Passenger strand of miR-145-3p acts as a tumor-suppressor by targeting MYO1B in head and neck squamous cell carcinoma

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Abstract. Analysis of the microRNA (miRNA) expression signature of head and neck squamous cell carcinoma (HNSCC) based on RNA sequencing showed that dual strands of pre-miR-145 (miR-145-5p, guide strand; and miR-145-3p, passenger strand) were significantly reduced in cancer tissues. In miRNA biogenesis, passenger strands of miRNAs are degraded and have no biological activities in cells. The aims of this study were to investigate the functional significance of the passenger strand of miR-145 and to identify miR-145-3p-regulated oncogenic genes in HNSCC cells. Expression levels of miR-145-5p and miR-145-3p were significantly down-regulated in HNSCC tissues and cell lines (SAS and HSC3 cells). Ectopic expression of miR-145-3p inhibited cancer cell proliferation, migration and invasion, similar to miR-145-5p, in HNSCC cells. Myosin 1B (MYO1B) was directly regulated by miR-145-3p, and knockdown of MYO1B by siRNA inhibited cancer cell aggressiveness. Overexpression of MYO1B was confirmed in HNSCC clinical specimens by analysis of protein and mRNA levels. Interestingly, high expression of MYO1B was associated with poor prognosis in patients with HNSCC by analysis of The Cancer Genome Atlas database (p=0.00452). Our data demonstrated that the passenger strand of miR-145 acted as an antitumor miRNA through targeting MYO1B in HNSCC cells. The involvement of dual strands of pre-miR-145 (miR-145-5p and miR-145-3p) in the regulation of HNSCC pathogenesis is a novel concept in present RNA research.

Introduction

Head and neck squamous cell carcinoma (HNSCC) occurs from the mucosa in the upper aerodigestive tract, including the oral cavity, oropharynx, hypopharynx and larynx, and this disease is the sixth most common cancer worldwide (1). Approximately 550,000 new patients are diagnosed, and 30,000 patients die of this disease annually (2). Due to the local recurrence and distant metastasis of HNSCC, the overall survival of patients with HNSCC has not improved in the last decade (3). Currently developed targeted molecular therapies are not sufficiently efficacious in the management of HNSCC (3). Therefore, improving our understanding of the molecular mechanisms of HNSCC aggressiveness is needed based on current genomic approaches.

MicroRNAs (miRNAs) are small noncoding RNAs (19-22 nucleotides in length) involved in the repression or degradation of target RNA transcripts in a sequence-dependent manner (4). One of the unique features of miRNAs is that a single miRNA regulates a vast number of protein-coding or noncoding RNAs in human cells (5). Thus, aberrant expression of miRNAs disrupts systematically regulated RNA networks in cancer cells. In fact, accumulating evidence has revealed that aberrant expression of miRNAs is deeply involved in the pathogenesis of human cancers (6).

In miRNA biogenesis, precursor miRNA (pre-miRNA) is cleaved in the cytoplasm, generating a miRNA duplex comprised of a guide strand and passenger strand. The guide strand of miRNA is thought to be incorporated into the RNA-induced silencing complex (RISC) to target mRNAs, whereas the passenger strand of miRNA is degraded and is not thought to have regulatory activity in cells (7). However, in contrast to this paradigm, we demonstrated that passenger strands of miRNAs, i.e., miR-144-5p, miR-139-3p, miR-150-3p and miR-145-3p, were downregulated and acted as antitumor miRNAs in several types of cancers (8-13). Moreover, dual strands of pre-miR-145 (miR-145-5p and miR-145-3p) coordinately target oncogenic
MTDH and UHRF1 in lung cancer and bladder cancer, respectively (10,11). The involvement of passenger miRNA strands and regulation of cancer networks by passenger miRNAs are novel concepts in cancer research.

Analysis of the miRNA expression signature of HNSCC by RNA sequencing revealed that miR-145-5p and miR-145-3p were significantly downregulated in cancer tissues. The guide strand miR-145-5p has been established as an oncogene in several cancers, including HNSCC (14). However, the functional significance of the passenger strand of miR-145 in HNSCC is still unknown. The aims of this present study were to investigate the antitumor function of miR-145-3p and to identify its target oncogenic genes in HNSCC cells. Elucidation of the antitumor roles of passenger strands of miRNAs and the cancer networks mediated by these miRNAs may provide insights into the molecular pathogenesis of HNSCC.

Materials and methods

Clinical HNSCC specimens, cell lines, and cell culture
A total of 22 clinical tissue specimens were collected from patients with HNSCC who underwent surgical resection at Chiba University Hospital between 2008 and 2014. The clinicopathological features of patients with HNSCC are summarized in Table 1. All patients in this study provided informed consent, and the study protocol was approved by the Institutional Review Board of Chiba University. TNM classification and tumor stage were determined by the Union for International Cancer Control (UICC) (15).

In this study, we used the following human HNSCC cells: SAS (derived from a primary lesion of tongue squamous cell carcinoma) and HSC3 (derived from human lymph node metastasis of tongue squamous cell carcinoma), as described previously.

Mature miRNA and small interfering RNA (siRNA) transfection into HNSCC cells. The following RNA species were used in this study: mature miRNAs, Pre-miR miRNA Precursors (hsa-miR-145-3p, assay ID: PM 13036; hsa-miR-145-5p, assay ID: PM 11480), negative control miRNA (assay ID: AM 17111) (both from Applied Biosystems, Foster City, CA, USA), siRNA ( Stealth Select RNAi siRNA; si-MYO1B P/N: HSS106714 and HSS106716; Invitrogen, Carlsbad, CA, USA). The transfection procedures were described previously (16-20).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The procedure for PCR quantification was described previously (16-19). TaqMan probes and primers for MYO1B (P/N: Hs00362654_m1; Applied Biosystems) were assay-on-demand gene expression products. Expression for miR-145-3p (P/N: 002149; Applied Biosystems) and miR-145-5p (P/N: 002278) was used to quantify the expression levels of miRNAs according to the manufacturer’s protocol. To normalize the data for quantification of mRNA and miRNAs, we used human GAPDH (P/N: Hs99999908_m1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (P/N: Hs02391390_m1) and RNU48 (assay ID: 001006) (all from Applied Biosystems). The relative expression levels were analyzed using the 2^{-\Delta\Delta CT} method.

