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ORIGINAL ARTICLE

# Tumor-suppressive microRNAs (*miR-26a/b*, *miR-29a/b/c* and *miR-218*) concertedly suppressed metastasis-promoting *LOXL2* in head and neck squamous cell carcinoma

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**In spite of considerable advances in multimodality therapy, including surgery, radiotherapy and chemotherapy, the overall survival rate for patients with head and neck squamous cell carcinoma (HNSCC) is very poor (only 15–45%). Understanding the molecular mechanisms of metastatic pathways underlying HNSCC using currently available genomic approaches might improve therapies for and prevention of the disease. Our previous studies showed that three tumor-suppressive microRNAs (miRNAs), *miR-26a/b*, *miR-29a/b/c* and *miR-218*, significantly inhibited cancer cell migration and invasion. Therefore, we hypothesized that these miRNAs-regulated target genes deeply contributed to cancer metastasis. These tumor-suppressive miRNAs directly regulate *LOXL2* expression in HNSCC cells by using *in silico* analysis and luciferase reporter assays. Overexpressed *LOXL2* was confirmed in HNSCC clinical specimens, and silencing of *LOXL2* inhibited cancer cell migration and invasion in HNSCC cell lines. Our present data showed that tumor-suppressive miRNAs regulation of *LOXL2* will provide new insights into the novel molecular mechanisms of HNSCC metastasis.**
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## INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world, and 650 000 new cases are diagnosed every year.[1] Despite considerable advances in multimodality therapy, including surgery, radiotherapy and chemotherapy, the overall survival rate for patients with HNSCC is only 15–45%.[2,3] Patients with HNSCC are usually diagnosed at a late stage, and local tumor recurrence and distant metastasis often occur after conventional therapies.[2,3] Metastatic disease is responsible for about 90% of deaths within 12 months of diagnosis in patients with HNSCC.[1] Therefore, understanding the molecular mechanisms of the metastatic pathways underlying HNSCC using currently available genomic approaches may improve therapies for and prevention of the disease.

MicroRNAs (miRNAs) are endogenous small noncoding RNA molecules (19–22 bases in length) that function to regulate protein-coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner.[4] To date, a substantial amount of evidence has suggested that miRNAs are aberrantly expressed in many human cancers and have significant roles in human oncogenesis and metastasis.[5–7] We sequentially identified tumor-suppressive miRNAs that contribute to cancer cell migration and invasion based on the miRNA expression signatures of HNSCC.[8–11] Among these tumor-suppressive miRNAs, *microRNA* (*miR*)*-26a/b*, *miR-29a/b/c* and *miR-218* significantly inhibit cancer cell migration and invasion through targeting of genes encoding extracellular matrix (ECM) components and integrins.[11–13]

The ECM is composed of secreted molecules that constitute the cell microenvironment, including a dynamic and complex array of glycoproteins, collagens, glycosaminoglycans and proteoglycans.[14] The ECM imparts spatial context for signaling events through various cell surface growth factor receptors and adhesion molecules, such as integrins.[14] Recent studies have shown that ECM components and ECM-related receptor proteins are aberrantly expressed in cancer tissues and that such dysregulation of the composition and organization of the ECM can induce activation of specific signaling pathways.[15] Integrins are a large family of cell surface receptors composed of two subunits (α and β), which bind to ECM components. Most types of cells require integrin-mediated signal pathways for proliferation, migration, invasion and survival.[16] Several studies have shown that overexpression or activation of integrin-mediated cancer signals

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promotes cancer cell progression and metastasis.[17–19] For example, the interaction between laminin-332 and α6β4 integrin triggers a number of signaling cascades in cancer cells, promoting both cell migration and cancer cell survival in HNSCC.[12,13] We also showed that tumor-suppressive function of *miR-218* and *miR-29a/b/c* in prostate cancer and renal cell carcinoma through targeting ECM component genes.[20,21]

In cancer cells, aberrant expression of miRNAs disrupts tightly controlled miRNA/protein-coding RNA networks. Therefore, identification of tumor-suppressive miRNAs that act together to regulate oncogenic genes is the first step in elucidating the mechanisms of human oncogenesis and metastasis. The aim of the current study was to identify miRNAs (*miR-26a/b*, *miR-29a/b/c* and *miR-218*) that function in concert to downregulate metastasis-promoting genes in HNSCC. Genome-wide gene expression data and *in silico* analysis revealed that several putative candidate genes were regulated by these tumor-suppressive miRNAs. Here we focused on the lysyl oxidase-like 2 (*LOXL2*) gene. Our present data showed that *LOXL2* was overexpressed in HNSCC clinical specimens and that silencing of the *LOXL2* gene significantly inhibited the migration and invasion of cancer cells. Moreover, *LOXL2* was a direct target of *miR-26a/b*, *miR-29a/b/c* and *miR-218* regulation, as shown using luciferase reporter assays. These data suggested that the pathway involving regulation of *LOXL2* by tumor-suppressive miRNAs may be a potential target in the development of novel therapies to treat HNSCC.

## MATERIALS AND METHODS

### Clinical HNSCC specimens

A total of 19 pairs of primary HNSCC and corresponding normal epithelial tissue were obtained from patients with HNSCC at Chiba University Hospital (Chiba, Japan) from 2008 to 2015. The patients' backgrounds and clinicopathological characteristics are shown in Table 1. The patients were classified according to the 2002 Union for International Cancer Control Tumor-Node-Metastasis (TNM) staging criteria before treatment. Written consent for tissue donation for research purposes was obtained from each patient before tissue collection. The protocol was approved by the Institutional Review Board of Chiba University. The specimens were immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at − 20 °C until RNA extraction.

**Table 1 Clinical features of 19 patients with HNSCC**

| *No.* | *Age* | *Sex* | *Location* | *T* | *N* | *M* | *Stage* | *Differentiaion* |
|---|---|---|---|---|---|---|---|---|
| 1 | 68 | M | Tongue | 2 | 0 | 0 | II | Well |
| 2 | 66 | M | Tongue | 2 | 0 | 0 | II | Moderate |
| 3 | 76 | F | Tongue | 1 | 0 | 0 | I | Well |
| 4 | 69 | M | Tongue | 1 | 0 | 0 | I | Well |
| 5 | 73 | F | Tongue | 1 | 0 | 0 | I | Well |
| 6 | 67 | M | Tongue | 4a | 2c | 0 | IVA | Moderate |
| 7 | 52 | M | Oropharynx | 3 | 2a | 0 | IVA | Moderate |
| 8 | 76 | M | Oropharynx | 2 | 0 | 0 | II | Moderate |
| 9 | 55 | M | Oropharynx | 4a | 2c | 0 | IVA | Moderate |
| 10 | 74 | M | Oropharynx | 2 | 0 | 0 | II | Well |
| 11 | 63 | M | Larynx | 3 | 0 | 0 | III | Moderate |
| 12 | 69 | M | Larynx | 3 | 0 | 0 | III | Well |
| 13 | 66 | M | Larynx | 4a | 0 | 0 | IVA | Moderate |
| 14 | 82 | M | Larynx | 3 | 0 | 0 | III | Poor |
| 15 | 68 | M | Hypopharynx | 4a | 0 | 0 | IVA | Moderate |
| 16 | 73 | M | Hypopharynx | 3 | 1 | 0 | III | Poor |
| 17 | 66 | M | Hypopharynx | 2 | 2c | 0 | IVA | Moderate |
| 18 | 68 | M | Hypopharynx | 2 | 2b | 0 | IVA | Poor |
| 19 | 65 | M | Hypopharynx | 1 | 2b | 0 | IVA | Moderate |

Abbreviation: HNSCC, head and neck squamous cell carcinoma.

