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กองทุนรัชดาภิเษกสมโภช

รายงานวิจัย

ความสัมพันธ์ระหว่าง polymorphisms ของ vitamin D receptor gene และโรคปริทันต์อักเสบชนิดเรื้อรังในคนไทย

โดย

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ผู้วิจัยขอขอบคุณ Dr. Kevin Tompkins (คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย) ที่ให้ความช่วยเหลือในการตรวจสอบบทความภาษาอังกฤษเพื่อส่งตีพิมพ์ และขอขอบคุณอาจารย์และเจ้าหน้าที่ของภาควิชาปริทันตวิทยา คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย และคณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล สำหรับความช่วยเหลือในการตรวจทางคลินิคในผู้เข้าร่วมโครงการ และการบันทึกข้อมูลที่ได้จากการตรวจ สุดท้ายนี้เราขอขอบคุณพนักงานการไฟฟ้าฝ่ายผลิตแห่งประเทศไทยที่เข้าร่วมและทำให้โครงการนี้ประสบความสำเร็จได้ด้วยดี

โครงการวิจัย ความสัมพันธ์ระหว่าง polymorphisms ของ vitamin D receptor gene และโรคปริทันต์อักเสบชนิดเรื้อรังในคนไทย

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บทคัดย่อ

ความหลากหลายทางพันธุกรรมในยีนวิตามินดีรีเซปเตอร์มีความสัมพันธ์กับความเสี่ยงในการเกิดการติดเชื้อและโรคกระดูก อย่างไรก็ตาม จากผลการศึกษาที่ผ่านมายังไม่สามารถสรุปได้อย่างชัดเจนว่าความหลากหลายทางพันธุกรรมในยีนนี้ มีความสัมพันธ์กับการเกิดโรคปริทันต์อักเสบหรือไม่ ดังนั้นทางคณะผู้วิจัยจึงทำการศึกษาแบบตัดขวาง เพื่อศึกษาความสัมพันธ์ระหว่างความหลากหลายทางพันธุกรรมในยีนวิตามินดีรีเซปเตอร์ และความเสี่ยงในการเกิดโรคปริทันต์อักเสบเรื้อรังในกลุ่มประชากรไทย เราได้ทำการเตรียมสารพันธุกรรมจากกลุ่มตัวอย่างคนไทยจำนวน 1,460 ราย ที่มีอายุระหว่าง 39-65 ปี โดยทำการจีโนไทป์ความหลากหลายทางพันธุกรรมในยีนวิตามินดีรีเซปเตอร์ 4 ตำแหน่งคือ *Fok*I, *Bsm*I, *Apa*I และ *Taq*I โดยใช้วิธีการเพิ่มปริมาณสารพันธุกรรมแบบเรียลไทม์ เราแบ่งกลุ่มตัวอย่างออกเป็น 3 กลุ่มตามสถานะของโรคปริทันต์ได้แก่ กลุ่มที่ไม่เป็นโรคหรือเป็นโรคระดับน้อย กลุ่มที่เป็นโรคระดับปานกลาง และกลุ่มที่เป็นโรคระดับรุนแรง การทดสอบทางสถิติเพื่อศึกษาความสัมพันธ์ระหว่างจีโนไทป์และโรคปริทันต์อักเสบ ใช้สถิติ multivariate logistic regression analysis โดยมีปัจจัยเสี่ยงอื่นๆที่ต้องควบคุมสำหรับโรคปริ

ทันต์อักเสบ คืออายุ เพศ การสูบบุหรี่ และโรคเบาหวาน ผลการทดลองพบว่า ลักษณะจีโนไทป์ CC หรือ CT ของ *Fok*I สัมพันธ์กับการเกิดโรคปริทันต์อักเสบเรื้อรังระดับปานกลางและรุนแรง โดยมีอัตราส่วนของโอกาสการเกิดโรค (odds ratio) เป็น 1.4 (95% CI 1.0-1.9) และ 2.0 (95% CI 1.3-2.9) ตามลำดับ แต่ไม่พบความสัมพันธ์ของการเกิดโรคปริทันต์อักเสบกับความหลากหลายตำแหน่งอื่นหรือ *Bsm*I-*Apa*I-*Taq*I haplotypes จากนั้นผู้วิจัยได้ศึกษาผลจากการทำงานร่วมกันของยีนและปัจจัยการสูบบุหรี่ โดยใช้กลุ่มตัวอย่างที่ไม่สูบบุหรี่และมีจีโนไทป์ TT ของ *Fok*I เป็นกลุ่มอ้างอิง พบว่ากลุ่มตัวอย่างที่สูบบุหรี่และมีจีโนไทป์ CC หรือ CT ของ *Fok*I มีโอกาสในการเกิดโรคปริทันต์อักเสบเรื้อรังระดับรุนแรงมากที่สุด โดยมีอัตราส่วนของโอกาสการเกิดโรคเป็น 10.4 (95% CI 4.9-22.1) ในขณะที่กลุ่มตัวอย่างที่สูบบุหรี่และมีจีโนไทป์ TT และกลุ่มตัวอย่างที่ไม่สูบบุหรี่ที่มีจีโนไทป์ CC หรือ CT ของ *Fok*I มีโอกาสในการเกิดโรคเพิ่มขึ้นเพียง 2.7 (95% CI 1.1-6.7) และ 2.0 เท่า (95% CI 1.2-3.4) ตามลำดับ ปฏิกิริยาของ*Fok*I และการสูบบุหรี่มีผลต่อการเกิดโรคปริทันต์อักเสบเรื้อรังระดับรุนแรงมากกว่าผลรวมเมื่อคิดแยกจากปัจจัยทั้งสอง บ่งบอกว่าปฏิกิริยาของทั้งสองปัจจัยเป็นแบบ additive interaction โดยสรุป การศึกษานี้ได้แสดงความสัมพันธ์ระหว่าง *Fok*I และระดับความรุนแรงในการเกิดโรคปริทันต์อักเสบ นอกจากนั้น ยังแสดงปฏิกิริยาของ *Fok*I และปัจจัยการสูบบุหรี่ ว่ามีผลเสริมกันต่อความเสี่ยงในการเกิดโรคปริทันต์อักเสบเรื้อรังอีกด้วย

