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Identification and validation of m⁶A RNA regulatory network in pulpitis

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Abstract

Background N⁶-methyladenosine (m⁶A) RNA modification regulators play an important role in many human diseases, and its abnormal expression can lead to the occurrence and development of diseases. However, their significance in pulpitis remains largely unknown. Here, we sought to identify and validate the m⁶A RNA regulatory network in pulpitis.

Methods Gene expression data for m⁶A regulators in human pulpitis and normal pulp tissues from public GEO databases were analyzed. Bioinformatics analysis including Gene ontology (GO) functional, and Kyoto encyclopedia of genes and genomes (KEGG) pathway analyses were performed by R package, and Cytoscape software was used to study the role of m⁶A miRNA-mRNA regulatory network in pulpitis. Quantitative real-time PCR (qRT-PCR) was performed to validate the expression of key m⁶A regulators in collected human pulpitis specimens.

Results Differential genes between pulpitis and normal groups were found from the GEO database, and further analysis found that there were significant differences in the m⁶A modification-related genes *ALKBH5*, *METTL14*, *METTL3*, *METTL16*, *RBM15B* and *YTHDF1*. And their interaction relationships and hub genes were determined. The hub m⁶A regulator targets were enriched in immune cells differentiation, glutamatergic synapse, ephrin receptor binding and osteoclast differentiation in pulpitis. Validation by qRT-PCR showed that the expression of methylases *METTL14* and *METTL3* was decreased, thus these two genes may play a key role in pulpitis.

Conclusion Our study identified and validated the m⁶A RNA regulatory network in pulpitis. These findings will provide valuable resource to guide the mechanistic and therapeutic analysis of the role of key m⁶A modulators in pulpitis.

Keywords Pulpitis, Gene expression, m⁶A modification, Inflammation

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Introduction

Pulpitis is a common disease in the oral cavity with a high incidence. Pulpitis is an inflammatory disease of the dental pulp which is caused by a progressive inflammatory reaction of the pulp-dentin complex caused by deep caries, trauma, etc. [1]. There is still no new breakthrough in the treatment of pulpitis at present [2]. The treatment for pulpitis is still root canal therapy to remove the pulp tissue. However, after the pulp tissue is removed from the affected tooth, there is no nutritional supply, and the brittleness increases, resulting in a decrease in the service life of the tooth. Pulpitis, as an inflammatory disease, is closely related to epigenetic modifications [3]. Research has shown that DNA methylation and RNA modification are involved in the occurrence and development of this disease [4, 5]. However, the mechanisms involved have not yet been fully elucidated, and the urgent search for the pathogenesis of pulpitis and new therapeutic targets is expected to bring good news to patients with pulpitis.

RNA in organisms can be post transcriptionally modified by more than 100 different chemical modifications, among which N⁶-methyladenosine (m⁶A) has been identified as the most abundant internal modification in eukaryotic mRNA [6]. m⁶A is associated with many diseases such as cancer [7], and viral infection and inflammation are also diseases affected by m⁶A [8, 9]. m⁶A modification is reversible and has become one of the current research hotspots. It is catalyzed by “writers” (METTL3, METTL14 and WTAP) to form a methyltransferase complex, which adds a methyl group to the N6 site of adenine in the RRACU sequence motif (R means G or A), while demethylation the enzymes ALKBH5 and FTO reverse this process, acting as an “eraser” [10]. When modified with m⁶A, transcripts can be recognized by “readers” (such as YTHDF1-3, YTHDC1 and IGF2BPs) to perform corresponding regulatory functions [11]. However, the role of m⁶A modification in the development of pulpitis disease remains unknown.

Bioinformatics studies on data from microarrays have emerged as promising and efficient tools for screening disease-related genes [12, 13]. This study used public datasets and bioinformatics analysis to identify the m⁶A RNA regulatory network in pulpitis. And the corresponding tissue samples were collected to further verify the expression of key related m⁶A regulators in pulpitis. This study can provide clues for further research on the occurrence and development mechanism of clinical pulpitis disease, which is crucial for identifying new targets for early detection and treatment of pulpitis.

Methods

Human samples

Human pulp tissue samples were obtained from patient dental specimens aged 18–35 years, all from completely

extracted third molars (12 women and 8 men; age 25.65±4.9 years). Pulpitis samples were obtained from 10 patients (6 women and 4 men; age 26.5±5.2 years) with teeth diagnosed as irreversible pulpitis with a previous history of spontaneous pain and caused by cold and/or heat testing of persistent pain. These teeth had clinical and radiographic evidence of deep carious lesions extending into the pulp cavity. Control samples were obtained from 10 patients (6 women and 4 men; age 24.8±4.8 years) with fully erupted teeth, intact crowns, and surrounded by healthy periodontal tissue, from orthodontic indications extracted third molar. The patients underwent tooth extraction after obtaining informed consent. This study is compliant with the Declaration of Helsinki. This study protocol was approved by the Ethics Committee of Jinhua Hospital Affiliated to Zhejiang University (No.S96; 16/05/2022).

