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Reduced Expression of Cyclin-dependent Kinase Inhibitor p27^{Kip1} Is Associated with Advanced Stage and Invasiveness of Gastric Carcinomas

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Reduced expression of a cyclin-dependent kinase inhibitor p27^{Kip1} has recently been shown to predict poor survival of patients with breast and colorectal cancers. We studied the expression of p27^{Kip1} in gastric carcinomas by northern blotting, western blotting and immunohistochemistry to determine whether lack of p27 has implications for aggressiveness of gastric cancer. Reduced expression of p27 was detected in 40% of the gastric carcinomas at the mRNA level, while it was detected in 57% at the protein level. No gross alterations of the *p27* gene were observed in any of the cases examined by Southern blot analysis. Immunohistochemical studies revealed that the expression of p27 was well preserved in most of the gastric adenomas, whereas it was so in only 26% of the gastric carcinomas. Fifty-six percent of the carcinomas showed almost no p27-positive cells. Decrease of p27-positive cells significantly correlated with advanced stage, depth of tumor invasion and lymph node metastasis. The expression of p27 showed an inverse correlation with the expression of cyclin E. These findings suggest that reduction of p27^{Kip1} protein may reflect the progression of gastric carcinomas and may be an indicator of high-grade malignancy.

Key words: CDK inhibitor — p27^{Kip1} — Gastric carcinoma — Northern blotting — Immunohistochemistry

Abnormalities in the cell cycle are deeply involved in the genesis and progression of cancers through unbridled cell growth and division.[1,2] Multiple cyclins and cyclin-dependent kinases (CDKs) are positive regulators of cell cycle progression. In gastric carcinomas, we have recently found that amplification and overexpression of the cyclin E gene are correlated with the aggressiveness.[3,4] On the other hand, several CDK inhibitors have been identified and potentially act as tumor suppressors.[5–10]

p27^{Kip1}, a member of the cip/kip family of CDK inhibitors, was first identified as a negative growth regulator present in cells rendered quiescent by contact inhibition, as well as in transforming growth factor β-treated cells.[6–8] p27 binds to a wide variety of cyclin/CDK complexes including CDK2 and CDK4, inhibits kinase activity, and blocks the cell cycle. We have recently found that growth suppression of interferon-β is associated with the induction of p27 in gastric carcinoma cells.[11] Although p27 is a candidate tumor suppressor, mutations of the *p27* gene are rarely found in human cancers.[12,13] Recently, striking findings regarding the significance of p27 in progression of breast and colorectal carcinomas have been reported.[14–16] Reduced expression of p27 protein significantly correlates with poor prognosis of these patients. Decreased levels of p27 protein are suggested to be brought about by proteasome-dependent degradation, while p27 mRNA levels are preserved in many cases.[16] Therefore, decreasing p27 expression measured immunohistochemically should be a significant predictor of poor survival. However, no study has been conducted to examine the expression of p27^{Kip1} in gastric carcinoma tissues in relation to biological behavior. In the present study, we examined the expression of p27^{Kip1} gastric carcinoma tissues by northern blotting, western blotting and immunohistochemistry and analyzed the correlation of p27 expression with tumor stage, depth of tumor invasion and lymph node metastasis.

In total, 68 adenocarcinomas and 11 adenomas of the stomach were studied. They were obtained by surgery and endoscopical removal at Hiroshima University Hospital and related facilities. For molecular analyses, tumor and non-neoplastic tissue samples obtained at the time of surgery were immediately frozen in liquid nitrogen and stored at −80°C. We confirmed histologically that tumor samples consisted mainly of carcinoma tissue. For immunohistochemistry, tissues were fixed in 10% buffered formalin and embedded in paraffin. One or two representative slides of the primary tumors, which included superficial, central and deep areas, from each case were analyzed. The definitions of stage grouping, histological classification and depth of tumor invasion were made according to the criteria of the Japanese Classification of Gastric Carcinoma.[17]

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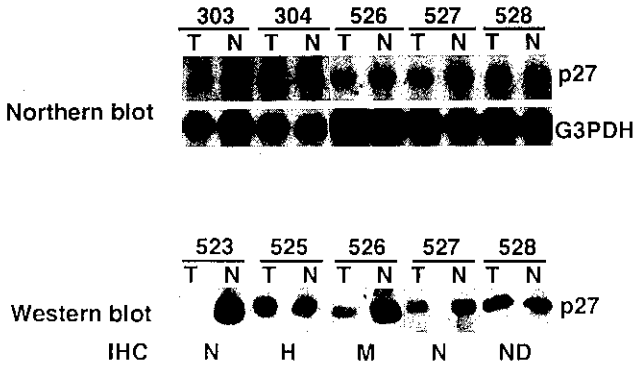

