Activation of the Notch1/STAT3/Twist signaling axis promotes gastric cancer progression

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Gastric carcinoma is one of the most common malignancies and a lethal cancer in the world. Notch signaling and transcription factors STAT3 (signal transducer and activator of transcription 3) and Twist regulate tumor development and are critical regulators of gastric cancer progression. Herein, the relationship among Notch, STAT3 and Twist pathways in the control of gastric cancer progression was studied. We found that Twist and phosphorylated STAT3 levels were promoted by the activated Notch1 receptor in human stomach adenocarcinoma SC-M1, embryonic kidney HEK293 and erythroleukemia K562 cells. Notch1 signaling dramatically induced Twist promoter activity through a C promoter binding factor-1-independent manner and STAT3 phosphorylation. Overexpression of Notch1 receptor intracellular domain (N1IC) enhanced the interaction between nuclear STAT3 and Twist promoter in cells. Gastric cancer progression of SC-M1 cells was promoted by N1IC through STAT3 phosphorylation and Twist expression including colony formation, migration and invasion. STAT3 regulated gastric cancer progression of SC-M1 cells via Twist. N1IC also elevated the progression of other gastric cancer cells such as AGS and KATO III cells through STAT3 and Twist. The N1IC-promoted tumor growth and lung metastasis of SC-M1 cells in mice were suppressed by the STAT3 inhibitor JSI-124 and Twist knockdown. Furthermore, Notch1 and Notch ligand Jagged1 expressions were significantly associated with phosphorylated STAT3 and Twist levels in gastric cancer tissues of patients. Taken together, these results suggest that Notch1/STAT3/Twist signaling axis is involved in progression of human gastric cancer and modulation of this cascade has potential for the targeted combination therapy.

Introduction

Gastric carcinoma is one of the most common malignancies and the second most lethal cancer worldwide (for reviews, see refs 1,2). A growing body of evidence indicates that diet, Helicobacter pylori infection, and specific genetic alterations are risk factors of human gastric cancer (1,2). Pathogenesis of gastric cancer is complex and associated with activation of oncogenes, inactivation of tumor suppressor genes and dysregulation of cellular signal pathways (1,2). The underlying molecular mechanisms responsible for aggressiveness of gastric cancer have not yet been clearly elucidated.

The activated Notch receptors may act as oncogenes or tumor suppressors to modulate tumorigenesis depending on cellular context and cross talk with other pathways (for a review, see ref. 6). In mammals, four Notch receptors (Notch1–4) and five Notch receptor ligands (Deltalike 1, 3, and 4 and Jagged1 and 2) have been identified (6). After binding with ligands, Notch receptors are activated by a series of cleavage events to release Notch receptor intracellular domains that modulate their target genes via both pathways dependent on and independent of C promoter binding factor-1 (CBF1)/recombination signal binding protein-JK (RBP-JK) (6). Notch receptors and Jagged1 are expressed in human gastric cancer (7,8) and Notch1 signaling promotes gastric cancer progression through induction of cyclooxygenase-2 (COX-2) expression (7).

Transcription factor Twist plays a pivotal role in embryogenesis and tumorigenesis (for reviews, see refs 9–11). It is overexpressed in several cancers and involved in tumor progression (10). Twist also regulates epithelial–mesenchymal transition in the development of metastatic cancer cells (for a review, see ref. 11). As demonstrated previously (12,13), elevation of Twist expression enhances tumorigenesis through activation of signal transducer and activator of transcription 3 (STAT3). Twist expression is also elevated in gastric cancer and correlated with clinicopathological parameters (14,15). Additionally, Twist is a critical regulator of gastric cancer progression (16,17).

STAT3 is activated by cytokines, growth factors and oncogenic proteins and mediates a variety of biological processes (for reviews, see refs 18,19). The aberrant activation of STAT3 is implicated in progression of numerous human cancers including gastric cancer (for reviews, see refs 18–20). Recent studies showed that Notch signaling participates in epithelial–mesenchymal transition (21,22) and induces Drosophila mesoderm subdivision via the control of Twist expression (27). Because all Notch, Twist and STAT3 pathways regulate tumorigenesis of gastric cancer, we tried to address whether STAT3/Twist axis is involved in modulation of gastric cancer progression enhanced by Notch1 signaling in the present study.

