LncRNA DUXAP8 promotes the progression of laryngeal cancer through targeting miR-384/POU2F1 axis

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Abstract

Background

Laryngeal cancer has become one of the most prevalent tumors of the upper respiratory tract, especially head and neck cancers[1]. Laryngeal cancer approximately accounts for 1% – 5% cancer-related incidence in the world[2]. Some patients diagnosed at an early stage can be well treated surgically.
following combination therapy[3]. However, because lack early diagnostic capabilities, most cases are diagnosed at an advanced stage and may suffer from painful symptoms such as persistent cough, stridor, bad breath, earache and difficulty swallowing[4]. Hence, the well understanding of specific molecular mechanisms involved in the development of laryngeal cancer is more urgent and will contribute to identify efficient targets for the diagnosis and treatment.

Long non-coding RNAs (lncRNAs) can play important regulatory roles (tumor suppressors or oncogenes) in many types of cancers[5, 6]. Recently, a series of lncRNAs involved in the progression of laryngeal cancer have been identified and may be potential biomarkers. For example, lncRNA MEG3 is downregulated in laryngeal cancer tissues and overexpression of MEG3 inhibits cell proliferation and induces apoptosis of laryngeal cancer cells[7]. LncRNA RGMB-AS1 is upregulated in laryngeal cancer tissues and downregulation of RGMB-AS1 inhibits proliferation and invasion of laryngeal cancer cells[8].

Pseudogenes, genomic DNA sequences which are similar to normal genes, are identified as defunct relatives of functional genes[9]. Double homeobox A pseudogene 8 (DUXAP8), a pseudogene derived IncRNA, is highly overexpressed and acts oncogenic role in multiple cancers. For instance, upregulation of DUXAP8 promotes cell growth by directly sponging Krüppel-like factor 2 (KLF2) in human hepatocellular carcinoma[10]. Knockdown of DUXAP8 inhibits cell proliferation and invasion of non-small-cell lung cancer cells through silencing EGR1 and RHOB[11]. Despite of crucial roles of DUXAP8 in human cancers, its function and specific mechanism in laryngeal cancer remains unclear.

MicroRNAs (miRNAs) also have emerging as newly identified non-coding regulatory RNAs with approximately with 22 nucleotides in length[12]. More and more reports reveals the essential functions of miRNAs in laryngeal cancer such as miR-141[13], miR-632[14], miR-144[15], and so on. Previous studies have demonstrated that miR-384 play crucial functions in various cancer including colorectal cancer[16], gastric cancer[17], and non-small cell lung cancer[18]. Recently, miR-384 has also been identified to act important roles in laryngeal cancer. Wang et al. reveals that overexpression of miR-384 inhibits cell proliferation and promotes cell apoptosis of laryngeal cancer cells in vitro[19]. Wang et al. also demonstrates that IncRNA SNHG3 is upregulated in laryngeal cancer tissues and knockdown of SNHG3 reduces cell viability, migration, and invasion of laryngeal cancer cell lines TU212 and TU686 through targeting miR-384/WEE1 axis[20]. These reports suggest that miR-384 may be act as a target of other IncRNAs to participate in the progression of laryngeal cancer.

POU domain class 2 transcription factor 1 (POU2F1), a ubiquitous transcription factor, can regulate the expression of downstream genes associated with cell cycle[21]. Increasing reports have revealed that high level of POU2F1 are closely related to the metastasis, apoptosis, growth and epithelial-mesenchymal transition (EMT) process of tumor cells such as cervical cancer[21], liver cancer[22], and hepatocellular carcinoma[23]. In addition, POU2F1 is highly expressed in head and neck squamous cell carcinoma cell lines, as well as in patient tumor tissues compared with adjacent normal tissues[24]. These studies suggest that POU2F1 may also play potential tumorigenic role in laryngeal cancer.
In this study, we analyzed the differentially expressed lncRNAs between laryngeal cancer tissues and adjacent normal tissues in GSE59652 dataset, and identified several abnormally expressed lncRNAs including DUXAP8. We found that DUXAP8 was highly expressed in laryngeal cancer tissues and cell lines. Further, knockdown of DUXAP reduced cell growth of laryngeal cancer cells both in vitro and in vivo. Bio-informatics analysis and rescue experiments determined that knockdown of DUXAP8 inhibited the progression of laryngeal cancer through targeting miR-384/POU2F1 axis. Our findings provide that DUXAP8 play an important tumorigenic role in laryngeal cancer and could act as potential therapeutic target.

