



Association between Estrogen, Vitamin D and Microrna17 Gene Polymorphisms and Periapical Lesions

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This study evaluated the association between polymorphisms in genes encoding estrogen receptors 1 (ESR1) and 2 (ESR2), vitamin D receptor (VDR) and in microRNA17 (which binds to ESR1 and VDR) with persistent apical periodontitis (PAP) after the endodontic treatment. We included 162 patients who completed endodontic treatment at least one year ago and presented apical periodontitis at the beginning of the root canal therapy. Clinical and radiographic exams were performed to evaluate the presence of PAP or healthy periradicular tissues (healed). Saliva samples were collected as a genomic DNA. The genotyping of ESR1 (rs2234693 and rs9340799), ESR2 (rs1256049 and rs4986938), VDR (rs739837 and rs2228570) and miRNA17 (rs4284505) were performed by real-time PCR. Chi-square test was used to the distribution of genotype and allele frequencies. Haplotype analysis was also performed. Eighty-nine patients were included in the "healed" group and 73 in the "PAP" group. No association was found between the allelic and genotypic polymorphisms studied and PAP ($p > 0.05$). Haplotype analysis also did not demonstrated an association ($p > 0.05$). In conclusion, the genetic polymorphisms in ESR1, ESR2, VDR and miRNA17 are not associated with PAP.

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Introduction

The apical periodontitis (AP) is an inflammatory and infection condition that results from a host reaction of anaerobic polymicrobial infection of the root canal system, which leads to necrosis of the pulp tissue (1-3). This inflammatory reaction causes destruction of periradicular tissues, which can be observed in periapical radiographs as radiolucent areas confined to the root apex (1-4). The complete healing of the apical periodontitis and the restoration of the function are the fundamental goals of non-surgical root canal therapy (5). However, the persistent apical periodontitis (PAP) after satisfactory root canal therapy may occur (6), and many studies have been demonstrating that genetic polymorphisms are involved in the PAP (3,7-13). Genetic polymorphisms can be defined as differences in the human DNA sequence, in more than 1% of the population, which influence the organism's susceptibility to disease and its responses to the environment (3,5-13).

Estrogen presents multifunctional in many tissues, mainly in bone. Estrogen in bone metabolism plays an important role in RANKL (Receptor activator of NF-

kB ligand) and OPG (osteoprotegerin) release (14) and in bone-regulating cytokines IL1 (interleukin 1), IL6 (interleukin 6), TNF-alpha (tumor necrosis factor), and prostaglandin-E2. These factors increase bone resorption and are downregulated by estrogen (15). Studies performed in estrogen deficiency rats with experimental apical periodontitis showed that they have a significantly increased bone loss (16-18). Estrogen binds to either ER α (estrogen receptor alpha) or ER β (estrogen receptor beta) stimulating ER-positive cell lines (19). The genes ESR1 and ESR2 encode ER α and ER β (20).

In addition, vitamin D is an important factor in estrogen biosynthesis of both women and men (21). It is worth mentioning that vitamin D is essential in bone formation and remodeling. The molecular mechanisms of the vitamin D system contribute to the maintenance of plasma calcium, phosphate and bone mineral homeostasis (22,23). Vitamin D receptor, which is encoded by VDR gene, is recognized as a member of the super-family of nuclear receptors that regulate genes expression and has a central role in the biology of vitamin D action (24). The VDR expression contribute to the maintenance of plasma calcium,

phosphate and bone mineral homeostasis (22,23). VDR is expressed by all three major bone cell types: osteoblasts, osteoclasts, and osteocytes. The ablation of the VDR gene produces hereditary vitamin-D-resistant rickets, alopecia, hypocalcaemia, hypophosphatemia, and rickets. This condition has been associated with bone loss and cortical bone porosity severe enough to initiate spontaneous bone fractures, showing that any defect in bone mineralization is largely dependent on the availability of plasma calcium and phosphate via VDR-mediated tissue absorption (22,23). Interestingly, a common oral manifestation in patients with vitamin D-resistant rickets is apical periodontitis (25).

MicroRNAs (miRNAs) are a family of small non-coding RNAs that regulate genes expression post-transcriptionally. The miRNAs can affect the stability and degradation of specific target mRNAs and, consequently, causing translational inhibition (26). miRNAs are important in the differentiation and proliferation of bone cells and is also pointed as potential novel diagnostic biomarkers for bone diseases and bone regeneration (27).

Although ERs, VDR and miRNAs have been widely studied in bone diseases, their role in apical periodontitis is still largely unknown. Therefore, the aim of this study was to evaluate if genetic polymorphisms in ESR1, ESR2, VDR and miRNA17 are associated with PAP.

