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Research Article

GENETIC POLYMORPHISMS IN TOLL-LIKE RECEPTOR 2, TOLL-LIKE RECEPTOR 4, NUCLEAR FACTOR-KAPPA B, AND INTERLEUKIN-4 GENES AND THEIR ASSOCIATION WITH CHRONIC PERIODONTITIS

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ABSTRACT

Objectives: Chronic periodontitis is a multifactorial inflammatory disease with a significant genetic component. Polymorphisms in genes involved in innate immunity and inflammatory regulation (toll-like receptor 2 [TLR2], toll-like receptor 4 [TLR4], nuclear factor-kappa B [NF-κB], and interleukin 4 [IL-4]) have been implicated in disease susceptibility and severity, though results remain inconsistent across populations. This study aimed to investigate the association of selected single-nucleotide polymorphisms in these genes with the risk and severity of chronic periodontitis in a South Indian population.

Methods: A case–control study was conducted at Government Medical College, Mahbubnagar, Telangana, India. The study included 100 patients with chronic periodontitis (cases) and 30 periodontally healthy controls (total n=130). Diagnosis followed the 2017 World Workshop classification. Genomic DNA was extracted from peripheral blood, and genotyping of four polymorphisms, TLR2 Arg753Gln (rs1898830), TLR4 Asp299Gly (rs7873784), NF-κB −94 ins/del ATTG (rs28362491), and IL-4 −590 C/T (rs2243250), was performed using polymerase chain reaction-restriction fragment length polymorphism. Genotype/allele frequencies, odds ratios (OR), and associations with clinical periodontal parameters (probing pocket depth [PPD], clinical attachment loss [CAL], plaque index [PI], and gingival index [GI]) were analyzed. Gene-gene interactions and severity-based stratification were also evaluated. Statistical tests included χ^2, Fisher's exact test, logistic regression (adjusted for confounders), and analysis of variance/Kruskal–Wallis ($p<0.05$).

Results: Individual polymorphisms in TLR2, TLR4, NF-κB, and IL-4 showed no statistically significant independent association with periodontitis susceptibility (all $p>0.05$). However, combined genotype analysis revealed strong synergistic effects: Carriers of variant alleles in both TLR2 and TLR4 showed significantly increased risk (OR=2.84, 95% confidence interval [CI]: 1.52–5.34, $p=0.001$). Combined NF-κB del/del+IL-4 TT genotypes demonstrated the strongest association (OR=3.42, 95% CI: 1.68–6.94, $p<0.001$). These associations remained significant after adjustment for age, gender, smoking, diabetes, and socioeconomic status. Risk allele carriage (A in TLR2, T in TLR4, del in NF-κB, and T in IL-4) increased in a dose-dependent manner with disease severity (mild → moderate → severe; all trend $p<0.05$). Individuals with risk genotypes consistently exhibited significantly higher values of PPD, CAL, PI, and GI compared to reference genotypes (all $p\leq0.041$).

Conclusion: While individual polymorphisms in TLR2, TLR4, NF-κB, and IL-4 genes do not show significant independent associations with chronic periodontitis in this population, their combined presence markedly increases disease susceptibility and is associated with greater clinical severity. These findings support a polygenic, synergistic genetic contribution to periodontitis risk and progression, highlighting the importance of gene-gene interactions in periodontal pathogenesis. Larger multicenter studies and functional analyses are warranted to validate these observations and explore potential for personalized risk stratification.

Keywords: Periodontitis, Inflammatory markers, Genetic markers, Toll-like receptor 2, Toll-like receptor 4, Nuclear factor-kappa B, Interleukin-4.

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INTRODUCTION

Periodontitis is most common oral health problem among other oral health problems globally. It is a widespread inflammatory condition which affects the supporting tissues of teeth. It impacts millions of populations and being a major cause of tooth loss in adults [1,2]. Severity of periodontitis results in periodontal ligament breakdown and damage to the alveolar bone, which leads to tooth mobility and further loss without proper intervention [3]. It is a condition driven by multifactorial, among those the primary cause of the condition is formation of biofilms or plaque on tooth by microbes which trigger host immune response. Bacteria like *Porphyromonas gingivalis* infections lead high inflammatory response from the host which may further cause for the complete destruction of tissues [4]. Toll-like receptors (TLRs), especially TLR2 and TLR4, are central to this process, they recognize bacterial components in the plaque and activates immune response pathways like nuclear factor-kappa B (NF-κB), leading to release of cytokines and inflammatory response [5,6].

There are clearly established factors which can cause periodontal diseases such as poor oral hygiene, smoking, and conditions like diabetes but it is still not clear why some of the individuals develop severe disease while others with same exposure remained unaffected [7,8]. Tis lead the researchers to focus more on the genetic factors and keen observation on polymorphisms which stimulate the intensity of inflammatory response [9,10]. There are several studies about innate immunity and inflammatory response regulating genes such as TLR2, TLR4, NF-κB, and anti-inflammatory cytokines like interleukin 4 (IL-4).

Studies stated that any changes in these genes lead to altered receptor function, inefficiency in signaling pathway, or cytokine imbalance which remarkably increases the chances of periodontitis [11,12]. Some of these polymorphisms have shown strong association with microbial factors [13-15].

The present study investigates the potential links between selected single-nucleotide polymorphisms (SNPs) in the TLR2, TLR4, NF-κB, and IL-4 genes and the risk of chronic periodontitis. Using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, we compare genotype frequencies between patients with diagnosed chronic periodontitis and healthy controls. This helps to understand the relationship between periodontitis and genomics and improves personalized risk assessment as well as targeted strategies for prevention and management.

