ANTICANCER RESEARCH *24*: 1071-1076 (2004)

Nuclear Factor-Î B p65 is a Prognostic Indicator in Gastric Carcinoma

NAOKI YAMANAKA[1], NOBUHIKO SASAKI[1], AKIRA TASAKI[1], HIROSHI NAKASHIMA[1], MAKOTO KUBO[1], TAKASHI MORISAKI[1], HIROKAZU NOSHIRO[2], TAKASHI YAO[3], MASAZUMI TSUNEYOSHI[3], MASAO TANAKA[2] and MITSUO KATANO[1]

Departments of [1]Cancer Therapy and Research, [2]Surgery and Oncology and [3]Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Abstract. *Background: In common with other investigators, we have reported the constitutive activation of transcription factor nuclear factor-Î B (NF-Î B) in a variety of carcinomas, but there is no definite information on its clinical significance. Materials and Methods: NF-Î B p65 activation was determined by immunohistochemical analysis of surgically resected specimens from 63 gastric carcinomas. The 63 patients were divided into a high NF-Î B group (21 patients) and a low NF-Î B group (42 patients). Forty-seven of the 63 patients underwent curative resection. The 47 patients consisted of 13 high NF-Î B patients and 34 low NF-Î B patients. Results: The high NF-Î B group demonstrated a shorter overall survival rate compared with the low NF-Î B group (p=0.015). In the 47 patients who underwent curative resection, the high NF-Î B group also showed a poor survival prognosis (p=0.032). Multivariate analysis indicated that NF-Î B activation is a potential prognostic factor in gastric carcinoma. Conclusion: Constitutive activation of NF-Î B p65 may be a new prognostic parameter in gastric carcinoma.*

The transcription factor nuclear factor-Î B (NF-Î B) is a heterodimeric complex of the Rel-family proteins (1, 2). The most common dimer is the RelA (p65)/NF-Î B1 (p50) heterodimer, *i.e.*, NF-Î B (3, 4). NF-Î B is sequestered in the cytoplasms in an inactive form through interaction with the inhibitor of nuclear factor-Î B proteins (IÎ Bs) (5, 6). Various stimuli such as cytokines and infectious agents induce phosphorylation, ubiquitination and degradation of IÎ Bs and allow nuclear translocation of RelA/NF-Î B (7, 8). Once in the nucleus, RelA/NF-Î B binds to DNA at its response elements and activates the expression of genes related to inflammatory response mediators, anti- and pro-apoptotic proteins and growth factors (9, 10).

We and other investigators have previously reported the constitutive activation of NF-Î B in a variety of carcinomas, including pancreatic carcinoma (11), breast carcinoma (12), colorectal carcinoma (13), hepatocellular carcinoma (14), prostate carcinoma (15) and gastric carcinoma (16). Because NF-Î B activates the expression of genes involved in the cell cycle, invasion, metastasis, angiogenesis and anti-apoptosis, constitutive activation of NF-Î B in carcinomas may play important roles in tumor development and progression (17). Our previous study using surgically resected carcinoma tissues showed that NF-Î B activity was related to tumor size, depth of invasion and lymphatic invasion in gastric carcinoma (16). However, there has been no definite evidence concerning the clinical significance of NF-Î B activation in carcinoma tissues. This is the first report showing a possible prognostic value of the constitutive activation of NF-Î B in gastric carcinoma tissue.

Materials and Methods

Clinical samples. The patients analyzed in this study are essentially the same as those used in our previous study (16). Sixty-four patients with gastric carcinoma, who gave informed consent before surgical treatment, underwent resection at Kyushu University, Japan and one associated hospital between 1995 and 1999. One missing patient was excluded and the remaining 63 patients were entered into the present study. All 63 surgically resected primary gastric carcinoma specimens were classified histologically according to TNM classification (International Union against Cancer) (18).

