Stathmin1 promotes lymph node metastasis in hypopharyngeal squamous cell carcinoma via regulation of HIF-1α/VEGF-A axis and MTA1 expression

YUQIAN WU, QIN LIU, JIAOJIAO TONG, CHUNHUI HU and DIANSHUI SUN

Cancer Center, The Second Hospital of Shandong University, Jinan, Shandong 250033, P.R. China

Received October 30, 2022; Accepted January 16, 2023

DOI: 10.3892/mco.2023.2617

Abstract. Extensive neck lymph node metastasis (LNM) is an important clinical feature of hypopharyngeal squamous cell carcinoma (HSCC). Stathmin1 (STMN1) is closely associated with LNM in numerous human cancers. In the present study, the association between STMN1 and neck LNM in HSCC and the underlying molecular mechanisms were explored. First, postoperative samples of HSCC were screened and the association between STMN1 and neck LNM in HSCC was analyzed. Then, cell functional experiments were performed to assess the potential of STMN1 to promote invasion and migration. Subsequently, the potential target genes and pathways of STMN1 were predicted using bioinformatics analysis. Finally, the obtained target genes and pathways of STMN1 were validated by reverse transcription-quantitative PCR (RT-qPCR) and western blot analyses to confirm the potential mechanisms by which STMN1 promotes LNM in HSCC. As a result, a total of 117 postoperative samples of HSCC were screened, and STMN1 was proven to be associated with neck LNM in HSCC. Further, cell functional experiments established that high expression of STMN1 could actually promote FaDu cell invasion and metastasis. Bioinformatics analysis revealed that high expression of STMN1 was associated with the activation of hypoxia inducible factor-1alpha (HIF-1α) pathway and increased expression of metastasis-associated protein 1 (MTA1). Finally, RT-qPCR and western blot analyses confirmed that STMN1 promotes the expression levels of HIF-1α/vascular endothelial growth factor (VEGF)-A and MTA1 in FaDu cell lines. In conclusion, it was found that high expression of STMN1 promoted neck LNM in HSCC and the potential mechanisms may be via regulation of the HIF-1α/VEGF-A axis and MTA1 expression.

Correspondence to: Professor Dianshui Sun, Cancer Center, The Second Hospital of Shandong University, 247 Beiyuan Road, Jinan, Shandong 250033, P.R. China
E-mail: sundianshui@163.com

Key words: HIF-1α/VEGF-A axis, hypopharyngeal squamous cell carcinoma, lymph node metastasis, metastasis-associated protein 1, stathmin1

Introduction

Hypopharyngeal squamous cell carcinoma (HSCC) is one of the most aggressive types of head and neck cancer (1-2). Neck lymph node metastasis (LNM) is an important clinical characteristic of HSCC. The incidence of neck LNM is 40-70% (3), which influences the prognosis and leads to poor survival of patients with HSCC (2,4). Thus, in order to improve the survival of patients with HSCC, neck lymph node dissection and irradiation are routinely performed during hypopharyngeal cancer surgery and radiotherapy, which are the two main treatment modalities for HSCC (5). Since the neck lymphatic drainage region in HSCC includes the whole neck region from the skull base to the supraclavicular region, extensive lymph node dissection and radiation are cumbersome. The difficulty of the surgical procedure and the irradiation plan prolong the time to recovery from these therapies, and at the same time increase the incidence of surgical complications and radiation damage, such as local dysfunction, pharyngeal fistula, neck edema, and so on (6). This seriously affects the quality of life of HSCC survivors (7). However, in clinical practice, there is a great variation in neck LNM in HSCC among different patients (8). Previous studies found that ~40-50% of patients who underwent neck lymph node dissection did not exhibit tumor metastases in postoperative pathologic specimens (9,10). Therefore, it is critical to identify HSCC patients at high risk of neck LNM so that proper lymph node dissection and irradiation can be performed only in selected patients. This will be beneficial in reducing treatment complications and improving the quality of life of HSCC survivors.

Molecular mechanisms underlying HSCC determine tumor development, progression, invasion and metastasis, and also determine neck LNM in HSCC (11-13). An improved understanding of the molecular mechanisms of neck LNM in HSCC and identification of tumor-specific biomarkers for the prediction of neck LNM are urgently needed to guide the treatment of neck LNM, and to improve the prognosis and the quality of life of patients with HSCC.

Stathmin1 (STMN1), also known as oncoprotein 18, is a cytoplasmic protein that self-regulates through a phosphorylation pathway, controlling the dynamic balance of the microtubule system, influencing cell mitosis and regulating cell cycle progression. This, in turn, affects tumor cell proliferation and motility (14,15). It has been widely

reported that STMN1 is overexpressed in numerous different human cancers and is closely associated with LNM (16,17). Cao *et al* (16) retrospectively analyzed eight studies and found that esophageal cancer patients with high STMN1 expression had a significantly higher risk of LNM than those with low STMN1 expression, further proving that high STMN1 expression was a risk factor for susceptibility to LNM in tumors. Chen *et al* (17) found that STMN1 was overexpressed in HSCC, and promoted FaDu cell proliferation and migration. However, whether STMN1 promotes neck LNM in HSCC and the underlying molecular mechanisms remain to be elucidated. In the present study, a correlation analysis was performed between neck LNM in HSCC and STMN1 expression level in a large sample of clinical cases. The molecular mechanisms underlying the promotion of neck LNM in HSCC by STMN1 were explored, which could perhaps guide treatment of neck LNM.

Materials and methods

Study case selection. To establish the association between STMN1 and neck LNM in HSCC, clinical cases of HSCC were selected and analyzed. Study inclusion criteria were as follows: Absence of distant metastases; patient had undergone radical hypopharyngeal cancer resection with neck lymph node dissection; pathological confirmation as HSCC; neck LNM status was pathologically evaluated after operation; patient had not undergone preoperative chemotherapy or radiotherapy; preoperative clinical data and paraffin-embedded specimens for immunohistochemical (IHC) staining were available. Eligible cases were recruited from the Second Hospital of Shandong University between January 2015 and December 2020 and from the Shandong Provincial Hospital between January 2018 and March 2021. A written informed consent was obtained from each patient before surgery.

IHC staining and evaluation of staining results. For eligible cases of HSCC, STMN1 expression and lymphatic vessel invasion (LVI) status in tumor tissues were evaluated with IHC staining. The detailed procedure of IHC was as follows:

IHC staining was performed on 4-μm paraffin tissue sections mounted on slides and dried for 8 h at 60˚C. The slides were deparaffinized in xylene and dehydrated conventionally, then pressure-cooked in sodium citrate buffer (pH 6.0) (LBP Med-Sci) to facilitate antigen retrieval. After natural cooling, endogenous peroxidase was blocked with 3% hydrogen peroxide. The sections were subsequently incubated with rabbit anti-STMN1 polyclonal antibody (1:75) (Wuhan Boster Biological Technology, Ltd.), or rabbit anti-D2-40 polyclonal antibody (1:75) (Wuhan Boster Biological Technology, Ltd.) (Anti-D2-40 antibody was used to stain lymphatic vessels, and in stained lymphatic vessels, the presence of tumor embolus represented LVI) overnight at 4˚C. After washing with PBS (LBP Med-Sci), the sections were incubated for 30 min with the two-step method followed by the poly-HRP anti-mouse/rabbit detection system (LBP Med-Sci). A DAB detection kit (Talent-Bio) was used for 5-10 min to show immunolabeling, resulting in a brown precipitate. Finally, the sections were re-stained with hematoxylin (Beijing Solarbio Science & Technology Co., Ltd.), differentiated in hydrochloric acid alcohol, and sealed with neutral balsam (Beijing Solarbio Science & Technology Co., Ltd.).

