APOLIPOPROTEIN E (RS429358, RS7412) GENOTYPES AND PERIODONTITIS RISK IN SOUTH INDIAN POPULATION - AN ASSOCIATION ANALYSIS

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ABSTRACT

BACKGROUND: Periodontitis is a chronic inflammatory disease. Periodontal pathogens are screened by Toll-like receptors (TLRs), and the inflammatory signaling pathway is regulated by apolipoprotein E (apoE).

AIM: The present study was to analyze the association of apoE (rs429358, rs7412) with stage II periodontitis, stage III/IV periodontitis, and healthy controls in the South Indian population.

MATERIALS AND METHODS: A total of 328 subjects were recruited for this study, among which 81 stage II periodontitis patients, 80 stage III/IV periodontitis patients, and 167 healthy controls. The genomic DNA was isolated from the peripheral blood. Three major haplotypes of the apoE gene were genotyped in control samples and periodontitis cases using Real-Time polymerase chain reaction (RT-PCR). The reaction was performed using the KAPA SYBR® FAST qPCR Kit.

RESULTS: Statistical analysis was done using the R package. Data were analysed by independent sample t-test and $\chi 2$ test. Our study showed a significant association between the E2/E3 genotype and stage II periodontitis in the South Indian population (OR 3.85; CI 1.03-15.62; p = 0.046). For stage III/IV periodontitis, there was no significant association with the E2/E4 genotype in the South Indian population (OR 0.33; CI 0.07-1.15; p = 0.085).

CONCLUSION: The present study has demonstrated that the E4 allele and the E4/E4 genotype are more prevalent in the study population. The E2/E3 genotype was associated with stage II periodontitis in the South Indian population.

KEYWORDS: Periodontitis, Apolipoprotein E, Polymorphism, Real-time PCR

INTRODUCTION

Periodontitis affects the tissues surrounding the teeth. The overall prevalence of periodontitis is approximately 11% among the world population, with around 743 million people affected, according to the Global Burden of Disease Study (GBD, 1990–2010) [1,2]. The major etiological factors in periodontal destruction are the gram-negative anaerobes. These microbial factors are screened by different Pattern Recognition Receptors (PRRs), such as Toll-like receptor (TLR4). The activation of these PRRs leads to the induction of an intracellular signaling cascade involving various signalling molecules like myeloid differentiation factor 88(MyD88) and IRAK, which, in turn, leads to the transcription of numerous inflammatory mediators [3]. TLR4 plays a significant role in the host's defence against invading pathogens. The TLR4 pathway sends signals through the myeloid differentiation factor 88 (MyD88), which in turn sends signals through IRAK1 and TRAF6. This ultimately stimulates the transcription factor nuclear factor kappa B, which enters the nucleus and transcribes the pro-inflammatory cytokine genes. The inactive pro-inflammatory cytokines, such as IL-1β and IL-18, are converted into their active forms by the inflammasome cascade of NLRP3. The signalling molecules of the TLR4 pathway, such as IRAK1 and TRAF6, are negatively regulated by microRNA-146a. The microRNA 146a is, in turn, regulated by apolipoprotein E (apoE) [4,5].

Apolipoprotein E (apoE) is a small molecular protein weighing 34 kDa with numerous biological properties. The isoforms of apoE include apoE2, apoE3, and apoE4. The apolipoprotein mediates the binding of lipoproteins or lipid complexes in the plasma or interstitial fluids to specific cell-surface receptors. These receptors internalize apoE-containing lipoprotein particles; thus, apoE participates in the distribution and redistribution of lipids among various tissues and cells of the body[6]. The other functions of apolipoprotein include the regulation of cytoskeletal assembly and stability, as well as mitochondrial integrity and function. It is one of several apolipoproteins associated with very low-density lipoproteins (VLDL), intermediate-density lipoproteins, chylomicron remnants, and certain subclasses of high-density lipoproteins (HDL). ApoE plays a key role in regulating the clearance of these lipoproteins from the plasma by serving as a ligand for surface receptors, such as LDL receptor family members and heparan sulfate proteoglycans. ApoE3, the most common of the three isoforms, is considered to be the normal form. ApoE2 and apoE4 differ from apoE3 by single amino acid substitutions at position 112 or 158. Human apoE is a 34.2 kDa glycoprotein with 299 amino acid residues[7]. The amino acid residues at positions 112 and 158 are either cysteine or arginine, which determine the three isoforms of apoE[8]. The three apoE isoforms differ among themselves in a single amino acid in their sequence, which alters the protein's structure and influences its function.

