Genetic polymorphisms of TNFA and IL-1A and generalized aggressive periodontitis


Abstract

Virulent bacteria could cause gingival fibroblasts apoptosis through lipopolysaccharide release during generalized aggressive periodontitis (GAgP) development and evolution. We showed that treatment with lipopolysaccharide (LPS, 1 μg/mL) for 30 days induced the decrease in the number of cultured rat gingival fibroblasts as compared to control group, which received no treatment. GAgP is considered to have also a genetic etiology, so the aim of our study was to evaluate if some polymorphisms of tumor necrosis factor-alpha (TNFA) and interleukin 1A (IL-1A) genes are associated with GAgP in a sample of Romanian population. We selected a group of 32 subjects (22 cases and 10 controls) for studying the TNFA (-857) polymorphism and 97 subjects (66 cases and 31 controls) for IL-1A (-889) polymorphism. The single nucleotide polymorphisms were genotyped by real-time polymerase chain reaction for all subjects. The genotype and allelic distribution tended to be equally between the cases and the controls group. Similar results were obtained for the dominant and recessive model. The data suggest that TNFA (-857) C/T and IL-1A (-889) C/T polymorphisms are not associated with susceptibility to GAgP in this Romanian population, potentially because of the small sample size. This is the first such study for Romanian northeastern population.

**Keywords:** single nucleotide polymorphism, periodontitis, TNFA, IL-1A.

Introduction

Most of the genetic research in oral disease has focused on gene polymorphisms that play a role in the immune response, tissue destructive process, or metabolic mechanism. In some situations, genetic polymorphisms could cause a change in the protein or its expressions, possibly resulting in alterations in innate and adaptive immunity and may thus be deterministic in disease progression [1]. It is thought that single nucleotide polymorphism (SNP) analyses will contribute to the identification of multiple genes associated with periodontitis as genetic markers and risk factors.

Periodontal disease is considered a complex disease associated with multiple genetic factors and oral environmental factors. It is also regarded as a multifactorial condition that occurs because of interplay between environmental, behavioral, microbial and genetic factors. Genetic studies revealed the polygenic nature of periodontitis. Genetic polymorphism in cytokine genes is regarded as a promising factor in inducing periodontal disease [2].

Apoptosis in gingival fibroblasts might be induced by lipopolysaccharide (LPS) [3]. *Porphyromonas gingivalis* is an oral bacterium that causes pathology in a number of dental infections that are associated with increased fibroblast cell death. Studies demonstrated that *P. gingivalis* stimulates cell death by apoptosis rather than necrosis. Some studies showed that apoptosis was induced independent of proteolytic activity and was also independent of caspase activity because a pan-caspase inhibitor, Z-VAD-fmk, had little effect. Moreover, *P. gingivalis* down-regulated caspase-3 mRNA levels and caspase-3 activity. The consequence of this downregulation was a significant reduction in tumor necrosis factor-alpha (TNFA)-induced apoptosis, which is caspase-3-dependent. Immunofluorescence and immunoblot analysis revealed *P. gingivalis*-induced translocation of apoptosis-inducing factor (AIF) from the cytoplasm to the nucleus. siRNA studies were undertaken and demonstrated that *P. gingivalis* stimulated cell death was significantly reduced when AIF was silenced (*p*<0.05). Treatment of human gingival fibroblasts with H-89, a protein kinase A inhibitor that blocks AIF activa-
tion also reduced \(P.\) gingivalis-induced apoptosis \((p<0.05)\). These results indicate that \(P.\) gingivalis causes fibroblast apoptosis through a pathway that involves protein kinase A and AIF, which is not dependent upon bacterial proteolytic activity and is also independent of the classic apoptotic pathways involving caspase-3 [4].