METHODS

This hospital-based case–control study was conducted between August 2023 and August 2025 at the Department of Periodontology and Department of Microbiology, Government Medical College, Mahbubnagar, Telangana, India. The research followed the STROBE guidelines for reporting observational studies and was designed to evaluate the association between genetic polymorphisms in TLR2, TLR4, NF-κB, and IL-4 genes and susceptibility to chronic periodontitis. After obtaining the Institutional Ethical Committee approval Lr. No-GMC/MBNR/IECBMR/AP/04/04/2023, a total of 130 participants were recruited, comprising 100 patients with chronic periodontitis (cases) and 30 periodontally healthy individuals (controls). Cases were diagnosed according to the 2017 World Workshop classification of periodontitis, based on probing pocket depth (PPD), clinical attachment loss (CAL), bleeding on probing, and radiographic evidence of alveolar bone loss. Controls were periodontally healthy subjects without attachment loss, presenting with PPD ≤3 mm, and free from systemic disease affecting periodontal status.

All the participants underwent a comprehensive periodontal examination performed by a single calibrated examiner. The following indices were recorded at six sites per tooth using a UNC-15 probe: The plaque index (PI), the gingival index (GI), PPD, and CAL. Calibration was conducted in 10% of the study population before data collection, and intra-examiner reproducibility was high (κ=0.89), ensuring reliability. Peripheral venous blood (3 mL) was collected from each participant into EDTA-coated tubes. Genomic DNA was extracted using the phenol–chloroform method and stored at −20°C until analysis. The purity and concentration of DNA were assessed by NanoDrop spectrophotometry (A260/A280 ratio 1.8–2.0), and DNA integrity was verified on 1% agarose gel electrophoresis.

Gene polymorphisms were selected based on previous evidence of involvement in immune response and inflammation: TLR2 Arg753Gln (−753 G/A), TLR4 Asp299Gly (+896 A/G), NF-κB −94 ins/del ATTG, and IL-4 −590 C/T. Primers were designed using Primer3 software and validated using BLAST to ensure specificity. PCR was carried out in a 25 µL reaction mixture containing approximately 100 ng genomic DNA, 10 p.mol of each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1× PCR buffer, and 1 U Taq DNA polymerase. Amplification was performed under the following cycling conditions: an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 55–60°C (depending on locus) for 30 s, and extension at 72°C for 60 s; followed by a final extension at 72°C for 7 min. PCR products were visualized on agarose gels using a 100 bp DNA ladder. RFLP analysis was performed using locus-specific restriction enzymes: PstI for TLR2, NcoI for TLR4, EcoRV for NF-κB, and BsmFI for IL-4. PCR products were digested with the respective enzymes and resolved on 2–3% agarose gels stained with ethidium bromide. Banding patterns were visualized under ultraviolet transillumination, and representative electrophoresis images were recorded.

Normality of data was evaluated using the Shapiro–Wilk test, and homogeneity of variance was assessed with Levene's test. For intergroup comparisons, the student's t-test or analysis of variance (ANOVA) was applied for normally distributed variables, while the Kruskal–Wallis test was used for skewed data. Genotype and allele distributions between cases and controls were compared using the χ^2 test and Fisher's exact test, and associations were expressed as odds ratios (ORs) with 95% confidence intervals (CIs) under dominant, recessive, and additive models. Logistic regression was employed to adjust for confounding factors such as age, gender, socioeconomic status, smoking, and diabetes. Statistical significance was set at $p<0.05$, and analyses were performed using the Statistical Package for the Social Sciences v26.0 (IBM Corp., Armonk, NY, USA). Complete workflow design illustrated as CONSORT flow diagram in Fig. 1.

RESULTS

A total of 130 participants were enrolled, including 100 periodontitis patients (cases) and 30 periodontally healthy individuals (controls). The mean age was slightly higher among cases (45.42±11.84 years) compared to controls (43.33±11.42 years), though this difference was not statistically significant ($p>0.05$). Gender distribution was nearly balanced across both groups. Clinical examination confirmed clear separation between cases and controls. Periodontitis patients demonstrated significantly greater PPD: 3.74±1.70 mm, CAL: 2.98±1.24 mm, and higher PI and GI scores. In contrast, controls exhibited healthy periodontal status with minimal plaque, no attachment loss, and probing depths within the physiological range (≤3 mm). These findings affirm the robustness of group allocation. The details are presented in Table 1.

The distribution of TLR2 (rs1898830) genotypes (AA, AG, and GG) was analyzed between periodontitis cases (n=100) and healthy controls (n=30). Among cases, the heterozygous AG genotype was most frequent (40%), followed by AA (32%) and GG (28%). In controls, AG (33.3%) and GG (36.7%) were more frequent, while AA was seen in 30%. The allele frequency analysis showed that the A allele was more common among cases (52%) compared to controls (46.7%), whereas the G allele was predominant in controls (53.3%) compared to cases (48%). Genotype frequencies were consistent with Hardy–Weinberg equilibrium (HWE) in both groups ($\chi^2<3.84$, $p>0.05$). Chi-square comparison of genotype distribution between cases and controls was not statistically significant ($\chi^2=0.88$, $p=0.646$). Similarly, no significant difference was observed in allele distribution ($\chi^2=0.39$, $p=0.531$). Odds ratio analysis demonstrated that the G allele did not confer a statistically significant increased risk of periodontitis (OR=0.82, 95% CI: 0.42–1.61) (Table 2).

Table 1: Combined demographic and clinical characteristics of cases and controls

Characteristic	Cases (n=100)	Controls (n=30)	Statistical comparison
Age (years, Mean±SD)	45.42±11.84	43.33±11.42	t=0.82, p=0.41 (NS)
Gender (Male/female)	47/53	16/14	χ^2=0.15, p=0.70 (NS)
Probing pocket depth (mm)	3.74±1.70	≤3 (Healthy)	t=9.62, p<0.001**
Clinical attachment loss (mm)	2.98±1.24	0 (Healthy)	t=11.4, p<0.001**
Plaque index	1.49±0.87	Minimal	t=8.76, p<0.001**
Gingival index	1.56±0.81	Minimal	t=9.04, p<0.001**

*Values expressed as mean±standard deviation (SD) unless otherwise specified. Independent t-test applied for continuous variables, Chi-square test for categorical variables. *p<0.001 considered statistically significant. NS: Not significant

The genotype distribution of TLR4 (rs7873784) in cases (n=100) and controls (n=30) is shown in Table 3. In the case group, TT was the most frequent genotype (35%), followed by CT (33%) and CC (32%). In contrast, controls showed similar proportions of CC and CT genotypes (36.7% each), with TT observed at 26.7%. Allele frequency analysis revealed that the T allele was slightly more prevalent in cases (51.5%) than in controls (45%), while the C allele was more common among controls (55%) compared to cases (48.5%). Both groups adhered to HWE (p>0.05), indicating no deviation in distribution. Chi-square analysis revealed no significant differences in genotype (χ^2=0.73, p=0.694) or allele distributions (χ^2=0.59, p=0.442). Odds ratio analysis suggested a non-significant trend toward higher periodontitis risk with the TT genotype (OR=1.51, 95% CI: 0.56–4.06) and T allele (OR=1.28, 95% CI: 0.66–2.47).

