indices (LI) were determined by counting the positively stained nuclei in at least 500 tumour cells.

STATISTICAL ANALYSIS

Fisher's exact test was used to evaluate the association between two dichotomous variables. The difference in the MIB-1, cyclin D1, cyclin A, cyclin E, p21 and p27 LIs between each of the groups was estimated by an unpooled *t*-test.

For statistical analyses, a *P*-value < 0.05 was considered to be significant.

Results

CLINICAL FINDINGS

The clinical data of LGFMS are summarized in Table 1. The patients' ages ranged from 11 to 51 years (average 31.5 years). Six patients were female and five were male. Seven cases occurred in the extremities (four in the buttock and one each in the thigh, the shoulder and the upper arm). Four arose in the neck, abdominal wall or back.

Tumour size was known in 10 cases and varied from 17 mm to 180 mm in maximum diameter, with an average of 68 mm. Nine tumours were situated in deep soft tissue (subfacial or even deeper), whereas two tumours were situated superficially (subcutis).

Three patients were initially treated by wide excision and eight patients received simple excision. Follow-up data were available in eight cases with a duration ranging from 24 to 147 months (mean 67.2 months). Local recurrence occurred in four cases, in which simple excision had been performed as the initial treatment. No local recurrence was observed in any of three patients who had been treated with wide excision. One patient (case 5) died of recurrent inoperable tumour, but no patients were affected by lung metastasis in this series.

Among 75 cases of MFS, 22 cases were categorized as FNCLCC grade 1, low-grade MFS. The clinical data of this group are summarized in Table 2. The patients included 13 males and nine females. The average age was 60.1 years (range 20–83 years). Tumour locations included the extremities in 18 patients (five in the thigh, four in the lower leg, three in the groin, two each in the shoulder and forearm, and one each in the scapular region and the buttock) and the chest wall or back in four patients. Tumour size was known in 19 cases, ranging from 10 mm to 120 mm, with an average of 50 mm.

Table 1. Clinical and pathological data of patients with low-grade fibromyxoid sarcoma

Case	Age (years)/sex	Location/depth	Size, mm	Treatment	Follow-up
1	32/M	Thigh/D	180	Wide excision	NED 24 months
2	31/M	Buttock/D	160	Excision	NA
3	13/M	Buttock/S	NA	Excision	Local recurrence at 2, 3, 5 years; wide excision: NED at 63 months
4	29/F	Back/D	75	Excision	Local recurrence at 36 months; wide excision: NED at 56 months
5	40/F	Neck/D	30	Excision	Local recurrence at 24 months; inoperable: DOD at 54 months
6	21/F	Buttock/D	50	Excision	NA
7	47/M	Buttock/D	30	Wide excision	NED at 64 months
8	11/F	Shoulder/D	45	Excision	NED at 34 months
9	25/F	Back/S	17	Excision	NA
10	47/F	Abdominal wall/D	40	Excision	Local recurrence at 96 months
11	51/M	Upper arm/D	55	Wide excision	NED at 147 months

D, Deeply situated (subfascial or even deeper) tumour; S, superficially situated (subcutis) tumour; DOD, died of disease; NED, no evidence of disease; NA, not available.

Table 2. Clinical and pathological data of low-grade myxofibrosarcoma

Case	Age (years)/sex	Location/depth	Size, mm	Treatment	Follow-up
1	63/F	Thigh/S	35	Excision	NED at 56 months
2	60/M	Lower leg/S	10	Wide excision	NED at 153 months
3	20/F	Thigh/S	40	Excision	NA
4	77/F	Shoulder/D	120	Wide excision	Died of other causes at 2 months
5	34/M	Scapular region/S	15	Excision	NED at 84 months
6	72/M	Lower leg/U	NA	Excision	NA
7	28/M	Lower leg/S	25	Excision	NA
8	62/M	Groin/S	45	Wide excision	Local recurrence at 31 months; wide excision: NED at 100 months
9	77/M	Thigh/U	NA	Excision	NA
10	41/F	Thigh/D	30	Excision	Local recurrence at 11 months; DOD at 48 months
11	77/M	Chest wall/D	90	Excision	Local recurrence at 55 months; died of other cause at 140 months
12	68/M	Shoulder/S	85	Wide excision	NED at 98 months
13	57/F	Chest wall/D	43	Wide excision	NED at 13 months
14	83/F	Chest wall/S	30	Wide excision	NA
15	54/M	Groin/D	45	Excision	Local recurrence at 48 months
16	75/M	Forearm/S	25	Excision	Local recurrence at 24 months; wide excision: NED at 149 months
17	69/F	Back/S	NA	Wide excision	NED at 57 months
18	75/F	Buttock/S	100	Excision	Local recurrence at 35 months
19	72/M	Thigh/S	90	Excision	NED at 48 months
20	66/M	Groin/S	35	Excision	Local recurrence at 9 months; wide excision: NED at 75 months
21	50/F	Forearm/D	60	Excision	Local recurrence at 37 months
22	43/M	Lower leg/S	30	Excision	Local recurrence at 5 months; wide excision: NED at 138 months

D, Deeply situated (subfascial or even deeper) tumour; S, superficially situated (subcutis) tumour; U, unknown; DOD, died of disease; NED, no evidence of disease; NA, not available.

Six tumours were deeply situated, and 14 were superficially situated. The depth of two tumours was unknown. Seven patients were treated with wide excision, whereas 15 were treated with simple excision. Follow-up data were available in 17 cases with a duration ranging from 2 to 153 months (mean 71.1 months). Local recurrence occurred in nine patients including one patient who had received wide excision and eight who had received simple excision. One patient died of lung metastasis (case 10).

As for a comparison between LGFMS and low-grade MFS, the patients with low-grade MFS were significantly older than those with LGFMS (MFS average 60.1 years; LGFMS average 31.5 years; $P < 0.0001$, unpooled *t*-test) and significantly more of them had tumours which were situated superficially (low-grade MSF 14/20; LGFMT 2/11; $P = 0.0077$, Fisher's exact test). However, no difference was observed with regard to tumour size.

PATHOLOGICAL FINDINGS

The cut surface of LGFMS presented a greyish-white collagenous mass with intermingling myxoid areas (Figure 4). The mitotic index ranged from 0 to 5 per 50 high-power fields (HPFs) (× 400) (mean 1.0 per 50 HPFs). None of the LGFMS contained tumour necrosis and all were FNCLCC grade 1 tumours. One tumour contained an area of giant rosettes (case 9). AJCC stage was evaluable in 10 cases. Six cases (60%) were considered to be stage IA and four cases (40%) stage IIA.

Grossly, low-grade MFS exhibited gelatinous tumour nodules, accompanied by white opaque nodules and haemorrhage (Figure 5). No cases contained solid areas. Only one case (4.5%) showed tumour necrosis of < 50% of the tumour, whereas in the remaining 21 cases tumour necrosis was absent. The mitotic index ranged from 0 to 6 per 10 HPFs (mean 1.4 per 10 HPFs). One patient who died with lung metastases (case 10) showed high-grade storiform–pleomorphic sarcoma in their recurrent tumour. AJCC stage was evaluable in 19 cases. Sixteen cases (84.2%) were considered to be AJCC stage I (A and B) and three (15.8%) stage IIA.

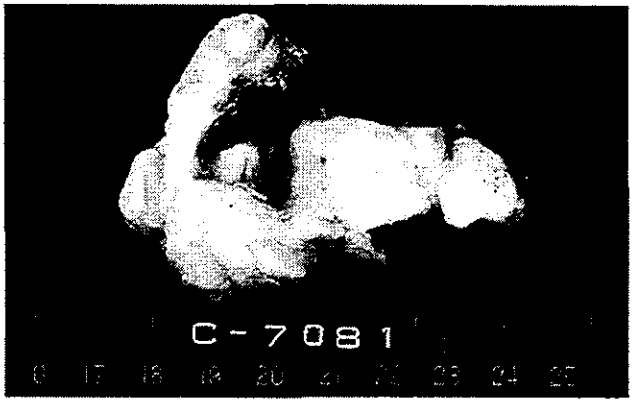

Figure 4. Gross findings of low-grade fibromyxoid sarcoma. The tumour shows firm yellow-white cut surfaces with a focal glistening appearance.

