

# The Activated Notch1 Signal Pathway Is Associated with Gastric Cancer Progression through Cyclooxygenase-2

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## Abstract

**Gastric carcinoma is one of the most common cancers and lethal malignancies worldwide. Thus far, the regulatory mechanisms of its aggressiveness are still poorly understood. To understand the pathogenesis and to develop new therapeutic strategies, it is essential to dissect the molecular mechanisms that regulate progression of gastric cancer. Herein, we sought to address whether Notch1 signal pathway is involved in the control of progression in gastric cancer. We found that expression of Notch ligand Jagged1 was correlated with aggressiveness of human gastric cancer. Patients with Jagged1 expression in gastric cancer tissues had a poor survival rate compared with those without Jagged1 expression. The Notch1 receptor intracellular domain (NIIC), the activated form of Notch1 receptor, promoted the colony-forming ability and xenografted tumor growth of human stomach adenocarcinoma SC-M1 cells. Migration and invasion abilities of SC-M1 cells were enhanced by NIIC. Furthermore, NIIC and C promoter-binding factor 1 (CBF1) bound to cyclooxygenase-2 (COX-2) promoter and elevated COX-2 expression in SC-M1 cells through a CBF1-dependent manner. The colony-forming, migration, and invasion abilities enhanced by NIIC were suppressed in SC-M1 cells after treatment with the COX-2 inhibitor NS-398 or knockdown of COX-2. These cellular processes inhibited by Notch1 knockdown were restored by prostaglandin E<sub>2</sub> or exogenous COX-2. Taken together, these results suggest that activation of Notch1 signal pathway promotes progression of gastric cancer, at least in part through COX-2. [Cancer Res 2009;69(12):5039–48]**

## Introduction

Notch signal pathway is involved in several cellular processes, such as proliferation, differentiation, apoptosis, cell fate decision, and maintenance of stem cells (1–3). It also plays an important role in the control of tumorigenesis (1–3). The activated Notch receptor may exhibit as an oncogene or tumor suppressor to modulate tumorigenesis. At present, the regulatory mechanisms of Notch signal pathway to activate or suppress tumorigenesis remain unclear.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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In the canonical Notch signal pathway, Notch receptor is activated and cleaved to release and translocate its intracellular domain into the nucleus after ligand binding. The cleaved Notch receptor intracellular domain regulates its downstream target genes via both C promoter-binding factor 1 (CBF1; also called RBP-Jκ)-dependent and -independent pathways (3).

Gastric carcinoma is one of the most common cancers and prevalent malignancies to cause death from cancer in the world (4, 5). It is evident that the risk factors of human gastric cancer include diet, *Helicobacter pylori* infection, and genetic alterations (6–8). For the curative treatment of gastric cancer without distant metastasis, it is potentially curable by the surgical resection of its primary tumor and control of lymph node metastasis (6). However, gastric cancer with distant metastasis is still incurable now.

The aggressiveness of this disease may be caused by activation of oncogenes, inactivation of tumor suppressor genes, and deregulation of growth factors and their receptors (9, 10). The activation of oncogenes, such as c-met, β-catenin, K-ras, and c-erbB2, and inhibition of tumor suppressor genes, such as p53, APC, E-cadherin, RUNX3, hMLH1, and p16, had been shown in human gastric cancer (6, 8, 11). Thus far, the regulatory mechanisms of aggressiveness in gastric cancer are not yet fully understood.

The elevated expression of cyclooxygenase-2 (COX-2) was found in gastric cancer (12–15). The mRNA expression of COX-2 in gastric carcinoma tissue is also associated with depth of invasion (16). Additionally, COX-2 overexpression is correlated with lymphatic vessel invasion, lymph node metastasis, and poor prognosis of human gastric carcinoma (17, 18). Inhibition of COX-2 expression has a promising effect in the prevention and treatment of gastric cancer (19–24).

Recently, it was found that Notch receptors (Notch1–Notch3) and Notch ligand Jagged1 are expressed in human gastric cancer (25–27). Notch signal pathway is also activated after the chronic infection of *H. pylori* in gastric cancer (28). Furthermore, the activation of Wnt pathway was also shown to promote gastric cancer progression (29). This β-catenin-mediated Wnt signaling is repressed by Notch1 receptor in keratinocytes (30). Therefore, we sought to address whether Notch signal pathway is involved in the control of progression in human gastric cancer. We also delineated the regulatory mechanisms of Notch1 receptor intracellular domain (NIIC), the activated form of Notch1 receptor, in progression of gastric cancer.

## Materials and Methods

**Surgical samples.** Human gastric adenocarcinoma tissues were obtained from gastric cancer patients who underwent gastric resection at the Department of Surgery, Taipei Veterans General Hospital. Informed

consent was obtained from all patients. None of the patients had undergone chemotherapy or radiotherapy before surgery. Tissue blocks were fixed overnight at 4°C with 4% neutrally buffered paraformaldehyde solution, dehydrated, cleared with Histo-Clear II (National Diagnostics), and then embedded in wax. Sections were used for H&E staining and immunostaining.

**Immunostaining of Notch1 receptor, Jagged1, and COX-2.** Notch1 receptor, Jagged1, and COX-2 in gastric cancer tissues were localized with the avidin-biotin-peroxidase technique (20). Tissue sections were incubated with goat anti-human Notch-1 COOH terminus antibody (Santa Cruz) at 1:20 dilution, goat anti-human Jagged1 antibody (Santa Cruz) at 1:50 dilution, or rabbit anti-COX-2 antibody (Cayman Chemical) at 1:100 dilution at 4°C overnight. Dilutions should be prepared using an antibody diluent (Dako Cytomation), and then biotinylated universal IgG was applied for 15 min, followed by streptavidin peroxidase conjugation for 15 min and substrate chromogen for 10 min, and then counterstained with hematoxylin for 5 min. Preimmune rabbit or mouse IgGs were used as the negative control.

The distribution of Jagged1, Notch1 receptor, and COX-2 in tissue specimens was evaluated by a semiquantitative system to calculate the percentage of positive neoplastic cells and estimated within the following arbitrary ranges: -, no positive cells; +, 1% to 25%; ++, 26% to 75%; +++, >75%.

