Effect of Notch1 Signaling on Cellular Proliferation and Apoptosis of Human Laryngeal Carcinoma

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Research Article

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Abstract

The pathological processes of occurrence and development of malignancies include the excessive proliferation and apoptosis resistance of neoplastic cells. The study aims to identify the effects of Notch1 signaling on the proliferation and apoptosis of laryngeal cancer cells in hypoxic microenvironment. Notch1 and Ki-67 expression in laryngeal squamous cell carcinoma (LSCC) tissue samples were detected by immunohistochemistry. The apoptotic index (AI) of LSCC was evaluated by TUNEL method. In laryngeal cancer cells, small interfering RNA (siRNA) technology was to inhibit Notch1 expression. Meanwhile, Real-time PCR detected Notch1, Hes1 and Hey1 mRNA expression, and Western blot detected Notch1 and Notch1 intracellular domain (N1ICD) protein expression. Annexin V-FITC/propidium iodide staining and Cell Counting Kit-8 methods measured cell apoptosis and proliferation, respectively. Notch1 expression was detected in 63.55%(68/107) of LSCC samples and was significantly related to the proliferation index (PI) ($P < 0.05$) and AI ($P < 0.05$) in LSCC tissues. Furthermore, it was confirmed that hypoxia could induce proliferation and inhibit apoptosis of laryngeal carcinoma cells ($P < 0.05$). Meanwhile, Notch1 expression and Notch1 signaling activity could be upregulated by hypoxia ($P < 0.05$). In contrast, suppression of Notch1 signaling activity in hypoxic neoplastic cells could obviously decrease cell proliferation and increase cell apoptosis (both $P<0.05$). Our study has demonstrated that hypoxia may promote cell proliferation and inhibit cell apoptosis of laryngeal carcinoma. Notch1 signalling may exert a pivotal role in regulating the proliferation and apoptosis resistance of laryngeal cancer cells under hypoxia.

Introduction

Laryngeal carcinoma has been considered as a common malignancy of the head and neck. Theoretically, the pathological processes of occurrence and development of malignancies include the excessive proliferation and apoptosis resistance of neoplastic cells. To date, the regulatory mechanism of the aberrant growth of laryngeal cancer cells has not been elucidated.

Hypoxia has been served as a basic feature of human solid tumor microenvironment. It is well-known that hypoxia has a variety of effects on the regulation of cell apoptosis and proliferation in human neoplasms, which might be mediated by a set of regulatory mechanisms [1–3]. Previously, a number of documents have already demontrated that hypoxia could participate in the regulation of multidrug resistance [4], stem-like biological properties [5] and metastasis [6] of laryngeal cancer cells. Unfortunately, the regulatory effect and mechanisms of hypoxia on the proliferation and apoptosis of laryngeal cancer cells are still unclear.

As we all know, Notch signaling serves as a highly conserved intercellular signaling pathway that can participate in regulating different biological behaviors of neoplastic cells under hypoxia, and is mediated by regulating downstream target genes expression [7, 8]. Almost in line with the research conclusion of Dai et al. [9], our previous data has observed that the expression level of Notch1 in laryngeal cancer tissues is significantly higher than that in normal mucosal tissues, and was positively associated with

lymph node metastasis and clinical stage [10], suggesting that aberrant Notch1 signaling may be involved in regulating the malignant process of laryngeal carcinoma. Up to now, a set of studies have indicated that the effects of Notch1 signaling on the apoptosis and proliferation of various neoplastic cells are still controversial [11–14]. Furthermore, the study of Jiao et al. [15] has confirmed that overexpression of Notch1 in Hep-2 cells could suppress cellular proliferation and induce cell apoptosis. On the contrary, Dai et al. [9] have confirmed that Notch1 expression in Hep-2 cells could promote cell growth and inhibit apoptosis by regulating Notch1 target genes. As mentioned above, the role of Notch1 signaling in regulating cellular proliferation and apoptosis of laryngeal carcinoma by in vitro studies with Hep-2 cells is also controversial.

