RNAi-mediated knockdown of Notch-1 leads to cell growth inhibition and enhanced chemosensitivity in human breast cancer

SHAOLEI ZANG[1*], FENG CHEN[1*], JIANJIAN DAI[1], DONGMEI GUO[1], WILLIAM TSE[3], XUN QU[2], DAOXIN MA[1] and CHUNYAN JI[1]

[1]Department of Hematology and [2]Institute of Basic Medical Sciences, Qilu Hospital, Shandong University, Jinan, Shandong Province 250012, P.R. China; [3]Division of Medical Oncology, University of Colorado, Denver, CO, USA

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Abstract. Notch signaling plays a critical role in determining cell fate such as proliferation, differentiation, and apoptosis. Accumulating evidence indicates that aberrant Notch signaling has tumor-promoting function in breast cancer. We hypothesized that Notch signaling may be a potential therapeutic target for human breast cancer. To address this issue, we down-regulated the expression of the Notch-1 receptor by siRNA in human breast cancer cells. We found that the down-regulation of Notch-1 signaling caused cancer cell growth inhibition by apoptosis induction. The effect of the down-regulation of Notch-1 may be through the inactivation of NF-κB. In addition, the down-regulation of Notch-1 signaling increased chemosensitivity to doxorubicin and docetaxel. Our results suggested that Notch signaling may be a promising target for breast cancer treatment.

Introduction

Breast cancer is one of the leading causes of cancer-related death in women. The lifetime risk of developing breast cancer is about one in eight for women around the world. For example, about 182,000 new cases are diagnosed with breast cancer and 43,300 die in America each year. Although the survival of the breast cancer patients has improved with the chemotherapy, radiation therapy as well as hormone therapy, the outcome still remains poor. Therefore, the development of novel therapy strategies and discovery of more effective therapeutic targets are required and will greatly contribute to the treatment of breast cancer.

Correspondence to: Dr Chunyan Ji, Department of Hematology, Qilu Hospital, Shandong University, 107 West Wenhua Road, Jinan, Shandong Province 250012, P.R. China
E-mail: jichunyan@sdu.edu.cn

*Contibuted equally

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Notch signaling is a pathway highly conserved through evolution which regulates various physiological processes, including stem cell maintenance, differentiation, proliferation and apoptosis. In mammals, key components of the Notch pathway include four transmembrane receptors (Notch 1-4) and five ligands (Dll1, Dll3, Dll4 and Jag-1, -2) (1,2). Direct binding of a ligand from a signaling cell to a Notch receptor on the membrane of the receiving cell initiates two successive proteolytic cleavages by TACE (TNF-α-converting enzyme) and the γ-secretase/presenilin complex, which ultimately results in the release of the intracellular domain (N-IC). N-IC then translocates into the nucleus and directly interacts with the DNA binding protein CBF1/Su (H)/Lag1 (CSF) that activates the transcription of target genes including the hairy/enhancer-of-split (HES-1) (3).

Accumulating evidence strongly indicates that aberrant Notch signaling has a tumor promoting function in breast cancer (4,5). A role for Notch signaling in human breast cancer has been suggested by the development of adenocarcinomas in the murine mammary gland following either pathway activation or the loss of Numb expression, a negative regulator of the Notch pathway (6,7). It was recently reported that the Notch signaling pathway also contributes to drug resistance in cancer cells. Inhibition of Notch signaling prevented drug resistance and sensitized myeloma cells to chemotherapy (8). Among the Notch Pathways, Notch-1 and Myc (a well studied oncogene) expression are positively correlated by immunostaining in 38% of examined human breast carcinomas (9). Notch-1 cross-talk has also been reported with other major cell growth and apoptotic regulatory pathways through modulating the activity of the transcription factor, for example, nuclear factor-κ B (NF-κB) (10). The existing evidence led us to hypothesize that Notch signaling may be a potential therapeutic target for human breast cancer.

In our earlier studies, we have shown that Notch-1 is overexpressed and highly activated in both human breast cancer cell lines and specimens (11). Thus, we tested our hypothesis on whether down-regulation of Notch-1 gene expression by small interfering RNA (siRNA) could inhibit cell growth and induce apoptosis.