Material and Methods

Sample Description

The protocol for this study was approved by the Research Ethics Committee of the University of São Paulo, Ribeirão Preto, São Paulo, Brazil (2.323.266) and by the Research Ethics Committee of the Fluminense Federal University, Rio de Janeiro, Brazil (1.029.674). Informed written consent was obtained with an assent document by all subjects.

A sample of 162 (117 females and 45 males) patients were evaluated in the present study. The recruitment of the subjects was previously described in Mazzi-Chaves et al (3) and in Petean et al (13). Only subjects with pulp necrosis associated with apical periodontitis at the beginning of the root canal therapy and those that attended regular follow-up visit were selected. This selection was based in previous studies (3,7,8,13). Patients with at least one root canal-treated in any permanent human teeth, no obvious reason for root canal failure. Clinical information regarding their general health, systemic disease, smoking and parafunctional habits was collected through an anamnesis from all subjects.

Phenotype Determination

During the regular follow-up appointments, the phenotype was determined based on the periapical radiographs and clinical aspects. Immediate postoperative

radiographs were compared with the recall radiographs after at least 1 year of the end of endodontic treatment, by the same calibrate observer as described in Petean et al. (13).

The treatment outcome was categorized in PAP and healed, according to Morsani et al. (7), in which PAP was defined as follow: a lack of healing with apparently well obturated root canal system(s) as determined by a radiographic examination; root canal therapy completed at least a year prior with signs and symptoms of the condition; the preexisting radiographic lesion persists; and the presence of a clinical signs or symptoms of periapical conditions, such as sinus tract, pain and swelling. Healed was considered as follow: the absence of pain and swelling; disappearance of the sinus tract; no loss of function and no evidence of tissue destruction.

Mirna and Polymorphisms Selection

The genetic polymorphisms in ESR1 (rs2234693 and rs9340799), ESR2 (rs1256049 and rs4986938) and VDR (rs739837 and rs2228570) genes were selected based on their function in bone biology (28-30) and in their minor allele frequencies, that should be higher than 10%.

The selection of the miRNA was performed through the miRanda software (31), which was used to scan miRNA-mRNA interactions. This software uses a target prediction algorithms and identified the miRNA17 as targets the mRNA of both genes, ESR1 and VDR, with high scores of predicted alignment (www.mirna.org). The genetic polymorphism rs4284505 in miRNA17 was selected due the fact that it was the only polymorphism which a minor allele frequency higher than 10%.

The characteristics of the selected genes and polymorphisms are presented in the Table 1.

DNA Extraction and Genotyping Analysis

Saliva samples were collected from all included subjects. Genomic DNA for the allelic discrimination analysis was extracted from buccal cells isolated from saliva as described in Mazzi-Chaves et al. (3) and Petean et al. (13).

Genotyping was performed by real-time polymerase chain reactions (PCR) using the TaqMan assay in the real-time PCR system ABI PRISM® 7500HT (Foster City, CA, USA). The probes and the master mix are from Applied Biosystems (Foster City, CA, USA).

Statistical Analysis

Data were analyzed using Plink. Age differences between groups were calculated using t-test. Fisher's exact and Pearson chi-square tests were used to compare the demographic difference (age, sex, ethnicity, healthy condition and habits) between groups. Pearson chi-square test was used to compare genotypes distributions between

'Healed' and 'PAP' in the additive, recessive and dominant model and the allele distributions. Haplotype analysis was performed with the polymorphisms rs2234693-rs9340799, rs1256049-rs4986938 and rs739837-rs2228570 in ESR1, ESR2 and VDR respectively.

All tests were performed with an established alpha of

0.05. Hardy-Weinberg equilibrium was evaluated using the chi-square test within each polymorphism in each population set.

Results

Among the included patients, 89 was included in the "healed" group and 73 in the "PAP" group. The subjects age ranged from 16 to 83. Age, gender and medical conditions were not associated with PAP ($p>0.05$).

All the studied genetic polymorphisms were in Hardy-Weinberg equilibrium (data not shown).

Table 2 demonstrated the genotype distribution of the studied polymorphisms in the healed and PAP groups. There was no association between the studied polymorphisms and PAP in the additive, recessive and dominant models ($p>0.05$).

Alleles frequencies distributions were not associated with PAP ($p>0.05$). Haplotype analysis of rs2234693-rs9340799, rs1256049-rs4986938 and rs739837-rs2228570 were not associated with PAP ($p>0.05$).