TNFA and interleukin-1alpha (IL-1A) are proinflammatory cytokines that participate in the establishment of inflammatory lesions in periodontitis. Recent reports have indicated that allelic variation of cytokines genes and factors regulating their expression may influence the clinical outcome, susceptibility and progression of periodontal disease. Dysregulation of cytokine gene expression may be responsible for the repeated cycles of tissue inflammation observed in these disorders [5].

The above-mentioned studies already examined the association between periodontitis and single nucleotide polymorphisms (SNPs) that affect cytokines productivity. Such reports on TNFA and IL-1A and periodontitis suggested a correlation between the high level of these cytokines production and the variant alleles of TNFA and IL-1A SNPs [3–5]. Therefore, we decided to study these SNPs in our population and their relation to a severe form of periodontitis, generalized aggressive periodontitis (GAgP).

GAgP is characterized by a rapid destruction of the periodontal tissues, which affects in general young people without any systemic disorders. The objective of the present study was to evaluate the association between TNFA (-857) and IL-1A (-889) gene polymorphisms and GAgP.

Materials and Methods

Fibroblast cultures and LPS treatment

Gingival fibroblasts were achieved as previously described [6] from 6-week-old male rats, 150–170 g body weight, from gingival explants, and grown up in specific culture medium, consisting of DMEM (Dulbecco’s Modified Eagle Medium), 10% fetal bovine serum (FBS), 100 U/mL Penicillin and 100 mg/mL Streptomycin in an atmosphere containing 5% CO\(_2\) at 37°C. Medium was supplemented with LPS treatment (1 \(\mu\)g/mL; LPS from \(Escherichia\) coli 055:B5; Sigma-Aldrich) for 30 days in culture. The study protocol was approved by the Ethic Research Committee of “Grigore T. Popa” University of Medicine and Pharmacy, Iassy, Romania.

Human subjects and clinical assessments

Thirty-two subjects were selected for the TNFA polymorphism study, including 22 with GAgP and 10 without periodontal disease from the ones who sought dental treatment in a private clinic. For the IL-1A polymorphism study, we selected a total of 97 adult subjects, 66 for the cases group and 31 for the control group under the same conditions. Informed consent was obtained from all individuals. The study protocol was approved by the Ethic Research Committee of “Grigore T. Popa” University of Medicine and Pharmacy, Iassy, Romania.

The clinical criterion was considered to be interproximal attachment loss affecting at least three permanent teeth other than a first molars and incisors. The clinical investigation included also smoking status, plaque and bleeding indexes, pocket depth and the presence of dental mobility [7]. Clinical measurements were performed at six sites/tooth and included probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP), meaning more than 30% of teeth with PD and/or CAL >5 mm or more than 60% of teeth with PD and/or CAL >4 mm.

The subjects that did not present loss of gingival attachment for more than one tooth, and the periodontal probe measurement was not deeper than 3 mm, did not present gingival bleedings and bone resorption or a history of periodontal disease, were considered healthy from periodontal point of view and included in the control group [6], meaning no sites with PD and/or CAL >3 mm and no more than 10% of sites with BOP.

Exclusion criteria included mental disorders, lack of judgment, minors, pregnant woman, other ethnic groups and immigrants. We also excluded the subjects with diabetes or the ones having antibiotic treatment in the last six months.

Saliva samples and DNA extraction

Saliva samples may serve as the best alternative sampling for extraction of genomic DNA for its high concentration and acceptable purity [8]. Therefore, non-invasive saliva samples (1 mL) were collected from all the subjects, both cases and control groups, in sterile tubes and frozen until processing. Genomic DNA was extracted out of the saliva samples using the kit Charge Switch gDNA Buccal Cell Kits (Invitrogen). Lysis, binding with magnetic beads, washing, eluting and quantifying DNA were processed accordingly to the manufacturer’s instructions. Then, the DNA was quantified using the spectrophotometer NanoDrop 2000 and frozen at -20°C until the samples were processed. Disease biomarkers in saliva are used often as a diagnostic tool to screen oral and systemic health.

