ASDJ
AINSHAMS DENTAL JOURNAL

Print ISSN 1110-7642

Online ISSN 2735-5039

AIN SHAMS DENTAL JOURNAL
Official Publication of Ain Shams Dental School
March 2021 • Vol. XXIV

Toll-like receptor-4 Single Gene Polymorphism and Chronic Periodontitis Susceptibility in a Sample of Egyptian Population: A Case-Control Study

Mihad M. Farouk [1] ,Nevine H. Kheir El Din [2] , Ola M. Ezzat [3] Olfat G. Shaker[4]

Abstract

Objectives: Toll-like receptors (TLR) are signal molecules that act as pattern recognition receptors (PRRs) to bacterial cell wall components. Due to this particular reason, differences in host susceptibility to periodontitis are owed mainly to functional polymorphisms located in PRRs. The aim of this study was to investigate relationship between periodontitis and variation in the TLR4 gene: Thr/Ile (rs4986791), Gly/Thr (rs2149356), Asp/Gly (rs4986790).\

Subjects and Methods: The study design was case-control performed in sample of Egyptians; 50 periodontitis (Cases) and 50 with healthy periodontium (Control). Clinical periodontal parameters were recorded and blood samples were taken for all participants. For genetic analysis Real time polymerase chain reaction (RT-PCR) was the method for recording different genotypes related to single nucleotide polymorphisms (SNP) in TLR4 gene.

Results: The group suffering from periodontitis had a significantly higher frequency of TT (mutant) genotype (30%), when compared to 8 % in the control group. Conversely, the control group showed a higher frequency of GT (heterozygous) genotype (62%), in comparison to 44% in periodontitis group. It was calculated that the relative risk of potential periodontal infection in patients with GT genotype is 0.935. The relative risk of potential periodontal infection in patients with TT genotype is 2.544. Thr/Ile (rs4986791), Asp/Gly (rs4986790) SNPs were not found in the study participants.

Conclusion: In our sample of the Egyptian population, TLR4 SNP rs2149356 was linked with periodontitis. Subjects with homozygous mutant genotype (TT) are more susceptible to periodontitis. While subjects with heterozygous genotype (GT) are at a less risk than homozygous mutant compared to heterozygous normal genotype (GG). TLR4 SNPs Asp/Gly rs4986790 and Thr/Ile rs4986791 do not exist in the Egyptian population.

Keywords: periodontitis, Toll-like receptor 4, single nucleotide polymorphisms

1. Teaching assistant of Oral Medicine, periodontology & Oral Diagnosis, Faculty of Dentistry, British University. Egypt
2. Professor of Oral l Medicine, periodontology & Oral Diagnosis, Faculty of Dentistry, Ain Shams University
3. Assistant Professor of Oral Medicine, periodontology & Oral Diagnosis, Faculty of Dentistry, Ain Shams University
4. Professor of Molecular Biology and Biochemistry, Faculty of Medicine, Cairo University

Introduction:

Periodontitis is an immune inflammatory disease of complex multifactorial etiology. Nevertheless, the presence of gram negative microbes is of paramount importance in the initiation and progression of periodontal diseases, individual distinction in the susceptibility to disease is affected by environmental and genetic influences.1

TLR4 is found on antigen presenting cells, fibroblasts and keratinocytes of the gingival epithelium. TLR4's main function is the recognition of lipopolysaccharides (LPS) of Gram-negative bacteria such as: Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans and Veillonella parvula. This recognition is completed via engaging several protein components such as lipopolysaccharide binding protein (LBP) and CD14.2 The previous process leads to cytokine activation through Nuclear Factor Kappa beta (NF-Kb) which are activated by inflammatory cells.3,4

Pro-inflammatory cytokines such as Interleukin 6 (IL-6), IL-8 and TNF-α and matrix metalloproteinase and prostaglandin E2 cause symptoms such as bone loss (Giannobile, 2008). Genetic variations of host's immune-related DNA molecules such as gene polymorphisms affect the occurrence and the patterns of diseases.5

