Roles of cyclin-dependent kinase 8 and β-catenin in the oncogenesis and progression of gastric adenocarcinoma

MUN-YOUNG KIM[1], SONG IY HAN[2] and SUNG-CHUL LIM[1,2]

[1]Department of Pathology and [2]Research Center for Resistant Cells,
Chosun University School of Medicine, Gwangju, Republic of Korea

Abstract. Gastric adenocarcinoma is a common cause of cancer-related death. The Wnt/β-catenin pathway plays an important role in various cancers. However, relatively little is known about the regulatory mechanism of β-catenin in stomach cancer. To determine the patterns of cyclin-dependent kinase (CDK) 8 and β-catenin expression and the relationship between CDK8 and β-catenin, we conducted a study of immuno-histochemical staining of tumor tissues (12 adenomas, 24 early gastric carcinomas, 24 advanced gastric carcinomas and 21 metastatic lymph nodes), together with Western blot analysis and CDK8 interference studies using gastric cancer cell lines. Gastric adenocarcinomas with CDK8 expression had distinct clinical, prognostic and molecular attributes. CDK8 expression and the delocalization of β-catenin expression showed a significant positive correlation with carcinogenesis and tumor progression, especially lymph node metastasis. Immunohisto-chemically, CDK8 expression in gastric adenocarcinoma was independently associated with β-catenin activation (p<0.05). β-catenin expression was suppressed by CDK8 interference in the gastric adenocarcinoma cell lines, SNU-601 and SNU-638. These data support the potential link between CDK8 and β-catenin, and suggest that CDK8 detection and β-catenin delocalization could be related to a poor prognosis. Moreover, the interference of CDK8 could be a promising therapeutic modality for gastric adenocarcinoma.

Introduction

Gastric cancer is the second leading cause of death associated with cancer worldwide (1). In East Asia, particularly in Korea, it is the most prevalent cancer (2,3). Numerous studies have reported the risk factors for gastric cancer together with the clinical and pathological characteristics of gastric cancer. Nonetheless, its etiology has not yet been elucidated. Over the years, on account of the improvement of molecular pathological techniques, molecular mechanisms involved in the development of gastric cancer as well as factors pertinent to prognosis, have been reported (4,5).

The Wnt/β-catenin pathway, which is involved in the early oncogenic course of various cancers, is also involved in the development of most colon (6,7) and gastric cancers (8,9). β-catenin is the central component in the Wnt signal trans-duction system. Wnt contributes to the stabilization of β-catenin. Once stabilized, β-catenin accumulates and migrates to the nucleus. The impaired control of the Wnt/β-catenin pathway plays an important role in the initiation of colon cancer (10).

Cyclin-dependent kinase (CDK) 8 is located on chromo-some 13, it pairs with transcriptional regulators, and participates in the control of the transcription of key pathways involved in the formation of colon cancer (11). In addition, for β-catenin-dependent transcription as well as oncogenesis, the activation of CDK8 is essential (12,13). Nevertheless, as far as we know, studies on CDK8 in gastric cancer have not yet been reported. Therefore, it is inferred that the elucidation of the correlation of CDK8 to β-catenin in gastric cancer cells would not only facilitate the understanding of the development as well as the progression of gastric cancer, but would also provide clues for effective therapeutic approaches in gastric cancer.

Therefore, we evaluated the immunohistochemical expression level or pattern of CDK8 and β-catenin according to various clinicopathological factors in gastric cancer tissues, and the correlation of CDK8 to β-catenin was examined. In addition, to validate the results of immunohistochemical studies using gastric cancer tissues, molecular pathological studies using gastric cancer cell lines were also performed.

Materials and methods

Case selection and tissue sampling. Forty-eight cases of gastric adenocarcinoma and 12 cases of gastric adenoma were included in this study. All the patients with adenocarcinoma [advanced gastric carcinoma (AGC), 24 cases; early gastric carcinoma (EGC), 24 cases] underwent gastrectomy from January 2005 to December 2006 at the Chosun University Hospital (Gwangju, Korea). Informed consent was obtained from all patients, and research protocols were approved by the

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Correspondence to: Professor Sung-Chul Lim, Department of Pathology, Chosun University Hospital, 588 Seosuk-dong, Dong-gu, Gwangju, Republic of Korea
E-mail: sclim@chosun.ac.kr

Table I. Clinical, epidemiological and histopathological characteristics of 60 patients with gastric neoplasm (%).

Characteristics	AGC	EGC	Adenoma	Total
No. of patients	24 (40)	24 (40)	12 (20)	60
Age (years)				
Mean	60.1	54.7	64.2	
Range	25-77	30-71	52-86	
Gender				
Male	19 (79)	15 (63)	11 (92)	45
Female	5 (21)	9 (37)	1 (8)	15
Lymph node metastasis				
Yes	18 (75)	3 (13)		
No	6 (25)	21 (87)		
Histopathological grade				
W/D	2 (8)	6 (25)	L/G 7 (58.3)	
M/D	10 (42)	6 (25)	H/G 5 (41.7)	
P/D	12 (50)	12 (50)		
Lauren's type				
Intestinal	13 (54)	16 (67)		
Diffuse	6 (25)	6 (25)		
Mixed	5 (21)	2 (8)		
Histologic type				
Tubular			11 (92)[a]	
Villous			0	
Villotubular			1 (8)	

W/D, well differentiated; M/D, moderately differentiated; P/D, poorly differentiated; L/G, low grade; H/G, high grade. [a]A case of focal malignant transformation was included.