We also explored whether the effects of down-regulation of Notch-1 were associated with the inactivation of NF-κB. In addition, we examined the effect of the down-regulation of Notch signaling on sensitizing breast cancer cells to chemo-

therapeutics. The results show that down-regulation of Notch-1 could be a novel therapeutic strategy for the treatment of human breast cancer.

Materials and methods

Cell culture and experimental reagents. Human breast cancer cell lines MDA-MB-231 and MCF-7 were cultured in RPMI-1640 (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37˚C. Notch-1 siRNA/siRNA control and Lipofectamine™ 2000 were purchased from Invitrogen (Carlsbad, CA). SYBR Green Real-time PCR Master Mix was purchased from Toyobo (Osaka, Japan). RevertAid™ First Strand cDNA Synthesis kit was from MBI (Fermentas, Hanover, MD). Annexin V-FITC kit was obtained from Jingmei Biotech (Shenzhen, China). Propidium Iodide was from Sigma (St. Louis, MO). Nuclear extract kit was purchased from Active Motif (Carlsbad, CA) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Amresco (Cleveland, OH). Daunorubicin (Pharmacia Italia, S.P.A) was dissolved in phosphate-buffered solution (PBS) to make a 100 μg/ml stock solution. Docetaxel (Aventis Pharmaceuticals, Bridgewater, NJ) was dissolved in ethanol to make a 4 μmol/l stock solution.

SiRNA transfection. MDA-MB-231 and MCF7 cells ($1.2x10^5$) were seeded in 6-well plates (or 4000 cells/well in 96-well plates). After incubation overnight, cells (30-50% confluence) were treated according to the protocol of the manufacturer with 40 nM siRNA (5'-UCGCAUUGACCAU UCAAACUGGUGG-3') or control siRNA (does not match any known mammalian genebank sequences) which had been precomplexed with Lipofectamine™ 2000. Seventy-two hours after siRNA transfection, cells were used for MTT, real-time PCR, Western blotting and other experiments.

Cell growth inhibition by MTT assay. MDA-MB-231 and MCF-7 cells were incubated overnight at a density of 4000 cells/well in 96-well plates, and subsequently transfected with Notch-1 siRNA or siRNA control. Seventy-two hours after tansfection, 20 μl of MTT was added per well. After an additional 4 h incubation, the media was removed, and 200 μl of DMSO was added per well, followed by 10 min thorough mixing, color development was measured on a microplate reader at 570 nm.

For the chemotherapeutic sensitivity assay, 24 h after transfection, cells were exposed to various doses of docetaxel or doxorubicin for an additional 48 h and the MTT assay was performed as described above.

Real-time RT-PCR. Total RNA was isolated from transfected cells by TRIzol (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized with RevertAid™ First Strand cDNA Synthesis kit. PCR reactions were carried out in a total of 25 μl reaction mixture (2 μl of cDNA, 12.5 μl of 2X SYBR Green PCR Master Mix, 1.5 μl of forward and reverse primers and 7.5 μl of H_2O). The PCR procedures were the following: preheating at 95˚C for 10 min; 45 cycles of 95˚C for 10 sec, 60˚C for 40 sec. Data were analyzed with com-parative Ct method and normalized by actin expression in each sample. Primer sequences for ß-actin, Notch-1, Hes1 are available on request.

Apoptosis assay. Seventy-two hours after transfection of Notch-1 siRNA or control siRNA, the transfected MDA-MB-231 and MCF7 cells were trypsinized, collected and washed twice with cold PBS. Cells were labeled by Annexin V and followed by PI. Annexin V-PI were measured by FACS Calibur and analyzed with the Modfit software. For the chemotherapeutic drug assays, 24 h after transfection, cells were exposed to 1.5 nM docetaxel or 75 ng/ml doxorubicin respectively for 48 h, then cells were collected and analyzed as above.

Western blot analysis. Cells were incubated at 4˚C for 20 min in lysis buffer [50 mmol/l Tris (pH 7.5), 100 mmol/l NaCl, 1 mmol/l EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/l sodium orthovanadate, 10 Al/ml protease inhibitor cocktail, 1 mmol/l phenylmethylsulfonyl fluoride]. The protein concentration was determined with the Bio-Rad assay system (Hercules, CA). Total proteins were fractionated using SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked with 5% non-fat dried milk in PBS buffer containing 0.1% Tween-20 and then incubated with appropriate primary antibodies. The primary polyclonal antibodies of activated Notch-1 and Hes1 (Abcam, Ltd., Cambridge, UK). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody, and the protein bands were detected using the enhanced chemiluminesence detection system (Amersham, Buckinghamshire, UK). The results shown are representative of three independent experiments.