Accordingly, the purpose of current research was to further explore the effects of Notch1 signaling on the regulation of apoptosis and proliferation of laryngeal cancer cells in the hypoxic microenvironment, clarifying the regulatory role of Notch1 signaling in tumor progression.

Materials And Methods

Laryngeal cancer tissue samples

Specimens from 107 cases of LSCC were collected from the Department of Otolaryngology, Head and Neck Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine between 1997 and 2020. Patients had been diagnosed as LSCC by histopathology. All patients included in this study had no history of radiotherapy or chemotherapy before surgery. The study was approved by the Ethics Committees of Xinhua Hospital. Meanwhile, written informed consent was gotten from the patients or their family.

Immunohistochemistry and TUNEL assay

All tissue samples were obtained from the archives of the department of pathology. Immunostaining for Notch1 was performed on representative 5 μm sections from LSCC tissue blocks. The following primary antibodies were to incubate tissue sections: rabbit anti-Notch1 monoclonal antibody (Epitomics, Inc, Burlingame, California, USA) at 1:100 dilution overnight at 4^{o}C, rabbit anti-Ki-67 polyclonal antibody (BA1508, Wuhan Boster, China) at 1:800 dilution overnight at 4^{o}C. The study was conducted by a two-step immunohistochemistry assay using the DAKO EnVision+System (DAKO, Carpinteria, CA, USA). AI was analyzed by TUNEL assay using an ApopTag Peroxidase In Situ Apoptosis Detection kit (S7100, Chemicon International, Inc., USA).

Evaluation of staining

All samples were independently reviewed by two senior pathologists, without prior knowledge of patients' information. The immunohistochemical staining of Notch1 was evaluated as mentioned previously [16]. Furthermore, Notch1 protein expression was defined as negative (absent or weak immunostaining) and positive (moderate or strong immunostaining). Ki-67 immunostaining and the apoptosis rate of cancer

cells measured by TUNEL method were obtained by reviewing a minimum of 1000 total cancer cells in the representative areas. Then, they were showed as the number of stained nuclei per 100 cells on a 400×objective.

Laryngeal cancer cell lines

The AMC-HN-8 and Tu212 cell lines were both obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. The cells were maintained in Dulbecco's modification of Eagle's medium (DMEM; Gibco Corporation, USA) with 10% fetal bovine serum (Hyclone, USA) and 1% penicillin/streptomycin (Invitrogen). For normoxic condition, cells were cultured in an incubator at 37°C in a humidified atmosphere of 21% O_2, 5% CO_2, and 74% N_2. For hypoxic condition, cells are placed in a hypoxic incubator (NuaireTM US autoflow CO_2 water jacketed incubator) at 37°C with 1% O_2, 5% CO_2 and 94% N_2.

Cell transfection

Double-stranded siRNA oligonucleotide targeting 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 synthesized by Shanghai Genepharma Co. Ltd. (China). After 24 hours of culture in antibiotics-free medium, Lipofectamine 2000 was used to transfect siRNA (100 nM) into laryngeal cancer cells. After transfection for 24 hours, laryngeal cancer cells were collected for further research.

Real-time PCR analysis

Total RNA was extracted from laryngeal cancer cells using Trizol reagent (Invitrogen). According to the protocol of reverse transcription kit, the cDNA was reverse-transcribed from the isolated RNA. Primer sequences 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'. GAPDH (internal control) forward, 5'-CATCTTCCAGGAGCGAGA-3' and reverse, 5'-TGTTGTCATACTTCTCAT-3'. As performed in our previous research [4], Real-time PCR assay used SYBR Green PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) to detect the mRNA expression of Notch1, Hes1, Hey1 and GAPDH genes. Data were analyzed according to the $2^{-\triangle\triangle CT}$ method [17].

