Notch1 Signaling Modulates Hypoxia-induced Multidrug Resistance of Human Laryngeal Cancer Cells

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Research Article

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Abstract

Background: Laryngeal carcinoma is one of the common malignant tumors of the head and neck. Multidrug resistance (MDR) remains a critical problem in the chemotherapy for patients with laryngeal cancer. This study aims to clarify the role and mechanisms of Notch1 signaling on MDR induced by hypoxia in laryngeal cancer cells.

Methods and Results: Laryngeal carcinoma cells were cultured under normoxia or hypoxia. *Notch1* expression was inhibited by small interfering RNA (siRNA). The expression of *Notch1*, *Hes1*, *Hey1*, *MDR1* and *survivin* mRNA was determined by Real-time PCR. The expression of Notch1, Notch1 intracellular domain (N1ICD), MDR1/P-gp and survivin protein was detected by Western blot. Current research showed that hypoxia could upregulate *Notch1* expression and the activity of Notch1 signaling. Furthermore, suppression of *Notch1* expression could effectively down-regulate the activity of Notch1 signaling and the expression of *MDR* and *survivin* genes in laryngeal cancer cells under hypoxia ($P<0.05$). Cell Counting Kit-8 (CCK-8) assay confirmed that the sensitivity of hypoxic laryngeal cancer cells to a variety of drugs could be up-regulated by suppressing Notch1 expression ($P<0.05$). Additionally, flow cytometry (FCM) showed that suppression of Notch1 expression significantly increased cisplatin-induced apoptosis and intracellular Rh123 (Rh123) accumulation in hypoxic laryngeal carcinoma cells ($P<0.05$).

Conclusions: Notch1 signalling could be regarded as a pivotal regulator for mediating hypoxia-induced MDR in laryngeal cancer cells by regulating survivin-mediated apoptosis resistance and MDR1/P-gp-mediated drug transport.

Introduction

Laryngeal carcinoma is one of the common malignant tumors of the head and neck. As we know, concurrent chemoradiation has been considered as the primary treatment for locally advanced laryngeal cancer. However, MDR remains a critical problem in the chemotherapy for patients with laryngeal cancer. Unfortunately, the regulatory mechanisms related to MDR of laryngeal carcinoma still remain unclear.

Hypoxia could be served as an essential character of the microenvironment within human solid tumors. It is well-known that hypoxia can cause a series of functional adaptive responses of tumor cells, includig MDR [1-3], which is mediated by a variety of mechanisms. Previously, our in vitro study has confirmed that hypoxia could significantly induce MDR of laryngeal cancer cells [4]. To our knowledge, the molecular mechanisms of hypoxia-induced MDR in laryngeal cancer cells are not fully elucidated.

Notch signaling is regarded as a highly conserved intercellular signaling pathway for the regulation of various biological behaviors in tumor cells under hypoxic microenvironment, which are achieved by regulating the expression of downstream target genes [5, 6]. So far, a series of documents have already demonstrated that aberrant expression of Notch receptors or ligands can be observed in a variety of malignancies, which might be involved in malignant progression [7-9]. Almostly consistent with the study

of Meng-Yuan Dai et al [10], our previous study has found that Notch1 expression in laryngeal cancer tissues was evidently higher than that of laryngeal normal tissues, and was related to lymph node metastasis and clinical stage [11], suggesting that Notch signaling might play a pivotal role in regulation of malignant progression of laryngeal cancer. Recently, a number of studies have confirmed that Notch1 signaling is involved in regulating MDR of various neoplasic cells [12-14]. Furthermore, several studies have indicated that Notch1 expression has a positive correlation with cisplatin [15, 16] and paclitaxel [16] resistance in head and neck squamous cell carcinoma. The above studies suggest that Notch1 signaling may be involved in regulating MDR of laryngeal cancer cells in the hypoxic microenvironment. Up to now, there is no relevant literature report.

In the current study, we were to investigate the regulatory role of Notch1 signaling in hypoxia-induced MDR of laryngeal cancer cells and clarify its possible molecular mechanisms.

