Asian Journal of Dental Sciences

Volume 7, Issue 1, Page 350-357, 2024; Article no.AJDS.126789

Salivary Tec and Nesprin-2 Levels in Post-orthodontic Patients

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/126789

Original Research Article

Received: 12/09/2024
Accepted: 16/11/2024
Published: 23/11/2024

ABSTRACT

Introduction: Tyrosine-protein kinase protein (Tec) is known in activating calcium signalling, which is significant in bone remodelling, while nuclear envelope spectrin repeat (Nesprin) 2, is an outer nuclear membrane protein that provides cells with mechanosensory functions, including in osteocytes. Osteocytes, in turn, take role in promoting bone resorption.
Aim: To quantify the levels of salivary Tec protein and Nesprin-2 among control and post-orthodontic patients, using an enzyme-linked immunosorbent assay (ELISA).
Study Design: A quasi-experimental study.
Place and Duration of Study: Centre for Paediatric Dentistry and Orthodontics Studies, Faculty of Dentistry, Universiti Teknologi MARA (UiTM), Sungai Buloh Campus, Sungai Buloh, Selangor, Malaysia, between September 2022 and September 2023.
Methodology: Collection of a 5 ml unstimulated whole saliva samples from each subject: 10 healthy individuals as the control group, and 10 post-orthodontic patients at the immediate debond stage. Concentrations of Tec protein and Nesprin-2 were determined using commercially available

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Cite as: *Omar, Nurul Nadira Binti, Rohana Ahmad, Saeid Reza Doustjalali, Negar-Shafiei Sabet, Khor Goot Heah, and Aida Nur Ashikin Abd Rahman. 2024. "Salivary Tec and Nesprin-2 Levels in Post-Orthodontic Patients". Asian Journal of Dental Sciences 7 (1):350-57. https://journalajds.com/index.php/AJDS/article/view/213.*

ELISA kits. An independent-sample t-test was conducted to compare the scores between the two groups.
Results: This study of 10 non-orthodontic and 10 post-orthodontically treated patients found significantly lower mean salivary Tec protein in the saliva of post orthodontic subjects compared to the healthy control group (P<0.05). Nesprin-2 level was slightly lower at immediate debond, but the difference was small without statistical significance. Female participants (60%) constituted the majority of the participants, aged between 18-33 years.
Conclusion: This study highlighted reduced activity of bone remodelling at the immediate debond stage, by decreased level of salivary Tec protein and Nesprin-2 at the immediate debond stage. These two proteins may be useful in orthodontic retention monitoring.
Clinical Significance: This study elucidated the potential to enhance comprehension of the relapse mechanism following orthodontic treatment by identifying and examining significant markers of Tec and Nesprin-2.

Keywords: Orthodontics; salivary proteins; retention; ELISA; saliva.

1. INTRODUCTION

Long-term orthodontic stability studies indicate a complex interaction among treatment modalities, retention protocols, and individual patient factors. Recent studies highlight the significance of retention strategies, and the diversity of outcomes associated with various orthodontic methods [1-3]. These factors have been demonstrated to lack reliability as predictors in long-term stability. Relapse can be defined as any unwanted change in tooth position that deviates from a corrected malocclusion following orthodontic treatment [4]. The relapse of lower incisor irregularity is a challenge in orthodontics, resulting in crowding. Research demonstrates that fixed retainers can reduce this issue; however, a certain level of relapse remains evident.

A study indicated that the Little Irregularity Index (LII) showed improvement post-treatment; however, it increased to a medium degree after two years, suggesting relapse [5]. Factors leading to this relapse include the detachment of retainers, changes in intercanine width, and the buildup of biofilm [5,6]. Some studies indicate that the relationship between treatment modalities and relapse is complex, suggesting that biological factors may significantly influence the stability of incisor alignment [7].

In past centuries, orthodontic relapse studies have centred on the reorganization of gingival and periodontal tissues following treatment, which could affect stability [8]. Nowadays, research on orthodontic biomarkers continues to advance, emphasizing their potential clinical application. Recent studies indicate that localized administration of osteoprotegerin (OPG) can markedly minimize relapse rates by suppressing osteoclast activity, thereby improving tooth stability following orthodontic treatment [9,10]. Moreover, alkaline phosphatase (ALP) levels in gingival crevicular fluid can be evaluated to monitor bone turnover during the retention phase; however, research suggested no direct correlation between ALP levels and relapse distance [11].