Project Title Association between vitamin D receptor gene polymorphisms and chronic periodontitis in Thais

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Abstract

Polymorphisms of the vitamin D receptor (VDR) gene have been implicated in the susceptibility to infections and bone-related diseases. However, their relationship with periodontal disease remains unclear. This cross-sectional study investigated whether the susceptibility to chronic periodontitis in a Thai population is associated with VDR polymorphisms. Genomic DNA was obtained from 1,460 subjects, aged 39-65 years. Genotyping of VDR polymorphisms (*Fok*I*, Bsm*I, *Apa*I, and *Taq*I) was performed using real-time polymerase chain reaction. Subjects were categorized into three groups; no/mild, moderate, and severe periodontitis. Multinomial logistic regression analysis was used to determine the degree of association between VDR polymorphisms and periodontal status adjusted for age, gender, education, smoking, and diabetes. The CC+CT genotypes of *Fok*I polymorphism were associated with moderate and severe periodontitis with odds ratios (OR) of 1.4 (95% CI 1.0-1.9) and 2.0 (95% CI 1.3-2.9),

respectively. There was no significant relationship between the other VDR polymorphisms or *Bsm*I-*Apa*I-*Taq*I haplotypes and periodontitis. To examine gene-smoking interaction, non-smokers with the TT genotype of *Fok*I polymorphism were used as the reference group for all comparisons. Current smokers who had the CC+CT genotypes presented the highest risk of severe periodontitis with an OR of 10.4 (95% CI 4.9-22.1), whereas their counterparts with the TT genotype and non-smokers bearing the CC+CT genotypes had an increased risk by 2.7 (95% CI 1.1-6.7) and 2.0 folds (95% CI 1.2-3.4), respectively. The combined effect of *Fok*I polymorphism and current smoking was 3.5 times (95% CI 1.3-9.9) greater than what would be expected from the sum of their individual effects, indicating a significant additive interaction. In conclusion, our data indicate that *Fok*I polymorphism of VDR gene was significantly associated with periodontal disease severity in this study group. We are also the first to demonstrate that *Fok*I polymorphism and smoking synergistically interacted in increasing the risk of chronic periodontitis.

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CHAPTER 1

INTRODUCTION

Chronic periodontitis is one of the most significant dental health problem found in Thailand. It is the main cause of tooth loss in adults. Although the primary etiology for disease initiation and progression is subgingival plaque bacteria, an individual's susceptibility to disease is influenced by genetic and environmental risk factors. Several previous studies have been reported other risk factors associated with severity and progression of chronic periodontitis including age, gender, education, oral hygiene status, smoking, and diabetes. Our previous study suggested that education, current smoking, diabetes, and the presence of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivali* in subgingival plaque were associated with chronic periodontitis. However, none of the genetic association studies with chronic periodontitis has ever been done in Thai population.

Many studies have identified potential gene polymorphisms that influence immune functions and bone or connective tissue metabolism as they are related to the pathogenesis of periodontal disease. Vitamin D receptor (VDR) is a nuclear receptor that binds to the active form of vitamin D. It is involved in a variety of biological processes, including bone metabolism, modulation of immune response, and regulation of cell proliferation and differentiation. Polymorphisms of VDR gene have been shown to be associated with bone mineral density, and the incidence of bone-related diseases,

particularly osteoporosis. They have also been linked to infectious diseases such as tuberculosis, and autoimmune diseases. Therefore, it is reasonable to hypothesize that polymorphisms of VDR gene may distribute an essential role in the susceptibility to chronic periodontitis. Previous studies have examined the association between VDR polymorphisms or combinations of these variants and periodontitis at FokI, BsmI, ApaI and TaqI restriction sites. The results have been inconsistent and it remains unclear which VDR polymorphisms influence disease susceptibility. Most of the association studies in Asians have been performed in Chinese, Japanese and Korean, but none has ever been done in Thai population. Therefore, the aim of the present study was to investigate the associations between four polymorphisms in VDR gene, including *Fok*I, *Bsm*I, *Apa*I, and *Taq*I, as well as their haplotypes, and the risk of chronic periodontitis in a Thai population. In addition, the possibility of interactions between these polymorphisms and smoking was examined.