### Cell lines

The following human HNSCC cell lines were used: FaDu (derived from the primary tumor in a patient with hypopharyngeal SCC) and SAS (derived from the primary tumor of a patient with tongue SCC). Both cell lines were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% $CO_2$ at 37 °C.

### RNA isolation

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA concentrations were determined spectrophotometrically, and molecular integrity was checked by gel electrophoresis. The quality of RNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

### Quantitative real-time reverse transcription polymerase chain reaction (qRT–PCR)

The expression levels of *miR-26a* (Assay ID: 000405), *miR-26b* (Assay ID: 000407), *miR-29a* (Assay ID: 002112), *miR-29b* (Assay ID: 000413), *miR-29c* (Assay ID: 000413) and *miR-218* (Assay ID: 000587) were analyzed by TaqMan qRT–PCR and normalized to *RNU48* (Assay ID: 001006). TaqMan probes and primers for *LOXL2* (P/N: Hs00158757_m1; Applied Biosystems, Foster City, CA, USA), *LOX* (P/N: Hs00942480_m1) and *GUSB* (P/N: Hs99999908_ml) as an internal control were obtained from Applied Biosystems (Assay-On-Demand Gene Expression Products).

### Immunohistochemistry

Three hypopharyngeal SCC tissue specimens were immunostained following the manufacture's protocol with the Ultra-Vision Detection System (Thermo Scientific, Fremont, CA, USA). Primary rabbit polyclonal antibodies against LOXL2 were diluted 1:1000. The slides were treated with biotinylated goat antibodies.

### Transfection with mature miRNAs and small-interfering RNA

The following mature miRNAs were used in this study: mirVana miRNA mimics for *hsa-miR-26a-5p* (product ID: PM10249), *hsa-miR-26b-5p* (product ID: PM12899), *hsa-miR-29a-3p* (product ID: MC12499), *hsa-miR-29b-3p* (product ID: MC10103), *hsa-miR-29c-3p* (product ID: MC10518) and *hsa-miR-218* (product ID: AM17100; Applied Biosystems). The following si-RNAs were used in this study: stealth select RNAi si-RNA, *si-LOXL2* (P/N: HSS180848; Invitrogen), and negative control miRNA/small-interfering RNA (P/N: AM17111, Applied Biosystems). Transfection methods were described previously.[8–13,20,21]

### Cell proliferation, migration and invasion assays

To investigate the functional roles of LOXL2, we performed cell proliferation, migration and invasion assays using *si-LOXL2*-transfected FaDu and SAS cells. The experimental procedures were performed as described in our previous studies.[9–13,20,21]

### Identification of putative target genes regulated by *miR-26a/b*, *miR-29a/b/c* and *miR-218*

To investigate putative target genes regulated by these miRNAs, we use *in silico* analysis. First, we screened genes using TargetScan Release 6.2 (http://www.targetscan.org/). To identify upregulated genes in HNSCC, we analyzed a publicly available gene expression data set in GEO (accession number 9638).

### Western blotting

Cells were collected 72 h after transfection and lysates were prepared. Next, 20 μg of protein lysates were separated on Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA, USA) and transfered to PVDF membranes. Immunoblotting was performed with rabbit anti-LOXL2 antibodies (1:1000; ab96233, Abcam, Cambridge, UK); anti-GAPDH antibodies (1:4000; ab8245, Abcam) were used to detect GAPDH as an internal loading control. Membranes were washed and incubated with anti-rabbit IgG horseradish peroxidase-linked antibodies (7074; Cell Signaling Technology, Danvers, MA, USA). Complexes were visualized

with Clarity Western ECL Substrate (Bio-Rad). The experimental procedures were described previously.[8–13,20,21]

### Plasmid construction and dual-luciferase assays

Partial wild-type sequences of the LOXL2 3′-untranslated region (UTR) or those with deleted *miR-26a/b*, *miR-29a/b/c* and *miR-218* binding sites were inserted between the XhoI-PmeI restriction sites in the 3′-UTR of the *hRuc* gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The procedure for the dual-luciferase reporter assay was described previously.[8–13,20,21]

### Identification of genes regulated by LOXL2 in HNSCC

To identify the genes regulated by *LOXL2*, we performed gene expression analysis using *si-LOXL2*-transfected FaDu cells. An oligo-microarray (human 60k v; Agilent Technologies) was used for gene expression studies.

### Statistical analysis

The relationships between two groups and the numerical values obtained by real-time RT–PCR were analyzed using paired *t*-tests. Spearman's rank test was used to evaluate the correlation between the expression of *miR-26/b*, *miR-29a/b/c* and *miR-218* and the expression of *LOXL2*. The relationships among more than three variables and numerical values were analyzed using Bonferroni-adjusted Mann–Whitney *U*-tests. All analyses were performed using Expert Stat View (version 4, SAS Institute Inc., Cary, NC, USA).

## RESULTS

### Selection of candidate genes regulated by tumor-suppressive miRNAs (*miR-26a/b*, *miR-29a/b/c* and *miR-218*) in HNSCC

To identify putative target genes regulated by tumor-suppressive miRNAs, particularly those inhibiting cancer cell migration and invasion in HNSCC cells (that is, *miR-26a/b*, *miR-29a/b/c* and *miR-218*), as described our previous studies,[11–13] we used *in silico* analysis and genome-wide gene expression analysis. Our strategy for selection of target genes is shown in Figure 1. We screened target genes using the TargetScan database and identified 334 genes. Next, these genes were then analyzed with a publicly available gene expression data set in GEO (accession number: GSE9638) and upregulated genes (log2 ratio $>1.5$) were chosen. As a result, 26 genes were identified as putative target genes regulated by *miR-26a/b*, *miR-29a/b/c* and *miR-218* (Table 2). To show the effectiveness of Table 2, we checked the public database. Previous studies showed that *HAPLN1* (hyaluronan an proteoglycan link protein 1) and *POFUT1* (protein O-fucosyltransferase 1) were upregulated in cancer tissues and act as oncogenes in oral squamous cell carcinoma.[22,23] The new knowledge of HNSCC might be offered by analyzing the gene included in this list. Among these candidate genes, we focused on *LOX* and *LOXL2* because these genes have been reported to promote metastasis in several types of cancers. Furthermore, our recent study of renal cell carcinoma showed that *miR-29a/b/c* significantly inhibited cancer cell migration and invasion directly targeting *LOXL2*.[21] These data suggest that *LOXL2* member deeply contribute to cancer cell metastasis.

