Viral MicroRNAs Identified in Human Dental Pulp

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Abstract

Introduction: MicroRNAs (miRs) are a family of noncoding RNAs that regulate gene expression. They are ubiquitous among multicellular eukaryotes and are also encoded by some viruses. Upon infection, viral miRs (vmiRs) can potentially target gene expression in the host and alter the immune response. Although prior studies have reported viral infections in human pulp, the role of vmiRs in pulpal disease is yet to be explored. The purpose of this study was to examine the expression of vmiRs in normal and diseased pulps and to identify potential target genes. **Methods:** Total RNA was extracted and quantified from normal and inflamed human pulps ($N = 28$). Expression profiles of vmiRs were then interrogated using miRNA microarrays (V3) and the miRNA Complete Labeling and Hyb Kit (Agilent Technologies, Santa Clara, CA). To identify vmiRs that were differentially expressed, we applied a permutation test. **Results:** Of the 12 vmiRs detected in the pulp, 4 vmiRs (including those from herpesvirus and human cytomegalovirus) were differentially expressed in inflamed pulp compared with normal pulp ($P < .05$). Using bioinformatics, we identified potential target genes for the differentially expressed vmiRs. They included key mediators involved in the detection of microbial ligands, chemotaxis, proteolysis, cytokines, and signal transduction molecules. **Conclusions:** These data suggest that miRs may play a role in interspecies regulation of pulpal health and disease. Further research is needed to elucidate the mechanisms by which vmiRs can potentially modulate the host response in pulpal disease. *(J Endod 2017;43:84–89)*

Key Words

Dental pulp, human, inflammation, microRNA, viral

Significance

The functions of viral microRNAs include evading the immune response, prolonging the longevity of infected cells, and regulating the switch to lytic infections. This study explores the expression of viral microRNAs in normal and inflamed human pulps.

MicroRNAs (miRs) are 21- to 24-nucleotide (nt) long, single-stranded, noncoding RNAs. By fine-tuning the transcriptome, they regulate most fundamental biological processes including cell differentiation, signaling, cell death, and pathogen response (1, 2). miRs are transcribed as long precursor transcripts called primary miRs (pri-miRs) by RNA polymerase II. The localized stem loop structure on pri-miR is recognized by Drosha to release ~60–80 nt pre-miRs in the nucleus. These imperfectly paired stem loops are exported to the cytoplasm and are processed by Dicer to enzymatically generate miR duplexes. Of the 2 strands, the mature miR strand is finally loaded onto RNA-induced silencing complexes, whereas the passenger (star) strand is generally degraded. The mature miR guides the protein machinery to regulate messenger RNAs and eventually affects translation.

Recent studies show that viruses, especially those with DNA genome, encode miRs in order to regulate their life cycle inside the host (3–5). The viral transcripts generated in the nucleus are recognized by the host proteins and processed in a way similar to the canonical miR pathway. Viral pri-miRs are primarily transcribed by polymerase II; however, it has been shown that polymerase III generates transfer RNA-like miR precursors in murine gamma herpesvirus type 68 (6). After the discovery of viral microRNAs (vmiRs) in Epstein-Barr virus (EBV), several herpesvirus families were later found to encode miRs. To date, more than 250 different miRs of viral origin have been identified, and the list continues to expand (7).

Herpesviruses are large, double-stranded DNA viruses that infect a range of invertebrate and vertebrate animals. There are 8 types that infect humans: herpes simplex viruses 1 and 2 (HSV-1 and -2), varicella-zoster virus, EBV, human cytomegalovirus (HCMV), human herpesvirus 6, human herpesvirus 7, and Kaposi sarcoma-associated herpesvirus (KSHV). The genomic locations of alpha (eg, HSV-1 and -2) and gamma (eg, EBV and KSHV) herpesviruses miRs are clustered generally in the vicinity of latency-associated regions; however, beta herpesvirus (eg, HCMV) is scattered throughout the viral genome. The expression profiles of vmiRs are restricted to specific stages of the virus life cycle. For instance, most of the miRs in KSHV and EBV are expressed during latency, whereas HSV-1 miR-H1 and few HCMV miRs are expressed during the productive stages of the infection.

vmiRs may regulate both viral and/or host transcripts. Several vmiRs target viral genes to which they are antisense (eg, EBV miR-BART2 targeting *BALF5* gene), whereas others control transcripts emanating from other genomic location (eg, HSV-1 miR-H6

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Supported by the American Association of Endodontists Foundation, NIH R01DE021052, and NIH R56DE025399.

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0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2016.10.006

targeting infected cell polypeptide 4) (3, 8). Target identification reveals that several vmiRs regulate the same target, a feature commonly observed in the mammalian system. For instance, HSV-1 miR-H3 and miR-H4 regulate the level of pathogenesis factor ICP34.5, whereas EBV latent membrane protein 1 is controlled by 3 different vmiRs (3).

Modulation of host genes is another function of viral miRs. Given the capacity to regulate hundreds of probable host transcripts, vmiRs have the potential to significantly influence the host transcriptome. Global transcriptome profiling has revealed the set of host genes under direct influence of vmiRs. Unlike metazoan miRs, vmiRs exhibit limited sequence conservation even in closely related families, indicating their functional divergence (9). Furthermore, emerging evidence shows that KSHV miRs exert a protective function by targeting NF-kappa-B inhibitor alpha and nuclear factor kappa B pathways and thus rescuing cell cycle progression and inhibiting apoptosis. Intricate interactions of KSHV miRs with other viral oncogenes are likely to maintain or break the fine balance between uncontrolled cell growth and cell homeostasis and are, therefore, essential for KSHV-induced cellular transformation (10).