Immunohistochemistry. Specimens were immunostained to evaluate nuclear translocation of RelA, as described previously (16, 19). Briefly, carcinoma specimens were fixed with 10% formalin and

Abbreviations: NF-Î B, nuclear factor-Î B; IÎ Bs, inhibitor of nuclear factor-Î B proteins; DAB, 3,3'-diaminobenzidine; PBS, phosphate-buffered saline.

Correspondence to: Mitsuo Katano, M.D., Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan. Tel: +81-92-642-6941, Fax: +81-92-642-6221, e-mail: mkatano@tumor.med.kyushu-u.ac.jp

Key Words: Nuclear translocation, RelA, immunohistochemistry, poor prognosis.

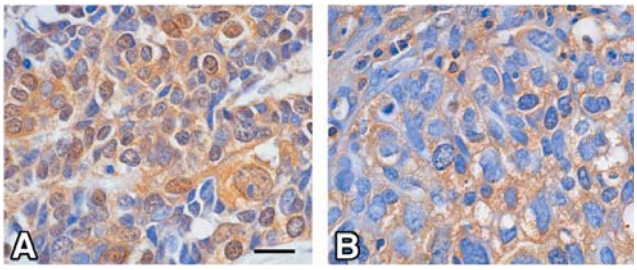

Figure 1. *Immunohistochemical staining of NF-Î B p65 in gastric carcinoma cells. A: A representative high NF-Î B case. Nuclear p65 staining is seen in 61% of carcinoma cells. B: A representative low NF-Î B case. Nuclear p65 staining is seen in only 1% of carcinoma cells. Bars, 20 μm.*

Table I. *Relationship between NF-Î B activation and clinicopathological parameters in 63 patients with gastric carcinoma who underwent resection.*

	NF-Î B > 25%	NF-Î B ≤ 25%	*P* value
Age (years)	64.95 ± 7.19	61.24 ± 14.07	0.215
Sex			
men	17	28	0.375
women	4	14	
Stage			
I, II	4	21	0.028
III, IV	17	21	
Depth of invasion			
T1	2	12	0.114
T2, 3, 4	19	30	
Nodal status			
node-negative	5	14	0.564
node-positive	16	28	
Histological type			
intestinal	11	17	0.427
diffuse	10	25	

embedded in paraffin. Slides were probed with anti-RelA (p65; 1:150; sc-109; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated with secondary antibodies (goat anti-rabbit immunoglobulin; Nichirei Co., Ltd., Tokyo, Japan). Finally, antibody binding was detected with a combination of 3,3'-diaminobenzidine (DAB, 40 mg/150 ml) in phosphate-buffered saline (PBS; Wako Pure Chemical Industries, Hyogo, Japan) and 0.06% hydrogen peroxide.

The specimens were photographed with a digital camera (Jenoptik, Binary Planner 4490, Jena, Germany) connected to a microscope (Olympus, BX51, Tokyo, Japan). The numbers of nuclear-positive cells in each specimen were counted. One hundred cells were counted for each section. Nuclear staining, which indicated nuclear translocation of RelA, was considered to be a marker of NF-Î B activation.

NF-Î B activation was dichotomized at 25% on the basis of the mean ± SD (22.5% ± 2.4%) percentage of nuclear RelA. Thus, 63 patients were divided into a high NF-Î B group (> 25%) and a low NF-Î B group (≤ 25%) (16).

Statistical analysis. Fisher's test was used for the statistical analyses that related the NF-Î B activation and clinicopathological parameters. Survival curves were calculated using the Kaplan-Meier method and analyzed using the log-rank test. The influence of each variable on survival was assessed by Cox's proportional hazard regression model. All calculations were carried out using StatView 5.0J (Abacus Concepts, Berkeley, CA, USA). All results with a p level less than 0.05 were considered significant.