CHAPTER 2

SURVEY OF RELATED LITERATURE

Chronic periodontitis is an inflammatory disease characterized by gradual destruction of connective tissues and alveolar bone. It is well established that chronic periodontitis is a multifactorial disease (Kornman 2008). Although the primary etiology for disease initiation and progression is subgingival plaque bacteria, an individual's susceptibility to disease is influenced by genetic and environmental risk factors. Our previous studies reported that age, gender, education, oral hygiene status, smoking, and diabetes are associated with chronic periodontitis in a Thai population (Torrungruang et al. 2005a; Torrungruang et al. 2005b). Moreover, the presence of *A. actinomycetemcomitans* and *P. gingivalis* in subgingival plaque increased the disease risk by 2.5 and 3.4 times, respectively (Torrungruang et al. 2009). In addition to these factors, several lines of evidence suggest that there is a substantial genetic component involved in the pathogenesis of chronic periodontitis (Laine et al. 2012). Furthermore, genetic factors may influence disease susceptibility in a complex way by acting in concert with environmental factors.

Many studies have identified gene polymorphisms that influence immune functions and bone or connective tissue metabolism as they are related to the pathogenesis of periodontal disease (Laine et al. 2012). Vitamin D receptor (VDR) is a nuclear receptor that binds to the active form of vitamin D. It is involved in various

biological processes, including bone metabolism, modulation of immune response, and regulation of cell proliferation and differentiation (Uitterlinden et al. 2004). Polymorphisms of the VDR gene have been shown to be associated with bone mineral density and the incidence of bone-related diseases, particularly osteoporosis (Thakkinstian et al. 2004; Ji et al. 2010). They have also been linked to *Mycobacterium tuberculosis* infections and chronic inflammatory diseases such as rheumatoid arthritis (Gao et al. 2010; Lee et al. 2011). Therefore, it is reasonable to hypothesize that VDR polymorphisms may play a role in the susceptibility to chronic periodontitis.

Previous studies have examined the associations between VDR polymorphisms at the FokI, BsmI, ApaI, and TaqI restriction sites or their combinations and periodontitis (de Brito Junior et al. 2004; Wang et al. 2009). The results have been inconsistent and it remains unclear which VDR polymorphisms influence disease susceptibility. In addition, only few studies have investigated the interaction between these polymorphisms and environmental factors, such as smoking, in relation to the risk of periodontitis (Nibali et al. 2008; Tanaka et al. 2013). A meta-analysis of 15 studies revealed that *Taq*I, *Apa*I, and *Bsm*I polymorphisms were associated with chronic periodontitis in Asians, but not in Caucasians, while there was no association between *Fok*I polymorphism and chronic periodontitis (Deng et al. 2011). A more recent meta-analysis including 18 studies came to a different conclusion that *Taq*I polymorphism was the only VDR polymorphism associated with chronic periodontitis in Asians (Chen et al. 2012). These conflicting

results indicate that larger studies should be conducted to clarify the role of VDR polymorphisms in chronic periodontitis. Therefore, the aim of the present study was to investigate the associations between four polymorphisms of the VDR gene, *Fok*I, *Bsm*I, *Apa*I, and *Taq*I, as well as their haplotypes, and the risk of chronic periodontitis in a Thai population. Moreover, the possibility of interactions between these polymorphisms and smoking was examined.

CHAPTER 3

PROCEDURE

Study participants

This cross-sectional study was part of a cohort study conducted among employees of the Electricity Generating Authority of Thailand (EGAT) (Vathesatogkit et al. 2012). The participants who worked at EGAT headquarters were enrolled in the study from June to November 2003. They had at least six teeth and did not require antibiotic prophylaxis for periodontal examinations. The study was approved by the local ethics committee and conforms to the STROBE guidelines. Written informed consent was obtained from each participant.

Examinations and sample collection

All participants received medical and dental examinations as previously described (Torrungruang et al. 2009). Probing depth (PD) and recession were measured at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual). Clinical attachment level (CAL) was calculated from PD and recession. Genomic DNA was extracted from peripheral blood lymphocytes using a standard phenol-chloroform method. DNA samples were diluted to a concentration of 5 ng/μl before genotyping.