Ethics Committee of Chosun University Hospital. The median age of the AGC and EGC patients was 60 years (range, 25-77) and 55 years (range, 30-71), respectively. The median age of the adenoma patients was 64 years (range, 52-86) (Table I). The histological grade of the adenocarcinomas was classified according to the guidelines of the American Joint Committee on Cancer (AJCC): well-differentiated (low grade, grade 1), moderately differentiated (intermediate grade, grade 2), and poorly differentiated (high grade, grade 3) (14). Metastatic lymph nodes and their corresponding primary gastric lesions in 21 regional lymph node-positive cases (AGC, 18 cases; EGC, 3 cases) were included in this analysis.

Histopathological analysis
Microscopic examination. Each tumor was re-evaluated by retrospect analysis of the medical records and the tissue slide file of the pathology department. Age, gender, tumor size, histological subtype and the degree of differentiation, the depth of tumor invasion, the status of lymph node metastasis and distant metastasis, were assessed. The stage was defined according to the TNM staging system of the AJCC (14).

Tissues to be examined were fixed in 10% neutral formalin, and the prepared paraffin-embedded tissues were sectioned 4-5 μm in thickness, and hematoxylin and eosin staining was performed. The cells were examined under a light microscope, the representative area suitable to the study purpose was selected, and slides were prepared for immunohistochemical analysis.

Immunohistochemical staining. Briefly, the 4-μm thick sections that were obtained after formalin fixation and paraffin-embedding, were deparaffinized in xylene and were rehydrated with distilled water through a graded series of ethanol solutions. The sections were then placed in a glass jar with 10 mM citrate buffer (pH 6.0) and irradiated in a microwave oven for 15 min, and were allowed to cool down in the jar at room temperature for 20 min. The slides were then rinsed with Tris-buffered saline (TBS). Blocking reagent was added for 10 min after quenching the endogenous peroxidase activity in 0.3% hydrogen peroxide for 10 min. The slides were then washed as before, and they were subsequently subjected to the primary antibody reaction. Mouse monoclonal β-catenin (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal CDK8 (dilution 1:200; Santa Cruz Biotechnology) were applied to the tissue sections and were allowed to incubate in a moist chamber for 1 h at room temperature. After washing with TBS, Polink-2 HRP plus mouse DAB detection system (Golden Bridge International, Inc., WA, USA) and Polink-2 HRP plus rabbit DAB detection system (Golden Bridge International) were used for β-catenin and CDK8, respectively. Counterstaining was performed with Mayer's hematoxylin.

The positive control for CDK8 was mammary invasive ductal carcinoma with strong nuclear staining, as shown by another study, and for β-catenin, normal gastric mucosa adjacent tumor tissue. Instead of the primary antibody, TBS was used for the negative control.
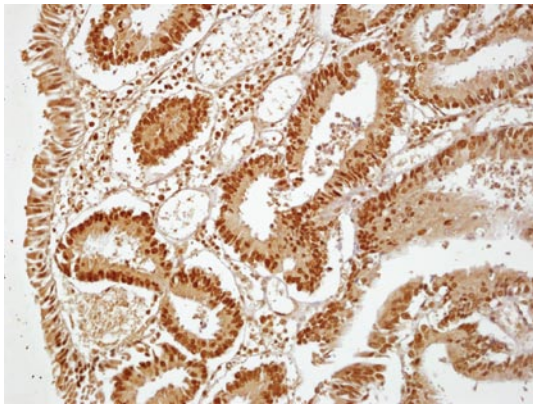
Figure 1. Gastric adenoma. Immunohistochemical staining for CDK8. Diffuse nuclear staining was identified. Polink-2 HRP plus rabbit DAB detection system, counterstained by hematoxylin.

Analysis and interpretation of the staining. In order to exclude subjectivity, a pathologist who did not know the clinical course of the subjects evaluated the staining results.

Scoring of β-catenin was based upon the distribution of β-catenin within the cell membrane (0-1), cytoplasm (0-2), and nucleus (0-2), with a total score of 0 reflecting cell membrane staining only, similar to that seen in normal colonic mucosa, up to an aggregate score of 5 for tumors with strong nuclear staining (score of 2), diffuse cytoplasmic staining (score of 2), and loss of cell membrane staining (score of 1). Total scores were then collapsed into two grades (inactive, 0-2; active, 3-5) (15).

CDK8 expression was determined by assessing the level of immunohistochemical staining in the tumor cell nuclei. Initially, the overall staining intensity was scored as none, weak, moderate or strong. Cases categorized as positive were those characterized by weak, moderate or strong staining, while cases categorized as negative were those with no nuclear staining (10).

Western blot analysis of CDK8 and β-catenin
Cell culture. The human-derived gastric cancer cell lines, SNU-1, SNU-601 and SNU-638 (Korean Cell Line Bank, Seoul, Korea), were seeded at a concentration of 1.5x10^6 cells in 10 cm Petri dishes and maintained in RPMI-1640 medium (Invitrogen) containing 10% FBS (Gibco BRL, Grand Island, NY, USA) and 1X antibiotic-antimycotic solution (Gibco BRL) at 37°C in 5% CO_2. After overnight incubation, the cells were treated with the indicated drugs at various time-points. These cell lines were established from gastric adenocarcinomas with no history of anti-cancer chemotherapy or radiotherapy.

Reagents. Polyclonal rabbit anti-human CDK8 (H-139), monoclonal anti-β-catenin (E-5) and the ECL system were purchased from Santa Cruz Biotechnology and monoclonal mouse anti-actin was purchased from Sigma (St. Louis, MO, USA). Unless specified, drugs were purchased from Gibco BRL.

