

Decreased expression of microRNA-30b promotes the development of pulpitis by upregulating the expression of interleukin-6 receptor

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Abstract. The present study aimed to examine the expression of interleukin-6 receptor (IL-6R) mRNA and protein in pulp tissues, blood and saliva from patients with pulpitis. It also investigated the association between IL-6R and microRNA (miR)-30b, as well as their effects on pulpitis. A total of 28 patients with pulpitis were recruited into the experimental group and 16 subjects with no pulpitis who also underwent tooth extraction were recruited into the control group. Pulp tissues, plasma and saliva were collected from all participants. Reverse transcription-quantitative polymerase chain reaction was used to determine the expression of IL-6R mRNA and miR-30b in all sample types. Western blot analysis was performed to examine the protein expression of IL-6R in pulp tissues, while ELISA was used to determine the contents of IL-6R protein in the plasma and saliva samples. A dual luciferase reporter assay was performed to verify the interactions between IL-6R and miR-30b. The expression of IL-6R mRNA in the pulp tissues, plasma and saliva was significantly increased in patients with pulpitis compared with the control group. Similarly, the IL-6R protein expression in the samples from patients with pulpitis were also significantly increased compared with the control group. Conversely, the expression of miR-30b was significantly reduced in the samples from patients with pulpitis compared with the control group. The dual luciferase reporter assay revealed that miR-30b may bind with the 3'-untranslated seed region of IL-6R mRNA to regulate its expression. The present study demonstrated that the upregulated expression of IL-6R in pulp tissues, plasma and saliva from patients with pulpitis was associated with the

downregulation of miR-30b expression. In addition, miR-30b may affect the progression of pulpitis via IL-6R and may be a potential genetic marker for the diagnosis of pulpitis.

Introduction

Pulpitis is an inflammation of the dental pulp and the most common type of pathological disease to affect the pulp tissue; it is characterized by pain, which can be severe (1). The diagnosis and treatment of pulpitis are difficult as there are multiple causes, which can involve various different microbes. Therefore, pulpitis has become a focus in clinical research. Multiple factors are responsible for the occurrence of pulpitis, including bacterial infection, physical and chemical stimulation and the immune response, although bacterial infection is the primary cause (2,3). Obligate anaerobes and facultative anaerobes are the primary pathogens that cause pulpitis (4). Following lysis, Gram-negative bacteria release bacterial endotoxin, which has strong cytotoxicity and antigenicity (5). Endotoxin may directly destroy the local tissues and induce an inflammatory reaction (6). It has been previously reported that endotoxin induces the release of prostaglandins, interleukin (IL), leukotrienes, transforming growth factor and tumor necrosis factor (TNF) by macrophages and pulp cells (7). IL-6 is a key cytokine in inflammatory processes and has been previously reported to serve an important role in the pathogenesis of pulpitis (8,9). However, IL-6 only exerts its biological effects after binding with the IL-6 receptor (IL-6R) to form an IL-6/IL-6R complex, which is subsequently combined with gp130 to form a high-affinity complex (10). However, the regulatory effects of IL-6R in pulpitis and the regulatory mechanism of the upstream genes of IL-6R have not been entirely identified.

MicroRNAs (miRNAs or miRs) are a type of non-coding, small RNA molecule made up of 18-22 nucleotides, which regulate the expression of proteins at mRNA level in eukaryotes (11-13). The pathogenesis of pulpitis is accompanied by changes in the expression of a variety of miRNA molecules and proteins, suggesting that miRNA may serve important roles in the regulation of proteins associated with the disease (14-16). In the present study, the expression of IL-6R mRNA and protein were examined in pulp tissues, blood and saliva from patients

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with pulpitis. The association between IL-6R and miR-30b was also investigated as well as their effects on pulpitis.

