Bioinformatics analysis of dysregulated exosomal microRNAs derived from oral squamous cell carcinoma cells

Tadashi Masaoka1), Keiji Shinozuka2,3), Kenshin Ohara2), Hiromasa Tsuda4), Kenichi Imai5), and Morio Tonogi2)

1) Division of Oral Structural and Functional Biology, Nihon University Graduate School of Dentistry, Tokyo, Japan
2) Department of Oral and Maxillofacial Surgery, Nihon University School of Dentistry, Tokyo, Japan
3) Division of Oral Maxillofacial Surgery, School of Medicine, Teikyo University, Tokyo, Japan
4) Department of Biochemistry, Nihon University School of Dentistry, Tokyo, Japan
5) Department of Microbiology, Nihon University School of Dentistry, Tokyo, Japan

Abstract

Purpose: The present study aimed to identify dysregulated exosomal miRNAs associated with diagnostic and therapeutic biomarkers in oral squamous cell carcinoma (OSCC).

Methods: Microarray analysis was used to compare expression profiles of exosomal miRNAs in the OSCC-derived cell lines HSC-2, HSC-3, Ca9-22, and HO-1-N1 with those in human normal keratinocytes (HNOKs). The identified OSCC-related miRNAs and their potential target genes were analyzed with bioinformatic analyses, and the data were subjected to Ingenuity Pathway Analysis (IPA) to clarify functional networks and gene ontologies of the identified exosomal miRNAs secreted by OSCC cells.

Results: Comparison with HNOKs detected 8 upregulated and 12 downregulated miRNAs in OSCC-secreted exosomes. The potential target miRNAs of these dysregulated miRNAs were suggested by IPA, and 6 significant genetic networks were indicated by genetic network analysis. Furthermore, 4 crucial upstream miRNAs—miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p—were identified. miR-125b-5p was a central node in the most significant network. Gene ontology analysis showed significant enrichment of genes with cancer-related functions, such as molecular mechanisms of cancer, cell cycle, and regulation of the epithelial-mesenchymal transition.

Conclusion: These results provide a comprehensive view of the functions of dysregulated exosomal miRNAs in OSCC, thus illuminating OSCC tumorigenesis and development.

Keywords: exosome, microarray, miRNA, oral squamous cell carcinoma, pathway analysis

Introduction

Despite progress in the understanding and treatment of oral cancer, it remains the sixth most diagnosed cancer worldwide and is responsible for approximately 145,000 deaths annually [1]. Oral squamous cell carcinoma (OSCC) accounts for 90% of all oral cancers [2]. Tobacco and alcohol consumption are the main risk factors OSCC, and human papilloma virus (HPV) infection is involved in a subset of cases [3]. Current therapeutic options for OSCC are surgery, chemotherapy, radiotherapy, and immunotherapy. However, despite diagnostic and therapeutic advances, the 5-year overall survival rate of OSCC (approximately 50% to 60%) has remained unchanged for decades, and the disease has high metastatic potential [4]. In addition, traditional cancer-screening techniques, such as detecting and imaging protein biomarkers, are inadequate for early OSCC detection and diagnosis [2]. Therefore, an enhanced understanding of the molecular mechanisms underlying OSCC tumorigenesis is crucial to ensure early diagnosis, improve prognosis prediction, and establish effective therapies.

Cells communicate with neighboring and distant cells via secretion of extracellular vesicles (EVs) [5]. Exosomes, first discovered in 1983, are 50-150 nm in diameter and are present in the extracellular space and in blood, urine, and saliva [5]. Exosomes carry functional molecules, including proteins, metabolites, lipid mediators, and nucleic acids (miRNAs, non-coding RNAs, and DNA) [5], and are involved in important processes such as immune responses and tissue repair. However, tumor cells can also secrete large numbers of exosomes, which alter their environment and enable tumor cells to grow and disseminate by triggering vascular permeability and conditioning premetastatic sites [6]. Therefore, exosomes have potential clinical applications as they may contain biomarkers and therapeutic targets, and their utility in OSCC diagnosis and treatment is receiving considerable attention.

