Original Article

microRNA-1266-5p directly targets DAB2IP to enhance oncogenicity and metastasis in oral cancer

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KEYWORDS
Oral cancer; MicroRNA-1266-5p; DAB2IP; Metastasis

Abstract Background/purpose: Oral cancer has been recognized as one of the most common malignancies worldwide and ranks the fifth leading cause of cancer death in Taiwan. A variety of studies have demonstrated that microRNAs are involved in the regulation of the hallmarks of oral carcinogenesis. Nevertheless, the effect of miR-1266-5p on the tumorigenesis of oral cancer has not been investigated, and not to mention, its functional role in oral cancer.

Materials and methods: The upregulation of miR-1266-5p in SASVO3 and SASM5 cells was identified by RNA-Seq and examined by qRT-PCR analysis. The phenotypic assays including proliferation activity, migration capacity, invasion, wound healing, and colony-forming abilities were conducted in oral cancer cells after knockdown of miR-1266-5p. Luciferase reporter and western blotting were used to validate DAB2IP was a direct target of miR-1266-5p in oral cancer.

Results: We identified that miR-1266-5p was significantly overexpressed in highly tumorigenic SASVO3 cells and metastatic SASM5 cells. qRT-PCR revealed that miR-1266 significantly
increased upregulated in oral cancer and lymph node metastatic tissues compared to normal counterparts. We found that downregulation of miR-1266-5p inhibited the proliferation and clonogenicity capacities of SASVO3 cells. Knockdown of miR-1266-5p also inhibited migration/invasion and self-renewal abilities in SASM5 cells. Moreover, we validated miR-1266-5p directly bound to the 3’UTR of DAB2IP in oral cancer cells. We found that DAB2IP knockdown reversed the inhibitory effects of self-renewal and migration mediated by silencing of miR-1266-5p.

**Conclusion:** miR-1266 functions as a biomarker in oral cancer patients, and downregulation of miR-1266 may ameliorate the oncogenic and metastasis potential of oral cancer by targeting DAB2IP.

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**Introduction**

Oral cancer refers to a malignant tumor that occurs in the mouth, mostly classified as oral squamous cell carcinoma (OSCC). The incidence and mortality of oral cancer are gradually increased worldwide, owing to the habit of betel nut chewing, alcohol drinking, and cigarette smoking. Since oral cancer has been characterized by high potential of metastasis and low survival rate, curing oral cancer is becoming tricky. Over the past decades, the prognosis of OSCC patients with level IV/V metastasis has not improved significantly despite sophisticated surgical, chemotherapy, and radiotherapeutic modalities. Therefore, the development of a strategy to inhibit cancer invasiveness becomes the most challenging issue in OSCC therapy.

MicroRNAs (miRNAs) have been considered to play crucial roles in carcinogenesis via binding to the 3’UTR of target genes and inhibiting their expression. Mounting data have shown that miR-1266 is abnormally expressed in several cancers. MIIR-1266 has been identified as one of the deregulated miRNAs in cervical cancer specimens and is positively correlated with poor prognosis. Su et al. reported that miR-1266-5p serves as a prognostic biomarker for hepatocellular carcinoma (HCC) and promotes tumorigenic properties of HCC cells. Overexpression of miR-1266 increased chemoresistance of pancreatic cancer and SOCS3, PTPN11, ITCH, and TNIP1 CADM1 were found to be the targets of miR-1266. However, the critical role of miR-1266 in oral cancer has not been explored. Therefore, we sought to examine and characterize the function of miR-1266-5p in oncogenicity and metastasis traits, and verify its target in oral cancer.

**Materials and methods**

**Oral cancer cell lines and OSCC tissues**

The OSCC cell lines SASVO3 and SASM5 were gifts from Dr. Jeng-Fan Lo (Institute of Oral Biology, College of Dentistry, National Yang Ming Chiao Tung University, Taipei, Taiwan) and were cultivated as previously described. OSCC (T), lymph node metastasis (LN) and normal paired noncancerous matched tissues (N) specimens were collected with written informed consents and all protocols were approved by The Institutional Review Board in Chung Shan Medical University Hospital.

**Quantitative real-time PCR (qRT–PCR) for miR-1266 expression detection**

To detect the level of miR-1266, qRT–PCR was performed using TaqMan miRNA assays with specific primer sets (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s instruction with a StepOne Plus real-time PCR system.