Materials And Methods

Cell lines and cell culture

Laryngeal carcinoma cell lines Hep-2 and AMC-HN-8 were gained from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. Neoplasic cells were cultured in DMEM (Gibco Corporation, USA) which was supplemented with 1% penicillin / streptomycin (Invitrogen) and 10% fetal bovine serum (Hyclone, USA). For normoxic conditions, cells were placed in an incubator at 37°C in an atmosphere of 21% O_2, 74% N_2 and 5% CO_2. For hypoxic conditions, cells were placed in a hypoxic incubator (NuaireTM US autoflow CO_2 water jacketed incubator) at 37°C containing 1% O_2, 94 % N_2 and 5% CO_2.

Cell transfection

The double-stranded siRNA oligonucleotide targeting human *Notch1* gene (Notch1-siRNA) (sense: 5'-CAGGGAGCAUGUGUAACAUTT-3', anti-sense: 5'-AUGUUACACAUGCUCCCUGTT-3') and the scrambled siRNA (sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3') were both synthesized by Shanghai Genepharma Co. Ltd. (China). After 24 hours of culture in antibiotic-free medium, laryngeal cancer cells were transfected with siRNA (100 nM) using Lipofectamine 2000. Then, cells should be collected for further examine after transfection for 24 hours.

Real-time PCR analysis

Trizol reagent (Invitrogen) extracted total RNA from neoplastic cells. According to reverse transcription kit instructions, cDNA synthesis was implemented. The primers for PCR were as follows: *Notch1* forward, 5'-CTACCTGTCAGACGTGGCCT-3' and reverse, 5'-CGCAGAGGGTTGTATTGGTT-3'. *Hes1* forward, 5'-TCTGAGCCAGCTGAAAACAC-3' and reverse, 5'-GGTACTTCCCCAGCACACTT-3'. *Hey1* forward, 5'-GGCTCCTTCCACTTACTGTCTC-3' and reverse, 5'- ACTTTCCCCTCCCTCATTCTAC-3'. *MDR1* forward, 5'-CTTCAGGGTTTCACATTTGGC-3' and reverse, 5'- GGTAGTCAATGCTCCAGTGG-3'. *Survivin* forward,

5'-CTTCATCCACTGCCCCAC-3' and reverse, 5'- ACTTTCTCCGCAGTTTCCTC-3'. *GAPDH* (internal control) forward, 5'-CATCTTCCAGGAGCGAGA-3' and reverse, 5'-TGTTGTCATACTTCTCAT-3'. As conducted in our previous study [4], Real-time PCR quantified the expression of *Notch1*, *Hes1*, *Hey1*, *MDR1*, *survivin* and *GAPDH* mRNA using SYBR Green PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). Real-time PCR results were analyzed by the $2^{-\triangle\triangle CT}$ method [17].

Western blot analysis

Laryngeal cancer cells were collected and lysed with RIPA lysis buffer for half an hour. Equal amounts of lysate proteins (25 μg) went electrophoresis in SDS-PAGE (5% stacking gel and 8% separating gel) and transferred to a PVDF membrane (Millipore), blocked with 5% skim milk solution for 2 hours at room temperature. Then, the membranes were incubated with primary antibodies (Notch1 1:1000, rabbit anti-human; N1ICD 1:1000, rabbit anti-human; Survivin 1:1000, rabbit anti-human; MDR1/P-gp 1:200, mouse anti-human; GAPDH, 1:1000, mouse anti-human) overnight at 4˚C, and the secondary antibodies (1: 5000; room temperature, 1 hour). Finally, the immunoreactive proteins were visualized by electrogenerated chemiluminescence.

Cell cytotoxicity assay

CCK-8 assay was to assess the sensitivity of neoplastic cells to adriamycin, paclitaxel, cisplatin, 5-FU and gemcitabine. The cells were placed in 96-well culture panels (5×10^3 cells/well). After 12 hours, cells were dealed with a certain dose of chemotherapeutic drugs and cultured for another 48 hours under hypoxia or normoxia. As mentioned in previous study [4], the drug concentration (IC_{50}) which lead to a 50% reduction in cell number could be calculated.

