MicroRNA-216a inhibits the growth and metastasis of oral squamous cell carcinoma by targeting eukaryotic translation initiation factor 4B

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Abstract. There is increasing evidence to suggest that microRNAs (miRNAs; miRs) are involved in the development of oral squamous cell carcinoma (OSCC). miR-216a has been identified as being involved in tumorigenesis, however, the mechanisms of miR-216a in various types of cancer, either as a tumor suppressor or as an oncogenic miRNA, and the specific regulatory role of miR-216a in OSCC remain to be elucidated. The present study demonstrated that the expression of miR-216a was significantly reduced in OSCC tissues and cell lines. Overexpression of miR-216a significantly suppressed the proliferation, colony formation, migration and invasion of the OSCC cells. In addition, eukaryotic translation initiation factor 4B (EIF4B) was identified as a direct target of miR-216a, which was observed to be upregulated in the OSCC tissues. Furthermore, overexpression of EIF4B significantly attenuated the antitumor effect of miR-216a, and a negative correlation was observed between miR-216a and EIF4B in the OSCC tissues. Taken together, these findings indicated that miR-216a has a suppressive role in OSCC cells by directly targeting EIF4B, and may function as a potential prognostic biomarker and novel therapeutic target.

Introduction

Oral cancer is one of most common types of malignancy worldwide, and ~42,440 new cases were diagnosed in the United States in 2014 (1). Oral squamous cell carcinoma (OSCC) accounts for ~90% of oral cancer cases. Despite significant advances in therapeutic strategies in the last few years, the overall 5-year survival rates of patients with OSCC is ~60% at the age of 62 years (2). Funk *et al* (3) reported that the 5-year survival rate is almost 80% in the early stages, however, this rate decreases to between 20 and 40% in advanced-stage OSCC. This indicates that early detection is essential for improving the survival rates and prognosis in OSCC. Therefore, an improved understanding of the molecular biology and pathogenesis of OSCC is essential for the development of novel biomarkers and therapies.

MicroRNAs (miRNAs) are a class of small non-coding RNAs of ~21-23 nucleotides in length, which regulate target genes through the 3'-untranslated regions (3'-UTRs) to induce mRNA degradation and inhibit mRNA translation (4). Accumulating evidence has reported that miRNAs are important in tumorigenesis due to their aberrant expression (5), and can function either as oncogenes or tumor suppressors in various types of cancer, depending on their downstream target genes (5,6). In addition, several miRNAs have been identified to be dysregulated, regulating the initiation and progression of OSCC (7). miR-216a has been observed to be downregulated and function as a tumor suppressor in several types of cancer (8-10). However, the role of miR-216a in the regulation of key genes and signaling pathways associated with OSCC remains to be elucidated.

In the present study, the expression levels of miR-216a in OSCC specimens and cell lines, and the effects on the growth and metastasis of OSCC cells were investigated. In addition, the present study aimed to identify whether eukaryotic translation initiation factor 4B (EIF4B) is a potential target of miR-216a and identify correlations with miR-216a in the OSCC tissues.

The current study may not only provide a novel understanding of the regulatory mechanism of miR-216a but additionally offer a novel target for the treatment of OSCC.

Materials and methods

OSCC specimens, cell lines and transfection. A total of 23 paired OSCC tissue samples and adjacent non-tumor tissue samples were collected from patients undergoing resection of OSCC at the No.1 People's Hospital (Jining, China) between June 2011 and December 2013. Of the patients, 15 were men and 8 were women, with a median age of 51 years (range, 22-83). No patients received chemotherapy or radiotherapy prior to surgery. The tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C. Histopathology was confirmed by two independent pathologists. The study was

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approved by the Hospital Ethical Committee and informed consent was obtained from each patient prior to commencement of the investigation.

The SCC-4 and CAL 27 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (GE Healthcare, Logan UT, USA). The HEK293 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and were maintained in Dulbecco's modified Eagle's medium. All the media were supplemented with 10% fetal bovine serum (FBS) at 37˚C under 5% CO_2. For transfection, cells were transfected using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Following 4 h incubation at 37˚C in FBS-free medium, cells were incubated for 24 h at 37˚C in 10% FBS medium.

