

Abnormal expression of HAX-1 is associated with cellular proliferation and migration in human hypopharyngeal squamous cell carcinoma

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Received August 28, 2016; Accepted May 15, 2017

DOI: 10.3892/mmr.2017.7155

Abstract. HCLS1-associated protein X-1 (HAX-1) is highly expressed or overexpressed in various types of human tumor, and its overexpression is associated with cancer metastasis and cellular proliferation. However, the precise molecular mechanism involved in HAX-1-associated proliferation and metastasis in hypopharyngeal carcinoma is unknown. The present study aimed to investigate the role of HAX-1 in the metastasis and proliferation of hypopharyngeal carcinoma. Reverse transcription-quantitative polymerase chain reaction analysis and western blotting indicated that HAX-1 was overexpressed in hypopharyngeal carcinoma specimens. MTT, clone formation and transwell assays were performed to detect the effects of HAX-1 knockdown or overexpression on the major oncogenic properties of the FaDu hypopharyngeal carcinoma cell line. Downregulation of HAX-1 was observed to significantly suppress cellular proliferation, migration and clonal. By contrast, overexpression of HAX-1 significantly promoted cellular proliferation, migration and clonal formation. Furthermore, HAX-1 knockdown markedly suppressed epithelial-mesenchymal transition. In conclusion, HAX-1 is a potential oncogene, and may promote the tumorigenesis and progression of hypopharyngeal carcinoma, as well as serve as a valuable molecular target for the treatment of hypopharyngeal carcinoma.

Introduction

As a type of head and neck squamous cell carcinoma, hypopharyngeal carcinoma has a poor prognosis and an incidence of

~10 cases/million people every year (1), and its overall survival rate is 15-45% (2). Although the locoregional control of the cancer has been significantly improved in previous decades due to the advent of various novel treatments, including surgery, radiotherapy and chemotherapy, there has been no significant improvement in the survival rates of patients with hypopharyngeal carcinoma. Therefore, developing novel prognostic markers to facilitate patient management is of the utmost importance.

HCLS1-associated protein X-1 (HAX-1) is a family of ubiquitously expressed proteins ranging between 26 and 35 kDa in size, which result from alternative splicing of the single HAX-1 gene (3). The human HAX-1 gene is located on chromosome 1 (1q21.3) (4). HAX-1 is involved in a variety of important physiological processes, including the regulation of apoptosis, cell motility and endocytosis, and combination with the 3' untranslated regions (5-8). Accumulating evidence has also proved that HAX-1 is highly expressed or overexpressed in various types of human tumor, including colorectal cancer (9), esophageal squamous carcinoma (10), oral squamous cell carcinoma (11), hepatocellular carcinoma (12), breast cancer, lung cancer (13), B lymphoma (14) and melanoma (15). In addition, HAX is associated with the pathogenesis of malignancies and other diseases, including psoriasis and systemic sclerosis (5). However, the expression pattern and possible roles of HAX-1 in hypopharyngeal carcinoma have not been investigated.

In the present study, the role of HAX-1 in the processes of cellular proliferation and tumorigenesis in hypopharyngeal carcinoma was investigated. HAX-1 was observed to be overexpressed in hypopharyngeal carcinoma tissues and cell lines. It was also observed that HAX-1 regulates the growth, proliferation and migration of the FaDu hypopharyngeal carcinoma cell line.

Materials and methods

Tissue samples and cell culture. A total of 24 pairs of primary hypopharyngeal squamous cell carcinoma and corresponding non-malignant tissue specimens were obtained from patients

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Key words: HCLS1-associated protein X-1, proliferation, migration, hypopharyngeal carcinoma

with hypopharyngeal squamous cell carcinoma. Among them, 9 male patients (age range, 48-78 years; average age 62.6 years) were recruited from the Hospital Affiliated to Nantong University (Nantong, China), and 15 (13 male and 2 female) patients (age range, 47-72 years; average age, 61.7 years) were recruited from the General Hospital of Jinan Military Area (Jinan, China), between July 2008 and June 2013. None of the patients received any type of treatment (radiation therapy, chemotherapy or immunotherapy) before surgery. Tissue samples were collected immediately following tumor excision during surgery. All specimens were subjected to histological diagnosis by a pathologist. The study protocol was approved by the Human Research Ethics Committee of the Affiliated Hospital of Nantong University and General Hospital of Jinan Military Region, and all patients provided written informed consent prior to participation in the study.