Methods

Microarray analysis

The microarray datasets including GSE59652 and GSE143224 available online were downloaded from the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI). Differentially expression analysis of GSE59652 was performed to screen differentially expressed lncRNAs between laryngeal cancer tissues and non-tumorous tissues by using the limma package of R language based on the criteria “P Value< 0.05 and |fold change| > 1.” The top 10 upregulated and top 10 downregulated lncRNAs were used to generate a heat-map by R language.

Tissues specimens

A total of 90 patients with laryngeal cancer were recruited in this study at Beijing Chaoyang Hospital of Capital Medical University from between 2011.01 and 2016.01. 90 matched cancerous and noncancerous tissues were tested by real-time qPCR. 90 pairs of tumor and adjacent normal tissues were obtained by surgical excision, and the histopathological diagnosis for each sample was confirmed by two pathologists independently. All participants have written to obtain their informed consents. All producers were approved by the human Ethics Committee of Beijing Chaoyang Hospital of Capital Medical University. The clinicopathological characteristics of 90 laryngeal cancer patients were shown in Table 1.

Cell culture

Human laryngeal carcinoma cell lines TU212, TU177 and AMC-HN-8 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HEK293T cells for the luciferase reporter assay were purchased from the Institute of Biochemistry and Cell Biology (Shanghai). All cells were cultured in DMEM Medium (Gibco, USA) supplementing with 10% fetal bovine serum (FBS) (Invitrogen), and 1% penicillin/streptomycin at 37°C with 5% CO₂.

Cell transfection

MiR-384 mimics, miR-384 inhibitor and corresponding negative controls miR-NC and inhibitor NC were purchased from RiboBio (Guangzhou, China). To overexpress POU2F1, the cDNA sequence of POU2F1
were amplified by PCR and then cloned into Expression Vector pcDNA3.1 (Invitrogen, CA, USA) to generate oe-POU2F1. Meanwhile, the empty vector pcDNA3.1 (oe-NC) was used as the negative control. Cell transfection for siRNAs and plasmids into TU212 and TU177 cells was performed by using Lipofectamine 2000 kit (Invitrogen, USA) according to the manufacturer’s instructions. After transfection for 48 h, cells were harvested and used for subsequent experiments.

**The construction of stable cell lines with downregulation of DUXAP8**

The short hairpin RNA targeting DUXAP8 (sh-DUXAP8) and negative control sh-NC were inserted into pLKO.1 lentiviral vector and then transfected into TU212 and TU177 cells with a lentiviral gene delivery system as previously described[25]. Subsequently, cells were treated with 1 μg/ml puromycin for 2 weeks to screen the stable cell lines. And the expression of DUXAP8 was determined by qRT-PCR analysis. The sequences used in this study as follows: sh-DUXAP8: 5′- GCAGCATACTCAAATTACAGCA -3′; sh-NC: 5′- UUCUCGAACGUGUCACGUTT-3′.

**qRT-PCR analysis**

Total RNA of tumor tissues and cultured cells was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Approximately 1.2 μg of total RNA was reversely transcribed into cDNA by a Reverse Transcription Kit (Takara, Dalian, China). Then qRT-PCR assay was performed by using the SYBR Green (Takara) based ABI 7500 Detection System (Applied Bioskyns, Foster City, CA, USA) with GAPDH and U6 as the internal references. The relative expression change of targets was analyzed by the 2^ΔΔCt method. The primers used as follows: DUXAP8 forward: 5′- AGGATGGAGTCTCGCTGTATTGC -3′; reverse: 5′- GGAGGTTTGTGTCTTTCTTTTTT -3′; POU2F1 forward: 5′- GCGAAGCTTGTTAAAATATTCAAAATGGCGGAC -3′; reverse: 5′ - GATTGCTCCTCCTACAGGCAC -3′; GAPDH forward: 5′- AGGGCTGCTTTTAACTCTGGT -3′, reverse: 5′- CCCACTTGATTTTGGAGGGA -3′; U6 forward: 5′- GCTTCGGCAGCACATATACCTGCTGTGTCAT -3′, reverse: 5′- CGCTTCACGAATTTTCGTGCAT -3′.

