Journal of Oral Biology and Craniofacial Research 14 (2024) 494–499

Contents lists available at ScienceDirect

Journal of Oral Biology and Craniofacial Research

journal homepage: www.elsevier.com/locate/jobcr

Overexpression of insulin-like growth factor-2 mRNA-binding protein 1 is associated with periodontal disease

Burra Anand Deepika [a], Jaiganesh Ramamurthy [a], Balachander Kannan [b], Vijayashree Priyadharsini Jayaseelan [c], Paramasivam Arumugam [b,*]

[a] Department of Periodontics, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, India
[b] Molecular Biology Lab, Centre for Cellular and Molecular Research, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, India
[c] Clinical Genetics Lab, Centre for Cellular and Molecular Research, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, India

ARTICLE INFO

Keywords:
Health
Periodontitis
Genetics
Novel m6A reader
IGF2BP1

ABSTRACT

Objective: To investigate the potential role of a novel m6A RNA regulator, Insulin-like Growth Factor-2 mRNA-binding protein 1 (IGF2BP1), in periodontal disease pathogenesis.
Materials and methods: Gingival tissue samples from 60 periodontitis patients and 60 healthy individuals were analyzed for IGF2BP1 mRNA and protein expression *via* real-time quantitative PCR (RT-qPCR) and Western blotting. Additionally, *Porphyromonas gingivalis* Lipopolysaccharide (Pg-LPS) -induced human gingival fibroblasts (HGFs) were evaluated for IGF2BP1 and proinflammatory cytokine expression. *In silico* functional analysis further explored potential molecular mechanisms.
Results: IGF2BP1 mRNA and protein levels were significantly higher in the periodontitis group compared to the healthy group. Functional analysis implicated IGF2BP1 in regulating the IL-17 signaling pathway, a key player in inflammation. Pg-LPS treatment upregulated IGF2BP1 and proinflammatory cytokines in HGFs, supporting this finding.
Conclusion: Our study suggests that IGF2BP1 overexpression contributes to periodontitis pathogenesis, potentially through IL-17 signaling. Further research is needed to elucidate the precise molecular mechanisms and explore IGF2BP1 as a potential therapeutic target or biomarker for this common oral disease.

1. Introduction

Periodontitis is a chronic inflammatory disease characterized by the progressive destruction of tissues surrounding the teeth. This leads to loss of connective tissue, collagen in the gums, periodontal ligament, and alveolar bone. Consequently, tooth roots become exposed, harboring bacterial biofilms that solidify as dental calculus. The slow, chronic nature of this disease culminates in tooth mobility, chewing difficulties, aesthetic concerns, and ultimately, tooth loss if left untreated. Periodontitis' impact extends beyond oral health, triggering low-grade systemic inflammation harmful to other organs.[1,2] It's a multifactorial disease influenced by both environmental and genetic factors, with the alveolar bone serving as a bridge linking it to systemic conditions like diabetes, liver issues, cardiovascular diseases, and osteoporosis.[3]

Porphyromonas gingivalis, a Gram-negative anaerobic bacterium, stands out as a major contributor to periodontitis. This resident of the subgingival plaque (biofilm on teeth below the gum line) possesses virulence factors like gingipains (proteinases), lipopolysaccharides, and fimbriae, allowing it to degrade host tissues, evade immune responses, and drive periodontal damage. Studies support this link: *P. gingivalis* prevalence is higher in periodontitis patients than in healthy individuals,[4] and animal models demonstrate its ability to induce inflammation and tissue destruction.[5] Targeting *P. gingivalis* emerges as a promising strategy for preventing and treating this disease.

Disease-modifying genes also play a role in periodontitis susceptibility. Aggressive periodontitis, with its Mendelian inheritance pattern, serves as a model for identifying genetic risk factors.[6] However, complex

* Corresponding author. Centre for Cellular and Molecular Research, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, 600077, TN, India.
E-mail address: paramasivama.sdc@saveetha.com (P. Arumugam).

https://doi.org/10.1016/j.jobcr.2024.06.001
Received 5 January 2024; Received in revised form 21 May 2024; Accepted 5 June 2024
Available online 2 July 2024

interactions between gene-gene, gene-environment, and environmental-lifestyle factors contribute to the disease's development.[7,8] Epigenetic changes, including DNA and RNA methylation, as well as post-transcriptional modifications, further influence susceptibility.