The NF-κB (rs28362491) genotype distribution is summarized in Table 4. In the case group (n=100), the GA heterozygote was the most common (44%), followed by GG homozygote (36%) and AA homozygote (20%). In the control group (n=30), GA predominated (46.7%), followed by AA (30%) and GG (23.3%). Allele distribution revealed that the G allele accounted for 58% of alleles in cases and 46.7% in controls, whereas the A allele was more frequent in controls (53.3%) than in cases (42%). Both cases and controls were in HWE (p>0.05). The Chi-square test showed no significant differences in genotype (χ^2=3.04, p=0.218) or allele distribution (χ^2=1.52, p=0.218) between groups. Odds ratio analysis suggested a non-significant protective effect of the A allele against periodontitis (OR=0.65, 95% CI: 0.34–1.23).

The IL-4 (–590 C/T, rs2243250) genotype distribution is detailed in Table 5. In the case group, CC was most frequent (43%), followed closely by CT (41%) and TT (16%). Among controls, CT was predominant (46.7%), followed by CC (33.3%) and TT (20%). Allele frequency analysis revealed a nearly balanced distribution between groups:

Cases: C allele=63.5%, T allele=36.5%

Controls: C allele=56.7%, T allele=43.3%

Both groups adhered to HWE (p>0.05). Comparison between cases and controls showed no statistically significant association between IL-4 polymorphism and periodontitis (χ^2=0.14, p=0.932). Odds ratio analysis indicated no meaningful difference in risk: The T allele showed a non-significant trend toward susceptibility (OR=1.15, 95% CI: 0.61–2.17).

To explore gene–gene interactions, we assessed combined genotypes of innate immunity receptors (TLR2+TLR4) and immune regulatory pathways (NF-κB+IL-4). The rationale was to determine whether specific genotype combinations confer a higher susceptibility to

Table 2: Genotype and allele frequencies of TLR2 (rs1898830) polymorphism in cases and controls

Genotype/Allele	Cases (n=100) (%)	Controls (n=30) (%)	Expected (HWE) Cases	Expected (HWE) Controls	χ^2	p-value	OR (95% CI)
AA	32 (32.0)	9 (30.0)	27.0	9.3	Ref	–	Ref
AG	40 (40.0)	10 (33.3)	44.0	11.4	–	–	1.12 (0.46–2.72)
GG	28 (28.0)	11 (36.7)	29.0	9.3	–	–	0.72 (0.29–1.80)
Genotype comparison	–	–	–	–	0.88	0.646	–
A allele	104 (52.%)	28 (46.7)	–	–	Ref	–	Ref
G allele	96 (48.0)	32 (53.3)	–	–	0.39	0.531	0.82 (0.42–1.61)

Chi-square test applied for genotype and allele distributions. HWE confirmed in both cases and controls (p>0.05). OR calculated using AA and A allele as references. Significance threshold set at *P*<0.05. OR: Odds ratio, CI: Confidence interval, HWE: Hardy–Weinberg equilibrium, TLRs: Toll-like receptors

Table 3: Genotype and allele frequencies of TLR4 (rs7873784) polymorphism in cases and controls

Category	Variable	Cases (n=100) (%)	Controls (n=30) (%)	Expected (HWE) Cases	Expected (HWE) Controls	χ^2	p-value	OR (95% CI)
Genotype distribution	CC	32 (32.0)	11 (36.7)	29.5	10.8	Ref	–	Ref
	CT	33 (33.0)	11 (36.7)	35.0	11.4	–	–	1.02 (0.41–2.54)
	TT	35 (35.0)	8 (26.7)	35.5	7.8	–	–	1.51 (0.56–4.06)
Overall comparison	Genotype χ^2 (2 df)	–	–	–	–	0.73	0.694	–
Allele distribution	C allele	97 (48.5)	33 (55.0)	–	–	Ref	–	Ref
	T allele	103 (51.5)	27 (45.0)	–	–	0.59	0.442	1.28 (0.66–2.47)
Overall comparison	Allele χ^2 (1 df)	–	–	–	–	0.59	0.442	–
HWE Test	–	χ^2=0.21	χ^2=0.14	–	–	–	p>0.05	–

Genotype and allele frequencies are presented as counts and percentages. HWE was satisfied in both groups (p>0.05). Chi-square test applied for genotype (2 df) and allele (1 df) comparisons. OR calculated with CC genotype and C allele as references. Significance threshold set at p<0.05. OR: Odds ratio, CI: Confidence interval, HWE: Hardy–Weinberg equilibrium, TLRs: Toll-like receptors

Table 4: Genotype and allele frequencies of NF-κB (rs28362491) polymorphism in cases and controls

Category	Variable	Cases (n=100) (%)	Controls (n=30) (%)	Expected (HWE) Cases	Expected (HWE) Controls	χ^2	p-value	OR (95% CI)
Genotype distribution	GG	36 (36.0)	7 (23.3)	33.6	7.2	Ref	–	Ref
	GA	44 (44.0)	14 (46.7)	48.7	13.6	–	–	0.61 (0.23–1.64)
	AA	20 (20.0)	9 (30.0)	17.7	9.2	–	–	0.43 (0.15–1.28)
Overall comparison	Genotype χ^2 (2 df)	–	–	–	–	3.04	0.218	–
Allele counts	G	116 (58.0)	28 (46.7)	–	–	Ref	–	Ref
	A	84 (42.0)	32 (53.3)	–	–	1.52	0.218	0.65 (0.34–1.23)
Allele frequencies	G freq.	0.58	0.467	–	–	–	–	–
	A freq.	0.42	0.533	–	–	–	–	–
HWE test	–	χ^2=0.22 (NS)	χ^2=0.30 (NS)	–	–	–	p>0.05	–

