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p21 Expression and Mutation in Gastric Carcinoma
: Analysis by Immunohistochemistry and PCR-SSCP

p21 protein has been reported to be a critical downstream effector of p53 and a potent inhibitor of cyclin-dependent kinases. Thus, the *p21* gene is thought to play a central role in tumor suppression. In this study we investigated p21 protein expression and mutation in gastric adenocarcinoma. A total of 76 primary gastric carcinoma specimens were immunohistochemically stained for p21 protein expression and evaluated the correlations between p21 expression and clinicopathologic features. In a proportion of them (20 cases), we also analyzed the possible presence of *p21* gene mutations using PCR-SSCP method. Fourty seven out of 76 cases (61.8%) were p21-negative, and the remaining twenty nine cases (38.2%) were p21-positive on immunostains. There was a correlation between the expression of p21, and the depth of tumor invasion and lymph node metastasis ($p<0.05$). No mutation of the *p21* gene was detected in all of 20 tumor tissues. These results suggest that the status of p21 expression may have prognostic value in gastrc adenocarcinoma.

Key Words : *Proto-oncogene protein p21; Stomach neoplasms; Carcinoma; Immunohistochemistry; PCR-SSCP*

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INTRODUCTION

The p21 protein, a universal cyclin-dependent kinase (CDK) inhibitor, was first identified as a cyclin-dependent kinase interacting protein 1 (CIP 1) from studies trying to characterize upstream regulation factors of CDK (1). The gene is located on chromosome 6 p (2-4). The p21 protein has been reported not only to inhibit CDK function, but also to interact with proliferation cell nuclear antigen (PCNA), bcl-2 and c-myc to inhibit DNA replication and to block cell cycle progression (5-9). Taken together, these data suggest that the p21 protein play a central role in cell cycle regulation. Mutations in the *p21* gene have been demonstrated in prostate cancer and abnormal p21 expression has been found in brain tumor and colon cancer, suggesting an involvement of this gene in malignancies (10, 11).

p21 was also cloned as a wild-type p53 activated fragment-1 (WAF1) from studies looking for downstream effectors of p53. The p53 protein can induce p21 expression by binding to an upstream regulatory site of the *p21* gene. This induction requires wild-type p53 activity, suggesting that p21 is a critical downstream effector of p53. Thus, it is reasonable to investigate the involvement of p53 function in cell cycle regulation by examining p21 protein expression.

In this study, we analyzed p21 protein expression by immunohistochemistry and *p21* gene mutation by PCR-SSCP in gastric carcinoma, and investigated a correlation between p21 expression and clinicopathologic features.

MATERIALS AND METHODS

Patients and tissue samples

Tumor specimens from 76 patients (45 males and 31 females) with primary gastric adenocarcinoma underwent curative surgery between 1993 and 1997 in Chun Chon Sacred Heart Hospital of Hallym University were analyzed. The median age of the gastric cancer patients was 59.5 years, with a range of 27 to 77 years. None of the patients had received preoperative adjuvant therapy (radiotherapy or chemotherapy).

Freshly removed tissue samples were fixed in 10% neutral formalin and embedded in paraffin wax. Two representative blocks were selected and serial sections were examined by immunohistochemistry.

According to the classification of the Korean Research Society for gastic cancer (12), these specimens were his-

tologically classified; these included 15 well-differentiated tubular; 35 moderately differentiated tubular; 14 poorly-differentiated tubular; 8 signet ring cell; and 4 mucinous carcinomas. The clinicopathologic data included age, sex, differentiation, depth of invasion, presence of lymphatic and venous invasion, and lymph node metastasis.

For PCR-SSCP, fresh tumor tissues from 20 patients with gastric cancer were saved in liquid nitrogen.