Cell proliferation, migration, and invasion assays. Cell proliferation, migration and invasion assays were described previously (16-19).

Incorporation of miR-145-3p or miR-145-5p into the RISC by Ago2 immunoprecipitation. SAS cells were transfected with 10 nM miRNA by reverse transfection. After 48 h, immunoprecipitation was performed using a human Ago2 miRNA isolation kit (Wako, Osaka, Japan) according to the manufacturer's protocol. Expression levels of miR-145-3p or miR-145-5p were measured by qRT-PCR. miRNA data were normalized to the expression of miR-150-5p (P/N: PM10070; Applied Biosystems), which was not affected by miR-145-3p and miR-145-5p transfection.

Western blot analysis. Cells were harvested and lysed 48 h after transfection. Each cell lysate (50 µg of protein) was separated using Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed with monoclonal anti-MYO1B antibodies (1:250 dilution; HPA013607; Sigma-Aldrich, St. Louis, MO, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (1:1,000 dilution; ab8245; Abcam, Cambridge, UK) were used as an internal control. The procedures were described in our previous studies (16-19).

Identification of putative genes regulated by miR-145-3p in HNSCC cells. Specific genes regulated by miR-145-3p were identified by a combination of in silico and genome-wide gene expression analyses. Genes regulated by miR-145-3p were listed using the TargetScan database. Oligo microarrays (Human GE 60K; Agilent Technologies) were used for gene expression analyses. The microarray data were deposited into GEO (http://www.ncbi.nlm.nih.gov/geo/), with accession number GSE82108. Upregulated genes in HNSCC were obtained from publicly available data sets in GEO (accession no. GSE9638). To identify signaling pathways regulated in silico, gene expression data were analyzed using the KEGG pathway categories with the GeneCodis program.

Regulation of targets downstream of MYO1B in HNSCC. We investigated pathways regulated by MYO1B in HNSCC cells. We analyzed gene expression using si-MYO1B-transfected SAS cells. Microarray data were used for expression profiling of si-MYO1B transfectants. The microarray data were deposited into GEO (accession no. GSE100746). We analyzed common downregulated genes using the GEO dataset.

Plasmid construction and dual-luciferase reporter assay. The partial wild-type sequence of the MYO1B 3'-untranslated region (3'-UTR) was inserted between the Xhol-Pmel restriction sites in the 3'-UTR of the hRluc gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). Alternatively, we used sequences that were missing the miR-145-3p target sites (position 88-94 or position 1117-1123). The synthesized DNA was cloned into the psiCHECK-2 vector. SAS cells were transfected with 20 ng of the vector, 20 nM microRNAs, and 1 µl Lipofectamine 2000 in 100 µl Opti-MEM (both from Invitrogen). The procedure of dual-luciferase reporter assay was described previously (16-19).
**Immunohistochemistry.** Formalin-fixed, paraffin-embedded (FFPE) tissues were used. Tissue sections were incubated overnight at 4°C with anti-MYO1B antibodies diluted 1:300 (HPA013607; Sigma-Aldrich). The procedure for immunohistochemistry was described previously (21).

**The Cancer Genome Atlas (TCGA)-HNSCC data analysis.** To explore the clinical significance of MYO1B in HNSCC, we used the RNA sequencing database in TCGA (https://tcga-data.nci.nih.gov/tcga/). The gene expression and clinical data were retrieved from cBioportal (http://www.cbioportal.org/, the provisional data downloaded July 1, 2017).

**Statistical analysis.** Relationships between two or three variables and numerical values were analyzed using Mann-Whitney U tests or Bonferroni-adjusted Mann-Whitney U tests. Spearman's rank tests were used to evaluate the correlations between the expression of miR-145-3p or miR-145-5p and target genes. Expert StatView software (version 5.0; SAS Institute Inc., Cary, NC, USA) was used for these analyses. Multivariate Cox proportional hazard regression models were used to determine independent factors for survival with JMP Pro 13.

**Results**

**Expression levels of miR-145-5p and miR-145-3p in HNSCC clinical specimens and cell lines.** To confirm our miRNA expression signatures in HNSCC by RNA sequencing, we validated the expression levels of miR-145-5p and miR-145-3p in HNSCC clinical specimens and cell lines. In Fig. 1, the expression levels of miR-145-5p and miR-145-3p were significantly reduced in cancer tissues compared with those in corresponding adjacent noncancerous epithelium (p<0.0001) (Fig. 1A). Additionally, the expression levels of miR-145-5p and miR-145-3p in SAS and HSC3 cells were markedly downregulated (Fig. 1A).

Spearman's rank test showed a positive correlation between the expression levels of miR-145-5p and miR-145-3p in clinical specimens (Fig. 1A).

**Effects of ectopic expression of miR-145-5p and miR-145-3p on cell proliferation, migration and invasion in HNSCC cell lines.** To validate the functional roles of miR-145-3p and miR-145-5p, we carried out gain-of-function assays using miRNA transfection into two HNSCC cell lines (SAS and HSC3). XTT assays revealed that cell proliferation was significantly inhibited in miR-145-5p and miR-145-3p transfectants in comparison with mock or miR-control transfectants (Fig. 1B). Similarly, migration assays showed that cell migration activity was significantly inhibited in miR-145-3p and miR-145-5p transfectants in comparison with mock and miR-control transfectants (Fig. 1C). Matrigel invasion assays also demonstrated that cell invasion activity was significantly inhibited in miR-145-3p and miR-145-5p transfectants in comparison with mock and miR-control transfectants (Fig. 1C).

