

ORIGINAL ARTICLE

Notch1 controls cell chemoresistance in small cell lung carcinoma cells

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Keywords

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Abstract

Background: Small cell lung carcinoma (SCLC) is characterized by a high rate of relapse and failure of chemotherapy because of the emergence of drug resistant cells. Notch signaling controls carcinogenesis in several human malignancies and could be involved in the resistance of cells to several chemotherapeutic agents. Herein, we analyzed the role of Notch1 signaling in the resistance of human SCLC cells to doxorubicin.

Methods: Small interfering ribonucleic acid technology was used to knock down (KD) *Notch1* in H69AR and SBC-3 SCLC cells. We detected the effect of inhibiting Notch1 on the expression of drug resistant related molecules: multidrug resistance-associated protein (MRP-1) and anti-apoptotic factor B-cell lymphoma-2, as well as to cell adhesion molecule E-cadherin, which contributes to the adhesion of SCLC cells to the extracellular matrix and confers chemoresistance in a process known as cell adhesion-mediated drug resistance (CAM-DR). We also observed the effect of KD *Notch1* on cell survival under high concentrations of doxorubicin treated media. **Results:** H69AR and SBC-3 cells expressed Notch1 protein and grew as adherent aggregates, which confer resistance to high concentrations of doxorubicin. On inhibiting *Notch1*, we observed activation of the apoptotic pathway in cells, possibly resulting from the loss of CAM-DR and, thus, SBC-3 cells showed a loss of chemoresistant ability. However, in H69AR cells with KD *Notch1*, the expression of MRP-1 was increased and, thus, sustained the chemoresistant ability of cells.

Conclusion: The Notch1 signaling pathway is involved in mediating the drug resistance phenotype of SCLC cells.

Introduction

Lung cancer is reported to be one of the leading causes of cancer-related death worldwide. Among its various histological types, small cell lung carcinoma (SCLC) is considered one of the most aggressive, because of its rapid growth and early metastasis.¹ SCLC does not respond well to surgery or radiotherapy, and, thus, chemotherapy remains the primary mode to treat SCLC patients.^{1,2} Although there is a good initial response to chemotherapeutic treatment, subsequent relapse is common, which necessitates new modalities for SCLC treatment.³ The high rate of relapse and failure of chemotherapy is believed to a large degree to result from the emergence of drug resistant cells during treatment.⁴ The

acquisition of resistance to anti-cancer drugs could be related to the expression of adenosine triphosphate (ATP) dependent transporters that detect and eject anti-cancer drugs from cells, such as multidrug resistance-associated protein-1 (MRP-1), the expression of anti-apoptotic factors which protect cells from drug-induced apoptosis, and the induction of drug detoxification mechanisms.⁵ Studying this drug resistance phenomenon in vitro is based on cellular models of SCLC, using sensitive and resistant cell lines to chemotherapy. H69 is a drug sensitive SCLC cell line, which, upon treatment with increasing concentration of doxorubicin, the surviving cells, named H69AR, develop multidrug resistant (MDR) phenotypes.⁶ Several factors are involved in the MDR phenotype of H69AR cells, such as over-expression of MRP-1

and/or the anti-apoptotic factor B-cell lymphoma-2 (Bcl-2).^{7,8} Another hypothesis stated that the adhesion of SCLC cells to the extracellular matrix (ECM) enhances tumorigenicity and confers resistance to chemotherapeutic agents, as a result of suppressing chemotherapy-induced apoptosis, a phenomenon referred to as cell adhesion-mediated drug resistance (CAM-DR).⁹ In this context, H69AR and SBC-3 cells, which are characterized with adherent growth patterns, are representative models for this drug resistance mechanism in SCLC.