Western blot analysis

Total protein from laryngeal cancer cells was extracted using RIPA lysis buffer. Then, total protein extracts went electrophoresis in SDS-PAGE (5% stacking gel and 8% separating gel) and transferred to PVDF membranes (Millipore), blocked with 5% skimmed milk solution at room temperature for 2 hours. Next, the membranes were immunoblotted with primary antibodies (Notch1 1:1000, rabbit anti-human; N1ICD 1:1000, rabbit anti-human; GAPDH, 1:1000, mouse anti-human) overnight at 4˚C, and incubated

with the secondary antibodies (1: 5000; room temperature, 1 hour). The proteins were visualized and quantified by electrogenerated chemiluminescence.

Cell proliferation assay

For the proliferation assay, cells were plated into 96-well culture panels at a dose of 5×10^3 cells/well. Cell Counting Kit-8 (CCK-8) method was used to assess the proliferation of AMC-HN-8 and Tu212 cells according to the manufacturer's protocol. The optical density (OD) was measured at 450 nm on a Microplate Reader (Bio-Rad Laboratories, Inc.).

Cell apoptosis analysis

The apoptosis index (AI) of neoplastic cells were evaluated by BD FACS Calibur cytometry using Annexin V-FITC Apoptosis Detection Kit (Bender Medsystems Inc. USA) according to the manufacturer's protocol. Cells were cultured in 6-well plates (4×10^5 cells/well) and incubated overnight at 37˚C. Then, after renewing the medium, the cells were cultured in hypoxia or normoxia for 48 hours. After that, neoplastic cells were washed in DMEM and resuspended in 190 µL of Tris-HCl buffer. Furthermore, the cell suspension added with 5 µL Annexin-V-FITC and 5 µl propidium iodide. The fluorescence intensity of the stained cells was assessed by FCM.

Statistical analysis

Continuous data were analyzed with Student's t-test or one-way ANOVA analysis. Statistical analyses were processed with SPSS20.0 software. $P<0.05$ was regarded as statistically significant.

Results

Immunoexpression of Notch1 in human LSCC tissues

LSCC samples from 107 laryngeal cancer patients were labeled for Notch1 by using immunohistochemistry method. The immunostaining for Notch1 was observed in 68 (63.55%) of 107 laryngeal cancer tissue samples, and was located in the membrane and cytoplasm of tumor cells (Fig. 1a).

Measurement of apoptotic index and proliferative index in LSCC tissues and its relation to Notch1 expression

The proliferation level (Ki-67 expression) and apoptosis index (TUNEL staining) (Fig. 1b and c) were both determined to examine the staining of cell nuclei. The average proliferative and apoptotic indices (PI and AI) in human LSCC tissues were 27.80 ± 3.82% and 1.47 ± 0.18%, respectively. In the current study,

Notch1 expression was negatively associated with AI in LSCC tissues (P<0.05) (Table 1). On the other hand, the expression of Notch1 was positively related to PI (P<0.05) (Table 1).

Table 1
Correlation between proliferative and apoptotic indices based on Notch1 expression in laryngeal squamous cell carcinoma

Indices (% ± SD)	Notch1 expression		
	Positive	Negative	*P*
Mean apoptotic index	1.31±0.16%	1.75±0.21%	<0.05
Mean proliferative index	30.48±4.16%	23.12±3.02%	<0.05

Hypoxia promoted proliferation and inhibited apoptosis of laryngeal cancer cells

AMC-HN-8 and Tu212 cells were incubated in normoxia or hypoxia for 48 hours to observe the effects of hypoxia on the proliferation and apoptosis of neoplasm cells. The apoptosis and proliferation of laryngeal cancer cells were detected by Annexin V-FITC/propidium iodide staining and CCK-8 methods, respectively. As can be seen from Fig. 2a, the proliferation of neoplasm cells were significantly up-regulated by hypoxia. On the other hand, the percent of cells apoptosis was decreased after incubation in hypoxia for 48 hours (Fig. 2b, P<0.05).