The FaDu hypopharyngeal carcinoma cell line was obtained from American Type Culture Collection (Manassas, VA, USA) and was cultured as a monolayer in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin, at 37°C in a humidified atmosphere containing 5% CO₂.

Small-interfering RNA (siRNA) transfection. For the siRNA silencing of HAX-1, RNA interference was performed using synthetic siRNA. HAX-1 siRNA and scrambled siRNA were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). HAX-1 siRNA1, 5'-CCACGATAACTTCGGCTTT-3'; siRNA2, 5'-GCCATTTTCATAGGTTTGGAT-3'; and siRNA3, 5'-GGACTCAATGCTTAAGTAT-3' and scrambled siRNA, 5'-GCAGATAGGTAGGCGTTAT-3'. HAX-1 siRNAs or scramble siRNA were transfected into FaDu cells using Lipofectamine RNAiMAX transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A total of 24 h prior to transfection, FaDu cells were plated in 6-well plates at a density of 2x10⁴ cells/well in antibiotic-free culture medium. The following day, 3 µl Lipofectamine RNAiMax transfection reagent was combined with 120 pmol siRNA (6 µl from a 20 µM stock) in a volume of 250 µl Opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc.) and incubated for 20 min; the complexes of Lipofectamine RNAiMax and siRNAs were then added directly to each well. After 24 h of transfection at 37°C in a humidified atmosphere containing 5% CO₂, cells were harvested for cellular proliferation, migration and colony formation assays.

Plasmid construction and transfection. HAX-1 overexpression vector was constructed using the polymerase chain reaction (PCR) method. Briefly, constructs containing human HAX-1-GFP fusions were generated by cloning the cDNA of the HAX1 coding region into a pEGFP-N1 vector (Clontech Laboratories, Inc., Mountainview, CA, USA). The HAX-1 cDNA was isolated from healthy human embryonic kidney 293 cells and the coding sequence was obtained using the following specific primers: Forward, 5'-GGAATTCGGGAA TGAGCCTCTTTG-3', containing an *EcoRI* restriction site; and reverse, 5'-CGGGATCCCGCCGGGACCGGAAC-3', containing a *BamHI* restriction site. PCR was then conducted

using a thermal cycler (MJ Research, Inc., Waltham, MA, USA), under the following conditions: A total of 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 70 sec. The PCR products were confirmed by direct DNA sequencing and cloned into the mammalian expression vector pEGFP-N1. FaDu cells were used for the overexpression studies. Stably-transfected clones were obtained by G418 selection (Promega Corporation, Madison, WI, USA). A stable transfectant of the pEGFP-N1 empty vector was used as a control. For transfection, complexes of Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and plasmid were prepared according to the manufacturer's protocol, and these complexes were directly mixed with cells in 24-well cell culture plates at a density of 4x10⁴ cells/well. The level of HAX-1 expression following transfection was assayed by western blot analysis.

Reverse transcription-quantitative PCR (RT-qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from the cells and tissue specimens. First-strand cDNA was synthesized from 1 µg total RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed in a total volume of 20 µl using SYBR-Green PCR Master mix (Roche Diagnostic GmbH, Mannheim, Germany) on a 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 63°C for 60 sec. All reactions were performed in duplicate. The mRNA expression levels of HAX-1 were normalized to GAPDH mRNA levels using the 2^{-ΔΔC_q} method (16). The PCR primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) and the sequences were as follows: HAX-1 forward, 5'-CCCAACCAGCACCAGACT-3' and reverse, 5'-GCTCCT CCACTATCCCATCT-3'; and GAPDH forward, 5'-CGGATT TGGTCGTATTGGG-3' and reverse, 5'-CGCTCCTGGAAG ATGGTGAT-3'.

