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Synergistic Effect of Notch-3-Specific Inhibition and Paclitaxel in Non-Small Cell Lung Cancer (NSCLC) Cells Via Activation of The Intrinsic Apoptosis Pathway

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Lung cancers are resistant to conventional chemotherapeutic interventions such as paclitaxel. Notch signaling is crucial in the chemoresistance of lung cancer cells. The Notch inhibitor gamma-secretase inhibitor (GSI) inhibits the Notch signaling pathway.

Material/Methods: Here, we evaluated how Notch-3 inhibition by GSI can enhance the sensitivity of lung cancer cells to paclitaxel. To study how Notch-3-specific inhibition affects non-small cell lung cancer (NSCLC), we compared the cell viability, apoptosis, and colony formation of A549 and H1299 cells treated with Notch-3 siRNA and GSI.

Results: The expression levels of Notch-3 or Notch intracellular domain 3 (NICD3) and apoptosis-related proteins were measured and compared between different groups. Notch-3 was significantly overexpressed in both cell lines, and Notch-3 expression was elevated after paclitaxel treatment, indicating activation of the Notch signaling pathway. Inhibition of the Notch signaling pathway by GSI and Notch-3 siRNA reduced cell proliferation and induced apoptosis in A549 and H1299 cells, thereby boosting sensitivity of the cell lines to paclitaxel. Concomitant treatment with paclitaxel and GSI or siRNA downregulated Bcl-2 expression and upregulated Bax expression levels.

Conclusions: These results indicate a synergistic effect of Notch-3-specific inhibition and paclitaxel through alteration of the intrinsic apoptosis pathway, which was involved in Notch-3-induced chemoresistance in NSCLC cells, and GSI inhibited Notch-3-induced chemoresistance in a concentration-dependent manner. This approach that combines Notch-3-specific inhibition and paclitaxel would be likely to apply in NSCLC.

MeSH Keywords: Amyloid Precursor Protein Secretases • Carcinoma, Non-Small-Cell Lung • Paclitaxel • Receptors, Notch • RNA, Small Interfering

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Background

Lung cancer is recognized to be one of the most common cancers. It remains the leading cause of global cancer deaths. The extremely invasive phenotype and rapid progression, as well as resistance to chemotherapy, of lung cancer contribute to the poor prognosis of this type of cancer [1]. The primary form of lung cancer is non-small cell lung cancer (NSCLC), which accounts for roughly 80% of all cancers of this type. It is highly invasive, resistant to chemotherapy, and shows rapid progression [1,2]. The main treatment involves cytoreductive surgery followed by a chemotherapy regimen with agents such as platinum and paclitaxel. Despite this, the results yielded are not satisfactory, and the prognosis remains poor with a low 5-year survival rate [2,3]. Although chemotherapy has seen considerable progress in recent years, recurrences are inevitable in the majority of patients, and most patients with lung cancers acquired chemoresistance [4]. The standard first-line therapy regimen advocated for patients with advanced NSCLC is paclitaxel with platinum, especially as a single agent in advanced and metastatic NSCLC [5]. However, most patients acquire resistance after a period of treatment [6]. Survival-related pathways in NSCLC have been confirmed to contribute to tumor progression and recurrence. Recent studies have also shown that combining molecular target and chemotherapy strategies may improve the treatment outcomes in these patients. For example, paclitaxel resistance is developed through several mechanisms, including alteration of high HDAC expression and tubulin mutations [7,8]. Many signaling pathways, such as epidermal growth factor receptor (EGFR), WNT, Notch, and Hedgehog, are involved in drug resistance in NSCLC [9]. Among them, the Notch signaling pathway was shown to play significant roles in tumor initiation, proliferation, differentiation, invasion, and apoptosis in cell lines and animal models [10,11]. Moreover, this pathway influences chemosensitivity to some chemotherapeutic agents such as GEM [12].

Notch signal pathway receptors (Notch 1–4) are transmembrane proteins that bind to ligands (Delta-like 1, 3, and 4 and Jagged1 and Jagged2) and undergo proteolytic cleavage by multiple enzymes, resulting in the production of Notch intracellular domain (NICD), which acts on the nucleus and drives the expression of Hes1 and Hey-1, acting as some transcriptional repressors and inhibitors, which are fundamentally inhibitory of positively acting bHLH proteins, including the ubiquitous E2A factors and the tissue-restricted bHLH inducers of differentiation including Mash1 (mammalian achaete-scute homolog-1), MyoD, and many others [13,14].

In recent years, studies have found that the Notch pathway is a target for the treatment of patients with chemoresistant and recurrent NSCLC [14]. However, due to the complexity of this pathway, studies have found it difficult to predict the outcome of Notch activation because of the multiple Notch receptors and ligands and the large number of target genes. In addition, there is potential crosstalk between Notch and other signaling pathways; this further complicates the system [15]. This has been illustrated by several studies reporting high Notch protein expression levels in cancers such as lung, ovarian, pancreatic, and gastric carcinomas [16–21].

In addition, the Notch-3 protein is highly expressed in NSCLC [22–25], and elevated Notch-3 expression is associated with resistance to chemotherapy and decreased survival rates among patients with ovarian cancer [26]. Although cytotoxic therapies inhibit the proliferation of cancer cells, researchers have demonstrated Notch3 overexpression in chemoresistant patients and hypothesized that Notch3 may in turn promote tumorigenic cell populations that can sustain tumor growth [27]. While this hypothesis is remains to be proven, some discoveries have confirmed that combining Notch pathway inhibition and conventional cytotoxic therapy can obviously inhibit tumor development.

