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Decreased expression of the m6A RNA methyltransferase METTL3 is associated with residual ridge resorption

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ABSTRACT

Objective: N6-methyladenosine (m6A) methylation and its regulators play crucial roles in the progression of osteoporosis (OP) by regulating the expression of osteoporosis-related genes. In this study, we have analyzed the expression of methyltransferase-like 3 (*METTL3*) and its target gene Runt-related transcription factor 2 (*RUNX2*) in patients with residual ridge resorption (RRR).

Materials and methods: A total 50 number of participants were included in this comparative study (RRR – n25 and healthy control – n25). Total RNA was extracted from peripheral blood and converted into cDNA. *METTL3* and *RUNX2* expression levels were quantified using RT-qPCR with *GAPDH* as the reference gene. Bioinformatics tools were used to identify gene functions and pathways.

Results: Real-time polymerase chain reaction (qPCR) revealed that *METTL3* and *RUNX2* expression was down-regulated in the RRR group compared to that in healthy controls ($P < 0.05$). *In silico* functional analysis provided information regarding the role of METTL3 in various biological processes.

Conclusion: Our findings suggest that *METTL3* dysregulation contributes to RRR pathogenesis. Further large-scale samples and functional studies are required to identify their therapeutic potential.

1. Introduction

The residual ridge is a portion of the alveolar bone along with its soft tissue covering that remains after tooth extraction. Even after wound healing, residual alveolar bone undergoes catabolic remodeling throughout its lifetime. The highest rate of resorption occurred in the initial 3–6 months, and then gradually declined. This unique phenomenon is termed as residual ridge resorption (RRR). The degree of resorption in the mandible is three or four times higher than that in the maxilla, which is attributed to a smaller denture-bearing area in the mandible, and thus a greater load per square centimeter.[1] However, the etiology and pathogenesis of RRR are not well understood. According to Atwood, RRR is most likely to be multifactorial. The possible etiological factors can be divided into four categories: anatomical, metabolic, functional, and prosthetic factors. The rates of resorption and bone loss vary from patient to patient, within the same patient at different times, and even at the same time in different parts of the ridge.[2]

Osteoporosis is a metabolic disorder affecting postmenopausal women. Low bone mass and bone tissue degradation are prominent symptoms of this condition, and may increase the risk of bone fractures. However, not all individuals with osteoporosis develop RRR. Animal studies have revealed a link among alveolar bone loss, delayed extraction, socket healing, and systemic osteoporosis. However, other studies have demonstrated a poor or no association. Several genes play crucial roles in bone formation and resorption, but the link between osteoporosis and jaw bone resorption remains unknown.[3] Several genes play crucial roles in bone formation and resorption. Previous studies have shown that certain genes such as *RANKL* and *Semaphorin 4D* (Sema4D), are associated with RRR. Inhibition of these genes improves alveolar bone loss.[4] Moreover, several single-nucleotide polymorphisms (SNP) and mutations in *IL10, NOD2, MMP-1,* and *VEGF* have been correlated with RRR.[5] These results indicate that these genes play a crucial role in

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RRR. Recent research has discovered that epitranscriptomics plays a crucial role in various diseases, including oral diseases and cancers. The most abundant epitranscriptomic modification of m6A methylation is associated with various biological processes including bone formation. Cellular biological processes such as cell differentiation, embryonic development, and disease occurrence are regulated by m6A modifications. Some methyltransferase and demethyltransferase enzymes control m6A methylation. In particular, METTL3 is an important m6A methyltransferase with multiple functions in humans, and dysregulated METTL3 expression is associated with various diseases.[6] However, the role and expression profile of METTL3 in RRR remains unclear. In this study, we analyzed *METTL3* expression in patients with RRR, and *RUNX2* gene expression to correlate with *METTL3* expression. In addition, the functional role of the METTL3 protein enrichment analysis pathway was assessed using bioinformatic software.

2. Materials and methods

2.1. Patient selection criteria and clinical measurement

The study included mandibular edentulous cases with complete edentulism only, although patients were excluded if they had any chronic or systemic disorders that could alter bone health. Bone height, defined as the "distance between the superior and inferior borders of the mandible, was measured for each patient in accordance with the American College of Prosthodontists' recommendation. The mandibular bone height was measured by two competent prosthodontists (examiners). Duplicate readings were taken. To improve intra-examiner reliability, all radiographs were re-assessed and some radiographs evaluated by one examiner were re-measured by the other. The average readings were computed and recorded.

2.2. Sample collection

This study was conducted at Saveetha Dental College and Hospitals, Chennai, from January 2022 to June 2022. Fifty subjects participated in this study, including RRR patients (n-25) and healthy volunteers (n-25). G power statistical software (version 3.1.9.6) was used to calculate the sample size for the present study, with the effect size, α error probability, and power. Peripheral blood samples (2 mL) were collected in ethylenediaminetetraacetic acid (EDTA) tubes, and plasma and WBC were immediately separated and stored at −80 °C until further processing. The study adhered to the guidelines of the Declaration of Helsinki and the rules of the Institutional Ethics Committee.