Materials and methods

Plasmids and plasmid construction

Expression construct pcDNA-HA-N1IC contains the complementary DNA (cDNA) encoding Notch1 receptor intracellular domain (N1IC) of human (12). The plasmids pcDNA-myc-N2IC-His, pcDNA-N3IC-myc-His and pcDNA-N4IC-myc-His direct expressions of Notch receptor intracellular domains [amino acid residues 1700 to 2471 of Notch2 receptor intracellular domain (N2IC), 1665 to 2321 of Notch3 receptor intracellular domain (N3IC) and 1468 to 2002 of Notch4 receptor intracellular domain (N4IC), respectively]. Construct pSG5Flag-RBP-JKvP16 contains the cDNA encoding the constitutively active RBP-JK mutant. Plasmids STAT3wt and STAT3dn express the wild-type and dominant negative mutant of STAT3, respectively (12). The pFlag-Twist expression construct contains the full-length Twist cDNA (29). Reporter plasmids containing human Twist promoter DNA fragments from nt –970 to +48, –970 to +140 and –95 to +48 were constructed in pGL3-basic vector. Reporter plasmid containing Twist promoter DNA fragment from nt –139 to +48 was described previously (29). Reporter plasmid 4×wtCBF1Luc contains four copies of CBF1-response elements in pGL3-promoter vector (30).

For knockdown of the endogenous STAT3 and Twist, the following target sequences were constructed in small interfering...
RNA (siRNA) vector pLKO.1; STAT3, 5′-GCAAAGAATCAAGTGCCACTTTT-3′ and Twist, 5′-TCCGCTGATCCTTAAGGAGACTCT-3′ (42) and 5′-GCAAGGACTCTAAGGATGCAAGAAGG-3′ (43). To knockdown Notch1 receptor, the target sequence was also constructed in siRNA vector pLKO.1 (7,31). For knockdown validation, the pLKO.1-shLuc siRNA vector against luciferase was used as a negative control (7,31).

Cell culture and transfection

Human stomach carcinoma SC-M1, AGS, AZ521, NUGC-3 and KATO III cells, human embryonic kidney HEK293 and 293T cells or erythroleukemia K562 cells were cultured in Dulbecco’s modified Eagle’s medium or RPMI 1640 medium with 10% fetal bovine serum. The established N1IC-expressing SC-M1 (SC-M1/HA-N1IC), K562 (K562/HA-N1IC) or HEK293 (HEK293/myc-N1IC) cells and their control cells (SC-M1/pCDNA3, K562/pCDNA3 or HEK293/pCDNA3) were described previously (7,28). To activate the endogenous Notch pathway, the stable COS-7 cell lines expressing the secreted form of Notch ligand Jagged1 (COS-7/Jagged1ext) and their control cells (COS-7/pCNA3, 1-myc-His) were established and described previously (32).

HEK293T, K562, SC-M1, AGS or KATO III cells were transiently transfected by the calcium phosphate coprecipitation method, SuperFect transfection reagent (Qiagen, Valencia, CA), or electroporation (28,33). For luciferase reporter gene assay, cells (5×10⁴) were seeded onto six-well plates and transfected for 2 days as described (28,33). Luciferase activities were quantified using the Dual-Luciferase™ reporter assay system (Promega, Madison, WI) and then normalized with Renilla luciferase activity for transfection efficiency.

For colony-forming, migration and invasion assays, SC-M1, AGS or KATO III cells were seeded after transfection for 2 days (7,33).

The γ-secretase inhibitor N-(3,5-difluorophenacetyl)-L-alanyl-S-phenylglycine t-butyl ester and STAT3 inhibitor Cucurbitacin/JSI-124 (Sigma-Aldrich, St Louis, MO) at the indicated concentration in dimethyl sulfoxide or in an equal volume of vehicle were added in the present study.

Western blot analysis

Whole-cell lysates were prepared and then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described before (28). Western blot analysis was performed with anti-N1IC (sc-6014; Santa Cruz Biotechnology, Santa Cruz, CA), anti-N2IC (sc-7423; Santa Cruz Biotechnology), anti-N3IC (sc-5995; Santa Cruz Biotechnology), anti-N4IC (sc-5994; Santa Cruz Biotechnology), anti-Twist (sc-15393; Santa Cruz Biotechnology), anti-cleaved Notch1 (sc2241; Cell Signaling Technology, Beverly, MA), anti-p-STAT3 (Tyr705) (#9131; Cell Signaling Technology), anti-p-STAT3 (Ser727) (#9134; Cell Signaling Technology), anti-Twist (#9132; Cell Signaling Technology) and anti-GAPDH antibodies (4699–9555; Biogenesis, Poole, UK).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed as described previously (31,33). Briefly, nuclear extracts were prepared for ChIP assay using protein A Sepharose-bound antibodies of anti-STAT3 and anti-IgG antibodies. By PCR amplification, the specific primers 5′-GAGGGGGACTGGAAAGC-3′ and 5′-GCTGGAACGGTGGCGG-3′ were used to amplify the 187-bp PCR products of DNA fragment of Twist promoter.