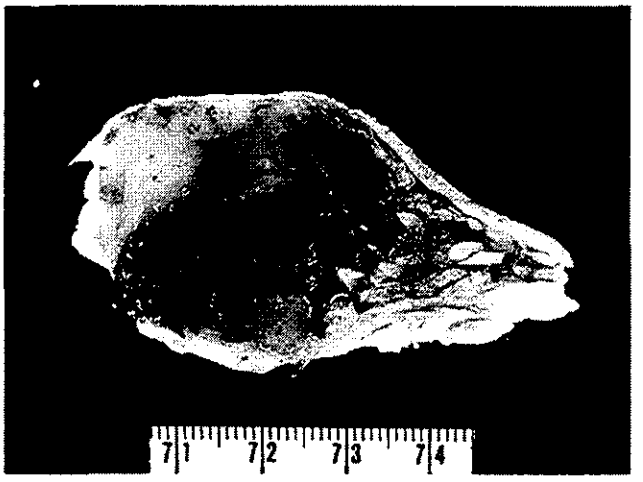

Figure 5. Cut surface of low-grade myxofibrosarcoma. The multinodular tumour located within the subcutis shows a gelatinous appearance admixed with focal greyish solid areas, accompanied by stromal haemorrhage.

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Among nine cases of LGFMS, tumour cells were positive for p53 and MDM2 in one case (11.1%) and three cases (33.3%), respectively. In all nine cases, p16 expression was normally preserved. As for 13 cases of low-grade MFS, positive immunoreactivity for p53 and MDM2 was observed in three (23.1%) and six cases (46.2%), respectively. p16 expression was abnormally reduced in three out of 13 cases (23.1%).

Immunoreactivity for MIB-1, p21, p27, cyclin A, cyclin D1 and cyclin E is summarized in Table 3. The MIB-1 LI ($P = 0.0084$) and cyclin E LI ($P = 0.0239$) in low-grade MFS (MIB-1 LI, mean 14.76; cyclin E LI, mean 11.55) were significantly higher than those in LGFMS (MIB-1 LI, mean 4.68; cyclin E LI, mean 3.38) (Table 3, Figure 6a,b). On the other hand, the p21 LI ($P = 0.0112$) and p27 LI ($P = 0.0363$) in low-grade MFS (p21 LI, mean 25.53; p27 LI, mean 42.68) were significantly lower than those of LGFMS (p21 LI, mean 42.74; p27 LI, mean,57.28) (Table 3, Figure 6c–f). There was no statistically significant difference between low-grade MFS and LGFMS with regard to p53, MDM2, p16, cyclin D1 or cyclin A expression. No significant relationship was observed between the expression of these cell cycle regulators and local recurrence in either LGFMS or low-grade MFS.

Discussion

Low-grade fibromyxoid sarcoma (LGFMS) is an uncommon soft-tissue tumour initially characterized by its

Table 3. Labeling index (LI) of MIB-1 and cell cycle regulators in low-grade fibromyxoid sarcoma (LGFMS) and low-grade myxofibrosarcoma (MFS)

Antibodies		LI			*P*-value
		Average	SD	SE	
MIB-1	LGFMS ($n = 9$)	4.68	4.01	1.34	0.0084*
	Low-grade MFS ($n = 13$)	14.76	9.73	2.70	
p21	LGFMS	42.74	17.69	5.89	0.0112*
	Low-grade MFS	25.53	11.29	3.13	
p27	LGFMS	57.28	18.80	6.27	0.0363*
	Low-grade MFS	42.68	11.81	3.28	
Cyclin A	LGFMS	3.75	2.46	0.82	0.1413
	Low-grade MFS	7.27	6.54	1.82	
Cyclin D1	LGFMS	21.20	12.21	4.07	0.1420
	Low-grade MFS	14.22	9.25	2.56	
Cyclin E	LGFMS	3.38	1.61	0.54	0.0239*
	Low-grade MFS	11.55	9.86	2.74	

SD, Standard deviation; SE, standard error.
*Statistically significant.

deceptively benign histological appearance and rather frequent metastasis. The original report by Evans demonstrated that seven out of 12 cases developed distant metastasis, with a follow-up ranging from 5.5 to 50 years.[2] However, a recent study reported less frequent metastasis, although its better prognosis may simply reflect the fact that this entity is now well known and is treated with aggressive surgery.[15] In the series of Goodlad *et al.*,[3] only one of 11 cases developed pulmonary metastasis, although the follow-up duration was not as long as that of Evans's cases. The high metastatic rate of early reports may be based on selectively reporting metastasizing cases or ascertaining cases after metastases had occurred. In our 11 cases, no patients developed distant metastasis and only one patient died of tumour, due to uncontrolled tumour recurrence. The low metastatic rate of our series may also be related to aggressive surgery and a rather short follow-up period.

LGFMS with giant collagen rosettes was initially described as 'hyalinizing spindle cell tumour with giant rosettes'.[14] A recent study demonstrated that the biological behaviour of LGFMS with and without giant rosettes is identical.[15] Moreover, cytogenetic study has shown that both tumours share a common t(7;16)(q34;p11) translocation.[18] In our small series, only one out of 11 cases revealed prominent giant collagenous rosettes.

Weiss and Goldblum[19] defined myxoid malignant fibrous histiocytoma (MFH) as MFH with myxoid areas in excess of 50% of the tumour, while Mentzel *et al.*[7] defined myxofibrosarcoma (MFS) as myxoid MFH including low-grade and high-grade tumours, with a myxoid area of at least 10%. Mentzel *et al.*[7] divided their MFSs into three groups: low, intermediate and high-grade lesions according to cellularity, nuclear pleomorphism and necrosis. The FNCLCC grading system is based on tumour differentiation, mitotic count and tumour necrosis and has been reported as being a reproducible histological grading system.[8] In this study, we used Weiss and Goldblum's diagnostic criteria for the diagnosis of MFS. Moreover, we employed the FNCLCC grading system and defined a low-grade MFS as FNCLCC grade 1 tumour.

Recently, Weiss and Goldblum described LGFMS as a fibromyxoid variant of fibrosarcoma and MFS as a myxoid variant of fibrosarcoma.[14] The distinctive histological features of MFS were myxoid matrix containing elongated curvilinear capillaries, and fusiform, round or stellate tumour cells with hyperchromatic atypical nuclei.[7] On the other hand, LGFMS shows contrasting fibrous and myxoid areas, a swirling