Genotype determination

Gene polymorphisms were analyzed by the real-time polymerase chain reaction (RT-PCR) technique. The two genotypes were identified by Tagman Genotyping Assays (Invitrogen, Applied Biosystems) according to the TaqMan protocol in 96 well plates, in order to detect the SNPs C>T (-857) rs 1799724 for TNFA gene and C>T (-889) rs 1800587 for IL1A gene.
We took 4 μL of each sample and we add 12.5 μL TaqMan Genotyping Master Mix, 1.25 μL SNP Genotyping Assay (specific for each SNP), 11.25 μL DNase-free water and the DNA samples. We obtained a PCR mix of 25 μL in each microtube. Then, we used a LightCycler 480 (Roche) thermocycler to analyze our 32 and respectively 97 samples at the following parameters: 94°C for 5 minutes, followed by 35 cycles – 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final incubation at 72°C by 7 minutes followed by a cooling to 4°C. A no template control (NTC) tube was used as quality control of the assay for each genotype.

Since allele B (rare) represents the hypothesis of the correlation with the pathogenesis of GaGp, we grouped genotypes according to the presence or the absence of allele B, in AA (wild type), AB (heterozygous) and BB (mutant). Mx Pro software (Mx3005P, Stratagene, Agilent Technologies) was used for processing the data obtained from the DNA samples and generate the genotyping results.

Statistical analyses

The frequency of alleles and the distribution of the genotype were compared among groups by chi-square test and Fisher’s exact test, and odds ratios and 95% confidence intervals were also determined. A 5% significance level was set for all the analyses performed.

Results

Lipopolysaccharide (LPS) fibroblasts treatment

The treatment with LPS (1 μg/mL) for 30 days reduced the total number of viable fibroblasts (assayed by Trypan blue technique) as compared to control group, which received no treatment. That means a 79.29±9.81% total decreasing of LPS treated cultured fibroblasts after 30 days (n=7 experiments). Figure 1 is showing the different density of cultured treated fibroblasts and the morphological appearance of apoptotic ones (evidenced as A →).

Genotype distribution

In what concerns the IL-1A (-889) genotype distribution, the frequency of homozygous for common allele (AA) was 9.37% for the patients with GaGp and 6.45% for healthy subjects, whereas the frequency of heterozygous (AB) was 25% for the patients with GaGp and 29.03% for healthy subjects. The homozygous for rare allele (BB) was also detected in similar values for the two groups, 65.62% for the patients with GaGp, respectively 64.51% for the healthy subjects. The data were not statistically significant (λ²=0.34, DF=2, p=0.84). Three samples from the cases group and one from the control group could not be classified (Figure 2).

For TNFA (-857) genotype distribution, the wild-type (CC) 66.66% was present in the study group and 62.5% in the control group, the heterozygous (CT) was present 28.57% in the study group and 37.5% in the control group, whereas the mutant (TT) was present only in the study group but only for 4.76% and has not been detected in the control group. The statistical analysis did not show significant results (λ²=0.54, DF=2, p=0.76). Two samples from the control group and one from the study group could not be classified (Figure 3).

We also constructed a dominant model (negative genotype CC, positive genotype CT+TT) and a recessive model of distribution (negative genotype CC+CT, positive genotype TT).

TNFA (-857) presented in the dominant model a prevalence of the negative genotype higher for the cases group (66.66%) than for the controls (62.5%), but for the positive genotype the prevalence was higher for the controls (37.5%) as compared to cases (33.33%). The statistical analyses of the contingency tables were not statistically significant (λ²=0.04, DF=1, p=0.83).

The negative genotype of the recessive model for TNFA (-857) was more prevalent in controls vs. cases (100% vs. 95.23%). The positive genotype of this model was absent in controls group, being present exclusively in cases group, which might show a trend for association with the susceptibility to GaGp, but in a small percentage (4.77%). The results were still not statistically significant (λ²=0.39, DF=1, p=0.52).

