A study on notch signaling in human breast cancer

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Breast cancer is one of the leading causes of cancer death in women. The Notch family of proteins plays crucial roles in determining cell fates such as proliferation, differentiation and apoptosis. A role for Notch signaling in human breast cancer has been suggested by the development of adenocarcinomas in the murine mammary gland. However, it is not clear currently whether Notch signaling is frequently expressed and activated in breast cancers. Here we show that Notch signaling is overexpressed and highly activated in breast cancers. More significantly, the attenuation of Notch signaling by γ-secretase inhibitor can inhibit the proliferation of breast cancer cells by both causing cell cycle arrest and apoptosis. Thus, targeting Notch signaling may be of therapeutic value in breast cancers.

Key words: Human breast cancer; Notch; γ-secretase inhibitor; Cell cycle; Apoptosis

Breast cancer is one of the most common malignancies in women. Clinically, it is treated by surgical resection and chemotherapy/radiation therapy. With the applying of hormone therapy that aimed at estrogen a molecular target, the survival of the breast cancer patients has been improved. Yet breast cancer remains one of the leading cancer death in female. A greater understanding of the molecular pathways involved in breast cancer cell proliferation will lead to more effective targeted therapies.

Notch genes encode large transmembrane proteins that act as receptors for the Delta, Serrate, Lag-2 (DSL) family of ligands. There are four different Notch proteins in mammals (Notch1-4) and five known ligands: Delta-like 1, Delta-like 3,Delta-like 4, Jagged 1 and Jagged 2 [1]. Direct binding of a ligand from a signaling cell to a Notch receptor on the plasma 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) [2] N-IC then translocates into the nucleus, where it directly interacts with the DNA binding protein CBF1/Su(H)/Lag1(CSF) and activates transcription of target genes including the hairy/enhancer-of-split (HES-1) [3]. Notch proteins play crucial role in cell fate determinations such as proliferation, differentiation and apoptosis [4,5]. Depending on the cell lineage, Notch proteins can either promote or block proliferation/differentiation [4,6,7]. Due to the fundemental roles of Notch proteins in balancing cell proliferation and differentiation, Notch signaling has been suggested to be involved in malignant transformation. To date, aberrant Notch signaling has been observed in hematologic malignancies and solid tumors such as cervix, colon, lung, pancreas, skin and brain carcinomas [2, 8-13].

The first indication that Notch signaling might play a role in neoplastic development of the mammary gland came from the characterization of a common insertion site for the mouse mammary tumour virus in Czech II mice [14]. In 20% of these tumors, the mouse mammary tumour virus was inserted within the Notch4/int-3 locus. Since then, similar mouse mammary tumor virus insertions into Notch1 have been described [15]. At both loci, insertion of the provirus leads to expression of a Notch protein that consists of the transmembrane and intracellular domains only, suggesting that deregulated Notch signaling leads to tumor. Several studies show that overexpression of consititutively activated notch1 or notch4 in normal human breast epithelial cells can induce transformation in vitro [16]. An mRNA transcript encoding the intracellular domain of Notch4 was detected in two human breast cancer lines [17], Notch1 protein was highly expressed

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in four breast tumors that overexpress H-ras [5]. These data suggest that Notch signaling may play an important role in human breast tumorigenesis. There is still a need to examine systematically whether aberrant Notch signaling occur in breast cancer,whether it is activated and what role it plays in tumor development.

Here we show that Notch and its ligands are overexpressed in human breast cancers compared with normal breast tissues at the margin of tumor sections. Notch is activated both in vivo and in vitro in breast cancers as we observed the accumulation of N-IC and the expression of known downstream target genes. More significantly, γ-secretase inhibitor- a pharmacologic agent known to block effectively Notch activation, can induce cell cycle arrest and apoptosis so to inhibit the proliferation of the breast cancer cells in vitro, suggesting that inhibition of Notch signaling may be a therapeutic strategy for this disease.

