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Expression of Cyclin Dependent Kinase Inhibitor $p21^{WAF1}$ alone and in Combination with $p27^{KIP1}$ shows Prognostic Value in Gastric Carcinoma

Evidence suggests that multiple molecular events, including alteration of cell cycle regulators are involved in the development and progression of gastric carcinoma. Recently, it has been reported that the expression of p21 and p27, integrating the effects of one or more cell cycle regulators, consequently, acting as a single indicator of several possible cell cycle gene alterations is associated with tumor suppression. In the present study, we studied the immunohistochemical expression of p21 and p27 in gastrectomy specimens from 84 patients with gastric adenocarcinoma, and analysed its correlation to clinicopathologic data, including patients survival. Loss of p21 and p27 expression was noted in 45 (53.6%) and 44 (52.4%) of the 84 gastric carcinoma tissues, respectively. The expression of p21 was significantly correlated with histological type ($p=0.005$), recurrence ($p=0.002$) and death ($p=0.002$) after surgery, and p27 expression ($p=0.001$). Kaplan-Meier survival plots showed p21 negative group ($p=0.0014$) or both p21 and p27 negative group ($p=0.0048$) was significantly poorer in overall survival than both p21 and p27 positive or one of both positive group. Our results suggest that the status of p21 and p27 expression in immunohistochemical stain may be a useful prognostic marker of gastric carcinoma.

Key Words : *Stomach neoplasms; Carcinoma; Cyclin-dependent kinases; Enzyme inhibitors; Proto-oncogene protein p21 (ras)*

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INTRODUCTION

Gastric carcinoma is a major cause of cancer death throughout the world, especially in Korea (1, 2). It is known that multiple oncogenes, growth factors and tumor suppressor genes are involved in gastric carcinogenesis and progression (3). Many of these are associated in the signal transduction pathway and feed back loops connecting the cell cycle machinery with extracellular signals (4).

Recent studies have shown that two families of cyclin-dependent kinases inhibitors (CDKNs) play a crucial role in mediating extracellular negative signal resulting in cell cycle arrest at different G1 points (5). One is the INK4 family, including $p16^{INK4a}$, $p15^{INK4b}$, p18 and p19 (6-8). Another group is the Kip/Cip family, including p21, p27, and p57 (9-12). Among them, p21 and p27 are structurally related proteins that act as universal inhibitor for most cyclin-CDK complexes. $p21^{WAF1/CIP1}$ is induced by p53 protein and act as effector of p53 tumor suppressor gene and also inhibit PCNA-dependent DNA replication (13-15). $p27^{KIP1}$ is induced by extracellular anti-mitogenic signals such as cell-cell contact, TGF-β, agent that elevate cyclic AMP, and rapamycin, and implicated in G1 phase arrest (16-17). Inactivation of these CDKIs may be an important cause of deregulation of the cell cycle and may be involved in tumorigenesis and/or progression of some tumor (18-20). Analysis of their functional status in cancer may confer useful information.

Although mutational inactivation of p21 and p27 has been reported to be a rare event, functional loss of the CDKIs was relatively frequent in human cancer (21-24). Immunohistochemical analysis of their expression, therefore, could be a simple and useful method for evaluating their functional status in tissue. A few immunohistochemical studies on the CDKIs in normal and neoplastic human tissues showed association with proliferation capacity (5, 26), and indicated that their alterations are

associated with disease progression (27-33).

In the present study, we characterized the immunohistochemical expression of p21 and p27 in gastric carcinoma of 84 patients and adjacent normal tissues, and analysed their relationship to the clinicopathologic data including patients survival.

MATERIALS AND METHODS

Clinical materials

A total of 84 gastrectomy specimens from patients (57 men and 27 women) with gastric adenocarcinoma was obtained from the routine files of the Department of Pathology, Hanyang University Hospital. Their ages ranged between 34 and 82 years (mean=59.6 years). No patient received chemotherapy before surgery. All patients underwent curative resection of the gastric cancer. All specimens were fixed in 10% formalin and embedded in paraffin wax. Clinicopathologic data from all cases were reviewed and the following features were evaluated; (a) location of the tumor, (b) type of the tumor, (c) status of lymph nodes, (d) TNM stage, (e) recurrence after surgery, and (f) survival. Hematoxylin-eosin stained slides were reviewed and the representative blocks which contained both normal mucosa and carcinoma were obtained.