**Table I. Clinical features of 22 patients with HNSCC.**

| No. | Age | Sex | Location | T     | N   | M  | Stage | Differentiation |
|-----|-----|-----|----------|-------|-----|----|-------|-----------------|
| 1   | 64  | F   | Oral floor | 4a    | 2c  | 0  | IV A  | Moderate        |
| 2   | 73  | M   | Tongue    | 3     | 2b  | 0  | IV A  | Poor            |
| 3   | 77  | M   | Tongue    | 2     | 2b  | 0  | IV A  | Poor            |
| 4   | 63  | F   | Oral floor | 2     | 2b  | 0  | IVA   | Basaloid SCC    |
| 5   | 59  | M   | Tongue    | 1     | 2a  | 0  | IV A  | Moderate        |
| 6   | 36  | F   | Tongue    | 3     | 1   | 0  | III   | Moderate        |
| 7   | 67  | M   | Tongue    | 3     | 0   | 0  | III   | Moderate        |
| 8   | 60  | F   | Tongue    | 2     | 1   | 0  | III   | Well            |
| 9   | 66  | M   | Tongue    | 2     | 0   | 0  | II    | Moderate        |
| 10  | 67  | M   | Tongue    | 2     | 0   | 0  | II    | Poor to moderate|
| 11  | 76  | F   | Tongue    | 1     | 0   | 0  | I     | Well            |
| 12  | 69  | M   | Tongue    | 1     | 0   | 0  | I     | Well            |
| 13  | 73  | F   | Tongue    | 1     | 0   | 0  | I     | Well            |
| 14  | 64  | M   | Tongue    | 1     | 0   | 0  | I     | Well            |
| 15  | 70  | M   | Tongue    | 1     | 0   | 0  | I     | Well            |
| 16  | 38  | M   | Tongue    | 1     | 0   | 0  | I     | Well            |
| 17  | 51  | M   | Tongue    | 1     | 0   | 0  | I     | Well            |
| 18  | 34  | F   | Tongue    | 1     | 0   | 0  | I     | Poor            |
| 19  | 70  | M   | Tongue    | 1     | 0   | 0  | I     | Moderate        |
| 20  | 71  | M   | Tongue    | 1     | 0   | 0  | I     | Well            |
| 21  | 82  | M   | Oral floor | 1     | 0   | 0  | I     | Well            |
| 22  | 81  | M   | Tongue    | 1     | 0   | 0  | I     | Extremely well   |

HNSCC, head and neck squamous cell carcinoma; F, female; M, male; TNM classification and tumor stage were determined by the Union for International Cancer Control (UICC).
transfectants in comparison with mock and miR-control transfectants (Fig. 1D).

Incorporation of miR-145-3p into the RISC in HNSCC cells. We hypothesized that the passenger strand miR-145-3p may be incorporated into the RISC and exert important effects in cancer cells. Accordingly, we performed immunoprecipitation with antibodies targeting Ago2, which plays an important role in the RISC. After transfection with miR-145-3p or miR-145-5p, Ago2-bound miRNAs were isolated, and qRT-PCR was carried out to determine whether miR-145-3p and miR-145-5p bound to Ago2. After transfection with miR-145-3p and immunoprecipitation by anti-Ago2 antibodies, miR-145-3p levels were significantly higher than those of mock- or miR-control-transfected cells and those of miR-145-5p-transfected SAS cells (p<0.0001) (Fig. 2A). Similarly, after miR-145-5p transfection, miR-145-5p was detected by Ago2 immunoprecipitation (p<0.0001) (Fig. 2B).

Identification of putative targets of miR-145-3p regulation in HNSCC cells. We performed in silico and gene expression analyses to identify genes targeted by miR-145-3p for regulation (Fig. 3). First, we selected putative miR-145-3p target genes using the TargetScan database and identified 3,164 genes. Next, we performed comprehensive gene expression analysis using miR-145-3p transfectants of SAS, with negative control miRNA transfectants serving as controls (accession no. GSE 82108). A total of 1,187 genes were
commonly downregulated (log₂ ratio<0). The gene set was then analyzed with a publicly available gene expression data set in GEO (accession no. GSE9638), and genes upregulated in HNSCC were chosen (fold-change >1.5). A total of 14 genes were identified as candidate targets of miR-145-3p regulation (Table II). Next, these genes were validated with TCGA database, and we investigated the correlations between survival

Table II. Putative targets of miR-145-3p regulation in HNSCC cells.

| Gene symbol | Gene name                              | Conserved site count | miR-145-3p transfection fold-change | HNSCC (high vs. low) fold-change | Prognosis (high vs. low) p-value |
|-------------|----------------------------------------|----------------------|------------------------------------|----------------------------------|---------------------------------|
| MYO1B       | Myosin IB                              | 2                    | -1.49                              | 1.72                             | 0.00452                         |
| C16orf74    | Chromosome 16 open reading frame 74    | 1                    | -0.88                              | 1.97                             | 0.014                           |
| SP9         | Sp9 transcription factor               | 1                    | -0.97                              | 2.38                             | 0.0277*                         |
| RBP1        | Retinol binding protein 1, cellular   | 1                    | -1.2                               | 2.6                              | 0.0316                          |
| LRRC3       | Leucine rich repeat containing 3      | 2                    | -0.86                              | 1.54                             | 0.0749                          |
| PSPH        | Phosphoserine phosphatase              | 1                    | -0.8                               | 1.95                             | 0.0804                          |
| CDC47L      | Cell division cycle associated 7-like  | 1                    | -0.88                              | 1.71                             | 0.107                           |
| CBS         | Cystathionine-β-synthase               | 2                    | -1.29                              | 1.54                             | 0.228                           |
| SH2D5       | SH2 domain containing 5               | 1                    | -1.87                              | 2.34                             | 0.301                           |
| PXDN        | Peroxidasin homolog (Drosophila)      | 1                    | -0.94                              | 1.63                             | 0.317                           |
| CYP27B1     | Cytochrome P450, family 27, subfamily B, polypeptide 1 | 2 | -0.86 | 2.65 | 0.531 |
| TNK2        | Tyrosine kinase, non-receptor, 2       | 1                    | -1.08                              | 2.3                             | 0.789                           |
| ALDH1L2     | Aldehyde dehydrogenase 1 family, member L2 | 1 | -1.19 | 2.3 | 0.855 |
| CCDC103     | Coiled-coil domain containing 103      | 1                    | -1.77                              | 2.03                             | 0.986                           |

HNSCC, head and neck squamous cell carcinoma; *poor prognosis with low expression.