Because of the complexity of the Notch pathway, a variety of inhibitors currently are being investigated to block it. Of these, γ-secretase inhibitors (GSIs) have been widely used in a variety of cancers [28]. Basic and clinical trials have reported the antitumor activity of GSIs, although these studies have been conducted on a small sample size of solid tumors [29]. Apart from inhibiting cell proliferation and inducing cell apoptosis by inhibiting the crack of Notch-3, GSI is associated with increasing the sensitivity of NSCLC cell lines to platinum chemotherapy, supporting the concept that GSI can be useful in chemotherapy treatment and recurrent cancer [30]. Thus, it is necessary to explore the molecular mechanisms in order to obtain greater insight into the role of GSI in the treatment of NSCLC.

Material and Methods

Cell culture

A549 and H1299 cell lines were originally obtained from the Nuclear Medicine Teaching and Research Department, Anhui Medical University. The two cell lines were cultured in 500 mL of Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technology Co., Shanghai, China) containing 50 mL of fetal bovine serum (FBS; Sijiqing, Hangzhou, China) and 5 mL of penicillin-streptomycin solution (Beyotime Institute, Shanghai, China). The cells were incubated at 37°C in an incubator containing 5% CO_2.

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Notch-3-specific siRNA transfection and GSI treatment

Briefly, a control scrambled siRNA (not homologous to any gene) and three different siRNAs targeting Notch-3 sequences selected by the results of Western blot (data not shown) were purchased from Ruibo (Guangzhou, China). The siRNA-Lipofectamine™ 2000 (Invitrogen) in Opti-MEM (Gibco) was premixed according to the manufacturer's instructions and then transfected into the two cell lines. After transfection, the cells were harvested and prepared for subsequent analysis. GSI (GSI-IX, D5924-5MG, Sigma) was diluted in dimethyl sulfoxide (DMSO, Sigma) to achieve a concentration of 10 mM and stored at –20°C. Before treatment, GSI was added to the medium at different concentrations as required. The concentration of DMSO in medium did not exceed 0.1%. The cells were cultured in different concentrations of GSI (0, 2, 5, and 20 μM) for 24 h, following which paclitaxel was added and the cells were cultured for 24, 48, and 72 h. The control cells were treated with 1 μL of DMSO in culture medium.

Groups

The cells were divided into groups: (a) NC, no treatment; (b) Control siRNA, 50 nM scrambled siRNA transfected cells; (c) Notch-3 siRNA, 50 nM Notch-3 siRNA transfected cells; (d) NC + paclitaxel, cells exposed to paclitaxel treatment; (e) Control siRNA + paclitaxel, cells treated with paclitaxel alone; (f) Notch-3 siRNA + paclitaxel; (g) 2 μM GSI, cells treated with 2 μM GSI; (h) 5 μM GSI, cells treated with 5 μM GSI; (i) 20 μM GSI, cells treated with 20 μM GSI; (j) DMSO, cells treated with 1 μL of DMSO; (k) GSI + paclitaxel, cells treated with different concentrations of GSI and paclitaxel (1 and 0.2 μM for A549 and H1299 cells, respectively).

MTT assay

Logarithmic-phase cells were transferred to the wells of 96-well plates at 3000 cells per well. They were then transfected with Notch-3 or control siRNA and GSI (2, 5, and 20 μM). After transfection and GSI treatment for 24 h, the cells were treated with paclitaxel for 24, 48, and 72 h. Tumor cells were determined with 10% of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) for 4 h, and then 150 μL of DMSO was used to terminate the reaction. Finally, the cell viability in each well was measured in terms of optical density at a wavelength of 490 nm. Each assay was performed six times.

Detection of apoptosis and colony-forming assays

The two cells were transfected with 50 nM siRNA and 20 μM GSI for 24 h, and then treated with or without paclitaxel. The apoptosis rate was detected using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Biomiga, Shanghai, China). According the specific method, cells were trypsinized, centrifuged, and washed with precooled phosphate-buffered saline (PBS). Each sample was treated with 400 μL of Annexin V-FITC for 10 min and 10 μL of propidium iodide (PI), according to the manufacturer's instructions. The samples were then mixed and incubated at 4°C in the dark, and analyzed within 1 h using a flow cytometer (Beckman, USA). The percentage of cell apoptosis was calculated using WinMDI29 and compared between the groups. All experiments were performed in triplicate. The effect of the inhibitor on colony formation was also evaluated. Cells were seeded at a density of 1×10^3 cells in six-well plates for one night. After transfection with 100 nM siRNA or 20 μM GSI for 24 h, the cells were processed with or without paclitaxel. After several days, colonies were washed, fixed with paraformaldehyde for 20 min, stained with hematoxylin for 30 min, and counted. Groups of cells >50 were scored as colonies.