Fig. 1. Expression of $p27^{Kip1}$ in gastric carcinomas and the corresponding non-neoplastic mucosa. Northern and western blot analyses were performed as described in the text. Applied amount and quality of samples were confirmed by reprobing with G3PDH. T, tumor tissue; N, corresponding non-neoplastic mucosa. The result of immunohistochemistry (IHC) is shown below the panel. Grades of p27 staining were classified as high (H), medium (M) and negative (N), as described in the text. ND, not determined.

RNAs were extracted by the standard guanidinium isothiocyanate/cesium chloride methods and poly(A)$^+$ selected RNAs were analyzed by northern blotting as described.[3] DNAs were extracted from fresh-frozen tissues by the phenol-chloroform method and Southern blot analysis was performed as described.[3] The cDNA fragment encoding $p27^{Kip1}$ was kindly provided by Dr. Massague (Howard Hughes Medical Institute, New York). The protein extraction and western blotting were carried out as described.[3] A monoclonal antibody to $p27^{Kip1}$ (K25020) was purchased from Transduction Laboratories (Lexington, Kentucky). The immune complex was visualized by using the ECL western blotting detection system (Amersham, Aylesbury, UK). The expression of p27 mRNA or protein was defined as decreased if more than 30% reduction of the signal intensities in the tumor tissues was detected in comparison with the corresponding non-neoplastic mucosa by one-dimensional densitometry on autoradiography.

A modification of the immunoglobulin enzyme bridge technique (ABC method) was employed as described.[18]

