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Clinicopathological significance and prognostic implication of nuclear factor-κB activation in colorectal cancer

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ABSTRACT

Objective: The aim of the present study was to evaluate the clinicopathological significance of phosphorylated nuclear factor-κB (pNF-κB) expression, and its impact on epithelial–mesenchymal transition and angiogenesis in colorectal cancer (CRC).

Methods: We carried out immunohistochemistry of pNF-κB on 261 human CRC tissues, and evaluated nuclear expression, regardless of cytoplasmic expression. We also investigated the correlation between pNF-κB expression and clinicopathological characteristics, survival, and epithelial–mesenchymal transition and angiogenesis-related markers in CRC.

Results: pNF-κB was expressed in the nuclei of 164 of the 261 CRC tissues (62.8%). Furthermore, pNF-κB was significantly correlated with frequent perineural invasion, lymph node metastasis, and higher pTNM stage. However, there was no significant correlation between pNF-κB expression and other clinicopathological parameters. Among the epithelial–mesenchymal transition markers examined, SNAIL expression was significantly correlated with pNF-κB expression ($P = 0.001$) but E-cadherin expression was not. CRC with pNF-κB expression had significantly higher SIRT1 expression levels and hypoxia-inducible factor-1α expression levels than CRC without pNF-κB expression ($P < 0.001$ and $P < 0.001$, respectively). However, there was no correlation between the expression levels of pNF-κB and VEGF. pNF-κB expression was significantly correlated with worse overall and recurrence-free survival rates ($P < 0.001$ and $P < 0.001$, respectively).

Conclusion: pNF-κB expression was significantly correlated with aggressive tumor behaviors and worse survival rates. Furthermore, pNF-κB expression may affect tumor invasion and progression through SNAIL-related epithelial–mesenchymal transition and SIRT1- and hypoxia-inducible factor-1α-induced angiogenesis.

1. Introduction

The proliferation and invasion of tumor cells are the important characteristics of cancer development and progression. Furthermore, tumor invasion and migration are associated with epithelial–mesenchymal transition and changes to the cell phenotype. Hypoxia is accompanied by tumor growth and invasion, and induces angiogenesis [1]. Cancer progression is induced by various extracellular and intracellular factors. Such factors include nuclear factor-κB (NF-κB), which belongs to the RelA family. It is a transcription factor that is responsible for oxidative stress in mammalian tissues [2–4]. NF-κB is also involved in cell proliferation, apoptosis, inflammation, and oncogenesis [4,5]. In malignant tumors, NF-κB encourages tumor proliferation and progression through the transcription of pro-proliferative and anti-apoptotic genes [5–8]. Furthermore, NF-κB is involved in epithelial–mesenchymal transition and angiogenesis in malignant tumors. Hypoxia-inducible factor-1 (HIF-1) is a key transcription factor that regulates blood vessel formation by affecting the expression of vascular endothelial growth factor (VEGF) [2]. A positive correlation between HIF-1α and NF-κB was shown in surgical colorectal cancer specimens [2]. In the previous study, epithelial–mesenchymal transition is associated with E-cadherin and SNAIL in colorectal cancer (CRC) [9]. The roles of NF-kB activation have been studied in various malignant tumors, including CRC [4,5,10–12]. In human CRC tissue, NF-κB expression levels are higher in tumor cells than in normal mucosal cells [13–15]. Although the clinicopathological significance of NF-κB has been studied in CRC, the roles of NF-κB activation are unclear by various NF-κB evaluation criteria.

In the present study, we investigated the correlation between

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phosphorylated NF-κB (pNF-κB) expression and clinicopathological characteristics and patient survival in CRC. We also evaluated the impact of pNF-κB on epithelial–mesenchymal transition and angiogenesis in CRC.

2. Materials and methods

2.1. Patients and tissue array methods

The files of 261 patients who had undergone surgical resection of CRC at the Eulji University Medical Center, between January 1, 2001 and December 31, 2010, were analyzed. We reviewed medical charts, pathological records, and glass slides in order to assess clinicopathological characteristics such as age, sex, tumor size, tumor location, tumor differentiation, vascular, lymphatic, and perineural invasion, depth of tumor, lymph node metastasis, distant metastasis, and pathologic tumor node metastatic (pTNM) stages. These cases were evaluated according to the 8th Edition of the American Joint Cancer Committee TNM classifications [16]. This protocol was reviewed and approved by the Institutional Review Board of Eulji University Hospital (Approval No. EMC 2018-10-021). Five array blocks containing a total of 261 resected CRC tissue cores obtained from patients were prepared. Briefly, tissue cores (2 mm in diameter) were taken from the center of individual paraffin-embedded CRC tissues (donor blocks) and arranged in recipient paraffin blocks using a trephine apparatus, as previously described [17]. A core was chosen from each case for analysis. An adequate case was defined as a tumor occupying more than 10% of the core area. Each block contained internal controls consisting of non-neoplastic colon tissue. Clinical outcomes were followed from the date of surgery to either the date of death or recurrence, resulting in a follow-up period ranging from 0 to 60 months.