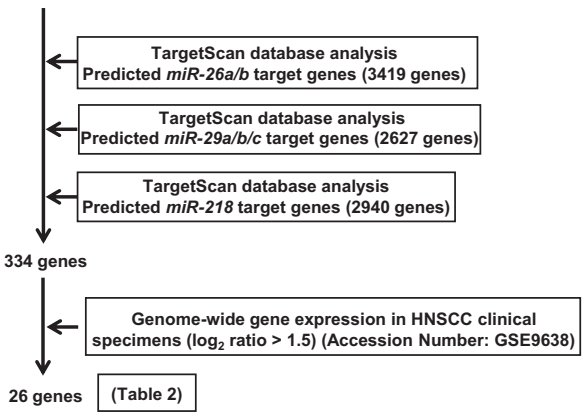


**Figure 1** Selection for putative target genes regulated by tumor-suppressive miRNAs (*miR-26a/b*, *miR-29a/b/c* and *miR-218*) in HNSCC. A total of 334 genes were identified as target genes containing binding sites for *miR-26a/b*, *miR-29a/b/c* and *miR-218*. Among these, 26 genes were upregulated in HNSCC clinical specimens (accession number: GSE9638). HNSCC, head and neck squamous cell carcinoma.

### Expression levels of LOXL2 in HNSCC clinical specimens and cell lines

Next, we evaluated the expression levels of *LOXL2* mRNA in HNSCC clinical specimens. The expression of *LOXL2* was significantly upregulated in cancer tissues compared with that in normal tissues ($P=0.0057$), as demonstrated by qRT–PCR (Figure 2a). We also determined the expression levels of LOXL2 protein in hypopharyngeal SCC specimens by immunohistochemical staining. LOXL2 was strongly expressed in several cancerous lesions, whereas no or low expression was observed in normal tissues (Figure 2b–d). The patients' backgrounds and clinicopathological characteristics are summarized in Table 1.

Finally, the expression levels of *LOX* in HNSCC clinical specimens were evaluated by qRT–PCR (Supplementary Figure 1). Overexpression of *LOX* was observed in clinical specimens. The mRNA expression of *LOX* was significantly repressed in *miR-29a/b/c* or *miR-218* transfectant compared with mock- or miR-control-transfectant cells. However, no suppressive effect was observed in *miR-26a/b* transfectant cells (Supplementary Figure 1). In addition, high expression of *LOX* was not found in cancer cell lines (FaDu and SAS). Therefore, we omitted this gene as a target of further analysis in the study.

### Expression levels of tumor-suppressive miRNAs in HNSCC clinical specimens and cell lines

The expression levels of *miR-26a*, *miR-26b*, *miR-29a*, *miR-29c* and *miR-218* were significantly lower in tumor tissues and cell lines (FaDu and SAS) than in corresponding normal epithelial tissues, as shown by qRT–PCR (Figures 3a, 4a and 5a). On the other hand, *miR-29b* expression was not significantly downregulated in clinical cancer tissues (Figure 4a).

Spearman's rank test showed negative correlations between the expression of *miR-26a*, *miR-26b*, *miR-29a*, *miR-29c* and *miR-218* and the expression of *LOXL2* in HNSCC clinical specimens (Figures 3b, 4b and 5b). However, there was no negative correlation between the expression levels of *miR-29b* and *LOXL2* (Figure 4b).

### LOXL2 was directly regulated by tumor-suppressive miRNAs (*miR-26a/b*, *miR-29a/b/c* and *miR-218*) in HNSCC cells

Next, we performed qRT–PCR and western blotting in FaDu and SAS cells to investigate whether *LOXL2* expression was downregulated by restoration of *miR-26a/b*, *miR-29a/b/c* or *miR-218*. The expression levels of LOXL2 mRNA and protein were significantly repressed in cells transfected with *miR-26a/b*, *miR-29a/b/c* or *miR-218* compared with that in mock- or miR-control-transfected cells (Figures 6a,b, 7a,b and 8a,b).

Furthermore, we performed luciferase reporter assay in SAS cells to determine whether *LOXL2* mRNA contained target sites for *miR-26a/b*, *miR-29a/b/c* and *miR-218*. We used vectors encoding

**Table 2 Putative target genes regulated by *miR-26a/b*, *miR-29a/b/c* and *miR-218***

| *miR-26a/b, miR-29a/b/c and miR-218 target genes* | *Representative transcript* | *Gene name* | *HNSCC log2 ratio* | *miR-26ab* | | *miR-29abc* | | *miR-218* | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *Conserved* | *Poorly* | *Conserved* | *Poorly* | *Conserved* | *Poorly* |
| *C11orf41* | NM_012194 | Chromosome 11 open reading frame 41 | 3.36 | 1 | 4 | 1 | 1 | 0 | 1 |
| *NTRK2* | NM_001018065 | Neurotrophic tyrosine kinase, receptor, type 2 | 3.33 | 0 | 1 | 0 | 2 | 0 | 3 |
| *RAB3B* | NM_002867 | RAB3B, member RAS oncogene family | 2.80 | 0 | 1 | 0 | 1 | 0 | 4 |
| *HAPLN1* | NM_001884 | Hyaluronan and proteoglycan link protein 1 | 2.67 | 1 | 1 | 1 | 0 | 2 | 0 |
| *ZIC5* | NM_033132 | Zic family member 5 | 2.58 | 1 | 1 | 1 | 0 | 0 | 1 |
| *ONECUT2* | NM_004852 | One cut homeobox 2 | 2.51 | 1 | 0 | 1 | 0 | 4 | 1 |
| *COL5A1* | NM_000093 | Collagen, type V, alpha 1 | 2.44 | 1 | 1 | 4 | 0 | 0 | 1 |
| *PNPLA3* | NM_025225 | Patatin-like phospholipase domain containing 3 | 2.40 | 0 | 2 | 0 | 1 | 0 | 1 |
| *LOXL2* | NM_002318 | Lysyl oxidase-like 2 | 2.39 | 2 | 0 | 1 | 1 | 0 | 1 |
| *FREM2* | NM_207361 | FRAS1 related extracellular matrix protein 2 | 2.34 | 0 | 1 | 2 | 1 | 0 | 2 |
| *FAM167A* | NM_053279 | Family with sequence similarity 167, member A | 2.29 | 0 | 1 | 1 | 1 | 0 | 1 |
| *ZNF469* | NM_001127464 | Zinc finger protein 469 | 2.17 | 1 | 0 | 1 | 0 | 0 | 1 |
| *TBC1D24* | NM_001199107 | TBC1 domain family, member 24 | 2.05 | 0 | 1 | 0 | 1 | 0 | 2 |
| *ENTPD7* | NM_020354 | Ectonucleoside triphosphate diphosphohydrolase 7 | 1.94 | 0 | 1 | 1 | 0 | 0 | 1 |
| *LOX* | NM_001178102 | Lysyl oxidase | 1.81 | 0 | 1 | 3 | 0 | 1 | 1 |
| *POFUT1* | NM_015352 | Protein O-fucosyltransferase 1 | 1.77 | 0 | 1 | 0 | 1 | 0 | 1 |
| *SCAI* | NM_001144877 | Suppressor of cancer cell invasion | 1.69 | 0 | 1 | 1 | 0 | 3 | 1 |
| *DENND5B* | NM_144973 | DENN/MADD domain containing 5B | 1.64 | 1 | 1 | 1 | 0 | 0 | 1 |
| *SCAMP5* | NM_001178111 | Secretory carrier membrane protein 5 | 1.63 | 0 | 1 | 1 | 0 | 1 | 1 |
| *PTPRD* | NM_001040712 | Protein tyrosine phosphatase, receptor type, D | 1.62 | 1 | 0 | 1 | 0 | 0 | 1 |
| *ZNF697* | NM_001080470 | Zinc finger protein 697 | 1.61 | 1 | 0 | 0 | 1 | 1 | 0 |
| *NAV1* | NM_001167738 | Neuron navigator 1 | 1.60 | 1 | 0 | 4 | 0 | 0 | 2 |
| *C11orf87* | NM_207645 | Chromosome 11 open reading frame 87 | 1.59 | 0 | 1 | 2 | 0 | 1 | 1 |
| *ACPL2* | NM_001037172 | Acid phosphatase-like 2 | 1.59 | 1 | 0 | 1 | 0 | 0 | 1 |
| *LMLN* | NM_001136049 | Leishmanolysin-like (metallopeptidase M8 family) | 1.58 | 1 | 0 | 1 | 0 | 1 | 0 |
| *ABL2* | NM_001136000 | v-abl Abelson murine leukemia viral oncogene homolog 2 | 1.57 | 3 | 2 | 0 | 1 | 1 | 1 |