Electrophoretic mobility shift assay for measuring NF-κB activity. Nuclear extracts from MDA-MB-231 and MCF7 cells were prepared according to the manufacturer's instructions using a nuclear extraction kit and quantified by the BCA method. The NF-κB probes were generated by annealing equimolar complementary oligonucleotides in TEN buffers at 95˚C for 10 min and then slowly cooling down to room temperature. The double-stranded probes were labeled with digoxigenin (Roche, Germany) using terminal transferase (Roche). For EMSA, 32 fmol of labeled probes were incubated at 25˚C for 20 min with 2 μg of nuclear extracts in the presence of 1 μg of poly (dI-dC), 0.1 μg of poly L-lysine, 30 mmol/l KCl, 20 mmol/l HEPES, 10 mmol/l $(NH_4)_2SO_4$, 1 mmol/l DTT, 0.2% v/v Tween-20, 1 mmol/l EDTA. Competition experiments were performed using a similar EMSA conditions as described above, except that the protein extracts were incubated with the probe in the presence of 250-fold molar excess of unlabeled double-stranded oligonucleotides as competitors. After the DNA-protein complexes were separated from the free probes by electrophoresis through a 5% non-denaturing polyacrylamide gel, the gel was transferred to a PVDF membrane and chemiluminescent detection was performed.

Densitometric and statistical analysis. The bidimensional optical densities of Notch-1 and ß-actin proteins on the film