2.2. Immunohistochemical staining and evaluation

Sections 4 μm in thickness for immunohistochemistry were cut from each tissue-array block, deparaffinized, and dehydrated. Immunohistochemical stainings were conducted following a compact polymer method using a VENTANA benchmark XT autostainer (Ventana Medical Systems, Inc., Tucson, AZ). Sections were then incubated with anti-pNF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-silent mating type information regulation 2 homolog 1 (SIRT1) (Santa Cruz Biotechnology), anti-SNAIL (Santa Cruz Biotechnology), anti-E-cadherin (Santa Cruz Biotechnology), anti-HIF-1α (Novus Biologicals, Littleton, CO), and anti-VEGF antibodies (Santa Cruz Biotechnology). Visualization was performed by treatment with OPTIVIEW universal 3,3′-diaminobenzidine kit (Ventana Medical Systems, Inc.). To confirm the reaction specificity of the antibody, a negative control stain without primary antibody was performed. All immunostained sections were lightly counterstained with Mayer's hematoxylin.

2.3. Evaluation of immunohistochemistry

pNF-κB positivity was defined as unequivocal brown nuclear pNF-κB staining in ≥5% of tumor cells [4]. Because NF-κB is constitutively expressed in the cytoplasm, tumor cells showing nuclear pNF-κB staining, regardless of cytoplasmic staining, were considered to show NF-κB activation.

Immunoreactivities for SNAIL and E-cadherin were observed in the nucleus and cell membrane, respectively. In addition, SIRT1, HIF-1α and VEGF expressions were evaluated in nucleus of tumor cells. The intensities of protein expression in the immunohistochemically-stained samples were scored from 0 to 3 (0 = negative; 1 = weak; 2 = moderate; and 3 = strong). The percentage of positively stained cells was categorized based on a scoring system from 0 to 4 (1 = 0–25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100%). An immunoreactive score (IRS) was calculated by multiplying the staining intensities scores with the percentages of positively stained cells [18]. In the assessments of SNAIL, E-cadherin, SIRT1, HIF-1α, and VEGF expressions, staining patterns were classified as low (IRS: 0 to 4) or high (IRS: 6 to 12).

2.4. Statistical analysis

Statistical analyses were performed using SPSS version 22.0 software (IBM Co., Chicago, IL). The significance of the correlation between pNF-κB and the clinicopathological characteristics was determined by χ^2 test. The comparisons between pNF-κB and age and tumor size were analyzed using the two-tailed Student's *t*-test Survival curves were estimated using the Kaplan-Meier product-limit method, and differences between the survival curves were determined to be significant based on the log-rank test. In addition, the multivariate analysis was conducted using cox proportional hazard model. Results were considered statistically significant for $P < 0.05$.

3. Results

3.1. Correlation between pNF-κB expression and clinicopathological characteristics

Nuclear pNF-κB expression was found in 62.8% (164 of 261). Fig. 1 shows representative images of pNF-κB immunostaining in CRC. pNF-κB expression was significantly correlated with perineural invasion, lymph node metastasis, and higher pTNM stage ($P = 0.006$, $P = 0.028$, and $P = 0.013$, respectively; Table 1). However, there was no significant correlation between pNF-κB expression and other pathological parameters.

3.2. Correlation between pNF-κB expression and epithelial-mesenchymal transition and angiogenesis

In comparison between pNF-κB expression and epithelial-mesenchymal transition markers, SNAIL expression was significantly higher in CRCs with positive pNF-κB expression than in those with negative pNF-κB expression ($P = 0.001$). However, there was no significant correlation between pNF-κB expression and E-cadherin expression ($P = 0.315$).