Rhodamine 123 accumulation assay

FCM assay was used to analyze the accumulation of Rh123 in Hep-2 and AMC-HN-8 cells as described previously [18]. The FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) evaluated the cell suspension by using 488 nm excitation. Then, Cell-Quest™ software (BD Biosciences) analyzed the experimental data.

Cell apoptosis analysis

Hep-2 (3×105 cells/well) and AMC-HN-8 (4×105 cells/well) cells were plated in six-well plates and cultured overnight at 37˚C. Then, cells were cultured in hypoxia or normoxia for 12 hours after culture medium was renewed. Next, cell culture further lasted 48 hours after adding cisplatin to each well until the concentration reached 2.5×10-9 M. As our previous research, the apoptosis index (AI) of cells was assessed by FCM and Annexin-V-FITC/propidium iodide (PI) staining method [4]. Finally, cell apoptosis rate was measured at the average fluorescence intensity.

Statistical analysis

The comparison of quantitative variables was assessed by Student's t-test analysis with SPSS20.0. That values of *P* less than 0.05 was regarded as statistically significant.

Results

Hypoxia up-regulated *Notch1* expression and the activity of Notch1 signaling in laryngeal carcinoma cells

Laryngeal cancer cells were cultured under normoxic or hypoxic conditions for 12, 24, 48 hours. Real-time PCR assay determined that hypoxia could obviously induce the expression of *Notch1*, Hes1, Hey1 mRNA in neoplastic cells ($P<0.05$) (Fig. 1A-C). Hes1 and Hey1 belong to the downstream target genes of Notch signaling, and are usually used to reflect the activity of Notch signaling. Similarly, Western blot assay showed that Notch1 and N1ICD expression in laryngeal cancer cells was up-regulated with exposure to hypoxia ($P<0.05$) (Fig. 1D, E). N1ICD could be considered as the active ingredient of Notch1 protein. Thus, the above data indicated that hypoxia could up-regulate Notch1 expression and the activity of Notch1 signaling.

Suppression of *Notch1* expression down-regulated the activity of Notch1 signaling in hypoxic laryngeal carcinoma cells

Real-time PCR exhibited that the expression of *Notch1*, *Hes1* and *Hey1* mRNA in Notch1-siRNA group was evidently less than that of control groups ($P<0.05$) (Fig. 2A-C). Meanwhile, Western blot assay revealed that Notch1 and N1ICD protein expression in Notch1-siRNA group was less than that of control groups ($P<0.05$) (Fig. 2D, E). The above data demonstrated that suppression of *Notch1* expression could down-regulate the activity of Notch1 signaling in hypoxic laryngeal cancer cells.

Suppression of *Notch1* expression inhibited multidrug resistance of laryngeal carcinoma cells under hypoxia

Our study compared the drug sensitivity of Notch1-siRNA group with that of control groups by CCK-8 method. As can be seen in Table 1 and 2, the results showed that the sensitivity of hypoxic Hep-2 and AMC-HN-8 cells to a variety of drugs was obviously enhanced by inhibiting Notch1 expression ($P<0.05$).

Suppression of *Notch1* expression inhibited the expression of *MDR1* and *survivin* genes in hypoxic laryngeal cancer cells

Real-time PCR assay exhibited that *MDR1* and *survivin* mRNA expression in Notch1-siRNA group was obviously less than that of control groups ($P<0.05$) (Fig. 3A-C). Besides, Western blot assay showed that MDR1/P-gp and survivin protein expression in Notch1-siRNA group was also less than that of control groups ($P<0.05$) (Fig. 3D, E). The above data indicated that MDR1 and survivin expression in hypoxic laryngeal cancer cells was down-regulated by inhibiting Notch1 expression.

Suppression of *Notch1* expression increased drug accumulation in hypoxic laryngeal cancer cells

FCM assay showed that the positive percentage of Rh123 in Notch1-siRNA group of Hep-2 cells was evidently higher than that of control groups (89.48±1.97% vs. 70.39±1.66%, 70.63±0.71%; $P<0.05$) (Fig. 4A); Besides, the positive percentage of Rh123 in Notch1-siRNA group of AMC-HN-8 cells was also higher than that of control groups (92.35±2.13% vs. 73.12±3.10%, 72.84±2.24%; $P<0.05$) (Fig. 4B). The above data revealed that suppression of Notch1 expression could enhance drug accumulation in hypoxic laryngeal cancer cells.

