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Expression of Cyclin D1 and Cyclin E in Human Gastric Carcinoma and its Clinicopathologic Significance

Cyclin D1 and cyclin E are the mammalian G1 cyclins that are both required and rate limiting for entry into S phase. Alterations in cell cycle regulators and subsequent deregulation of the cell cycle are frequently involved in tumorigenesis and/or tumor progression. We investigated the expression of cyclin D1 and cyclin E protein in 84 gastric carcinoma by immunohistochemical staining and also the relevance of each cyclin expression to the clinical outcomes. Overexpression of cyclin D1 and cyclin E was noted in 21 of 84 (25.0%) and 34 of 84 (40.5%) gastric cancer tissues, respectively. There was a significant correlation between overexpression of cyclin E and lymph node metastasis (p=0.003), recurrence (p=0.043), disease free survival (p= 0.0378) and overall survival (p=0.0319), but no correlation was noted between overexpression of cyclin D1 and other clinicopathologic variables. These findings suggest that overexpression of cyclin E and cyclin D1 is a frequent finding in gastric cancer and immunohistochemical analysis for cell cycle regulators, especially cyclin E might be a useful prognostic indicator in gastric cancer.

Key Words : *Stomach neoplasms; Carcinoma; Cyclin D1; Cyclin E*

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INTRODUCTION

Gastric cancer is one of the most common cancers in the world. Although it has been reported that many oncogenes, tumor suppressor genes, and growth factors are involved in the development of gastric cancer (1-5), their carcinogenic mechanism has not been fully explored. Recent evidence indicates that a set of cyclin-dependent kinases (cdk) and their regulatory subunits, cyclins, play a key role in cell cycle regulation (6, 7). Cyclin D1, functionally forming a complex with cdk4/cdk6, is involved in G1/S transition and its alteration can cause deregulation of the cell cycle resulting in oncogenesis (6-8). Overexpression of cyclin D1 has been reported in various human cancers, including breast cancer, esophageal cancer, hepatocellular carcinoma and pancreatic cancer and is associated with poor prognosis (9-12). Cyclin E is a late G1 cyclin, which along with its catalytic subunit, the protein kinase cdk2, is required for entry into S phase (13, 14). It has been also reported that overexpression of cyclin E is frequently found in breast cancer and also has a prognostic significance (15, 16).

The pathways through which these cyclins play a role suggest a possible implication in cancer, where their deregulation, such as untimely and excessive expression, could have severe consequences (17). These cyclin D1 or cyclin E gene amplifications are very frequently associated with each gene overexpression in many human cancers (10, 18-21). Alterations of the cell cycle regulators such as cyclin D1, cyclin E and p16 have been reported in gastric carcinoma, their clinical relevance has not been fully determined yet (22-24). Increased expression of cyclin D1 and cyclin E, although which is associated with gene amplification, in the absence of gene amplification also occurs in some of the human cancers including gastric cancer (22, 25). Immunohistochemical analysis of their expression, therefore, could be a simple and useful method for evaluating their status in the tissue level. To investigate the prognostic significance of cell cycle regulators, cyclin D1 and cyclin E in gastric cancer, we analyzed 84 gastric carcinoma tissues using immunohistochemical method.

MATERIALS AND METHODS

Tissue samples and patient population

Gastric cancer tissues were obtained from each of 84 patients undergoing surgery (Hanyang University, Seoul,

Korea) between 1988 and 1992. The group of patients consisted of 27 female and 57 male patients. Information concerning diagnosis date, tumor size and clinical stage, lymph node status and deaths was obtained from retrospective study. Subjects were followed until the earliest of the following: their date of death, the date they were last known to be alive, or the end of the follow-up period. Observations were censored at either the date of last known follow-up or the end date of the follow-up period if death had not occurred. The median follow-up period was 38 months.

Immunohistochemical staining

Formalin-fixed paraffin embedded tissue blocks were sectioned (4 μm thickness) and immunostain was performed following previously described methods (22). Sections were dewaxed and heated in a microwave oven for 10 minutes to retrieve the antigens. Endogenous peroxidase was blocked with a 3% hydrogen peroxide in methanol solution. All slides were preincubated with 10% normal goat serum for 20 minutes. Mouse monoclonal IgG antibody (1:200, Santa Cruz Biotec, U.S.A.) for cyclin D1 and rabbit polyclonal IgG antibody (1:200, Santa Cruz Biotec, U.S.A.) for cyclin E were incubated for 2 hours at room temperature, respectively. Each of the biotinylated secondary antibodies was added for 30 min followed by the streptavidin-biotin peroxidase reagent (DAKO, Santa Babara, CA, U.S.A.) for an additional 30 min. After washing with phosphate buffered saline, staining was achieved by a 3,3-diaminobenzidine (DAKO, Santa Babara, CA, U.S.A.).