After IHC staining, the association between the expression of STMN1 and neck LNM, and the association between the expression of STMN1 and LVI status were both analyzed.

Cell culture and transfection. Human HSCC cell lines (FaDu cells) were purchased from Procell Life Science & Technology Co., Ltd., and were identified by short tandem repeat (STR) and free of mycoplasma contamination. Subsequently, they were cultured in PMI150410-MEM medium (Procell Life Science & Technology Co., Ltd.) with 10% fetal bovine serum (FBS; Shanghai VivaCell Biosciences, Ltd.) and 1% penicillin/streptomycin (Shanghai Basal Media Technologies Co., Ltd.) in a humidified incubator containing 5% CO_2 at 37˚C. Cells in favorable growth condition were collected for subsequent experiments.

Human short hairpin RNA (shRNA) was packaged and synthesized by Shanghai GeneChem Co., Ltd. The shRNA sequences targeting STMN1 are shown in Table I and different sequences were synthesized to inhibit STMN1. The negative control (NC) shRNA was used as a blank control. According to the infection multiplicity (MOI=10) of the FaDu cell line provided in the pre-experiment, an aliquot of 2 ml FaDu cell suspension at a cell concentration of $5x10^4$ cells/ml was seeded in a six-well plate (Corning, Inc.), when the cell density roughly reached 30%, the culture medium was replaced, and then the diluted 10 μl shRNA and 40 μl transfection enhancer P (GeneChem, Inc.) were added and placed at 37˚C for 48 h for transfections. After transfections, the transfection efficiencies were evaluated with quantitative reverse transcription-polymerase chain reaction (RT-qPCR) and western blot analyses.

After a week, the cell lines, which reached satisfactory transfection efficiency and were readily cultured, were selected for the following cell functional and mechanism research experiments.

RNA extraction and RT-qPCR. RT-qPCR was used to test relative expression levels of genes analyzed in FaDu cells. Total RNA was extracted from FaDu cells using Esciece RNA Extraction Kit (www.esunbio.com), according to the manufacturer's instructions. Evo M-MLV Mix Kit and SYBR Green Premix Pro Taq HS qPCR Kit (Accurate; https://agbio.com.cn) were used for RT-PCR and qPCR, according to the manufacturer's instructions. The first step of RT-PCR was to remove genomic DNA under the reaction condition of 42˚C for 2 min, and then reverse transcription reaction was performed at 37˚C for 15 min and 85˚C for 5 sec. The qPCR reaction system included SYBR Green Pro Taq HS Premix, cDNA sample, RNase-free water and primers. The primers were synthesized by Shanghai GeneChem Co., Ltd and the sequences are presented in Table II. Reaction steps were as follows: 30 sec pre-denaturation at 95˚C followed by 40 cycles of 95˚C for 5 sec, and 60˚C for 30 sec, all of which were conducted on the Quantstudio TM5 System (Thermo Fisher Scientific, Inc.). The relative expression levels of the genes were calculated using the comparative $2^{-\Delta\Delta Cq}$ method (18) with GAPDH as the reference.

Table I. Sequences of shRNAs targeting STMN1.

Name	Sequence
NC shRNA	Blank sequence
STMN1-shRNA1	PSC71180-1 CCGGAACTGGAACGTTTGCGAGAGACTCGAGTCTCTCGCAAACGTTCCAGTTTTTTG
STMN1-shRNA2	PSC71181-1 CCGGGAAGAGAAACTGACCCACAAACTCGAGTTTGTGGGTCAGTTTCTCTTCTTTTTG
STMN1-shRNA3	PSC71182-1 CCGGCCAGGTGAAAGAACTGGAGAACTCGAGTTCTCCAGTTCTTTCACCTGGTTTTTG

STMN1, stathmin1; NC, negative control; shRNA, short hairpin RNA.

Table II. Primer sequences of genes analyzed for reverse transcription-quantitative PCR.

Gene name	Primer sequences (5'→3')	Product length (bp)
STMN1	F: TGATTCTCAGCCCTCGGTCAA R: GCTTCATGGGACTTGCGTCTT	133
HIF-1α	F: GGCAGCAACGACACAGAAAC R: TTTTCGTTGGGTGAGGGGAG	86
VEGF-A	F: CTTGCAGATGTGACAAGCCG R: GTCGATGGTGATGGTGTGGT	150
MTA1	F: ACCGAGTCGCTCAAGTCCTA R: ACAAGTCGGTGATGTCTGCC	142
GAPDH	F: GCACCGTCAAGGCTGAGAAC R: TGGTGAAGACGCCAGTGGA	138

STMN1, stathmin1; HIF-1α, hypoxia inducible factor-1alpha; VEGF-A, vascular endothelial growth factor; MTA1, metastasis-associated protein 1.

Cell functional experiments

Cell proliferation assay. Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology) assay was performed to assess the proliferation capacities of FaDu cells after transfections with different shRNA sequences targeting STMN1. An aliquot of 100 μl of FaDu cell suspension per well was seeded in 96-well culture plates (Corning, Inc.) at a cell concentration of $3x10^4$ cells/ml in an incubator. After the cells adhered to the wall and showed evidence of growth. Culture medium was replaced with 110 μl of fresh culture medium containing 10 μl of CCK-8 and placed in the incubator at 37˚C for 1.5 h. The absorbance was measured at 450 nm with a spectrophotometer (Tecan Group, Ltd.) and expressed as the optical density (OD). The operation was repeated every 24 h for three consecutive days.

Matrigel invasion assay. Matrigel invasion assay was performed to assess the invasion capacities of FaDu cells after transfections with different shRNA sequences targeting STMN1. During the procedures of Matrigel invasion assay, an aliquot of 200 μl of serum-free FaDu cell suspension that contains $5x10^4$ cells was seeded in the upper chamber lined with Matrigel (Corning, Inc.) in a 24-well Transwell device (8-μm pore size; Jet Bio-Filtration,) which was pretreated at 37˚C for 1 h, then 600 μl of 10% FBS culture medium was added to the lower chamber, and placed in the incubator at 37˚C for 36 h. The cells that invaded through the Matrigel to reach the membrane of the chambers were fixed in 4% formaldehyde and methanol, and subsequently, were stained with 0.1% crystal violet (Beijing Solarbio Science & Technology Co., Ltd.) for 1 h. Finally, images of the cells that invaded through the Matrigel to reach the membrane of the chambers were captured with a DMI8 inverted microscope (Leica Microsystems GmbH), following which the invasive cells were counted with ImageJ software (Java 1.8.0; National Institutes of Health).

Cell scratching assay. Cell scratching assay was performed to assess the migration capacities of FaDu cells after transfections with different shRNA sequences targeting STMN1. During the procedures of cell scratching assay, an aliquot of 2 ml of FaDu cell suspension that contains $5x10^5$ cells was seeded in a six-well plate (Corning, Inc.), and after the cells covered the plates, a 200 μl pipette tip was used to draw a straight line on the plates. Then, 2 ml of serum-free medium was added and the cells were placed in a 5% CO_2 incubator. Finally, images of the migration area were captured with a DMI8 inverted microscope at 0, 24 and 48 h, and densitometric analysis was performed using ImageJ software.