Immunohistochemical stain

Immunohistochemistry was performed using the avidin-biotin-peroxidase complex described by Hsu et al. (13), using primarily monoclonal antibodies raised against p21 (diluted 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Briefly, dewaxed 4-μm sections were heated in a microwave oven for 20 minutes to retrieve antigens. Endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol. Tissue sections were preblocked for 20 minutes with 10% rabbit serum, after which the primary antibody described above was added for 60 min. After washing, the sections were incubated with the secondary antimouse immunoglobulin conjugated with biotin for 30 minutes at room temperature, followed by incubation with streptavidin-peroxidase complex for 30 minutes. The reaction products of peroxidase were visualized by incubation with 0.05 M Tris-HCl buffer (pH 7.6) containing 20 mg 3,3'-diaminobenzidine and 100 μl 5% hydrogen peroxide per 100 mL. Finally, the slides were counter-stained for nuclei by methyl green stain.

To examine the specificity of immunostaining, the primary antibody was replaced by mouse normal IgG at 1:100 dilution and Tris-buffered saline. Control slides were invariably negative for immunostaining. To obtain constant immunohistochemical findings, appropriate control slides of gastric carcinoma were stained at the same time.

The sections were assessed independently by two investigators without knowledge of the patients clinical outcome. Nuclear staining of cancer cells in more than 5% was considered positive evidence of p21 expression. Cytoplasmic staining was excluded in this study.

PCR and SSCP

Single-strand conformational polymorphism (SSCP) analysis was performed by PCR amplification of the two coding exons of the *p21* gene. Exon 2 of the *p21* gene was amplified with oligonucleotides 5'-GCGCCATGTCAGAACCGGC and 5'-GAGAATCCTGGTCCCTTAC, and exon 3 was amplified with oligonucleotides 5'-GCCCCCCACTGTCTTCCT and 5'-GCGCTTCCAGGACTGCAGG (14). PCRs were performed in a volume of 50 μl containing 50 ng of DNA, 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 200 μM dGTP, dCTP, dTTP and dATP; 0.75 μM each primer; and 1 unit of Taq polymerase (Dynazyme, Finland). Amplification was performed in a Perkin-Elmer 9600 thermal cycle and consisted in l0 min, denaturation step at 95℃ followed by 37 cycles of 30 seconds at 95℃, 30 seconds at 62℃, and 30 seconds at 72℃.

Ten microliters of each PCR product was diluted in 10 μl of denaturing loading buffer (98% formamide; 0.05% bromphenol blue; and 0.05% xylene cyanol FF), heated at 95℃ for 3 min, and immediately placed on ice for 3-5 min. Total 20 μl solution was electrophoresed on 8% nondenaturing polyacrylamide gels containing 5% glycerol and 1x Tris-borate-EDTA (TBE). Gels were run in 1x TBE, respectively, at 200 V for 7-15 hrs, using a cooling chamber. After electrophoresis, the gel was dried. Silver staining was performed by a staining kit (Promega, USA).

All SSCP analyses were repeated twice each under electrophoresis conditions. Negative assay controls consisted of water and extraction buffer blanks for PCR.

Statistical analysis

Data were summarized as mean$\pm$SD. The data were analyzed using the Kruskai-Wallis test, Spearman correlation coefficients, Wilcoxon rank test and regression analysis. $P<0.05$ was considered statistically significant.

RESULTS

Immunohistochemical staining of p21 protein

p21 was positively detected in nuclei but not detected in cytoplasm. Because cancer cells usually showed heterogeneity of the nuclear staining in the same section, tumor specimens were considered positive for the p21 expression when there was evidence of nuclear staining in more than 5% of the tumor cells.

p21 positive cells were observed in foveolar epithelial cells of the normal gastic mucosa (Fig. 1A). The cells in the proliferative zone as well as the cells of the fundic glands and pyloric glands, did not show p21 positivity. And also metaplastic mucosa copying the intestine (intestinal metaplasia) showed a similar pattern (Fig. 1B). But the cells located in lower parts of the crypts (one-third from the bottom) showed p21 positivity.

