Association analysis of *FAS*-670A/G and *FASL*-844C/T polymorphisms with risk of generalized aggressive periodontitis disease

REZVAN ASGARI[1], KHEIROLLAH YARI[1,2], KAMRAN MANSOURI[1] and MITRA BAKHTIARI[3]

[1]Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah 67148-69914; [2]Molecular Genetics Laboratory, Zagros Bioidea Company, Incubator Center of Razi University, Kermanshah 67146-73175; [3]Department of Anatomical Sciences and Cell Biology, Kermanshah University of Medical Sciences, Kermanshah 67148-69914, Iran

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Abstract. The interaction of FAS/FAS ligand (FASL) serves an important role in the upregulation of apoptotic processes through different mechanisms in cells. Previous studies have established that the polymorphisms *FAS*-670A/G and *FASL*-844C/T are associated with risk of generalized aggressive periodontitis (GAP) in different ethnic populations. Therefore, in the present study, it was investigated for the first time whether *FAS*-670A/G and *FASL*-844C/T polymorphisms were associated with risk of GAP in Iran. This case-control study performed the polymerase chain reaction-restriction fragment length polymorphism method in 25 patients with GAP and 110 normal subjects as controls. The results indicated that there was no significant difference in *FAS*-670A/G genotype frequency between the GAP and control groups. A higher frequency of the combined genotype (AG+GG) was observed in the GAP patients (96.0%) compared with the control subjects (90.9%), though this was not significant [χ^2=0.705, degrees of freedom (df)=1, P=0.401]. Similarly, the prevalence of the G allele was non-significantly higher in the GAP group (62.0%) compared with that in the controls (60.0%; χ^2=0.012, df=1, P=0.913). For FASL-844C/T polymorphism, the frequency of the combined genotype (CT+TT) was higher in the GAP group (96.0%) when compared with the control subjects (91.8%); however its association was not statistically significant (χ^2=0.519, df=1, P=0.471). The frequency of the T allele only marginally differed between the groups, being 60.0% in the GAP group and 50.9% in the controls (χ^2=3.627, df=1, P=0.057). These results indicated that there were no significant associations between the *FAS*-670A/G and *FASL*-844C/T polymorphisms and the risk of disease in GAP patients when compared with normal individuals.

Correspondence to: Dr Kheirollah Yari, Medical Biology Research Center, Kermanshah University of Medical Sciences, Daneshgah Avenue, Kermanshah 67148-69914, Iran
E-mail: kyari@kums.ac.ir

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Introduction

Periodontal disease is a heterogeneous infection of the tissues supporting the teeth, primarily characterized by gingival inflammation (1), and may lead to tooth loss and destruction of the tissues surrounding and supporting the teeth (2,3). It is established that certain microorganisms serve important roles in the development of periodontal disease, and the disease has been associated with a number of infectious diseases, as well as chronic diseases including cancer (4). There are two types of periodontitis disease, namely aggressive (localized and generalized) and chronic, both of which involve loss of supporting structures of the teeth (5). Aggressive periodontitis is characterized by fast rates of progression, tissue attachment-loss and tissue destruction (6). Due to its increasing prevalence worldwide, aggressive periodontitis is regarded as a major health problem. The pathogenesis and development of periodontitis disease are associated with an interaction of immunological, genetic and environmental variables (7). In particular, genetic background has been significantly associated with susceptibility to periodontitis disease (3,8). There have been several studies on genetic factors implicated in the pathogenesis and severity of the diseases (3,9,10), and a number of key genes have been demonstrated to be involved in disease susceptibility and severity, or in determining the clinical course (11). The process of apoptosis serves a critical role in the development of periodontitis disease; therefore, genes related to this process are common candidates in genetic analyses regarding possible associations with disease course (12).