Bioinformatics analysis. Firstly, the expression levels of STMN1 in HSCC tissues and normal tissues were analyzed. The data of the gene chip GSE2379 were used for HSCC containing STMN1 from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) for this. Eventually, the gene chip data of four normal tissues and 14 HSCC tissues were included in the present study. The expression differences of STMN1 among the HSCC tissues, metastases tissues, and normal tissues were analyzed using R4.2.0 software (http://www.R-project.org).

In order to explore the molecular mechanisms underlying the promotion of neck LNM in HSCC by STMN1, a bioinformatics analysis was performed to identify potential target genes and pathways of STMN1. The gene sequencing data of

Table III. Clinicopathological characteristics of the included cases.

Clinicopathological characteristics	Number of cases	Expression level of stathmin1	
Sex[a]		High (n=80)	Low (n=37)
Male	109	78	31
Female	8	2	6
Median age, years (range)	60 (40-76)		
>60	55	42	13
≤60	62	38	24
Pathological stage[b]			
I-III	41	24	17
IV[c]	76	56	20
Pathological differentiation			
Well	66	39	27
Poor	51	41	10
Lymphatic vessel invasion			
Negative	51	19	32
Positive	66	61	5
Lymph node metastasis			
Negative	27	4	23
Positive	90	76	14

[a]The incidence rate of hypopharyngeal squamous cell carcinoma in male is obviously higher than that in female in the authors' country, it was confirmed that there is no bias in the selection of the patients, the difference in data for sex represents the real situation.
[b]American Joint Committee on Cancer (AJCC) TNM staging system for hypopharyngeal carcinoma (Eighth edition, 2017).
[c]Without distant metastasis.

HSCC are unavailable in The Cancer Genome Atlas (TCGA). Since HSCC is a representative cancer of head and neck SCC (HNSCC), HSCC exhibits common biological feature with HNSCC; therefore, the gene sequencing data of HNSCC were screened from TCGA. Eventually, a total of 504 HNSCC gene sequencing data were obtained. The data were divided into two groups (high and low expression groups) according to the expression levels of STMN1, and an enrichment analysis was performed using Molecular Signature Database v7.5.1 in the GSEA software (https://www.gsea-msigdb.org/gsea). The pathways with $P<0.05$ and false discovery rate (FDR) <0.25 were considered as significant enrichment pathways. At the same time, in order to obtain other potential target genes of STMN1, a target gene prediction analysis was performed using the Rv4.0.3 package from the Assistant for Clinical Bioinformatics website (www.aclbi.com).

After the bioinformatics analysis, the potential target genes and pathways of STMN1 promoting neck LNM in HSCC were identified. Subsequently, RT-qPCR and western blot analyses were used to further validate these potential target genes and pathways via a series of cell assays *in vitro*.

Western blot analysis. Western blot analysis was used to test the transfection efficiency and validate the potential target genes and pathways of STMN1. The FaDu cells of each group were lysed with RIPA lysis solution (Beyotime Institute of Biotechnology) and target proteins were extracted. Equal amounts of proteins (30 μg) were added for 10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane (Pall Life Sciences), then blocked with 5% skimmed milk at room temperature for 1.5 h. Subsequently, membranes were incubated with the following primary antibodies against: STMN1 (1:1,000; cat. no. PB9560; Wuhan Boster Biological Technology, Ltd.), hypoxia inducible factor-1alpha (HIF-1α; 1:1,000; cat. no. A6265; ABclonal Biotech Co., Ltd.), vascular endothelial growth factor (VEGF)-A (1:1,000; cat. no. ab214424; Abcam), metastasis-associated protein 1 (MTA1; 1:1,000; cat. no. ab288765; Abcam) and β-actin (1:2,000; cat. no. CPA9066; Cohesion Biosciences) overnight at 4˚C, and then washed with Tris-buffered saline and Tween-20 (TBST, 0.01 M Tris, 0.15 M NaCl, 0.1% Tween-20, pH=7.4) for 30 min. The membranes were incubated with the HRP-conjugated secondary antibody goat anti-rabbit/mouse IgG (1:5,000; cat. nos. E-AB-1001/E-AB-1003; Elabscience Biotechnology, Inc.) at room temperature for 1 h. After washing again with TBST, ECL luminescent solution was added and images were captured with Tanon chemiluminescence imager (Tanon Science and Technology Co., Ltd.). Finally, gray values of protein bands were quantitatively analyzed with ImageJ software, and β-actin was used as an internal reference.

Statistical analysis. Statistical analyses were performed using SPSS 20.0 software (IBM Corp.) and GraphPad prism 8.0 software (GraphPad Software, Inc.). All cell experiments were

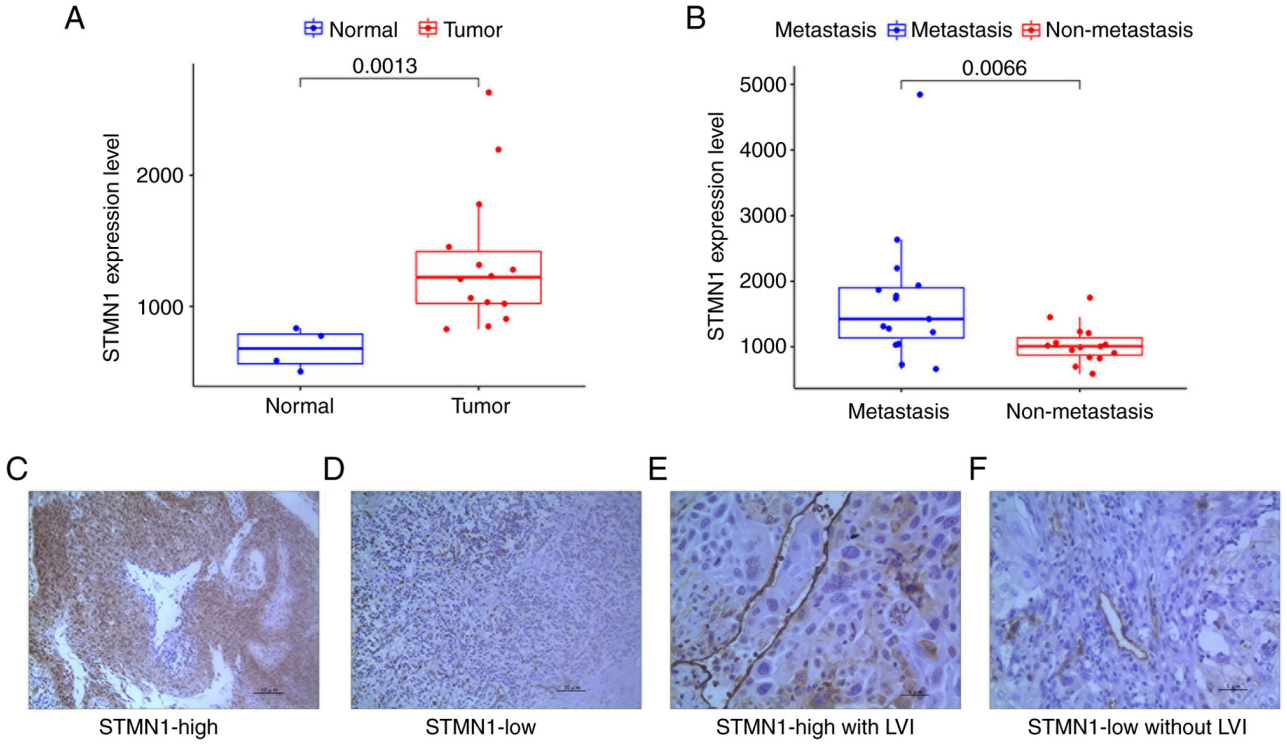

Figure 1. STMN1 expression differences and immunohistochemical staining for STMN1 and LVI. (A and B) STMN1 expression level was increased in HSCC tissues compared with normal hypopharyngeal tissues, and was further increased in HSCC tissues of patients with metastases. (C and D) STMN1 expression status in different HSCC samples (magnification, x200). (E and F) LVI statuses in different HSCC samples along with STMN1 expression statuses (magnification, x400). STMN1, stathmin1; LVI, lymphatic vessel invasion; HSCC, hypopharyngeal squamous cell carcinoma.

