controlling expression of genes involved in cell growth, differentiation, cytokine production, as well as in apoptosis, neoplastic transformation, adhesion, and migration [12]. Five subunits of NF-κB have been characterized: p50, p52, p65/RelA, RelB and c-Rel. They form either heterodimers or homodimers [13]. The most intensively studied NF-κB form is the heterodimer composed of the subunits p65 and p50. NF-κB in its inactive form is located in cytoplasm bound to the inhibitory molecule known as IκB protein. In a response to a wide variety of stimuli, including radiotherapy and chemotherapy such as 5-FU, IκB proteins are phosphorylated, ubiquitinated and degraded by the ubiquitin-proteasome pathway [14]. NF-κB released from its inhibitory molecule is translocated to the nucleus resulting in expression of target genes [15].

In cancer cells NF-κB very often loses its transient inducibility and becomes constitutively activated as shown in pancreatic adenocarcinoma [16], prostate cancer [17], breast cancer [18] and in many others. Recently, we intensively studied NF-κB in colorectal cancer. We showed in our *in vitro* experiments on colorectal cancer cell lines that activated NF-κB is associated with resistance to irradiation [19], inhibition of NF-κB leads to radiosenzitization [20] and enhances the cytotoxic effect of 5-FU [21]. Moreover, we reported in a clinical study that NF-κB/p65 positivity in single cancer cells displaying dissociated pattern of growth found after radiotherapy in rectal cancer patients may be associated with worse clinical outcome in terms of shortened disease-free interval and overall survival [22].

The aim of the study was to assess expression of NF-κB in tumor specimens before and after treatment of the rectal cancer patients undergoing neoadjuvant (chemo)radiotherapy and surgery. Furthermore, we studied whether there may exist a relationship between expression of NF-κB/p65 subunit before and after (chemo)radiotherapy, other tumor characteristics and the clinical outcome in terms of disease-free interval and overall survival. Moreover, we wanted to know if NF-κB/p65 correlates with pathologic response to preoperative (chemo)radiotherapy.

Patients and methods

Patients and tissue specimens. The study was performed in a series of patients with locally advanced rectal cancer who had been diagnosed and treated at the Charles University Third Faculty of Medicine Faculty Hospital Kralovske Vinohrady in Prague, Czech Republic. The criteria for selection included availability of a preoperative biopsy suitable for immunohistochemical analysis and surgery performed at the above mentioned hospital. The study was performed according to the Institutional Guidelines and approved by the Institutional Ethics Committee as described in our previous study [22].

Patients with localized, histologically confirmed adenocarcinoma of the rectum were evaluated by X-ray examination (chest) and computed tomography (abdomen and pelvis). All of them underwent pre-operative radiotherapy conducted with 45 Gy in 25 fractions at 1.8 Gy/fraction or with 34.5 Gy in 15 fractions at 2.3 Gy/fraction. Some patients received pre-operative chemoradiotherapy based on 5-FU regimen. Approximately 6 weeks after radiotherapy, the surgery with removal of the affected part of the rectum was performed. Each patient was followed up at regular basis according to standardized protocol as described in our previous study [22]. Development of local disease recurrence and/or metastatic relapse was assessed. Clinical data were obtained from the patient's medical records.

In each patient, two tissue samples were available for this study 1) from the diagnostic endoscopic biopsy before preoperative (chemo)radiotherapy and 2) from the surgically removed rectum after completed (chemo)radiotherapy. All tissue sections were matched to routine haematoxylin-eosin stained slides used for evaluation of the presence of cancer. The sections were reviewed by a single pathologist (VR).

Immunohistochemistry for NF-κB/p65. Formalin fixed, paraffin-embedded tumor tissue samples (4 μm slides) were obtained for immunohistochemistry. After deparaffinisation in xylene and graded alcohols, the slides were incubated in 0.3% pepsin/HCl solution at 37°C for 30 min. Endogenous peroxidase was blocked with 0.03% hydrogen peroxide for 5 min. The slides were rinsed in distilled water and covered with a protein block solution for 5 min. They were then incubated with primary mouse monoclonal antibody raised against amino acids 1-286 at the N-terminus of p65 subunit of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA) in dilution 1:50 for 30 min and developed using the Dako CSA II System as described by the manufacturer (DakoCytomation, Carpentaria, CA). In the Dako CSA II System procedure, the mouse primary antibody was first detected with a peroxidase-conjugated anti-mouse secondary antibody. The next step utilized the bound peroxidase to catalyze oxidation of a fluorescein-conjugated phenol, which then precipitated onto the specimen. The procedure was continued with detection of the bound fluorescein by a peroxidase-conjugated anti-fluorescein. Staining was completed when sections were treated with 3,3'-diaminobenzidine as chromogen for 5 min and counterstained with haematoxylin-eosin. The slides were dehydrated and mounted with glass coverslips.

