Comprehensive analysis of an lncRNA-miRNA-mRNA competing endogenous RNA network in pulpitis

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Background. Pulpitis is a common inflammatory disease that affects dental pulp. It is important to understand the molecular signals of inflammation and repair associated with this process. Increasing evidence has revealed that long noncoding RNAs (lncRNAs), via competitively sponging microRNAs (miRNAs), can act as competing endogenous RNAs (ceRNAs) to regulate inflammation and reparative responses. The aim of this study was to elucidate the potential roles of lncRNA, miRNA and messenger RNA (mRNA) ceRNA networks in pulpitis tissues compared to normal control tissues.

Methods. The oligo and limma packages were used to identify differentially expressed lncRNAs and mRNAs (DElncRNAs and DEmRNAs, respectively) based on expression profiles in two datasets, GSE92681 and GSE77459 , from the Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) were further analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. Protein-protein interaction (PPI) networks and modules were established to screen hub genes using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) and the Molecular Complex Detection (MCODE) plugin for Cytoscape, respectively. Furthermore, an lncRNA-miRNA-mRNA-hub genes regulatory network was constructed to investigate mechanisms related to the progression and prognosis of pulpitis. Then, quantitative real-time polymerase chain reaction (qRT-PCR) was applied to verify critical lncRNAs that may significantly affect the pathogenesis in inflamed and normal human dental pulp.

Results. A total of 644 upregulated and 264 downregulated differentially expressed genes (DEGs) in pulpitis samples were identified from the GSE77459 dataset, while 8 up- and 19 downregulated probes associated with lncRNA were identified from the GSE92681 dataset. Protein-protein interaction (PPI) based on STRING analysis revealed a network of DEGs containing 4,929 edges and 623 nodes. Upon combined analysis of the constructed PPI network and the MCODE results, 10 hub genes, including IL6, IL8, PTPRC, IL1B, TLR2, ITGAM, CCL2, PIK3CG, ICAM1, and PIK3CD, were detected in the network. Next, a ceRNA regulatory relationship consisting of one lncRNA (PVT1), one miRNA (hsa-miR-455-5p) and two mRNAs (SOCS3 and PLXNC1) was established. Then, we constructed the network in which the regulatory relationship between ceRNA and hub genes was summarized. Finally, our qRT-PCR results confirmed significantly higher levels of PVT1 transcript in inflamed pulp than in normal pulp tissues (p=0.03).

Conclusion. Our study identified a novel lncRNA-mediated ceRNA regulatory mechanisms in the pathogenesis of pulpitis.

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17 Abstract
18 **Background**. Pulpitis is a common inflammatory disease that affects dental pulp. It is important
19 to understand the molecular signals of inflammation and repair associated with this process.
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21 sponging microRNAs (miRNAs), can act as competing endogenous RNAs (ceRNAs) to regulate
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32 Detection (MCODE) plugin for Cytoscape, respectively. Furthermore, an lncRNA-miRNA-
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34 progression and prognosis of pulpitis. Then, quantitative real-time polymerase chain reaction
35 (qRT-PCR) was applied to verify critical lncRNAs that may significantly affect the pathogenesis
36 in inflamed and normal human dental pulp.
37 **Results**. A total of 644 upregulated and 264 downregulated differentially expressed genes
38 (DEGs) in pulpitis samples were identified from the GSE77459 dataset, while 8 up- and 19
39 downregulated probes associated with lncRNA were identified from the GSE92681 dataset.

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42   and the MCODE results, 10 hub genes, including IL6, IL8, PTPRC, IL1B, TLR2, ITGAM,
43   CCL2, PIK3CG, ICAM1, and PIK3CD, were detected in the network. Next, a ceRNA regulatory
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46   regulatory relationship between ceRNA and hub genes was summarized. Finally, our qRT-PCR
47   results confirmed significantly higher levels of PVT1 transcript in inflamed pulp than in normal
48   pulp tissues (p=0.03).
49   **Conclusion.** Our study identified a novel lncRNA-mediated ceRNA regulatory mechanisms in
50   the pathogenesis of pulpitis.
51
Introduction
53   Inflammation of the dental pulp (pulpitis) is associated with microbial infection of the root canal
54   system and the host response, which is characterized by spontaneous or provoked pain *(Bender,*
55   *2000; Rocas et al., 2015)*. Acute pulpitis can be an extremely painful condition and is believed to
56   be one of the main reasons patients seek emergency dental treatment *(Currie et al., 2017)*.
57   Depending on the state of inflammation, various treatment regimens are currently applied
58   clinically, including nonsurgical root canal treatment (such as pulpotomy and pulpectomy) and
59   surgical endodontic treatment *(European Society of Endodontology, 2006)*. If the issue cannot be
60   solved in time or appropriately, pulpitis may progress to pulp necrosis, periapical periodontitis,
61   or even severe oral and maxillofacial space infections *(Bertossi et al., 2017)*, ultimately causing a
62   significant medical and economic burden in terms of treatment costs.
63   Pulp inflammation represents a complex physiological response to harmful stimuli, such
64   as bacterial infections and physical and chemical injuries *(Yu & Abbott, 2016; Larsen & Fiehn,*
65   *2017; Pedano et al., 2018)*. The balance between inflammation and reparative processes in host
66   defense reactions can determine the extent of pulp inflammation, in which multiple signaling
67   pathways are involved *(Cooper, Holder & Smith, 2014; Farges et al., 2015)*. Studies over the
68   past decade have provided much information on the role of epigenetic modifications in
69   inflammatory diseases such as pulpitis and periodontitis. These studies have investigated
70   modifications of histones, methylation of DNA, and regulation of noncoding RNAs (ncRNAs),
71   among other epigenetic mechanisms *(Kearney et al., 2018)*.
72   Due to the application of sequencing and bioinformatics approaches, recent evidence has
73   identified previously unannotated transcripts and ncRNAs involved in pathologic mechanisms,
74   such as microRNAs (miRNAs), long noncoding RNAs (lncRNAs), pseudogenes and circular
75   RNAs, which were previously believed to have no biological functions *(Kaikkonen & Adelman,*
76   *2018)*. MiRNAs are small (~21-24 nucleotides in length), single-stranded ncRNA molecules that
77   can bind to complementary sequences within the 3' untranslated regions (UTRs) of target
78   messenger RNAs (mRNAs), resulting in mRNA degradation or repression *(Afonso-Grunz &*
79   *Muller, 2015)*. LncRNAs, which are ncRNAs of >200 nucleotides, function as competing