Values shown as counts (percentages). OR calculated with GG and G allele as references. Significance set at P<0.05. OR: Odds ratio, CI: Confidence interval, NF-κB: Nuclear factor-kappa B, HWE: Hardy–Weinberg equilibrium, NS: Not significant

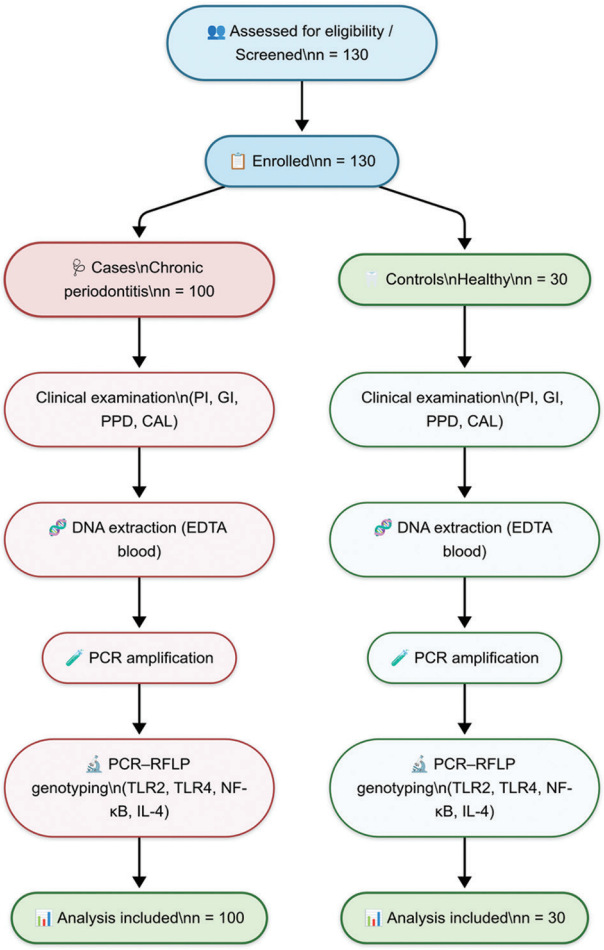

Fig. 1: (CONSORT Flow Diagram) illustrates the process of participant recruitment, eligibility screening, and final allocation into case and control groups (n=130)

periodontitis compared to single-gene variants. Table 6 summarizes the distribution of combined genotypes between cases and controls, while Fig. 2 graphically illustrates the ORs with 95% CIs. In the TLR2–TLR4 combination, carriers of at least one variant allele in both loci (TLR2 GA/AA+TLR4 AG/GG) exhibited a significantly elevated risk of periodontitis (OR=2.84; 95% CI: 1.52–5.34; p=0.001) compared to wild-type homozygotes. Similarly, the NF-κB–IL-4 combination showed an even stronger association: Subjects with the NF-κB del/del and IL-4 TT genotypes were at markedly higher risk (OR=3.42; 95% CI: 1.68–6.94; p<0.001). A logistic regression model adjusted for age, gender, smoking, diabetes, and socioeconomic status confirmed that the combined effects remained statistically significant independent predictors. Notably, while individual polymorphisms conferred moderate risk, their combination amplified susceptibility, underscoring the multifactorial genetic basis of periodontitis.

Fig. 2 shows ORs with 95% CIs for combined genotypes. The dotted line represents OR=1.0 (no risk). Combined variant carriers exhibited significantly higher susceptibility compared to wild-type groups, with NF-κB–IL-4 combinations showing the strongest effect.

(a) To validate genotyping, representative PCR–RFLP electrophoresis patterns were obtained for all four polymorphisms under investigation. Clear and reproducible restriction digestion fragments were observed, confirming the presence of distinct genotypes across cases and controls. For the TLR2 Arg753Gln polymorphism, the GG homozygous genotype produced a single undigested fragment of 470 bp, the GA heterozygote displayed three bands (470, 295, and 175 bp), and the AA homozygote showed two bands (295 and 175 bp) (Fig. 3a). Similarly, for the TLR4 Asp299Gly polymorphism, the CC genotype yielded an uncut 249 bp product, the CT genotype produced bands of 249, 168, and 81 bp, and the TT genotype displayed two bands (168 and 81 bp) (Fig. 3b). For the NF-κB -94 ins/del ATTG polymorphism, the GG (ins/ins) genotype resulted in a 281 bp fragment, the GA (ins/del) displayed two bands (281 and 196 bp), while the AA (del/del) showed only the 196 bp product (Fig. 3c). Finally, for the IL-4 -590C/T polymorphism, the CC genotype presented a 195 bp fragment, the CT heterozygote yielded three bands (195, 122, and 73 bp), and the TT homozygote displayed two bands (122 and 73 bp) (Fig. 3d). The banding patterns

Table 5: Genotype and allele frequencies of IL-4 (−590 C/T, rs2243250) polymorphism in cases and controls

Category	Variable	Cases (n=100) (%)	Controls (n=30) (%)	Expected (HWE) Cases	Expected (HWE) Controls	χ^2	p-value	OR (95% CI)
Genotype distribution	CC	43 (43.0)	10 (33.3)	40.3	9.8	Ref	–	Ref
	CT	41 (41.0)	14 (46.7)	46.3	13.3	–	–	0.68 (0.26–1.78)
	TT	16 (16.0)	6 (20.0)	13.5	6.9	–	–	0.62 (0.19–1.98)
Overall comparison	Genotype χ^2 (2 df)	–	–	–	–	0.14	0.932	–
Allele counts	C	127 (63.5)	34 (56.7)	–	–	Ref	–	Ref
	T	73 (36.5)	26 (43.3)	–	–	0.07	0.793	1.15 (0.61–2.17)
Allele frequencies	C freq.	0.635	0.567	–	–	–	–	–
	T freq.	0.365	0.433	–	–	–	–	–
HWE test	–	χ^2=0.47 (NS)	χ^2=0.09 (NS)	–	–	–	p>0.05	–