As an internal positive control we used nuclear NF-κB/p65 positivity in lymphocytes found in the stroma of tumor tissue

samples. As negative controls, samples of the same specimens were processed by the same immunohistochemistry method except with the omission of the primary antibody. Negative controls did not show any staining and were used during optimization of the method.

Scoring method for NF-κB/p65. NF-κB/p65 immunohistochemistry was scored by a single pathologist (VR) blinded with regard to the clinical-pathological characteristics of the patients.

Cytoplasmic NF-κB/p65 activation was considered positive when the cells showed diffuse or localized brown cytoplasmic staining. Presence of brown color within the nucleus was described as nuclear NF-κB/p65 positivity. Intensity of NF-κB/p65 expression was determined as undetectable (negative), low or high.

It is important to emphasize that the only activated form of NF-κB/p65 was detected by immunohistochemistry because monoclonal antibody that we used could detect NF-κB/p65 protein released from its inhibitory molecule IκB.

Statistical analysis. Fisher exact test and Chí square test were used to test the association of NF-κB/p65 expression with other variables.

Table 1. Characteristics of patients

Characteristics of patients	No. of patients	%
Total number of patients	50	100
Total number of tumor specimens	100	
Specimens before treatment	50	
Specimens after treatment	50	
Age		
Median	67	
Range	37 – 82	
Gender		
Male	38	76
Female	12	24
Staging		
Dukes A	19	38
Dukes B	19	38
Dukes C	7	14
Dukes D	5	10
Grading before treatment		
G1	19	38
G2	28	56
G3	3	6
Grading after treatment		
G1	13	26
G2	25	50
G3	12	24

Staging: Dukes A is superficially growing tumor; Dukes B is invasion through the bowel wall, lymph nodes not involved; Dukes C lymph nodes involved; Dukes D metastatic disease

Grading: G1 means well differentiated (low grade) tumor; G2 moderately differentiated (intermediate grade) tumor; G3 poorly differentiated (high grade) tumor

Analyses were performed to determine disease-free survival period (DFS) and overall survival period (OS). DFS was defined as the time from diagnosis to disease recurrence or until the date of the last follow-up. Data from patients who were alive without disease at the time of analysis were censored. OS was defined as the time from diagnosis to death. When the date of death was not available, the last follow-up date was used. Data from patients who had not died were censored. An association between NF-κB/p65 and DFS or OS was tested by comparing the Kaplan-Meier survival curves with log-rank tests used to test differences in survival distribution.

All p values were two-sided. All statistical analyses were performed at a 0.05 statistical significance level using the Statistica software (StatSoft, Prague, Czech Republic).

Results

Patient characteristics. From the selected series of the patients, both samples of tumor tissue were suitable for immunohistochemistry staining in 50 patients. Table 1 describes characteristics of the patients. The median age was 67 years (ranging from 37 to 82). Of 50 patients, 38 (76%) patients were men and 12 (24%) patients were women. All samples were classified as adenocarcinomas. Clinical stage represented by Dukes classification was as follows: Dukes A in 19 patients (38%), Dukes B in 19 patients (38%), Dukes C in 7 patients (14%), and Dukes D in 5 patients (10%), where Dukes A means the tumor is superficially growing, there may be invasion into but not through the bowel wall; Dukes B is invasion of the tumor through the bowel wall penetrating the muscle layer but not involving lymph nodes; Dukes C involvement of lymph nodes; and Dukes D metastatic disease [23].

