Specific inhibitor of Notch-3 enhances the sensitivity of NSCLC cells to gemcitabine

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Abstract. Notch-3 is a receptor of the Notch signaling pathway and plays an important role in regulating self-renewal, differentiation and apoptosis in cancer cells. Overexpression of Notch-3 has been proved to be associated with resistance to gemcitabine (GEM) and poor patient prognosis for various malignant tumors. In the present study, two non-small cell lung cancer (NSCLC) cell lines, H1299 and A549, were induced with GEM for two months and then were treated with various concentrations of a Notch signaling blocker, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), with the goal of reducing expression of Notch intracellular domain 3 (NICD3). Both cell lines were subsequently treated with either DAPT or DAPT combined with GEM and then viability, apoptosis, colony formation and cell count assays were performed. DAPT treatment effectively downregulated the expression of NICD3 in both cell lines. DAPT combined with GEM also significantly reduced the percentage of viable cells in both cell lines, while increasing the percentage of apoptotic cells, compared with GEM alone. In the clonogenicity assays, the combination of DAPT and GEM led to a decrease in clone numbers and significantly greater inhibition of the H1299 and A549 cells compared to treatment with DAPT or GEM alone. Meanwhile, levels of the apoptosis-related proteins, Bcl-2 and Bax, were found to be affected by the various treatments. Thus Notch-3 appears to be a promising target for gene therapy and DAPT is able to mediate a strong antitumor effect in NSCLC cells that overexpress Notch-3. Further studies of a combined treatment regimen with DAPT and GEM are warranted and may provide greater efficacy and safety in the treatment of NSCLC patients.

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Introduction

Lung cancer generally has a poor prognosis and it remains the leading cause of cancer-related deaths worldwide (1). Non-small cell lung cancer (NSCLC) represents approximately 85% of all newly diagnosed lung cancer cases (2). Currently, chemotherapy with cytotoxic agents is used for the treatment of lung cancer patients (3). Significant advances have also been made in the availability of targeted molecules for the inhibition of critical pathways in NSCLC, such as the targeting of epidermal growth factor receptor (EGFR) by afatinib (4). However, the 5-year survival rate for lung cancer still remains very low.

Gemcitabine (GEM) is a type of deoxycytidine analogue that has exhibited strong antitumor activity (5) and has been widely approved for the treatment of advanced lung cancer, pancreatic cancer and ovarian cancer (6). However, some studies have demonstrated that GEM resistance often limits its efficacy (7). Thus, new treatment strategies for NSCLC are needed. Considering the advances that have been made in the development of targeted therapies, a better understanding of lung cancer cell biology is needed to facilitate further advances (8,9).

Notch is a protein that plays an important role in embryogenesis and organogenesis by regulating cell proliferation and differentiation (10). This transmembrane heterodimeric receptor has four distinct forms (Notch1-4) in both rodents and humans. In particular, Notch-3 is a receptor of the Notch signaling pathway and it plays an important role in regulating self-renewal, differentiation and apoptosis in cancer cells. Consequently, it represents a promising target for the

development of novel therapies for the treatment of aggressive cancers such as NSCLC (11). Clinical studies have revealed that overexpression of Notch-3 is common in NSCLC and it correlates with a shorter progression-free period and shorter overall disease-free survival (12). Clinical studies have also revealed that a high level of Notch-3 expression is a poor prognostic factor for NSCLC (13). Additionally, Notch-3 overexpression has been reported to be related to the proliferative and apoptotic capacity of cancer cells (14). Overall survival is significantly lower for patients with Notch-3-positive tumors compared with those with Notch-3-negative tumors (15) and high levels of Notch-3 expression have been associated with drug resistance in NSCLC and ovarian cancer (16,17). Thus, Notch-3 may be a potential target for cancer therapies, either alone or in combination with GEM.

Based on our current understanding of the structure, function and regulation of the Notch signaling pathway, there are several steps which have been identified as potential targets for inhibiting this pathway, as well as Notch-3 activity. Accumulating evidence has revealed that activation of Notch proteins largely depends on γ-secretase activity (18). Thus, γ-secretase is a promising target for Notch-3 inhibition (19). *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) is a γ-secretase that is often referred to as a 'Notch inhibitor' in oncology. Moreover, DAPT is widely considered to act as an inhibitor in terms of biological activity and to mediate cytotoxic activities in various types of cancer cells (20). In clinical trials, DAPT has had a variety of indications (21). To investigate whether DAPT is an effective treatment for GEM-resistant tumors, DAPT inhibition of Notch-3 activity was tested. DAPT was found to effectively downregulate protein levels of NICD3 and to potentially target Notch-3 in gene therapy experiments conducted *in vitro* (22). Therefore, in the present study, a combined treatment involving DAPT and GEM was examined for its potential to effectively mediate antitumor activity in NSCLC cells.

Materials and methods

Cell lines. The NSCLC cell lines, H1299 and A549, were purchased [American Type Culture Collection (ATCC), Manassas, VA, USA] and cultured as recommended. Dulbecco's modified Eagle's medium (Gibco™ DMEM; Thermo Fisher Scientific, Inc., Shanghai, China) was supplemented with 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, China) and a 5-ml penicillin/streptomycin solution (100X; Beyotime Institute of Biotechnology, Shanghai, China) for each 500 ml of medium. The cells were cultured at 37˚C in a humidified incubator containing 5% CO_2.

Treatment. DAPT was purchased from Abcam (Shanghai, China) (120633) and GEM was a gift from Jiangsu Hansen Pharmaceutical Co. Ltd. (Jiangshu, China). The cells in this study received: i) no treatment (NC group); ii) 1 μl dimethyl sulfoxide (DMSO, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); iii) 20 μM DAPT (DAPT); iv) GEM (0.05 and 0.1 μM for A549 and H1299 cells, respectively); v) 1 μl DMSO + GEM (0.05 and 0.1 μM for A549 and H1299 cells, respectively); vi) 20 μM DAPT + GEM (0.05 and 0.1 μM for A549 and H1299 cells, respectively).