Genetic polymorphisms and especially single nucleotide polymorphisms (SNPs) that cause allelic differences in genes encoding host defense, could affect the susceptibility and progression of periodontal disease.6Hence, it is important to review different forms and phenotypes of immune-related molecules such as TLR4 for the prevention and treatment of periodontitis.7

TLR4 SNPs cause altered microorganism recognition and increase the susceptibility to periodontitis and leads to an aggravated periodontal infection and severe bone loss.8

Many TLR4 SNPs have been identified. The most recent is the G/T substitution which leads to replacement of glycine (Gly) with threonine (Thy) on the intron of TLR4 receptor.9

The aim of this case-control study was to investigate the association between the genetic variations of TLR-4 and periodontal disease susceptibility, specifically TLR-4 Thr/Ile (rs4986791)] SNPs.

METHODOLOGY:

One hundred Egyptian subjects were recruited from the outpatient clinic of Oral Medicine, Periodontology, and Oral Diagnosis department, Faculty of Dentistry, Ain Shams University and Faculty of Oral and Dental Medicine, British University in Egypt during the period from February 2018 to April 2019. The study was designed as a case control study; 50 Egyptian periodontitis patients (Cases) and 50 Egyptian patients with healthy periodontium (Control). Both genders with an age range from 30 years to 50 years were included in this study. Unrelated Egyptians from a close socio-economic status (SES) and residing in the same geographical area and suffering from Periodontitis stage II and/or stage III), affecting more than 30% of total sites) and graded as Grade B periodontitis were classified as cases. Patients with

history or current manifestation of serious systemic diseases, Vulnerable groups such as pregnant females, Patients who had received periodontal therapy and smokers in the past six months were excluded. The proposal was reviewed by the faculty of Dentistry, Ain Shams University Research Ethics Committee (FDASU-REC). Both cases and controls signed an informed consent after understanding the purpose of the research and received standard periodontal treatment after sample withdrawal. Genetic samples were not used for any other purposes and individual patients' results were kept confidential.

Patients who met the eligibility criteria were assessed clinically for clinical parameters such as: PI, GI, CAL, BOP% using the university of Michigan's O probe with William's markings periodontal probe. Venous blood samples (*5 ml) from each patient were collected by trained laboratory technicians using a sterile technique; whole blood was collected in EDTA-containing vacutainer tubes and stored at -80 C until needed for molecular assays.SNP genotyping in TLR4 gene was carried out by Real Time Polymerase Chain Reaction (RT-PCR). Genomic DNA was prepared from peripheral blood lymphocyte using a QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Extracted genomic DNA was amplified in a 25-ll solution using TaqMan Universal PCR Master Mix assay, primers: TLR-4 (rs2149356) Cat. # 4351379, and TaqMan probe (FAM) dye mix and DNase-free, sterile-filtered water (Applied Biosciences Hispania, Alcobendas, Madrid, Spain), supplied by Clinilab, Cairo, Egypt. The amplification conditions were as follows: after a denaturation time of 10 min at 96 C, 45 cycles at 60 C for 90 s and at 92 C for 15s for annealing and extension were carried out and fluorescence was measured at the end of every cycle and at the endpoint.

Results:

Demographic characteristics of the study sample:

The study sample included 100 Egyptian subjects: 50 periodontitis patients [26 females (52%) and 24 males (48%)], with mean age 40.4±7 years; and 50 patients with healthy periodontium (control) [26 females (52%) and 24 males (48%)], with mean age 36.3±6.4 years. Independent t-test showed a remarkable variance between groups' age (p=0.003) revealing that the group suffering from periodontitis were older than the control group. Patients and control subjects were matched with no significant difference between them regarding gender distribution.

Genetic analysis

Genotypes:

Frequencies and percentages (%) for Gly/Thr (rs2149356) are presented in Table (1).