Colony-forming, migration and invasion assays

As described (7,33), 4000 parental cells (SC-M1, AGS and KATO III cells) or 8000 SC-M1/pCNA3 and SC-M1/HA-N1IC cells were used for assay of anchorage-independent growth in soft agar for 14 days. Migration and invasion assays were performed in 24-well plates by Millipore tissue culture inserts (Millipore, Bedford, MA) for 12h and BD BioCoat Matrigel Invasion assay were performed in 24-well plates by Millicell tissue culture plate well inserts (Millipore, Bedford, MA) for 12h and BD BioCoat Matrigel Invasion Chambers (Becton Dickson, Mountain View, CA) for 20h, respectively.

Xenografted tumorigenicity assay in nude mice

According to protocols with the approval of ethical committee, 5-week-old BALB/c nu/nu mice (National Science Council Animal Center, Taipei, Taiwan) were subcutaneously injected with 6×10⁴ viable cells into both hind limbs (7,33). Tumor volume was estimated by caliper measurements of the length and width of xenografts every 3 days. On day 12 postinjection of tumor cells, tumor sizes reached about 100–150 mm³. Then the mice were randomized (10 animals/group; 2 tumors/animal) and dosed intraperitoneally (i.p.) with either JSI-124 at a dose of 1 mg/kg or vehicle in a total volume of 0.15 ml of 20% DMSO daily for 5 days. On day 27 after implantation, the mice were killed and the subcutaneous tumors were excised.

In vivo tail vein metastasis assay

The viable cells (1.5×10⁴) in a total volume of 0.1 ml of phosphate-buffered saline were injected into female non-obese diabetic severe combined immune-nodedeficience mice (National Taiwan University, Taipei, Taiwan) aged 8 weeks by tail vein injection (33). On day 1 after cancer cell injection, the mice were randomized (7 mice/group) and administered i.p. with either JSI-124 at a dose of 1 mg/kg or vehicle in a total volume of 0.15 ml of 20% DMSO once every other day from day 1 to day 9. After 6 weeks, the mice were killed and the lung metastatic nodules were counted by gross and microscopic examination.

Surgical samples

Tissues of gastric adenocarcinoma from gastric cancer patients were collected at the Department of Surgery, Taipei Veterans General Hospital. Informed consent was obtained from each patient before study. Among these patients, there were no persons undergoing chemotherapy or radiotherapy before surgery. Tissue sections were prepared as described elsewhere (7).

Immunostaining of phosphorylated STAT3 and Twist

As described previously (34), the avidin–biotin–peroxidase technique was used to localize phosphorylated STAT3 (p-STAT3) (Tyr705) and Twist in gastric cancer tissues. Tissue sections were incubated with rabbit anti-p-STAT3 (Tyr705) antibody (sc-9145; Cell Signaling Technology) at 1:100 dilution or rabbit anti-Twist antibody (sc-15393; Santa Cruz Biotechnology) at 1:100 dilution at 4°C overnight. Preimmune rabbit or mouse IgGs were used as the negative control.

The distribution of p-STAT3 (Tyr705) and Twist in tissue specimens was evaluated by a semiquantitative system to calculate the percentage of positive neoplastic cells. Then it was estimated within the following arbitrary ranges: −, no positive cells; +, 1–25%; ++, 26–75%; ++++, >75%. Correlation between Jagged1, Notch1 receptor, p-STAT3 (Tyr705), Twist and COX-2 levels was determined using linear regression.

Statistical analysis

Data analysis was performed by Chi-square test in analysis of various clinico-pathological factors. Statistical analysis was also done using Student’s t-test for simple comparison of two values. The difference of results was considered statistically significant if the P value was less than 0.05.

Results

Notch1 signaling enhances Twist expression and STAT3 phosphorylation

To assess the possibility that Notch1 signaling promotes cancer progression through modulation of Twist expression, the N1IC-expressing SC-M1/HA-N1IC, K562/HA-N1IC and HEK293/myc-N1IC cells were used to detect Twist expression by western blot analysis. Results showed that the exogenous N1IC increased Twist expression in N1IC-expressing SC-M1/HA-N1IC, K562/HA-N1IC and HEK293/myc-N1IC cells compared with SC-M1/pCNA3, K562/pCNA3 and HEK293/pCNA3 control cells (Figure 1A). Because Twist expression is increased by STAT3 phosphorylation on Tyr705 residue (12), we also evaluated the levels of p-STAT3 and STAT3 in the N1IC-expressing cells. Compared with control cells, the forced expression of N1IC elevated level of p-STAT3 (Tyr705) but not p-STAT3 (Ser727) and STAT3 (Figure 1A).