**miR-1266-5p and DAB2IP knockdown**

Scramble (Scr) and miR-1266 inhibitor control were purchased from Applied Biosystems. Lipofectamine™ 3000 transfection reagent was used according to the manufacturer’s protocol. The pLV-RNAi vector was purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). The method of cloning the double-stranded shRNA sequence will follow the manufacturer’s protocol. Oligonucleotide sequence of lentiviral vectors expressing shRNA that targets human DAB2IP was synthesized and cloned into pLV-RNAi to generate a lentiviral expression vector.

**Self-renewal assay**

OSCC cells were dissociated and cultured as moral spheres in modified DMEM/F-12. For the self-renewal ability evaluation, single cells will be obtained from accutase treated spheroids and the cell density of passage will be 1000 cells/ml in the serum-free medium.

**DAB2IP as the direct target of miR-1266-5p**

To confirm the exact binding site of miR-1266-5p, we manipulated the sequence on the 3’UTR of DAB2IP using pMIR-REPORT plasmid (Life Technologies, Grand Island, NY, USA) by point mutation, and the luciferase reporter assay was conducted to examine the relationship between DAB2IP and miR-1266-5p.
Oncogenic phenotypic assays

The oncogenic phenotypic assays of OSCC cells after transfection with miR-Scr. or miR-1266-5p inhibitors were characterized, including the proliferation, migration, invasion, and wound healing abilities. All procedures followed the previously described protocol.\textsuperscript{11}

Statistical analysis

Statistical Package of Social Sciences software (version 13.0) (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data from at least triplicate analysis were shown as mean ± SD. Student’s t-test was used to determine the statistical significance of the differences between experimental groups. P-value less than 0.05 was considered statistically significant.

Results

In this study, we selected SASVO3 cells for their highly tumorigenic feature and SASM5 cells for their metastatic capacity.\textsuperscript{8,9} To investigate the function of miR-1266-5p in various malignant SAS-derived OSCC cell lines, we utilized RNA -sequencing (RNA-seq) to identify the differential expression of miR-1266 among SAGFP, SASVO3 and SASM5 cells. Results showed that miR-1266-5p was significantly upregulated in SASVO3 and SASM5 cells (Fig. 1A). Similarly, to verify this finding, qRT-PCR was conducted and we showed that miR-1266-5p was significantly increased in SASVO3 and SASM5 cells (Fig. 1B). Next, we collected mucosa from normal (N) subjects, OSCC (T) and OSCC with lymph node metastasis (LN) patients to analyze the relative gene expression of miR-1266-5p. The results showed that the level of miR-1266-5p was upregulated in T and LN groups (Fig. 1C). We showed that miR-1266 was over-expression in oral cancer cell lines and tumor tissues.

To further ascertain the functional role of miR-1266-5p in SASVO3 cells, we transfected SASVO3 cells with a scramble and miR-1266-5p inhibitor. As shown in Fig. 2A, SASVO3 cells transfected with miR-1266-5p inhibitor reduced the relative cell growth in a time-dependent manner. Similarly, the relative C.F.U ability was significantly diminished (Fig. 2B). These results indicated that miR-1266-5p may influence the tumorigenic ability of SASVO3 cells.

Subsequently, the migration (Fig. 3A), invasion (Fig. 3B), wound healing (Fig. 3C), and self-renewal (Fig. 3D) capacities were significantly decreased in miR-1266-5p transfected group, indicating the anti-metastasis potential of miR-1266 in SASM5 cells. These results suggest that miR-1266-5p plays a tumor suppressor role in OSCC.

In light of the bioinformatic analysis, we predicted that DAB2IP might be the potential target of miR-1266-5p. The result showed that miR-1266-5p significantly inhibited the activity of the luciferase reporter gene (Fig. 4B). Also, the protein level of DAB2IP was significantly increased in SASM5 cells with miR-1266-5p inhibitor (Fig. 4C). We further observed that repression of DAB2IP reversed the inhibitory effects of miR-1266 knockdown on self-renewal (Fig. 5A) and migration (Fig. 5B) capacities in oral cancer cells.
Discussion

In the past few years, numerous studies dedicate to figuring out the participation of miRNA in the progression of oral cancer.\textsuperscript{12,13} At present, the prognosis of OSCC patients with level IV/V metastasis remains unsatisfactory. Aside from the diagnostic delays or a lack of oral cancer knowledge, the major determinant of the poor prognosis of oral cancer is metastasis. Once patients are diagnosed with advanced tumors, there is a high occurrence of invasion and distant metastases.