**Plasmids and plasmid construction.** The expression construct of pcDNA-HA-NIIC contains cDNA, encoding the intracellular domain of human Notch1 receptor with an HA tag at the NH<sub>2</sub> terminus (31). To knock-down Notch1 receptor and c-Myc, the target sequences were constructed in small interfering RNA (siRNA) vectors pLKO.1 and pSilencer 3.1-H1 neo, respectively (32). For COX-1 and COX-2 knockdowns, the following target sequences were constructed in vector pLKO.1: COX-1, 5'-CGCAA-GAGGTTTGGCATGAAA-3' and COX-2, 5'-GCTGAATTTAACACCTCTAT-3' (#1) and 5'-CCATCTCCTTGAAGGACTT-3' (#5). A siRNA vector against luciferase (pLKO.1-shLuc) was used as a negative control for knock-down validation. Reporter plasmid pCOX-2-Luc (-1334/-1) containing human COX-2 promoter in front of the luciferase gene in pGL3 basic vector (33). Expression construct pcDNA-COX-2 contains a 1.9-kb cDNA fragment of human COX-2. The expression construct pSG5Flag-RBP-VP16 expresses a constitutively active RBP-VP16 fusion protein.

**Cell culture and transfection.** Human stomach adenocarcinoma SC-M1 cells (34) and erythroleukemia K562 cells were cultured in RPMI 1640 with 10% fetal bovine serum. For the establishment of stable SC-M1 cells expressing HA-NIIC fusion protein (SC-M1/HA-NIIC), SC-M1 cells were transfected with pcDNA-HA-NIIC expression plasmid and then screened by Western blot analysis using both anti-HA (Santa Cruz) and anti-Notch1 COOH terminus antibodies (31). For the control, the linearized pcDNA3-HA plasmid was also electroporated into SC-M1 cells to establish the stable cells SC-M1/pcDNA3. The COS-7 cells expressing the secreted form of human Jagged1 (COS-7/Jagged1<sup>ext</sup>) and their control cells (COS-7/pcDNA3.1-myc-His) were previously established (35).

SC-M1 and K562 cells were transiently transfected by electroporation and SuperFect transfection reagent (Qiagen), respectively. For luciferase reporter assay, K562 cells ( $5 \times 10^5$ ) were seeded onto six-well plates and transfected for 2 d. Luciferase activities were measured and normalized (31, 36). For colony-forming assay, SC-M1 or SC-M1/pcDNA3 cells were seeded after 2 d of transfection.

To suppress the endogenous Notch signal pathway or COX-2 activity, 50  $\mu$ mol/L DAPT [*N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester] or NS-398 (Sigma-Aldrich) in DMSO were added, respectively. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>; Sigma-Aldrich) in ethanol was used at 2  $\mu$ g/mL.

**Western blot analysis.** Whole-cell lysates were prepared, as described previously (31), and Western blot analysis was performed with anti-HA, anti-Notch1 COOH terminus, anti-c-Myc, anti-cyclin D1, anti-cyclin D3, anti-p21, anti-COX-1 (Santa Cruz), anti-COX-2, anti-cleaved Notch1 (Cell Signaling), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (Biogenesis).

**Colony-forming assay.** The 4,000 SC-M1; 8,000 SC-M1/pcDNA3; or 8,000 SC-M1/HA-NIIC cells were used for assay of anchorage-independent growth in soft agar. After incubating at 37°C for 14 d, cells were stained

and the colonies were counted under the microscope from 10 random fields (32, 36).

**Tumorigenicity assay in nude mice.** Five-week-old BALB/c nu/nu mice were purchased from National Science Council Animal Center and allowed free access to food and water. All animal experiments were carried out with the approval of the ethical committee. By s.c. injection into both hind limbs, nude mice were inoculated with  $3 \times 10^6$  viable SC-M1/HA-NIIC or SC-M1/pcDNA3 cells in a total volume of 0.1 mL of PBS. Tumor volume was estimated as previously described (35).

**Migration and invasion assays.** Abilities of migration and invasion were examined in 24-well plates by Millicell tissue culture plate well inserts (Millipore) and BD BioCoat Matrigel invasion chambers (Becton Dickinson), respectively. Briefly, cells ( $1 \times 10^4$ ) in 200  $\mu$ L of serum-free medium were seeded onto the upper chamber (8- $\mu$ m pore size), and the lower chamber contained 900  $\mu$ L of complete medium for 12 h of migration assay. For 20 h of invasion assay, cells ( $5 \times 10^4$ ) in 500  $\mu$ L of serum-free medium were seeded onto the upper chamber (8- $\mu$ m pore size) and the lower chamber contained 750  $\mu$ L of complete medium. After incubation, cells on the upper surface of the membrane were removed with a cotton swab. Then cells that migrated or invaded to the lower surface of the membrane were fixed with methanol and stained with 0.005% crystal violet in PBS for 1 h. Numbers of migrated or invaded cells were counted under the microscope from 10 random fields.

**Real-time PCR analysis.** Total RNA was isolated and used to synthesize cDNA, as described previously (32). The 305-bp cDNA of human COX-2 was amplified with primers 5'-TTCAAATGAGATTGTGGGAAAAT-3' and 5'-AGATCATCTCTGCCTGAGTATCTT-3'. The 176-bp cDNA of internal control GAPDH was amplified with primers 5'-AAATCCCATCAC-CATCTTCC-3' and 5'-TCACACCATGACGAACA-3'. Quantitative real-time PCR was performed, and the relative quantification of mRNA expression level was normalized (32). All data are representative of the mean values and SDs from three independent experiments.

**Chromatin immunoprecipitation assay.** Chromatin immunoprecipitation (ChIP) assay was performed to amplify DNA fragments in chromosomal DNAs using protein A-Sepharose-bound anti-Notch1 COOH terminus and anti-CBF1 (Chemicon) antibodies (32). The specific primers 5'-GCTTACGCAATTTTTTAAGG-3' and 5'-CCTGACGCTCACTG-CAAGTCG-3' for PCR amplification were used to amplify the 166-bp DNA fragment of COX-2 promoter. The specific primers 5'-GTGAGTTTCT-CATCTAGG-3' and 5'-CGTCTGAACCACATACC-3' for PCR amplification were used to amplify the 231-bp DNA fragment of COX-1 promoter.