The periodontitis group had a significantly higher frequency of TT (mutant) genotype (30%), in comparison to 8 % in the control group. In contrast, the group of controls showed a higher frequency of GT (heterozygous) genotype (62%), in comparison to 44% in periodontitis group. difference between groups was statistically significant (p=0.018) as shown in Figure(1).

Table (1) Frequencies and percentages (%) for Gly/Thr (rs2149356)

Groups		Genotype (rs2149356) Gly/Thr			Total
		GG	GT	TT	
Periodontitis	Count	13	22	15	50
	% within Groups	26.0%	44.0%	30.0%	100.0%
Control	Count	15	31	4	50
	% within Groups	30.0%	62.0%	8.0%	100.0%
Total	Count	28	53	19	100
	% within Groups	28.0%	53.0%	19.0%	100.0%

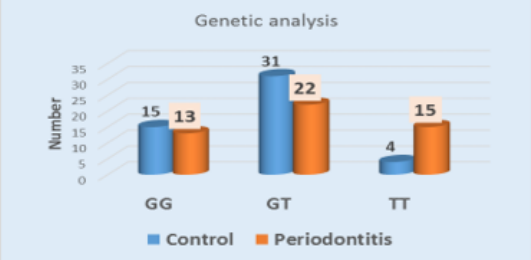

Figure (1): Bar chart showing frequency of different genotypes in control and periodontitis group.

Alleles:

Frequencies and percentages (%) for different alleles in periodontitis and control subjects are presented as shown per **Table (2).** The periodontitis group had a higher frequency of T allele (the mutant allele) (52%), in comparison to 39 % in the control group. In contrast, the group of controls showed a higher frequency of G (normal allele) allele (61%), in comparison to 48% in periodontitis group. Chi square test revealed that the difference between groups was not statistically significant (p=0.064). **(Figure 2).**

Table (2) Frequencies and percentages (%) for different alleles

Groups		Allele		X^2	P-value
		G	T		
Periodontitis	Count	48	52		
	% within Groups	48%	52%	3.41	0.064
Control	Count	61	39		
	% within Groups	61%	39%		
Total	Count	109	91		
	% within Groups	54.5%	45.5%	-----	

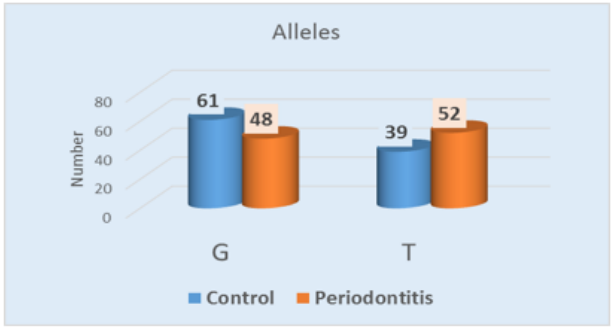

Figure (2): Bar chart showing frequency of different alleles in control and periodontitis group.

Relative risk

The relative risk of getting periodontitis in patients with GT genotype is 0.935 (95% confidence interval [C.I.] from 0.67 to 1.29). The relative risk of getting periodontitis in patients with TT genotype is 2.544 (95% confidence interval [C.I.] from 0.997 to 6.49). ***(Table 3)***

Table (3): Relative risk (RR) estimation amongst different genotypes.

	Genotype	Periodontitis	Control	RR	95% C.I.
GT vs GG	GT	22 (44%)	31 (62%)	0.935	0.67 to 1.29
	GG	13 (26%)	15 (30%)		
TT vs GG	TT	15 (30%)	4 (8%)	2.544	0.997 to 6.49
	GG	13 (26%)	15 (30%)		

Discussion:

In the current case control study patients and control subjects were matched without noteworthy difference amongst them in the matter of gender distribution. However, a significant difference between groups' age was found revealing that the group suffering from periodontitis were significantly older than the controls**.** These finding are supported by the findings of ***Tadjoedin et al.***[11] that periodontitis (formerly chronic periodontitis) has a tendency to increase with age.