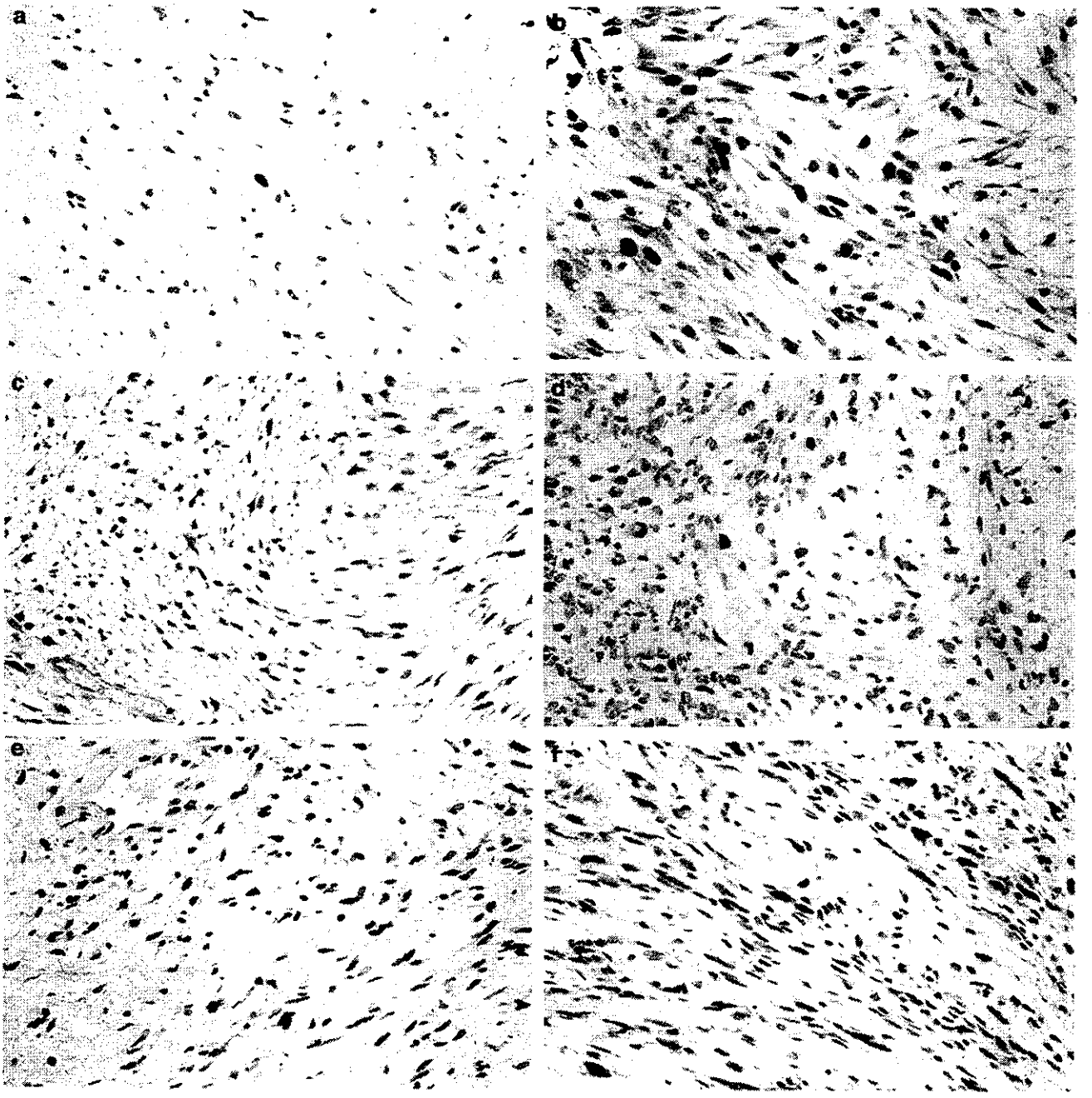

Figure 6. a, Low-grade fibromyxoid sarcoma (LGFMS) showing low MIB-1 labelling index (LI) of 3.7 (case 10). b, Low-grade myxofibrosarcoma (MFS) exhibiting high MIB-1 LI of 37.0 (case 4). c, LGFMS with high p21 LI of 70.5 (case 6). d, Low-grade MFS with low p21 LI of 7.4 (case 12). e, LGFMS with high p27 LI of 87.3 (case 10). f, Low-grade MFS with low p27 LI of 32.1 (case 17).

or whorled growth pattern of bland and deceptively benign appearing fibroblastic spindle cells.[2] The morphological distinction between LGFMS and low-grade MFS is sometimes difficult.[7] Compared with low-grade MFS, LGFMS is histologically made up of bland appearing, uniform spindle cells which have no striking nuclear atypia and which are arranged in a whorled pattern within a more prominent collagenous stroma.[2,3,7] Therefore, most tumours may be distinguished on histological grounds. Moreover, LGFMS has been reported to have a tendency to occur within the deep soft tissue of young adults.[3] In the current study we demonstrated that patients with LGFMS are significantly younger than those with low-grade MFS and that LGFMS is situated more frequently in deep soft tissue compared with low-grade MFS.

The alterations of *INK4A* and *INK4B* genes are reported to be restricted to high-grade adult soft-tissue sarcomas and correlated with poor prognosis.[20] In our study, all nine cases of LGFMS and 12 out of 13 cases of low-grade MFS showed preservation of p16 expression, immunohistochemically. Reduced expression of p16 seems therefore to be a rare event in such low-grade soft tissue sarcomas.

p21, p27 and p53 proteins are important regulators of the cell cycle. Wild-type p53 is a critical participant in G_1 cell cycle arrest through induction of the p21 gene product that shares partial structural homology with p27.[21,22] p27 is a negative regulator of the cell cycle and potential tumour suppressor gene.[23,24] In the current study, we found that MIB-1 LI and cyclin E LI in low-grade MFS are higher than those of LGFMS, whereas p21 LI and p27 LI in low-grade MFS are lower than those of LGFMS. The difference in the expression of these cell cycle regulator proteins may be useful for the distinction between LGFMS and low-grade MFS, in addition to the differences in histological features, patients' ages and tumour depth. These immunohistochemical differences of cell cycle regulators support the fact that both tumours are distinctive clinicopathological entities. However, the reason for the rather frequent rate of metastasis reported for LGFMS is still unknown.

In conclusion, low-grade MFH and LGFMS are distinctly different clinicopathological entities and the assessment of the immunohistochemical expression of cell cycle regulators such as cyclin E, p21 and p27 LGFM may be helpful to distinguish low-grade MFS from LGFMS in addition to conventional clinicopathological features.

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Nuclear Factor-κB p65 is a Prognostic Indicator in Gastric Carcinoma

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

The transcription factor nuclear factor-κB (NF-κB) is a heterodimeric complex of the Rel-family proteins (1, 2). The most common dimer is the RelA (p65)/NF-κB1 (p50) heterodimer, *i.e.*, NF-κB (3, 4). NF-κB is sequestered in the cytoplasms in an inactive form through interaction with the inhibitor of nuclear factor-κB proteins (IκBs) (5, 6). Various stimuli such as cytokines and infectious agents induce phosphorylation, ubiquitination and degradation of IκBs and allow nuclear translocation of RelA/NF-κB (7, 8). Once in the nucleus, RelA/NF-κB 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-κB 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-κB activates the expression of genes involved in the cell cycle, invasion, metastasis, angiogenesis and anti-apoptosis, constitutive activation of NF-κB in carcinomas may play important roles in tumor development and progression (17). Our previous study using surgically resected carcinoma tissues showed that NF-κB 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-κB activation in carcinoma tissues. This is the first report showing a possible prognostic value of the constitutive activation of NF-κB 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-κB, nuclear factor-κB; IκBs, inhibitor of nuclear factor-κB proteins; DAB, 3,3'-diaminobenzidine; PBS, phosphate-buffered saline.

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

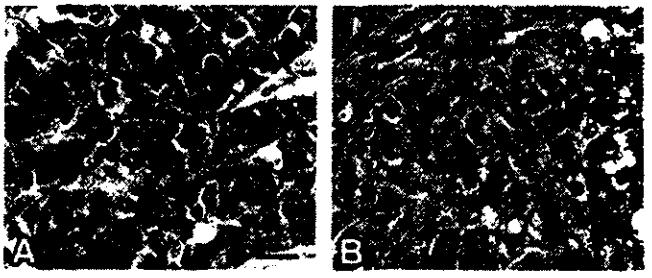

Figure 1. *Immunohistochemical staining of NF-κB p65 in gastric carcinoma cells. A: A representative high NF-κB case. Nuclear p65 staining is seen in 61% of carcinoma cells. B: A representative low NF-κB case. Nuclear p65 staining is seen in only 1% of carcinoma cells. Bars, 20 μm.*

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-κB activation.

NF-κB 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-κB group (> 25%) and a low NF-κB group (≤ 25%) (16).

Statistical analysis. Fisher's test was used for the statistical analyses that related the NF-κB 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-κB group and 42 in the low NF-κB group. Representative carcinoma specimens showing high NF-κB activation and low NF-κB activation are shown in Figure 1. Table I shows the correlation between NF-κB activation and the clinicopathological parameters. A significant correlation was found between NF-κB activation and tumor stage (p=0.028). The prognosis of the high NF-κB group was significantly worse than that of the low NF-κB group

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

	NF-κB > 25%	NF-κB ≤ 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	

(p=0.015, Figure 2). Multivariate analysis indicated that NF-κB 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-κB patients in this group were worse than those of the 34 low NF-κB patients (p=0.032, Figure 3). No correlation was found between NF-κB activation and the clinicopathological parameters examined (Table III). Although multivariate analysis indicated that the NF-κB activation may be a potential prognostic factor (hazards ratio 2.442), NF-κB activation was not an independent prognostic factor in this group (p=0.104, Table IV).

Finally, the prognostic value of NF-κB 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-κB patients in this group were worse than those of the 21 low NF-κB patients (p=0.007, Figure 4). No correlation was found between NF-κB 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-κB in gastric carcinomas. Patients showing high NF-κB activation in carcinoma tissue did not survive as long as those with low NF-κB activation.