Western blotting. Total protein was extracted using lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Igepal, 0.1% SDS, 0.25% sodium deoxycholate, 1 mM EDTA pH 7.4 and protease inhibitors (Promega Corporation) on ice for 1 h. Total protein concentration was determined using a bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of extracted protein samples (20 µg) were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following blocking in 5% non-fat milk for 1 h at room temperature, the membrane was incubated with the following primary antibodies at 4°C overnight: Anti-HAX-1 (1:500; cat. no. sc-28268; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-E-cadherin (1:1,000; cat. no. ab1416; Abcam, Cambridge, MA, USA), anti-vimentin (1:1,000; cat. no. ab92547; Abcam), anti-N-cadherin (1:500; cat. no. ab18203; Abcam) and anti-β-actin (1:2,000; cat. no. A1978; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The membrane was washed thrice with TBS containing 0.1% Tween-20 and then incubated with the following horseradish peroxidase-conjugated

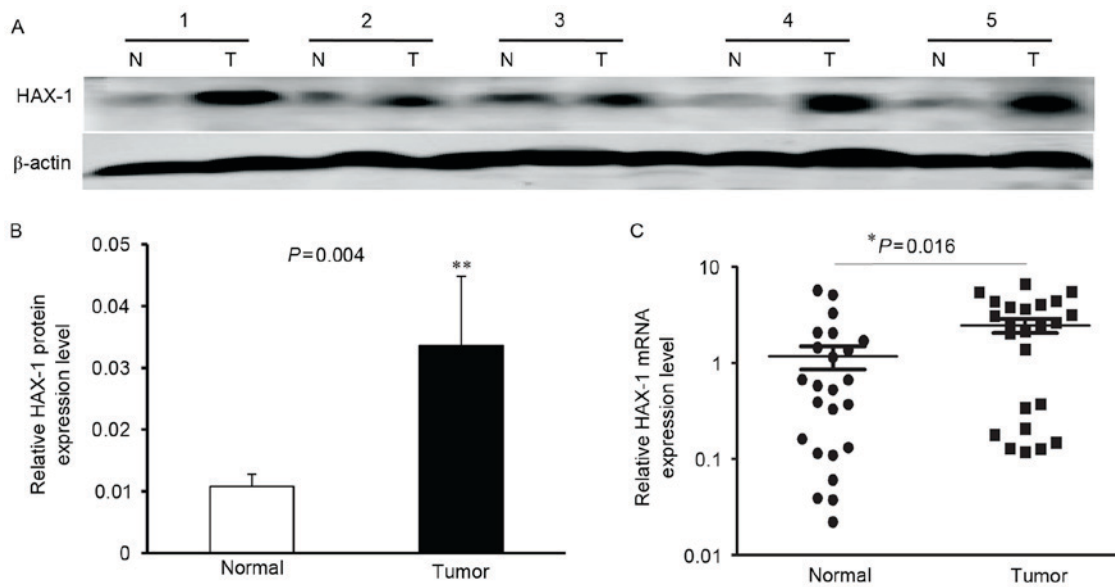


Figure 1. Expression of HAX-1 in tumor tissues as evaluated by western blotting and reverse transcription-quantitative polymerase chain reaction. (A) Representative results of HAX-1 protein expression in five paired T and N samples. (B) HAX-1 protein expression level was increased in T samples compared with the corresponding adjacent N samples. (C) The relative mRNA expression of HAX-1 was significantly increased in the T samples compared with the matched adjacent N samples. Data are presented as the mean \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$, as indicated. HAX-1, HCLS1-associated protein X-1; T, tumor tissue; N, non-tumor tissue.

secondary antibodies for 2 h at room temperature: Goat anti-mouse immunoglobulin (Ig) G (1:10,000; cat. no. 31430; Thermo Fisher Scientific, Inc.) and goat anti-rabbit IgG (1:10,000; cat. no. 31460; Thermo Fisher Scientific, Inc). The protein bands were detected using enhanced chemiluminescence (GE Healthcare Life Sciences, Little Chalfont, UK) and blots were semi-quantified using ImageJ software version 1.41 (National Institutes of Health, Bethesda, MD, USA).

MTT assay. After 1, 2, 3, 4 and 5 days of transfection, FaDu cells were digested, diluted to a density of 3×10^4 cells/ml and plated in 96-well plates (100 μ l cell suspension/well). The cells were cultured at 37°C for 24 h, and cell proliferation was evaluated using an MTT assay (Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol. A total of 10 μ l (5 mg/ml) MTT solution was added to the culture medium, and cells were incubated for an additional 4 h. The formazan precipitates were dissolved in 200 μ l dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA). The absorbance of each sample was determined at a wavelength of 490 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Colony formation assay. For the colony formation assay, a total of 800 cells were plated in complete growth media and allowed to grow until visible colonies formed (14 days). Cells in the plates were fixed with 100% methanol for 10 min at room temperature and stained with 0.5% crystal violet for 20 min at room temperature, and the number of colonies (>50 cells) was counted under an optical microscope. The experiment was performed in triplicate and repeated three times.

