Downregulation of Notch1 induces apoptosis and inhibits cell proliferation and metastasis in laryngeal squamous cell carcinoma

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Abstract. Notch signaling plays a key role in a wide variety of human tumors; it can be an oncogene or a tumor-suppressor gene depending on the tissue context. The functions of Notch1 in laryngeal squamous cell carcinoma (LSCC) are largely unknown. We investigated the role of Notch1 in LSCC cell growth, apoptosis and metastasis. We analyzed Notch1 expression in clinical LSCC samples using quantum dot immunohistochemistry (QD-IHC) and conventional IHC. Human laryngeal carcinoma HEp-2 cells were transfected with Notch1-specific short hairpin RNA (shRNA), and cell proliferation, apoptosis, and migration and invasion were evaluated using the cell counting assay, flow cytometry and wound healing and Transwell assays, respectively; western blotting was used to validate the expression of Notch1 target genes. Compared with normal tissues, Notch1 was upregulated in LSCC tissues; compared with LSCC tissues without metastasis, Notch1 upregulation was enhanced in LSCC tissues with metastasis ($P<0.05$). Transfection downregulated Notch1 mRNA and protein expression levels in the Notch1 shRNA group. There was a significantly greater decrease in cell proliferation in the Notch1 shRNA group than cell proliferation in the non-transfected ($P<0.05$) and negative shRNA groups ($P<0.05$). Furthermore, Notch1 knockdown induced apoptosis in the HEp-2 cells. Additionally, the number of migrated and invasive cells in the Notch1 shRNA group was decreased ($P<0.05$). Notch1 knockdown in the HEp-2 cells greatly inhibited phosphorylated extracellular signal-related kinase (p-ERK), phosphorylated protein kinase B (p-AKT), c-Myc, Bcl-2, p21, cyclin D1, cyclin-dependent kinase 4 (CDK4) and cyclin E expression levels and increased Bax expression. Altogether, our findings indicate that Notch1 expression is increased in human LSCC and correlates with tumorigenesis and metastasis, while in HEp-2 cells, Notch1 knockdown inhibited cell growth, induced apoptosis and inhibited migration and invasion by regulating Notch1 target genes, suggesting it may be a potential therapeutic target for treating LSCC.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most common head and neck cancers worldwide. In China, its incidence has been increasing gradually, particularly in the northeastern region. While surgery or radiotherapy can often cure early-stage LSCC, the outcome has not improved greatly for most patients with advanced disease in the last two decades despite the advances in therapy (1). The poor prognosis of laryngeal carcinoma is attributed to postoperative recurrence and distant metastasis (2). Therefore, it is crucial to develop a better understanding of the molecular mechanisms involved in the progression of laryngeal carcinoma to improve treatment efficacy.

The Notch signaling pathway is highly conserved evolutionally, playing central roles in embryonic development and adult life. In mammals, the Notch receptors (Notch1-4) are transmembrane proteins. Notch receptor activation takes place when the receptor binds with a ligand from the Jagged (Jagged1 and 2) or Delta family (DLL-1, -3 and -4), which are themselves attached to their respective cell membranes (3,4). Therefore, Notch receptor-ligand interaction occurs between two adjacent cells. Notch proteins are synthesized as precursors in the endoplasmic reticulum, and then the Notch intracellular domain (NICD) is released, translocating to the nucleus, driving the expression of HEY1, HES1, Myc, CCND1, Bcl-2 and other cellular process-regulating genes involved in proliferation, differentiation, stem cell maintenance and apoptosis (5).

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The Notch pathway is genetically altered in many hematopoietic and solid tumors; Notch signaling can be oncogenic or tumor-suppressive in tumorigenesis. A recent review (4) demonstrated that the Notch pathway plays an oncogenic role in T cell acute lymphoblastic and chronic lymphocytic leukemias, breast, pancreatic and colorectal cancer, and lung adenocarcinoma; a tumor-suppressive role was observed in B cell acute lymphoblastic and acute myeloid leukemias, ovarian carcinoma and small cell lung cancer. Moreover, Notch signaling has opposite effects in the same type of cancer (6-9). For example, Notch exhibits both tumor-promoting and inhibitory functions in lung carcinoma, depending on cell type (10,11). Clearly, Notch activity regulates tumor biology context-dependently and in a complex manner.

The roles of the Notch pathway in human LSCC remain unclear. Early studies suggested a pro-tumorigenic role in head and neck squamous cell carcinoma (HNSCC), mainly based on the increased Notch1 and Notch2 expression levels in HNSCC tissues (12-14). Recent exome sequencing data revealed that NOTCH1 is the second most frequently mutated gene in HNSCC, unexpectedly raising questions regarding the accepted role of Notch in HNSCC (15). Therefore, detailed clarification of the role of Notch1 in LSCC is urgent. In the present study, we analyzed Notch1 expression in clinical LSCC samples using quantum dot immunohistochemistry (QD-IHC) and conventional IHC. Using short hairpin RNA (shRNA), we silenced Notch1 expression in a human laryngeal carcinoma HEp-2 cell line to elucidate the effects of Notch1 on LSCC cell proliferation, apoptosis, migration and invasion.

