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The function of Notch1 intracellular domain in the differentiation of gastric cancer

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Abstract. Due to the complex function of the Notch signal pathway in gastric cancer (GC), the association between Notch homolog 1 (Notch1) intracellular domain (NICD) and differentiation of GC remains unknown. The present study aimed to investigate the potential association between NICD and GC differentiation, and demonstrated that poorly differentiated GC expressed increased NICD levels compared with well differentiated GC. A γ-secretase inhibitor inhibited the growth of AGS cells through downregulating NICD level. Additional data suggested that a COX-2 inhibitor caused a marked reduction of NICD level in comparison with a control group treated with dimethyl sulfoxide. Combined administration of γ-secretase and COX-2 inhibitor produced a marked inhibition of growth in AGS cells, which suggests that patients with poorly differentiated GC may benefit from the blockage of NICD, which potentially serves a role in GC differentiation.

Introduction

The Notch signaling pathway comprises Notch transmembrane receptors, Notch ligands, DNA-binding protein C-promotor Binding Factor 1 (CBF-1)/J κ-recombination signal-binding protein/Suppressor of hairless (H)/lin-12 and glp-1 (CSL), several effector molecules and regulatory molecules (1). A hallmark of Notch signaling that distinguishes it from other conserved signaling pathways is its mechanism of signal transduction. When Notch ligands, including Jagged (JAG)1, JAG2, delta like canonical notch ligand (DLL)1, DLL3 and DLL4, interact with Notch transmembrane receptors, for example Notch homolog (Notch) 1-4, on adjacent cells, this binding induces the cleavage of Notch receptor by proteases, including a disintegrin and metalloproteinase proteases or γ-secretase, to release Notch1 intracellular domain (NICD) (1-3). Then, NICD travels to the nucleus and binds to DNA binding proteins, such as CSL, to assemble a transcription complex that activates downstream target genes, including Hairy and enhancer of split HES1, HES5 and Hairy/enhancer-of-split related with YRPW motif protein 1 (1-3). This core signal transduction pathway is used in the majority of Notch-dependent processes and is known as the canonical CSL-NICD-Mastermind-like pathway (4,5). In addition, depending on the cellular context, the amplitude and duration of Notch activity may be additionally regulated at various points in the pathway (1-3). Activated Notch signaling has been demonstrated to serve an important role in the development and homeostasis of tissues by regulating cell-fate decisions, proliferation, differentiation and apoptosis (1,6). These features confer susceptibility of Notch signaling subversion by cancer cells.

Gastric cancer (GC) is one of the most common malignant diseases and the third leading cause of cancer-associated mortalities worldwide in 2012, with age-standardized incidence rates highest in eastern Asia (7). A previous study identified that high expression of Notch1-4 mRNA was associated with unfavorable overall survival in 876 patients with GC over a 20-year period (8). Activated Notch1 was a poor prognostic factor for patients with GC (9) and closely associated with an advanced tumor stage, tumor metastasis and overall patient survival (10). A previous study indicated that Notch1 may maintain the cancer stem-like phenotype of diffuse type GC through inducing CD133 gene expression, and that inhibiting Notch1 may be an effective treatment for CD133-positive diffuse type GC (11). ββ-Dimethylacrylshikonin and Sirtuin 3 may inhibit GC cell growth through downregulating Notch1 (12,13). In addition, Notch1 inhibition may also impair the invasion capability of GC cells (14). Certain previous studies suggested that Notch1 expression may be repressed by several microRNAs (miRNA/miR), including miR-124, miR-935 and miR-34 during GC progression (15-17). Concurrently, Notch1 and miR-151-5p interact with p53 in a reciprocal regulation loop to control gastric tumorigenesis (18). In addition, Notch2 and Notch3 receptor expression was also associated with gastric cancer development, and Notch4 receptor promoted gastric cancer growth (19-22). An additional study indicated that NICD was associated with the presence of lymph

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node metastasis and worse survival (9,10). However, few studies have examined the association between NICD and differentiation of GC.