Immunohistochemistry

Immunohistochemical staining was used to study p21 and p27 protein expression in gastric tissues. Formalin fixed, paraffin embedded tissue blocks were sectioned 4 μm thickness and immunohistochemistry was performed using previously described methods (34). Briefly, sections were dewaxed and heated in a microwave oven for 10 minutes to retrieve the antigens. Endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol. After blocking with 10% normal goat serum, rabbit polyclonal IgG antibody against p21 protein (Santa Cruz Biotec., USA, 1:200) and mouse monoclonal IgG antibody against p27 protein (Transduction Laboratory, USA, 1:200) were incubated with tissue sections at room temperature for 2 hours and at 4℃ for 18 hours, respectively. After three washes with phosphate buffered saline, the sections were treated with biotinylated anti-rabbit and anti-mouse IgG, followed by streptavidin-biotin peroxidase reagent (DAKO, Santa Babara, CA, USA). Finally, 3,3-diaminobenzidine (DAKO, Santa Babara, CA, USA) as chromogen and 1% hydrogen peroxide were applied. The slides were counterstained with hematoxylin.

Multiple microscopic fields were examined and immunohistochemical expression of p21 and p27 in normal and cancer tissue were classified semiquantitatively into five categories as follows: score 0; less than 5% of cells were positive, score 1; 5-25% of cells were positive, score 2; 26-50% of cells were positive, score 3; 51-75% of cells were positive, and score 4; more than 75% of cells were positive.

Statistical analysis

A Pearsons chi-square analysis was used to test the strength of association between clinicopathologic parameters and immunoexpression of p21 and p27 in gastric cancer tissues. Survival curves were calculated using the Kaplan-Meier method and differences in survival between groups were tested for statistical significance by the log-rank test. All statistical analyses were conducted using a SPSS (WIN 97) statistical software program.

RESULT

Immunohistochemical expression of p21 and p27 in normal gastric mucosa adjacent to the carcinoma were consistently positive in more than 25% of the cells, score 2. Lymphoid tissues infiltrated in the gastric mucosa also showed high expression of p27 but not of p21. Therefore, we classified the tumors into two groups: Score 0 or 1 was defined as a negative group and score 2, 3 and 4 as a positive group. The immunohistochemical results of 84 gastric carcinomas were listed in Table 1. A total of 45 (53.6%) and 44 (52.4%) gastric carcinomas showed decreased or loss of p21 and p27 expression compared to their normal counterparts, respectively (Fig. 1, 2).

There was no difference between p21 positive and negative group in age, sex, location of the tumor, histologic grade, TNM stage, and number of lymph node metastasis. p21 expression status significantly correlated with histological types (p=0.005). Thirty one (67.4%) of the 46 diffuse-type carcinoma showed loss of p21 expression, whereas 14 (36.8%) of the 38 intestinal-type carcinoma revealed loss of p21 expression (Table 2). Recurrence of

Table 1. Results of immunohistochemical staining in 84 gastric carcinoma

Category	p21	p27
0	14	8
1	31	36
2	21	20
3	18	13
4	0	7
Loss of expression	45/84 (53.6%)	44/84 (52.4%)

