Notch inhibition suppresses nasopharyngeal carcinoma by depleting cancer stem-like side population cells

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Abstract. The cancer stem cell (CSC) is responsible for the initiation, proliferation and radiation resistance. Side population (SP) cells are a rare subset of cells enriched with CSCs. The targeting of key signaling pathways that are active in CSCs is a therapeutic approach to treating cancer. Notch signaling is important for the self-renewal and maintenance of stem cells. Our previous studies demonstrated that downregulation of Notch signaling could enhance radiosensitivity of nasopharyngeal carcinoma (NPC) cells. In this study, we found that Notch signaling was highly activated in SP cells compared with that of non-SP (NSP) cells of NPC. Therefore, Notch inhibition could reduce the proportion of SP cells. As SP cells decreased, proliferation, anti-apoptosis and tumorigenesis were also decreased. This study shows that Notch inhibition may be a promising clinical approach in CSC-targeting therapy for NPC.

Introduction

Nasopharyngeal carcinoma (NPC) arises from epithelial cells that cover the surface and line of the nasopharynx. NPC shows marked variations between ethnic populations. The incidence of NPC is higher in China, particularly in some regions of Southern China with an incidence rate of up to 54.7 per 100,000 (1). Symptoms related to the primary tumor include epistaxis, nasal obstruction, otitis media and tinnitus. Because nasal and aural symptoms are non-specific and full clinical examination of the nasopharynx is not easily performed, the majority of NPC patients are diagnosed after the tumor reaches advanced stages. Therefore, the treatment effect of the majority of patients with NPC is not ideal.

The cancer stem cell (CSC) theory proposes that a small subset of cells is responsible for the initiation, proliferation and metastasis of cancer. CSCs generate a heterogeneous population of cells that constitute the cancer (2). The existence of CSCs and their ability to self-renew, differentiate into multiple lineages, resist apoptosis and extensively proliferate results in these cells being particularly detrimental. Thus far, CSCs have been distinguished from the bulk-tumor population by their expression pattern of cell surface proteins (e.g., CD24, CD44 and CD133) and cellular activities such as the efflux of Hoechst dye (3). The latter method, which we exploit in our studies, is the so-called side population (SP) identification and separation. The SP phenotype has been proven to be invaluable for stem cell isolation in the absence of definitive cell surface markers (4-6).

Targeting key signaling pathways that are active in CSC self-renewal is a therapeutic approach to treating cancer (7). Notch signaling is important for the self-renewal and maintenance of stem cells and is receiving increased attention as a target to eliminate CSCs (8). It has been found that the Notch signaling pathway is highly activated in NPC (9). Another study found that Notch signaling is mainly activated in human primary NPC cells that express embryonic stem cell proteins (10). In our previous study, we demonstrated that downregulation of Notch signaling by the Notch inhibitor N-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester (DAPT) could enhance radiosensitivity of NPC cells (11). On the other hand, CSCs contribute toward radiation resistance (12-14). Thus, we hypothesized that the Notch signaling pathway may be crucial to the maintenance of CSCs in NPC, and Notch inhibition may suppress NPC by depleted SP cells. To understand the underlying mechanism, we investigated the expression and activation of Notch signaling in SP and non-SP (NSP) cells from the NPC cell lines, and examined the effect of DAPT on the proportional changes of SP cells and on the inhibition of NPC cells.

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Materials and methods

Cell culture. Human NPC cell lines (CNE1 and CNE2) were obtained from the Xiangya Central Experiment Laboratory, Central South University, China. Cells were cultured in RPMI-1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Sijiqing, China), 100 U/ml penicillin G and 100 U/ml streptomycin (Gibco, Carlsbad, CA, USA) under standard conditions.

Proliferation assay. The effect of Notch inhibition on cell proliferation was determined by an MTT assay, as described in our previous studies (15). In brief, CNE1 cells were treated with various concentrations of DAPT or the same volume of DMSO (control) for 1-3 days at 37˚C. For the proliferation assay, the MTT dye was added to each well and incubated for 2 h at 37˚C according to the manufacturer's instructions (GenMed, China). Cell proliferation was expressed as the optical density (OD) of each well.