Figure 2. Both strands of miR-145-5p and miR-145-3p were incorporated into the RISC. (A and B) Expression levels of miR-145-5p and miR-145-3p after transfection with miR-145-5p or miR-145-3p following immunoprecipitation by Ago2 (*p<0.0001).

Figure 3. Flow chart illustrating the analysis strategy for miR-145-3p targets in head and neck squamous cell carcinoma (HNSCC) cells. A total of 3,164 genes were putative target genes of miR-145-3p in TargetScan database analysis (release 7.1). Finally, 14 genes were selected as putative targets of miR-145-3p in HNSCC cells.
rates and target genes with high or low expression. In this study, 3 genes (MYO1B, C16orf74 and RBP1) were selected as genes that affected the patient's overall survival (Table II and Fig. 4). Among them, MYO1B was found to have the greatest effect on the overall survival rate (p=0.00452). In this study, we focused on MYO1B as a candidate target gene of miR-145-3p regulation and investigated the functional roles of HNSCC cells.

Direct regulation of MYO1B by miR-145-3p in HNSCC cells. Next, we investigated whether the expression of MYO1B decreased in miR-145-3p-transfected HNSCC cells. MYO1B mRNA levels were significantly reduced by miR-145-3p transfection compared with the mock or miR-control transfectants (Fig. 5A). Furthermore, MYO1B protein levels were also reduced by miR-145-3p transfection compared with mock or miR-control transfectants (Fig. 5B). In contrast, miR-145-5p transfectants did not show altered expression of MYO1B mRNA or protein (Fig. 5A and B).

We then carried out luciferase reporter assays with a vector that included the 3'-UTR of MYO1B to confirm that miR-145-3p
directly regulated MYO1B in a sequence-dependent manner. TargetScan Human database predicted that there were two binding sites for miR-145-3p in the 3'-UTR of MYO1B (positions 88-94 and 1117-1123) (Fig. 5C). Cotransfection with miR-145-3p and vectors significantly reduced luciferase activity in comparison with those in mock and miR-control transfected cell lines in position 1117-1123 of the MYO1B 3'-uTR (Fig. 5D).

Effects of MYO1B knockdown on cell proliferation, migration, and invasion in HNSCC cell lines. A loss-of-function assay using siRNA was performed to examine the function of MYO1B in HNSCC cell lines. The expression levels of MYO1B mRNA and protein were reduced by si-MYO1B in HNSCC cell lines (Fig. 6A and B). Furthermore, we investigated effects of MYO1B knockdown on cell proliferation, migration, and invasion in HNSCC cell lines. Cancer cell proliferation was significantly reduced in si-MYO1B transfectants in comparison with that in mock- or miR control-transfected cell lines (Fig. 6C). Additionally, migration activities were significantly suppressed in si-MYO1B transfectants in comparison with that in mock- or miR control-transfected cell lines (Fig. 6D). Invasion activity was also significantly inhibited in si-MYO1B transfectants in comparison with that in mock- or miR control-transfected cell lines (Fig. 6E).
Expression of MYO1B in HNSCC clinical specimens. Next, we investigated the mRNA expression levels of MYO1B in 22 HNSCC clinical specimens by qRT-PCR. MYO1B was significantly upregulated in HNSCC tumor tissues (Fig. 7A). Spearman’s rank test showed a negative correlation between the expression of MYO1B and miR-145-3p (p=0.0025, R=−0.461) (Fig. 7B). Furthermore, we also examined the expression levels of MYO1B in HNSCC clinical specimens by immunostaining. MYO1B was strongly expressed in several cancer tissues (Fig. 7C: 1, patient no. 2; 2, no. 3; 3, no. 7 in Table I).

Correlation between MYO1B expression and clinicopathological characteristics in prognostic prediction in HNSCC specimens. We collected clinical data from TCGA database and analyzed clinicopathological factors and expression of MYO1B as a prognostic predictive factor. The multivariate cox proportional hazards model was used to validate independent predictors for overall survival, including MYO1B expression, clinical T stage, clinical N stage, age, sex and histologic grade. As a result, high expression of MYO1B was an independent predictive factor for survival [hazard ratio (HR), 1.68; 95% confidence interval (CI), 1.13-2.49; p=0.01] (Fig. 8).

Downstream genes affected by silencing of MYO1B in SAS cells. Finally, we performed genome-wide gene expression analysis using si-MYO1B in SAS cells to investigate which genes were mediated by MYO1B signaling. A SurePrint G3 Human GE 60K v3 microarray was used for genome-wide expression analysis. We submitted the raw data to the GEO database (accession no. GSE100746). In this study, we focused on significantly downregulated genes by both si-MYO1B-1 and si-MYO1B-2 transfection (log2 [si-MYO1B/mock] <1.5). MYO1B was the most significantly downregulated gene, indicating that the array data were worthy of evaluation. Genes
significantly downregulated by silencing of MYO1B are listed in Table III. Among MYO1B downstream genes, expression of 5 genes (ANXA10, TRIM9, TCTN3, BTBD16 and CYP19A1) was significantly associated with poor prognosis in patients with HNSCC based on TCGA database (Fig. 9).

