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Interleukin 1 receptor antagonist (IL1RN) genetic variations condition post-orthodontic external root resorption in endodontically-treated teeth

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Summary. External pical root resorption (EARR) is a frequent iatrogenic problem following orthodontic treatment in endodontically-treated teeth, about which the literature reports substantial variability in post-orthodontic treatment EARR responses. The main focus of the present study is to clarify whether variants in the interleukin-1 receptor antagonist gene coding for the IL-1ra protein have a positive/negative influence on EARR of endodontically-treated teeth. Ninety-three orthodontic patients were genetically screened for a single nucleotide polymorphism (SNP:rs419598) in the *IL1* cluster. The sample was classified into 2 groups: group 1 (affected-group) showed radiographic EARR of more than 2mm; group 2 (control-group), had no EARR or EARR≤ to 2mm following orthodontic treatment on root-filled teeth. Logistic regression analysis was performed to obtain an adjusted estimate between the SNPs studied and EARR. Genotype distributions, allelic frequencies, adjusted odds ratios (OR) and 95% confidence intervals were also calculated. We found that subjects homozygous [1/1(TT)] for the *IL1RN* gene [OR:10.85; p=0.001;CI:95%] were at risk of EARR in root-filled teeth. Genetic variants in the antagonist axis balance of the *IL1RN*(rs419598) have a direct repercussion on the predisposition to post-orthodontic EARR in root-filled teeth. Variants in allele 1 of the interleukin-1 receptor antagonist gene(rs419598) are associated(p=0.001**) with an increased risk of suffering post-orthodontic EARR in root-filled teeth.

Key words: External root resorption, Interleukin 1 receptor antagonist, Endodontically treated teeth, Orthodontics, Orthodontic treatment

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Introduction

Many clinicians consider orthodontic tooth movement of root-filled teeth to be clinically challenging because of their variable behavior towards external root resorption (EARR) (Wickwire et al., 1974; Spurrier et al., 1990). With respect to the prevalence of EARR in vital teeth in the context of orthodontic treatment, it has been established that one third of orthodontic patients are affected by moderate EARR and some 2-5% of cases by severe EARR (Taithongchai et al., 1996; Hartsfield et al., 2004). Although endodontically-treated teeth respond to tooth movement rates in a similar way to vital teeth, many professionals consider them to be even more prone to EARR secondary to tooth movement (Wickwire et al., 1974; Spurrier et al., 1990). As it is a relatively common secondary effect, understanding the aetiology, prognostic and predictive factors implicated in the EARR process is a major topic of interest and the subject of considerable research (Brezniak and Wasserstein, 1993, 2002; Hartsfield et al., 2004; Krishnan, 2005). Recent research reported that duration of treatment and total apical

displacement during orthodontic tooth movement were the factors most closely associated with the EARR process (Segal et al., 2004). Other clinical circumstances, such as the application of heavy orthodontic forces, type of malocclusion, gender, root shape, age, and even emotional stress levels, have also been associated in some way with reduced or increased root resorption (Brezniak and Wasserstein 1993, 2002; Weltman, 2010). Nevertheless, different prognoses for EARR have been observed for many root-filled teeth subject to orthodontic movement which had been apparently treated in same way, following identical protocols (Wickwire et al., 1974; Spurrier et al., 1990; Mah et al., 1996).

In this context, apart from clinically-related factors, genetic profiling plays a decisive role in the aetiology, prognosis and prediction of pathology in most medical specialties (Shuang et al., 2010; Kwiatkowski et al., 2011). Accordingly, one study based on white families found evidence of an association between EARR susceptibility following orthodontic treatment and a genetic marker (+3954) in the interleukin 1beta (*IL1B*) gene (Al-Qawasmi et al. 2003a) and in the *TNFRSF11A* locus(Al-Qawasmi et al. 2003b). More recently, it was reported that homozygous subjects for allele 1 (1/1) of the *IL1B* gene (+3594T) were at an increased risk of suffering EARR (Bastos-Lages et al. 2009). Additional polymorphisms were mapped in the *IL1* gene cluster and a direct association was likewise discovered, in different populations, between a single nucleotide polymorphism in the *IL1A* gene (-899) and EARR (Gülden et al. 2009).