Patients and methods

Patients. A total of 28 patients with pulpitis (12 male and 16 female; age range, 20–51 years; median age, 37.6 years) who underwent tooth extraction at Nanjing Stomatological Hospital (Nanjing, China) and The First Affiliated Hospital of Nanjing Medical University (Nanjing, China) between June 2014 and December 2016 were recruited into the experimental group. A total of 16 subjects who had no pulpitis but also underwent a tooth extraction operation (6 male and 10 female; age range, 19–52 years; median age, 36.9 years) were recruited into the control group. The exclusion criteria were: Intake of non-steroidal drugs, use of antibiotics and drinking alcohol or smoking within two weeks prior to diagnosis. Pulp tissues were collected from all subjects under sterile conditions, washed with saline and stored in liquid nitrogen (-196°C) prior to use. Fasting peripheral blood was collected from all subjects on the morning of the tooth extraction and plasma was separated from venous blood (30 ml) by centrifugation at $1,000 \times g$ at 4°C for 10 min. Saliva was collected from all subjects prior to the operation on the day of tooth extraction and stored under -80°C prior to its further investigation. All procedures were approved by the Ethics Committee of Nanjing University (Nanjing, China) and written informed consent was obtained from all patients or their families prior to their inclusion within the study.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Pulp tissues (100 mg) were ground into a powder in liquid nitrogen and lysed using 1 ml TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Plasma (100 μl) or saliva (100 μl) were directly lysed using 1 ml TRIzol reagent. Total RNA was extracted using the phenol chloroform method (17). RNA (1 μg) was reverse-transcribed into cDNA at 42°C for 60 min and stored at -20°C prior to further experimentation. The TIANScript II cDNA First Strand Synthesis Kit (Tiangen Biotech Co., Ltd., Beijing, China) was used for RT of mRNA and the miRcute miRNA First-strand cDNA Synthesis kit (Tiangen Biotech Co., Ltd.) was used for the reverse transcription of miRNA.

To detect IL-6R mRNA expression the SuperReal PreMix (SYBR-Green) RT-qPCR kit (Tiangen Biotech Co., Ltd.) was used and GAPDH was used as the internal reference gene. The primer sequences of IL-6R were forward, 5'-TGGTGGATGTTCCCCCGAG-3' and reverse, 5'-TCCTGGGAATACTGGCACGG-3'. The sequences of GAPDH were forward, 5'-AAGGCTGTGGGCAAGG-3' and reverse, 5'-TGGAGGAGTGGGTGTCG-3'. The reaction system (20 μl) was composed of RT-qPCR-Mix (10 μl), upstream primer (0.5 μl), downstream primer (0.5 μl), cDNA (2 μl) and ddH₂O (7 μl). The PCR protocol was as follows: Initial denaturation at 95°C for 30 sec; denaturation at 95°C for 5 sec and 39 cycles for elongation at 60°C for 20 sec (iQ5; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The $2^{-\Delta\Delta\text{Cq}}$ method (18) was used to calculate the relative expression of IL-6R mRNA against GAPDH. Each sample was examined in triplicate.

The miRcute miRNA RT-PCR kit (Tiangen Biotech Co., Ltd.) was used to detect the expression of miR-30b and U6

was used as the internal reference. The sequences of the miR-30b primers were forward, 5'-CGCGCTGTAAACATCTACAC-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'. The sequences of the U6 primers were forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTTCAT-3'. The reaction system (20 μl) was the same as above. The reaction protocol was as follows: Initial denaturation at 95°C for 5 min; 95°C for 10 sec and 40 cycles at 60°C for 20 sec; and elongation at 72°C for 10 sec. The $2^{-\Delta\Delta\text{Cq}}$ method was used to calculate the relative expression of miR-30b against U6. Each sample was tested in triplicate.

Western blot analysis. Tissue samples in each group were lysed with 600 μl precooled radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Following lysis for 30 min on ice, the mixture was centrifuged at $1,200 \times g$ for 10 min at 4°C . Protein concentration was determined from the supernatant using a bicinchoninic acid determination kit (cat. no. RTP7102; Real-Times Biotechnology Co., Ltd., Beijing, China). The protein samples were subsequently mixed with 5X SDS loading buffer. Following denaturation in boiling water for 5 min, the 20 μg samples were loaded onto 10% SDS-PAGE for electrophoresis. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (100 V for 2 h) and blocked with 5% skimmed milk at room temperature for 1 h. The membranes were incubated with rabbit anti-human IL-6R (1:1,000; cat. no. ab128008) and β -actin (1:5,000; cat. no. ab8227) polyclonal primary antibodies (Abcam, Cambridge, UK) at 4°C overnight. Following washing 5 times (5 min each time) with PBS with Tween-20 (PBST), the membranes were incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3,000; cat. no. ab6721; Abcam) for 1 h at room temperature prior to washing again with PBST 5 times (5 min each time). The membranes were subsequently developed using an enhanced chemiluminescence detection kit (cat. no. 11520709001; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for imaging. Image Lab v3.0 software (Bio-Rad Laboratories, Inc.) was used to acquire and analyze the imaging signals. The relative content of IL-6R protein was expressed against β -actin.