VDR genotyping

Genotyping of VDR polymorphisms, *Fok*I (rs2228570), *Bsm*I (rs1544410), *Apa*I (rs7975232), and *Taq*I (rs731236), was performed on LightCycler® 480 (Roche Diagnostics, Indianapolis, IN, USA) using the TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). Real-time polymerase chain reactions was carried out in 384-well plates using a total volume of 6 μl, consisting of 2 μl of DNA, 2.5 μl of 2x TaqMan Universal PCR Master Mix, 0.125 μl of 40x target-specific probes and primers, and 1.375 μl of DNase/RNase-free water. Cycling parameters comprised initial denaturation at 95°C for 10 minutes and 40 cycles of denaturation at 95°C for 15 seconds and annealing with extension at 60°C for 1 minute.

Statistical analysis

Subjects were categorized into three groups: no/mild, moderate, and severe chronic periodontitis, using three different case definitions: (i) percentage of sites with CAL ≥5 mm, (ii) mean CAL, and (iii) the CDC/AAP case definition (Page and Eke 2007). For (i) and (ii), subjects were grouped according to the extent and severity of attachment loss, respectively, using the 25th and 75th percentiles as cut-off points.

Independent variables known to affect periodontal disease severity were included as follows: age (<50, ≥50), gender, education (<Bachelor degree, ≥Bachelor degree), smoking status (non-smokers, former smokers, current smokers), and being diabetics (Torrungruang et al. 2005a). The diagnosis of diabetes was determined by fasting blood sugar of ≥126 mg/dl or taking anti-diabetic drugs during the past two

weeks. Associations between different degrees of disease severity and each independent variable were analyzed using the χ^2 test. Genotype distributions of the VDR polymorphisms were tested for Hardy-Weinberg equilibrium using the χ^2 test. The association between genotype/allele distribution and periodontitis was assessed by the Fisher exact test in three models: allele 1 versus allele 2, genotype 11 versus 12+22, and genotype 11+12 versus 22. The strength of linkage disequilibrium between each pair of polymorphisms was measured as linkage disequilibrium coefficients (D') using Haploview version 4.2 (Barrett et al. 2005). The *Bsm*I-*Apa*I-*Taq*I haplotypes were constructed from individual genotypes by an expectation maximization algorithm using PHASE version 2.1 (Stephens et al. 2001). P <0.05 was considered statistically significant.

To further investigate the degree of association, multinomial logistic regression was used. The outcome was periodontal disease severity, where the risk for having moderate or severe periodontitis was predicted using individuals with no/mild periodontitis as the reference group. Independent variables included VDR polymorphisms and factors known to be associated with periodontal disease. To build the multivariate model, all variables were entered, and those with P >0.05 for both moderate and severe periodontitis groups were removed one by one from the model. Crude and adjusted odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated for each independent variable.

The interactions between VDR polymorphisms and smoking were examined with regard to the risk of periodontitis. The multiplicative interaction was estimated by introducing an interaction term into the model. For the additive interaction, three measures and their 95% CI were calculated: (i) relative excess risk due to interaction (RERI); (ii) attributable proportion due to interaction (AP); and (iii) synergy index (S) (Andersson et al. 2005). RERI =0, AP =0, and S =1 indicate no interaction or strict additivity, whereas RERI >0, AP >0, or S >1 indicates positive interaction or more than additivity. If the 95% CI of any of these measures did not include the null values (0 for RERI/AP or 1 for S), the additive interaction was considered statistically significant. Excluding the calculation of linkage disequilibrium, all statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA).

CHAPTER 4

RESULT

The study population comprised 1,460 individuals, aged 39-65 years (mean±SD: 47±4). Using percentages of sites with CAL ≥5 mm as the outcome variable, the participants were categorized into three groups: 370 (25.3%) were classified with no/mild periodontitis, 725 (49.7%) with moderate periodontitis, and 365 (25.0%) with severe periodontitis. The associations between periodontal disease severity and basic characteristics of the study subjects are presented in Table 1. Periodontitis was more severe in older and less educated individuals, males, current and former smokers, and those with diabetes (P <0.001).

Genotype distributions of VDR polymorphisms in the subjects with no/mild periodontitis were consistent with the assumption of Hardy-Weinberg equilibrium (P >0.001). Frequencies of the VDR genotypes and alleles are displayed in Table 2. A significant difference was found only in the distribution of the *Fok*I risk genotypes (CC+CT) between the subjects with no/mild and moderate periodontitis (P =0.038) and between the subjects with no/mild and severe periodontitis (P =0.001). The frequency of the *Fok*I risk genotypes increased with increased disease severity, from 71.4% in the no/mild periodontitis group to 77.2% and 81.7% in moderate and severe periodontitis groups, respectively. The other VDR polymorphisms were not significantly associated with periodontal disease severity.

*Fok*I polymorphism was not in strong linkage disequilibrium with any VDR polymorphisms (D' <0.80). In contrast, *Bsm*I, *Apa*I, and *Taq*I polymorphisms exhibited strong linkage disequilibrium (D': 0.85-1.00). Therefore, the haplotypes consisting of these three polymorphisms were constructed including GCT, GAT, AAC, AAT, GAC, and GCC with the frequencies of 66.1%, 23.2%, 7.2%, 2.3%, 1.1%, and 0.1%, respectively. The associations between periodontal disease severity and the haplotypes with a frequency of more than 1% were examined. However, none of these *Bsm*I-*Apa*I-*Taq*I haplotypes were significantly associated with periodontal disease severity (P >0.05) (data not shown).