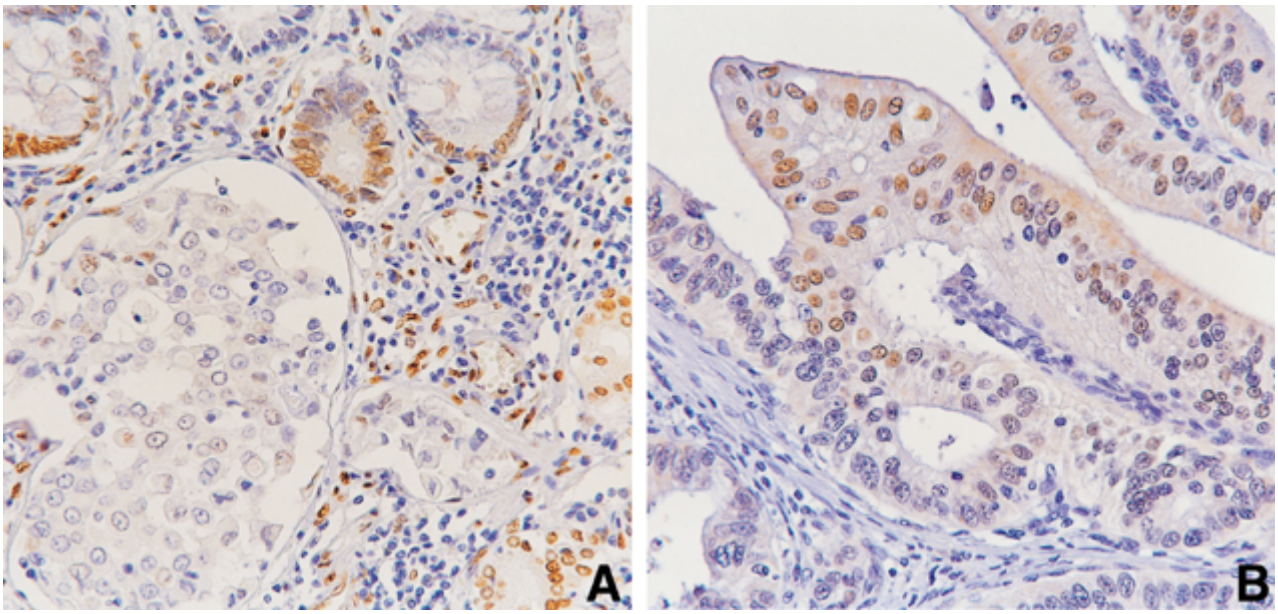

Fig. 1. Immunostain for p21. Diffuse-type gastric adenocarcinoma in the lymphatic spaces showed minimal staining compared with adjacent normal mucosa (A). Intestinal-type carcinoma showed some nuclear staining (B).

the disease was noted in 29 of 84 patients and among those 29 recurrences, 22 (75.9%) tumors showed loss of p21 expression. Thirty-four patients died during median 42.5 follow-up period, among those 34 deaths, 25 (73.5 %) tumors showed loss of p21 expression. Among 45 patients with p21 negative tumors, 22 (48.9%) and 25 (55.6%) patients revealed disease recurrence and death during the follow-up period, respectively. In contrast, 39 patients with p21 positive tumors showed disease recurrence and death only in 7 (18.9%) and 9 (24.3%) patients, respectively (Table 4).

There was a significant correlation between loss of p21 expression and disease free (p=0.0011) or overall survival (p=0.0014) (Fig. 3). After adjustment of survival data according to types of tumor and TNM stage, there was also a significant correlation between loss of p21 expres-