80   endogenous RNAs (ceRNAs) that interact with mRNAs, serving as miRNA sponges to restrain
81   miRNA function by competing for miRNA response elements (MREs) *(Tay, Rinn & Pandolfi,*
82   *2014)*. Increasing evidence has illustrated the essential roles of differentially expressed miRNAs
83   and lncRNAs (DEmiRNAs and DElncRNAs, respectively) in a variety of cellular and pathologic
84   processes associated with pulpitis, in which abnormal expression occurs *(Zhong et al., 2012;*
85   *Huang & Chen, 2018)*. However, there have been few comprehensive analyses of pulpitis-
86   associated lncRNAs and miRNAs in the context of a ceRNA network. The discovery of ceRNA
87   network interactions could provide important insights to advance our understanding of the
88   pathogenesis of pulpitis.
89   In this work, we first performed an integrated analysis and identified differentially
90   expressed genes (DEGs) between pulpitis and matched normal pulp tissues from two gene
91   expression profiles in the Gene Expression Omnibus (GEO) database. Then, Gene Ontology
92   (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis
93   were further conducted to analyze the major biological functions of the DEGs. Next, we
94   examined potential crosstalk in a constructed lncRNA-miRNA-mRNA ceRNA network that
95   involved one core lncRNA, one miRNA, and two mRNAs. Moreover, ten hub genes related to
96   pulpitis were identified as being aberrantly expressed in the protein-protein interaction (PPI)
97   network. Ultimately, the DElncRNA plasmacytoma variant translocation gene 1(PVT1), which
98   was significantly upregulated in inflamed pulp tissue, was validated by quantitative real-time
99   polymerase chain reaction (qRT-PCR).To the best of our knowledge, this study is the first
100 attempt to apply bioinformatics approaches to investigate the differential expression profiles of a
101 specific lncRNA-mediated ceRNA network in inflamed pulp.
102
Materials & Methods
104
Microarray Data
106 Microarray data from the GSE92681 and GSE77459 datasets were downloaded from the GEO
107 (https://www.ncbi.nlm.nih.gov/geo) of NCBI. GSE92681 including 7 pulpitis and 5 matched
108 normal pulp tissues was associated with the GPL16956 platform (Agilent-045997 Arraystar
109 Human LncRNA Microarray V3) *(Huang & Chen, 2018)*. GSE77459 contained 6 pulpitis tissue
110 specimens and 6 matched normal samples, and was associated the GPL17692 platform
111 (Affymetrix Human Gene 2.1 ST Array) *(Galicia et al., 2016)*. The DEmiRNAs were retrieved
112 as reported by *Zhong et al. (2012)*; until now, that data was the only known DEmiRNAs between
113 normal and inflamed human pulp tissue. All data were freely accessible online.
114
Data Processing
116 The oligo package was used to read the microarray and normalize the expression data *(Carvalho*
117 *& Irizarry, 2010)*. After the raw data were transformed to the expression files, the expression
118 files were further processed with the Linear Models for Microarray data (limma) R/Bioconductor
119 package (http://www.bioconductor.org) for analysis of the DEGs between pulpitis samples and

120   control samples with biological replication; the Benjamin and Hochberg method was applied for
121   multiple testing corrections *(Ritchie et al., 2015)*. Genes that met the cutoff criteria, a corrected
122   *p*-value < 0.05 and a |log$_2$ fold change (FC)| > 1, were considered DEGs.
123

Function and Pathway Enrichment Analysis of the DEmRNAs
125   To investigate pulpitis progression at the functional level, GO and pathway enrichment analyses
126   of the identified DEmRNAs were performed in this study. The identified DEGs were enriched
127   for terms in the GO biological process (BP), molecular function (MF), and cellular component
128   (CC) categories *(Ashburner et al., 2000)*. KEGG analysis was utilized to interpret the potential
129   functions and pathways of the aberrantly expressed genes *(Kanehisa et al., 2010)*. In addition,
130   the above data were analyzed using the clusterProfiler package in R *(Yu et al., 2012)*.
131

Protein-Protein Interaction (PPI) Network Analysis
133   The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, https://string-db.org/)
134   was used to identify PPIs among the selected DEGs. A combined score ≥0.4 was chosen for PPI
135   network construction *(Szklarczyk et al., 2015)*. Cytoscape software was used to visualize the
136   established DEGs network, while the MCODE plugin and CentiScaPe in Cytoscape were used to
137   select significant modules and calculate the connective of genes from the PPI network *(Shannon*
138   *et al., 2003; Scardoni et al., 2014)*.
139

Construction of the ceRNA Network
141   The lncRNA-associated ceRNA network in pulpitis was next constructed. First, miRNA-lncRNA
142   interactions were evaluated using the starBase (version 2.0) database with the default parameters
143   (clade: mammal, genome: human, assembly: hg19, number of supporting experiments: ≥ 1) *(Li et*
144   *al., 2014)*. Next, starBase (version 2.0), TargetScan (release 7.1), and miRDB (last modified:
145   May 03, 2016) were used to retrieve miRNA-targeted mRNAs *(Li et al., 2014; Agarwal et al.,*
146   *2015; Wong & Wang, 2015)*. The main steps of the method are shown in Fig. 1.
147

Validation Based on Clinical Samples of Human Dental Pulp
149   To further verify the expression of the DElncRNAs, qRT-PCR was performed. Briefly, 4 human
150   inflamed pulp tissue samples and 4 normal pulp tissue samples were obtained from Xiangya
151   Stomatological Hospital. The normal pulp tissues were collected from healthy third molars
152   extracted for orthodontic purposes. The inflamed pulp tissues were extracted from teeth
153   diagnosed with irreversible pulpitis in accordance with the endodontic diagnosis system from the
154   American Association of Endodontists. All patients gave written informed consent. The Ethics
155   Committee of Xiangya Stomatological Hospital of Central South University granted Ethical
156   approval to carry out the study within its facilities (Ethical Approval number: 20180026). Next,
157   RNA from all tissue samples, which were previously preserved in liquid nitrogen, was extracted
158   with TRIzol reagent (Thermo Fisher, USA). Reverse transcription was performed with a
159   Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). qRT-PCR was used to monitor the