One of the most important cell signaling systems is the Notch pathway. The human Notch is a transmembrane receptor protein encoded by one of four Notch genes (Notch1–Notch4).¹⁰ Upon binding with its ligands, Notch generates an intracellular domain (ICD) that translocates into the nucleus and drives the expression of various target genes, including the HES/HEY families, c-Myc, protein kinase B (Akt) and others.¹¹

Notch signaling has been implicated in several malignancies. In lung carcinoma, we previously showed that Notch signaling exhibits both tumor promoting and inhibiting functions, depending on cell type.^{12,13} Moreover, we demonstrated that Notch1 signaling is responsible for mediating cell adhesion in SCLC, and that Notch1 has an inhibitory tumor function, especially in the context of switching off cell invasion and metastasis.¹³ There have been several reports of the involvement of Notch signaling in the resistance of different cancer cells to chemotherapeutic reagents.^{14–17} However, the role of Notch signaling in SCLC chemoresistance has not been thoroughly reported. Thus, the present study examined the significance of Notch1 expression in regulating the chemoresistant ability of SCLC cells against doxorubicin, which may provide new therapeutic strategies to combat SCLC.

Materials and methods

Cell lines

The following human SCLC cell lines were purchased from American Type Cell Collection (Rockville, MD, USA): H69,

H69AR, H889, and HI668. The SBC-3 cell line was a generous gift from Dr. Makato Suzuki (Department of Respiratory Surgery, Graduate School of Medical Sciences, Kumamoto University, Japan). SBC-3 cells were grown in Eagle's minimum essential medium with 10% fetal bovine serum (FBS). H69AR cells were grown in RPMI 1640 medium; supplemented with 2 mM L-glutamine, 10 nM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 20% FBS. The remaining cells were grown in similar RPMI 1640 medium, but were supplemented with 10% FBS. All cells were incubated at 37°C in 5% CO₂ and saturated humidity.

Transfection with small interfering ribonucleic acid

Cells were transfected with *Notch1* specific siRNA and Stealth RNAi Negative control (Invitrogen, Carlsbad, CA, USA) using Lipofectamine RNAiMAX (Invitrogen) as previously described.¹² The *Notch1* siRNA sequence was: sense strand 5'-UCG CAU UGA CCA UUC AAA CUG GUGG-3' and antisense strand 5'-CCA CCA GUU UGA AUG GUC AAU GCGA-3'. The cells were harvested at 48 hours post-transfection.

Western blotting analysis

Cells were prepared for Western blotting as previously described.¹² Table 1 contains a list of the primary antibodies used. The membrane was then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence substrate (Amersham Pharmacia Biotech, Buckinghamshire, UK) for one hour and the immune complex was visualized using the ECL system (Santa Cruz Technology, Santa Cruz, CA, USA).

Immunofluorescence microscopy

Cells were prepared for immunofluorescence (IFA) as previously described.¹² Following subsequent washing with phosphate buffered saline (PBS) ×3 (5 minutes each), cells were

Table 1 Antibodies for western blot, immunofluorescence and immunocytochemistry

Primary antibodies	Reference	Lot	WB	IFA
Rabbit anti-Notch1 ICD	Cell signaling, Danvers, MA, USA Chemicon, Temecula, CA, USA	Val1744, D3B8 AB5709	1:500	
Mouse anti-E-cad	BD Transduction Laboratories, NJ, USA	61018	1:5 000	1:50
Rabbit anti-CCsp 3	Cell signaling, Danvers, MA, USA	9662	1:200	
Rabbit anti-Bcl-2	Cell signaling, Danvers, MA, USA	50E3	1:1 000	
Mouse anti-MRP-1	R&D, Minneapolis, MN, USA	MAB19291,QCRL-1	1:500	1:100
Mouse anti-β actin	Sigma Aldrich, St. Louis, MO, USA	AC-15	1:20 000	

References, quantities, and working dilutions are indicated. CCsp 3, cleaved caspase 3; E-cad, E-cadherin; ICD, intracellular domain; IFA, immunofluorescence; MRP-1, multidrug resistance-associated protein-1; SC, Santa Cruz Biotechnology; WB, western blot.

incubated with the appropriate secondary antibodies (Alexa Flour 568 donkey anti-goat immunoglobulin G; Molecular Probes, Eugene, OR, USA) and diluted in Dako Real Antibody Reagent (Dako, Glostrup, Denmark). After washing, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, MO, USA) for five minutes, washed with PBS $\times 3$ (5 minutes each), mounted in Gel Mount Aqueous Mounting Medium (Sigma Aldrich) and examined by fluorescent microscope (Olympus, Tokyo, Japan).