Results

Of the 63 specimens evaluated, 21 were included in the high NF-Î B group and 42 in the low NF-Î B group. Representative carcinoma specimens showing high NF-Î B activation and low NF-Î B activation are shown in Figure 1. Table I shows the correlation between NF-Î B activation and the clinicopathological parameters. A significant correlation was found between NF-Î B activation and tumor stage ($p=0.028$). The prognosis of the high NF-Î B group was significantly worse than that of the low NF-Î B group ($p=0.015$, Figure 2). Multivariate analysis indicated that NF-Î B activation ($p=0.033$) was an independent prognostic factor in this group (Table II).

Next, we evaluated the prognoses of 47 of the 63 patients who underwent curative resection. The prognoses of the 13 high NF-Î B patients in this group were worse than those of the 34 low NF-Î B patients ($p=0.032$, Figure 3). No correlation was found between NF-Î B activation and the clinicopathological parameters examined (Table III). Although multivariate analysis indicated that the NF-Î B activation may be a potential prognostic factor (hazards ratio 2.442), NF-Î B activation was not an independent prognostic factor in this group ($p=0.104$, Table IV).

Finally, the prognostic value of NF-Î B activation was analyzed in 25 patients with relatively early-stage carcinoma (stages I and II) of the 47 patients who underwent curative resection. The prognoses of the 4 high NF-Î B patients in this group were worse than those of the 21 low NF-Î B patients ($p=0.007$, Figure 4). No correlation was found between NF-Î B activation and the clinicopathological parameters examined (Table V).

Discussion

In the present study, we showed for the first time the prognostic value of the constitutive activation of NF-Î B in gastric carcinomas. Patients showing high NF-Î B activation in carcinoma tissue did not survive as long as those with low NF-Î B activation.

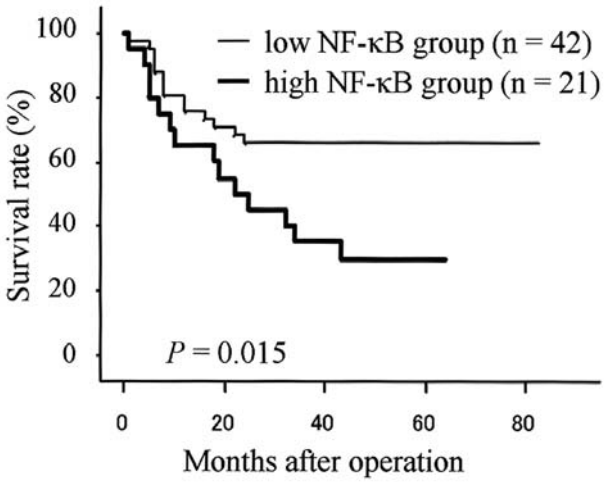

Figure 2. *Survival curves of the 63 patients with gastric carcinoma who underwent resection, according to NF-Î B activation in the carcinoma specimens.*

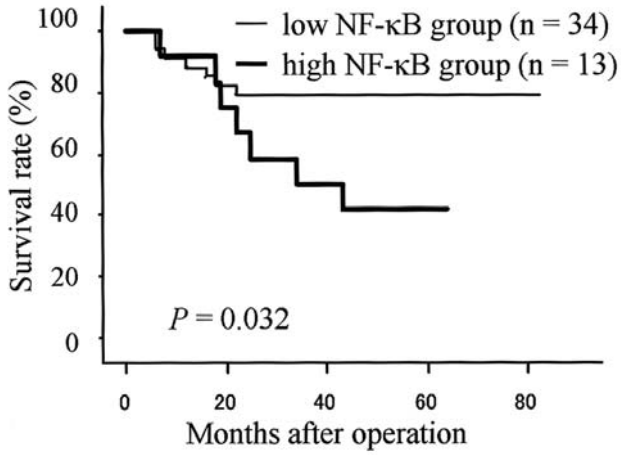

Figure 3. *Survival curves of the 47 patients with gastric carcinoma who underwent curative resection, according to NF-Î B activation in the carcinoma specimens.*

Table II. *Multivariate analyses of risk factors affecting survival rate in 63 patients with gastric carcinoma who underwent resection.*

