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Synergetic Effect of TLR4 Gene (D299G and T399I) Polymorphisms in Susceptibility to Pulmonary Tuberculosis

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Abstract **Background:** Toll-like receptor 4 (TLR4) is an essential component of the innate immune response to various microorganisms. Genetic polymorphisms in TLR4 are often associated with susceptibility to mycobacterial infection. **Objective:** The present study examines the influence of TLR4 896 A/G (Asp299Gly) and 1196 C/T (Thr399Ile) polymorphism in the susceptibility to develop pulmonary tuberculosis (PTB) in Indians. Materials & Methods: Single nucleotide polymorphisms (SNPs) of the TLR4 gene were genotyped using PCR-RFLP and sequenced in 198 patients with PTB and 60 healthy controls. Results: The frequency of mutant allele 299Gly and 399Ile of the TLR4 gene (14.4%; $p < 0.048$; RR=1.919; OR=2.074 & 14.6%; $p < 0.020$, RR=2.197; OR=2.402) was predominant in PTB patient as compared to healthy controls (7.5% & 6.7%), respectively. Data was further analysed under the dominant model [CT+TT (Thr/Ile + Ile/Ile)] at 399 position revealed preponderance of mutant allele in PTB patients (25.8%; $p < 0.045$; OR=2.555). On the other hand, combined evaluation of polymorphisms showed heterozygous/heterozygous (AG299+CT399) genotype was higher (16.2% vs 6.7%; p=0.063) in PTB patients than in control subjects. However homozygous/homozygous (GG299+TT399) genotype was observed only in PTB patients (3%).Conclusion: The current observation suggests the contribution of mutant allele 'Gly' & 'Ile' towards the increased risk of development of tuberculosis in North Indian population. As per our knowledge, this is first Indian study indicating the synergetic effect of two mutant (Asp299Gly and Thr399Ile) / alleles influencing the PTB infection. Further investigation on association of TLR-4 as an innate immunity biomarker with tuberculosis diseases need to be evaluated.

Keywords TLR4, Polymorphism, Pulmonary Tubeculosis

1. Introduction

Incidence of Pulmonary Tuberculosis (PTB) cases in India, in 2012 was 2.0-2.4 million. As per WHO, 2013 consensus[1] India alone, accounted for 26% of global cases. Genetic and non-genetic factors of both the bacterium and the host influence the host immune response to *M.Tuberculosis*. Many host susceptibility genes have been identified in the last decade. Association of numerous polymorphisms of innate immunity candidate genes implicated in host susceptibility to TB has been revealed by various studies and Toll-like receptor (TLR) 4 is one of them [2]. TLRs are trans-membrane receptors that activate cells of the innate immune systems upon recognition of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan, liporpotease etc. and they form the crucial link between the innate and adaptive immune responses. As an innate immune act, TLRs trigger signal transduction upon binding of ligands, that results in the expression of pro-inflammatory cytokines and the functional maturation of antigen presenting cells, which intern activate adaptive immunity [2,3].

Toll-like receptor 4 (TLR4) plays a vital role in immunity to tubercle bacillus and its gene polymorphisms are supposed to affect tuberculosis susceptibility. Several TLRs, particularly TLR2, TLR4 and TLR9, are known to be important in mycobacterial infections. Many *in-vivo* studies testify the protective role of TLR4 in the host defence against mycobacterial infections although there are also conflicting results [3–10]. While the genuine causes of discrepant results remain to be determined, there are still many other *in-vitro* studies supporting the role of TLR4 in mycobacterial infections [2,11–13]. Changes in TLRs and signalling molecules that result from polymorphisms are often associated with susceptibility to various infectious diseases [12]. Ten functional TLR members (TLR1–TLR13) have

been identified in humans [10–14]. Human TLR-4 gene (Gene ID=7099), located on chromosome 9 (9q33.1) spans a genomic region of 13.3 kb, has three exons and encodes a 839 amino acid protein and highly expressed on monocytes, lymphocytes, splenocytes and polymorphonuclear leucocytes [15]. Polymorphisms A+896G (rs4986790) and C+1196T (rs4986791) in TLR-4 are associated with susceptibility to tuberculosis and crucial among various reported studies in various populations [13,14,16,17]. These Single nucleotide polymorphisms (SNPs) affect TLR-4 extracellular domain as glycine at position 299 is replaced with aspartic acid (Asp299Gly) and threonine at 399 with isoleucine (Thr399Ile) [15].

The risk allele for rs4986790 is 'G' and for rs4986791 is 'T'. The SNP rs4986790 also known as 896A/G (also Asp299Gly) in the TLR4 gene is often studied along with a co-segregating SNP known as Thr399Ile, rs4986791 at C-T 1196 position [18]. Thr399Ile SNP is a non-synonymous C-T transition. Together, wide variety of both non-infectious and infectious diseases associated with these SNPs have been reported, although some cases reported conflicting or even contradictory results [12,13].

According to several studies, endotoxin-hyporesponsive phenotype is associated with the synergic effect of two variants of the TLR4 gene, Asp299Gly and Thr399Ile within the fourth exon. [11–13]. Moreover, the human alveolar macrophages or primary airway epithelial cells obtained from individuals with airway hypo responsiveness possess TLR4 Asp299Gly polymorphism. Extensive studies confirmed association of these TLR4 gene polymorphisms and resistance or susceptibility to many diseases and infections [2,11–13,16,17]. At the molecular level, it has been shown that the TLR 4 896 A>G mutation interferes with TLR4 interaction with MyD88 and other downstream messengers [18]. These mutations also appear to affect the levels of functional TLR 4 expression, leading to a 2-fold reduction [12,19].