The interleukin 1 gene (*IL1*) cluster on chromosome 2 contains genes that not only code for the pro-inflammatory cytokines (IL-1alpha and IL-1beta), but also for the anti-inflammatory cytokines (IL-1ra). The interleukin-1 receptor antagonist (IL-1ra) is a naturally-occurring cytokine that inhibits IL-1 activity (Arend, 1991). An imbalance in IL-1/IL-1ra levels has been referenced in various inflammatory pathologies (Tountas et al., 1999). Specific polymorphisms of the gene coding for IL-ra, the interleukin receptor antagonist (*IL1RN*) (Steinkasserer et al. 1992; Patterson et al. 1993) have been described as modulating IL-1ra secretion and also IL-1ß synthesis (Santtila et al., 1998; Rafiq et al., 2007). Moreover, several well researched studies have reported an association between specific polymorphisms in the *IL1RN* gene and various pathological processes (Persson et al., 2011).

Genetic biomarkers defining an increased/reduced susceptibility to suffering post-orthodontic EARR have been reported in recent years (Al-Qawasmi et al., 2003b). Nevertheless, all findings relating to a predisposition to EARR, mentioned above, refer to vital teeth undergoing orthodontic treatment. Whether biomarkers exist that would also define positive/negative susceptibility in the case of root-filled-teeth is a question that has not been yet clarified. The present study sets out to determine whether, variants in the interleukin-1 receptor antagonist gene (rs419598) coding for the IL-1ra protein, have a positive/negative impact on post-orthodontic EARR of endodontically-treated teeth.

Materials and methods

Sample

Ninety-three Caucasian patients, selected consecutively from the Orthodontics Unit were invited to participate in the study. All patients had had root canal treatment in one or more of the upper premolars and, at least 6 months later, had received comprehensive orthodontic treatment (straight-wire technique). Subjects were divided into two groups, based on the presence or absence of more than 2 mm EARR following orthodontic treatment, taken from radiographic measurements: group 1 consisted of patients with EARR (EARR≥2mm), and group 2 of patients without EARR (EARR<2mm). The following criteria were observed for subjects to be included in the study: all had had orthodontic treatment; there was complete root formation; there was no alteration in the incisal edge between measurements; there had been no subsequent orthodontic retreatment; systemic pathologies had not modified the hard tissue biology; root canal treatment had been performed on one tooth measured. There were also none of the exclusion criteria, specified elsewhere, in this sample (Morsani et al., 2011; Iglesias-Linares et al., 2012a). This study was carried out with the full knowledge and written consent of each subject, and in accordance with the ethical principles governing medical research and human subjects, as laid down in the Helsinki Declaration (2002 version, www.wma.net/e/policy/b3.htm), as well as approval for experimentation granted by the relevant institutional ethical committee.

DNA collection and detection of the IL-1RN genotype

DNA analysis was performed from an intraoral scrape obtained from the sample, using a sterile buccal swab. The selected *IL1* single nucleotide polymorphism (SNP) (rs419598) was obtained using an extensively validated diagnostic test for screening *IL1* DNA (GenoType®, PST®plus, Hain Life Science GmbH, Nehren, Germany) (Eickholz et al., 2008; Gruica et al., 2004; Feloutzis et al., 2003; Iglesias-Linares et al., 2012b). In summary, DNA was extracted and sequences of the *IL1* gene cluster were amplified by polymerase chain reaction, using HotStar Taq polymerase (Qiagen, Hilden, Germany) and a final 2.5 mM concentration of MgCl2. Specific probes to perform reverse hybridization were used to determine patients' genotypes for the studied marker, according to the manufacturer's instructions.

