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# Downregulation of miR-218-5p promotes invasion of oral squamous cell carcinoma cells via activation of CD44-ROCK signaling

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ABSTRACT

The invasion front of oral squamous cell carcinoma (OSCC) harbors the most aggressive cells of the tumor and is critical for cancer invasion and metastasis. MicroRNAs (miRNAs) play important roles in OSCC progression. In this study, we modelled the OSCC invasion front on a microfluidic chip, and investigated differences in miRNA profiles between cells in the invasion front and those in the tumor mass by small RNA sequencing. We found that miR-218-5p was downregulated in invasion front cells and negatively regulates OSCC invasiveness by targeting the CD44–ROCK pathway. Thus, miR-218-5p may serve as a useful therapeutic target for OSCC. Moreover, invasion front cell isolation based-on microfluidic technology provided a useful strategy for cancer invasion study.

## 1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity [1,2]. Most cases show aggesive growth pattern with metastasis to cervical lymph nodes occurring at their early stages. Distant metastasis of OSCC usually occurs at a later stage and is associated with poor prognosis [3]. Clarifying the mechanisms underlying OSCC invasion and metastasis is critical for the development of new strategies for improving patient outcome.

The invasion front of tumor tissues comprises a subgroup of cells at the edge of the tumor mass where the most aggressive cells presumably reside. Invasion front cells in OSCC were frequently observed to undergo epithelial-to-mesenchymal transition (EMT) with enhanced invasion and metastasis [4,5]. Accordingly, epithelial cadherin (E-CADHERIN) expression was downregulated in the invasion front relative to central/superficial areas of OSCC samples, and this was linked to histological invasiveness [4]. MiRNAs are well known as an epigenetic level to regulate cancer progression. Recent studies have shown that miRNAs play an important role in the invasion and metastasis of OSCC via targeting different proteins and pathways [6–8]. However, there is limited information on miRNAs expression in OSCC invasion front cells.

So far, the difficulty of isolating viable invasion front cells from tumor tissues has hindered elucidation of the characteristics of these highly invasive cells. Recent advances in microfluidics technology have provided a new platform for modeling cancer cell invasion [9,10]. We previously reproduced growth factor- and stromal cell-induced cancer cell invasion on microfluidic chips [11,12]. In the present study, we used a microfluidic chip to model isolated OSCC invasion front cells. These cells showed increased invasiveness, mesenchymal property and reduced miR-218-5p expression, which was responsible to the activation of CD44-Rho-associated protein kinase (ROCK) signaling pathway.

## 2. Materials and methods

### 2.1. Cell culture

An OSCC cell line, UM-SCC6, was a kind gift from Peking Union Medical University. Cells were cultured in DMEM high glucose culture medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Science cell), 100 U/ml penicillin and 100 U/ml streptomycin (Hyclone) at 37 °C with 5% $CO_2$.

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### 2.2. Invasion assay based on a microfluidic chip and isolation of the invasion front cells

Cell invasion was assessed by a microfluidic chip. Both fabrication of the microfluidic chip and cell invasion assay method were described in our previous study [13]. Briefly, Matrigel™ (Corning) was loaded into the matrix channel. Cells were seeded into the cell culture channel and cultured with serum-free medium. Medium containing 20% FBS was introduced into the stimulation channel. Both invasion distance and area were used to indicate cell invasion ability.

Cells that invaded through the matrix channel and migrated into the stimulation channel were termed as the invasion front cells in our study. These invasion front cells were harvested by trypsinization, then expanded to generate the second round invasion front cells by repeating above steps. The third round invasion front cells of UM-SCC6 were obtained and named as UM-SCC6-M.

### 2.3. Wound healing assay

Cell motility was evaluated by wound healing assay. Three wounds were created by pipette tip 24 h after seeding and the debris was removed. After culturing for another 48 h, wound areas were recorded and calculated with Image-Pro Plus 6.0. Cell migration rate was determined by the decrease of the wound area normalized to the starting time point.