Sensitivity of H69AR and SBC-3 to doxorubicin

Doxorubicin (Millipore, Hamburg, Germany) solutions of 1 mg/mL in ultra-pure water were prepared, and cells were treated, as previously detailed, with varying concentrations of doxorubicin (0.1, 1, 10, and 10 000 μM) for 24 hours.¹⁸ After drug exposure, each cell culture was collected, stained with trypan blue, and counted. Once the effect of different concentrations of doxorubicin on H69AR and SBC-3 was recorded, the same experiment was repeated using corresponding cells transfected with siRNA against Notch1 and cells transfected with the RNA interference (RNAi) negative control. We first plated 100×10^4 cells in three sets of 60 mm dishes. After 24 hours, two sets of cells were transfected with Notch1 specific siRNA and the RNA interference (RNAi) negative control, as previously described. The third set was left without transfection as a control set. The three sets of cells were washed 48 hours after transfection and refed with new medium containing a high concentration of doxorubicin (10 000 μM), and left for another 24 hours, after which each cell culture was washed, stained with trypan blue, and counted. The criteria for cellular integrity included: trypan blue exclusion, an intact nucleus, and an intact cell membrane. The experiments were repeated separately three times.

Statistical analysis

The differences in the mean values between the two groups were statistically analyzed using Student's *t*-test. All *P* values were based on two-tailed statistical analysis. By conventional criteria, if the *P* value is less than 0.05, the difference between the two samples is considered to be statistically significant. All statistical analysis was performed with the JMP9 software program (SAS Institute Inc., Cary, NC, USA).

Results

Notch 1 expression, cell adhesion and chemoresistance in H69AR and SBC-3 cells

H69AR and SBC-3 showed strong Notch1 expression by Western blot analysis (Fig. 1a). Both cells grew as adherent

aggregates to each other and to the culture dish (Fig. 1b) and showed strong E-cadherin (E-cad) expression (Fig. 1a). We postulated that such adhesive cell growth patterns could play a role in the CAM-DR phenotype of both cells. In addition, both cells expressed the anti-apoptotic Bcl-2, which was stronger in H69AR cells (Fig. 1a) and could participate in cell chemoresistance. Moreover, among the SCLC cells used, H69AR cells were the only cells to display the MRP-1 protein (Fig. 1a).

We postulated that both cells could possess chemoresistant ability. To test this hypothesis, we observed the ability of both H69AR and SBC-3 cells to resist different concentrations of doxorubicin. We found that both cells maintained survival when using low concentrations of doxorubicin (0.1, 1 and 10 μM), but decreased by approximately 50% when treated with 10 000 μM of doxorubicin, for 24 hours (Fig. 1c).

Knockdown *Notch1* abolished cell adhesion growth pattern and induced apoptosis

Twenty-four hours after *Notch1* siRNA and negative control transfection, we examined the expression of Notch1 ICD in cells by Western blot and IFA, to detect the efficacy of transfection. After confirming the successful KD of Notch1 in cells, we observed a loss of E-cad, a decrease in Bcl-2, and the induction of cleaved caspase 3 (CCsp 3), especially in H69AR cells with KD *Notch1*, which coincided with loss of the CAM-DR phenotype and activation of the apoptotic pathway. In addition, in H69AR cells with KD *Notch1*, MRP-1 protein expression was increased, as detected by Western blot and IFA, but SBC-3 cells showed no expression of MRP-1, regardless of the condition of Notch1 protein expression (Fig. 2a,b).