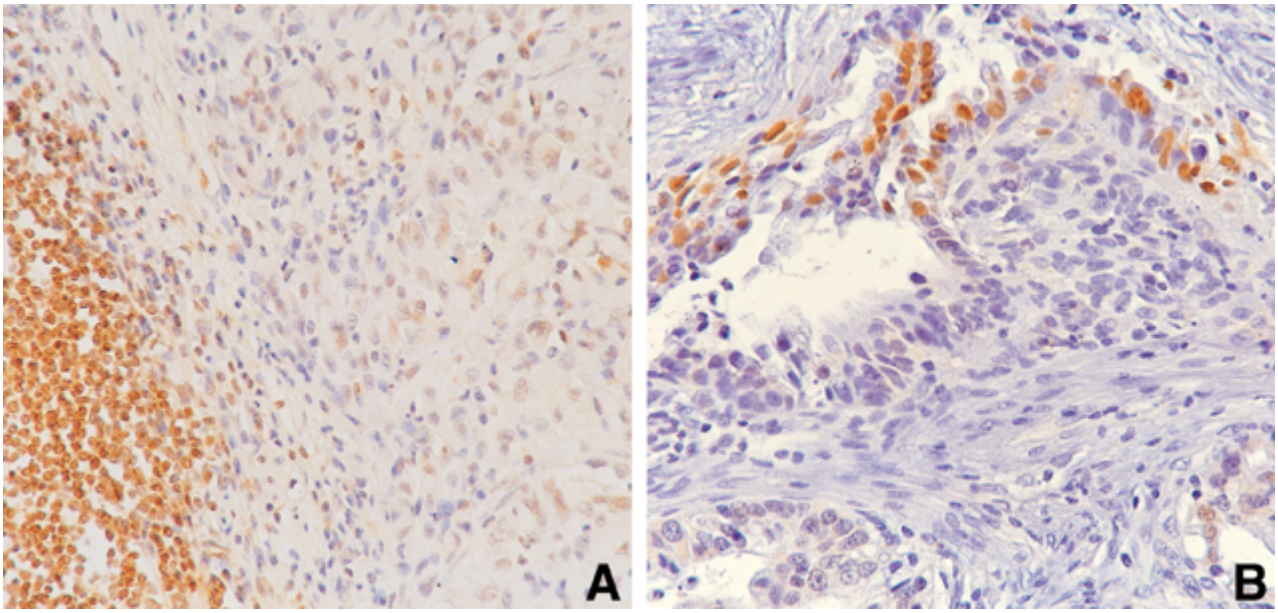

Fig. 2. Immunostain for p27. Diffuse-type gastric adenocarcinoma showed markedly decreased expression compared with adjacent lymphoid tissue (A). Intestinal-type carcinoma showed few nuclear staining (B).

Table 2. Expression of p21 protein and clinicopathologic characteristics of patients with gastric carcinoma

	p21 expression		p value
	Negative (n=45)	Positive (n=39)	
Age (years)			
<40 (n=15)	6	9	0.254
>40 (n=69)	39	30	
Sex			
Male (n=57)	30	27	0.802
Female (n=27)	15	12	
Tumor location			
Upper (n=4)	2	2	0.846
Middle (n=14)	9	5	
Lower (n=53)	27	26	
Diffuse (n=13)	7	6	
TNM stage			
I (n=4)	4	0	0.130
II (n=21)	9	12	
IIIa (n=39)	20	19	
IIIb (n=17)	9	8	
IV (n=3)	3	0	
Type			
Diffuse (n=46)	31	15	0.005
Intestinal (n=38)	14	24	
Lymph node metastasis			
Presence (n=73)	38	35	0.473
Absence (n=11)	7	4	
Mean (n=7.45)	7.68	7.20	

Table 3. Expression of p27 protein and clinicopathologic characteristics of patients with gastric carcinoma

	p27 expression		p value
	Negative (n=45)	Positive (n=39)	
Age (years)			
<40 (n=15)	7	8	0.625
>40 (n=69)	37	32	
Sex			
Male (n=57)	31	26	0.593
Female (n=27)	13	14	
Tumor location			
Upper (n=4)	4	0	0.846
Middle (n=14)	6	8	
Lower (n=53)	25	28	
Diffuse (n=13)	9	4	
TNM stage			
I (n=4)	4	0	0.120
II (n=21)	12	9	
IIIa (n=39)	16	23	
IIIb (n=17)	11	6	
IV (n=3)	1	2	
Type			
Diffuse (n=46)	27	19	0.202
Intestinal (n=38)	17	21	
Lymph node metastasis			
Presence (n=73)	34	39	0.798
Absence (n=11)	10	1	
Mean (n=7.45)	7.68	7.20	

sion and disease free or overall survival. Thirty-one patients with p21 negative diffuse-type carcinoma and 14 patients with p21 negative intestinal-type carcinoma showed shorter mean survival time (49 and 45 months) than positive counterparts (69 and 85 months), respectively (p=0.0056). Mean survival time of p21 negative tumor of TNM stage I, II, IIIa, IIIb and IV were 77, 61, 39, 47 and 10 months, respectively. There was no p21 positive tumor in stage I and stage IV groups. All of 12 patients with p21 positive tumor in stage II were alive. In contrast, mean survival time of TNM stage IIIa and IIIb was 69 and 52 months, respectively.