SP cell analysis and isolation. SP cell analysis and isolation were performed by fluorescence-activated cell sorting (FACS) (Beckman Coulter, Epic Altra, New York, NY, USA) as described elsewhere (16). Before SP cell analysis, cells were pretreated with 10 μmol/l DAPT or DMSO for 1-3 days. Cells at 80% confluence were washed twice with calcium/magnesium free phosphate-buffered saline (PBS), detached with 0.25% trypsin-EDTA (Gibco) and suspended at $1x10^6$ cells/ml in PBS supplemented with 2% FBS. Then, cells were incubated with 5 μg/ml Hoechst 33342 (Sigma, St. Louis, MO, USA) either alone or with 100 μg/ml verapamil (Sigma) at 37˚C in the dark for 70 min. Cells were washed, centrifuged and resuspended in cold PBS supplemented with 2% FBS, and then 1 μg/ml propidium iodide (PI; Sigma) was added. All cells were kept at 4˚C in the dark before FACS using dual wavelength analysis. Verapamil is traditionally used as a guiding parameter to determine the boundary between SP and NSP cells. Isolated cells were analyzed for purity, which was typically >99.5%.

Soft agar colony formation assay. Before the soft agar colony formation assay, CNE1 and CNE2 cells were pretreated with DAPT or DMSO for 2 days. The soft agar colony formation assay was performed in 12-well plates using a soft agar colony formation assay kit (GenMed) according to the manufacturer's instructions. DAPT- and DMSO-pretreated CNE1 and CNE2 cells (2,500 per well) were plated in the soft agar gel with 10 μmol/l DAPT added to the nutrient solution of the continuously treated cells. Every cell group (treated, continuously treated and DMSO) were plated in triplicate wells. Cells were maintained at 37˚C in a humidified incubator. On Day 14 after seeding, colonies were washed twice with PBS and stained with MTT dye. After washing out the dye, colonies >0.1 mm in diameter were counted under a microscope in five selected fields (left upper, right upper, left inferior, right inferior and central).

Apoptosis assay. The percentage of apoptotic cells was assessed using the Annexin V-FITC/PI method with FACS as described in our previous studies (15). CNE2 cells were randomly assigned into three groups: DMSO, 48 and 72 h. Before the apoptosis assay, CNE2 cells were incubated in 35-mm dishes with 10 μmol/l DAPT or the same volume of DMSO for 48 and 72 h. Then, Annexin V-FITC/PI staining of the cells was performed according to the manufacturer's instructions (Biouniquer, China).

In vivo tumor formation assay. Ethics approval for this protocol was obtained from The Institutional Animal Care and Use Committee at Fudan University (SYXK20090082). All animal experiments were performed in accordance with institutional guidelines for animal welfare. Five-week-old female immune-deficient nude mice (BALB/C-nu) were purchased from Shanghai Sino-British SIPPR/BK Lab Animal Co., Ltd., and housed in microisolator individually ventilated cages with access to water and food. Mice were randomly divided into DMSO- and DAPT-treated groups (n=6, each group). CNE2 cells were pretreated with 10 μmol/l DAPT or DMSO for 48 h, then $2x10^6$ CNE2 cells were subcutaneously injected into the right flank of the mice. After 4 weeks, xenografts were removed and processed into paraffin-embedded sections.

Real-time PCR. Total RNA was extracted from SP and NSP cells isolated from CNE2 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and converted to cDNA using MMLV reverse transcriptase (Promega, Madison, WI, USA). The primers and the methods of real-time PCR were described in our previous studies (15).

Western blot analysis. Western blotting was performed as described elsewhere (17). Briefly, the concentration of protein extracted from SP and NSP cells isolated from CNE2 cells was determined by the Lowry method. Equal amounts of protein were separated by 10% SDS-PAGE and electrophoretically transferred onto polyvinyldifluoridine membranes (Millipore, Billerica, MA, USA). Rabbit anti-human Notch1 intracellular domain (NICD) antibody (1:500; Cell Signaling Technology, Danvers, MA, USA) and mouse anti-human Hes-1 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to detect the expression of cleaved Notch1 and Hes-1. β-actin or Erk were used as an internal control.

