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Analysis of Transforming Growth Factor-Beta1 Gene Polymorphisms in Macedonian Patients with Chronic Periodontitis

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Abstract

Background. The existing conflicting data in the literature about the significance of *TGF-beta1* polymorphisms in periodontitis.

Aim. To determine whether *TGF-beta1* polymorphisms are associated with periodontitis in Macedonian population.

Material and methods. The sample consisted of 301 healthy unrelated individuals, and 132 patients with periodontitis. All individuals were of Macedonian origin and nationality and residents of different regions of the Republic of Macedonia. DNA was isolated from peripheral blood leukocytes by the phenol-chlorophorm extraction method. Cytokine polymorphism genotyping was performed by PCR-SSP (Heidelberg kit). The population genetics analysis package (PyPop) was used for analysis of the data. Crude odds ratios, were calculated with 95% confidence interval. $P<0.05$ was considered statistically significant.

Results. Significant negative (protective) association between the Macedonian patients with periodontitis and: 1) cytokine genotypes *TGF-beta1 cdn 10/C:T* and *TGF-beta1 cdn 25/G:G;* 2) cytokine haplotypes *TGF-beta1/TG* and cytokine haplotype zygotes *TGF-beta1/CG:TG* were found. Positive (susceptible) association was found with: 1) cytokine alleles *TGF-beta1 cdn 10/C* and *TGF-beta1 cdn 25/C;* 2) cytokine genotypes *TGF-beta1 cdn 10/C:C* and *TGF-beta1 cdn 25/C:G;* and 3) cytokine haplotypes *TGF-beta1/CC* and cytokine haplotype zygotes *TGF-beta1/CC:CG*.

Conclusion. It is concluded that polymorphisms of *TGF-beta1* gene are associated with an increased risk of chronic periodontitis in Macedonian population.

Introduction

Periodontitis is a widespread chronic inflammatory disease, caused by the interaction between periodontal bacteria and host immune response. Both environmental and genetic factors contribute to the pathogens of periodontitis, which is major cause of tooth loss among adults. Genetic factors may account for a considerable part of individual variations in the host response to periodontitis (1, 2). Genetic heterogeneity associated with disease could be attributed to genetic predisposition as well as social-cultural behavioural differences between populations (e.g. smoking, oral hygiene, access to dental treatment) (3). The importance of studying this disease can be seen from the frequency of periodontitis that affects up to 15% of the population.

The role of T cells in the pathogenesis of periodontitis has been investigated as they play a key role in the regulation of immune responses (4, 5). Genetic studies have shown that the presence of slight DNA variations in cytokine genes, called polymorphisms, seem to be related to increased risk for developing some chronic inflammatory diseases, such as periodontitis (6). Levels of cytokine expression are regulated by genetic polymorphisms and these variations interfere with the progression of disease (7).

Transforming growth factor-beta (TGFB) is a multifunctional peptide that controls proliferation, differentiation, and other functions in many cell types. TGFB acts synergistically with transforming growth factor-alpha TGFA (MIM 190170) in inducing transformation. It also acts as a negative autocrine growth factor. Dysregulation of TGFB activation and signaling may result in apoptosis. Many cells synthesize TGFB and almost all of them have specific receptors for this peptide. TGFB1, TGFB2 (MIM 190220), and TGFB3 (MIM 190230) all function through the same receptor signaling systems (8).

TGFB1 is multifunctional protein that control proliferation, differentiation, and other functions in many cell types. Many cells synthesize TGFB1 and essentially all of them have specific receptors for this protein. It regulates the actions of many other growth factors and determines a positive or negative direction of their effects. It plays an important role in bone remodeling. It is a potent stimulator of osteoblastic bone formation, causing chemotaxis, proliferation and differentiation in committed osteoblasts (8).