Scientists have identified distinct gene expression profiles and mutations/single nucleotide polymorphisms in individuals with periodontitis, differentiating them from healthy individuals. Notably, these genes regulate diverse biological and cellular processes, including cell growth and bone formation.[9–11] Intriguingly, a new N6-methyladenosine (m6A) RNA regulator, IGF2BP1, is strongly linked to inflammation, particularly bacterial-induced. Studies revealed its role in activating the pro-inflammatory NF-κB pathway and cytokine production in human macrophages and monocytes.[12] Further research suggests a connection between IGF2BP1 and miR-1246 in periodontal disease.[13] However, its expression in *P. gingivalis*-infected periodontal tissues remains unexplored. Our study delves into this knowledge gap by analyzing IGF2BP1 expression in both periodontal infected and healthy periodontal tissue samples. Additionally, we explore its function through cell culture experiments and *in silico* analyses using a bioinformatic platform.

2. Materials and methods

2.1. Patients and sample collection

This study aimed to investigate the role of IGF2BP1 in periodontitis. Between January 2022 and March 2023, we recruited 60 periodontitis patients and 60 healthy individuals from Saveetha Dental College and Hospitals, Chennai. We calculated the sample size using a preliminary pilot study using G Power (version 3.1) software. Participants with other systemic or genetic diseases or on medication during sample collection were excluded. Following periodontal pocket measurements, gingival tissue samples were collected and immediately stored at −80 °C. The Institutional Human Ethical Committee (IHEC Ref No: IHEC/SDC/PERIO-2002/22/080) approved the study, which adhered to the Declaration of Helsinki for human research. All participants or their guardians provided informed consent for participation in this study. We diagnosed chronic periodontitis based on established clinical criteria by American Academy of Periodontology. To obtain additional control tissue, we collected gingival tissue from healthy volunteers undergoing tooth extraction for orthodontic or wisdom tooth removal.

2.2. RNA extraction and cDNA conversion

Total RNA was extracted from gingival samples using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) following standard protocols.[14] The purity and quantity of RNA were assessed using a NanoDrop One (Thermo Fisher Scientific, USA). Subsequently, 2 μg RNA was converted into cDNA using a Takara single-strand cDNA synthesis kit (Takara, Tokyo, Japan). All samples were stored at −20 °C until further analysis.

2.3. Cell culture

Human gingival fibroblasts (HGFs) were derived from gingival tissues as previously described. After cultivation, HGFs were stimulated with different concentrations of Pg-LPS (0, 1, and 5 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 24 h in a CO2 incubator. RNA and proteins were extracted from stimulated and control cells using standard protocols. IGF2BP1 mRNA and protein expression and pro-inflammatory cytokine mRNA levels (IL-1β, IL-6, and TNF-α) were analyzed in Pg-LPS-stimulated and control HGF samples.

2.4. RT-qPCR mRNA expression analysis

Quantification of mRNA expression was conducted using a Bio-Rad CFX Opus 96 RT-qPCR system (Bio-Rad, Hercules, CA, USA) employing a standard SYBR Green qPCR Master Mix (Takara, Japan), as previously described.[15] The $2^{-\Delta\Delta Ct}$ method, with GAPDH as the reference gene, was utilized to estimate relative mRNA levels. Primer sequences are provided in Table 1.