**Western blot**

Total protein of cultured cells was extracted by using by using RIPA lysis buffer (Beyotime Institute of Biotechnology). Approximately equal amounts of protein were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore). After blocking with 5% skim milk, the membranes were incubated with primary antibodies POU2F1 (#8157S, 1:1000, CST, USA), GAPDH (ab181602, 1:5000, Abcam, UK) overnight at 4°C. Of which, GAPDH was considered as the internal reference. Then the membranes were exposed to HRP-conjugated secondary antibody at room temperature for another 1 h. ECL chromogenic substrate was used for quantification by densitometry (Quantity One software; Bio-Rad).

**Subcellular fractionation**
To determine the cellular localization of DUXAP8, cytosolic and nuclear fractions of TU212 cells were separated by the Nuclear/Cytosol Fractionation Kit (BioVision, Milpitas, CA, USA). Then, RNA from the cytosolic and nuclear fractions was extracted by using TRIzol reagent, and the expression level of DUXAP8 in cytoplasm and nucleus was evaluated by qRT-PCR method.

**CCK-8 assay**

CCK-8 assay was performed by using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). In brief, 1,000 TU212 and TU177 cells were seeded into 96-well plates overnight. And 10 ul of CCK-8 reagent was added to each well at 0, 24, 48, 72 and 96 h for another 2 h. The absorbance at 450 nm was detected with a microplate reader.

**Colony formation assay**

For colony formation assay, 1,000 TU212 and TU177 cells /well were seeded into 6-well plates and cultured for two weeks. Subsequently, cells were fixed with 4% paraformaldehyde and stained with crystal violet, then colonies were photographed and counted under a light microscope.

**EdU staining assay**

EdU staining assay was performed by using an EdU Apollo DNA *in vitro* kit (RIBOBIO, Guangzhou, China) according to the manufacturer’s instructions. Briefly, cells were incubated with 100 μl of 50 μM EdU/well at 37 °C for 2 h, and then visualized by using a fluorescence microscopy.

**Luciferase reporter assay**

Starbase v2.0 was applied to predict the potential binding sites between DUXAP8 and miR-384, as well as miR-384 and POU2F1. To determine the interactions among them, the wild type (WT) and MUT type (MUT) 3’-UTR of DUXAP8 and POU2F1 were amplified by PCR and inserted into psiCheck2 luciferase reporter vector. Then luciferase reporter plasmids were co-transfected with miR-384 mimics or miR-NC into HEK 293T cells. After transfection for 48 h, cells were lysed and relative luciferase activity was measured by using a dual luciferase reporter system.

**RNA immunoprecipitation (RIP) assay**

RIP assay was carried out by using a Thermo Fisher RIP kit (Thermo Fisher Scientific, MA, USA). In brief, the whole lysates of TU212 cells were incubated with magnetic beads conjugated to anti-Ago2 antibody or the negative control anti-IgG (Abcam) overnight at 4°C. On the next day, 30 μL of magnetic beads were added and incubated for another 2 h at 4°C, and immune-precipitated RNA was purified by TRIzol reagent and used for qRT-PCR analysis.

**Immunohistochemistry (IHC) assay**
To evaluate the cell proliferation *in vivo*, tumor tissues from mice were paraffin-embedded and cut into 5 µm-thick sections. Hematoxylin and eosin (H&E) staining was used to select the representative areas as previously described[26]. Then antibody against Ki-67 (1:400, Abcam, ab15580, USA) was used to detect the proliferation. The staining positivity was quantified in three different high-power fields of each section.

**Xenograft tumor model**

BALB/c nude mice (approximately 4 weeks old) were purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China). 2 × 10^6 TU212 cells stably transfected with sh-DUXAP8 or sh-NC were subcutaneously injected into the mice to construct the *in vivo* model as previously described (N = 6). Tumor volume was calculated with the following formula: \( V (\text{mm}^3) = (L \times W^2) / 2 \) every week for 4 weeks. On the day 28, mice were sacrificed through cervical dislocation and tumor weight was evaluated. All animal experiments were approved by the animal Ethics Committee of Beijing Chaoyang Hospital of Capital Medical University.

**Statistical analysis**

All data were presented as mean ± SD derived from at least three independent times. Statistical analysis was performed by using SPSS v.20.0 (Abbott Laboratories, Chicago, IL, USA). Difference was determined by Student's t test (two groups) or one-way ANOVA (among three or more groups). Kaplan–Meier method was used to compare the survival difference of laryngeal cancer patients based on the expression median of DUXAP8. Spearman correlation analysis was used to evaluate the correlation between groups. P < 0.05 was considered as the significant threshold.