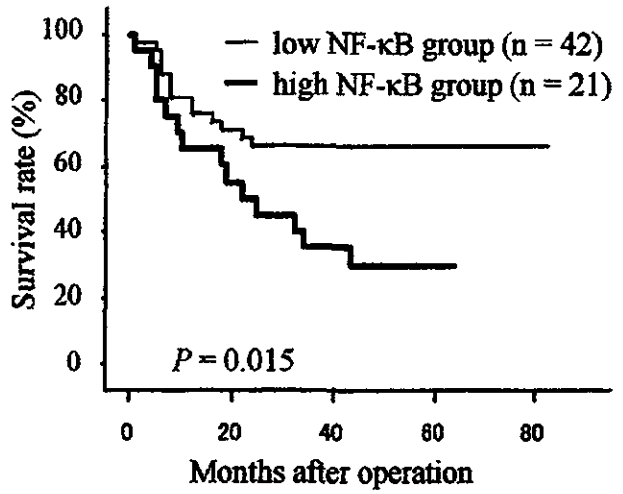

Figure 2. *Survival curves of the 63 patients with gastric carcinoma who underwent resection, according to NF-κB activation in the carcinoma specimens.*

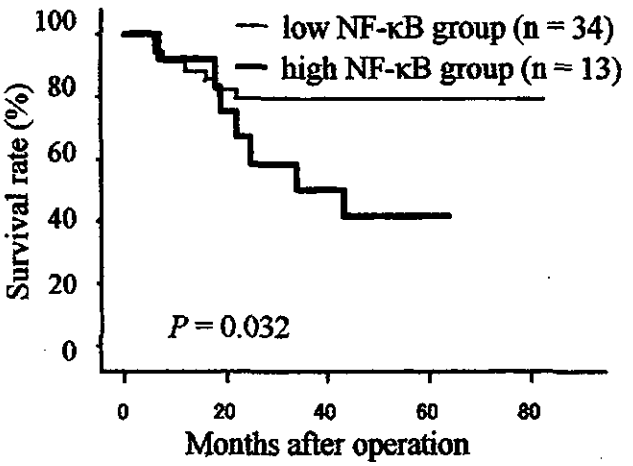

Figure 3. *Survival curves of the 47 patients with gastric carcinoma who underwent curative resection, according to NF-κB activation in the carcinoma specimens.*

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-κB activation		
≤ 25% *vs* > 25%	2.275	0.033

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

	NF-κB > 25%	NF-κB ≤ 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-κB 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-κB activation in carcinoma tissues has remained unclear. In the present study, therefore, we focused on the prognostic value of NF-κB 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-κB activation is a new prognostic parameter in gastric carcinoma.

We next examined whether NF-κB 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-κB activation		
≤ 25% *vs* > 25%	2.442	0.104

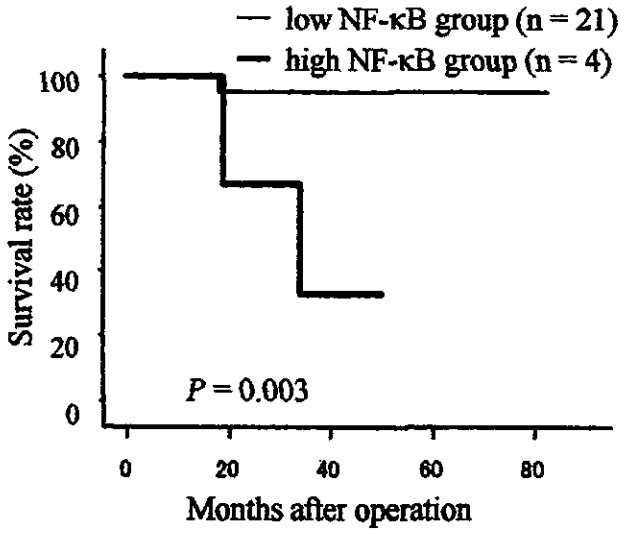

Figure 4. *Survival curves of the 25 patients with stage I and II gastric carcinoma who underwent curative resection, according to NF-κB activation in the carcinoma specimens.*

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

We found a significant correlation between NF-κB activation and lymphatic invasion (p=0.036 in the 63 patients, p=0.005 in the 47 patients). However, NF-κB

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

	NF-κB > 25%	NF-κB ≤ 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	

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).

Four of the 21 high NF-κB 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-κB 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-κB nuclear translocation rate of 25%. These results suggest a significant correlation between NF-κB 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-κB activation are high-risk patients (Figure 4). Though it is still unknown whether NF-κB 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-κB in gastric carcinoma.

Acknowledgements

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Interleukin 1β Enhances Invasive Ability of Gastric Carcinoma through Nuclear Factor-κB Activation

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ABSTRACT

Purpose: **We examined the role of interleukin (IL)-1β in activation of nuclear factor κB (NF-κB) and the biological function of activated NF-κB in gastric carcinoma cells.**

Experimental Design: **Human gastric carcinoma cell line GCTM-1 was used to examine NF-κB activation by immunostaining and electrophoretic mobility shift assay. Matrix metalloproteinase (MMP)-9 expression, which plays an important role in tumor invasion, was assessed by semiquantitative reverse transcription-PCR, Western blotting, and immunostaining. The invasive ability of GCTM-1 cells was measured by Matrigel invasion assay. *In vivo* expression of IL-1β and MMP-9 and activation of NF-κB in 10 surgically resected gastric carcinoma specimens were examined immunohistochemically.**

Results: **IL-1β enhanced NF-κB activation, MMP-9 expression, and the invasive ability of GCTM-1. A NF-κB inhibitor, pyrrolidine dithiocarbamate, suppressed both MMP-9 expression and invasiveness of IL-1β-treated GCTM-1 cells. IL-1β did not increase the invasive ability of GCTM-1 cells transfected with MMP-9 antisense oligonucleotide. Concomitant expression of IL-1β and nuclear NF-κB was observed in 3 of 10 gastric carcinoma specimens. Cells producing IL-1β were tumor-infiltrating macrophages in two specimens and gastric carcinoma cells in one specimen.**

Conclusions: **One of the molecules that may play a role in NF-κB activation in some gastric carcinomas is IL-1β. The present results suggest that IL-1β increases the invasive ability of carcinoma cells through activation of NF-κB and the resulting MMP-9 expression.**

INTRODUCTION

Nuclear factor κB (NF-κB) is a transcription factor that is involved in inflammation (1), cell proliferation (2), angiogenesis (3), and apoptosis (4). In most unstimulated normal cells, NF-κB is sequestered in the cytoplasm and complexed with inhibitor proteins (inhibitor of NF-κB; Ref. 5). Stimulation of cells leads to phosphorylation and degradation of inhibitors of NF-κB and allows translocation of NF-κB, *i.e.*, NF-κB (p65) and/or NF-κB1 (p50), to the nucleus, resulting in expression of target genes (6, 7). In most cases, NF-κB activation is evaluated as the nuclear translocation of NF-κB, especially p65. Recent studies revealed that NF-κB is constitutively activated in several types of carcinoma, including pancreatic (8), breast (9), colorectal (10), and hepatocellular carcinoma (11). We reported previously that NF-κB is constitutively activated in gastric carcinoma and suggested that NF-κB activation is related to the invasive ability of carcinoma cells (12). However, little information is available concerning the *in vivo* roles of NF-κB activation and the mechanisms by which NF-κB is activated in carcinoma tissue.

Generalized or local inflammation may transform the carcinoma cells to more aggressive cells. It has been suggested that chronic inflammation produces a procancer microenvironment that favors survival and growth of carcinoma cells (13–16). Inflammatory infiltrates are thought to produce large amounts of proinflammatory cytokines such as interleukin (IL)-1β at the tumor site. IL-1β induces NF-κB activation in macrophages and neutrophils (17, 18). We hypothesized that IL-1β produced at the tumor site enhances NF-κB activation in carcinoma cells and that NF-κB activation accelerates invasiveness of carcinoma cells by increasing expression of invasion-related molecules. We investigated activation of NF-κB and the resulting expression of matrix metalloproteinase (MMP)-9 in response to IL-1β in a gastric carcinoma cell line.

MATERIALS AND METHODS

Reagents and Antibodies. Recombinant human IL-1β was purchased from Diaclone (Besancon, France). Pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB translocation, was purchased from Sigma (Deisenhofen, Germany). The primary antibodies used for immunohistochemistry and Western blot analysis were anti-NF-κB p65 (sc-109; Santa Cruz Biotechnology), anti-MMP-9 (sc-6840; Santa Cruz Biotechnology), and anti-IL-1β (BD Biosciences, Heidelberg, Germany). Secondary antibodies for immunohistochemistry were purchased from Nichirei (Tokyo, Japan), and those labeled with FITC for West-

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ern blot analysis were purchased from Santa Cruz Biotechnology.

