International Journal of Current Advanced Research
ISSN: O: 2319-6475, ISSN: P: 2319-6505, Impact Factor: 6.614
Available Online at www.journalijcar.org
Volume 10; Issue 03 (A); March 2021; Page No.23976-23981
DOI: http://dx.doi.org/10.24327/ijcar.2021.23981.4752

Research Article

EVALUATION OF EXTERNAL APICAL ROOT RESORPTION USING CBCT AND ITS ASSOCIATION WITH IL-6 SNP (RS 1800796) AS A RISK FACTOR AFTER ORTHODONTIC TREATMENT IN LOCAL POPULATION

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A R T I C L E I N F O

Article History:
Received 6th December, 2020
Received in revised form 15th January, 2021
Accepted 12th February, 2021
Published online 28th March, 2021

Key words:
EARR, IL-6 SNP,

A B S T R A C T

Aims and Objectives: To evaluate the relationship of IL-6 (rs 1800796) GC with external apical root resorption using DNA sequencing and to accurately measure the extent of external apical root resorption using cone beam computed tomography.

Methods: DNA samples of 30 subjects with non-syndromic tooth agenesis collected from the department were used for the study. CBCT of maxillary central incisor before and after treatment of 30 patients were also recorded. The extracted DNA samples were subjected to Polymerase chain reaction in which amplification of the selected gene segments was carried out; later these amplified products were subjected to DNA sequencing. Results were documented in the form of electropherograms.

Results:

- The mean amount of EARR seen across all 30 subjects was 0.45mm which was statistically significant ($p<0.001$).
- EARR of >1mm was seen is 6 subjects of which 2 subjects showed a positive expression of IL-6 SNP GC (22.2 %, with p value=0.59) which was not statically significant.
- EARR of ≤1mm was seen is 11 subjects of which 5 subjects showed a positive expression of IL-6 SNP GC (45.4%, with p value=0.59) which was not statically significant.

Conclusion

1. The mean amount of EARR seen across all 30 subjects was 0.45mm which was statistically significant. The association of gender with EARR showed a higher predilection towards females than males.
2. EARR of >1mm was seen is 6 subjects of which 2 subjects showed a positive expression of IL-6 SNP GC which was not statically significant.
3. EARR of ≤1mm was seen is 11 subjects of which 5 subjects showed a positive expression of IL-6 SNP GC. Hence showing only a mild correlation of IL-6 SNP GC as a risk factor following orthodontic treatment which was not statistically significant.

INTRODUCTION

Root resorption was first described by Bates in 1856, and it was later correlated to orthodontics by Ottolengui in 1914. External apical root resorption (EARR) is a reduction of root structure involving the apices. EARR occurrence has been reported with and without orthodontic treatment and can be diagnosed by orthodontists during routine diagnostic procedures and radiographs. It has been reported that 5 mm or more of apical root resorption may occur in 5% of orthodontic patients.[6]Newman *et al* reported in 1975 that EARR exhibits a familial aggregation, more studies have described the association of EARR with genetics.[46]

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Al-Qawasmi *et al* showed that the IL-1beta polymorphism plays a role in the genetic influence on EARR and that the IL-1beta allele 1 is a risk factor for EARR.[12]

Although heritability estimates do not provide information about the number of possible genes contributing to the phenotype, the pivotal report of Harris *et al* indicated that there is probably an important genetic predisposition to EARR.[5]

An important breakthrough in bone biology was the identification of the role of cytokines in bone remodelling. Cytokines are involved in initiating, amplifying, perpetuating, and resolving inflammatory responses. They are key mediators for tissue damage and play an important role in tooth movement. Cytokines are classified as pro-inflammatory and

anti-inflammatory. Pro-inflammatory ones are tumour necrosis factor, interleukin 1(IL-1, interleukin 2 (IL-2), interleukin 6 (IL-6).IL-6 acts as both a Pro-inflammatory and Anti-inflammatory cytokine and was found to be increased in root resorption tissue.[15]

In most studies, the amount of root resorption was obtained using two-dimensional x-ray films, such as periapical films, panoramic radiographs, and lateral cephalograms. However, root resorption occurs three dimensionally. Katona and Yu *et al* proved that neither periapical film nor panoramic radiograph was accurate enough for studying EARR, this might also explain the various discrepancies among the studies on EARR.[19]

In recent years, cone-beam computed tomography (CBCT) has been used extensively in oral clinical work. Because CBCT can measure the width and length of the tooth at any three dimensional section accurately, studies on EARR with CBCT have demonstrated much improved accuracy and sensitivity.[32] Despite many studies on EARR, great controversies remain with regard to its risk factors, which may be attributed to 2 things: an inaccurate measuring method and the multifactorial nature of EARR.[46]

Therefore, in this study, a CBCT was taken to accurately measure the amount of root resorption of the maxillary incisors and multiple linear regression analysis was performed to explore the genetic association of IL-6 SNP GC and root resorption associated with orthodontic treatment.