Data collection and differentially analysis

The gene expression profile of human pulpitis was downloaded from GSE77459 through Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>), which includes 6 normal dental pulp tissues and 6 inflamed pulp tissues. “Limma” R package were used to screen the differentially expressed genes (DEGs) and LogFC>0.7 and P<0.05 were set as the criterion. The volcano plot was visualized by “ggplot2” R package. The m⁶A regulators including “writers”: METTL3, METTL14, WTAP, ZC3H13, METTL16, ZCCHC4, RBM15 and RBM15B, “erasers”: FTO and ALKBH5, and “readers”: HNRNPA2B1, HNRNPC, IGF2BP1, IGF2BP2, IGF2BP3, YTHDC1, YTHDC2, YTHDF1, YTHDF2 and YTHDF3. The targets of differentially expressed m⁶A genes were acquired from m⁶A2Target database (<http://m6a2target.canceromics.org>).

Functional enrichment analysis

We further performed the Kyoto Encyclopedia of Genes and Genomes (KEGG) [14–16] and Gene Ontology (GO) pathway enrichment analyses including biological processes (BP), cellular components (CC) and molecular functions (MF) analysis of the targets of differential m⁶A genes by “clusterProfiler”, “org.Hs.eg.db” and “GOplot” R package.

Construction of miRNA-mRNA network of pulpitis-related m⁶A regulators

Upon the pulpitis-related m⁶A regulators identified, we also predicted the potential interacted miRNA through miRwalk database (<http://mirwalk.umm.uni-heidelberg.de/>). Then we constructed and visualized the miRNA-mRNA network which showed the miRNAs interacting with the key m⁶A regulators in human pulpitis by Cytoscape software.

RNA isolation and real-time PCR analysis

Total RNA from dental pulp tissue was extracted using TRIzol (Vazyme, Nanjing, China). Reverse transcription reagents (Vazyme, Nanjing, China) were used for cDNA synthesis. Real-time PCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using the SYBR Green PCR protocol. Relative mRNA expression of target genes was normalized to GAPDH using the $2^{-\Delta\Delta CT}$ method and expressed as the mean \pm SD of the number of replicates. The primers and primer sequences used in this experiment are listed in Table 1.

Statistical analysis

The statistical analyses were carried out using Graphpad software. The data were manifested as means \pm SD. The independent-sample t-test was applied for the comparisons between the normal and pulpitis group. P value <0.05 was set as statistically significant.

Results

Identification of pulpitis-associated m⁶A regulators

To explore the functional m⁶A regulators in pulpitis, we preformed the differential analysis of GSE77459 dataset of pulpitis. Firstly, PCA analysis was performed and we found that the differential genes of the normal group and the pulpitis group were significantly grouped (Fig. 1A). Further differential expression analysis of genes between pulpitis tissue and normal pulp tissue was performed, as shown in the volcano plot (Fig. 1B). Then, the m⁶A-related genes were selected from these genes, including “writers”, “erasers” and “readers”, and further analysis was performed, showing the changes in m⁶A regulator gene expression between the pulpitis group and the normal group in Fig. 1C. Finally, the correlation analysis of these genes was carried out (Fig. 1D), and it was found that these genes were basically significantly correlated in pulpitis.

We further compared the expression of m⁶A-related genes in the normal group and the pulpitis group and made corresponding statistical analysis (Fig. 2). The

Table 1 Primers for PCR analysis

Genes	Forward primer 5'-3'	Reverse primer 5'-3'
ALKBH5	CGGCGAAGGCTACACTTACG	CCACCAGCTTTTGGATCACCA
METTL3	TTGTCTCCAACCTCCGTAGT	CCAGATCAGAGAGGTG-GTGTAG
METTL14	GAACACAGAGCTTA-AATCCCA	TGTCAGCTAAACCTA-CATCCCTG
METTL16	TTCTGTCAAGGTCGGACAATG	CAGCACCACGAATGTTATGGG
RBM15B	TACACGGAGGCTACAGTACA	GTCGTACAGCCGTAGTAGTC
YTHDF1	ACCTGTCCAGCTATTACCGG	TGGTGAGGTATGGAATCGGAG
YTHDF3	TCAGAGTAACAGCTATC-CACCA	GGTTGTCAGATATGGCATAG-GCT
ZCCHC4	CCCTACGGACCCACTCTT	GCAAGTCTAGCTCCTGACAAC

results showed that there were significant differences in the genes ALKBH5, METTL14, METTL3, METTL16, RBM15B and YTHDF1 between normal group and the pulpitis group. It indicates that these genes may play specific roles in pulpitis.