Cells. Human gastric adenocarcinoma cell line GCTM-1 was established in our laboratory from ascites of a cancer patient with peritoneal dissemination (19). GCTM-1 cells were maintained in RPMI 1640 (*Life Technologies*, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in an atmosphere of 5% CO_2 in air.

Gastric Carcinoma Specimens. Carcinoma specimens from 10 gastric carcinoma patients who gave informed consent before surgical treatment were fixed with liquid nitrogen immediately after surgery.

Immunohistochemistry. For immunostaining of carcinoma specimens, frozen sections (4 μm) were fixed in acetone for 10 min. For immunostaining of cultured carcinoma cells, GCTM-1 cells (1×10^5 cells/well) were grown on 8-chamber glass slides overnight. Cells were air-dried and fixed in 100% methanol for 5 min at −20°C and then incubated in PBS containing 0.2% Triton X-100 for 10 min. Slides were immersed in 3% H_2O_2 in methanol or 10% normal goat or rabbit serum (Santa Cruz Biotechnology) for 30 min, and each section was then incubated with the optimal concentration of primary antibody for 24–48 h at 4°C. After the sections were incubated with appropriate secondary antibodies, immune complexes were detected with a combination of 3,3′-diaminobenzidine (40 mg/150 ml in PBS; Wako Pure Chemical Industries, Hyogo, Japan) and 0.06% hydrogen peroxide. Specimens were photographed with a digital camera (Binary Planner 4490; Jenoptik, Jena, Germany) and attached to a microscope (BX51; Olympus, Tokyo, Japan). Nuclear staining, which reflects nuclear translocation of p65, was considered a marker of NF-κB activation.

Electrophoretic Mobility Shift Assay. Preparation of nuclear extracts of GCTM-1 cells was performed as described previously (12). Briefly, 1×10^6 GCTM-1 cells cocultured with IL-1β for the indicated times were collected and washed once with PBS. GCTM-1 cells were then homogenized in hypotonic buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1% NP40, and 5% protease inhibitor mixture] incubated for 10 min on ice. Nuclei were collected by centrifugation at 800 × *g* for 5 min, washed once with hypotonic buffer, and resuspended in low-salt buffer [20 mM HEPES (pH 7.9), 0.02 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, and 5% protease inhibitor mixture]. An equal volume of high-salt buffer [20 mM HEPES (pH 7.9), 800 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, and 5% protease inhibitor mixture] was added by vortex mixing. Nuclei were incubated for 30 min on ice and centrifuged at 13,000 × *g* for 30 min. The supernatants were collected.

Nuclear protein extracts of GCTM-1 cells were analyzed by electrophoretic mobility shift analysis for NF-κB nuclear translocation as described previously (12). Nuclear protein extracts of 1×10^6 cells were incubated for 30 min at 37°C with binding buffer [60 mM HEPES (pH 7.5), 180 mM KCl, 15 mM $MgCl_2$, 0.6 mM EDTA, and 24% glycerol], poly(deoxyinosinic-deoxycytidylic acid) (Amersham Pharmacia Biotech ABs, Uppsala, Sweden), and ^{32}P-labeled double-stranded oligonucleotide containing the NF-κB binding motif (5′-AGT-TGA-GGG-GAC-TTT-CCC-AGG-C-3′; Promega). Mixtures were loaded onto 4% polyacrylamide gels and separated by electrophoresis in 0.25× Tris-borate EDTA running buffer. Oligomer-protein complexes were visualized by autoradiography. Intensity of the NF-κB band was determined with NIH Image version 1.60 software (NIH Division of Computer Research and Technology).

Semiquantitative Reverse Transcription-PCR. Total RNA was extracted from GCTM-1 cells with the guanidinium thiocyanate-phenol-chloroform single-step method (20) and quantified by spectrophotometry (Ultrospec 2100 Pro; Amersham Pharmacia Biotech, Cambridge, United Kingdom). IL-1β sense (5′-CAG-TGA-AAT-GAT-GGC-TTA-TTA-C-3′) and antisense (5′-CTT-TCA-ACA-CGC-AGG-ACA-GGT-3′) primers yield a 548-bp product (21). MMP-9 sense (5′-TGG-GCT-ACG-TGA-CCT-ATG-ACA-T-3′) and antisense (5′-GCC-CAG-CCC-ACC-TCC-ACT-CCT-C-3′) primers yield a 172-bp product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense (5′-CCA-CCC-ATG-GCA-AAT-TCC-ATG-GCA-3′) and antisense (5′-TCT-AGA-CGG-CAG-GTC-AGG-TCC-ACC-3′) primers gave rise to a 593-bp product (22). IL-1β amplification conditions consisted of an initial denaturation for 2 min at 95°C followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, and 1 min at 72°C (21). MMP-9 and GAPDH amplification conditions comprised an initial denaturation for 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C (22). Amplification of each gene was in the linear range. The PCR products were separated on ethidium bromide-stained 2% agarose gels. Semiquantitative analysis was done with Molecular Imager FX Pro (Bio-Rad) to obtain MMP-9/GAPDH ratios.

Western Blot Analysis. GCTM-1 cells (5×10^6) cultured as described above (23) were collected and lysed with extraction buffer [10 mM HEPES (pH 7.9), 1.5 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1% SDS, 0.5 mM phenylmethylsulfonyl fluoride, and double-distilled water]. After 30 min at 4°C, cell debris was removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was collected. Protein concentrations were measured with an Ultrospec 2100 Pro. Protein (100 μg) was mixed with SDS sample buffer, separated by SDS-PAGE (10% acrylamide), transferred to polyvinylidene difluoride membrane (Bio-Rad), and blocked with 0.1% Tween 20/5% skim milk for 1 h at room temperature. Membranes were incubated with anti-MMP-9 antibody for 1 h. Membranes were washed three times with 0.1% Tween 20 in PBS and stained with the appropriate secondary antibody. For quantification, the bands were scanned with Molecular Imager FX Pro, and densitometry was done with NIH Image version 1.60 software.

MMP-9 Antisense Oligonucleotide Treatment. MMP-9 antisense oligonucleotide (5′-CAG-GGG-CTG-CCA-GAG-GCT-CAT-3′; Sigma Genosys Japan, Hokkaido, Japan) or scramble oligonucleotide (5′-GCG-AGC-TAG-GAC-TGT-GCA-GCC-3′) was transfected into GCTM-1 cells with LipofectAMINE Plus Reagent (Invitrogen) following a procedure protocol (24). Oligonucleotide-LipofectAMINE complexes were added to GCTM-1 cells (1×10^6) suspended in serum-free medium and incubated for 5 h. An equal volume of 15% fetal bovine serum-RPMI 1640 was then added, and cells were incubated for 24 h.

Matrigel Invasion Assay. Invasiveness of GCTM-1 cells was assessed as the invasion of cells through Matrigel-

coated transwell inserts (22). Briefly, the upper surface of the filter (pore size, 8.0 μm; BD Biosciences) was coated with basement membrane Matrigel (BD Biosciences) at a concentration of 250 μg/cm^2 and air-dried overnight at room temperature. GCTM-1 cells were suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 with 10% fetal bovine serum and treated with IL-1β (5 ng/ml) for 12 h. Cells were washed twice with PBS to remove IL-1β, 1×10^5 cells were added to the upper chamber, and 2% Matrigel-containing RPMI 1640 (400 μl) was added to the lower chamber. Cells were incubated in a water-saturated 5% CO_2 atmosphere at 37°C for 48 h. Cells that transverse the Matrigel adhere to the opposite surface of the filter. After incubation, the filter was fixed with 100% methanol and stained with Giemsa, after which cells on the upper surface were removed completely with a cotton swab. GCTM-1 cells that had migrated from the upper to the lower side of the filter were counted under light microscopy at a magnification of ×200. Tumor cell invasiveness was defined as the mean cell number of cells in five microscopic fields.

Statistical Analysis. Student's *t* test was used for statistical analyses. Calculations were carried out with StatView software (Abacus Concepts). All results with a $P < 0.05$ were considered statistically significant.