By somatic cell hybridization and in situ hybridization, *TGFB* gene was assigned to 19q13.1-q13.3 in man (9). It was determined that the *TGFB1* precursor gene contains 7 exons and very large introns (10). Until now, 232 single nucleotide polymorphisms (SNPs) are deposited in dbSNP: a database of single nucleotide polymorphisms (11). We investigated polymorphisms of *TGF-beta1 cdn10* (refSNP ID rs1800470, C/T, Pro/Leu) and *TGF-beta1 cdn25* (refSNP ID rs1800471, C/G, Pro/Arg), which are non synonymous coding types located in exon-1, codon 2 of the *TGFB1* gene (11). Conflicting results are published in the literature about the association of *TGF-beta1* polymorphisms with periodontitis (12-14).

The aim of this study was to evaluate *TGF-beta1* gene polymorphisms in Macedonian patients with periodontitis and healthy population.

Material and methods

Selection criteria

Selection criteria for participation in this study have been identical for the both groups and referred to the following characteristics: all the examinees were from Macedonian nationality to the second generation, unrelated, spoke Macedonian language and were residents from different geographical regions in Republic of Macedonia. Individuals with less than 20 natural teeth were excluded from the study. All of the participants signed an informed consent to participate in this study.

Groups

The total studied sample consisted of 433 individuals, divided into two groups as follows: patients with periodontitis and healthy individuals.

Patients with periodontitis. The group of patients with periodontitis consisted of 132 subjects, age 38.97 ± 10.124 years, recruited in the study at the Department of Periodontology, University Clinical Centre of Stomatology in Skopje. Patients with: i) diseases of oral soft and hard tissues in oral cavity, excluding caries and periodontitis; ii) presence of orthodontic apparatuses (prosthesis) in mouth; iii) usage of systemic antibiotics in period of three months before engagement in this study; iv) pregnancy and lactation; v) diabetes; vi) appliance of immune-suppressive therapy; and vii) history of any general disease which violates functions of immune system, were excluded from the study. All subjects in examined group were not current or former smokers.

All the patients from examined group had diagnosis chronic (adult) periodontitis (CP), which was established clinically and by x-ray verification, in accordance to criteria of American Academy of Periodontal Disease (AAP, 1999) (15): presence of chronic gingivoragia, bleeding on probing, presence of probing pocket depth, clinical attachment loss (CAL), and horizontal or vertical loss of alveolar bone. Degree of clinical attachment loss, in all patients was defined with confirmed periodontal probe. For each tooth, maximal CAL (distance between the cement-enamel junction and the bottom of the pocket) were derived by measuring six sites around each tooth and recording the maximum values. Patients with generalized CP were selected if they showed attachment loss in e” 30% of the teeth, with a minimum CAL of 3 mm.

Degree of gingival inflammation was fortified pursuant to Loe-Silness' index (16). 0 – normal gingival (pale pink color, with hard and paltry granular consistency); 1 – mild inflammation (marginal gingiva is mildly

red, with small oedema and does not bleed on mild provocation); 2 – modest inflammation (gingiva with red color, with emphasized oedema on the free gingival, there is bleeding on mild pressure with plug); 3 – strong inflammation (gingiva with clearly red color, with lot of oedemas, with tendency for spontaneous bleedings). Basic clinical parameters of the patients with chronic periodontitis are given in Table 1.

Table 1: Basic clinical parameters of the patients with periodontitis.

Parameter (n=121 patients with periodontitis)	Value
Female	41.90%
Male	58.10%
Age (years)	38.97 ± 10.124*
Loe-Silnes Index (GI)	2.38 ± 0.675
Bleeding on probing (BOP %)	82.52 ± 8.143
Clinical attachment loss (CAL)	5.18 ± 0.716

*, Mean ± SD.

Healthy individuals. The control group consisted of 301 unrelated individuals. Control subjects were age and sex non-matched healthy individuals who attended the Institute of Immunobiology and Human Genetics for DNA donation between May 1, 2001 and April 25, 2002 (17-19).

All of the patients and healthy individuals included in this study were matched by ethnicity and signed a written consent to participate in the study which was approved by the Committee of the Ministry of Education and Science from Republic of Macedonia (No 087405), and Ethical Committee of the Medical Faculty in Skopje. Individuals with declared presence of periodontitis in the written consent were excluded from the investigation.