m⁶A-related genes molecular interaction network and core genes interaction network

To explore the hub m⁶A regulators in pulpitis, we further constructed the molecular interaction network of m⁶A-related genes, and Cytoscape was used to extract the interaction network of core genes (Fig. 3), which further suggested that these genes RBM15B, METTL16, METTL14, YTHDF3 and ALKBH5 may be the main regulators in pulpitis m⁶A-modified genes.

Potential functions of genes modified by m⁶A regulators associated with pulpitis

To explore the role of m⁶A regulators in pulpitis, we further analyzed the downstream target molecules of core genes with statistical significance and intersected these molecules with differential genes in GEO (Fig. 4A), suggesting that these intersected molecules are m⁶A regulated molecules in pulpitis. And the number of intersected gene between GSE77459 and RBM15B targets, GSE77459 and YTHDF1 targets and GSE77459 and YTHDF3 is 110, 418, 70, respectively. We performed GO enrichment analysis and KEGG enrichment analysis at the same time (Fig. 4B-C), and the results showed that these further screened downstream genes were mainly enriched in biological processes such as cell adhesion, T cell activation, glutamatergic synapse, ephrin receptor binding, cytokine receptors, and osteoclasts differentiation. These results suggested that m⁶A regulators in pulpitis are mainly associated the biological processes of immune cell activation, glutamatergic synapse, cytokine activity and osteoclast differentiation, which are important for pulpitis progression.

Molecular interaction network of upstream miRNAs regulating the core m⁶A genes

Pulpitis is a ubiquitous chronic inflammation in the dental pulp, and miRNAs have been found to act as promoters or inhibitors of oral inflammation [17]. To explore the potential upstream regulators of hub m⁶A genes in pulpitis, we used the miRwalkdatabase to predict the upstream miRNAs of the core m⁶A genes, including has-miR-4709-3p, has-miR-5010-3p, hsa-miR-4257, hsa-miR-550b-2-5p, has-miR-7977, has-miR-29b-2-5p, has-miR-149-3p, has-miR-363-5p, has-miR-329-3p, hsa-miR-329-3p, and made a molecular interaction network (Fig. 5).