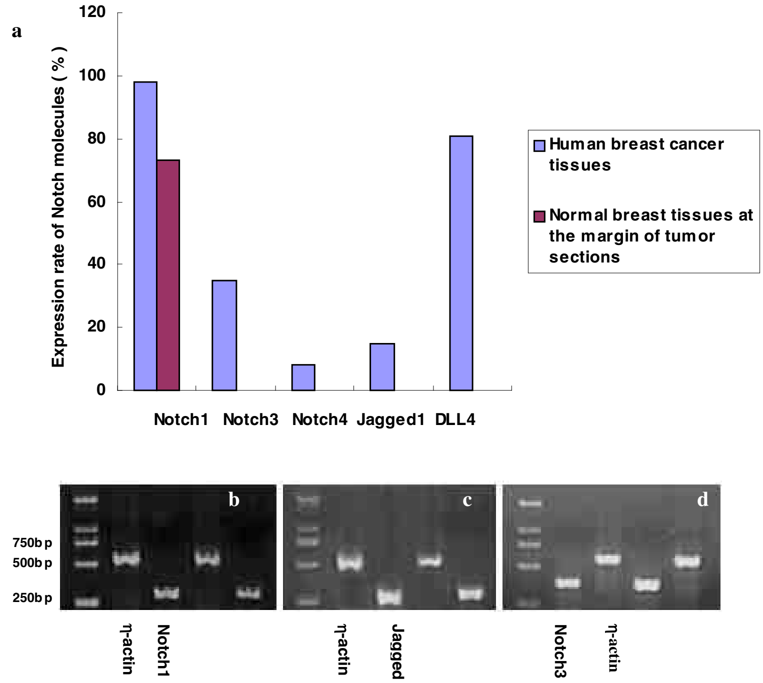

Figure 1. Expression of Notch molecules in human breast cancer. a, the expression rate of Notch1,3,4 and Jagged1, DLL4 in human breast cancer samples and normal breast tissues from the margin of tumor section. b, the expression of Notch1 and b-actin(inner control) in human breast cancer cell lines MDA-MB-231 (left two lines) and MDA-MB-435 (right two lines). c, the expression of Jagged1 and b-actin in human breast cancer cell lines MDA-MB-231 (left two lines) and MDA-MB-435 (right two lines). d, the expression of Notch3 and b-actin in human breast cancer cell lines MDA-MB-231 (left two lines) and MDA-MB-435(right two lines).

Materials and methods

Cell culture. Human breast cancer cell lines MDA-MB-231 and MDA-MB-435 were cultured in RPMI medium 1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (Lanzhou Hyclone Bio-engineering Co, China), penicillin-streptomycin (100U/ml). All cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

RT- PCR. 62 breast cancer specimens and 22 normal breast tissues from the margin of tumor sections were derived from the patients treated at the Shandong University Qilu hospital and Jinan Central Hospital from Oct, 2004 to Dec, 2005. They were hospitalized as mammary gland lump, and were finally diagnosed as breast cancer by pathological examination. The breast tissues from the margin of tumor sections were confirmed to be normal tissues by pathologist. Subsequently written informed consent was obtained from the women before surgery. Cells were grown to near confluence in 100ml flasks. Total RNA from tissues and cells was extracted by means of TRIZOL (Omega, UK) according to the manufacturer's instructions. Synthesis of first-strand cDNA was carried out with RevertAid™ First Strand cDNA Synthesis Kit (MBI, Fermentas, USA). Primers for Notch1, Notch3, Notch4, Jagged1 and DLL4 are shown in Table 1. Primers specific for β-actin were used to normalize cDNA yield.

Immunostaining. Formalin-fixed, paraffin-embedded tissue sections (5mm thick) were deparaffined in xylene, rehydrated in grade alcohols, and briefly microwaved in 0.001mol/L citrate buffer, PH 6.0, to optimize antigen retrieval. Alternatively, cultured cells grown on cover slides were fixed in 95% ethanol. Endogenous peroxidases were quenched with 3% H_2O_2 in methanol for 10 minutes. Sections were then used to detect Notch1-IC using Histostain-plus kit (JINGMEI BIOTECH, Shenzhen, China) according to the manufacturer's instructions. The primary antibody of activated Notch1 (Notch1-IC, Abcam Ltd, Cambridge, UK) was diluted 1:500. Immunostaining was visualized by using Olympus IX81.