Abbreviation: HNSCC, head and neck squamous cell carcinoma.

either a partial wild-type sequence or a sequence in which the miRNA binding site was deleted from the 3′-UTR of *LOXL2* mRNA. We found that the luminescence intensity was significantly reduced by co-transfection with *miR-26a/b*, *miR-29a/b/c*, *miR-218* and the vector carrying the wild-type 3′-UTR of *LOXL2* mRNA (Figures 6c, 7c and 8c).

### Effects of silencing LOXL2 on cell proliferation, migration and invasion in HNSCC cell lines

To investigate the functional role of *LOXL2*, we performed loss-of-function studies using *si-LOXL2* transfectants. First, we evaluated the knockdown efficiency of *si-LOXL2* transfection in FaDu and SAS cells. Western blotting and qRT–PCR indicated that the si-RNAs effectively downregulated LOXL2 expression in FaDu and SAS cells (Figure 9a,b).

XTT assays demonstrated that cell proliferation was significantly inhibited in *si-LOXL2-1* transfectants compared with that in mock- or *si-control*-transfected SAS cells. On the other hand, proliferation was not inhibited in FaDu cells (Figure 9c). Migration and invasion assays demonstrated that cell migration and invasion activities were significantly inhibited in *si-LOXL2* transfectants compared with that in mock or *si-control* transfectants for both cell lines (Figure 9d,e).

### Identification of downstream pathways regulated by LOXL2

Few reports have described the functional significance of LOXL2 in HNSCC. Therefore, we investigated the molecular pathways regulated by LOXL2 in FaDu cells using genome-wide gene expression analysis in *si-LOXL2* transfectants. Twenty-five genes were downregulated (log2 ratio $< -1$) in response to *si-LOXL2* transfection and upregulated (log2 ratio $>1$) in HNSCC clinical specimens (accession number: GSE9638; Table 3).

## DISCUSSION

The highly invasive properties of cancer cells frequently cause locoregional recurrence and distant metastasis in patients with HNSCC, leading to an expected 5-year survival rate of ~50% for patients with advanced disease.[24,25] Therefore, understanding the molecular mechanisms of metastatic pathways underlying HNSCC using current genomic approaches, including noncoding RNA networks, would facilitate the development of novel therapies for and prevention of the disease. For elucidation of novel metastatic RNA networks in HNSCC, we sequentially identified tumor-suppressive miRNAs that regulated oncogenic genes and HNSCC-related pathways.[11–13] Our previous studies of the functional significance of downregulated miRNAs in HNSCC expression signatures showed that six miRNAs (that is, *miR-26a/b*, *miR-29a/b/c* and *miR-218*) significantly inhibit cancer cell migration and invasion,[11–13] suggesting that these miRNAs act to suppress metastasis in human cancers. Therefore, we hypothesized that these tumor-suppressive miRNAs (that is, *miR-26a/b*, *miR-29a/b/c* and *miR-218*) would regulate genes with key functions in HNSCC metastasis.

Previous studies have shown that *miR-26a* and *miR-26b* act as tumor suppressors by targeting oncogenic genes in several types of cancers, such as breast cancer, nasopharyngeal carcinoma and hepatocellular carcinoma. More recently, the overexpression of

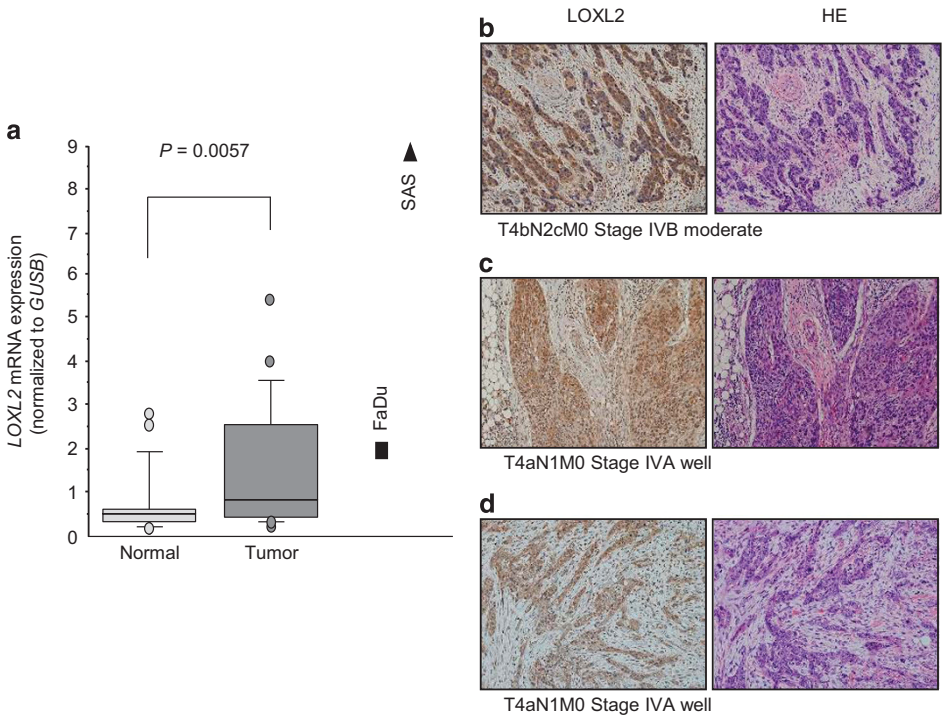


**Figure 2** Expression levels of LOXL2 in HNSCC clinical specimens and cell lines. (**a**) Expression levels of *LOXL2* mRNA in HNSCC clinical specimens and cell lines (FaDu and SAS). *GUSB* was used as an internal control. (**b**–**d**) Immunohistochemical staining for detection of LOXL2 and HE staining in three patients with hypopharyngeal squamous cell carcinoma. HNSCC, head and neck squamous cell carcinoma.