The presence of miRNAs and microRNAs (miRNAs) in exosomes was first reported in 2007 [7]. The profile of exosomal miRNAs (which are 22-25 nucleotides long) is similar to that of cellular miRNAs. These molecules play roles in stem cell differentiation, hematopoiesis, exocytosis, differentiation, organogenesis, and tumorigenesis [7]. The specificity and distinct signatures of tumor exosomal miRNAs, as well as their involvement in tumorigenesis, invasion, angiogenesis, progression, metastasis, and chemoresistance, have been reported [7]. Fukumoto et al. reported that in OSCC tumors, miRNA-26a and miRNA-26b enhance cell migration and invasion by regulating expression of transmembrane protein 184B [8]. Kawakubo et al. demonstrated that exosomal miR-200c-3p induces an invasive phenotype in previously noninvasive cells within the OSCC tumor mass [9]. Thus, exosomal miRNAs have potential for diagnostic and therapeutic applications. Because exosomes can be isolated with minimal invasive methods, their use as novel molecular diagnostic tools in cancer therapy has recently sparked interest in the use of liquid biopsies. Exosomes may provide more-sensitive biomarkers for cancer detection, as exosomal miRNAs have greater stability than cellular miRNAs [9]. Several studies have reported clinical applications of liquid biopsies, including assessment of exosomal miRNAs in head and neck squamous cell carcinoma (HNSCC). Lin et al. reported increased expression of miR-21 and miR-24 in HNSCC [10], and Sommerer et al. suggested that high levels of miR-142, miR-186, miR-195, miR-374b, and miR-574 are prognostic biomarkers for the disease [11].

Although several exosomal miRNAs have been reported as potential biomarkers and their biological functions characterized, no systematic analysis of OSCC exosomal miRNAs has been published. The aim of this study was to identify exosomal miRNAs associated with OSCC and discover their functions and clinical significance.

Materials and Methods

Cell culture

Human normal oral keratinocytes (HNOKs) were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA; catalog number 2610) and cultured in an oral keratinocyte medium (ScienCell Research Laboratories) in accordance with the manufacturer’s instructions. The OSCC-derived cell lines HSC-2 (catalog number JCRB0622), HSC-3 (JCRB0623), Ca9-22 (JCRB0625), and HO-1-N1 (JCRB0831) were obtained from the Human Science Research Resources Bank (Osaka, Japan). All cell lines were tested and declared free of mycoplasma infection by the company and used for experimentation within 3 months after purchase.

All OSCC-derived cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM). For differentiation, the cells were cultured in 0.5% or 1% type I collagen gel. Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO2 at 90% humidity. The medium was changed every 48 hours, and the spent medium was collected for analysis.

Response to Dr. Keiji Shinozuka, Department of Oral and Maxillofacial Surgery, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan
Fax: +81-3-3219-8356 E-mail: shinozuka.keiji@nihon-u.ac.jp

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Correspondence to Dr. Keiji Shinozuka, Department of Oral and Maxillofacial Surgery, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan
Fax: +81-3-3219-8356 E-mail: shinozuka.keiji@nihon-u.ac.jp

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Fax: +81-3-3219-8356 E-mail: shinozuka.keiji@nihon-u.ac.jp

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cell lines were maintained in Dulbecco’s modified Eagle’s medium/F-12 HAM (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Co.) and 50 U/mL of penicillin-streptomycin (Sigma-Aldrich Co.). All cell lines were cultured at 37°C in a humidified incubator in the presence of 5% CO₂.

Isolation of exosomal miRNA from culture media

Cells were cultured without FBS or penicillin-streptomycin for 48 h and the culture media was collected. Each sample of medium (10 mL) was centrifuged for 5 min at 3,200 × g to remove cell debris, and the supernatant was used for exosome isolation. Exosomes were isolated by column purification and a miRCURY Exosome Isolation Kit (Exiqon, Woburn, MA, USA), which recovers all RNA species including miRNAs, in accordance with the manufacturer’s instructions. The quality and quantity of the extracted RNA were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Samples with optical density (OD)₂₆₀/OD₂₈₀ ratios of 1.60-2.20 were used in microarray experiments.

MiRNA expression profiling

MiRNA expression profiling was performed on 10 samples (HNOK: n = 2; OSCC-derived cell lines HSC-2, HSC-3, Ca9-22, and HO-1-N1: n = 2 each). The sixth-generation miRCURY LNA miRNA polymerase chain reaction (PCR) system (Exiqon) containing miRNome human panels I + II was used (V.4, Exiqon) and consists of primer sets against 752 established human miRNAs, in accordance with the manufacturer’s recommended protocol. Briefly, first, miRNAs were reverse-transcribed from total RNA (200 ng) by using miRNA-specific reverse transcription primers. The cells produced much lower yields, with RNA concentrations of approximately 50 ng/µL each. Next, the reverse-transcribed miRNAs were amplified with a locked nucleic acid-enhanced PCR primer anchored in the miRNA sequence and a universal PCR primer [12]. The raw data were received as normalized miRNA expression profiles. To identify deregulated miRNAs, this study focused on miRNAs that were differentially expressed between OSCC-derived cell lines and HNOKs by ≥1.5 fold.

