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# Down-regulation of Notch1 by gamma-secretase inhibition contributes to cell growth inhibition and apoptosis in ovarian cancer cells A2780

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ABSTRACT

The release of Notch intracellular domain (NICD) is mediated by γ-secretase. γ-Secretase inhibitors have been shown to be potent inhibitors of NICD. We hypothesized that Notch1 is acting as an oncogene in ovarian cancer and that inhibition of Notch1 would lead to inhibition of cell growth and apoptotic cell death in ovarian cancer cells. In this study, expressions of Notch1 and hes1 in four human ovarian cancer (A2780, SKOV3, HO-8910, and HO-8910PM), and one ovarian surface (IOSE 144) cell lines were detected by Western blot and quantitative real-time RT-PCR. The effects of γ-secretase inhibition (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester, DAPT) were measured by MTT assay, flow cytometry, ELISA and colony-forming assay. Our results showed that Notch1 and hes1 were found in all the four human ovarian cancer and IOSE 144 cell lines, and they were significantly higher in ovarian cancer cells A2780 compared to another four ovarian cells. Down-regulation of Notch1 expression by DAPT was able to substantially inhibit cell growth, induce G1 cell cycle arrest and induce cell apoptosis in A2780 in dose- and time-dependent manner. In addition, hes1 was found to be down-regulated in dose- and time-dependent manner by DAPT in A2780. These results demonstrate that treatment with DAPT leads to growth inhibition and apoptosis of A2780 cells in dose- and time-dependent manner. These findings also support the conclusion that blocking of the Notch1 activity by γ-secretase inhibitors represents a potentially attractive strategy of targeted therapy for ovarian cancer.



## Introduction

Ovarian cancer is the second most common gynecologic cancer among women and the first leading cause of death from gynecologic malignancy worldwide. This is due to absence of symptoms in early stages of this disease and lack of a reliable method for early detection. Despite advances in chemotherapy and radical surgery, ovarian cancer remains the most deadly gynecologic malignancy [1]. This disappointing outcome strongly suggests that innovative research is needed to control this deadly disease.

The Notch signaling pathway plays a key role in the proliferation and differentiation of many tissues. It is an evolutionarily conserved pathway that regulates critical cell fate decisions [2]. In mammals, the Notch family consists four receptors (Notch1–Notch4) and five ligands (Jagged-1, Jagged-2, Delta-like-1, Delta-like-3, and Delta-like-4) [3]. Notch ligands and receptors are type I membrane proteins that regulate cell fate during cell–cell contact [2,4]. Receptor–ligand interaction between two neighboring cells leads to γ-secretase-mediated proteolytic release of the Notch intracellular domain (NICD) [5]. NICD then translocates into the nucleus, in which it interacts with the transcriptional cofactor CBF1 and transactivates gene targets such as those in hes and hey families, which in turn affect numerous pathways involving cell-fate determination [6].

Abnormal Notch signaling has been documented in many cancers and has been associated with tumorigenesis [7]. Recent data indicates that Notch3 amplification activate oncogenes in ovarian cancer [8] and Jagged-1/Notch3 interaction constitutes a juxtacrine loop promoting proliferation and dissemination of ovarian cancer cells [9]. In addition, Notch1 has oncogenic function in human ovarian carcinogenesis [10] and its expression correlates with tumor differentiation status in ovarian carcinoma [11]. Reports show that down-regulation of Notch1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells [12] and γ-secretase inhibitors are able to inhibit cell growth and induce cell apoptosis in some cancer cells through Notch signaling [13]. It has also been reported inactivating the Notch signaling by γ-secretase inhibitors may provide a targeted therapy for those tumors with Notch activation [14]. Therefore, we hypothesized that γ-secretase inhibitors may inhibit Notch1 activation in ovarian cancer cells leading of inhibition of cell growth and apoptotic cell death. Consequently,

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in this report, we tested our hypothesis on whether down-regulation of Notch1 gene expression by γ-secretase inhibitor could inhibit cell growth and induce cells apoptosis.

Our findings indicated that down-regulation of Notch1 inhibited cell growth with concomitant induction of apoptosis. Our data also showed that γ-secretase inhibitor down-regulated the expression of Notch1 and its downstream molecule hes1, suggesting that blocking of the Notch1 activity by γ-secretase inhibitors represents a potentially attractive strategy of targeted therapy for ovarian cancer.

## Materials and methods

*Cell lines culture.* Four human ovarian cancer (A2780, SKOV3, HO-8910, and HO-8910PM), and one ovarian surface (IOSE 144) cell lines were maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Sigma).

*γ-Secretase inhibitor treatment.* *N*-[*N*-(3,5-Difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT, Sigma, St. Louis, MO, USA), a potent γ-secretase inhibitor, was used to block Notch1-mediated signal transduction in A2780 cell line. Cells in logarithmic growth were seeded at densities of $1 \times 10^5$ cells/mL and cultured in the presence of different concentrations of DAPT (25, 50, and 75 μmol/L) for up to 3 days. Control cells were treated with 0.1% dimethyl sulfoxide (DMSO) in culture medium. At various time points after treatment onset, the expression of Notch1 and hes1, cell growth inhibition, cell cycle distribution, cell apoptosis, and cell colony-forming were analyzed.