Materials and methods

Laryngeal carcinoma specimens and cell lines. Formalin-fixed, paraffin-embedded tissue samples were collected from Renmin Hospital of Wuhan University, China, from January 2009 to December 2010, from patients aged 39-81 years (median, 58 years). There were 31 normal vocal polyp tissues and 55 LSCC samples (18 cases with metastasis, 37 cases without metastasis). A tissue microarray of 40 LSCC cases (5 cases with metastasis and 35 cases without metastasis) was purchased from AiLiNa Technologies (Xian, China). We obtained written informed consent from the patients. The Ethics Committee of Renmin Hospital of Wuhan University approved the study protocol.

The HEp-2 cell line was obtained from the China Center for Type Culture Collection (Wuhan, China). HEp-2 cells were cultured in RPMI-1640 culture medium (HyClone, Logan, UT, USA) and supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 20 µg/ml streptomycin in a fully humidified incubator at 37°C and in an atmosphere of 5% CO_2 in air.

shRNA transfection. The enhanced green fluorescent protein (EGFP)-V-RS-Notch1 shRNA plasmid was purchased from Wuhan XiMa Technologies Co. Ltd. (Wuhan, China). The shRNA plasmid structure is as follows: stop-mir30 flanking-shRNA1-mir30 flanking-EGFP-CMV-U6 shRNA2-stop; the shRNA sequences are as follows: Notch1 shRNA1, 5'-CCGGG ACATCACGGATCATAT-3'; Notch1 shRNA2, 5'-CGCTGCC TGGACAAGATCAAT-3'. Three treatments were designed for

the present study. Untreated HEp-2 cells were considered as the blank control, and termed the non-transfected group. The plasmid EGFP-V-RS-Notch1 shRNA, containing Notch1-specific shRNA, and the plasmid EGFP-V-RS-negative shRNA, containing non-specific shRNA (Wuhan XiMa Technologies Co., Ltd.), were the Notch1 shRNA and negative shRNA groups, respectively. These plasmids were transfected into HEp-2 cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

QD-IHC. The collected tissue samples and tissue microarray were used for QD-based immunofluorescence histochemical detection. For antibody binding, slides were incubated with rabbit anti-human Notch1 (1:200; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. For QD conjugation, the slides were incubated with zinc sulfate-capped cadmium selenide QDs conjugated to anti-rabbit immunoglobulin G probes with an emission wavelength of 605 nm (1:50 in 2% bovine serum albumin; Jiayuan Quantum Dot Co., Ltd., Wuhan, China) for 30 min at 37°C. Following incubation, the slides were vigorously washed with phosphate-buffered saline (PBS), mounted with neutral glycerol and stored at 4°C for observation. The QDs were excited by blue light (excitation wavelength of 450-480 nm under U-MWB filters) and present red light under excitation. IHC staining was observed under light microscopy, and positive cells appeared brown-yellow and granular, primarily to prevent drying of tissues. The staining results were scored according to existing standards: negative, no staining; 1+, weak staining; 2+, strong staining. The IHC results were graded as negative, positive or strongly positive as follows: negative, no staining or 1+ staining in ≤30% of cells; positive, 1+ staining in >30% of cells or 2+ staining in ≤50% of cells; strongly positive, 2+ staining in >50% of cells.

IHC. The collected tissue samples were used for IHC detection. Rabbit anti-human Notch1 (1:200; Cell Signaling Technology) was used to detect Notch1 expression. The immunohistochemistry protocols have been described elsewhere (16). Immunostaining intensity was estimated semi-quantitatively according to signal intensity and distribution. The staining results were assessed on a three-tier scale: negative, no staining; 1+, weak staining, 2+, strong staining. The IHC results were graded as negative, positive, or strongly positive as follows: negative, no staining or 1+ staining in ≤30% of cells; positive, 1+ staining in >30% of cells or 2+ staining in ≤50% of cells; strongly positive, 2+ staining in >50% of cells.

Quantitative reverse transcription-PCR (qRT-PCR). Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and reverse-transcribed into complementary DNA (cDNA) using a PrimeScript RT reagent kit (Takara, Kyoto, Japan). The primer sequences used were as follows: Notch1 forward and reverse, 5'-ATGACCAGTGGCTACGTG TG-3' and 5'-CGGCAACGTCGTCAATACAC-3', respectively; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward and reverse, 5'-GAAAGCCTGCCGGTGACTAA-3' and 5'-AGGAAAAGCATCACCCGGAG-3', respectively. The cDNA was used as the template for qRT-PCR detection of the expression of the genes of interest with SYBR Premix Ex Taq™ (Takara). Data were analyzed according to the

comparative threshold cycle value ($2^{-\Delta\Delta Ct}$) method. Relative mRNA expression was normalized to GAPDH expression.

Cell proliferation assay. Cells were plated into 96-well culture plates at 3,000 cells/well and transfected with shRNA mentioned above. At the same time each day for 24, 48 and 72 h, the original culture medium was removed and 10 μl Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) and 90 μl fresh RPMI-1640 medium were added to each well. The cells were incubated at 37˚C for 1 h, and the absorbance of the medium at 450 nm was measured.