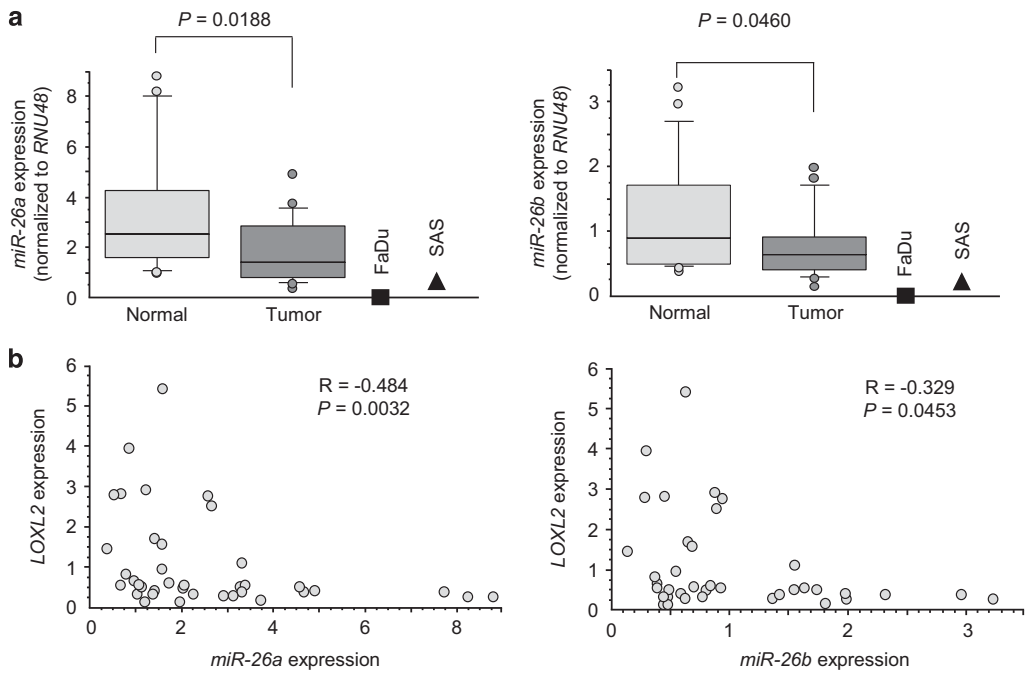


**Figure 3** Expression levels of *miR-26a* and *miR-26b* in HNSCC clinical specimens and cell lines. (**a**) Expression levels of *miR-26a* and *miR-26b* in clinical specimens and cell lines (FaDu and SAS). *RNU48* was used as an internal control. (**b**) Correlations between the expression levels of *LOXL2* and *miR-26a* or *miR-26b* were determined in HNSCC clinical specimens. Spearman's rank test was used to evaluate the correlations. HNSCC, head and neck squamous cell carcinoma.

*miR-26a* has been shown to inhibit tongue SCC cell proliferation and promote cell apoptosis.[11,26] Decreased expression of *miR-29a/b/c* has been observed in cholangiocarcinoma, nasopharyngeal cancer, non-small cell lung cancer, hepatocellular carcinoma, malignant peripheral nerve sheath tumors and mantle cell lymphoma.[27] Our recent studies of miRNA expression signatures in urothelial cancers and prostate cancer showed that *miR-218* is frequently downregulated in cancer tissues and functions as a tumor suppressor.[20,28] The tumor-suppressive

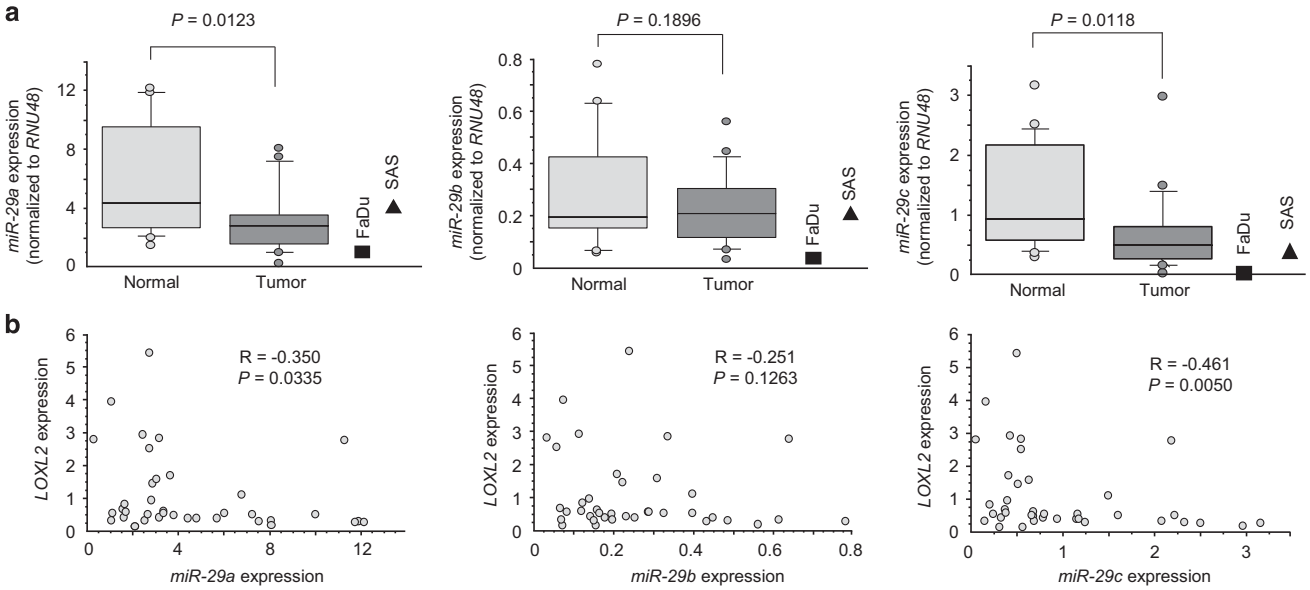


**Figure 4** Expression levels of *miR-29a*, *miR-29b* and *miR-29c* in HNSCC clinical specimens and cell lines. (**a**) Expression levels of *miR-29a*, *miR-29b* and *miR-29c* in clinical specimens and cell lines (FaDu and SAS). *RNU48* was used as an internal control. (**b**) Correlations between the expression levels of LOXL2 and *miR-29a*, *miR-29b* or *miR-29c* were determined in HNSCC clinical specimens. Spearman's rank test was used to evaluate the correlations. HNSCC, head and neck squamous cell carcinoma.