*Quantitative real-time RT-PCR.* Total RNA was extracted from the four human ovarian cancer and IOSE 144 cell lines, using Trizol reagent (Gibco BRL). cDNA was synthesized from RNA, using an PrimeScript™ RT reagent Kit (TaKaRa). The cDNA specimens were amplified using an SYBR Premix Ex Taq™ II (TaKaRa). Notch1 primers were: forward 5′-TCAGCGGGATCCACTGTGAG-3′ and reverse 5′-ACACAGGCAGGTGAACGAGTTG-3′. hes1 primers were: forward 5′-TGGAAATGACAGTGAAGCACCTC-3′ and reverse 5′-TCGTTCATGC ACTCGCTGAAG-3′. The internal control β-actin primers were: forward 5′-TGGCACCCAGCACAATGAA-3′ and reverse 5′-CTAAGTCAT AGTCCGCCTAGAAGCA-3′. PCR amplification was done on the ABI 7500 system (Applied Biosystems) using SYBR Green II (TaKaRa). We used β-actin to normalize mRNA. Relative quantitation of mRNA expression levels was determined using the relative standard curve method according to the manufacturer's instructions (Applied Biosystems).

*Western blot analysis.* Total proteins from the four human ovarian cancer and IOSE 144 cell lines were lysed in lysis buffer by incubating for 15 min at 4 °C. The protein concentrations were determined using the Bio-Rad assay system (Bio-Rad). Total proteins were fractionated using sodium dodecyl sulfate polyacrylamide (10%) gels for electrophoresis (SDS–PAGE), and the gels were transferred onto nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 and then incubated with appropriate primary antibodies overnight at 4 °C. Horseradish peroxidase-conjugated anti-goat IgG was used as the secondary antibody, and the protein bands were detected using the electrochemiluminescence (ECL) method (Amersham Biosciences). Western blot analyses were quantified by using laser densitometry, and the results were presented as the mean of three independent experiments with error bars representing the standard deviation. Membranes were incubated for 30 min at 50 °C in buffer that contained 2% SDS, 62.5 mmol/L Tris (pH 6.7), and 100 mmol/L 2-mercaptoethanol. The membranes were then washed and incubated with the desired primary antibody.

*Cell growth inhibition studies by MTT assay.* The A2780 cells ($5 \times 10^3$) were seeded in a 96-well culture plate and subsequently were treated with 25, 50, and 75 μmol/L DAPT for 24, 48, and 72 h. Control cells were treated with 0.1% dimethyl sulfoxide (DMSO) in culture medium. After treatment, the cells were incubated with MTT reagent (0.5 mg/mL) at 37 °C for 4 h. The resulting formazan crystals were solubilized by the addition of 150 μL DMSO to each well. The optical density at 570 nm was measured and cell viability was determined by the formula: cell viability (%) = (absorbance of the treated wells − absorbance of the blank control wells)/(absorbance of the negative control wells − absorbance of the blank control wells) × 100%. All MTT experiments were performed in triplicate and repeated at least three times.

*Flow cytometry and cell cycle analysis.* The cell cycle was analyzed by flow cytometry. Briefly, cells ($1 \times 10^6$) were collected and washed in PBS, then fixed in 75% alcohol for 30 min at 4 °C. After washing in cold PBS three times, cells were resuspended in 1 mL of PBS solution with 40 μg of propidium iodide (Sigma) and 100 μg of RNase A (Sigma) for 30 min at 37 °C. Samples were then analyzed for their DNA content by FACS (BD Immunocytometry Systems, San Jose, CA). Each experiment was repeated for at least three times.

*Cell death (apoptosis) assay.* The cell death detection ELISA Kit was used for investigating apoptosis in treated cells according to the protocol of the manufacturer. Briefly, cell culture supernatants were washed away to remove fragmented DNA from necrotic cells, then cells were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with antihistone antibody. Samples were incubated with anti-DNA peroxidase followed by color development with ABTS substrate. Finally, the absorbances of the samples were determined with a microplate reader (SLT, Spectra LabInstruments Deutschland GmbH, Crailsheim, Germany) at 405 and 490 nm (reference wavelength).

*Colony-forming assay.* A2780, and A2780-DAPT cells ($5 \times 10^4$/mL) were plated in six-well plates according to the manufacturer's instructions. After 14 days of incubation at 37 °C in a humidified atmosphere containing 5% CO2 in air, colonies were counted using an inverted microscope (Leica, Heidelberg, Germany).

*Statistical analysis.* Results were expressed as means ± standard error. Student's *t* test was performed for estimation of statistical significance. Significant changes within the 95% confidence interval ($P < 0.05$) are marked by an asterisk.

## Results

### *Notch1 and hes1 are highly expressed in ovarian cancer cells A2780*

Our studies were done to examine the relative levels of Notch1 and hes1 in four human ovarian cancer (A2780, SKOV3, HO-8910, and HO-8910PM), and one ovarian surface (IOSE 144) cell lines, by real-time RT-PCR and Western blot analysis. We used real-time RT-PCR and Western blot analysis to measure expression of Notch1 mRNA and protein level in all the five ovarian cells. Our data indicated that Notch1 was found in all the five ovarian cell lines, Notch1 mRNA and protein level were significantly higher in ovarian cancer A2780 and HO-8910 cells compared to IOSE 144 (Fig. 1A and B). Then, we used real-time RT-PCR and Western to analyze hes1 mRNA and protein level in the five cells. Our results showed that hes1 was higher in ovarian cancer A2780 compared to IOSE 144 (Fig. 1C and D). These findings suggest that Notch1 and hes1 are highly expressed in ovarian cancer cells A2780. So, we choose A2780 to finish the subsequent studies.

### *Inhibition of Notch1 signaling*

In order to test whether DAPT could regulate the expression of Notch1, we used real-time RT-PCR to detect the Notch1 level in A2780 cells treated with DAPT. Our study revealed that different concentrations of DAPT (25, 50, and 75 μmol/L) resulted in signif-