Apoptosis assay. Cells were plated into 6-well culture plates and transfected with shRNA. After 72 h, apoptosis was analyzed using a FACScan instrument (Becton-Dickinson, San Jose, CA, USA). The cells were harvested and washed in PBS, and then stained with adenomatous polyposis coli (APC) and 7-amino actinomycin D (7-AAD) (Lianke, Hangzhou, China). The stained cells were analyzed with a FACSCanto™ II spectrometer (Becton-Dickinson). Data were analyzed using FlowJo 7.6.5 software.

Scratch wound-healing motility assay. Cells were transfected with shRNA for 24 h, and then plated into 6-well culture plates. Then, a scratch was made by running a pipette tip through the dish; the cells were then cultured under standard conditions for 24 h. The plates were washed twice with fresh medium to remove non-adherent cells and then photographed. The number of cells that had migrated from the edge of the wound was counted.

In vitro cell invasion and migration assays. For the cell invasion assays, Transwell membranes (Corning Inc., New York, NY, USA) were coated with Matrigel (2.5 mg/ml). Twenty-four hours after transfection, cells were serum-starved for 8 h, and were then collected in RPMI-1640 medium containing 3% FBS. Cells were seeded onto the top chambers of the precoated Transwells in the same medium alone at 1.0x10^5 cells/well. The bottom chambers of the Transwells contained 600 μl RPMI-1640 medium and 10% FBS. After 48 h, the Matrigel and cells in the top chamber were swabbed with a Q-tip, and the membranes were stained with crystal violet for 10 min. Cells in at least five random fields were counted and photographed under microscopy (x200).

Cell migration assays were also performed using Transwell membranes (Corning). The procedure used was similar to that of the cell invasion assay, except the Transwell membranes were not coated with Matrigel.

Western blotting. Total protein from the plasmid-transfected HEp-2 cells was extracted using radioimmunoprecipitation assay buffer [1 mM MgCl$_2$, 10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40]; and protein expression was analyzed by western blotting. GAPDH was used as the loading control. Total protein extracts were separated by SDS-polyacrylamide gel electrophoresis on 12% gels and transferred to polyvinylidene difluoride membranes. The proteins were visualized and were quantified using Odyssey Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ, USA).

Table I. P-values for the western blot analysis (Student's t-test).

	P-value (Notch1-shRNA vs. non-transfection)	P-value (Notch1-shRNA vs. negative-shRNA)
p-ERK	0.0100	0.0219
t-ERK	0.5230	0.5968
c-Myc	0.0012	0.0016
Bcl-2	0.0048	0.0089
Bax	0.0467	0.0439
CDK4	0.0483	0.0307
Cyclin E	0.0183	0.0272
p-Akt	0.0304	0.0231
t-Akt	0.8955	0.6798
p21	0.0263	0.0313
Cyclin D1	0.0389	0.0443

Statistical analysis. All data are expressed as the mean ± standard deviation (SD); the data were analyzed with SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). The QD-IHC and IHC results were analyzed using the rank-sum test. The results from the PCR, western blotting, apoptosis assay and *in vitro* cell migration/invasion assays were analyzed using the Student's t-test. P<0.05 was considered statistically significant (Table I).

Results

Notch1 is upregulated in LSCC. Compared with normal samples, Notch1 is both overexpressed and downregulated in human cancers. In the present study, we investigated Notch1 expression in LSCC and normal vocal polyp tissues using QD-IHC and conventional IHC. QD-IHC has better image quality and sensitivity than conventional staining methods, and detected Notch1 expression in a total of 95 LSCC and 31 normal vocal polyp specimens. Fig. 1 and Table II show no or very low Notch1 expression in the normal vocal polyp tissues, but higher expression in the LSCC tissues. There was gradual Notch1 upregulation in the normal vocal polyp tissues and in LSCC without and with metastasis. We determined that Notch1 was localized to the cytoplasm and nucleus. To confirm our results, we characterized Notch1 expression levels using IHC. Among the 55 LSCC tissues, 48 samples (85.4%) had high Notch1 expression; among the normal vocal polyp tissues, Notch1 expression was high in only 12 samples (38.7%). Notch1 expression levels were higher in LSCC with metastasis than in LSCC without metastasis (Fig. 1 and Table III). These results indicate that Notch1 is upregulated in LSCC tissues and plays an important role in LSCC tumorigenesis and metastasis.