repeated three times. The expression levels of genes, OD_{450} values, relative number of invasive cells, and relative migratory ratios were expressed as the mean ± standard deviation (SD). The differences in numerical variables between two groups were compared using the independent-samples t-test. The correlations between the expression status of STMN1 and neck LNM and LVI status were analyzed using the Spearman's rank correlation test. All P-values were two-sided, and a value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological features of the included cases. A total of 117 HSCC cases from the two hospitals were eligible, as per the inclusion criteria, and were included in the present study. The expression levels of STMN1 and LVI status for eligible cases were evaluated with IHC staining. The clinicopathological features of the included cases are presented in Table III.

STMN1 is highly expressed in HSCC tissues and is associated with LNM. The analyses of included data from gene chips GSE2379 revealed that STMN1 expression was evidently increased in HSCC tissues compared with normal hypopharyngeal tissues, and further increased in HSCC tissues of patients with metastases ($P<0.01$, Fig. 1A and B).

Subsequently, IHC staining analysis of the 117 eligible cases in the present study demonstrated that STMN1 was highly expressed in the majority of cases (Fig. 1C and D); moreover, high expression of STMN1 was evidently associated with neck LNM in HSCC (Correlation Coefficient: 0.631, $P<0.001$). Further analysis revealed that high expression of STMN1 was also associated with LVI (Correlation Coefficient: 0.588, $P<0.001$, Fig. 1E and F). These results indicated that high expression of STMN1 in HSCC tissues promoted neck LNM in HSCC and LVI.

Knockdown of STMN1 in FaDu cell lines. After performing the aforementioned clinical analyses, the functions of STMN1 in HSCC were further verified in FaDu cell lines. As STMN1 showed high expression in FaDu cell lines, STMN1 was knocked down using different targeting sequences of shRNA. After the transfections, the green fluorescent protein, which was used to label the shRNA sequences, was observed under a fluorescence microscope to confirm the success of the transfection (Fig. 2). Then, RT-qPCR and western blot analyses were performed to further verify the efficacy of the STMN1 knockdown. The results showed that the shRNA1 and shRNA2 sequences had improved efficacy in STMN1 knockdown than the shRNA3 sequence, compared with the NC shRNA sequence (Fig. 3A and B). At the same time, the cell lines transfected with the shRNA1 and shRNA2 sequences had improved growth conditions than those transfected with the shRNA3 sequence. Therefore, the FaDu cell lines of STMN1 knockdown with shRNA1 and shRNA2 sequences were used in the following cell functional experiments.

Knockdown of STMN1 reduces the proliferation, invasion and migration of FaDu cells. In cell functional experiments,

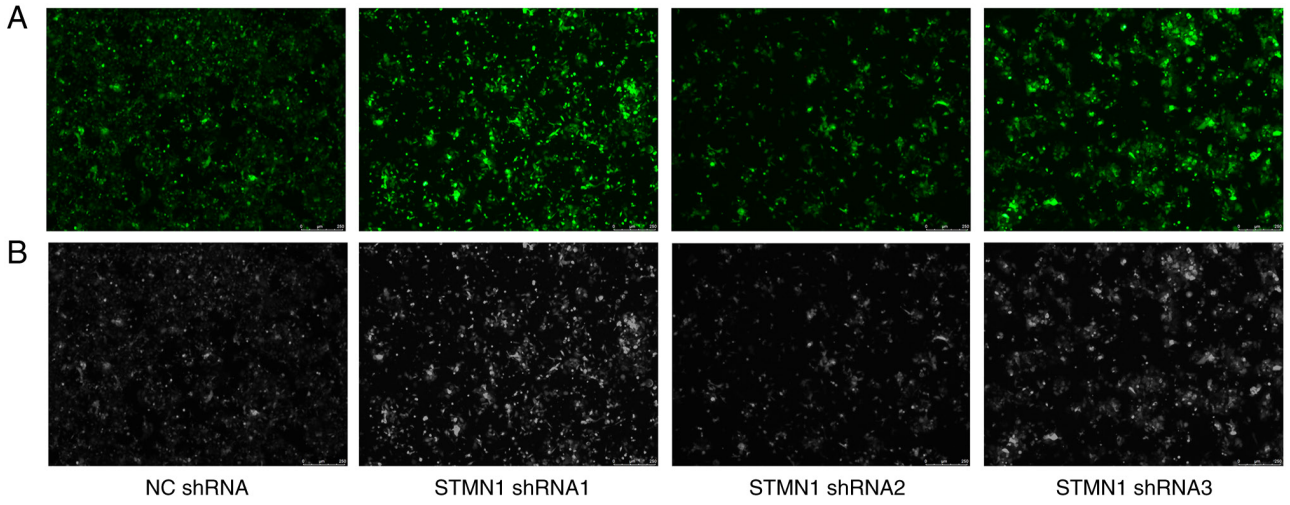

Figure 2. Transfection images with different shRNA sequences. (A) The fluorescent pictures labeled with green fluorescent protein (scale bar, 250 μm). (B) The ordinary optical light pictures of corresponding fields (scale bar, 250 μm). All showed that the transfections were successful. shRNA, short hairpin RNA.