Several studies (*in-vivo &in-vitro*) have demonstrated the protective role of TLR4 in the host defence against mycobacterial infection [6,13,20] and genetic factors play a major role in the susceptibility to tuberculosis [21,22]. Only few Indian studies reported the frequency of polymorphism (Asp299Gly) of TLR 4 gene was significantly increased in PTB patients as compared to the healthy controls [2]. So, there has been great interest regarding the association of the SNPsTLR4896 A>*G*and TLR41196 C>*T* to susceptibility for mycobacterial infection or tuberculosis disease states. The present study investigated the potential role of TLR-4 polymorphisms namely Asp299Gly and Thr399Ile as a risk factor in the development of pulmonary tuberculosis infection in North Indian population.

2. Materials and Methods

2.1. Study Design

We conducted this study at National Institute of Tuberculosis and Respiratory Diseases, New Delhi, India during the period of 2012-2014. The inclusion criteria for the recruitment of PTB patients were defined as follows: patients had to present with clinical manifestations suggestive of tuberculosis, i.e., the presence of productive cough, low-grade fever, night sweats, weight loss and chest pain. Pulmonary tuberculosis was diagnosed on the basis of the presence of clinical symptoms or chest radiography or the presence of acid fast bacilli (AFB) in sputum smear or *M. tuberculosis* positive culture on Löwenstein-Jensen medium. A detailed clinical history, sex, and age were also collected from the requisition form that accompanied with specimens. On the other hand the healthy control population was recruited from people who come for general health examinations or non TB cases (absence of clinical symptoms of active PTB, asymptomatic with no previous history of TB or ATT) or attendants of patients or volunteers with normal chest X-ray. Healthy controls were further subjected to Mantoux test and categorized into PPD negative and PPD positive.

2.2. Collection of Clinical Specimens

Current study was approved by Research and Ethics Committee of the Institute. The study groups involved 258 subjects consisting of 198 individuals with PTB and 60 healthy controls. Peripheral blood samples were collected in EDTA tubes from all PTB patients and unrelated healthy controls after obtaining consent as per ethical guidelines.

2.3. TLR4 Genotyping

Genomic DNA was extracted from EDTA anticoagulated peripheral blood leukocytes by salting out procedure (Miller et al., 1988). Single nucleotide polymorphisms (SNPs) of TLR4gene (896 A/G; Asp299Gly and 1196 C/T; Thr399Ile) were detected using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to previously described method (2,23)]. Amplification of human DNA was performed by PCR in a total 25 µl reaction volume containing 1X PCR buffer, 0.2 mM dNTPs, 50 ng of each primer (Table 1) (TLR 4 Asp299Gly and Thr399Ile), 1U of *Taq* polymerase (Genei, India) and 50-100 ng of extracted DNA. The amplification protocol used was; denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec. The amplified products were visualized on 2% agarose gels. Amplified fragments of TLR 4 Asp299Gly and TLR4 Thr399Ile polymorphism were digested overnight at 37°C with 1U of *Nco*I and *Hinf*I restriction endonucleases, respectively. The restricted amplicons were visualized on 3% agarose gel.

Table 1. Table showing the primer sequences to amplify the two SNPs and restriction products of TLR4 gene

TLR4 SNPs	Primer sequences (5'-3')	PCR product (bp)	Restriction Enzyme	Restriction product size (bp)
Asp299Gly	F: GATTAGCATACTTAGACTACTACCTCCATG	249	NcoI	Wild type (allele A): 249 Mutant (allele G): 223 + 26
	R: GATCAACTTCTGAAAAAGCATTCCCAC			
Thr399Ile	F: GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA	406	HinfI	Wild type (allele C): 406 Mutant (allele T): 377 + 29
	R: ACCTGAAGACTGGAGAGTGAGTTAAATGCT			

2.4. TLR4 Amplicon Sequencing

For the confirmation of TLR 4 Asp299Gly (rs4986790) and Thr399Ile (rs4986791) polymorphisms, ten randomly selected PCR products were purified and got sequenced commercially with the reverse primers using ABI Prism Big Dye terminator Cycle sequencing system (PE Applied Biosystem, USA). The chromatograms were visualized using BioEdit [24]. Sequence alignments were done using Clustal OMEGA [25].

2.5 Statistical Analysis

To analyze the association of two polymorphisms (Asp299Gly and Thr399Ile) of TLR-4 gene, the allelic and genotypic frequencies of both polymorphisms were compared between the PTB patients and the healthy subjects and assessed using MSTAT statistical software by Chi-square ($\chi 2$) and Fischer test. Chi-square test was used to compare the difference in the distribution of mutations between PTB affected individuals and the control group. Strength of association between mutations and the disease/ control group was computed by Odds ratio (95% confidence interval). The p values < 0.05 was considered to be statistically significant.

3. Results

In this study, we investigated the role of TLR4 polymorphism at position A896G (Asp299Gly) and C1196T (Thr399Ile) in the study groups involved 258 subjects consisting of 198 individuals with tuberculosis (age range: 18-76 years; mean age 34.4; 178 men and 20 women) and 60 healthy controls (age range: 18-65; mean age: 44; 36 men and 24 women) from the outdoor and wards National Institute of Tuberculosis & Respiratory Diseases, Delhi, using PCR-RFLP method.

3.1. Distribution of Genotypic and Allelic Frequency of TLR 4 (Asp299Gly and Thr399Ile) Polymorphism in Healthy Controls vs PTB Patients

Population distribution of the TLR 4 alleles revealed that two mutations occurred with a frequency of nearly 7% in the healthy Indian population (Asp 299 Gly, 7.5% and Thr 399 Ile, 6.7%) (Tables 2 & 3)

3.1.1. Asp299Gly Genotyping

The restriction digestion for mutant allele at position 896 A/G (Asp299Gly) yielded two fragments of size 223 bp and 26 bp (Fig. 1). The allelic and genotypic frequencies of TLR4 mutation were analyzed in patients with PTB and healthy controls (Fig. 2; Table 2). The allelic frequency of Asp299Gly was found to be significantly increased (14.3%) in PTB patients compared with healthy control (7.5%) group [($\chi 2$ value = 3.924; Odds ratio = 2.074, p value = 0.048, 95% CI = 0.954-4.65; RR = 1.919 (0.960-4.084)]. The genotypic frequency of heterozygous (AG) and homozygous mutant (GG) was also found to be increased in PTB patients as compared to the healthy controls (21.5% vs 11.7%; p = 0.084) and (3.5% vs 1.7%; p = 0.464), respectively but not significant.