ELISA. An IL-6R ELISA kit (Shanghai Joe Feather Biological Science and Technology Co., Ltd., Shanghai, China) was used to determine the concentration of IL-6R in plasma and saliva samples. In 96-well microplates, the standards (50 μl) and samples (10 μl liquid samples and 40 μl diluent) were added into the predefined wells, while the blank wells were left empty. In the wells for standards and samples, HRP-labeled conjugates (100 μl) were added prior to sealing the plates for incubation at 37°C for 1 h. Following washing of the plates 5 times, the substrates A (50 μl) and B (50 μl) were added into each well. After incubation at 37°C for 15 min, the stop solution (50 μl) was added into each well and the absorbance of each well was measured at 450 nm within 15 min.

Dual luciferase reporter assay. Bioinformatics prediction is a powerful tool used to study the various functions of miRNAs. To understand the regulatory mechanism of IL-6R miRanda (microrna.org/microrna/home.do), TargetScan (targetscan.org), PiTa (genie.weizmann.ac.il/pubs/mir07/mir07_data.

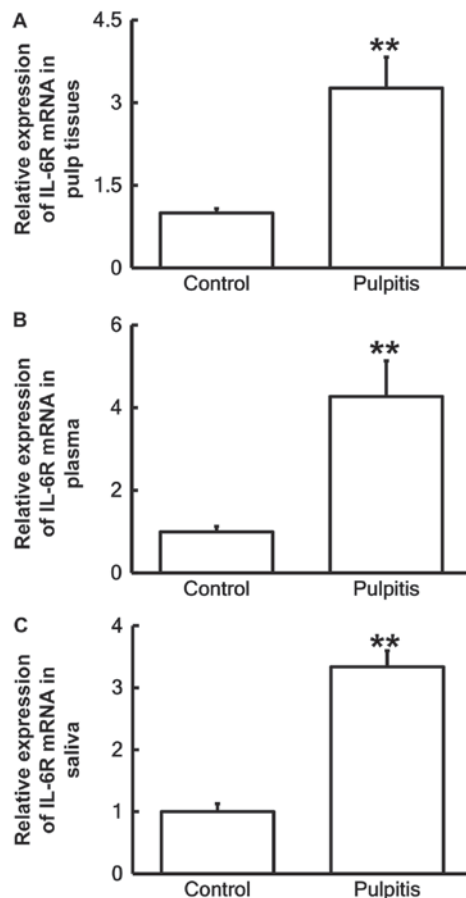


Figure 1. Expression of IL-6R mRNA in (A) pulp tissues, (B) plasma and (C) saliva from control subjects and patients with pulpitis. Reverse transcription-quantitative polymerase chain reaction was used to determine the expression of IL-6R mRNA. **P<0.01 vs. the control group. IL, interleukin.

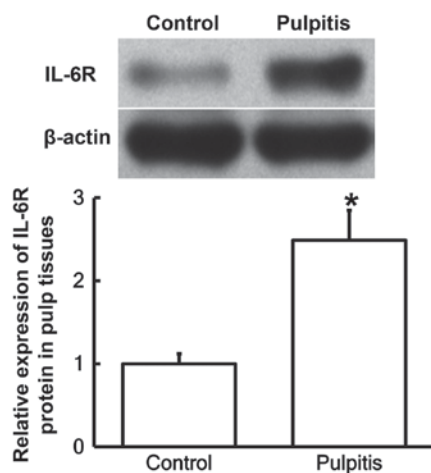


Figure 2. Expression of IL-6R protein in pulp tissues from control subjects and patients with pulpitis. Western blot analysis was performed to determine the protein expression of IL-6R in pulp tissues. *P<0.05 vs. the control group. IL-6R, interleukin-6 receptor.

html), RNAhybrid (bibiserv.techfak.uni-bielefeld.de/rnahybrid/) and PICTA (pictar.mdc-berlin.de/) were used to predict the miRNA molecules that may regulate IL-6R. The databases revealed that miR-30b may potentially regulate IL-6R.

