MicroRNA-335-5p Plays Dual Roles in Periapical Lesions by Complex Regulation Pathways

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Abstract

Introduction: MicroRNA-335-5p has been reported to regulate osteogenic and chondrogenic differentiations of mesenchymal stem cells. The aim of this study was to explore the function and regulation mechanism of miR-335-5p in apical periodontitis (AP). **Methods:** Total RNAs were extracted from human periodontal ligament fibroblasts (HPDLFs), 10 AP tissues, and 6 healthy periodontal ligament tissues using lysis buffer. Gene expression was detected using real-time polymerase chain reaction. The Dual Luciferase Assay (Promega, Madison, WI) was used to test miR-335-5p directly targeted urokinase-type plasminogen activator receptor (uPAR) and the receptor activator of nuclear factor kappa-B ligand (RANKL). Western Blot was used to detect protein expressions of RANKL, uPAR, and the fragile X–related 1 gene (FXR1). The enzyme-linked immunosorbent assay was used to detect the secretions of interleukin 6, tumor necrosis factor alpha, and RANKL. Data were analyzed using the Student *t* test. **Results:** miR-335-5p acted as a positive mediator in HPDLF inflammation ($P < .05$). Two targets of miR-335-5p, uPAR and RANKL, were identified. Interestingly, uPAR was repressed by miR-335-5p at the basal level, but it can be relieved from miR-335-5p–mediated repression, which is called derepression, when HPDLFs were subjected to lipopolysaccharide stimulation. miR-335-5p promoted RANKL in HPDLFs regardless of whether or not it was under inflammatory conditions ($P < .05$). We proved FXR1 was responsible for the derepression of uPAR from miR-335-5p ($P < .01$). Both FXR1 and uPAR were positive mediators in HPDLF inflammation ($P < .05$). miR-335-5p, uPAR, RANKL, and FXR1 had the same expression profiles in HPDLF inflammation and AP tissues ($P < .05$). **Conclusions:** Our data showed that miR-335-5p may play dual roles in AP, and it might be considered as a target for therapeutic potency in clinical applications. *(J Endod 2017;■:1–6)*

Key Words

Apical periodontitis, fragile X–related 1 gene, microRNA-335-5p, receptor activator of nuclear factor kappa-B ligand, urokinase-type plasminogen activator receptor

Significance

We found miR-335-5p may play dual roles of promoting bone resorption and inflammation in AP. Thus, miR-335-5p could become a potential new target for therapeutic intervention in apical periodontitis through directly targeting uPAR and RANKL.

MicroRNAs (miRNAs) are small, noncoding RNAs emerging as new regulators in diverse biological progression, including differentiation, proliferation, and apoptosis (1, 2). Dysregulation of miRNAs plays major roles in various diseases like atherosclerosis, diabetes, rheumatoid arthritis, cancers, and so on (3). In addition to being presented in intracellular miRNAs, extracellular/circulating miRNAs packaged in extracellular vesicles/exosomes are detected in 12 human body fluids, including peripheral blood, plasma, serum, and even saliva. Moreover, extracellular miRNAs are remarkably stable, making their isolation and analysis easy (4–6). Thus, miRNAs have been highlighted because they are attractive biomarkers and potential therapeutic targets in many disorders.

miR-335-5p is encoded by its host gene mesoderm-specific transcript, which encodes 2 miRNAs (miR-335-5p and miR-335-3p); miR-335-5p is the more commonly studied product and is also referred to as miR-335. It is initially described as a tumor suppressor in breast cancer by directly targeting SRY-related HMG-box4 (*SOX4*) and tenascin C (7). Next, researchers demonstrated miR-335-5p was a tumor suppressor in gastric cancer, osteosarcoma cells and pancreatic cancer (8–10) while a tumor oncogene in astrocytoma and meningiomas (11, 12). Recently, miR-335-5p has been reported to regulate osteogenic and chondrogenic differentiations of mesenchymal stem cells (MSCs) (13, 14). Some studies reported significant up-regulation of miR-335-5p in adipose tissue inflammation, whereas others found a dramatic down-regulation in the inflammatory response of human mesenchymal stem cells (hMSCs) and osteoarthritis tissues (14–16). Persistent immune responses and alveolar bone resorption in the apical area are the main characteristics of apical periodontitis (17). To date, the function and regulation mechanism of miR-335-5p in periapical periodontitis has not been explored.