NF-κB/p65 expression by immunohistochemistry in rectal tissue samples. Stained by immunohistochemistry, 50 pairs of patient-matched rectal tissue samples with the tumor were examined. In normal rectal tissue neither nuclear nor cytoplasmic positivity of NF-κB/p65 was observed (Fig. 1A). In tumor tissue only cytoplasmic NF-κB/p65 positivity of tumor cells was found. The cytoplasmic positivity showed different patterns ranging from perinuclear, or granular to diffuse throughout the cytoplasm. More NF-κB/p65 positive tumor cells were found in invasive margins of the tumor. Positive nuclear NF-κB/p65 staining was found only in reactive lymphocytes in the tumor stroma. It seems that positive NF-κB/p65 expression was typical for tumor tissue only. Normal rectal tissue was negative for NF-κB/p65.

Thirty-seven (74%) of the pre-treatment tumor samples and 30 (60%) of the post-treatment tumor samples exhibited low cytoplasmic NF-κB/p65 expression (Fig. 1B). Nine (18%) of the pre-treatment tumor samples and 16 (32%) of the post-treatment tumor samples exhibited high cytoplasmic NF-κB/p65 expression (Fig. 1C). NF-κB/p65 expression was undetected in 4 (8%) of the pre-treatment samples and in 4 (8%) of the post-treatment samples.

In patient-matched tumor samples, NF-κB/p65 cytoplasmic staining was undetected in both pre- and post-treatment samples in 2 (4%) patients and was positive in both pre- and post-treatment samples in 44 (88%) patients. In 2 patients, cytoplasmic staining was undetected in pre-treatment and was positive in post-treatment specimens, in the remaining 2 patients cytoplasmic staining was positive in pre-treatment and was undetected in post-treatment specimens.

Out of 9 specimens with high cytoplasmic NF-κB/p65 pre-treatment positivity 2 (22%) turned to low and 7 (78%) stayed at high NF-κB/p65 positivity after treatment, out of 41 specimens with low or undetectable cytoplasmic NF-κB/p65 pre-treatment positivity 32 (78%) stayed after (chemo)radiotherapy at low or undetectable level and 9 (22%) changed to high level of NF-κB/p65. Thus it seems that the treatment did not change in most cases the level of NF-κB/p65 cytoplasmic positivity (p=0.003).

NF-κB/p65 expression and correlation with clinical-pathological parameters. Evaluating pre-(chemo)radiotherapy specimens, of 19 well differentiated tumors 19 (100%) dis-