Discussion

Accumulating evidence has shown that aberrant expression of miRNAs disrupts the well-ordered RNA networks in cancer cells and is involved in the pathogenesis of human cancers (22). Based on the miRNA expression signatures of human cancers, we have sequentially identified antitumor miRNAs that regulate novel cancer networks (16,23‑26). Analyses of our miRNA signature of HNSCC by RNA sequencing showed that several passenger strands of miRNAs were significantly downregulated in cancer tissues (8). Our recent study demonstrated that both strands of pre-miR‑150 (miR‑150‑5p, guide strand; and miR‑150‑3p, passenger strand) had antitumor functions and that these miRNAs cooperatively regulated oncogenic ITGA3, ITGA6 and TNC in HNSCC cells (8). Our other studies showed that the passenger strand of miR‑150 acted as an antitumor miRNA in several types of cancers, such as esophageal cancer and prostate cancer (9,27). These findings suggested that miRNA passenger strands also contribute substantially to cancer pathogenesis and that identification of RNA networks mediated by miRNA passenger strands may provide novel insights into the pathogenesis of HNSCC.

Based on our miRNA signature of HNSCC, we focused on the passenger strand miR‑145‑3p in this study. Similarly,
Table III. Identification of \textit{MYO1B} downstream genes in HNSCC cells.

| Gene symbol | Gene name | Log$_2$ (si-MYO1B-1/ mock) | Log$_2$ (si-MYO1B-2/ mock) | Average Log$_2$ (si-MYO1B/ mock) |
|-------------|-----------|----------------------------|----------------------------|---------------------------------|
| MYO1B       | Myosin IB | -4.007414                  | -4.668526                  | -4.337970                       |
| ANXA10      | Annexin A10 | -3.842131                | -2.857582                  | -3.349857                       |
| MATN3       | Matrilin 3 | -4.224010                  | -2.082490                  | -3.153250                       |
| SOHLH1      | Spermatogenesis and oogenesis specific basic helix-loop-helix 1 | -4.337910 | -1.851334 | -3.094622 |
| SMAD1-AS1   | SMAD1 antisense RNA 1 | -3.191749 | -2.902154 | -3.046952 |
| KRT6B       | Keratin 6B, type II | -3.626921 | -2.042663 | -2.834793 |
| KLK13       | Kallikrein-related peptidase 13 | -3.540325 | -1.954660 | -2.747493 |
| PAX6        | Paired box 6 | -2.130839 | -3.085356 | -2.608098 |
| C5orf66-AS1 | C5orf66 antisense RNA 1 | -2.704701 | -2.114864 | -2.409783 |
| PDGFRB      | Platelet-derived growth factor receptor, β polypeptide | -2.886416 | -1.846569 | -2.366492 |
| HSD17B2     | Hydroxysteroid (17-β) dehydrogenase 2 | -2.325892 | -2.381436 | -2.355188 |
| SP140       | SPI140 nuclear body protein | -2.470807 | -2.107213 | -2.289010 |
| OR9G4       | Olfactory receptor, family 9, subfamily G, member 4 | -2.296291 | -2.127879 | -2.210825 |
| FOXD3-AS1   | FOXD3 antisense RNA 1 (head to head) | -1.846461 | -2.412832 | -2.129647 |
| MAGEB17     | Melanoma antigen family B, 17 | -2.394958 | -1.745608 | -2.070283 |
| AMHD1       | Amidohydrolase domain containing 1 | -2.223687 | -1.916799 | -2.070243 |
| IGFBP1      | Insulin-like growth factor binding protein 1 | -2.512259 | -1.592634 | -2.054246 |
| MMP1        | Matrix metallopeptidase 1 (interstitial collagenase) | -1.821691 | -2.272875 | -2.047283 |
| EN1         | Engrailed homeobox 1 | -1.834606 | -2.220468 | -2.027537 |
| FGF13-AS1   | FGF13 antisense RNA 1 | -2.349053 | -1.690626 | -2.019840 |
| ZC3H12D     | Zinc finger CCCH-type containing 12D | -2.232335 | -1.807164 | -2.019749 |
| KRT6A       | Keratin 6A, type II | -2.307169 | -1.631724 | -1.969448 |
| FAM196B     | Family with sequence similarity 196, member B | -1.855253 | -2.031945 | -1.943599 |
| DNMT3B      | DNA (cytosine-5-)‑methyltransferase 3β | -1.530055 | -2.334314 | -1.932185 |
| LIN28A      | Lin-28 homolog A (\textit{C. elegans}) | -2.292716 | -1.548248 | -1.920438 |
| ZNF501      | Zinc finger protein 501 | -1.788270 | -2.042201 | -1.915235 |
| REC114      | REC114 meiotic recombination protein | -2.230243 | -1.557493 | -1.893868 |
| TRIM9       | Tripartite motif containing 9 | -2.078308 | -1.691343 | -1.884826 |
| ZBED3-AS1   | ZBED3 antisense RNA 1 | -2.140241 | -1.610560 | -1.875401 |
| PHKA2-AS1   | PHKA2 antisense RNA 1 | -2.028810 | -1.706765 | -1.867776 |
| ZDHHC22     | Zinc finger, DHHC-type containing 22 | -2.044774 | -1.670274 | -1.857524 |
| SCAND2P     | SCAN domain containing 2 pseudogene | -1.924617 | -1.758662 | -1.841642 |
| SPRR1B      | Small proline-rich protein 1B | -1.967544 | -1.702868 | -1.853203 |
| SLC35D3     | Solute carrier family 35, member D3 | -1.638228 | -2.005418 | -1.821823 |
| ANO1-AS2    | ANO1 antisense RNA 2 (head to head) | -1.763001 | -1.854457 | -1.808723 |
| C22orf23    | Chromosome 22 open reading frame 23 | -1.741993 | -1.847972 | -1.794896 |
| TCTN3       | Tectonic family member 3 | -1.880425 | -1.697651 | -1.789038 |
| FAM198A     | Family with sequence similarity 198, member A | -1.940336 | -1.603225 | -1.771781 |
| TG          | Thyroglobulin | -1.719962 | -1.822218 | -1.771090 |
| RASGEF1A    | RasGEF domain family, member 1A | -1.865154 | -1.672245 | -1.770689 |
| KATNAL2     | Katanin p60 subunit A-like 2 | -1.982902 | -1.529477 | -1.754425 |
| KLHL14      | Kelch-like family member 14 | -1.575037 | -1.902098 | -1.738568 |
| NANOS1      | Nanos homolog 1 (\textit{Drosophila}) | -1.785024 | -1.677882 | -1.731453 |
| BTBD16      | BTB (POZ) domain containing 16 | -1.769118 | -1.670396 | -1.719729 |
| APOL4       | Apolipoprotein L, 4 | -1.542360 | -1.880493 | -1.714227 |
| ZNF385C     | Zinc finger protein 385C | -1.792121 | -1.621945 | -1.707324 |
| ABO         | ABO blood group | -1.624970 | -1.780649 | -1.702810 |
| CD200R1     | CD200 receptor 1 | -1.778945 | -1.529047 | -1.653996 |
| VWA3A       | Von Willebrand factor A domain containing 3A | -1.527752 | -1.740674 | -1.634180 |
| CYP19A1     | Cytochrome P450, family 19, subfamily A, polypeptide 1 | -1.572258 | -1.670624 | -1.621441 |
| ZNF880      | Zinc finger protein 880 | -1.604488 | -1.632158 | -1.618323 |
| NKX6-2      | NK6 homeobox 2 | -1.597461 | -1.625215 | -1.611336 |
| GPR157      | G protein-coupled receptor 157 | -1.583724 | -1.613383 | -1.598554 |
| ST3GAL5-AS1 | ST3GAL5 antisense RNA 1 (head to head) | -1.526179 | -1.554262 | -1.540221
miR-145-5p, the guide strand of miR-145, was significantly reduced in this signature. Downregulation of miR-145-5p is frequently observed in many types of cancer, and prior studies have confirmed the antitumor function of miR-145-5p by demonstration of its effects on several types of oncogenes in cancer cells (10,11). Several studies have shown that downregulation of miR-145-5p is caused by hypermethylation of the promoter region of pre-miR-145 in prostate cancer (28). Importantly, the tumor suppressor p53 has been shown to directly bind p53-response elements in the promoter region of pre-miR-145 and to control the expression of miR-145-5p (29). p53 mutations are found in >50% of patients with HNSCC (30). Thus, downregulation of miR-145-5p and miR-145-3p may be dependent on p53 inactivation in cancer cells.