Table 1. Primers used in this study

Target gene	Primer sequence	Product size (bp)
Notch1	(F) 5'-GGG TCC ACC AGT TTG AAT GG-3' (R) 5'-GTT TGC TGG CTG CAG GTT CT-3	306
Notch3	(F) 5'-CAA CCC GGT GTA CGA GAA GT-3' (R) 5'-ACA ACG CTC CCA GGT AGT CA-3'	443
Notch4	(F) 5'-TCT CCC TCT CCA TTG ACA CC-3' (R) 5'-TCC TGG AAG CAC TCG TTG-3'	326
Jagged1	(F) 5'-CTCATCAGCCGTGTCTCAAC-3' (R) 5'-GGCACACACACTTAAATCCG-3'	297
DLL-4	(F) 5'-AAG GCT GCG CTA CTC TTA CC-3' (R) 5'-ATC CTC CTG GTC CTT ACA GC-3'	538
HES-1	(F) 5'- TGA TTT GGA TGC TCT GAA GAA AGA TA-3' (R) 5'-GCT GCA GGT TCC GGA GGT. -3'	100
β-actin	(F) 5'-CGG GAC CTG ACT GAC TAC CT-3' (R) 5'-AAG CAT TTG CGG TGG A-3'	578

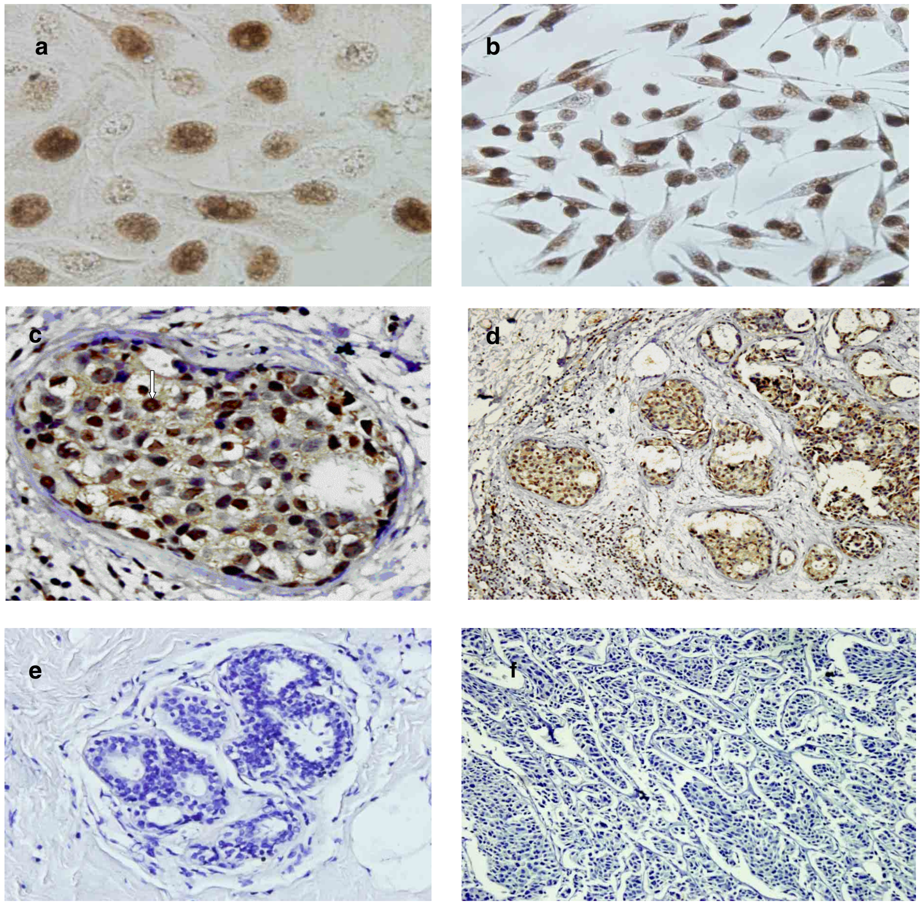

Figure2: Immunostaining for Notch1-IC in human breast cancer cell lines and breast cancer tissues. Human breast cancer cell lines MDA-MB-231 and MDA-MB-435 are both strongly stained(a,b), indicating that Notch is highly activated in both cell lines. Normal breast tissues are negative for Notch1-IC staining (e), whereas most breast cancer tissues are positive for Notch1-IC staining(c,d), mainly localized in the nuclear (shown by the arrows in c), indicative of Notch activation. Only 1 case in our study showed no positive staining for Notch1-IC (f), suggesting that Notch signaling was not involved in all human breast cancers.