KD *Notch1* and chemoresistant ability of H69AR and SBC-3 cells

When using a high concentration (10 000 μM) of doxorubicin for 24 hours on cells with KD *Notch1*, we observed a statistically significant decrease in the number of SBC-3 cells with KD *Notch1*, when compared with either cells transfected with the negative control or the control set ($P < 0.05$ each), indicating that KD *Notch1* abolished the chemoresistant ability of SBC-3 cells. On the other hand, there was no statistically significant difference between the number of H69AR cells with KD *Notch1* and either cells transfected with the negative control or the control set ($P > 0.05$ each) (Fig. 2c), indicating that KD *Notch1* preserved the chemoresistant ability of H69AR cells, possibly through the induction of MRP-1 expression.

Discussion

Despite rapidly accumulating information, the role of Notch signaling in oncogenesis is not fully understood, because of

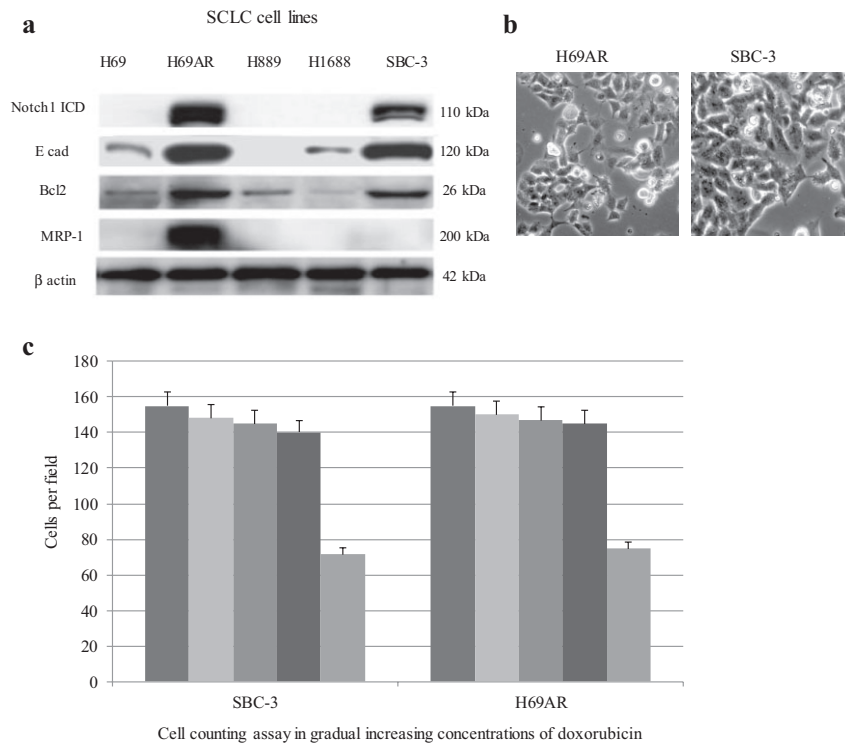


Figure 1 Cytological characteristics of H69AR and SBC-3 cells. **(a)** Western blot analysis of Notch1 intracellular domain expression and chemoresistant related molecules (E-cadherin, B-cell lymphoma-2, and multidrug resistance-associated protein-1) in human small cell lung carcinoma cells. **(b)** Representative photos of the adhesive growth pattern of H69AR and SBC-3 cells, through light microscope. Note, cell adhesion to each other and to the culture dish (×20). **(c)** Cell counting par graph represents the average number of viable cells (×10⁴); plotted on Y axis, cultured for 24 hours, with increasing doxorubicin concentration. (■) No treatment, (■) 0.1 μM, (■) 1 μM, (■) 10 μM, (■) 10 000 μM.

the dual functions of Notch as either a tumor suppressor or oncogene, depending on the cellular context.¹⁹ Our present report sheds some light on the possible involvement of Notch1 signaling in mediating the chemoresistant ability of SCLC cells.