**Results**

**LncRNA DUXAP8 was significantly upregulated in laryngeal cancer**

To evaluate the expression of lncRNAs in laryngeal cancer, a non-coding RNA profiling dataset (GSE59652) was used to analyze differentially expressed lncRNAs between laryngeal cancer samples and adjacent normal samples. A total of 447 differentially expressed lncRNAs between the two groups were identified (Fig. 1A). Further, the heat map of top 10 significantly upregulated lncRNAs and downregulated lncRNAs were shown in Fig. 1B. Among these significantly and differentially expressed lncRNAs, the function of highly overexpressed DUXAP8 in laryngeal cancer has not been reported. To confirm its high expression in laryngeal cancer, we detected the expression level of DUXAP8 between tumor tissues and adjacent normal tissues. The results showed that DUXAP8 was markedly upregulated in tumor tissues compared with adjacent tissues (n = 90) (p < 0.001) (Fig. 1C). Next, 90 patients with laryngeal cancer were divided into two groups (high DUXAP8 group and high DUXAP8 group) according to the expression median value of DUXAP8. Kaplan-Meier survival curves indicated that laryngeal cancer patients with high DUXAP8 level had a worse prognosis than that with low DUXAP8 level (n = 90) (p =
These results suggested that DUXAP8 might play a potential tumorigenic role in laryngeal cancer.

**Knockdown of DUXAP8 inhibited proliferation of laryngeal cancer cells in vitro**

To explore the potential role of DUXAP8 in laryngeal cancer, we then evaluated the expression level of DUXAP8 in laryngeal carcinoma cell lines TU212, TU177 and AMC-HN-8. The results showed that DUXAP8 was highly expressed in TU212 (p < 0.001) and TU177 cells (p < 0.001) compared with that in AMC-HN-8 cells (Fig. 2A), suggesting that DUXAP8 was reliably upregulated in laryngeal cancer and TU212, TU177 cells were used for the subsequent experiments. Then stable cell lines of TU212 and TU177 with downregulation of DUXAP8 were constructed. We found that sh-DUXAP8 significantly decreased the level of DUXAP8 compared with sh-NC both in TU212 cells (p < 0.001) and TU177 cell (p < 0.001) (Fig. 2B). CCK-8 assay showed that sh-DUXAP8 obviously decreased the cell growth of both TU212 cells (p < 0.001) and TU177 cell (p < 0.05) compared with sh-NC (Fig. 2C). Colony formation assay indicated that sh-DUXAP8 significantly decreased the number of colonies both in TU212 cells (p < 0.001) and TU177 cell (p < 0.001) (Fig. 2D). EdU staining assay indicated that sh-DUXAP8 obviously decreased the EdU-positive cells of both TU212 cells (p < 0.05) and TU177 cell (p < 0.01) compared with sh-NC (Fig. 2E). These results revealed that knockdown of DUXAP8 obviously inhibited proliferation of laryngeal cancer cells in vitro.

**DUXAP8 positively regulated POU2F1 in laryngeal cancer**

To determine the subcellular localization of DUXAP8, subcellular fractionation was performed based on qRT-PCR analysis. The results showed that most of the positives orientated in the cytoplasm and minority in the nucleus (Fig. 3A), suggesting that DUXAP8 was located in the cytoplasm. Then StarBase online database (http://www.starbase.sysu.edu.cn) was used to search for potential ceRNA networks and we found that DUXAP8 might function as sponges of 49 target mRNAs. We then analyzed these 49 genes in GSE143224 dataset, and found that the levels of seven genes (ANXA4, CREBBP, LPP, PFDN4, POU2F1, PTPRG and SEC63) were significantly upregulated in laryngeal cancer tissues compared with normal tissues (Fig. 3B). To further search for the downstream genes of DUXAP8, the expression of seven candidates in DUXAP8-knockdown TU212 cells. QRT-PCR analysis revealed that sh-DUXAP8 significantly decreased POU2F1 expression compared with sh-NC (p < 0.001), while had no obvious effect on other candidates (Fig. 3C). Similarly, sh-DUXAP8 also downregulated POU2F1 expression compared with sh-NC in TU177 cells (p < 0.001) (Fig. 3D). Meanwhile, sh-DUXAP8 decreased the protein level of POU2F1 compared with sh-NC both in TU212 cells and TU177 cells (Fig. 3E). Moreover, the level of POU2F1 in tumor tissues was significantly upregulated compared with adjacent normal tissues (n = 90) (p < 0.001) (Fig. 3F). In addition, spearman correlation analysis showed a positive correlation between DUXAP8 and POU2F1 level in laryngeal cancer (n = 90) (r = 0.445, p < 0.001) (Fig. 3G). These results suggested that DUXAP8 positively regulated POU2F1 in laryngeal cancer, and the role of DUXAP8 might be mediated by POU2F1.