MiRNA target gene prediction and gene ontology analysis

To identify the biological functions of differentially expressed miRNAs and the signaling pathways involving their potential targets, Ingenuity Pathway Analysis (IPA, QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis, accessed on November 10, 2020) was performed [13-15]. Genes were categorized in relation to their location, cellular components, and reported or suggested molecular functions. The identified genes were also mapped to genetic networks available at the IPA database, and a score based on the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone was obtained. A score of 3, which indicates a 1/1000 chance that a gene is in a network by chance (i.e. a 99.9% confidence level), was used as the threshold when identifying gene networks.

Results

Exosomal miRNA expression in OSCC-derived cell lines

To identify exosomal miRNAs differentially expressed between OSCC-derived cell lines and HNOKs, miRNA array expression profiling was performed. As compared with HNOK-derived exosomes, HSC2, HSC3, Ca9-22, and HO-1-N1 cell-derived exosomes contained 183, 129, 179, and 139 upregulated (Fig. 1A) and 111, 82, 136, and 99 downregulated (Fig. 1B) miRNAs, respectively. Comparison with HNOKs identified 8 upregulated and 12 downregulated miRNAs in all 4 OSCC-derived cell lines.

Genetic network and ontology analysis

First, the IPA miR target filter was used to identify potential miRNA targets of the 20 differentially expressed miRNAs. The analysis revealed 236 potential mRNA targets with experimental evidence. Next, genetic network analysis of the regulated miRNAs and their target genes was performed by using IPA. These networks show functional relationships between gene products, as indicated by published interactions. Four highly significant networks were observed with some common biological functions, including cellular development, cellular growth and proliferation, and cell death and survival (Table 1). The IPA tool then associated these networks with known biological pathways. Four networks were significant in OSCC (i.e. they were composed of more identified genes than would be expected by chance). The network with the highest score (network 1) was centered around miR-125b-5p (Fig. 2), which was linked to most genes in the network. To strengthen the gene regulatory network data and deepen insight regarding the functions of target genes and their regulation, the relationships of the 4 upstream miRNAs (miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p) that were most relevant in IPA upstream regulator analysis were combined into an integrated target gene regulatory network (Table 2). MiRNA expression profiling results for the top 4 upstream miRNAs are shown in Table 2. Gene ontology analysis was also performed by using IPA downstream effect analysis. Cancer was the most significantly enriched functional category (Table 3). The ranges of P-values are attributable to the presence of several annotations within the Molecular Function categories. Enriched biological processes in the integrated network (Fig. 3) included advanced malignant tumor, epithelial-mesenchymal transition (EMT), and primary solid tumor and contained several important cancer-related targets. The associated downstream targets included transforming growth factor beta receptor 2 (TGFB2R2), vascular endothelial growth factor A (VEGFA), vimentin (VIM), E2F2 transcription factor 1 (E2F1), RB transcriptional corepressor 1 (RB1), BCL2 apoptosis regulator (BCL2), signal transducer and activator of transcription 3 (STAT3), tumor necrosis factor, and zinc finger and BTB domain containing 7A (ZBTB7A). The upregulated targets included phosphatase and tensin homolog (PTEN), zinc finger protein, FOG family member 2 (ZFPM2), E2F transcription factor 3 (E2F3), stearoyl-CoA desaturase, erb-b2 receptor tyrosine kinase 3 (ERBB3), and tumor protein p53 (TP53).
Discussion

In this study, the potential regulatory effects of dysregulated exosomal miRNAs released by OSCC-derived cell lines were assessed. miRNA microarray analysis revealed 20 (8 upregulated and 12 downregulated) exosomal miRNAs that were differentially expressed in all 4 OSCC-derived cell lines, as compared with HNOKs. Analyzing the 20 miRNAs and their potential corresponding target genes, 6 genetic networks (Table 1) that included 4 miRNAs (miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p) that were significantly associated with cancer were detected. miR-125b was a central node in the network of highest significance; therefore, its related network was used for pathway construction. The enriched canonical pathways confirmed the relationships between the identified exosomal miRNA networks and cancer. Thus, these 4 miRNAs may have crucial roles in OSCC, and further investigations into their biological activities will aid in understanding tumorigenesis and tumor development.