160   expression of PVT1 (Fw, 5′-TGAGAACTGTCCTTACGTGACC-3′; Rev, 5′-

161   AGAGCACCAAGACTGGCTCT-3′) and GAPDH (Fw, 5′-

162   GACAGTCAGCCGCATCTTCTT-3′; Rev, 5′-AATCCGTTGACTCCGACCTTC-3′);

163   GAPDH was used as the housekeeping gene for normalization. Amplification was performed

164   with LightCycler 480 SYBR Green I Master (Roche, USA) on a real-time PCR system

165   (LightCycler 480, Roche, USA). Threshold cycle values (CT) were determined, and the data

166   were analyzed with Roche software with the $2^{-\Delta\Delta CT}$ method. An unpaired Wilcoxon test was

167   conducted to compare lncRNA expression at the transcriptional level between normal and

168   inflamed pulp tissues.

169

170   **Results**

171

172   **Identification of DEGs in Pulpitis**

173   Based on the criteria of $p < 0.05$ and $|\log_2 FC| > 1$, a total of 908 DEGs were screened from

174   GSE77459, including 264 downregulated genes and 644 upregulated genes in pulpitis. In the

175   GSE92681 dataset, 27 probes associated with DElncRNA, including 19 downregulated and 8

176   upregulated probes, were identified. A volcano plot for GSE77459 and a heatmap for GSE92681

177   are shown in Fig. 2. The detailed differential expression profiles are summarized in

178   Supplementary file 1.

179

180   **GO and KEGG Analysis of the DEGs**

181   GO function and KEGG pathway enrichment analyses of the DEGs were performed using the

182   clusterProfiler package in R. The results of GO analysis illustrated that the DEGs were enriched

183   for BP terms including leukocyte migration, the adaptive immune response, the immune

184   response-regulating cell surface receptor signaling pathway, regulation of leukocyte activation,

185   and the immune response-activating cell surface receptor signaling pathway. CC analysis showed

186   that the DEGs were significantly enriched for the side of membrane, secretory granule

187   membrane, and external side of plasma membrane, tertiary granule, and specific granule terms.

188   For the MF category, the DEGs were enriched in antigen binding, serine-type peptidase activity,

189   serine hydrolase activity, serine-type endopeptidase activity, and cytokine activity (Fig. 3).

190   Based on KEGG pathway analysis, the DEGs were significantly enriched in pathways

191   associated with cytokine-cytokine receptor interaction, the chemokine signaling pathway, cell

192   adhesion molecules, *Staphylococcus aureus* infection, and the hematopoietic cell lineage (Fig.

193   4). The detailed results of the GO enrichment and KEGG pathway analyses are provided in

194   Supplementary file 2.

195

196   **PPI Network Construction and Hub Genes Identification**

197   Protein interactions among the DEGs were detected with the online STRING program with a

198   cutoff score of ≥0.4. In total, 4,929 edges and 623 nodes were involved in the PPI network. One

199   significant module containing 528 edges and 33 nodes was selected from the PPI network by the

200 MCODE plugin in Cytoscape (Fig. 5). In this module, we found that most of the genes were
201 mainly enriched for and associated with chemokines (Tables 1 and 2).
202   In addition, we screened the top 10 hub mRNAs from the PPI network. These main hub
203 genes were IL6, IL8, PTPRC, IL1B, TLR2, ITGAM, CCL2, PIK3CG, ICAM1, and PIK3CD.
204 Hence, the key genes associated with pulpitis could be predicted by our network.
205
206 **Construction and Analysis of the ceRNA Network in Pulpitis**
207 To identify correlations among the DElncRNAs in the ceRNA network, we used starBase to
208 search for interactions between miRNAs and lncRNAs. The target mRNAs of the miRNAs in the
209 network were predicted with starBase, TargetScan, and miRDB. The ceRNA network was
210 constructed based on coexpressed lncRNAs/miRNAs, miRNAs/mRNAs, and lncRNAs/mRNAs.
211 Overlapping datasets were visualized using Venn diagrams (Fig. 6A). As shown in Fig. 6B, the
212 lncRNA-miRNA-mRNA network was composed of one lncRNA (PVT1), one miRNA (hsa-
213 miR-455-5p), and two mRNAs (suppressor of cytokine signaling 3, SOCS3 and Plexin C1,
214 PLXNC1). In addition, a comprehensive analysis of the relationships among PVT1, hsa-miR-
215 455-5p, SOCS3 and PLXNC1 and the top 10 downstream connected genes is depicted in Fig.
216 6B.
217
218 **Validation of PVT1 expression in Clinical Samples of Human Dental Pulp**
219 We evaluated the expression of PVT1 by qRT-PCR in pulpitis tissues compared to normal pulp
220 tissues. As depicted in Fig. 7, PVT1 transcript levels were significantly higher in inflamed pulp
221 than in normal pulp ($p < 0.05$).
222

Discussion

224 Pulpitis is considered a tightly regulated process involving microorganisms and host immune
225 events mediated by molecular factors *(Renard et al., 2016)*. Inflammation of the dental pulp is
226 characterized by opportunistic infection of the pulp space by commensal oral microorganisms,
227 such as *Porphyromonas* and *Streptococcus* species *(Rocas et al., 2016)*. The most common route
228 for microorganism invasion is through dental caries. Other possible portals include tooth damage
229 from trauma, exposed dentinal tubules or the apical foramen *(Raslan & Wetzel, 2006)*. Upon
230 irritation, cells in human dental pulp, for example, endothelial cells, odontoblasts and
231 macrophages, immediately trigger immune responses to pathogens and their virulent factors,
232 potentially stalling the spread of infection, preventing injury-related signaling, and launching
233 reparative processes *(Rechenberg, Galicia & Peters, 2016)*. However, if the delicate balance
234 between the immune-inflammatory response and dental tissue healing is disrupted, irreversible
235 pulpitis could result from uncontrollable inflammation caused by the invading bacteria.
236   Over the past decades, ncRNAs, including lncRNAs and short ncRNAs (such as
237 miRNAs), have gained increasing attention for their roles in physiological and pathologic
238 responses. In diverse biological processes, gene expression is regulated by posttranscriptional
239 mechanisms involving ncRNAs through their binding to the 3'-UTRs of target mRNAs, leading