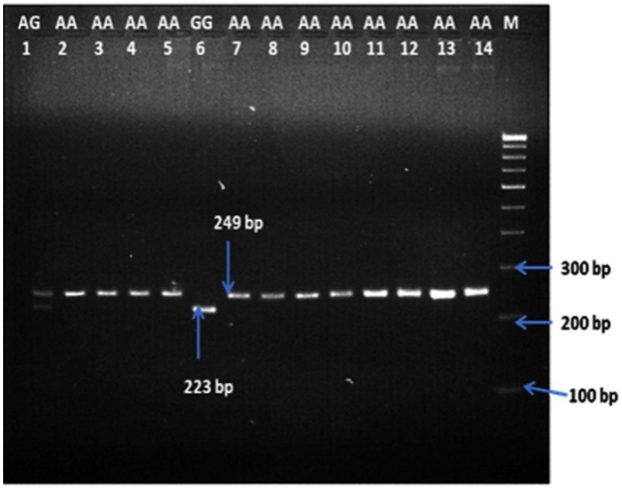

Figure 1. Gel image showing 896 A/G polymorphism in the TLR4 gene (corresponding to Asp299Gly mutation). Lanes 1 – heterozygous (AG); 2–5 & 7–14 – wild type (AA); 6 – homozygous mutant (GG) genotypes; M – 100 bp DNA ladder

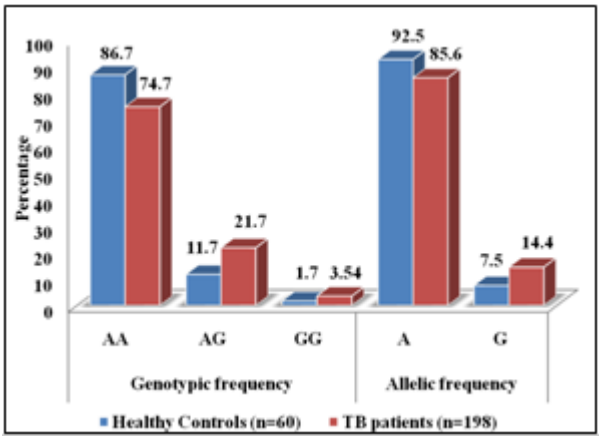

Figure 2. Graphical representation of genotypic and allelic frequencies of 896 A/G (Asp299Gly) polymorphism in the TLR4 gene in PTB patients; AA – Wild type; AG – Heterozygous mutant; GG – Homozygous mutants; *p value = 0.048 (significant for G allele) 95% CI = 0.954-4.65; RR = 1.919 (0.960-4.084).

Table 2. Genotypic & allelic frequencies (risk allele 'G') of the present and reported study for the *TLR4* 896 A>*G* SNP and association with tuberculosis disease.

Population	Control genotype (Healthy individual)						Case genotype (PTB patients)						Conclusion reported	References
	No. studied	A/A	A/G	G/G	Allele frequency A	Allele frequency G	No. studied	A/A	A/G	G/G	Allele frequency A	Allele frequency G		
Indian	250	206	44	0	91.2%	8.8 %	129	95	34	0	86.8%	13.2%	Increased Susceptibility	Najmi et al 2010
Indian	207	151	53	3	85.7%	14.3%	204	153	47	4	86.5%	13.5%	No association	Selvaraj et al 2010
Iranian	149	146	3	0	99%	1%	124	122	2	0	99.2%	0.8%	No association	Jahantigh et al 2013
Colombian	207	151	53	3	85.7%	14.3%	204	153	47	4	86.5%	13.5%	No association	Sanchez et al 2012
Gambian	298	235	58	5	88.6%	11.4%	307	241	62	4	88.6%	11.4%	No association	Newport et al 2004
Mexican	114	110	4	0	98.2%	1.8%	104	94	10	0	95.2%	4.8%	No association	Rosas-Taraco et al 2007
Indian	60	52 (86.7%)	7 (11.7%)# / 8/60 (13.3%)##	1 (1.7%)**	92.5 % (111/120)	7.5%* (9/120)	198	148 (74.7%)	43 (21.7%)# / 50/198 (25.3%)##	7 (3.54%)**	85.6% (339/396)	14.4%* (n=57/396)	Increased Susceptibility	Present study

*$\chi 2$ value =3.924; Odds ratio =2.074, P-value =0.048, 95% CI=0.954-4.65; RR= 1.919 (0.960-4.084)
**$\chi 2$ value =0.535; Odds ratio =2.162, P-value =0.464, 95% CI=0.259-47.724; RR= 2.121 (0.273-45.849)
#$\chi 2$ value =2.977; Odds ratio =2.100, P-value =0.084, 95% CI=0.842-5.461; RR= 1.861 (0.872-4.407)
$\chi 2$ value =3.754; Odds ratio =2.196, P-value =0.053, 95% CI=0.926-5.385; RR= 1.894 (0.942-4.187)
Dominant Model = AG + GG vs AA or heterozygous + homozygous mutant vs wild type

Table 3. Genotypic & allelic (risk allele 'T') frequencies of the present and reported studies for the *TLR4* 1196 C>TSNP (Thr399Ile) and association with tuberculosis disease.