Statistical analysis. All experimental procedures were performed in triplicate. Data from *in vivo* tumor formation assays were analyzed for statistical significance using χ^2 with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Other data were analyzed for statistical significance using analysis of variance (ANOVA) or t-tests with the same software. A P-value <0.05 was considered statistically significant.

Results

DAPT inhibits NPC cell proliferation. In our previous studies (15), we found that DAPT could inhibit CNE2 growth and the lowest applicable concentration was 10 μmol/l. CNE2 is a poorly differentiated squamous cell carcinoma line. To further determine whether the Notch inhibitor (DAPT) could inhibit NPC cell proliferation and the lowest applicable concentration, we examined the effect of DAPT on the proliferation of CNE1 cells. CNE1 is well-differentiated squamous cell carcinoma cell line. CNE1 cells were also treated with various

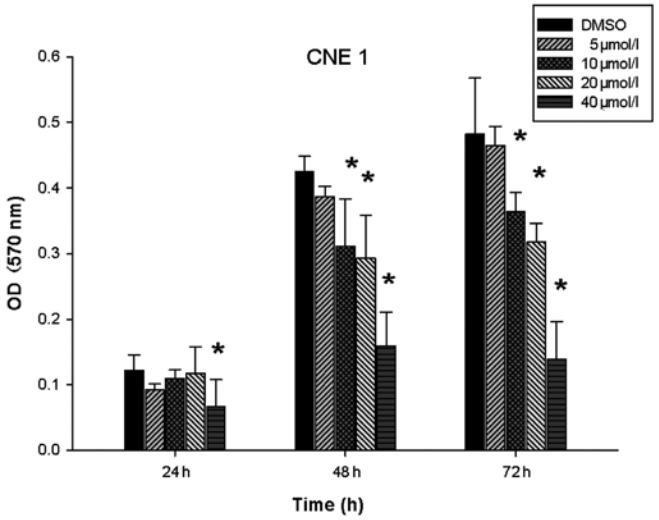

Figure 1. DAPT inhibits the proliferation of the NPC cell line CNE1. CNE1 cells were treated with DAPT at the indicated concentrations for 24-72 h. Control cells were treated with the same volume of DMSO. Each bar represents the mean and standard deviation (SD) of three separate experiments. *P<0.05.