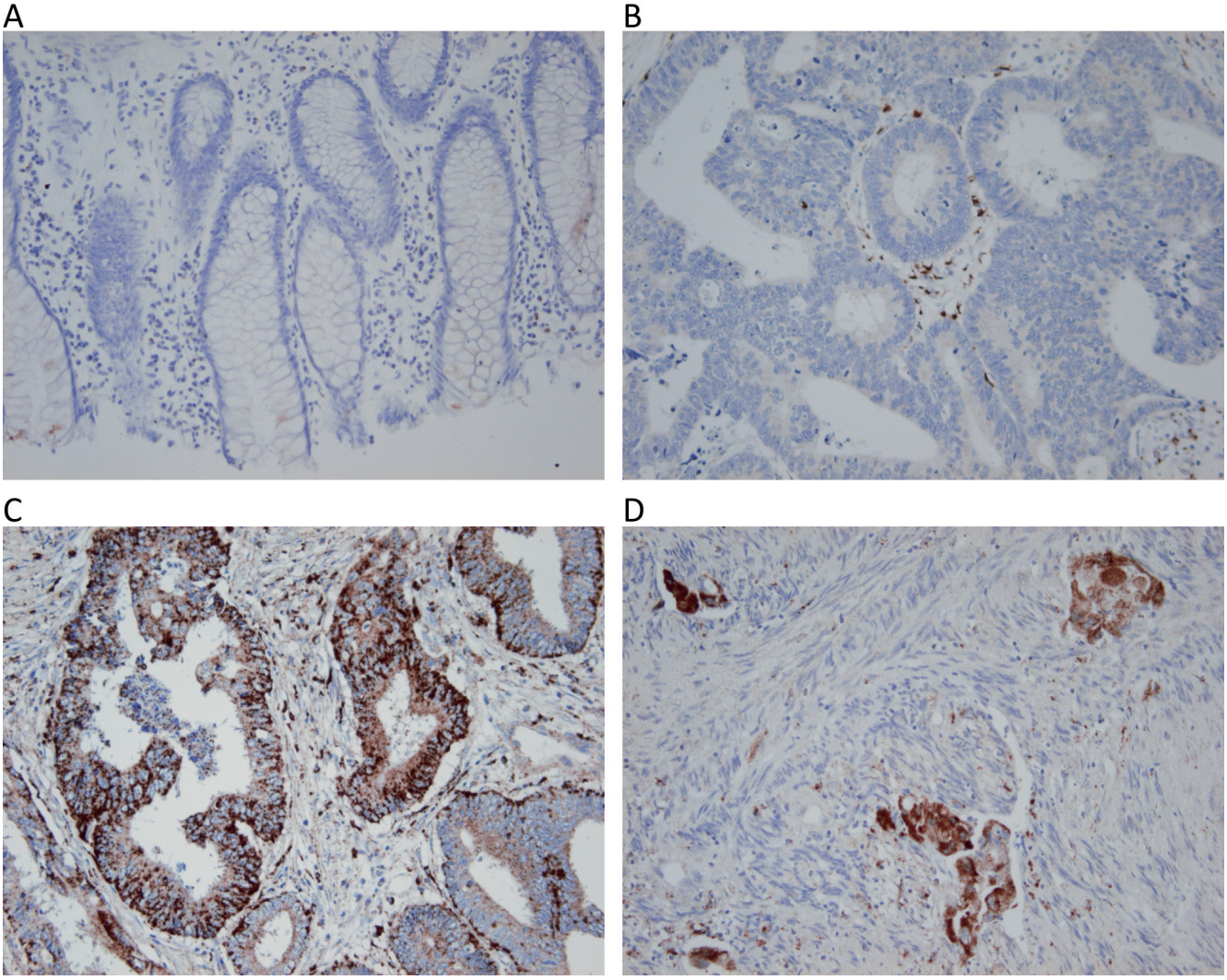

Figure 1. A. Normal rectal tissue with NF-κB/p65 negative epithelial cells. Immunohistochemistry for NF-κB/p65 using primary mouse monoclonal antibody against activated form of p65 subunit was performed. Presence of brown staining within the nucleus was described as nuclear NF-κB/p65 positivity. Presence of diffuse or localized brown cytoplasmic staining was considered as cytoplasmic NF-κB/p65 positivity. In normal rectal tissue neither nuclear nor cytoplasmic positivity of NF-κB/p65 was observed. B. Adenocarcinoma of the rectum, grade 2. Low NF-κB/p65 positivity is seen in epithelial cells. Tumor cells displayed low level of cytoplasmic NF-κB/p65 positivity characterized by presence of light brown staining. C. Adenocarcinoma of the rectum, grade 2. High NF-κB/p65 positivity is seen in epithelial cells. Tumor cells displayed high level of cytoplasmic NF-κB/p65 positivity characterized by presence of dark brown staining. D. High NF-κB/p65 positivity in deeply invasive high-grade cancer cells. In some of post-(chemo) radiotherapy specimens single tumor cells or small clones of them deeply infiltrating the wall of the rectum were present. These cells displayed high level of cytoplasmic NF-κB/p65 positivity characterized by presence of dark brown staining. D. published in our previous study [22].

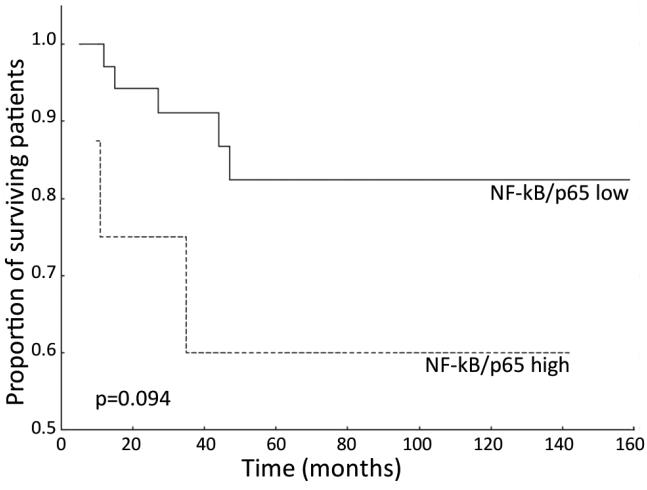

Figure 2. Kaplan–Meier curves and disease-free interval according to NF-κB/p65 expression in pre-treatment tumor samples. Disease-free survival in patients showing low NF-κB/p65 tumor expression ("NF-κB/p65 low") and high NF-κB/p65 tumor expression ("NF-κB/p65 high"). High NF-κB/p65 expression in tumor cells was associated with shortened disease-free survival (p=0.094).