**Statistical analysis.** Data were analyzed by  $\chi^2$  test in the analysis of various clinicopathologic factors. Survival rate was calculated by the Kaplan-Meier method. The significance of the survival differences was assessed using Cox's proportion hazard regression model to measure the independent contribution of each variable to overall survival. The difference was considered to be significant when *P* value was <0.05.

## Results

**Clinical relevance of Notch1 receptor, Jagged1, and COX-2 expressions in gastric cancer tissues.** To study whether Notch1 signal pathway is involved in the progression of human gastric cancer, immunohistochemical stains of Notch1 receptor and Jagged1 were performed on gastric cancer tissues to examine their clinical relevance. Seventy-two of 96 gastric cancer patients (75%) expressed Jagged1 in cancer tissues. These patients were associated with advanced cancers, especially Borrmann types III and IV cancers (Table 1; Supplementary Table S1), and consequently had poor survival rate (Fig. 1A). These results suggest that Jagged1 expression is correlated with the progression of gastric cancer. Furthermore, 57 of 90 gastric cancer patients (63.3%) expressed Notch1 receptor in cancer tissues (Supplementary Table S2). These patients had no specific clinicopathologic relevance.

It was shown that COX-2 expression is involved in the regulation of growth and metastasis of human gastric cancer (16–18).

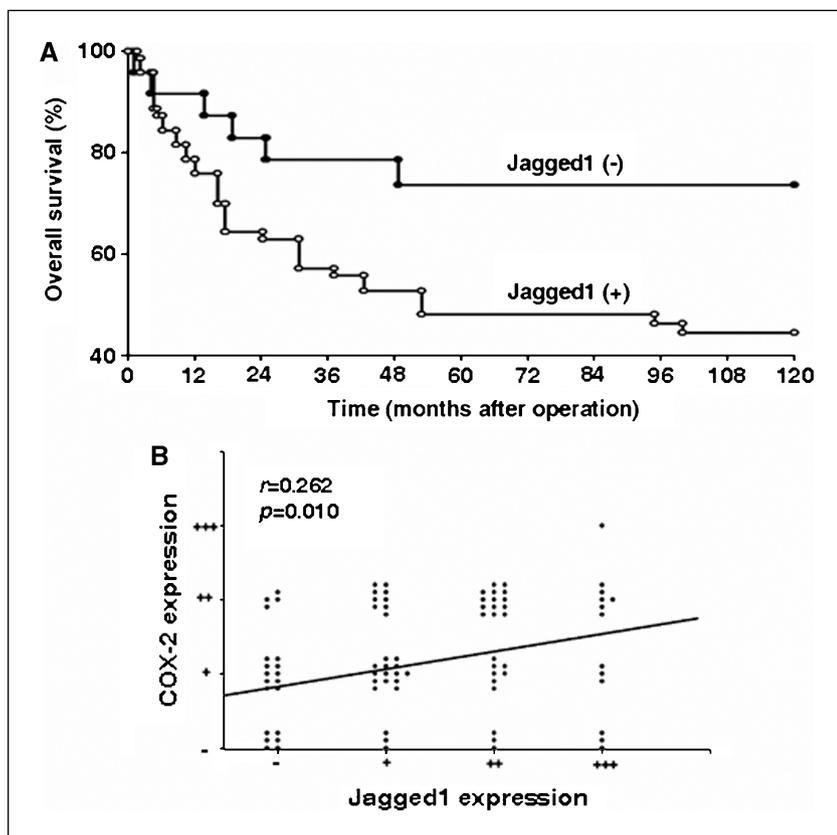
**Table 1.** Clinicopathologic features in gastric cancer patients with and without Jagged1 expression

	Jagged1 expression		Univariate analysis, $P$ ( $\chi^2$ )	Multivariate analysis, $P$
	Negative ( $n = 24$ )	Positive ( $n = 72$ )		
Age (y)				
<65	9	30		
>65	15	42	0.719	0.963
Sex				
Male	17	59		
Female	7	13	0.246	0.517
Tumor size				
<4 cm	11	18		
4-8 cm	9	39		
>8 cm	4	15	0.172	
Site of tumor				
Upper stomach	4	6		
Middle stomach	6	13		
Lower stomach	14	44	0.176	
Whole stomach	0	9		
Cell grade				
Poor differentiated	16	41		
Moderate and well differentiated	8	31	0.401	
Borrmann type				
Superficial type	15	10		
I + II type	2	15		
III + IV type	7	47	<0.001	<0.001*
Stromal reaction				
Medullary type	15	15		
Intermediate type	3	34		
Schirrhous type	6	23	<0.001	0.427
Ming's histologic classification				
Expanding type	6	26		
Infiltrative type	18	46	0.317	
Lauren's histologic class				
Intestinal type	12	39		
Diffuse type	12	33	0.723	
Lymph node metastasis				
Negative	16	37		
Positive	8	35	0.192	
Lymphovascular invasion				
Negative	12	8		
Positive	12	64	<0.001	0.289
Peritoneal dissemination				
Negative	22	62		
Positive	2	10	0.725	
Depth of cancer invasion				
Mucosa, submucosa	13	9		
Propria muscle, subserosa	3	14		
Serosa (infiltration) exposed	8	49	<0.001	0.555
Tumor-node-metastasis stage				
I	16	16		
II	1	22		
III	3	15		
IV	4	19	0.001	0.367

\* $P < 0.05$ , statistical significance.

Therefore, statistical analysis of COX-2 expression was compared with Jagged1 expression in cancer tissues. Results showed that Jagged1 expression was correlated with COX-2 expression in gastric cancer tissues (Fig. 1B).

**The expressions of Notch target genes are elevated by NIIC in SC-M1 human gastric cancer cells.** To further investigate the role of Notch1 signal pathway in the control of progression in gastric cancer, the NIIC-expressing gastric cancer cells were established



**Figure 1.** Gastric cancer patients without Jagged1 expression had better survival rate than those with Jagged1 expression. *A*, overall survival rates of gastric cancer patients with ( $n = 72$ ) or without ( $n = 24$ ) Jagged1 expressions were evaluated.  $P = 0.035$  compared with those without Jagged1 expression. *B*, statistical analysis of COX-2 expression was compared with Jagged1 expression in cancer tissues.  $P = 0.010$ .

to constitutively activate Notch1 signaling. Owing to >95% of malignancies of stomach are adenocarcinomas; the human stomach adenocarcinoma SC-M1 cells were used to establish NIIC-expressing cells (SC-M1/HA-NIIC #4, #7, and #12 cells) in the present study. Compared with SC-M1/pcDNA3 control cells, the HA-NIIC fusion protein was detected in SC-M1/HA-NIIC cells by Western blot analysis using anti-Notch1 COOH terminus and anti-HA antibodies (Fig. 2A, left; Supplementary Fig. S1).