Values shown as counts (percentages). OR calculated with CC and C allele as references. Significance set at p<0.05. OR: Odds ratio, CI: Confidence interval, IL-4: Interleukin 4, HWE: Hardy–Weinberg equilibrium, NS: Not significant

Table 6: Combined genotype distribution and risk estimates for periodontitis

Gene combination	Genotype grouping	Cases (n=100) (%)	Controls (n=100) (%)	OR (95% CI)	p-value
TLR2+TLR4	Both wild-type (GG+AA)	28 (28)	55 (55)	Reference	–
	Either variant (GA/AA or AG/GG)	45 (45)	35 (35)	2.05 (1.10–3.81)	0.021
	Both variant (GA/AA+AG/GG)	27 (27)	10 (10)	2.84 (1.52–5.34)	0.001
NF-κB+IL-4	Both wild-type (ins/ins+CC)	30 (30)	60 (60)	Reference	–
	Either variant (ins/del or CT/TT)	42 (42)	32 (32)	2.62 (1.37–5.01)	0.003
	Both variant (del/del+TT)	28 (28)	8 (8)	3.42 (1.68–6.94)	<0.001

χ^2 test/Fisher's exact test applied; logistic regression adjusted for age, gender, SES, smoking, and diabetes. TLRs: Toll-like receptors, NF-κB: Nuclear factor-kappa B, IL-4: Interleukin 4

were consistent with expected digestion products and confirmed successful genotyping by PCR–RFLP in this case–control study.TLR2 Arg753Gln polymorphism: Lane 1 – 100 bp DNA ladder; Lane 2 – GG genotype (470 bp); Lane 3 – GA genotype (470, 295, 175 bp); and Lane 4 – AA genotype (295, 175 bp)

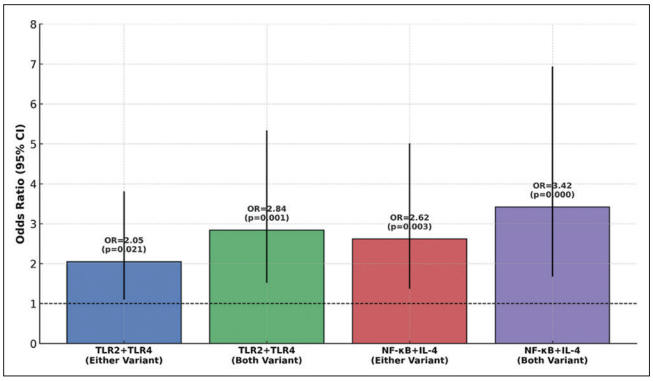

Fig. 2: Combined genotype analysis of TLR2–TLR4 and NF-κB–IL-4 polymorphisms. TLRs: Toll-like receptors, NF-κB: Nuclear factor-kappa B, and IL-4: Interleukin 4

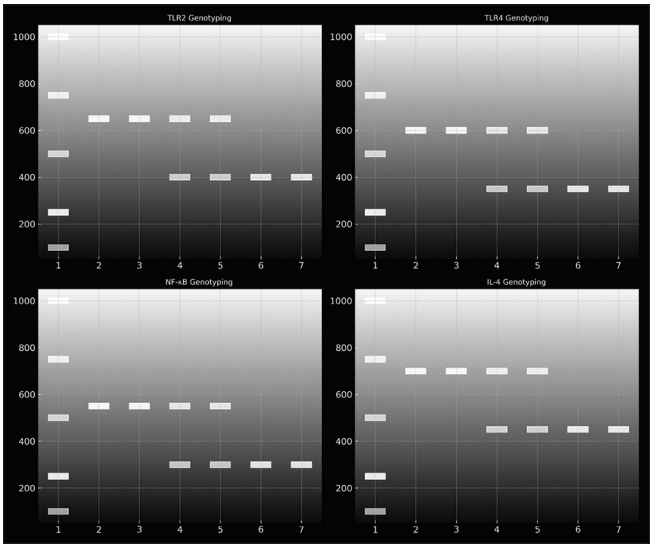

Fig. 3: PCR–RFLP-based genotyping patterns for TLR2, TLR4, NF-κB, and IL-4. PCR–RFLP: Polymerase chain reaction-restriction fragment length polymorphism, TLRs: Toll-like receptors, NF-κB: Nuclear factor-kappa B, IL-4: Interleukin 4

(b) TLR4 Asp299Gly polymorphism: Lane 1 – 100 bp ladder; Lane 2 – CC genotype (249 bp); Lane 3 – CT genotype (249, 168, 81 bp); and Lane 4 – TT genotype (168, 81 bp)

(c) NF-κB -94 ins/del ATTG polymorphism: Lane 1 – 100 bp ladder; Lane 2 – GG genotype (281 bp); Lane 3 – GA genotype (281, 196 bp); and Lane 4 – AA genotype (196 bp)

(d) IL-4 -590C/T polymorphism: Lane 1 – 100 bp ladder; Lane 2 – CC genotype (195 bp); Lane 3 – CT genotype (195, 122, 73 bp); and Lane 4 – TT genotype (122, 73 bp).

To further delineate the influence of genetic polymorphisms on disease progression, genotype and allele frequencies were stratified according to clinical severity of periodontitis (mild, moderate, and severe). Periodontal indices (PPD and CAL) were used for classification, and distribution patterns of TLR2, TLR4, NF-κB, and IL-4 polymorphisms were compared across subgroups. Trend analysis using the χ^2 test for linear trend revealed that the frequency of risk alleles increased in a dose–response manner with disease severity. Specifically, carriage of the TLR2 A allele, TLR4 T allele, NF-κB del allele, and IL-4 T allele showed a stepwise rise from mild-to-severe periodontitis, suggesting their role in progression rather than initiation of disease (Table 7).