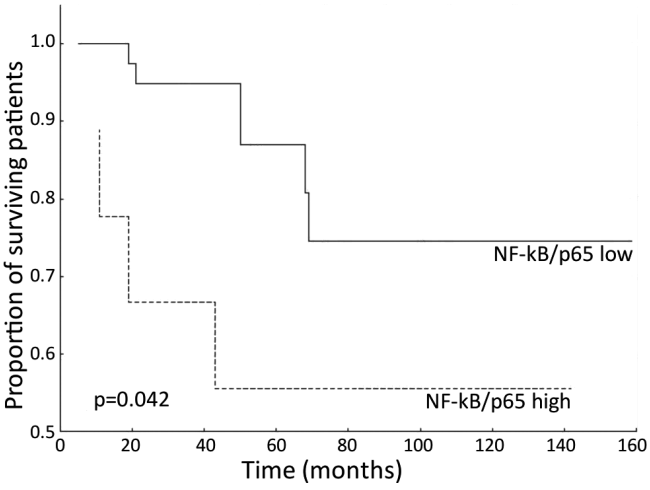

Figure 3. Kaplan–Meier curves and overall survival according to NF-κB/p65 expression in pre-treatment tumor samples. Overall survival in patients showing low NF-κB/p65 tumor expression ("NF-κB/p65 low") and high NF-κB/p65 tumor expression ("NF-κB/p65 high"). Overall survival of patients with high NF-κB/p65 expression in tumor cells was significantly shortened (p=0.042).

played low or undetectable NF-κB/p65 positivity, whereas in 31 moderately and poorly differentiated tumors 22 (71%) had low and 9 (29%) high NF-κB/p65 positivity. There can be seen association between NF-κB/p65 positivity and differentiation of the tumors. Low or undetectable expression of NF-κB/p65 in pre-(chemo)radiotherapy specimens was significantly associated with well differentiated tumors (p=0.009).

With regards to the expression of NF-κB/p65 in post-(chemo)radiotherapy specimens, of 13 well differentiated tumors 11 (85%) displayed low or undetectable NF-κB/p65 positivity, whereas in 37 moderately and poorly differentiated tumors 23 (62%) had low and 14 (38%) high NF-κB/p65 positivity. There was no statistically significant association (p=0.18) between expression of NF-κB/p65 and grading after (chemo)radiotherapy.

Furthermore, there was no association between NF-κB/p65 pre-(chemo)radiotherapy as well as NF-κB/p65 post-(chemo) radiotherapy expression and patients' sex and age.

NF-κB/p65 expression and pathological response to preoperative (chemo)radiotherapy. There was 1 patient (2%) with no pathological response to (chemo)radiotherapy, 12 patients (24%) with weak pathological response, 18 patients (37%) with moderate pathological response, and 18 patients (37%) with strong pathological response to (chemo) radiotherapy. One patient was not evaluated. The only strong pathological response was characterized by extensive necrosis with ulceration and residual tumor structures. However, tumor cells were identified in every post-(chemo)radiotherapy specimen.

When considering NF-κB/p65 expression either before or after treatment, 5 (28%) of 18 patients achieving strong pathological response had high tumor NF-κB/p65 expression, and 12 (39%) of 31 patients with no response to moderate response had high NF-κB/p65 expression (p=0.54). When considering NF-κB/p65 expression before treatment only, statistical significance was not reached (p=0.71) as well as when considering NF-κB/p65 expression after treatment only (p=0.75) in association with response to pre-operative therapy. Statistical significance of the association between NF-κB/p65 expression and pathological response to preoperative (chemo) radiotherapy was not reached.

NF-κB/p65 expression and clinical outcome. On univariate analysis, when considering only pre-treatment tumor samples, high NF-κB/p65 expression was associated with shortened DFS (p=0.094), although statistical significance was not reached (Fig. 2). DFS was evaluated in 45 patients, five patients were excluded from the analysis because of presence of distant metastases at diagnosis. During the follow-up time, 4 (50%) of 8 patients with high NF-κB/p65 expression developed a relapse compared to 8 (22%) of 37 patients with low or undetectable NF-κB/p65 expression.