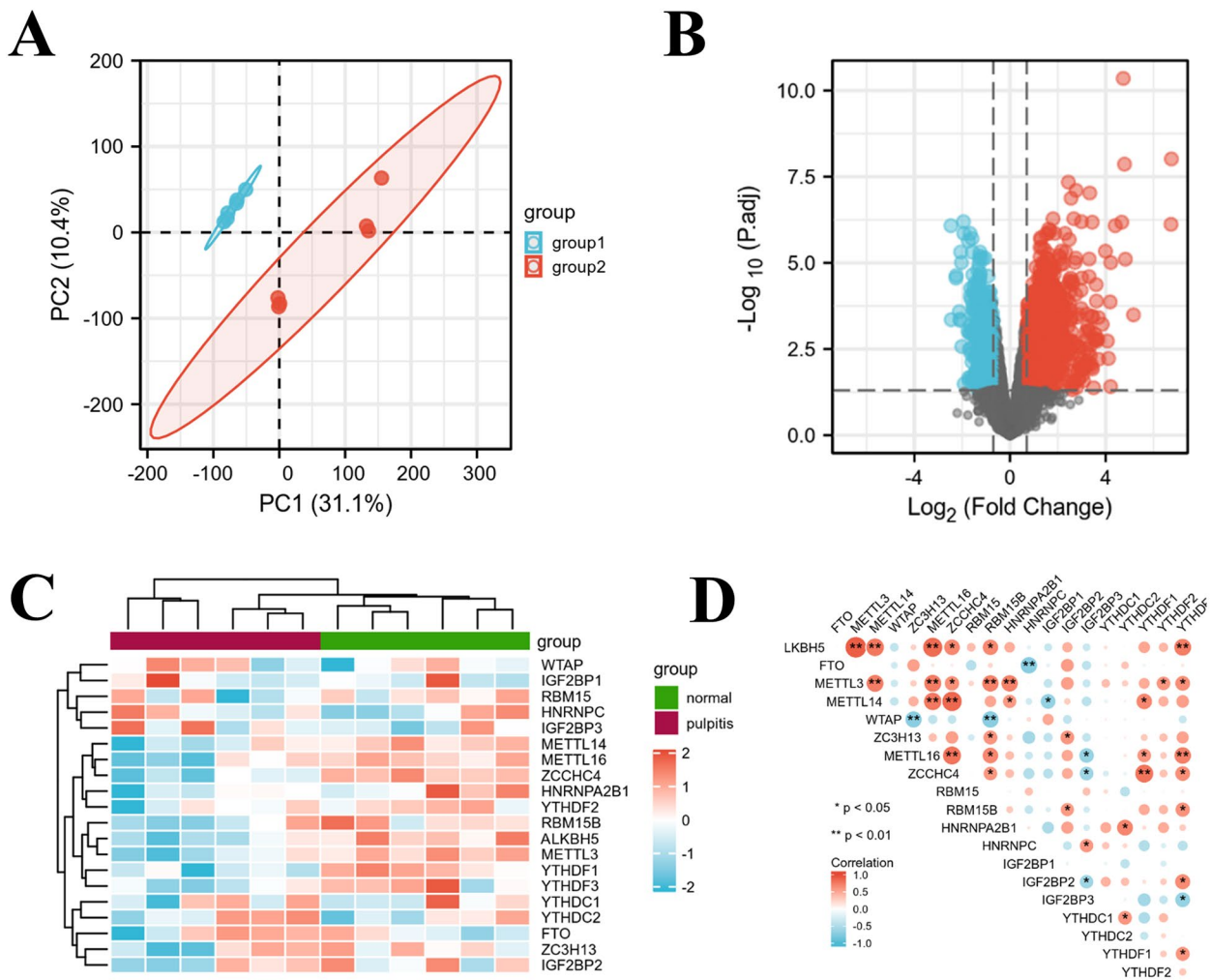


Fig. 1 Identification of pulpitis-associated m⁶A regulators. **(A)** PCA plot: Differential gene significant grouping between normal group and pulpitis group. **(B)** Volcano plot: Differentially expressed genes between pulpitis group and normal group. Red indicates up-regulated mRNAs in pulpitis, blue indicates down-regulated mRNAs in pulpitis, and black indicates no differentially expressed mRNAs. **(C)** Heatmap of gene expression in pulpitis and normal groups. **(D)** Correlation analysis among m⁶A-related genes in pulpitis

Validation of key m⁶A regulators in pulpitis

To further validate the key m⁶A regulators in pulpitis, the expression of ALKBH5, METTL3, METTL14, METTL16, RBM15B, YTHDF1, YTHDF3 and ZCCHC4 genes was detected by PCR on collected clinical tissue samples. METTL14 mRNA was confirmed to be significantly downregulated in pulpitis samples compared with controls (Fig. 6). However, YTHDF1, YTHDF3, METTL16 and METTL3 gene expression were not statistically different between the pulpitis group and the control group, and RBM15B, ZCCHC4 and ALKBH5 were up-regulated (Fig. 6). These results suggest that METTL14 may be a key regulator in pulpitis, further validating the differential expression of m6A-related genes between pulpitis and normal groups.

Discussion

Pulpitis is a chronic progressive inflammatory and destructive disease that exists and occurs globally, affecting people’s quality of life to a certain extent [18]. The specific pathogenesis of the disease has not yet been elucidated [19], and it is believed to be related to a variety of factors.

In recent years, m⁶A regulators have been found to play an important role in the occurrence, development and prognosis of various human diseases [20, 21]. Studies have shown that m⁶A modification plays a role in inflammatory diseases [22], but the role of m⁶A modification in pulpitis has not been elucidated. This is the first systematic bioinformatics analysis and validation of m⁶A regulators in pulpitis disease, which can provide directions for