Western blot analysis. Cells were plated at $1x10^5$ cells per well in 6-well plates and treated as described above. The cells were subsequently harvested with lysis buffer (RIPA, Beyotime Institute of Biotechnology) and an equal volume of 1X SDS buffer (Beyotime Institute of Biotechnology) was added to each protein sample. After the samples were placed in boiling water for 10 min, they were subsequently separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to 0.45-μm or 0.22-μm nitrocellulose membranes. The membranes were blocked in Tris-HCl buffered saline Tween (TBST) containing 0.5% dry milk and then were incubated with antibodies recognizing Notch-3 and NICD3 (1:5,000; cat. no. ab23426; Abcam, Cambridge, UK), and anti-Bcl-2 and anti-Bax (1:1,000; cat. nos. YM3041 and YT0459, respectively; ImmunoWay Biotechnology Co., Plano, TX, USA), overnight. The membranes were then incubated with appropriate goat anti-mouse IgG secondary antibodies (1:10,000; cat. no. ZB-2305; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 1 h. Bound antibodies were visualized with enhanced chemiluminescence reagents and imaged on film (Kodak X-ray film). Actin was detected with a mouse anti-actin monoclonal antibody (1:5,000; cat. no. ZM-0001; Beijing Zhongshan Jinqiao Biotechnology, Co., Ltd., Beijing, China) as an internal control for protein quantification. Each experiment was repeated 3 times and similar results were obtained by the ImageJ bundled with Java 1.8.0_101 (imagej.nih.gov/ij/download/).

Cultivating higher expression of Notch-3. H1299 and A549 cells were plated at $1x10^6$ cells per well in 6-well plates. Twenty-four hours later, GEM was added to the culture medium. After 4 days, both sets of cells were harvested, subjected to protein extraction, and expression of Notch-3 was detected by western blot analysis. In parallel, an additional set of cells from each cell line were cultured with GEM for two months. These cells were also harvested, subjected to protein extraction, and expression of Notch-3 was detected by western blot analysis. Each experiment was repeated 3 times and expression of Notch-3 was compared with and without GEM treatment. The cells induced with GEM for two months were used in subsequent experiments.

Cell viability assay. Tumor cells were plated in 96-well plates with 3,000 cells per well and were allowed to attach overnight. To detect the sensitivity of GEM, H1299 and A549 cells were incubated with various concentrations of GEM. Different concentrations of DAPT were subsequently added to the two cell lines for 48 h, and then 24, 48 and 72 h later, 10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well. After 4 h, cell viability for each well was measured based on optical density measurements obtained at a wavelength of 490 nm. Each cell viability assay was performed six times.

H1299 and A549 cells overexpressing Notch-3 were plated in 96-well plates (3,000 cells/well) and were allowed to attach overnight. The cells were then treated with DMSO or 20 μM DAPT for 24 h, followed by treatment with a specific concentration of GEM (0.05 or 0.1 μM for A549 and H1299 cells, respectively). Two days later, cell viability was evaluated with the addition of MTT as described above.