Hypoxia enhanced Notch1 expression and Notch1 signaling activity in laryngeal cancer cells

AMC-HN-8 and Tu212 cells were incubated under normoxia or hypoxia for 48 hours. The data of Real-time PCR confirmed that hypoxia significantly up-regulated Notch1, Hes1, Hey1 mRNA expression in tumor cells (P<0.05) (Fig. 3a-c). The data of Western blot assay demonstrated that the expression of Notch1 and N1ICD protein in laryngeal cancer cells could be enhanced by hypoxia (P<0.05) (Fig. 3d, e). The above results revealed that Notch1 expression and Notch1 signaling activity in laryngeal cancer cells could be enhanced by hypoxia.

Inhibition of Notch1 expression reduced the activity of Notch1 signaling in laryngeal cancer cells under hypoxia

Real-time PCR results showed that Notch1, Hes1 and Hey1 mRNA levels in Notch1-siRNA group were significantly lower than those in control groups (P<0.05) (Fig. 4a-c). Likewise, Western blot results confirmed that the expression levels of N1ICD and Notch1 protein in Notch1-siRNA group were lower than those in control groups (P<0.05) (Fig. 4d, e). The above results indicated that Notch1 signaling activity in hypoxic laryngeal cancer cells could be down-regulated by inhibition of Notch1 expression.

Inhibition of the activity of Notch1 signaling downregulated proliferation and induced apoptosis of hypoxic laryngeal carcinoma cells

To explore the effects of Notch1 signaling on cell proliferation and apoptosis in hypoxia, AMC-HN-8 and Tu212 cells were evaluated by using CCK-8 method and Annexin V-FITC/propidium iodide staining method. As can be seen in Fig. 5, suppression of the activity of Notch1 signaling in AMC-HN-8 and Tu212 cells by transfected with siRNA could evidently decrease cell proliferation and increase the rate of apoptosis (both $P<0.05$).

Discussion

To our knowledge, abnormal proliferation and apoptosis of neoplastic cells always underly the pathological process of carcinogenesis and progression in various malignancies [18]. However, the regulatory mechanisms related to the apoptosis and proliferation of human neoplasm cells still remain unclear. Thus, it is significant to explore the regulatory mechanisms of the disturbance between cell apoptosis and proliferation in human laryngeal carcinoma.

Hypoxia, as an essential feature of tumor microenvironment, induces a set of functional adaptive responses of neoplastic cells, includig cell proliferation and apoptosis [1–3], which is mediated by a series of molecular mechanisms. Likewise, a number of literatures have already demonstrated that hypoxia is engaged in regulating a variety of malignant biological phenotypes of laryngeal carcinoma cells [4–6]. Further, to explore whether hypoxia takes part in the regulation of the proliferation and apoptosis of laryngeal cancer cells, our study detected and analyzed the proliferation and apoptosis status of AMC-HN-8 and Tu212 cells under normoxia and hypoxia. Consequently, it was observed that hypoxia significantly enhanced proliferation and apoptosis resistance of laryngeal carcinoma cells.

Notch signaling, as a core molecular signaling pathway, takes a vital role in regulating the expression of downstream target genes under hypoxia, which regulates a set of biological phenotypes of human neoplasms [7, 8]. Previously, the study by Dai et al. [9] and our data [10] have both elucidated that Notch1 expression is up-regulated in LSCC tissues and is obviously related to lymph node metastasis. Moreover, the present study exhibited that Notch1 expression and Notch1 signaling activity in Tu212 and AMC-HN-8 cells could be enhanced by hypoxia. The above data revealed that, in the hypoxic microenvironment, Notch1 signaling might participate in regulating the malignant process of laryngeal cancer.