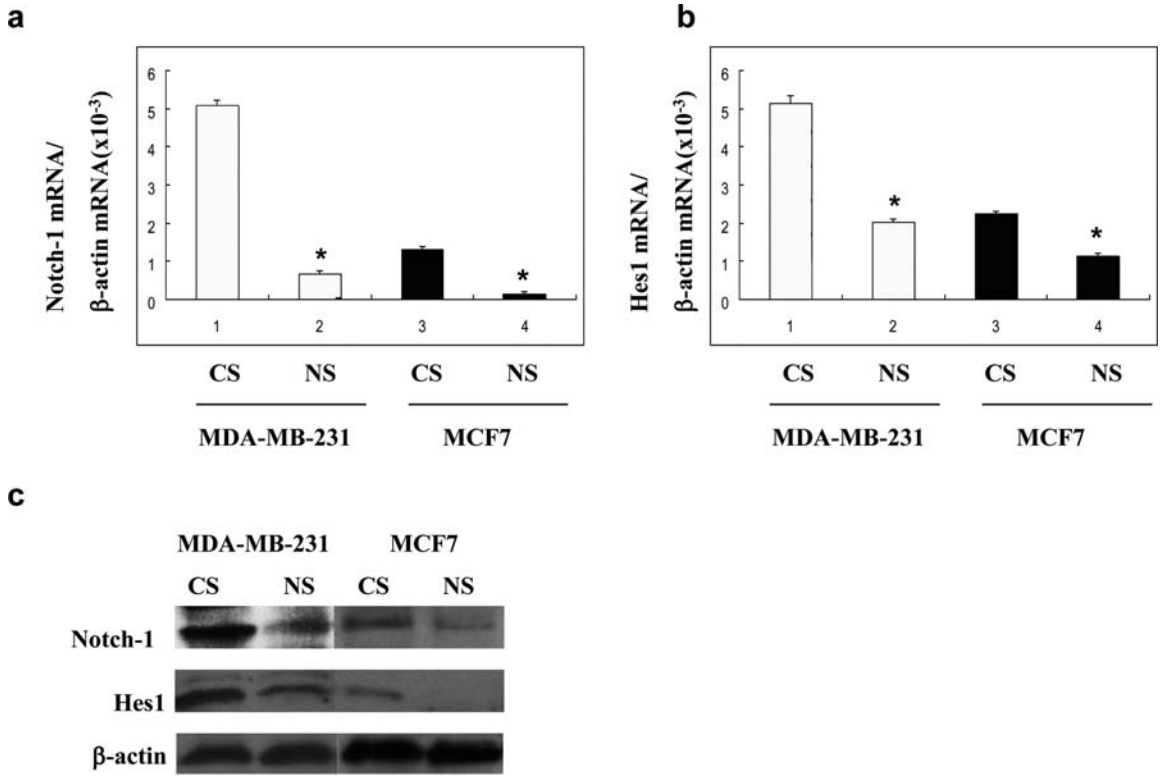

Figure 1. Down-regulation of Notch-1 and its target gene Hes1 by siRNA in human breast cancer cell lines MDA-MB-231 and MCF7. CS, control siRNA; NS, Notch-1 siRNA. (a) and (b), Notch-1 and Hes1 mRNA level was measured by real-time RT-PCR. (c), Notch-1 and Hes1 protein level was measured by Western blotting. The data are from three independent experiments, *P<0.05.

were quantified and analyzed by Molecular Analyst software (Bio-Rad). The ratios of Notch-1 against ß-actin were calculated. The cell growth inhibition by transfection or the combination of transfection and drug treatment was evaluated statistically by using Stat-Mate software (GraphPad Software, Inc., San Diego, CA). P<0.05 was considered statistically significant.

Results

Notch-1 siRNA effectively down-regulated the expression level of Notch-1 in human breast cancer cells. To definitely test the down-regulation effect of RNAi on Notch signaling pathway, two types of human breast cancer cell lines with high levels expression of Notch-1, MDA-MB-231 (estrogen receptor negative) and MCF-7 (estrogen receptor positive) were chosen in this study. We initially examined knockdown efficiency of different concentrations of siRNA on Notch-1 mRNA expression and chose the concentration of 40 nM with the highest interfering efficiency for further analysis. After transfected with Notch-1 siRNA or control siRNA for 72 h, the cell lines were collected to examine the expression of Notch-1 and Hes1 at the mRNA and protein levels by real-time RT-PCR and Western blot analysis, respectively. As shown in Fig. 1a and b, Notch-1 and Hes1 mRNA were decreased by about 90% in both MDA-MB-231 and MCF7 cells. Protein levels were also greatly reduced in Notch-1 siRNA-transfected cells compared with control siRNA-transfected cells (Fig. 1c). To exclude the possibility of RNAi off-target effects, we performed the experiments with different siRNAs and obtained similar results, indicating that the effects indeed resulted from Notch-1-RNAi interfere. On the basis of these results, we concluded that Notch-1 siRNA can effectively down-regulate the expression level of Notch-1 in human breast cancer cells.

SiRNA-mediated down-regulation of Notch-1 inhibits cell growth and induces apoptosis in human breast cancer. To investigate whether Notch-1 could be an effective therapeutic target for breast cancer, the effect of Notch-1 siRNA on cell growth was evaluated by MTT assay. We found that the down-regulation of Notch-1 expression caused an ~40% reduction of cell growth in both breast cancer cell lines at 3 days after siRNA transfection (Fig. 2). To investigate whether the growth-inhibitory effects of Notch-1 RNAi were related to the induction of apoptosis, cells transfected after 72 h were stained with Annexin V-PI and analyzed by flow cytometry. Given the fact that late apoptosis contains necrosis cells, although total apoptosis rate changed apparently, only early apoptosis is taken into account in our study. Compared with 1.25 and 0.94% in control siRNA transfected cells, the early apoptosis rates were 5.64 and 2.88% in Notch-1 siRNA transfected MDA-MB-231 and MCF7 respectively (Fig. 3). These data suggested that the growth inhibition induced by Notch-1 siRNA was partially due to an increase in cell apoptosis.