Of the 76 gastric adenocarcinomas studied, 29 (38.2%) showed positive staining within the cell nucleus with the p21 antibody (Fig. 2B). Fig. 2A showed p21 negative cells.

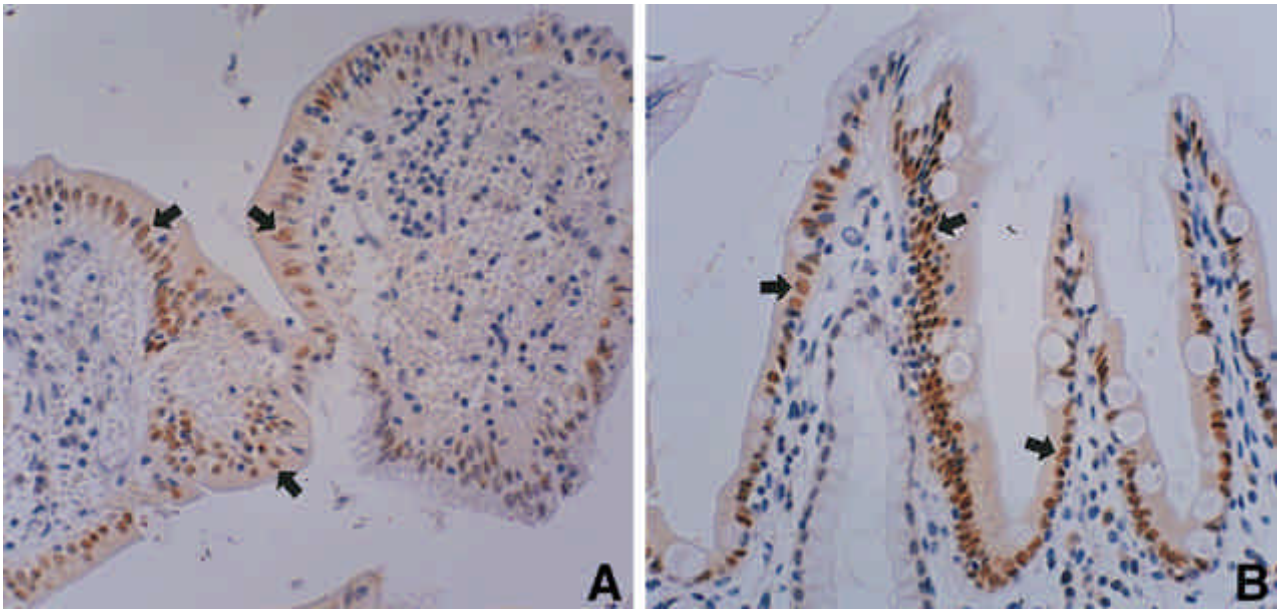

Fig. 1. Immunostaining of p21 in non-neoplastic gastric mucosa. The expression of p21 is seen frequently in the foveolar epithelial cells (A) and metaplastic glandular cells (B). Brown nuclei indicate p21 positive cells (pointed by arrows)(×100).

Table 1. Association of p21 expression with clinicopathologic parameters of gastric carcinoma

Parameters	p21*		p value
	Negative	Positive	
Age			p=NS
>50	9 (11.8)	4 (5.3)	
≤50	38 (50.0)	25 (32.9)	
Sex			p=NS
M	29 (38.2)	16 (21.1)	
F	18 (23.7)	13 (17.1)	
Depth of invasion			p<0.05
T1	5 (6.6)	11 (14.5)	
T2	11 (14.5)	9 (11.8)	
T3	8 (10.5)	3 (4.0)	
T4	23 (30.3)	6 (7.9)	
Lymph node metastasis			p<0.05
Absence	12 (17.1)	16 (22.9)	
Presence	24 (34.3)	18 (25.7)	
Differentiation			p=NS
Tub. well	8 (10.5)	7 (9.2)	
Tub. mod	22 (29.0)	13 (17.1)	
Tub. poor	10 (13.2)	4 (5.3)	
Signet	4 (5.3)	4 (5.3)	
Mucinous	3 (4.0)	1 (1.3)	
Lymphatic invasion			p=NS
Absence	9 (12.5)	12 (16.7)	
Presence	34 (47.2)	17 (23.6)	
Venous invasion			p=NS
Absence	14 (19.4)	15 (20.8)	
Presence	29 (40.3)	14 (19.4)	

p<0.05 are by chi-square test.
NS: not significant.