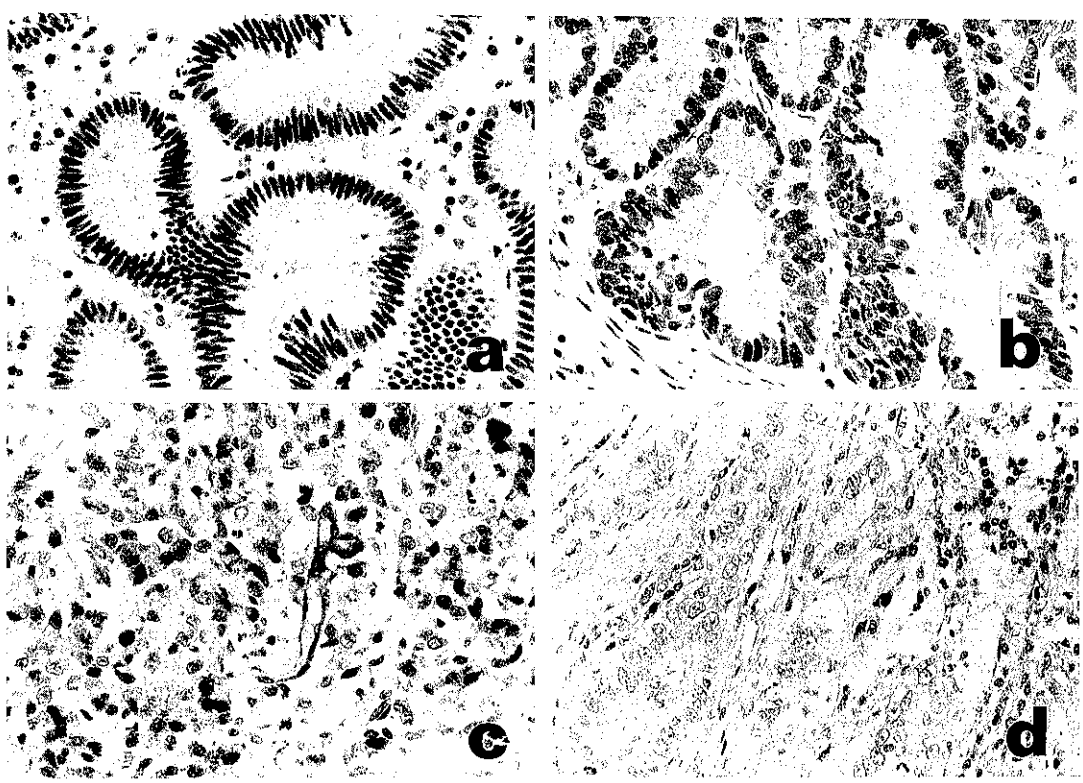

Fig. 2. Immunostaining of $p27^{Kip1}$ in gastric adenoma and adenocarcinoma. a, Adenoma (graded as high, ×230). p27 staining is observed in nuclei of almost all adenoma cells. b, Well differentiated tubular adenocarcinoma (early cancer, graded as high, ×230). c, Poorly differentiated adenocarcinoma (early cancer, graded as high, ×275). Many carcinoma cells are positive to p27 in their nuclei. d, Moderately differentiated tubular adenocarcinoma (advanced cancer, graded as negative, ×230). Although infiltrating lymphocytes are p27-positive, tumor cells show no p27 staining.

Microwave pretreatment in citrate buffer was performed for 10×3 min to retrieve the antigenicity. The primary reaction with anti-p27 antibody (diluted 1 : 500) or anti-cyclin E antibody (14591A, Pharmingen, San Diego, California, diluted 1 : 200) was conducted at room temperature for 120 min. Peroxidase staining was performed for 10–15 min and the sections were weakly counterstained with hematoxylin. For adenomas and superficial carcinomas, the entire stained sections were observed. For large tumors of advanced cases, at least ten fields including superficial, central and deeply invasive areas were observed and the number of stained cells and the staining intensity were estimated. The levels of p27 staining were graded as high (over 30% of tumor cells showed moderate to intense immunoreactivity), medium (5–30% of the tumor cells showed moderate immunoreactivity) or negative (less than 5% of the tumor cells showed weak immunoreactivity or no staining). The cyclin E staining was classified as positive and negative as described.[18]

First, we examined the expression of p27 mRNA in gastric carcinomas by northern blot analysis. Fresh-frozen tissues were available in 20 carcinoma cases, most of which were advanced cases. Among them, 8 (40%) expressed p27 mRNA at lower levels in tumor tissues than in non-neoplastic mucosa. Representative cases are shown in Fig. 1. However, no gross alterations of the *p27* gene were detected in any carcinoma tissues examined (data not shown). By western blotting, we analyzed the expression of p27 protein in gastric carcinomas and compared the result with that in non-neoplastic mucosa. Four (57%) of the 7 carcinomas expressed lower levels of p27 protein than corresponding non-neoplastic mucosa. Some carcinomas expressed considerable levels of p27 mRNA, whereas the p27 protein levels were low or undetectable (e.g., case 526 in Fig. 1). These findings suggest that posttranslational modification may influence the level of p27 protein in carcinoma tissues.

We next immunohistochemically examined the expression of p27 protein in gastric carcinomas and adenomas. In non-neoplastic components of the gastric tissues, p27 protein was consistently positive in the non-neoplastic mucosa, including intestinal metaplasia (strongly in the bottom layer), inflammatory cells, fibroblasts, smooth muscle cells and endothelial cells. p27 protein was localized in the nucleus of these cells. Therefore, p27 staining in these components can be regarded as an internal positive control of the immunohistochemistry. If normal staining was not even, we excluded the case from the analysis. More than 90% of the gastric adenomas showed p27 staining in most of the tumor cells, as shown in Fig. 2. On the other hand, of the 68 gastric carcinomas, 18 (26%) showed p27 staining of high grade, whereas 38 (56%) were negative to p27. The grades of p27 staining were compatible with the levels of p27 protein detected by western blotting in 80% of the cases examined (Fig. 1). The discrepancy found in some cases might be due to the expression of p27 by the stromal cells described above that gave positive signals in western blotting, but could be excluded in immunohistochemical evaluation.

Table I. Expression of p27 in Gastric Adenomas and Adenocarcinomas and Its Correlation with Clinicopathological Parameters

	Case no.	p27-positive cells[a] (%)			*P* value[d]
		high	medium	negative	
Adenoma	11	10 (91)	0	1 (9)	0.0002
Adenocarcinoma	68	18 (26)	12 (18)	38 (56)	
Histology[b]					0.1561
well	39	12 (31)	9 (23)	18 (46)	
poorly	29	6 (21)	3 (10)	20 (69)	
Stage[b]					0.0142
1	27	14 (52)	4 (15)	9 (33)	
2	5	1 (20)	1 (20)	3 (60)	
3	21	1 (5)	4 (19)	16 (76)	
4	15	2 (13)	3 (20)	10 (67)	
Depth of invasion[b]					<0.0001
m, sm	20	13 (65)	4 (20)	3 (15)	
mp, ss	22	4 (18)	3 (14)	15 (68)	
se, si	26	1 (4)	5 (19)	20 (77)	
Lymphnode metastasis					0.0013
negative	29	14 (48)	4 (14)	10 (34)	
positive	39	4 (10)	8 (21)	27 (69)	
Cyclin E expression[c]					0.0057
negative	13	8 (62)	1 (8)	4 (31)	
positive	47	8 (17)	9 (19)	30 (64)	