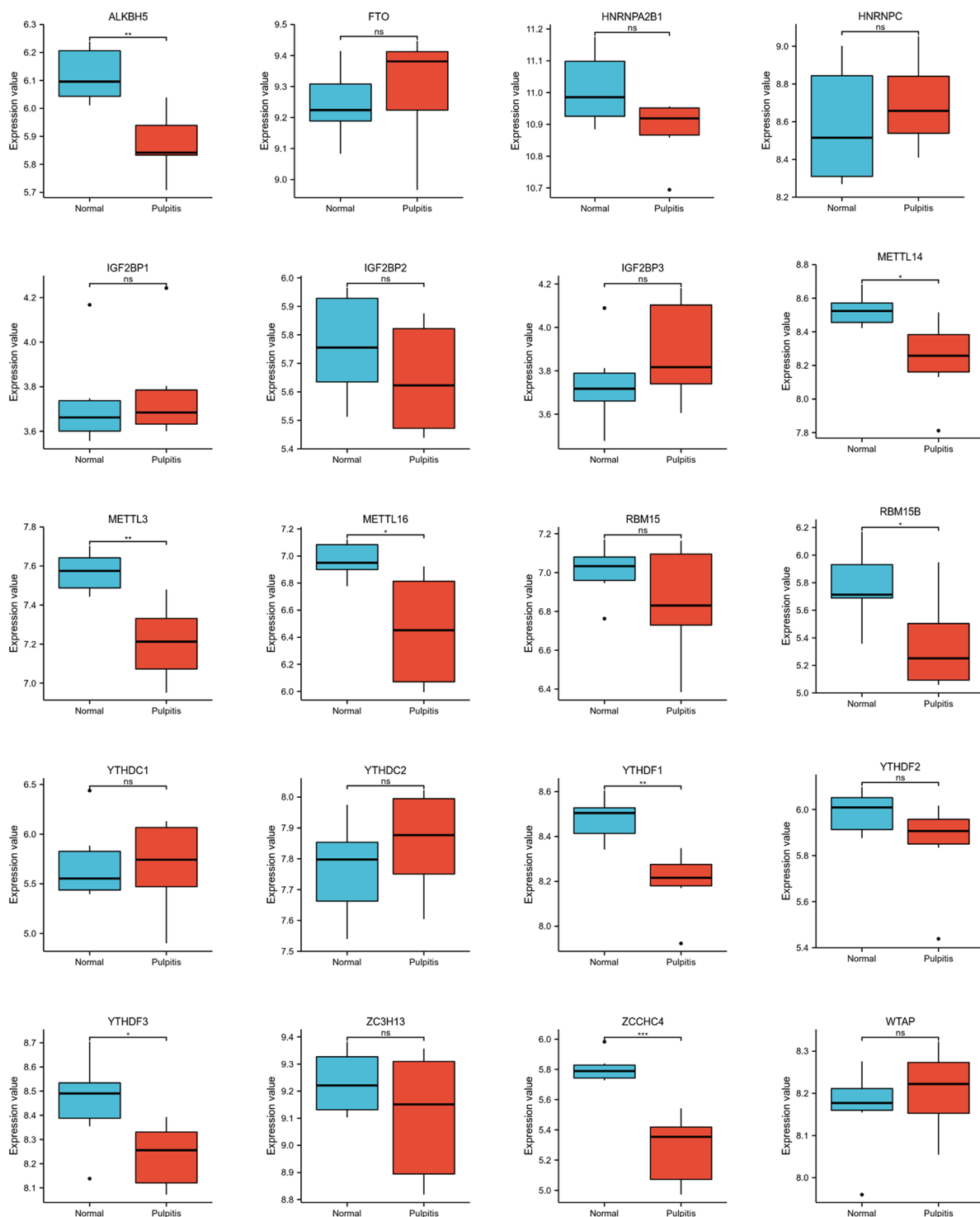


Fig. 2 Box plots for m⁶A-related genes expression between normal group and pulpitis group. *P < 0.05, **P < 0.01, ***P < 0.001

the development mechanism of pulpitis and the targeted therapy of pulpitis.

We were the first to analyze and find the differences of m⁶A-related genes between pulpitis and normal groups, among which *ALKBH5*, *METTL14*, *METTL3*,

METTL16, *RBM15B* and *YTHDF1* were significantly different, suggesting that these genes may be involved in the occurrence of pulpitis important role in development. These hub genes have been reported in other inflammatory diseases such as rheumatoid arthritis [23],