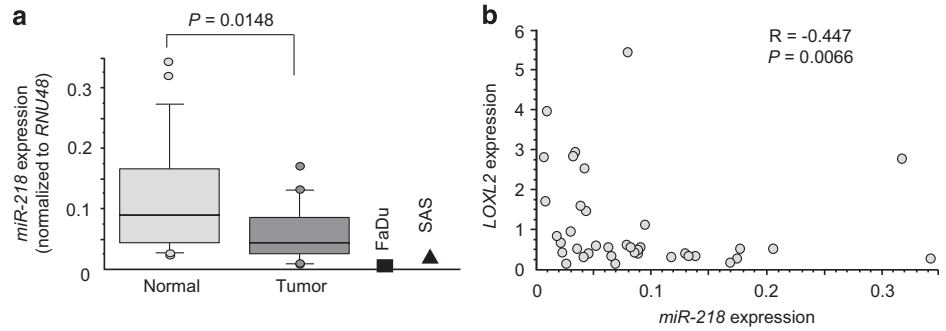


**Figure 5** Expression levels of *miR-218* in HNSCC clinical specimens and cell lines. (**a**) Expression levels of *miR-218* in clinical specimens and cell lines (FaDu and SAS). *RNU48* was used as an internal control. (**b**) The correlation between *LOXL2* and *miR-218* expression was determined in HNSCC clinical specimens. Spearman's rank test was used to evaluate the correlation. HNSCC, head and neck squamous cell carcinoma.

functions of *miR-218* in several types of cancers have been described by other research groups.[29]

To better understand cancer cell metastasis, we sequentially identified tumor-suppressive miRNAs mediating novel metastatic pathways using genome-wide gene expression analysis and *in silico* analysis.[11–13] Among the putative targets of *miR-26a/b*, *miR-29a/b/c* and *miR-218* regulation in HNSCC, we focused on the *LOX* and *LOXL2* genes as metastatic regulators in HNSCC cells. Importantly, our previous data in renal cell carcinoma showed that the *LOXL2* gene is a direct target of tumor-suppressive *miR-29a/b/c* regulation.[21] Past studies have shown that *LOX* and *LOXL2* are overexpressed in several types of cancers.[30–33] In the present study, we confirmed the overexpression of *LOX* and *LOXL2* in HNSCC clinical specimens. *LOXL2* was directly regulated by *miR-26a/b*, *miR-29a/b/c* and *miR-218*. *LOXL2* was directly regulated by *miR-26a/b*, *miR-29a/b/c* and *miR-218*. However, *LOX* was regulated by *miR-29a/b/c* and *miR-218*, not by *miR-26a/b* in HNSCC cells (Supplementary Figure 1). Thus, we focused on *LOXL2* and investigated the functional significance of *LOXL2* regulation in HNSCC. Our data showed that silencing of *LOXL2* significantly inhibited cancer cell migration and invasion, consistent with the function of LOX-family proteins in covalent crosslinking of collagen and/or elastin in the ECM.[34]

Collagens are the most abundant ECM component, and excessive ECM deposition has been observed in many cancers.[35–37] In cancer cells, collagens regulate the physical and biochemical properties of the cancer cell microenvironment, which modulates cancer cell polarity, migration and signaling.[38] Collagen crosslinking is extracellularly initiated by the LOX family of secreted enzymes, which are regulated by hypoxia-inducible factor and are induced under hypoxic conditions.[39] Hypoxic conditions also induce the expression of collagen-degrading proteins and matrix metalloproteinases (MMPs), thereby contributing to ECM remodeling; these mechanisms are mediated by several families of proteinases that have been suggested to promote cancer cell invasion.[40] In this study, we investigated the pathways downstream of *LOXL2* in HNSCC cells following knockdown of *LOXL2* using *si-LOXL2*. Among the putative *LOXL2* downstream genes, *PLOD3* encodes enzymes that mediate collagen lysine hydroxylation,[41] and *SERPINH1* encodes a member of the serpin superfamily of serine proteinase inhibitors, which has a role in

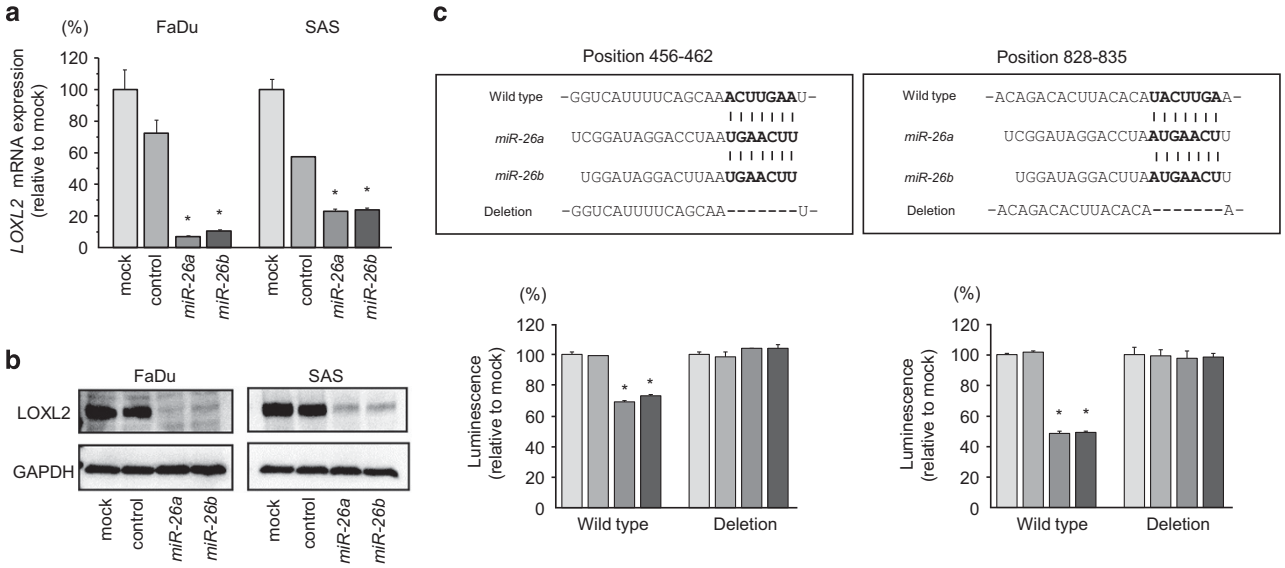


**Figure 6** Direct regulation of *LOXL2* by *miR-26a* and *miR-26b*. (**a**) *LOXL2* mRNA expression 72 h after transfection with *miR-26a* and *miR-26b*. *GUSB* was used as an internal control. (**b**) LOXL2 protein expression 72 h after transfection with *miR-26a* and *miR-26b*. GAPDH was used as a loading control. (**c**) *miR-26a* and *miR-26b* binding sites in the 3′-UTR of *LOXL2* mRNA. Luciferase reporter assays used vectors that included the wild-type sequence of putative *miR-26a* and *miR-26b* binding sites or mutants in which the binding sites were deleted (Deletion). Renilla luciferase signals were normalized to firefly luciferase signals. UTR, untranslated region.