2.3. RNA extraction and RT-qPCR

Total RNA was extracted from the WBC samples using TRIzol (Thermo Fisher Scientific, USA) and quantified using NanoDrop One (Thermo Fisher Scientific). A PrimeScript First-Strand cDNA Synthesis Kit (Takara, Japan) was used for cDNA conversion from 2 μg total RNA. Bio-Rad CFX96 RT-qPCR (Bio-Rad, USA) was used for *METTL3* and *RUNX2* gene expression analysis. The primer sequences used are listed in Table 1. The composition of each qPCR reaction was 200 ng templates cDNA, 50 μm of forward and reverse primer, 10 μl of 2× SYBR, and DDH20 for final volume adjustment up to 20 μl. The qPCR was performed at specific temperatures. Initial denaturation was performed at 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s and 58 °C for 30 s. GAPDH was used as a reference gene for comparison with the target gene. The automated Bio-Rad CFX maestro 1.0 software (version 4.0.2325.0418) was used for relative quantification.

Table 1
Primers used for RT-qPCR analysis.

Gene	Primer	Primer sequence (5′→3′)
METTL3	Forward	CGTACTACAGGATGATGGCTTTC
METTL3	Reverse	TTTCATCTACCCGTTCATACCC
RUNX2	Forward	TTCCAGACCAGCAGCACTC
RUNX2	Reverse	CAGCGTCAACACCATCATT
GAPDH	Forward	TCCAAAATCAAGTGGGGCGA
GAPDH	Reverse	TGATGACCCTTTTGGCTCCC

2.4. In silico functional analysis and statistical analysis

Furthermore, we performed an *in silico* functional analysis of METTL3 using bioinformatic tools and platforms. First, we assessed the protein-protein interactions of METTL3 using STRING (https://string-db.org/). Furthermore, we collected all proteins that interacted with METTL3 for pathway analysis using Metascape (http://metascape.org). The SRAMP software was used for m6A prediction of the full-length sequence (5540 bp) of *RUNX2*. Finally, SPSS software, version 23 (IBM, Chicago, Illinois, United States) was used for two-way ANOVA, Tukey's test, and Student's t-test was performed to analyze the gene expression results of patients with RRR and healthy controls.

3. Results

In this study, we recruited 50 participants: 25 from the RRR group and 25 from the healthy group; the clinical information is listed in Table 2. We then analyzed the expression of *METTL3* and *RUNX2* in residual ridge resorption and healthy control samples. METTL3 is a crucial methyltransferase involved in m6A methylation. We found that *METTL3* and *RUNX2* expression levels were significantly reduced ($p < 0.01$, $p < 0.001$, respectively; Fig. 1A and B) in patients with RRR compared to those in the healthy group. *METTL3* downregulation significantly correlated with reduced *RUNX2* expression ($p < 0.05$). Furthermore, we correlated with height of mandibular bone, age, and sex. However, there were significant differences in mandibular bone height or age or sex. In addition, SRAMP software was used for m6A prediction of the full-length sequence (5540 bp) of RUNX2. As presented in Fig. 1C, the m6A modification peak with very high confidence in the RUNX2 transcript suggests that m6A modification and its regulator METTL3 are linked to RRR by regulating the RUNX2 signalling pathway in an m6A-dependent manner. Furthermore, protein-protein interactions (Fig. 1D), and *in silico* functional analysis revealed that the METTL3 protein is involved in m6A RNA methylation along with the regulation of mRNA processing (Fig. 1E). Therefore, it might be associated with bone formation and osteogenesis and is linked to RRR.

4. Discussion

In humans, RRR is characterized by the loss of mandibular or maxillary bones, which lasts for a long time after tooth removal. It deteriorates the oral habitat and contributes to the development of systemic disorders. However, the molecular processes and risk factors for RRR progression remain unknown. Moreover, postmenopausal

Table 2
Patient demographic and clinical information.

Details	Residual Ridge Resorption (n = 25)	Normal Healthy subjects (n = 25)
Gender ratio (Male: Female)	1:4 Male: 5 Female: 20	1:4 Male: 5 Female: 20
Age Range	40–60 years	40–60 years
Any Systemic/Chronic diseases	None	None
Edentulous state	Complete edentulism	None
Mandibular Bone height (mean)	16.03 mm	N/A

N/A: not available.