To check whether Notch signal pathway is activated in SC-M1/HA-NIIC cells, expressions of its downstream target genes were assessed, including c-Myc, cyclin D1, cyclin D3, and p21. Western blot analysis was performed to analyze expressions of these genes in SC-M1/HA-NIIC cells. The data showed that the exogenous NIIC induced expressions of targets of Notch signal pathway in SC-M1/HA-NIIC cells (Supplementary Fig. S1).

**The activation of Notch1 signaling promotes tumor growth of SC-M1 cells.** To study the role of Notch1 signaling in the progression of SC-M1 cells, colony-forming assay was performed in soft agar. As shown in Fig. 2A (right), colony-forming ability of SC-M1 cells was increased by NIIC expression. The colony area was enlarged in SC-M1/HA-NIIC cells compared with control cells (Supplementary Fig. S2A).

To check whether the endogenous Notch signaling is also involved in the control of progression of SC-M1 cells, colony-forming ability was determined after treatment with the inhibitor of Notch signaling or secreted form of Notch ligand and knockdown of Notch1 receptor by siRNA method. After treatment with 50  $\mu\text{mol/L}$  DAPT, the colony-forming ability of SC-M1 cells was decreased (Fig. 2B, left). Furthermore, transient transfection with siRNA vector against Notch1 receptor could knockdown the en-

dogenous Notch1 receptor of SC-M1 cells compared with siRNA vector against luciferase (Fig. 2B, right). After Notch1 knockdown, colony-forming ability of SC-M1 cells was also lowered (Fig. 2B, middle). Additionally, colony-forming ability of SC-M1/pcDNA3 control cells was evaluated after treatment with conditioned media of the single cell-derived stable COS-7 cells constitutively expressing the secreted form of Notch ligand Jagged1 (COS-7/Jagged1<sup>ext</sup>) to activate the endogenous Notch signal pathway (35). Colony-forming ability of SC-M1/pcDNA3 cells was promoted in the presence of the secreted form of Jagged1 (Supplementary Fig. S2B).

We further investigated whether NIIC also enhances tumor growth in the case of s.c. implanted NIIC-expressing gastric cancer cells in nude mice. Tumor sizes of SC-M1/HA-NIIC cells were augmented to 7.5-fold on day 27 after tumor inoculation (Fig. 2C).

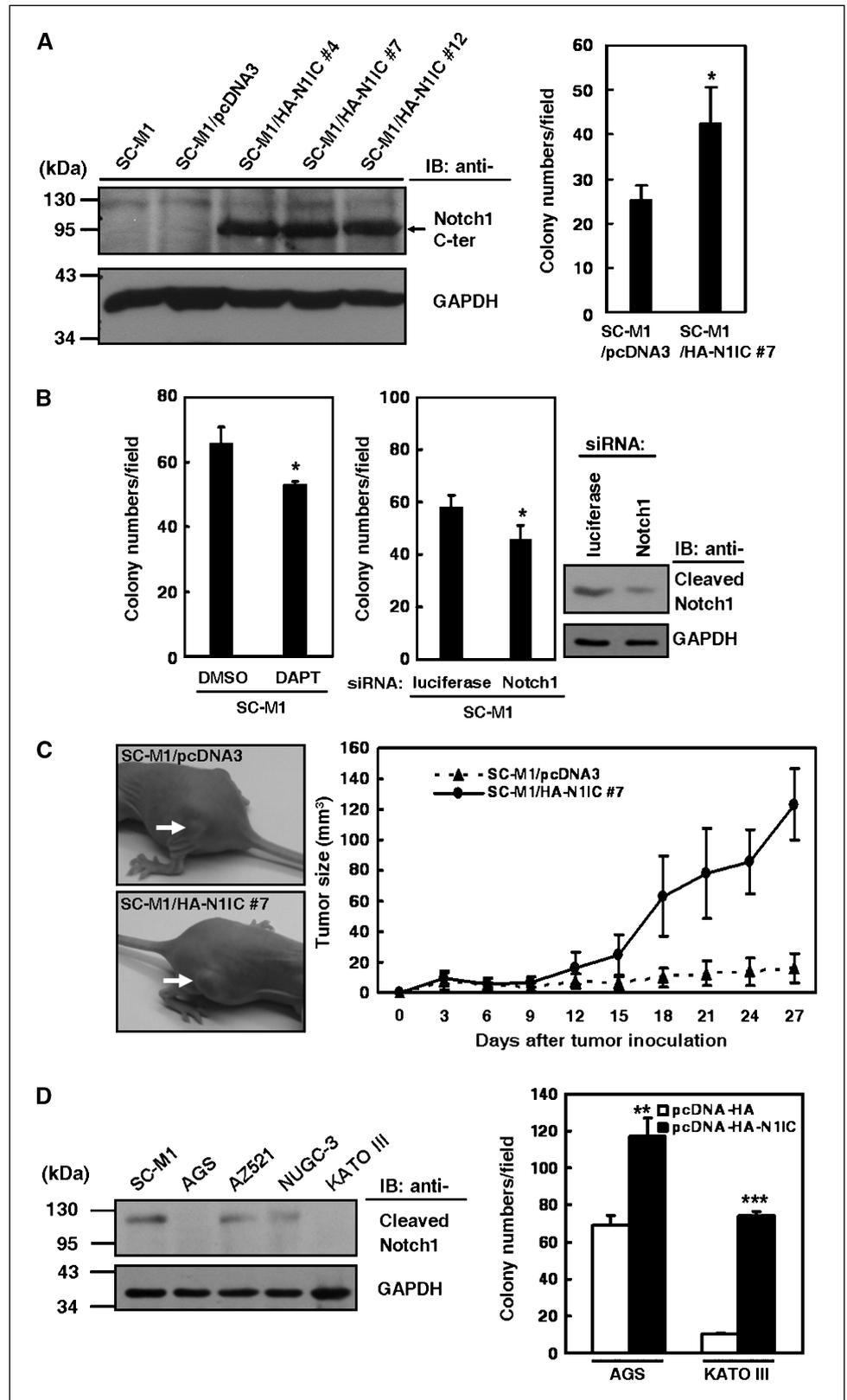
To identify gastric cancer cells with or without activated Notch1 signaling, Western blot analysis was performed using anti-cleaved Notch1 antibody. These results showed that Notch1 signaling was activated in SC-M1, AZ521, and NUGC-3 cells with the detectable cleaved Notch1 receptor (Fig. 2D, left). Whereas, it was not significantly activated in AGS and KATO III cells that negatively or weakly expressed the cleaved Notch1 receptor. Additionally, transfection with NIIC expression construct enhanced colony formation ability in AGS and KATO III cells (Fig. 2D, right).