Expression levels of passenger strand of miR-145-3p was lower than miR-145-5p as a guide strand miRNA in HNSCC clinical specimens and cell lines. Our previous studies of bladder, lung, and prostate cancers showed that expression levels of miR-145-3p was lower than miR-145-5p in each cancer (10,11,31). The results of the present data of HNSCC was similar to our previous data. Explanation is incomplete as to in what kind of molecular mechanisms the expression of the two miRNAs differ. This problem is an important issue in miRNA biosynthesis.

Our functional assays showed that miR-145-3p had antitumor functions similar to miR-145-5p in HNSCC cells. We have also demonstrated miR-145-3p is downregulated in cancer tissues and acts as an antitumor miRNA in bladder, lung, and prostate cancers by targeting several oncogenic genes (10,11,31). Previous studies demonstrated that several oncogenic genes were regulated by miR-145-5p in several types of cancers (32-34). There are few studies for target genes by miR-145-3p regulation in cancer cells, including HNSCC cells. Thus, we evaluated miR-145-3p regulatory oncogenic networks in HNSCC cells; a total of 14 putative targets of miR-145-3p in HNSCC cells were identified in this study. Among these candidates, MYO1B, C16orf74, SP9 and RB1 were found to be associated with poor prognosis in patients with HNSCC by TCGA data analyses.

In this study, we focused on MYO1B because high expression of MYO1B was strongly associated with poor prognosis in patients with HNSCC. Myosins are actin-associated
molecular motor proteins that regulate membrane tension, anchor membrane proteins and organelles, and transport intercellular vesicles (35,36). We have demonstrated that antitumor miRNAs inhibited cancer cell migration and invasion through targeting several actin-binding proteins and actin-associate proteins, e.g., FSCN1, LASP1, ARPC5 and ANLN (37-40). Overexpression of these proteins has been detected in cancer tissues and has been shown to contribute to cancer cell aggressiveness.

Our present data of restoration of miR-145-5p or miR-145-3p showed the inhibition of cancer cell proliferation. However, inhibition of cell proliferation was weak by knockdown of MYOIB in HNSCC cells. These data suggest that miR-145-5p or miR-145-3p inhibit cell proliferation genes and pathways which do not rely on MYOIB in HNSCC cells. MYOIB belongs to a member of the membrane-associated class I myosin family and functions as a linker between membranes and the actin cytoskeleton in several cellular processes (41). Previous studies have demonstrated other functions of MYOIB. For example, MYOIB is localized in the endocytotic compartment and has pivotal roles in endocytosis (42). MYOIB couples with the actin assembly to organelles and controls membrane remodeling at the trans-Golgi network (43). In cancer cells, MYOIB is highly expressed in PC-3 metastatic prostate cancer cells, and knockdown of MYOIB affects the cytoskeleton and cell migration (44). Another study showed that knockdown of MYOIB significantly inhibits migratory and invasive abilities of HNSCC cells in vitro and in vivo (35). Our present data confirmed these findings and suggested that MYOIB may be an effective target for the treatment of HNSCC.