All slides were coded and evaluated without knowledge of patient identity or clinical status by an experienced pathologist. Each experiment was independently performed twice. Results of immunostaining were classified semiquantitatively into four groups, according to the number of positively nuclear-stained tumour cells: Score 0: negative or < 5% nuclear or cytoplasmic stain; Score 1: 5-25% nuclear stain; Score 2: 26-50% nuclear stain; Score 3: 51-75% nuclear stain; Score 4: > 75% nuclear stain. Adjacent normal gastric mucosa provided negative internal control for the cyclin D1 and E protein expression.

Statistical analysis

Frequencies of each cyclin overexpression were compared with various clinical characteristics and pathological variables using Pearsons chi-square test. Survival curves were calculated using the Kaplan-Meier method and compared with the frequencies of each cyclin overexpression using log-rank test. Univariate analysis and multivariate stepwise Cox-regression analysis were performed to identify prognostic factors for survival. All statistical analyses were two-sided at a significance level of $p=0.05$, and performed using SPSS 7.5® statistical software.

RESULTS

Using the anti-cyclin D1 and cyclin E antibody, normal gastric mucosa adjacent to the cancer showed immu-

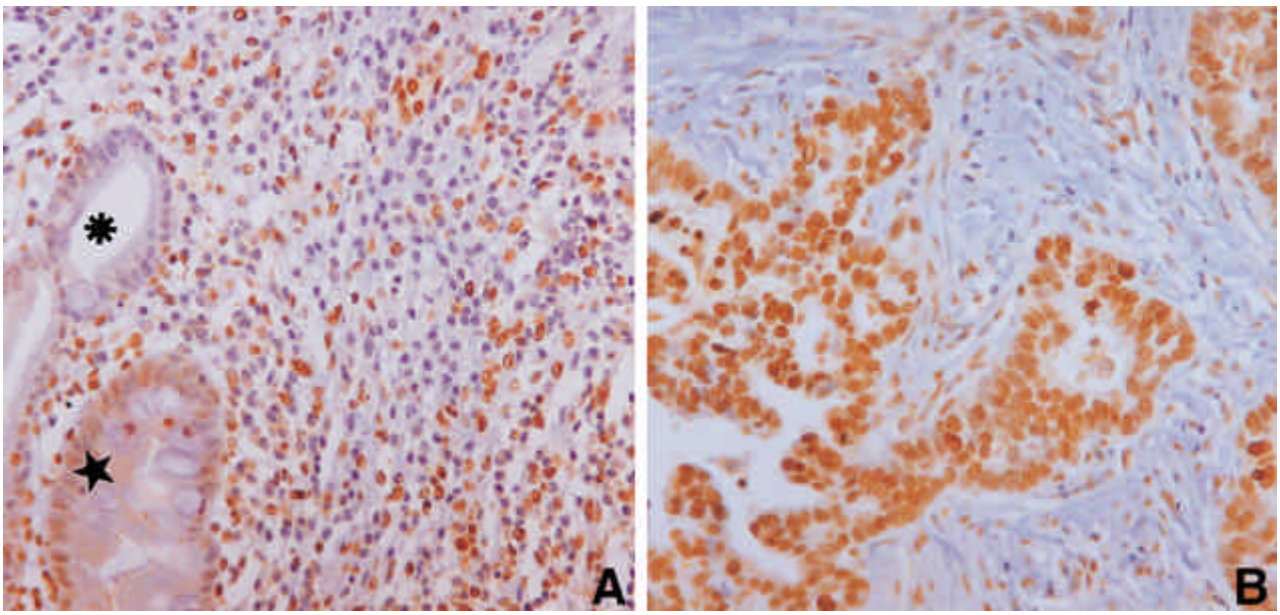

Fig. 1. Nuclear immunostaining for cyclin D1 in diffuse type (A) and intestinal type adenocarcinoma (B). Normal gastric mucosa (*) and intestinal metaplasia (★) showed occasional weak nuclear staining (×200).