All participants of periodontitis group (100%) were predetermined as grade B as per the inclusion criteria to have moderate rate of progression with case phenotypes that destruction commensurate with biofilm deposits according to ***Tonetti, 2018***.[1] Regarding stage, Stage II showed a higher frequency (80%), compared to Stage III (20%); This reveals that moderate destruction rate periodontitis is more common than severe destruction rate periodontitis in this sample of the population.

All subjects included in this study (100%) were predetermined to be medically free, non-smokers this was done by previous authors[12]to eliminate confounding factors. For the periodontitis group 50% of the patients' chief complaint was halitosis while 30% complained of mobility and 20% complained of pain which are known complaints associated with periodontal diseases.

In the current case control study frequencies and percentages (%) for TLR-4 Gly/Thr (rs2149356), Asp/Gly (rs4986790) and Thr/Ile (rs4986791) were assessed in periodontitis and control subjects. No evidence of The genetic alterations of Asp/Gly rs4986790 and Thr/Ile rs4986791 was found in either the cases or the controls.

Mutation of Thr/Ile (T/C) rs4986791 was not found in the whole subjects, only the homozygous normal wild type (CC) was found in 100 % of periodontitis patients and controls with healthy periodontium. Neither the heterozygous (CT) nor the mutant (TT) were found in either any of the cases or the controls.

Mutation of Asp/Gly (A/G) rs4986790 was not found in the whole subjects, only the homozygous normal wild type (GG) was found in 100% of periodontitis patients and controls with healthy periodontium. Neither the heterozygous (AG) nor the mutant (AA) were found in either any of the cases or the controls.

These results are consistent with another Egyptian study assessing the association of TLR4 gene polymorphisms in Systemic lupus (SLE) and Rheumatoid Arthritis patients (RA). No evidence of the genetic alterations of Asp299Gly and Thr399Ile polymorphisms in the RA patients group, the SLE patients group, or control population. Both The patients group and control population had simmilar genotypes so this verifies that these two polymorphisms are not found in a group of Egyptian population**.**[13]

Frequencies and percentages (%) for Gly/Thr (rs2149356) indicated an association between SNP, rs2149356 (TT/GG+GT, p=0.018), and periodontitis respectively.

The patient's suffering from periodontitis showed a considerably greater frequency of TT (mutant) genotype (30%), when compared to (8 %) in the control group. The controls expressed a greater occurrence of GT (heterozygous) genotype (62%), when compared to 44% in periodontitis group. The difference between groups was statistically substantial (p=0.018) as per Chi square test. Mutant genotype (TT) is predominant among the periodontitis group, while it's least prevalent in the control group. The normal homozygous genotype (GG) occurs at a higher incidence for the controls rather than the periodontitis group. The heterozygous genotype (GT) occurs more in the control group than the periodontitis group.

The previous findings indicated that SNP of Toll-like receptor 4 rs2149356 (TT/GG+GT) is linked with severe loss of periodontal attachement in Egyptian patients suffering from periodontitis. These findings are consistent with those of ***Li et al.***[8] that stated that the genetic variations of rs4986790 and rs4986791 were not noted in neither of the study groups. But proved that there was an association between TLR4 SNP rs2149356 (TT/GG+GT, OR D 7.60, p < 0:001), and severe loss of periodontal attachement in north Chinese subjects [8].

The allele frequency was also measured to further ensure the association of the mutant allele to periodontitis as proposed by ***Berdeli et al.***[6]The allele with the higher frequency in the Periodontitis group is T which is the mutant allele, while for the control group, it is the G allele which is the normal allele at a statistically significant value (p=0.064). These results suggested that the mutant allele is associated with periodontitis, these results are consistent with those of ***Qing et al.***[14]***; Li et al.***[8]

In order to test susceptibility to periodontitis, ***Reddy et al.***[15]suggested calculating the relative risk (OR). The relative risk of getting periodontitis in patients with TT genotype is 2.544 (95% confidence interval [C.I.] from 0.997 to 6.49). This denotes that the subjects with homozygous mutant genotype (TT) are 2.544 times at a higher relative risk for periodontitis, and thus are more susceptible to periodontitis.