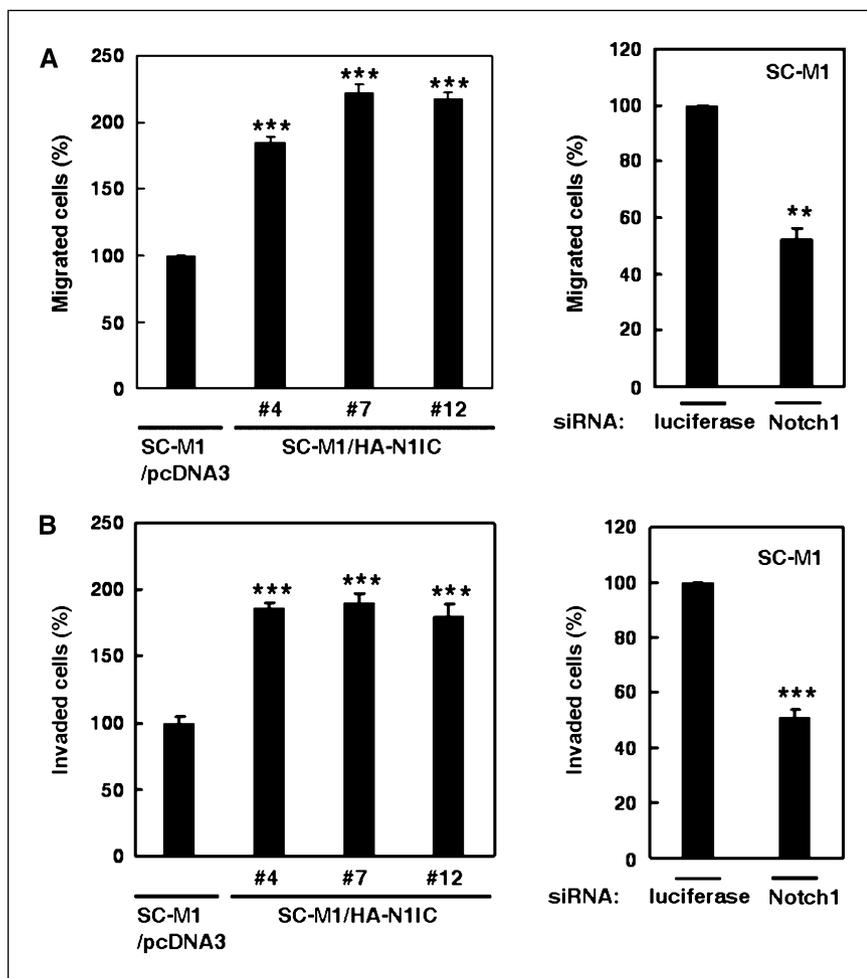
**The activation of Notch1 signaling enhances migration and invasion abilities of SC-M1 cells.** To check whether NIIC is involved in metastasis of gastric cancer, abilities of migration and invasion were evaluated in SC-M1 cells. As shown in Fig. 3A and B (left), migration and invasion abilities of SC-M1 cells were increased by NIIC. Furthermore, Notch1 knockdown suppressed migration and invasion abilities of SC-M1 cells (Fig. 3A and B, right).

After transfection with NIIC expression construct, migration and invasion abilities were enhanced in AGS and KATO III cells that negatively or weakly expressed the cleaved Notch1 receptor (Supplementary Fig. S3).

As described above, the exogenous NIIC induced expressions of targets of Notch1 signaling in SC-M1 cells, such as c-Myc (Supplementary Fig. S1). We also evaluated whether c-Myc is involved in Notch1-mediated tumor progression. The NIIC-enhanced abilities

**Figure 2.** NIIC promotes tumor growth of SC-M1 cells. **A**, whole-cell extracts prepared from SC-M1 cells, HA-N1IC fusion protein-expressing SC-M1/HA-N1IC cells (#4, #7, and #12), and SC-M1/pcDNA3 control cells were used for Western blot analysis (left). The SC-M1/HA-N1IC #7 cells were seeded for colony-forming assay (right). **B**, SC-M1 cells were treated with 50  $\mu\text{mol/L}$  DAPT (left) and transfected with siRNA vectors against Notch1 receptor or Luciferase (middle) for colony-forming assay. Whole-cell extracts were also analyzed by Western blot analysis after transfection with siRNA vectors in SC-M1 cells (right). **C**, the viable HA-N1IC fusion protein-expressing SC-M1/HA-N1IC #7 cells were s.c. inoculated into nude mice. Tumor sizes were measured at the time indicated. Data are from six nude mice and from a representative experiment that was performed thrice with identical results. \*,  $P < 0.05$  compared with control cells, vehicle, or mock. **D**, whole-cell extracts of SC-M1, AGS, AZ521, NUGC-3, and KATO III cells were used for Western blot analysis (left). Abilities of colony formation were evaluated in AGS and KATO III cells after transfection with NIIC-expressing construct (pcDNA-HA-N1IC) or control vector (right). \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  compared with mock.





**Figure 3.** N1IC enhances migration and invasion abilities of SC-M1 cells. *A*, SC-M1/HA-N1IC cells (*left*) and SC-M1 cells transfected with siRNA vectors against Notch1 receptor (*right*) were seeded and incubated to evaluate migration ability. *B*, the SC-M1/HA-N1IC cells (*left*) and SC-M1 cells transfected with siRNA vectors against Notch1 receptor (*right*) were seeded and incubated to evaluate invasion ability. Columns, mean of three independent experiments. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  compared with control cells or mock.

of colony formation, migration, and invasion were attenuated after transfection with siRNA vector against c-Myc in SC-M1 cells (Supplementary Fig. S4).