Up to now, a number of documents have demonstrated that the effects of Notch1 signaling on cell apoptosis and proliferation in different kinds of human neoplasms are variable [11–14]. The inconsistency of research conclusions may be attributed to the regulatory heterogeneity among different types of tumor cells. Moreover, the study of Jiao et al. [20] has indicated that Notch1 signaling could inhibit cell proliferation and induce apoptosis of Hep-2 cells. On the contrary, an in vitro study of Dai et al. [9] has demonstrated that Notch1 signaling could promote proliferation and inhibit apoptosis of Hep-2

cells. The conclusions of the above two studies on laryngeal cancer cells are also full of controversy. Current research indicated that Notch1 expression in laryngeal carcinoma tissues was negatively correlated with AI and positively associated with PI. Meanwhile, our in vitro study indicated that suppression of the activity of Notch1 signaling in Tu212 and AMC-HN-8 cells under hypoxia could obviously induce cell apoptosis and decrease cell proliferation. Consistent with the conclusion of Dai et al. [9], it can be regarded that Notch1 signaling contributes to cellular proliferation and apoptotic resistance in laryngeal carcinoma.

Conclusion

In summary, current research demonstrates that Notch1 signaling might play a critical role in regulating the apoptosis and proliferation of laryngeal cancer cells in the hypoxic microenvironment. Further research is needed to explore the mechanisms of Notch1 signaling in regulating the apoptosis and proliferation of human laryngeal carcinoma cells.

Declarations

Acknowledgments

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Statements and Declarations

Funding

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

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

Data Availability

The datasets generated during and/or analysed during the current study are not publicly available due to [REASON(S) WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.].

Ethics approval

LSCC samples collection and the study protocol were approved by the Ethics Committees of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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Figures

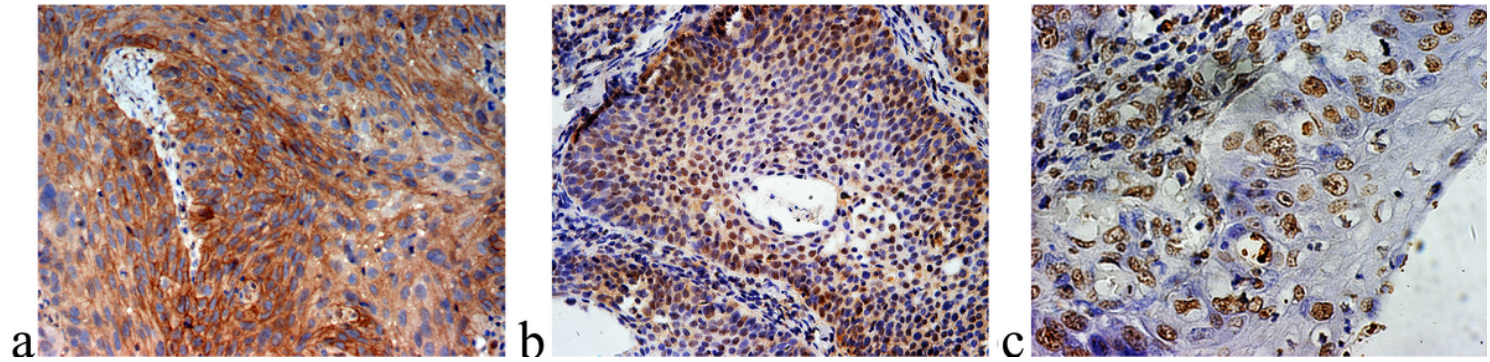

Figure 1

Immunohistochemical staining of Notch1 and Ki-67, TUNEL positive cells in laryngeal cancer tissues. a Notch1 was observed in the cytoplasm and membranes of cancer cells (×200). b Ki-67 was found in the nuclei of neoplastic cells (×400). c Apoptotic cells were assessed by TUNEL method (×400)