Cellular migration assay. For the migration assays, FaDu cells (1×10^5 cells/well) were added to the upper chambers of Transwell inserts (BD Biosciences, Franklin Lakes, NJ, USA;

8- μ m pore size). Cells were incubated in medium without serum and medium with 10% fetal bovine serum served as a chemoattractant in the lower chambers. After 24 h of incubation at 37°C in a humidified atmosphere containing 5% CO₂, cells that did not migrate through the pores were carefully wiped out with cotton wool. Cells on the lower membrane were fixed with 100% methanol for 10 min and stained with 0.2% crystal violet for 20 min at room temperature. Images of the inserts were captured and migrated cells were counted in 5 randomly selected fields under an inverted microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis. Statistical calculations were performed using the SPSS software package (version 17.0; SPSS, Inc., Chicago, IL, USA). The Student's t-test was used to evaluate the differences between two groups. One-way analysis of variance was used to analyze the comparison of means in more than two groups. Data are presented as the mean \pm standard error of the mean. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

HAX-1 overexpression in hypopharyngeal carcinoma. To investigate whether HAX-1 is expressed in hypopharyngeal carcinoma, western blot analysis was used to analyze the 24 hypopharyngeal carcinoma tissues and matched non-tumor hypopharyngeal epithelial samples (Fig. 1A). The mean HAX-1 protein level in hypopharyngeal carcinoma tissues was demonstrated to be significantly increased compared with in the adjacent non-tumor tissues ($P = 0.004$; Fig. 1B). Furthermore, RT-qPCR analysis was performed in the 24 tumor tissues and the corresponding non-tumor samples. The results demonstrated that the expression of HAX-1 was significantly increased in hypopharyngeal carcinoma samples