Population	Control genotype (Healthy individual)						Case genotype (PTB patients)						Conclusion reported	References
	No. studied	C/C	C/T	T/T	Allele frequency C	Allele frequency T	No. studied	C/C	C/T	T/T	Allele frequency C	Allele frequency T		
Indian	250	206	43	1	91%	9 %	135	95	34	0	86.8%	13.2%	No association	Najmi et al 2010
Indian	203	152	46	5	86.2%	13.8%	203	150	49	4	86%	14.0%	No association	Selvaraj et al 2010
Iranian	149	141	7	1	97%	3%	124	112	10	2	94.4%	5.6%	No association	Jahantigh et al 2013
Colombian	299	272	26	1	95.3%	4.7%	466	429	36	1	95.9%	4.1%	No association	Sanchej et al 2012
Indian	60	52 (86.7%)	8 (13.3%) # / 8/60 (13.3%)##	0**	93.3% (112/120)	6.7%* (8/120)	198	147 (74.2%)	44 (22.2%)# / 51/198 (25.8%)##	7 (3.54%)**	85.4% (338/396)	14.6%* (n=58/396)	Increased Susceptibility	Present Study

*$\chi 2$ value =5.257; Odds ratio = 2.402; 95% CI= 1.066-5.623; P-value =0.020; RR=2.197 (1.057-4.897)
**$\chi 2$ value =2.180; 95% CI= 0.387-Inf; P-value =0.140; RR=Inf (0.399-Inf)
#$\chi 2$ value =2.261; Odds ratio = 1.857; 95% CI= 0.777-4.584; P-value =0.133; RR=1.667 (0.820 -3.714)
##$\chi 2$ value =4.030; Odds ratio = 2.555; 95% CI= 0.952-5.525; P-value =0.045; RR=1.932 (0.963 -4.266)
Dominant Model = CT+TT vs CC or heterozygous + homozygous mutant vs wild type

3.1.2. Thr399Ile Genotyping

The restriction of mutant allele at position 1196 C/T (Thr399Ile) yielded two fragments of sizes 377 bp and 29 bp (Fig. 3). The distribution of Thr399Ile polymorphism alleles and genotypes in PTB patients and healthy controls was analysed (Fig. 4; Table 3). The frequency of the mutant T (399Ile) allele was 14.5% in the PTB patients as compared with 6.7% in controls and were significantly different [$\chi 2$ value =5.257; Odds ratio = 2.402; 95% CI= 1.066-5.623; p-value =0.020; RR=2.197 (1.057-4.897)]. An upsurge was also observed in the genotypic heterozygous frequency (CT) in the PTB patients as compared to healthy controls of CT (22% vs 13.3%, p=0.133) and TT genotypes (3.5% vs 0%, p=0.140) but it was found statistically non significant.

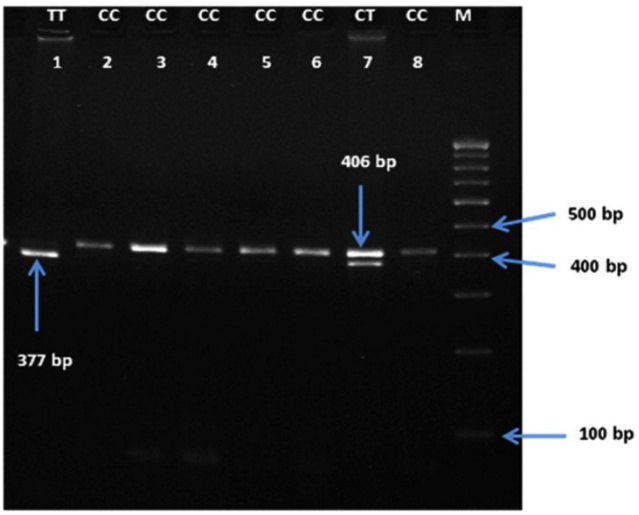

Figure 3. Gel image showing the 1196 C/T polymorphism in the TLR4 gene (corresponding to Thr399Ile mutation); Lanes 1 – homozygous mutant (TT); 2–6 & 8 – wild type (CC); 7 – heterozygous (CT) genotypes; M – 100 bp DNA ladder

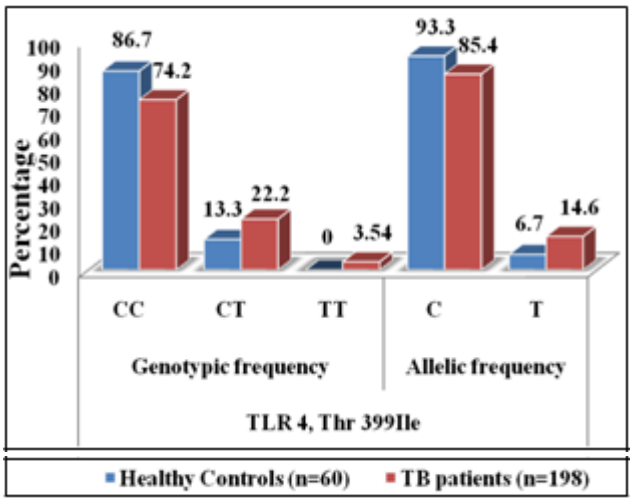

Figure 4. Graphical representation of genotypic and allelic frequencies of the 1196 C/T (Thr399Ile) polymorphism in the TLR4 gene in PTB patients; CC – Wild type; CT – Heterozygous mutant; TT – Homozygous mutants; *p value = 0.020 (significant for T allele) 95% CI= 1.066-5.623; RR=2.197 (1.057-4.897).