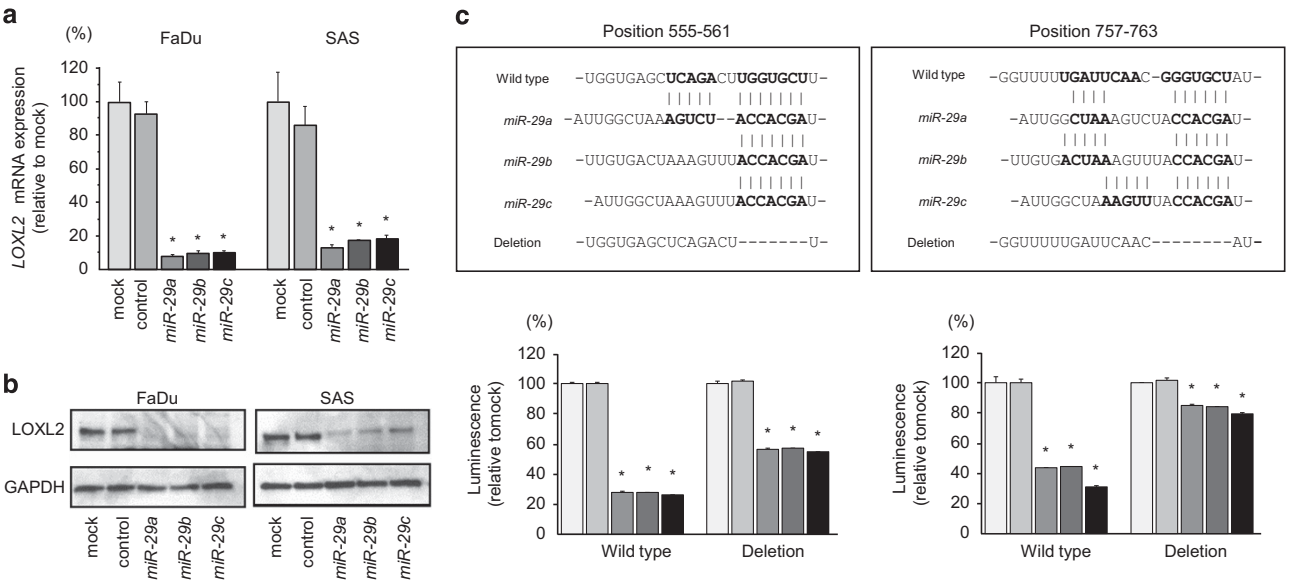


**Figure 7** Direct regulation of *LOXL2* by *miR-29a*, *miR-29b* and *miR-29c*. (**a**) *LOXL2* mRNA expression 72 h after transfection with *miR-29a*, *miR-29b* and *miR-29c*. *GUSB* was used as an internal control. (**b**) LOXL2 protein expression 72 h after transfection with *miR-29a*, *miR-29b* and *miR-29c*. GAPDH was used as a loading control. (**c**) *miR-29a*, *miR-29b* and *miR-29c* binding sites in the 3′-UTR of *LOXL2* mRNA. Luciferase reporter assays used vectors that included wild-type sequences of putative *miR-29a*, *miR-29b* and *miR-29c* binding sites or mutants in which the binding sites were deleted. Renilla luciferase signals were normalized to firefly luciferase signals. UTR, untranslated region.

collagen biosynthesis as a collagen-specific molecular chaperone.[42] Moreover, *MMP1*, which is also downstream of *LOXL2*, encodes a secreted enzyme that breaks down the interstitial collagens, that is, types I, II and III.[43] Thus, these proteins increase the crosslinking of collagens and other ECM components, subsequently promoting matrix stiffness. Moreover, *LOXL2*-mediated ECM deposition seems to function in synergy with MMP activity, which may lead to remodeling of the ECM in such a way as to increase metastasis.[40]

The intracellular function of LOXL2 is still not clear. Several transcription factors are upregulated in metastatic cells that are undergoing the epithelial-mesenchymal transition, including Snail, TWIST, ZEB and others.[44] Transforming growth factor-β has a critical role in activating Snail, which in turn downregulates E-cadherin; this process promotes the epithelial-mesenchymal transition.[45] Interestingly, past studies have shown that nuclear LOXL2 interacts with the transcription factor Snail1, represses E-cadherin and induces the epithelial-mesenchymal transition.[46] These findings indicated that LOXL2 deeply contribute to activation of metastatic pathways in HNSCC. Recent study showed that LOXL2 binds *NOTCH1* promoter region and repressed NOTCH1 transcription.[47] Several studies

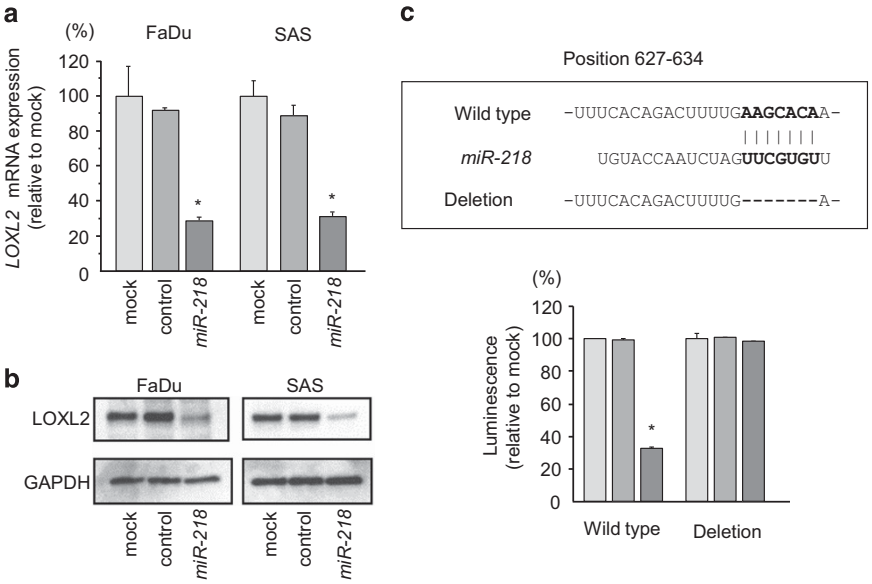


**Figure 8** Direct regulation of *LOXL2* by *miR-218*. (**a**) *LOXL2* mRNA expression 72 h after transfection with *miR-218*. *GUSB* was used as an internal control. (**b**) LOXL2 protein expression 72 h after transfection with *miR-218*. GAPDH was used as a loading control. (**c**) *miR-218* binding sites in the 3′-UTR of *LOXL2* mRNA. Luciferase reporter assays used vectors that included the wild-type sequence of the putative *miR-218* binding site or a mutant in which the binding sites were deleted. Renilla luciferase signals were normalized to firefly luciferase signals. UTR, untranslated region.