Notch1 shRNA downregulates Notch1 mRNA and protein expression effectively in HEp-2 cells. The efficacy of Notch1 shRNA in HEp-2 cells was analyzed using RT-PCR and western blotting. Compared with the negative shRNA group, Notch1 mRNA (Fig. 2A) and protein (Fig. 2B and C) expression in the Notch1 shRNA group was decreased by 72.6 and 76.8%, respectively. There were no significant changes in the
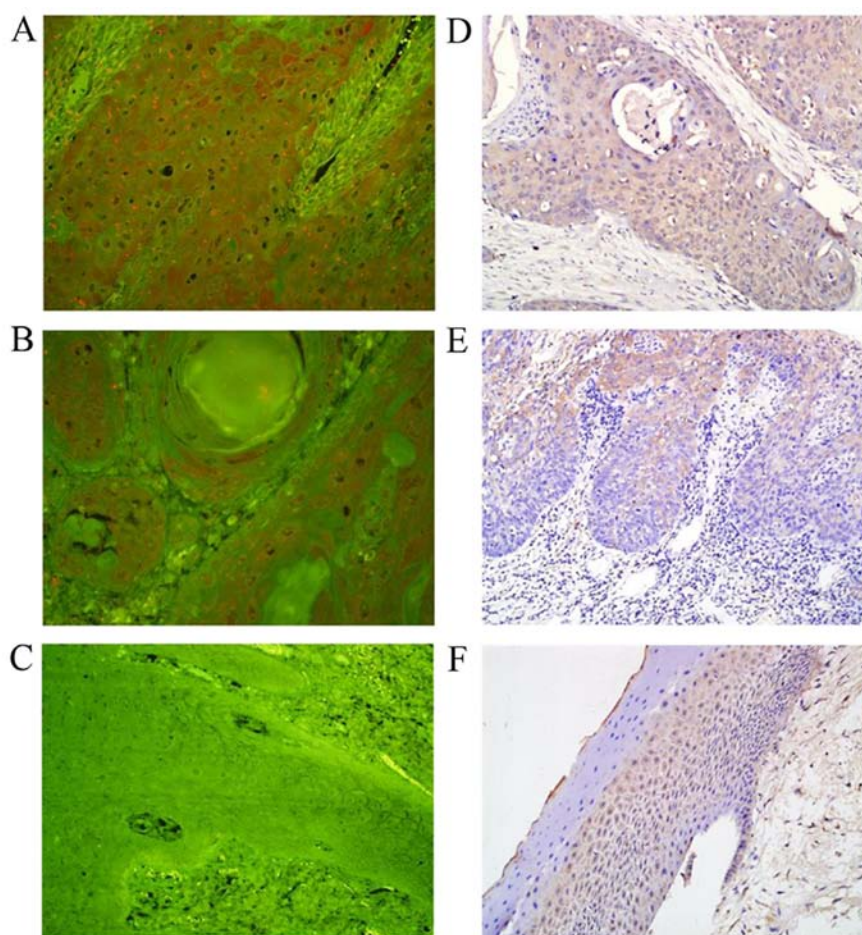
Figure 1. Notch1 is upregulated in LSCC. (A-C) Representative QD-IHC images of Notch1 expression in LSCC with metastasis (A) or without metastasis (B) and in normal vocal polyp tissues (C). (D-F) Representative IHC images of Notch1 expression in LSCC with metastasis (D) or without metastasis (E) and in normal vocal polyp tissues (F). Original magnification, x200.

Table II. QD-IHC detection of Notch1 expression in LSCC and normal vocal polyp tissues.

Sample	Case	Negative	Positive	Strong positive	P-value
Normal vocal polyps	31	24	5	2	
LSCC	95	11	44	41	<0.01[a]
Without metastasis	72	11	39	22	<0.01[a]
With metastasis	23	0	4	19	<0.01[b]

[a]When compared with normal vocal polyp tissues. [b]When compared with normal vocal polyp tissues and LSCC without metastasis. QD-IHC, quantum dot immunohistochemistry; LSCC, laryngeal squamous cell carcinoma.

Table III. IHC detection of Notch1 expression in LSCC and normal vocal polyp tissues.

Sample	Case	Negative	Positive	Strong positive	P-value
Normal vocal polyps	31	19	7	5	
LSCC	55	7	21	27	<0.01[a]
Without metastasis	37	5	19	13	<0.01[a]
With metastasis	18	2	2	14	<0.01[b]