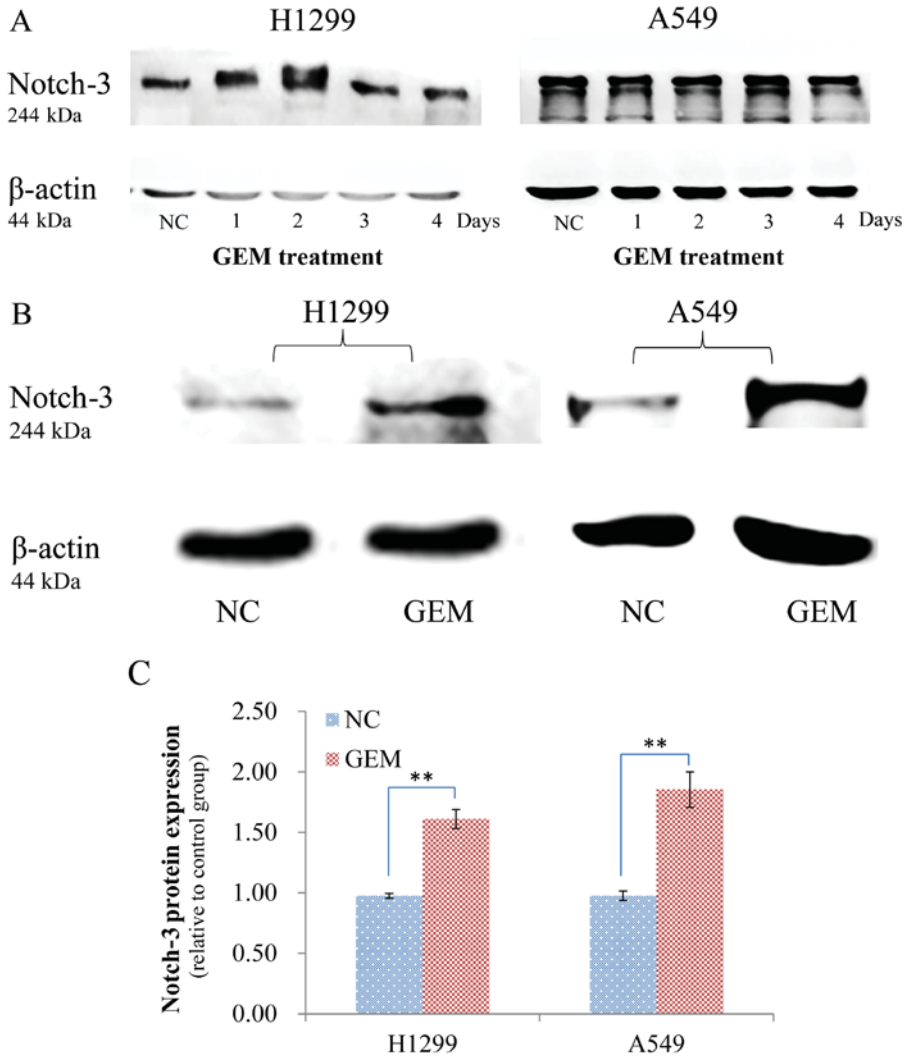

Figure 1. Notch-3 expression in human NSCLC cells. (A) Western blot analysis was performed to detect levels of Notch-3 in H1299 and A549 cells with and without treatment with GEM for 4 consecutive days. β-actin was detected as a loading control. (B) Notch-3 was markedly upregulated after H1299 and A549 cells were exposed to GEM for two months. (C) Protein levels were quantified with ImageJ software and subjected to densitometric analysis. **P<0.01 vs. control. NSCLC, non-small cell lung cancer; GEM, gemcitabine.

Apoptosis assay. H1299 and A549 cells overexpressing Notch-3 were plated in 6-well plates (1x10^5 cells/well) and were allowed to attach overnight. The cells were then incubated with 1 μl DMSO or 20 μM DAPT for 24 h, then they were treated with GEM at varying concentrations (e.g., H1299 cells received 10 μM GEM and A549 cells received 1 μM GEM). Two days later, the cells were trypsinized, collected, centrifuged, and washed with pre-cooled phosphate-buffered saline (PBS). To each sample (10^5-10^6 cells each), 5 μl Annexin V-FITC (BestBio, Shanghai, China) and 10 μl propidium iodide (PI) were added. The samples were mixed and then incubated at room temperature in the dark. After 15 min, fluorescence was determined using a flow cytometer (Beckman, USA) within 1 h. The percentages of cells staining for apoptosis were calculated with WinMDI 2.9 (http://facs.scripps.edu) and compared. All of these experiments were performed in triplicate.

Cell colony assay. H1299 and A549 cells overexpressing Notch-3 were seeded in 96-well plates (3,000 cells/well) and were allowed to attach overnight. The next day, DMSO or 20 μM DAPT was added to each well. Twenty-four hours later, the cells were treated with GEM at varying concentrations. Two days later, the number of colonies that formed were counted. After an additional 15 days, the colonies were washed with PBS, fixed with formaldehyde for 20 min, stained with hematoxylin for 30 min, and counted. Clusters of cells containing >50 cells were scored by fluorescence electron microscope (Shanghai Changfang Optical Instrument, Co., Ltd., Shanghai, China) as colonies.

Cell count assay. H1299 and A549 cells were cultured in 24-well plates (10^4 cells/well). According to the treatment groups, all of the wells were treated with DMSO or 20 μM DAPT on the second day after plating. Twenty-four hours later, GEM was added. Cells were subsequently counted each day for 7 days. Counting was performed by adding 100 μl of pancreatic enzyme (0.25% Gibco; Thermo Fisher Scientific, Inc.) to each well. After the cells were detached and dissociated from one another, 100 μl medium was added to terminate digestion. All of the samples were subsequently counted with cell counter. Each group was counted 3 times.