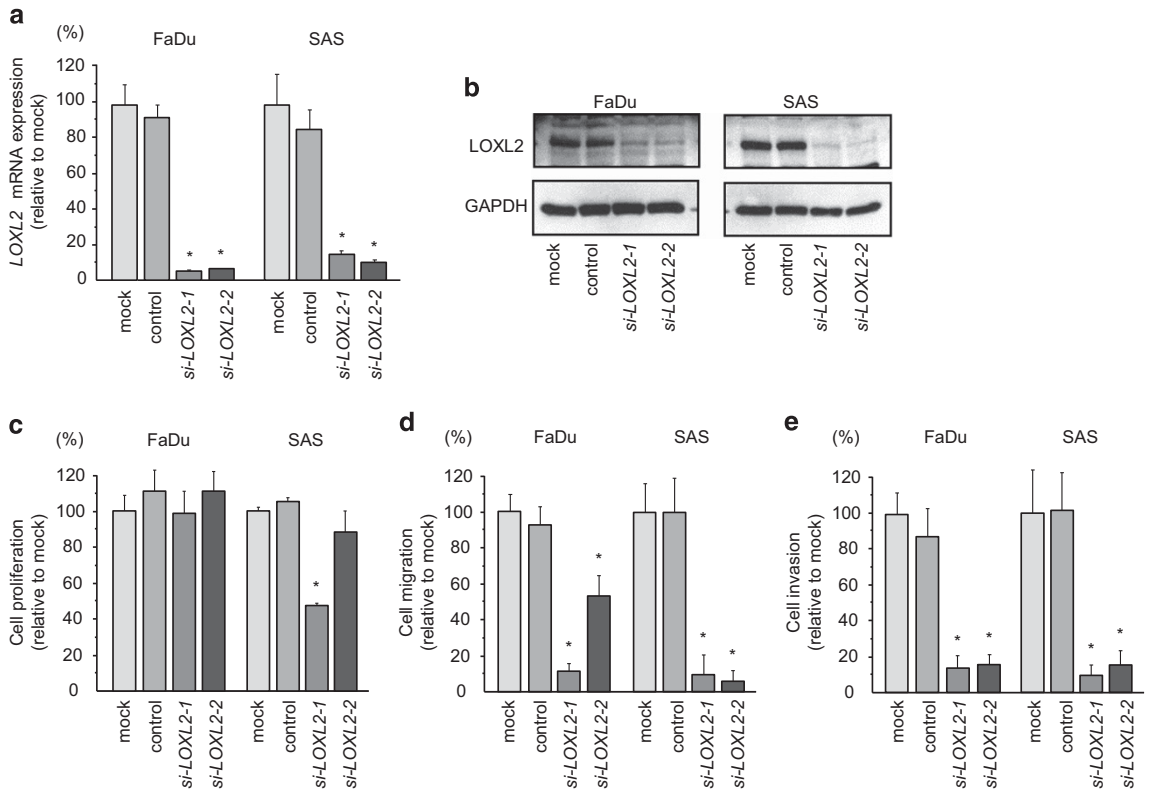


**Figure 9** Effects of si-*LOXL2* transfection on HNSCC cell lines. (**a**) Expression of *LOXL2* mRNA 72 h after transfection with si-*LOXL2* in FaDu and SAS cells. *GUSB* was used as an internal control. (**b**) Protein expression of LOXL2 72 h after transfection with si-*LOXL2*. GAPDH was used as a loading control. (**c**) Cell proliferation 72 h after transfection with 10 nM si-*LOXL2*. (**d**) Cell migration 72 h after transfection with 10 nM si-*LOXL2*. (**e**) Cell invasion 72 h after transfection with 10 nM si-*LOXL2*. HNSCC, head and neck squamous cell carcinoma.

indicated that NOTCH mutation and NOTCH-mediated signal pathways can have either an oncogenic or a tumor-suppressive effects.[48] A further study is necessary about LOXL2-NOTCH signaling pathways and HNSCC oncogenesis.

In conclusion, downregulation of *miR-26a/b*, *miR-29a/b/c* and *miR-218* was frequently observed in HNSCC clinical specimens. These miRNAs functioned as tumor suppressors, inhibiting cancer cell migration and invasion. Our present study showed that *LOXL2* was

**Table 3 Downregulated genes after transfection with *si-LOXL2* in FaDu cells**

| *Gene symbol* | *Gene name* | *Cytoband* | *si-LOXL2 TF (log2 ratio)* | *Clinical HNSCCs (log2 ratio)* |
|---|---|---|---|---|
| *LOXL2* | Lysyl oxidase-like 2 | 8p21.3 | −3.70 | 2.39 |
| *NMB* | Neuromedin B | 15q25.2 | −2.68 | 2.36 |
| *SLC44A1* | Solute carrier family 44 (choline transporter), member 1 | 9q31.1 | −2.56 | 1.11 |
| *NXNL2* | Nucleoredoxin-like 2 | 9q22.1 | −1.74 | 1.47 |
| *TRIP13* | Thyroid hormone receptor interactor 13 | 5p15.33 | −1.71 | 2.00 |
| *MCM7* | Minichromosome maintenance complex component 7 | 7q22.1 | −1.60 | 1.65 |
| *SERPINH1* | Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1) | 11q13.5 | −1.39 | 1.75 |
| *PRSS21* | Protease, serine, 21 (testisin) | 16p13.3 | −1.37 | 1.50 |
| *LEPREL1* | Leprecan-like 1 | 3q28 | −1.36 | 1.05 |
| *GINS1* | GINS complex subunit 1 (Psf1 homolog) | 20p11.21 | −1.34 | 1.55 |
| *PYCR1* | Pyrroline-5-carboxylate reductase 1 | 17q25.3 | −1.30 | 2.08 |
| *COL12A1* | Collagen, type XII, alpha 1 | 6q13 | −1.28 | 1.53 |
| *GAL* | galanin/GMAP prepropeptide | 11q13.3 | −1.27 | 3.54 |
| *MLLT11* | Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 11 | 1q21.3 | −1.24 | 2.53 |
| *MARVELD1* | MARVEL domain containing 1 | 10q24.2 | −1.22 | 1.39 |
| *CBX2* | Chromobox homolog 2 | 17q25.3 | −1.19 | 2.31 |
| *GRB10* | Growth factor receptor-bound protein 10 | 7p12.1 | −1.18 | 1.61 |
| *DHRS2* | Dehydrogenase/reductase (SDR family) member 2 | 14q11.2 | −1.18 | 2.18 |
| *FAM122B* | Family with sequence similarity 122B | Xq26.3 | −1.13 | 1.25 |
| *C12orf75* | Chromosome 12 open reading frame 75 | 12q23.3 | −1.11 | 1.77 |
| *MMP1* | Matrix metallopeptidase 1 (interstitial collagenase) | 11q22.2 | −1.06 | 5.32 |
| *CDCA7L* | Cell division cycle associated 7-like | 7p15.3 | −1.06 | 1.71 |
| *CENPM* | Centromere protein M | 22q13.2 | −1.02 | 1.67 |
| *PLOD3* | Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 | 7q22.1 | −1.01 | 1.04 |
| *PRR11* | Proline rich 11 | 17q22 | −1.00 | 1.18 |

Abbreviation: HNSCC, head and neck squamous cell carcinoma.

a direct target of all of these tumor-suppressive miRNAs in HNSCC cells. Moreover, *LOXL2* was upregulated in HNSCC clinical specimens and contributed to cancer cell migration and invasion, indicating that LOXL2 promoted metastasis. The identification of novel metastatic pathways and targets regulated by the tumor-suppressive miRNA-LOXL2 axis may lead to a better understanding of HNSCC and the development of new therapeutic strategies to treat this disease.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)