The cyclooxygenase (COX) enzyme exists in two forms: COX-1 and COX-2. COX-1 is constitutively produced, while COX-2 is an inducible form. Despite being previously explored as a pro-inflammatory molecule, several data indicated the vital role of COX-2 in GC (23), and in other types of cancer (24,25). A previous study suggested that the activation of Notch1 signal pathway may promote the progression of gastric cancer through COX-2 (26). Notch2 may also induce COX-2 expression, and the suppression of tumor progression by Notch2 knockdown in GC cells may be reversed by exogenous COX-2 (20). Nevertheless, the effect of COX-2 on Notch activity in GC cells has not yet been studied. The present study revealed that poorly-differentiated GC expressed increased levels of NICD compared with well-differentiated GC, indicating potential involvement of NICD in GC differentiation. The selective COX-2 inhibitor NS-398 may enhance antitumor activity of N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a non-specific inhibitor of Notch, in poorly-differentiated GC cells via downregulating NICD level.

Materials and methods

Specimens. Human GC tissues and adjacent normal gastric tissues (>5 cm from tumor) were obtained from 12 patients (between 40 and 80 years old, with a median age of 70; including 3 females and 9 males) who underwent gastric resection at the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) without pre-operative chemotherapy or radiation between May 2012 and July 2012. Normal gastric tissue (n=1) was collected from a healthy patient by gastroscopy. Written informed consent was obtained from each patient and approval was obtained from the Ethical Committee on Human Research of Wenzhou Medical University. Each tumor sample was assigned at a histological grade based on the World Health Organization (WHO) classification criteria of tumors of the digestive system (27).

Robust Multi-Array mveraging (RMA) normalized basal expression profiles. RMA normalized basal expression profiles for AGS cell were downloaded from the Genomics of Drug Sensitivity in Cancer Project (GDSC; version 6.1, March 2017; http://www.cancerrxgene.org/). The GDSC is a collaboration between the Cancer Genome Project at the Wellcome Trust Sanger Institute (Hinxton, UK) and the Center for Molecular Therapeutics, Massachusetts General Hospital Cancer Center (Boston, USA). The expression profiles contained the expression of 12,687 genes of 1,019 cell lines (28).

Cell culture. The human poorly differentiated GC AGS cell line (the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) was cultured in F12 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml-1 penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated at 37˚C with 5% CO_2 in humidified air.

Immunocytochemistry staining (ICC). $1x10^6$ AGS cells were seeded onto glass coverslips and cultured in 37˚C humidified air overnight. Cells were fixed with 4% paraformaldehyde for 30 min. The following steps were completed according to the manufacturer's protocol of a rabbit polymer detection kit (cat. no. PV6001; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). The details are as follows: Cells were incubated with endogenous peroxidase blockers (included in the kit) for 10 min at room temperature, the slides were probed with an rabbit anti-human NICD antibody (used for recognizing the active form of the Notch1 receptor, exposed following cleavage by γ-secretase; 1:100 dilution; cat. no. 07-1231; EMD Millipore, Billerica, MA, USA), at 4˚C overnight and followed by incubation with horseradish peroxidase (HRP)-labeled goat anti rabbit IgG polymer (included in the kit) at room temperature for 20 min. Finally, slides were stained by diaminobenzidine (DAB; cat. no. ZLI-9017; Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 3 min and hematoxylin (cat. no. ZLI-9610; Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 1 min. PBS was used to replace the primary antibody in for the negative control. The slides were examined under a fluorescence microscope (BX-50; Olympus Corporation, Tokyo, Japan).

Western blot analysis. Whole-cell lysates were prepared from human specimens or AGS cells using cultured cell protein extraction reagent (cat. no. AR0103; Boster Biological Technology Co., Ltd., CA, USA) or mammalian tissue protein extraction reagent (cat. no. AR0101; Boster Biological Technology Co., Ltd.). The concentration of proteins were determined by using BCA Protein Assay kit (cat. no. P0012; Beyotime Biotech, Nantong, China). 80 μg protein were separated using a 10% gel and SDS-PAGE and then transferred to polyvinylidene fluoride membranes (EMD Millipore). Subsequent to blockage of non-specific binding sites with 5% non-fat milk at room temperature for 90 min, the membranes were incubated with rabbit anti-NICD antibody (1:500), rabbit anti-HES1 (1:500 dilution; cat. no. ab71559; Abcam, Cambridge, UK), rabbit anti-HES5 (1:500 dilution; cat. no. ab194111; Abcam), rabbit anti-GAPDH antibody (1:500 dilution; cat. no. AB-P-R 001; Hangzhou Goodhere Biotechnology Co., Ltd., Hangzhou, China) or rabbit anti-β-actin antibody (1:500 dilution; cat. no. Ab8227; Abcam) at 4˚C overnight. Following washing in TBST three times, membranes were incubated at room temperature for 60 min with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:3,000 dilution; cat. no. ZB-2301; Zhongshan Golden Bridge Biotechnology Co., Ltd.) and detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). Signal intensities were quantified by Quantity One v4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH and β-actin were used as loading controls.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from AGS cells treated with DAPT (20 μM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), NS-398 (50 μM; Sigma-Aldrich), DAPT+NS-398 or dimethyl sulfoxide for 48 h using TRIzol® (cat. no. 15596026; Thermo Fisher Scientific, Inc.), and then RNA was reversed to cDNA by using M-MLV Reverse