To investigate whether the endogenous Notch signaling is also involved in promotion of Twist and p-STAT3 (Tyr705) levels, conditioned media of the secreted form of Jagged1-expressing COS-7/Jagged1 cells were used to activate the endogenous Notch pathway (32). After treating SC-M1/pCNA3, K562/pCNA3 and HEK293/pCNA3 cells with conditioned media of COS-7/Jagged1 cells, activity of reporter gene containing CBF1-response elements (Supplementary Figure S1 is available at Carcinogenesis Online) and Twist and p-STAT3 (Tyr705) levels were elevated (Figure 1B). Consistently, Twist and p-STAT3 (Tyr705) levels but not p-STAT3 (Ser727) and STAT3 were decreased in gastric cancer cells including SC-M1, AZ521 and NUGC-3 cells after treatment with the γ-secretase inhibitor N-(3,5-difluorophenacetyl)-L-alanyl-S-phenylglycine t-butyl ester to block endogenous Notch signaling (Figure 1C).

Therefore, the endogenous Notch signaling also enhanced Twist and p-STAT3 (Tyr705) levels.

To check whether four Notch receptors are involved in the induction of Twist and p-STAT3 (Tyr705) levels, constructs of the intracellular domains of four Notch receptors (N1IC, N2IC, N3IC and N4IC) were transfected into HEK293T cells for western blot analysis. Data showed that only the exogenous N1IC significantly enhanced Twist and p-STAT3 (Tyr705) levels in HEK293T cells (Figure 1D).
N1IC induces Twist promoter activity through STAT3 phosphorylation

To address whether N1IC enhances Twist expression through inducing Twist promoter activity, reporter gene assay was performed in K562 cells. Twist promoter activity was induced after cotransfection with N1IC-expressing construct and reporter plasmids containing the human Twist promoter DNA fragments from nt −970 to +48 or nt −139 to +48 (Figure 2A). Therefore, the DNA sequence from nt −139 to +48 of Twist promoter is sufficient for its transactivation by N1IC.

To further clarify whether activation of Twist promoter by N1IC is through CBF1-dependent or CBF1-independent pathways, reporter plasmids containing Twist promoter or CBF1-response elements were cotransfected with construct of the constitutively active RBP-Jκ-VP16 fusion protein for reporter gene assay. Although RBP-Jκ-VP16 fusion protein strikingly promoted activity of the CBF1-dependent reporter gene (4×wtCBF1Luc), it slightly elevated activity of reporter gene containing Twist promoter (Figure 2A).

Alternatively, N1IC-expressing plasmid and reporter plasmid containing Twist promoter were cotransfected with expression construct of CBF1 or siRNA vector against CBF1 (32) into SC-M1 cells for reporter gene assay. Data showed that overexpression and knockdown of CBF1 did not significantly affect N1IC-mediated Twist promoter activity (Supplementary Figure S2A and B is available at Carcinogenesis Online). Furthermore, reporter gene assay was performed after transfection with three expression constructs of CBF1 mutants, including RLI261AAA expressing the normal pattern of nuclear staining and EEF233AAA and KLV249AAA expressing cytosolic staining (35). These CBF1 mutants also did not significantly affect N1IC-mediated Twist promoter activity (Supplementary Figure S2C is available at Carcinogenesis Online). These results suggest that Twist promoter activity is induced by Notch1 pathway mainly through a CBF1-independent manner.

To further map the critical regions of Twist promoter transactivated by N1IC, reporter plasmids containing various lengths of Twist promoter were cotransfected with reporter gene assay in K562 cells. Reporter gene activity was induced by N1IC after transfection with reporter plasmid containing Twist promoter from nt −139 to +48, but not those containing Twist promoter from nt −970 to −140 or nt −95 to +48 of Twist promoter.
to +48 (Figure 2B). Thus, the DNA sequence from nt −139 to −96 of Twist promoter is necessary for its transactivation by N1IC. Moreover, constructs of N1IC, N2IC, N3IC or N4IC were cotransfected with reporter plasmids containing Twist promoter in K562 cells for reporter gene assay. Data showed that overexpression of N1IC but not the others induced Twist promoter activity (Supplementary Figure S3A is available at Carcinogenesis Online). Twist promoter activity was also elevated after treatment with conditioned media of COS-7/Jagged1ext cells in SC-M1/pcDNA3, K562/pcDNA3 and HEK293/pcDNA3 cells using reporter gene assay (Supplementary Figure S3B is available at Carcinogenesis Online). Nuclear extracts of SC-M1/HA-N1IC, SC-M1/pcDNA3 and the transfected KATO III and AGS cells were prepared for ChIP assay using anti-STAT3 and anti-IgG antibodies. The immunoprecipitated DNAs were used to amplify the 187-bp PCR products in the region of Twist promoter.