Suppression of *Notch1* expression enhanced cisplatin-induced apoptosis of hypoxic laryngeal cancer cells

Annexin-V/PI staining method showed that AI of Hep-2 cells in Notch1-siRNA group was obviously higher than that of control groups (46.69±0.68% vs. 20.56±0.85%, 20.40±0.58%; $P<0.05$) (Fig. 4C); Likewise, AI of AMC-HN-8 cells in Notch1-siRNA group was also higher than that of control groups (51.67±0.69% vs. 31.27±0.54%, 31.19±0.57%; $P<0.05$) (Fig. 4D). The above data demonstrated that suppression of Notch1 expression enhanced cisplatin-induced apoptosis of hypoxic laryngeal cancer cells.

Discussion

Notch signaling is a crucial signal transduction pathway for the regulation of biological behaviors of neoplasic cells under hypoxia [5]. Previously, the data of Meng-Yuan Dai et al. [10] and our work [11] have demonstrated that high expression of Notch1 in laryngeal cancer tissues was associated with lymph node metastasis. Furthermore, current research exhibited that hypoxia could enhance *Notch1* expression and the activity of Notch1 signaling in laryngeal cancer cells. The above results suggested that, in the hypoxic microenvironment of laryngeal cancer tissue, Notch1 signaling might take an important part in regulation of malignant phenotypes.

Up to date, a number of studies in other oncology fields have shown that Notch1 signaling is involved in regulating MDR of various neoplastic cells [12-14]. Furthermore, the studies of Zuping Zhang et al. [16] and Feng Gu et al. [15] demonstrated that Notch1 expression was positively correlated with chemotherapy resistance of head and neck carcinoma. And then, the present work showed that the sensitivity of hypoxic laryngeal cancer cells to a variety of chemotherapy drugs was obviously enhanced by restraining the activity of Notch1 signaling. That is to say, Notch1 signaling might take a significant part in mediating hypoxia-induced MDR in laryngeal cancer cells.

MDR1/P-gp, as a critical drug transporter, affects on the regulating of intracellular drug concentrations. MDR1/P-gp has been confirmed as an important regulator of MDR in laryngeal cancer cells [19, 20]. Furthermore, our previous work has suggested that MDR1/P-gp could serve a significant role in regulating hypoxia-induced MDR in laryngeal carcinoma cells through cellular drug effluxing mechanism [21]. Recently, Jiayuan Huang et al. [22] have indicated that Notch-1 signaling may play a role in regulating chemoresistance in lung adenocarcinoma by mediating *MDR1* expression. Likewise, our present work has elucidated that suppression of *Notch1* expression could down-regulate *MDR1* expression in hypoxic laryngeal carcinoma cells, and reduce the drug efflux ability of neoplastic cells.

Consequently, it is suggested that Notch1 signaling might participate in the regulation of MDR1/P-gp-mediated drug transport in laryngeal cancer cells under hypoxia.

Survivin belongs to the inhibitor of apoptosis family and participates in the apoptosis regulation of laryngeal cancer cells [23, 24]. Besides, the study of Himani Sharma et al. [25] has indicated that survivin takes part in the regulation of drug sensitivity of head and neck squamous cell carcinoma cells, including Hep-2 cells. Recently, our research has already confirmed that survivin might play a regulatory role in hypoxia-induced MDR of laryngeal carcinoma cells by regulating apoptosis resistance [26]. Moreover, several studies have identified that Notch-1 signaling might regulate *survivin* expression in basal breast cancer cells [27] and lung cancer cells [28]. In this series, our work has confirmed that suppression of *Notch1* expression could down-regulate *survivin* expression in hypoxic laryngeal carcinoma cells, and enhance cisplatin-induced apoptosis of neoplastic cells. Accordingly, it is indicated that Notch1 signaling might be involved in the regulation of survivin-mediated apoptosis resistance of laryngeal cancer cells under hypoxia.