240   to translational repression or target degradation *(Vasudevan, 2012)*. Accumulating studies have
241   provided evidence supporting the ceRNA hypothesis, which holds that lncRNAs harboring
242   MREs can competitively bind to certain miRNAs, thus regulating miRNA-mediated downstream
243   target gene silencing at the posttranscriptional level *(Khorkova, Hsiao & Wahlestedt, 2015)*.
244   Although the functional relevance of several lncRNAs and miRNAs in pulpitis has been proven
245   in the scientific literature, an lncRNA-based ceRNA network involved in pulpitis has yet to be
246   defined *(Zhong et al., 2012; Huang & Chen, 2018)*. In the current study, bioinformatics analyses
247   were applied to explore the expression profiles of lncRNAs, miRNAs, and mRNAs in patients
248   with pulpitis. Furthermore, an lncRNA-related ceRNA network was constructed by integrating
249   data from GEO pulpitis expression profiles. These data might shed light on how lncRNA-
250   miRNA-mRNA interactions are involved in pulpitis. Immune response can be triggered by
251   inflammatory mediators or cytokines produced by infectious cells through the transcriptional or
252   post-transcriptional regulations. The expression of ncRNAs may be closely related to the
253   development of immune cells (particularly monocytes, macrophages, NK cells, T helper cells,
254   CD8$^+$ T cells, and Treg cells) and the maintenance of immune system homeostasis *(Cortez et al.,*
255   *2019; Imamura & Akimitsu, 2014)*. For instance, lincRNA-Cox2 regulates the release of
256   inflammatory mediators and cytokines by stimulation of TLR2 in bacterial infections *(Carpenter*
257   *et al. 2013)*, and miR-155 exerts an antiproliferative effect on CD8$^+$ T cells in response to the
258   type I interferon signaling *(Gracias et al., 2013)*.
259   LncRNAs and miRNAs, which are closely correlated with inflammatory mediator
260   production and pulp repair, could be affected by the inflammatory status in dental pulp *(Zhong et*
261   *al., 2012; Galicia et al., 2016; Huang & Chen, 2018)*. In this study, our findings indicated that
262   lncRNA PVT1might regulate the progression of pulpitis by acting as a sponge for miR-455-5p.
263   Emerging studies have proven that PVT1 plays an important role in inflammation and
264   tumorigenesis. For example, down-regulation of PVT1 correlates with the differentiation of
265   Th17 cells and the duration of multiple sclerosis, a chronic immune-mediated disease
266   *(Eftekharian et al., 2017)*. Additionally, PVT1 promotes the production of inflammatory
267   cytokines to aggravate the progression of IL-1β-stimulated osteoarthritis *(Zhao et al., 2018)*.
268   Overexpressed PVT1 can enhance the expression of IL-6 and IL-1β in a nonbinding manner by
269   regulating the nuclear factor-κB (NF-κB) pathway, which is considered a critical mechanism in
270   the regulation of immune and inflammatory processes, and ultimately aggravating septic acute
271   kidney injury *(Huang et al., 2017)*. Moreover, some studies have revealed that PVT1, which is
272   located on 8q24.21 and contains the *myc* proto-oncogene, is upregulated in certain human tumors
273   *(Shtivelman & Bishop, 1989)* and is correlated with pathologic stage in multiple cancers *(Chai et*
274   *al., 2018)*. In this study, our qRT-PCR results indicated that PVT1 was upregulated in pulpitis
275   tissue compared with matched normal pulp tissue, suggesting that PVT1 is an important factor
276   contributing to pulpitis. However, the exact role of PVT1 in the occurrence and development of
277   pulp inflammation and the underlying mechanism require further investigation.
278   MiR-455-5p is one subtype of the mature miR-455, which is located in the sense strand
279   of chromosome 9q32, and is a tumor-associated miRNA molecule. The other subtype is miR-

280   455-3p *(Arai et al., 2019)*. MiR-455 is involved in a variety of biological processes through the
281   repression of specific mRNA at the posttranscriptional level. For example, miR-455–5p regulates
282   the posttranscriptional repression of UDP-glucuronosyltransferase (UGT) 2B expression by
283   binding to MREs in 3'-UTR of UGT2B, which plays a role in drug glucuronidation
284   *(Papageorgiou & Court, 2017)*. Moreover, in colorectal cancer, miR-455 is up-regulated to
285   inhibit the protein expression of RAF proto-oncogene serine/threonine protein kinase (RAF1)
286   other than affects mRNA level, which regulates the cellular proliferation and invasion *(Chai et*
287   *al., 2015)*. In pulpitis, the precise posttranscriptional mechanisms of miR-455-5p remain to be
288   explored. Previous studies have revealed that miR-455-5p can serve as a prognostic biomarker
289   and therapeutic target for patients with certain cancers, such as non-small cell lung cancer and
290   basal cell carcinoma *(Sand et al., 2012; Wang et al., 2017)*. Moreover, miR-455 represents novel
291   regulator of the immune and inflammatory response.MiR-455-5p plays an anti-inflammatory role
292   in multiple sclerosis. MiR-455-5p is negatively correlated with the activation of the NF-κB
293   pathway, thus inhibiting the expression of IL-1β, IL-6 and IL-8 and in turn ameliorating the
294   severity of multiple sclerosis *(Torabi et al., 2019)*. Macrophage polarization is an essential
295   component of immunity and homeostasis, and miR-455-5p overexpression reverses the
296   polarization of macrophages to the M1 phenotype to reduce the secretion of proinflammatory
297   cytokines, such as IL-1β and TNF-α *(Chi et al., 2018)*. The accumulation of miR-455 regulates
298   the innate immune response in E2F1-deficient mice, leading to reduced inflammation induced by
299   LPS *(Warg et al., 2012)*. In patients infected with the hepatitis B virus, serum miR-455-3p levels
300   are decreased during chronic disease progression *(Singh et al., 2018)*. The algorithms in our
301   study predicted that miR-455-5p was downregulated (Fig. 6B) in pulpitis samples compared to
302   control samples. Further work will be needed with multiple clinical samples to clarify the action
303   of miR-455-5p in pulpitis and thus to uncover the mechanisms underlying the effects of miR-
304   455-5p on proinflammatory processes.
305   Based on the module produced by the STRING analysis, two core mRNAs, identified as
306   SOCS3 and PLXNC1, can be considered important target genes of miR-455-5p. SOCS3 is a
307   major suppressor of inflammation and is known as a feedback inhibitor of the JAK/STAT3
308   signaling pathway *(Carow & Rottenberg, 2014)*. SOCS3 can inhibit JAK2 activity to attenuate
309   STAT3 phosphorylation, inhibit the NF-κB pathway and trigger the expression of various genes
310   in response to cytokines (the IL-6 family and IL-10), consequently affecting cell proliferation,
311   differentiation, and apoptosis *(Gao et al., 2018)*. SOCS3 deficiency results in increased alveolar
312   loss with high levels of IL-1β, IL-6, and IL-8 in periodontitis *(Papathanasiou et al., 2016)*.
313   Furthermore, induction of SOCS3 expression may markedly reduce cell adherence by inhibiting
314   TNF-α-stimulated ICAM1 expression in lung inflammation *(Lee et al., 2013)*. PLXNC1 is an
315   endogenous receptor of the neuronal guidance protein semaphorin 7a (Sema 7a). Previous
316   studies have suggested that PLXNC1 might play an important role in immunological and
317   proinflammatory responses. A study by *Konig et al. (2014)* on PLXNC1^{-/-} mice provided
318   evidence that PLXNC1 depletion results in a reduced inflammatory response and decreased
319   cytokine IL-1β production *in vivo*. We hypothesize that PLXNC1 and SOCS3 play important