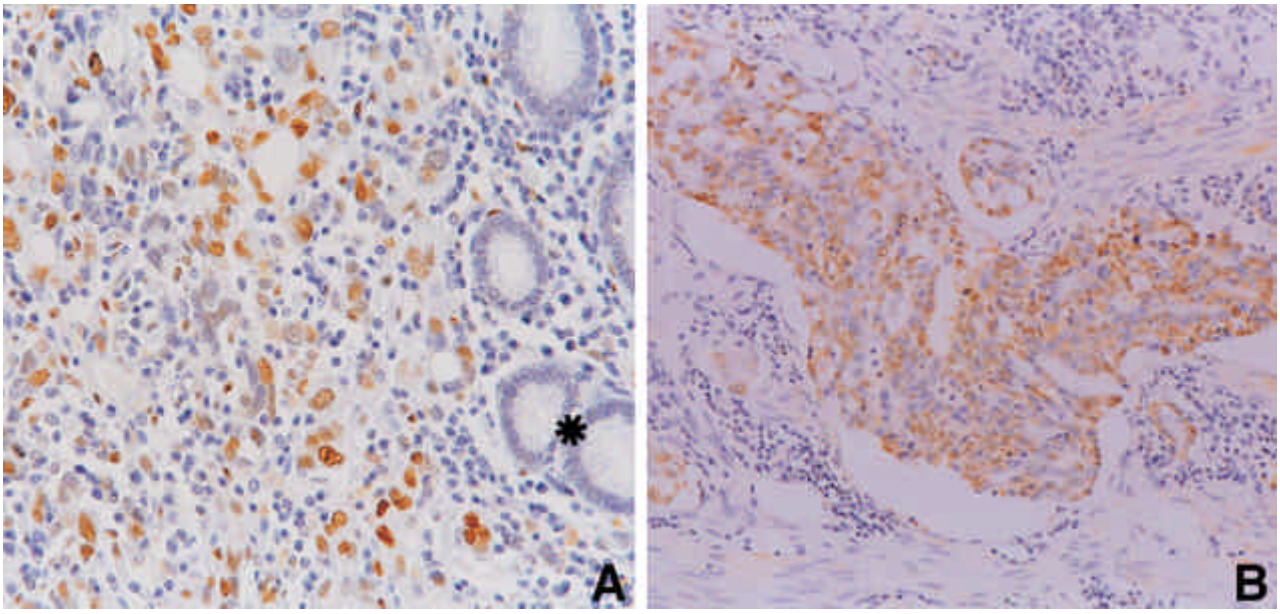

Fig. 2. Nuclear immunostaining for cyclin E in diffuse type (A) and intestinal type adenocarcinoma (B) with lymphatic invasion. Adjacent normal gastric mucosa (*) showed no nuclear staining (×200).

nostaining in less than 25% (Score 1) of the cells. Therefore, we defined overexpression in cancer as that over Score 2. Overexpression of cyclin D1 and cyclin E was noted in 21 of 84 (25.0%), and 34 of 84 (40.5%) gastric cancer tissues, respectively (Table 1). Representative results are shown in Fig. 1 and 2. Excluding few exceptional cases, the gastric epithelium with intestinal metaplasia showed similar stainability with normal gastric epithelium in anti-cyclin D and anti-cyclin E antibodies. Therefore, we did not count the immunostaining pattern of the metaplastic epithelium separately. Overexpression of cyclin E in gastric carcinoma was associated with the number of lymph node metastasis (p=0.003) (Table 2) and frequent relapse (p=0.043) (Table 3). However, we could not find correlation with sex, age, histological grade, tumor location, tumor type and TNM stage. After a median follow-up period of 38 months, there was a significant correlation between the overexpression of cyclin E and disease free survival (p=0.0378) and overall survival (p=0.0319) (Fig. 3).