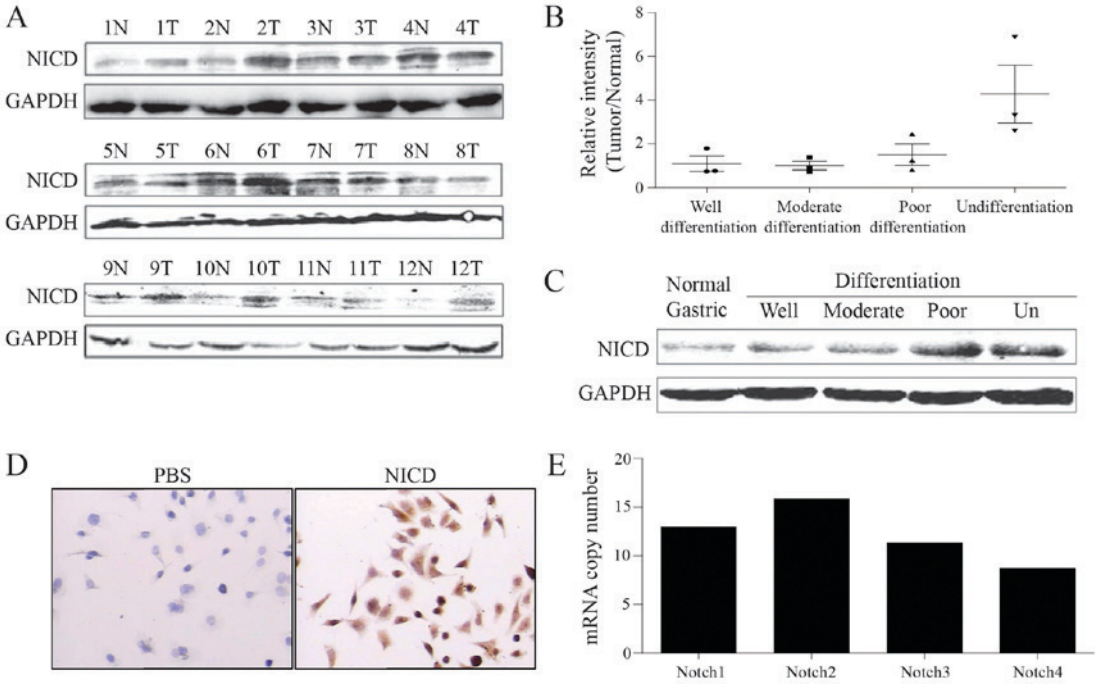

Figure 1. Levels of NICD in GC. (A) Level of NICD proteins in GC specimens. (B) Densitometric data from (A). (C) Level of NICD in different differentiated GC tissues. Normal gastric tissue was collected from the healthy patients by gastroscopy. (D) Representative images from immunohistochemical analysis of AGS cells. NICD is expressed in the nucleus of the AGS cell line (magnification, x400). (E) Data from Genomics of Drug Sensitivity in Cancer database. NICD, Notch1 intracellular domain; GC, gastric cancer; N, normal gastric tissue; T, gastric cancer tissue.

Transcriptase (cat. no. 28025021; Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was performed using iQ™ SYBR® Green Supermix (cat. no. 170-8882; Bio-Rad Laboratories, Inc.) according to manufacturer's protocol. The thermocycling conditions for PCR amplification were as follows: 95˚C for 2 min (pre-denaturation), 40 cycles of 95˚C for 15 sec (denaturation) and 60˚C for 30 sec (annealing and elongation), using the following primers: HES1 forward, 5'-ACACGACAC CGGATAAACCAA-3' and reverse, 5'-CGAGTGCGCACC TCGGTA-3'; and GAPDH forward, 5'-TCCCATCACCAT CTTCCAGG-3' and reverse, 5'-GATGACCCTTTTGGCTCC C-3' (GeneCore BioTechnologies Co., Ltd., Shanghai, China). The relative genomic copy number was calculated using the comparative Cq method (29). GAPDH mRNA levels were measured as a housekeeper gene for normalization of HES1 mRNA expression values. The fold change from control group was set at 1-fold.

