Hypoxia can be an essential characteristic of the microenvironment within human solid tumors. It is well known that hypoxia can induce a series of functional adaptive responses in tumor cells, including MDR, which is mediated by a variety of mechanisms. Previously, our in vitro study has confirmed that hypoxia could significantly induce MDR in laryngeal cancer cells. To our knowledge, the molecular mechanisms of hypoxia-induced MDR in laryngeal cancer cells are incompletely elucidated.

The Notch signaling pathway is regarded as a highly conserved intercellular signaling pathway for the regulation of various biological behaviors in tumor cells in a hypoxic microenvironment; this function is achieved via regulation of downstream target gene expression. To date, a series of documents have already demonstrated that Notch receptors or ligands exhibit aberrant expression in a variety of malignancies, a phenomenon that might be involved in malignant progression. Almost consistent with the findings of a study by Meng-Yuan Dai et al.
we previously found that Notch1 expression in laryngeal cancer tissues was evidently higher than that in laryngeal normal tissues and was related to lymph node metastasis and clinical stage [11], suggesting that Notch1 signaling might play a pivotal role in regulation the malignant progression of laryngeal cancer. Recently, a number of studies have confirmed that Notch1 signaling is involved in regulating MDR of various neoplastic 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 findings suggest that Notch1 signaling may be involved in regulating MDR in laryngeal cancer cells in the hypoxic microenvironment. Up to now, there have been no relevant literature reports.

In the current study, we were to investigate the regulatory role of Notch1 signaling in hypoxia-induced MDR in 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. Neoplastic cells were cultured in DMEM (Gibco Corporation, USA) 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% O₂, 5% CO₂ and 94% N₂. For hypoxic conditions, cells were placed in a hypoxic incubator (NuaireTM US generating chemiluminescence). Real-time PCR was conducted to quantify the expression of Notch1, Hes1, Hey1, MDR1, survivin and GAPDH mRNA using SYBR Green PCR kit (Takara Bio-technology Co., Ltd., Dalian, China). Real-time PCR data analyses were performed by the 2^−∆∆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), transferred to a PVDF membrane (Millipore), and blocked with 5% skim milk solution for 2 h at room temperature. Then, the membranes were incubated with primary antibodies (anti-Notch1, 1:1000, rabbit anti-human; anti-Hes1, 1:1000, mouse anti-human; anti-Hey1, 1:1000, rabbit anti-human; anti-survivin, 1:1000, mouse anti-human; anti-MDR1/P-gp, 1:200, mouse anti-human; anti-GAPDH, 1:1000, mouse anti-human) overnight at 4˚C and with secondary antibodies (1: 5000; Sigma-Aldrich) for 1 h at room temperature. Finally, immunoreactive proteins were visualized by electrogenerated chemiluminescence.

Cell cytotoxicity assay.

A CCK-8 assay was to assess the sensitivity of neoplastic cells to adriamycin, paclitaxel, cisplatin, 5-FU and gemcitabine. Cells were seeded in 96-well culture plates (5 × 10³ cells/well). After 12 h, the cells were treated with a certain dose of chemotherapeutic drugs and cultured for another 48 h under hypoxia or normoxia. As mentioned in a previous study [4], the drug concentration that led to a 50% reduction in the cell number (IC_{50}), was 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]. A FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze the
cell suspension with an excitation wavelength of 488 nm. Then, Cell-Quest™ software (BD Biosciences) was used to analyze 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, the cells were cultured under hypoxic or normoxic conditions for 12 h after the culture medium was refreshed. Paclitaxel or cisplatin was added to each well to a concentration of 5.0 × 10⁻⁹ M or 2.5 × 10⁻⁹ M, respectively. After that, the cells were cultured for another 48 h. As in our previous research, the apoptosis index (AI) of cells was assessed by FCM and Annexin-V-FITC/propidium iodide (PI) staining method [4]. Finally, the apoptosis rate was measured as the average fluorescence intensity.

Statistical analysis

Quantitative variables were compared by Student’s t test with SPSS 20.0. P values of less than 0.05 were regarded as statistically significant.

Results

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

The drug sensitivity of the Notch1-siRNA group with that of the control groups was compared by a CCK-8 assay in our study. The results showed that the sensitivity of hypoxic Hep-2 and AMC-HN-8 cells to a variety of drugs was obviously enhanced by inhibition of Notch1 expression (P < 0.05) (Supplementary Tables 1 and 2).

Suppression of Notch1 expression inhibited the mRNA expression of MDR1 and survivin in hypoxic laryngeal cancer cells.

Real-time PCR analysis showed that the MDR1 and survivin mRNA expression levels in the Notch1-siRNA group were obviously lower than those in the control groups (P < 0.05) (Fig. 3 A-C). In addition, Western blot analysis showed that the MDR1/P-gp and survivin protein expression levels in the Notch1-siRNA group were lower than those in the control groups (P < 0.05) (Fig. 3D and E). The above data indicated that MDR1 and survivin expression in hypoxic laryngeal cancer cells were downregulated by inhibition of Notch1 expression.