Down-regulation of Notch-1 expression by siRNA reduces NF-κB DNA-binding activity. Both Notch signaling and NF-κB pathway are involved in cell-fate determination and differen-

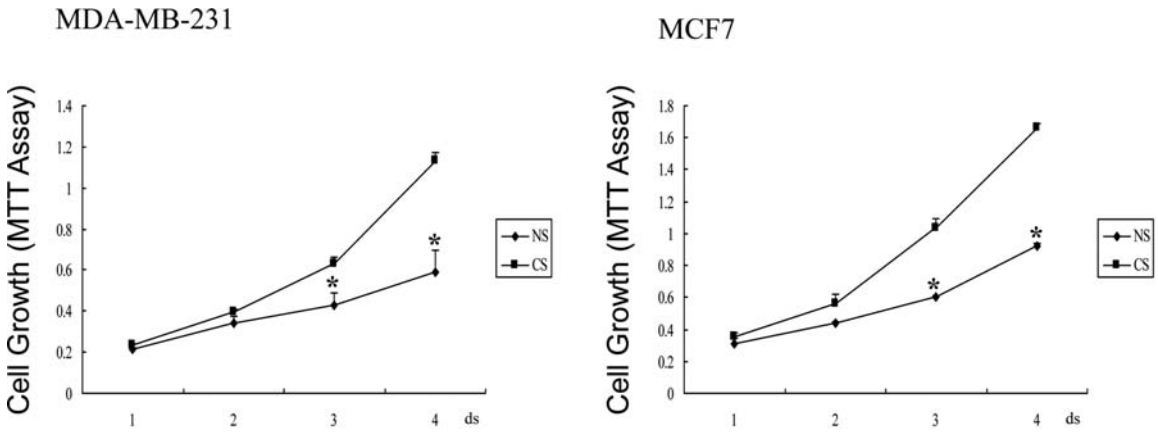

Figure 2. Effect of down-regulation of Notch-1 by siRNA on breast cancer cell growth. Cell growth inhibition tested by MTT assay in MDA-MB-231 and MCF7. *P<0.05, **P<0.01. Data were from three independent experiments. ds, days after transfection.

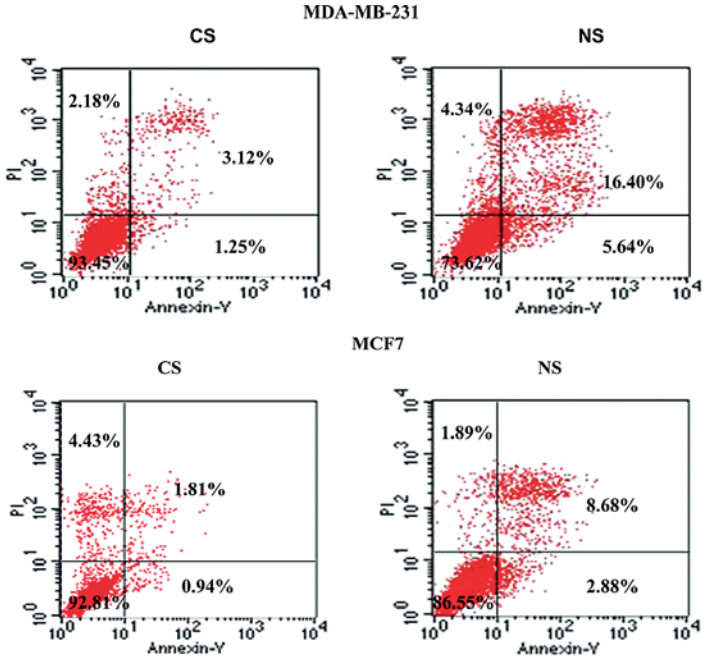

Figure 3. Effect of the down-regulation of Notch-1 on cell apoptosis. Transfected MDA-MB-231 and MCF7 cells were harvested and stained with Annexin V-PI. Apoptosis rates were analysised by flow cytometry. Down-regulation of Notch-1 increased the apoptosis rate of both MDA-MB-231 and MCF7 cells. We only took the early apoptosis rates into account because it is more representative. Each experiment was repeated twice to confirm the result.

tiation in different organisms and cell types. It was reported that there is cross-talk between these two pathways in human cervical and pancreatic cancer and that Notch signaling may be the upstream regulator (10,12). We hypothesized that down-regulation of the downstream effects of Notch-1 could be related to the activity of the NF-κB pathway in human breast cancer. To address this hypothesis, we examined the NF-κB DNA-binding activity by EMSA. Down-regulation of Notch-1 significantly inhibited NF-κB DNA-binding activity compared with control cells in both MDA-MB-231 and MCF7 cells (Fig. 4). These results provide evidence that cross-talk also exists between Notch-1 and NF-κB in breast cancer.