In summary, current research indicates that Notch1 signaling may play an important role in regulating hypoxia-induced MDR in laryngeal cancer cells by regulating survivin-mediated apoptosis resistance and MDR1/P-gp-mediated drug transport. Further study is needed to determine the role and mechanisms of Notch1 signaling in hypoxia-induced MDR of laryngeal carcinoma cells through in vivo experiments.

Declarations

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Conflicts of interest/Competing interests The author reports no conflicts of interest/Competing interests in this work.

Availability of data and material Available.

Code availability Not applicable.

Authors' contributions Dawei Li, study execution, data acquisition, analysis and interpretation, manuscript drafting and revising, final approval, and accountability for all aspects of the work; Dan Xu,

study execution, data acquisition, analysis and interpretation, manuscript drafting and revising, final approval, and accountability for all aspects of the work; Penghui Chen, study execution, data acquisition, analysis and interpretation; Jin Xie, study design, data analysis and interpretation, manuscript revising, final approval, and accountability for all aspects of the work.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors approved to publish this article.

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Tables

Table 1 Effect of Notch1 silencing on chemosensitivity in *hypoxic* Hep-2 cells

Drug	IC_{50} (μg/ml)		
	Blank control	Negative control	Notch1-siRNA
Paclitaxel	37.71×10-3±0.18	38.52×10-3±0.29	4.34×10-3±0.13*
5-Fu	243.04±0.84	241.24±0.88	26.17±0.27*
Doxorubicin	3.91±0.07	3.78±0.11	1.08±0.21*
Gemcitabine	38.26±0.29	39.12±0.53	11.46±0.12*
Cisplatin	8.91±0.11	8.73±0.21	2.03±0.18*

IC_{50} is the concentration of each drug that caused a 50% reduction in the number of cells.

Mean ± SD of three individual experiments are shown. *: *P*□0.05 vs. Blank control and Negative control.

Table 2 Effect of Notch1 silencing on chemosensitivity in *hypoxic* AMC-HN-8 cells

Drug	IC_{50} (μg/ml)		
	Blank control	Negative control	Notch1-siRNA
Paclitaxel	35.18×10-3±0.78	36.12×10-3±0.21	3.06×10-3±0.31*
5-Fu	227.07±1.24	228.28±1.37	23.61±0.32*
Doxorubicin	3.68±0.35	3.83±0.41	0.81±0.42*
Gemcitabine	35.31±0.25	36.25±0.48	9.36±0.28*
Cisplatin	8.61±0.17	8.55±0.31	1.56±0.34*

IC_{50} is the concentration of each drug that caused a 50% reduction in the number of cells.

Mean ± SD of three individual experiments are shown. *: *P*□0.05 vs. Blank control and Negative control.