The apoE affects both innate and acquired immune responses in vitro, as demonstrated by its ability to suppress lymphocyte proliferation, generate cytolytic T-cells, and stimulate cultured neutrophils[9,10]. ApoE regulates the innate immune response through PU.1, which in turn regulates the synthesis of miR146a [11].

The apoE gene maps to chromosome 19 in a cluster with the related apolipoprotein C1 and C2 genes. Mutations in this gene result in familial dysbetalipoproteinemia, also known as type III hyperlipoproteinemia (HLP III), in which

increased plasma cholesterol and triglycerides are the consequence of impaired clearance of chylomicron and VLDL remnants[12]. The apoE gene is located on chromosome 19 at 19q13.32. The apoE gene has six exons. There are no studies in the literature associating apolipoprotein E polymorphism with stage II or Stage III/IV periodontitis. Thus, the present study aimed to investigate the association between apolipoprotein E polymorphism (rs429358, rs7412) and stage II periodontitis, stage III/IV periodontitis, and healthy controls in the South Indian population. The main objectives of this study include the evaluation of the association between the apoE gene polymorphism and stage II/III periodontitis, as well as to identify the genotypic association of apoE with clinical parameters such as probing depth, clinical attachment level, and gingival index.

Materials and Methods

A minimum sample size of 72 per test group was calculated, assuming an Δ of 1.5 (fold change). This is calculated based on a 50% change in gene expression between the two groups, corresponding to a two-sided test at $\propto\alpha = 0.05$ and $z\beta = 1.28$, which corresponds to 90% power. The number of test subjects was assumed to be double the control group size. A current trend in the genetics field is to increase the number of control subjects to provide a more robust analysis from a statistical viewpoint. The present case-control study comprised 81 patients with stage II periodontitis, 80 patients with stage III/IV periodontitis, and 167 healthy controls. The study was approved by the Institutional Ethical Committee of Saveetha University, Chennai (Ethics Committee number 017/10/2013/IEC/SU). Subjects who reported to the Department of Periodontics - Saveetha Dental College were recruited for the study. The South Indian population includes people who speak Tamil, Telugu, Malayalam, and Kannada as their mother tongue and were included in this study. All other people were excluded from the study. After obtaining informed consent from the subjects, 5 milliliters of peripheral blood samples were collected by venipuncture from each participant in EDTA vacutainers and stored at -20 °C. The patients were classified into stage II and stage III/IV periodontitis groups based on the 2017 AAP classification. The inclusion criteria for control samples included clinically healthy gingiva without bleeding on probing, no sites with probing pocket depths≥3 mm, and no clinical attachment loss in any tooth. The inclusion criteria for periodontitis patients include the presence of at least 20 teeth in situ, ≥ six teeth with a probing pocket depth of ≥6 mm, and loss of attachment, along with radiographic evidence of bone loss. The exclusion criteria for all groups include smokers, pregnant women, lactating mothers, immunocompromised individuals, diabetic patients, and patients under systemic antibiotics.

The genomic DNA was isolated from the peripheral blood using the QIAamp DNA Blood Mini Kit (USA) according to the manufacturer's instructions. The isolated DNA was quantified using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Germany). Three major haplotypes of the apoE gene were genotyped in control samples and periodontitis cases using Real-Time PCR.

Reagents:

The Real-Time PCR was carried out using a Life Technologies - Quant Studio 6 Flex Real-Time PCR System, ThermoFisher, USA. The reaction was performed using the KAPA SYBR® FAST qPCR Kit Master Mix (2X) (Kapa Biosystems, USA), which contains all the PCR components, including SYBR Green dye. The primers (5 ng/μL) in three different combinations were used in reaction mixtures to achieve an amplification product of 173 bp, as shown in Tables 1 and 2.