Based on the bioinformatics results, wild-type (WT) and mutant seed regions of miR-30b in the 3'-untranslated region (UTR) of the IL-6R gene were chemically synthesized *in vitro* by Sangon Biotech Co., Ltd., Shanghai, China). The two ends were subsequently attached using the *SpeI* and *HindIII* restriction sites and cloned into pMIR-REPORT luciferase reporter plasmids (Ambion) using Lipofectamine 2000 (both Thermo Fisher Scientific, Inc.). Plasmids (0.8 μ g) with either the negative control (NC), WT or mutant 3'-UTR DNA sequences were co-transfected with agomiR-30b (100 nM; Sangon Biotech Co., Ltd.) into 293T cells. Following incubation at 37°C for 24 h, the cells were lysed using a dual luciferase reporter assay kit (Promega Corporation, Fitchburg, WI, USA) according to the manufacturer's manual. Fluorescence intensity was measured using a GloMax 20/20 luminometer (Promega Corporation). The fluorescence values of each group of cells were measured using *Renilla* fluorescence activity as the internal reference.

Statistical analysis. The results were analyzed using SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). The data were expressed as the mean \pm standard deviation. The data were tested for normality and multi-group data were analyzed using one-way analysis of variance. In cases of homogeneity of variance the Least Significant Difference and Student-Newman-Keuls post hoc methods were used; in cases of heterogeneity of variance the Tamhane's T2 or Dunnett's T3 post hoc methods were used. Comparison between two groups was performed using a Student's t-test. Each test was repeated at least 3 times. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of IL-6R mRNA is significantly elevated in pulp tissues, plasma and saliva from patients with pulpitis. RT-qPCR was performed to measure the expression of IL-6R mRNA in pulp tissue, plasma and saliva. The data revealed that the IL-6R mRNA levels in the pulp tissue (Fig. 1A), plasma (Fig. 1B) and saliva (Fig. 1C) from patients with pulpitis were significantly increased compared with the control groups (P<0.01 for all).

Protein expression of IL-6R is significantly elevated in pulp tissues from patients with pulpitis. To determine the protein expression of IL-6R in the pulp tissues, western blot analysis was performed. The results demonstrated that IL-6R protein expression was significantly increased in pulp tissues from patients with pulpitis compared with the control group (P<0.05; Fig. 2).

IL-6R protein is significantly increased in the plasma and saliva from patients with pulpitis. ELISA was used to examine the level of IL-6R protein in the plasma and saliva. The results revealed that the IL-6R protein content in the plasma and saliva of patients with pulpitis was significantly increased compared with the control group (both P<0.05; Fig. 3).

Levels of miR-30b are reduced in the pulp tissue, plasma and saliva of patients with pulpitis. RT-qPCR was used to measure the expression of miR-30b in the three sample types. The results demonstrated that the level of miR-30b in the pulp

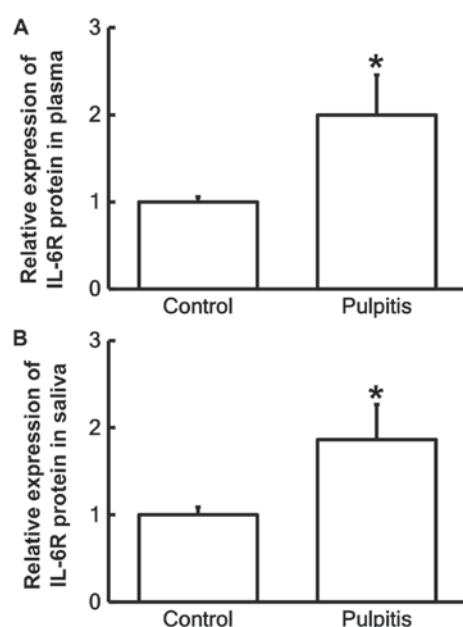


Figure 3. Presence of IL-6R protein in the (A) plasma and (B) saliva of patients with pulpitis and control participants. An ELISA assay was performed to determine the contents of the protein in the plasma and saliva. * $P < 0.05$ vs. the control group. IL-6R, interleukin-6 receptor.