Relationship between p21 expression and clinicopathologic parameters

Expression of p21 protein was analyzed in relation to clinicopathologic parameters, including age, sex, histologic differentiation, tumor depth, lymph node metastasis, lymphatic, and venous invasion (Table 1). According to chi-square test, tumor depth and lymph node metastasis correlated significantly with p21 expression ($r=0.36$; $p<0.05$ and $r=0.29$; $p<0.05$). And also Spearman's correlation coefficients ($p<0.05$) showed a significant correlation between the p21 expression and lymphatic and venous invasion (Table 2).

PCR-SSCP and DNA sequencing of p21

PCR-SSCP of p21 gene (exon2 and exon 3) showed no abnormal mobility shifting in the twenty cancer tissues of the patients. We performed DNA sequencing of the same cases for confirmation and found no mutation (Fig 3).

Table 2. Spearman's correlation coefficients($p<0.05$) between p21 expression and pathological parameters

Parameters	Depth of invasion	Lymph node metastasis	Lymphatic invasion	Venous invasion
p21	-0.36026	-0.28998	-0.22065	-0.19166

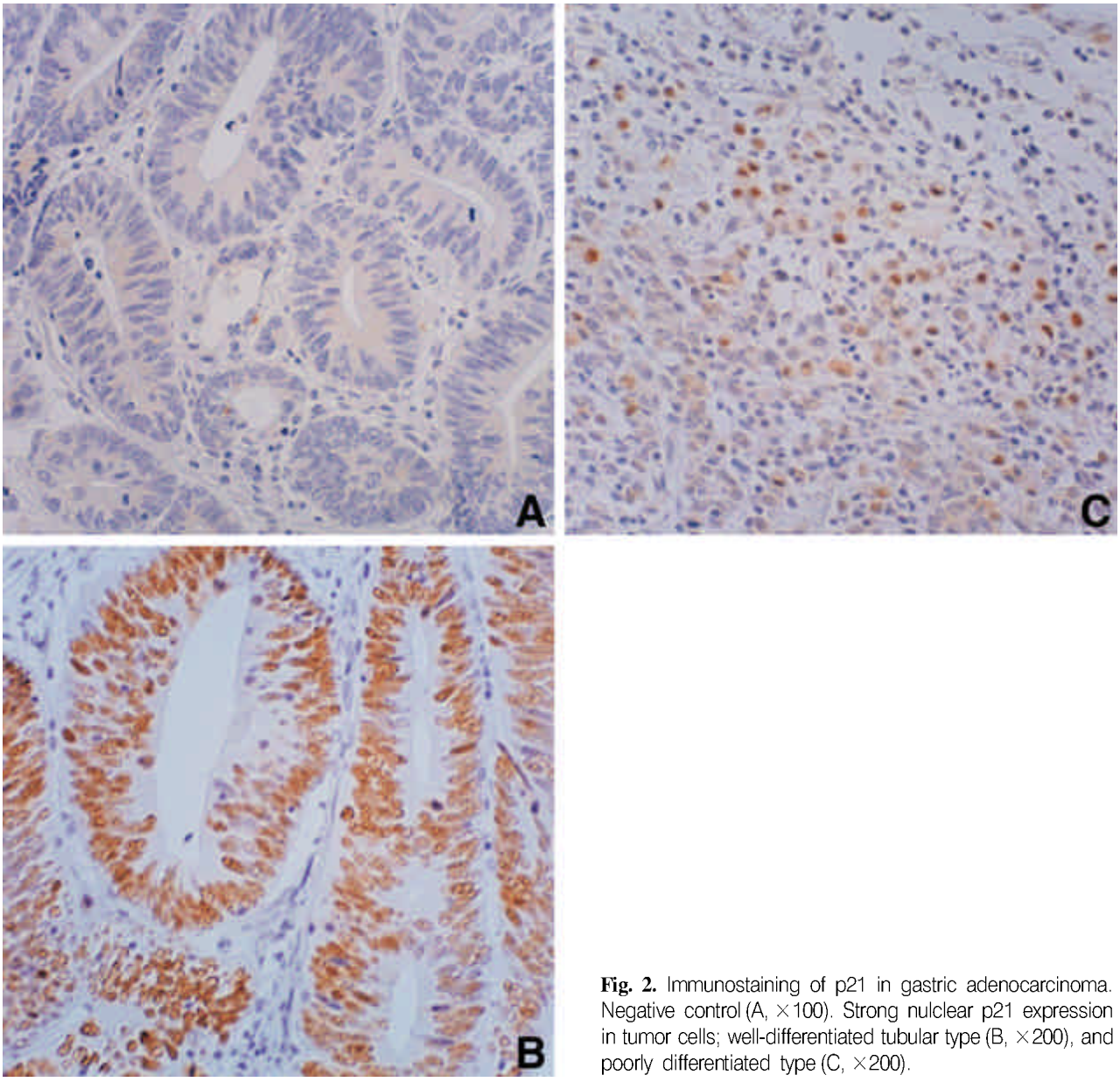

Fig. 2. Immunostaining of p21 in gastric adenocarcinoma. Negative control (A, ×100). Strong nulclear p21 expression in tumor cells; well-differentiated tubular type (B, ×200), and poorly differentiated type (C, ×200).

DISCUSSION

Recently, several regulators of the cell cycle have been identified and have received much attention as prognostic factors in the evaluation of the malignancy potential of tumors. Of these regulators, p53 and p21, negative regulator of the cell cycle, are well known as a marker of some malignant diseases. However, the clinical importance of p21 has not been clarified, although p53 has been studied by many investigators. The expression of *p21* gene is regulated by two pathways, namely, p53-independent and dependent mechanisms (1, 3, 5, 15-17). However, since p21 is induced mainly by a p53-dependent pathway, p21 seems to directly reflect the growth-suppressive function of p53 and would be a more reliable marker for the assessment of behavior of cancers than p53. To evaluate p21 as prognostic marker of gastric cancer, we studied its expression in relation to the clinicopathologic parameters of the disease.

Epithelial cells exit the cell cycle as they move toward the mucosal surface in the normal mucosa of the stomach. Consequently, these cells are fully differentiated and will be shed to the gastric lumen by renewal mechanisms (18). In this study, p21 positive cells were detected in superficial areas of normal gastric mucosa and intestinal metaplastic glands. These findings support the view that p21 can be induced during differentiation. Furthermore p21 might be associated with the cellular senes-