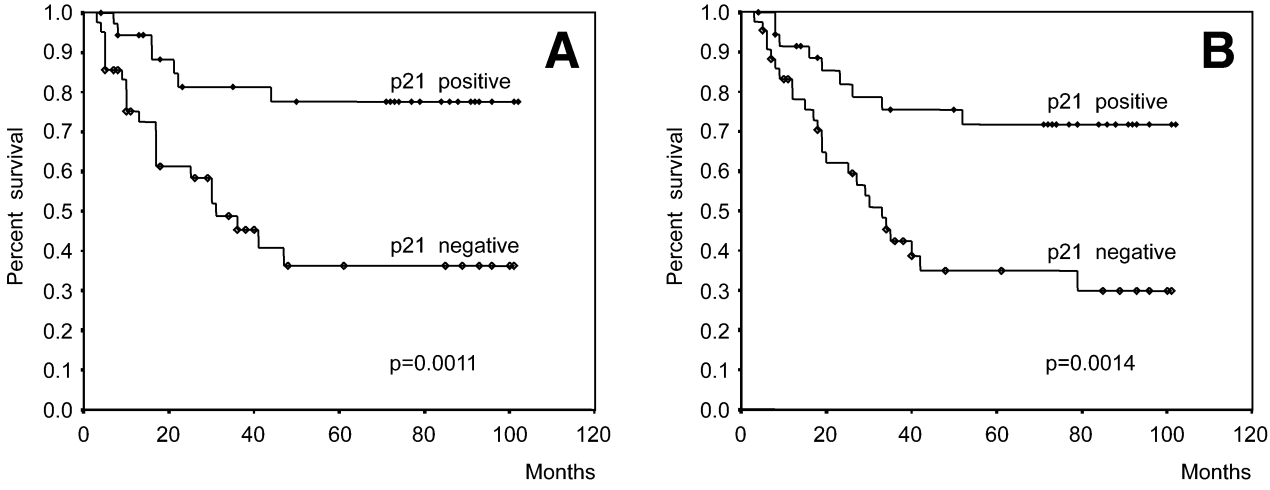

Fig. 3. Disease free (A) and overall (B) survival of patients with p21 positive (n=39) and negative (n=45) carcinoma.

Table 4. Recurrence and death rates in patients with gastric carcinoma according to the expression of p21 and p27

	Expression of p21			Expression of p27		
	Negative (n=45)	Positive (n=39)	p value	Negative (n=44)	Positive (n=40)	p value
Recurrence*	22 (52.3)	7 (18.9)	0.002	16 (40.0)	13 (33.3)	0.539
Death†	25 (58.1)	9 (24.3)	0.002	20 (48.8)	14 (35.9)	0.244

*Information was not available for five cases; †Information was not available for four cases.

Table 5. Correlation between p21 and p27 expression

		p21		Total	p value
		Negative (%)	Positive (%)		
p27	Negative	31 (36.9)	13 (15.5)	44	0.001
	Positive	14 (16.7)	26 (31.0)	40	
	Total	45	39	84	

Although loss of p27 expression was a frequent feature (52.4%) in gastric carcinoma, significant correlation was not found between p27 and clinicopathologic data including age, sex, location of the tumor, histologic grade, TNM stage (Table 3), and recurrence or death rate (Table 4).

In our series, there was a close correlation between p21 and p27 expression (p=0.001) (Table 5). Although p27