Parameter	Hazard ratio	*P* value
Depth of invasion		
T1 *vs* T2, 3, 4	0.533	0.315
Lymph node metastasis		
negative *vs* positive	0.303	0.064
Histological type		
intestinal *vs* diffuse	1.440	0.379
NF-Î B activation		
≤ 25% *vs* > 25%	2.275	0.033

Table III. *Relationship between NF-Î B activation and clinicopathological parameters in 47 patients with gastric carcinoma who underwent curative resection.*

	NF-Î B > 25%	NF-Î B ≤ 25%	*P* value
Age (years)	64.38 ± 8.48	61.21 ± 13.89	0.349
Sex			
men	10	23	0.461
women	3	11	
Stage			
I, II	4	21	0.101
III, IV	9	13	
Depth of invasion			
T1	2	11	0.301
T2, 3	11	23	
Nodal status			
node-negative	3	14	0.321
node-positive	10	20	
Histological type			
intestinal	7	16	0.752
diffuse	6	18	

Although a large number of investigations strongly suggest that NF-Î B is constitutively activated in gastric and other carcinomas (11-16) and is an important transcriptional factor related to biological malignant characteristics such as anti-apoptosis and invasiveness of carcinomas (17, 20), the biological significance of NF-Î B activation in carcinoma tissues has remained unclear. In the present study, therefore, we focused on the prognostic value of NF-Î B activation in gastric carcinoma tissue.

Because all the carcinoma specimens were collected at Kyushu University, Japan and only one associated hospital, from 1995 to 1999, and all analyses were performed without knowledge of the corresponding clinical and pathological data, the patient population was quite homogeneous in terms of the surgical procedures performed, the postoperative therapeutic schedules and the follow-up schedules. As data from our previous report indicated (16), data from the present study confirmed that NF-Î B activation is a new prognostic parameter in gastric carcinoma.

We next examined whether NF-Î B is independent of traditional pathological parameters. Of the traditional clinicopathological parameters, lymph node metastasis seems to be among the more important risk factors for predicting overall survival in gastric carcinoma patients (21, 22). Lymph node metastasis was also a potential prognostic factor in all of the 63 patients entered in the present study (p=0.064).

Table IV. *Multivariate analyses of risk factors affecting survival rate in 47 patients with gastric carcinoma who underwent curative resection.*

Parameter	Hazard ratio	*P* value
Depth of invasion		
T1 *vs* T2, 3	0.571	0.487
Lymph node metastasis		
negative *vs* positive	0.439	0.320
Histological type		
intestinal *vs* diffuse	1.171	0.776
NF-Î B activation		
≤ 25% *vs* > 25%	2.442	0.104

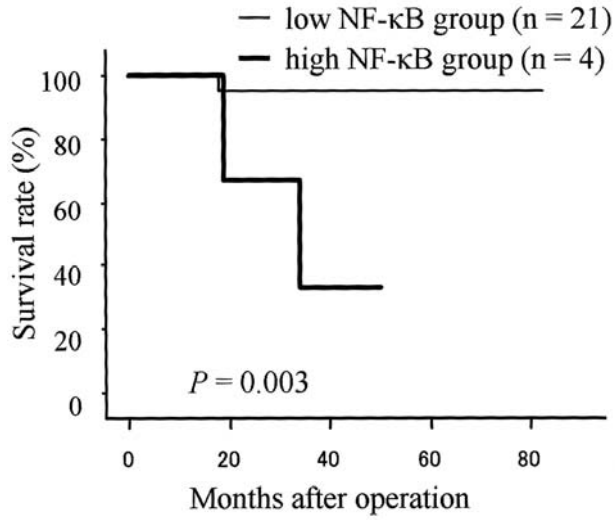

Figure 4. *Survival curves of the 25 patients with stage I and II gastric carcinoma who underwent curative resection, according to NF-Î B activation in the carcinoma specimens.*

However, there was no significant relationship between lymph node metastasis and NF-Î B activation (Table I), suggesting independence of NF-Î B from lymph node metastasis. In fact, multivariate analysis showed that only NF-Î B was a prognostic parameter and that NF-Î B was independent of the traditional pathological parameters, including lymph node metastasis, in all 63 patients (Table II). However, the independence of NF-Î B disappeared when the 47 patients who underwent curative resection were analyzed (Table IV), whereas the hazards ratio (2.442) of NF-Î B was the highest of the four parameters examined (Table II).