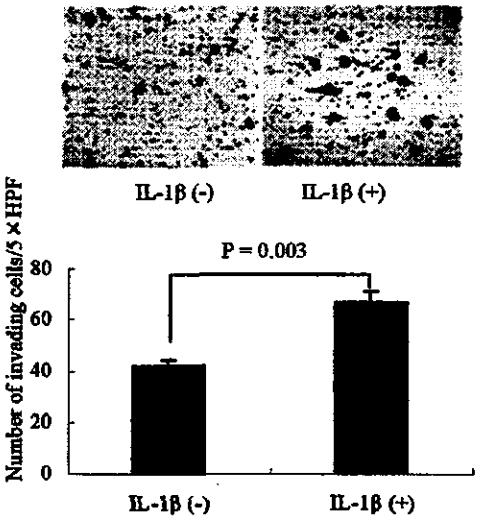

Fig. 2 Effect of interleukin (IL)-1β on the *in vitro* invasiveness of GCTM-1 cells. GCTM-1 cells were incubated with or without IL-1β (5 ng/ml) for 12 h. After incubation, cells were washed with PBS to remove IL-1β. IL-1β-treated GCTM-1 cells (2×10^5 cells/well) were seeded in a Matrigel-precoated invasion chamber and incubated at 37°C for 48 h. The number of cells that invaded the Matrigel were examined and counted under light microscopy (×200). Data are expressed as the mean ± SD of the number of cells counted in five randomly selected fields. Representative data from one of three independent experiments are shown.

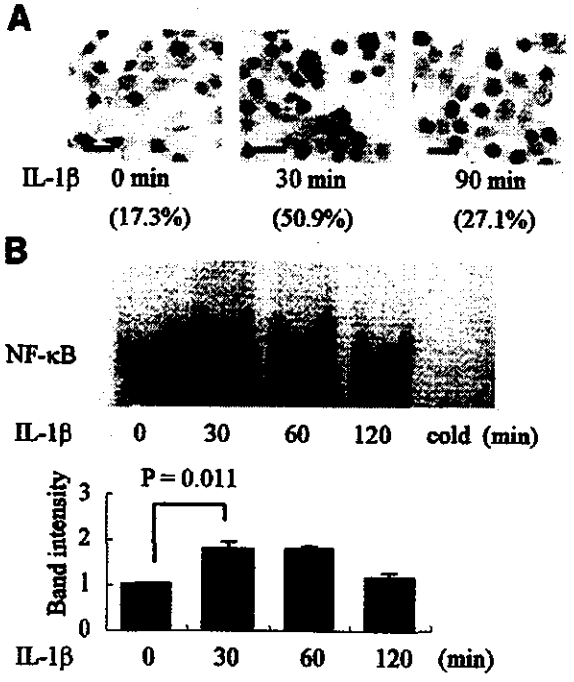

Fig. 1 Activation of nuclear factor-κB (NF-κB) p65 in interleukin (IL)-1β-stimulated gastric carcinoma cell line GCTM-1. *A*, photographic images of immunohistochemical staining of p65 at various points after stimulation of GCTM-1 cells with 5 ng/ml IL-1β. Cells showing nuclear translocation of NF-κB were counted and presented as the percentage of positive cells. *Bar*, 20 μm. *B, top panel*, NF-κB binding activity of GCTM-1 cells treated with IL-1β (5 ng/ml) for the indicated times was examined by electrophoretic mobility shift analysis. *B, bottom panel*, band intensities of NF-κB were quantified with NIH Image, and the value represents the mean ± SD from three different experiments. The control band intensity was indicated as 1.0.

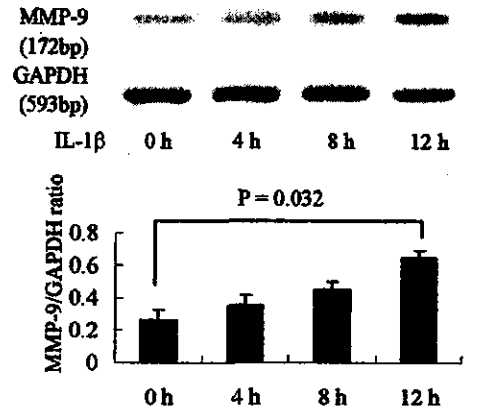

Fig. 3 Effect of interleukin-1β on expression of matrix metalloproteinase (MMP)-9 mRNA levels in GCTM-1 cells. *Top panel*, total RNA of MMP-9 isolated from GCTM-1 cells treated with interleukin-1β (5 ng/ml) for the indicated times was assessed by semiquantitative reverse transcription-PCR. *Bottom panel*, band intensities were quantified with NIH Image, and the data are presented as the ratio of MMP-9 to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The value represents the mean ± SD from three different experiments.

RESULTS

IL-1β Induced NF-κB Activation in GCTM-1 Cells. On the basis of our previous study, 5 ng/ml IL-1β was used throughout this study (12). Nuclear staining of NF-κB p65 in GCTM-1 cells was enhanced within 30 min of GCTM-1 exposure to 5 ng/ml IL-1β (Fig. 1*A*). We also investigated the effect

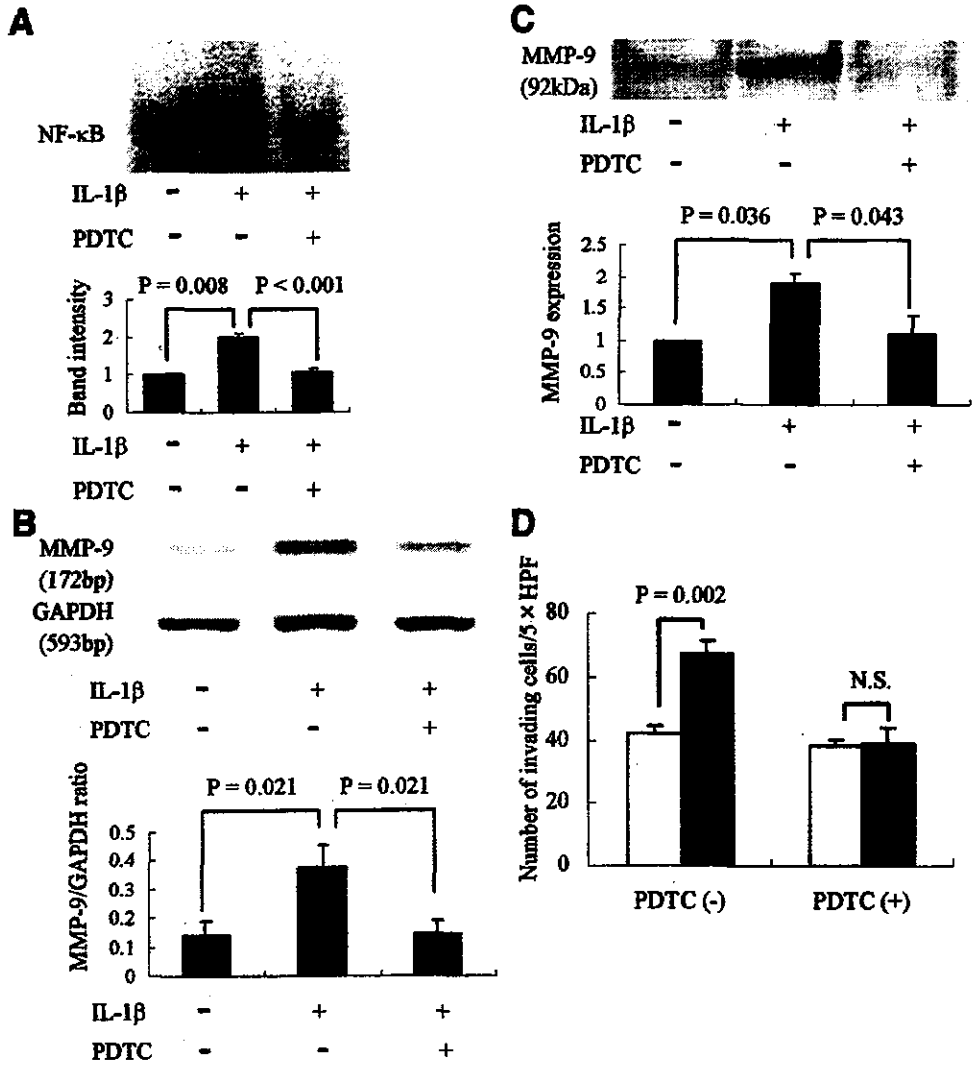

Fig. 4 Inhibition of interleukin (IL)-1β-induced matrix metalloproteinase (MMP)-9 expression and invasion of GCTM-1 cells by PDTC. GCTM-1 cells were preincubated with PDTC (10 μM) for 1 h before the addition of IL-1β (5 ng/ml). *A, top panel,* nuclear factor-κB binding activity 30 min after the addition of IL-1β was examined by electrophoretic mobility shift analysis. *A, bottom panel,* band intensities of nuclear factor-κB were quantified with NIH Image, and the values represent the mean ± SD from three different experiments. The control band intensity was indicated as 1.0. *B, top panel,* total RNA of MMP-9 isolated from GCTM-1 cells 12 h after the addition of IL-1β was assessed by semiquantitative reverse transcription-PCR. *B, bottom panel,* band intensities were quantified with NIH Image, and the data are presented as the ratio of MMP-9 to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Data represent the mean ± SD from three different experiments. *C, top panel,* MMP-9 expression 18 h after the addition of IL-1β was examined by Western blotting. *C, bottom panel,* band intensities were quantified with NIH Image, and the data represent the mean ± SD from three different experiments. The control band intensity was indicated as 1.0. *D,* invasiveness of cells was assessed by a Matrigel invasion assay. Cells that had invaded the Matrigel were counted under light microscopy (×200). Data are expressed as the mean ± SD of the number of cells counted in five randomly selected fields (□, control cells; ■, IL-1β-treated cells). Representative data from one of three independent experiments are shown.