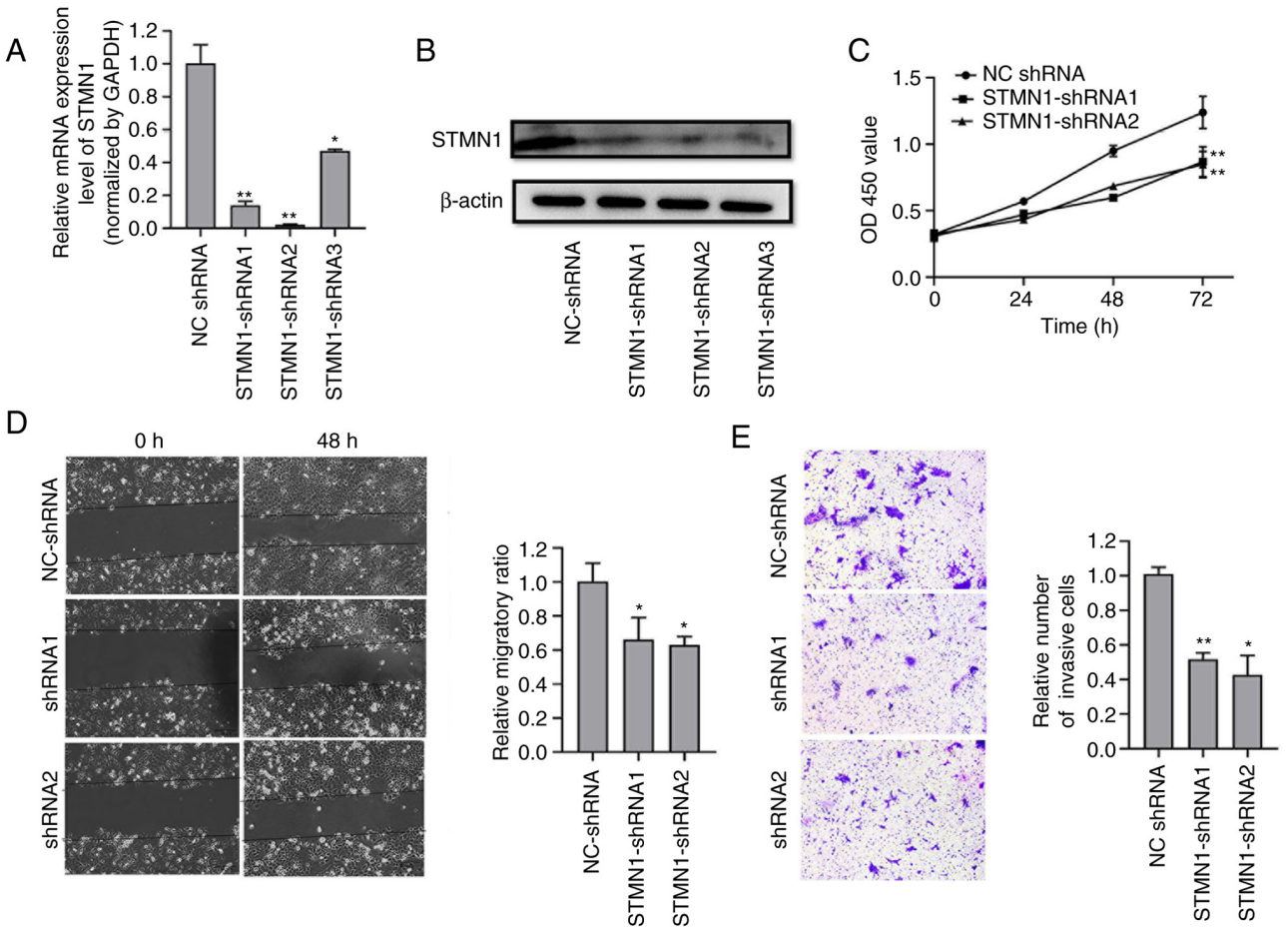

Figure 3. The transfection efficacy evaluation of STMN1 knockdown and the results of cell functional experiments. (A and B) The relative mRNA and protein expression levels of STMN1 in different groups. All of them were evidently decreased after STMN1 knockdown with different shRNA sequences, compared with the NC shRNA group. (C-E) The OD_{450}, the relative migratory ratio at 48 h, and the relative number of invasive cells were all significantly decreased after STMN1 knockdown with different shRNA sequences; all were compared with the NC shRNA group. The magnification of picture D and picture E were 100 times and 200 times, respectively. *P<0.05 and **P<0.01. STMN1, stathmin1; shRNA, short hairpin RNA; NC, negative control.

compared with the cells in the NC shRNA group, the cells in the STMN1 shRNA groups had lower OD_{450} value (P<0.01); the relative migratory ratios were significantly reduced (P<0.05), and the relative number of invasive cells in the lower chamber were significantly decreased (P<0.05, Fig. 3C-E). These cell functional experimental results revealed that knockdown of

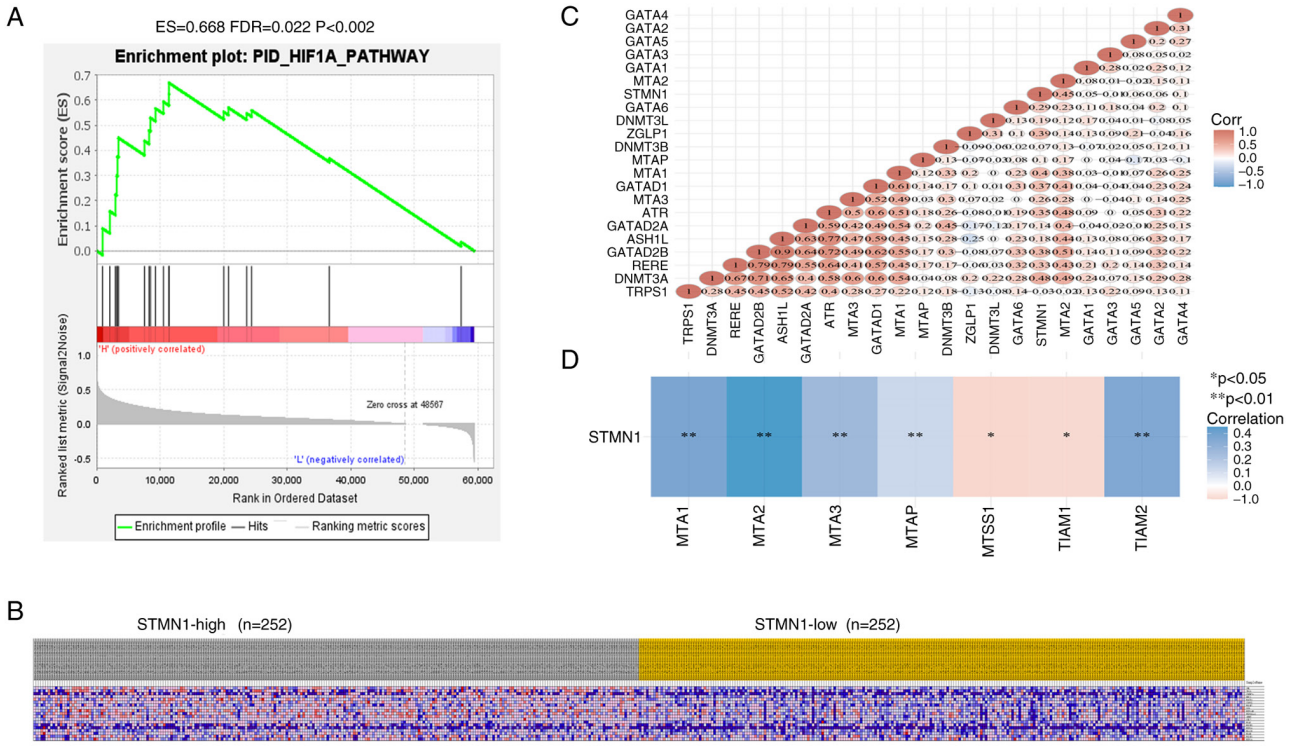

Figure 4. Bioinformatics analyses for target genes and pathways of STMN1. (A) STMN1 was evidently enriched in the HIF-1α pathway in head and neck squamous cell carcinoma. (B) Gene expression levels of HIF-1α pathway were different between cases with high expression of STMN1 and those with low expression of STMN1. (C and D) Target gene prediction analysis from website https://www.aclbi.com, which demonstrated that the expression level of MTA1 was significantly correlated with that of STMN1. STMN1, stathmin1; HIF-1α, hypoxia inducible factor-1alpha; MTA1, tumor metastasis-associated protein 1.