As the frequency of variant genotypes of both of the polymorphisms was quite low, we adopted a dominant model and the association of tuberculosis was calculated. The Ile (Thr/ Ile plus Ile/Ile vs Thr/Thr) carriers at position Thr399Ile were significantly associated with PTB infection (p-value =0.045; $\chi 2$ value =4.030; Odds ratio = 2.555; 95% CI= 0.952-5.525; RR=1.932 (0.963 -4.266), respectively when compared with the control group. The Gly (Asp/Gly plus Gly/Gly vs Asp/Asp) carriers at position Asp299Gly were not significantly associated ($\chi 2$ value =3.754; Odds ratio =2.196, p-value =0.053, 95% CI=0.926-5.385; RR= 1.894 (0.942-4.187) as shown in table 2 & 3.

3.2. Combined Effect of Asp299Gly and Thr399Ile

Further analysis of the combined effect of Asp299Gly and Thr399Ile in our study indicated that overall in 8 PTB patients, either risk allele 'T' for Thr399Ile or risk allele 'G' for Asp299Gly of TLR4 gene were present which may be associated with susceptibility to PTB in North Indian population. Interestingly, 6 PTB patients were carrying both homozygous mutant GG of 299 A/G TLR4 (Asp299Gly) & TT of 399 C/T TLR4 (Thr399Ile), simultaneously. And the frequency of homozygous mutant (GG+TT) in PTB patients (N = 6/198; 3%; p = 0.172) was increased as compared to healthy controls (N = 0/60; 0%). Similarly, frequency of heterozygous mutant (AG + CT) polymorphism were also increased in PTB patient (N = 32/198; 16.2%; p = 0.063) as compared with controls (N = 4/60; 6.7%), respectively. Statistically frequency for both homozygous & heterozygous mutant was found not significant.

3.3. DNA Sequencing for TLR4 Asp299Gly and Thr399Ile Polymorphism

The PCR products harbouring the TLR4variants from RFLP analysis were confirmed through DNA sequencing. These included four samples from PTB patients (one heterozygous and 3 homozygous mutants) for both Asp299Gly & Thr399Ile polymorphisms harbouring the variant. The sequencing results demonstrated that all sequenced samples had Asp (codon GAT) at amino acid 299 in wild type and Gly (codon GGT) in heterozygous and in homozygous mutants. The sequence from the heterozygous mutant revealed the "T" nucleotide was replaced with T/C (Fig. 5a) and with "C" in homozygous mutant (Fig. 5b). The sequencing results confirmed that all sequenced samples had Thr at amino acid 399 (codon ACC) in wild type and Ile (codon ATC) in heterozygous and homozygous mutant. The wild type G nucleotide in heterozygous sample was replaced with G/A nucleotide (Fig. 6a) and with "A" nucleotide in homozygous mutant (Fig. 6b) in samples by DNA sequencing. There was 100% concordance between the results of RFLP typing and DNA sequencing.

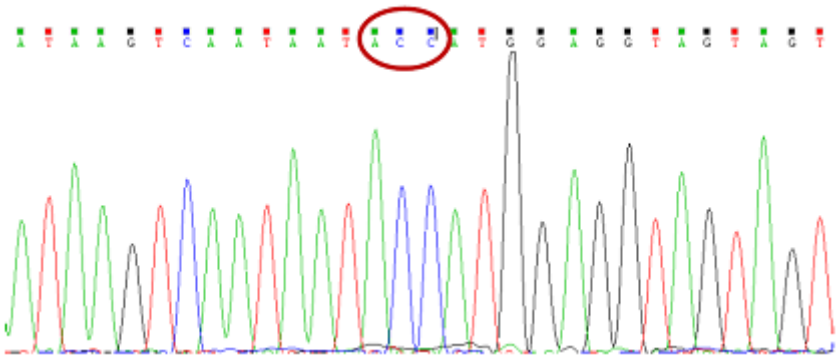

Figure 5a. Sequence analysis of *TLR4* Asp299Gly polymorphisms in tuberculosis patients (homozygous mutant genotype GG; TB-131). The circled area demonstrates the nucleotide base changes from GAT=Asp to GGT=Gly; T (A on –ve strand) nucleotide in the wild-type was replaced with a peak of C (G on –ve strand) nucleotide in Asp299Gly polymorphism.

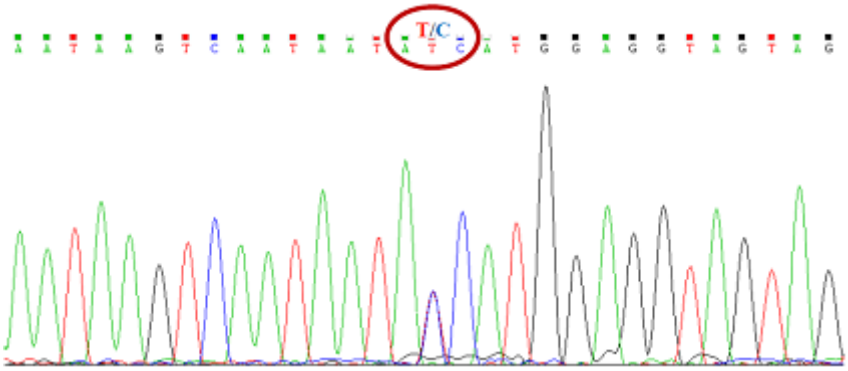

Figure 5b. Sequence analysis of *TLR4* Asp299Gly polymorphism in tuberculosis patient (heterozygous mutant genotype AG; TB-180). The circled area demonstrates overlapping peaks of the wild nucleotide base T (A on –ve strand) and peak of mutant C (G on –ve strand) nucleotide representing heterozygous condition of Asp299Gly polymorphism.