Inhibition analysis. Briefly, $4^{*}10^{4}$ cells of MDA-MB-231 and MDA-MB-435 were plated in a 96-well plate and allowed to proliferate overnight. The cells were then treated with increasing concentrations of γ-secretase inhibitor I (0-5μ mol/l) (EMD Bioscience, CA) or DMSO as a control, and after 24h, 20μl of MTT (Amresco, CA) was added per well. After additional 4h incubation, remove the liquid, 200μl DMSO was added per well. The plate was shaken thoroughly for 10 mins, and color development measured on a microplate reader at 570 nm.

Analysis of Cell cycle. Briefly, $1^{*}10^{6}$ cells were plated in 100 ml culture flasks and allowed to proliferate till 70%-80% confluent. Then, the cells were treated with γ-secretase inhibitor I (3μmol/L) or DMSO (15μl) as a control, and after 24h, cells were harvested and washed in PBS, then fixed in 75% alcohol for 30 min at 4°C. After washing in cold PBS for three times, cells were resuspended in 1 ml of PBS solution with 40 μg of propidium iodide (PI, Sigma) and 100 μg of RNase A (Sigma) for 30 min at 37°C. Samples were then analyzed for their DNA content by FACS Calibur (Becton Dickinson, CA).

Detection of apoptosis. Cells were treated with γ-secretase inhibitor I (3μmol/L) or DMSO (15μl) as described above. To measure apoptosis, Annexin-FITC Apoptosis Detection Kit (JINGMEI BIOTECH, Shenzhen, China) was used according to its instructions. Precisely, fresh cells were labled with 1:500 annexin V-biotin conjugated with fluorescein isothiocyanate (FITC) followed by 1:1000 PI. Annexin V-PI were measured by FACS Calibur (Becton Dickinson, CA) and analyzed with Modfit Softwere.

Statistical Analysis. The expression rates of Notch molecules were analyzed by Wilcoxon rank sum test. The Student t test was used to determine the statistical significance of the data obtained and to compare the means between groups. A $P<0.05$ represented a statistically significant difference.

Results

Notch and its ligands are overexpressed in human breast cancer. Of 62 breast cancer samples, 61 cases are positive for Notch1 expression, 22 cases are positive for Notch3 expression, 5 cases are positive for Notch4 expression, 50 cases are positive for Jagged1 expression, 45 cases are positive for DLL4 expression. Of 22 normal breast tissues at the margin of tumor sections, only 16 cases are positive for Notch1 expression, other Notch receptors and ligands are not detected at mRNA level. The expression rates of Notch1, 3, 4, Jagged1 and DLL4 gene mRNA in cancer specimen and normal breast tissue at the margin of tumor sections are 98%,35%,8%,15%, 81% and 73%,0,0,0 , 0, respectively. The expression rate of Notch1 mRNA in the cancer specimens is significantly higher than that in the specimens at the margin of tumor sections(P<0.05). The relative coefficient of the cancer specimens expressing Notch1 mRNA is significantly higher than that of the normal breast tissues at the margin of tumor sections, and the medians were 1.23 and 0.75 respectively.(P<0.05, Wilcoxon rank sum test)(Figure 1a)

Notch1, 3 and JAG1 were all expressed in human breast cancer cell lines MDA-MB-231 and MDA-MB-435. (Figure 1b)

Notch1 is activated in human breast cancer. We used the antibody which recognizes only the activated form of Notch1 (N1-ICD, the cleaved intracellular form) to detect its activation. We initially surveyed two breast cancer cell lines MDA-MB-231 and MDA-MB-435. In both cell lines, we observed a clear accumulation of N1-ICD in both the cytoplasm and nuclear, with the later finding being indicative of Notch activation. (Figure 2a, b)