Down-regulation of Notch-1 expression by siRNA increases chemosensitivity. Since Notch signaling pathway has been recently shown to contribute to drug resistance in cancer cells, we examined if the down-regulation of Notch-1 could sensitize breast cancer cells to frequently used chemotherapeutic drugs such as doxorubicin and docetaxel. We tested the effect of the combination of various doses of doxorubicin/docetaxel and Notch-1 siRNA on MDA-MB-231 cells. Compared to control siRNA transfected cells, down-regulation of Notch-1 led to more cell death in MDA-MB-231 (data not shown). We chose 1.5 nM doxorubicin and 75 ng/ml docetaxel for further experiments.

The effect of down-regulation of Notch-1 combined with 1.5 nM doxorubicin or 75 ng/ml docetaxel on the growth of

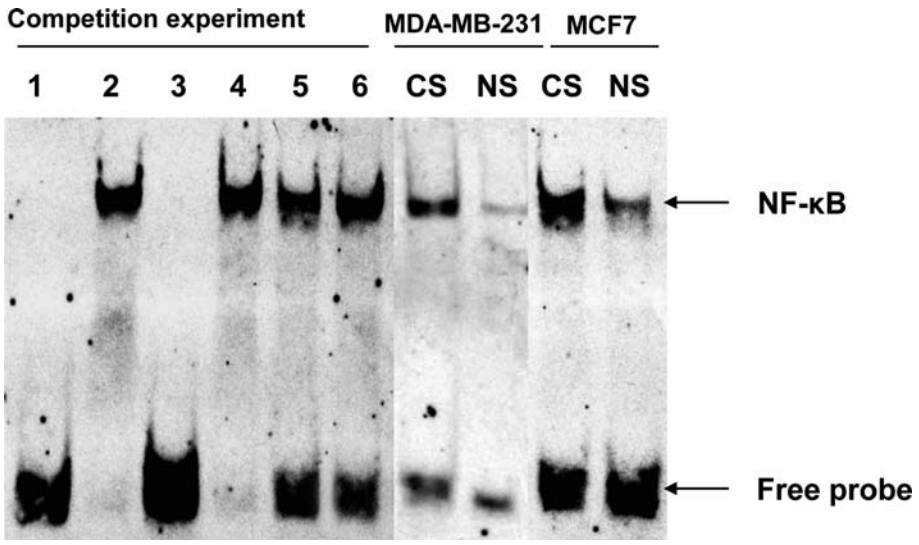

Figure 4. Effect of the down-regulation of Notch-1 on NF-κB DNA binding activity. Nuclear proteins from control siRNA and Notch-1 siRNA transfected cells were subjected to analysis for NF-κB activity tested by EMSA. Lane 1, labeled NF-κB specific oligonucleotides without nuclear extracts; lanes 2-6, labeled NF-κB specific oligonucleotides with nuclear extracts plus 250-fold excess amount of unlabeled NF-κB specific oligonucleotides in lane 3, unlabeled mutant NF-κB oligonucleotides in lane 4, unlabeled heterogenous sequence in lane 5 and unlabeled ARE in lane 6. All the competitive assay confirmed the specificity of NF-κB binding to the DNA consensus sequence. The NF-κB DNA binding activity was greatly reduced by Notch-1 siRNA compared with control in both MDA-MB-231 and MCF7 cells.