Apoptosis is among the main physiological types of cell death that is involved in different physiological and pathological functions including maintenance of tissue homeostasis, regulating cell numbers and eliminating unwanted or potentially dangerous cells during organism development (13). Therefore, alterations in apoptotic pathways and abnormal regulation of apoptosis contributes to the pathogenesis of various human

diseases including cancer and inflammatory diseases (14). In previous reports, there has been focus on the *FAS*/*FASL* axis as a key and major pathway involved in apoptosis induction, in the prevention of autoimmune diseases and in the maintenance of immune tolerance (12,15,16). The *FAS* and *FASL* genes are located on chromosome 10q24.1 and 1q23, respectively. FAS also known as tumor necrosis factor (TNF) receptor superfamily member 6, cluster of differentiation (CD)95 or apoptosis antigen 1 is a type I cell membrane receptor protein that is expressed in a variety of tissues and serves a critical role in apoptotic signaling in many cell types (17). FASL (also known as TNF superfamily member 6 or CD95 ligand), as a natural ligand of FAS, is a type II transmembrane protein belonging to the TNF superfamily that can trigger cell death signal cascades by cross-linking with FAS (18). Several previous studies have reported that decreased expression of *FAS* and/or elevated expression of *FASL* may be associated with numerous types of human cancer and immunological diseases (13,19-21).

Substitution of A to G at position -670 in the promoter region of *FAS* and C to T substitution at position -844 in the promoter region of *FASL* are among the major single-nucleotide polymorphisms to have been identified in the genes (22). The *FAS* polymorphism -670A/G is situated within the signal transducers and activators of transcription 1 transcription factor binding sites; thus, presence of the -670G allele diminishes promoter activity and decreases *FAS* gene expression (21,23). The *FASL* 844C/T polymorphism is located in a putative binding motif of CAAT/enhanced-binding protein β element (24).

Due to the association of apoptosis with aggressive periodontitis and FAS/FASL, the aim of the present study was to evaluate whether the *FAS*-670A/G and *FASL*-844C/T polymorphisms were associated with disease course in a Kurdish population with generalized aggressive periodontitis (GAP) in Western Iran.

Materials and methods

Materials. The DNA extraction kit, agarose, Taq polymerase and dNTPs used for polymerase chain reaction (PCR) experiments were purchased from Zagros Bioidea Co. (Incubator Center of Razi University, Kermanshah, Iran). The *Mva*I and *Bsr*DI restriction enzymes were supplied by New England BioLabs, Inc. (Ipswich, MA, USA). All other chemicals and reagents of analytical grade were supplied by Merck KGaA (Darmstadt, Germany). Forward and reverse primers were synthesized by SinaClon Bioscience Co. (Tehran, Iran).

Sample collection. A total of 25 patients with GAP were enrolled, comprising of 87% females and 13% males with a mean age of 21.0±3.5 years (3). All subjects were enrolled from the School of Dentistry, Qazvin University of Medical Sciences (Qazvin, Iran) from November, 2011 to May, 2012. As a control group, 110 men with the same ethnic background as patients, who were free from signs of periodontitis (mean age, 33.91±7.43 years), were also enrolled. All patients and control individuals were free of general and genetics diseases. Periodontal disease was diagnosed according to clinical and radiographic parameters that included bleeding on probing, probing pocket depth and clinical attachment loss. Aggressive periodontitis was defined by inter-proximal attachment loss affecting at least three teeth that were either first molars or incisors (3,6,25). For the patient and control groups, the exclusion criteria were a history of HIV and/or hepatitis infection, diabetes, usage of anti-inflammatory and/or immunosuppressive drugs, pregnancy or lactation, and smoking. The current research procedures were approved by the ethics committee of Kermanshah University of Medical Sciences (Kermanshah, Iran). All subjects enrolled in the study were informed of the aim and procedures of the research and provided written informed consent in accordance with the Helsinki II declaration.

DNA extraction. From each subject, 5-ml blood samples were collected in micro-tubes containing EDTA. DNA was extracted from whole blood using the DNA isolation kit (Zagros Bioidea Co.) (26). The purified genomic DNA was analyzed and verified by electrophoresis on 1% agarose gel. The DNA concentration and purity were assessed with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) at wavelengths of 260 and 280 nm (27,28).

Genotyping. *FAS* and *FASL* gene polymorphisms were assessed with a PCR-based restriction fragment length polymorphism (PCR-RFLP) method (19).