MATERIAL AND METHODS

The present study aimed at investigating external apical root resorption and its genetic association with IL-6 SNP GC (rs 1800796) which was detected using the Polymerase Chain Reaction (PCR) test followed by DNA Sequencing. Automated DNA sequencing procedure was selected for the sequencing of DNA where each nucleotide was labelled with fluorescent dyes. Thus when the DNA fragments were placed on the electrophoresis gel and passed through a laser beam, the DNA sequence was detected more precisely and accurately on an electropherogram unlike other sequencing techniques.

A CBCT of the maxillary central incisors was taken both before and after treatment, the amount of resorption was calculated by measuring the root length of the maxillary central incisor before and after treatment from the data collected.

Source of the data

The sample consisted of 5ml of saliva which was collected from 30 patients reporting to the Department of Orthodontics and Dentofacial Orthopedics, D.A.P.M.R.V Dental College and Hospital, Bengaluru. Information of each patient was collected in the form of a detailed case history. CBCT of maxillary central incisors before and after treatment was recorded.

Inclusion Criteria

1. Patients treated with a Pre-adjusted edgewise technique.
2. The incisor crown undamaged during orthodontic treatment.
3. Completed root development of the maxillary incisors.

Exclusion criteria

1. Functional appliance used during treatment.
2. Obvious root resorption of the maxillary central incisors before treatment.
3. Periodontitis.
4. Abnormal root of the maxillary central incisors.
5. Developing maxillary central incisor root.
6. Surgery needed.

Dnaisolation

Materials required:- centrifuge tubes, micro centrifuge tubes, centrifuge, water bath, vortex, micropipettes electrophoresis tank and gel doc.

Reagents required:- DNazol, Isopropanol, 70% ethanol and 1X TE (Tris EDTA).

Protocol

1. Saliva samples were transferred into fresh 30 ml centrifuge tubes.
2. Centrifuged at 6,000 rpm for 5 minutes at RT. Supernatant was discarded.
3. Pellet was dispersed gently and added 2ml of DNazol lysis buffer.
4. Incubated at 65ºC for 1hr.
5. Centrifuged at 10,000rpm for 10min.
6. Add equal volumes of Isopropanol ,mix gently
7. Centrifuged at 10,000 rpm for 20 minutes, supernatant was discarded. To the pellet added 500 µl of 70% Ethanol, again centrifuged at 10,000 rpm for 5 minutes.
8. Supernatant was drained out and air dried the pellet.
9. Added 50µl of 1X TE to the pellet and resuspend by finger flicking

Column purification protocol

Materials required:-Column, collecting tubes, micropipettes and micro centrifuge.

Reagents required:-Equilibration buffer (5M GuHCL-Guanidine hydrochloride), 8M GuHCl, wash buffer I (25% ethanol, GuHcl 2M), wash buffer II (Ethanol 75% Tris-Cl 40mM) and 1X TE.

1. The column was placed with a collection tube in vial stand, added 400µl of equilibration buffer and centrifuged at high speed for 1 minute (10000 rpm). Discarded the collected buffer. Added 400 µl of equilibration buffer to the DNA sample, mixed gently loaded to the column, centrifuged at high speed for 1 minute. Collected the flow through.
2. Added 500µl of wash buffer 1, centrifuged at high speed for 1 minute. Collected the wash.(wash1)
3. Added 500µl of wash buffer, centrifuged at high speed for 1 minute. Collected the wash.(wash2)
4. Again the column was centrifuged with empty collection tube to completely remove the wash buffer for 2 minutes.
5. The column was placed in new collection tube and added 50 µl of pre-warmed Elution buffer at the

centre of the filter membrane. Incubated for 2 minutes and centrifuged at high speed for 1 minute.
6. Repeated step 6.
7. Removed the column, closed the lid of collection vial – This purified Genomic DNA was ready to use for PCR.

Amplification of RS1800796 gene

Materials required: PCR tubes, micropipettes, PCR machine, electrophoresis tank and gel doc.

Reagents required:- Nuclease free water, rs1800796 Forward primer (10pmol/µl), rs1800796 Reverse Primer (10pmol/µl), 2X PCR master mix (Taq Pol ,Taq Pol assay buffer, dNTP's) Template DNA's.

Primer sequence

Il6 FP: GCAGCAGCCAACCTCCTCTAAG
Il6 RP: CAGTGACCAGATTAACAGGCTAG
Amplification of the **rs1800796** gene was performed using rs1800796 primers.