Figures

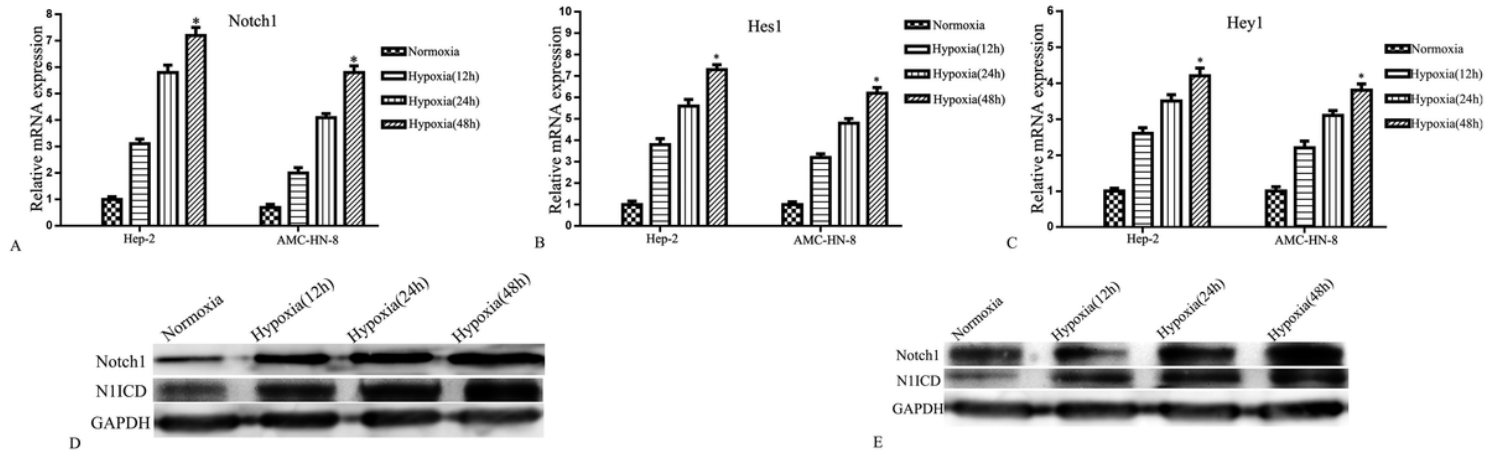

Figure 1

Changes in Notch1 expression and the activity of Notch1 signaling in laryngeal carcinoma cells under normoxia or hypoxia. Real-time PCR estimated the expression of Notch1 (A), Hes1 (B) and Hey1 (C) mRNA in neoplastic cells. Western blot evaluated the expression of Notch1 and N1ICD protein in Hep-2 (D) and AMC-HN-8 (E) cells. *P<0.05, versus control groups

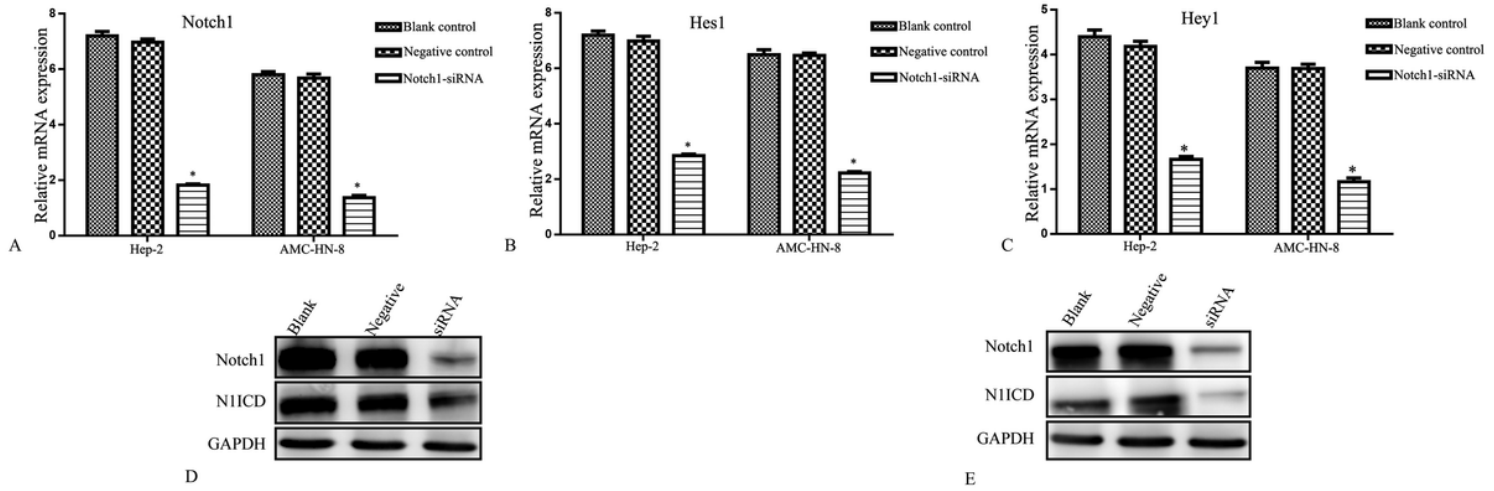

Figure 2

Effects of Notch1-siRNA on the activity of Notch1 signaling in hypoxic laryngeal carcinoma cells. Neoplastic cells were transfected with the scrambled siRNA or Notch1-siRNA. Real-time PCR assessed the expression of Notch1 (A), Hes1 (B) and Hey1 (C) mRNA in Hep-2 and AMC-HN-8 cells under hypoxia. Western blot estimated the expression of Notch1 and N1ICD protein in Hep-2 (D) and AMC-HN-8 (E) cells under hypoxia. *P<0.05, versus control groups