The PCR was conducted to amplify the *FAS* and *FASL* polymorphisms with the following primers: For *FAS*-670A/G, forward, 5'-GGCTGTCCATGTTGTGGCTGC-3' and reverse, 5'-CTACCTAAGAGCTATCTACCGTTC-3' (22); and for *FASL*-844C/T, forward, 5'-CAGCTACTCAGGAGGCCA AG-3' and reverse, 5'-GCTCTGAGGGGAGAGACCAT-3' (20). The PCR thermal cycling conditions were as follows: For *FAS*, one cycle of denaturation at 94˚C for 5 min followed by 30 cycles at 94˚C for 30 sec, 61˚C for 30 sec and 72˚C for 30 sec, with a final extension at 72˚C for 6 min; and for *FASL*, one cycle of denaturation at 94˚C for 5 min followed by 30 cycles at 94˚C for 30 sec, 67˚C for 30 sec and 72˚C for 30 sec, with a final extension at 72˚C for 6 min. PCR was performed in a total volume of 25 μl containing 0.5 μM of each primer, 2 μl DNA (100-300 ng) as the template, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase and 2.5 μl 10X PCR buffer (200 mM Tris HCl, pH 8.4, 500 mM KCl).

For *FAS*-670A/G, the obtained PCR product (331 bp) was digested with *Mva*I at 37˚C for 15 h. The resulting fragment sizes were as follows: 189, 98 and 44 bp for the -670AA genotype, and 233 and 98 bp for the -670GG genotype. For *FASL*-844C/T, the obtained PCR product (401 bp) was digested with *Bsr*DI at 37˚C for 15 h. In the presence of the -844CC genotype, the PCR product was digested into two fragments of 233 and 168 bp, while in the presence of the -844T allele a fragment of 401 bp was observed (19). Digested PCR products were identified on a 2% agarose gel stained with Gel Red DNA stain under ultraviolet light.

Statistical analysis. The Pearson's χ^2 test was used to compare differences in frequency distributions of alleles and genotypes of the *FAS* and *FASL* polymorphisms. All statistical tests were two-sided, and $P<0.05$ was assumed to indicate statistical significance. SPSS statistical software version 21.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses.

Table I. Frequency of *FAS*-670A/G genotypes and alleles in GAP patients and controls.

Genotype/alleles, n (%)	GAP group (n=25)	Control group (n=110)
-670A/G genotype		
AA	(4.0) 1	10 (9.1)
AG	17 (68.0)	68 (61.8)
GG	7 (28.0)	32 (29.1)
(χ^2=0.501, df=2, P=0.489)		
AG+GG	24 (96.0)	100 (90.9)
(χ^2=0.705, df=1, P=0.401)		
-670A/G alleles		
A	19 (38.0)	88 (40.0)
G	31 (62.0)	132 (60.0)
(χ^2=0.012, df=1, P=0.913)		

GAP, generalized aggressive periodontitis; df, degrees of freedom.

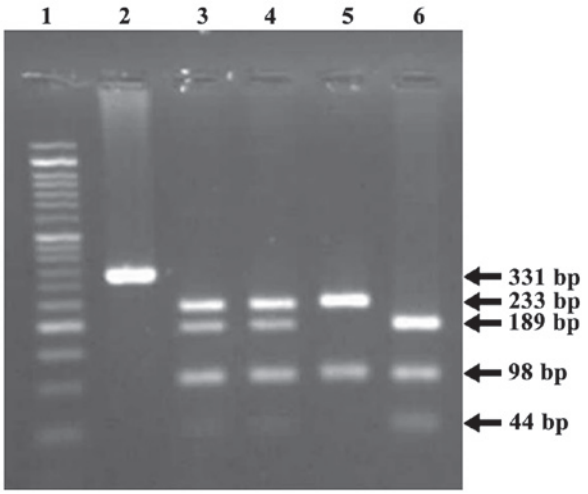

Figure 1. Agarose gel electrophoresis pattern of *FAS*-670A/G genotype products digested with *Mva*I. From left to right: Lane 1, marker (50-bp ladder); lane 2, PCR product of 331 bp; lanes 3 and 4, AG genotype (233, 189, 98 and 44 bp); lane 5, GG genotype (233 and 98 bp); lane 6, AA genotype (189, 98 and 44 bp).