Root resorption analysis

The methodology was divided into three main steps;

1. CBCT scans of the maxillary central incisors.
2. Identification of the reference points.
3. Measurement of root length pre and post treatment

CBCT scan of the maxillary incisors using CBCT Machine: Kodak 9500 U.S model was used to take CBCT scans with FOV of 18x21 cm. Images were reconstructed using the Dolphin software (11.8 premium version)

1. Reference point at the root tip
2. Reference point at the CEJ
3. Distance measured between the two points was taken as the root length all measurements were marked and calculated with Dolphin software 11.8.

Statistical Analysis

Statistical Package for Social Sciences [SPSS] for Windows, Version 22.0. Released in 2013. Armonk, NY: IBM Corp., was used to perform statistical analyses.

Descriptive Statistics

Descriptive analysis includes expression of all the explanatory and outcome in terms of Mean & SD for continuous variables, whereas in terms of frequency and proportions for categorical variables.

Inferential Statistics

Student Paired t Test was used to compare the mean Root length (in mm) between Pre and Post treatment periods.
Mann Whitney Test was used to compare the mean Root resorption (in mm) between genders.

Chi Square Test was used to compare the root resorption with IL-6 GC expression and also gender wise comparison of IL-6 GC expression among study subjects.

The level of significance [P-Value] was set at P<0.05.

Sample Size of Estimation

Sample Size Estimation

Analysis:	A priori: Compute required sample size		
Input:	Tail(s)	=	Two
	Effect size dz	=	0.53
	α err prob	=	0.05
	Power (1-β err prob)	=	0.80
Output:	Noncentrality parameter δ	=	2.9029296
	Critical t	=	2.0452296
	Df	=	29
	Total sample size	=	30
	Actual power	=	0.8011297

The sample size has been estimated using the software GPower v. 3.1.9.2

Considering the effect size to be measured (dz) at 53% for Two-tailed hypothesis, power of the study at 80% and the margin of the error at 5%, the total sample size needed is 30.

RESULTS

In the present study, the relationship between IL-6 SNP GC with root resorption after orthodontic treatment was done using measurements taken on CBCT scans and using polymerase chain reaction test followed by DNA sequencing for identification of IL-6 SNP GC.

The mean amount of EARR seen across all 30 subjects was 0.45mm which was statistically significant (p<0.001).The association of gender with EARR showed a higher predilection towards females than males.

EARR of >1mm was seen is 6 subjects of which 2 subjects showed a positive expression of IL-6 SNP GC (22.2 %, with p value=0.59) which was not statically significant.

EARR of ≤1mm was seen is 11 subjects of which 5 subjects showed a positive expression of IL-6 SNP GC (45.4%, with p value=0.59) which was not statically significant.

DISCUSSION

External apical root resorption is a common complication in orthodontic treatment and its possible risk factors include genetics, age, sex, orthodontic treatment and its duration. EARR is a frequent iatrogenic outcome associated with orthodontic treatment, especially in the maxillary incisors, and may also occur in the absence of orthodontic treatment[1].

Newman *et al* reported in 1975 that EARR exhibits a familial aggregation, more studies have described the association of EARR with genetics. Al-Qawasmi *et al* showed that the IL-1beta polymorphism plays a role in the genetic influence on EARR and that the IL-1β allele 1 is a risk factor for EARR[46.]. Although heritability estimates do not provide information about the number possible genes contributing to the phenotype, the pivotal report of Harris *et al* indicated that there is probably an important genetic predisposition to EARR[5]. A recent study by Yugio *et al* showed a correlation between IL-6 SNP GC and EARR[46]. In our study to show the correlation IL-6 SNP GC we found that, >1mm was seen is 6 subjects of which 2 subjects showed a positive expression of IL-6 SNP GC (22.2 %, with p value=0.59) which was not statically significant and EARR of <1mm was seen is 11 subjects of which 5 subjects showed a positive expression of IL-6 SNP GC (45.4%, with p value=0.59) which showed a mild correlation.

IL-6 and its receptors mainly activate 2 signal pathways: the JAK kinase, signal transduction and transcriptional activation (JAK/STAT) pathway, and the mitogen activating protein kinases (MAPK) pathway. The STAT pathway mediates antiproliferation, apoptosis, and osteolysis signal, and the MAPK pathway mediates mitogenic, antiapoptosis, antiosteogenic, or antiosteogenesis signals; these 2 pathways counteract each other[46].