Next, to evaluate the role of NF-κB in angiogenesis of CRC, the correlations between pNF-κB expression and angiogenesis-related markers were investigated. Immunohistochemistry for SIRT1, HIF-1α, and VEGF were performed among angiogenesis-related markers. pNF-κB expression was significantly correlated with SIRT1 and HIF-1α expressions ($P < 0.001$ and $P < 0.001$, respectively). However, there was no significant correlation between pNF-κB expression and VEGF expression ($P = 0.652$).

3.3. Correlation between pNF-κB expression and survival

The prognostic role of NF-κB activation in CRC was investigated. pNF-κB expression was significantly correlated with poor overall and recurrence-free survivals ($P < 0.001$ and $P < 0.001$, respectively: Fig. 2). In addition, in cox-regression test, nuclear pNF-κB expression was significantly correlated with worse overall and recurrence-free survivals ($P < 0.001$ and $P < 0.001$, respectively). In CRCs with pNF-κB expression, there was no impact of SIRT1 and HIF-1α on overall and recurrent-free survivals. In addition, there was no significant correlation between patients' survival and VEGF expression ($P = 0.699$) or HIF-1α ($P = 0.341$).

4. Discussion

NF-κB plays important roles in the proliferation and progression of malignant tumors [4–9]. Elucidating the roles of NF-κB could improve our understanding of various intracellular signaling pathways and assist