Western blot analysis

For Western blot analysis, the treated cells were washed twice with ice-cold PBS and incubated in a cell lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 1% phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, for 15 min at 4°C. Standard sodium dodecyl sulfate (SDS) sample buffer was added to the protein products, which were boiled for 10 min and centrifuged at 12,000×*g* for 10 min at 4°C. The sample proteins were then separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, Massachusetts, USA), which was blocked using 5% skim milk for 2 h at room temperature, and incubated with the specific antibodies against Notch3 and NICD3 (ab23426; Abcam, Cambridge, UK, 1: 5000) and anti-Bcl-2 and anti-Bax antibodies (YM3041 and YT0459 respectively; Immunoway, USA, 1: 1000) for one night. Mouse antibodies against β-actin were purchased from ZSGB-BIO (Beijing, China). Horseradish peroxidase (HRP)-conjugated IgG was used as the secondary antibody. Western blot analysis of β-actin on the same membrane served as the loading control. The protein bands were densitometrically analyzed using ImageJ software. All experiments were performed in triplicate.

Statistical analysis

All experiments were carried out in triplicate. Data were processed as the mean ± standard deviations (SDs). Differences among the treatments were compared using analysis of the *t* test. Statistical significance was determined at a P value of <0.05.

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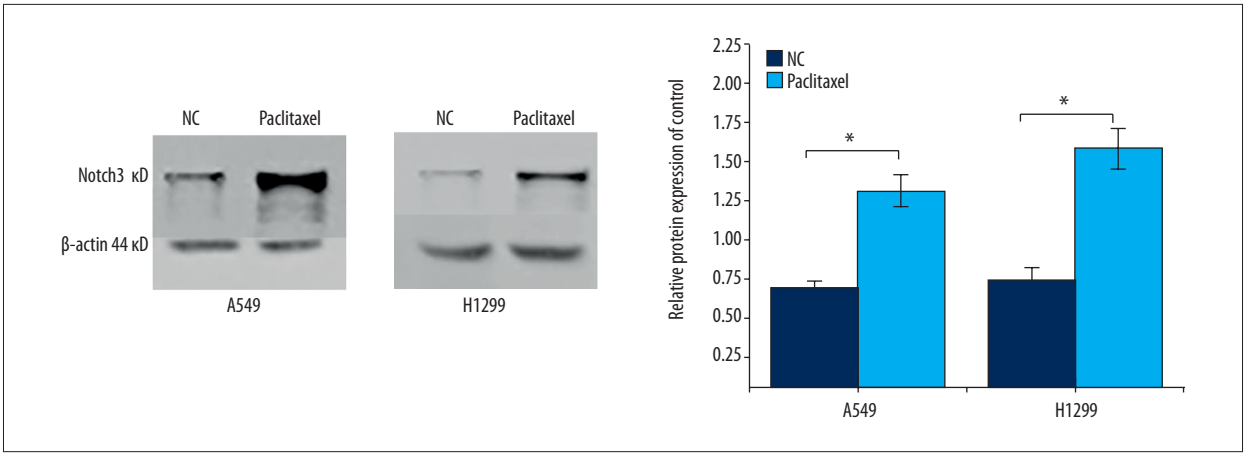

Figure 1. Notch-3 expression in human non-small cell lung cancer (NSCLC) cells. Protein bands in representative blots with mean densitometry values. β-Actin was used as the loading control. Experiments were repeated in triplicate and yielded similar results. Effects of paclitaxel on two cell lines with and without Notch-3 siRNA. Statistical analysis is also shown; Notch-3 was obviously upregulated after paclitaxel treatment ($P<0.05$). * $P<0.05$ *vs.* control.

Results

Paclitaxel treatment upregulates Notch-3 expression

We previously demonstrated that Notch-3 expression levels are higher in NSCLCs compared with normal lung tissue [25]. Results of the Western blot analysis of A549 and H1299 cells revealed obvious upregulation of Notch-3 ($P<0.05$; Figure 1). The result indicated that the paclitaxel induced the activation of Notch signaling.

Notch-3 inhibition sensitizes A549 and H1299 cells to paclitaxel treatment, inhibiting tumor growth

To investigate the effect after paclitaxel therapy, A549 and H1299 cells were exposed to 0–20 and 0–12.8 μM paclitaxel for an additional 48 h. Knockdown of Notch-3 by specific siRNA inhibited cell viability and sensitized A549 and H1299 cells to paclitaxel (Figure 2A, 2B). Cell viability was assessed using the MTT assay to assess the effect of Notch-3 inhibition on paclitaxel sensitivity of both cell lines. Following stable siRNA transfection, the IC_{50} was calculated from the MTT assay. The IC_{50} of paclitaxel was reduced in both the cell lines treated with Notch-3-specific siRNA compared to the untransfected control cells. We also examined the inhibition rates of paclitaxel treatment alone and paclitaxel plus GSI for different durations. GSI-treated A549 and H1299 cells cultured with 2 μM and 0.2 μM paclitaxel exhibited decreased viability compared to cells treated with paclitaxel alone at all the GSI concentrations and time durations tested. Notably, the cell viability decreased with increasing concentrations of GSI (2–20 μM). The inhibition rate significantly increased in the paclitaxel plus GSI treatment group compared with the paclitaxel group in both cell lines (Figure 2C, 2D).