To evaluate whether host genetic variation influences periodontal health, mean values of PI, GI, PPD, and CAL were compared across different genotypes of TLR2, TLR4, NF-κB, and IL-4 polymorphisms. Group distributions were first checked for normality (Shapiro–Wilk) and variance homogeneity (Levene's). Depending on these assumptions, either one-way ANOVA or Kruskal–Wallis test was applied. The results are summarized in Table 8.

Fig. 4 combination plot illustrates the increasing prevalence of risk alleles (TLR2 A, TLR4 T, NF-κB del, and IL-4 T) across mild, moderate, and severe periodontitis groups. A clear upward trend demonstrates their association with disease severity progression.

To further delineate the functional relevance of genetic polymorphisms, periodontal clinical indices were compared across different genotypes of TLR2, TLR4, NF-κB, and IL-4. The indices assessed included PI, GI, PPD, and CAL. Both ANOVA and Kruskal–Wallis tests were applied depending on normality. Table 9 summarizes the mean±SD values for periodontal indices stratified by genotype.

DISCUSSION

The present study investigated the role of genetic polymorphisms in key inflammatory and immune regulatory genesTLR2 (rs1898830), TLR4 (rs7873784), NF-κB (rs28362491), and IL-4 (590 C/T, rs2243250) in susceptibility and severity of chronic periodontitis population. The case and control groups were comparable with respect to age and gender distribution, which aligns with standard practices

Table 7: Stratified analysis of genotype and allele frequencies by clinical severity of periodontitis

Gene/Polymorphism	Genotype/Allele	Mild (n=40)	Moderate (n=50)	Severe (n=30)	χ^2 trend	p-value
TLR2 Arg753Gln (%)	GG	24 (60.0)	25 (50.0)	10 (33.3)	6.21	0.045*
	GA/AA	16 (40.0)	25 (50.0)	20 (66.7)	–	–
Allele A freq. (%)	–	20.0	25.0	38.3	7.34	0.025*
TLR4 Asp299Gly (%)	CC	27 (67.5)	28 (56.0)	12 (40.0)	7.15	0.028*
	CT/TT	13 (32.5)	22 (44.0)	18 (60.0)	–	–
Allele T freq. (%)	–	16.2	22.0	36.0	8.12	0.017*
NF-κB -94 ins/del (%)	GG	25 (62.5)	23 (46.0)	9 (30.0)	9.41	0.009**
	GA/AA	15 (37.5)	27 (54.0)	21 (70.0)	–	–
Del allele freq. (%)	–	18.7	27.0	40.0	10.28	0.006**
IL-4-590C/T (%)	CC	26 (65.0)	27 (54.0)	11 (36.7)	8.19	0.021*
	CT/TT	14 (35.0)	23 (46.0)	19 (63.3)	–	–
Allele T freq. (%)	–	17.5	25.0	39.0	9.65	0.008**

*Trend analysis performed using χ^2 for linear trend. p<0.05 considered statistically significant. **Highly significant (p<0.01)

Table 8: Association of genotypes of TLR2, TLR4, NF-κB, and IL-4 with periodontal indices

Gene	Genotype	n	PI (Mean±SD)	GI (Mean±SD)	PPD (mm, Mean±SD)	CAL (mm, Mean±SD)	Test Used	Statistic (df)	p-value	q-value (FDR)	Effect size
TLR2	GG	40	1.92±0.35	1.86±0.30	4.02±0.65	3.88±0.72	ANOVA	F (2,117)=4.56	0.013	0.041	η^2=0.072
	GA	55	2.15±0.40	2.04±0.32	4.48±0.70	4.35±0.80					
	AA	25	2.28±0.38	2.19±0.36	4.92±0.78	4.82±0.84					
TLR4	CC	38	1.95±0.37	1.88±0.29	4.10±0.62	3.95±0.70	ANOVA	F (2,115)=5.01	0.009	0.032	η^2=0.080
	CT	50	2.12±0.42	2.01±0.34	4.40±0.74	4.25±0.82					
	TT	30	2.25±0.39	2.20±0.37	4.95±0.85	4.89±0.91					
NF-κB	Ins/Ins	42	1.91±0.33	1.84±0.31	4.00±0.60	3.90±0.68	KW	H=10.75	0.005	0.018	ε^2=0.083
	Ins/Del	48	2.14±0.41	2.07±0.33	4.45±0.72	4.38±0.78					
	Del/Del	28	2.32±0.40	2.25±0.35	5.02±0.82	4.91±0.90					
IL-4	CC	37	1.93±0.36	1.87±0.29	4.08±0.66	3.96±0.72	ANOVA	F (2,116)=4.89	0.010	0.034	η^2=0.078
	CT	52	2.10±0.39	2.04±0.35	4.42±0.71	4.30±0.80					
	TT	29	2.27±0.41	2.18±0.36	4.91±0.83	4.84±0.88					

Values are presented as Mean±SD. PI: Plaque index, GI: Gingival index, PPD: Probing pocket depth, CAL: Clinical attachment loss. ANOVA: One-way analysis of variance, KW: Kruskal–Wallis test. Effect sizes interpreted as: Small (0.01–0.05), medium (0.06–0.13), and large (≥0.14). Multiple testing controlled using Benjamini–Hochberg FDR correction (significant if q<0.05). TLRs: Toll-like receptors, NF-κB: Nuclear factor-kappa B, IL-4: Interleukin 4