STMN1 significantly reduced the abilities of cell proliferation, migration and invasion.

Potential target genes and pathways of STMN1. To explore the potential mechanisms by which STMN1 promotes LNM in HSCC, evaluation of the potential target genes and pathways of STMN1 based on a target gene prediction analysis from the website https://www.aclbi.com and pathway enrichment analysis from TCGA database were performed. The results showed that the genes in the HIF-1α pathway were enriched in the STMN1 high expression group (Fig. 4A and B). At the same time, the expression level of tumor metastasis-associated protein 1 (MTA1) correlated well with that of STMN1 (Fig. 4C and D). These results indicated that STMN1 potentially promoted LNM in HSCC via regulation of MTA1 expression and the HIF-1α pathway activation.

Knockdown of STMN1 inhibits the activity of the HIF-1α/VGEF-A signal axis. After bioinformatics analysis, the mRNA and protein levels of HIF-1α in FaDu cells with STMN1 knockdown were detected by RT-qPCR and western blot analyses, respectively. It was identified that the relative mRNA expression level and protein expression level of HIF-1a were both significantly decreased after STMN1 knockdown with different shRNA sequences (Fig. 5A-C). These findings indicated that silencing of STMN1 played an important role in the HIF-1α pathway. VEGF-A is one of the downstream target genes regulated by HIF-1α and is associated with LNM. Therefore, at the protein level, the relative expression level of VEGF-A in FaDu cells was also detected by western blot analysis. The results revealed that the relative expression level of VEGF-A was also significantly decreased along with the decline in HIF-1α after STMN1 knockdown with different shRNA sequences (Fig. 5B and D). These results indicated that STMN1 knockdown could inhibit the activity of the HIF-1α/VEGF-A axis.

Knockdown of STMN1 inhibits the expression of MTA1. After bioinformatics analysis, the relative mRNA and protein expression levels of MTA1 in FaDu cells with STMN1 knockdown were also detected by RT-qPCR and western blot analyses. Consistent with the authors' predictions, the relative expression level of MTA1 in FaDu cells was significantly decreased after STMN1 knockdown with different shRNA sequences (Fig. 5B, E and F). These results indicated that STMN1 knockdown could also inhibit the expression of MTA1.

Discussion

HSCC is the dominant histological subtype in hypopharyngeal carcinoma (HC), accounting for more than 95% of HC cases, particularly in China. The tendency toward extensive neck LNM is the most important clinical feature of HSCC, which influences the prognosis and leads to poor survival in HSCC (2,3). Strategies to control neck LNM in HSCC treatment, including extensive neck lymph node dissection and irradiation, cause immense pain to HSCC survivors (6,7). Effective prediction of the possibility of neck LNM will reduce unnecessary neck lymph node disposal, which will evidently improve the quality of life of HSCC survivors. However, there is a paucity of effective biomarkers for predicting neck LNM

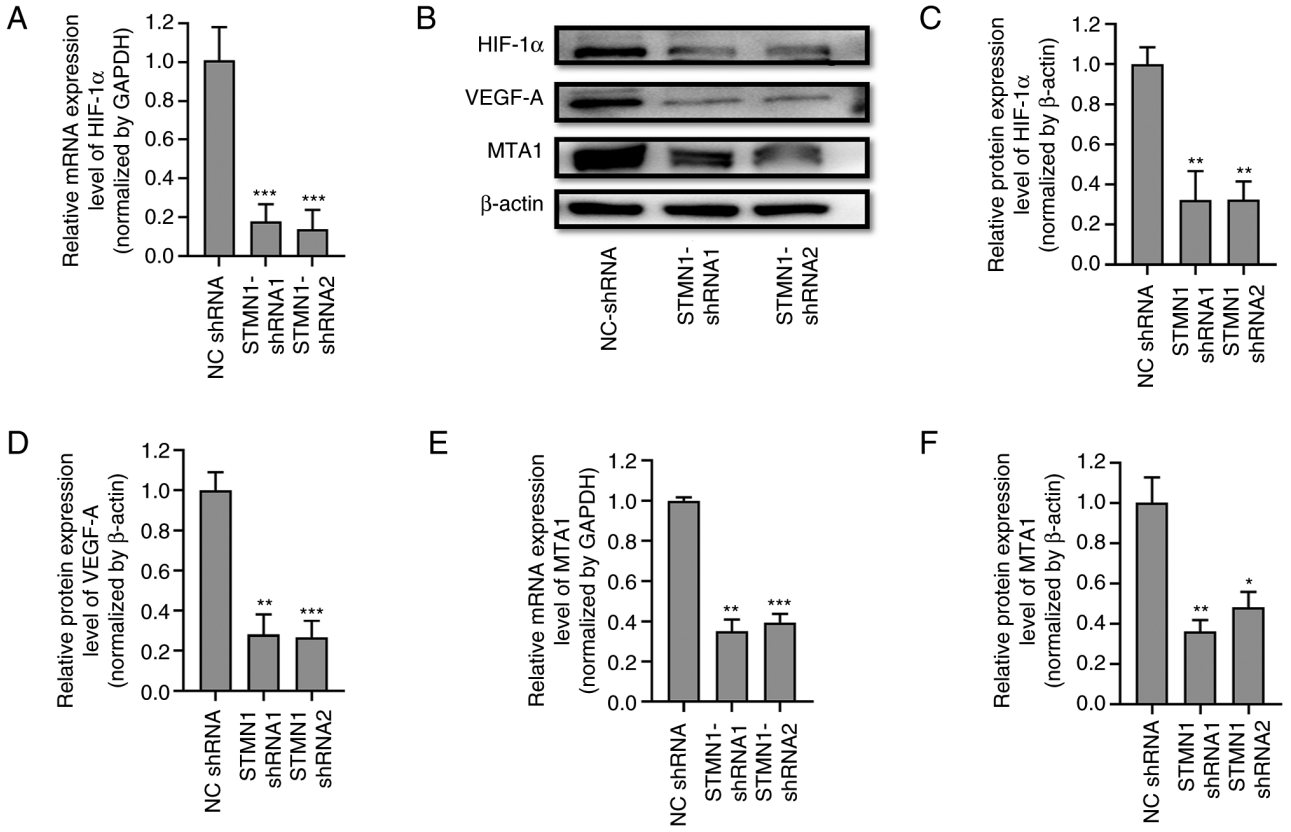

Figure 5. The mRNA and protein expression levels of the target genes of STMN1 in FaDu cells. (A-C) The relative mRNA and protein expression levels of HIF-1α were signifiicantly decreased after STMN1 knockdown with different shRNA sequences. (D) The relative expression level of VEGF-A, the downstream protein of HIF-1α, was significantly decreased after STMN1 knockdown. (E and F) Both relative mRNA and protein expression levels of MTA1 significantly decreased after STMN1 knockdown. All were compared with the NC shRNA group. *P<0.05, **P<0.01 and ***P<0.001. STMN1, stathmin1; HIF-1α, hypoxia inducible factor-1alpha; shRNA, short hairpin RNA; VEGF, vascular endothelial growth factor; MTA1, tumor metastasis-associated protein 1; NC, negative control.

in HSCC in current clinical practice. In the present study, for the first time to the best of our knowledge, the association between STMN1 and neck LNM in HSCC and the molecular mechanisms underlying the promotion of neck LNM in HSCC by STMN1 were explored.