Effect of Notch-3 pathway inhibition on apoptosis and colony-forming ability

We next determined whether enhancement of paclitaxel sensitivity by inhibition of the Notch pathway is related to apoptosis. Flow cytometric analysis showed significant increases in the apoptosis rates of A549 cells treated with paclitaxel plus siRNA or GSI (Figure 3), whereas the difference in the apoptosis rate of H1299 cells treated with siRNA or GSI was marginally significant (Figure 3). Specifically, A549 and H1299 cells treated with paclitaxel and 20 μM GSI showed an increase in apoptosis compared to those treated with paclitaxel alone. The results of the colony-forming assay showed that both Notch-3 siRNA and GSI significantly inhibited colony growth. The number of colonies formed from A549 cells treated with a combination of paclitaxel plus either 20 μM GSI or siRNA were lower than those from paclitaxel-treated A549 cells (Figure 4A, 4C). Similarly, there were also significantly fewer H1299 cells in the paclitaxel plus 20 μM GSI or Notch-3 siRNA groups compared with H1299 cells treated with paclitaxel alone (Figure 4B, 4D).

Intrinsic pathway is involved in Notch inhibition-induced apoptosis, and related protein levels are changed by Notch-3 inhibition

Notch-3 expression was downregulated in cells treated with Notch-3 inhibitors. Bcl-2 expression was significantly decreased and Bax expression was increased at 48 h, as well as at 72 h, in the 20 μM GSI group compared to the 2 and 5 μM GSI-treated groups (Figure 5C, 4D). Treatment with Notch-3 siRNA significantly reduced the Bcl-2 level and increased the Bax level (Figure 4A, 4B). Moreover, we further examined the expression of proteins involved in the intrinsic apoptosis pathway in paclitaxel resistance. Bax expression was obviously

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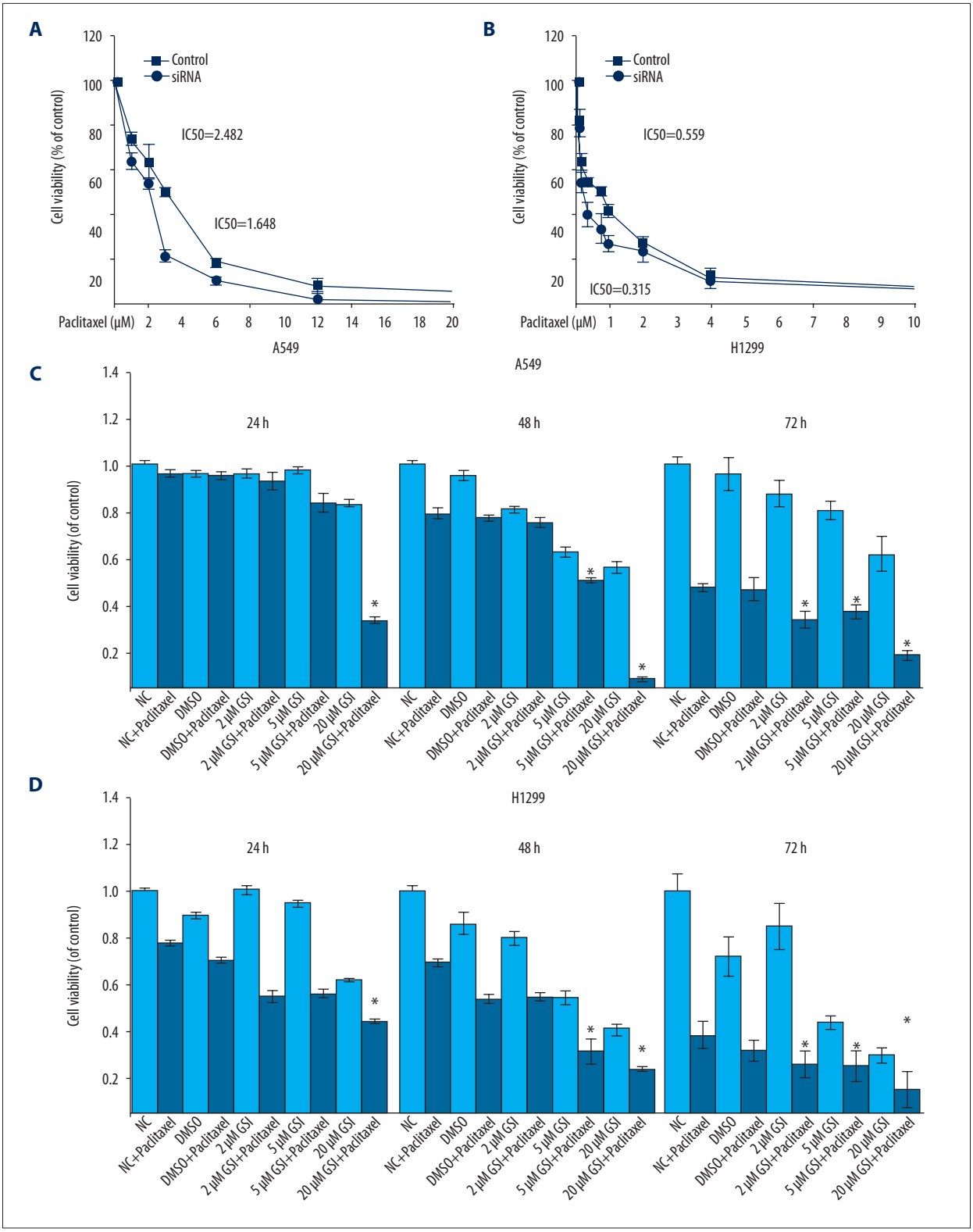

Figure 2. Notch-3 inactivation promotes paclitaxel-induced cytotoxicity in A549 and H1299 cells. (**A, B**) Knockdown of Notch-3 by 50 nM siRNA affects the sensitivity of A549 and H1299 cells to paclitaxel. Treatment with Notch-3-specific siRNA decreased the paclitaxel IC_{50} of each cell line (P<0.05). Experiments were performed in triplicate with eight determinations per condition. (**C, D**) Cell viability was assessed by the MTT assay after inhibition of Notch-3 with 2, 5, and 20 μM GSI. * P<0.05 *vs.* control.