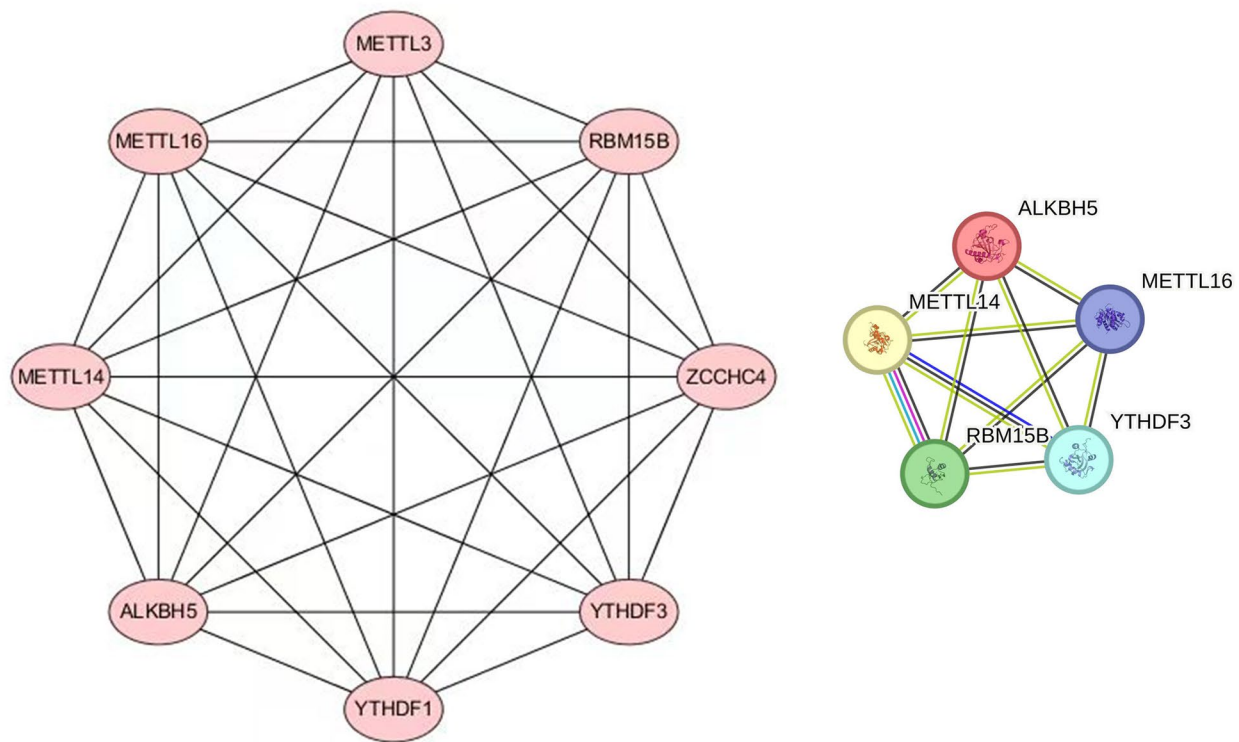


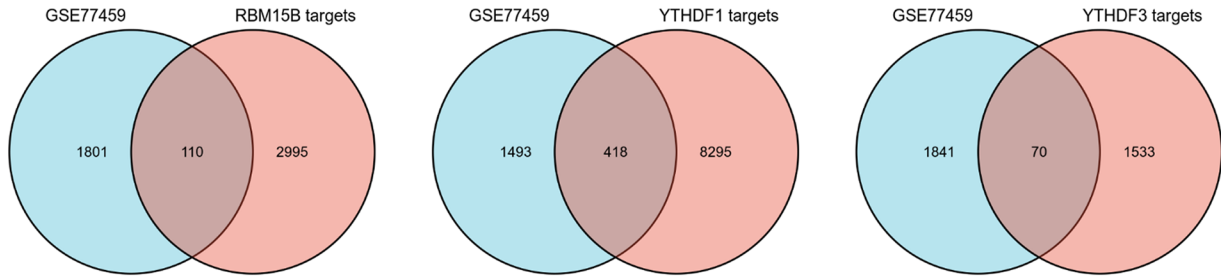
Fig. 3 m⁶A-related genes molecular interaction network and core genes interaction network

systemic lupus erythematosus [24] and scleroderma [25], further illustrating the importance and necessity of our study of the role of m⁶A in pulpitis. We analyzed functional enrichment analysis of key m⁶A target genes and found that these genes were mainly enriched in biological processes such as cell adhesion, T cell activation, glutamatergic synapse, ephrin receptor binding, cytokine receptors, and osteoclasts differentiation. These biological processes play an important role in the occurrence and development of pulpitis. Studies have shown that cell adhesion is related to the inflammatory progression of pulpitis and affects pulp regeneration [2]. Cytokine receptors can recruit stem cells and promote the differentiation of stem cells, which can promote the regeneration of dental pulp [26]. Invasion of caries-associated bacteria into dentin tissue resulted in enhanced osteoclastogenesis and inhibition of odontoblastogenesis [27],