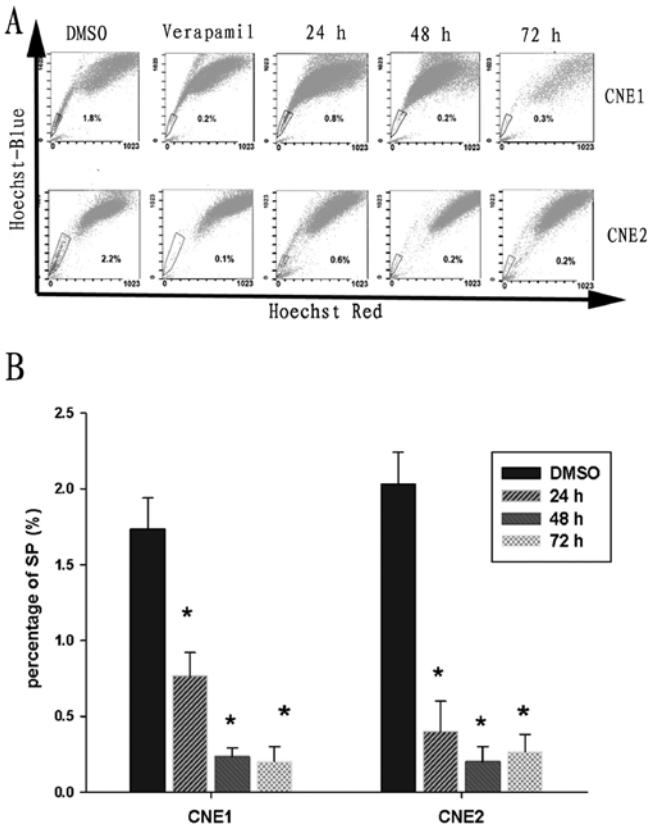

Figure 2. DAPT reduces the proportion of SP cells in CNE1 and CNE2 cell lines. CNE1 and CNE2 cells were treated with DMSO or 10 μmol/l DAPT for 24, 48 and 72 h. The proportion of SP cells in the two cell lines was determined by FACS in the presence or absence of verapamil. (A) Representative examples of FACS analysis. (B) After DAPT treatment for 24, 48 or 72 h, the percentage of SP cells significantly decreases in the two cell lines. Results are shown as the mean ± SD from three samples. *P<0.05.

DAPT concentrations (0, 5, 10, 20 or 40 μmol/l). The MTT assay revealed that DAPT could inhibit CNE1 proliferation in a dose-dependent manner. For CNE1 cells, 40 μmol/l DAPT inhibited proliferation after 24 h (P<0.05), and 10 μmol/l DAPT inhibited proliferation after 48 h (P<0.05, Fig. 1). Similarly to the results in CNE2 cells, 10 μmol/l was the lowest concentration that inhibited CNE1 proliferation. Thus, DAPT inhibited proliferation in poorly as well as in well-differentiated NPC cells.

DAPT depletes SP cells in CNE1 and CNE2 cell lines. CSC theory states that CSCs are responsible for cancer growth. We hypothesized that DAPT inhibition of NPC cell proliferation may be mediated by decreasing the number of CSCs. Therefore, we analyzed the percentage of SP cells after the two NPC cell lines were pretreated with DAPT. For CNE1 cells, the percentage of SP cells was 1.73±0.21%, while the percentage of SP cells in the CNE2 cell line was 2.03±0.21%. After pretreatment with 10 μmol/l DAPT for 24 h, the percentage of SP cells was significantly decreased in both cell lines based on FACS analysis. Few SP cells were detected in CNE1 and CNE2 cell lines after pretreatment with DAPT for 48 h (Fig. 2). Taken together, these data suggest that Notch inhibition significantly depletes SP cells in the CNE1 and CNE2 cell line.

DAPT reduces colony formation and induces apoptosis in NPC cells. It is well known that differentiation and the anti-apoptotic ability of CSCs is higher than those of non-CSCs, and SP cells of NPC demonstrate higher colony formation ability (16). Thus, after the proportion of SP to NSP cells decreases, the colony formation and anti-apoptotic abilities of NPC cells may also decrease. After CNE1 and CNE2 cells were pretreated with DAPT (10 μmol/l) for 2 days, a soft agar colony formation assay was performed. The colony formation ability of DAPT-pretreated cells was significantly decreased (P<0.05, Fig. 3C and D). After the two cell lines were continuously treated with DAPT, there were few colonies formed. The colony formation assay results were easily observed with the naked-eye and under a microscope (Fig. 3A and B). We further examined whether reduced colony formation was due to apoptosis induced by DAPT. In our previous studies (15), we found that DAPT had no obvious impact on the apoptotic fraction when the cells were only treated for 2 days. So, we investigated the apoptotic fraction in the presence of DAPT for a longer time. The result demonstrated that DAPT had an obvious impact on the percentage of apoptotic cells after DAPT pretreatment for 72 h (P<0.05, Fig. 3E). Therefore, DAPT induces apoptosis in NPC cells and decreases colony formation in continuously treated groups, which may be associated with apoptosis induced by DAPT.

