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 (N1IC), 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 N1IC. Furthermore, N1IC 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 N1IC 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 inhibitor NS-398 or knockdown of COX-2. These cellular processes inhibited by Notch1 knockdown were restored by prostaglandin E2 or exogenous COX-2.

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.

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-Js)–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 (N1IC), 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 resection at the Department of Surgery, Taipei Veterans General Hospital. Informed consent was obtained from each patient. The study was approved by the institutional review board of Taipei Veterans General Hospital.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).
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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 in 4% 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 Notch1 COOH terminus antibody (Santa Cruz) at I:20 dilution, goat anti-human Jagged1 antibody (Santa Cruz) at I:50 dilution, or rabbit anti-COX-2 antibody (Cayman Chemical) at I: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-N1IC contains cDNA, encoding the intracellular domain of human Notch1 receptor with an HA tag at the NH2 terminus (31). To knockdown 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-GAGGTTGCGCATGAAA-3′ and COX-2, 5′-GCTGATTTACACCTCCTAT-3′ (#1) and 5′-CATCTCCTCTGAGAAAGACTT-3′ (#5). A siRNA vector against luciferase (pLKO.1-siLuc) was used as a negative control for knockdown validation. Reporter plasmid pCDN-OX2-Luc (−133/−1) containing human COX-2 promoter in front of the luciferase gene in pGPl3 basic vector (33). Expression construct pcDNA-COX-2 contains a 1.9-kb cDNA fragment of human COX-2. The expression construct pG53Flag-RBP-VIP16 expresses a constitutively active COX-2-VIP16 fusion protein.

**Cell culture and transfection.** Human stomach adenocarcinoma M1-SC (34) and erythroblleukemia K562 cells were cultured in RPMI 1640 with 10% fetal bovine serum. For the establishment of stable SC-M1 cells expressing HA-N1IC fusion protein (SC-M1/HA-N1IC), SC-M1 cells were transfected with pCDNA-HA-N1IC 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/Jagged1ext) and their control cells (COS-7/pcDNA3.1) were previously established (35). For colony-forming assay, SC-M1 or SC-M1/pcDNA3 cells were seeded after growth in soft agar. After incubating at 37°C for 14 d, cells were stained with 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′-TCAATGAGTTGGAGAATTAT-3′ and 5′-AGATCATCTCCTGCTGAGATCTT-3′. The 176-bp cDNA of internal control GAPDH was amplified with primers 5′-AAATCCCATCACATCCTTCC-3′ and 5′-TCAACGCTGACCA-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′-GCTTACGCAATTTTTTTAAGG-3′ and 5′-CTGACGTCACCATGCAGTC-3′ were used to amplify the 166-bp DNA fragment of COX-2 promoter. The specific primers 5′-GGTATTTCATCATCAGT-3′ and 5′-GGTTGACCAACCACATAC-3′ for PCR amplification were used to amplify the 231-bp DNA fragment of COX-1 promoter.

**Statistical analysis.** Data were analyzed by χ2 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).
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 N1IC 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 N1IC-expressing gastric cancer cells were established.
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 N1IC-expressing cells (SC-M1/HA-N1IC #4, #7, and #12 cells) in the present study. Compared with SC-M1/pcDNA3 control cells, the HA-N1IC fusion protein was detected in SC-M1/HA-N1IC 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-N1IC 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-N1IC cells. The data showed that the exogenous N1IC induced expressions of targets of Notch signal pathway in SC-M1/HA-N1IC 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 N1IC expression. The colony area was enlarged in SC-M1/HA-N1IC cells compared with control cells (Supplementary Fig. S1).

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 μmol/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 endogenous 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/Jagged1ext) 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 N1IC also enhances tumor growth in the case of s.c. implanted N1IC-expressing gastric cancer cells in nude mice. Tumor sizes of SC-M1/HA-N1IC cells were augmented to 7.5-fold on day 27 after tumor inoculation (Fig. 2C).

The activation of Notch1 signaling enhances migration and invasion abilities of SC-M1 cells. To check whether N1IC 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 N1IC. Furthermore, Notch1 knockdown suppressed migration and invasion abilities of SC-M1 cells (Fig. 3A and B, right).

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.
After transfection with N1IC 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 N1IC 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 N1IC-enhanced abilities...
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. 4A (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. S5A). The mRNA expression of COX-2 was also elevated by N1IC in SC-M1 cells (Fig. 4A, 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. 4B). Moreover, COX-2 promoter activity was also evaluated after coculture with Jagged1-expressing COS-7/Jagged1 cells (Supplementary Fig. S5B). 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-Jκ-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-Jκ-VP16 fusion protein in a dose-dependent manner (Fig. 4C). 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. 4D, 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 enhanced 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 PGE2 or transfection with COX-2

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**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-Jκ mutant-expressing plasmid (pSG5Flag-RBP-VP16) for reporter gene assay. **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 < 0.001 compared with control cells or mock.
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 β-catenin–mediated Wnt signal pathway promotes gastric cancer
progression (29) and cross-talks with Notch and COX-2 pathways (30, 38). Nuclear factor-κB also mediates COX-2 expression to regulate cell proliferation of human gastric cancer cells (39) and interacts 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-β-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 N1H could up-regulate COX-2 expression. Whether N1C 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 signaling. Although there are side effects of COX-2 inhibitors, 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 γ-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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