Genomic DNA Isolation and Storage

DNA was isolated from peripheral blood leukocytes by either phenol-chlorophorm extraction method or using the BioRobot EZ1 workstation (QIAGEN) (20). The quality and quantity of DNA was analyzed by GeneQuant (Pharmacia Biotech, Uppsala, Sweden). DNA samples were stored in the Macedonian Human DNA Bank at the Institute of Immunobiology and Human Genetics, Faculty of Medicine, Skopje, Republic of Macedonia (21).

Typing Method

TGF-beta1 polymorphism genotyping (*TGF-beta1 cdn10*, refSNP ID rs1800470 and *TGF-beta1 cdn25,* refSNP ID: rs1800471) was performed by PCR-SSP (Heidelberg kit). Briefly, PCR-SSP typing Heidelberg kit consists of 48 PCR primer mixes aliquotted in 96 well PCR plates (two typings per plate). Master mix, which was supplied along with the reagents and consisted of $MgCL_2$, buffer, dNTP's, and glycerol was mixed with 1.2 - 3.0 µg DNA and 20 U Taq polymerase and dispensed in the 48 wells (22). Agarose gel electrophoresis on a 2% gel revealed a positive or negative signal for specific amplification in each well. Subsequently, the results were analyzed according to the interpretation scheme provided with the kit.

Statistical Methods

The population genetics analysis package, PyPop, was used for TGF-beta1 polymorphism genotyping data in this study (23-25). Allele frequencies for each polymorphism were determined (26, 27). Comparisons of different genotypes for two groups were tested by the Fisher exact test. Crude odds ratios (OR), as estimates of the relative risk, were calculated within 95% CI. $P<0.05$ was considered statistically significant.

Results

Frequencies of polymorphic *TGF-beta1* alleles and genotypes, Fisher exact p-value, Odds ratio and Wald's 95% confidence interval in patients with periodontitis and healthy Macedonians are shown In Table 2.

Significant differences in alleles and genotypes distribution between patients with periodontitis and control group were found in *TGF-β1 cdn10* and *TGF-β1 cdn25* polymorphism, except for the *TGF-β1 cdn10/T:T* genotype. Positive association with periodontitis was found for *TGF-β1 cdn10/C:C* and *TGF-β1 cdn25/*

Table 2: *TGF beta1* gene alleles and genotypes frequency, Odds ratio, Wald's 95% confidence interval, and Fisher exact p-value, in patients with periodontitis and healthy Macedonian population.

Cytokine polymorphism	Allele/ Genotype	PARO		CONTROL		Fisher exact p-value	Odds ratio	Wald's 95% CI
		N	F	N	F			
TGF-β1 cdn10	*T*	84	0.404	282	0.502	0.015*	0.67	0.48-0.93
	C	124	0.596	280	0.498	0.015*	1.49	1.08-2.05
TGF-β1 cdn25	*G*	167	0.803	532	0.947	<0.001*	0.23	0.14-0.38
	C	41	0.197	30	0.053	<0.001*	4.35	2.63-719
TGF-β1 cdn10	*C:C*	42	0.404	65	0.231	0.001*	2.25	1.39-3.64
	C:T	40	0.385	150	0.533	0.001*	0.55	0.34-0.86
	T:T	22	0.212	66	0.235	0.630	0.87	0.50-1.51
TGF-β1 cdn25	*C:G*	37	0.356	30	0.107	<0.001*	4.62	2.66-8.02
	G:G	65	0.625	251	0.893	<0.001*	0.19	0.12-0.34
	C:C	2	0.019	0	0	&	&	&

Abbreviations: PARO = periodontitis; N = absolute number; F = frequency; CI = Confidence Interval; &, cannot be calculated; *, statistically significant.

C:G genotypes (odds ratio 2.25 and 4.62, respectively). Negative association with periodontitis was found for *TGF-β1 cdn10/T* and *TGF-β1 cdn25/G* alleles (odds ratio 0.67 and 0.23, respectively), as well as for *TGF-β1 cdn10/C:T, TGF-β1 cdn10/T:T, and TGF-β1 cdn25/G:G* genotypes (odds ratio 0.55, 0.87 and 0.19, respectively). *TGF-β1 cdn25/G:G* genotype was not found in controls, but in patients with periodontitis was found for very small frequency (0.019) (Table 2).