We found a significant correlation between NF-Î B activation and lymphatic invasion ($p=0.036$ in the 63 patients, $p=0.005$ in the 47 patients). However, NF-Î B activation showed no significant correlation to venous invasion ($p=0.064$ in the 63 patients, $p=0.186$ in the 47 patients). Because it is generally accepted that lymphatic invasion is well correlated to lymph node metastasis (23, 24), there is a possibility that many of our patients have occult micro-lymph node metastasis and that these patients have a poor prognosis. In fact, recent investigations have indicated that occult micro-lymph node metastasis, which is defined by immunostaining or genetic procedures, is a potent prognostic indicator in the early-stage of gastric carcinoma without histopathological lymph node metastasis (25).

Table V. *Relationhip between NF-Î B activation and clinicopathological parameters in 25 patients with stages I and II gastric carcinoma who underwent curative resection.*

	NF-Î B > 25%	NF-Î B ≤ 25%	*P* value
Age (years)	68.50 ± 5.51	62.19 ± 13.54	0.144
Sex			
men	3	14	>0.999
women	1	7	
Depth of invasion			
T1	2	11	>0.999
T2	2	10	
Nodal status			
node-negative	2	14	0.602
node-positive	2	7	
Histological type			
intestinal	2	13	>0.999
diffuse	2	8	

Four of the 21 high NF-Î B patients in the group of 63 were relatively early-stage patients (stages I and II). All 4 patients showed positive lymphatic invasion, but 2 patients were free from lymph node metastasis. Of these 4 patients, one died 19 months (node-negative patient) and one died 34 months (node-positive patient) after surgery. However, 21 of the 42 low NF-Î B patients were relatively early-stage patients. Of these 21 patients, 7 patients showed positive lymphatic invasion. Only one patient (node-negative) died, 18 months after surgery. He showed negative lymphatic invasion and an NF-Î B nuclear translocation rate of 25%. These results suggest a significant correlation between NF-Î B activation and lymphatic invasion ($p=0.039$). Because there are no satisfactory parameters apart from lymph node status for evaluating survival prognosis of early-stage gastric carcinoma patients, we hypothesize that patients with early-stage gastric carcinoma showing high NF-Î B activation are high-risk patients (Figure 4). Though it is still unknown whether NF-Î B activation itself plays a significant role in the aggressive characteristics of carcinoma such as lymphatic invasion, the present data showed for the first

time a clinical significance, *i.e.*, prognostic value, for the constitutive activation of NF-Î B in gastric carcinoma.

Acknowledgements

We thank Nobuhiro Torada, Takaaki Kanemaru and Kaori Nomiyama for their skillful technical assistance. This study was supported in part by a Grant for Scientific Research (13470240) from the Ministry of Education Culture, Sports, Science and Technology, Japan.