**DUXAP8 positively regulated POU2F1 through sponging miR-384**
Starbase v2.0 was applied to predict the potential targets of DUXAP8, and the results showed that there was a putative binding site between DUXAP8 and miR-384 (Fig. 4A). Meanwhile, luciferase reporter assay was performed and indicated that miR-384 mimic significantly decreased relative luciferase activity in DUXAP8-wt (p < 0.001), while exhibited no obvious effect in DUXAP8-mut in HEK-293T cell lines (Fig. 4A). Further, bio-information analysis predicted a putative binding site between miR-384 and POU2F1 (Fig. 4B), which was consistent of our result above that DUXAP8 positively regulated POU2F1. Luciferase reporter assay indicated that miR-384 mimic significantly decreased relative luciferase activity in POU2F1-wt (p < 0.001), while exhibited no obvious effect in POU2F1-mut in HEK-293T cell lines (Fig. 4B).

To determine whether DUXAP8 and miR-384 were in the same RNA-induced silencing complex (RISC), RIP assay was performed and the results revealed that levels of DUXAP8 and miR-384 were both higher in the anti-Ago2 group than that in the anti-normal IgG group (Fig. 4C). Meanwhile, we found that knockdown of DUXAP8 significantly increased the expression of miR-384 compared with sh-NC both in TU212 cells (p < 0.001) and TU177 cells (p < 0.001) (Fig. 4D). Further, TU212 and TU177 cells were co-transfected with miR-384 mimics, miR-NC, miR-384 inhibitor or inhibitor NC. miR-384 mimics markedly decreased the expression of DUXAP8 compared with miR-NC both in TU212 cells (p < 0.001) and TU177 cells (p < 0.001), and miR-384 inhibitor increased the expression of DUXAP8 compared with inhibitor NC both in TU212 cells (p < 0.01) and TU177 cells (p < 0.001) (Fig. 4E). MiR-384 mimics decreased the protein level of POU2F1 compared with miR-NC, and miR-384 inhibitor increased POU2F1 expression compared with inhibitor NC both in TU212 cells and TU177 cells (Fig. 4F). Moreover, the level of miR-384 in tumor tissues was higher than that in adjacent normal tissues (n = 90) (p < 0.001) (Fig. 4G). In addition, spearman correlation analysis showed that there was an obviously negative correlation between DUXAP8 and miR-384 level (r = -0.5545, p < 0.001) (Fig. 4H), as well as between miR-384 and POU2F1 level (r = -0.4045, p < 0.001) (Fig. 4I) in laryngeal cancer tissues (n = 90). These results suggested that DUXAP8 positively regulated POU2F1 through sponging miR-384 in laryngeal cancer.

**DUXAP8 promoted proliferation of laryngeal cancer cells by regulating POU2F1 expression in vitro**

Next, we explore whether the effect of DUXAP8 in laryngeal cancer was mediated by miR-384/POU2F1 axis. TU212 and TU177 cells stably downregulating DUXAP8 were transfected with oe-POU2F1 or oe-NC. Western blot assay showed that sh-DUXAP8 decreased the expression of POU2F1 compared with sh-NC both in TU212 and TU177 cells; Oe-POU2F1 obviously increased the expression of POU2F1 both in TU212 and TU177 cells compared with oe-NC; Co-transfection of sh-DUXAP8 and oe-POU2F1 obviously attenuated the inhibitory effect of sh-DUXAP8 on the expression of POU2F1 both in TU212 and TU177 cells (Fig. 5A). Cell proliferation was also evaluated by CCK-8 assay (Fig. 5B), colony formation assay (Fig. 5C) and EdU staining assay (Fig. 5D): sh-DUXAP8 significantly inhibited proliferation of both TU212 and TU177 cells (p < 0.01); Oe-POU2F1 enhanced proliferation of both TU212 and TU177 cells (p < 0.01); While co-transfection of sh-DUXAP8 and oe-POU2F1 obviously attenuated the inhibitory effect of sh-DUXAP8 on the cell proliferation of both TU212 and TU177 cells (p < 0.05). These results revealed that DUXAP8 promoted proliferation of laryngeal cancer cells by regulating POU2F1 expression in vitro.