Similarly, the OS of patients with high NF-κB/p65 expression was significantly shortened (p=0.042) (Fig. 3). During the follow-up time, 4 (44%) of 9 patients with high NF-κB/p65 expression had died from cancer compared with only 6 (15%) of 41 patients with low or undetectable NF-κB/p65 expression.

Interestingly, when considering only post-treatment tumor samples, high NF-κB/p65 expression was neither associated with shortened DFS (p=0.123) nor with shortened OS (p=0.684).

NF-κB/p65 expression and metastatic disease. Regardless of tumor NF-κB/p65 cytoplasmic expression, in some

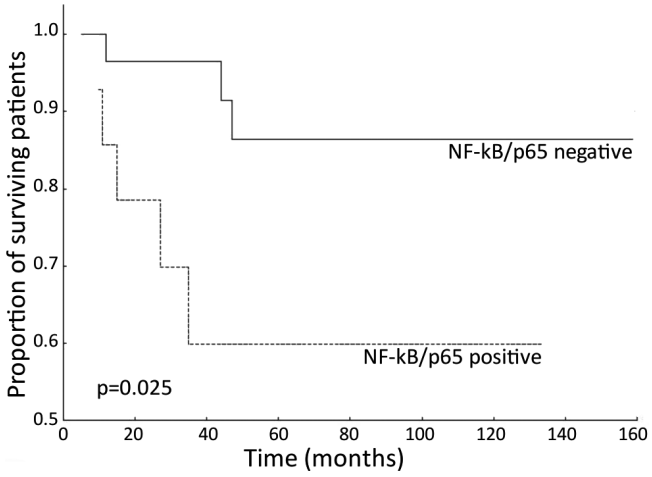

Figure 4. Kaplan–Meier curves and disease-free interval according to presence of dissociated cells in post-treatment samples. Disease-free survival in patients showing presence ("NF-κB/p65 positive") or absence ("NF-κB/p65 negative") of dissociated invasively growing cancer cells in post-treatment tissue samples. Presence of the cells was significantly associated with shortened disease-free survival (p=0.025). These cells displayed high level of cytoplasmic NF-κB/p65 positivity characterized by presence of dark brown staining.

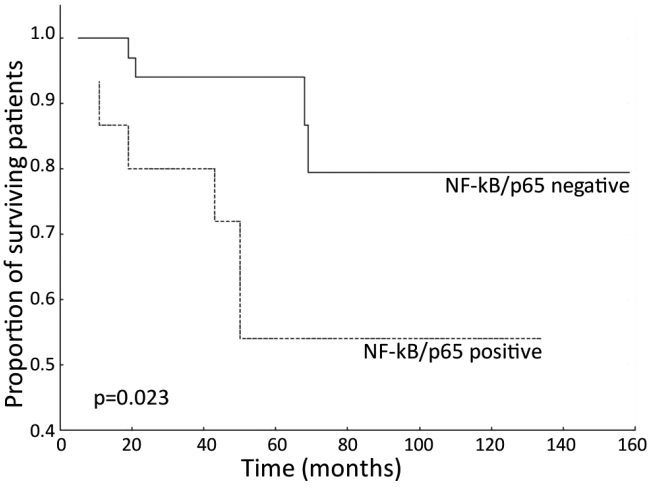

Figure 5. Kaplan–Meier curves and overall survival according to presence of dissociated cells in post-treatment samples . Overall survival in patients showing presence ("NF-κB/p65 positive") or absence ("NF-κB/p65 negative") of dissociated invasively growing cancer cells in post-treatment tissue samples. Presence of the cells was significantly associated with shortened overall survival (p=0.023). These cells displayed high level of cytoplasmic NF-κB/p65 positivity characterized by presence of dark brown staining.

of post-(chemo)radiotherapy specimens there was possible to observe single tumor cells or small clones of them deeply infiltrating the wall of the rectum (Fig. 1D). These cells were characterized by high cytoplasmic NF-κB/p65 positivity that allowed their identification. Their presence in post-(chemo)radiotherapy samples was significantly associated with shortened DFS (p=0.025) (Fig. 4). DFS was evaluated in 45 patients, five patients were excluded from the analysis because of presence of distant metastases at diagnosis. During the follow-up time, 7 (50%) of 14 patients with presence of the single tumor cells or small clones in their post-(chemo)radiotherapy samples developed a relapse compared to 5 (16%) of 31 patients with no presence of these cells.