For the second SNP, IL-1A (-889), in the dominant model the negative genotype was predominant in the cases group (9.37% vs. 6.45% in controls group) and the positive genotype was predominant in the controls.
(93.55% vs. 90.63% in cases group). It did not reach statistic significance either ($\chi^2=0.23$, DF=1, $p=0.63$).

In what concerns the recessive model, the negative genotype presented similar results for both groups (34.37% in cases vs. 35.48% in controls), as well as the positive genotype (65.63% in cases vs. 64.52% in controls), the difference being too low to be significant ($\chi^2=0.01$, DF=1, $p=0.91$).

### Allele discrimination

For the TNFA (-857) SNP, the frequency of allele C (common) was 80.95% in the cases groups vs. 81.25% in controls group. Very close values were observed also for allele T (rare), 19.05% for cases vs. 18.75% for controls, and not statistically significant ($\chi^2=0.001$, DF=1, $p=0.97$).

The IL-1A (-889) wild-type allele C (21.87% in cases vs. 20.96% in controls) and mutant T (78.13% in cases vs. 79.04% in controls) were almost equally distributed among the two groups accordingly to chi-square test ($\chi^2=0.02$, DF=1, $p=0.88$).

Statistical analyses revealed no significant differences in genotype and allele distributions for either gene between the two groups. No associations were observed between GAgP and TNFA (-857) and IL-1A (-889) gene polymorphisms in Romanian patients.

### Discussion

Although bacteria are essential for the initiation of any form of periodontitis, the quality and types of bacteria have not been sufficient to explain the differences in disease severity. Therefore, it is considered now that some genetic variations (SNPs) commonly found in our population can represent factors which can amplify the inflammatory processes and make individuals more susceptible to an increased severity of periodontitis [9].

Since certain cytokines (TNF-α, IL-1α) are key regulators of the inflammatory response and are important in periodontitis, we investigated the relationship between some genetic variations associated with cytokine production in generalized aggressive periodontitis.

A single genetic variation may play only a moderate or limited role in common diseases, but it may have important interactions with other genetic variations or environmental factors [1]. Not only gene–gene interactions, but also gene–environment interactions form a complex network in which the disease can initiate and progress. Using a decision tree analysis, TNF-857 and IL-1A-889 SNPs were identified as discriminators between periodontitis and non-periodontitis [10].

The cytokine TNF-α has been found at high levels in gingival crevicular fluid and gingival tissues from periodontitis lesions [11]. Variability in the promoter and coding regions of the TNF gene may modulate the magnitude of its secretory response [12]. In 2003, it was evidenced that TNF-α (-857) SNP variant allele carrier frequency of Japanese subjects (who carried at least one variant allele among severe periodontitis patients) was significantly higher than in healthy subjects [13].

Many investigators have, however, demonstrated that IL-1 regulates the degradation of the extracellular matrix and bone of the periodontal tissue. The gingival fluid levels of IL-1α have been repeatedly associated with periodontitis. In addition, IL-1 is a strong enhancer of tissue levels of PGE2 and TNF-α [6]. SNPs from regulatory regions (promoter region), like IL-1A (-889), can cause changes in gene expression and are essential for the regulation of the transcription of the coding region. R-allele of IL-1A (-889) will result in up-regulating of protein production [7].

It was reported an increased composite genotype of the R-alleles of the IL-1A, IL-1B and IL-1RN genes in non-smoking patients in whom P. gingivalis and A. actinomycetemcomitans could not be detected [14]. These results suggest that IL-1 gene polymorphisms may play a role in the absence of other (putative) risk factors [15]. However, the prevalence of genotype-positive IL-1A (-889) in different ethnic groups and their correlation to clinical manifestations of GAgP had displayed contradictory results.