[a]When compared with normal vocal polyp tissues. [b]When compared with normal vocal polyp tissues and LSCC without metastasis. IHC, immunohistochemistry; LSCC, laryngeal squamous cell carcinoma.
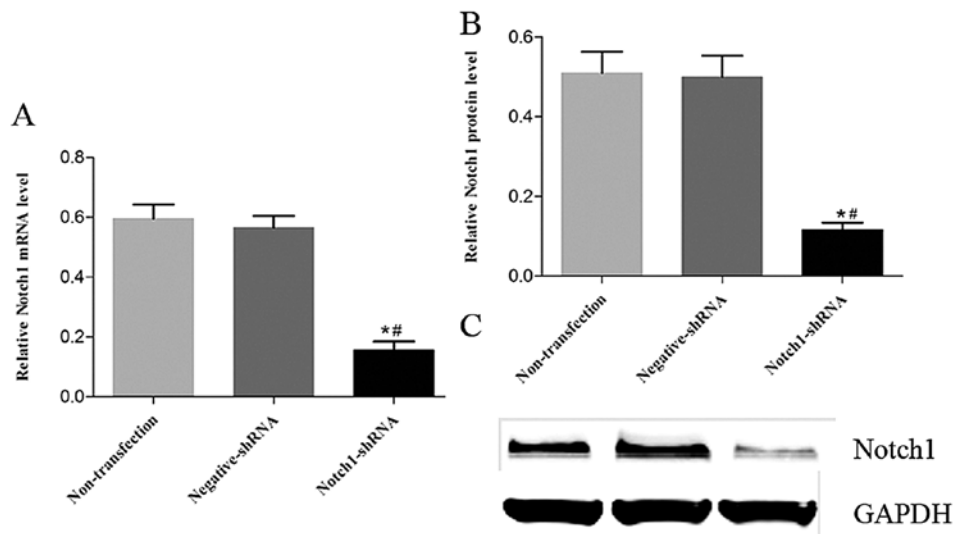
Figure 2. shRNA knockdown of Notch1 in HEp-2 cells. (A) RT-PCR determination of Notch1 mRNA expression; GAPDH was used as the internal control. (B and C) Western blot analysis of Notch1 protein expression normalized to GAPDH. Data presented are the means ± SD. *P<0.05 compared with the non-transfected group; #P<0.05 compared with the negative shRNA group.
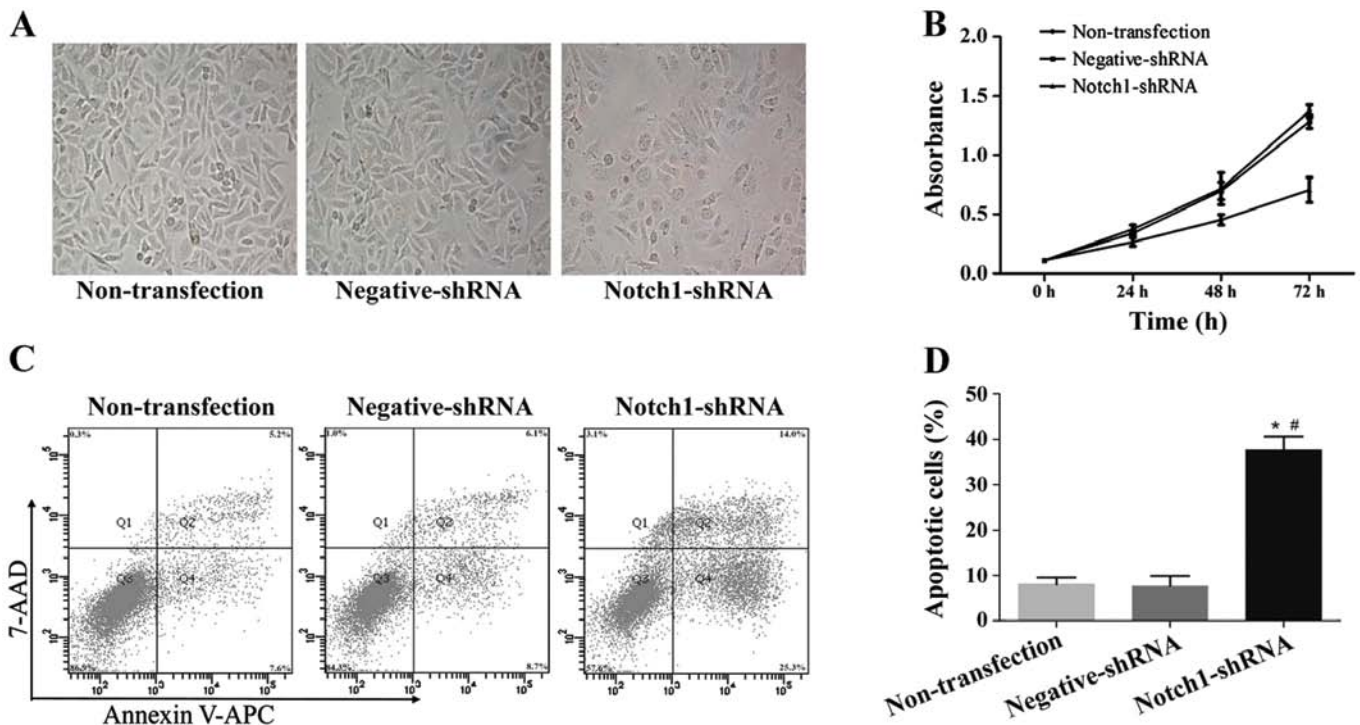

Figure 3. Notch1 knockdown induces HEp-2 cell morphological changes, inhibits proliferation and induces apoptosis. (A) Cell morphology at 72 h post-transfection (magnification, x400). (B) CCK-8 assay of HEp-2 cell proliferation. (C) At 72 h after shRNA transfection, HEp-2 cells were stained with APC and 7-AAD, and the apoptotic cells were analyzed by flow cytometry. (D) Percentage of early and late apoptotic cells. The data represent the results of three independent experiments. *P<0.05 compared with the non-transfected group; #P<0.05 compared with the negative shRNA group.

Notch2 mRNA or protein levels in the non-transfected and negative shRNA groups (P>0.05).

Notch1 knockdown induces morphological changes, inhibits proliferation and induces apoptosis in HEp-2 cells. To investigate the effect of Notch1 on cancer cell morphology, proliferation and apoptosis, we used shRNA to knock down Notch1 in HEp-2 cells. Following a 72-h Notch1 shRNA transfection in HEp-2 cells, the cells were examined and

photographed under phase contrast microscopy at x400 to observe the cell morphological changes. Fig. 3A shows that the cell morphological changes were consistent with the reduced cell numbers in culture. Notch1-specific shRNA significantly inhibited HEp-2 cell growth at 24, 48 and 72 h post-transfection (Fig. 3B, 24 h, P<0.05; 48 and 72 h, P<0.01).