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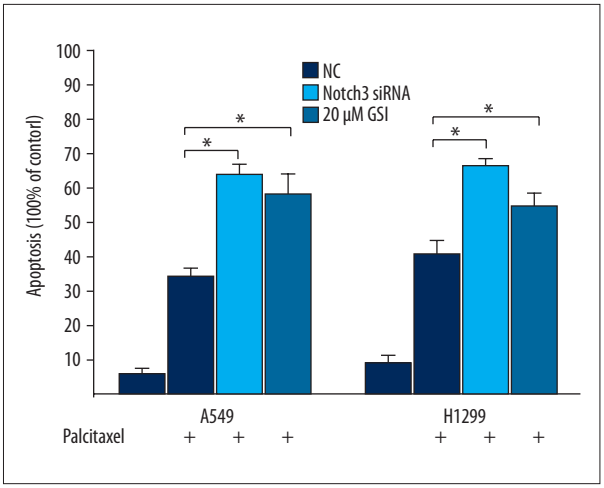

Figure 3. Effect of Notch-3 inactivation on NSCLC cells. Apoptotic cells were assessed by fluorescence-activated cell sorting (FACS) after 20 μM GSI and 50 nM Notch-3 siRNA treatment for 48 h in A549 cells and H1299 cells. The apoptotic fraction of cells exposed to 20 μM paclitaxel. Experiments were performed in triplicate. * P<0.05 *vs.* control.

increased in cells treated with paclitaxel in combination with GSI or Notch-3 siRNA compared to cells treated with paclitaxel alone or parent cells. In addition, Bcl-2 expression was also decreased in the cell lines treated with paclitaxel in combination with Notch-3 siRNA or GSI.

Discussion

One of the critical challenges in the treatment of lung cancer is the development of chemoresistant recurrence. Although the current therapy involves platinum-based chemotherapy with cytoreductive surgery, significant progress has been made in molecular targeted therapy. However, due to the specificity of the tumor cells, the problem of acquired resistance remains to be addressed, which ultimately results in recurrence and low survival rates [31]. Moreover, molecular targeted therapy also need further research. Paclitaxel cytotoxicity caused specifically by paclitaxel results in cell death [32,33]. Therefore, development of new targeted therapies represents a critical challenge. Different studies have shown that drug resistance occurs via a range of mechanisms, including apoptosis-related protein Bcl-2, interference with survival pathways by factors such as EMT-related proteins and transforming growth factor (TGF)-β, DNA repair activity, and regulation of cancer stem cells (CSCs) by CD44 and CD24 [34,35]. Here, Notch-3 inhibition downregulated the expression levels of its target molecules, including apoptosis-associated proteins, resulting in reduced tumorigenesis and proliferation, thus responding to the paclitaxel treatment [36,37]. Furthermore, some results indicated that Notch-3 inhibition could help enhance the sensitivity to paclitaxel [38,39]. Recent findings have suggested that the Notch pathway is one of the most important signal pathways in tumor development or progression [40]. Overall, other results also have proved that Notch-3 protein significantly affects the development and prognosis of lung cancer. Together with the findings of other studies, our study found that Notch-3 was the most significantly overexpressed Notch family member in NSCLCs, and it seems to be more closely related to chemoresistance and recurrence than any of the other receptors in ovarian cancer [41].

The results of this study provide comprehensive evidence that the Notch-3 protein alters the sensitivity of A549 and H1299 cells to paclitaxel. Notch-3 was significantly overexpressed in both cell lines. Notch-3 was activated during paclitaxel treatment, and treatment with Notch-3 siRNA and GSI in turn resulted in downregulation of Notch-3. Notch-3 overexpression was reported in relation to chemotherapeutic drugs to treat ovarian, pancreatic, and hepatocellular carcinoma [12,42–44]. Together with these results, our findings suggest that acquisition of paclitaxel resistance is possibly related to Notch-3 overexpression in NSCLC [45]. Thus, we assessed the efficacy of Notch-3-specific inhibition and GSI on paclitaxel-acquired resistance in NSCLCs in this study. In the present research, we analyzed the effect of Notch-3-specific inhibition on the chemosensitivity of paclitaxel in some experiments including cell viability, apoptosis, and colony formation of cells treated with paclitaxel in combination with either Notch-3 siRNA or GSI. Though this mechanism occurs via the regulation of apoptosis, it does not rule out the existence of other mechanisms. We then compared the expression levels of apoptosis-related proteins between the groups. Inhibition of Notch-3 with siRNA was comparable to the effect seen with GSI treatment, specifically the decrease in cell viability, colony formation, and increased apoptosis in response to paclitaxel. This result illustrating the sensitization of A549 and H1299 cells to paclitaxel was consistent with previous findings reported in other tumors. Notch-3 siRNA was found to reduce the IC_{50} of paclitaxel in A549 and H1299 cells. Moreover, cells treated with 20 μM GSI exhibited a greater reduction in cell viability than cells treated with 2 and 5 μM GSI in A549, and similar reductions were observed in H1299 at 72 h after treatment. This difference may be because H1299 cells are more sensitive to paclitaxel following exposure for a longer duration. These findings are in line with the results of another study, in which Notch-3 was reported to be related to the regulation of platinum resistance in NSCLC [46]. In our study, the Notch-3 inhibitor sensitized lung cancer cells to a paclitaxel-based chemotherapeutic agent.