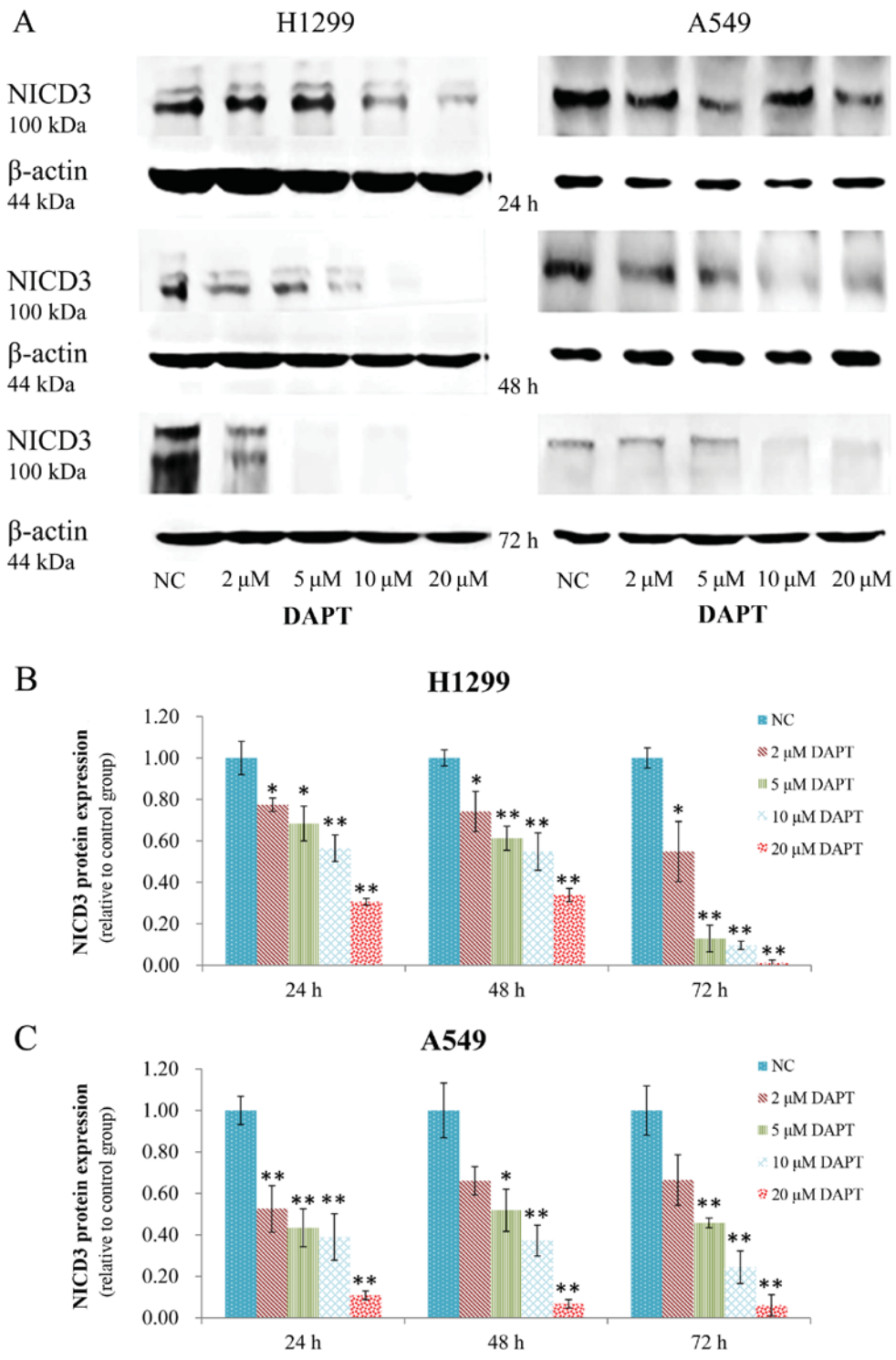

Figure 2. NICD3 expression in NSCLC cells after treatment with DAPT. (A) Significant inhibition of Notch-3 was observed in the H1299 and A549 cells that were treated with 2, 5, 10, and 20 μM DAPT for 24, 48 and 72 h. (B and C) Protein levels were quantified with ImageJ software and subjected to densitometric analysis. Experiments were repeated in triplicate. **P<0.01 vs. control; *P<0.05 vs. control. NSCLC, non-small cell lung cancer; NICD3, Notch intracellular domain 3; DAPT, γ-secretase inhibitor and indirect inhibitor of Notch.

Statistical analysis. Data are presented as the mean ± standard deviation (SD). Comparisons between the treatment groups were performed by applying a t-test. Multiple factors were analyzed by using the Mann-Whitney U-test. Values with a P-value <0.05 were considered statistically significant (*P<0.05, **P<0.01 vs. control; as indicated in the figures).

Results

Induction of Notch-3 overexpression in tumor cells. GEM resistance currently represents a major challenge to cancer treatments. A majority of the research that has been conducted regarding this resistance has been at a nonclinical research stage. However, valuable experimental experience has been

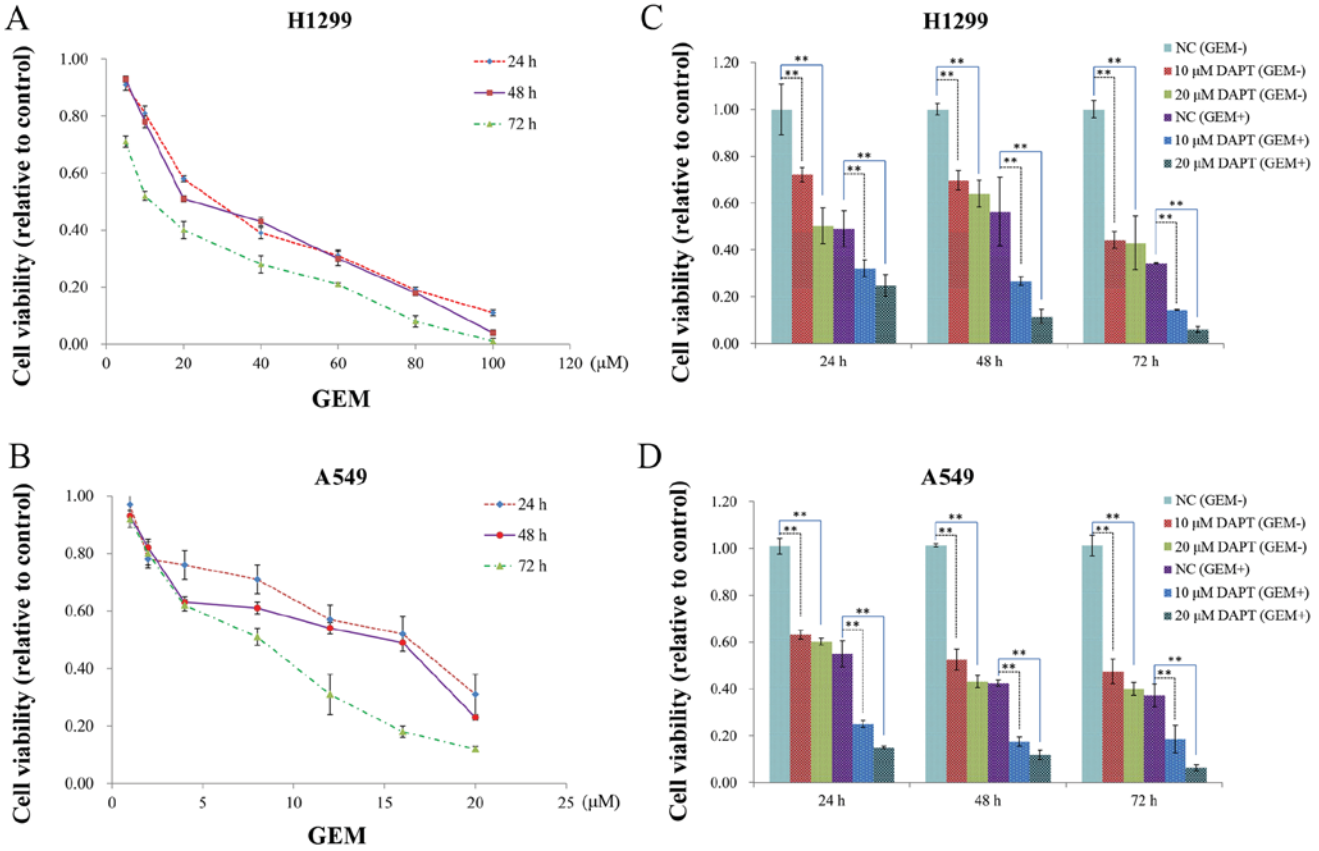

Figure 3. Notch-3 inactivation promotes GEM-induced cytotoxicity in H1299 and A549 cells. IC_{50} values for GEM against H1299 and A549 cells at time-points of 24, 48 and 72 h were calculated from cell viability data (A and B). Cell viability was assessed by MTT assays after Notch-3 was inhibited by 0, 10 and 20 μM DAPT (C and D). Cell viability data from experiments performed with 10 and 20 μM DAPT treatments with and without GEM are quantitated are shown. Experiments were performed in triplicate. **P<0.01 vs. control. GEM, gemcitabine; DAPT, γ-secretase inhibitor and indirect inhibitor of Notch.