320   roles in conditions associated with inflammation. Both might therefore be potential targets to
321   prevent damage due to excessive tissue inflammation.
322   GO and pathway analyses were used to infer the potential functions of the DEGs in
323   pulpitis. GO annotation revealed that the top GO terms in the BP and MF categories for the
324   DEGs between the inflamed and healthy pulp tissue were mainly associated with the
325   immune/inflammation system (Fig. 3), suggesting that the cascade of cellular events in pulpitis is
326   precisely regulated by migrating leukocytes and their surrounding microenvironments and also
327   supporting other previous theories explaining the occurrence of pulpitis *(Cooper et al., 2017)*.
328   Furthermore, KEGG pathway analysis indicated that the cytokine-cytokine receptor interaction
329   pathway was the most highly enriched pathway for the upregulated lncRNAs between pulpitis
330   and normal dental tissues, while other classic inflammatory signaling pathways, including the
331   chemokine signaling pathway, cell adhesion molecules (CAMs), *Staphylococcus aureus*
332   infection, the hematopoietic cell lineage, the TNF signaling pathway, and osteoclast
333   differentiation were also enriched. These pathways were mainly involved in immune regulation,
334   intercellular signaling, defense mechanisms, osteoclast differentiation, and hematopoiesis.
335

336   **Conclusions**
337   To the best of our knowledge, there have been no studies on lncRNA-associated ceRNA
338   networks in pulpitis. Here, we used bioinformatics methods to elucidate the lncRNA-miRNA-
339   mRNA ceRNA network associated with dental pulp inflammation. Our analysis suggests a
340   potential mechanism by which PVT1 competes with miR-455-5p to regulate the expression of
341   SOCS3 and PLXNC1. Upregulation of PVT1, which has been validated in inflamed pulp by
342   qRT-PCR, could increase inflammation and cytokine and chemokine production in inflamed
343   pulp tissue compared with healthy pulp tissue. In addition, overexpression of PVT1 reduced
344   miR-455-5p expression and indirectly promoted SOCS3 and PLXNC1 expression and the
345   cytokine cascade. In addition, SOCS3 and PLXNC1 could regulate the hub genes (including IL-
346   1β, IL-6, IL-8 and ICAM1), which play a critical role in pulp inflammation. Upon
347   comprehensive analysis of the lncRNA-related ceRNA network, some novel and crucial
348   characteristics of pulpitis were revealed. These findings provide new insights into the
349   pathogenesis of endodontic lesions and might identify potential diagnostic and therapeutic
350   strategies for future studies.
351

352   **References**
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Main steps of the construction of the regulatory network in pulpitis.

Step 1: We identified the differentially expressed mRNAs, lncRNAs and miRNAs. Step 2: The GO and KEGG enrichment analyses were conducted and the PPI network was constructed. Then, we identified the hub genes from the PPI network. Step 3: The ceRNA regulatory relationships were predicted using online tools. Step 4: The ceRNA network with hub genes was constructed.