Table 1: Primer combinations for RT-PCR

ApoE haplotype	PRIMER COMBINATIONS
ApoE2	ApoE 112C and ApoE 158C
ApoE3	ApoE 112C and ApoE 158R
ApoE4	ApoE 112R andApoE 158R

Table 2: Nucleotide Sequences for ApoE Genotyping

REACTION PRIMER FOR	PRIMER SEQUENCE
ApoE 112C-Forward	5'-CGGACATGGAGGACGTGT-3'
ApoE 112R-Forward	5'-CGGACATGGAGGACGTGC-3'
ApoE 158C-Reverse	5'-CTGGTACACTGCCAGGCA-3'
ApoE 158R-Reverse	5'-CTGGTACACTGCCAGGCG-3'

The genomic DNA, diluted to a concentration of 10 ng/μL using 0.5X TE buffer, was used as the template for this reaction. The RT-PCR reaction setup was performed in a 384-well optical plate for the three different reaction mixtures. A final reaction volume of 10.0 μL for each sample was achieved by using the following components: 5.0 μL of SYBR FAST qPCR Master Mix (2X), 1.0 μL of each primer, 2.0 μL of nuclease-free water, and 1.0 μL of genomic DNA. Non-template controls were also included by using the same reaction mixtures without template DNA. The PCR amplification was performed with the following reaction conditions: initial DNA Polymerase activation at 95 °C for 10 minutes, followed by 40 cycles with denaturation at 95 °C for 15 seconds, and combined annealing and extension at 62 °C for 1 minute. Amplification was performed using the Quant Studio 6 Flex Real-Time PCR System with the comparative Ct (ΔCt) method (quantitation experiment setup).

Fluorescence measurement at each cycle, as the amplification progresses, allows for the quantification of the template. It is based on the fluorescent signal from a fluorescent reporter molecule (such as a double-stranded DNA-binding SYBR Green dye) that appears during the exponential phase of amplification. The fluorescence intensity increases proportionally with each amplification cycle in response to the increased amplicon concentration.

The cycle number at which the instrument can discriminate the amplification-generated fluorescence as being above the ambient background signal is called the "Ct" or threshold cycle. This Ct value is directly correlated to the initial sample concentration. The Quant Studio 6 Flex Real-Time Manager Software determines the Ct value for each sample based on specific user-defined parameters.

The ApoE haplotype present in the individuals tested was determined by comparison of differential amplification attained in the three different amplification setups.

RESULTS

R package software was used for the statistical analysis. Data was analysed by independent sample t-test and χ2 test. The association between genotypes of apoE (rs429358, rs7412) with periodontitis was assessed by computing the odds ratio (OR) with 95% confidence intervals (95% Cis). The level of significance was kept at P<0.05. The genotypic distribution of the test and control groups was evaluated for Hardy-Weinberg Equilibrium (HWE). Data on frequency distribution collected for stage II periodontitis and stage III/IV periodontitis patients was pooled into a single periodontitis group and analyzed statistically. One-way ANOVA was performed to compare clinical variables, including pocket depth (PD), clinical attachment level (CAL), and gingival index of Loe and Silness,[14] with apoE polymorphism, as shown in Table 3.

Table 3. Comparison of APOE genotypes and alleles with clinical parameters

Stage II periodontitis	***E2/E2 (n=8)***	***E2/E3 (n=13)***	***E2/E4 (n=30)***	***E3/E3 (n=7)***	***E3/E4 (n=9)***	***E4/E4 (n=14)***	***F-statistic***	***p-value***
Probing Depth (PD)	5.00±0.77	5.33±0.47	5.13±0.60	4.69±0.61	4.88±0.55	5.15±0.88	1.14	0.35
Clinical Attachment Level (CAL)	4.25±0.76	4.50±0.55	4.38±0.70	4.09±0.40	4.19±0.42	4.56±0.65	0.84	0.52
Gingival Index (GI)	1.51±0.42	1.89±0.37	1.88±0.27	1.45±0.24	1.67±0.40	1.70±0.38	3.43	0.01
Stage III/IV periodontitis	**E2/E2 (n=12)**	**E2/E3 (n=22)**	**E2/E4 (n=26)**	**E3/E3 (n=1)**	**E3/E4 (n=3)**	**E4/E4 (n=16)**	**F-statistic**	**p-value**
Probing Depth (PD)	5.65±1.27	4.62±0.00	5.79±1.51	5.77±1.19	5.17±0.60	5.67±0.95	3.25	0.01
Clinical Attachment Level (CAL)	4.75±0.89	4.82±0.00	4.76±0.99	4.85±0.84	4.46±0.70	4.79±1.05	0.11	0.99
Gingival Index (GI)	1.44±0.30	1.22±0.00	1.49±0.32	1.56±0.37	1.43±0.22	1.43±0.31	2.86	0.02
PERIODONTITIS	**E2/E2 (n=20)**	**E2/E3 (n=35)**	**E2/E4 (n=56)**	**E3/E3 (n=8)**	**E3/E4 (n=12)**	**E4/E4 (n=30)**	**F-statistic**	**p-value**
Probing Depth (PD)	5.39±1.12	5.24±0.50	5.30±0.88	5.37±1.14	5.04±0.59	5.39±0.94	0.38	0.86
Clinical Attachment Level (CAL)	4.55±0.85	4.54±0.52	4.47±0.75	4.57±0.80	4.33±0.60	4.66±0.86	0.45	0.81
Gingival Index (GI)	1.47±0.35	1.81±0.41	1.78±0.32	1.52±0.33	1.54±0.33	1.58±0.37	4.39	0.0009