DAPT reduces tumor formation of NPC cells in immune-deficient nude mice. Previous studies show that SP cells in NPC possess higher tumorigenicity *in vivo* compared with that of NSP cells (16). In the colony formation assay, we showed that DAPT inhibited NPC cell proliferation *in vitro*, but the cytotoxic effect of 10 μmol/l DAPT was rather limited after treatment for 2 days. In *in vivo* tumor formation assays, only 1 out of 6 mice formed xenografts in DAPT-treated groups. In contrast, 5 out of 6 mice formed xenografts in DMSO-treated groups (P<0.05, Fig. 4A

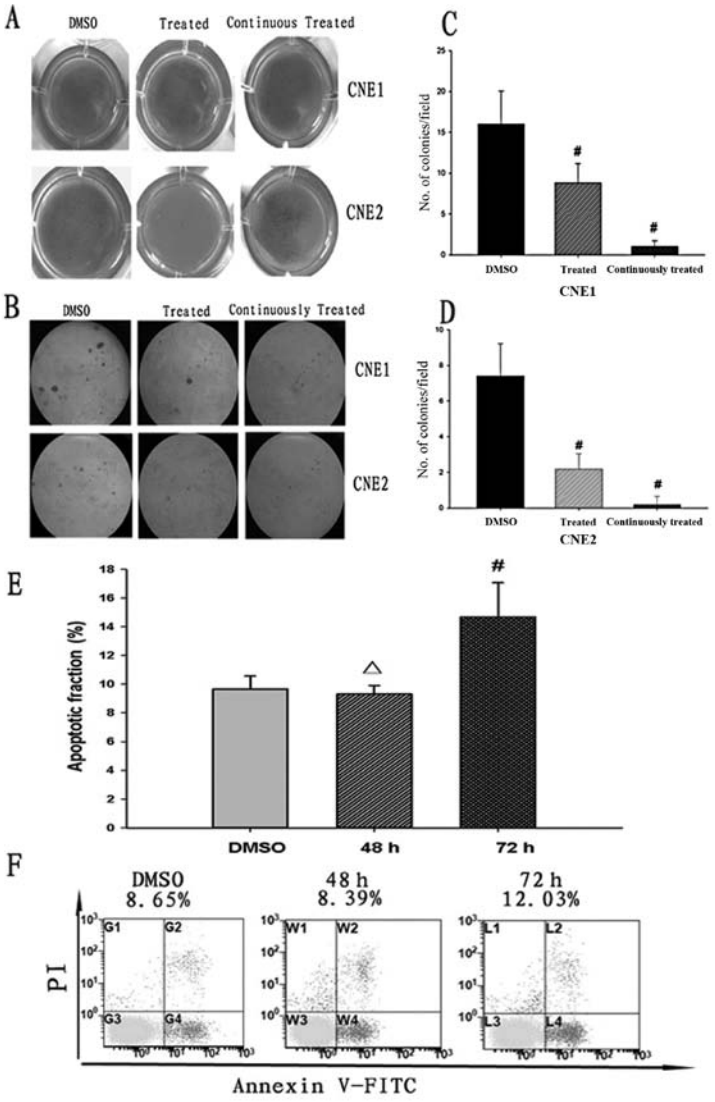

Figure 3. DAPT reduces colony formation and induces apoptosis in NPC cells. (A) Macro observation of colonies formed by experimental groups. (B) Representative microscopic images of colonies. DAPT significantly reduces the clonogenic potential of CNE1 (C) and CNE2 (D) cell lines. DAPT induces apoptosis after treatment for 72 h, but not 48 h (E). Results are shown as the mean ± SD from three samples. $^{\#}P<0.05$; $^{\Delta}P<0.05$. (F) Representative examples of FACS analysis.

and B). Histological analysis suggested that all xenografts showed the distinct pathological features of poorly differentiated cells (Fig. 4C and D). These results demonstrate that DAPT decreases tumor formation and reduces the tumorigenicity of NPC cells.

Notch signaling is highly activated in SP cells, but not in NSP cells in the CNE2 cell line. To determine the underlying mechanism of DAPT-mediated depletion of SP cells, the activation status of Notch signaling in the SP and NSP cells of the CNE2 cell line was examined by real-time PCR and western blot analyses. NICD and Hes-1 are important signaling molecules in the Notch signaling pathway. NICD is the activated form of Notch1 and is cleaved by γ-secretase from the cell membrane. Hes-1 is a downstream target gene of Notch signaling. As shown in Fig. 4E-G, Notch1 and Hes-1 expression levels were higher in SP cells compared with those of NSP cells at both mRNA and protein levels. Combined with the above results, these data suggest that Notch signaling is required for the maintenance of SP cells.

