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MicroRNA-29b regulates migration in oral squamous cell carcinoma and its clinical significance

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SUMMARY

Objectives: MicroRNA (miRNA) machinery regulates cancer cell behavior, and has been implicated in patients' clinical status and prognosis. We found that microRNA-29b (miR-29b) increased significantly in advanced migratory cells. However, miR-29b controls the migration ability, and its regulatory mechanism in oral squamous cell carcinoma (OSCC) remains unknown.

Materials and methods: We triggered miR-29b expression in OSCC patients and cell lines by conducting real-time quantitative PCR. We determined the functions of miR-29b in the migration of OSCC cells by using gain- and loss-of-function approaches. We elevated the target genes of miR29b through software predictions and a luciferase report assay. We used an orthotopic OSCC animal model to investigate the effects of miR29b on OSCC cell metastasis in vivo.

Results: The clinical data revealed that miR-29b expression was correlated with lymph node metastasis and an advanced tumor stage in 98 OSCC patients. Furthermore, multivariate analysis revealed that miR-29b expression was significantly correlated with recurrence, and indicated poor survival. MiR-29b promoted OSCC cell migration and downregulated CX3CL1, a cell–cell adhesion regulator, which plays an essential role in miR-29b-regulated OSCC cell migration machinery. Furthermore, we found that CX3CL1 expression was correlated with lymph node metastasis and an early tumor stage in OSCC patients, and negatively correlated with miR-29b expression.

Conclusion: MiR-29b acts as an oncomir, promoting cell migration through CX3CL1 suppression, and could be a potential therapeutic target for preventing OSCC progression.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common type of head and neck cancer. With more than half a million patients being diagnosed (5% of all cancers) each year worldwide, OSCC is the sixth leading cause of cancer death in Taiwan, with nearly 5400 new cases and 2200 deaths per year [1,2]. Despite the advancement of management strategies, the overall survival of patients has not improved significantly during the last 20 y, with the 5-y survival rate at 62.1% [3]. The major reason for the high mortality rate from OSCC is metastasis to adjacent tissues. The rate of local recurrence at the primary site, and regional recurrence of metastasis at the peripheral lymph node range from 20% to 40% [3–5]. However, the underlying molecular mechanisms in OSCC lymph node metastasis are barely understood.

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MicroRNAs (miRNAs) are small, single-stranded, evolutionarily conserved non-coding RNAs consisting of 21–24 nucleotides. miRNAs have been widely acknowledged to act as novel regulators of various genes involved in fundamental cell processes such as in development, differentiation, proliferation, survival, and death [6]. Multitudinous miRNAs have been implicated in numerous aspects of tumor progression including epigenetic regulation, tumorigenesis, apoptosis inhibition, tumor migration, invasion, and metastasis [7–10]. To date, up to 2555 human miRNAs have been identified and cataloged in the miRBase database [11], and miRNAs are believed to regulate over 60% of all human genes in the human genome [12]. To investigate the miRNA profile in oral cancer progression, we found that miR-29b was up-regulated in the highly motile cells. Knowledge regarding the function of miR-29b in oral cancer is scant. This study examines the correlation between miR-29b expression in oral cancer and clinical outcomes. We overexpressed or silenced miR-29b in oral cancer cells to investigate the function of miR-29b on oral cancer metastasis in vitro and in vivo, as well as to identify the downstream effector(s) of miR-29b.

Materials and methods

Cell lines and clinical specimens

Oral cancer and head and neck cell lines SAS and TW2.6 were cultured in, respectively, DMEM and F12/DMEM medium supplemented with 10% FBS, 1% antibiotics at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. TW2.6 MS-10 was a subline selected from parental TW2.6 by Transwell after ten generation of selection.

Oral squamous cell carcinoma specimens were collected as previously described [13]. Briefly, OSCC specimens were collected at the time of surgery from previously untreated patients who underwent surgical resection at the National Taiwan University Hospital from September 1, 1993, to August 31, 1997. IRB approval of this study was reviewed and approved by the Research Ethics Committee of National Taiwan University Hospital Organization. The histologic identification of oral cancer was determined as recommended by the World Health Organization [14]. The final disease stage was determined by a combination of surgical and pathologic findings, according to the current tumor–node–metastasis staging system for oral cancer.