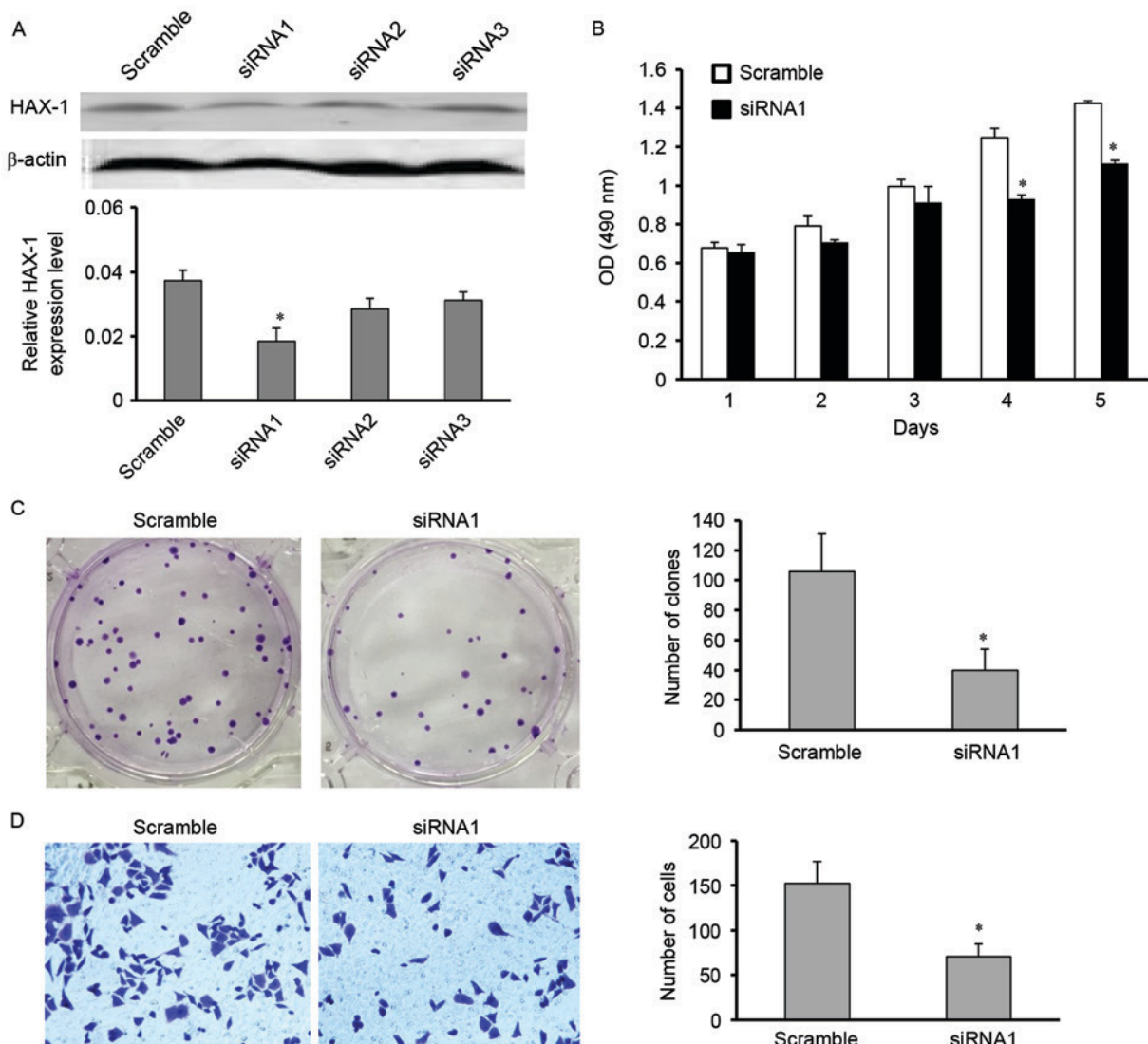


Figure 2. siRNA-mediated knockdown of HAX-1 expression inhibits cell viability, proliferation and migration *in vitro*. (A) Western blot analysis demonstrated HAX-1 protein expression levels. A total of three different HAX-1 siRNAs and an siRNA scramble were used, and the results demonstrated that HAX-1 siRNA1 decreased the level of HAX-1 expression more effectively than the scramble and other siRNAs. (B) Cell viability was evaluated using an MTT assay. (C) Colony numbers of the siRNA1- and scramble-transfected FaDu cells from plate colony formation assays. (D) Transwell assays demonstrated that silencing of HAX-1 expression by siRNA1 inhibited migration in FaDu cells (magnification, $\times 200$). Data are presented as the mean \pm standard error of the mean from three independent experiments. * $P < 0.05$ vs. scramble siRNA-transfected cells. siRNA, small-interfering RNA; HAX-1, HCLS1-associated protein X-1.

compared with in the corresponding non-tumor samples ($P = 0.016$; Fig. 1C).

Downregulation of HAX-1 suppressed viability and proliferation in FaDu cells *in vitro*. Western blot analysis demonstrated that HAX-1 siRNA1 decreased the level of HAX-1 expression more effectively than scramble and other siRNAs (Fig. 2A). To measure the effect of HAX-1 downregulation on the viability and proliferative ability of FaDu cells *in vitro*, MTT and colony formation assays were performed. The MTT assay demonstrated that HAX-1 siRNA1 significantly inhibited the viability of the FaDu cells compared with the scrambled siRNA (Fig. 2B; $P < 0.05$). As presented in Fig. 2C, the colony formation assay demonstrated the plate colony number in the HAX-1 siRNA1 group was decreased compared with the scrambled siRNA group ($P < 0.05$). These results indicated that the downregulation of HAX-1 expression inhibited the viability and proliferative ability of FaDu cells.

Downregulation of HAX-1 inhibited the migratory potential of FaDu cells *in vitro*. To investigate the effect of HAX-1 downregulation on the migratory potential of FaDu cells, transwell assays were performed. The results demonstrated that the number of migratory cells in the HAX-1 siRNA1 treatment group was significantly decreased compared with the scramble siRNA treatment group (Fig. 2D; $P < 0.05$). These results revealed that downregulation of HAX-1 expression suppressed the migratory ability of FaDu cells.

Overexpression of HAX-1 promoted the viability, proliferation and migration of FaDu cells *in vitro*. Western blot analysis demonstrated that endogenous HAX-1 expression was significantly increased in the HAX-1 overexpression vector group compared with the control vector group (Fig. 3A). The effects of HAX-1 on cell viability and proliferation were analyzed by MTT and colony formation assays, respectively, and the results indicated that upregulation of HAX-1 expression enhanced the