Frequencies of polymorphic *TGF-beta1* haplotypes and diplotypes, Fisher exact p-value, Odds ratio and Wald's 95% confidence interval in patients with periodontitis and healthy Macedonians are shown in Table 3.

Table 3: *TGF-beta1* gene haplotypes and diplotypes frequency, Odds ratio, Wald's 95% confidence interval, and Fisher exact p-value, in patients with periodontitis and healthy Macedonian population. First nucleotide from haplotypes (*C* or *T*) belongs to *TGF-beta1 cdn10*, and second nucleotide (*C* or G) belongs to *TGF-beta1 cdn25*. Haplotypes from parents (diplotypes) are separated by colon (:).

Cytokine polymorphism	Haplotype/ Diplotype	PARO		CONTROL		Fisher exact p-value	Odds ratio	Wald's 95% CI
		N	F	N	F			
TGF-β1 Haplotype	*CC*	39	0.188	30	0.053	<0.001*	4.09	2.47-6.79
	CG	85	0.409	250	0.444	0.370	0.86	0.62-1.19
	TG	82	0.394	282	0.502	0.010*	0.65	0.47-0.89
	TC	2	0.010	0	0	&	&	&
TGF-β1 Diplotype	*CG:CG*	11	0.106	49	0.174	0.090	0.56	0.26-1.17
	CG:TG	32	0.308	136	0.484	0.001*	0.47	0.29-0.78
	TG:TG	22	0.212	66	0.235	0.630	0.87	0.49-1.56
	TG:CC	6	0.058	14	0.005	0.760	1.17	0.39-3.37
	CC:CG	29	0.279	16	0.057	<0.001*	6.40	3.16-13.10
	TC:CG	2	0.019	0	0	&	&	&
	CC:CC	2	0.019	0	0	&	&	&

Abbreviations: PARO = periodontitis; N = absolute number; F = frequency; CI = Confidence Interval; &, cannot be calculated; *, statistically significant.

Significant positive association with periodontitis was found for *TGF-beta1/CC* haplotype (p<0.001, odds ratio 4.09, CI 2.47-6.79) and for *TGF-beta1/CC:CG* diplotype (p<0.001, odds ratio 6.40, CI 3.16-13.10). Negative association with periodontitis was found for *TGF-beta1/TC* haplotype (p<0.01, odds ratio 0.65, CI 0.47-0.89) and for *TGF-beta1/CG:TG* diplotype (p=0.001, odds ratio 0.47, CI 0.29-0.78). TGF-beta1/TC haplotype, TGF-beta1/TC:CG, and TGF-beta1/CC:CC were found only in patients with periodontitis (Table 3).

Cumulative positive (susceptible) association of cytosine (C) at different positions in *TGF-beta1 cdn10* gene polymorphism with periodontitis is shown on Fig.1.

From the Fig. 1 we can see that the presence of cytosine (C) in *TGF-beta1 cdn10/C* allele has OR of 1.49, in *TGF-beta1 cdn10/C:C* genotype has OR 2.25,

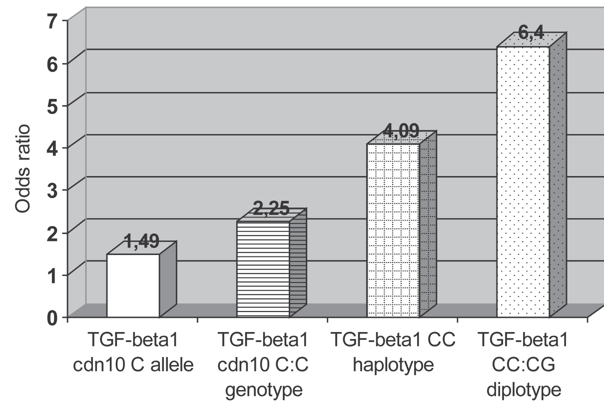

Figure 1: Cumulative positive (susceptible) association of cytosine (C) at different positions in TGF-beta1 cdn10 *gene polymorphism with periodontitis.*

in *TGF-beta1/CC* haplotype has OR of 4.09, and the highest OR has *TGF-beta1/CC:CG* diplotype of 6.40.