Fig. 2. N1IC induces Twist promoter activity through STAT3 phosphorylation. (A) Reporter plasmids containing various lengths of Twist promoter (−970/+48, −970 to +48; −139/+48, −139 to +48) and containing four copies of CBF1-response elements (4×wtCBF1Luc) were cotransfected with N1IC-expressing construct (N1IC), constitutively active RBP-Jκ mutant-expressing plasmid (RBP-Jκ-VP16) or control vector (-) into K562 cells for reporter gene assay. (B) Reporter plasmids containing various lengths of Twist promoter (−970 to −140, −970 to −140; −139 to +48, −139 to +48; −95 to +48, −95 to +48) were cotransfected with N1IC-expressing construct (N1IC) or control vector (-) into K562 cells for reporter gene assay. (C) Reporter plasmid containing Twist promoter from nt −970 to +48 was cotransfected with N1IC-expressing construct (N1IC) and wild-type STAT3-expressing construct (STAT3wt) or dominant negative STAT3 mutant-expressing construct (STAT3dn) into K562 cells for reporter gene assay. The islet shows results of STAT3 expressions after transfection with STAT3-expressing constructs into HEK293T cells by western blot analysis. (D) Whole-cell extracts of K562 cells treated with 0.5 μM JSI-124 or vehicle (DMSO) for 24 h were subjected to western blot analysis (left). After cotransfection with reporter plasmid containing Twist promoter from nt −970 to +48 and N1IC-expressing construct (N1IC) or control vector (-) into K562, SC-M1, KATO III and AGS cells, the transfected cells were treated with 0.5 μM JSI-124 or DMSO for reporter gene assay (right). Values are mean from at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. #P < 0.05, ##P < 0.01. (E) N1IC-expressing construct (N1IC) or control vector (-) were transfected into KATO III and AGS cells. Nuclear extracts of SC-M1/HA-N1IC, SC-M1/pcDNA3 and the transfected KATO III and AGS cells were prepared for ChIP assay using anti-STAT3 and anti-IgG antibodies. The immunoprecipitated DNAs were used to amplify the 187-bp PCR products in the region of Twist promoter.

STAT3 binds on Twist promoter and fully activates the promoter activity (12). This STAT3 response element is located in the sequence of Twist promoter from nt −139 to −96, which is indispensable for its transactivation by N1IC. Therefore, it is possible that N1IC enhances STAT3 phosphorylation and then p-STAT3 binds on Twist promoter for induction of Twist expression. To clarify this possibility, reporter plasmid containing Twist promoter was cotransfected with constructs of N1IC and wild-type (STAT3wt) or dominant negative mutant (STAT3dn) of STAT3 into K562 cells for reporter gene assay. The N1IC-induced Twist promoter activity was further enhanced by wild-type STAT3 but suppressed by dominant negative one (Figure 2C). In addition, both p-STAT3 (Tyr705) and Twist levels were inhibited by STAT3 inhibitor JSI-124 for 24 h in K562 cells (Figure 2D, left). After cotransfection with

Supplementary Figure S3C is available at Carcinogenesis Online.
reporter plasmid containing Twist promoter from nt −970 to +48 and N1IC expression construct in erythroleukemia K562 cells and gastric cancer cells including SC-M1, KATO III and AGS cells, the N1IC-induced Twist promoter activities were attenuated by JSL-124 treatment (Figure 2D, right). The exogenous N1IC also increased protein–DNA interaction between nuclear STAT3 and Twist promoter in KATO III, AGS and N1IC-expressing SC-M1/HA-N1IC cells by ChIP assay (Figure 2E).

**N1IC promotes gastric cancer progression through STAT3 phosphorylation**

To explore whether N1IC promotes gastric cancer progression through STAT3, SC-M1 cells were cotransfected with constructs of N1IC and STAT3 for evaluation of colony formation, migration and invasion abilities. Results showed that the N1IC-enhanced gastric cancer progression of SC-M1 cells was further augmented by wild-type STAT3 but relieved by dominant negative one (Figure 3A). To check whether the endogenous STAT3 is also involved in gastric cancer progression enhanced by N1IC, colony formation, migration and invasion abilities of N1IC-expressing SC-M1 cells were determined after STAT3 knockdown. STAT3 expression in SC-M1 cells was knocked down after transfection with siRNA vector against STAT3 (Supplementary Figure S4A is available at Carcinogenesis Online). The N1IC-enhanced gastric cancer progression of SC-M1 cells was abolished by STAT3 knockdown (Figure 3B). Furthermore, the N1IC-induced p-STAT3 (Tyr705) and Twist levels were abrogated by JSL-124 (Supplementary Figure S4B is available at Carcinogenesis Online). The N1IC-stimulated gastric cancer progression of SC-M1 cells was alleviated by JSL-124 treatment (Figure 3C). These results suggest that STAT3 phosphorylation is essential for the N1IC-elevated gastric cancer progression of SC-M1 cells.