2.5. Western blot

Gingival tissue protein extraction followed established protocols. Total protein was quantified using a BCA protein assay kit (Pierce, Thermo Fisher Scientific, USA) as per the manufacturer's guidelines. Equal protein amounts were resolved on 10 % SDS-PAGE gels and transferred to PVDF membranes. Blocking occurred with 5 % BSA for 2 h, followed by overnight incubation at 4 °C with primary antibodies against IGF2BP1 and GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA). After washing, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 2 h. Protein visualization was achieved by the Bio-Rad Chemi doc XRS + using clarity max ECL substrate (Bio-Rad, USA), and quantification was performed using Image Lab software (Bio-Rad, USA). The protocol was followed based on previous literature.[14]

2.6. In silico functional analysis

The IGF2BP1 gene interaction network was analyzed using GeneMania (http://genemania.org/),[16] and protein interaction networks were analyzed using STRING (https://string-db.org/)[17] databases. Functional pathway analysis of IGF2BP1 was performed using Metascape (https://metascape.org/)[18] using its network genes and proteins. Furthermore, the SRAMP software (http://www.cuilab.cn/sramp/)[19] was used for m6A prediction of the full-length IL-17 mRNA sequence.

2.7. Statistical analysis

Statistical analyses were conducted using SPSS statistics 27 software (IBM Corp.). We used the student's t-test to compare gene expression levels between the two groups, as it is appropriate for normally distributed data. In cases where the data did not meet normality assumptions, we employed the Mann-Whitney *U* test to ensure the robustness of our statistical comparisons. A p-value <0.05 was considered statistically significant. All experiments were conducted in duplicate or triplicate.

3. Results

3.1. Higher IGF2BP1 levels in periodontitis

Our initial focus was on IGF2BP1 expression in individuals with periodontitis compared to healthy controls. Both qPCR and Western blot analyses revealed a significant upregulation of IGF2BP1 at both the mRNA and protein levels in the periodontitis group ($p < 0.05$; Fig. 1A–C). These findings suggest a potential role for IGF2BP1 in the pathogenesis of periodontal disease.

3.2. Pg-LPS induces IGF2BP1 overexpression in human gingival fibroblasts

To explore the potential influence of bacterial factors, we utilized HGFs treated with Pg-LPS. Notably, Pg-LPS exposure at 5 μg/ml for 24 h did not alter HGF morphology (Fig. 2A). However, both qPCR and Western blot analyses revealed a striking increase in IGF2BP1 expression in Pg-LPS-treated HGFs compared to controls ($p < 0.05$; Fig. 2B–D). This finding strengthens the link between IGF2BP1 and periodontal disease progression.

Table 1
Primer sequence for qPCR.

Gene	Forward Primer	Reverse primer
IGF2BP1	5′-TAGTACCAAGAGACCAGACCC-3′	5′-GATTTCTGCCCGTTGTTGTC-3′
IL-1β	5′-CCACAGACCTTCCAGGAGAATG-3′	5′-GTGCAGTTCAGTGATCGTACAGG-3′
IL-6	5′-ACTCACCTCTTCAGAACGAATTG-3′	5′-CCATCTTTGGAAGGTTCAGGTTG-3′
TNF-α	5′-CCTCTCTCTAATCAGCCCTCTG-3′	5′-GAGGACCTGGGAGTAGATGAG-3′
GAPDH	5′-TCCAAAATCAAGTGGGGCGA-3′	5′-TGATGACCCTTTTGGCTCCC-3′