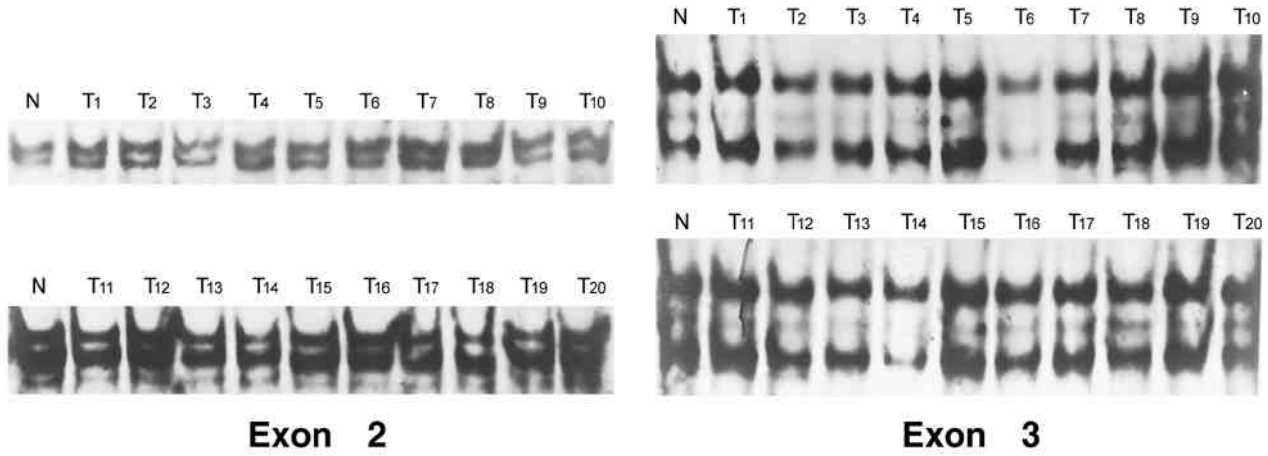

Fig. 3. Single-strand conformational polymorphism of PCR products for *p21* (exon 2 and exon 3) gene in tumor tissue (T1-T20) shows no abnormal band or mobility shifting. N: Normal as control, T: Tumor sample.

cence of non-neoplastic gastric mucosa.

p21 function has been studied by some groups in cultured cell lines (19, 20). In irradiated cells, p21 regulates the cell cycle and arrests the cell at the G1 checkpoint. p21 positive cells proceeded to DNA repair or apoptosis in cell lines (2, 21, 22). Moreover, a direct effect of p21 as an inhibitor of cell proliferation has been demonstrated by its transient expression in tumor cells and in normal fibroblasts (1, 23). However, p21 expression in gastric carcinoma in vivo has not been sufficiently elucidated.

Wataru et al. reported that the expression of p21 was detected in 81 of 343 gastric adenocarcinomas (24%). In this study, p21 expression was detected in 29 of 76 gastric adenocarcinomas (38.2%). We compared the clinicopathologic features of patients with p21-positive and negative primary tumors. Although there was no relationship between p21 expression and age, sex, and histologic differentiation, there was a correlation with depth of tumor invasion, lymph node metastasis ($p<0.05$), lymphatic, and venous invasion (Spearman's correlation coefficent $r=0.22$, $r=0.19$). Moreover, the majority of the patients with p21-negative tumors had advanced lesions. And a strong association with disease progression was also demonstrated.

Differences in p21 expression among histological types were not found. Other investigators have also shown that the loss of p21 correlates positively with the depth of cancer invasion and also stage progression in primary prostate cancer (24). Similar abnormalities have been reported in studies of other cell cycle-regulating factors such as p53 (13), Rb (25), cyclins (26), and PCNA (27). Our study and others suggest that cell cycle deregulation is an important factor in gastric cancer progression.

The biologic significance of p21 overexpression has not been elucidated sufficiently. Recent studies have demonstrated a correlation with poor prognosis in colon (11), breast (28, 29), and ovary (30) carcinoma patients. In this study, p21 negative tumor significantly correlated with depth of invasion and lymph node metastasis. These results strongly suggest that the loss of p21 is secondary to disease progression, as cancer cells with high proliferative activity may have a higher potential for metastasisc than those with low proliferative activity (27, 31).

Immunoreactive p21 protein might be the wild, nonmutated type, because a recent study failed to detect *p21* gene mutations in a large series of various human tumors (32). We analyzed p21 gene mutation (exon 2 and exon 3) of 20 gastric cancer tissue using PCR-SSCP. No mutation was detected.

Although futher studies on a larger scale will be necessary to elucidate the relevance of p21 expression to the biological behavior of cancers, this retrospective study suggests that p21 expression correlated with disease progression and may have prognostic value in gastric adenocarcinoma.

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