**N1IC promotes gastric cancer progression through Twist**

Next, we sought to unravel whether N1IC promotes gastric cancer progression via activating Twist expression. Endogenous Twist of SC-M1 cells could be knocked down after transfection with siRNA vectors against Twist (Supplementary Figure S5 is available at Carcinogenesis Online). The N1IC-enhanced gastric cancer progression of SC-M1 cells was relieved by Twist knockdown (Figure 4A). Both p-STAT3 (Tyr705) and Twist levels of SC-M1 cells were suppressed by inhibition of Notch1 signaling after transfection with siRNA vector against Notch1 receptor (Figure 4B, left). The suppressed gastric cancer progression by Notch1 knockdown was attenuated after transfection with Twist expression construct (Figure 4B, right).

**STAT3 regulates gastric cancer progression through Twist**

Moreover, the role of STAT3/Twist axis in gastric cancer progression was also examined. Results showed that gastric cancer progression suppressed by treatment with JSL-124 or transfection with siRNA vector against STAT3 was restored by exogenous Twist in SC-M1 cells (Supplementary Figure S6 is available at Carcinogenesis Online).

**N1IC also elevates gastric cancer progression of AGS and KATO III cells through STAT3 and Twist**

Besides SC-M1 cells, expressions of the cleaved Notch1 receptor, STAT3, p-STAT3 (Tyr705) and Twist in other gastric cancer cells were also investigated such as AGS, AZ521, NUGC-3 and KATO III cells.

![Fig. 3. N1IC promotes gastric cancer progression through STAT3 phosphorylation.](https://academic.oup.com/carcin/article-abstract/33/8/1459/2463696)
Data of western blot analysis showed that expression of the cleaved Notch1 receptor was positively correlated with p-STAT3 (Tyr705) and Twist levels in these cells (Supplementary Figure S7A is available at Carcinogenesis Online). The results suggest that the activation of Notch1 signaling is associated with p-STAT3 (Tyr705) and Twist levels in gastric cancer cells.

To study whether N1IC also promotes gastric cancer progression through STAT3 and Twist in AGS and KATO III cells that negatively or weakly expressed the cleaved Notch1 receptor, N1IC expression construct was cotransfected with siRNA vector against Notch1 receptor and Twist-expressing construct (Twist). The transfected cells were also seeded for the subsequent colony-forming, migration and invasion assays (right). Means of three independent experiments performed in triplicate are shown. *P < 0.05, **P < 0.01, ***P < 0.001. #P < 0.05, ##P < 0.01.

Fig. 4. N1IC promotes gastric cancer progression through Twist. (A) After transfection with siRNA vectors against Twist (#42 and #43) into SC-M1/HA-N1IC or SC-M1/pcDNA3 cells, the transfected cells were seeded and incubated for colony-forming, migration and invasion assays. (B) Whole-cell extracts of SC-M1 cells transfected with siRNA vector against Notch1 receptor were analyzed by western blot analysis (left). SC-M1 cells were cotransfected with siRNA vector against Notch1 receptor and Twist-expressing construct (Twist). The transfected cells were also seeded for the subsequent colony-forming, migration and invasion assays (right). Means of three independent experiments performed in triplicate are shown. *P < 0.05, **P < 0.01, ***P < 0.001. #P < 0.05, ##P < 0.01.

N1IC promotes tumor growth and metastasis of SC-M1 cells through phosphorylated STAT3 and Twist

Furthermore, animal models were used to address whether N1IC promotes tumor growth and metastasis of gastric cancer through p-STAT3 and Twist. Nude mice were subcutaneously implanted with N1IC-expressing SC-M1/HA-N1IC cells. Consistent with previous results (7), the xenografted tumor sizes of SC-M1/HA-N1IC cells were larger than those of control cells at day 12. The nude mice were daily i.p. injected with JSI-124 for 5 days after implantation for 12 days. Results showed that the N1IC-promoted tumor growth of SC-M1 cells was suppressed by JSI-124 treatment (Figure 5A).