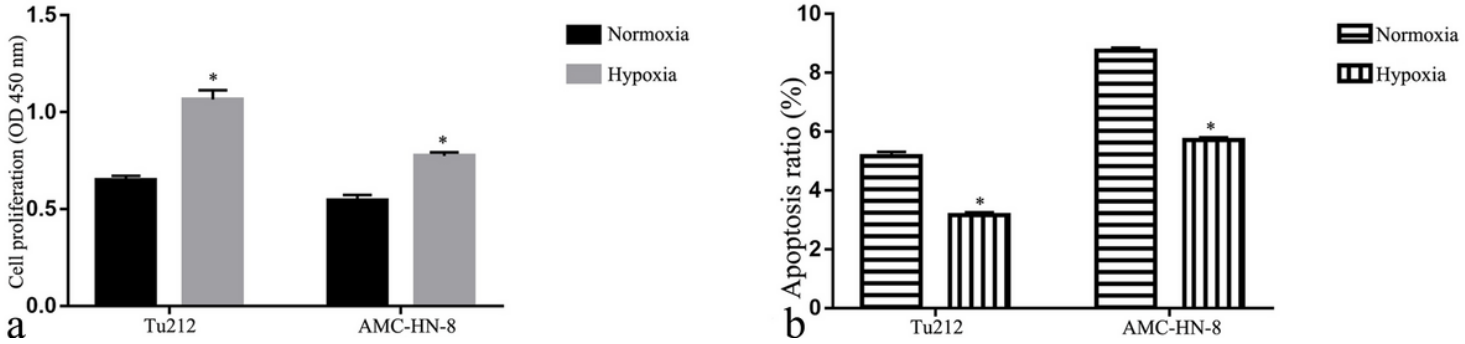

Figure 2

Changes in cell proliferation and apoptosis of laryngeal carcinoma cells under normoxic and hypoxic conditions. It was observed that hypoxia could significantly promote proliferation (a) and suppress apoptosis (b) of laryngeal carcinoma cells. *P<0.05, versus normoxia group

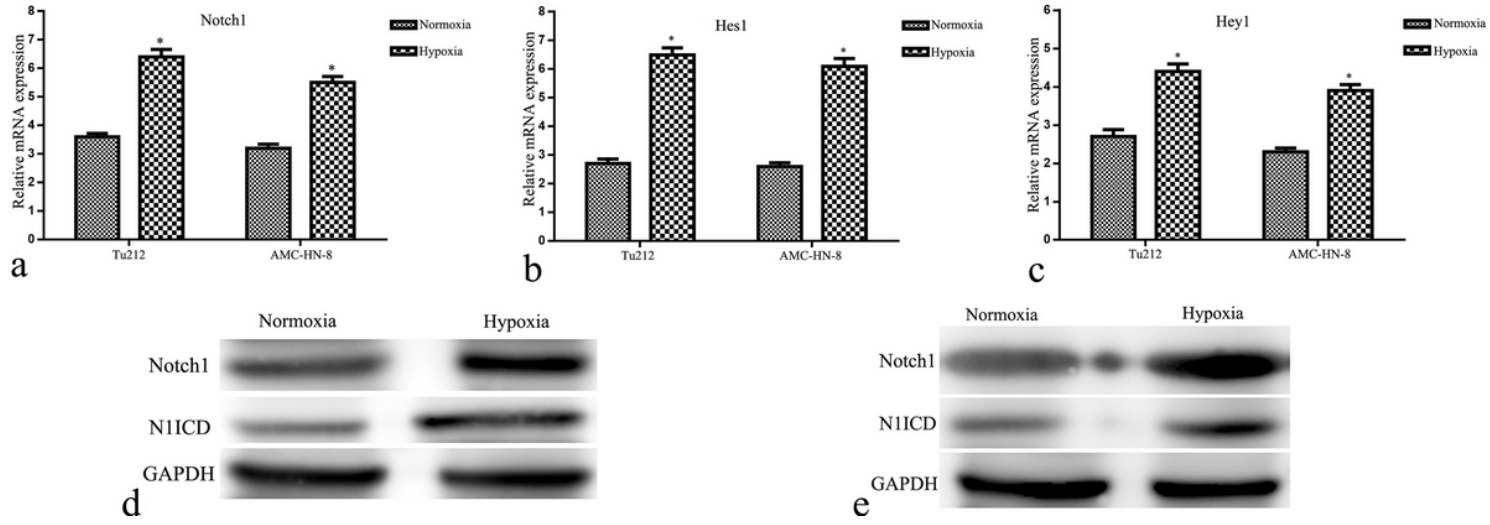

Figure 3

Changes in Notch1 expression and Notch1 signaling activity in laryngeal cancer cells under normoxia and hypoxia. Real-time PCR assay estimated Notch1 (a), Hes1 (b) and Hey1 (c) mRNA expression in laryngeal cancer cells. Western blot evaluated the expression of Notch1 and N1ICD protein in Tu212 (d) and AMC-HN-8 (e) cells. *P<0.05, versus control groups