Table 1. Results of immunohistochemical staining in 84 gastric cancers

Score	Cyclin D1	Cyclin E
0	39	21
1	24	29
2	8	9
3	7	19
4	6	6
Overexpression	21/84 (25.0%)	34/84 (40.5%)

Table 2. Patients characteristics in gastric cancer according to the cyclin E expression

	Cyclin E expression		
	Overexpression (n=34)	Negative (n=50)	p value
Age (years)			
< 40 (n=15)	7	8	0.590
> 40 (n=69)	27	42	
Sex			
Male (n=57)	19	38	0.053
Female (n=27)	15	12	
Histologic grade			
Well differentiated (n=2)	1	1	0.960
Moderately differentiated (n=22)	9	13	
Poorly differentiated (n=60)	24	36	
Tumor location			
Upper (n=4)	2	2	0.412
Middle (n=14)	8	6	
Lower (n=53)	18	35	
Diffuse (n=13)	6	7	
TNM stage			
I (n=4)	0	4	0.204
II (n=21)	10	11	
IIIa (n=39)	13	26	
IIIb (n=17)	9	8	
IV (n=3)	2	1	
Type			
Diffuse (n=46)	21	25	0.288
Intestinal (n=38)	13	25	
Lymph node metastasis			
Presence (n=73)	30	43	0.766
Absence (n=11)	4	7	
Mean* (m=7.45)	10.79	5.18	0.003

*Mean: mean numbers of lymph node metastasis.

While the overexpression of cyclin D1 was noted in 25.0% of gastric cancer, there was no correlation between overexpression of cyclin D1 and other clinicopathologic variables (Table 4). Also there was no significant correlation between overexpression of cyclin D1 and disease free (p=0.7406) or overall survival (p=0.8425). In our series of 84 tissues from gastric cancer, we were able to demonstrate a close correlation between cyclin D1 expression and cyclin E expression (p=0.021) as shown in Table 5. A multivariate analysis with Cox regression analysis identified two independent predictors with regard to survival. The analysis revealed that tumor stage and cyclin E expression status were independent prognostic factors (Table 6).

Table 3. Patients characteristics in gastric cancer according to the cyclin D1 expression

	Cyclin D1 expression		
	Overexpression (n=21)	Negative (n=63)	p value
Age (years)			
< 40 (n=15)	5	10	0.411
> 40 (n=69)	16	53	
Sex			
Male (n=57)	13	44	0.500
Female (n=27)	8	19	
Histologic grade			
Well differentiated (n=2)	1	1	0.457
Moderately differentiated (n=22)	7	15	
Poorly differentiated (n=60)	13	47	
Tumor location			
Upper (n=4)	0	4	0.435
Middle (n=14)	2	12	
Lower (n=53)	15	38	
Diffuse (n=13)	4	9	
TNM stage			
I (n=4)	0	4	0.616
II (n=21)	6	15	
IIIa (n=39)	10	29	
IIIb (n=17)	5	12	
IV (n=3)	0	3	
Type			
Diffuse (n=46)	9	37	0.206
Intestinal (n=38)	12	26	
Lymph node metastasis			
Presence (n=73)	20	53	0.191
Absence (n=11)	1	10	
Mean* (m=7.45)	10.14	6.56	

*Mean: mean numbers of lymph node metastasis.

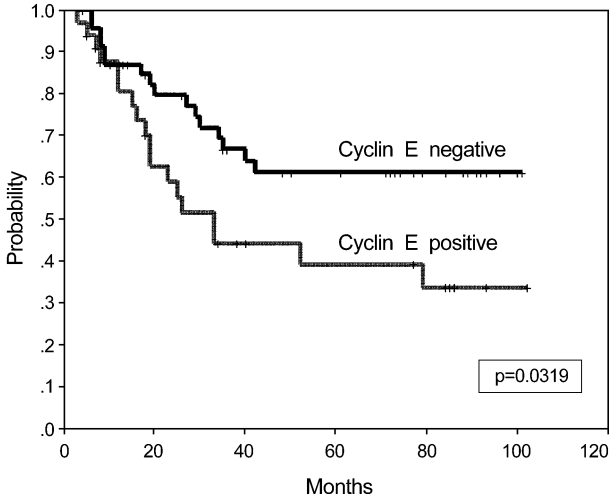

Fig. 3. Overall survival for patients with (n=34) or without (n=54) cyclin E overexpression.