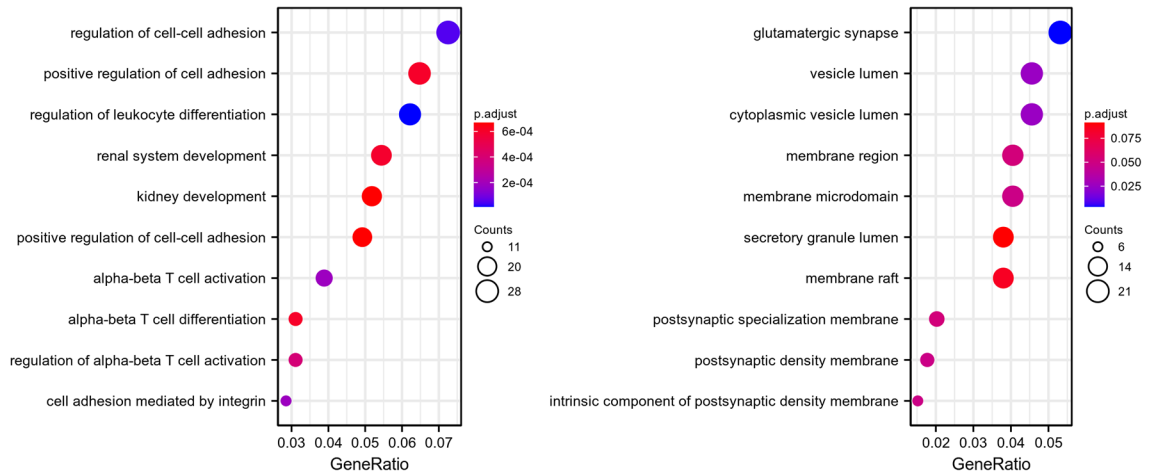
suggesting that osteoclast differentiation also plays an important role in the progression of pulpitis. These indicate that m⁶A is also very important for the progression of pulpitis. At the same time, we have made miRNAs that may regulate m⁶A, indicating that these miRNAs may also be important non-coding RNAs in the regulation of pulpitis.

In addition, the gene expression levels of *ALKBH5*, *METTL3*, *METTL14*, *METTL16*, *RBM15B*, *YTHDF1*, *YTHDF3* and *ZCCHC4* were detected in the collected human dental pulp tissue samples, and it was found that the expression of *METTL14* in pulpitis was lower than that in the control group. The result indicates that *METTL14* may be particularly important in pulpitis, and can be used as a biomarker for the progress of pulpitis, which provides a new idea for future therapeutic targets and drug development of pulpitis.

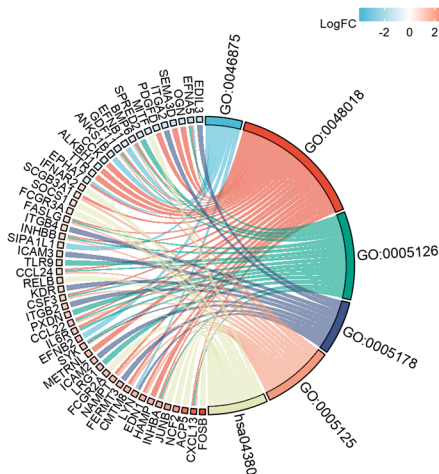
A



B



C



ONTOLOGY	ID	Description
MF	GO:0046875	ephrin receptor binding
MF	GO:0048018	receptor ligand activity
MF	GO:0005126	cytokine receptor binding
MF	GO:0005178	integrin binding
MF	GO:0005125	cytokine activity
KEGG	hsa04380	Osteoclast differentiation

Fig. 4 (A) The intersection of the downstream target molecules of the above core genes and the differential genes in GEO. (B-C) GO enrichment analysis and KEGG analysis