To investigate the effect of JSI-124 treatment on the N1IC-modulated metastatic colonization of gastric cancer cells, SC-M1/HA-N1IC cells were intravenously injected into lateral tail vein of non-obese diabetic severe-combined immunodeficiency mice. Mice were administered with JSI-124 by i.p. injection once every other day on days 1–7 and then killed on day 47 for evaluation of metastatic nodules in lungs. Mice injected with SC-M1/HA-N1IC cells had numerous large metastases in lung compared with those injected with control cells (Figure 5B). After treatment with JSI-124, mice injected with SC-M1/HA-N1IC cells had fewer and smaller lung metastatic nodules compared with those injected with vehicle. These results indicated that the N1IC-promoted ability of SC-M1 cells to form metastatic nodules in lungs was attenuated by JSI-124 treatment. Likewise, the N1IC-augmented tumor growth of SC-M1 cells was alleviated in nude mice after knockdown of Twist (Figure 5C). The N1IC-elevated ability of SC-M1 cells to form metastatic nodules in lungs was ameliorated by Twist knockdown in non-obese diabetic severe-combined immunodeficiency mice (Figure 5D).
Clinical relevance of Notch1 receptor, Jagged1, p-STAT3 and Twist levels in gastric cancer tissues

To assess whether the Notch1/STAT3/Twist signaling is involved in progression of human gastric cancer, the clinical relevance of Notch1 receptor, Jagged1, p-STAT3 (Tyr705) and Twist levels was examined on gastric cancer tissues using immunohistochemical staining. Sixty three out of 98 gastric cancer specimens (64%) expressed Twist in cancer tissues (Supplementary Table S1 is available at Carcinogenesis Online). Multivariate analysis showed that intestinal type Lauren’s histology type and Borrmann I and II types were significantly correlated with Twist expression in gastric cancer tissues (Supplementary Table S2 is available at Carcinogenesis Online). Furthermore, data of the statistical analysis of Notch1 receptor, Jagged1, p-STAT3 (Tyr705), Twist and COX-2 levels in gastric cancer tissues were compared with each other. Results showed that Jagged1 and Notch1 expressions were also associated with p-STAT3 (Tyr705), Twist and COX-2 levels in gastric cancer tissues (Table 1).

Discussion

All Notch, STAT3 and Twist pathways exhibit essential functions in many common cellular processes modulating tumorigenesis. In the present study, the in vitro and in vivo results showed that Notch1/STAT3/Twist cascade is involved in gastric cancer progression. Furthermore, Notch1 receptor, Jagged1 and p-STAT3 (Tyr705) levels in gastric cancer tissues of patients were correlated with each other (Table 1). To our knowledge, this is the first report regarding the linkage of Notch signaling and STAT3/Twist axis in regulation of gastric cancer progression.

We show herein that N1IC enhanced STAT3 phosphorylation at the residue of Tyr705 but not at the residue of Ser727 (Figure 1). Phosphorylation of STAT3 at Tyr705 residue leads to its dimerization and nuclear translocation, whereas phosphorylation at its Ser727 residue is required in transcriptional activation (36). Notch ligands, Delta-like 4 and Jagged1, induce STAT3 phosphorylation located at

![Fig. 5. N1IC promotes tumor growth and metastasis through phosphorylated STAT3 and Twist. (A) The viable SC-M1/HA-N1IC or SC-M1/pcDNA3 cells were subcutaneously injected into nude mice (n = 10 per group) for measurement of tumor sizes at the time indicated. On day 12 after implantation, mice received daily i.p. injections of vehicle (20% DMSO) or JSI-124 (1 mg/kg in 20% DMSO) for 5 days. On day 27, the mice were killed and subcutaneous tumors were excised. Data are representative of three experiments. (B) For measurement of metastatic nodules in lungs, non-obese diabetic severe-combined immunodeficiency mice (n = 7 per group) were injected with the viable SC-M1/HA-N1IC cells or SC-M1/pcDNA3 cells by tail vein injection. On day 1 after cancer cell injection, mice were administered with daily i.p. injections of JSI-124 (1 mg/kg in 20% DMSO) or vehicle (20% DMSO) once every other day from day 1 to day 9. After 6 weeks, the mice were killed and the metastatic nodules in the lungs were counted by gross and microscopic examination. Data are from a representative experiment that was performed three times with identical results. (C) and (D) As described above, SC-M1/HA-N1IC or SC-M1/pcDNA3 cells transfected with siRNA vectors against Twist (#42) or luciferase were injected into nude mice (n = 5 per group) for measurement of tumor sizes (C) and non-obese diabetic severe-combined immunodeficiency mice (n = 5 per group) for measurement of metastatic nodules in lungs (D). Data are representative of 2 (D) or 3 (C) experiments with similar results. *P < 0.05, ***P < 0.001. ##P < 0.01.
cancer progression through HES-1 requires further study.

It is possible that HES-1 is upregulated by Notch1 signaling and in turn activates the kinases and induces STAT3 phosphorylation on tyrosine residues of Ser727 but not Tyr705 in fetal neural stem cells (37). During differentiation of neural stem cells, Notch signaling collaborates with ciliary neurotrophic factor in STAT3 phosphorylation on Tyr705 and Ser727 residues (23). These data showed that the activation of Notch signaling exhibits distinct biological functions through phosphorylation at different residues of STAT3.