Radiographic measurements

Based on valuable research published previously (Al-Qawasmi et al., 2003a; Harris et al., 1997) upper

endodontically treated premolars were measured on pre and post-treatment lateral cephalometric and panoramic radiographs. All pre and post-treatment images were calibrated beforehand and a correction factor for magnification applied in all cases. Measurements were taken on digital radiographs, using diagnostic software that enabled the image filters to give the most precision when localizing the terminal points of the roots. The dependent variable of interest for the subject was set on the root-filled tooth with the highest EARR value. As described in previous studies (Bastos-Lages et al., 2009), proportional measurements were also performed (Iglesias-Linares et al., 2012a). If, during treatment, the root length had diminished, the degree of EARR was r1 r2- [c1/c2]).

Statistics

To prevent interobservational error, all the previously defined procedures were carried out by the same trained operator (I.L.A) using blinded assessment. Simple and weighted *k* statistics were computed to determine intra-examiner agreement on the radiographic assessment of control and affected subjects. Additionally, error on measurements from the panoramic and lateral cephalometric radiographs was statistically determined by comparing double measurements for 15 randomly chosen patients with a two week interval. Calculations were made using the Student's t-test for paired samples, with absence of significance being regarded as indicative of concordance between mean values. Method error was calculated from the equation: $SE=\sqrt{(\Sigma d2/2n)}$, where *d* is the difference between the double measurements and *n* the number of paired double measurements (Houston, 1983).

Univariate analysis of the results consisted of a descriptive analysis of quantitative and categorical variables. Binary logistic regression analysis was used to assess any independent interference of clinical parameters on post-orthodontic EARR. A logistic regression analysis was also performed to obtain an adjusted estimate between post-orthodontic EARR and the *IL1* cluster SNP. The risk of EARR associated with alleles or genotypes was calculated using adjusted odds ratios (OR), with a 95% confidence interval, and statistical significance set at $p<0.05$. Data analysis was performed by means of SPSS software (version 17.0; LEAD Technologies, USA).

Results

The accuracy of the method used shows intraobservational error for tooth length and crown height measurements of 0.22mm and 0.36mm from lateral and panoramic radiographies, respectively. There were no statistically significant differences between repeat and original measurements ($p>0.05$), and the concordance indices (k=87; k=91) for measurements on lateral and panoramic radiographies respectively were very favourable (Siegel and Castellan, 1998).

The clinical characteristics of the sample describe a mean age of 24 years / 1 month old (±5 years/5months), with an average treatment time for comprehensive orthodontic treatment of 27.21 months (±4.9 months). Independent assessment of the influence of clinical parameters using binary logistic regression analysis revealed there was no association between post-orthodontic EARR and any of the clinical features in our sample ($p<0.05$), as seen in Table 1.

The SNP frequencies analyzed for the *IL1RN* gene were distributed as follows for EARR-affected subjects (gene, rs number, most frequent > less frequent nucleotide, minor allele frequency): *IL1RN* (+2018), rs419598, T>C, 2.6% (Table 2). Note that, there was a substantial increase in the commonest allele frequencies of the SNP studied in the *IL1* gene cluster in the affected subjects. Meanwhile, there was a more than one-fold increment of the most frequent allele of the *IL1RN* gene

Table 1. The clinical features of examined patients.

Clinical parameters	>2mm EARR* patients (n= 39)	Controls (n=54)	95% CI for OR			
			OR	lower	upper	p value**
Sex [n (%)]			0.696	0.298	1.628	0.403
female	18 (46.2%)	30 (55.6%)				
male	21 (53.8%)	24 (44.4%)				
Angle classification [n (%)]			-	-	-	0.697
Class I	18 (46.2%)	28 (51.9%)	1.111	0.285	4.325	0.879
Class II	16 (41.0%)	19 (35.2%)	0.748	0.185	3.016	0.683
Class III	5 (12.8%)	7 (13.0%)				
Treatment [n (%)]			1.724	0.722	4.117	0.220
extraction	14 (35.9%)	26 (48.1%)				
non-extraction	25 (64.1%)	28 (51.9%)				
Mean age [years]	24.54±5.853	23.89+5.729	0.981	0.900	1.053	0.504

*: at least one maxillary premolar; **: Binary logistic regression analysis. Dependent variable: control vs >2mm EARR patients.