gained in the construction of drug-resistant cell models as a result of these efforts. Most *in vitro* studies of NSCLC have focused on A549 cells, a human lung adenocarcinoma cell line. Here, two commonly used NSCLC cell lines (H1299 and A549) were compared, with a focus on Notch-3 protein expression in response to different durations of GEM induction. In the western blot assays performed, no significant differences in protein expression were observed between the no treatment group and induction with GEM for 1, 2, 3 and 4 days (Fig. 1A). However, significant differences in Notch-3 expression were detected in both cell lines after they were induced with GEM for 2 months (Fig. 1B and C). Therefore, H1299 and A549 cells that were subjected to the latter induction method were analyzed in subsequent experiments.

DAPT efficiently downregulates NICD3 expression. H1299 and A549 cells overexpressing Notch-3 were incubated with various concentrations of DAPT (2, 5, 10, and 20 μM). As a result, they exhibited a significant dose-dependent decrease in NICD3 protein expression. Moreover, marked decreases in expression were most notably detected at 24, 48 and 72 h after treatment with 10 μM DAPT or 20 μM DAPT. These results suggest that DAPT effectively reduced expression of NICD3 (Fig. 2).

Treatment with GEM and DAPT affects cell growth. When A549 and H1299 cells were incubated with GEM, an inhibitory effect on cell growth was observed 24, 48 and 72 h after the start of GEM treatment (Fig. 3A and B). Both time-dependent decreases and decreases in the half maximal inhibitory concentration (IC_{50}) values for GEM in each cell line were observed at the three time points assayed. In cell viability assays that were conducted with both cell lines in the presence of DAPT and DAPT+GEM, the inhibitory effect of GEM was further enhanced compared to the control groups at the 48 h time-point (Fig. 3C and D). It is generally believed that drug inhibition of tumor cells is related to apoptosis rates and colony numbers. Therefore, we subsequently investigated the apoptosis rate and colony growth of these 2 cell lines.

DAPT enhances GEM sensitivity to increase apoptosis and colony numbers. The percentage of cells undergoing apoptosis was significantly increased in both the H1299 and A549 cells that were treated with DAPT compared with no treatment (Fig. 4A). Moreover, when these two cells lines were treated with DAPT+GEM vs. GEM alone, a marked increase in the percentage of apoptotic cells was also observed (Fig. 4A). Correspondingly, the number of colonies formed following treatment of the H1299 and A549 cells with DAPT+GEM was markedly lower than the number of colonies formed following the treatment of H1299 and A549 cells with GEM alone (Fig. 4B and C). These results suggest that DAPT is able to enhance the efficacy of GEM and this positive impact is consistent with our hypothesis that DAPT enhances the drug