Colony formation is an important factor governing tumor progression. Previous studies have reported that Notch-3

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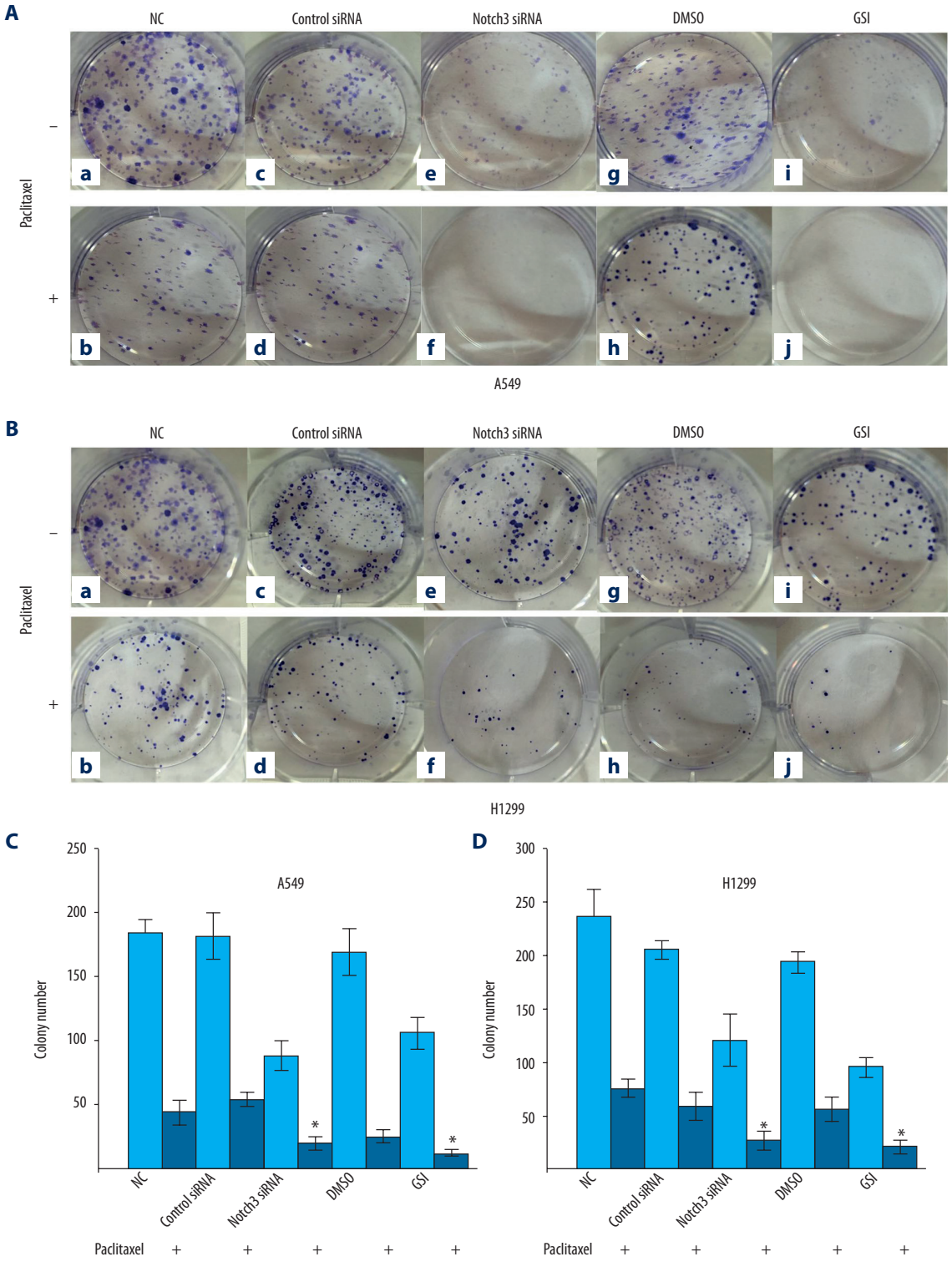

Figure 4. The number of colonies formed following treatment of A549 cells (**A, C**) and H1299 cells (**B, D**) with 20 μM GSI and 50 nM Notch-3 siRNA with or without paclitaxel. (**a**) NC, (**b**) NC + paclitaxel, (**c**) control siRNA, (**d**) control siRNA + paclitaxel, (**e**) Notch-3 siRNA, (**f**) Notch-3 siRNA + paclitaxel, (**g**) DMSO, (**h**) DMSO + paclitaxel, (**i**) 20 μM GSI, and (**j**) 20 μM GSI + paclitaxel. The graph represents the mean decrease in colonies after treatment of each group. * P<0.05 *vs.* control.