Western blot analysis. Electrophoretically separated proteins were transferred onto an NC membrane, blocked in 5% skim-milk/TBST, and incubated with primary antibodies to CDK8,

β-catenin and β-actin. The membranes were then incubated with HRP-conjugated secondary antibody and visualized using the SuperSignal Chemiluminescence kit (Pierce Biotechnology), and finally the signals were acquired by an image analyzer (Image station 4000MN, Kodak).

RNA interference. CDK8 siRNA 5'-GUU UUU GCC GGU UGU CAA A(dTdT)-3'/(S) 5'-UUU GAC AAC CGG CAA AAA C(dTdT)-5' (AS) and scrambled RNA (Ctrl RNAi) sequence 5'-CCU ACG CCA CCA AUU UCG U(dTdT)-3' (S)/5'-ACG AAA UUG GUG GCG UAG G(dTdT)-3' (AS) were designed and synthesized by Bioneer (Daejeon, Korea). SNU-1 cells (10^6) were transfected with 5 μg siRNA using JET-PEI reagent (Polyplus-Transfection, France). SNU-601 and SNU-638 cells (10^6) were transfected with 5 μg siRNA using the Amaxa transfection system (Lonza, Levallois-Perret, France). Then cells were grown for 48 h prior to total protein extraction.

Statistical analysis. The StatView software package (Abacus Concepts, Berkeley, CA, USA) was used for statistical analysis. The χ^2 test, Fisher's exact test, ANOVA, and logistic regression analysis were used to determine the correlation between clinicopathological parameters and expression patterns of CDK8 and β-catenin. The log-rank test was used for the analysis of Kaplan-Meier survival curves. Statistical significance was determined at a value of p<0.05.

Results

Immunoreactivity for CDK8
i) Gastric adenoma. Among the 12 cases of adenoma, 8 cases (66.7%) were positive for CDK8 (Fig. 1), which was a statistically significant (p<0.01). It could be considered that CDK8 is involved in the development of adenoma, and considering that gastric adenocarcinoma was derived from adenoma in some cases, CDK8 could be considered to be involved in the early stages of the development of gastric adenocarcinoma. When cases shown to be positive were classified, 4 cases were weakly positive, 3 cases were moderately positive and 1 case was strongly positive. All 3 moderately positive cases were high grade adenoma cases, and the case shown to be strongly positivity was the high grade adenoma case showing focal malignant transformation. Therefore, it was found that when the adenoma grade increased, becoming more malignant, the expression of CDK8 was elevated (Table II).

ii) EGC
Negative lymph node metastasis group. Twenty cases excluding 1 case (95.2%) were CDK8-positive (Fig. 2). When positive cases were classified, 7 cases were weakly positive, 9 cases were moderately positive, and 5 cases were strongly positive (Table II).

Positive lymph node metastasis group. All 3 cases (100%) were positive for CDK8. When positive cases were classified, 1 case was moderately positive and 2 cases were strongly positive. As regards the modulation of the expression of CDK8 according to the presence or absence of lymph node metastasis in the EGC group, lymph node metastasis in this group was rare, and thus comparative evaluation was not feasible. None-

Table II. Immunoreactivity for CDK8 in gastric neoplasm and metastatic lymph node (%).

Immuno- reactivity	AGC		EGC			Meta-LN	
	LN⁻	LN⁺	LN⁻	LN⁺	Adenoma[a]	AGC	EGC
Negative	0	0	1 (4.8)	0	4 (33.3)	0	0
Positive[b]	6 (100)	18 (100)	20 (95.2)	3 (100)	8 (66.7)	18 (100)	3 (100)
Weak	2	4	7	0	4	0	0
Moderate	1	7	9	1	3	8	0
Strong	3	7	5	2	1[c]	10	3
Total	6	18	21	3	12	18	3

Meta-LN, metastatic lymph node; LN⁻, negative lymph node metastasis; LN⁺, positive lymph node metastasis. [a]Immunoreactivity was statistically significant in the adenoma group patients, $p<0.01$. [b]Immunoreactivity had a statistically significant positive correlation with lymph node metastasis in the AGC group, $p<0.001$. [c]A case of focal malignant transformation was included.
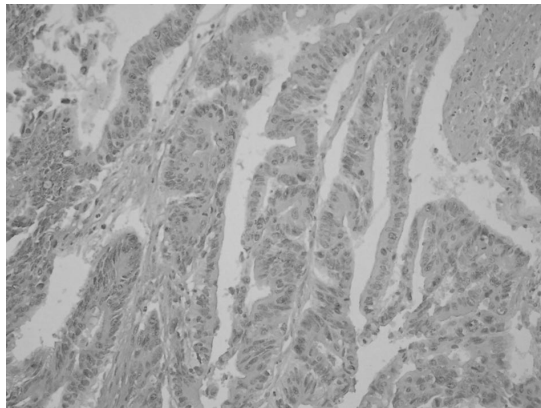

Figure 2. Early gastric adenocarcinoma. Immunohistochemical staining for CDK8. Weak nuclear staining was identified. Polink-2 HRP plus rabbit DAB detection system, counterstained by hematoxylin.

theless, in the group with lymph node metastasis, a trend in the enhancement of the intensity of CDK8 staining was shown (Table II).

iii) AGC
Negative lymph node metastasis group. All 6 cases (100%) were CDK8-positive. Classifying the positive group, 2 cases were weakly positive, 1 case was moderately positive, and 3 cases showed strong positivity (Table II).