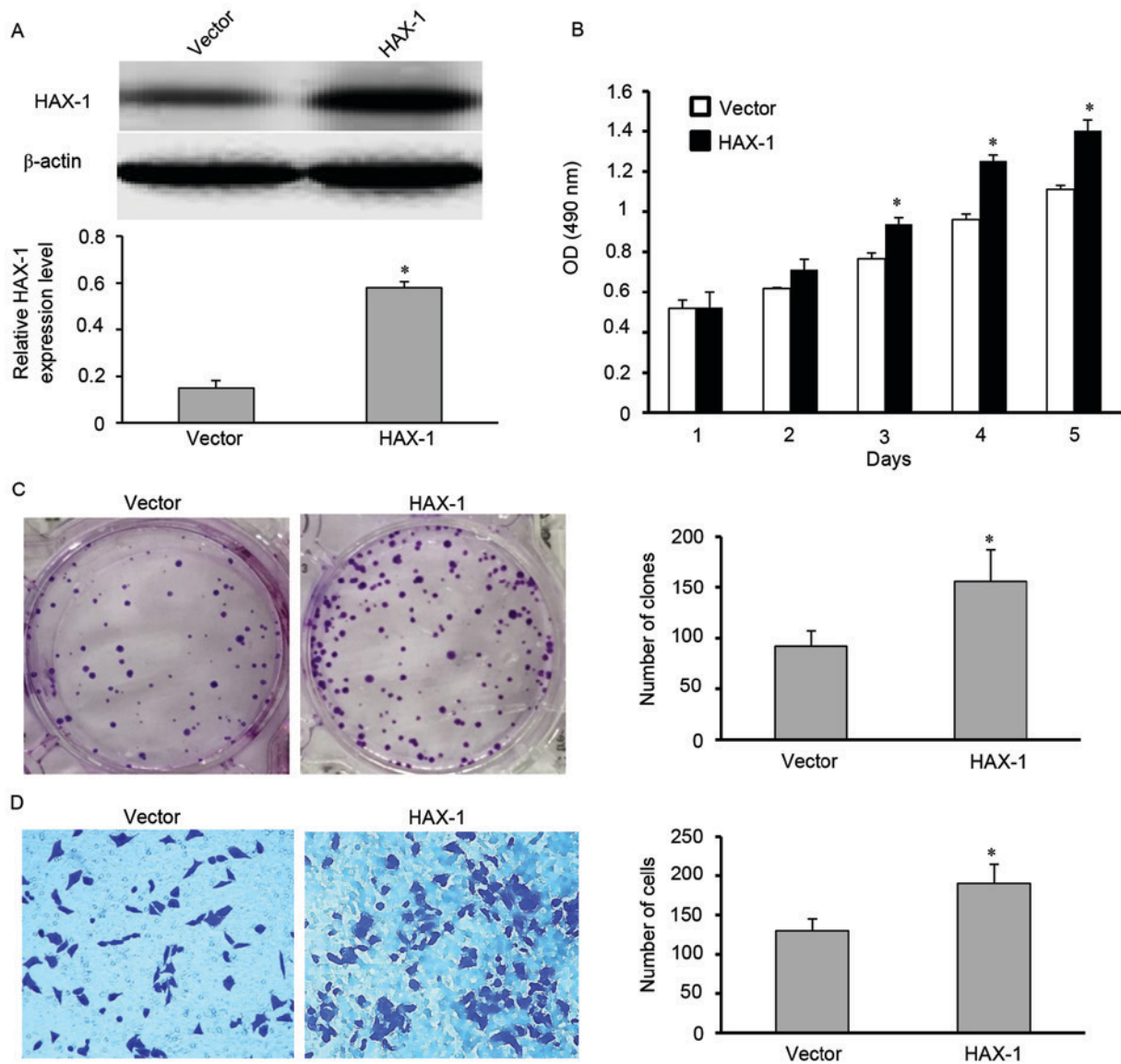


Figure 3. Overexpression of HAX-1 promotes the viability, proliferation and migration of FaDu cells *in vitro*. (A) Western blot analysis demonstrated HAX-1 protein expression levels. HAX-1 overexpression plasmid and empty control vectors were used. (B) Cell viability was evaluated using the MTT assay. (C) Colony numbers of the HAX-1 overexpression plasmid- and empty vector-transfected FaDu cells in plate colony formation assays. (D) Transwell assays demonstrated that overexpression of HAX-1 in FaDu cells promoted cell migration compared with the empty vector (magnification, x200). Data are presented as the mean \pm standard error of the mean from three independent experiments. * $P < 0.05$ vs. empty vector-transfected cells. HAX-1, HCLS1-associated protein X-1.

viability of FaDu cells and increased the number of colonies of FaDu cells compared with the control cells (Fig. 3B and C, respectively; $P < 0.05$). In addition, overexpression of HAX-1 significantly increased the number of migratory FaDu cells by transwell assay (Fig. 3D; $P < 0.05$).

