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Profiling of Salivary Exosomal Micro RNAs in Burning Mouth Syndrome Patients

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Purpose: The exact causes of burning mouth syndrome (BMS) is unclear so far. There are many studies to elucidate the relation between oral disease and genetic predisposition. In this study, we first tried to investigate salivary exosomal genetic components that could play an important role for diagnosing and elucidating the progression of BMS.

Methods: We compared salivary exosomal micro RNAs (miRNAs) of BMS Patients to those of control using next generation sequencing (NGS). Unstimulated whole saliva from 15 patients with BMS and 10 control subjects were divided into two sets. Isolated exosomes and their total RNAs were subject to NGS for the screening of miRNAs.

Results: There were up-regulated 10 exosomal miRNAs (hsa-miR-1273h-5p, hsa-miR-1273a, hsa-miR-1304-3p, hsa-miR-4449, hsa-miR-1285-3p, hsa-miR-6802-5p, hsa-miR-1268a, hsa-miR-1273d, hsa-miR-1273f, and hsa-miR-423-5p) and down-regulated 18 exosomal miRNAs (hsa-miR-27b-3p, hsa-miR-16-5p, hsa-miR-186-5p, hsa-miR-142-3p, hsa-miR-141-3p, hsa-miR-150-5p, hsa-miR-374a-5p, hsa-miR-93-5p, hsa-miR-29c-3p, hsa-miR-29a-3p, hsa-miR-148a-3p, hsa-miR-22-3p, hsa-miR-27a-3p, hsa-miR-424-5p, hsa-miR-19b-3p, hsa-miR-99a-5p, hsa-miR-548d-3p, and hsa-miR-19a-3p) in BMS patients comparing with those of control subjects.

Conclusions: We show that there are 28 differential expression of miRNAs between the patients with BMS and those of control subjects. The specific function of indicated miRNAs should be further elucidated.

Key Words: Burning mouth syndrome; miRNA; Mi-RNAs; Next generation sequencing

INTRODUCTION

Burning mouth syndrome (BMS) is a kind of chronic pain disease characterized by burning sensation or pain felt in the oral mucosa. Although many studies have been done in the past, it is still difficult to diagnose and treat clearly. The International Classification of Headache Disorders defines BMS accordingly as "an recurring burning or dysesthetic sensation in oral cavity, continuing for more than 2 hours per day and more than 3 months without clinically evident causative lesions" [1]. In the recent study, the female to male ratio was 5:1 and the age-adjusted female to male ratio was also 5:1. The mean age was 59.4 years (range, 25-90 years) and the highest prevalent age was the range of 70-79 years (0.38%) [2]. The main symptomatic area was the tongue (especially tongue tip) and 81.9% of patients felt burning sensation in the tongue alone or with other site. The demographic data in the recent studies were comparable to those in previous studies, and BMS was found most commonly in postmenopausal women [2].

The etiology of BMS is multifactorial. Various local factors have been mentioned in many studies in the meantime. The most relevant associations are oral parafunctional habit, salivary gland dysfunction and local nerve trauma [3,4].

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Systemic factors also affect the development and prevalence of BMS. Significant contributing factors are menopausal disorders, diabetes, and nutritional deficiency [3-5]. Several studies suggest psychologic factors, such as depression, anxiety and stress play an important role [4,6]. The prevalence of somatic symptoms is higher in the patients with psychologic problem than in the general population [7]. However, at present, histological, pharmacological, physiological, psychological and imaging evidence infers that BMS is based on the neuropathic mechanism [8]. Tongue biopsy from BMS revealed change of morphology and density in the epithelial and subpapillary nerve fibers [9]. Relief of pain using topical neuroactive drug [10] and raised trigeminal nerve sensitivity [11] suggest neuropathic background of BMS. Besides, there is an opinion that disorder of the nigrostriatal dopaminergic system would alter nociception of the trigeminal system [11]. Recently, many studies have reported dysregulation of microRNAs in patients suffering from pain disorder including complex regional pain syndrome, irritable bowel syndrome and fibromyalgia. MicroRNAs have an important role through the post-transcriptional regulation of proteins and the target is modulators in pain processing, such as γ-aminobutyric acid-α1, cyclooxygenase 2, TRPV1 and Na^+ and Ca^{2+} channels [12].

BMS is a pain syndrome confined to the oral mucosa, so consideration of saliva is important in studying BMS. Human saliva is complex secretion from major salivary glands, minor salivary glands and gingival crevice fluid [13]. Saliva contains a variety of hormones, cytokines, antibodies, enzymes, protein, peptide, DNAs, and RNAs. These various components of saliva are similar to those of plasma, because of interaction between saliva and blood [13,14]. They give clues about physiologic, psychologic, endocrinal and metabolic conditions for oral and systemic diseases [13,15]. In addition, saliva collection is easy and non-invasive compared with other methods. However, there are several disadvantages in the research using saliva. They are variability, possibility of contamination and the presence of proteins, such as amylase that covers other proteins. To overcome these problems, the research about salivary exosomes is needed [16].