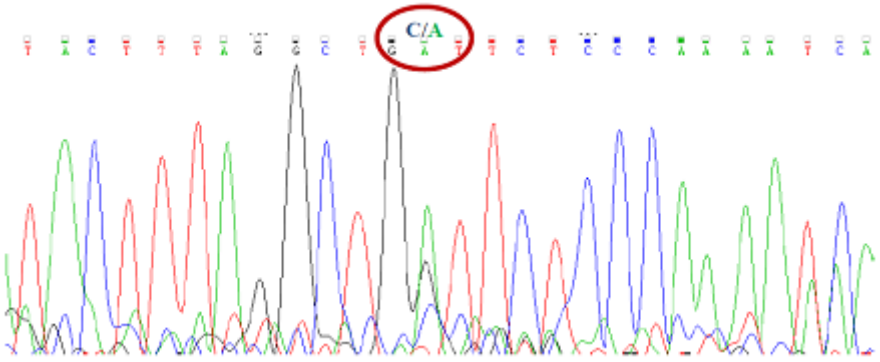

Figure 6a. Sequence analysis showing the double peak for heterozygous mutant genotype CT (Thr399Ile) polymorphisms in TLR4 gene in representative sample TB-173. The circled area demonstrates the nucleotide base changes; G nucleotide in the wild-type was substituted with doublet of G and A nucleotide in Thr399Ile polymorphism (ACC=Thr to ATC=Ile)

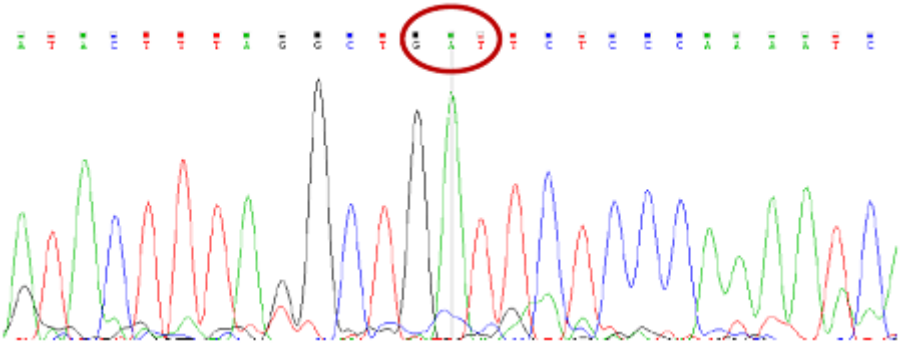

Figure 6b. Sequence analysis showing the homozygous mutant genotype TT (Thr399Ile) polymorphisms in in TLR4 gene in representative sample TB-131. The circled area demonstrates the nucleotide base changes; G nucleotide in wild-type was substitute with A nucleotide in Thr399Ile polymorphism (ACC=Thr to ATC=Ile).

Table 4. Combined effect of TLR4 polymorphisms Thr399Ile (A>G) and Asp299Gly (C>T)in pulmonary tuberculosis infection (risk allele 'T' & 'G')

Genotypes (299+399)	Number of subjects		Statistical values		
	Healthy Controls (n=60) (%)	PTB patients (n=198) (%)	P value*	Odds Ratio	Relative Ratio
AA+ CC	49 (81.7%)	136 (68.7%)	0.051	0.492 (0.224-1.061)	0.841 (0.738-1.017
AA + CT	3 (5%)	11 (5.6%)	0.868	1.118 (0.276-5.243)	1.111 (0.301-4.960)
AA + TT	0	1 (0.51%)	0.581	Inf (0.017-Inf)	Inf (0.018-Inf)
AG +CC	3 (5%)	11 (5.6%)	0.868	1.118 (0.276-5.243)	1.111 (0.301-4.960)
AG+CT	4 (6.7%)	32 (16.2%)	0.063	2.699 (0.859-9.432)	2.424 (0.878-7.955)
AG+TT	absent	absent		-	-
GG+CC	absent	absent		-	-
GG+CT	1 (1.7%)	1 (0.51%)	0.369	0.299 (0.008-11.145)	0.303 (0.008-11.045)
GG+TT	0	6 (3%)	0.172	Inf (0.322-Inf)	Inf (0.333-Inf)

*P value <0.05 was considered to be significant

4. Discussion

The susceptibility to tuberculosis in connection with TLR4 is focussed on two SNPs. The first is 896th base adenine substitution with guanine of 299th codon, which results in replacement of aspartic acid by glycine (Asp299Gly), and the other is at 1,196 bp substitution of cytosine with thymine, which results in a threonine to isoleucine replacement at 399th codon (Thr399Ile) [26,27]. Evidence suggests that polymorphisms within TLRs may reduce its affinity to *M. tuberculosis* derived ligands causing an impairment of the immune response against TB by altering the signal transduction of anti-MTB immune responses[22].

In this regard, the present study provides the evidence that polymorphisms in TLR4 gene contribute to PTB susceptibility in North Indians. The combined effect of two common polymorphisms (Asp299Gly and Thr399Ile) of TLR4 gene is being reported first time in this study, which reports the increase in the likelihood to acquire PTB. Our results indicated that in distribution of population of the TLR 4 alleles revealed that two mutations occurred with a frequency of nearly 7% in the healthy Indian population (Asp299Gly, 7.5% and Thr399Ile, 6.7%) (Tables 2 & 3; Fig. 2 & 4). Our results are in concordance with the published study by Najmi et al., (2010)[2] in North Indian population.