Cell growth assay. AGS cells ($8x10^3$ cells/well) were plated into 96-well plates in triplicate, and then treated with DAPT (5, 10 and 20 μM) or NS-398 (25, 50 and 100 μM). Cell survival rate was assessed at 12, 24, 48 or 72 h following treatment using a commercial Cell Counting kit (CCK8; cat. no. C0037; Beyotime Institute of Biotechnology, Haimen, China) according to the protocol of the manufacturer.

Cell apoptosis assay. A cell apoptosis assay was conducted by flow cytometry with Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (cat. no. 556547; BD Biosciences, San Jose, CA, USA). AGS cells ($4x10^5$ cells/well) were plated onto 6-well plates. Following attachment at 37˚C overnight, cells were treated with 50 μM NS-398, 20 μM DAPT or 50 μM NS-398 combined with 20 μM DAPT for 48 h. Subsequent to dissociation and centrifugation at 4˚C and 1,000 x g for 5 min, cells were resuspended in combined buffer solution and double-stained with Annexin V-FITC and propidium iodide. Apoptosis was measured by using flow cytometry (BD Biosciences) and analyzed by using WinMDI 2.9 analysis software (30).

Statistical analysis. All experiments were repeated in triplicate. The data were processed by the SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA), and presented as means ± standard deviation. A one-way analysis of variance with post hoc contrasts using Dunnett's test was used to assess statistical significance of difference between treatment groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Poorly differentiated GC expresses increased levels of NICD. Previous study has indicated that Notch signaling was closely associated with GC (8-22). Western blot analysis was used to detect NICD protein levels in 12 GC specimens. The data indicated that the NICD amplification rate in poor differentiation GC was increased compared with well-differentiated GC (Fig. 1A and B; Table I). The level of NICD proteins in GC specimens of different differentiation levels and normal gastric epithelial tissues was additionally analyzed through western blot analysis. The data demonstrated that the poorer the level of differentiation of the GC tissue, the higher the level of NICD proteins it possessed (Fig. 1C). In the poorly differentiated GC AGS cell line, NICD proteins were also expressed

in a high level (Fig. 1D). GDSC database was employed, and it was identified that Notch1 was also expressed in AGS cells (Fig. 1E). All the aforementioned results suggested that the level of NICD may be associated with the differentiation of GC.

γ-secretase inhibitor DAPT inhibits growth of poorly differentiated GC AGS cell line. γ-secretase serves a key function in the Notch signal pathway: γ-secretase blockage may suppress the cleavage of Notch receptor and block signaling transduction (1-3). The data indicated that the γ-secretase inhibitor DAPT downregulated the level of NICD in a dose-dependent manner (Fig. 2A). The ICC results indicated that DAPT may also decrease the level of nuclear NICD (Fig. 2B). Considering the biological function of NICD and Notch1 signaling, a CCK8 kit was used to detect the growth inhibition of DAPT in the poorly differentiated GC AGS cell line. Results suggested that DAPT may decrease the growth of AGS in a dose- and time-dependent manner. Following treatment with DAPT for 48 and 72 h, the half-maximal inhibitory concentration was 8.8±1.1 and 14.6±5.3 μM, respectively (Fig. 3A and B).

DAPT induces apoptosis of AGS. Notch signal pathway may also regulate cell apoptosis. In the present study, flow cytometry was applied for the investigation of the effect of DAPT on apoptosis in AGS cells. The data suggested that DAPT may significantly induce cell apoptosis in AGS cells in a dose-dependent manner. A total of 20 μM DAPT caused levels of early and late apoptosis of ~14.55 and 36.16%, respectively (Fig. 4A and B).