Discussion

In this study we studied 132 Macedonian patients with periodontitis in comparison with controls, and found several positive associations between *TGF-beta1* alleles, genotypes, haplotypes, and diplotypes and an association between the polymorphisms in this gene and periodontitis was found.

We analysed *TGF-beta1* polymorphism at two positions: *TGF-beta1 cdn10* and *TGF-beta1 cdn25*, and we were able to investigate haplotypes and haplotype combinations (diplotypes) of this cytokine gene. We found that *TGF-beta1 cdn10/T* and *TGF-beta1 cdn25/G* alleles were negatively associated with periodontitis. The cumulative effects of cytosine (C) position in *TGF-beta1 cdn10* changed from the lowest in *TGF-beta1 cdn10/C* allele (OR=1.49), bigger in *TGF-beta1 cdn10/C:C* genotype (OR=2.25), almost doubled in *TGF-beta1 cdn10/CC* haplotype (OR=4.09) and biggest in *TGF-beta1 cdn10/CC:CG* haplotype combination (OR=6.40).

Our findings are in agreement with authors who found *TGF-beta 1 cdn25* more frequently in the control group in comparison with patient with periodontitis in German population (12). *TGF-beta1 cdn25/G:G* (Arg(25)/Arg(25)) genotype was detected more frequently in control subjects than in periodontitis patients (OR = 0.459, 95% CI = 0.230-0.920, P=0.042), and concluded that *TGF-beta1 cdn25* polymorphisms are associated with susceptibility to chronic periodon-

titis in the population studied (12). *TGF-beta1* gene polymorphisms in a Turkish population with different forms of periodontitis were evaluated and association between *TGF-beta1* genotype and clinical periodontal parameters were investigated. Their findings suggest that the *TGF-beta1 cdn25* polymorphic allele is associated with chronic periodontitis in the Turkish population (13). Contrary to that, association with examined polymorphisms of *TGF-beta1* gene (*TGF-beta1 -800G/A, -509C/T, cdn10* and *cdn25* polymorphisms) and chronic periodontitis in Czech population was not found and there was no association between any polymorphisms in the *TGF-beta1* gene, severity of periodontitis and the smoking status in their study (14).

Genetic background influences a lot of functions that refer to inflammation, inflammatory response, innate or acquired immunity, bacteria colonization and expression of periodontitis (28). Periodontitis is considered as a complex disease where many genes interact with environment and participate in the disease onset and severity. Gene variations (polymorphisms), which participate in complex infectious diseases, are prevalent in every population and vary among healthy and diseased individuals. Many of these polymorphisms can be functionally neutral, but some have direct functional effect in gene transcription, RNA stability or coding of the proteins themselves (29).

Our findings suggest the role of polymorphism of *TGF-beta1* gene as a marker for phenotype expression of TGF, which is in correlation to degree of bone destruction. TGF, as anabolic cytokine in periodontal tissue, increases as a result of reaction to chronic presence of lipoid-polysaccharides (LPS) from bacteria (that mainly come from monocytes and fibro-regions). TGF participate in conduction of rebuilding of extracellular matrix through secretion of collagen and glikozaminoglycols.

The role of TGF is very important for wound healing and regenerative therapy in the treatment of periodontitis (30). TGF as a part of acquired immunity has role in ethiopatogenesis of chronic diseases, especially in regenerative abilities of the tissue. Cumulative positive association of *TGF-beta1* gene with periodontitis, found in this study, suggests possible marker for degree of destruction in periodontal tissue, i.e. phenotype variations of periodontitis (31).

In summary, our data show a significant association between *TGF beta1* polymorphisms and periodontitis in Macedonian population and support previous studies which implicated TGF beta1 polymorphisms as important players in the susceptibility to periodontitis.

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