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

The FCM results showed that the percentage of Rh123-positive Hep-2 cells in the Notch1-siRNA group was evidently higher than that in the control groups (89.48 ± 1.97% vs. 70.39 ± 1.66% and 70.63 ± 0.71%; P < 0.05) (Fig. 4A). In addition, the percentage of Rh123-positive AMC-HN-8 cells in the Notch1-siRNA group was higher than that in the control groups (92.35 ± 2.13% vs. 73.12 ± 3.10% and 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 drug-induced apoptosis in hypoxic laryngeal cancer cells.

Annexin-V/PI staining assay showed that the apoptosis rate of Hep-2 or AMC-HN-8 cells induced by cisplatin in the Notch1-siRNA group was obviously higher than that in the control groups (P < 0.05) (Fig. 4 C, 4D). Likewise,
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has indicated that survivin participates in the regulation of apoptosis [10]. Moreover, several studies have identified that survivin expression can downregulate Notch1 expression in hypoxic laryngeal carcinoma cells [11]. Hence, our work has already confirmed that survivin might play a regulatory role in hypoxia-induced MDR in laryngeal carcinoma cells by regulating apoptosis resistance [26].

Survivin belongs to the inhibitor of apoptosis family and participates in the regulation of apoptosis in laryngeal cancer cells [23, 24]. Besides, a study of Himani Sharma et al. [25] has indicated that survivin participates in the regulation of drug sensitivity in head and neck squamous cell carcinoma cells, including HEP-2 cells. Recently, our research has confirmed that survivin might play a regulatory role in hypoxia-induced MDR in laryngeal carcinoma cells by regulating apoptosis resistance [26]. Moreover, several studies have identified that Notch-1 signaling might play an important role in regulating hypoxia-induced MDR in laryngeal cancer cells

**Discussion**

Notch signaling is a crucial signal transduction pathway for the regulation of biological behaviors of neoplastic cells under hypoxia [5]. Previously, the findings 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 the regulation of malignant phenotypes.

Up to date, a number of studies in other cancers have shown that Notch1 signaling is involved in regulating MDR in 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 chemoresistance of head and neck carcinoma. Then, the present work showed that the sensitivity of hypoxic laryngeal cancer cells to a variety of chemotherapeutic drugs was obviously enhanced by suppressing Notch1 signaling activity. That is, Notch1 signaling might play a significant role in mediating hypoxia-induced MDR in laryngeal cancer cells.

MDR1/P-gp, a crucial drug transporter, affects the regulation 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] 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 downregulate MDR1 expression in hypoxic laryngeal carcinoma cells and reduce the drug efflux ability of neoplastic cells. Consequently, these findings suggest that Notch1 signaling might participate in the regulation of MDR1/P-gp-mediated drug transport in hypoxic laryngeal cancer cells.

Survivin belongs to the inhibitor of apoptosis family and participates in the regulation of apoptosis in laryngeal cancer cells [23, 24]. Besides, a study of Himani Sharma et al. [25] has indicated that survivin participates in the regulation of drug sensitivity in 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 in 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 of studies, our work confirmed that suppression of Notch1 expression can downregulate survivin expression in hypoxic laryngeal carcinoma cells and enhance drug-induced apoptosis in neoplastic cells. Accordingly, these findings indicate that Notch1 signaling might be involved in the regulation of survivin-mediated apoptosis resistance in hypoxic laryngeal cancer cells.

In summary, the current research indicates that Notch1 signaling might play an important role in regulating hypoxia-induced MDR in laryngeal cancer cells by regulating survivin-mediated apoptosis resistance and
Availability of data and material Available.

Code Availability Not applicable.

Declarations

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

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors approved the publication of this article.

Fig. 4 Effects of Notch1-siRNA on the drug efflux capacity and drug-induced apoptosis of hypoxic laryngeal carcinoma cells. FCM assay detected intracellular Rh123 accumulation and the apoptosis rate of cells. (A) The positive percentage of Rh123 in Hep-2 cells. (B) The positive percentage of Rh123 in AMC-HN-8 cells. (C) The apoptosis rate of Hep-2 cells induced by cisplatin. (D) The apoptosis rate of AMC-HN-8 cells induced by cisplatin. (E) The apoptosis rate of Hep-2 cells induced by paclitaxel. (F) The apoptosis rate of AMC-HN-8 cells induced by paclitaxel. *P < 0.05, versus the control groups.

MDR1/P-gp-mediated drug transport. Further study through in vivo experiments is needed to determine the role and mechanisms of Notch1 signaling in hypoxia-induced MDR in laryngeal carcinoma cells.

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