To identify MYOIB-mediated HNSCC pathways, we performed genome-wide gene expression analyses using si-MYOIB transfectants. A total of 54 genes were found to be mediated by MYOIB in HNSCC. Among them, 5 genes (ANXA10, TRIM9, TCTN3, BTBDB16 and CYP19A1) were significantly associated with poor prognosis in patients with HNSCC by TCGA database analyses. Annexin family proteins are calcium-dependent phospholipid-binding proteins that regulate cell growth and signal transduction (45). Overexpression of ANXA10 has been reported in oral squamous cell carcinoma, and expression of ANXA10 promotes cancer cell proliferation through regulating mitogen-activated protein kinase signaling pathways (46). Exploration of novel MYOIB-mediated pathways may improve our understanding of the aggressiveness of this disease.

In conclusion, downregulation of miR-145-3p was observed in HNSCC clinical specimens, and this passenger strand acted as an antitumor miRNA through targeting MYOIB in HNSCC cells. MYOIB was highly expressed in HNSCC clinical specimens and was found to promote cancer aggressiveness in functional assays. Elucidation of the pathways mediated by the miR-145-3p/MYOIB axis is expected to contribute to further analyses of oncogenesis mechanisms and treatment strategies in HNSCC.

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References

1. Jemal A, Siegel R, Xu J and Ward E: Cancer statistics, 2010. CA Cancer J Clin 60: 277-300, 2010.
2. Jou A and Hess J: Epidemiology and molecular biology of head and neck cancer. Oncol Res Treat 40: 328-332, 2017.
3. Sawicki M, Szudy A, Szczeryk M, Krawczyk P and Klatka J: Molecularly targeted therapies in head and neck cancers. Otalaryngol Pol 66: 307-312, 2012.
4. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
5. Filipowicz W, Bhattacharyya SN and Sonenberg N: Mechanisms of post-transcriptional regulation by microRNAs: Are the answers in sight? Nat Rev Genet 9: 102-114, 2008.
6. Adams BD, Kasinski AL and Slack FJ: Aberrant regulation and function of microRNAs in cancer. Curr Biol 24: R762-R776, 2014.
7. Mah SM, Buske C, Humphries RK and Kuchenbauer F: miRNA*: A passenger stranded in RNA-induced silencing complex? Crit Rev Eukaryot Gene Expr 20: 141-148, 2010.
8. Koshizuka K, Nohata N, Hanazawa T, Kikkawa N, Arai T, Okato A, Fukumoto I, Katada K, Okamoto Y and Seki N: Deep sequencing-based microRNA expression signatures in head and neck squamous cell carcinoma: Dual strands of pre-miR-150 as antitumor miRNAs. Oncotarget 8: 30288-30304, 2017.
9. Okato A, Arai T, Kojima S, Koshizuka K, Osako Y, Idichi T, Kurozumi A, Goto Y, Kato M, Naya Y, et al.: Dual strands of pre-miR-150 (miR-150-5p and miR-150-3p) act as antitumor miRNAs targeting SPOCK1 in lung squamous cell carcinoma. Oncotarget 51: 245-256, 2017.
10. Matakai H, Seki N, Mizuno K, Nohata N, Kamikawaji K, Kumamoto T, Koshizuka K, Goto Y and Inoue H: Dual-strand tumor-suppressor microRNA-145 (miR-145-5p and miR-145-3p) coordinately targeted MTDH in lung squamous cell carcinoma. Oncotarget 7: 72084-72098, 2016.
11. Matsuhashita R, Yoshino H, Enokida H, Goto Y, Miyamoto K, Yonemori A, Inoguchi S, Nakagawa M and Seki N: Regulation of UHRF1 by dual-strand tumor-suppressor microRNA-145 (miR-145-5p and miR-145-3p): Inhibition of bladder cancer cell aggressiveness. Oncotarget 7: 28460-28487, 2016.
12. Matsuhashita R, Seki N, Chiyomaru T, Inoguchi S, Ishihara T, Goto Y, Nishikawa R, Matakai H, Tatarano S, Itsako T, et al.: Turn-over-suppressive microRNA-144-5p directly targets CCNE1/2 as potential prognostic markers in bladder cancer. Br J Cancer 113: 282-289, 2015.
13. Yonemori M, Seki N, Yoshino H, Matsuhashita R, Miyamoto K, Nakagawa M and Enokida H: Dual tumor-suppressors miR-139-5p and miR-139-3p targeting matrix metalloproteinase 11 in bladder cancer. Cancer Sci 107: 1233-1242, 2016.
14. Karatas OF, Yuceturk B, Suer I, Yilmaz M, Cansiz H, Solak M, Ittmann M and Ozen M: Role of miR-145 in human laryngeal squamous cell carcinoma. Head Neck 38: 260-266, 2016.
15. Huang SH and O'Sullivan B: Overview of the 8th edition TNM classification for head and neck cancer. Curr Treat Options Oncol 18: 40-20, 2017.
16. Fukumoto I, Hanazawa T, Kinoshita T, Kikkawa N, Chiyomaru T, Goto Y, Nishikawa R, Chiyomaru T, Nakagawa M, et al.: MicroRNA expression signature of oral squamous cell carcinoma: Functional role of microRNA-26a/b in the modulation of novel cancer pathways. Br J Cancer 112: 891-900, 2015.
17. Koshizuka K, Hanazawa T, Fukumoto I, Kikkawa N, Matsuhashita R, Matakai H, Mizuno K, Okamoto Y and Seki N: Dual-receptor (EGFR and c-MET) inhibition by tumor-suppressive miR-1 and miR-206 in head and neck squamous cell carcinoma. J Hum Genet 62: 113-121, 2017.
18. Nohata N, Sone Y, Hanazawa T, Fuse M, Kikkawa N, Yoshino H, Chiyomaru T, Kawakami K, Enokida H, Nakagawa M, et al.: miR-1 as a tumor suppressive microRNA targeting TAGLN2 in head and neck squamous cell carcinoma. Oncotarget 2: 29-42, 2011.
19. Goto Y, Kojima S, Nishikawa R, Enokida H, Chiyomaru T, Tanaka K, Nakagawa M, Naya Y, Ichikawa T and Seki N: Targeting CCNE1/2 as potential prognostic markers in bladder cancer. Br J Cancer 112: 113-121, 2015.
20. Yonemori M, Seki N, Yoshino H, Matsuhashita R, Miyamoto K, Nakagawa M and Enokida H: Dual tumor-suppressors miR-139-5p and miR-139-3p targeting matrix metalloproteinase 11 in bladder cancer. Cancer Sci 107: 1233-1242, 2016.
21. Koshizuka K, Hanazawa T, Fukumoto I, Kikkawa N, Matsuhashita R, Matakai H, Mizuno K, Okamoto Y and Seki N: Dual-receptor (EGFR and c-MET) inhibition by tumor-suppressive miR-1 and miR-206 in head and neck squamous cell carcinoma. J Hum Genet 62: 113-121, 2017.
22. Nohata N, Sone Y, Hanazawa T, Fuse M, Kikkawa N, Yoshino H, Chiyomaru T, Kawakami K, Enokida H, Nakagawa M, et al.: miR-1 as a tumor suppressive microRNA targeting TAGLN2 in head and neck squamous cell carcinoma. Oncotarget 2: 29-42, 2011.
23. Goto Y, Kojima S, Nishikawa R, Enokida H, Chiyomaru T, Tanaka K, Nakagawa M, Naya Y, Ichikawa T and Seki N: Targeting CCNE1/2 as potential prognostic markers in bladder cancer. Br J Cancer 112: 891-900, 2015.
24. Fukumoto I, Hanazawa T, Kinoshita T, Kikkawa N, Chiyomaru T, Goto Y, Nishikawa R, Chiyomaru T, Nakagawa M, et al.: MicroRNA expression signature of oral squamous cell carcinoma: Functional role of microRNA-26a/b in the modulation of novel cancer pathways. Br J Cancer 112: 891-900, 2015.
Kurozumi A, Goto Y, Matsushita R, Fukumoto I, Kato M, Nishikawa R, Sakamoto S, Enokida H, Nakagawa M, Ichikawa T, et al.: Tumor-suppressive microRNA-223 inhibits cancer cell migration and invasion by targeting IFGA/ITGB1 signaling in prostate cancer. Cancer Sci 107: 84-94, 2016.