Salivary exosomes are small lipid bilayer vesicles with an average diameter of 30-100 nm and released into the extracellular environment through fusion of multivesicular bodies with the plasma membrane. Exosomes contain various proteins, mRNA and noncoding small RNA [14]. Exosomes interact with other cells, transfer protein and RNA into cells, and allow transferred RNA to be expressed in target cells [17]. The types of small RNA include microRNAs (mi-RNAs), ribosomal RNAs (r-RNAs), transfer RNAs (t-RNAs), piwi-interacting RNAs (pi-RNAs), small nuclear RNAs (sn-RNAs) and small nucleolar RNAs (sno-RNAs). Among these, miRNA play an essential role in the various functions of nucleic acids and proteins, such as transcription from DNA to RNA, and translation of mRNA into proteins, which can lead to various diseases if its function is wrong [18].

We hypothesize that the use of exosomal miRNA biomarkers will help early detection and establishing the pathogenesis of BMS. To profile exosomal miRNA, extracted total RNAs are subject to next-generation Sequencing (NGS) analysis. We suggest several salivary exosomal miRNAs which can serve as candidates for pathogenesis and diagnosis of BMS.

MATERIALS AND METHODS

1. Research Subjects and Collection of Saliva

Fifteen randomized Korean patients with BMS (mean±standard deviation [SD] age, 66.1±11.5 years) who visited the Department of Oral Medicine, Kyungpook National University Dental Hospital were enrolled in this study. All the patients fulfilled the clinical criteria of primary BMS. In brief, all patients do not showed any problems, such as hormonal changes, allergies, dry mouth-related disease (Sjogren) or drugs or radiation therapy, anti-depressant, nutritional changes, infections, or acidic reflux. Age-sex matched 10 patients without BMS or any other oral mucosal pain diseases were recruited as control group after their dental and medical examination. Unstimulated whole saliva samples were collected after mouthwash with distilled water for 1 minute and waiting for additional 5 minutes. This study was approved by the Institutional Review Board of Kyungpook National University (IRB no. KNU 2016-0113) and informed consents were obtained.

2. Exosome and RNA Purification

Exosomes were obtained using ExoQuick precipitation methods (System Biosciences [SBI], Mountain View, CA, USA). A 500 μL of whole saliva was mixed with 500 μL of ExoQuick solution, incubated overnight at 4°C, and centrifuged at 1,500 g and 4°C for 15 minutes. The pellet was undergone resuspension in PBS and used for RNA isolation. In extraction of total RNA from the purified exosomes of both patients with BMS and control subjects, we used TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and miRNeasy Mini Kit (QIAGEN, Valencia, CA, USA), according to the manufacturers' protocols. NanoDrop (Thermo Scientific, Wilmington, DE, USA) was used to checking the quality and quantity of total RNA. For microarray analysis, RNA samples of each group were evaluated to get sufficient amount of RNA for the experiment.

3. Small RNA NGS Sequencing Analysis

A 50-100 ng RNA was used for NGS analysis in each group. The experimental and control samples were compared on the basis of fold changes and performing independent t-test. The false discovery rate was controlled by making an adjustment the p-value with the Benjamini–Hochberg algorithm. All data and genetic differential expressions were analyzed through R programming language v3.0.2 (R Development Core Team, 2013; http://www.r-project.org).

4. Statistical Analysis

All data were presented as the SDs in the Results. Mann–Whitney U-test has been used to compare differences between BMS group and control group with Origin 8.0 (OriginLab, Northhampton, MA, USA), and probability values less than 0.05% were considered to be statistically significant.

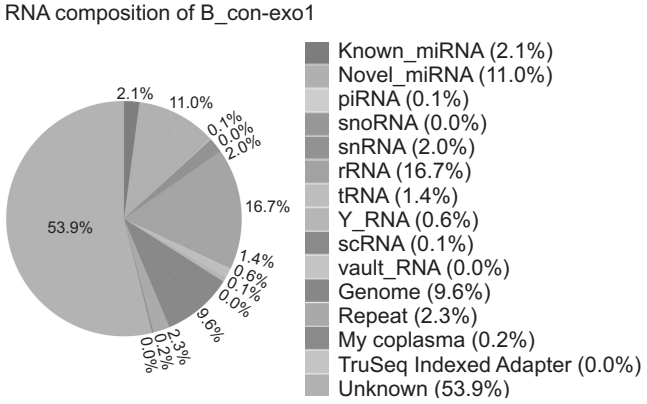

Fig. 1. Representative diagram of small RNA composition in salivary exosome.