tissue ($P < 0.05$; Fig. 4A), plasma ($P < 0.01$; Fig. 4B) and saliva ($P < 0.01$; Fig. 4C) of patients with pulpitis was significantly reduced compared with normal individuals.

miR-30b may bind with the 3'-UTR seed region of IL-6R mRNA to regulate its expression. A dual luciferase reporter assay was performed to identify any interactions between miR-30b and the 3'-UTR of IL-6R mRNA as predicted by bioinformatics. The fluorescence value of the cells co-transfected with the agomiR-30b and pMIR-REPORT-WT luciferase reporter plasmids was significantly reduced compared with the NC group ($P < 0.01$; Fig. 5). By contrast, the fluorescence value of the cells co-transfected with the agomiR-30b and pMIR-REPORT-mutant luciferase reporter plasmids was not significantly different from the NC group. These results indicate that miR-30b binds with the 3'-UTR seed region of IL-6R mRNA to regulate its expression.

Discussion

IL-6 is a cytokine with a wide variety of functions and its expression is typically elevated as part of the immune response (19). The production of IL-6 is stimulated by foreign bodies, including bacteria, endotoxin and dust particles (20). Elevated IL-6 expression in the body can lead to inflammatory diseases, including rheumatoid arthritis and Crohn's disease (21). In rheumatoid arthritis IL-6 stimulates the secretion of inflammatory mediators, including IL-1, by T lymphocytes and B lymphocytes, promotes the maturation and differentiation of B lymphocytes and enhances the effects of IL-1 β and TNF- α (22). It has been reported that IL-6 has a chemotaxis effect on other inflammatory cells during inflammatory responses, including neutral lymphocytes and mononuclear macrophages (23). IL-6 also induces the body to produce C-reactive protein and fibrinogen during inflammation

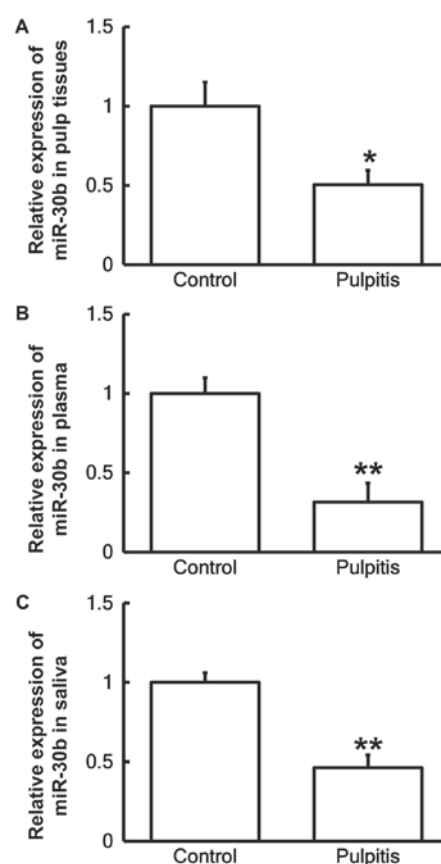


Figure 4. Expression of miR-30b in (A) pulp tissues, (B) plasma and (C) saliva from control individuals and patients with pulpitis. Reverse transcription-quantitative polymerase chain reaction was used to determine the expression of miR-30b. * $P < 0.05$ and ** $P < 0.01$ vs. the control group. miR, microRNA.

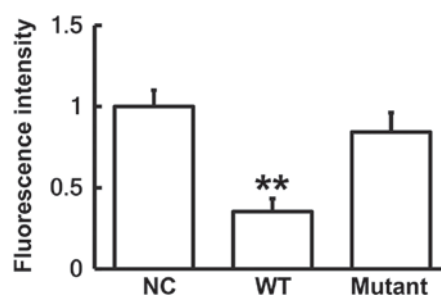


Figure 5. Identification of interactions between microRNA-30b and interleukin-6R mRNA using a dual luciferase reporter assay. Plasmids (0.8 μ g) with NC, WT or mutant 3'-untranslated region DNA sequences were co-transfected with agomiR-30b into 293T cells. Following cultivation for 24 h the cells were lysed using a dual luciferase reporter assay kit and the fluorescence intensity was measured. The fluorescence values of each group was measured and *Renilla* fluorescence activity was used as the internal reference. ** $P < 0.01$ vs. the NC group. NC, negative control; WT, wild type.