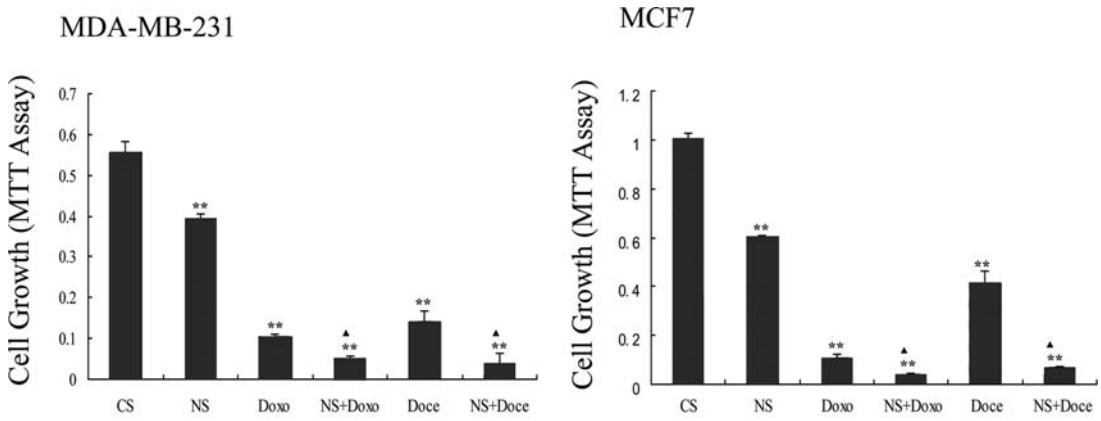

Figure 5. Inhibition of cancer cell growth tested by MTT assay. We analysed 75 μg/ml doxorubicin or 1.5 nM docetaxel with combination with Notch-1 down-regulation in MDA-MB-231 and MCF7 cells. CS, control siRNA; NS, Notch-1 siRNA; Doxo, 75 μg/ml doxorubicin; NS+Doxo, Notch-1 siRNA and 75 μg/ml doxorubicin combination; Doce, 1.5 nM docetaxel; NS+Doce, Notch-1 siRNA and 1.5 nM docetaxel combination. **P<0.01, compare with control; ▲P<0.05 compare with mono-treatment; n=3.

both cell lines is shown in Fig. 5. We found that the combination of Notch-1 down-regulation and docetaxel or doxorubicin led to a 50 or 70% enhancement in growth inhibition respectively as compared to single chemotherapeutic treatment in MDA-MB-231 cells. Similar results were found in MCF7 cells.

To further clarify the mechanism of enhanced growth inhibition of breast cancer cells by the combination of down-regulation of Notch-1 and chemotherapeutic agents, we detected apoptosis rates induced by doxorubicin and docetaxel after the down-regulation of Notch-1. It was showed that the combination of Notch-1 down-regulation with doxorubicin increased the early apoptosis rate 4- and 2.5-fold in MDA-MB-231 and MCF7 cells, respectively. Similarly, down-regulation of Notch-1 combined with docetaxel increased the early apoptosis rate 5- and 6-fold in MDA-MB-231 and MCF7 cells, respectively. Taking together, the Notch-1 siRNA transfected MDA-MB-231 and MCF7 cells were significantly more sensitive to docetaxel and doxorubicin-induced apoptosis (Fig. 6).

Discussion

Notch signaling plays important roles in maintaining the balance among cell proliferation, differentiation and apoptosis. The aberrant activation of Notch signaling has been reported in many human malignancies including human T-ALL, cervical cancer, and prostate cancer (12-14). Recently, Notch receptor expression levels have been found to be elevated in human breast cancer cell lines and breast cancer specimen