These biomarkers, present in saliva and gingival crevicular fluid, can offer new perspectives on bone remodelling and treatment-related complications, thereby emphasizing the potential of biomarkers in predicting relapse. Effective biomarkers must be determined in readily accessible bodily fluids, including urine, serum, blood, gingival crevicular fluid (GCF), saliva, and cerebrospinal fluid (CSF). Saliva has become recognized as an important medium owing to its non-invasive collection, affordability, and capacity to indicate systemic changes in the body [12]. It functions as a diagnostic instrument for various medical issues, including cancers and infectious diseases, by identifying biomarkers such as RNA, protein, and DNA. In orthodontic therapy, salivary biomarkers can signify alterations active orthodontic tooth movement, thereby providing a non-invasive diagnostic instrument [13]. Saliva encompasses biomarkers indicative of bone deposition and resorption, facilitating pain management and treatment efficacy [14].

Tyrosine-protein kinase (Tec) is a tyrosine kinase encoded by the TEC gene. Tec comprises five domains: the N-terminal pleckstrin homolog (PH) domain, the Tec homology (TH) domain, the Src homology (SH3) domain, the Src homology (SH2) domain, and the C-terminal protein tyrosine kinase (PTK) domain. Tec participates in the intracellular signalling pathways of cytokine

receptors, lymphocyte surface antigen heterotrimeric G-protein-coupled receptors, and integrin molecules. Tec is a crucial regulator of the immune system [15,16]. Tec has demonstrated involvement in RANKL-induced osteoclastogenesis. Shinohara, et al. [17] demonstrated that osteoclasts, rather than osteoblasts, exhibit the highest expression levels of Tec mRNAs. This result was validated through quantitative polymerase chain reaction (qPCR) and immunoblot analysis. The group proposed that Tec is the protein molecule that links the RANK and immunoreceptor tyrosine-based activation motif (ITAM) pathways, along with the Btk molecule, to initiate calcium signalling, which is crucial for bone remodelling (Fig. 1).

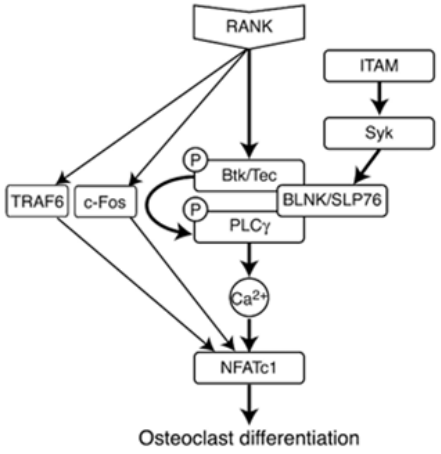

Fig. 1. Integration of the RANK and ITAM Signals by Tec Kinases Adapted from Shinohara et al. [17]

Nesprin-2 is a protein which is encoded by the SYNE2 gene in humans. Nesprins are structured proteins that feature a central extended spectrin-repeat (SR) rod domain and a C-terminal Klarsicht/ANC-1/Syne homology (KASH) transmembrane domain that functions as a NE-targeting motif. The internal integrity of the nucleus is maintained by the binding of Nesprin-2 (Nesp2) to cytoplasmic F-actin, which anchors the nucleus to the cytoskeleton. 'Mechanosensory function' is the primary function of Nesprin-2. Through actin filaments, the protein establishes a connection between the nuclear envelope cytoskeletons. The connection enables the nucleus of the cell to detect and respond to mechanical difficulties during cellular stresses, as well as to maintain the cell nucleus's position [18]. In the event of orthodontic tooth movement and relapse, osteoclasts are among the cells observed in the alveolar bone. Osteocytes are widely recognized as the mechanosensing cells of the bone located within the lacunar-canalicular system and known to play a vital role in bone mechanobiology and regulating bone homeostasis [19,20], by playing role to facilitate bone resorption by secreting RANKL and engaging in apoptosis [21].

The potential of Tec and Nesprin as biological markers of stability has been emphasized by Awang-Kechik et al. [22] who have utilised saliva samples from post-orthodontic patients. While the study utilised Liquid Chromatography-Mass Spectrometry (LC-MS) for qualitative analysis, no previous research has quantified the amount of Tec and Nesprin-2. Therefore, the objective of the investigation was to quantify the stability and relapse of Tec and Nesprin2 in post-orthodontic patients by employing an enzyme-linked immunosorbent assay (ELISA). We hypothesised that there is no difference in the level of concentration of Tec and Nesprin-2 proteins between non-orthodontically and orthodontically treated groups.