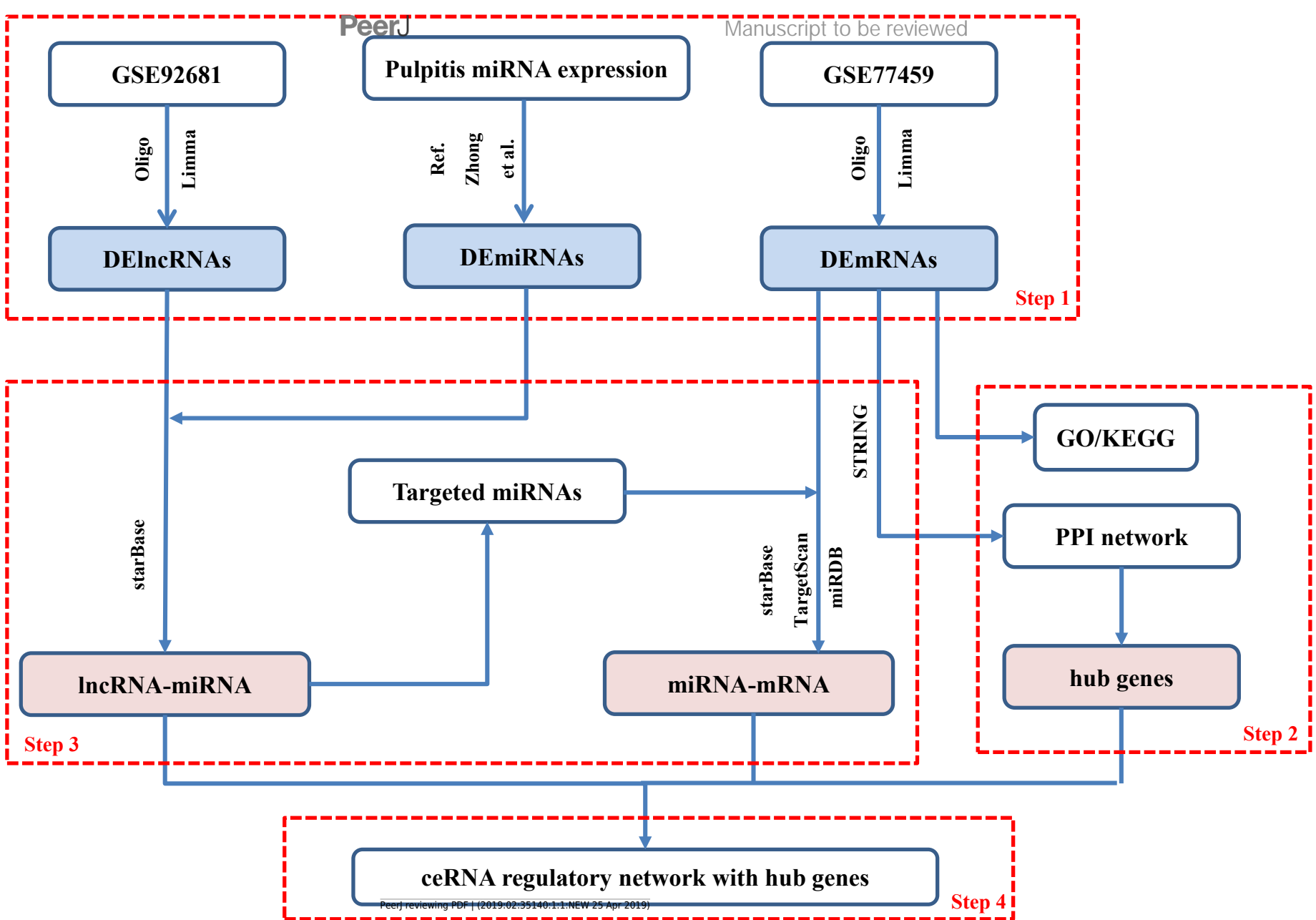
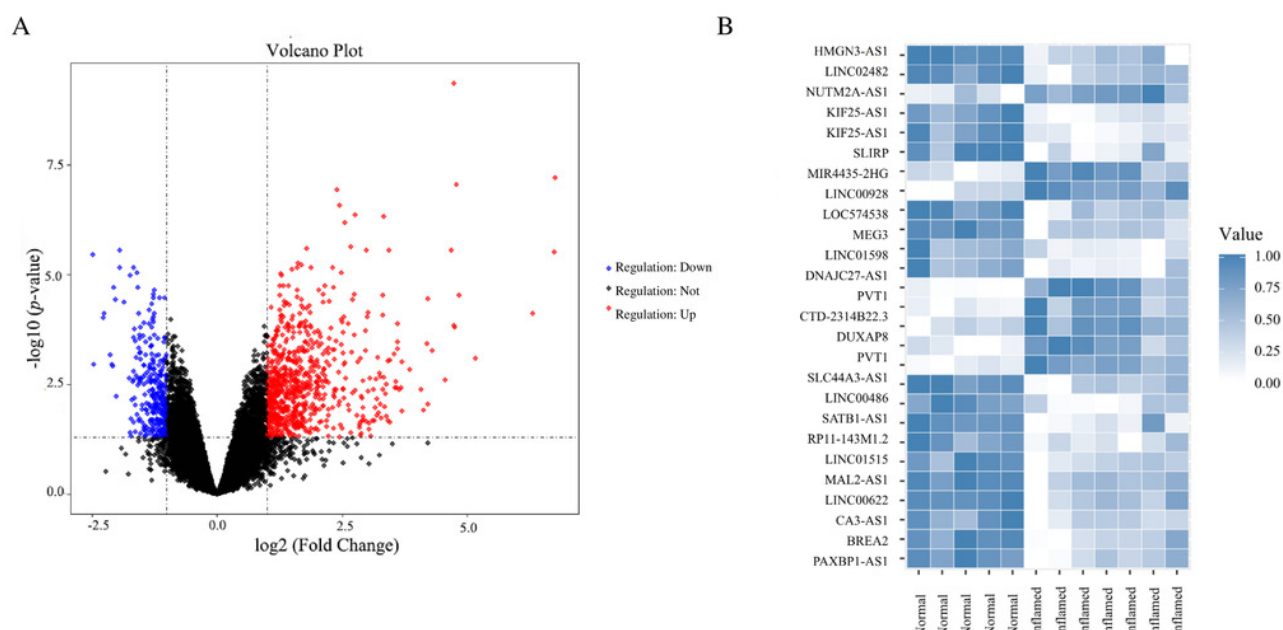
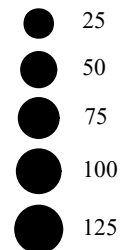
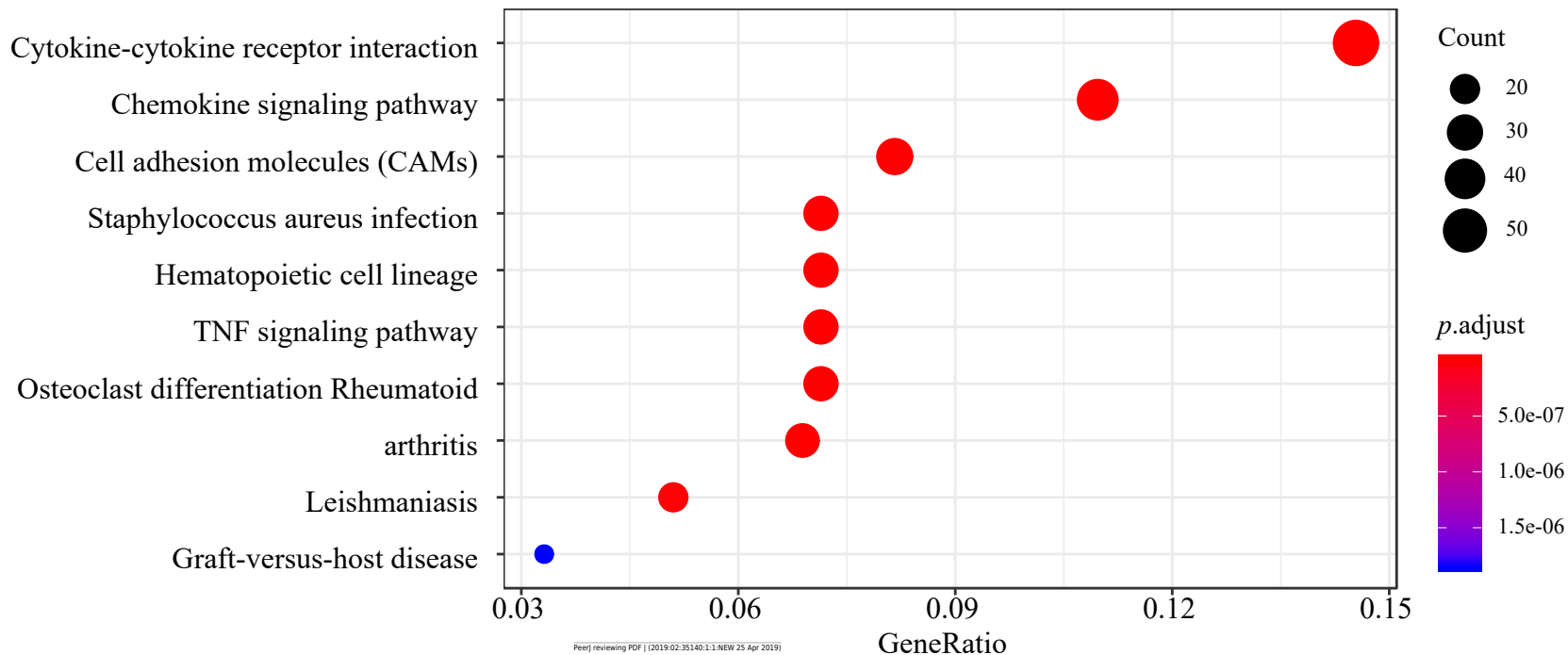