Table 2. Interleukin 1 gene cluster genotype SNP (rs419598) genotypes, alleles frequencies distribution and risk associated with external apical root resorption secondary to orthodontic treatment in root filled teeth.

Gene SNP > 2mm EARR patients (n=39)		Controls (n=54)		Risk of EARR > 2mm			
Genotype fr [n(%)]	Allele fr [n(%)]	Genotype fr [n(%)]	Allele fr [n(%)]	Genotypes compared	OR	95% CI	p Value
IL1-RN +2018 (rs419598)							
TT [32 (82.1)]	T [70 (89.7)]	TT [16 (29.6)]	T [59 (54.6)]	TT Vs CC/TC	10.857	3.97 - 29.6	0.001***
CT [6 (15.4)]	C [8 (10.2)]	CT [27 (50.0)]	C [49 (45.3)]	CT Vs CC/TT	0.18	0.06 - 0.50	0.001***
CC [1 (2.6)]		CC [11 (20.4)]		CC Vs CT/CC	0.10	0.13 - 0.83	0.011**

EARR: external apical root resorption; SNP: single nucleotide polymorphism; fr: frequencies; CI: confidence interval; OR: odds ratio; **: p > 0.05; ***: p=0.001.

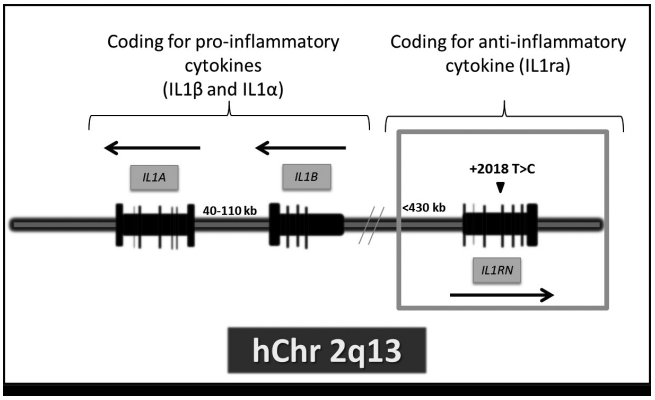

Fig. 1. Representative image of *IL1* cluster gene SNPs positioned on human chromosome 2q13 (hCHr 2q13), where three genes are represented. The selected gene, the interleukin 1 receptor antagonist, *IL1RN* (red square), which encodes the anti-inflammatory protein IL-1ra; and two genes, interleukin 1 alpha (*IL1A*) and interleukin 1 beta (*IL1B*), which encode two pro-inflammatory cytokines, IL-1ß, IL-1α (two distinct proteins performing IL-1functions), respectively. The *IL1RN* gene contains different polymorphisms, although the schema represents only the functional SNP studied in this research (+2018). The plus sign (+) denotes the location of the polymorphism relative to the site where messenger RNA transcription begins. Vertical bars indicate the part of the gene that encodes the proteins. Arrows indicate the direction of transcription of the DNA sequence into messenger RNA.

when compared to the control group and, conversely, a decrease of 17.8 % of the C allele (Table 2).

The genotypes of *IL1RN* were as follows: 2.6% had a 2/2 genotype (CC), the least frequent genotype; 15.4% of the sample had a 1/2 genotype (CT); and interestingly, 82.1% had a 1/1 genotype (TT) (Table 2). It should be noted that there was a marked decrease in frequency of the most common genotypes in the control group when compared to the EARR group (82.1%<29.6%). This data represents a more than two-fold increase in the frequency of the most common genotype of *IL1RN* (p<0.05*) in the EARR-affected group, when compared to the control group.

The sample was classified on the basis of the presence or absence of the pathology; it was noted that 66.6% of subjects homozygous for the T allele (32 out of 48)-present in the 1/1 *IL1RN* genotype-suffered EARR. Similarly 8.3% of homozygous subjects with the 2/2 genotype for the C allele (1out of 12) also presented EARR (Table 2). There is accordingly a significant statistical difference for homozygous carriers of the most common T allele for *IL1RN* SNP (1/1), compared to other genotypes. These subjects had a 10.85 times (p=0.001**) higher risk of suffering post-orthodontic EARR in root-filled teeth, with a 95% confidence interval [OR: 10.85 (p=0.001**)] (Table 2).