and may promote thrombosis (24). In addition, IL-6 also serves an important role in the occurrence and development of cell differentiation, coagulation and various types of cancer (25). The expression of IL-6 is significantly elevated during inflammatory responses, which are caused by injury, trauma, stress and infection. To have an effect in cells, IL-6 must first bind with the membrane receptor IL-6R, which is only expressed in hepatocytes, mononuclear cells, macrophages and certain

lymphocytes (26). This receptor-ligand complex then combines with gp130 to form a dimer, which initiates intracellular signals (27). Therefore, IL-6R is an important regulator for the biological functions of IL-6.

In the present study, it was revealed that the expression of IL-6R mRNA and protein was significantly increased in the pulp tissues, plasma and saliva of patients with pulpitis compared with the control group. This suggests that there is inflammation associated with pulpitis and upregulation of IL-6R is caused by the activation of mononuclear cells and lymphocytes, which secrete abundant IL-6 to produce antigenic immune responses. This is consistent with immune responses observed in other areas of the body (28).

The regulation of mRNA transcription and expression is a complex process, which is affected by multiple factors (29). The present study focused on miRNA molecules as the upstream regulatory factors of IL-6R. Bioinformatics was used to predict the upstream genes that regulate IL-6R. Literature searches suggested that miR-30b is an upstream regulator of IL-6R. Notably, miRNA molecules exert negative feedback effects on their target mRNA molecules by cutting the mRNA and serving as a translation restraint. Therefore, miRNA molecules are important regulatory factors in normal development, physiology and disease. A number of miRNA molecules have been identified as biomarkers of specific diseases (30,31). miR-30b is a member of the miR-30 family, which includes miR-30a, miR-30b, miR-30c, miR-30d and miR-30e (32). These five miRNA molecules are encoded by genes located on different chromosomes; however, they have similar seed sequences (33). It has been previously reported that miR-30b may be involved in a number of processes, including inflammatory responses, malignant tumor development and epithelial mesenchymal transition (34-37), and may have positive effects on nerve repair, the inhibition of apoptosis and blood vessel regeneration (34-37). A previous study has demonstrated that miR-30b is associated with the occurrence and development of human neural tube cells and their tumors; its expression is also associated with schizophrenia (38). In melanoma miR-30b regulates the expression of acetylglucosamine transferase, which promotes tumor metastasis (39). In lung cancer, trastuzumab inhibits the growth of cancer cells by upregulating miR-30b (40). In addition, miR-30b affects the development and degeneration of the mammary gland (41) and regulates the receptivity of the human endometrium (42). Therefore, miR-30b is associated with the growth, development, differentiation and migration of cells. The results of the present study were consistent with these previous findings as they reveal that miR-30b was significantly downregulated and IL-6R was significantly upregulated in the pulp tissues of patients with pulpitis. This suggests that the immune system may negatively regulate the cutting effect of miR-30b on IL-6R through the downregulation of miR-30b and also promote the expression of IL-6R, which participates in the immune response. The results of the expression of miR-30b and IL-6R in the plasma and saliva were similar to the results observed in the pulp tissues. This indicates that the changes to miR-30b and IL-6R levels may reflect the status of the inflammatory response and tissue damage in pulpitis. The present study has some limitations, including the small sample size and the patients primarily being Chinese.

In conclusion, the present study demonstrates in patients with pulpitis the decreased expression of miR-30b in pulp

tissues, plasma, and saliva regulates the expression of IL-6R, which serves a crucial biological role in the occurrence and development of the disease. The gene assessed in the present study may be used as a genetic marker for pulpitis detection in the future. However, further studies are required to fully assess its use in the diagnosis and treatment of the disease.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Authors' contributions

NZ, WY and LM conceived and designed the study. NZ, QZ, NW, SW, JG, XL and JW performed experiments. NZ and QZ wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Nanjing University (Nanjing, China) and written informed consent was obtained from all patients or their families prior to their inclusion within the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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