Figure 2

DEGs between pulpits samples and normal samples.

(A) Volcano plot for the DEGs in dataset GSE77459. The x-axis indicates the log FC, and the y-axis indicates the log10 (adjusted p-value). The red dots represent upregulated genes, and the blue dots represent downregulated genes. The DEGs were screened on the basis of a |fold change|>1.0 and an adjusted p-value of < 0.05. The black dots represent genes with no significant difference. FC, fold change. (B) Heatmap of the DEGs in dataset GSE92681. The relative expression values were normalized to fall in a range from zero to one. Genes expressed at high levels are shown in blue, while those expressed at low levels are shown in white.

Top 10 processes revealed in GO enrichment to influence biological process (BP), molecular function (MF), and cellular component (CC).

The colored dots represent the term enrichment: blue indicates low enrichment, and red indicates high enrichment. The sizes of the dots represent the numbers of genes in each GO category.

Top 10 enriched KEGG pathways for the upregulated DEGs.

The y-axis shows the KEGG pathway names. The colored dots represent the term enrichment: blue indicates low enrichment, while red indicates high enrichment. The sizes of the dots represent the numbers of genes.

The most significant module in the PPI network of the DEGs.

The most significant module includes 528 edges and 33 nodes. The circles represent genes, and the lines represent interactions between the proteins encoded by the genes.