a) Grades of p27-positive cells were classified as high (> 30%), medium (5–30%) and negative (5% >) as described in the text.
b) According to the criteria of the Japanese Classification of Gastric Cancer.[17]
c) Cyclin E expression was defined as positive and negative as described.[18]
d) The correlation was analyzed by Fisher's exact test and *P* values are shown. *P* values less than 0.05 were regarded as statistically significant.

We then analyzed the relationship between p27 staining and clinicopathological parameters (Table I). High p27 expression was frequently found in early stage cases, superficial carcinomas and carcinomas without metastasis. On the other hand, reduced expression of p27 was associated with advanced stage, deep invasion and nodal metastasis. Typical was the relation between reduced p27 protein and depth of tumor invasion. High p27 expression was observed in 65% of the carcinomas limited to the submucosa, whereas it was detected in only 18% and 4% of the carcinomas invading the muscularis propria and of those with serosal exposure or invasion to other organs, respectively. There was a significant correlation

between reduced p27 expression and tumor stage, invasiveness and lymph node metastasis ($P<0.05$). The expression of p27 was heterogenous in some carcinomas and some of them showed a tendency to express less p27 in the deeply invasive portion. Moreover, the expression of p27 correlated inversely with the expression of cyclin E. Sixty-two percent of cyclin E-negative carcinomas showed high p27 expression, while 64% of cyclin E-positive tumors were negative to p27. Although p27 expression was a little stronger in well differentiated adenocarcinomas than in poorly differentiated adenocarcinomas, the correlation between reduced p27 expression and invasiveness as well as advanced stage was significant when only well differentiated adenocarcinomas were analyzed (data not shown). Furthermore, a relationship between reduced expression of p27 and depth of tumor invasion or lymphnode metastasis was also evident in poorly differentiated adenocarcinomas.

Among various cell cycle regulators, genetic alterations in $p27^{Kip1}$ as well as $p21^{WAF1/CIP1}$ are rare in human cancers, in contrast to the frequent abnormalities in *p53* and *p16* genes.[9, 12, 13] Three recent reports from independent groups shed light on the role of p27 in cancer biology.[14–16] They all found that reduced expression of p27 protein predicted poor patient survival, but for different cancers, such as breast and colorectal cancers. The present results also strongly suggest that reduced expression of p27 protein may participate in the progression of gastric carcinoma. Therefore, reduced p27 expression should be a candidate prognostic factor for gastric cancer patients as well. The prognostic value of this phenomenon needs to be confirmed.

It has been shown that the regulation of the cellular abundance of p27 in the cell cycle occurs at the post-translational level.[19] In the present study, a number of carcinomas expressed considerable levels of p27 mRNA, but reduced or no p27 protein. Reduced expression of p27 in colorectal carcinoma is suggested to result from ubiquitin-mediated proteasomal degradation rather than altered gene expression or genetic abnormalities.[16] We also found no gross alterations of the *p27* gene in gastric carcinoma tissues. Therefore, a similar mechanism might afford the reduced p27 protein levels in gastric carcinomas.

We have found that gene amplification and overexpression of cyclin E are associated with biological malignancy of gastric carcinomas.[3, 4] The expression of p27 and cyclin E has been reported to correlate with survival in young breast cancer patients.[14] Here we have also demonstrated an inverse correlation between the expressions of p27 and cyclin E, although we do not know at present which is the causative alteration. The proliferation and progression of cancer cells should be regulated not only by p27 and cyclin E, but also by a balance of other negative and positive regulators. We have recently found that the expression of $p21^{WAF1/CIP1}$ positively correlates with malignant behavior of gastric carcinomas, although p21 is a negative regulator of the cell cycle.[20] According to the present results, loss of p27 function and gain of cyclin E may overweigh the increase of p21 in the progression of gastric carcinomas.

In conclusion, the reduction of $p27^{Kip1}$ protein may be an indicator of high-grade malignancy and a candidate prognostic factor of gastric carcinomas.

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