Discussion

With the development of novel therapeutic technologies, the 5-year survival rate (SR) of NPC has improved, but in some regional areas the 5-year SR is still <50% and the 10-year SR is <30% (18). Thus, it is important to explore new strategies to decrease the mortality rates of NPC. CSCs are thought to contribute toward therapeutic resistance, recurrence and metastasis. If the proportion of CSCs could be decreased, then the mortality rates of cancer may also decrease. SP cells are a rare subset of cells enriched with CSCs. In a previous study, SP cells were isolated from NPC cell lines and found to possess high proliferative, self-renewal, differentiation and tumorigenic abilities compared with those of NSP cells (16). In the present study, we found that Notch signaling is highly

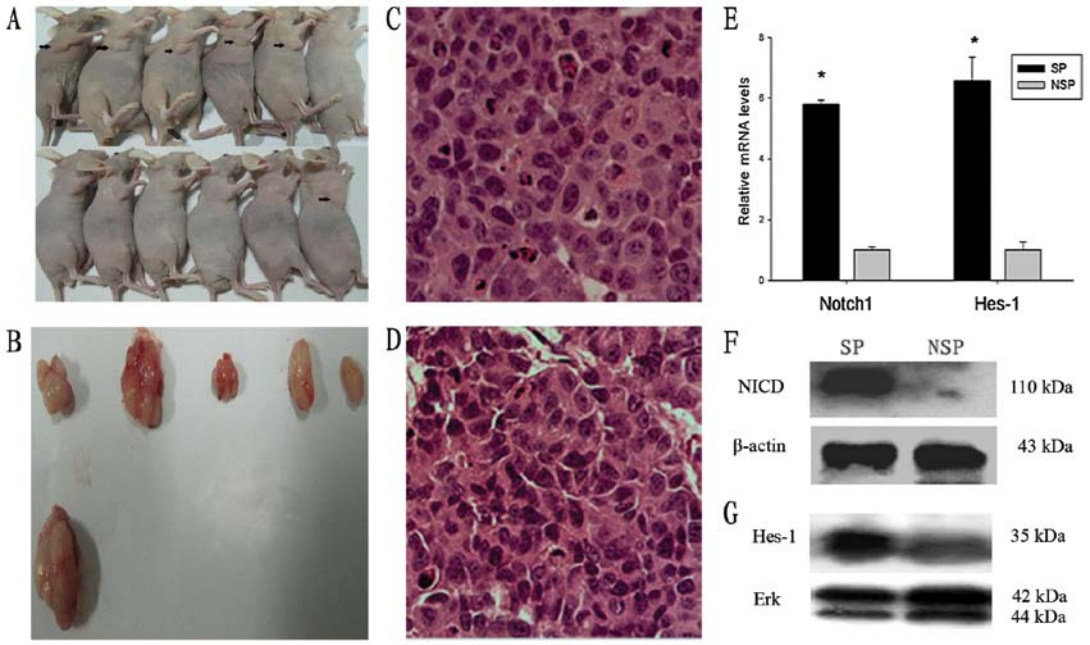

Figure 4. DAPT inhibits tumor formation and the Notch pathway in SP and NSP cells of CNE2. (A) Representative subcutaneous xenografts (arrows) produced by CNE2 cells. (B) Dissected xenografts from the two groups. Hematoxylin-eosin staining of xenografts derived from DMSO- (C) and DAPT-treated (D) groups (magnification, x400). (E) The expression of Notch1 and Hes-1 at mRNA levels was detected by real-time PCR in SP and NSP cells. Error bars represent the SD of three independent experiments. *P<0.05. Western blot analysis shows that SP cells express higher amounts of NICD (F) and Hes-1 (G) compared with those of NSP cells. β-actin or Erk were used as loading controls.

activated in SP cells compared with that of NSP cells in NPC cells. Therefore, Notch inhibition significantly reduces the proportion of SP cells in NPC cells. Additionally, the abilities of high proliferation, anti-apoptosis and tumorigenesis of NPC cells are inhibited with the decrease of SP cells.