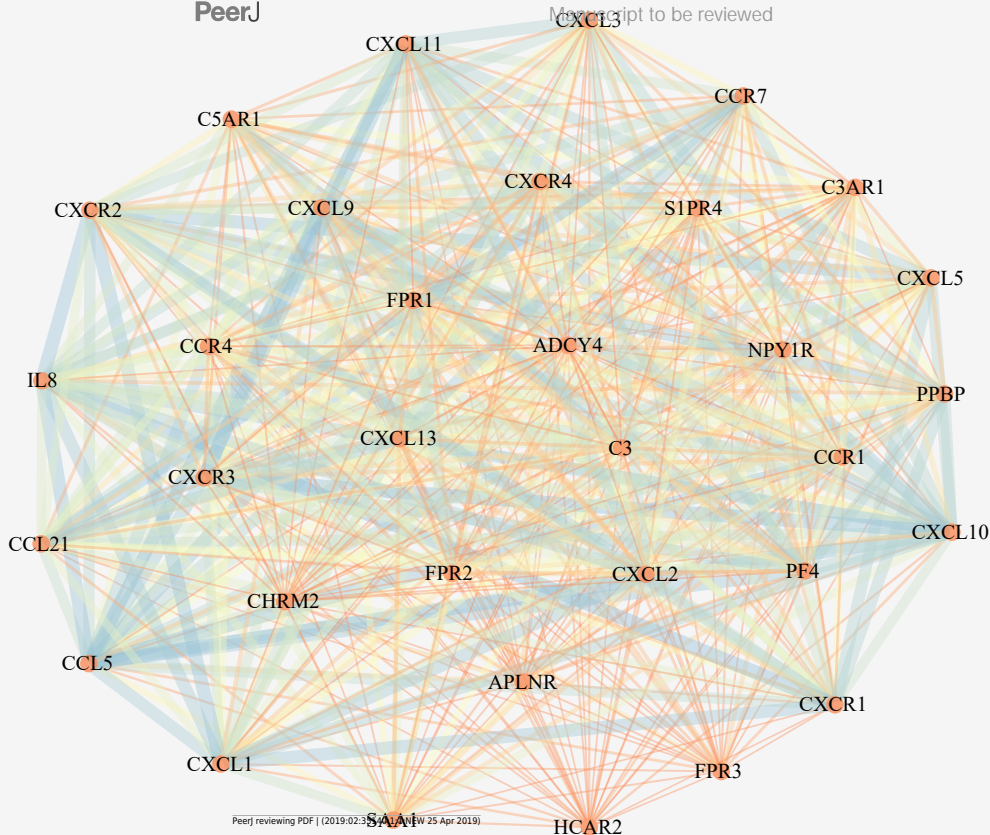
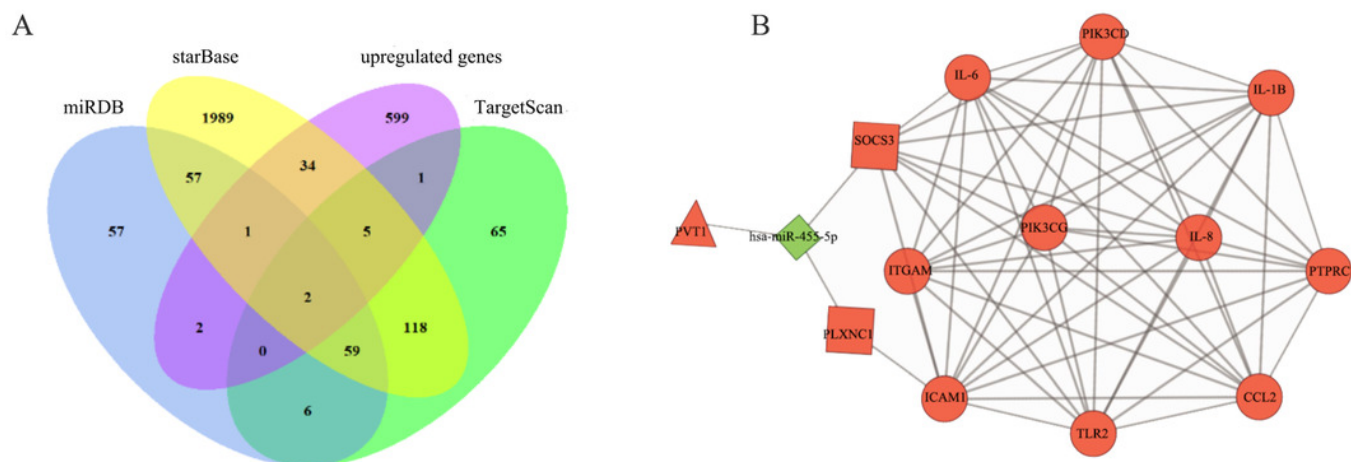
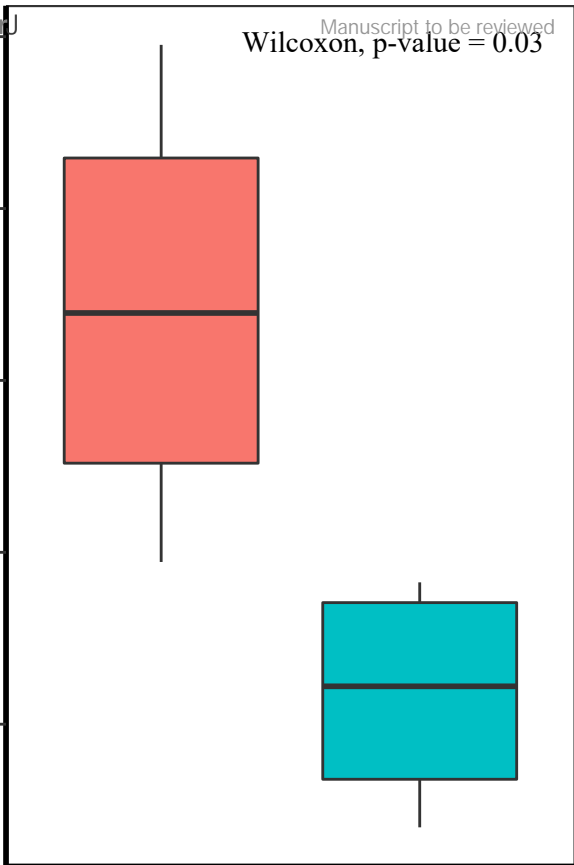



Figure 6

Construction of ceRNA network in pulpitis.

(A) Venn diagram showing the number of distinct and overlapping RNAs among the upregulated genes and the RNAs identified with miRDB, starBase, and TargetScan. The overlapping areas show the upregulated genes identified by three online tools. (B) Interaction of RNAs in the PVT1-associated ceRNA network. The triangle node and the diamond node represent the lncRNA and miRNA, respectively. The rectangle nodes represent miR-455-5p-targeted mRNAs. The round nodes are the top 10 hub DEmRNAs in the network. The up- and downregulated genes are colored in red and green, respectively.

Relative expression levels of PVT1 in inflamed and normal pulp tissue.

The transcript levels of PVT1 were determined by qRT-PCR and normalized to those of the reference RNA GAPDH. *p*-value =0.03

Top 15 enriched GO terms of the DEGs.

1   **Table 1: Top 15 enriched GO terms of the DEGs.**

Terms	Pathway description	Count	*P*-Value
GO.BP:0070098	chemokine-mediated signaling pathway	17	4.46E-35
GO.BP:0060326	cell chemotaxis	21	2.18E-33
GO.BP:0002690	positive regulation of leukocyte chemotaxis	17	2.84E-30
GO.BP:0050921	positive regulation of chemotaxis	18	7.66E-30
GO.BP:0006954	inflammatory response	23	5.00E-29
GO.CC:0005615	extracellular space	15	1.79E-07
GO.CC:0009897	external side of plasma membrane	6	0.000948
GO.CC:0005886	plasma membrane	19	0.00174
GO.CC:0071944	cell periphery	19	0.00174
GO.CC:0005887	integral component of plasma membrane	10	0.0144
GO.MF:0045236	CXCR chemokine receptor binding	11	5.28E-25
GO.MF:0008009	chemokine activity	13	2.39E-24

GO.MF:0001664	G-protein coupled receptor binding	15	8.21E-20
GO.MF:0048248	CXCR3 chemokine receptor binding	5	4.67E-12
GO.MF:0008528	G-protein coupled peptide receptor activity	9	2.18E-11

Top 5 enriched KEGG pathways of the DEGs.

1 **Table 2:Top 5 enriched KEGG pathways of the DEGs.**

Category	Pathway description	Count	*P*-Value
KEGG:map04062	chemokine signaling pathway	21	1.49E-33
KEGG:map04060	cytokine-cytokine receptor interaction	20	4.10E-28
KEGG:map05150	*Staphylococcus aureus* infection	6	1.78E-08
KEGG:map04080	neuroactive ligand-receptor interaction	9	2.16E-08
KEGG:map04668	TNF signaling pathway	6	9.52E-07