Downregulation of HAX-1 suppressed epithelial-mesenchymal transition (EMT). EMT has recently been linked to tumor progression, through which epithelial cells acquire mesenchymal properties, and exhibit reduced intercellular adhesion and increased motility (17). Through western blot analysis, downregulation of HAX-1 was observed to inhibit the protein expression of epithelial (E)-cadherin, and induce the protein expression of vimentin and neural (N)-cadherin, compared with the scramble siRNA group (Fig. 4A and B; $P < 0.05$). Whereas, in the HAX-1 overexpression cells, vimentin and

N-cadherin expression was upregulated, and E-cadherin expression was downregulated (Fig. 4C and D; $P < 0.05$).

Discussion

HAX-1 is ubiquitously expressed in human and murine tissues, and is reported to be localized to the mitochondria, nuclear membrane and endoplasmic reticulum (11). HAX-1 is characterized by the activation and permeabilization of mitochondria, resulting in the release of cytochrome *c* and other proapoptotic molecules into the cytosol (18). A previous study demonstrated that HAX-1 comprises a family of apoptotic regulators with antagonistic roles in response to oxidative stress (19). Previously, a study confirmed that HAX-1 expression is a predictor of tumorigenesis, growth, progression, invasion and metastasis in multiple human malignancies, and

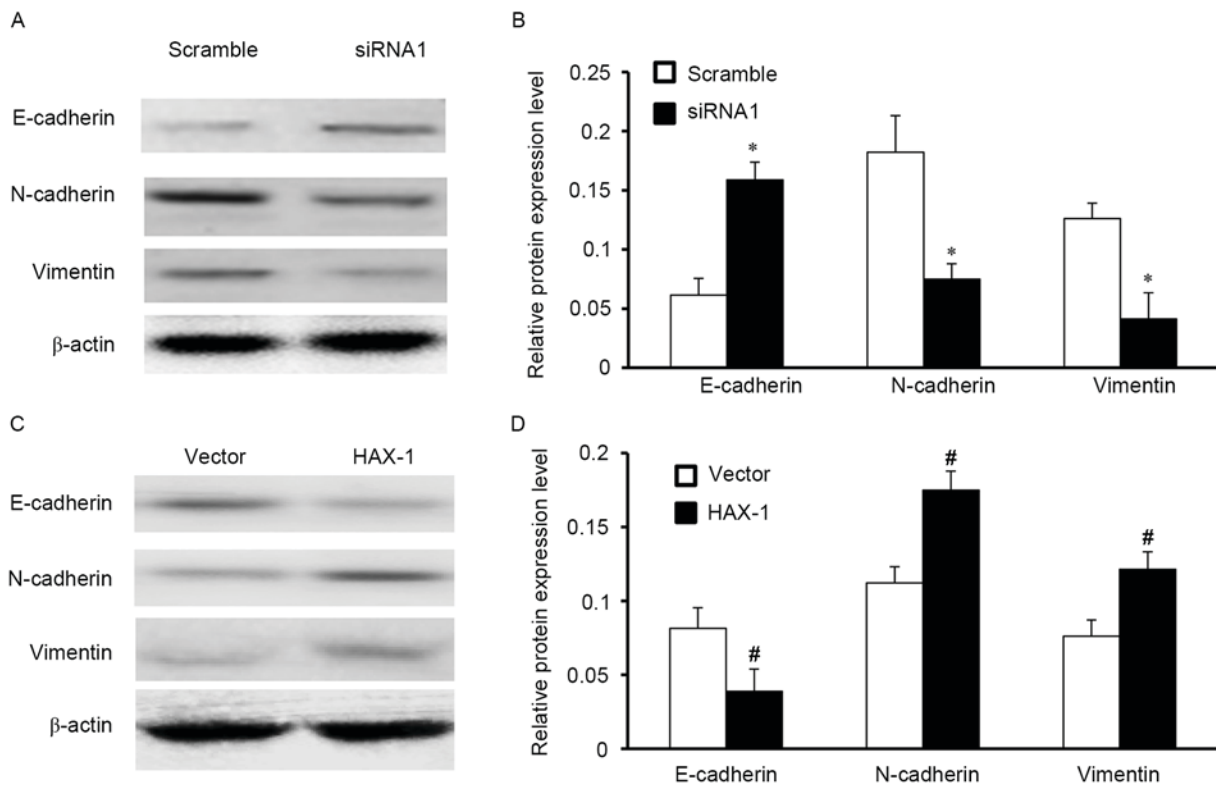


Figure 4. Effect of HAX-1 expression on epithelial-mesenchymal transition. (A) Western blot analysis demonstrated that HAX-1 knockdown increased E-cadherin expression, and suppressed vimentin and N-cadherin expression in FaDu cells. (B) Quantification of the western blotting of siRNA-transfected cells. E-cadherin protein expression was increased in the siRNA group compared with the scramble group. N-cadherin protein expression was decreased in the siRNA group compared with the scramble group. Vimentin protein expression was decreased in the siRNA group compared with the scramble group. (C) Western blot analysis demonstrated that HAX-1 overexpression decreased E-cadherin expression, and promoted vimentin and N-cadherin expression in FaDu cells transfected with HAX-1 overexpression plasmid compared with the empty vector group. (D) Quantification of the western blotting of plasmid-transfected cells. E-cadherin protein expression was decreased in the HAX-1 overexpression group compared with the vector control group. N-cadherin protein expression was increased in the HAX-1 overexpression group compared with the vector control group. Vimentin protein expression was increased in the HAX-1 overexpression group compared with the vector control group. Data are presented as the mean \pm standard error of the mean. * $P < 0.05$ vs. scramble siRNA-transfected cells; # $P < 0.05$ vs. empty vector-transfected cells. HAX-1, HCLS1-associated protein X-1; E-cadherin, epithelial cadherin; N-cadherin, neural cadherin; siRNA, small-interfering RNA.