Positive lymph node metastasis group. All 18 cases (100%) were CDK8-positive (Fig. 3). Classifying the positive group, 4 cases were weakly positive, 7 cases were moderately positive, and 7 cases showed strong positivity. As regards the modulation of the expression of CDK8 according to the presence or absence of lymph node metastasis in AGC group, in the group with lymph node metastasis, a tendency of the significant enhancement of the intensity of CDK8 staining was shown ($p<0.001$) (Table II).

iv) Metastatic lymph node
EGC. All 3 cases (100%) were CDK8 positive, and all showed strong positivity.

AGC. All 18 cases (100%) were CDK8-positive. Of the cases shown to be positive, 8 cases were moderately positive and 10 cases showed strong positivity. Concerning the modulation of the expression of CDK8 in the metastatic lymph node EGC and AGC groups, metastasis in the EGC group was rare and thus comparative analysis was not feasible. Nonetheless, it was determined that even in EGC, in cases strongly expressing CDK8, lymph node metastasis could readily occur (Table II).

Immunoreactivity for β-catenin. In normal mucosa, β-catenin was found along the cell membrane. In tumor cases, delocalization in areas other than the cell membrane was observed. Therefore, the modulation was examined primarily focusing on the intranuclear expression of β-catenin, intracytoplasmic expression, and cell membrane expression.

i) Gastric adenoma
Intranuclear expression. No intranuclear expression was observed in any of the 12 cases (0%) (Table III).

Intracytoplasmic expression. No intracytoplasmic expression was observed in any of the 12 cases (0%) (Table III).

Membranous expression. In 9 cases (75%), cell membrane expression was maintained. In 3 cases (25%), the cell membrane expression was reduced (Fig. 4). The 3 cases with the reduced cell membrane expression were high grade adenomas, including high grade adenomas showing focal malignancy (Table III).

β-catenin total score (active). β-catenin was not activated in any of the 12 cases (100%) (Table III).

ii) EGC
Negative lymph node metastasis group. a) Intranuclear expression: Intranuclear staining was observed in 1 case (5%) (Table III). b) Intracytoplasmic expression: Intracytoplasmic staining was observed in 6 cases (29%). Among them, 4 cases (19%) showed weak expression, and the other 2 cases (10%) showed moderate/strong expression (Table III). c) Membranous expression: The cell membrane expression was maintained in 10 cases (48%), and was lost in 11 cases (52%) (Table III). d) β-catenin total score (active): It was activated in 3 cases (14%) (Table III).

Positive lymph node metastasis group. a) Intranuclear expression: No intranuclear expression was observed in any of
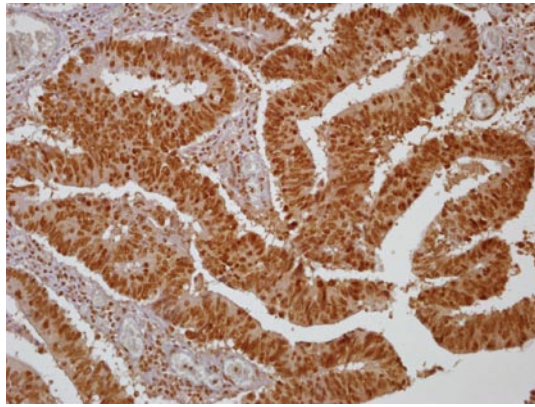
Figure 3. Advanced gastric adenocarcinoma. Immunohistochemical staining for CDK8. Diffuse strong nuclear staining was identified. Polink-2 HRP plus rabbit DAB detection system, counterstained by hematoxylin.
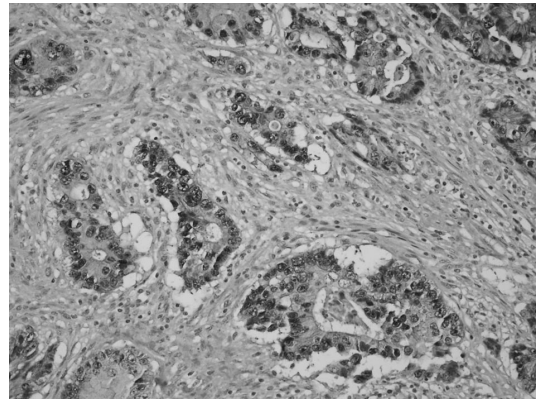

Figure 5. Advanced gastric adenocarcinoma. Immunohistochemical staining for β-catenin. Delocalization of β-catenin in the nucleus was noted. Polink-2 HRP plus mouse DAB detection system, counterstained by hematoxylin.
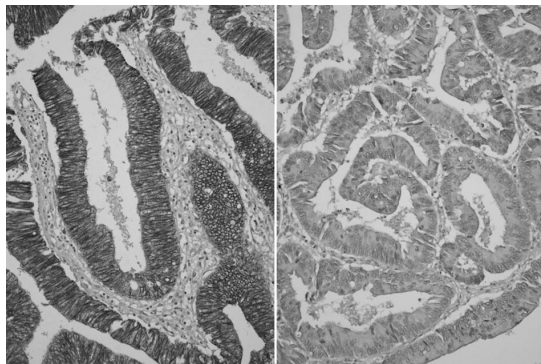