**N1IC binds to COX-2 promoter and elevates COX-2 expression in SC-M1 cells through a CBF1-dependent pathway.** We further studied whether N1IC modulates tumor progression of gastric cancer through induction of COX-2. Western blot analysis was performed to analyze COX-2 expression in SC-M1/HA-N1IC cells. As shown in Fig. 4*A* (*left*), the exogenous N1IC induced expression of COX-2, but not COX-1, in SC-M1 cells. COX-2 expression was inhibited after treatment with DAPT or transfection with siRNA vector against Notch1 receptor (Supplementary Fig. S5*A*). The mRNA expression of COX-2 was also elevated by N1IC in SC-M1 cells (Fig. 4*A*, *right*).

Additionally, reporter gene assay was performed to check whether N1IC can activate COX-2 expression through enhancing COX-2 promoter activity. Because of the low transfection efficiency of SC-M1 cells, K562 cells were used for this study. After transfection with reporter plasmid containing human COX-2 promoter [pCOX-2-Luc (-1334/-1)], N1IC expression activated reporter gene activity (Fig. 4*B*). Moreover, COX-2 promoter activity was also evaluated after coculture with Jagged1-expressing COS-7/Jagged1<sup>ext</sup> cells (Supplementary Fig. S5*B*). The results showed that COX-2 promoter activity was enhanced by induction of endogenous Notch signaling.

To further investigate whether activation of COX-2 promoter by Notch signal pathway is CBF1-dependent or CBF1-independent, pCOX-2-Luc (-1334/-1) reporter plasmid was cotransfected with various amounts of expression construct of the constitutively active RBP-J $\kappa$ -VP16 fusion protein (pSG5Flag-RBP-VP16) for reporter gene assay in K562 cells. Activity of reporter gene containing COX-2 promoter was significantly induced by RBP-J $\kappa$ -VP16 fusion protein in a dose-dependent manner (Fig. 4*C*). These results suggest that COX-2 expression is activated by Notch signal pathway through a CBF1-dependent pathway.

We surmised that N1IC and CBF1 might bind to the DNA of COX-2 promoter to modulate reporter gene activity in the context of living cells. To delineate this possibility, we examined DNA-binding ability of these proteins on COX-2 promoter by ChIP assay using anti-IgG, anti-Notch1 COOH terminus, and anti-CBF1 antibodies in SC-M1/HA-N1IC cells. The immunoprecipitated DNA was used to amplify PCR products of COX-2 promoter, COX-1 promoter, and promoter of Hes-1, a target gene of CBF1-dependent Notch signal pathway. The amplified PCR products of COX-2 promoter were present in the samples immunoprecipitated with anti-Notch1 COOH terminus and anti-CBF1 antibodies, but not with anti-IgG antibody (Fig. 4*D*, *left*). The amplified PCR products of COX-1 promoter were not present in those immunoprecipitated with anti-Notch1 COOH terminus, anti-CBF1, and anti-IgG antibodies. Furthermore, the amplified PCR

products of Hes-1 promoter were also present in those immunoprecipitated with anti-Notch1 COOH terminus and anti-CBF1 antibodies, but not with anti-IgG antibody. Percentage of the immunoprecipitated COX-2 promoter fragments was also quantified by real-time PCR (Fig. 4D, right). These results suggest that N1IC and CBF1 bind to COX-2 and Hes-1 promoters in chromosomal DNA of SC-M1/HA-N1IC cells.

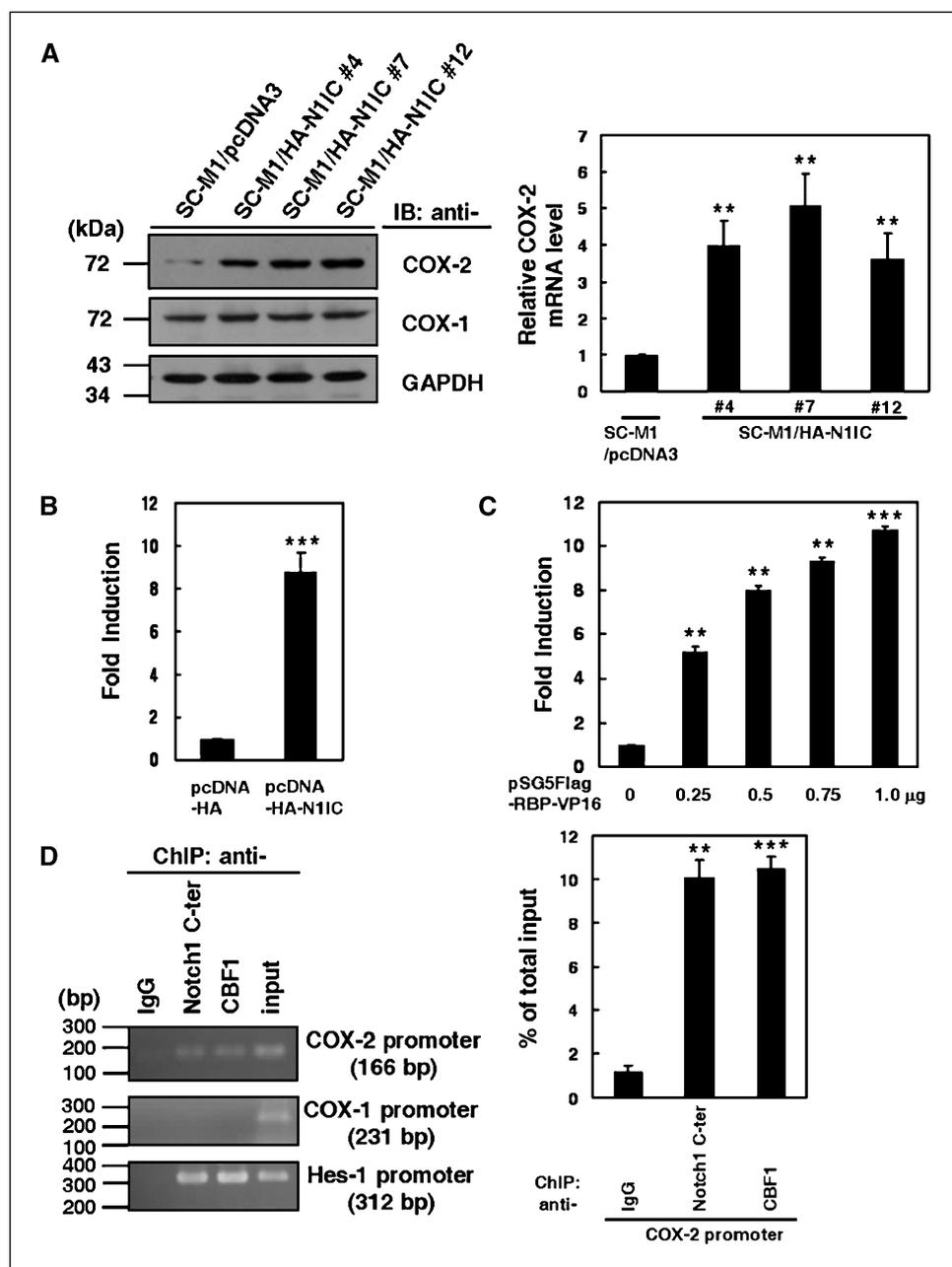
**COX-2 is involved in tumor progression enhanced by N1IC in SC-M1 cells.** To address whether the tumor progression promoted by N1IC is through COX-2 in gastric cancer, SC-M1/HA-N1IC cells were treated with COX-2 inhibitor NS-398 to block COX-2 activity. As compared with control cells, the increment of colony-forming ability in SC-M1/HA-N1IC cells was suppressed through a dose-dependent manner after treatment with NS-398 (Fig. 5A, left). Additionally, the migration and invasion abilities en-