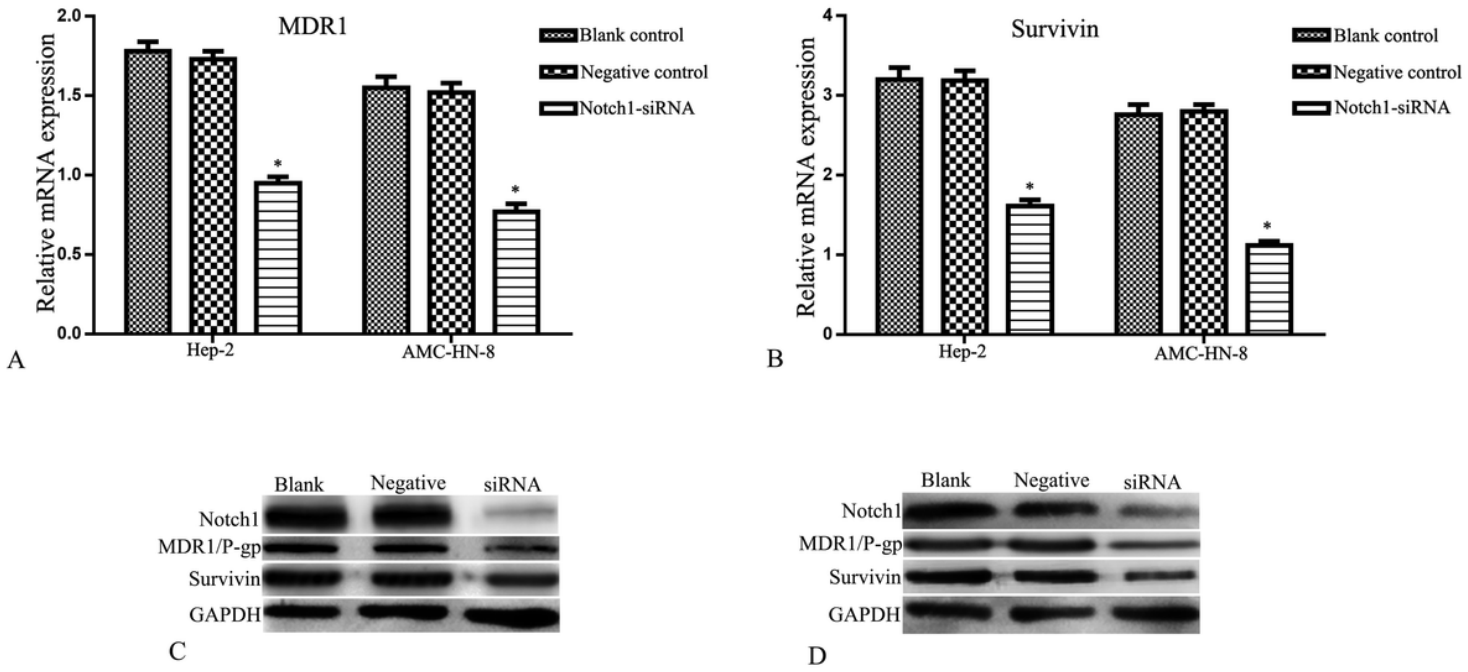

Figure 3

Effects of Notch1-siRNA on MDR1 and survivin expression in hypoxic laryngeal cancer cells. The cells were transfected with the scrambled siRNA or Notch1-siRNA. Real-time PCR assessed the expression of MDR1 (A) and survivin (B) mRNA in neoplastic cells under hypoxia. Western blot estimated the expression of MDR1/P-gp and survivin protein in Hep-2 (C) and AMC-HN-8 (D) cells under hypoxia. *P<0.05, versus control groups