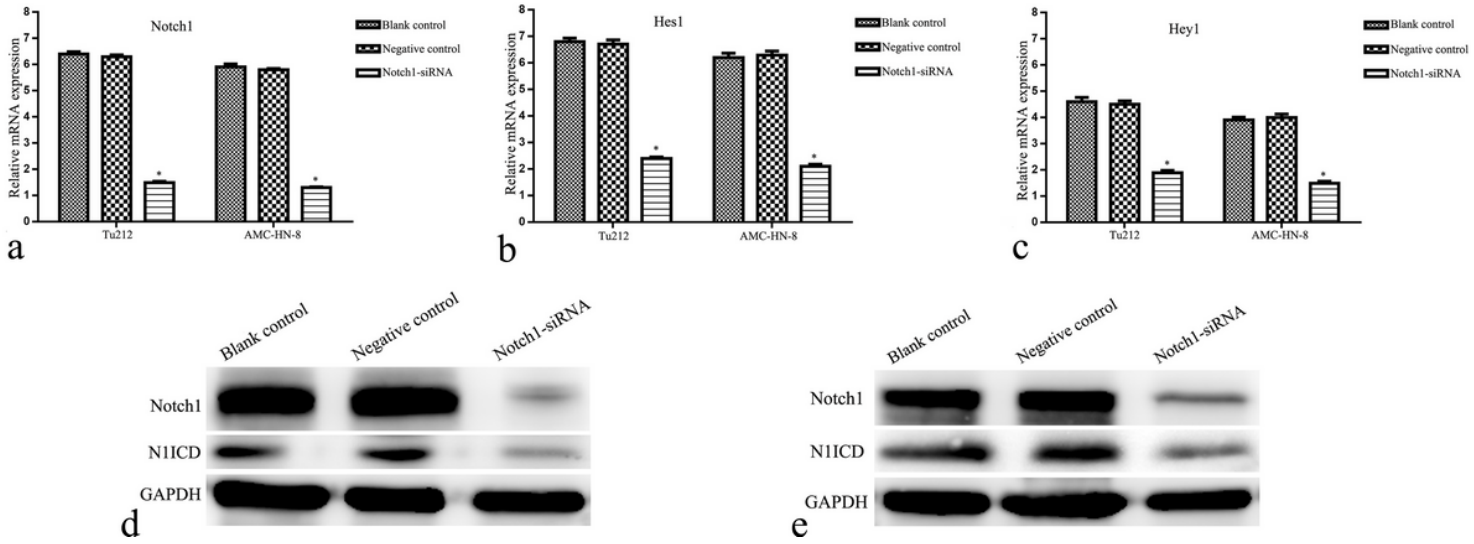

Figure 4

Effects of Notch1-siRNA on the activity of Notch1 signaling in laryngeal cancer cells under hypoxia. Real-time PCR assay assessed Notch1 (a), Hes1 (b) and Hey1 (c) mRNA expression in Tu212 and AMC-HN-8 cells under hypoxia. Western blot estimated the expression of Notch1 and N1ICD protein in Tu212 (d) and AMC-HN-8 (e) cells under hypoxic condition. *P<0.05, versus control groups