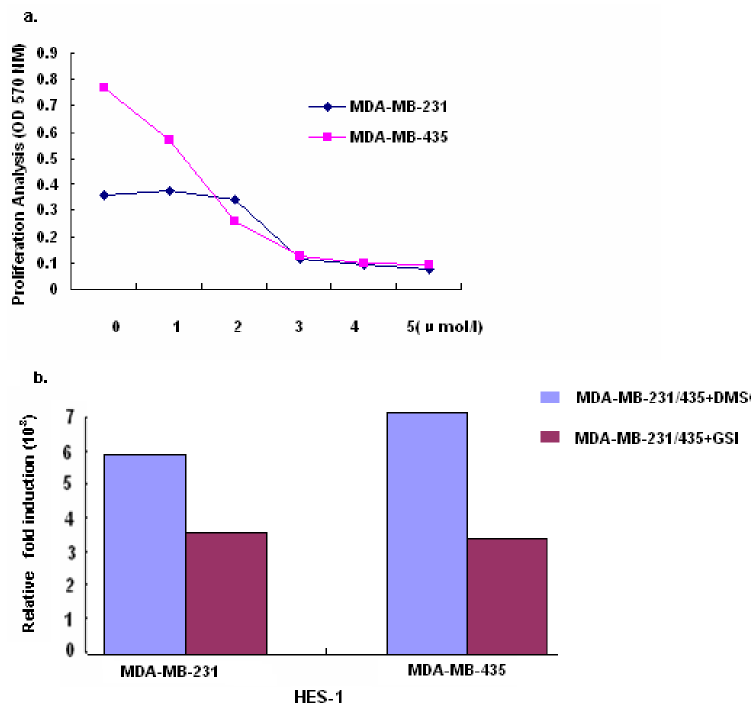

Figure 3: γ-secretase inhibitor I inhibits the proliferation of human breast cancer cell lines MDA-MB-231 and MDA-MB-435 through the inhibition of Notch signaling. a, after treated with increasing concentration of γ-secretase inhibitor I (0,1,2,3,4,5µmol/l) or DMSO as control,the proliferation of MDA-MB-231 and MDA-MB-435 cells were measured with MTT method as described in "Material and Method". Both cell lines that treated with γ-secretase inhibitor I had a decreasing proliferation rated with the increasing concentration of γ-secretase inhibitor I. b, γ-secretase inhibitor I significantly decreased transcription level of HES-1,a known down-stream target gene. p<0.01, data are means of three independent experiments.

To detect the activation of Notch signaling in actual tumors, we extended the analysis to include 10 various types of breast cancer samples and 4 normal breast tissues from the margin of tumor sections. Just as the cell lines, we saw accumulation of N1-ICD in most (9 in 10) tumor samples analysed, only 1 sample showed negative staining. Whereas, no N1-ICD staining was detected in all the normal breast tissues. (Figure 2c, d, e,f)

γ-secretase inhibitor I can inhibit the proliferation of breast cancer cells. The clear aberrant activation of Notch signaling in both breast cancer cell lines and tumor tissue samples suggests that it may play a significant role in tumor development. Consequently, we explored what the inhibition of Notch signaling may result in breast cancer cell lines MDA-MB-231 and MDA-MB-435. γ-secretase inhibitor I, a pharmacologic agent known to block effectively Notch activation, was used to evaluate the effect of Notch inhibition on breast cancer cells. We observed the proliferation of both cell lines decreased with the increasing dosage of γ-secretase inhibitor I (1-5µmol/l) (Figure 3a). The inhibition of Notch signaling was verified by the significant decrease in mRNA level of HES1-a known down-stream target of Notch signaling. (Figure 3b)

γ-secretase inhibitor I can cause cell cycle arrest in breast cancer cell lines. As observed above, inhibition of Notch signaling can inhibit the proliferation of breast cancer cells in vitro, we then explored the mechanisms underlying the growth inhibition.

Because cell proliferation and death are closely linked to progression of cell cycle, we analysed cell cycle distribution in breast cancer cell lines MDA-MB-231 and MDA-MB-435. Representative cell cycle profile of γ-secretase inhibitor I -treated cells and DMSO-treated cells as controls are shown in Figure 4, with data expressed as mean percentage of cells in each cell cycle phase derived from three independent experiments. γ-secretase inhibitor I-treated cells of MDA-MB-231 and MDA-MB-435 showed a higher proportion of cells in G2/M phase compared with control (29.9% vs 15.99%, and 45.5% vs 16.63% in MDA-MB-231 and MDA-MB-435 respectively) and a decrease in the proportion of cells in S phase relative to that observed in controls (19.82% vs 32.7% and 29.98% vs