On the other hand, the distribution of allelic frequency varies from the range of various frequency of allele has been reported in different population (1 – 14.3% for TLR4 299 and 3 – 13.8% for TLR4 399) [12,16]. Further, the comparative analysis revealed that the allelic frequencies of mutant 299Gly and 399Ile of TLR4 gene were significantly raised [14.4% (P< 0.048; RR=1.919; OR=2.074) & 14.6% (p<0.020, RR=2.197; OR=2.402)] in PTB patients as compared to healthy controls (7.5% & 6.7%), respectively (Tables 2 & 3). Our results demonstrated the possible association or involvement of mutant alleles towards the susceptibility of TB infection. Various studies conducted earlier also revealed the similar findings [6,11,28]. Notably in contrast, some studies reported that no association was found between these polymorphisms and susceptibility to PTB [11,16,17,29]. In our study we observed an upsurge in the genotypic frequencies of mutant allele at both positions (Asp299Gly & Thr399Ile) of TLR 4 gene in PTB patients as compared to healthy controls but were not statistically significant. The second statistical approach which we adopted in current study is the dominant model and the association with PTB. The Ile (Thr/ Ile + Ile/ Ile vs Thr/Thr) genotype frequency of Thr399Ile polymorphism was significantly higher [(25.8% vs 13.3%; p <0.045; χ2 value = 4.030; OR = 2.555; 95% CI= 0.952-5.525; RR=1.932 (0.963 -4.266)] in PTB patients as compared to healthy controls, respectively and conferred a 2-fold risk for susceptibility to tuberculosis infection, respectively (Table-3). On the other hand, Gly (Asp/Gly + Gly/Gly vs Asp/Asp) genotype frequency of Thr399Ile polymorphism frequency was also increased [(25.3% vs 13.3%; p <0.053; OR=2.196; χ2 value =3.754; 95% CI=0.926-5.385; RR= 1.894 (0.942-4.187)] in PTB patients (with positive chest bacillary load) but we could not find this statistically significant as compared to healthy controls (Table-2). An earlier study [22] also reported the significant association with tuberculosis for TLR9 gene after adopting the dominant model and additive model. In contrast, Verma et al., (2011)[30] adopted a recessive model where the frequency of variant genotypes of both the polymorphism of TLR 4 gene was quite low and found the significantly increased risk of Neurocysticercosis.

Furthermore, the combined evaluation of both mutant genotype (299Gly; GG and 399Ile; TT) showed that the frequency of heterozygous/heterozygous (AG+ CT) genotype [16.2% vs 6.7% (p = 0.063)] and homozygous/homozygous (GG+TT) genotype was higher (3% vs 0%; p = 0.172) in PTB patients than in control subjects but this difference failed to reach the level of

statistical significance between the PTB patients & healthy control (Table 4). Both TLR4 gene polymorphisms act in a dependent manner by affecting each other to impart susceptibility to tuberculosis. It has also been reported previously that TLR4 Asp299Gly polymorphism occurred in combination with the polymorphism at Thr399Ile amino acid position [12,16,31]. Another study also reported the synergetic effect of genotypes TLR4 C1196T and TLR9 T1486C polymorphisms & found increased risk of PTB susceptibility [11].

Sequencing results demonstrated 100% concordance with genotyping results of both of Asp299Gly and Thr399Ile polymorphisms. GAT (Asp) → GGT (Gly) and ACC (Thr) → ATC (Ile) substitution in heterozygous and homozygous mutant was observed at nucleotide position 896 and 1196, respectively. The same polymorphisms were reported to have a role to play not only in tuberculosis, but also in other diseases too [12,27,32].

It may be feasible that, in cases of tuberculosis, both of these polymorphisms are involved in altering the immune function, which includes control of inflammatory cascades, elaboration of effect or molecules, and interaction with adaptive immune responses. The current observation highlight the contribution of mutant allele 'Thr' towards the increased risk of development of tuberculosis in North Indian population.

5. Conclusions

In conclusion, the present study suggests that Asp299Gly and Thr399Ile polymorphisms of TLR4 gene are involved as risk factors for PTB in our population. The presence of Gly and Ile increased the risk for the development of tuberculosis. Therefore, the results prove to be an effort in the direction of providing relevant information that the carriers of these mutations or genetic variations in TLR4 could contribute to the likelihood of developing PTB. This incidence of both homozygous as well as heterozygous polymorphisms to an extent could be of diagnostic value in the evaluation of genetic disease patterns. As per our knowledge this is the first Indian study reporting synergetic effect of two mutant allele of TLR4 in PTB infection. Further studies are needed to interpret the better role of TLRs in conjunction with other biomarkers.

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REFERENCES

[1] WHO | Global tuberculosis report 2014 [Internet]. WHO. [cited 2014 Nov 13]. Available from: http://www.who.int/tb/publications/global_report/en/

[2] Najmi N, Kaur G, Sharma SK, Mehra NK. Human Toll-like receptor 4 polymorphisms TLR4 Asp299Gly and Thr399Ile influence susceptibility and severity of pulmonary tuberculosis in the Asian Indian population. Tissue Antigens. 2010 Aug;76(2):102–9.

[3] Branger J, Leemans JC, Florquin S, Weijer S, Speelman P, Van Der Poll T. Toll-like receptor 4 plays a protective role in pulmonary tuberculosis in mice. Int Immunol. 2004 Mar;16(3):509–16.

[4] Means TK, Wang S, Lien E, Yoshimura A, Golenbock DT, Fenton MJ. Human toll-like receptors mediate cellular activation by Mycobacterium tuberculosis. J Immunol Baltim Md 1950. 1999 Oct 1;163(7):3920–7.

[5] Means TK, Jones BW, Schromm AB, Shurtleff BA, Smith JA, Keane J, et al. Differential effects of a Toll-like receptor antagonist on Mycobacterium tuberculosis-induced macrophage responses. J Immunol Baltim Md 1950. 2001 Mar 15;166(6):4074–82.

[6] Abel B, Thieblemont N, Quesniaux VJF, Brown N, Mpagi J, Miyake K, et al. Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. J Immunol Baltim Md 1950. 2002 Sep 15;169(6):3155–62.

[7] Reiling N, Hölscher C, Fehrenbach A, Kröger S, Kirschning CJ, Goyert S, et al. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. J Immunol Baltim Md 1950. 2002 Oct 1;169(7):3480–4.

[8] Shim TS, Turner OC, Orme IM. Toll-like receptor 4 plays no role in susceptibility of mice to Mycobacterium tuberculosis infection. Tuberc Edinb Scotl. 2003;83(6):367–71.