Table II. Frequency of FASL-844C/T genotypes and alleles in GAP patients and controls.

Genotype/alleles, n (%)	GAP group (n=25)	Control group (n=110)
-844C/T genotype		
CC	1 (4.0)	9 (8.2)
CT	18 (72.0)	90 (81.8)
TT	6 (24.0)	11 (10.0)
(χ^2=3.897, df=2, P=0.142)		
CT+TT	24 (96.0)	101 (91.8)
(χ^2=0.519, df=1, P=0.471)		
-844C/T alleles		
C	20 (40.0)	108 (49.1)
T	30 (60.0)	112 (50.9)
(χ^2=3.627, df=1, P=0.057)		

GAP, generalized aggressive periodontitis; df, degrees of freedom.

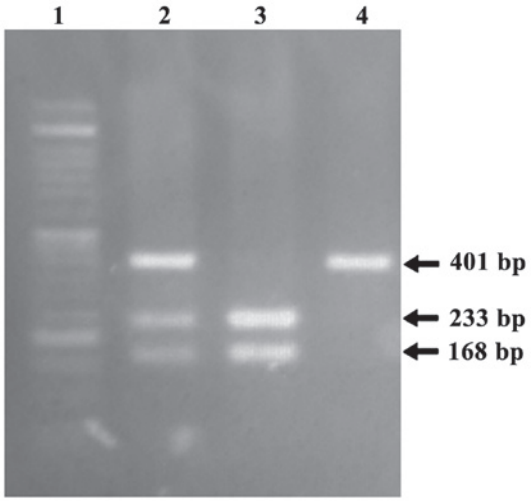

Figure 2. Agarose gel electrophoresis pattern of *FASL*-844C/T genotype products digested with *Bsr*DI. From left to right: Lane 1, marker (50-bp ladder); lane 2, CT genotype (401, 233 and 168 bp); lane 3, CC genotype (233 and 168 bp); lane 4, TT genotype (401 bp).

Results

FAS-670A/G and FASL-844C/T products. Fragments of 331 and 401 bp in size corresponding to the *FAS* and *FASL* genes, respectively, were amplified by PCR. Figs. 1 and 2 depict the fragment separation pattern of the *FAS*-670A/G and *FASL*-844C/T genotypes, respectively, following agarose gel electrophoresis.

FAS-670A/G genotype frequencies. The allele frequencies of the *FAS* and *FASL* genotypes were in Hardy-Weinberg equilibrium. The frequencies of the *FAS*-670A/G genotypes and alleles among the GAP and control subjects are listed in Table I. A higher frequency of the combined genotype (AG+GG) was observed in the GAP patients (96.0%) compared with the control subjects (90.9%), though the difference was not significant [χ^2=0.705, degrees of freedom (df)=1, P=0.401]. Similarly, the prevalence of the G allele was non-significantly higher in the GAP group (62.0%) compared with that in the controls (60.0%; χ^2=0.012, df=1, P=0.913).

FASL-844C/T genotype frequencies. For *FASL*-844C/T genotype, as indicated in Table II, the frequency of the TT genotype was non-significantly higher in the GAP group (24.0%) when compared with the control subjects (10.0%; χ^2=3.897, df=2, P=0.142). Additionally, the frequency of the combined genotype (CT+TT) was higher in the GAP group (96.0%) compared with the controls (91.8%); however, its association was not statistically significant (χ^2=0.519, df=1, P=0.471). The frequency of the T allele was 60.0% in the GAP group and

50.9% in the controls, though was deemed to not differ significantly between the groups (χ^2=3.627, df=1, P=0.057).

Discussion

The present study assessed the potential association between two known polymorphisms in cell death pathway genes (*FAS*-670A/G and *FASL*-844C/T) and the presentation of GAP in Western Iranian patients. The absence of a significant association between the *FAS*-670A/G polymorphism with GAP risk was observed. Additionally, the study presented here confirmed no significant associations between -844C/T alleles of *FASL* polymorphism with GAP. Therefore, the study indicated that -844C/T variants of *FASL* and -670A/G variants of *FAS* were not associated with GAP in the Iranian population studied.