The patients with presence of these cells in post-(chemo) radiotherapy specimens have worse clinical outcome in term of overall survival (p=0.023) (Fig. 5). During the follow-up time, 6 (40%) of 15 patients with presence of the single tumor cells or small clones in their post-(chemo)radiotherapy samples had died from cancer compared to 4 (11%) of 35 patients with no presence of these cells.

Discussion

The role of NF-κB in cancer origin and progression has been intensively studied. The present clinical study is a part of our complex research focused on the association of NF-κB, colorectal cancer and anticancer treatment. In our previous *in vitro* experiments [19] we described that colorectal cancer cell lines display different level of constitutive NF-κB activity. Moreover, we showed that irradiation causes secondary NF-κB activation and activated NF-κB is associated with resistance to irradiation [19]. On the contrary, inhibition of NF-κB leads to radiosenzitization [20] and enhances the cytotoxic effect of 5-FU [21]. In both, constitutive and radiation-induced, activation of NF-κB, p65 subunit plays an important role. Our data suggest that the level of constitutive NF-κB activity may predict the level of secondary, radiation-induced NF-κB activity and radiosensitivity of colorectal cancer cells [19].

These findings we wanted to confirm on tumor samples in the clinical study. A series of patients with the diagnosis of rectal carcinoma undergoing neoadjuvant (chemo)radiotherapy and surgery was selected for the study. We focused on assessment of NF-κB/p65 expression in tumor specimens before and after treatment. Furthermore, immunohistochemistry, a routinely used method in clinical medicine, was used for detection of NF-κB/p65 in the samples to assess feasibility of the method.

In the present study, immunohistochemistry performed on tissue samples revealed an increased expression of NF-κB/p65 in rectal adenocarcinomas but not in normal rectal tissue. The findings were the same before (chemo)radiotherapy and after (chemo)radiotherapy as well. Increased expression of NF-κB/p65 was found in the cytoplasm of the tumor cells displaying different intensity and different patterns ranging from perinuclear or granular to diffuse throughout the cytoplasm. More NF-κB/p65 positive tumor cells were found in invasive margins of the tumor. Positive nuclear NF-κB/p65 staining was found only in reactive lymphocytes in the tumor stroma. Normal rectal tissue was negative for NF-κB/p65.

In the process of optimization of immunohistochemistry we focused on the cytoplasmic positivity and nuclear positivity of NF-κB/p65 in tumor cells. We used different immunohistochemistry methods, different antibodies, and different detection systems. However, these modifications showed the same result: various levels of cytoplasmic positivity and no nuclear positivity in tumor cells. Potential false negativity of the immunohistochemistry method may be excluded because nuclear positivity of NF-κB/p65 was found in reactive lymphocytes around the cancer structures.

NF-κB/p65 expression found in our samples is consistent with results of previous reports of other authors. Colorectal carcinoma tissue samples examined by immunohistochemistry in the study of Evertsson and Sun [24] displayed nuclear positivity in a few cells from the whole specimen. Different methodologies including fixation and microwave treatment used in the study of these authors [24] increased the staining of the cytoplasm, but did not affect the nuclear staining. Yu et al. [25, 26] detected in colorectal carcinoma specimens positive for NF-κB/p65 only 10–20% in the nucleus. The majority of NF-κB/p65, which is freed from its inhibitory molecule IκB, remains within the cytoplasm [27]. Even under long-term activation, only 10–20% of NF-κB/p65 can be localized in the nucleus and 80–90% of NF-κB/p65 still remains in the cytoplasm [28]. Monoclonal antibody used for immunohistochemistry in our study could detect only activated form of NF-κB/p65 protein released from its inhibitory molecule IκB. In our study [22] we discussed cytoplasmic positivity of NF-κB/p65 found in tumor cells in detail.