COX-2 inhibitor NS-398 may enhance the inhibitory effect of DAPT to Notch1 signaling. A number of previous studies have demonstrated the association between COX-2 and Notch signaling: It has been suggested that Notch signal may upregulate the expression of COX-2 (20,26). A small number of studies have focused on the effect of COX-2 on Notch signaling: The downregulation of NICD, HES1 and HES5 indicated the inhibition of Notch1 signaling (31-33). HES1 and HES5 proteins were employed to reveal the effect of the treatment in the present study to Notch1 signaling. The present study identified that 50 μM NS-398 may significantly downregulate the expression levels of NICD and its target genes HES1 and HES5 (Fig. 5A and B). Additional data indicated that combination treatment of NS-398 and DAPT may result in decreased expression levels of NICD, HES1 and HES5 compared with DAPT treatment alone (Fig. 5A and B). In addition, HES1 was selected and detected by RT-qPCR. The results of this analysis suggested that combination treatment may also inhibit Notch1 signaling to a greater extent compared with single-agent treatment with DAPT or NS-398 (Fig. 5C). Therefore, reduced NICD, HES1 and HES5 protein expression suggests that the inhibition of growth may be attributed to Notch1 signal blockage. These outcomes suggested that COX-2 inhibition may significantly increase the Notch1 blockage level in AGS cells, and that patients with GC may benefit from this combination treatment.

Combination treatment of DAPT and NS-398 notably suppresses growth in AGS cells. Finally, the antitumor activity of this combination plan in AGS cells was assessed by employing CCK8 and flow cytometry. Compared with single drug treatment, combination treatment induced an increased level of growth inhibition in AGS cells following treatment for 12, 24 and 48 h (Fig. 6A). Data from the flow cytometry analysis indicated a similar synergistic effect, as the rate of cell apoptosis caused by the combination treatment group was increased compared with the monotherapy (Fig. 6B).

Table I. Rate of high NICD expression in gastric cancer tissues.

Gastric cancer subtype	Rate of high NICD expression, %
Undifferentiated	100.00 (3/3)
Low differentiation	66.70 (2/3)
Moderate differentiation	33.30 (1/3)
Well-differentiated	33.30 (1/3)

NICD, Notch1 intracellular domain.

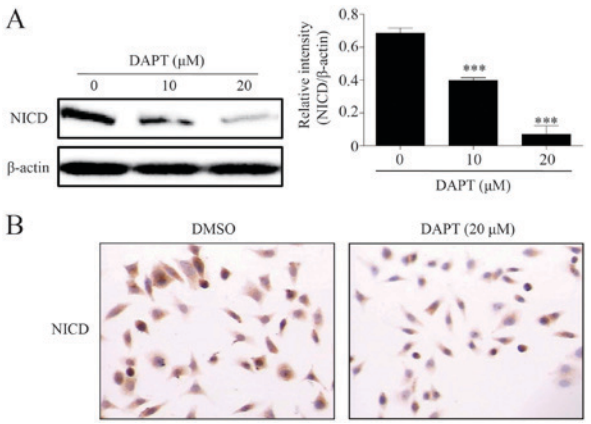

Figure 2. DAPT downregulated NICD level in AGS cells. (A) Western blot analysis detected the effect of DAPT in AGS cells. Cells were treated with DAPT (10 and 20 μM) for 48 h, and NICD level was investigated. β-actin was used as a loading control. ***P<0.001 vs. 0 μM group. (B) Immunocytochemistry in DAPT-treated AGS cells. Cells were treated with DAPT for 48 h and brown staining indicates NICD expression (magnification, x400). DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; NICD, Notch1 intracellular domain.

Discussion

Despite a decline in the overall incidence, gastric carcinoma remains a critical global health problem (7). Previous data have indicated that the tumor cellular differentiation degree has a close association with the prognosis of patients with GC (34-37). Certain studies indicated that the poorly differentiated adenocarcinoma subtype progressed to lymph node metastasis more easily, and was demonstrated to be poor prognostic factors in patients with gastric cancer with bone metastases (35,36). The rate of poor differentiation was significantly increased in younger cases compared with older patients, particularly in young female patients (37). The degree of cell differentiation is also an important predictor of survival in advanced GC (38). Therefore, exploration of the molecular mechanisms and