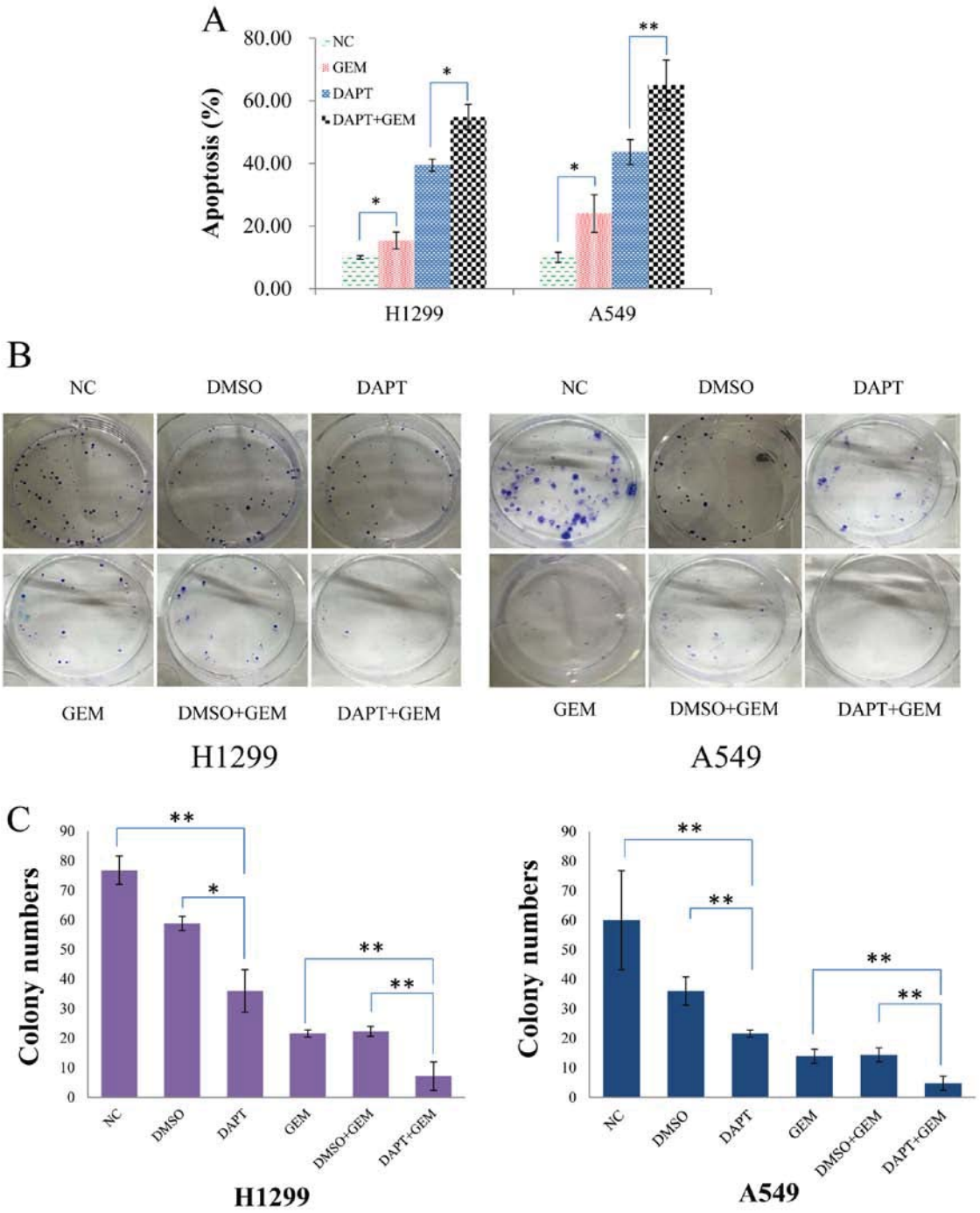

Figure 4. Effect of Notch-3 inactivation on GEM-induced apoptosis and colony numbers in NSCLC cell lines. (A) The percentage of apoptotic H1299 and A549 cells increased after treatment with 20 μM DAPT and GEM for 48 h. (B and C) Colony numbers of H1299 and A549 cells were decreased after treatment with 20 μM DAPT and GEM for 48 h. Experiments were performed in triplicate. **P<0.01 vs. control; *P<0.05 vs. control. NSCLC, non-small cell lung cancer; GEM, gemcitabine; DAPT, γ-secretase inhibitor and indirect inhibitor of Notch.

sensitivity of NSCLC cell lines, thereby delaying or blocking GEM resistance.

Inhibition of DAPT specifically increases lung cancer cell sensitivity to GEM. Cell proliferation was assayed for H1299 and A549 cells that were treated with and without DAPT and GEM individually and in combination. An obvious effect of DAPT on cell proliferation was observed in both sets of cells (Fig. 5A and B), while treatment with DAPT + GEM significantly enhanced the effect of GEM on cell proliferation (Fig. 5C and D). These results revealed that DAPT positively promoted the pharmacological effects of GEM.

Role of apoptosis-related proteins in DAPT-enhanced GEM sensitivity. It has been shown that Bcl-2 family proteins can be regulated by direct interactions with Bax, Bak, Bcl-2, and Bcl-xL to allow mitochondrial outer membrane permeabilization (MOMP) and apoptosis to occur. Expression levels of Bcl-2 and Bax have also been found to be related to GEM resistance.

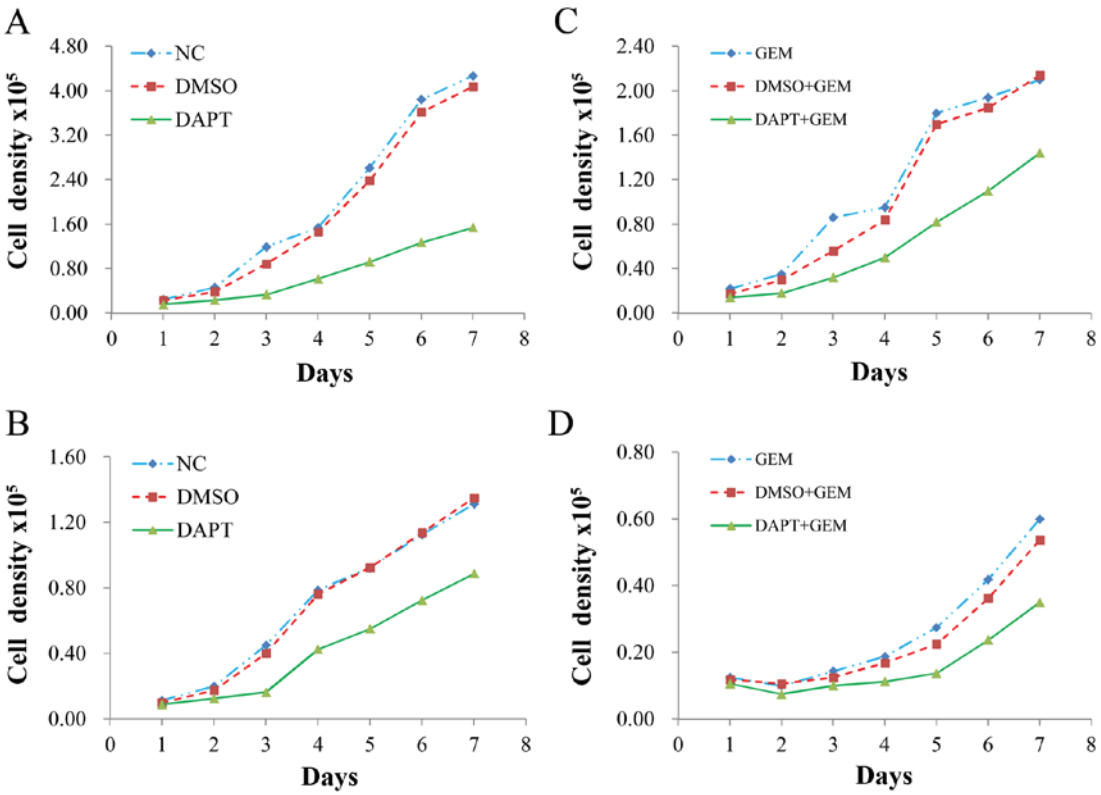

Figure 5. DAPT treatment increased the sensitivity of lung cancer cells to GEM. Cell numbers were reduced after H1299 (A) and A549 (B) cells were treated with 20 μM DAPT vs. DMSO or no treatment (NC). DAPT+GEM treatment had a greater effect on the proliferation of H1299 (C) and A549 (D) cells compared with GEM with or without DMSO. GEM, gemcitabine; DAPT, γ-secretase inhibitor and indirect inhibitor of Notch.