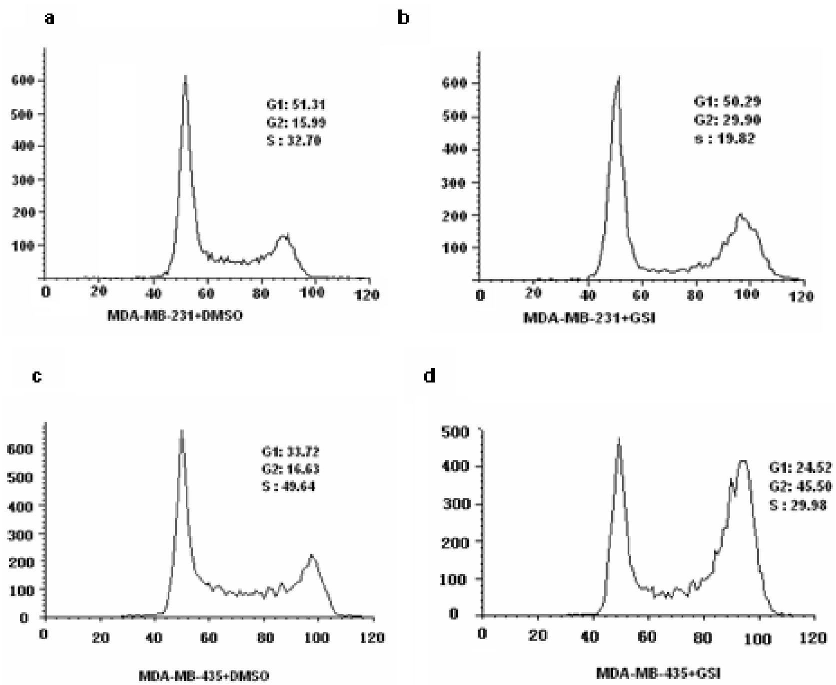

Figure 4: γ-secretase inhibitor I alters the cell cycle kinetics of human breast cancer cells. MDA-MB-231 and MDA-MB-435 cells were harvested 24 h after treated with γ-secretase inhibitor I or DMSO as control, and PI staining was used to analyse cell cycle distribution. γ-secretase inhibitor I caused G2/M arrest in MDA-MB-231 and MDA-MB-435 cells.

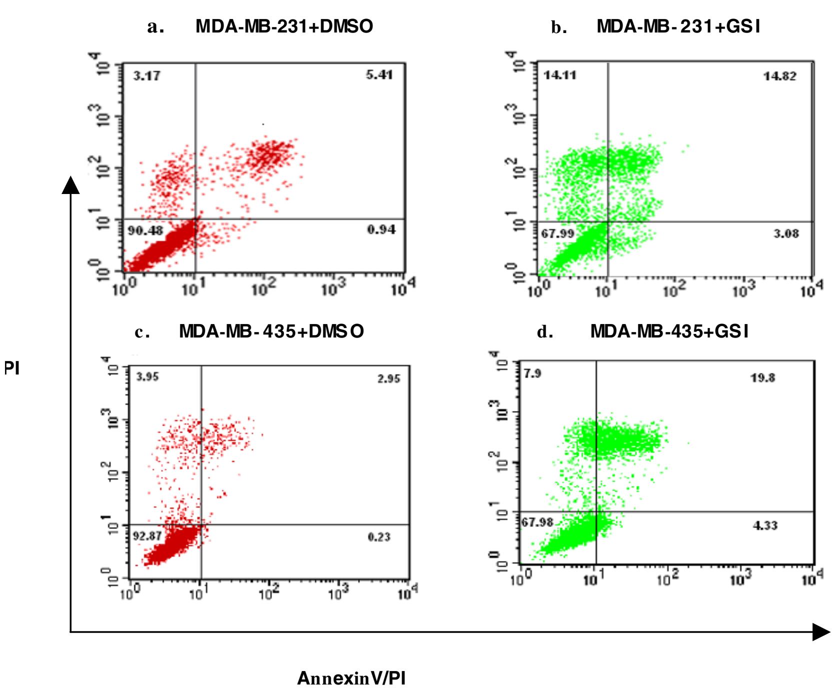

Figure 5: γ-secretase inhibitor I caused apoptosis in human breast cancer cells. After treated with γ-secretase inhibitor I or DMSO as control, MDA-MB-231 and MDA-MB-435 cells were harvested and stained with annexinV/PI to evaluate apoptosis. Result showes that the percentage of apoptotic cells treated with γ-secretase inhibitor I is significantly higher than the control cells.

49.64% in MDA-MB-231 and MDA-MB-435 respectively). Cell cycle distribution analysis showed that the increase in G2/M phase cells observed in γ-secretase inhibitor I -treated populations was significant ($p<0.05$), suggesting that inhibition of Notch signaling induces G2/M phase cell cycle arrest in breast cancer cell lines MDA-MB-231 and MDA-MB-435.