is increased in numerous types of tumor (11). In the present study, using RT-qPCR and western blot analysis, HAX-1 expression was observed to be increased in hypopharyngeal carcinoma tissues.

A previous study reported that there was HAX-1 overexpression in esophageal squamous cell carcinoma samples, and that the level of HAX-1 mRNA is a risk factor for lymph node metastasis and indicator of survival in patients with esophageal squamous cell carcinoma (10). Another study suggested that HAX-1 expression was elevated in hepatocellular carcinoma samples, and that high expression of HAX-1 was associated with histological grade, tumor size, cirrhosis and poor clinical outcome (12). Furthermore, high HAX-1 expression in breast cancer is associated with poor prognosis (20). It has also been demonstrated that upregulated HAX-1 is associated with advanced clinicopathological characteristics and prognosis in colorectal cancer (9). A previous study reported on HAX-1-mediated chemotherapeutic resistance in T-cell leukemia and melanoma (21). Furthermore, previous results suggested a critical scaffolding role for HAX-1 in lysophosphatidic acid-stimulated Ras-related C3 botulinum toxin substrate 1-cortactin interaction and subsequent ovarian cancer cell migration (22). It has also been demonstrated that reduction of HAX-1 levels by siRNA suppresses $\alpha_v\beta_6$ -dependent

carcinoma cell migration by downregulating $\alpha_v\beta_6$ endocytosis via a clathrin-mediated pathway (11). The present study demonstrated that downregulation of HAX-1 in FaDu cells significantly inhibits cell viability, proliferation and migration *in vitro*. Furthermore, downregulation of HAX-1 expression resulted in the increase of epithelial marker E-cadherin, and the decrease of mesenchymal markers vimentin and N-cadherin. The results of the present study were corroborated by previous findings where HAX-1 is overexpressed in a number of cancer types and exhibits a number of oncogenic functions.

In conclusion, to the best of the author's knowledge, the results of the present study demonstrated for the first time that HAX-1 is overexpressed in hypopharyngeal carcinoma. Furthermore, knockdown of HAX-1 may inhibit the viability, proliferative and migratory abilities of FaDu cells *in vitro*. These results provide important information for the identification and characterization of a novel molecular marker, and a target for hypopharyngeal carcinoma therapy.

Acknowledgements

The present study was supported by the Jiangsu Postdoctoral Science Foundation (grant no. 1402202C), the Jiangsu 'Six Talent Peaks' Foundation (grant no. 2014-WSW-030), the

Nantong Science and Technology Project (HS2014008) and the Project of the Affiliated Hospital of Nantong University (grant no. TDFY0306).

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