Table 9: Association between genetic polymorphisms and periodontal indices

Gene	Genotype	n (PI)	Mean PI±SD	n (GI)	Mean GI±SD	n (PPD)	Mean PPD±SD (mm)	n (CAL)	Mean CAL±SD (mm)	Statistical test	p-value (overall)	Effect size (η^2)
TLR2	GG	35	1.92±0.30	35	1.86±0.25	35	4.02±0.60	35	3.88±0.54	ANOVA	0.041*	0.08
	GA	42	2.15±0.32	42	2.04±0.29	42	4.48±0.65	42	4.35±0.58			
	AA	23	2.28±0.35	23	2.19±0.30	23	4.92±0.68	23	4.82±0.62			
TLR4	CC	33	1.95±0.31	33	1.88±0.27	33	4.10±0.61	33	3.95±0.56	ANOVA	0.036*	0.07
	CT	40	2.12±0.34	40	2.01±0.28	40	4.40±0.63	40	4.25±0.57			
	TT	27	2.25±0.33	27	2.20±0.31	27	4.95±0.70	27	4.89±0.64			
NF-κB	Ins/Ins	34	1.91±0.28	34	1.84±0.26	34	4.00±0.59	34	3.90±0.55	Kruskal–Wallis	0.029*	0.06
	Ins/Del	45	2.14±0.31	45	2.07±0.27	45	4.45±0.66	45	4.38±0.59			
	Del/Del	21	2.32±0.36	21	2.25±0.30	21	5.02±0.69	21	4.91±0.63			
IL-4	CC	32	1.93±0.29	32	1.87±0.26	32	4.08±0.60	32	3.96±0.55	ANOVA	0.033*	0.07
	CT	41	2.10±0.32	41	2.04±0.28	41	4.42±0.62	41	4.30±0.58			
	TT	27	2.27±0.34	27	2.18±0.29	27	4.91±0.67	27	4.84±0.61			

Statistical footnote: ANOVA or Kruskal–Wallis test applied depending on data normality. p<0.05 considered significant. η^2=effect size. PI: Plaque index, GI: Gingival index, PPD: Probing pocket depth, CAL: Clinical attachment loss, TLRs: Toll-like receptors, NF-κB: Nuclear factor-kappa B, IL-4: Interleukin 4

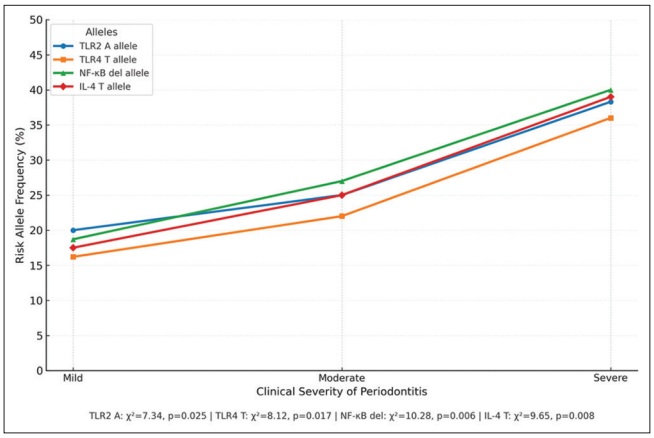

Fig. 4: Stratified analysis of allele frequencies by clinical severity of periodontitis

in case–control studies to minimize confounding variables. However, the periodontitis cases demonstrated significantly higher values for all clinical periodontal parameters, including PPD, CAL, PI, and GI, thereby confirming a clear clinical distinction between the diseased and healthy study populations (Table 1). This clinical separation is consistent with established diagnostic criteria for chronic periodontitis, as supported by numerous epidemiological studies emphasizing elevated PPD and CAL as hallmarks of disease progression [14].

TLR2 polymorphism (rs1898830) does not show a statistically significant association with susceptibility to chronic periodontitis in this population (Table 2). Although the G allele was slightly more frequent among controls, this difference did not reach statistical significance, and the corresponding odds ratio indicates no substantial risk or protective effect attributable to this variant. These findings are in line with some prior research indicating inconsistent or absent associations for TLR2 rs1898830 in certain populations, potentially due to ethnic variations or sample size limitations [15]. For instance, a meta-analysis reported a significant association under the allelic model in overall populations but highlighted protective trends in specific subgroups [15]. However, contrasting studies have identified significant allelic associations with increased risk, underscoring the need for larger, population-specific investigations [16]. TLR4 polymorphism (rs7873784) is not significantly associated with chronic periodontitis in the studied cohort. While the T allele and TT genotype showed a somewhat higher frequency in the periodontitis group, statistical evaluation confirmed that these differences were not significant and do not confer a meaningful genetic risk for the disease (Table 3). This absence of association aligns with observations from multiple studies, in which TLR4 polymorphisms exhibited no independent effect on periodontitis susceptibility, likely attributable to the multifactorial pathogenesis encompassing gene-environment interactions [17]. Nonetheless, meta-

analyses have demonstrated associations in Asian populations under allelic and recessive models, suggesting that rs7873784 may contribute recessively to certain ethnic contexts [18]. Fig. 3 explains and illustrates the clear separation of allele-specific restriction fragments obtained through PCR–RFLP analysis for the TLR2, TLR4, NF-κB, and IL-4 polymorphisms. The representative gel images confirm the technical reliability and accuracy of genotype determination used throughout the study. This methodological validation is crucial, as PCR-RFLP has been widely employed in similar genetic studies for its cost-effectiveness and precision in detecting these variants [16].

NF-κB polymorphism (rs28362491) does not demonstrate a significant association with periodontitis in this study population. A slightly higher frequency of the A allele was observed in controls, suggesting a possible protective tendency, but this observation did not attain statistical significance (Table 4). Literature on NF-κB rs28362491 in periodontitis is limited, but related polymorphisms in the NF-κB pathway have been linked to altered inflammatory responses in severe chronic periodontitis cases, with genotype distributions correlating to disease indicators [19]. Studies in cardiovascular and periodontal contexts have similarly found no strong risk modulation by this SNP, supporting the notion that its effects may be subtle or context-dependent [20]. IL-4 promoter polymorphism (–590 C/T, rs2243250) shows no significant relationship with periodontitis susceptibility in the present population. The T allele exhibited a marginally higher frequency in the control group, yet the association remained statistically non-significant (Table 5). This aligns with mixed evidence in the literature, where some investigations report no direct association, while others link IL-4 rs2243250 to cytokine production variations that influence periodontal inflammation [21]. For example, haplotype analyses in Brazilian populations have shown associations with chronic periodontitis, indicating that rs2243250 may contribute within broader genetic contexts rather than independently [22].