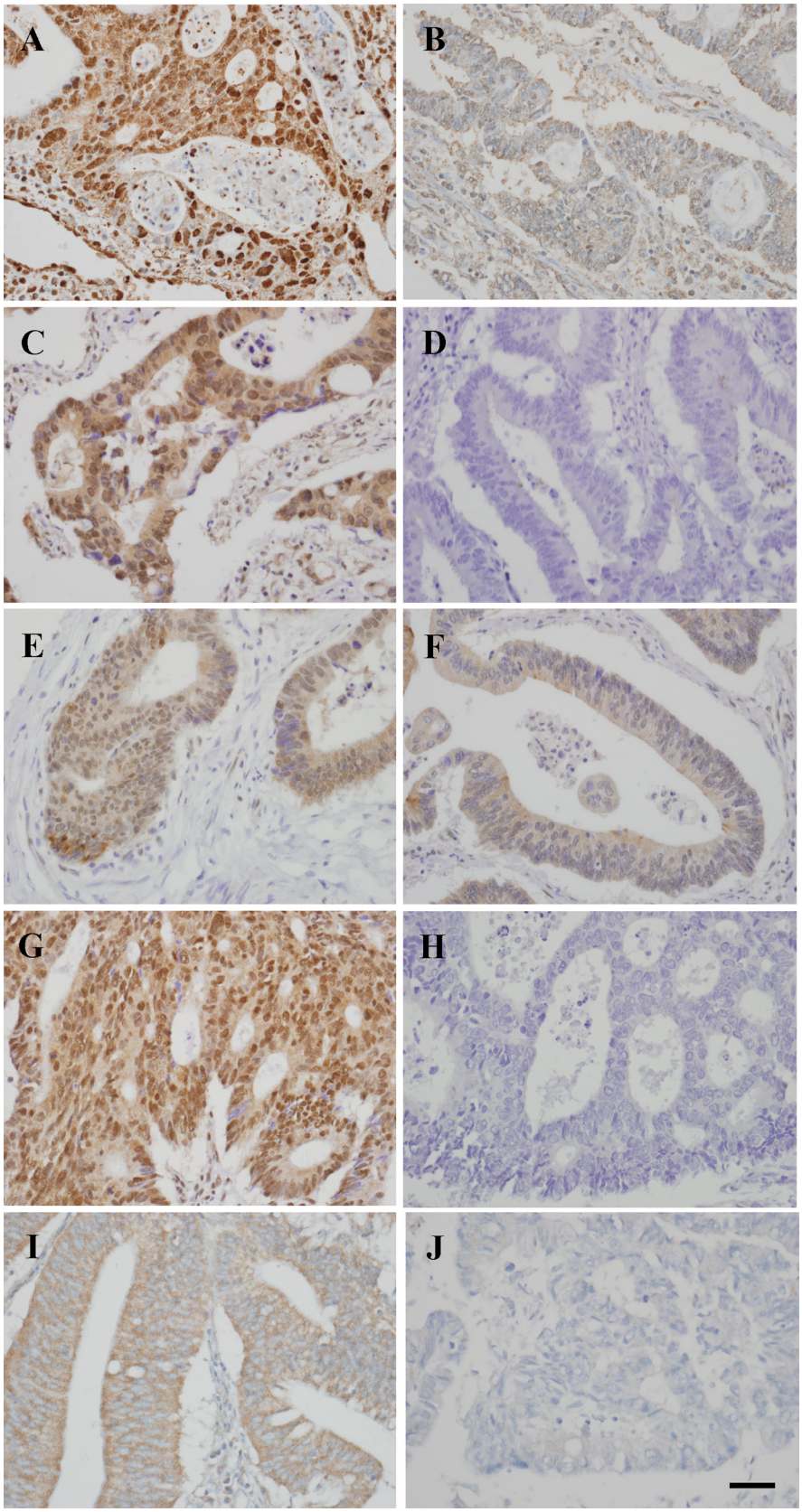

Fig. 1. Representative images showing phosphorylated NF-κB p65 expression (A: positive and B: negative), SNAIL (C: positive and D: negative), HIF-1α (E: positive and F: negative), SIRT1 (G: positive and H: negative), and VEGF (I: positive and J: negative) in colorectal cancer (×400). (Scale bar = 500 μm).

Table 1
The correlation between pNF-κB expression and clinicopathological parameters in colorectal cancers.

	pNF-κB expression		*P-value*
	Positive	Negative	
Total (n = 261)	164 (62.8)	97 (37.2)	
Age (years)	63.34 ± 13.26	63.42 ± 12.36	0.960
Sex			0.936
Male	82 (50.0)	48 (49.5)	
Female	82 (50.0)	49 (50.5)	
Tumor size			0.255
≤ 5 cm	61 (37.2)	54 (55.7)	
> 5 cm	103 (62.8)	43 (44.3)	
Tumor size (cm)	5.58 ± 2.10	5.31 ± 2.06	0.311
Location of tumor			0.376
Right colon	82 (50.0)	43 (44.3)	
Left colon and rectum	82 (50.0)	54 (55.7)	
Tumor differentiation			0.651
Well or Moderate	128 (78.0)	78 (80.4)	
Poorly	36 (22.0)	19 (19.6)	
Vascular invasion			0.484
Present	16 (9.8)	7 (7.2)	
Absent	148 (90.2)	90 (92.8)	
Lymphatic invasion			0.614
Present	41 (25.0)	27 (27.8)	
Absent	123 (75.0)	70 (72.2)	
Perineural invasion			***0.006***
Present	35 (21.3)	8 (8.2)	
Absent	129 (78.7)	89 (91.8)	
pT stage			0.523
pT1	5 (3.0)	1 (1.0)	
pT2	19 (11.6)	15 (15.5)	
pT3	122 (74.4)	73 (75.3)	
pT4	18 (11.0)	8 (8.2)	
Lymph node metastasis			***0.028***
Present	99 (60.4)	45 (46.4)	
Absent	65 (39.6)	52 (53.6)	
Distant metastasis			0.051
Present	23 (14.0)	6 (6.2)	
Absent	141 (86.0)	91 (93.8)	
pTNM stage			***0.013***
I–II	62 (37.8)	52 (53.6)	
III–IV	102 (62.2)	45 (46.4)	

Numbers in parentheses represent percentage.

the development of therapeutic agents. However, there is no conclusive information on the role of NF-κB in human CRC tissue. Furthermore, the prognostic implication of pNF-κB is controversial. To the best of our knowledge, the present study is the first attempt to provide detailed information on the clinicopathological significance and prognostic role of pNF-κB expression, and the impact of pNF-κB on epithelial–mesenchymal transition and angiogenesis through immunohistochemical analysis using human CRC tissue.

Mammalian NF-κB comprises five subunits: RelA (p65), RelB, cRel, p50 (NF-κB1), and p52 (NF-κB2) [3]. NF-κB is activated through two pathways: the canonical pathway and the alternative pathway [4,19]. The NF-κB-IκBα complex is inactive and is mainly located in the cytoplasm [7,9,20,21]. In the canonical pathway, phosphorylation of IκBα leads to dissociation of the NF-κB-IκBα complex and translocation of NF-κB into the nucleus [3,12]. The phosphorylation of the NF-κB subunits is also required to activate NF-κB. Non-phosphorylated NF-κB subunits are spontaneously exported, and have no functional effect [4,20]. In the present study, we used an antibody for phosphorylated NF-κB p65, and evaluated its expression in the nucleus, but not in the cytoplasm. That is, cytoplasmic pNF-κB p65 expression was considered negative. Although there has been some research into the prognostic value of cytoplasmic NF-κB expression, further studies are required [22].