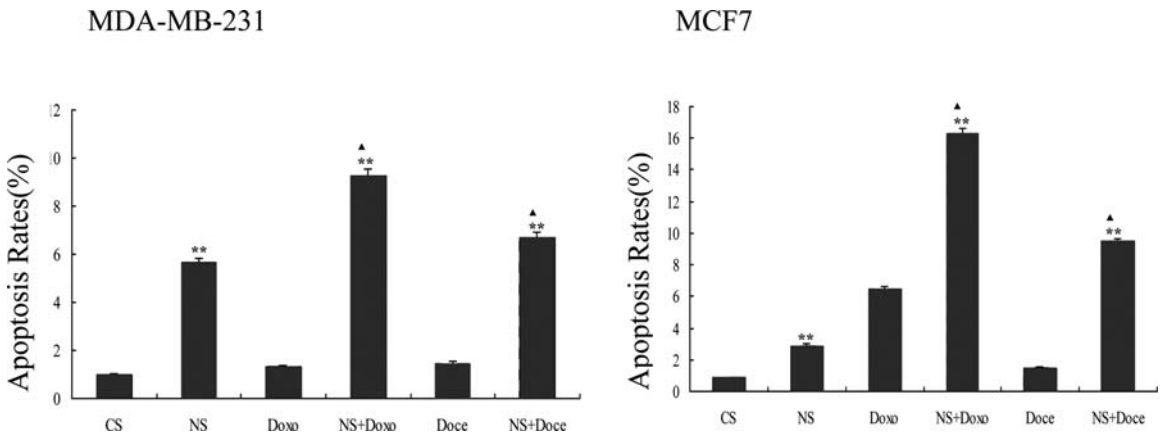

Figure 6. Induction of apoptosis in cancer cells tested by flow cytometry. CS, control siRNA; NS, Notch-1 siRNA; Doxo, 75 μg/ml doxorubicin; NS+Doxo, Notch-1 siRNA and 75 μg/ml doxorubicin combination; Doce, 1.5 nM docetaxel; NS+Doce, Notch-1 siRNA and 1.5 nM docetaxel combination. **P<0.01, compare with control; ▲P<0.05 compare with mono-treatment.

(6,15). A study on human breast cancer showed that 50% of human breast tumors expressed reduced protein level of Numb, a negative regulator of Notch signaling (7). These findings highlight an oncogenic role for Notch signaling in human breast cancer.

In the present study, we tested whether Notch signaling could be a therapeutic target for breast cancer. We found that the down-regulation of Notch-1 inhibited the growth of human breast cancer cells by inducing apoptosis. The effect of the down-regulation of Notch-1 may be through the inactivation of NF-κB. In addition, the down-regulation of Notch-1 can sensitize human breast cancer cells to chemotherapeutics such as docetaxel and doxorubicin. Our results provided *in vitro* evidence that Notch-1 is a therapeutic target of human breast cancer and may be a promising target in combination with other chemotherapeutics.

It has been reported that the Notch gene suppresses apoptosis and promotes cell proliferation through a growth factor-mediated survival pathway (16). Down-regulation of Notch-1 was found to cause apoptosis in adult T-ALL and pancreatic cancer (17,18). Yet there is discrepancy about the effect of down-regulation of Notch-1 on human breast cancer. Yamaguchi *et al* demonstrated that down-regulation of Notch-1 by RNA interference had little or no suppressive effect on the proliferation of either ERbB2-positive or ErbB2-negative cell line (19). However, in a recently published work, Notch-1 knockdown or γ-secretase inhibition was found to decrease cyclins A and B1, causing G2 arrest, and eventually cell death of breast cancer cells (20). In the present study, we again demonstrated that both MDA-MB-231 and MCF7 breast cancer cells have reduced growth and increased apoptosis after down-regulation of Notch-1. This is further evidence that Notch-1 is a potent therapeutic target for human breast cancer.

It was reported that $CD24^{-/Low}/CD44^{+}$ cancer initiating cells, which were isolated from MDA-MB-231 and MCF7 breast cancer monolayer culture and propagated as mammospheres were more resistant to radiation than cells grown as monolayer cultures (21). Radiation increased the number of these cells through induction of Jagged-1 expression and subsequent activation of Notch-1. We found that siRNA transfected cells were more sensitive to chemotherapeutics such as docetaxel and doxorubicin. The previous and our current study suggested that Notch-1 activation was involved in radiation and chemotherapy resistance. This finding has an important implication for clinical significance because docetaxel and doxorubicin are frequently used in various cancers including breast cancer. To achieve greater inhibitory effects on cancer cells, the combination of two or more chemotherapeutic agents is commonly considered. Our results provide *in vitro* evidence that the down-regulation of Notch-1 in combination with other chemotherapeutics could be a novel strategy to achieve better treatment outcomes in human breast cancer.

NF-κB is another major cell growth and apoptosis regulatory pathway. NF-κB mediates survival signals that inhibit apoptosis and promote cancer cell growth. However, NF-κB is activated by both chemotherapeutics and radiation, in many cases this response may inhibit the ability of cancer therapies to induce cell death. There is known to be cross-talk between the NF-κB and Notch signaling pathways. In hemopoietic progenitor cells, NF-κB activity is regulated by Notch-1 via transcriptional control of NF-κB (22). It was also shown that, in Notch-1 induced T-cell leukemia, NF-κB was identified as one of the major mediators of Notch-1 induced transformation (23). We observed that the activity of NF-κB decreased with the down-regulation of Notch-1 signaling in MDA-MB-231 and MCF7 cells. The result suggests that the down-regulation of Notch-1 may be partly mediated by the NF-κB pathway. Future work exploring the connections between the Notch and NF-κB signaling pathways may elucidate more potential therapeutic targets.

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