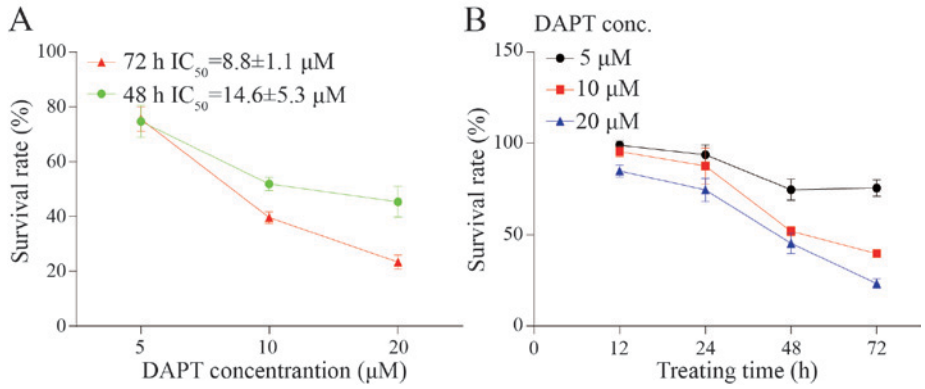

Figure 3. DAPT suppressed the growth of AGS cells. (A) DAPT inhibited the growth of AGS cells in a dose-dependent manner. The cell viabilities of AGS cells treated with DAPT was measured by a Cell Counting kit 8. The concentration of DAPT was 5, 10 and 20 μM. Cells were treated with DAPT for 48 or 72 h. (B) DAPT inhibited the growth of AGS cells in a time-dependent manner. Cells were treated with various concentrations of DAPT (5, 10 and 20 μM) for 12, 24, 48 or 72 h. DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester.

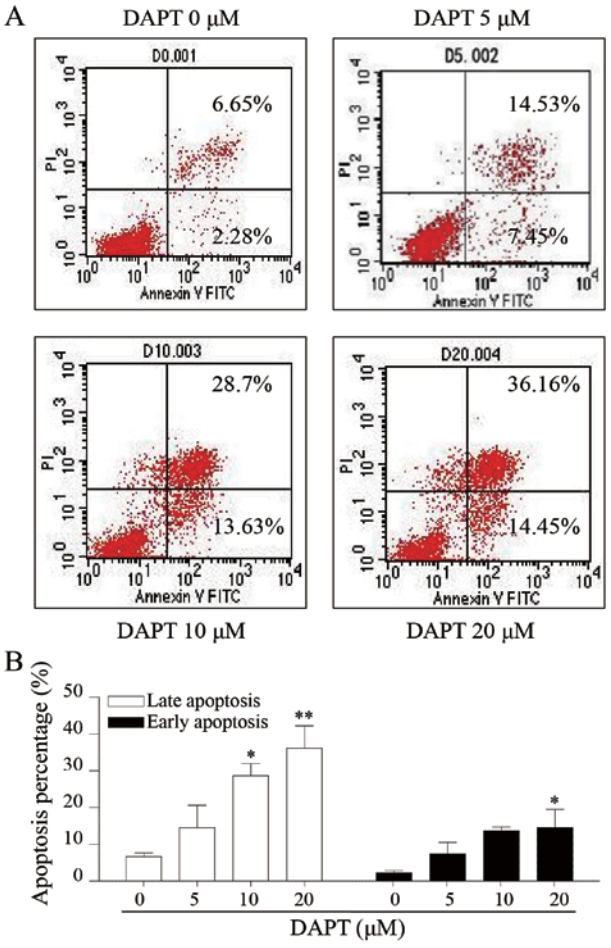

Figure 4. DAPT induced apoptosis in AGS cells. (A and B) AGS cells were treated with DAPT (5, 10 and 20 μM) for 48 h, and flow cytometry was used to detect apoptosis. *P<0.05 and **P<0.01 vs. control group. DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester.

identification of the phenotype of GC differentiation will facilitate the identification of novel targets and the development of personalized therapies in GC.

A previous study indicated that the differentiation induced by modulating Musashi/Numb/Notch signaling in cancer cells may be a novel therapeutic target for advanced leukemia and other solid carcinomas (39). Multiple studies have demonstrated that Notch signaling is likely to serve an oncogenic role in several cancer cell types, as it favors development and differentiation in various cell types, including myeloid cells or secretory cells (40,41). Notch1 and Notch2 inhibited by hypoxia may induce neuroendocrine differentiation in prostate cancer (42). In addition, Notch signaling may induce aberrant differentiation in several types of cancer, including pancreatic cancer, medulloblastoma and mucoepidermoid carcinoma (43). In the present study, a poorly differentiated GC cell line was employed to reveal the potential association between Notch1 signaling and GC differentiation. Convincing evidence indicated that Notch1 and NICD was highly expressed in the AGS cell line (10,44). An additional study indicated that there was no significant difference between the levels of Notch2 in the normal gastric epithelial AGS and GES-1 cell lines (45). For Notch3 and Notch4, there have been no studies that have observed a difference in Notch3 or Notch4 expression between the AGS cell line and normal gastric cells. However, Ji *et al* (17) also detected the expression of Notch3 and Notch4 in AGS cell lines. The present study examined the GDSC database (28) and it was confirmed that all 4 Notch receptors were expressed in AGS cells It was also observed that NICD was expressed in gastric cancer tissues. Furthermore, the present study identified that the high level of NICD was significantly associated with poor differentiation. In addition, the poorly differentiated human gastric adenocarcinoma AGS cell line also harbored amplified NICD protein (Fig. 1). These results suggested that the therapeutic potential of NICD to treat patients with poorly differentiated GC.