Firstly, in 117 postoperative samples and gene chip data of HSCC, it was found that STMN1 expression was increased in HSCC tissues; the high expression of STMN1 was associated with high incidence of neck LNM. This result confirmed the authors' hypothesis that high expression of STMN1 in HSCC promoted neck LNM, providing the basis for further research, as also guaranteeing the value of the present study. Afterwards, in the clinical cases, the correlation between STMN1 expression and LVI in tumor tissues was further analyzed; LVI is considered to be the initial step in LNM and indicates the great possibility of LNM (19). Eventually it was revealed that high expression of STMN1 was associated with high incidence of LVI, which proved that high expression of STMN1 promoted LVI in HSCC. This provided evidence that STMN1 promoted neck LNM in HSCC possibly via promotion of LVI in HSCC.

In subsequent cell functional and mechanistic experiments, different shRNA sequences targeting STMN1 were synthesized to inhibit STMN1 expression in FaDu cell lines to guarantee the reliability and repeatability of the experimental results. Eventually, in the cell functional experiments, it was found that high expression of STMN1 could actually promote FaDu cell invasion and metastasis. These results of cell experiments further verified the aforementioned clinical findings and confirmed the reliability of the present study.

In the following cell mechanistic experiments, the molecular mechanisms underlying promotion of neck LNM in HSCC by STMN1 were investigated. A bioinformatics analysis was first performed to identify potential target genes and pathways of STMN1, and subsequently further validated these potential target genes and pathways. After bioinformatics analysis, it was revealed that high expression of STMN1 was associated with the activation of and gene enrichment of the HIF-1α pathway. At the same time, it was also revealed that the expression level of STMN1 was associated with that of MTA1. These results indicated that STMN1 may influence neck LNM in HSCC via regulation of the activity of the HIF-1α pathway and the expression of MTA1.

HIF-1α is the most ubiquitously expressed protein and functions as a key regulator of oxygen homeostasis in numerous cell types, mediating transcriptional activation of lymphangiogenesis and LNM under hypoxic conditions. It is a critical feature of the tumor microenvironment that promotes invasion and metastasis (20). Promotion of LNM by HIF-1α has been widely reported in human tumors. Zhao *et al* (21) and Méndez-Blanco *et al* (22) found that HIF-1α was associated with LNM in breast cancer and hepatocellular carcinoma in large samples of clinical cases. Yang *et al* (23) reported

that UBE2C mediated HIF-1α activation to promote LNM in HNSCC. Further, the underlying molecular mechanisms by which HIF-1α promotes LNM were considered to be via regulation of signaling cascades, such as VEGF-A/-C/-D, TGF-β, C/EBP-δ and Prox-1 (20). Among them, the HIF-1α/VEGF-A/-C/-D signaling axis has been considered to be the most important molecular mechanism that promotes LNM (20,24).

VEGFs stimulate lymphangiogenesis by activating VEGF receptor (VEGFR) tyrosine kinases in endothelial cells. Intratumoral hypoxia upregulates the expression of VEGFs, and importantly, hypoxia selects a subpopulation of tumor cells with an invasive and metastatic phenotype that have the capacity to escape from the primary tumors (25,26). The secreted glycoproteins VEGF-C or VEGF-D activate VEGFR-3, a cell surface receptor tyrosine kinase on lymphatic endothelium, leading to growth of lymphatic vessels at the periphery of and occasionally inside the primary tumor, thereby promoting spread of tumor cells to lymph nodes. At present, the VEGF-C/VEGF-D/VEGFR-3 pathway is the most well-understood pathway regulating lymphangiogenesis and LNM (27). VEGF-A, another VEGF family member, initially identified as an important promoter of angiogenesis, primarily binds to VEGFR-1 and VEGFR-2. VEGF-A is known to exert potent lymphangiogenic activity by activating VEGFR-2, thereby facilitating metastatic spread (28). In addition, VEGF-A enhances the heterodimerization of VEGFR-3 with VEGFR-2 and the phosphorylation of VEGFR-3. This, in turn, provides proliferative stimuli to lymphatic endothelial cells (29), which are activated in lymphatic metastasis of tumors (19). In the present study, it was found that HIF-1α/VEGF-A was significantly increased in FaDu cells with high expression of STMN1. Moreover, after STMN1 knockdown with different interference sequences, the expression levels of HIF-1α/VEGF-A were evidently decreased. These results led to the conclusion that STMN1 promotes neck LNM in HSCC via the HIF-1α/VEGF-A axis.

MTA1 has been considered as a transcriptional regulator and was identified in a screen for genes expressed in metastatic cells (30). MTA1 is overexpressed in a variety of tumors and is closely related to LNM and cancer cell invasion. Li *et al* (31) reported that the expression level of MTA1 was strongly associated with the depth of invasion and lymphatic metastasis in gastrointestinal cancer in a meta-analysis. Liu *et al* (32) considered MTA1 to be a novel marker predicting survival and LNM in cervical cancer. In HNSCC, Roepman *et al* (33) found that MTA1 overexpression was associated with invasion and neck LNM. In the bioinformatics analysis performed in the present study, the expression of MTA1 was considered to be associated with STMN1 expression. In the following cell experiments, MTA1 was also found to be increased in FaDu cells with high expression of STMN1; concurrently, with STMN1 knockdown with different interference sequences, the expression levels of MTA1 were evidently decreased. These results indicated that STMN1 may promote neck LNM in HSCC by affecting the expression of MTA1 in some way. However, the mechanisms by which MTA1 promotes LNM were unclear. Guo *et al* (34) reported that MTA1 expression was correlated with HIF-1α expression and lymphangiogenesis in esophageal cancer. In HSCC, whether MTA1 promotes neck LNM by regulating HIF-1α expression is worthy of further research.

In summary, based on a clinical sample analysis, it was identified that high expression of STMN1 promoted neck LNM in HSCC and LVI. Subsequent cell experiments confirmed that high expression of STMN1 could actually promote FaDu cell invasion and metastasis. The studies on underlying molecular mechanisms revealed that STMN1 promotes neck LNM in HSCC via the HIF-1α/VEGF-A axis and by promoting the expression of MTA1. It would be worthwhile to elucidate the detailed mechanisms further in future studies.

Acknowledgements

The authors appreciate the support and help from the Pathology Department of the Shandong Provincial Hospital.

Funding

The present study was supported by the Natural Science Foundation of Shandong Province (grant no. ZR2019MH017) and the Development Foundation of the Second Hospital of Shandong University for Postgraduate Supervisor (grant no. 6010220073).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

DS and YW contributed to the study concepts, study design, and the manuscript editing and review. YW contributed to the experimental studies, data acquisition and analysis. QL contributed to the literature research. JT and CH contributed to the data collection of eligible clinical cases and data analysis. DS and YW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. KYLL-2022LW141) by the Ethics Committees of the Second Hospital of Shandong University (Jinan, China). Written informed consent was obtained from each patient before surgery.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Aupérin A: Epidemiology of head and neck cancers: An update. Curr Opin Oncol 32: 178-186, 2020.