To validate the effect of Notch1 on apoptosis, we detected apoptotic cells using APC and 7-AAD staining and flow cytometry following Notch1 shRNA transfection
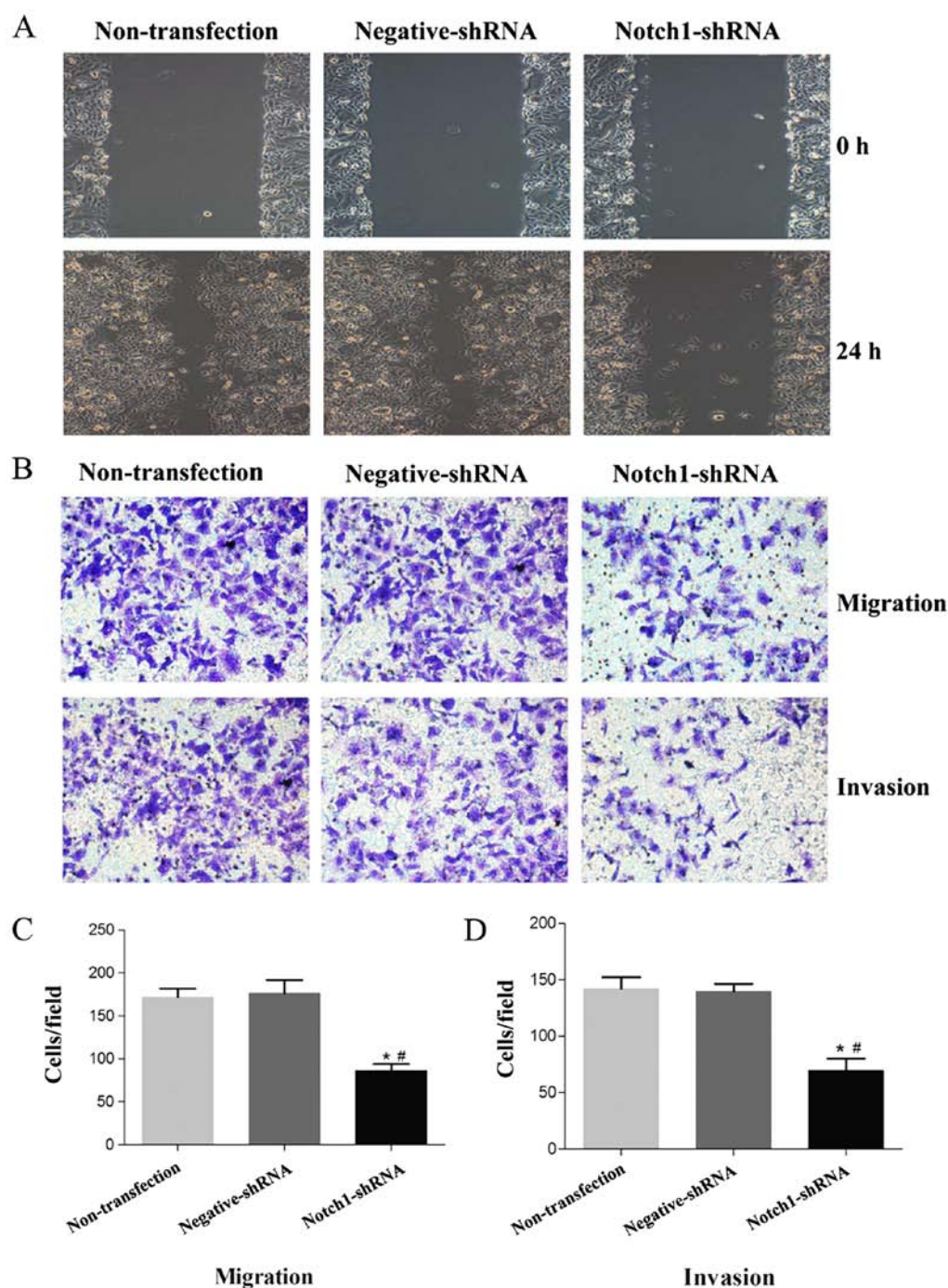
Figure 4. Notch1 knockdown inhibits HEp-2 cell migration and invasion abilities. (A) Photomicrograph of scratches made in the shRNA-transfected HEp-2 cell layer showing inhibition of cellular motility in the Notch1-silenced cells as compared with the non-transfected and negative shRNA groups. (B) Representative images of the Transwell assay without (top panel) or with (bottom panel) Matrigel coating after shRNA transfection. (C) Migration assay results showing the number of HEp-2 cells that had migrated through the non-Matrigel coated filters. (D) Invasion assay showing the number of cells that had passed through the Matrigel-precoated filters. The cell counts presented are the mean values/field from at least five randomly selected low-power fields (magnification, x200) from three independent experiments (error bars, means ± SD). *P<0.05 compared with the non-transfected group; #P<0.05 compared with the negative shRNA group.

in HEp-2 cells. At 72 h after transfection, there were higher percentages of both early apoptotic cells (APC-positive, 7-AAD-negative) and late apoptotic cells (APC-positive, 7-AAD-positive) in the Notch1 shRNA-transfected HEp-2 cells as compared with the non-transfected and negative shRNA HEp-2 cells (Fig. 3C and D, P<0.05). These results confirmed that Notch1 is an oncogene in LSCC and may contribute to LSCC cell proliferation and apoptosis.