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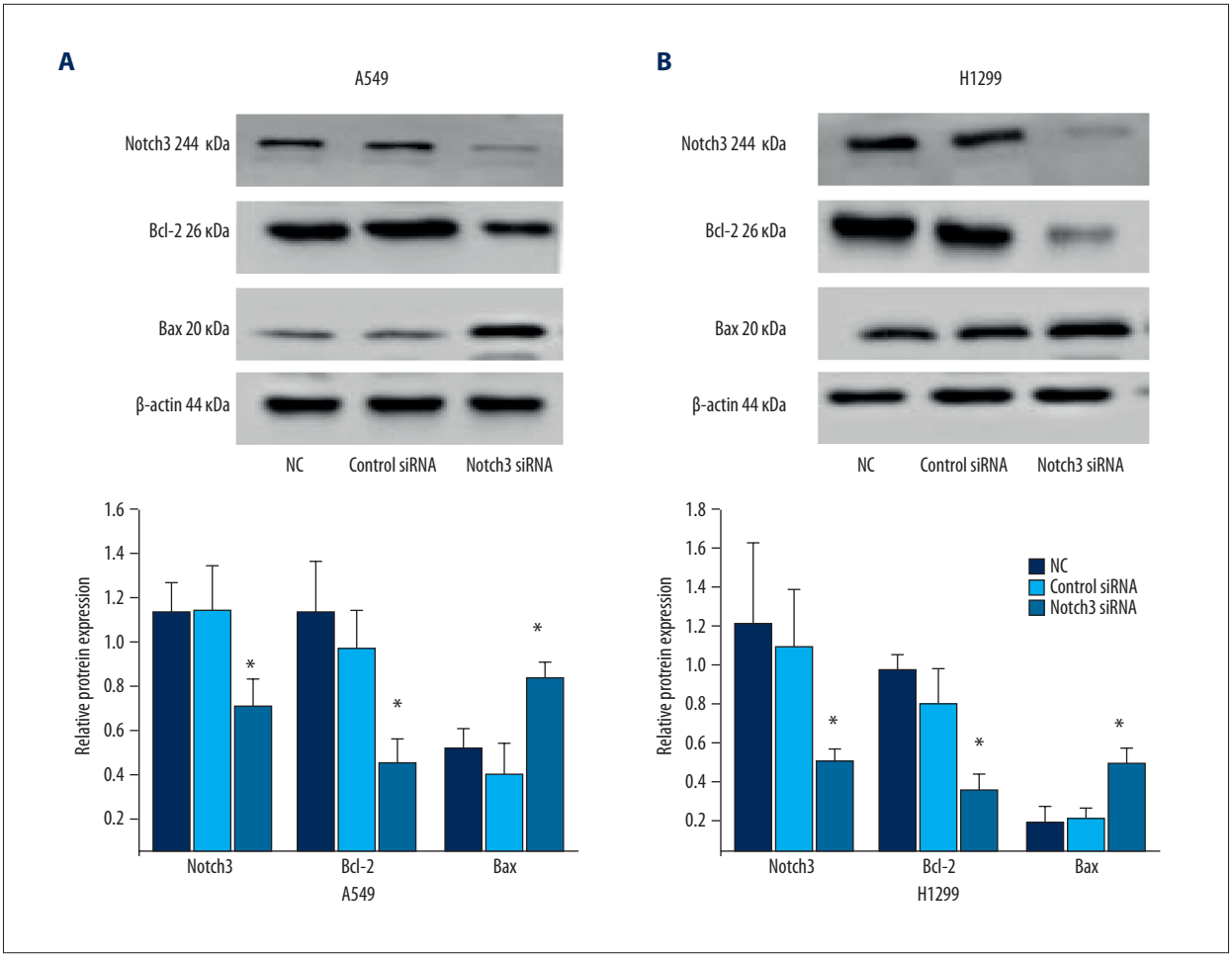
overexpression is correlated with NSCLC progression. In our study, the colony numbers of cells incubated with GSI and Notch-3 siRNA plus paclitaxel were lower than those of cells treated with GSI, Notch-3 siRNA, or paclitaxel alone. Both inhibitors exhibited similar inhibitory effects on cancer cell colony formation in A549 and H1299 cells. Most recently, it has been reported that the combination of GSI with paclitaxel reduced the survival in NSCLC cells, suggesting a relationship between GSI and paclitaxel. However, there is a need for further research in preclinical and clinical investigations.

Chemotherapy-related apoptosis protein plays a crucial role in acquisition of drug resistance [47]. On the other hand, the Notch signaling pathway has been suggested to be closely associated with apoptosis in some malignancies [38]. In the present study, treatment with Notch-3 inhibition (either via siRNA or GSI) in combination with paclitaxel significantly increased apoptosis, thereby boosting sensitivity to paclitaxel. Therefore, inhibition of apoptosis may be an important factor in Notch-induced chemoresistance to paclitaxel. The apoptosis pathway is extensively regulated by the Bcl-2 family, which includes pro-apoptotic and anti-apoptotic factors such as Bax and Bcl-2, respectively [48]. Compared with the no treatment group, paclitaxel treatment with Notch-3 inhibition resulted in decreased expression of Bcl-2 and marked upregulation in Bax expression in both the cell lines tested. These results indicated the potential role of alterations in the intrinsic apoptosis pathway in Notch-induced paclitaxel resistance of NSCLC cells.