The relative risk of getting periodontitis in patients with GT genotype is 0.935 (95% confidence interval [C.I.] from 0.67 to 1.29). This denotes that the subjects with heterozygous genotype (GT) are 0.935 times at a higher relative risk, and thus are more susceptible to periodontitis than patients with heterozygous normal genotype (GG). ***Li et al.***[8] found that patients with mutant genotype TT were at 7.6 times at a higher relative risk for periodontitis.

Well-designed studies related to caucasian populations linked the genetic variation known as polymorphism with increased sucseptibility to periodontitis though at wavering degrees.[16]

Differences in the pressure of environmental pathogens on human populations explains the varying distribution of the previously mentioned polymorphisms that leads to their precise geographical spread.[17]

The relationship of TLR4 Asp299Gly with periodontal disease is debatable as per several authors.The prevelance of periodontitis has been

reported to elevated by some researchers or decreased by others when linked to TLR4 Asp299Gly SNP.[18,19]Some researches concluded the lack of a relationship between periodontal disease and TLR4 Asp299Gly SNP.[6,20] Based on the latters' findings , there is an inverse relationship between the carriage of P. gingivalis and TLR4 Asp299Gly SNP.

In researches assesing the Mongoloid race , it was concluded that the genotypes Asp299Gly(rs4986790) and Thr399Ile (rs4986791) do not exisist.[16] Studies reviewing Japanese and Chinese populations had the same interpretations, at which there was no occurrence of these alleles in any patient suffering from periodontal disease.[21,22,23]The same can be said about the Egyptian population as studies by ***Taha et al.***[13]related to Systemic Lupus Erythematosis (SLE) and ***Sadik et al.***[10]concerning Hepatitis C Virus (HCV) patients; have found only the wild type of these genotype among their study subjects. However, SNP TLR4 rs2148356-T/T was associated with both diseases.[10,13]

Rs2149356 was also not only found in the Egyptian population but in an aaray of Asian populations, such as Japanese , Korean as well as Chinese. [14,23,24,25] The polymorphism exisits in Europeans and other Caucasians.[26] It was also linked positively with different disease: such as type 2 diabetes mellitus, gouty arthritis, it also affected the response of HCV patients to ribavirin therapy.[10]The exsistance of the missense mutation of SNPs was affected by the difference in race.[27]

The link between TLR4 SNPs polymorphism and periodontal disease in a multitude of ethnicities was evaluated by different studies. Seven meta-analyses have been conducted, but there were inconsistencies in the information acquired moreover, two systematic reviews have been conducted, and all came up with varying conclusions.[16,22]The variation of ethnic backgrounds and the pressure exerted by of confounding factors such as smoking and systemic disease are the main contributing factors in the difference in outcomes in the previous clinical studies.[28]

As far as we know, this is the pioneer study to assess the link between these three SNPs with susceptibility of periodontitis regarding the Egyptian population. The location of SNP rs2149356 is in the intron of TLR4 gene, the regulation of TLR4 mRNA expression is its main scope. The expression of the TLR4 gene is regulated by the intronic sequence and a critical role in the gene expression process is played by rs2149356 polymorphism . Despite not directly participating in the process of transcription initiating amino acid substitutions, like rs4986791,[12] it impacts the expression of genes in different methods such as augmenting the part of mRNA transcripts, merging and/or causing a decreaase in the proper merging regardless of amino acid substitution. Inverse associations are shown in subjects with TLR4 mRNA rs2149356.[14,29]