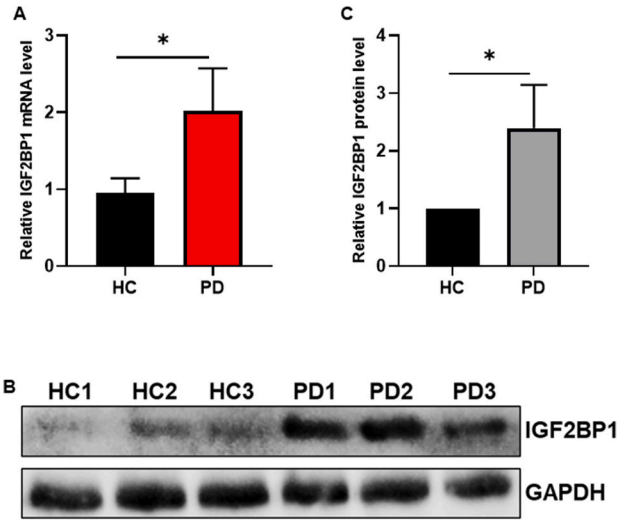

Fig. 1. Differences in IGF2BP1 expression in the periodontal disease and healthy groups. (A) IGF2BP1 mRNA expression levels in the periodontal healthy group and the periodontal disease group were detected by real-time PCR (RT-qPCR). (B) IGF2BP1 protein expression levels in the periodontal healthy group and the periodontal disease group were detected by Western blot. (C) IGF2BP1/GAPDH densitometry ratio histogram (Western blot) of two groups (periodontal healthy and periodontal disease groups). *Significant ($p < 0.05$) difference between the periodontal healthy and periodontal disease groups. Groups: HC - Healthy Control, PD - Periodontal Disease.

3.3. Elevated proinflammatory cytokines in Pg-LPS-stimulated HGFs

Since inflammation is a key feature of periodontitis, we further investigated proinflammatory cytokine expression in Pg-LPS-treated HGFs. Levels of *IL-1β, IL-6,* and *TNF-α* were significantly elevated compared to controls ($p < 0.05$; Fig. 3A–C), implying involvement of these inflammatory mediators in the disease process.

3.4. In silico functional enrichment of IGF2BP1 is associated with IL-17 regulation

The interaction between the IGF2BP1 gene and protein was identified using online databases such as Genemania (Fig. 4A) and STRING (Fig. 4B). Interacting genes and proteins were collected and analyzed for pathway enrichment analysis using the Metascape database. Notably, these analyses revealed interactions between IGF2BP1 and gene/protein networks associated with diverse functions, including, mRNA stabilization, metabolic processes, translation initiation, MAPK6/MAPK4, Interleukin-17 (IL-17) (pro-inflammatory cytokines), and negative regulation of canonical Wnt signaling pathways (Fig. 4C). These findings suggest that IGF2BP1 role in periodontitis may involve regulating IL-17 signaling and other cellular processes, providing intriguing avenues for further investigation.

Previous research has established the crucial role of IL-17 in both inflammation and tissue destruction within periodontal disease. Notably, a recent study revealed that the IGF2BP family stabilizes IL-17 mRNA through m6A modifications, acting as a driving force in inflammatory diseases. Building upon this knowledge, our study utilized SRAMP software to predict m6A modifications within the full-length sequence of IL-17. Remarkably, as depicted (Fig. 4D), a high-confidence m6A modification peak was identified in the IL-17 transcript. This compelling finding suggests a strong link between m6A modification and its regulator, IGF2BP1, in controlling periodontal disease progression through modulation of the IL-17 signaling pathway in an m6A-dependent manner.

4. Discussion

Our study identified IGF2BP1 as significantly upregulated in periodontitis patients and Pg-LPS-treated human gingival fibroblasts. This suggests a potential role for IGF2BP1 in disease development and response to bacterial factors. Furthermore, the observed elevation of proinflammatory cytokines like IL-1β, IL-6, and TNF-α in Pg-LPS stimulated HGFs strengthens the link between IGF2BP1 and inflammation. Interestingly, *in silico* analysis revealed interactions between IGF2BP1 and networks associated with IL-17 regulation, a key player in periodontal disease. Additionally, a predicted m6A modification site within the IL-17 transcript suggests a potential mechanism involving IGF2BP1, m6A modification, and IL-17 signaling in the pathogenesis of periodontitis. These findings warrant further investigation into the precise role of the IGF2BP1-m6A-IL-17 axis in periodontal disease progression (Fig. 5).

Chronic periodontitis affects a significant portion of the adult population globally, and its complex pathogenesis involving genetic and epigenetic factors remains incompletely understood. Our findings contribute to this understanding by highlighting the potential role of IGF2BP1 dysregulation in the disease. Previous studies have linked IGF2BP1 to inflammatory responses in various disease conditions. *In vitro* studies by independent researchers further suggest IGF2BP1 overexpression involvement in periodontitis through the NF-κB signaling pathway [17]However, this is the first study to report elevated IGF2BP1 expression at the mRNA and protein levels in periodontitis patients.