### 2.4. Transwell invasion assay

Transwell invasion assay was performed using Transwell plates (8-μm pore size, Corning). The chamber inserts were coated with Matrigel™ (Corning). Cells were seeded into the top chambers, and medium supplemented with 20% FBS was used as a chemoattractant in the lower chamber. After 24 h' culturing, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The cells adhering to the upper surface of the membrane were removed by a cotton swab. The cells on the lower side of the membrane were counted.

### 2.5. Cell proliferation assay

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8, Dojindo). CCK-8 solution was added at different time points. The absorbance at 450 nm was measured by a microplate spectrophotometer (Enspire2300, Perkin Elmer).

### 2.6. Immunofluorescence staining

Cells were seeded on cell culture slides to reach confluency, then the cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. After blocking with goat serum, cells were stained with primary antibodies (Table S1) at 4 °C overnight. Primary antibodies were visualized by a TRITC-conjugated goat anti-rabbit IgG (Jackson). DAPI (Roche Diagnostics) was used to visualize nuclei.

### 2.7. Western blotting

Total protein was extracted using RIPA buffer (Sigma Aldrich). Proteins were separated by SDS-PAGE and transferred to polyvinylidenedifluoride (PVDF) membrane (Millipore). After blocking in non-fat milk, the membranes were incubated with primary antibodies (Table S2), then washed and incubated with secondary antibodies. Finally, the immunoreactive protein bands were detected by ECL detection system (Bio Rad). GAPDH was used as a loading control.

### 2.8. Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from cells using Trizol reagent (Takara). cDNA was synthesized using 5X All-In-One RT Master Mix (Abmgood). The mature miRNA was reverse transcribed with specific primers (Table S3) using Prime Script™ RT Master Mix (Takara). qPCR was performed using KAPA SYBR® FAST Universal qPCR Kits (KAPA). GAPDH and U6 were used as controls to normalize mRNA and miRNA, respectively. Relative quantification was calculated based on the $2^{-\Delta\Delta Ct}$ method. All reactions were carried out in triplicate.

### 2.9. Small RNA sequencing and data analysis

Small RNA sequencing and data analysis were done by Novogene. Briefly, sequencing libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB) and index codes were added to attribute sequences to each sample. Sequencing was performed on an Illumina Hiseq 2500/2000 platform and 50bp single-end reads were generated.

Raw data (raw reads) of fastq format were firstly processed through custom perl and python scripts. The small RNA tags were mapped to reference sequence by Bowtie [14]. Predicting the target gene of miRNA was performed by miRanda [15]. MiRNA expression levels were estimated by transcript per million (TPM) through the following criteria [16]:

Normalized expression = mapped readcount/Total reads*$10^6$.

Differential expression analysis of two samples was performed using the DEGseq (2010) R package. P-value was adjusted using q value [17]. Q value < 0.01 and |log2 (fold change)| > 1 was set as the threshold for significantly differential expression by default. KOBAS [18] software was used to test the statistical enrichment of the target gene candidates in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

### 2.10. Cell transfection

MiR-218-5p mimics and inhibitor were purchased from GenePharma. Cells were transfected with miR-218-5p mimics, miR-218-5p inhibitor, or negative control using Lipofectamine™ 2000 (Invitrogen Life Technologies), respectively. Transfection efficiency and further analyses were performed after 48 h.

### 2.11. Luciferase reporter assay

According to the miRanda predict website (http://www.microrna.org), two predicted binding region of miR-218-5p in the 3′ UTR of CD44 (CD44-P1 and CD44-P2) were amplified from human genomic DNA and inserted into the psiCHECK2 luciferase reporter vector (Ambion) (Table S4). For site-specific mutagenesis, the CD44-P1 region complementary to the seed sequence of miR-218-5p was mutated and obtained (psi-CD44-MutP1). Firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was normalized with renilla luciferase activity. Each assay was repeated in triplicate.

### 2.12. Statistical analyses

Statistical analysis was performed using SPSS version 16.0 for Windows. Student's *t* test was used to confirm comparisons of binary variables. Statistical significance was defined as $P < 0.05$.

## 3. Results

### 3.1. Microfluidic chip-based model of the invasion front

A microfluidic chip was used to model invasion front *in vitro* (Fig. 1A). The matrix channel indicating the maximum invasion distance for the cells was designed with a length of 500 μm. Matrigel in the