Plasmid construction. The pre-miR-216a plasmid was constructed using the following synthetic oligonucleotides that were obtained from SangonBiotech, Shanghai, China: Sense, 5'-AATTCGATGGCTGTGAGTTGGCTTAATCTCAGCTG GCAACTGTGAGATGTTCATACAATCCCTCACAGTGGT CTCTGGGATTATGCTAAACAGAGCAATTTCCTAGCCC TCACGAA-3'; anti-sense, 5'-AGCTTTCGTGAGGGCTAGG AAATTGCTCTGTTTAGCATAATCCCAGAGACCACTGT GAGGGATTGTATGAACATCTCACAGTTGCCAGCT GAGACCAAGCCAACTCACAGCCATCG-3'. These oligonucleotides were then cloned into the pcDNA6.2-GW vector at the *Eco*RIand *Hind*III sites (Promega Corporation, Madison, WI, USA) using TargetScan, version 6.2 (www.targetscan.org/) to predict the target of miR-216a. Furthermore, the complimentary sites in the 3'-UTRs of the wild-type EIF4B (EIF4B-WT) and mutant EIF4B (EIF4B-MT) of miR-216a were synthesized (SangonBiotech) and cloned into the pmirGLO dual-luciferase reporter vector at the *Sac*1 and *Xho* 1 sites (Promega Corporation). The EIF4B expression plasmid was obtained from GeneCopoeia, Inc. (Rockville, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was isolated from the OSCC tissues and cells using TRIzol reagent (Invitrogen Life Technologies). All the reagents used for RT-qPCR were obtained from Tiangen Biotech Co., Ltd. (Beijing, China). For mRNA analyses, first-strand cDNA was synthesized using a FastQuant RT kit (Tiangen Biotech Co., Ltd.). The mature miRNA was reverse transcribed using specific primers for miR-216a. Subsequently, qPCR was performed using SuperReal PreMix Plus (SYBR Green) on an ABI7500 PCR machine (Applied Biosystems Life Technologies, Foster City, CA, USA). The relative expression levels of mRNA and miRNA were calculated based on the $2^{-\Delta\Delta Ct}$ method. β-actin and U6 were used as controls for mRNA 1 and miRNA, respectively. The following primers that were synthesized by SangonBiotech were used in the qPCR analysis: RT 5'-GTCGTATCCAGTGCGTGTCGT GGAGTCGGCAATTGCACTGGATACGACTCACAGT-3' for miR-216a; miR216a, forward 5'-ATCCAGTGCGTGTCGTG-3' and reverse 5'-TGCTTAATCTCAGCTGGCA-3'; and EIF4B, forward 5'-AGCGTCAGCTGGATGAGCCAA-3' and reverse 5'-TGTCCTCGACCGTTCCCGTT-3'.

Cell proliferation assay. The transfected cells were seeded into 96-well plates at a density of 3,000 cells/well. At 24, 48 and 72 h following transfection, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) assay was used to determine the cell proliferation. The MTT solution (5 mg/ml; 200 μl) was added to the cells and incubated for 4 h at 37˚C. Following the removal of the culture medium, the remaining crystals were dissolved in 150 μl dimethyl sulfoxide, and the absorbance at 492 nm was measured (FluoStar Optima; BD Biosciences, Franklin Lakes, NJ, USA).

Colony formation assay. The cells were cultured in 12-well plates at a density of 2,000 cells/well between 24 h and 15 days. The clones were washed with phosphate-buffered saline (PBS) and stained using 0.1% crystal violet (Beyotime Institute of Biotechnology, Haimen, China) for 20 min. Images of the colonies were captured and the numbers of cells were counted under a microscope (CK2; Olympus, Tokyo, Japan).

Migration and invasion assays. The present study then performed cell migration and invasion assays using uncoated or coated Matrigel, respectively. Briefly, the cells ($5x10^4$) were added to the upper chamber of Transwell inserts in serum-free medium containing 0.1% FBS, and a medium supplemented with 10% FBS was added to the lower chamber. The cells were cultured for 24 h at 37˚C in 5% CO_2. The Matrigel and non-invading cells were then gently removed, and the migrated and invaded cells in the lower membrane were fixed with 4% paraformaldehyde (Nanjing Chemical Material Corporation, Nanjing, China), stained using 0.1% crystal violet and counted under a light microscope (CK2; Olympus).

Dual luciferase reporter assays. The HEK293 cells were seeded in 96-well plates (3,000 cells/well) and co-transfected with 0.1 mg pmirGLO reporter plasmid (EIF4B-WT/EIF4B-MT) and 0.1 mg pre-miR-216a plasmid or control miRNA. Cells were incubated at 37˚C for 24 h and then lysed with a mixture of Dual-Glo Luciferase reagent and buffer (Promega Corporation). The luciferase activities were measured using the FluoStar Optima.

Western blot analysis. The total protein was extracted from the transfected cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's instructions. The total proteins (50 μg) were separated using 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), which were then blocked in 5% non-fat milk in Tris-buffered saline with 0.05% Tween-20 for 2 h at 37˚C, followed by incubation with rabbit monoclonal anti-EIF4B antibody (1:1,000; 17917-1-AP; Protein Tech Group, Inc., Wuhan, China). β-actin was used as a loading control.

Statistical analysis. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. All data are presented as the mean ± standard error of the mean of at