Both arms of the reaction of the immunnity, the innate and adaptive responses, are started by TLRs.Both act together to combat infection in mammals. The response of the innate immunity offers instant protection. Still, its mode of

attack on pathogens is rather non specific, sometimes this leads to healthy tissue damage in cases where the innate immune response persists for a long time. A common signaling pathway is activated by TLRs which results in stimulation of pro-inflammatory cytokines such as tumor necrosis factor (TNF) a, and interleukin (IL-6, IL-1b and IL-12), alternative pathways are also activated .The former prompt suitable effector responses as a response to different types of pathogens.[30,31]

Interactions with Toll/interleukin (IL)-1 receptor have been reported by TLR4 forming (TIR) domains. Four adaptor proteins MyD88-adaptorlike/TIR domain-containing adaptor protein [MAL/TIRAP], myeloid differentiation factor [MyD88],[32] TIR domain-containing adaptor inducing interferon-b [TRIF] and TRIF-related adaptor molecule [TRAM].[33,34]

The previously mentioned cause signal transduction from the different TIR domains and involve downstream signaling proteins.

TLR4 signaling was categorised into MyD88 dependent and MyD88 independent. The MyD88-dependent pathway is signaled by IL-1 receptor-associated kinase (IRAK) 1, IRAK4, tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6, and transforming growth factor-b-activated kinase (TAK) 1, which triggers downstream of mitogen-activated protein kinase (MAPK) pathways[35]. The previously mentioned events lead to stimulating the transcription factors nuclear factor (NF-kB) and activator protein (AP)- 1, respectively, and regulate the expression of pro-inflammatory cytokines and other genes related to the immune system. The MyD88-independent pathway is interceded by TRIF, which stimulates interferon regulatory factor (IRF) 3, prompts interferon (IFN)-b expression, IFN-responsive genes,moreover it leads to the NF-kB and MAPK late-phase activation.[36]

The elevated incidence of the TLR4 rs2148356-T/T genotype in the group suffering from periodontitis and its connotation with PD can be elucidated by the fact that this elevated rs2148356-T/T TLR4 SNP incidence, phenotypically expressed as an exaggereated immune-inflammatory reaction and adverse side effects, is owed to changes in the function that render people more prone to be less responsive to LPS leading to an diminished efficiency of LPS signaling and a decreased capacity to cause inflammation. The previously mentioned reasons include an elevated threat of severe infection vulnerability by pathogenic bacteria.[8,37]

It was suggested by ***Schroder et al.*** [18] that Asp299Gly (rs4986790) and Thr399Ile (rs4986791) mutations showed no discrepancy in LPS signaling. Hence, different SNPs such as: rs2148356 as an alternative to rs4986790 or rs4986791 may be responsible for altering the host vulnerability toward periodontal disease in Asian population.[8] As a consequence the same can be said for the Egyptian population based on our findings.

This information is consistent with two studies that concluded that gingival epithelial cells with TLR4 SNPs were hypo-responsive to *P. gingivalis* related to the production of pro-inflammatory cytokines at both protein and mRNA

levels. The epithelial cells were hyporesponsive functionally when affected by SNPs , demonstrated by changes in TLR expression by real-time PCR when compared to normal, and by pro-inflammatory and chemokine cytokines reaction to P.gingivalis challenge confirmed by protein analyses.[38,39]

Decreased activation of NF-kβ and pro-inflammatory cytokine expression has been related to the T allelle which subsequently cause an intensified destruction due to adverse products of periodontal pathogens.[40,41,42] Elevated destruction in periodontitis group in our results, as well as elevated relative risk of periodontitis to patients carrying that allele are explained by these findings.

Conclusion:

There is an association between TLR4 SNP, rs2149356 and periodontitis in our sample of the Egyptian population. Subjects with homozygous mutant genotype (TT) are more susceptible to periodontitis. While subjects with heterozygous genotype (GT) are at a less risk than homozygous mutant, they are more susceptible to periodontitis than patients with heterozygous normal genotype (GG). TLR4 SNPs Asp/Gly rs4986790 and Thr/Ile rs4986791 were not found in the whole sample and thus they do not exist in the Egyptian population.

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