Discussion

Post-orthodontic external apical root resorption is a iatrogenic effect of endodontically treated teeth, with permanent loss of dental root structure secondary to clinical or therapeutic factors (Hartsfield, 2009). Nevertheless, other factors may play a decisive role in conditioning the way specific patients respond differently to standard therapy protocols, or in determining the emergence and severity of the pathology (Al-Qawasmi et al., 2003a; Hartsfield, 2009). Mechanical forces transmitted by orthodontic appliances to the root-filled teeth are still required to initiate the inflammatory response in the patient, required for tooth movement to take place (Wang et al., 2007), although other factors may modulate or augment the inflammatory response so that the clinical scenario varies and facilitates the onset of the pathology (Rogus et al., 2008). Many recent studies have started the process of defining and evaluating the decisive role of specific genetic profiles as risk factors in the development of certain pathologies, such as post-orthodontic EARR in the vital teeth (Bastos-Lages et al., 2009; Gülden et al., 2009; Hartsfield, 2009). With regard to root-filled teeth, recent research has focused on a genetic predisposition to EARR (Iglesias-Linares et al., 2012a) and there is another study about the predisposition to persistent apical periodontitis in the context of variants in the interleukin 1 cluster genes (Morsani et al., 2011). However, as far as we are aware, no previous study in the literature has evaluated the acquired genetic risk of post-orthodontic EARR in root-filled teeth associated with interleukin 1 receptor

antagonist (*IL1RN*) variants.

Classifying the sample according to the presence or absence of EARR (root resorption ≥2mm) (Al-Qawasmi et al., 2003a) took place after a randomized blinded assessment of radiographic images before and after orthodontic treatment by the same experienced examiner (I.L.A). Our objective was to assess observed variations in calibrated pre and post-treatment images, which had been taken using the same standardized position, trained operator and method. Additionally, we chose a previously published measurement method because it enabled us to make an overall comparison between our results and previous study data in the same field of research. However, there might still be errors of absolute accuracy, in spite of the very good reproducibility of our measurements, (k=87; k=91) until such time as an accurate 3-D imaging system becomes the standard test (Durack et al., 2011). However, although present day approaches in the field demonstrate excellent accuracy, the radiation dose remains a concern when using conventional computer tomography (CT) or even cone beam computer tomography (CBCT) for 3D diagnosis (Durack et al., 2011).

The fact that the establishment and severity of EARR in orthodontic patients can be so unpredictable and variable makes it important to trace fresh diagnostics of common molecular profiles. In this regard, variations in specific genes coding for pro-inflammatory cytokines, including IL1α and IL-1ß, have been associated with increased/decreased levels of inflammatory proteins (Kahraman et al., 2006; Rogus et al., 2008) and have somehow been positively or negatively correlated with susceptibility to post-orthodontic EARR (Al-Qawasmi et al., 2003b; Bastos-Lages et al., 2009; Gülden et al., 2009; Iglesias-Linares et al., 2012a). It is important to mention that interleukin 1 is one of the first molecular mediators implicated in the inflammation of the vessel wall at the beginning of and during tooth movement (Krishnan and Davidovitch, 2009), a prerequisite for tooth movement to occur. This could contribute to facilitate bone remodeling and the subsequent reduction in radicular stress, since apical stress is a major factor directly correlating with EARR during orthodontic tooth movement (Weltman et al., 2010; Hayashi et al., 2012).