We explored the relationship between Notch1 and some of the mechanisms that have been linked to cell chemoresistant ability, such as MRP-1 protein expression, anti-apoptotic Bcl-2 protein expression, and the CAM-DR mechanism.⁷⁻⁹ MRP-1 is an ATP-binding cassette transporter, which can transport metabolic products and drugs across extracellular membranes.⁷ H69AR and SBC-3 cells are characterized by an adhesive growth pattern, which could provide CAM-DR. However, the present study showed that H69AR cells differ from SBC-3 in the context of MRP-1 expression. H69AR cells developed from H69 cells after treatment with an increasing concentration of doxorubicin, leading to new cytological characteristics, such as Notch1 and MRP-1 protein expression, stronger Bcl-2 expression, and an adhesive cell growth pattern.⁶ We have previously shown that inhibiting Notch1 in H69AR and SBC-3 cells leads to decreased Bcl-2 and the induction of apoptosis, at the same time leading to decreased

E-cad expression and a loss of cell adhesion.^{12,13} In the present study, we showed that such apoptosis activation could be related to a loss of cell adhesion, indicating that inhibiting Notch1 can lead to the loss of the CAM-DR phenotype, which was similar to Kraus *et al*'s results.²⁰ However, in H69AR cells with KD *Notch1*, activation of the epithelial-mesenchymal transition (EMT) phenotype, as we previously reported, increased the expression of MRP-1 protein, maintaining the drug resistance of cells.¹³ These findings point to a possible inverse relationship between Notch1 and MRP-1 expression in H69AR cells, particularly after Western blot analysis revealed that the induction of Notch1 in H69 cells did not induce MRP-1 protein expression (data not shown). On the other hand, in SBC-3 cells, although KD *Notch1* activated the EMT phenotype, the drug resistant ability of cells decreased, possibly as a result of the absence of ATP-binding cassette transporters, such as MRP-1.¹³ All of these observations suggest that Notch1 signaling in SCLC could participate in the chemoresistant ability of cells in vitro, possibly through mediating cell adhesion, expression of anti-apoptotic factors, and through expression of ATP dependent transporters, such as MRP-1 (Fig. 3). However, because of the complex nature of