γ-secretase inhibitor I can cause apoptosis in breast cancer cell lines. We found that a portion of cells treated with γ-secretase inhibitor I, which normally grow as tightly adherent monolayer, became detached and exhibited a rounded shape, meanwhile, a portion of apoptotic body were observed (not shown). These morphological changes were not observed in control cells, and lead us to speculate that inhibition of Notch may lead to apoptosis of breast cancer cell lines. To detect apoptosis, MDA-MB-231 and MDA-MB-435 cells were treated with γ-secretase inhibitor I and DMSO as controls for 24h, then stained with annexin V/PI. (Figure 5a) The fraction of apoptotic cells in γ-secretase inhibitor I-treated populations was significantly higher than that observed in controls (0.94% vs 3.03%, 0.23% vs 4.33% in MDA-MB-231 and MDA-MB-435 respectively, $p<0.05$, data are mean percentage of apoptotic cells from three independent experiments, demonstrating that inhibition of Notch signaling could also induce apoptosis in breast cancer cells.

Discussion

Although it is clear that Notch is involved in the genesis of diverse tumor types, the function of Notch in cancer is complex [18,19]. In humans, a truncated, activated form of Notch1 expression has been identified as a causative factor in the development of T-cell acute lymphoblastic leukemia and lymphomas [20]. An overexpression of Notch molecules is also observed in solid human tumors such as colon, lung, pancreas, skin and brain cancers, suggesting a possible causative role for deregulated Notch signaling in tumor pathogenesis. However, expression of activated Notch1 causes growth inhibition of human papillomavirus-positive cervical carcinoma cells, prostate cancer cell and liver carcinoma cells, suggesting a tumor suppressing role [21,22,6].

Several studies have shown that Notch signaling is involved in human breast cancer [23,24]. Reedijk et al. proved a direct relationship between high level of JAG1/NOTCH1 expression and poor overall patient survival in human breast cancer, and predicted that a JAG1/Notch1 loop is functioning to promote tumor formation and progression [23]. Stylianou's study showed that increased Notch signaling was sufficient to transform normal breast epithelial cells and attenuation of Notch signaling could revert the transformed phenotype of human breast cnacer cell lines [24]. Here we show that Notch receptors (Notch1, 3, 4) and ligands (JAG1 and DLL4) are highly and widely expressed in human breast cancers. Meanwhile, the Notch signaling is activated in human breast cancer tissue samples and cell lines as we observed accumulation of N1-ICD localized both in the plasma and nuclear, mostly in the latter, whereas N1-ICD was undetectable in normal breast tissues at the margin of tumor sections, indicating that Notch pathway is aberrantly activated in human breast cancer. We observed none of the Notch ligands were expressed in normal breast tissues from the margin of tumor sections. This is in contrast to the former report that Notch ligands Jagged1 and Jagged2 are expressed in the luminal epithelium of normal human breast tissue [23]. These data again suggest the carcinogenesis role of Notch in breast cancers.

Inhibition of Notch signaling by γ-secretase inhibitor I could cause growth arrest in breast cancer cells through cell cycle arrest in G2/M phase. γ-secretases are known to mediate proteolysis of Notch receptors and release the activated form of Notch. Thus, γ-secretase inhibitor can inhibit the activation of Notch signaling [25]. Studies show that γ-secretase inhibitor cause apoptosis in Kaposi's sarcoma and melanoma [26,27]. To the best of our knowledge, this is the first time in literature showing that γ-secretase inhibitor can inhibit the proliferation of breast cancer cells, suggesting that inactivation of Notch may be a potential therapeutic approach for this malignant disease. We also observed apoptosis after the inhibition of Notch signaling, it would be interesting to know the molecular interface between cell cycle arrest and apoptosis upon inhibition of Notch signaling in the cells.

Despite much work has been done, an overall understanding of Notch signaling in human breast cancer still demand further exploration. First, there are three Notch receptors expressed in most breast cancers, which one play the main role in the tumorigenesis? Do they cooperate or antagonize? Second, what role do the Notch ligands-JAG1, DLL4 play in human breast cancer? Do they just activate the receptor? Since there is report that the ligands CTFs (c-terminal fragments) compete with an active form of Notch for cleavage by γ-secretase and can thus inhibit Notch signaling in vitro [28]. Third, what are molecular events underlying cell cycle arrest and apoptosis following the inhibition of Notch signaling? All this will aid a better understanding of the molecular pathways involved in breast cancer cell proliferation, providing even new targets for treatment.

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