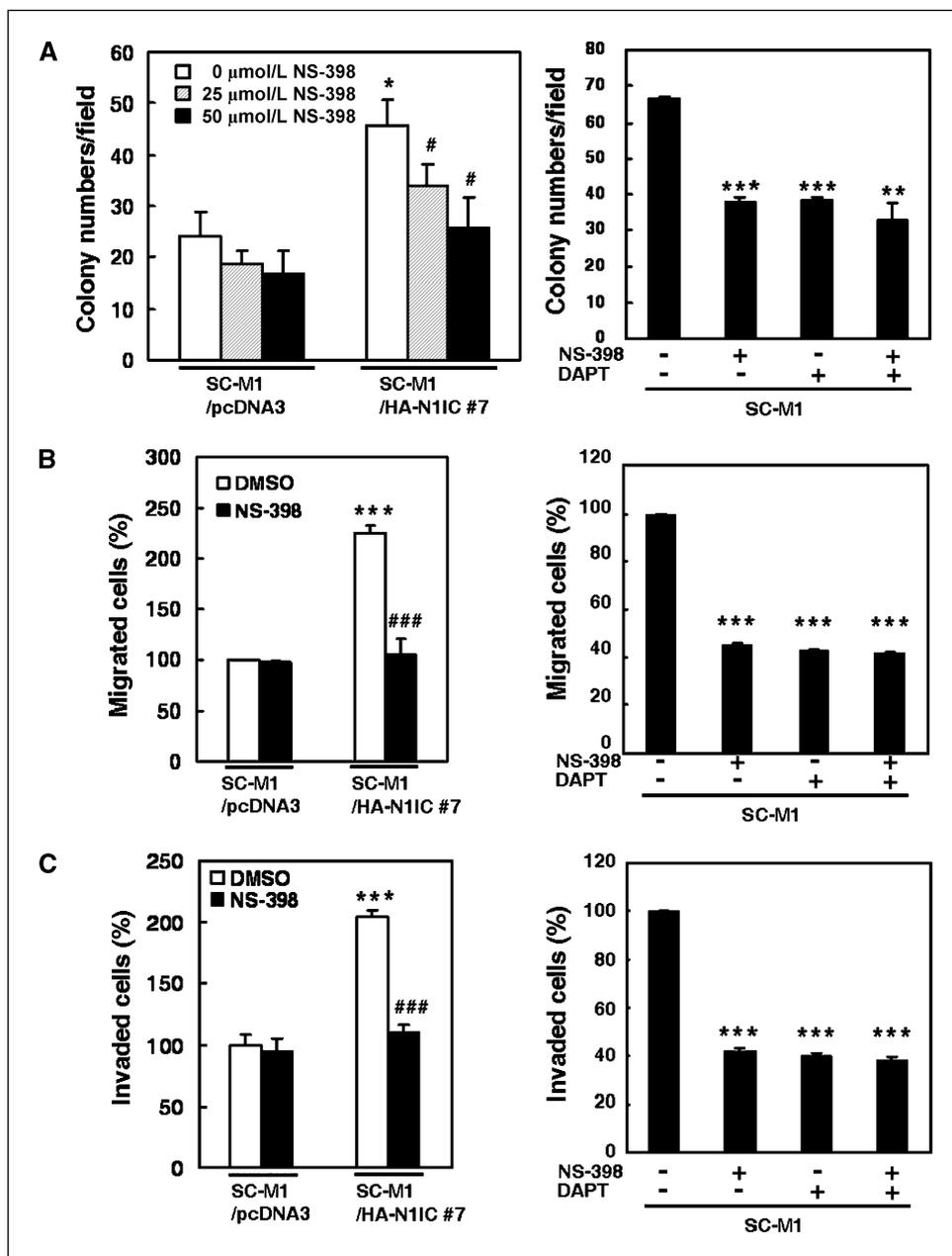
hanced by N1IC in SC-M1 cells were suppressed after treatment with NS-398 (Fig. 5B and C, left).

To further study the relationship between COX-2 activity and Notch1 signaling in the control of tumor progression in SC-M1 cells, we treated with NS-398 and DAPT to block COX-2 activity and Notch signal pathway, respectively. Treatment with NS-398 or DAPT alone reduced the colony-forming, migration, and invasion abilities of SC-M1 cells (Fig. 5A–C, right). However, treatment with NS-398 in combination with DAPT did not further block these biological functions of SC-M1 cells to a higher extent than treatment with NS-398 or DAPT alone.