The degree of association between VDR polymorphisms or *Bsm*I-*Apa*I-*Taq*I haplotypes and periodontal disease severity was tested using multinomial logistic regression (Table 3). When regressed univariately, the *Fok*I risk genotypes (CC+CT) were significantly associated with periodontitis, whereas the other VDR polymorphisms and *Bsm*I-*Apa*I-*Taq*I haplotypes showed no significant association with disease. When regressed multivariately, the *Fok*I risk genotypes remained significantly associated with both moderate and severe periodontitis with adjusted ORs of 1.4 (95% CI 1.0-1.9; P =0.033) and 2.0 (95% CI 1.3-2.9; P =0.001), respectively. The other VDR polymorphisms and *Bsm*I-*Apa*I-*Taq*I haplotypes did not reach statistical significance and were not included in the multivariate model. Repeating the analyses using mean CAL or the CDC/AAP case definition as outcome variables confirmed that the *Fok*I risk genotypes

were correlated with severe periodontitis, although at a lower significance level (data not shown).

The association between *Fok*I polymorphism and periodontal disease severity, stratified by smoking status is presented in Table 4. Non-smokers carrying the TT genotype were used as the reference group for all comparisons. Current smokers who had the CC+CT genotypes experienced the greatest risk of developing severe periodontitis with an adjusted OR of 10.4 (95% CI 4.9-22.1; P <0.001), whereas their counterparts with the TT genotype and non-smokers bearing the CC+CT genotypes had an increased risk by 2.7 (95% CI 1.1-6.7; P <0.05) and 2.0 folds (95% CI 1.2-3.4; P <0.05), respectively. The interaction between *Fok*I polymorphism and smoking status was not significantly associated with periodontitis on a multiplicative scale (P =0.320). However, there was a significantly additive interaction between the *Fok*I risk genotypes (CC+CT) and current smoking for the risk of severe periodontitis, as indicated by the 95% CI of AP and S values that did not include the null values (Table 5). The estimate for the AP value was 0.7 (95% CI 0.4-0.9), meaning that 70% of severe periodontitis cases in subjects who had *Fok*I risk genotypes and currently smoked were due to the interaction of these two factors. Additionally, the S value of 3.5 (95% CI 1.3-9.9) indicated that the combined effect of the *Fok*I risk genotypes and current smoking was 3.5 times greater than expected based on the sum of the individual effects of these two

factors. Neither multiplicative nor additive interactions were observed between the other VDR polymorphisms or *Bsm*I-*Apa*I-*Taq*I haplotypes and smoking (data not shown).

Table 1. Basic characteristics of the study subjects according to periodontal disease severity

Characteristics	Total	Periodontitis			*P* value*
	N = 1460	No/mild	Moderate	Severe	
Age, N (%)					
≥ 50 (50-65)	423	63 (14.9)	203 (48.0)	157 (37.1)	<0.001
< 50 (39-49)	1037	307 (29.6)	522 (50.3)	208 (20.1)	
Gender, N (%)					
Male	1044	193 (18.5)	535 (51.2)	316 (30.3)	<0.001
Female	416	177 (42.5)	190 (45.7)	49 (11.8)	
Education, N (%)					
< Bachelor degree	680	123 (18.1)	311 (45.7)	246 (36.2)	<0.001
≥ Bachelor degree	780	247 (31.7)	414 (53.1)	119 (15.3)	
Smoking status, N (%)	250	26 (10.4)	104 (41.6)	120 (48.0)	<0.001
Current smokers	337	56 (16.6)	185 (54.9)	96 (28.5)	
Former smokers	873	288 (33.0)	436 (49.9)	149 (17.1)	
Non-smokers					
Diabetes, N (%)					
Yes	86	9 (10.5)	38 (44.2)	39 (45.3)	<0.001
No	1374	361 (26.3)	687 (50.0)	326 (23.7)	

* Associations between different degrees of periodontal disease severity and each independent variable were analyzed using the χ^2 test.

Table 2. Distribution of VDR genotypes and alleles according to periodontal disease severity