In this study, we showed the expression profiles of miR-335-5p in human periapical lesion tissues and acute lipopolysaccharide (LPS)-induced human periodontal ligament fibroblast (HPDLF) inflammation. Furthermore, we primarily illustrated the function and intricate regulation mechanisms of miR-335-5p in HPDLF inflammation, which could become a potentially new target for therapeutic intervention in apical periodontitis.

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TABLE 1. Primer Sequences Used in Real-time Polymerase Chain Reaction

Primer	Sequence 5′–3′
IL-6	F: 5′-GTGCCTCTTTGCTTTCAC-3′ R: 5′-GGTACATCCTCGACGGCATCT-3′
TNF-α	F: 5′-CAC AGTGAAGTGCTGGCAAC-3′ R: 5′-AGGAAGGCCTAAGGTCCACT-3′
RANKL	F: 5′-GCT CATCAACCACCCCAAT C-3′ R: 5′-ATGCAGCTCAGACACTCCTC-3′
uPAR	F: 5′-GCTCATCAACCACCCCAATC-3′ R: 5′-ATGCAG CTCAGACACTCCTC-3′
FXR1	F: 5′-GCTCATCAACCACCCCAATC-3′ R: 5′-AT GCAGCTCAGACAC TCCTC-3′
GAPDH	F: 5′-CCAAGGAGTAAGACCCCTGG-3′ R: 5′-TGGTTGA GCACAGGGTACTT- 3′
miR-335-5p	5′-CCTCAAGAG CAATAACGAAAAATGT-3′

F, forward; FXR1, fragile X–related 1 gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; R, reverse; RANKL, receptor activator of nuclear factor kappa-B ligand; TNF-α, tumor necrosis factor alpha; uPAR, urokinase-type plasminogen activator receptor.
MicroRNA Universal Primer and U6 primer were contained in the kit.

Materials and Methods

Tissue Samples, Cell Culture, Media, and Reagents

This study was approved by the ethics committee of West China College of Stomatology, Sichuan, China, and informed consent was obtained from each patient. As we previously reported (18), 10 apical periodontitis tissue samples and 6 healthy periodontal ligament tissue samples were obtained, and HPDLFs were isolated and cultured in Dulbecco modified Eagle medium (Gibco, Wien, Austria) supplemented with 10% fetal bovine serum (Gibco), 50 mg/mL streptomycin, and 100 U/mL penicillin (Sigma-Aldrich, St Louis, MO). HPDLFs were stimulated with LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich) at 1 μg/mL.

Real-time Polymerase Chain Reaction

Total RNA were extracted from HPDLFs or tissue samples using trizol (Life Technologies, Carlsbad, CA) lysis methods; mRNAs were reverse transcribed to complementary DNA (cDNA) using the PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan); miRNAs were reverse transcribed using the miScript II RT Kit (Qiagen, Germantown, MD). Real-time polymerase chain reaction (PCR) amplifications were run in the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using the SYBR Premix Ex Taq II kit (TaKaRa) for messenger RNAs and the miScript SYBR Green PCR Kit (Qiagen) for miRNAs. The primer sequences are provided in Table 1. Glyceraldehyde-3-phosphate dehydrogenase and RNU6B (U6) (Qiagen) were used as controls for mRNA and miRNA detection, respectively.