Following the treatment of H1299 and A549 cells with GEM, Bcl-2 was found to be upregulated, while expression of Bax exhibited no obvious changes (Fig. 6A and B). However, when these two cell lines were incubated with various concentrations of DAPT (2, 5, 10 and 20 μM) for 48 h, expression of Bcl-2 decreased in both cell lines, while expression of Bax increased (Fig. 6C-E).

Discussion

To date, there are many methods available that provide inhibition of Notch signaling pathway activity. Various approaches include the targeting of Notch ligands, Notch receptors, ADAM-mediated cleavage of Notch, γ-secretase-mediated cleavage of Notch, and specific targeting of Notch-3 (19). Targeting of Notch-3 by silencing RNA (siRNA) has been reported and it is an important method. Moreover, treatment with DAPT can decrease cleavage of Notch-3. Direct comparisons of Notch-3-targeted siRNAs and DAPT treatments have shown that both approaches can achieve a similar silencing effect, although other aspects of the Notch signaling pathway appear to be affected as well (23).

In the present study, the γ-secretase inhibitor, DAPT, was selected to target Notch-3. Our results demonstrated that DAPT treatment is able to effectively inhibit NICD3 expression at the protein level and can significantly inhibit cell proliferation, with higher concentrations associated with stronger inhibitory effects. These results provide evidence that DAPT is able to effectively inhibit Notch-3 secretion, and Notch-3 is a highly important target for cancer treatment as demonstrated in previous studies. Many articles have also reported that effective inhibition of Notch-3 activity by DAPT results in a decrease in cell proliferation (24). *In vivo*, Tammam *et al* demonstrated that tumor growth was inhibited in a mouse model that received systemic administration of DAPT (22). In the present study, DAPT inhibited the proliferation of cells overexpressing Notch-3, it significantly decreased the percentage of cells undergoing apoptosis, and the numbers of colonies formed were decreased. Thus, Notch-3 appears to be important for cell viability by promoting cell proliferation and inhibiting cell apoptosis. Specific mechanisms involving the influence of Notch-3 on tumor biology have been widely studied. Notch-3 has been found to regulate many signaling pathways related to tumor development, as well as other important proteins in cells, and to promote tumorigenesis or inhibit tumor progression (25). Moreover, in our recent clinical study, high levels of Notch-3 expression were detected in immunohistochemistry assays and this was identified as a poor prognostic factor for NSCLC patients regardless of treatment (26).

In the clinic, chemotherapeutic drugs and molecular-targeted drugs (e.g. inhibitors of EGFR) are used to treat NSCLC. However, only a few chemotherapeutic drugs are available for long-term treatment of NSCLC due to the potential for chemoresistance (27). Thus, a combination of molecular-targeted drugs and chemotherapy drugs may be important for the treatment of cancer. In several clinical studies, chemoresistance of NSCLC was found to be related to the overexpression of certain proteins, including EGFR, Notch and RIPK1 (28-31). It has been recognized that Notch-3 expression is related to the efficacy of GEM. Moreover, a

