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Genome-wide profiles of metastasis-associated mRNAs and microRNAs in salivary adenoid cystic carcinoma

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ABSTRACT

Salivary adenoid cystic carcinoma (SACC) is often accompanied with poor prognosis due to local recurrence, distant metastasis, and perineural invasion. The mechanism involved in SACC metastasis is not yet fully understood. In this study, we profiled the expression of messenger RNA (mRNA) and microRNA (miRNA) in a SACC cell line, ACC-2, and a highly metastatic SACC cell line, ACC-M, using high-throughput sequencing. We discovered that: (1) differentially expressed (DE) mRNAs and DE miRNAs are potentially involved in SACC metastasis; (2) multiple regulatory interactions between DE miRNAs and DE mRNAs exist; and (3) miR-338–5p/3p target LAMC2 to impair motility and invasion of ACC-M and MDA-MB-231 cells. In conclusion, our study integrated the regulatory effects of miRNAs and mRNAs on SACC metastasis and provided a potential application for miRNAs in future therapeutic intervention.

1. Introduction

SACC represents approximately 10% of all salivary neoplasms, accounting for 25% of malignant tumors in the major salivary glands and 50% in the minor glands [1,2]. The poor prognosis and low overall survival rate associated with SACC is the primary result of local recurrence and distant metastasis, particularly metastasis to the nerves and lungs, causing an inherent resistance to complete surgical resection, systemic chemotherapy, and conventional radiotherapy [3,4]. Thus, a significant obstacle to the treatment of SACC is the lack of an in-depth understanding of the biological mechanisms involved in the metastasis associated with this type of cancer.

Several previous studies have revealed the gene expression differences between metastatic and non-metastatic SACC. Specific genes were shown to be involved in the migration and/or invasion of SACC during metastasis, including *Notch-4*, *EMMPRIN*, *ß-catenin*, *MMPs*, *cyclin D1*, etc. [5–7]. Furthermore, miRNAs were also thought to function in the progression of SACC metastasis. Numerous metastasis-related miRNAs that might be directly or indirectly involved in the metastatic progression of SACC have been identified [8]. Although previous studies have reported mRNAs and miRNAs to be essential in the progression of SACC, genome-wide profiling of mRNAs and miRNAs in elucidating the metastatic mechanism of SACC remains scarce. In this study, we combined mRNA and miRNA profiles in low and highly metastatic SACC cell lines for the first time. Our work provided available mRNA and miRNA signatures for diagnosis or prognosis and suggests that multiple mRNA-miRNA regulatory networks are involved in the progression of malignant tumors.

2. Material and methods

2.1. Cell lines and tissue specimens

ACC-2 and ACC-M cells were obtained from the West China Hospital of Stomatology (Sichuan, China). 95C, 95D, MCF7, MDA-MB-231, HepG2 and HCCLM3 cells were purchased from the China Center for Type Culture Collection (CCTCC, China). ACC-2, ACC-M, 95C and 95D were maintained in RPMI-1640 medium, while MCF7, MDA-MB-231, HepG2 and HCCLM3 cells were cultured in DMEM medium. All cells were maintained at 37 °C in a

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humidified atmosphere of 5% CO2 in medium supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin–streptomycin (Invitrogen).

The Institutional Review Board at Zheng Zhou University approved this study and informed consent was obtained from all patients. 22 non-metastasis SACC patients and 15 metastasis SACC patients were diagnosed in the Pathology Department of the First Affiliated Hospital of Zhengzhou University. All tumor or corresponding non-tumor tissue samples were collected and immediately stored in liquid nitrogen for further use.

2.2. RNA preparation, miRNA and mRNA analysis, and data analysis

Total RNA of all kinds of cells and tissue samples was isolated using TRIzol reagent (Life Technologies, USA) following the manufacturer's instructions. Small RNAs sequencing and miRNA expression evaluation were performed as previously described [9]. mRNA purification, cDNA synthesis, adaptor ligation, purification, and library construction were performed according to the manufacturer's instructions. mRNA sequencing was performed on an Illumina NextSeq500 platform. Following quality control and annotation with the reference database (http://www.ensembl.org), the gene expression levels were estimated with RPKM (Reads Per Kilobase per Million mapped reads). mRNAs or miRNAs with fold change (FC) ≥ 2 or FC ≤ 0.5 were treated as DE mRNAs or miRNAs in the two cells lines.

2.3. qRT-PCR analyses and data statistics

A miScript Reverse Transcription Kit and miScript SYBR Green PCR Kit (Qiagen, Germany) were used for qRT-PCR of miRNA, and RNU6 was used as an endogenous control. A reverse transcription system kit (Takara, Japan) and IQ SYBR Green Supermix were used for mRNA expression analysis, andβ-actin was set as an internal control. All qRT-PCRs were conducted using an iCycler system (Bio-Rad, USA) and relative expression levels were determined using the $(1 + E_T)^{(\Delta Ct)}/(1 + E_R)^{(\Delta Ct)}$ method.