Figure 4. Gastric adenoma. Immunohistochemical staining for β-catenin. Left, Distinct membranous staining was demonstrated; right, loss of membranous staining was identified. Polink-2 HRP plus mouse DAB detection system, counterstained by hematoxylin.
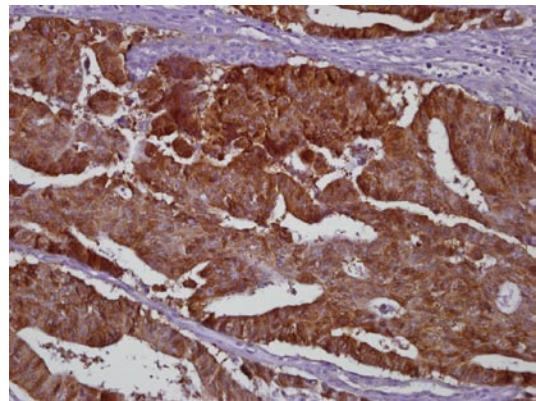

Figure 6. Advanced gastric adenocarcinoma. Immunohistochemical staining for β-catenin. Delocalization of β-catenin in the cytoplasm was noted. Polink-2 HRP plus mouse DAB detection system, counterstained by hematoxylin.

the 3 cases (0%) (Table III). When compared to the group without lymph node metastasis, due to the limited number of cases and all cases being negative, the correlation of the intranuclear expression of β-catenin to lymph node metastasis could not be evaluated. b) Intracytoplasmic expression: Weak intracytoplasmic expression was observed in 1 case (33%) (Table III). When compared to the group without lymph node metastasis, intracytoplasmic expression of β-catenin did not correlate significantly to lymph node metastasis. c) Membranous expression: The cell membrane expression was maintained in 1 case (33%), and was lost in 2 cases (67%) (Table III). When compared to the group without lymph node metastasis, the loss of the β-catenin cell membrane expression correlated significantly with lymph node metastasis (p<0.01). Particularly, in EGC, lymph node metastasis positively correlated with the β-catenin cell membrane expression. d) β-catenin total score (active): It was not activated in all 3 cases (100%) (Table III). When compared to the group without lymph node metastasis, due to the limited number of cases and the fact that it was not activated in any of the cases, the correlation of the total score (activation) of the expression of β-catenin to lymph node metastasis could not be evaluated.

iii) AGC
Negative lymph node metastasis group. a) Intranuclear expression: Weak intranuclear staining was observed in 2 cases (33%) (Fig. 5 and Table III). b) Intracytoplasmic expression: Weak intracellular staining was observed in 2 cases (33%) (Fig. 6 and Table III). c) Membranous expression: The cell membrane expression was maintained in 2 cases (33%), and was lost in 4 cases (67%) (Table III). d) β-catenin total score (active): It was activated in 2 cases (33%) (Table III).

Positive lymph node metastasis group. a) Intranuclear expression: Intranuclear staining was observed in 3 cases (17%), 2 cases showed weak expression, and 1 case showed moderate/strong expression (Table III). b) Intracytoplasmic expression: Intracytoplasmic staining was observed in 9 cases (50%). Among them, 6 cases (33%) showed weak expression and the other 3 cases (17%) showed moderate/strong expression (Table III). When compared to the group without lymph node metastasis, the intracytoplasmic expression of β-catenin correlated significantly with lymph node metastasis (p<0.05). Particularly, in AGC, lymph node metastasis positively correlated with the intracytoplasmin expression of β-catenin as well as the expression intensity. c) Membranous expression: The cell membrane expression was maintained in 5 cases

Table III. Immunoreactivity for β-catenin in gastric neoplasm and metastatic lymph node (%).

Immuno-reactivity	AGC		EGC			Meta-LN	
	LN⁻	LN⁺	LN⁻	LN⁺	Adenoma[a]	AGC	EGC
Nucleus							
-	4 (67)	15 (83)	20 (95)	3 (100)	12 (100)	14 (78)	2 (67)
1⁺	2 (33)	2 (11)	1 (5)	0	0	3 (17)	1 (33)
2⁺	0	1 (6)	0	0	0	1 (5)	0
Cytoplasm[b]							
-	4 (67)	9 (50)	15 (71)	2 (67)	12 (100)	8 (44)	2 (67)
1⁺	2 (33)	6 (33)	4 (19)	1 (33)	0	6 (33)	1 (33)
2⁺	0	3 (17)	2 (10)	0	0	4 (22)	0
Membrane[c]							
Expressed	2 (33)	5 (28)	10 (48)	1 (33)	9 (75)	3 (17)	1 (33)
Lost (1⁺)	4 (67)	13 (72)	11 (52)	2 (67)	3 (25)[a]	15 (83)	2 (67)
Overall score[d]							
0-2 (inactive)	4 (67)	12 (67)	18 (86)	3 (100)	12 (100)	10 (56)	2 (67)
3-5 (active)	2 (33)	6 (33)	3 (14)	0	0	8 (44)	1 (33)
Total	6	18	21	3	12	18	3