Tumors with aberrantly activated oncogenes were frequently dependent on oncogene-associated signaling pathways (46). In the present study, when cells were treated with DAPT, their growth and survival were markedly inhibited. These data suggested that the Notch1 pathway may be a major signaling pathway for survival of AGS cells, and a potential target for poorly differentiated GC. Nevertheless, specific targeted therapies often require specific patient conditions in order to be effective. For example, human epidermal growth factor receptor 2 (HER2) expression is a validated predictive biomarker for anti-HER2 target therapy, and patients with lung

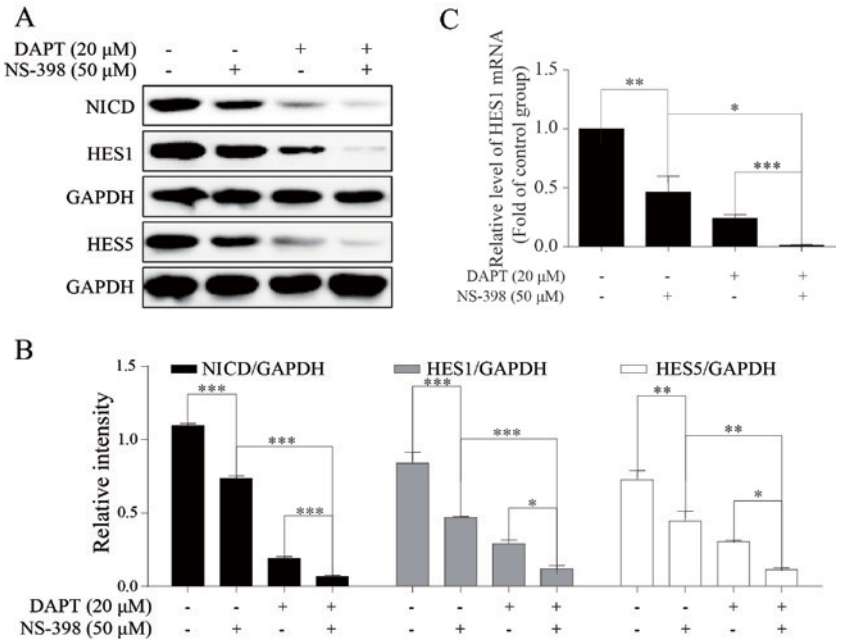

Figure 5. Effect of DAPT and NS-398 on NICD expression in AGS cells. (A and B) AGS cells were treated with drugs as indicated for 48 h. Levels of NICD, HES1 and HES5 were analyzed by western blot analysis. Quantitative analyses of each average gray value of the preparations following normalization with that of individual GAPDH levels. The average gray values presented as mean ± standard deviation (n=3). (C) The mRNA level of HES1 was evaluated by reverse transcription quantitative polymerase chain reaction. AGS cells were treated with DAPT (20 μM), NS-398 (50 μM), DAPT + NS-398 or dimethyl sulfoxide for 48 h, then RNA was extracted and detected. Data are the means of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001. DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; NICD, Notch1 intracellular domain; HES, Hes family BHLH transcription factor.