Esquela-Kerscher A and Slack FJ: Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6: 259-269, 2006.

Nohata N, Hanazawa T, Kikkawa N, Sakurai D, Fujimura L, et al.: Identification of tumour suppressive microRNA-874 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 105: 833-841, 2011.

Fukumoto I, Kinoshita T, Hanazawa T, Kikkawa N, Chiyomaru T, Ishida Y, Ichikawa T, Naya Y, et al.: Identification of tumour suppressive microRNA-451a in hypopharyngeal squamous cell carcinoma based on microRNA expression signature. Br J Cancer 111: 386-394, 2014.

Goto Y, Kojima S, Nishikawa R, Kurozumi A, Kato M, Enokida H, Matsushita R, Yamazaki K, Ishida Y, Nakagawa M, et al.: MicroRNA expression signature of castration-resistant prostate cancer: The microRNA-221/222 cluster functions as a tumour suppressor and disease progression marker. Br J Cancer 113: 1055-1065, 2015.

Goto Y, Kurozumi A, Nohata N, Kojima S, Matsushita R, Yoshino H, Yamazaki K, Ishida Y, Ichikawa T, Naya Y, et al.: The microRNA signature of patients with metastic failure: Regulation of UHRF1 pathways by microRNA-101 in renal cell cancer. Oncotarget 7: 59070-59086, 2016.

Osako Y, Seki N, Koshizuka K, Okato A, Idichi T, Arai T, Omoto I, Sasaki K, Uchikado Y, Kita Y, et al.: Regulation of SPOCK1 by dual strands of pre-miR-150 inhibit cancer cell migration and invasion in esophageal squamous cell carcinoma. J Hum Genet: Jun 29, 2017. (Epub ahead of print). doi: 10.1038/ jhg.2017.69.

Xia W, Chen Q, Wang J, Mao Q, Dong G, Shi R, Zheng Y, Xu L and Jiang Z: Long noncoding microRNA-145 is a potential prognostic marker in patients with lung adenocarcinoma. Sci Rep 5: 13359. 2015.

Yang P, Yang Y, An W, Xu J, Zhang G, Jie J and Zhang Q: The long noncoding RNA-ROR promotes the resistance of radiotherapy for human colorectal cancer cells by targeting the p53/miR-145-150 pathway. J Gastroenterol Hepatol 32: 837-845, 2017.

Wood NB, Kotelnikov V, Caldarelli DD, Hutchinson J, Panje WR, Hegde P, Leurgans S, LaFalotte S, Taylor SG IV, Preisler HD, et al.: Mutation of p53 in squamous cell cancer of the head and neck: Relationship to tumor cell proliferation. Laryngoscope 107: 827-833, 1997.

Goto Y, Kurozumi A, Arai T, Nohata N, Kojima S, Okato A, Kato M, Yamazaki K, Ishida Y, Naya Y, et al.: Impact of novel miR-145-3p regulatory networks on survival in patients with castration-resistant prostate cancer. Br J Cancer 117: 409-420, 2017.

Pashaei E, Guzel E, Ozgurses ME, Demirel G, Aydin N and Ozen M: A Meta-analysis: Identification of common mir-145 target genes that have similar behavior in different GEO datasets. PLoS One 11: e0161491, 2016.

Sachdeva M and Mo YY: miR-145-mediated suppression of cell growth, invasion and metastasis. Am J Transl Res 2: 170-180, 2010.