2. MATERIALS AND METHODS

2.1 Sample Characterization

The sample size was determined based on a prior study [23]. The calculation was performed using G* Power 3.1.9.7, with a significance level of 0.05, statistical power of 0.8, and an effect size of 1.34. A sample size of 10 patients was necessary for each group. As a result, 15 patients currently receiving treatment at the Centre of Paediatric and Orthodontic Studies, Faculty of Dentistry, Universiti Teknologi MARA (UiTM) were recruited for this study. On the other hand, 10 untreated patients were recruited. Prior to the study, informed consent was acquired. The protocol received approval from the UiTM Research Ethics Committee (REC/08/2022 (PG/MR/197).

2.2 Selection Criteria

The post-orthodontic patients were selected from individuals nearing the debonding phase. They underwent orthodontic treatment involving the extraction of four premolars, utilizing MBT prescription 0.022 x 0.028-inch slot pre-adjusted edgewise fixed orthodontic appliances (Victory SeriesTM, 3M Unitek, Germany). All patients were generally healthy and exhibited good periodontal status. Individuals with unsatisfactory oral hygiene, those who smoke, pregnant individuals, and those with bonded retainers

were excluded from. Ten healthy non-orthodontic individuals were chosen as the control group.

2.3 Saliva Sampling Protocol

A thorough scaling was performed one week ahead to ensure optimal oral health before saliva collection. Patients were instructed to refrain from any oral activities, specifically abstaining from food consumption for a minimum of 1.5 hours prior to the procedure [24].

Saliva samples were consistently collected within a standardized time frame, specifically between 10 and 11 am, to mitigate circadian variation. Saliva sampling was done by single operator which was N.N. Saliva samples were obtained from each patient immediately following the removal of fixed appliances. Patients were told to sit upright and instructed to rinse their mouths with distilled water, followed by a 5-minute rest prior to saliva collection. Five millilitres of unstimulated whole saliva was collected by passive drooling into a 50 ml sterile centrifuge tube [25]. Patients were instructed to refrain from speaking or moving their tongues during the collection process. The head was inclined downward to allow the saliva to collect in the mouth. Saliva samples were obtained over a duration of 7 minutes. All samples were maintained on ice throughout the procedure. Saliva samples were subsequently centrifuged at 10,000 rpm and 4° C for 10 minutes to eliminate insoluble materials, cells, and debris. The supernatant was obtained and aliquoted into 10 mL centrifuge tubes, each containing a volume of 100 µL, to ascertain protein concentration. The pellets were discarded. Each sample was preserved at -80° C until subsequent analysis to maintain protein biomarkers. The identical saliva collection procedures were implemented for the control group.

2.4 Elisa Protocol

The ELISA was conducted following the protocol provided by an ELISA kit from BlueGene Biotech (China). A standard curve was established by plotting the logarithm of Tec and Nesprin-2 concentrations against the logarithm of the mean absorbance for each standard, with the optimal fit line determined through regression analysis (Microsoft Excel 2024). The Tec concentration in each sample was ascertained by comparing the optical density (OD) of the samples to a standard curve established for each Tec analysis. The sensitivity of the ELISA kit was 1.0 ng/mL.

2.5 Statistical Analysis

All data were analysed utilizing SPSS version 22.0. The Cronbach's Alpha for intra-examiner agreement regarding incisor irregularities was assessed. The normality of the data was determined using the Shapiro-Wilk test. Subsequently, the data were analysed using an independent samples t-test to ascertain the statistical differences between the mean protein levels of the control group and the immediate debond stage. Differences were deemed significant when $P<0.05$.

3. RESULTS AND DISCUSSION

A total of 25 Malaysian patients aged between 18 and 33 years were recruited. Among these, 60% were female and 40% were male. The number of female patients exceeded that of male patients. Out of 15 recruited patients, only 10 returned for retention review. Hence the total sample for debond group is 10, while another 10 participants formed the non-orthodontically treated group. The gender distribution in this study is largely reflective of the relative gender distribution of those seeking orthodontic treatment, with females being more likely to seek orthodontic treatment than males [26-31]. A recent study found that 87.9% of the sample consisted of female patients, with 72.6% citied aesthetic improvement as their primary motivation [27]. Another study involving 126 patients revealed that 82.5% of patients seeking orthodontic treatment were female, motivated by factors such as social harassment and a desire for enhanced appearance [29]. Moreover, Oh, et al. [31] reported that positive attitudes and perception regarding orthodontic treatment largely depended on age, gender and socioeconomic status.