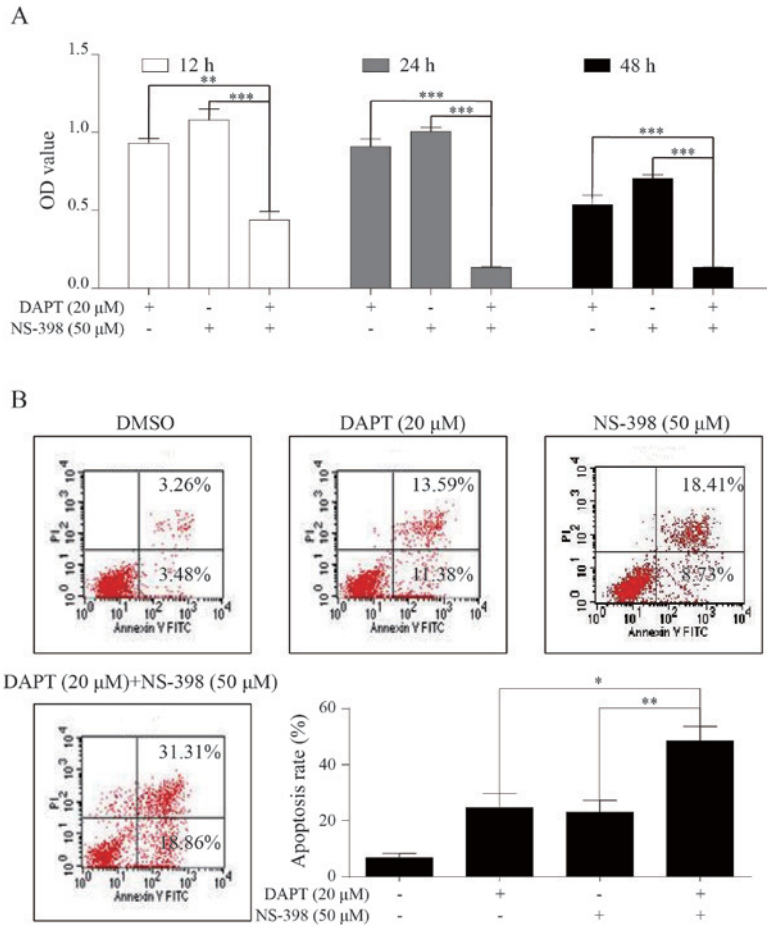

Figure 6. Antitumor activity of combination treatment with DAPT and NS-398 in AGS cells. (A) Combined treatment of DAPT and NS-398 significantly increased the growth inhibition of AGS cells compared with monotherapy. Cells were treated as indicated for 12, 24 or 48 h. Cell viability was measured by CCK8. **P<0.01 and ***P<0.001. (B) Combined treatment of DAPT and NS-398 induced an increased level of apoptosis compared with monotherapy. Cells were treated as indicated for 48 h. *P<0.05 and **P<0.01. DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester.

cancer who possessed the FGFR1 gene amplification benefitted more from FGFR inhibitors than those not exhibiting the FGFR1 gene amplification (47,48). Consequently, a precise genotype classification of patients with GC may improve the success rate of Notch-inhibiting treatment.

Amplified COX-2 was an independent prognostic factor of GC (25). Previous studies have primarily focused on the effect of Notch signaling on COX-2 (20,26). In the present study, NS-398 treatment was used to block COX-2 activity, and NS-398 could significantly inhibit the growth of GC cells. It was also identified that NS-398 also markedly downregulated the level of NICD and HES1. However, the underlying mechanism for the crosslinking of these two pathways is not clear, and merits additional study. Concomitant with the volume of evidence that associates the activation of Notch signaling with oncogenesis, there is much supporting evidence for a tumor-suppressive role of Notch in certain situations: Guo *et al* (49) indicated that Notch2 may negatively regulate cell invasion by inhibiting the Phosphoinositide 3-kinase/Protein kinase B signaling pathway in gastric cancer. Zhou *et al* (50) also suggested that Notch1 regulated Phosphatase and tensin homolog expression through CBF-1, and served a pro-apoptotic role in gastric cancer cells (50). The present study only identified that combination therapy of NS-398 and DAPT demonstrated a more improved antitumor activity in poorly differentiated GC cells than monotherapy of NS-398 or DAPT; this observation was not verified in other GC cell lines, for example in MKN7, a well differentiated GC cell line (51). Therefore, the effect of DAPT and NS-398 in well-differentiated GC cell lines requires additional study. Taken together, COX-2 inhibition in turn suppressed Notch1 signal pathway transduction, and the combination treatment of γ-secretase and COX-2 inhibitors may have therapeutic potential in patients with poorly-differentiated GC.

In summary, the expression of NICD was associated with the differentiation of GC. A limitation of the present study was the small sample size, therefore, additional studies concerning the association between NICD and GC are required. In addition, combined with NS-398, treatment of DAPT supported the novel strategies for cancer therapy in patients with poorly-differentiated GC.

Acknowledgements

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