**Knockdown of DUXAP8 inhibited tumor growth in vivo**
To further confirm the tumorigenic role of DUXAP8 in laryngeal cancer, xenograft tumor model in vivo was performed by using TU212 cells stably transfected with sh-DUXAP8 and sh-NC. The representative images of tumors were shown in Fig. 6A, indicating that sh-DUXAP8 obviously inhibited tumor growth compared with sh-NC. Meanwhile, knockdown of DUXAP8 significantly decreased tumor weight (p < 0.001) (Fig. 6B) and tumor volume (p < 0.001) (Fig. 6C) compared with sh-NC. IHC assay showed that xenograft tumors from sh-DUXAP8 group exhibited a significant reduction of Ki-67 positive cells compared with that in the sh-NC group (p < 0.01) (Fig. 6D), suggesting that knockdown of DUXAP8 inhibited the proliferation of tumor cells in vivo. In addition, the expression of DUXAP8, miR-384 and POU2F1 in tumor tissues was evaluated and the results indicated that sh-DUXAP8 significantly decreased the expression of DUXAP8 (p < 0.01) and POU2F1 (p < 0.01), while increased miR-384 expression (p < 0.01) compared with sh-NC (Fig. 6E). These results indicated that knockdown of DUXAP8 inhibited tumor growth in vivo through modulating miR-384/POU2F1 axis.

Discussion

Due to its highly aggressive nature, laryngeal cancer has becoming a common type of cancers with an unacceptable higher mortality[27]. Therefore, it is essential to explore the specific molecular mechanisms involved in laryngeal cancer in detail, and develop novel therapeutic or diagnostic targets. In the present study, we demonstrated that DUXAP8 was markedly upregulated in laryngeal cancer tissues and cell lines (TU212 and TU177 cells). High level of DUXAP8 predicted a worse prognosis of patients with laryngeal cancer than low DUXAP8 level. The key findings of this study was that lncRNA DUXAP8 promoted laryngeal cancer progression by modulating miR-384/POU2F1 axis.

Increasing evidences identified a large number of lncRNAs participated in various biological processes such as proliferation, invasion, migration and EMT process during the development of laryngeal cancer. For example, lncRNA SNHG1 promotes cell proliferation in laryngeal cancer through regulating Notch1 signaling pathway[28]. IncRNA DLX6-AS1 promotes the proliferation of laryngeal cancer cells via targeting the miR-26a/TRPC3 axis[29]. LncRNA TUG1 promotes cell proliferation and migration of laryngeal cancer cells[30]. LncRNA LINC00152 knockdown significantly inhibits the proliferation, migration and invasion and induce apoptosis of laryngeal cancer cells[31]. In this study, we identified top 10 significantly upregulated lncRNAs (DUXAP8, AC008753.2, AL353608.1, AC078842.2, AL139275.2, LINC02044, AC025572.1, AC021683.1, AC023794.3 and FAM242C) between tumor tissues and adjacent normal tissues, suggesting that these upregulated lncRNAs might play potential tumorigenic roles in laryngeal cancer. Although the function of DUXAP8 in multiple cancers have been well studied[32, 33], its role in laryngeal cancer remains unclear. Hence, we focused on DUXAP8 due to its highest expression and further explored its functions in laryngeal cancer. Here, we verified that DUXAP8 was significantly upregulated in laryngeal cancer tissues and cell lines. Moreover, knockdown of DUXAP8 could efficiently inhibited cell growth of laryngeal cancer cell lines TU212 and TU177 cells. These results suggest that DUXAP8 is an oncogene in laryngeal cancer.
It has been reported that IncRNAs can function as competing endogenous RNAs (ceRNAs) for miRNAs to promote or suppress the progression of human cancers[34]. For DUXAP8, recent studies have identified several direct target miRNAs in various cancers, such as miR-577 in colorectal cancer[35], miR-126 in renal cell carcinoma[33], miR-422a in hepatocellular carcinoma[36]. Here, bioinformatics prediction, luciferase reporter gene assay and RIP assay were performed to confirm the directly negative correlation between DUXAP8 and miR-384. Previous studies have revealed that miR-384 is downregulated and functions crucial roles as a tumor suppressor in a series of human cancers including renal cell carcinoma[37], osteosarcoma[38], and colorectal cancer[16]. In addition, miR-384 is also significantly downregulated in laryngeal cancer tissues and knockdown of miR-384 promotes the progression of laryngeal cancer through targeting WISP1 signaling pathway[19]. Our results provide a new regulatory mechanism of miR-384 in laryngeal cancer, extending the understanding of IncRNA-miRNA network in human cancers.