Notch signaling plays a pivotal role in the regulation of numerous fundamental cellular processes, such as proliferation, stem cell maintenance and differentiation during embryonic and adult development (19,20). Dysfunction of Notch signaling can result in a variety of developmental defects and adult pathologies (21), including tumorigenesis. Notably, the Notch signaling pathway is frequently activated in several human malignancies such as glioma (22), breast cancer (23), colon cancer (24) and NPC (9,10). Subsequent studies have found that Notch signaling contributes toward the maintenance of CSCs (25-27). In the present study, we found that Notch signaling is highly activated in SP cells compared with that in NSP cells of NPC, which is consistent with previous studies (28-31).

The Notch receptor is a transmembrane protein expressed on the cell surface as a heterodimer with an EGF-like repeat-rich extracellular domain and an intracellular domain with a single pass transmembrane domain (19). Signaling is initiated by ligand-receptor binding, thereby inducing a series of cleavages named S2, S3 and S4. Among them, the S3/4 cleavage is an intramembranous cleavage mediated by presenilin-dependent γ-secretase, which results in translocation of the NICD into the nucleus. Nuclear NICD then interacts with the transcriptional factor CSL (known as CBF1/RBPJκ in mammals) to activate downstream target genes such as Hes-1 (32,33). γ-secretase is a multi-subunit enzyme with specificity for cleavage of intramembrane substrates such as Notch (34). Therefore, γ-secretase inhibitors (GSIs) can block Notch activation. The advantage of using GSIs is that all Notch receptors require γ-secretase for processing and signaling (35). siRNA can inhibit Notch signaling, but the approach cannot be easily used in the clinic. Therefore, in the present study, we used the GSI DAPT to inhibit Notch signaling and investigate the effects.

Based on the CSC theory, CSCs possess a capacity to self-renew and generate a certain number of CSCs, which maintains the balance between CSCs and non-CSCs (36). During this process, Notch and other signaling pathways, such as Hedgehog (Hh) and Wnt, are key factors in the regulation of self-renewal (2). In the present study, we found that Notch signaling is highly activated in CSCs. In our previous study, we found that DAPT inhibits Notch signaling in NPC cells (11). After the Notch signaling pathway is inhibited, CSC differentiation to non-CSCs increases and the percentage of CSCs decreases, which is similar to the effect of inhibiting the Wnt signaling pathway (37).

Notch inhibitors are not traditional cytotoxic drugs. In the present study, the cytotoxic effects of DAPT at 10 mol/l were rather limited. However, 10 μmol/l DAPT significantly inhibited the Notch signaling pathway and decreased the proportion of CSCs. After CSCs have been depleted in cell lines, it is plausible that proliferation, antiapoptosis and tumorigenesis of the cell lines will be impaired. Furthermore, we found that the volume of single tumors in DAPT-treated groups was similar to those of DMSO-treated groups in the *in vivo* tumor formation assays. These results demonstrate that Notch inhibition can only decrease the tumor formation of NPC cells, but has no effect on long-term tumor growth. Therefore, to maximize the efficacy of Notch inhibition, it will likely be necessary to administer a Notch inhibitor in combination with other treatments such as radiation therapy and other chemotherapies (29).

For NPC treatment, Notch inhibition combined with radiation therapy may be a good therapeutic approach. In our previous study, we found that Notch inhibition enhances the radiosensitivity of NPC cells (11).

In conclusion, our data indicate that Notch signaling is highly activated in SP cells compared with that of NSP cells in NPC. Thus, Notch inhibition significantly depletes SP cells. With the decrease of SP cells, proliferation, anti-apoptosis and tumorigenesis are also decreased. These results indicate that Notch inhibition may be a promising preclinical application in CSC-targeting therapy for NPC. Certainly, the application of Notch inhibition is not limited to NPC because the Notch signaling pathway is not only critical for the regulation of CSCs in NPC, but also of CSCs in other cancers.

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