Several oral microorganisms, such as *P. gingivalis*, are specifically linked to periodontal infections due to their virulence factors and lipopolysaccharide (LPS), which can modify human inflammatory responses and related genes. The MAPK signaling pathway is crucial in the innate immune response to bacterial invasion. This pathway influences the periodontal environment by regulating inflammatory cytokine production, bone resorption, and osteoclast differentiation. Proinflammatory cytokines like IL-17, IL-1β, IL-6, TNF-α, and Toll-like receptors (TLRs) are key players in bacterial infection-induced inflammation, primarily controlled by the MAPK pathway.[20] Studies by Xu et al. (2017) suggest that increased IGF2BP1 regulates MAPK signaling activation through mRNA translation.[21] This suggests a potential role for IGF2BP1 in influencing periodontitis inflammation through the MAPK pathway and subsequent proinflammatory cytokine production.

Existing research demonstrates that IL-17 overexpression in periodontitis patients plays a crucial role in disease progression.[22,23] Moreover, pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α significantly increase during periodontal infection and exhibit a strong association with IL-17 levels.[24] Our *in silico* analyses, using functional enrichment and SRAMP software, suggest a compelling link between m6A methylation modification, its regulator IGF2BP1, and IL-17

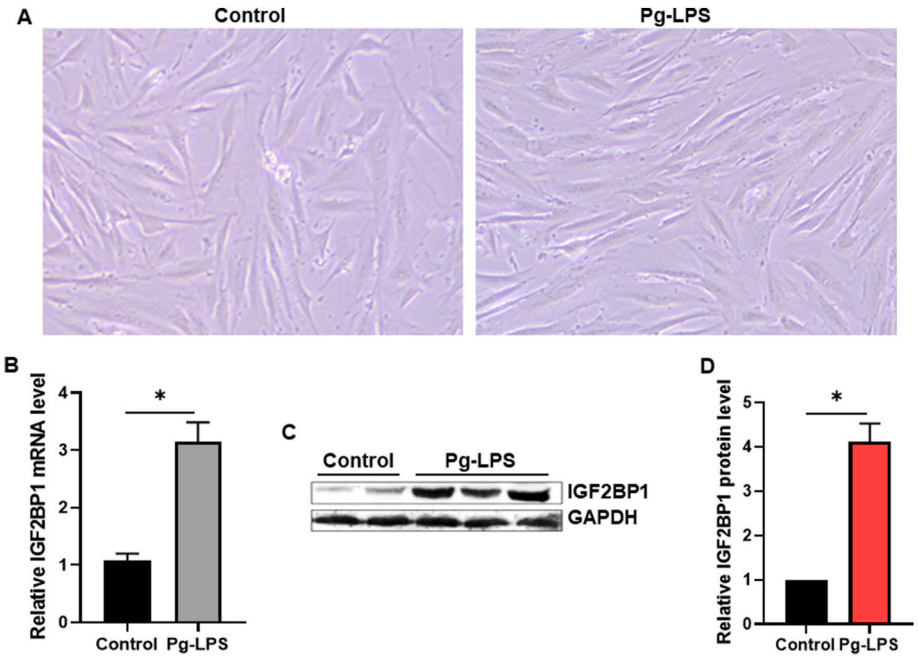

Fig. 2. IGF2BP1 expression in HGFs after stimulation with Pg-LPS. (A) HGFs morphology in control (without Pg-LPS) and Pg-LPS stimulated (5 μg/ml) at 24 h. (B) The changes of *IGF2BP1* mRNA expression in control and Pg-LPS stimulated HGFs at 24 h were detected by RT-qPCR. (C) The changes of IGF2BP1 protein expression in control and Pg-LPS stimulated HGFs at 24 h were detected by Western blot. (D) IGF2BP1/GAPDH densitometry ratio histogram of two groups (control and Pg-LPS stimulated HGFs). *Significant ($p < 0.05$) difference between control and Pg-LPS stimulated HGFs.