Paclitaxel resistance has been an important problem in the treatment of NSCLC; as a result, the progress of therapeutic strategies to overcome paclitaxel resistance continues to pose a challenge. This is the first report researching the expression of Notch-3 signaling molecules in relation to paclitaxel sensitivity in NSCLC. However, limited studies focus on the detailed mechanism of the Notch3 protein in improving the sensitivity of paclitaxel-related chemotherapy. Therefore, further research with *in vivo* tumor xenografts is required to confirm the results obtained in this study, in order to determine the role of Notch-3 signaling in paclitaxel resistance and to better apply these findings in the clinical setting.



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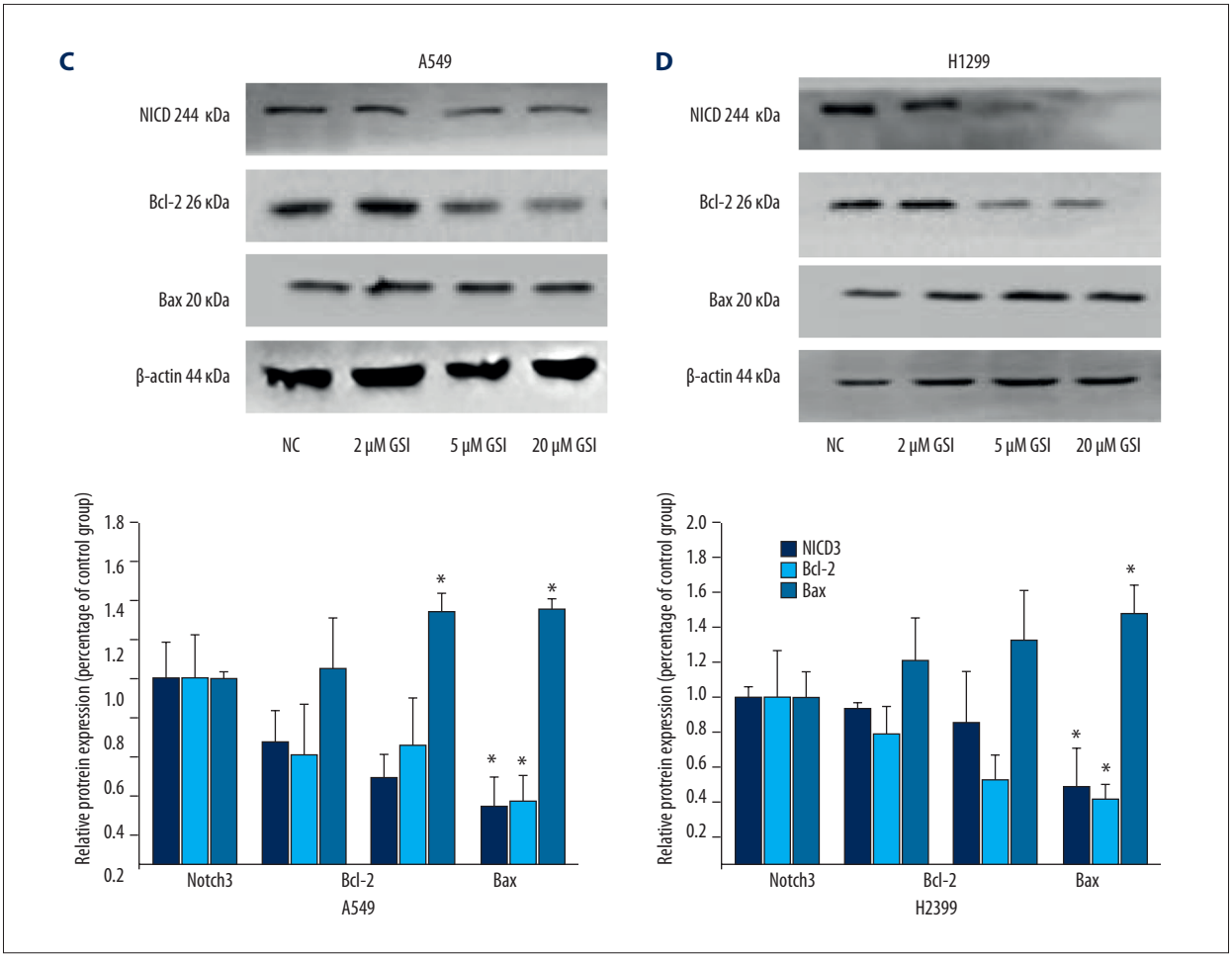

Figure 5. Expression of apoptosis-related proteins in A549 and H1299 cells after Notch-3 knockdown with siRNA and Notch-3 inhibition with GSI. Target proteins, including Notch3, Bcl-2, and Bax, showed a significant difference in expression after Notch-3 siRNA treatment at 48 h in both A549 and H1299 cells (**A, B**). Expression of NICD3, Bcl-2, and Bax following treatment with 2, 5, and 20 μM GSI for 48 h in both cell lines (**C, D**). Proteins were quantified by densitometric analysis and analyzed using ImageJ software. * P<0.05 *vs.* control.

Conclusions

Currently, our results serve as a basis for the targeted treatment of the Notch pathway in chemoresistant lung cancers. Importantly, clinical trials are needed to provide evidence of the connection between drug resistance and the Notch pathway. In this study, we only demonstrated that siRNA knockdown or inhibition of Notch-3 could sensitize paclitaxel and inhibit cell proliferation and apoptosis. Therefore, the synergistic effect of Notch-3-specific inhibition and paclitaxel represents one of the primary chemotherapeutic agents against NSCLC. In the future, we aim to validate the findings of this research in animals and humans.

Conflict of interest

None.

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