[9] Hölscher C, Reiling N, Schaible UE, Hölscher A, Bathmann C, Korbel D, et al. Containment of aerogenic Mycobacterium tuberculosis infection in mice does not require MyD88 adaptor function for TLR2, -4 and -9. Eur J Immunol. 2008 Mar;38(3):680–94.

[10] Kleinnijenhuis J, Oosting M, Joosten LAB, Netea MG, Van Crevel R. Innate immune recognition of Mycobacterium tuberculosis. Clin Dev Immunol. 2011;2011:405310.

[11] Jahantigh D, Salimi S, Alavi-Naini R, Emamdadi A, Owaysee Osquee H, Farajian Mashhadi F. Association between TLR4 and TLR9 gene polymorphisms with development of pulmonary tuberculosis in Zahedan, southeastern Iran. ScientificWorldJournal. 2013;2013:534053.

[12] Ziakas PD, Prodromou ML, El Khoury J, Zintzaras E, Mylonakis E. The role of TLR4 896 A>G and 1196 C>T in susceptibility to infections: a review and meta-analysis of genetic association studies. PloS One. 2013;8(11):e81047.

[13] Zaki HY, Leung KH, Yiu WC, Gasmelseed N, Elwali NEM, Yip SP. Common polymorphisms in TLR4 gene associated with susceptibility to pulmonary tuberculosis in the Sudanese. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2012 Jul;16(7):934–40.

[14] Ferwerda B, Kibiki GS, Netea MG, Dolmans WMV, van der

Ven AJ. The toll-like receptor 4 Asp299Gly variant and tuberculosis susceptibility in HIV-infected patients in Tanzania. AIDS Lond Engl. 2007 Jun 19;21(10):1375–7.

[15] Santini D, Angeletti S, Ruzzo A, Dicuonzo G, Galluzzo S, Vincenzi B, et al. Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms in gastric cancer of intestinal and diffuse histotypes. Clin Exp Immunol. 2008 Dec;154(3):360–4.

[16] Selvaraj P, Harishankar M, Singh B, Jawahar MS, Banurekha VV. Toll-like receptor and TIRAP gene polymorphisms in pulmonary tuberculosis patients of South India. Tuberc Edinb Scotl. 2010 Sep;90(5):306–10.

[17] Sánchez D, Lefebvre C, Rioux J, García LF, Barrera LF. Evaluation of Toll-like receptor and adaptor molecule polymorphisms for susceptibility to tuberculosis in a Colombian population. Int J Immunogenet. 2012 Jun;39(3):216–23.

[18] Figueroa L, Xiong Y, Song C, Piao W, Vogel SN, Medvedev AE. The Asp299Gly polymorphism alters TLR4 signaling by interfering with recruitment of MyD88 and TRIF. J Immunol Baltim Md 1950. 2012 May 1;188(9):4506–15.

[19] Prohinar P, Rallabhandi P, Weiss JP, Gioannini TL. Expression of functional D299G.T399I polymorphic variant of TLR4 depends more on coexpression of MD-2 than does wild-type TLR4. J Immunol Baltim Md 1950. 2010 Apr 15;184(8):4362–7.

[20] Fremond CMC, Nicolle DMM, Torres DS, Quesniaux VFJ. Control of Mycobacterium bovis BCG infection with increased inflammation in TLR4-deficient mice. Microbes Infect Inst Pasteur. 2003 Oct;5(12):1070–81.

[21] Yim J-J, Selvaraj P. Genetic susceptibility in tuberculosis. Respirol Carlton Vic. 2010 Feb;15(2):241–56.

[22] Torres-García D, Cruz-Lagunas A, García-Sancho Figueroa MC, Fernández-Plata R, Baez-Saldaña R, Mendoza-Milla C, et al. Variants in toll-like receptor 9 gene influence susceptibility to tuberculosis in a Mexican population. J Transl Med. 2013;11:220.

[23] Lorenz E, Frees KL, Schwartz DA. Determination of the TLR4 genotype using allele-specific PCR. BioTechniques. 2001 Jul;31(1):22–4.

[24] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95–8.

[25] Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol. 2011;7:539.

[26] Lorenz E, Frees KL, Schwartz DA. Determination of the TLR4 genotype using allele-specific PCR. BioTechniques. 2001 Jul;31(1):22–4.

[27] Wang XH, Wang FR, Tang YF, Zou HZ, Zhao YQ. Association of miR-149C>T and miR-499A>G polymorphisms with the risk of hepatocellular carcinoma in the Chinese population. Genet Mol Res. 2014;13:5048–54.

[28] Rosas-Taraco AG, Revol A, Salinas-Carmona MC, Rendon A, Caballero-Olin G, Arce-Mendoza AY. CD14 C(-159)T polymorphism is a risk factor for development of pulmonary tuberculosis. J Infect Dis. 2007 Dec 1;196(11):1698–706.

[29] Xue Y, Zhao ZQ, Wang HJ, Jin L, Liu CP, Wang Y, et al. Toll-like receptors 2 and 4 gene polymorphisms in a southeastern Chinese population with tuberculosis. Int J Immunogenet. 2010 Apr;37(2):135–8.

[30] Verma A, Prasad KN, Cheekatla SS, Nyati KK, Paliwal VK, Gupta RK. Immune response in symptomatic and asymptomatic neurocysticercosis. Med Microbiol Immunol (Berl). 2011 Nov;200(4):255–61.

[31] Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet. 2000 Jun;25(2):187–91.

[32] Davoodi H, Seow HF. Variant Toll-like receptor4 (Asp299Gly and Thr399Ile alleles) and Toll-like receptor2 (Arg753Gln and Arg677Trp alleles) in colorectal cancer. Iran J Allergy Asthma Immunol. 2011 Jun;10(2):91–9.