Isolation of RNA, reverse transcription and real-time PCR quantification

Total RNA was extracted using Trizol RNA isolation kit according to the manufacturer's instructions (Invitrogen Corporation, Calsbad, CA). miRNA reverse transcription was synthesized using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The reactions were incubated at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The sample was normalized to reference control gene RNU6B or β-actin.

Migration assay

Cell migration assays were performed using Transwell chambers (8 μM pore size; Millicell, Millipore). After 24 h, the cells migrated to the bottom chamber were stained with crystal violet. Images of ten different fields were captured from each membrane, and the number of migratory cells was counted. *In vitro* wound healing assays were performed using culture-insert (400 micrometer ±50 micrometer; ibidi Gmbh). Images of cell migrated toward the wounded area were captured at different time point depending on the cell types.

Plasmids, transient transfection and stably transfected clone selection

All transient transfection was performed using Lipofectamine™ 2000 (Invitrogen Corporation, Calsbad, CA). Briefly, the plasmids [pLemiR, pLemiR-miR-29b, pmiRZip-scramble control, pmiR-Zip-amiR-29b (Invitrogen Corporation, Calsbad, CA); pLKO, pLKO-shCX3CL1, pLKO-shCCND2, pLKO-shCOL5A3, pLKO-shCOL4A3 (RNAi core, Academia Sinica)] and Lipofectamine™ 2000 were mixed for 20 min before adding the cells. Selection of stable transfectants was achieved with Gentamicin (G418) at a concentration of 0.4–1.2 mg/mL, or puromycin at a concentration of 3–10 μg/mL. After 2 weeks of selection, resistant clones were maintained in 100 μg/mL G418 or 1 μg/mL puromycin.

3′UTR luciferase reporter assay

The 3′UTR and seed region mutant of human CX3CL1 were cloned into pMIR-REPORT™ Luciferase vector (Promega Corporation, Madison, WI). This construct was co-transfected with control plasmid or plasmids expressing miR-29b and Renilla plasmid into SAS and TW2.6 cells. Luciferase activity was measured 48 h after transfection using the Dual-luciferase reporter assay system (Promega Corporation, Madison, WI).

Western blotting

Western blot analysis was performed as previously described [15], proteins were separated on 10% polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were probed with anti-CX3CL1 (R&D System Inc., Minneapolis, MN), anti-β-actin, or anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), overnight at 4 °C. Antibody–protein complexes were detected with enhanced chemiluminescence reagents (Millipore, Bedford, MA) and image of the blot was obtained using LAS-4000 (Fujifilm, Tokyo, Japan).

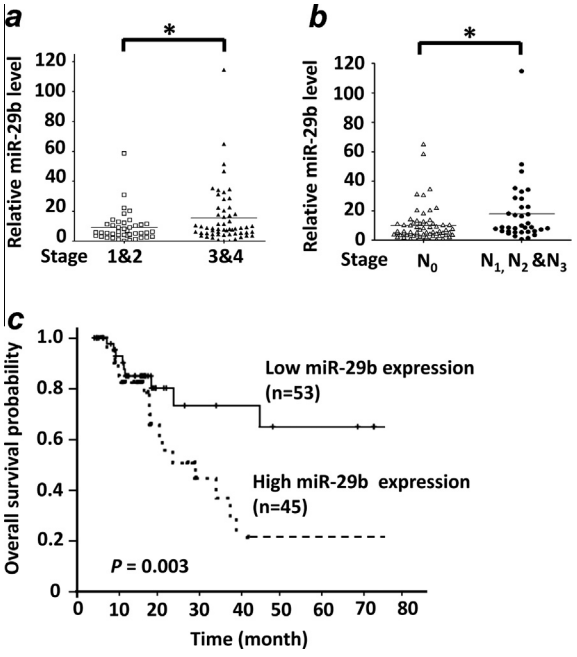

Figure 1. MiR-29b correlates to advanced TNM stage, lymph node involvement, and poor survival probability in OSCC patients. (a and b) Real-time quantitative RT-PCR of miR-29b in oral squamous cell carcinoma patients ($n = 98$). Patient samples were grouping by TNM stage ($P < 0.001$) and lymph nodule involvement ($P < 0.01$). (c) Kaplan–Meier survival curves for OSCC patients plotted on miR-29b. The log rank tests show statistical difference in survival between patients with high (fold change >8.76) and low miR-29b expression (fold change ≦ 8.76; $P = 0.003$).