Meta-LN, metastatic lymph node; LN⁻, negative lymph node metastasis; LN⁺, positive lymph node metastasis. [a]A case of focal malignant transformation was included. [b]Cytoplasmic immunoreactivity had a statistically significant positive correlation with metastasis in the AGC LN⁺ and AGC Meta-LN groups, $p<0.05$. [c]Loss of membranous immunoreactivity had a statistically significant positive correlation with metastasis in the EGC LN⁺ and AGC Meta-LN groups, $p<0.01$. [d]β-catenin score was calculated as the sum of nuclear (0-2), cytoplasmic (0-2) and membrane (0-1) scores as described in Materials and methods. Overall score had a statistically significant positive correlation with metastasis in the AGC Meta-LN group, $p<0.05$.
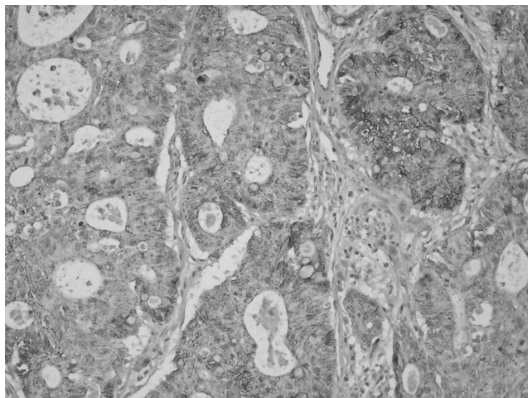

Figure 7. Advanced gastric adenocarcinoma. Immunohistochemical staining for β-catenin. Loss of membranous β-catenin expression was identified. Polink-2 HRP plus mouse DAB detection system, counterstained by hematoxylin.

(28%) and was lost in 13 cases (72%) (Fig. 7) (Table III). When compared to the group without lymph node metastasis, the loss of the β-catenin cell membrane expression correlated significantly with lymph node metastasis. d) β-catenin total score (active): β-catenin was activated in 6 cases (33%) (Table III). When compared to the group without lymph node metastasis, the total score (activation) of the expression of β-catenin correlated significantly with lymph node metastasis.

iv) Metastatic lymph node
EGC. a) Intranuclear expression: Weak intranuclear staining was observed in 1 case (33%) (Table III). b) Intracytoplasmic expression: Weak expression was shown in 1 case (33%). c) Membranous expression: The cell membrane expression was maintained in 1 case (33%), and was lost in 2 cases (67%). d) β-catenin total score (active): β-catenin was activated in 1 case (33 %) (Table III).

AGC. a) Intranuclear expression: Intranuclear staining was observed in 4 cases (22%), 3 cases showed weak expression, and 1 case showed moderate/strong expression (Table III). b) Intracytoplasmic expression: Intracytoplasmic staining was observed in 10 cases (55%). Among them, 6 cases (33%) showed weak expression, and the other 4 cases (22%) showed moderate/strong expression (Table III). When compared to the EGC group, in the AGC group, the intracytoplasmic expression of β-catenin correlated with lymph nodes metastasis ($p<0.05$). Particularly, it could be predicted that in AGC, if strong intracytoplasmic expression of β-catenin is observed, lymph node metastasis could readily occur (Table III). c) Membranous expression: The cell membrane expression was maintained in 3 cases (17%), and was lost in 15 cases (83%) (Table III). When compared to the EGC group, the AGC group significantly correlated with the loss of the β-catenin cell membrane expression ($p<0.01$). d) β-catenin total score (active): β-catenin was activated in 8 cases (44%). When compared to the EGC group, the AGC group correlated significantly with the total score of the expression of β-catenin (active) ($p<0.05$) (Table III).

Table IV. Relation between the immunoreactivity of β-catenin and CDK8 in EGC (%).

β-catenin	CDK8			
	Weak (n=7)	Moderate (n=10)	Strong (n=7)	P-value
Nucleus				
- (n=23)	7 (100)	10 (100)	6 (86)	
+ (n=1)	0	0	1 (14)	
Cytoplasm				
- (n=17)	7 (100)	6 (60)	4 (57)	
+ (n=7)	0	4 (40)	3 (43)	
Membrane				<0.001[a]
Expressed (n=11)	6 (86)	4 (40)	1 (14)	
Lost (n=13)	1 (14)	6 (60)	6 (86)	

[a]Loss of membranous staining of β-catenin and CDK8 stainability revealed a statistically significant positive correlation in the EGC group, p<0.001.

Table V. Relation between the immunoreactivity of β-catenin and CDK8 in AGC (%).

β-catenin	CDK8			
	Weak (n=7)	Moderate (n=10)	Strong (n=7)	P-value
Nucleus				<0.05[a]
- (n=19)	6 (100)	6 (75)	7 (70)	
+ (n=5)	0	2 (25)	3 (30)	
Cytoplasm				<0.005[b]
- (n=13)	5 (83)	6 (75)	2 (20)	
+ (n=11)	1 (17)	2 (25)	8 (80)	
Membrane				<0.001[c]
Expressed (n=7)	6 (100)	1 (13)	0	
Lost (n=17)	0	7 (87)	10 (100)	

[a]β-catenin delocalization in the nucleus and CDK8 stainability revealed a statistically significant positive correlation in the AGC group, p<0.05. [b]β-catenin delocalization in the cytoplasm and CDK8 stainability revealed a statistically significant positive correlation in the AGC group, p<0.005. [c]Loss of membranous staining of ß-catenin and CDK8 stainability revealed a statistically significant positive correlation in the AGC group, p<0.001.

Relation of immunohistochemical expression of β-catenin and CDK8
EGC. The expression pattern of β-catenin according to the expression level of CDK8 was examined. It was observed that it did not statistically significantly correlate with the intra-nuclear and intracytoplasmic staining of β-catenin. Nonetheless, as the level of the expression of CDK8 became enhanced, a tendency of the delocalization of β-catenin (intranuclear staining, intracytoplasmic staining) was shown. However, the loss of the β-catenin cell membrane expression according to the expression level of CDK8 was statistically significant (p<0.001), and thus, as the expression level of CDK8 in early

Table VI. Relation between the immunoreactivity of β-catenin and CDK8 in the metastatic lymph node (%).