Polymorphisms (allele1/allele2)*	Genotype/ Allele	Periodontitis, N (%)			Model	*P* value†	
		No/Mild	Moderate	Severe		Moderate Periodontitis	Severe Periodontitis
*Fok*I (C/T)	CC	111 (30.0)	211 (29.1)	113 (31.0)	1 VS 2	0.278	**0.032**
	CT	153 (41.4)	349 (48.1)	185 (50.7)	11 VS 12+22	0.779	0.810
	TT	106 (28.6)	165 (22.8)	67 (18.4)	11+12 VS 22	**0.038**	**0.001**
	Allele C‡	375 (50.7)	771 (53.2)	411 (56.3)			
	Allele T	365 (49.3)	679 (46.8)	319 (43.7)			
*Bsm*I (G/A)	GG	303 (81.9)	589 (81.2)	291 (79.7)	1 VS 2	0.816	0.535
	AG	67 (18.1)	136 (18.8)	74 (20.3)	11 VS 12+22	0.806	0.512
	Allele G	673 (90.9)	1314 (90.6)	656 (89.9)	11+12 VS 22	1.000	1.000
	Allele A‡	67 (9.1)	136 (9.4)	74 (10.1)			
*Apa*I (C/A)	CC	177 (47.8)	322 (44.4)	152 (41.6)	1 VS 2	0.631	0.226
	AC	146 (39.5)	320 (44.1)	167 (45.8)	11 VS 12+22	0.305	0.103
	AA	47 (12.7)	83 (11.5)	46 (12.6)	11+12 VS 22	0.554	1.000
	Allele C	500 (67.6)	964 (66.5)	471 (64.5)			
	Allele A‡	240 (32.4)	486 (33.5)	259 (35.5)			
*Taq*I (T/C)	TT	314 (84.9)	606 (83.6)	309 (84.7)	1 VS 2	0.571	1.000
	CT	53 (14.3)	111 (15.3)	53 (14.5)	11 VS 12+22	0.602	1.000
	CC	3 (0.8)	8 (1.1)	3 (0.8)	11+12 VS 22	0.759	1.000
	Allele T	681 (92.0)	1323 (91.2)	671 (91.9)			
	Allele C‡	59 (8.0)	127 (8.8)	59 (8.1)			

*Annotation data from the February 2009 human reference sequence (GRCh37) was retrieved on January 20, 2015 from the UCSC Genome Browser database (Karolchik et al. 2014).

†Associations between different degrees of periodontal disease severity and genotype/allele distribution were analyzed using the Fisher exact test. A significant association (P <0.05) is indicated in bold.

‡Risk allele is indicated as an allele with higher frequencies in individuals with moderate and severe periodontitis compared to those with no/mild periodontitis.

Table 3. Crude and adjusted odds ratios (OR) and 95% confidence intervals (CI) for the risk of moderate and severe periodontitis

Variables	Crude OR (95% CI)		Adjusted OR (95% CI)*	
	Moderate Periodontitis	Severe Periodontitis	Moderate Periodontitis	Severe Periodontitis
Age ≥ 50	1.9 (1.4 - 2.6)§	3.7 (2.6 - 5.2)§	1.8 (1.3 - 2.4)‡	3.3 (2.3 - 4.8)§
Male	2.6 (2.0 - 3.4)§	5.9 (4.1 - 8.5)§	2.0 (1.5 - 2.7)§	3.1 (2.1 - 4.8)§
< Bachelor degree	1.5 (1.2 - 2.0)‡	4.2 (3.1 - 5.6)§	1.3 (1.0 - 1.7)	2.8 (2.0 - 4.0)§
Current smokers	2.6 (1.7 - 4.2)§	8.9 (5.6 - 14.2)§	1.7 (1.1 - 2.8)†	4.2 (2.5 - 7.1)§
Former smokers	2.2 (1.6 - 3.0)§	3.3 (2.3 - 4.9)§	1.4 (1.0 - 2.1)	1.7 (1.1 - 2.6)†
Diabetes	2.2 (1.1 - 4.6)†	4.8 (2.3 - 10.1)§	1.7 (0.8 - 3.6)	2.6 (1.2 - 5.9)†
*Fok*I (CC+CT)	1.4 (1.0 - 1.8)†	1.8 (1.3 - 2.5)‡	1.4 (1.0 - 1.9)†	2.0 (1.3 - 2.9)‡
*Bsm*I (AA+AG)	1.0 (0.8 - 1.4)	1.2 (0.8 - 1.7)	-	-
*Apa*I (AA+AC)	1.1 (0.9 - 1.5)	1.3 (0.9 - 1.7)	-	-
*Taq*I (CC+CT)	1.1 (0.8 - 1.6)	1.0 (0.7 - 1.5)	-	-
GCT haplotype	1.1 (0.8 - 1.6)	1.0 (0.7 - 1.6)	-	-
GAT haplotype	1.1 (0.9 - 1.4)	1.2 (0.9 - 1.6)	-	-
GAC haplotype	1.3 (0.5 - 3.2)	0.9 (0.3 - 2.6)	-	-
AAT haplotype	0.9 (0.5 - 1.7)	1.4 (0.7 - 2.6)	-	-
AAC haplotype	1.1 (0.8 - 1.6)	1.0 (0.7 - 1.6)	-	-

*OR and 95% CI, adjusted for age, gender, education, smoking and diabetes, determined by multinomial logistic regression analysis using individuals with no/mild periodontitis as the reference group.

† P <0.05 and ≥0.01.

‡ P <0.01 and ≥0.001.

§ P <0.001.