The average concentration of Tec and Nesprin-2 is presented in Table 1.

Table 1. Descriptive analysis of protein concentration

Proteins	Mean (SD)(ng/mL)	
	Control group	Debond group
Tec	5.35(±1.83)	3.92(±1.18)
Nesprin-2	3.04 (±1.96)	2.61 (±2.34)

The results indicated that both protein levels in the control group were higher than those in the debond group. A mean concentration of Tec level nearly twice as high was observed in the control

Table 2. Independent-sample t-test of Tec and Nesprin-2 in control and debond group

Proteins	Mean differences (SD) (ng/mL)	95% Confidence Interval (CI)	t value (df)	*p*-value
Tec	-1.42 (±0.69) *	-0.026, 2.875	2.06	0.05
Nesprin-2	-0.43 (±0.97)	-1.60	2.46	0.66

*Note *Significant (P<0.05), n=10*

group. The average salivary Tec protein concentration was substantially lower in the saliva of post-orthodontic individuals compared to the healthy control group *(P*<0.05). The Nesprin-2 level was marginally reduced at immediate debond, yet the difference was minimal and lacked statistical significance. Therefore, the null hypothesis was rejected due to the differences found in between control and debond group.

No studies have yet examined the levels of Tec and Nesprin-2 in the saliva of post-orthodontic patients utilizing the ELISA technique. Prior research examined the concentrations of diverse biomarkers in the gingival crevicular fluid. OPG and bone alkaline phosphatase (BALP) have the potential to detect alveolar bone formation, while receptor activator of nuclear factor kappa-B ligand (RANKL) serves as a marker for resorption [32]. Meanwhile, the administration of raloxifene has recently been shown to diminish relapse, although this research was conducted using a rodent relapse model [33].

Preventing relapse following active orthodontic tooth movements presents a clinical challenge. The alveolar bone undergoes ongoing remodelling after orthodontic tooth movement, resulting in the formation of a new compression zone in the direction opposite to the tooth movement. Nevertheless, 73% of relapses occurred merely one day post-appliance removal [34] while 98% of relapses were noted two weeks following retention [33]. A recent histological examination has recorded a significant quantity of osteoclasts on the twentieth day of relapse in rabbit models, indicating a correlation between osteoclast count and orthodontic relapse occurring at day 20 [35]. This is consistent with the findings of Aoki et al., who found most of relapse occurred on day 1 of relapse observation [36]. The aforementioned evidence are corroborated by our findings, whereby both proteins exhibited a declining trend, indicating potential reductions in their levels at immediate debond. Nonetheless, only Tec protein achieved statistical significance (P,0.05), suggesting that we can be confident that this reduction is not due to random variation. Although Nesprin-2 trend did not achieve statistical significance, the noted decrease may still hold biological relevance and could necessitate further exploration to validate its potential impact. We postulated that as saliva samples were collected immediately post-debonding, the biological alterations in the alveolar bone may not have commenced. It is hypothesized that an extended retention period will lead to an increased concentration of Tec and Nesprin-2 as the alveolar bone and periodontal fibres restore their structural integrity, eventually resulting in relapse.

This study is the first to identify the novelty of salivary Tec and Nesprin-2 during the orthodontic retention phase. An increasing number of studies have indicated the potential of various biological markers, specifically BALP, OPG, RANKL, and OPN [32]. However, these studies utilized gingival crevicular fluid samples to assess alveolar bone remodelling in relation to the efficacy of orthodontic treatment. Comparing these studies with ours is challenging due to the differing methodologies employed in qualitative and quantitative measurements.

Given the constraints of the saliva sampling size and a single time-point sample collection, the application of these results in a clinical context necessitates additional research over an extended observational period. Our data may provide a foundation for the future quantification of Tec and Nesprin-2. The current study presents opportunities for additional efforts to identify suitable candidate proteins aimed at detecting teeth at risk of relapse, to be utilized clinically, in tandem with personalised orthodontics. A longitudinal study is recommended to monitor the patterns of Tec and Nesprin-2 in relation to changes in relapse severity.

4. CONCLUSION

This study emphasized diminished bone remodelling activity at the immediate debond stage, evidenced by reduced levels of salivary Tec protein and Nesprin-2 during this phase.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

CONSENT AND ETHICAL APPROVAL

All the patients who agreed to participate in this study signed the consent form. This study was approved by the UiTM Research Ethics Committee (REC) REC/08/2022 (PG/MR/197) and follows the Helsinki Declaration.

COMPETING INTERESTS

Authors have declared that no competing interests.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/126789