Table 1. Genes and markers studied in the present study

Gene	Chromosome	Genetic Polymorphism	Functional consequence	Base Change	Global MAF
ESR1	Chr. 6	rs2234693	Transition Substitution	C/T	0.55
ESR1	Chr. 6	rs9340799	Transition Substitution	A/G	0.72
ESR2	Chr. 14	rs1256049	Intron variant	C/T	0.87
ESR2	Chr. 14	rs4986938	Intron variant	C/T	0.74
VDR	Chr. 12	rs739837	intron variant	G/T	0.49
VDR	Chr. 12	rs2228570	Unknown	A/G	0.33
miRNA17	Chr. 14	rs4284505	Intron variant	A/C	0.47

Note: MAF means minor allele frequency. Data obtained from databases: <http://www.ncbi.nlm.nih.gov/snp/>; <http://genome.ucsc.edu/>; <http://www.thermofisher.com/>.

Table 2. Genotype distributions among the groups

Genes	Polymorphisms	Genotypes	Groups		p-values		
			Healed n (%)	PAP n (%)	Additive model	Recessive model	Dominant model
ESR1	rs2234693	CC	18 (18.0)	16 (16.0)	0.98	0.99	0.85
		CT	36 (35.5)	31 (31.5)			
		TT	27 (27.5)	25 (24.5)			
	rs9340799	AA	34 (37.2)	35 (31.9)	0.50	0.38	0.28
		AG	31 (29.6)	24 (25.4)			
		GG	12 (10.2)	7 (8.7)			
rs1256049	CC	72 (87.8)	63 (0.90)	0.66	-	-	
	CT	10 (12.2)	7 (0.10)				
	CC	43 (41.5)	34 (35.5)				0.20
rs4986938	CT	33 (30.7)	24 (26.3)				
	TT	8 (11.8)	14 (10.2)				
	GG	18 (15.7)	11 (13.3)				
VDR	rs739837	GT	38 (37.9)	32 (32.1)	0.57	0.61	0.43
		TT	28 (30.3)	28 (25.6)			
		AA	8 (8.31)	7 (6.7)			
	rs2228570	AG	43 (40.9)	31 (33.2)	0.77	0.88	0.52
		GG	35 (36.9)	32 (30.1)			
		AA	18 (18.5)	15 (14.5)			
MiRNA 17	rs4284505	AG	48 (43.7)	30 (34.3)	0.33	0.84	0.18
		GG	22 (25.8)	24 (10.2)			
		GG	22 (25.8)	24 (10.2)			

Note: for the polymorphism rs1256049 the recessive and dominant model were not calculated because none of the individual presented TT genotype.

Discussion

In the past years, studies of the genetic factors associated with oral phenotypes have been emerging (32), including studies that evaluated the association of genetic polymorphisms in the apical periodontitis pathogenesis and phenotype (3,8-13,33). The current evidence supports the concept that PAP is a multifactorial condition, in which many genes play an important role.

In previous study (13), genetic polymorphisms in RANK and RANKL were associated with PAP. Bone processes are controlled mainly by the interaction between RANK, RANKL and OPG (34,35). The balance between RANKL and OPG expression is determinant on the bone remodeling process, and studies in animal model

(36,37) and humans (38,39) have demonstrated that RANK, RANKL and OPG triad are important in the development, progression and repair of AP. The bone resorption process is mainly controlled by the RANK / RANKL / OPG signaling pathway, which are considered regulators of the bone remodeling process. RANKL is a soluble mediator, synthesized by osteoblasts, bone marrow stromal cells, and endothelial cells. Although there is a physiological expression of RANKL, this is a molecule abundantly produced by T lymphocytes throughout inflammatory processes. In addition, RANKL is the mediator responsible for binding to RANK, a surface-expressed receptor of osteoclasts and their progenitors, macrophages present in the bone marrow that after this binding receive the stimulation of maturation and conversion into active osteoclasts. The OPG is a soluble receptor secreted by osteoblast anabolic stimuli through which competes with RANK for binding to RANKL agent, thereby preventing this link, so as to inhibit osteoclast genesis (13,34–39). Therefore, we hypothesize that genetic polymorphisms in genes that encode molecules involved in this triad are candidates to PAP in humans.