Notch1 knockdown inhibits HEp-2 cell migration and invasion abilities. We examined whether Notch1 plays a role in LSCC cell migration and invasion. The wound healing assay determined that Notch1 knockdown significantly decreased HEp-2 cell motility and migration (Fig. 4A). To confirm this, we performed *in vitro* Transwell cell migration and invasion assays following a 48-h Notch1 shRNA transfection. There were significantly decreased numbers of migrating and invasive
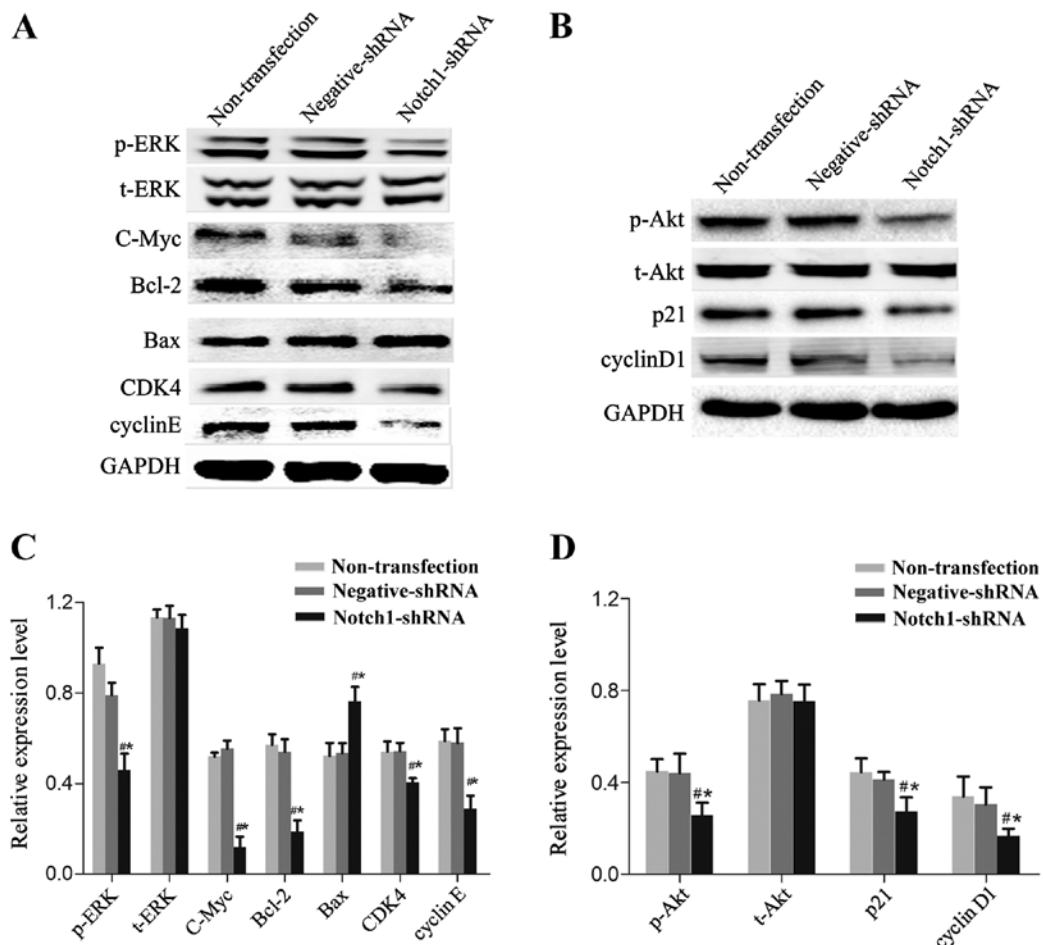
Figure 5. Notch1 knockdown inhibits the expression of proteins related to cell proliferation and apoptosis. (A and B) Western blot analysis of p-ERK, t-ERK, c-Myc, Bcl-2, Bax, CDK4, cyclin E, p-Akt, t-Akt, p21 and cyclin D1 protein expression at 72 h after shRNA transfection; results were normalized to GAPDH. (C and D) Data presented are the means ± SD. *P<0.05 compared with the non-transfected group; #P<0.05 compared with the negative shRNA group.

Notch1 shRNA-transfected HEp-2 cells compared with the non-transfected and negative shRNA cells (Fig. 4B-D, P<0.05). These results indicate that Notch1 is crucial for inhibiting laryngeal cancer cell migration and invasion *in vitro*.

Notch1 knockdown inhibits the expression of proteins related to cell proliferation and apoptosis. We measured the expression of Notch pathway-related proteins using western blotting to investigate the underlying molecular mechanisms. Notch1 knockdown in HEp-2 cells inhibited phosphorylated extracellular signal-related kinase (p-ERK), phosphorylated protein kinase B (p-AKT), c-Myc, Bcl-2, p21, cyclin D1, cyclin-dependent kinase 4 (CDK4) and cyclin E expression (Fig. 5, P<0.05) and increased Bax expression (Fig. 5, P<0.05). There was no obvious change to t-ERK and t-Akt protein expression (Fig. 5, P>0.05).