Table 6. Multivariate analysis of factors predicting survival

Factors	p value	Odds ratio
Stage	0.0095	3.53
Cyclin E	0.0284	2.13

Table 4. Recurrence and death rate in gastric cancer patients with or without G1 cyclin expression

	Expression of cyclin D1		p value	Expression of cyclin E		p value
	Overexpression (%)	Negative (%)		Overexpression (%)	Negative (%)	
Recurrence	9 (42.8)	20 (31.7)	0.373	16 (47.0)	13 (26.0)	0.043*
Death	9 (42.8)	25 (39.7)	0.749	18 (52.9)	16 (32.0)	0.068

Table 5. Correlation between cyclin D1 and cyclin E expression

	Cyclin E		Total	p value
	Overexpression (%)	Negative (%)		
Cyclin D1 overexpression	13 (15.5)	8 (9.5)	21	0.021
Negative	21 (25.0)	42 (50.0)	63	
Total	34	50	84	

DISCUSSION

It has been reported that cyclin D1 or cyclin E is deregulated in many types of human tumor cells including breast, liver, esophageal, and pancreatic cancer, and the amplification and/or overexpression of these cyclins may contribute to oncogenic transformation of cells in vitro and in vivo (9-12, 15). Recently, these G1 cyclins have recently been proposed as a prognostic marker for several human cancers (15, 16). This prompted us to investigate the potential involvement of the cyclin D1 and cyclin E in human gastric cancer, which is one of the most common cancers in Korea.

In the present study, overexpression of cyclin D1 was noted in 25% of gastric cancers, compared with adjacent normal gastric mucosa suggesting that cyclin D1 protein expression also may be deregulated in gastric cancer. Although overexpression of cyclin D1 was noted in a sizeable portion of gastric cancer studied, no clear correlation could be determined with other clinicopathological findings. Sasaki et al. reported that no amplification of cyclin D1 gene was found in gastric cancer (24). Although there is a correlation between gene amplification and gene expression, this conflicting result should be elucidated from our ongoing study of cyclin D1 gene amplification.

Cyclin E overexpression was detected in 50.4% of gastric cancers by immunohistochemistry. Recently, Akama et al. reported that cyclin E gene amplification was noted in about 15% of gastric cancer and all the gastric cancers with overexpression of cyclin E also showed gene amplification (22). Sasaki et al. reported that cyclin E gene amplification was observed in 6 of 42 (15%) of gastric cancer tissues (24) and Yokozaki et al. also reported that cyclin E overexpression was noted in 7 out of 45 (15.6%) gastric carcinoma (23). Although the overexpression rate was low compared to our results, it can be assumed that overexpression of cyclin E may be a consistent finding in gastric cancer. These conflicting results in overexpression rate of cyclin E gene might be attributed to the different kind of antibody or different technique in immunohistochemistry. For cyclin E expression, in our study rabbit polyclonal IgG antibody (1:200) was used, whereas Akama et al. used mouse monoclonal antibody (1:250). Interestingly, Akama et al. also reported that cyclin E gene amplification and overexpression is often associated with well differentiated advanced gastric cancer. However, we were not able to show a significant correlation between overexpression of cyclin E and sex, age, histologic grade, tumor type, location, and stage except the number of lymph node metastasis. This conflicting result should be elucidated. Comparative studies of cyclin E expression and clinical features revealed a significant correlation between poor disease free or overall survival and overexpression of cyclin E protein in our series of 84 gastric cancer, in agreement with other reports on breast cancer (16). These findings indicate that cyclin E overexpression may be involved in abnormal cell proliferation and seems to be an early event in tumorigenesis since there was no statistical difference in the level of expression between early and advanced tumor stages. Our findings demonstrated that by multivariate analysis cyclin E expression status is an independent prognostic factor with regard to survival for patients with gastric cancer as well as those with breast carcinoma (15, 16). In our analysis, both cyclin E and cyclin D1 were overexpressed in a large number of gastric cancers, but only cyclin E was associated with poor prognosis. It is not clear that some important regulators are frequently mutated in tumors, while others are affected through other pathways. Thus, a search for a set of cell-cycle proteins that are representative of independent regulatory pathways and that also provide the most important prognostic marker in individual tumors will be warranted.

In conclusion, overexpression of cyclin D1 and cyclin E is a frequent finding in gastric cancer. Along with the observation that cyclin E overexpression was associated with poor survival rates, it can be suggested that cyclin E overexpression might be useful for prognostic measure in gastric cancer. Further studies to determine whether other cell cycle regulators are involved in deregulation of cyclin D1 and cyclin E in gastric carcinogenesis are needed.

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