Dual Luciferase Assay

Target genes of miR-335-5p were predicted through miRanda (www.microrna.org) by selecting miR-335 as the key word and Homo sapiens as the species. We identified 2 potential targets, plasminogen activator, urokinase receptor and tumor necrosis factor superfamily member 11, whose mirSVR scores were −0.8611 and −0.8975, respectively. Even though plasminogen activator, urokinase receptor (PLAUR) and tumor necrosis factor superfamily member 11 (TNFSF11) are the official symbols, in most studies, they are known as urokinase-type plasminogen activator receptor (uPAR) and receptor activator of nuclear factor kappa-B ligand (RANKL), respectively. The fragments containing their putative binding sequences or mutant sequences

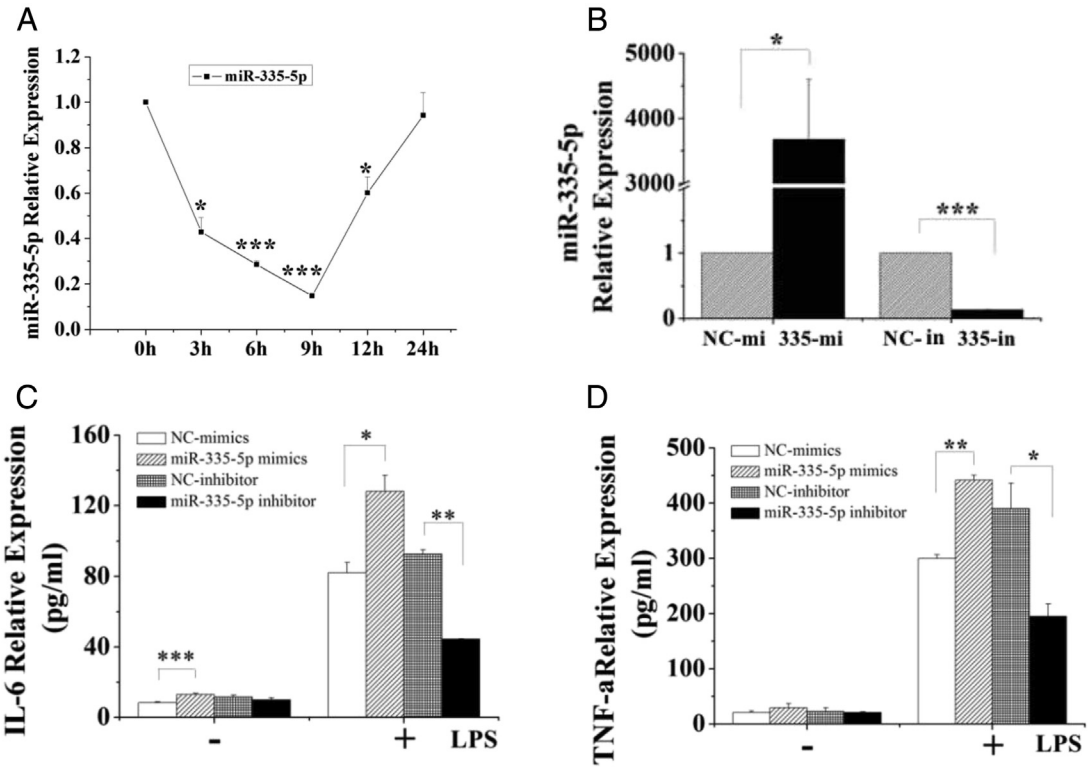

Figure 1. The expression and function of miR-335-5p in HPDLF inflammation. (*A*) Real-time PCR analysis of miR-335-5p expression in HPDLFs stimulated with LPS at different time points. U6 was used as the normalization control. (*B*) miR-335-5p expression in HPDLFs after transfection with miR-335-5p mimics (335-mi), inhibitor (335-in), or each negative control (NC-mi, NC-in) for 48 hours. (*C* and *D*) Enzyme-linked immunosorbent assay analysis of IL-6 and TNF-α in HPDLFs at the basal level and the 3-hour LPS exposure level after transfection with miR-335-5p mimics, inhibitor, or each negative controls for 48 hours. *P < .05. **P < .01. ***P < .001.