The study group consisted of 81 stage II periodontitis patients with an average age of 36.65 ± 5.22 years, 80 stage III/IV periodontitis patients with an average age of 28.34 ± 3.12 years, and 167 healthy subjects with a mean age of 32.25 ± 5.14 years. One-way ANOVA revealed a significant association between ApoE genotypes and the gingival index in both periodontitis groups. There was a significant association between probing depth and ApoE genotypes in severe (stage III/IV) periodontitis cases. The pocket depth, ranging from 4.62mm to 5.79mm, was found to be associated with stage III/IV periodontitis and the apoE polymorphism. No significant difference was found between the periodontitis group and the controls concerning age. The frequency distribution of apoE (rs429358, rs7412) genotypes in stage II periodontitis patients, stage III/IV periodontitis patients, and healthy controls is demonstrated in Table 4 and was found to follow the Hardy-Weinberg Equilibrium (HWE).

Table 4. Comparison of APOE genotype and alleles with stage II, stage III/IV periodontitis and healthy controls

				Control vs. Periodontitis			Control vs. Stage II Periodontitis			Control vs Stage III/IV periodontitis		
	Control (n=167)	Stage III/IV (n=80)	Stage II periodontitis (n=81)	OR	(95% CI)	p-value	OR	(95% CI)	p-value	OR	(95% CI)	p-value
Genotype												
E3/E3	37	22	13	1	NA	NA	1.00	NA	NA	1.00	NA	NA
E2/E2	13	12	8	1.61	0.70-3.82	0.26	1.74	0.57-5.22	0.32	1.54	0.59-4.04	0.37
E4/E4	70	26	30	0.85	0.47-1.52	0.57	1.21	0.57-2.68	0.62	0.63	0.31-1.26	0.19
E2/E3	5	1	7	1.66	0.50-6.15	0.41	3.85	1.03-15.62	0.04	0.38	.01-2.66	0.36
E2/E4	16	3	9	0.80	0.32-1.93	0.61	1.59	0.55-4.54	0.38	0.33	0.07-1.15	0.08
E3/E4	26	16	14	1.22	0.60-2.47	0.58	1.52	0.61-3.85	0.36	1.04	0.45-2.36	0.93
Allele												
E3	105	61	47	1	NA	NA	1.00	NA	NA	1.00	NA	NA
E2	47	28	32	0.97	0.58-1.16	0.26	1.52	0.86-2.68	0.15	1.03	0.58-1.80	0.92
E4	182	71	83	0.65	0.78-1.98	0.36	1.02	0.66-1.57	0.93	0.67	0.44-1.02	0.06

The genotype and allele frequencies were not significantly different between the cases (pooled periodontitis) and control groups ($p > 0.05$). Also, the frequency distribution among stage III/IV periodontitis patients and controls was similar and lacked any significant association. However, there was a significant association between the ApoE genotypes and stage II periodontitis in the South Indian population. The E2/E3 genotype was significantly associated with stage II periodontitis in this population.

DISCUSSION

Although apolipoprotein E is primarily involved in lipid metabolism, it also has additional functions, such as regulating innate immunity.[15] The role of apoE in innate immune regulation is mainly through inducing the transcription of miR146a [16,17]. The present study analyzed the association of apoE polymorphisms with periodontitis in a South Indian population. Our study showed a significant association between the E2/E3 genotype and stage II periodontitis in a south Indian population (OR 3.85; CI 1.03-15.62; $p = 0.046$). For the severe (stage III/IV) periodontitis, there was no significant association with the E2/E4 genotype in the south Indian population (OR 0.33; CI 0.07-1.15; $p = 0.085$). Our study results are in contradiction to those of Linhartova et al.'s 2015 study, which was conducted in the Czech population. Linhartova et al.'s study did not find any significant association between apoE genotypes and chronic periodontitis in the Czech population. In their study, the severe periodontitis group was not included. Our study was the first to investigate the association between apoE polymorphisms and stage III/IV periodontitis. The possible reason for the association of apoE genotypes with moderate stage II periodontitis, but not a significant association in the case of severe periodontitis, might be its role in regulating the inflammatory pathway in the initial stages of periodontal pathogenesis. As the disease progresses, its role may be minimal in regulating periodontal disease pathogenesis. A thorough analysis of the biological plausibility of this finding is required.