Table 4. Association between *Fok*I polymorphism and periodontal disease severity stratified by smoking status

	Frequency, N				Adjust OR (95% CI)*	
	*Fok*I (CC+CT)		*Fok*I (TT)		*Fok*I (CC+CT)	*Fok*I (TT)
	Cases	Controls	Cases	Controls		
Moderate Periodontitis						
Current smokers	74	15	30	11	2.7 (1.4 - 5.2)‡	1.4 (0.7 - 3.1)
Former smokers	145	42	40	14	1.9 (1.1 - 3.1)†	1.6 (0.8 - 3.2)
Non-smokers	341	207	95	81	1.4 (1.0 - 1.9)	Reference
Severe Periodontitis						
Current smokers	99	15	21	11	10.4 (4.9 - 22.1)§	2.7 (1.1 - 6.7)†
Former smokers	74	42	22	14	2.9 (1.5 - 5.6)‡	2.6 (1.1 - 6.2)†
Non-smokers	125	207	24	81	2.0 (1.2 - 3.4)†	Reference

*OR and 95% CI, adjusted for age, gender, education, and diabetes, determined by multinomial logistic regression analysis. A multiplicative interaction between *Fok*I polymorphism and smoking status was not significantly associated with chronic periodontitis ($P = 0.320$).

† $P < 0.05$ and ≥ 0.01.

‡ $P < 0.01$ and ≥ 0.001.

§ $P < 0.001$.

CI, confidence intervals; OR, odds ratio.

Table 5. Measures of the additive interaction between *Fok*I polymorphism and smoking status in relation to the risk of chronic periodontitis

	RERI (95% CI)	AP (95% CI)	S (95% CI)
Moderate Periodontitis			
Current smokers	0.9 (-0.9 – 2.8)	0.3 (-0.2 – 0.9)	2.2 (0.4 – 11.5)
Former smokers	-0.1 (-1.3 – 1.2)	0.0 (-0.7 – 0.6)	1.0 (0.3 – 3.6)
Severe Periodontitis			
Current smokers	6.71 (-0.1 – 13.6)	**0.7 (0.4 – 0.9)***	**3.5 (1.3 – 9.9)***
Former smokers	-0.7 (-3.0 – 1.7)	-0.2 (-1.0 – 0.6)	0.8 (0.3 – 1.9)

* A significant additive interaction, as indicated by the 95% CI of these measures that do not include the null values (0 for AP and 1 for S).

AP, attributable proportion due to interaction; CI, confidence intervals; RERI, relative excess risk due to interaction; S, synergy index.

CHAPTER 5

DISCUSSION

In the present study, we examined the associations between VDR polymorphisms and the risk of chronic periodontitis in a Thai population. The results showed that *FokI*, but not *Bsm*I, *Apa*I, or *Taq*I, polymorphism was significantly associated with periodontal disease severity. In addition, there was an additive interaction between the *Fok*I risk genotypes and current smoking for the risk of severe periodontitis.

Our study demonstrated that the risk genotypes (CC+CT) of *Fok*I polymorphism were associated with both moderate and severe chronic periodontitis. A positive association between the CC genotype of *Fok*I polymorphism and aggressive periodontitis was reported in Korean (Park et al. 2006) and eastern Chinese (Li et al. 2008) populations, while no association was observed between *Fok*I polymorphism and chronic periodontitis in Japanese (Tachi et al. 2003) and southern Chinese (Wang et al. 2009) populations. The discrepancy between these findings may be related to ethnicity as the distribution of VDR polymorphisms varies among different populations. We found that the minor allele frequency for *FokI* (T allele) in the subjects with no/mild periodontitis was 49.3%, which was higher than those reported in Chinese (44.2%) and Japanese (32.6%) populations (dbSNP 2014 Oct 16). Additionally, the lack of statistical power and heterogeneity in periodontitis case definitions may contribute to the differences

observed between studies. The sample size used in the present study was much larger than those of previous studies, thereby providing sufficient statistical power to detect mild genetic effects in chronic periodontitis. Furthermore, we used three different case definitions in our analyses, which consistently demonstrated that the risk genotypes of *Fok*I polymorphism were related to enhanced susceptibility to disease.

Consistent with previous studies (Gunes et al. 2008; Tanaka et al. 2013), strong linkage disequilibrium was found between *Bsm*I, *Apa*I, and *Taq*I polymorphisms. Therefore, the combined analysis of these three variants could be more powerful in identifying individuals at risk for periodontitis. Nevertheless, our results indicated that none of the *Bsm*I-*Apa*I-*Taq*I haplotypes were significantly associated with periodontal disease severity. A similar finding was observed in a previous study in Japanese subjects (Tanaka et al. 2013). However, different findings were reported in Brazilian and Turkish populations. The GT haplotype of *Bsm*I and *Taq*I polymorphisms was associated with chronic periodontitis in Brazilians (de Brito Junior et al. 2004). In addition, the GAT haplotype of *Bsm*I, *Apa*I, and *Taq*I polymorphisms increased the susceptibility to severe chronic periodontitis in Turkish subjects (Gunes et al. 2008). It should be noted that the *Bsm*I, *Apa*I, and *Taq*I polymorphisms by themselves are not functional polymorphisms, but they are markers for unidentified functional alleles located elsewhere in the gene (Uitterlinden et al. 2004). The functional alleles that are in linkage disequilibrium with

these markers are likely to be different among ethnic groups. This could partly explain the varying results observed in the association studies.