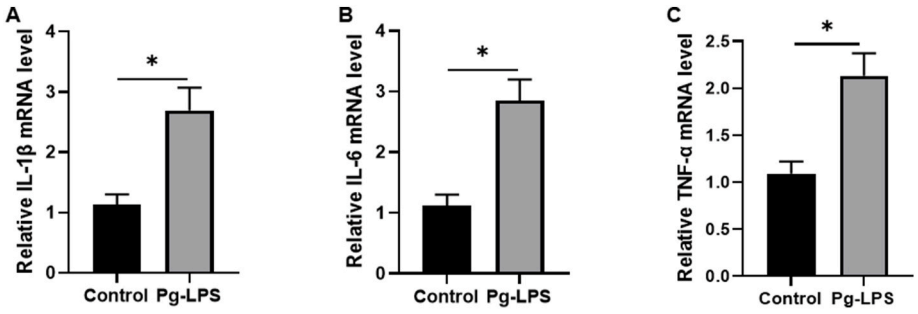

Fig. 3. Proinflammatory cytokines expression in HGFs after stimulation with Pg-LPS. mRNA expression of proinflammatory cytokines, including IL1β (A), IL6 (B), TNFα (C) in control and Pg-LPS treated HGFs at 24 h were detected by RT-qPCR. *Significant ($P < 0.05$) difference between control and Pg-LPS stimulated HGFs.

signaling in periodontitis. This suggests a potential regulatory network where IGF2BP1 may influence IL-17 expression through m6A-dependent mechanisms, contributing to periodontal pathogenesis.

Canonical Wnt signaling regulates cell proliferation, differentiation, and fate, playing a critical role in periodontal tissue homeostasis and cell function.[25] It also exhibits opposing effects on bone formation (positive) and cementum production (negative).[26] Interestingly, recent studies have identified IGF2BP1 as a direct target of the Wnt signaling pathway.[27] In our *in silico* analysis, IGF2BP1 and its associated genes/proteins linked to MAPK6/MAPK4, IL-17, and negative regulation of canonical Wnt signaling. This suggests that IGF2BP1 expression may impact periodontal pathogenesis by interacting with multiple signaling pathways, including Wnt and MAPK, potentially influencing inflammation and cell behavior.

Recent evidence highlights the importance of m6A methylation and its regulatory proteins in inflammatory responses and various inflammatory diseases. IGF2BP1, an oncofetal mRNA-binding protein and recently identified m6A binding protein, plays a crucial role in mRNA stabilization and translation, including pro-inflammatory genes. Studies have shown that abnormal IGF2BP1 expression is associated with various signaling pathways, making it a potential therapeutic target for diverse diseases.[28–30] Several studies have indicated that abnormal expression of IGF2BP1 is linked to various signaling pathways, and targeting IGF2BP1 is a better therapeutic option for various diseases. For instance, silencing of IGF2BP1 in RAW264.7 macrophage inhibited ox-LDL-induced lipid deposition and inflammation by suppressing RUNX1 expression and promoting autophagy.[28] Furthermore, another study showed that IGF2BP1 inhibition significantly reduced the proliferation of mesenchymal stem cells (MSCs), decreased expression of c-MYC and GLI1, and increased expression of p21 [29]. These results suggested that IGF2BP1 is linked to both inflammation and cell proliferation. IGF2BP1 expression may play a potential role in periodontal pathogenesis by regulating multiple pathways.