Figure 2  Ectopic expression of miR-1266-5p inhibitor declined the ability of growth rate and colony formation in SASVO3 cells. (A) Relative growth and (B) C.F.U ability of SASVO3 cells transfected with miR-Scr. or miR-1266-5p inhibitor. *$p<0.05$ compared to miR-Scrambled (miR-Scr.).

Figure 3  Ectopic expression of miR-1266-5p inhibitor suppressed the ability of migration, invasion, and self-renewal in SASM5 cells. (A) Relative migration, (B) invasion, (C) wound healing and (D) self-renewal ability of SASM5 cells transfected with miR-Scr. or miR-1266-5p inhibitor. *$p<0.05$ compared to miR-Scrambled (miR-Scr.).
metastasis. Aberrant expression of miRNAs is tightly related to tumor development and metastasis in oral cancer. For instance, the increased expression of miR-21 in blood samples is positively correlated with advanced stage and lymph node status in oral squamous cell carcinoma.\(^\text{14}\) Compared with non-cancerous tissues, miR-211 was found to be upregulated in OSCC tissue samples with nodal metastasis and vascular invasion.\(^\text{15}\) miR-211 can promote metastasis by targeting CCNG2 transforming growth factor \(\beta\) type II receptor (TGF\(\beta\)RII). MicroRNA-155-5p was upregulated in metastatic OSCC and reported to have prognostic potential.\(^\text{16}\) It has been shown that upregulation of miR-134 led to an elevation of nodal metastasis and mortality in oral carcinomas through targeting the WW domain-containing proteins.

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**Figure 4** MiR-1266-5p directly targets the 3′ UTR of DAB2IP. (A) Sequence of miR-1266-5p, the putative binding sequence along with the mutant sequence at the 3′-untranslated region (3′UTR) of DAB2IP; (B) Luciferase activity decreased when cells were co-transfected with Wt-DAB2IP and miR-1266-5p mimics; (C) The protein expression of DAB2IP in SASM5 cells transfected with miR-Scr. or miR-1266 inhibitor. *\(p<0.05\) compared to miR-Scr.

**Figure 5** The inhibitory effects of silencing DAB2IP on self-renewal and migration abilities were reversed by inhibition of miR-1266-5p. Self-renewal (A) and migration (B) capacities of cells transfected with miR-1266-5p inhibitor with or without silence of DAB2IP were evaluated. *\(p<0.05\) compared with control group; #\(p<0.05\) compared with miR-1266-5p inhibitor only group.
Our data found that the expression of miR-1266 was elevated in lymph node metastatic oral cancer tissues compared with non-cancerous tissues.

DAB2IP, a new of the Ras GTPase-activating protein family, has been implicated in the regulation of cell proliferation, survival, apoptosis, epithelial-to-mesenchymal transition (EMT), cancer stem cell phenotype, radiation, and chemotherapy resistance. DAB2IP was first reported to interact with DOC2/DAB2 and functioned as a tumor suppressor in multiple cancer types. Several studies have revealed that DAB2IP modulated the cancer stemness properties. DAB2IP significantly inhibited self-renewal in colorectal carcinoma stem-like cells through c-Myc degradation. Another study showed that CRISPR/Cas9-mediated deletion of DAB2IP acquired the cancer stemness properties. DAB2IP significantly decreased in the tumor specimens relative to the nontumor tissue. In associated with these findings, our data showed that DAB2IP rescued the inhibitory effects on oral cancer oncogenicity and metastasis abilities with miR-1266-5p knockdown (Fig. 5A). These studies suggested that we may be able to eliminate miR-1266/DAB2IP axis to reduce cancer stemness.

In conclusion, we have found that miR-1266-5p expression was increased in oral cancer cells. Knockdown of miR-1266-5p repressed proliferation, migration, and self-renewal characteristics. We have identified DAB2IP as the direct target of miR-1266-5p. Our study suggested that miR-1266-5p/DAB2IP axis may represent a therapeutic target in oral cancer.

Declaration of competing interest

All authors have no conflicts of interest relevant to this article.

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