The interleukin 1 receptor antagonist (IL1ra) is a protein that neutralizes the pro-inflammatory function of interleukin 1 (Hurme and Santtila, 1998). Genetic variants in the gene coding for this protein, *IL1RN*, have been associated with an alteration in IL1ra levels. The +2018T>C polymorphism in exon 2 of the gene is in a 100% linkage disequilibrium with a penta-allelic 86 bp variable-number-of-tandem-repeat (VNTR) polymorphism in intron 2 of the gene, which is strongly related to increased production of IL-1ra (Hurme and Santtila, 1998) and IL-1ß *in vitro* (Santtila et al., 1998). In the clinical setting, these genetic variants have been described as having a direct impact on many chronic inflammatory diseases, such as Barrett's oesophagus and gastric or oesophageal cancer, among others (Persson et al., 2011), although as yet, there is no established association with EARR. On the basis of the results shown in the present study, the strong association between genotype 1/1 (TT) of the *IL1RN* gene SNP [OR: 10.85; p=0.001**] and post-orthodontic EARR in root-filled teeth could be consistent with an increase in IL-1ra protein levels, which previous *in vitro* and *in vivo* studies in other fields have underlined (Danis et al., 1995; Vamvakopoulos et al., 2002). In line with these results, recent research into post-orthodontic EARR in the vital teeth, using a southern European sample, found an association between *ILRN* gene variants at position +2018 and an increased risk of suffering EARR secondary to orthodontic treatment (Iglesias-Linares et al., 2012a). In the earlier research, subjects homozygous for the cytosine allele were found to have a predisposition to EARR in their vital teeth (OR: 6.75; P=0.001; CI: 95%) and, on the basis of the results of this study, the same trend, although even more obvious, was observed in endodontically-treated teeth (OR: 10.85; p=0.001;CI: 95%). The fact of vital pulp being replaced by inorganic filling material in the tooth seems to increase the risk of suffering orthodontic EARR. Although no definitive scientific evidence has so far been provided and many clinical factors could modify the observed results, some authors have nevertheless proposed that a graft-versus-host response towards the filling material after initial EARR has begun could be the key factor triggering an enhanced EARR process in root-filled teeth subject to orthodontic movement (Weltman et al., 2010). Similar to the observed results, another recent study conducted in the Czech Republic, confirmed and supplied new findings, in which an increased risk of suffering EARR was associated with variations in the interleukin antagonist gene (86 bp variable-number-of-tandem-repeat (VNTR) polymorphism in intron 2 of the gene) which were in 100% linkage disequilibrium with the studied +2018T>C polymorphisms. This study concluded that VNTR gene variants were associated with EARR in the vital teeth, especially in a sub-group of Czech girls of Caucasian origin (OR=2.50, 95% CI: 1.13-5.53; p=0.020) (Linhartova et al., 2012). One plausible hypothesis for the genetic predisposition in vital and root-filled teeth would be that the increase in the competitive antagonist, IL-1ra, of the inflammatory mediators, IL-1ß and IL-1α, would be associated with a difficult alveolar bone remodelling rate during root-filled tooth movement. Subsequently, the imbalance on the IL-1ra/IL-1ß axis could have a direct impact on the enhancement of radicular stress during orthodontic tooth movement (Al-Qawasmi et al., 2003b), as exemplified exceptionally in a genetically modified knockout mouse model *IL-1B*-/-, which described an aggressive EARR secondary to force application (Al-Qawasmi et al., 2004). Some authors have even suggested that the *IL1RN* allele 1-and not the *IL1B* SNPs themselves-could be the key regulator of IL1ß production, at least in the *in vitro* model and in some inflammatory diseases (Santtila et al., 1998;

Fragoso et al., 2010). Taken together, the data suggests that the known allelisms in the *IL1RN* gene could be engaged in cross-talk with their competitive agonist-or some unidentified allele strongly associated with it-subsequently exerting a directly negative impact on EARR and possibly playing a decisive role in controlling IL-1ß production.

Conclusion

In conclusion, the results described in the present study, indicate that variants in a specific allele (allele 1) of the interleukin 1 cytokine antagonist gene (rs419598) (or some unknown linked allele) are directly associated (p=0.001**) with an increased risk of suffering post-orthodontic EARR in root-filled teeth. To the best of our knowledge, this is the first paper to describe an association between an IL1RN polymorphism and the variability of EARR in patients with endodontically-treated teeth who undergo orthodontic treatment.

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