Discussion

Many aspects of cancer biology, including cell renewal, proliferation, tumor angiogenesis and metastasis are regulated by the Notch signaling pathway (17). Notch signaling is implicated in the tumorigenesis of both solid and hematological malignancies, regulating tumor biology context-dependently and in a complex manner. Notch signaling has oncogenic effects in T cell acute lymphoblastic leukemia, breast and colorectal cancer, where Notch upregulation has been observed in these tumor types as compared with normal samples. Notch signaling also has tumor-suppressive effects, such as in B cell acute lymphoblastic leukemia, ovarian carcinoma and small cell lung cancer, in which Notch is downregulated in these cancer tissues or cells (3,18-20). However, Notch signaling pathway molecular alterations in LSCC are less well defined. A recent report found that QD-IHC has good correlation and consistency with conventional IHC and in comparison, has better image quality and sensitivity (21). Therefore, we used both QD-IHC and IHC to comprehensively confirm Notch1 expression levels in LSCC tissues. Notch1 was upregulated in LSCC tissues as compared with normal vocal polyp tissues; the upregulation was even higher in LSCC tissues with metastasis when compared with LSCC tissues without metastasis, indicating Notch1 may be oncogenic in LSCC tumorigenesis and metastasis.

The role of Notch1 in cellular proliferation and apoptosis has been described in many cell types, and the results have been controversial (5). In the present study, we used shRNA interference to silence the NOTCH1 gene in human laryngocarcinoma HEp-2 cells. RT-PCR and western blotting

detected successful NOTCH1 knockdown in the HEp-2 cells, where the shRNA inhibited Notch1 expression effectively at the mRNA and protein levels. We then examined the effect of Notch1 silencing on HEp-2 cell proliferation. Notch1 shRNA suppressed the proliferative potential of HEp-2 cells compared with that of the non-transfected and negative shRNA-transfected HEp-2 cells. Furthermore, Notch1 knockdown induced apoptosis in the HEp-2 cells at a rate of 37%, which was significantly higher than the rate of apoptosis in the non-transfected and negative shRNA-transfected groups.

Notch1 promotes or suppresses tumorigenesis by regulating different target genes in specific tissue environments and in cancer microenvironments. Abdel Aziz *et al* (22) found that downregulating NOTCH1 and its target gene HES1 significantly decreased the rate of hepatocellular carcinoma (HCC) HepG2 cell proliferation. Moriyama *et al* (23) confirmed that Notch signaling acts through HES1 to play an important role in immature melanoblast survival by preventing apoptosis. Li *et al* (24) found that Notch1 mediates the proliferation of smooth muscle cells via HEY2. We examined the conventional Notch target genes in HEp-2 cells, and found that silencing of Notch1 downregulated p-ERK, p-Akt, c-Myc, Bcl-2, p21, cyclin D1, CDK4 and cyclin E and upregulated Bax; t-ERK and t-Akt expression levels were unchanged.

Among the validated genes, Bcl-2 is a well-known anti-apoptosis gene (25), and Bax is a pro-apoptosis protein (26); these findings were consistent with the increased number of apoptotic cells following Notch1 knockdown. Furthermore, we found downregulation of p-Akt after Notch1 knockdown, confirming that deactivation of the PI3K/Akt pathway attenuated Notch1-induced apoptosis. Knockdown of Notch1 deactivated Akt phosphorylation, decreased Bcl-2 and increased Bax expression, and induced cell apoptosis. ERK is a major effect target of the mitogen-activated protein kinase (MAPK) pathway, and p-ERK was downregulated following Notch1 knockdown, which also inhibited cell proliferation and promoted apoptosis. At the same time, c-Myc was downregulated. c-Myc activates Bax, which induces cytochrome *c* release from the mitochondria into the cytoplasm, resulting in caspase cascade-induced apoptosis. c-Myc also activates transcription factor p53, ARF and apoptosis regulation indirectly, inhibiting MAPK and nuclear factor κB activity, which is sensitive to tumor necrosis factor-mediated signaling, promoting apoptosis (27). In the present study, it was also found that p21, cyclin D1, CDK4 and cyclin E protein expression was downregulated, indicating that Notch1 induces cell cycle arrest and thereby inhibits cell proliferation in HEp-2 cells. This is consistent with previous studies in different carcinoma cells. In small cell lung cancer cells, Notch signaling can increase p21 and p27 expression and hence induce cell cycle arrest (28). In human glioma cells, Notch signaling induces cell cycle arrest by downregulating MCM2 and p21 protein expression (29). In the present study, downregulation of Notch1 inhibited HEp-2 cell proliferation by altering cell cycle-related protein expression. Metastasis and invasion are two important factors affecting LSCC prognosis and recurrence. We found that Notch1 controls HEp-2 cell migration and invasion, although the underlying molecular mechanism remains unclear. Luo *et al* found that inhibition of NICD decreased tumor invasion in gastric cancer (30). Zhou *et al* reported that downregulation of Notch1 decreased HCC cell migration and invasion by regulating E-cadherin and CD44v6 (31). These results all demonstrate that Notch1 contributes to the migration and invasion of cancer cells. Nevertheless, further studies are warranted to more precisely determine the molecular mechanisms of Notch1 in LSCC invasion and metastasis.

Acknowledgements

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