of IL-1β on nuclear translocation of NF-κB p65 by electrophoretic mobility shift analysis. When GCTM-1 cells were treated with IL-1β for 30 min, NF-κB binding to DNA clearly increased (Fig. 1*B*).

IL-1β Enhanced the Invasive Ability of GCTM-1 Cells through MMP-9 Expression. We examined whether IL-1β enhances invasive ability of GCTM-1 cells using a Matrigel invasion assay. Pretreatment of GCTM-1 cells with 5 ng/ml IL-1β significantly increased the invasive ability of these cells ($P = 0.003$, Fig. 2). We then examined whether the enhanced invasive ability of GCTM-1 cells induced by IL-1β is associated with MMP-9. IL-1β increased levels of MMP-9 mRNA in GCTM-1 cells within 8 h (Fig. 3).

NF-κB Inhibitor, PDTC, Suppressed MMP-9 Expression and Invasive Ability of GCTM-1 Cells Induced by IL-1β. To confirm the relation between NF-κB activation, MMP-9 expression, and enhanced invasive ability, we examined the effect of a specific NF-κB inhibitor, PDTC, on IL-1β-induced changes (25). PDTC (10 μM) had no effect on cell viability (data not shown). PDTC (10 μM) was added to GCTM-1 cells 1 h before treatment with IL-1β, and expression of MMP-9 was analyzed. PDTC inhibited nuclear translocation

of NF-κB p65 induced by IL-1β (Fig. 4*A*). In the Matrigel invasion assay, PDTC was added to GCTM-1 cultures 1 h before treatment with IL-1β for 12 h. GCTM-1 cells were washed immediately before the Matrigel invasion assay to remove both PDTC and IL-1β. PDTC suppressed MMP-9 expression at both the mRNA and protein levels (Fig. 4, *B* and *C*). Enhanced invasive ability of GCTM-1 cells induced by IL-1β was also suppressed by PDTC (Fig. 4*D*).

IL-1β Did Not Increase the Invasive Ability of GCTM-1 Cells Treated with MMP-9 Antisense Oligonucleotide. To verify the effect of MMP-9 on the enhanced invasive ability of GCTM-1 cells induced by IL-1β, we performed Matrigel invasion assays with MMP-9 antisense oligonucleotide-transfected GCTM-1 cells. The invasive ability of MMP-9-antisense oligonucleotide-transfected GCTM-1 cells was not affected by IL-1β (Fig. 5).

Macrophages Infiltrating Gastric Carcinoma Tissue and/or Gastric Carcinoma Cells Produced IL-1β. Finally, we examined whether IL-1β is produced at the tumor site. IL-1β immunoreactivity was clearly observed in three of the 10 carcinoma specimens. Cells producing IL-1β were tumor-infiltrating macrophages in two specimens and gastric carcinoma cells in one specimen. In these three specimens, both NF-κB activation and MMP-9 expression were found (Fig. 6).

DISCUSSION

Here we report that in gastric carcinoma tissue IL-1β produced at the tumor site may play a role in NF-κB activation and that this activation increases expression of MMP-9, resulting in increased invasiveness of carcinoma cells.

We reported previously that NF-κB is constitutively activated in gastric carcinoma and suggested that NF-κB activation may be linked to the invasive abilities of gastric carcinoma cells (12). In fact, several researchers have described a close relation between NF-κB activation and increased expression of invasion-related molecules, such as MMPs, by carcinoma cells *in vitro* (22, 26, 27). In the present study, we first examined constitutive activation of NF-κB in gastric carcinoma cells. Because IL-1β induces NF-κB activation in gastric carcinoma HTB-135 cells (12), we hypothesized that IL-1β might activate NF-κB in gastric carcinoma tissue. IL-1β in fact induced NF-κB activation in gastric carcinoma GCTM-1 cells. It is generally accepted that microbial and nonmicrobial products induce IL-1β secretion by macrophages (28, 29) and that macrophages frequently infiltrate carcinoma tissue (30–33). It has also been suggested that tumor-infiltrating macrophages are activated by transforming growth factor β1 secreted by tumor cells to produce IL-1β (34). IL-1β is secreted by a variety of tumors, including melanoma (35), sarcoma (36), hepatoblastoma (37), ovarian carcinoma (38), colorectal carcinoma (30, 39), pancreatic carcinoma (40), and gastric carcinoma (41, 42). Although these findings suggest that IL-1β may be secreted locally at the site of the tumor, direct evidence that IL-1β activates NF-κB in gastric carcinoma tissue has not been reported. GCTM-1 cells do not express IL-1β at both the mRNA and protein levels (data not shown). In 10 surgically resected gastric carcinoma specimens, IL-1β secretion from tumor-infiltrating macrophages and gastric carcinoma cells was observed in two specimens and one specimen, respectively (Fig. 6). These findings provide evidence of an association between autocrine secretion of IL-1β from carcinoma cells and activation of NF-κB in gastric carcinoma tissue.

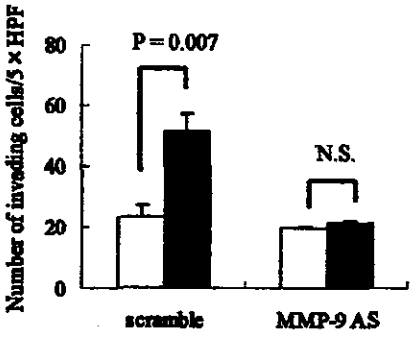

Fig. 5 Effect of interleukin (IL)-1β on the invasive ability of matrix metalloproteinase 9 antisense oligonucleotide-transfected GCTM-1 cells. GCTM-1 cells treated with scramble or matrix metalloproteinase 9 antisense oligonucleotide were incubated with IL-1β (5 ng/ml) for 12 h. After incubation, cells were washed with PBS to remove the IL-1β. IL-1β-treated tumor cells (2×10^5 cells/well) were then seeded into a Matrigel-precoated invasion chamber and incubated at 37°C for 48 h. Cells that had invaded the Matrigel were examined and counted under light microscopy (×200). Data are expressed as the mean ± SD of the number of cells counted in five randomly selected fields (□, control cells; ■, IL-1β-treated cells). Representative data from one of three independent experiments are shown.

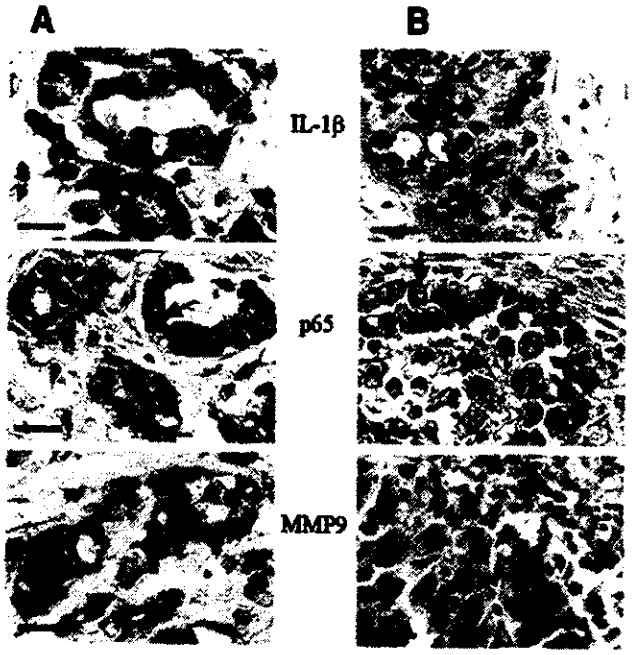

Fig. 6 Expression of nuclear factor-κB (NF-κB) p65, interleukin (IL)-1β, and matrix metalloproteinase (MMP)-9 at the tumor site of gastric carcinoma specimens (*A* and *B*). Expression of these three molecules was examined by immunostaining. *A*, photographic images of autocrine IL-1β secretion by gastric carcinoma cells, NF-κB p65 nuclear translocation in gastric carcinoma cells (*arrow*), and MMP-9 expression by gastric carcinoma cells. *B*, photographic images of paracrine IL-1β secretion by tumor-infiltrating macrophages (*arrow*), NF-κB p65 nuclear translocation in gastric carcinoma cells (*arrow*), and MMP-9 expression by gastric carcinoma cells and stromal cells. *Bar*, 20 μm.