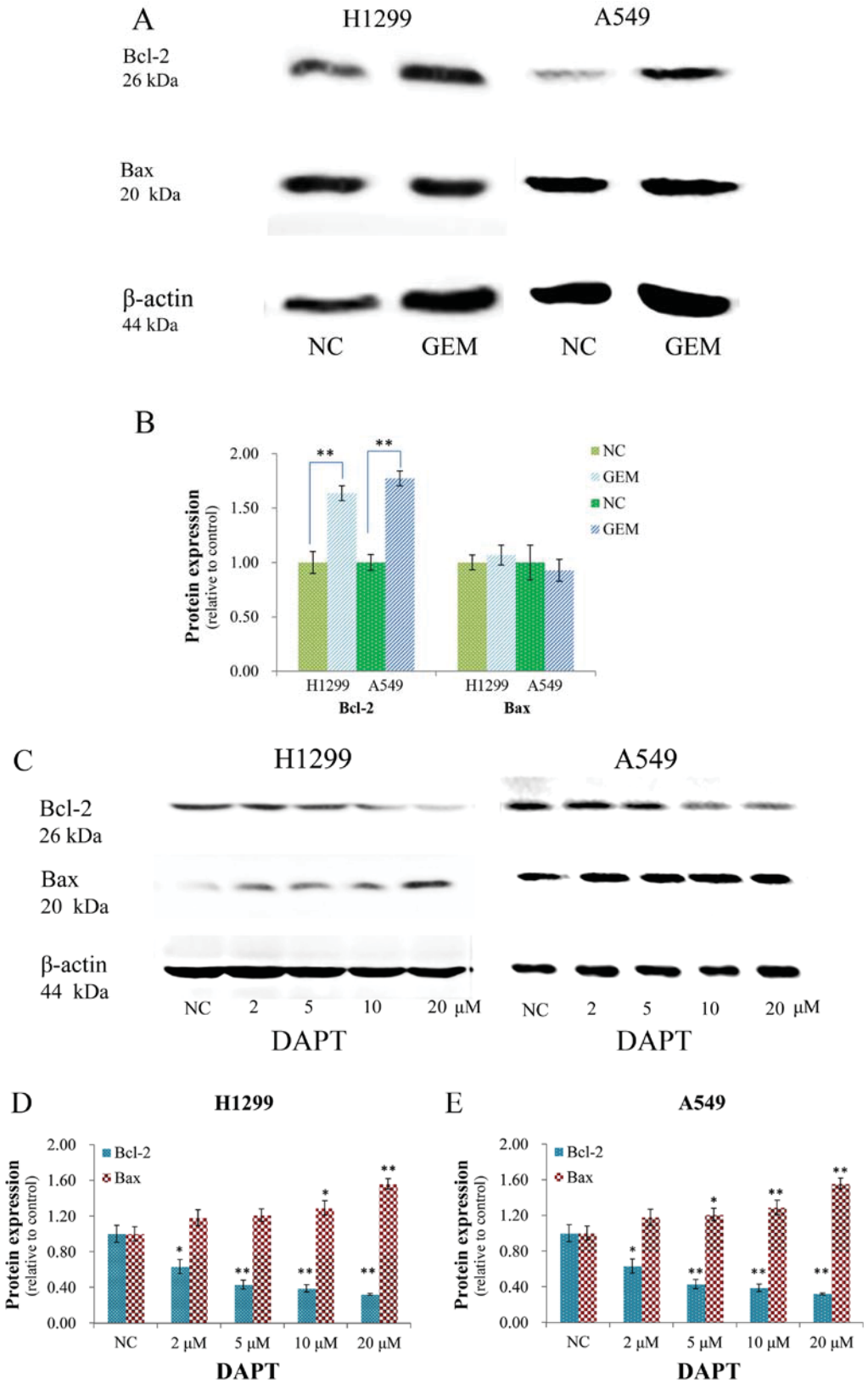

Figure 6. Expression of apoptosis-related proteins in H1299 and A549 cell lines. (A and B) Bcl-2 and Bax exhibited significant differences in expression after H1299 and A549 cells were treated with GEM for two months. (C) Expression levels of Bcl-2 and Bax were detected following treatment of H1299 and A549 cells with 2, 5, 10, and 20 μM DAPT for 48 h. (D and E) Protein levels were quantified with ImageJ software and subjected to densitometric analysis. **P<0.01 vs. control; *P<0.05 vs. control. GEM, gemcitabine; DAPT, γ-secretase inhibitor and indirect inhibitor of Notch.

role for Notch-3 in tumor development has been established; indeed, high levels of Notch-3 expression have been associated with resistance to chemotherapy drugs in various types of cancers (23,32,33). GEM is used in the clinic due to its structural similiarities with deoxycytidine, a molecule which affects cells and promotes cell apoptosis. In some clinical

studies of patients with cholangiocarcinoma, overexpression of Notch-3 was found to potentially correlate with resistance to GEM-based chemotherapy and poor survival (34). Consistent with these data, preclinical studies have shown that Notch-3 is related to GEM sensitivity in other tumors (30,35). However, the mechanism responsible for GEM's ability to enhance Notch-3 expression in specific cells remains unclear. Thus, the role of Notch-3, as well as Notch1, in predicting GEM efficacy is an active area of research. Meanwhile, it is apparent that Notch family proteins affect the effectiveness of GEM for NSCLC and enhancement of GEM-mediated resistance by Notch-3 overexpression has been observed in other types of cancer, including pancreatic cancer.

Previously, Aoki *et al* reported that poor survival was associated with Notch-3 expression in cases of extrahepatic cholangiocarcinoma (34). The results of the present study confirm that cells with long-term exposure to GEM have enhanced expression of Notch-3 and increased resistance to GEM. There are multiple genes that are involved in mediating induced resistance that results from continuous exposure to GEM. In the present study, H1299 and A549 cells were exposed to GEM to induce overexpression of Notch-3 and to avoid the controversy mentioned above. Our results further confirmed that Notch-3 is a major determinant in mediating the cytotoxic effect of GEM in NSCLC cells. Further research is still needed to explain how GEM is able to enhance expression of Notch-3 and suppress secretion of Notch-3 to improve sensitivity to GEM chemotherapy. The results of previous studies indicate that the process of apoptosis may be involved (36). The results of the present study are consistent with this hypothesis, since GEM treatment was found to significantly improve Notch-3 expression, and Bcl-2 expression was also increased. Bcl-2 family proteins are major regulators of apoptosis that primarily act in mitochondria where the mitochondrial apoptotic pathway involves a caspase-9-dependent caspase signaling cascade (37). One component of this cascade, caspase-3, is usually activated by caspase-9 and this leads to the cleavage and inactivation of key cellular proteins such as PARP and DNA fragmentation factor (38). Some members of the Bcl-2 family modulate the activation of caspases (39). For example, Bcl-2 inhibits the release of cytochrome *c* by mitochondria, thereby preventing cell death (40,41). Therefore, in the present study, we focused on Bcl-2 in a preliminary investigation of a possible role for apoptosis in GEM resistance. Based on the results obtained, further in-depth studies of the mechanistic details involved will be pursued.

Thus, further evidence is provided that the drug resistance and sensitivity of GEM in relation to Notch-3 involve apoptosis. Moreover, support for the inhibition of Notch-3 by DAPT for the treatment of patients with NSCLC was demonstrated in the present study, especially for patients presenting with resistance to GEM. Furthermore, we demonstrated that DAPT could be used alone or in combination with GEM.

In summary, Notch-3 is not only important for regulating cell viability, but it is also an important target for GEM-mediated antitumor effects. Therefore, Notch-3 may be a good candidate for use in the development of new treatment strategies for cancer patients who can undergo GEM-based chemotherapy and overexpress Notch-3. Furthermore, based on the results obtained, a prospective study and clinical treatment are under consideration, with the latter including a combined treatment region of DAPT and GEM for tumor patients with GEM-resistant and Notch-3-overexpressing tumors.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author upon.

Authors' contributions

YBZ designed the research. BD and JG performed the research and wrote the manuscript. All authors analyzed the data and were involved in writing the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study does not contain any studies involving human participants or animals that were performed by any of the authors.

Consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests to report.

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