2.4. miRNA target predication and miRNA-gene interaction analysis

The DE miRNAs in both cell lines were selected for target prediction. Three web-based miRNA target prediction databases were used to predict the potential targets of the DE miRNAs, including TargetScan (https://www.targetscan.org/vert60/), Miranda (http://www.microrna.org/microrna/home.do), and Microcosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). The open source software platform Cytoscape version 3.2.0 was used to analyze the miRNA-gene interactions.

2.5. Plasmid construction, cell transfection, and western blotting

A 3'UTR fragment of human LAMC2 gene containing two putative binding sites of miR-338–5p and miR-338–3p was amplified by PCR using the primers 5'-GGTTCTTGGGATACAGATCTC-3′ and 5′-TGGAGAACATGTTCACACCC-3'. The PCR product was then subcloned into the psiCHECK-2 vector (Promega, Madison, WI, USA) at the Xho1 site and named psiCHECK-2-LAMC2. HEK293 cells cultured in a 24-well plate were transfected with 100 ng psiCHECK-2-LAMC2 plasmids and 50 ng miR-338–5p agomir or miR-338–3p agomir (Guangzhou, China) with Lipofectamie 3000 (Invitrogen, USA). Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and Renilla luciferase activity was normalized with firefly luciferase activity.

Protein levels were evaluated with Western blotting. Fibronectin (ab32419) and LAMC2 (ab210959) antibodies were purchased from Abcam (Cambridge, United Kingdom), and β-actin antibodies (sc-47778) were obtained from Santa Cruz Biotechnology (CA, USA). Total cells protein was extracted with RIPA lysis buffer containing protease inhibitor and separated on a 10% or 12% SDS-PAGE gel. Protein was then transferred to polyvinylidene fluoride membranes and immunoblotted against the corresponding antibodies. Signals were visualized using an enhanced chemiluminescence detection system (Thermo Fisher Scientific, USA).

2.6. In vitro cell migration and invasion assays

Wound-healing assay was performed to evaluate cell migration ability as previous [10]. Cells invasion ability was determined using *in vitro* transwell invasion assays (Millipore, USA). 48 h later after agomir transfection, 1×10^5 cells were seeded in the upper chamber coated with Matrigel (BD Biosciences). At 48 h after invasion, the attached cells in the lower chamber were stained with violet purple. The number of invasive cells was determined using an inverted microscope (Leica) from four random fields. MDA-MB-231 cells migration ability was measured as same way but without Matrigel. All experiments including the migration and invasion assays were repeated independently at least three times.

2.7. Data analysis and statistical methods

All data were analyzed by one-way ANOVA and presented as the mean ± standard error of mean (SEM) using the statistical package SPSS (version 16.0). Significant differences were accepted at $^{*}P < 0.05$ or $^{**}P < 0.01$.

3. Results

3.1. MiRNA and mRNA expression profiling in ACC-2 and ACC-M

A previous study showed that ACC-M cells were derived from parental ACC-2 by a combination of *in vivo* selection and *in vitro* cloning, and exhibited a highly metastatic capacity [11]. Significantly enhanced cell motility in ACC-M comparison to ACC-2 was observed (Fig. 1A and B). Transwell assay showed the ACC-M cells exhibited higher invasion ability than the ACC-2 cells (Fig. 1C and D). These results indicated that the ACC-M cells could serve as a suitable model for the analysis of the molecular mechanisms of SACC metastasis.

ACC-2 and ACC-M cells were then subjected to RNA sequencing respectively. We obtained 1405 miRNAs in total, in which 251 miRNAs were ACC-2 specific, and 212 were only detected in the ACC-M cells (Fig. 1E). 42 miRNAs were identified to be dominantly expressed in ACC-2, and the expression of 25 of the miRNAs was upregulated in ACC-M (Fig. 1F). The top 15 DE miRNAs in low (ACC-2) and high (ACC-M) metastatic SACC cell lines were listed (Table S1). A total of 15,152 mRNAs were identified in ACC-2 and ACC-M cells, in which 960 and 1826 were revealed to be cell type specific expressed (Fig. 1G). 296 mRNAs exhibited increased expression, while the expression of 240 mRNAs was found to be downregulated in ACC-2 cells in comparison to the ACC-M cells (Fig. 1H). The top 15 DE miRNAs in ACC-2 and ACC-M cells were listed in Table S2. These results indicated that although ACC-2 and ACC-M are derived from the same type of tumor, a significant difference in the expression levels of miRNAs and mRNAs exists, which might possibly contribute towards their different cellular behaviors, particularly in the context of metastasis.