Before investigating the clinicopathological significance of NF-kB activation, a comparison between the expression of NF-kB in normal mucosa and tumors was necessary. NF-kB activation is significantly greater in CRC cells than in the adjacent normal mucosal cells [5,7,23]. Furthermore, CRC tumor cells exhibit higher Cyclin D1, VEGF-A, and MMP-9 gene expression levels [7]. The activation of NF-κB is involved in various cellular functions, including cell proliferation, tumor progression, angiogenesis, and metastasis. These functions have been studied in various malignant tumors, such as stomach, breast, ovarian, and prostate cancers [2,4,24–27]. Various factors, including PI3K/Akt, can activate NF-κB as an upstream signaling factor in CRC [24]. Tumor progression can be evaluated by assessing the correlation between NF-κB activation, and tumor stage and distant metastasis. In the present study, pNF-κB expression was significantly correlated with higher pTNM stage, which was consistent with the results from a previous study [7].

We investigated the impact of NF-κB activation on epithelial–mesenchymal transition and angiogenesis in CRC because they are important for tumor proliferation and progression. In the present study, we evaluated epithelial–mesenchymal transition through the immunohistochemistry of SNAIL and E-cadherin. There was a significant correlation between nuclear pNF-κB expression and SNAIL expression, but not between nuclear pNF-κB expression and E-cadherin expression. Angiogenesis is necessary for metastasis and the progression of malignant tumors [28], and hypoxia occurs when cell proliferation outpaces the rate of angiogenesis [29,30]. Hypoxia induces the expression of HIF-1α, which regulates several gene transcription factors [31]. HIF-1α is frequently overexpressed in malignant tumors, and is associated with radiotherapy and chemoresistance [31–33]. HIF-1α is significantly correlated with poor survival in various cancers [32]. However, detailed evaluation in the present study revealed that VEGF expression had no significant effect on the rate of survival in CRC. The authors of a previous study reported that NF-κB expression is significantly correlated with HIF-1α expression and VEGF expression in stage III CRCs [7]. However, in contrast to the results of that study [7], there was no significant correlation between NF-κB activation and VEGF expression in the present study. In addition, microvessel density was significantly correlated with VEGF expression, but not pNF-κB expression ($P = 0.005$ and $P = 0.632$, respectively; data not shown). Furthermore, in subgroup analysis based on pTNM stage, there was no significant correlation between pNF-κB expression and VEGF expression. As described above, NF-κB activation has been evaluated using various criteria in previous studies [7]. Although our results may differ from those of previous studies, NF-κB activation is important in epithelial–mesenchymal transition and angiogenesis in CRC. In addition, because immunohistochemistry using tissue microarray methods was performed in the present study, intra-tumoral heterogeneity could be present.

The authors of several previous studies, including a meta-analysis, have reported the prognostic role of NF-κB activation in CRC [34–37]. Our results were consistent with those of the meta-analysis. However, Lewander et al. reported that nuclear NF-κB expression was not correlated with worse survival rates [22], and O'Neil et al. used immunohistochemical analysis of the NF-κB p50 subunit to evaluate NF-κB activation [35]. The discrepancies in the results of the assessment of the prognostic value of NF-κB may be caused by variations in the populations and tumor stage studied, and in the evaluation criteria used [22,30,34,36]. Lewander et al. reported that cytoplasmic pNF-κB expression was significantly correlated with worse survival in CRC, but nuclear pNF-κB expression was not [22]. As above described, because NF-κB is a transcription factor, the nuclear translocation of NF-κB is required. Therefore, the nuclear expression of NF-κB, regardless of whether it is in the phosphorylated or non-phosphorylated form, is comparable to NF-κB activation. Lewander et al. compared the cytoplasmic and nuclear expression levels of NF-κB, and found that cytoplasmic NF-κB expression was significantly correlated with nuclear NF-κB expression [22]. Because the discrepancy between the rates of nuclear and cytoplasmic NF-κB expression was 42.3%, it is unclear whether cytoplasmic NF-κB expression is proportional to the transcriptional