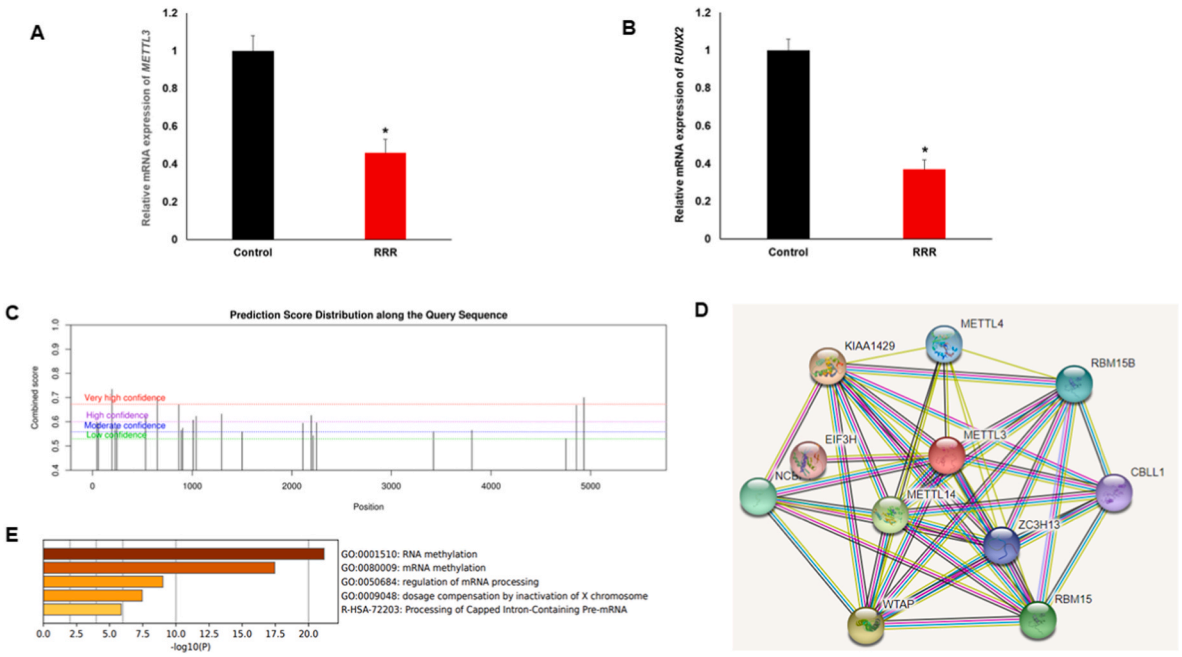

Fig. 1. Analysis of mRNA expression of METTL3 (A) and RUNX2 (B) in RRR and control groups was performed using real-time polymerase chain reaction (real-time PCR). RUNX2 sequence-based m6A modification site prediction using SRAMP software combined with the prediction score (C). Protein-protein interaction network between METTL3 and its target proteins in the STRING dataset (D). Functional enrichment analysis or pathway enrichment analysis using the Metascape database (E). * indicates statistical significance *($P < 0.05$)*.

osteoporosis causes deterioration of the bone tissue microarchitecture and poor bone mineral density. According to several clinical studies, the RRR increases in osteoporotic edentulous patients. However, other studies have found no statistical association between edentulous jaw resorption and osteoporosis. Therefore, the association between RRR and osteoporosis remains controversial and has not yet been thoroughly investigated.[3] Bone morphogenetic proteins (BMPs), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) are required for bone development and repair.[4]

The role of m6A methylation in cellular function and destiny control has been demonstrated in emerging research in which abnormal changes in methyltransferases and demethylases result in dysfunction or illness. Reduced m6A alterations in bone marrow mesenchymal stem cells (BMSCs) impair the osteogenic and adipogenic responses caused by parathyroid hormone, resulting in severe bone loss and excessive accumulation of adipose tissue. Previous studies have shown that METTL3 deficiency reduces the osteogenic differentiation capability of bone marrow stem cells. However, little is known about the role of METTL3 in osteoblast function during inflammation.[7] Furthermore, an animal model study demonstrated that METTL3 knockdown in mice results in reduced bone formation, inadequate osteogenic differentiation potential, and increased marrow adiposity.[6] This indicated that METTL3 may be associated with bone formation and osteogenic differentiation. Similarly, our results indicated that *METTL3* expression was significantly reduced ($p < 0.05$, Fig. 1A) in patients compared to that in healthy controls. This strongly indicates that METTL3 plays a role in bone formation and resorption in RRR.