STAT3 is a substrate of Janus-activated kinases, growth factor receptor kinases, Src, breast tumor kinase and c-Fes family kinases (18,19,38). Binding of cell surface receptors with their cytokines activates the kinases and induces STAT3 phosphorylation on tyrosine residue for modulation of tumorigenesis (for reviews, see refs 18 and 19). For example, pathways of Interleukin-6 and epidermal growth factor receptor activate STAT3/Twist axis and in turn promote cancer metastasis (12,13). How does the activated Notch1 receptor enhance STAT3 phosphorylation as shown in Figure 1? Recent study showed that the basal and inflammation-induced Interleukin-6 levels are increased by Notch signaling (39). In contrast, the CBF1-dependent Notch signal-downstream strategy for treatment of gastric cancer in the near future.

Table I. Clinical relevance of Notch1 receptor, Jagged1, p-STAT3 (Tyr705), Twist and COX-2 levels in gastric cancer tissues

<table>
<thead>
<tr>
<th>Spearman’s rho coefficient</th>
<th>Jagged1</th>
<th>Notch1</th>
<th>p-STAT3 (Tyr705)</th>
<th>Twist</th>
<th>COX-2</th>
</tr>
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<tr>
<td>Correlation coefficient</td>
<td>1.000</td>
<td>0.349</td>
<td>0.718</td>
<td>0.273</td>
<td>0.375</td>
</tr>
<tr>
<td>Significance</td>
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<td>0.003</td>
<td>0.000</td>
<td>0.014</td>
<td>0.001</td>
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<tr>
<td>Patient numbers</td>
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<td>73</td>
<td>77</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Notch1 Correlation coefficient</td>
<td>0.349</td>
<td>1.000</td>
<td>0.221</td>
<td>0.339</td>
<td>0.275</td>
</tr>
<tr>
<td>Significance</td>
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<td>—</td>
<td>0.045</td>
<td>0.001</td>
<td>0.018</td>
</tr>
<tr>
<td>Patient numbers</td>
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<td>87</td>
<td>83</td>
<td>87</td>
<td>73</td>
</tr>
<tr>
<td>p-STAT3 (Tyr705) Correlation coefficient</td>
<td>0.718</td>
<td>0.221</td>
<td>1.000</td>
<td>0.172</td>
<td>0.503</td>
</tr>
<tr>
<td>Significance</td>
<td>0.000</td>
<td>0.045</td>
<td>—</td>
<td>0.096</td>
<td>0.000</td>
</tr>
<tr>
<td>Patient numbers</td>
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<td>83</td>
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<td>94</td>
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<tr>
<td>Twist Correlation coefficient</td>
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<td>0.172</td>
<td>1.000</td>
<td>0.338</td>
</tr>
<tr>
<td>Significance</td>
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<td>0.001</td>
<td>0.096</td>
<td>—</td>
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<tr>
<td>COX-2 Correlation coefficient</td>
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<td>0.275</td>
<td>0.503</td>
<td>0.338</td>
<td>1.000</td>
</tr>
<tr>
<td>Significance</td>
<td>0.001</td>
<td>0.018</td>
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<td>77</td>
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</tbody>
</table>

Figure 3, the N1IC-enhanced tumor progression of gastric cancer cells was dramatically and significantly suppressed after treatment with JSI-124. Thus, the N1IC-promoted tumor growth was attenuated, at least in part, through suppression of STAT3 phosphorylation in tumor cells.

Based on the results of Figure 2A, Notch1 signaling enhances Twist promoter activity, primarily through a CBF1-independent pathway. However, there was slight increment of Twist promoter activity via a CBF1-dependent manner by the constitutively active RBP-Jκ-VP16 fusion protein (Figure 2A). Our current findings imply that both the CBF1-dependent and CBF1-independent Notch1 pathways can upregulate Twist promoter activity simultaneously. Furthermore, the mammalian target of rapamycin modulates differentiation through STAT3/p63/Jagged1/Notch1 axis (44). It is interesting to delineate the possibility that Notch1 signaling and STAT3 cross talk in a reciprocal regulatory loop in control of gastric cancer progression. Therefore, the regulatory mechanism of network containing Notch1, STAT3 and Twist pathways is complicated.

Numerous studies have validated STAT3 or Notch pathways as targets for cancer therapy by a variety of strategies (6,45). Evidence implicates that modulation of the RTK/PI3K/AKT/mTOR/STAT3/p63/Jagged1/Notch1/HE1 network has therapeutic potential of the targeted combination therapy against tumorigenesis (44). Thus, the cross talk between Notch and STAT3/Twist pathways suggests a potentially combinatorial strategy for treatment of gastric cancer in the near future.

Supplementary material
Supplementary Tables S1 and S2 and Figures S1–S7 can be found at http://carcin.oxfordjournals.org/

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References

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