MiR-125b is an established oncogenic miRNA. It is upregulated in various cancers [16-18] and its downregulation inactivates the tumor suppressor function of TP53 in human lung fibroblasts [19] and oral cancer [20]. MiR-125b is significantly downregulated in tongue squamous cell carcinoma, as compared with normal adjacent tissue, and this finding matches patterns observed for other oral cavity carcinomas [20]. Henson et al. [21] reported that miR-125b downregulation is important for OSCC development and progression. Furthermore, in a previous study [22], downregulation of miR-125b was observed in OSCC-derived cell lines and samples. MiR-125b-transfected cells showed a decreased proliferation rate and enhanced radiosensitivity to X-ray irradiation. Moreover, miR-125b expression correlated with OSCC tumor staging and survival. These findings indicate that dysregulating miR-125b expression or activity might contribute to tumorigenesis by promoting OSCC proliferation.

Fig. 2 The highest-scoring miRNA–target network (network 1). Functional relationships between dysregulated miRNAs and their target gene products, as indicated by known interactions, are shown. Node shapes indicate the functional class of each gene product. Node color intensity indicates the degree of upregulation (red) and downregulation (green) in oral squamous cell carcinoma-derived cell lines, as compared with human normal keratinocytes.

Canonical pathway analysis

To further understand the relationships between miRNAs and their target mRNAs, the target genes were categorized according to signaling pathway with the IPA tool. The top 30 canonical pathways are listed in Fig. 4. Several pathways related to cancer were among the most significant, followed by cell cycle: G1/S checkpoint regulation; network analysis also identified cell cycle regulation as a crucial cancer-related function of OSCC exosomes.
inhibits autophagy under hypoxia. In addition, miR-17-5p has been linked to the development of many other cancers, including colorectal [25], prostate [26], and lung [27] cancers, and its expression level is associated with cancer aggressiveness and therapy resistance [28]. In glioma cells, overexpression of miR-17-5p decreases Beclin 1-mediated autophagy [29].

A role for miR-23a has been demonstrated in prostate cancer [30]. In OSCC, Chen et al. [31] revealed that miR-23a suppresses tumor proliferation and invasion and promotes apoptosis. Using bioinformatic analyses, they predicted that these effects were exerted via targeting fibroblast growth factor 2. Moreover, miR-23a is overexpressed in several tumors and promotes invasion and metastasis [32-34].

MiR-200b is an important regulator of EMT [35]. The induction and effects of MiR-200b were consistent with those of a previous report, which found that miR-200b was significantly upregulated in World Health Organization (WHO) grade II/III OSCC, as compared with WHO grade I OSCC [36]. In human tongue cancer cells, miR-200b regulates chemotherapy-induced EMT by targeting the BMI1 proto-oncogene, polycomb ring finger [37]. In head and neck cancer, miR-200b overexpression is associated with disease progression and poor outcomes [36,38].

These studies indicate that miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p function as oncogenes. Therefore, these 4 miRNAs could be effective diagnostic markers and/or therapeutic targets for OSCC. In particular, a previous study reported that miR-125b is a promising target for OSCC treatment [22].

In conclusion, this is the first study to use bioinformatic analysis to investigate differences in exosomal miRNAs in culture supernatants of OSCC cell lines and HNOKs. Uptake of tumor exosome miRNAs affects premetastatic or progressive organ stromal cells, thus producing a tumor-supportive microenvironment [39]. The comprehensive miRNA-target gene expression profiling-assisted pathway analysis method used is an appealing approach for identifying candidate genes and pathways involved in the carcinogenesis of OSCC and other cancers. The 4 dysregulated miRNAs identified—miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p—were associated with tumor development, EMT regulation, and...
tumor genesis. Identification of novel molecular pathways and targets regulated by these miRNAs may improve understanding of OSCC and its tumorogenesis and lead to development of new therapeutic strategies for its treatment. Wang et al. reported that miR-23a can be used as a therapeutic tool in cancer management [40]. While these predictions require experimental validation in future studies, the present results provide new avenues for future studies of the potential of miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p as prognostic biomarkers and therapeutic targets in OSCC.

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Conflict of interest

The authors declare that they have no competing interests.

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