As described above, inhibition of Notch signaling reduced COX-2 expression (Supplementary Fig. S5A). The suppressed abilities of colony formation, migration, and invasion by Notch1 knockdown were reversed after treatment with PGE<sub>2</sub> or transfection with COX-2

**Figure 4.** N1IC elevates COX-2 expression in SC-M1 cells through a CBF1-dependent pathway. *A*, whole-cell extracts prepared from SC-M1/HA-N1IC cells were used for Western blot analysis (left). The COX-2 transcript levels in SC-M1/HA-N1IC cells were measured by quantitative real-time PCR (right). *B*, reporter plasmid containing full-length COX-2 promoter was cotransfected with N1IC-expressing construct (*pcDNA-HA-N1IC*) or its control vector (*pcDNA-HA*) into K562 cells for reporter gene assay. *C*, K562 cells were cotransfected with reporter plasmid containing full-length COX-2 promoter and various amounts of constitutively active RBP-Jk mutant-expressing plasmid (pSG5Flag-RBP-VP16) for reporter gene assay. Columns, mean from at least three independent experiments. *D*, SC-M1/HA-N1IC #7 cells were harvested for the ChIP assay (left). The percentages of immunoprecipitated DNAs were also quantified by real-time PCR and normalized to total input DNA (right). \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  compared with control cells or mock.





**Figure 5.** COX-2 is involved in tumor progression enhanced by N1IC in SC-M1 cells. *A*, SC-M1/HA-N1IC #7 cells were treated with various concentrations of NS-398 (*left*). SC-M1 cells were treated with 50  $\mu\text{mol/L}$  NS-398 or 50  $\mu\text{mol/L}$  DAPT (*right*). The treated cells were seeded for colony-forming assay. *B* and *C*, SC-M1/HA-N1IC #7 cells were treated with 50  $\mu\text{mol/L}$  NS-398 (*left*) and SC-M1 cells were treated with 50  $\mu\text{mol/L}$  NS-398 or 50  $\mu\text{mol/L}$  DAPT (*right*) to evaluate migration (*B*) or invasion (*C*) abilities. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  compared with vehicle in SC-M1 or SC-M1/pcDNA3 control cells. #,  $P < 0.05$ ; ###,  $P < 0.001$  compared with vehicle in SC-M1/HA-N1IC #7 cells.

expression construct (Supplementary Fig. S6 and S7). The enhanced abilities of colony formation, migration, and invasion by Notch1 overexpression were attenuated after transfection with siRNA vectors against COX-2 (Supplementary Fig. S8). These data suggest that Notch signal pathway modulates gastric cancer progression through a COX-2-dependent manner.

## Discussion

The role of Notch signal pathway in gastric cancer is very complicated, and its regulatory mechanism remains elusive at present. We show herein that Notch1 signaling contributes to tumor progression of human gastric cancer through induction of COX-2 expression. Furthermore, N1IC binds to COX-2 promoter and elicits the enhancement of COX-2 promoter activity through

a CBF1-dependent pathway. To our knowledge, this is the first report regarding the linkage of Notch signal pathway and COX-2 expression in regulation of gastric cancer progression.

In this study, we noted that Notch1 receptor and Jagged1 were expressed in gastric cancer tissues. Furthermore, we found that patients with Jagged1 expression in cancer tissues had more advanced cancer, especially Borrmann types III and IV cancers (Table 1), and consequently had poor survival than those without Jagged1 expression (Fig. 1A). These results suggest that Notch1 signal pathway plays an important role in gastric cancer progression.

COX-2 expression is an independent prognostic factor of gastric cancer (17). There are several signal pathways converged onto COX-2 promoter (37). These signalings regulate COX-2 expression through an independent or synergistic manner. Besides, the  $\beta$ -catenin-mediated Wnt signal pathway promotes gastric cancer

progression (29) and cross-talks with Notch and COX-2 pathways (30, 38). Nuclear factor- $\kappa$ B also mediates COX-2 expression to regulate cell proliferation of human gastric cancer cells (39) and interplays with Notch signaling (40).

Recently, activation of Notch signal pathway was found to participate in epithelial-mesenchymal transition (EMT) in development and tumorigenesis. For example, Notch signaling up-regulates Snail-1 expression and elevates EMT in cardiac development, kidney tubular cell differentiation, and hypoxia (41–43). The Jagged1-activated Notch signaling also promotes EMT through E-cadherin repressed by Slug (44). Jagged1 and Hey1, a target gene of Notch signal pathway, are also involved in mediating transforming growth factor- $\beta$ -induced EMT (42). Notch3 activation up-regulates N-cadherin expression, but not vimentin, in HEK293 cells (45).

In addition to COX-2, Twist was also shown to regulate cell motility and invasion in gastric cancer cell lines, probably through N-cadherin and fibronectin (46). Possibly, these EMT mediators induced by NIC could up-regulate COX-2 expression. Whether NIC induces expression of COX-2 to modulate metastasis in gastric cancer through EMT mediators remains unknown. Further studies are needed to unravel the COX-2 expression regulated by Notch signaling.

NS-398 treatment was used to block COX-2 activity in this study (Fig. 5). Notably, NS-398 may cause side effects in cell growth and development through COX-2-independent pathway (47, 48). Herein, we had confirmed the effect of Notch1 signaling on gastric cancer progression through COX-2-dependent manner by PGE<sub>2</sub> treatment, COX-2 overexpression, and COX-2 knockdown (Supplementary Figs. S6, S7, and S8).

It had been documented that genetic or pharmacologic manipulation of Notch signal pathway may have therapeutic potential for

the treatment of this signaling-associated diseases (for a review, see ref. 49). For example,  $\gamma$ -secretase inhibitors may act as a new target-based therapy for those diseases through inhibiting Notch signaling. Although there are side effects of  $\gamma$ -secretase inhibitors for treatment of cancers, including thymus atrophy and intestinal goblet cell hyperplasia, it was suggested that they are promising anticancer drugs and will be applied for treatment with gastric cancer and colorectal cancer (50). COX-2 inhibitors also have a promising role in the prevention and treatment of gastric cancer despite of their substantial gastrointestinal toxicity and increased cardiovascular risk (21–23). Therefore, COX-2 inhibitors in combination with  $\gamma$ -secretase inhibitors in lower doses may be a new strategy for treatment with gastric cancer in the foreseeable future.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## The Activated Notch1 Signal Pathway Is Associated with Gastric Cancer Progression through Cyclooxygenase-2

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