Hao et al. (2022) demonstrated that *P. gingivalis*, a key periodontal pathogen, alters host gene expression and promotes inflammation in RAW 264.7 cells, increasing pro-inflammatory cytokines and inhibiting AhR expression.[31] Similarly, other studies have shown that periodontal pathogens can modulate host gene responses and inflammation.[32] Our findings align with these studies, showing that Pg-LPS increases IGF2BP1 and pro-inflammatory cytokine levels. Additionally, bacterial LPS has been shown to influence m6A modification and its regulatory proteins in other disease contexts.[30] The accumulating evidence,

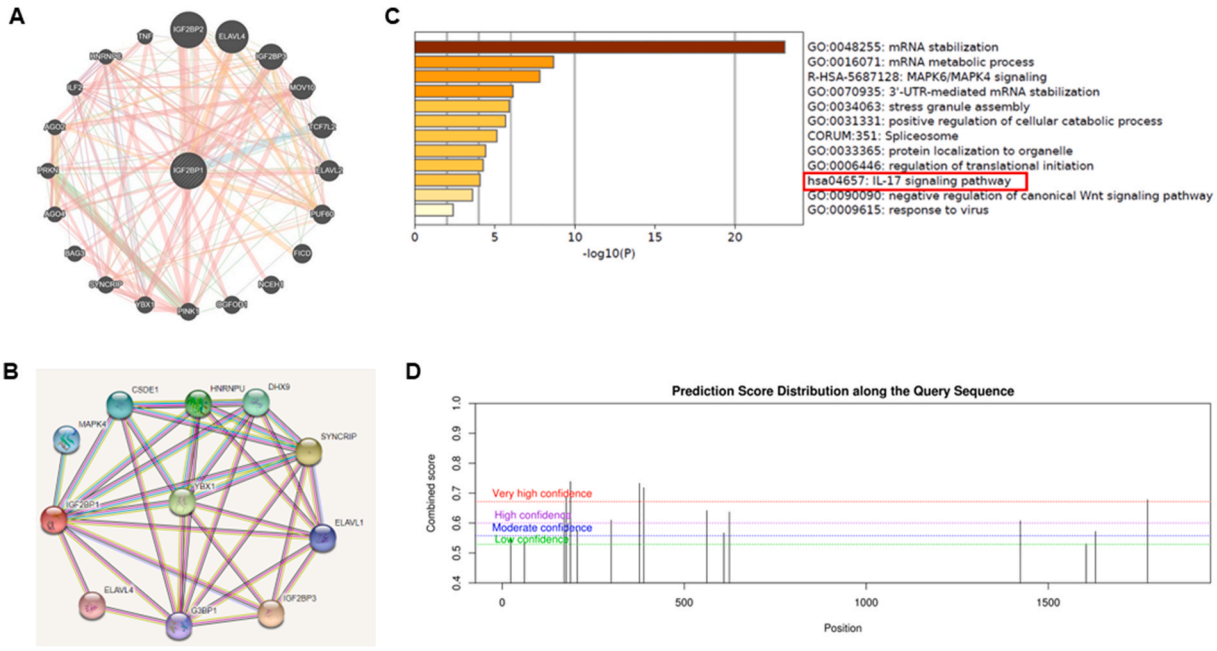

Fig. 4. Functional enrichment analysis. *IGF2BP*1 gene interactions were identified using GeneMANIA database (A). IGF2BP1 protein interactions were analyzed using STRING database (B). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and process enrichment analyses were performed (C). IL-17 sequence-based m6A modification site prediction using SRAMP software combined with the prediction score (D).