Periodontal disease as an inflammatory illness is a primary cause of tooth loss. GAP, as a sub-type of periodontitis disease, affects the supporting tissue of the teeth with inflammatory infection (3). Aggressive periodontitis as local or general-type generally presents in individuals between the ages of 20 and 35 years old, as damage to the first incisive and molar alveolar bone (9). Previous studies have demonstrated that microbial agents, certain environmental risk factors including living conditions and dietary habits, and genetic susceptibility are necessary for the pathogenesis and development of periodontal disease (10,29). Additionally, previous findings also indicated that genetic factors were significantly associated with progression of periodontal disease in various ethnic populations (29).

The present study indicated that there were no significant associations between the *FAS*-670A/G and *FASL*-844C/T polymorphisms and the presentation of GAP in patients when compared with control individuals.

Apoptosis, as a form of programmed cell death, is a fundamental biological process that occurs in multi-cellular organisms to maintain tissue turnover (17). Interactions of FAS/FASL may serve a crucial role in preventing autoimmune diseases and maintaining immune tolerance (17,30). FAS and FASL, as type-II transmembrane proteins that belong to the TNF family, have been identified in inflammatory infiltrates in patients with periodontitis disease (19). In general, FASL has been implicated in the induction of T-cell apoptosis (11). Alterations in the regulation of FAS/FASL may contribute to the pathogenesis of various human diseases including cancer and inflammatory diseases such as periodontitis (31).

A number of studies have been conducted to analyze the potential relationship between the main polymorphisms of candidate genes with susceptibility to periodontitis diseases. For instance, Kazemi *et al* (10) reported an association between manganese superoxide dismutase (Val-9Ala) genotype with risk of GAP. Ayazi *et al* (3) identified a positive association between interleukin-1β gene polymorphism and risk of periodontitis disease. Darvishi *et al* (9) determined that there was no significant association between the TNF-α-1031(T/C) genotypes and GAP in an Iranian population. Furthermore, Menezes and Colombo (32) in Brazil and Heidari *et al* (33) in Iran reported that there was no significant association between chronic periodontitis with TNF-α (-308G/A) gene polymorphism. Certain studies have reported an association of the major polymorphisms *FAS*-670A/G and *FASL*-844C/T with different human diseases including prostate, bladder, gastric and cervical cancers, esophageal squamous cell carcinoma, systemic lupus erythematosus and infertility disorders (11,19,20). Wu *et al* (21) observed that *FAS*-670A/G gene polymorphism was associated with the severity of villous atrophy in coeliac disease. Sun *et al* (20) reported that polymorphisms in the *FAS* and *FASL* genes were associated with an increased risk of developing esophageal squamous-cell carcinoma in Chinese individuals. Wu *et al* (24) identified the major polymorphism -844C/T in the promoter region of *FASL* in African American patients with systemic lupus erythematosus. Additionally, they observed that -844C genotype may lead to increased risk of autoimmunity with enhanced expression levels of *FASL*, which led them to conclude that differences in the regulation of *FASL* expression may contribute to the development of the autoimmune phenotype (24).

To the best of our knowledge, there are no previous studies on the association of *FAS* and *FASL* polymorphisms with risk of periodontitis disease. However, Wohlfahrt *et al* (11) reported no significant association between selected candidate gene polymorphisms and severe chronic periodontitis in white North American individuals. Additionally, Brozovic *et al* (34) reported that *Porphyromonas gingivalis*, a Gram-negative anaerobe associated with severe periodontal disease, enhanced *FASL* expression through up-regulation of nuclear factor-κB-mediated gene transcription and induced apoptotic cell death in human gingival epithelial cells.

In conclusion, the current study, to the best of our knowledge, was the first to analyze the putative association of *FAS*-670A/G and *FASL*-844C/T polymorphisms with risk of GAP in an Iranian population; however, the results indicated that there were no significant associations of these polymorphisms with the presentation of GAP in patients when compared with normal individuals. Nevertheless, the present results may be used as a basis for studies on other major polymorphisms of candidate genes related to periodontitis disease. The results should now be confirmed by studies on larger groups in the future.

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