2. Newman JR, Connolly TM, Illing EA, Kilgore ML, Locher JL and Carroll WR: Survival trends in hypopharyngeal cancer: A population-based review. Laryngoscope 125: 624-629, 2015.
3. Buckley JG and MacLennan K: Cervical node metastases in laryngeal and hypopharyngeal cancer: A prospective analysis of prevalence and distribution. Head Neck 2: 380-385, 2000.
4. Cooper JS, Porter K, Mallin K, Hoffman HT, Weber RS, Ang KK, Gay EG and Langer CJ: National Cancer Database report on cancer of the head and neck:10-year update. Head Neck 31: 748-758, 2009.
5. Eckel HE and Bradley PJ: Treatment options for hypopharyngeal cancer. Adv Otorhinolaryngol 83: 47-53, 2019.
6. Sewnaik A and Baatenburg de Jong RJ: Sequelae and complications of treatment for hypopharyngeal cancer: Minimising the risks. Adv Otorhinolaryngol 83: 109-117, 2019.
7. Mahalingam S and Spielmann P: Quality of life outcomes following treatment of hypopharyngeal cancer. Adv Otorhinolaryngol 83: 126-134, 2019.
8. van den Bosch S, Czerwinski M, Govers T, Takes RP, de Bree R, Al-Mamgani A, Hannink G and Kaanders JHAM: Diagnostic test accuracy of sentinel lymph node biopsy in squamous cell carcinoma of the oropharynx, larynx, and hypopharynx: A systematic review and meta-analysis. Head Neck 44: 2621-2632, 2022.
9. Horváth A, Prekopp P, Polony G, Székely E, Tamás L and Dános K: Accuracy of the preoperative diagnostic workup in patients with head and neck cancers undergoing neck dissection in terms of nodal metastases. Eur Arch Otorhinolaryngol 278: 2041-2046, 2021.
10. Freiser ME, Ojo RB, Lo K, Saint-Victor S, Bollig C, Nayak CS and Sargi ZB: Complications and oncologic outcomes following elective neck dissection with salvage laryngectomy for the N0 neck. Am J Otolaryngol 37: 186-194, 2016.
11. Hardisson D: Molecular pathogenesis of head and neck squamous cell carcinoma. Eur Arch Otorhinolaryngol 260: 502-508, 2003.
12. Rickman DS, Millon R, De Reynies A, Thomas E, Wasylyk C, Muller D, Abecassis J and Wasylyk B: Prediction of future metastasis and molecular characterization of head and neck squamous-cell carcinoma based on transcriptome and genome analysis by microarrays. Oncogene 27: 6607-6622, 2008.
13. Elsheikh MN, Rinaldo A, Hamakawa H, Mahfouz ME, Rodrigo JP, Brennan J, Devaney KO, Grandis JR and Ferlito A: Importance of molecular analysis in detecting cervical lymph node metastasis in head and neck squamous cell carcinoma. Head Neck 28: 842-849, 2006.
14. Gupta KK, Li C, Duan A, Alberico EO, Kim OV, Alber MS and Goodson HV: Mechanism for the catastrophe-promoting activity of the microtubule destabilizer Op18/stathmin. Proc Natl Acad Sci USA 110: 20449-20454, 2013.
15. Hsieh SY, Huang SF, Yu MC, Yeh TS, Chen TC, Lin YJ, Chang CJ, Sung CM, Lee YL and Hsu CY: Stathmin1 overexpression associated with polyploidy, tumor-cell invasion, early recurrence, and poor prognosis in human hepatoma. Mol Carcinog 49: 476-487, 2010.
16. Cao S, Zhang W, Shen P and Xu R: Low STMN1 is associated with better prognosis in Asian patients with esophageal cancers: A meta-analysis. J Gastroenterol Hepatol 35: 1668-1675, 2020.
17. Chen Y, Zhang Q, Ding C, Zhang X, Qiu X and Zhang Z: Stathmin1 overexpression in hypopharyngeal squamous cell carcinoma: A new promoter in FaDu cell proliferation and migration. Int J Oncol 50: 31-40, 2017.
18. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
19. Zhang Z, Helman JI and Li LJ: Lymphangiogenesis, lymphatic endothelial cells and lymphatic metastasis in head and neck cancer-a review of mechanisms. Int J Oral Sci 2: 5-14, 2010.
20. Ji RC: Hypoxia and lymphangiogenesis in tumor microenvironment and metastasis. Cancer Lett 346: 6-16, 2014.
21. Zhao Z, Mu H, Li Y, Liu Y, Zou J and Zhu Y: Clinicopathological and prognostic value of hypoxia-inducible factor-1α in breast cancer: A meta-analysis including 5177 patients. Clin Transl Oncol 22: 1892-1906, 2020.
22. Méndez-Blanco C, Fernández-Palanca P, Fondevila F, González-Gallego J and Mauriz JL: Prognostic and clinicopathological significance of hypoxia-inducible factors 1α and 2α in hepatocellular carcinoma: A systematic review with meta-analysis. Ther Adv Med Oncol 13: 1758835920987071, 2021.
23. Yang YF, Chang YC, Tsai KW, Hung MH and Kang BH: UBE2C triggers HIF-1α-glycolytic flux in head and neck squamous cell carcinoma. J Cell Mol Med 26: 3716-3725, 2022.
24. Hartiala P and Saarikko AM: Lymphangiogenesis and lymphangiogenic growth factors. J Reconstr Microsurg 32: 10-15, 2016.
25. Li S and Li Q: Cancer stem cells, lymphangiogenesis, and lymphatic metastasis. Cancer Lett 357: 438-447, 2015.
26. Achen MG and Stacker SA: Molecular control of lymphatic metastasis. Ann NY Acad Sci 1131: 225-234, 2008.
27. Gogineni A, Caunt M, Crow A, Lee CV, Fuh G, van Bruggen N, Ye W and Weimer RM: Inhibition of VEGF-C modulates distal lymphatic remodeling and secondary metastasis. PLoS One 8: e68755, 2013.
28. Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF and Detmar M: VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. J Exp Med 201: 1089-1099, 2005.
29. Alam A, Herault JP, Barron P, Favier B, Fons P, Delesque-Touchard N, Senegas I, Laboudie P, Bonnin J, Cassan C, *et al*: Heterodimerization with vascular endothelial growth factor receptor-2 (VEGFR-2) is necessary for VEGFR-3 activity. Biochem Biophys Res Commun 324: 909-915, 2004.
30. Toh Y and Nicolson GL: Identification and characterization of metastasis-associated gene/protein 1 (MTA1). Cancer Metastasis Rev 33: 837-842, 2014.
31. Li P, Cao W, Ding R, Cheng M, Xu X, Chen S, Chen B, Cao G and Xiong M: Expression and prognostic significance of metastasis-associated protein 1 in gastrointestinal cancer. Front Oncol 10: 542330, 2020.
32. Liu T, Yang M, Yang S, Ge T, Gu L and Lou G: Metastasis-associated protein 1 is a novel marker predicting survival and lymph nodes metastasis in cervical cancer. Hum Pathol 44: 2275-2281, 2013.
33. Roepman P, de Jager A, Groot Koerkamp MJ, Kummer JA, Slootweg PJ and Holstege FC: Maintenance of head and neck tumor gene expression profiles upon lymph node metastasis. Cancer Res 66: 11110-11114, 2006.
34. Guo X, Chen Y, Fang W, Yang W, Shi L and Zhu R: Metastasis associated protein 1 correlates with Hypoxia inducible-factor 1 alpha expression and lymphangiogenesis in esophageal cancer. Thorac Cancer 4: 312-317, 2013.