References

1 Karin M and Lin A: NF-Î B at the crossroads of life and death. Nat Immunol *3*: 221-227, 2002.
2 Karin M, Cao Y, Greten FR and Li ZW: NF-Î B in cancer: from innocent bystander to major culprit. Nat Rev Cancer *2*: 301-310, 2002.
3 Ghosh S, May MJ and Kopp EB: NF-Î B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol *16*: 225-260, 1998.
4 Santoro MG, Rossi A and Amici C: NF-Î B and virus infection: who controls whom. EMBO J *22*: 2552-2560, 2003.
5 Barnes PJ and Karin M: Nuclear factor-Î B – a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med *36*: 1066-1071, 1997.
6 Vermeulen L, De Wilde G, Notebaert S *et al*: Regulation of the transcriptional activity of the nuclear factor-Î B p65 subunit. Biochem Pharmacol *64*: 963-970, 2002.
7 Bharti AC and Aggarwal BB: Nuclear factor-kappa B and cancer: its role in prevention and therapy. Biochem Pharmacol *64*: 883-888, 2002.
8 Haefner B: NF-Î B: arresting a major culprit in cancer. Drug Discov Today *7*: 653-663, 2002.
9 Chen F, Castranova V and Shi X: New insights into the role of nuclear factor-Î B in cell growth regulation. Am J Pathol *159*: 387-397, 2001.
10 Li X and Stark GR: NFÎ B-dependent signaling pathways. Exp Hematol *30*: 285-296, 2002.
11 Wang W, Abbruzzese JL, Evans DB *et al*: Overexpression of urokinase-type plasminogen activator in pancreatic adenocarcinoma is regulated by constitutively activated RelA. Oncogene *18*: 4554-4563, 1999.
12 Biswas DK, Dai SC, Cruz A *et al*: The nuclear factor kappa B (NF-Î B): a potential therapeutic target for estrogen receptor negative breast cancers. Proc Natl Acad Sci USA *98*: 10386-10391, 2001.
13 Lind DS, Hochwald SN, Malaty J *et al*: Nuclear factor-kappa B is upregulated in colorectal cancer. Surgery *130*: 363-369, 2001.
14 Tai DI, Tsai SL, Chang YH *et al*: Constitutive activation of nuclear factor Î B in hepatocellular carcinoma. Cancer *89*: 2274-2281, 2000.
15 Chen CD and Sawyers CL: NF-Î B activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. Mol Cell Biol *22*: 2862-2870, 2002.
16 Sasaki N, Morisaki T, Hashizume K *et al*: Nuclear factor-Î B p65 (RelA) transcription factor is constitutively activated in human gastric carcinoma tissue. Clin Cancer Res *7*: 4136-4142, 2001.
17 Arlt A, Vorndamm J, Müerköster S *et al*: Autocrine production of interleukin 1β confers constitutive nuclear factor Î B activity and chemoresistance in pancreatic carcinoma cell lines. Cancer Res *62*: 910-916, 2002.
18 Sobin LH: Digestive system tumours: stomach. *In*: TNM Classification of Malignant Tumours, 5th ed (Wittekind Ch, eds). New York, Wiley-Liss. 1997, pp 59-62.
19 Zabel U, Henkel T, Silva MS *et al*: Nuclear uptake control of NF-Î B by MAD-3, an IÎ B protein present in the nucleus. EMBO J *12*: 201-211, 1993.
20 Wang JH, Manning BJ, Wu QD, Blankson S, Bouchier-Hayes D and Redmond HP: Endotoxin/lipopolysaccharide activates NF-Î B and enhances tumor cell adhesion and invasion through a β1 integrin-dependent mechanism. J Immunol *170*: 795-804, 2003.
21 Yokota T, Kunii Y, Teshima S *et al*: Significant prognostic factors in patients with early gastric cancer. Int Surg *85*: 286-290, 2000.
22 Kim JP, Lee JH, Kim SJ *et al*: Clinicopathologic characteristics and prognostic factors in 10,783 patients with gastric cancer. Gastric Cancer *1*: 125-133, 1998.
23 Matsumoto M, Natsugoe S, Nakashima S, Nakajo A *et al*: Lymph node micrometastasis and lymphatic mapping determined by reverse transcriptase-polymerase chain reaction in pN0 gastric carcinoma. Surgery *131*: 630-635, 2002.
24 Yokota T, Saito T, Teshima S *et al*: Probability of lymph node metastasis in small gastric cancer tumor: is it an indication for limited surgery? Int Surg *86*: 206-209, 2001.
25 Lee E, Chae Y, Kim I *et al*: Prognostic relevance of immunohistochemically detected lymph node micrometastasis in patients with gastric carcinoma. Cancer *94*: 2867-2873, 2002.

Received November 11, 2003
Accepted February 5, 2004