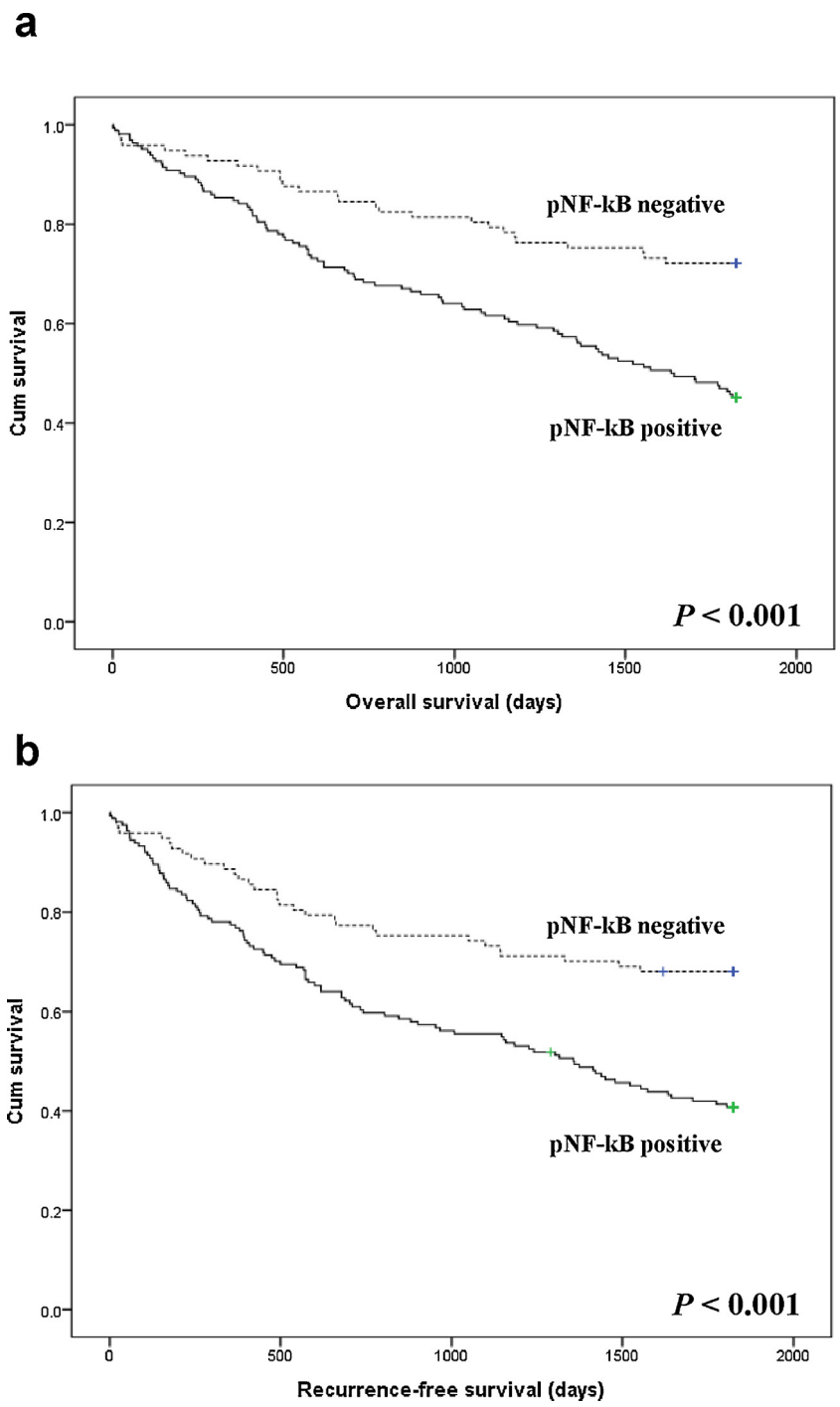

Fig. 2. Kaplan-Meier curves for overall (A) and recurrence-free survivals (B) according to phosphorylated NF-κB p65 expression. Patients with positive (solid line) and negative (dotted line) phosphorylated NF-κB p65 expression showed overall and recurrence-free survivals.

activity of NF-κB. Therefore, the prognostic value of cytoplasmic non-phospho-NF-κB expression is unclear.

In conclusion, our data showed that pNF-κB expression is significantly correlated with aggressive tumor behaviors and worse survival rates. Furthermore, there were significant correlations between pNF-κB expression and epithelial–mesenchymal transition and angiogenesis. The evaluation of pNF-κB expression may be useful for patient prognoses and the prediction of tumor behavior in CRC.

Conflict of interest

The authors declare that there are no conflicts of interest or financial disclosures.

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