The oral cavity is an ecological niche of diverse microbial species. Several bacterial and viral species are known to coexist in the same oral environment. Both gram-positive and gram-negative bacteria are known as causal agents for various oral diseases. Herpesviruses are the predominantly detected family of viruses in such infections. Under "healthy" conditions, this indigenous microflora remains benign and assists the host by maintaining basal host immunity, thereby establishing a symbiotic association. However, influenced by various external and internal factors, this equilibrium may be disturbed, leading to modulated immune responses (11).

Although viruses have been found in diseased pulp and periapical tissues, a causal relationship between viral infection and endodontic disease is yet to be established. Ferriera et al (12) reported that 48% of apical abscesses samples had KSHV infections, whereas Chen et al (13) detected HCMV in 29% of the patients with similar disease. A more recent study suggested that B cells and plasma cells in inflamed granulomas are a major source of EBV infection and that EBV could play a pivotal role in controlling immune cell responses in periapical granulomas (11). The prevalence of herpesviruses varies across different reports; nonetheless, it is recognized that they may act as opportunistic infections. The presence of herpesviruses in human immunodeficiency virus–infected patients further supports the notion that these pathogens are activated when the host's immunity is compromised (14).

Although the role of these viruses in augmenting the disease has been proposed, there is little information on how they contribute toward pathogenesis. It is likely that one mechanism by which they augment pulpal and periapical disease is by reshaping the host immune response via vmiRs. To explore this, we first compared the viral encoded miR profiles in healthy and diseased human pulps. We then used *in silico* target prediction of the differentially expressed vmiRs to identify the potential host target genes that are vital in defense against pathogens.

Materials and Methods

Study Participants and Sample Collection

This study was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill, Chapel Hill, NC, and conforms to the Strengthening the Reporting of Observational Studies guidelines for reporting observational studies (http://www.strobe-statement.org). Written informed consent was obtained from 28 participants who were recruited from the School of Dentistry, University of North Carolina at Chapel Hill. The inclusion criteria were ≥12 years old and American Association of Anesthesiologists status I or II. Patients who had a compromised immune system or those who were taking medications known to influence the immune response were excluded from the study. Participants were enrolled into 2 groups based on the pulpal status of the teeth being treated. Normal pulps were extirpated from healthy third molars or teeth extracted for orthodontic purposes. These did not include teeth with carious lesions or deep (≥5 mm) probing defects. Diseased pulps were extirpated from carious teeth diagnosed with irreversible pulpitis defined with either carious pulpal exposures or the presence of spontaneous pain and an exaggerated and lingering response to cold (1,1,1,2-tetrafluoroethane) (15).

After obtaining informed consent, local anesthesia was administered. For teeth diagnosed with normal pulps, the teeth were extracted and the root canal system immediately accessed. The pulp was then extirpated using a sterilized barbed broach or Hedstrom hand file. For teeth diagnosed with diseased pulps, rubber dam isolation was obtained, and the tooth and rubber dam were disinfected with 0.2% chlorhexidine gluconate. The carious tooth structure was removed, and then the root canal system was accessed. Pulp tissue was collected using a sterilized barbed broach or Hedstrom hand file. Pulp tissue was gently separated from the instrument and placed in a sterile Eppendorf tube with 0.5 mL RNAsafer Stabilizer Reagent (VWR, Bridgeport, NJ). All samples were stored at −80°C until processing.

RNA Isolation and MicroRNA Microarray

Samples were thawed on ice and centrifuged at 4°C for 2 minutes at 12,000 rpm to remove the stabilizer reagent. Total RNA was extracted using the miRNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA was quantitated using the NanoDrop (Thermo Scientific, Wilmington, DE), and RNA integrity was assessed using the 2100 Bioanalyzer (Agilent, Foster City, CA). The microRNA (miRNA) expression profiles of normal and diseased pulps were interrogated using human miRNA microarrays (V3) and the miRNA Complete Labeling and Hyb Kit (both from Agilent Technologies, Santa Clara, CA). The microarrays consist of glass slides containing 8 identical 15K oligonucleotide microarrays incorporating probes for 866 human and 89 human viral miRNAs represented from the Sanger miRBase 12.0. The procedure was performed as described previously (16). Briefly, 300 ng total RNA samples were dephosphorylated, denatured by dimethyl sulfoxide, and then immediately transferred to an ice-water bath for ligation. The samples were incubated with T4 RNA ligase at 16°C in a circulating water bath for 2 hours. Labeled miRNAs were desalted through Micro Bio-spin 6 columns (Bio-Rad, Hercules, CA) for purification. Samples were hybridized at 55°C for 20 hours at 20 rpm in a rotating hybridization oven. The microarray slides were then washed using fresh wash buffer. Finally, the slides were scanned using the Agilent Microarray Scanner and the Agilent Feature Extraction Software version 10.5.1.1 (Agilent).

Statistical Analysis

For microarray data analysis, any expression value that was lower than the reported error for that particular gene (which includes negative expression values) was set to be equal to the estimated error rate. Quantile normalization was applied to the expression data. To identify genes that were differentially expressed in each group, we applied a permutation test to test the null hypothesis that the mean expression of each gene was the same in both groups. An exact hypothesis test was used because the sample size was small. We used the resulting *P* values to estimate the false discovery rate q-value when the differential expression of each miR is called "significant." Because we performed multiple