The mechanism by which *Fok*I polymorphism influences the susceptibility to periodontal disease has not been clarified. The *Fok*I polymorphism is the only known polymorphism that affects the structure of VDR proteins (Uitterlinden et al. 2004). This polymorphism is located eight nucleotides upstream of the initiation start site in exon 2. The T variant creates an additional start codon (ACG to ATG), thus forming a protein with three amino acids longer than the C variant (427 instead of 424 amino acids). In transfection experiments, the presence of short VDR (C variant) lead to a higher activity of immune-specific transcription factors NF-κB and NFAT, compared to the presence of long VDR (T variant) (van Etten et al. 2007). Concordantly, human immune cells with a short VDR genotype had a greater phytohemagglutinin-stimulated proliferation and higher expression of cytokines than did the cells with a long VDR genotype. Furthermore, in patients with multiple sclerosis, a short VDR genotype was associated with an increased RANKL/OPG ratio in serum (Mirzaei et al. 2012). Taken together, the short VDR appears to transmit stronger bone resorption and inflammatory signals, which are the hallmark for the pathogenesis of periodontitis.

Periodontitis is a multifactorial disease, in which genetic and environmental factors may interact in increasing an individual's susceptibility to disease. Evidence suggests that smoking is the most important environmental factor for periodontitis

(Johannsen et al. 2014). Smoking has been shown to affect both serum and local levels of several inflammatory mediators, mainly through activation of NF-ΚB (Yang et al. 2007; Johannsen et al. 2014). Smoking also affects bone metabolism as smokers have been reported to have an increased RANKL/OPG ratio in serum and in periodontal tissues compared with non-smokers (Cesar-Neto et al. 2007; Lappin et al. 2007). Considering that VDR polymorphisms and smoking work along a similar pathogenesis pathway, one could speculate that the interaction between these two factors may synergistically increase the risk of periodontitis.

To date, only few studies have examined the interaction between VDR polymorphisms and smoking that affects periodontal disease. A multiplicative interaction was found between *Taq*I polymorphism and smoking with respect to the presence and progression of periodontitis in Caucasians (Nibali et al. 2008). Another study reported an additive interaction between *Apa*I polymorphism and smoking for the risk of periodontitis in Japanese women (Tanaka et al. 2013). However, these previous results differ from our study that found neither multiplicative nor additive interactions between *Taq*I or *Apa*I and smoking. Instead, we observed a significantly additive interaction between *Fok*I polymorphism and smoking in relation to the risk of chronic periodontitis. Compared with genotype-negative (TT) non-smokers, positivity for the risk genotypes (CC+CT) alone increased the risk of severe periodontitis by 2.0-fold, while current smoking alone increased the disease risk by 2.7-fold. The combined effect of being genotype positive

and smoking further increased the disease risk by 10.4-fold, which was 3.5 times greater than what would be expected from the sum of their individual effects. Despite the conflicting results between our study and others, these data provide supportive evidence of synergistic interactions between VDR polymorphisms and smoking in the pathogenesis of chronic periodontitis. Whether smoking differentially interacts with these four VDR polymorphisms in different populations, and how this interaction is mechanistically involved in the pathogenesis pathway of periodontitis remain to be elucidated.

Several factors have been shown to be associated with periodontal disease severity, including age, gender, education, smoking, and diabetes (Torrungruang et al. 2005a). These confounders were controlled for in our study using multinomial logistic regression. However, it is possible that our results may be confounded by other factors not included in the model such as serum vitamin D and the amount of vitamin D intake of each subject. Another limitation of our study was that the study population was not a random sample, thereby it cannot be said to be representative of the Thai population. Nevertheless, the distribution of VDR polymorphisms in this study is comparable to those reported in different groups of Thais (Chaimuangraj et al. 2006; Wananukul et al. 2012). Therefore, we can rule out the possibility of selection bias associated with genotype distribution in our study.

CHAPTER 6

CONCLUSION

In conclusion, our results demonstrated that the risk genotypes (CC+CT) of *Fok*I polymorphism were related to enhance susceptibility to chronic periodontitis in a Thai population. Analysis of VDR polymorphisms is useful for identifying individuals at the risk of developing disease so that appropriate preventive and treatment strategies could be tailored-made for each individual. In addition, the gene-smoking interaction observed in our study suggests that smoking cessation should be emphasized in individuals bearing the *Fok*I risk genotypes because quitting smoking will reduce the risk of periodontitis that would have been caused not only by smoking but also by the interaction of the two factors.

SUGGESTION FOR FURTHER WORK

As chronic periodontitis is a multifactorial disease chronic so other genetic predisposing may involve in the disease severity and progression. Further study should investigate in more gene polymorphisms and their interactions as well as with other environmental risk factors.

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