Our clinical study [22] focused on patients with rectal cancer who required neoadjuvant radiotherapy demonstrated in a relatively small number of patients that NF-κB/p65 high positivity in pre-radiotherapy tumor samples showed borderline significant association with shortened overall survival. Post-radiotherapy NF-κB/p65 positivity allowed identification of deeply infiltrating single tumor cells that can be otherwise easily omitted in haematoxylin-eosin stained slides. Presence of these highly NF-κB/p65 positive tumor cells deeply infiltrating rectum wall was significantly associated with worse clinical outcome as represented by shortened disease-free interval and overall survival. Regarding the role of NF-κB activation in patients with colorectal cancer, another data are available in a limited number of studies.

The present study focused on enlarged group of patients with the same diagnosis clearly showed that NF-κB/p65 expression examined in pre-(chemo)radiotherapy as well as in post-(chemo)radiotherapy tumor samples has its clinical implication. Low expression of NF-κB/p65 in pre-(chemo)radiotherapy tumor specimens as determined by immunohistochemistry was significantly associated with well differentiated tumors, high NF-κB/p65 expression with moderately and poorly differentiated tumors. Moreover, NF-κB/p65 correlated with clinical outcome. High expression of NF-κB/p65 in pre-(chemo)radiotherapy tumor samples was correlated with shortened overall survival. Interestingly, this was not demonstrated in post-(chemo)radiotherapy tumor samples. Small study performed by Puvvada et al. [29] on metastatic colorectal cancer patients showed similar results. Activation status of NF-κB correlated with clinical outcome. NF-κB activation had prognostic importance because of its association with a significantly shorter overall survival.

O'Neil et al. [30] designed their clinical study to evaluate NF-κB activation in patients with rectal cancer undergoing chemoradiotherapy in order to assess whether NF-κB status correlated with prognosis in these patients. The authors found that NF-κB nuclear expression determined by immunohistochemistry at baseline in rectal cancer was prognostic for decreased overall survival but not predictive of the response to radiotherapy. This is in agreement with the results of our study. In our patients, NF-κB/p65 expression in tumors either before or after (chemo)radiotherapy was not predictive of the response to the therapy. We were not able to demonstrate any association between expression of NF-κB/p65 and response to (chemo)radiotherapy. Interestingly, we did not confirm results of our *in vitro* study [19]. Of course, we have to think of transferability of the *in vitro* experiments, different conditions including repeated irradiation of rectal cancer patients and time interval between irradiation and NF-κB/p65 expression assessment as well as different methods used for NF-κB/p65 identification.

Berardi et al. [31] published study on locally advanced rectal cancer patients receiving neoadjuvant chemoradiotherapy. Biopsies and tumor samples were examined by immunohistochemistry. A significant correlation between a positive NF-κB expression, both in biopsies and in tumor samples, and a worse overall survival was observed. Moreover, median time to progression was significantly shorter in the NF-κB positive subgroup of patients. The authors suggest that NF-κB could represent an important parameter able to predict the outcome in patients receiving neoadjuvant treatment for rectal cancer. It also could be useful in order to select patients to receive adjuvant chemotherapy, intensifying the adjuvant therapy and, in the future, obviating the use of drugs involving NF-κB system in their mechanism of action in NF-κB positive patients [31].

O'Neil et al. [30] suggest that NF-κB might play an important role in tumor metastasis but not in resistance to chemoradiotherapy. The lack of correlation between the prognostic marker and the pathologic response to preoperative RT with concurrent chemotherapy could suggest that NF-κB affects survival by the promotion of the metastatic process and perhaps alters chemotherapy sensitivity rather than by affecting RT sensitivity primarily [30]. Our findings of single cells in post-(chemo)radiotherapy samples characterized by high NF-κB/p65 expression and by invasive pattern of growth may support the conclusions of O'Neil et al. [30]. Post-radiotherapy samples positive for those cells were powerful tool for correlation of DFS prognosis in our patients, more effective than NF-κB/p65 expression in pre-(chemo)radiotherapy tumor samples. These cells could be a source of a later progress of the

disease. Thus, they may help to identify rectal cancer patients being at risk of cancer progression and requiring more aggressive anti-cancer therapy.

In conclusion, our data on rectal cancer patients undergoing neoadjuvant (chemo)radiotherapy before surgery suggest that high level of expression of activated NF-κB/p65 subunit, in spite of its cytoplasmic positivity only, is associated with more aggressive features of the tumor, higher metastatic potential, and shortened overall survival. Although NF-κB/p65 does not correlate in our study with resistance to (chemo)radiotherapy, level of NF-κB/p65 may help to select those patients who have poor prognosis.

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