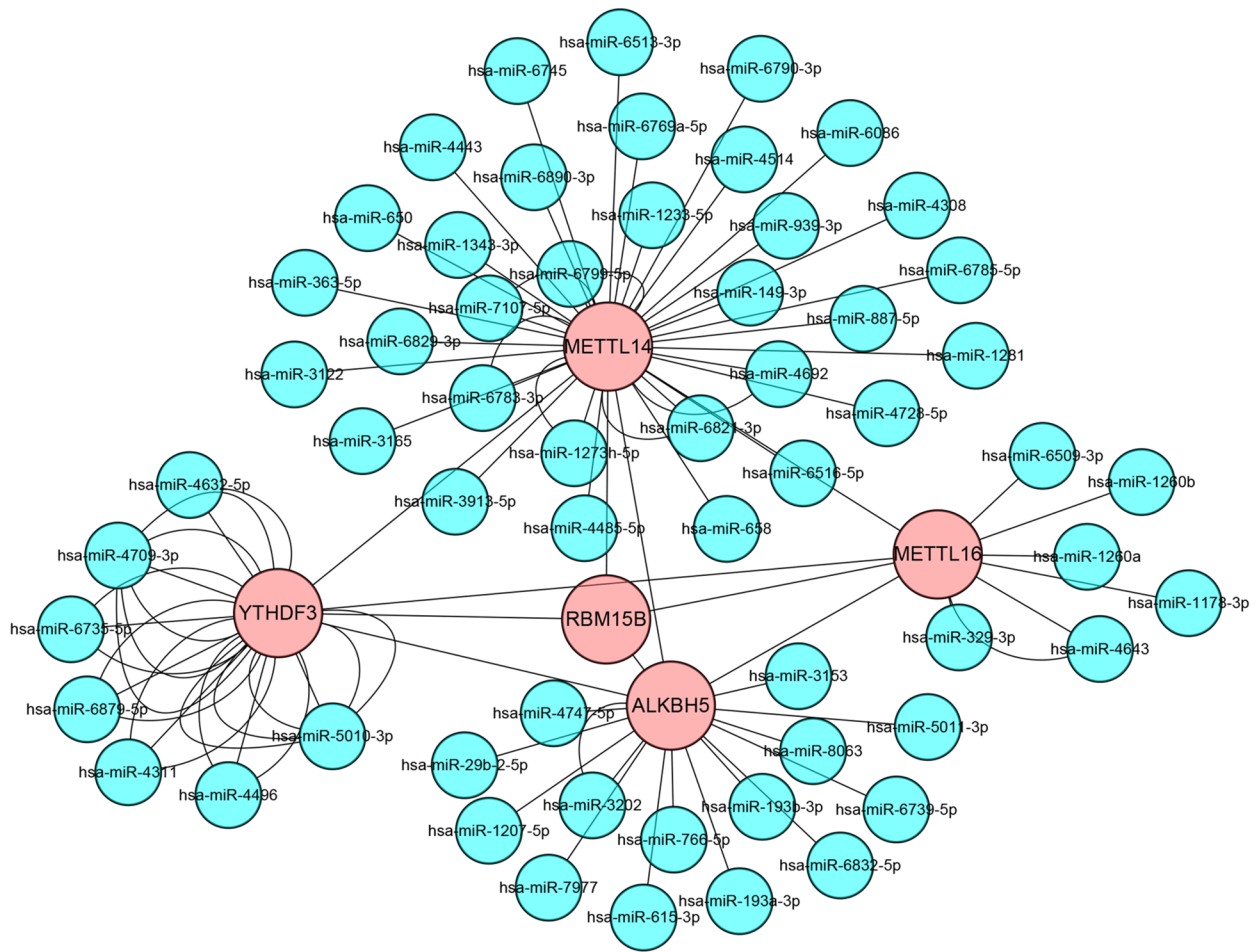


Fig. 5 Molecular interaction network of upstream miRNAs regulating the core m⁶A genes

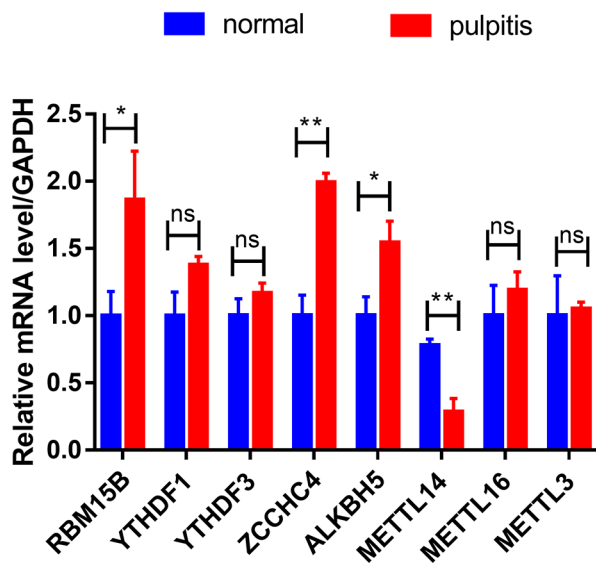


Fig. 6 The gene expression of ALKBH5, METTL3, METTL14, METTL16, RBM15B, YTHDF1, YTHDF3 and ZCCHC4 in normal group and pulpitis tissue samples was analyzed by quantitative real-time PCR. * $P < 0.05$ was considered statistically significant

Abbreviations

m ⁶ A	N6-methyladenosine
GO	Gene ontology
KEGG	Kyoto encyclopedia of genes and genomes
qRT-PCR	Quantitative real-time PCR
BP	Biological processes
CC	Cellular components
MF	Molecular functions

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Not applicable.

Authors' contributions

Hui Xu conducted the research and drafted the article. Guangjin Chen analyzed the data and drafted the article. Pengcheng Wang, Zhi Xu and Chunhui Chen collected specimens. Jiaying Zhou and Xukang Zhou revised the article. Fengyuan Lv and Xiaofang Li designed the study and revised the article.

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Data Availability

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

Declarations

Ethical approval and consent

All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols was approved by the Ethics Committee of Jinhua Hospital Affiliated to Zhejiang University (No.S96; 16/05/2022). Written informed consent was obtained for all subjects after explaining the purpose of the study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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