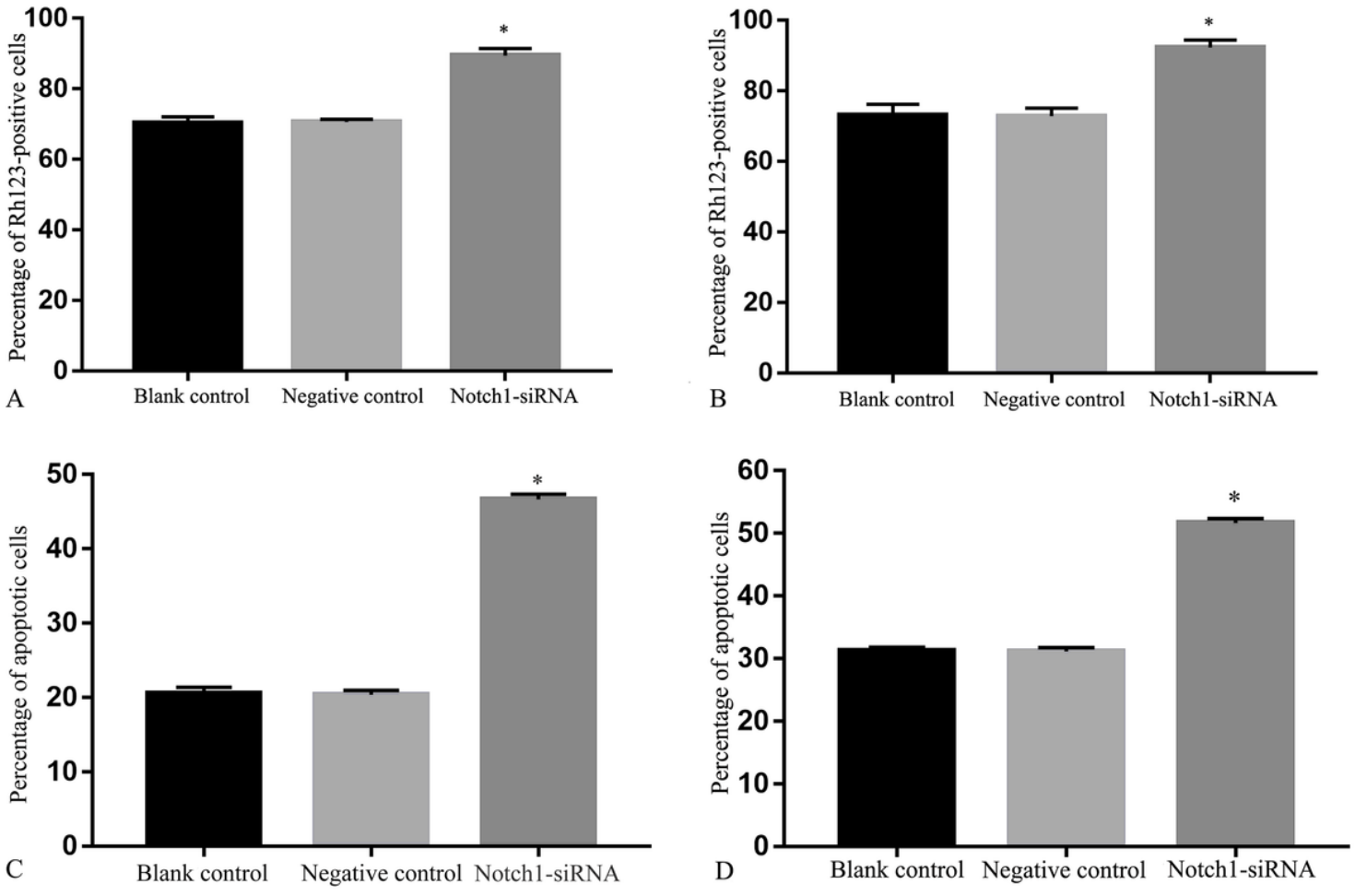

Figure 4

Effects of Notch1-siRNA on drug efflux capacity and cisplatin-induced apoptosis of hypoxic laryngeal carcinoma cells. FCM assay was used to detect intracellular Rh123 accumulation and apoptosis rate of neoplastic cells. Suppression of Notch1 expression could increase the positive percentage of Rh123 in Hep-2 (A) and AMC-HN-8 (B) cells, and enhance the apoptosis rate of Hep-2 (C) and AMC-HN-8 (D) cells induced by cisplatin. *P<0.05, versus control groups