RESULTS

1. Small RNA Composition in Salivary Exosome

Saliva samples (15 BMS patients, 10 control subjects) were divided into two sets for NGS analysis of extracted exosomal RNAs. The extracted RNAs are passed quality screening. Fig. 1 show the representative diagram of small RNAs composition using NGS analysis. There are known- and novel-miRNAs and other small RNAs, such as pi-, sn-, sc- (small conditional) RNAs. Most of small unknown RNAs are thought to originate from Streptococcus pneumonia.

2. Up- and Down-Regulated miRNAs Counts

In BMS group, there are total 73 miRNAs which are up-regulated more than 2-fold and 101 miRNAs which are down-regulated (Fig. 2). Of these, only 10 miRNAs are up-regulated and 18 miRNAs are down-regulated with

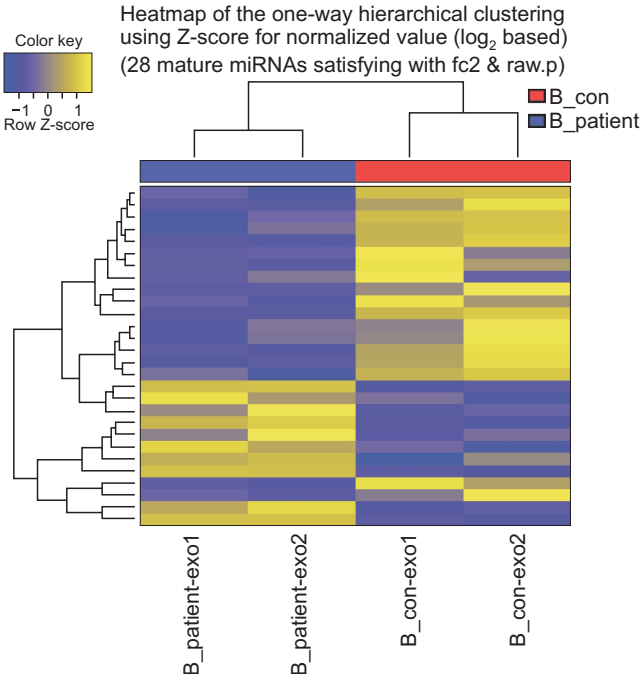

Fig. 2. Heatmap of exosomal miRNAs in the burning mouth syndrome patients and those of controls.

statistical significance in the BMS group (Fig. 2). Fig. 2 shows the heatmap of 28 miRNAs as the target for diagnostic or pathogenic candidates in the progression of BMS. The heatmap represents high-dimensional data as a two-dimensional image with colors displaying the intensity values. It shows the expression level of several genes consistence with other study [19,20].

3. Lists of Target miRNAs

Fig. 3 shows the name of up-regulated 10 miRNAs (hsa-miR-1273h-5p, hsa-miR-1273a, hsa-miR-1304-3p, hsa-miR-4449, hsa-miR-1285-3p, hsa-miR-6802-5p, hsa-miR-1268a, hsa-miR-1273d, hsa-miR-1273f, and hsa-miR-423-5p) and down-regulated 18 miRNAs (hsa-miR-27b-3p, hsa-miR-16-5p, hsa-miR-186-5p, hsa-miR-142-3p, hsa-miR-141-3p, hsa-miR-150-5p, hsa-miR-374a-5p, hsa-miR-93-5p, hsa-miR-29c-3p, hsa-miR-29a-3p, hsa-miR-148a-3p, hsa-miR-22-3p, hsa-miR-27a-3p, hsa-miR-424-5p, hsa-miR-19b-3p, hsa-miR-99a-5p, hsa-miR-548d-3p, and hsa-miR-19a-3p) in BMS patient group, comparing to those of control group.

DISCUSSION

The exosome contains miRNA from the origin cell selectively and then releases into the blood or other body fluid. The exosome is captured by the recipient cell, and the exosome-mediated miRNA affects the biological process of the recipient cell [21]. Exosomal miRNA profiling is possible only with high quality miRNA. Compared to cellular miRNA, exosomal miRNA is more stable and more resistant to physical degradation during storage or freeze because of lipoprotein structure of exosome [21,22]. MiRNA is a class of non-coding single stranded RNA composed of 19-24 neucleotides and has an ability to regulate a large portion of genome post-transcriptionally [20,23]. miRNAs have been known to regulate many pathophysiological processes and play an important role as biomarkers in many diseases. Recently, dysregulation of miRNAs have been identified to make pathologic condition, such as cancer, neurodegenerative diseases, cardiovascular disease and multiple pain disorders including complex regional pain syndrome (CRPS) and fibromyalgia [12]. CRPS is chronic neuropathic pain disorder that occurs at a specific site after trauma and characterized by accompanying autonomic nervous system dysfunctions, skin changes, and functional disorders. In one study of the micromodulation in CRPS, 18 miRNAs were observed. These significantly expressed miRNAs can have potential to modulate mRNAs in relation with ion channel and inflammatory mediators of CRPS. Patient stratification using miRNA profiling was also interesting in this study. Stratification of CRPS patients into subgroups according to