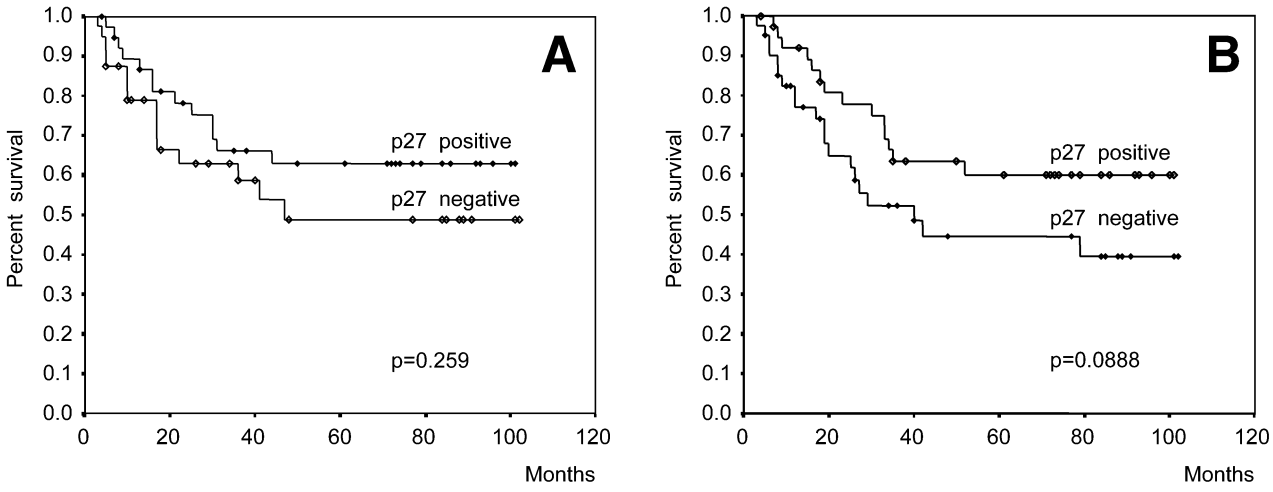

Fig. 4. Disease free (A) and overall (B) survival of patient with p27 positive (n=40) and negative (n=44) carcinoma.

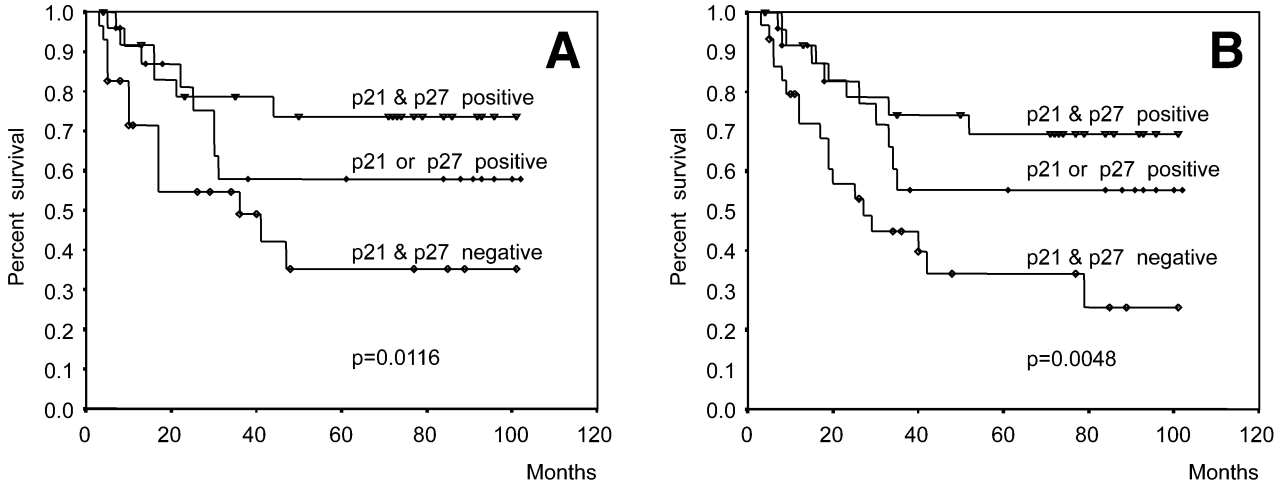

Fig. 5. Disease free (A) and overall (B) survival of patient with both p21 and p27 positive (n=26), p21 or p27 positive (n=27), and both negative (n=31) carcinoma.

expression showed that it did not affect patient survival (Fig. 4), a striking stratification of mortality risk was identified when three different combinations of p21 and p27 expression were evaluated for their relative effects on survival (Fig. 5). Thirty-one (36.9%) carcinomas showed loss of expression of both p21 and p27. The recurrence and death rates of patients with both p21 and p27 negative cancer were significantly higher than those of patients with one of or both p21 and p27 positive carcinomas ($p=0.042$).