Studies have found that IL-6 in the gingival crevicular fluid is increased in orthodontic patients Han *et al* established root resorption on rats and found that IL-6 mRNA is mainly expressed in fibroblasts, osteoblasts bone cells, and cementoblasts, and that positive expression of IL-6 mRNA was significantly enhanced in root resorption tissue and was higher than that of normal tissue. Although these studies suggest that IL-6 might play an important role in alveolar bone absorption and root resorption during orthodontic treatment ,nevertheless, it was still unclear how the 2 signal pathways mediated by IL-6 work in this process. On 1 hand, IL-6 could promote the secretion of inflammatory factors such as IL-1, exerting an inflammatory effect; on the other hand, it could also promote the form of anti-inflammatory factor such as IL-1, exerting an anti-inflammatory effect. Therefore, as a multifunctional cytokine with both inflammatory and anti-inflammatory effects, IL-6 might play an important role in EARR[15].

Study results on the relationship between sex and EARR are controversial. Most studies showed no correlation, but some reported that the average amount of root resorption in female subjects is greater than that in male subjects. The association of gender with EARR showed a higher predilection towards females than males[45]. Whether sex is correlated to EARR remains unclear. The independent sample t test showed that the average volume of root resorption of female subjects is greater than that of male subjects, but the difference was not significant.

We chose the maxillary left central incisor in our study. The tissue segmentation would not be accurate enough if the shape of the tooth was too complex, so to ensure the exact measurement of root resorption, we chose the incisor, whose morphology was simpler and more regular[46]. In most studies, the amount of root resorption was obtained using 2-dimensional x-ray films, such as periapical films, panoramic radiographs, and lateral cephalometrics. However, root resorption occurs 3 dimensionally and may occur at any site on the root. Katona and Yu *et al* also proved that neither periapical film nor panoramic radiograph was accurate enough for studying EARR clinically; this might also explain the discrepancies among the studies on EARR[19]. In recent years, cone-beam computed tomography has been used extensively in oral clinical work, because CBCT can measure the width and length of the tooth at any 3-dimensional section accurately[46].
Studies on EARR with CBCT have demonstrated much improved accuracy and sensitivity. Waneal proved that CBCT could accurately measure tooth and root resorption volumes and was more accurate and reliable measuring method for studying EARR[28]. Therefore, in this study, a CBCT reconstruction was used for the measurement of root resorption and measured with dolphin software (11.8) for both pre and post treatment measurements. We found the mean amount of EARR seen across all 30 subjects was 0.45mm which was statistically significant ($p<0.001$). EARR of >1mm was seen is 6 subjects and EARR of ≤1mm was seen is 11 subjects which was statistically significant showing a strong correlation between orthodontic treatment and root resorption.

Although both Sharab *et al* and Pereira *et al* indicated that long treatments are greatly associated with EARR[46], we did not include treatment length as a variable for the following reasons. First, treatment length could be affected by many other factors such as cooperation of the patient and the complexity of the case. Second, we chose the central incisor as our target, although in some cases during the treatment time, the posterior tooth was mainly moved and the incisor was slightly moved; if we included treatment length as a variable, it might have made the result less credible. Finally, root resorption occurred when the tooth was under orthodontic force, but treatment length was not coincident with affecting the time of orthodontic force on the incisor. So we did not include treatment length.

Few studies have reported the relationship between tooth movement and EARR. Although the average retraction distance for the central incisor was measured by the U1-NA (millimetres) and U1-NA angle (degrees) in the study of Sharab *et al*[46], they did not find any correlation between the retraction distance and EARR. However, some studies showed that root resorption was more likely in tooth extraction and deep overbite cases, and tooth movement in these situations is relatively large, indicating that the amount of tooth movement might correlate with EARR.

EARR is a complex condition influenced by many factors. Defining the genetic contributions to EARR is an important factor in understanding the contribution of other environmental factors such as habits and therapeutic biomechanics, type of malocclusion and treatment duration. In this study we were able to accurately measure the amount of EARR with the help of a CBCT which showed resorption in 17 subjects with a mean of 0.45mm of EARR. IL-6 SNP GC showed a mild correlation with EARR, further investigation with a larger sample size and inclusion of other genes is required to further understand the correlation of genetics with EARR.

CONCLUSION

The conclusions drawn from this study are:-

1. The mean amount of EARR seen across all 30 subjects was 0.45mm which was statistically significant .The association of gender with EARR showed a higher predilection towards females than males.
2. EARR of >1mm was seen is 6 subjects of which 2 subjects showed a positive expression of IL-6 SNP GC which was not statically significant
3. EARR of ≤1mm was seen is 11 subjects of which 5 subjects showed a positive expression of IL-6 SNP GC. Hence showing only a mild correlation of IL-6 SNP GC as a risk factor following orthodontic treatment which was not statistically significant.
4. Further investigation with an inclusion of a larger sample size, therapeutic biomechanics, type of malocclusion, treatment duration and inclusion of more genes is required to better understand the multifactorial nature of EARR.

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