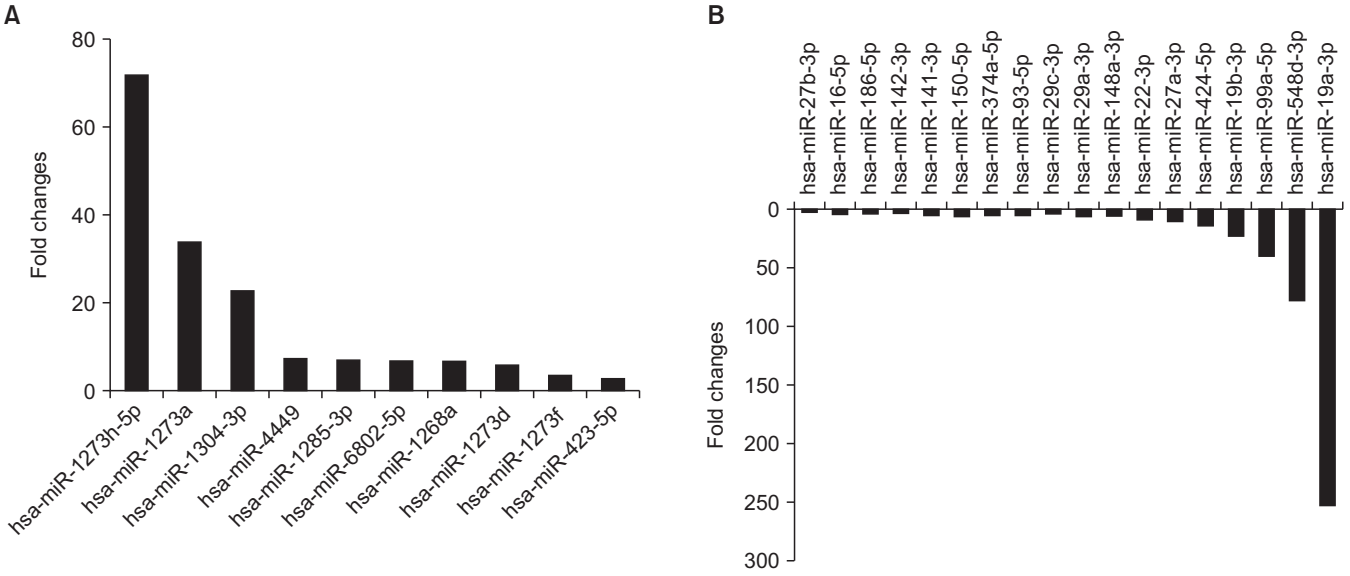

Fig. 3. The lists of target miRNAs with (A) up-regulated and (B) down-regulated differential expression changes in comparison between burning mouth syndrome patients and those of controls.

miRNA enable additional biomarkers to be regarded as significant, which were insignificant in the entire CRPS group, and more successful treatment would be possible via observations of various miRNAs affecting the disease [19]. Moreover, has-miR-150 was observed in whole blood like our study and confirmed to be related with headache. This could be applied to study of BMS in the future.

In the Alzheimer's disease, miR-29c-3p and miR-19b-3p were significantly lower in the patients compared with control subjects. miR-29c-3p and miR-19b-3p were thought to play an important role in cognitive function and regarded as useful biomarkers in the serum of Alzheimer's disease [24]. In our BMS group, miR-29c-3p and miR-19b-3p were also down-regulated, but we have not yet identified the exact role. In multiple sclerosis, miR-142-3p is related with glutamatergic synaptic alteration and a key player in IL-1ß-mediated synaptic dysfunction. Inhibition of miR-142-3p could be neuroprotective in multiple sclerosis [25]. MiR-142-3p was also down-regulated in our study of BMS and we need additional conformation to see if it plays similar role as in multiple sclerosis. In one research about CFA-induced inflammatory pain of murine muscles, miR-99a was significantly repressed in ipsilateral trigeminal ganglion neurons. Down-regulated miR-99a allowed pro-inflammatory and pro-nociceptive proteins to express and facilitated the development of inflammation and allodynia [26]. Mir-16-5p downregulated in our study was significantly increased in rheumatoid arthritis and breast cancer [27,28]. However, we don't know how it works in BMS yet.

Our study identified 28 miRNAs of salivary exosomes and this finding may accelerate the identification of potential targets for BMS. However, further research is needed to clarify the exact roles of suggested miRNA in scaled-up and more refined group. Manipulation of the miRNAs within exosomes may provide an effective tool for target therapy to specific cells and organs. We hope that additional research can give new insight about molecular mechanism underlying BMS.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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