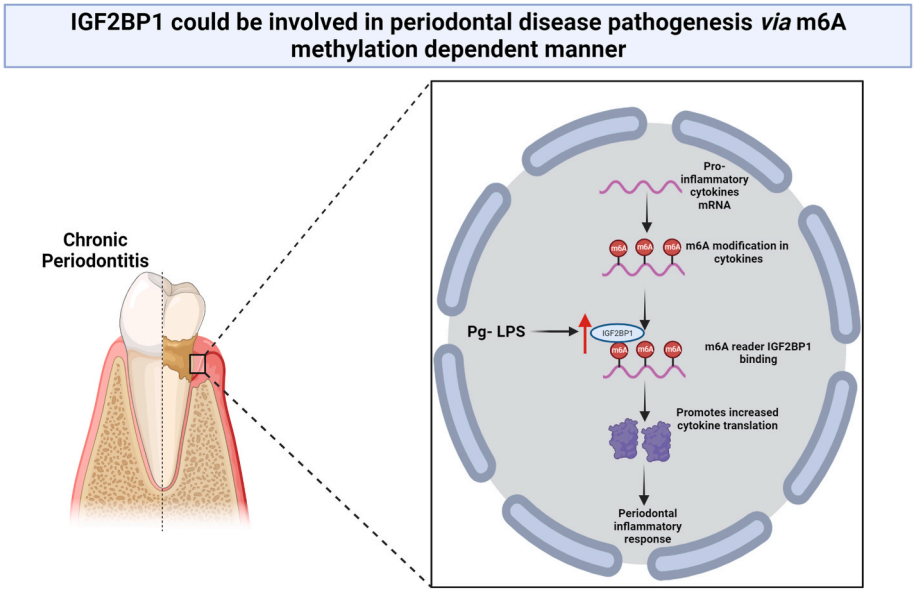

Fig. 5. The schematic representation suggests that IGF2BP1 is associated with periodontal disease pathogenesis in an m6A-dependent manner.

including our current findings, strongly suggests a significant link between IGF2BP1 dysregulation and periodontal disease development.

This study presents intriguing initial findings on IGF2BP1 role in periodontitis pathogenesis, yet several limitations need addressing in future research. The small sample size may limit the generalizability of the observed correlations between IGF2BP1 expression and disease status. While human gingival fibroblasts (HGFs) are useful *in vitro*, *in vivo* models or organ cultures could better elucidate IGF2BP1 role in the gingival tissue microenvironment. Although the study links IGF2BP1 to inflammatory responses, further functional assays, such as IGF2BP1 knockdown or overexpression experiments, are necessary to confirm its causal role in disease progression. *In silico* analysis provides insights into potential interactions and pathways, but these require validation through functional studies. Additionally, further research is needed to validate the regulatory role of a putative m6A modification site on the IL-17 transcript in periodontitis and its contribution to the m6A-IGF2BP1-IL-17 axis.

5. Conclusion

In conclusion, our results indicate that IGF2BP1 overexpression is associated with periodontal disease pathogenesis. Further functional studies may discover the potential role of IGF2BP1 in bacterial mediated periodontal inflammation. Therefore, targeting IGF2BP1 may be helpful for the treatment of various inflammatory diseases, including periodontitis. Furthermore, IGF2BP1 may be a potential biomarker for

periodontal infection. The present study does have some potential limitations. The major limitation is that we did not use specific knock-out cells and animal models to discover the molecular mechanisms of IGF2BP1 involving periodontal pathogenesis.

6. CRediT authorship contribution statement

Burra Anand Deepika: Validation, Formal analysis, Investigation, Data curation, Writing - original draft, review. Balachander Kannan: Data curation, formal analysis, investigation, writing - original draft, and review. Vijayashree Priyadharsini Jayaseelan: Methodology, formal analysis, writing-review. Jaiganesh Ramamurthy: Methodology, formal analysis, writing, reviewing, and editing. Paramasivam Arumugam: Conceptualization, methodology, formal analysis, writing, reviewing, and editing.

FUNDING information

The study was self-funded by the authors' institution.

Conflict of interest

The authors declare no conflicts of interest related to this study.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical statement

This study was approved by the Institutional Ethical Committee of the Saveetha Dental College and Hospital (IHEC/SDC/PERIO-2002/22/080). All participants signed an informed consent form.

Consent statementstatement

This study was approved by the Institutional Ethical Committee of the Saveetha Dental College and Hospital (IHEC/SDC/PERIO-2002/22/080). All participants signed an informed consent form.

Informed consent in regional language Tamil or English was received from the patients included in the study and a patient information sheet in regional language Tamil or English explaining about the aim and benefits of the study was also given to the patients. After receiving the informed consent from the patients, the samples were collected.

Acknowledgments

This study was supported and funded by Saveetha Dental College and Hospitals, Chennai. We would like to thank the Centre for Cellular and Molecular Research, Saveetha Dental College and Hospitals, Chennai, for providing laboratory support. The authors acknowledge the participants, clinical assistants, and laboratory assistants for their contribution to this work.

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