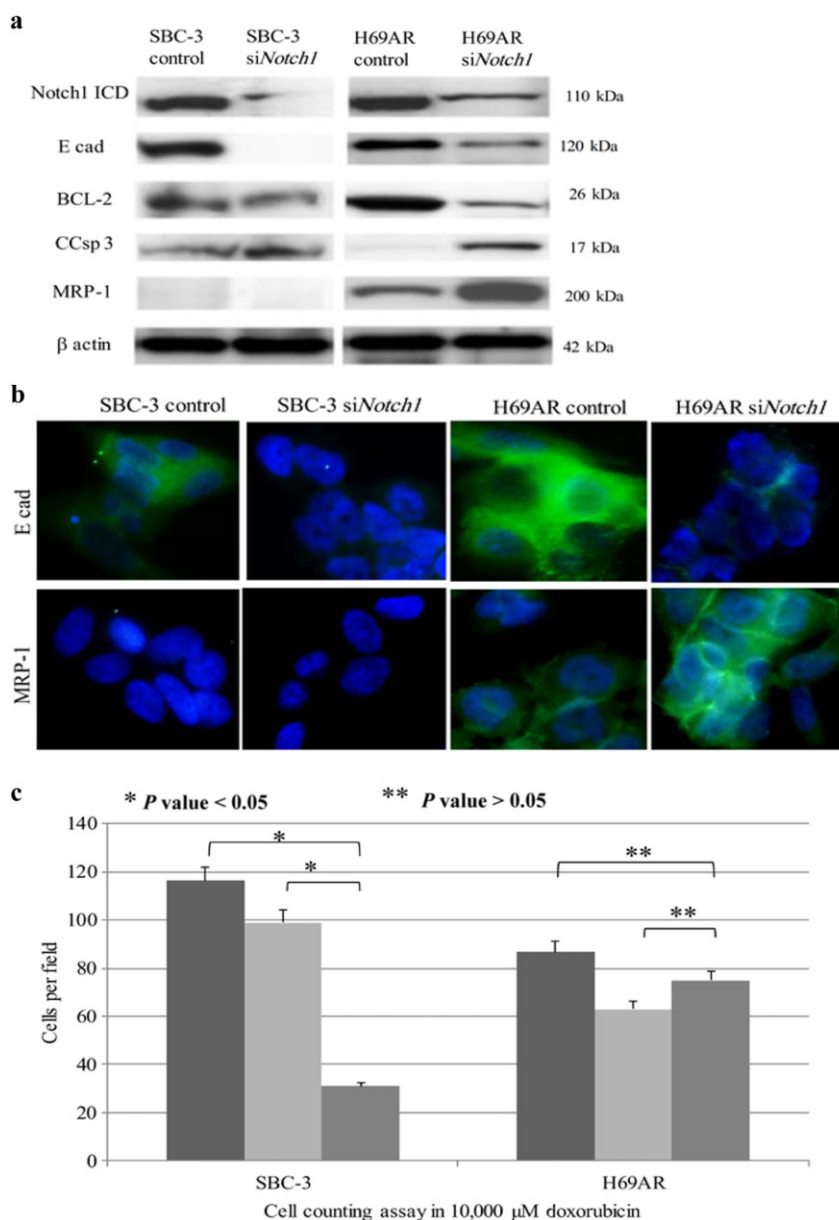


Figure 2 Effect of knockdown *Notch1* on the expression of chemoresistant related molecules (E-cadherin, B-cell lymphoma-2, and multidrug resistance-associated protein-1) and on cell survival under a high concentration of doxorubicin. **(a)** Western blot analysis of apoptosis and drug resistant related molecules, 48 hours after *Notch1* small interfering ribonucleic acid transfection. **(b)** Representative photos of immune-fluorescence staining of E-cadherin and multidrug resistance-associated protein-1. Each molecule was detected using a specific antibody. **(c)** Cell counting par graph represents the average number of viable cells ($\times 10^4$); plotted on Y axis, cultured for 24 hour at a high concentration (10 000 μM) of doxorubicin. (■) Control set (No transfection), (■) si-ve, (■) si*Notch1*.

Notch signaling, it is necessary to identify other Notch signaling components to better understand its role in SCLC.

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Disclosure

No authors report any conflict of interest.

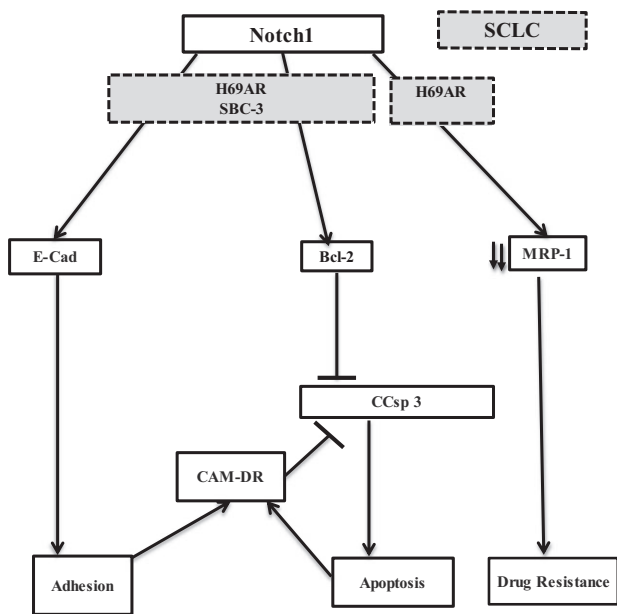


Figure 3 Molecular pathway of Notch1 signaling in small cell lung carcinoma (SCLC). Effect of Notch1 expression in SCLC. Bcl-2, B-cell lymphoma-2; CAM-DR, cell adhesion-mediated drug resistance; CCsp 3, cleaved caspase 3; E-cad, E-cadherin; MRP-1, multidrug resistance-associated protein-1.

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