β-catenin	CDK8			
	Weak (n=7)	Moderate (n=10)	Strong (n=7)	P-value
Nucleus				<0.05[a]
- (n=16)	0	7 (88)	9 (69)	
+ (n=5)	0	1 (12)	4 (31)	
Cytoplasm				<0.01[b]
- (n=10)	0	8 (100)	2 (15)	
+ (n=11)	0	0	11 (85)	
Membrane				<0.005[c]
Expressed (n=4)	0	4 (50)	0	
Lost (n=17)	0	4 (50)	13 (100)	

[a]β-catenin delocalization in the nucleus and CDK8 stainability revealed a statistically significant positive correlation in the metastatic lymph node, p<0.05. [b]β-catenin delocalization in the cytoplasm and CDK8 stainability revealed a statistically significant positive correlation in the metastatic lymph node, p<0.01. [c]Loss of membranous staining of β-catenin and CDK8 stainability revealed a statistically significant positive correlation in the metastatic lymph node, p<0.005.

gastric adenocarcinoma cells was increased, the loss of the β-catenin cell membrane expression was shown (Table IV).

AGC. The expression pattern of β-catenin according to the expression level of CDK8 was examined. It was observed that it correlated statistically significantly not only with the intra-nuclear and intracytoplasmic staining of β-catenin, but also with the loss of the cell membrane expression (p<0.05, p<0.005 and p<0.001, respectively). Particularly, as the expression level of CDK8 was increased, we not only observed intranuclear staining of β-catenin and intracytoplasmic staining, but also the loss of the cell membrane expression in advanced gastric adenocarcinoma cells (Table V).

Metastatic lymph node. The expression pattern of β-catenin according to the expression level of CDK8 was examined. It correlated statistically significantly not only with the intra-nuclear staining and intracytoplasmic staining of β-catenin but also with the loss of the cell membrane expression (p<0.05, p<0.01 and p<0.005, respectively). Particularly, as the expression level of CDK8 increased, we not only observed the intranuclear as well as the intracytoplasmic staining of tumor cells within the metastatic lymph nodes, but also the loss of the cell membrane expression (Table VI).

Gastric cancer cell line study
Western blot analysis. a) Expression of CDK8: Gastric cancer cell lines were cultured and CDK8 was measured. It was expressed strongly in the SNU-638 cell line. Nonetheless, it was weakly expressed in the SNU-1 and SNU-601 cell lines (Fig. 8). Similar to the results of immunohistochemical staining, this suggests that CDK8 is involved in gastric adenocarcinoma. b) Expression of β-catenin: Gastric cancer cell lines were cultured and their expression of β-catenin was measured. It was strongly expressed in the SNU-638 cell line. Nevertheless, the expression of β-catenin in SNU-1 and SNU-601 was very
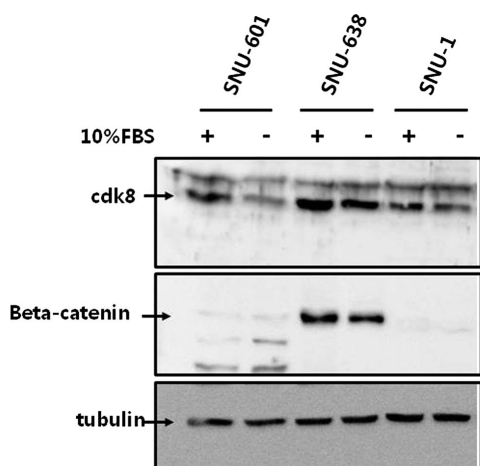
Figure 8. Gastric cancer cell lines. Western blot analysis for CDK8 and β-catenin. Each cell line expressed CDK8 and β-catenin. After serum starvation for 24 h, the CDK8 level was decreased in each cell line.

Figure 9. Gastric cancer cell lines. Effect of CDK8 interference on β-catenin expression. Significant suppression of β-catenin was identified in each cell line.

weak in comparison to SNU-638 (Fig. 8). As shown by the results of immunohistochemical staining, it could be considered that β-catenin may be expressed in association with CDK8. c) Experiments in the absence of serum: To clarify the relationship of CDK8 with β-catenin, gastric cancer cell lines were cultured in the absence of 10% FBS for 24 h. CDK8 was slightly decreased in the SNU-1, SNU-601 and SNU-638 cell lines in the absence of serum factors. Nonetheless, β-catenin was expressed weakly regardless of the presence or absence of serum factors (Fig. 8).

Expression of β-catenin after treatment of CDK8 siRNA. The interference of CDK8 was induced, and the expression of β-catenin in the gastric cancer cell lines, SNU-638 and SNU-601, was examined. It was found that the expression of β-catenin was decreased, and it was noticeable in the SNU-638 cells. Nonetheless, in these cases, it was not completely suppressed by the inteference of CDK8. In addition, in the SNU-1 cases, the expression of β-catenin was very weak even in the control group, and thus the effect of the interference of CDK8 could not be evaluated appropriately (Fig. 9).

Discussion

Tumors are induced by the dysregulation of the Wnt signal transduction system, and they are caused by β-catenin, adenomatous polyposis coli, glycogen synthase kinase 3β, axin, mutation of *Wnt* gene, etc. Among them, a type of multifunctional plasmosin, β-catenin is not only a critical regulator but also a major component of the cadherin complex (16-18). In normal cells, β-catenin contributes to cellular adhesion by binding to E-cadherin (19-21). When the Wnt signal is activated, the phosphorylation and degradation of β-catenin are suppressed. β-catenin accumulates in the cytoplasm, ultimately migrates to the nucleus, binds to the transcription factor, and contributes to cellular proliferation (22).