The carriage rate of R-allele of polymorphic IL-1A (-889) varies from 34% to 64% for patients and 35% to 60% for controls for Caucasian subjects [16]. For Romanian population, R-allele of IL-1A (-889) was almost equal in frequency in patients group (78.31%) and controls group (79.04%). The carriage rate for TNF-α (-857) was also similar between the two groups for the T allele, 21.878% for cases groups and 20.96% for controls.
We need more samples in order to reduce the effects of population, potentially because of the small sample size. Aggressive periodontitis in this northeastern Romanian population is not associated with susceptibility to generalized periodontitis. Further functional analysis is needed to elucidate the molecular mechanism of periodontitis. Combining different studies and research methods may help identify new research targets, this being the quintessence of personalized medicine of the future. With nowadays advances in technology and growth in genetic knowledge, a worldwide database could be prepared in the near future with a summary of various genomic markers and their clinical implications in various types of periodontitis, allowing this way to screen susceptible individuals and to develop new therapeutic strategies.

Conflict of interests

The authors declare that they have no conflict of interests.

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Regarding the CC, CT and TT genotypes, similar distributions were observed among the groups as well. Our data indicated that for TNFα SNP, CC was more prevalent in cases (66.66% vs. 62.5%) than controls, CT was more frequent in controls (37.5% vs. 28.57%) than in cases group and TT was absent in controls, being evidenced only in cases group (4.76%). Therefore, no significant differences among groups were found. The sample size for this SNP was small (32), thus careful interpretation of the data is necessary.

Our data indicated that IL-1α for CC genotype was more prevalent in cases (9.37%) as compared to controls (6.45%), CT genotype was more frequent in controls (29.03% vs. 25%) than in cases and TT genotype was almost equal distributed between the two groups (65.62% in cases vs. 64.51% in controls). Moreover, none of these differences was significant.

Lack of association between genotypes and clinical status may be due to small sample size (97), particularly for alleles of low prevalence [17]. A lack of significant association was observed for the dominant, as well as for the recessive model. Very few studies reported some correlation between these polymorphisms and periodontitis. Other TNFα and IL-1α SNPs were also investigated in relation to periodontitis.

IL-1α and TNFα polymorphisms seem to be equally distributed in the Romanian population between cases and controls, fact that could not permit any conclusions regarding its effect on GAgP. These results are in concordance with other studies of the same polymorphisms, but including other population groups [18].

The increased prevalence of periodontitis in young smokers was evidenced since 1993 [19]. Also, some studies confirmed the importance of smoking as a factor in severe loss periodontal attachment in AgP [20]. In our study, smoking could not be statistically correlated with GAgP.

The frequency of genetic polymorphisms may vary considerably among distinct ethnic groups, so the application of such markers for diagnosis and prognosis of periodontitis should be examined in different populations. The inconsistent results observed in the literature could be attributed to several factors related to the definition of disease, population heterogeneity, environmental and confounding risk factors [21].

Finally, the genetic basis for periodontitis may not be related to a single genetic variant, but may be influenced by multiple genes acting synergistically with environmental factors to increase or decrease the likelihood of developing a disease [22].

Conclusions

First of all, we showed that treatment with lipopolysaccharide (1 μg/mL) for 30 days induced the decrease in the number of cultured rat gingival fibroblasts and the increase of apoptotic index as compared to control group, which received no treatment. Secondly, the data suggest that TNFα (-857) C/T and IL-1α (-889) C/T polymorphisms are not associated with susceptibility to generalized aggressive periodontitis in this northeastern Romanian population, potentially because of the small sample size. We need more samples in order to reduce the effects of sampling variation and to define reliably the association between TNFα and IL-1α polymorphisms and generalized aggressive periodontitis. Further functional analysis is needed to elucidate the molecular mechanism of periodontitis. Combining different studies and research methods may help identify new research targets, this being the quintessence of personalized medicine of the future. With nowadays advances in technology and growth in genetic knowledge, a worldwide database could be prepared in the near future with a summary of various genomic markers and their clinical implications in various types of periodontitis, allowing this way to screen susceptible individuals and to develop new therapeutic strategies.

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