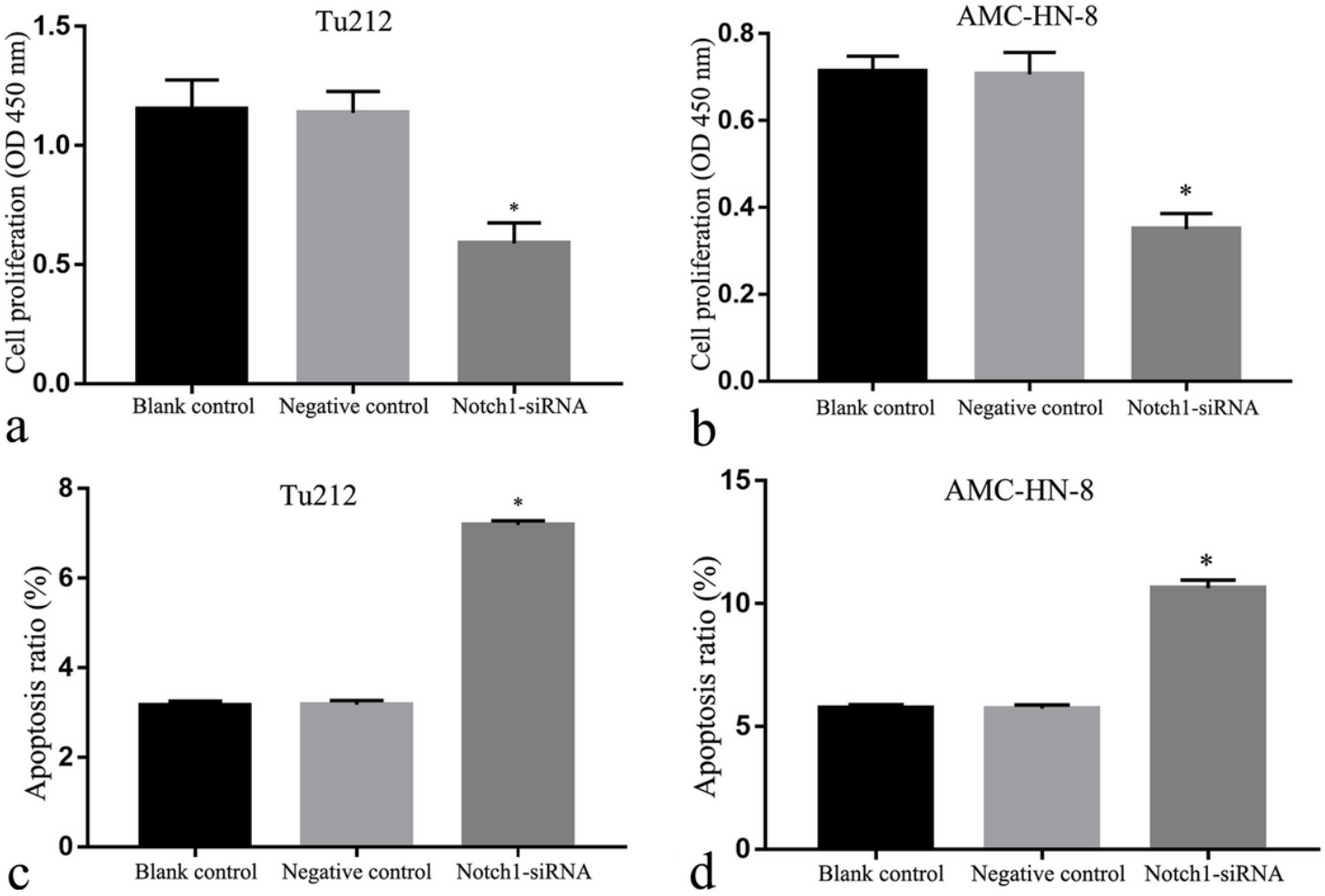

Figure 5

Effects of Notch1 signaling on apoptosis and proliferation of hypoxic laryngeal carcinoma cells. Suppression of the activity of Notch1 signaling could decrease cell proliferation of Tu212 (a) and AMC-HN-8 (b) cells under hypoxia. Suppression of the activity of Notch1 signaling could increase apoptosis of Tu212 (c) and AMC-HN-8 (d) cells under hypoxic condition. *P<0.05, versus control groups