In the Czech population, the E3 allele (83%) and the E2/E3 genotype (70%) were found to be more common in both the chronic periodontitis group and the controls. The E2/E2 genotype was found to be the least prevalent in their population (1.4%). However, our study has found that the E4 allele is more prevalent in our population (55% in controls and 50% in periodontitis cases). The most common genotype in the South Indian population has been found to be E4/E4, with 42% in controls and around 35% in periodontitis cases. This is a significant finding. Regarding the association of ApoE alleles with periodontitis, the E4 allele has shown an inverse association with severe periodontitis in our population, with an odds ratio of 0.67 and a p-value of 0.06 (nearing significance). The E4 allele has been shown to be highly unstable due to the methylation of the cytosine nucleotide. This allele and the corresponding genotype have been consistently linked to cardiovascular diseases and atherosclerosis in numerous studies. We analyzed the apoE polymorphisms using the SYBR green dye method and obtained distinct results, with the E4 allele and E4/E4 genotype being more prevalent in the South Indian ethnicity. This finding needs to be confirmed with a larger sample size and in different racial groups.

The genetic origin of the three common isoforms of the human apolipoprotein E (apoE) protein, known as E2, E3, and E4, was understood in 1981, and since then, numerous studies have explored its role in various diseases. The three common variants e2, e3, and e4 of the apoE gene are determined by three of the four haplotypes resulting from the combination of the alleles of the two SNPs rs429358 (C3937 T) and rs7412 (C4075 T) at the apoE locus—the haplotype T nucleotide at positions

3937 and 4075 leads to ε2 isoform. The haplotype T nucleotide at position 3937 and the C nucleotide at position 4075 lead to the ε3 isoform. The haplotype C nucleotide at both positions 3937 and 4075 leads to the ε4 isoform. The haplotype C nucleotide at position 3937 and the T nucleotide at position 4075 lead to the fourth isoform, known as ε3r. The fourth isoform is very rare in many races. In the APOE gene, both SNPs rs429358 and rs7412 lie within CpG islands, which are ''hot-spot'' mutation sites for the cytosine methylation and its deamination to thymine (Cytosine CH3 Thymine+ Nh3). Due to cytosine methylation, the ε4 isoform is unstable since both the nucleotides at positions 3937 and 4075 are cytosine nucleotides. The ε2 isoform is more stable, with ε3 exhibiting intermediate stability[20].

The apolipoprotein E isoforms suppress the production of pro-inflammatory mediators such as TNF-α and IL-1β by macrophages in an isoform-specific manner (E2 > E3 > E4)[21] The production of pro-inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) was higher in human APOE ε4 transgenic (Tg) mice than in APOE ε3 Tg ones. In addition to the immunoregulatory role of ApoE, there is evidence for the isoform-specific protective or detrimental effects of ApoE in relation to hepatitis C, HIV, herpes group of viruses, and the COVID-19 virus.[22,23,24,25] The role of viruses in the etiopathogenesis of periodontal disease is a known fact. The basic research into the linking mechanism between ApoE genetic changes, its immunomodulatory role, and the effect on microbes could shed light on understanding the etiopathogenesis of periodontitis.

The present study has shown that the E4 allele and E4/E4 genotype were more common in the study population. Although the E4 allele and E4/E4 genotype were more prevalent in the test and control groups, the E2/E3 genotype was significantly associated with moderate (stage II) periodontitis. This is a significant finding that shows the link between the apoE polymorphisms and susceptibility to periodontal disease. Several studies should be conducted to establish the link between apoE genotypes and periodontal diseases across different ethnicities.

CONCLUSION:

The present study concludes that the apoE genotypes E2/E3 are significantly associated with stage II periodontitis, and further studies should be conducted to establish the link or biological role of these findings.

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