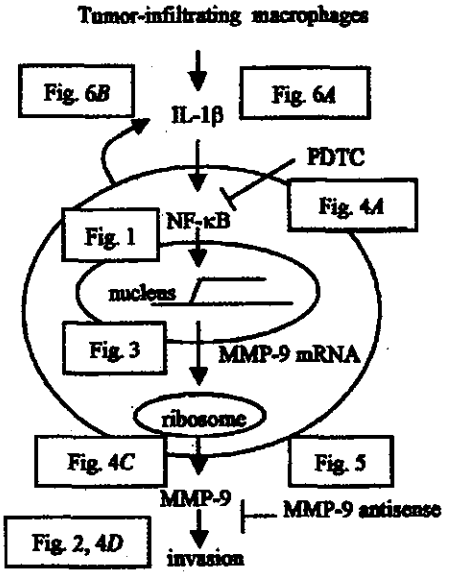

Fig. 7 Proposed mechanism for the enhancement of invasive ability of carcinoma cells by interleukin 1β. In some carcinoma cells, interleukin 1β secreted in a paracrine and/or autocrine fashion activates nuclear factor-κB and promotes the invasive ability through production of matrix metalloproteinase 9.

We then sought to clarify the biological function of IL-1β-induced activation of NF-κB induced in GCTM-1 cells. Arlt *et al.* (40) reported that NF-κB activation induced by IL-1β protects pancreatic carcinoma cell lines from apoptosis. Several studies have shown a close relation between NF-κB activation and expression of invasion-related molecules (26), suggesting that invasive ability is modified by NF-κB activation induced by IL-1β. Many factors, including MMP-2, MMP-9 (43, 44), urokinase-type plasminogen activator (45), and plasminogen activator inhibitor-1 (46), contribute to tumor invasion. It has been reported that an inhibitor of MMP-9 reduces invasion by some carcinoma cells (47–49), suggesting an essential role of MMP-9 in invasion. However, it was not known whether IL-1β increases MMP-9 expression or the invasive ability of carcinoma cells. We show for the first time that IL-1β increases both MMP-9 expression and the invasive ability of GCTM-1 cells and that these phenomena are both suppressed by NF-κB inhibitor PDTC (Fig. 4, *A–C*). IL-1β had no effect on the invasive ability of GCTM-1 cells treated with MMP-9 antisense oligonucleotide (Fig. 5). Our findings are summarized in Fig. 7. Briefly, tumor-infiltrating macrophages and/or carcinoma cells express IL-1β at the site of the tumors. IL-1β induces NF-κB activation, which then induces transcription of MMP-9 mRNA and production of MMP-9 protein. Up-regulation of MMP-9 enhances the ability of GCTM-1 cells to invade a collagen matrix.

In conclusion, NF-κB activation in gastric carcinoma cells is enhanced by IL-1β secreted by tumor-infiltrating macrophages (paracrine) and/or carcinoma cells (autocrine). NF-κB activation induced by IL-1β contributes to increased invasive ability of gastric carcinoma cells through overexpression of MMP-9. These findings suggest that inflammation at the site of a tumor may play some roles in the metastasis of tumor cells. In cases in which IL-1β may play a significant role in NF-κB activation of tumor cells, NF-κB inhibitor, neutralizing antibody to IL-1β, or anti-inflammatory agents may offer new therapeutic strategies against invasive gastric carcinoma.

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Death-Associated Protein Kinase (*DAP Kinase*) Alteration in Soft Tissue Leiomyosarcoma: Promoter Methylation or Homozygous Deletion Is Associated With a Loss of DAP Kinase Expression

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The *death-associated protein kinase* (*DAP kinase*) was initially identified as a positive mediator of programmed cell death induced by interferon-γ. To investigate the potential role and the alteration of the *DAP kinase* gene in soft tissue leiomyosarcoma (LMS), we first searched for homozygous deletion and promoter hypermethylation in 45 LMSs for which genomic DNA was available, using differential PCR and methylation-specific PCR, respectively. Promoter methylation was recognized in 10 of 45 cases (22%), and homozygous deletion was detected in 3 of 45 cases (7%). *p53* mutation was detected in 11 of 45 LMS cases (24%). Cases with *DAP kinase* alteration or *p53* mutation showed a close correlation with high French Federation of Cancer Centers grade or with poor prognosis ($P = 0.0244$, $P = 0.0491$, respectively). Next, to determine that *DAP kinase* promoter methylation or homozygous deletion is involved in the down-regulation of DAP kinase expression, we examined the expression of DAP kinase protein by immunohistochemistry. Decreased expression of DAP kinase protein was recognized in 13 of 45 LMS cases (29%). Seven of 13 cases (54%) with decreased expression of DAP kinase protein revealed promoter methylation or homozygous deletion of *DAP kinase*, and the methylation status or homozygous deletion of its gene showed a close correlation with decreased DAP kinase expression ($P = 0.0300$). In conclusion, although *DAP kinase* alteration was relatively rare, *DAP kinase* alteration and/or *p53* mutation may associate with tumor progression in soft-tissue LMSs. Furthermore, although further detailed analyses are necessary, promoter methylation or homozygous deletion status of *DAP kinase* may present a major alternative mechanism of a loss of or decrease in DAP kinase expression. HUM PATHOL 35:1266-1271. © 2004 Elsevier Inc. All rights reserved.

Key words: death-associated protein kinase, leiomyosarcoma, promoter hypermethylation, homozygous deletion.

Abbreviations: DAP kinase, death-associated protein kinase; LMS, leiomyosarcoma; FNCLCC, French Federation of Cancer Centers; AJCC, American Joint Committee on Cancer; SSCP, single-strand conformation polymorphism; PCR, polymerase chain reaction; HPF-high-power field.

Soft tissue sarcoma represent a heterogenous group of mesenchymal malignancies, and the majority of the previous scientific studies concerning the alteration of apoptosis-related factors in soft tissue sarcomas have dealt with broad categories of different type of tumors. As a consequence, data concerning single subtype of sarcomas are limited.

The *death-associated protein kinase* (*DAP kinase*) gene, located on chromosome 9p34.1, was initially isolated as a calcium/calmodulin-dependent positive mediator of INF-γ–induced apoptosis and is associated with the cell cytoskeleton.[1-3] Loss of DAP kinase expression has been reported in bladder and renal cell carcinoma,[4] lung cancer,[5] head and neck carcinoma,[6] and B-cell malignancies,[7] and it is thought to lead to immortalization of cells and development of cancers. Promoter hypermethylation of the *DAP kinase* CpG island was also detected in some malignancies, in which the range of methylation was variable,[4,5,7,8] and the methylation status has been found to be associated with silencing of the *DAP kinase* gene in these malignancies. Simpson et al[8] have reported that the inactivation of *DAP kinase* is strongly correlated with homozygous deletion or promoter hypermethylation in pituitary tumors. Furthermore, the presence of *DAP kinase* methylation is associated with lymph node involvement or advanced disease stage in some tumors.[9-11] *DAP kinase* was recently characterized as an upstream regulator of *p53*.[12] Some investigators have shown that some apoptosis-related genes, such as p53 and bcl-2, have an important role in the progression of soft tissue sarcomas.[13-15] However, the frequency of DAP kinase expression and the mechanism of inactivation of its gene are not clear in soft tissue sarcomas.

To elucidate the potential role or the alteration of *DAP kinase*, we analyzed promoter methylation and homozygous deletion in a histologically homogenous series of 45 cases of soft-tissue leiomyosarcomas (LMSs),

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