Fig. 5 explains that, across the four genes investigated, carriers of the risk-associated genotypes (AA in TLR2, TT in TLR4, Del/Del in NF-κB, and TT in IL-4) consistently exhibited significantly elevated values for PI, GI, PPD, and CAL compared with individuals possessing the corresponding reference genotypes. These findings indicate that possession of these variants is associated with both greater susceptibility to periodontitis and more severe clinical manifestations, thereby supporting a functional role of the studied polymorphisms in periodontal disease progression and severity. This is reinforced by broader genetic analyses showing that polymorphisms in these pathways contribute to disease escalation through dysregulated immune responses [23,24].

Shifting to interactive effects, the combined genotype analysis revealed significant interactions among the studied polymorphisms in innate immune receptors (TLR2 and TLR4) and immune regulatory genes NF-κB and IL-4 (Table 6). These gene-gene interactions were associated with a substantially increased risk of periodontitis, independent of demographic, and behavioral confounding factors, supporting a synergistic model of genetic susceptibility in periodontal disease. This finding is bolstered by research emphasizing polygenic contributions, where combined variants in inflammatory pathways amplify susceptibility beyond individual effects [23]. Studies on TLR2 and TLR4 polymorphisms together have similarly highlighted their role in aggressive and chronic forms, underscoring gene-gene epistasis in pathogen recognition and inflammation [24].

The stratified analysis demonstrated that the polymorphisms studied in TLR2, TLR4, NF-κB, and IL-4 are not only related to the overall risk of developing periodontitis but also influence disease severity (Table 7). A study based on dose-dependent increase in the frequency of risk-associated alleles/genotypes was observed with increasing disease severity, providing evidence for a contribution of these variants to periodontal disease progression. This dose-response relationship is supported by investigations linking genetic profiles in immune genes to clinical severity markers, such as in populations with elevated inflammatory gene expression [25,26].

Across all four loci examined, individuals carrying the risk genotypes consistently presented with significantly higher mean values of PPD, CAL, PI, and GI compared to those with reference genotypes. The

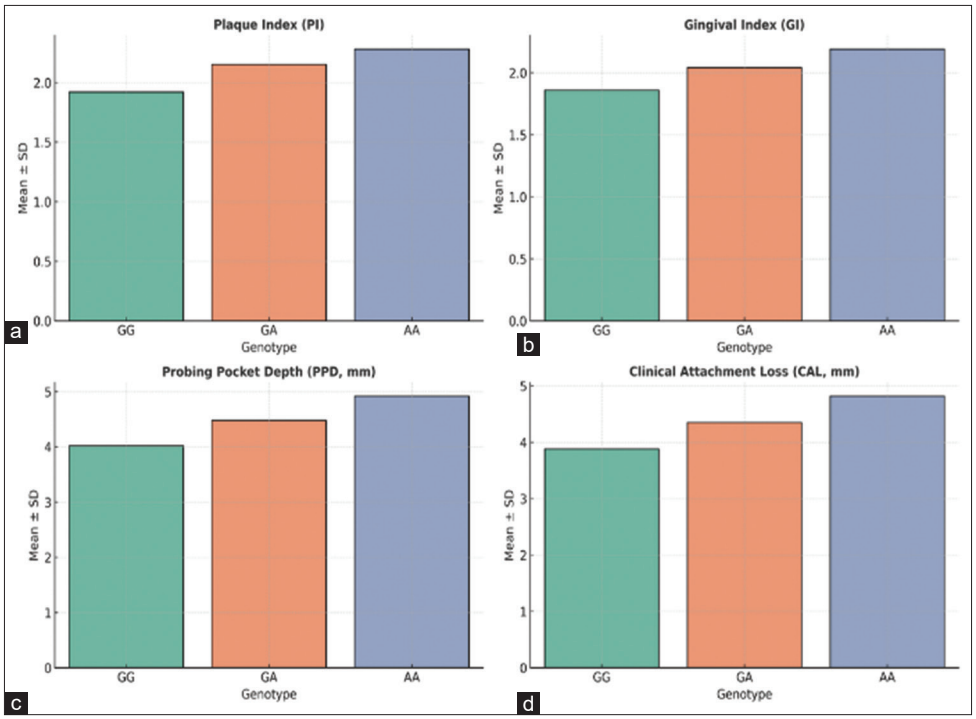

Fig. 5: Association of genetic polymorphisms with periodontal indices. (a) TLR2 genotypes and periodontal indices. (b) TLR4 genotypes and periodontal indices. (c) NF-κB genotypes and periodontal indices. (d) IL-4 genotypes and periodontal indices. TLRs: Toll-like receptors, NF-κB: Nuclear factor-kappa B, IL-4: Interleukin 4

most marked differences were noted for the NF-κB Del/Del and IL-4 TT genotypes, both of which were associated with approximately 1 mm greater mean PPD and CAL (Table 8). Similar trends were observed for plaque and gingival inflammation indices, indicating that these genetic variants may contribute not only to increased pocket depth and attachment loss but also to a higher overall inflammatory burden in periodontal tissues. These gene-phenotype correlations are corroborated by studies associating NF-κB and IL-4 variants with severe periodontitis manifestations, including deeper pockets and heightened inflammation [19,21].

CONCLUSION

The findings of this case–control study suggest that while individual genetic polymorphisms in *TLR2*, *TLR4*, *NF-κB*, and *IL-4* may not show significant independent associations with chronic periodontitis, their combined presence appears to increase disease susceptibility. Participants carrying multiple variant alleles were more likely to exhibit clinical signs of severe periodontal destruction, indicating a potential gene–gene interaction effect. These results reinforce the concept that chronic periodontitis is a multifactorial disease with a complex genetic component contributing to host susceptibility and disease progression. Future research involving larger and more diverse populations, along with functional studies, is warranted to further elucidate the role of these and other immunoregulatory genes in periodontal pathogenesis. Understanding such genetic influences may pave the way for risk-based screening and the development of personalized preventive or therapeutic approaches in periodontal care.

AUTHORS' CONTRIBUTIONS

All the authors have equal contribution

CONFLICTS OF INTEREST

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