To further explore the specific mechanism of DUXAP8 in laryngeal cancer, StarBase online database was used to predict the potential ceRNA network of DUXAP8 and further found that there were seven genes (ANXA4, CREBBP, LPP, PFDN4, POU2F1, PTPRG and SEC63) highly expressed in laryngeal cancer tissues compared with normal tissues in GSE143224 dataset. These results suggest that the seven genes might be indirectly regulated by DUXAP8. Annexin A4 (ANXA4) is highly expressed in laryngeal cancer and overexpression of ANXA4 promotes the progression of tumor, as well as chemoresistance of laryngeal cancer[39]. Except for POU2F1 and ANXA4, the roles of other five candidates in laryngeal cancer have not been well studied. Somatic mutations in CREBBP occur frequently in B-cell lymphoma, and the inactivation of CREBBP enhances the development of HDAC3-dependent lymphomas[40]. LPP is a Src substrate which is required for invadopodia formation and efficient lung metastasis of breast cancer[41]. Prefoldin 4 (PFDN4) is a transcriptional factor that controls the cell cycle and significantly upregulated in breast tumor[42]. Protein tyrosine phosphatase receptor type G (PTPRG) is a crucial tumor suppressor in multiple human cancers[43]. Interestingly, we found that knockdown of DUXAP only decreased the expression of POU2F1 and exhibited no obvious change on other candidates, suggesting that DUXAP8 might act as ceRNA of POU2F1. Overexpression of POU2F1 efficiently attenuated the inhibitory effect of sh-DUXAP8 on cell proliferation. Furthermore, function experiments revealed that DUXAP8 positively regulated POU2F1 expression by directly sponging miR-384.

Although our study demonstrated that knockdown of DUXAP8 inhibited tumor growth in vivo through targeting miR-384/POU2F1 axis, rescue experiments including downregulation of miR-384 or overexpression of POU2F1 whether reversed the protective effect of sh-DUXAP8 in laryngeal cancer in vivo should be performed to confirm the role of DUXAP8.

Conclusion

In summary, the present study demonstrated that IncRNA DUXAP8 was significantly upregulated in laryngeal cancer. Moreover, DUXAP8 promoted the progression of laryngeal cancer through upregulating
POU2F1 by directly sponging miR-384. Our results suggested that DUXAP8 might be a novel target for the treatment of laryngeal cancer.

**Abbreviations**

Not applicable

**Declarations**

**Ethics approval and consent to participate**

This study was approved by Beijing Chaoyang Hospital of Capital Medical University Ethics Committee and all the patients or parents/guardians of patients provided written informed consent.

**Consent for publication**

Not Applicable.

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

No potential conflicts of interest were disclosed.

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**Authors’ contributions**

NW designed the study. ZY carried out experiments and wrote the manuscript, NW revised the paper, XW, JD, JL and PH collected patient specimens and related information. ZY, XW, JD, JL and PH contributed to analysing the data. All authors reviewed the results and approved the final version of the manuscript.

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Not applicable
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Table 1: The clinicopathological characteristics of 90 laryngeal cancer patients.

| Characteristics       | No. of cases (%) |
|-----------------------|------------------|
| Sex                   |                  |
| Male                  | 49 (54.44)       |
| Female                | 41 (45.56)       |
| Age                   |                  |
| <60                   | 46 (51.11)       |
| ≥60                   | 44 (48.89)       |
| Smoking               |                  |
| Never                 | 6 (6.67)         |
| Ever                  | 84 (93.33)       |
| Drinking              |                  |
| Never                 | 16 (17.78)       |
| Ever                  | 74 (82.22)       |
| Primary location      |                  |
| Supraglottic          | 42 (46.67)       |
| Glottic               | 48 (53.33)       |
| Clinical stage        |                  |
| I–II                  | 50 (55.56)       |
| III–IV                | 40 (44.44)       |