It has been reported that CDK8 activation is essential for the β-catenin-dependent oncogenesis by which adenocarcinoma is developed in the colon mucosa (12,13). Nevertheless, as far
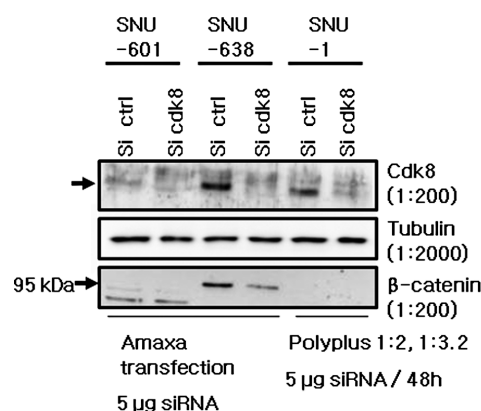
as we know, such studies on the stomach have not yet been conducted. However, through preclinical studies, it has been reported that CDK suppressors induce apoptosis, accelerate cell differentiation, suppress angiogenesis, and control transcription (23). In clinical trials using combination therapy with conventional chemotherapeutic agents, CDK suppressors have shown positive effects have been reported (24-26). Based on this, the understanding of the role of CDK8 and β-catenin in the oncogenesis, progression and metastasis of human gastric cancer as well as their correlation, not only provides a broad understanding of the etiology of gastric cancer, but may also provide important clues for effective prevention as well as therapeutic methods.

According to our results, CDK8 is expressed significantly in gastric adenoma. This expression was significantly enhanced in the gastric adenocarcinoma group including EGC, and thus it was speculated that CDK8 is involved in the early stages of the development of gastric adenocarcinoma. In addition, the expression of CDK8 showed a positive correlation with the presence or absence of lymph node metastasis, which shows that it is also involved in the progression, and particularly, in the metastasis of gastric adenocarcinoma.

However, the delocalization of β-catenin correlated significantly with adenocarcinoma compared to adenoma, and with the presence of lymph node metastasis in adenocarcinoma compared to the absence of lymph node metastasis. Particularly, intracytoplasmic staining correlated with lymph node metastasis in AGC and with the metastatic lymph node of AGC. The loss of cell membrane expression correlated significantly with lymph node metastasis in EGC and with metastatic lesions of AGC. Therefore, it is evident that the delocalization of β-catenin, particularly intracytoplasmic expression as well as the loss of cell membrane expression, are involved in the early oncogenesis and progression of gastric adenocarcinoma, particularly metastasis.

Examining the correlation of CDK8 to β-catenin, in EGC, the intensity of CDK8 expression and the loss of β-catenin cell membrane expression showed a statistically significant correlation. In AGC and metastatic lymph nodes, not only the staining intensity of CDK8 as well as the intranuclear or intracytoplasmic staining, but also the level of cell membrane staining showed a statistically significant positive correlation

with β-catenin. This shows that in the progression of adeno-carcinoma, particularly in metastasis, CDK8 and β-catenin are deeply involved, and that the delocalization of β-catenin plays an important role. In addition, it also shows that with the enhancement of the expression of CDK8, the delocalization of β-catenin may be induced. These findings are in agreement with the reports that the components of CDK8 module MED12 and MED13 act as a regulator of the activation of β-catenin (10,27,28) and that CDK8 suppresses E2F1 that suppresses β-catenin and thus enhances β-catenin indirectly (13). Therefore, based on the results from immunohistochemical analysis, studies examining the feasibility of the control of the WNT/β-catenin signaling pathway through CDK8 are required.

In experiments using gastric adenocarcinoma cell lines performed to confirm the correlation of CDK8 to β-catenin, all gastric cell lines expressed CDK8, and β-catenin showed CDK8-dependent expression. Thus it was strongly expressed in SNU-638 that also expressed CDK8 strongly. In Western blot analysis performed after omitting serum factors, CDK8 was decreased when serum factors were excluded, and β-catenin was also decreased in association, which shows that the expression of β-catenin is influenced mainly by CDK8. In addition, the interference of CDK8 was induced by the use of siRNA and the modulation of the expression of β-catenin was analyzed, and it was shown to be noticeably decreased. Such results support the claim of Firestein et al (12) that only CDK8 could play the role of post-translational modulator of β-catenin in colon cancer. It has also been reported that it would be justified to revise the strong suggestion of the possibility of the treatment of colon cancer by the regulation of CDK8 based on the claim of Firestein et al (12) as a theory limited to specific types of colon cancer (29). According to our results similar to colon cancer, it was found that the possibility of the treatment of gastric adenocarcinoma through the regulation of CDK8 is a theory applicable to specific types of gastric adeno-carcinoma.

Based on our study, it was found that CDK8 and β-catenin are deeply involved in the oncogenesis, tumor progression and metastasis of gastric adenocarcinoma. The expression of CDK8 closely correlates with the expression of β-catenin, and the expression of β-catenin is controlled by CDK8. Our results show that CDK8 and β-catenin should be considered as etiological factors of gastric adenocarcinoma, and that they could be applied not only as prognostic factors but also as possible candidates in the therapeutic approach of gastric adenocarcinoma by the control of β-catenin through CDK8.

Acknowledgements

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