Estrogen are involved in the triad RANK, RANKL and OPG (14,40), in which estrogen exert anti-resorptive effects on bone, at least in part, by stimulating ERs and OPG expression in osteoblasts (40). In addition, ER α is involved in the RANKL/OPG ratio (41). Additionally, the importance of ERs on cortical and trabecular bone, and also in different bone cell types have been widely explored (42). Although the studied genetic polymorphism rs2234693 and rs9340799 in ESR1 and rs1256049 and rs4986938 were not associated with PAP in the present study, they were previously associated with many different bone conditions (28,43) and oral conditions (43–45). The genetic polymorphisms rs2234693 and rs9340799 in ESR1 have been largely studied. The evidence linking these polymorphisms and its haplotype to bone mineral density (28,46). Also they were associated with postmenopausal osteoporosis (28,43). According to Rivadeneira et al (47), genetic polymorphisms in ESR2 alone and in interaction with ESR1 can also influence the risk of bone fracture in postmenopausal women.

It is important to emphasize although the studied genetic polymorphisms were not associated with PAP, it is possible that estrogen play an important role in this endodontic phenotype. Estrogen deficiency after ovariectomy or menopause plays an important role in the early alterations in the turnover of cancellous bone, leading to both the early and late forms of osteoporosis in women (48) and in animals with induced apical periodontitis, they presented a larger lesion (16–18). Therefore, it is possible that estrogen deficiency in humans could be involved in the PAP.

Recent data with an osteoblast-specific VDR knockout

mouse model demonstrated impaired RANKL expression and activity, confirming the crucial role of VDR in osteoblasts during the regulation of osteoclast genesis (22). The function of VDR has also been examined in osteocytes with an osteocyte-specific VDR deletion mouse model (22) and the data showed that high vitamin D levels in the plasma can act on osteocytes to inhibit bone mineral deposition in addition to stimulating bone resorption increasing RANKL expression by osteoblasts and possibly osteocytes (22). On the other hand, the adequate levels of vitamin D reduced bone RANKL expression and bone resorption, while also slightly prolonging the bone formation period of osteoblast (22). VDR genetic polymorphisms have been associated with bone mineral density (49,50) and in immune function (51,52). Furthermore, genetic polymorphisms in VDR have been associated with oral conditions (53–56). The genetic polymorphism rs731236 was associated with external apical root resorption in orthodontic patients (54). A meta-analysis of 15 studies evaluating the association between and aggressive and chronic periodontal disease genetic polymorphisms in VDR also found that this polymorphism was associated with periodontitis (57). However, in our study, both genetic polymorphisms in VDR were not associated with PAP.

It is also possible that post-transcriptional regulatory factors such as miRNAs are also involved in PAP. In fact, a study performed by Chan et al (58) explored the role of miRNAs in endodontic disease and providing novel insight into the genetic regulation of endodontic periapical pathogenesis. They observed that multiples miRNAs were down or up regulated in the diseased periapical tissues. In our study, a genetic polymorphism in miRNA17 was selected due the fact that it targets the miRNA of two candidate genes: ESR1 and VDR. The polymorphism rs4284505 was not associated with PAP, however, it is possible that other genetic polymorphisms in miRNAs are associated with PAP.

Briefly, more genetic polymorphisms should be investigate, so in a near future, they can provide information to allow a personalized treatment of patients with apical periodontitis.

In conclusion, genetic polymorphisms in ESR1 (rs2234693 and rs9340799), ESR2 (rs1256049 and rs4986938), VDR (rs739837 and rs2228570) and miRNA17 (rs4284505) were not associated with persistent apical periodontitis.

Resumo

Este estudo avaliou a associação entre polimorfismos em genes que codificam os receptores de estrogênio 1 (ESR1) e 2 (ESR2), receptor de vitamina D (VDR) e no microRNA17 (que se liga à ESR1 e VDR) e a periodontite apical persistente (PAP) após o tratamento endodôntico. Foram incluídos 162 pacientes com tratamento endodôntico concluído há pelo menos um ano e que apresentavam periodontite apical no início

da terapia endodôntica. Exames clínicos e radiográficos foram realizados para avaliar a presença de PAP ou tecidos perirradiculares saudáveis (cicatrizados). As amostras de saliva foram coletadas como fonte de DNA genômico. A genotipagem de ESR1 (rs2234693 e rs9340799), ESR2 (rs1256049 e rs4986938), VDR (rs739837 e rs2228570) e miRNA17 (rs4284505) foram realizadas por PCR em tempo real. O teste do qui-quadrado foi utilizado para a distribuição das frequências genotípicas e alélicas. A análise de haplótipos também foi realizada. Oitenta e nove pacientes foram incluídos no grupo "curado" e 73 no grupo "PAP". Não foi encontrada associação entre os polimorfismos alélicos e genotípicos estudados e a PAP ($p>0,05$). Conclui-se que os polimorfismos genéticos em ESR1, ESR2, VDR e miRNA17 não estão associados à PAP.

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