Several studies have shown that METTL3 is crucial for m6A methylation. Interestingly, m6A methylation regulates osteoporosis and bone remodeling through various signaling pathways, such as Wnt/β-catenin, PTH/Pth1r, PI3K-Akt, and other signaling pathways. These pathways are crucial for regulating bone homeostasis. Furthermore, m6A regulates the expression of ALP, VEGF, RUNX2, and Osterix, which are involved in osteoblasts, osteoclasts, and BMSCs. This indicates that METTL3 may be associated with bone health by regulating m6A methylation.[8] RUNX2 is a key transcription factor that regulates chondrocyte and osteoblast differentiation as well as bone production. RUNX2 is required for dental development. It governs the alveolar remodeling process, which is required for tooth eruption, and may play a role in periodontal ligament maintenance.[9] In this study, RUNX2 expression was significantly reduced and correlated with METTL3 expression. *METTL3* may control *RUNX2* expression, leading to the risk of RRR and bone-related disease pathogenesis. Knockdown of METTL3 in mice suppresses m6A methylation and RUNX2 expression.[10] Accumulating evidence suggests that METTL3 plays a key role in osteogenesis by regulating m6A methylation and controlling various genes related to bone development, such as RUNX2. The role of METTL3 in bone formation and RRR is through the regulation of m6A methylation in RUNX2, which is schematically represented in Fig. 2.

This is the first study to report epitranscriptomic alterations during residual ridge resorption. Our pilot study results highlight that epitranscriptomic alterations are significantly associated with osteoclasts and residual ridge resorption. *METTL3* and *RUNX2* gene expression was significantly correlated and downregulated in RRR patients. Previous studies have suggested that METTL3 is involved in bone formation by regulating RUNX2 through m6A methylation. The current study was limited by the small sample size and gene expression, and further protein expression and functional studies are required. However, our study may be helpful for further research in this area to identify the molecular mechanism of RRR pathogenesis and may be useful as a therapeutic target for bone degradation or osteoclast-related diseases, including RRR.

5. Conclusion

Our observations provide important insights into the dysregulation of METTL3 and suggest that m6A may be a risk factor for RRR pathogenesis. *RUNX2* expression also correlates with *METTL3* expression, which results in dysregulated m6A, leading to a huge pathway involved in RRR pathogenesis and bone remodeling. Further functional studies are required to determine the molecular mechanisms underlying the expression of RRR and METTL3.

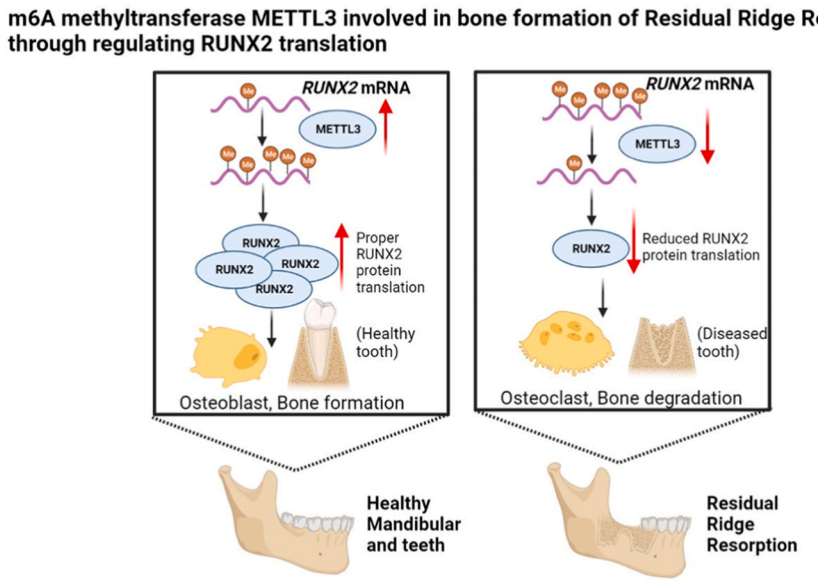

Fig. 2. Schematic representation of METTL3 role in RRR via regulation of RUNX2 translation. Methyltransferase like-3 (METTL3) is a crucial protein involved in m6A methylation that regulates various biological and cellular processes in humans. METTL3 is primarily responsible for methylation, which determines mRNA fate. Decreased METTL3 expression reduces the methylation of *RUNX2* mRNA, which results in reduced RUNX2 translation. RUNX2 is a crucial protein in bone formation, and reduced RUNX2 expression is associated with osteoclasts and bone degradation in RRR.

Ethical approval

This study was approved by the Institutional Ethical Committee of the Saveetha Dental College and Hospital. All participants signed an informed consent form.

CRediT authorship contribution statement

Harini Sri Krishnamoorthy: Validation, Formal analysis, Investigation, Data curation, Writing - original draft, review. **Balachander Kannan:** Data curation, investigation, writing - original draft, and review. **Dhanraj Ganapathy:** Methodology, formal analysis, and review. **Vijayashree Priyadharsini Jayaseelan:** Methodology, formal analysis, writing-review. **Paramasivam Arumugam:** Conceptualization, methodology, formal analysis, writing, reviewing, and editing.

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