DISCUSSION

We characterized the immunohistochemical expression of p21 and p27 in gastric carcinoma. Loss of p21 and p27 expression was found in 45 (53.6%) and 44 (52.4%) of 84 gastric carcinomas, respectively, which is similar to other investigators (31-33). The status of expression was compared to clinicopathologic data and with mortality after a median follow-up of 42.5 months. Although it has been reported that mutational inactivation is a rare event, functional loss of p21 and p27 are relatively frequent in human cancer (23, 35). Immunohistochemical analysis, therefore, may be of practical use for evaluating the functional status of CDKNs in human cancers. Recent reports showed that the practical clinical relevance of p21 and p27 expression is related to its prognostic significance in many human cancers, including breast cancer (27, 28), prostate cancer (29), colon cancer (30), and gastric carcinoma (31-33). p21, an effector protein of p53 tumor suppressor gene, and p27 are major negative regulators of cyclin-CDK complexes of cell cycle engine, and loss of their inhibitory control may be an important event in cancer development and progression (4-7).

In our series, although p21 and p27 expression were not found to correlate with age, sex, location of the tumor, TNM stage, and lymph node status, there was a correlation between the loss of p21 expression and recurrence and death rates of gastric cancer. Twenty two (75.9%) of 29 recurrent and 25 (73.5%) of 34 death cases showed loss of p21 expression. Kaplan-Meier plots of disease free and overall survival also showed increased recurrence and mortality risk associated with loss of p21 expression. These findings suggest that the status of p21 expression appeared to have prognostic value. Ogawa et al. and Mori et al. reported that loss of p21 and p27 expression is correlated with, in addition to survival, aggressiveness of gastric tumor represented by depth of invasion, lymphatic and vascular permeation, stage of the tumor, lymph node metastasis (31, 32). This disaccordance should be elucidated, although it may be explained in part by the finding that most of our cases were Stage III gastric carcinoma with lymph node metastasis. It has been proposed that diffuse-type gastric carcinoma is different from intestinal type in the genetic pathways of pathogenesis, epidemiology and biological behavior (3). Immunohistochemical studies revealed that diffuse-type gastric carcinoma showed more frequent loss of p21 expression than the intestinal-type (31, 32), and the level of p21 expression in a gastric cancer cell lines is associated with p53 gene abnormality (35). Our results showed differences in p21 expression between two histological types, supporting the difference in the molecular mechanism of cancer development between the two types.

In our series, although loss of p27 expression showed no statistically significant correlation with clinicopathologic data, there was a tendency of more frequent recurrence, death and poorer survival in patients with loss of p27 expression. Reduction in p27 levels are associated with increased cell proliferation (26), although other changes likely contribute to altered cell kinetics during carcinogenesis at this site.

There was good correlation between p21 and p27 expression. This result may suggest that certain molecular events cause alterations of inhibitory control of both p21 and p27 in the gastric carcinoma. Interestingly, a striking stratification of mortality risk was identified when three different combinations of p21 and p27 protein status of the tumor were compared. The recurrence and death rates of the patients with loss of both p21 and p27 expression were significantly higher than those patients with loss of one p21 and p27, or normal expression group. These tendencies were also found in Kaplan-Meier plots of disease free and overall survival. These findings may reflect that accumulation of alterations in cell-cycle regulatory proteins may associate with development of more aggressive phenotype (27). Certain tumors including hepatoma increased in total numbers of genetic alteration which is associated with tumor aggressiveness (36). Many positive growth regulators are finally linked with cyclin D, whereas negative growth regulators may act as a major inhibitor of cell proliferation through a cyclin-CDK inhibitors, including p21 and p27 (37, 38). Expression of p21 and p27 were also influenced by ubiquitin-proteosome pathway degrading the molecules, in addition to p53 induced by DNA damage or their upstream regulator such as TGF-β (30, 39). Underexpression of p21 and p27, whatever the mechanism, may result in increased CDK activity, and it is tempting to postulate that their effect in tumors is similar because they are independent regulators of the same pathway. The usefulness of p21 and p27 is that their expression integrates the effects of one or more cell cycle regulators and thereby a single indicator of several possible cell cycle gene

alterations.

In conclusion, loss of p21 and p27 expression is a relatively frequent finding in gastric carcinoma, especially in the diffuse type and the status of p21 and p27 expression by immunohistochemical stain may have a useful prognostic value.

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