

**Research Article**

## **NO ASSOCIATION OF TOLL-LIKE RECEPTOR4 (A896G) VARIANT IN LEPROSY**

**Naveen Chandra S., Venkata Sanjeev Kuamr N. and \*Anandaraj M.P.J.S.**

*LEPRA India – Blue Peter Public Health & Research Centre, Cherlapally, Hyderabad, India*

*\*Author for Correspondence*

### **ABSTRACT**

*Mycobacterium leprae* is the etiological agent of human leprosy; human infection with *M. leprae* offers a unique opportunity to link innate and adaptive immune responses to specific host genes. Identifying and functionally exploring the genetic and immunological factors that underlie human susceptibility to leprosy have yielded important insights into *M. leprae* pathogenesis. TLR's play a crucial role in identification of pathogens. TLR4 is a limiting factor in lipopolysaccharide (LPS) signal transduction. Genetic variations of *TLR4* may change the function of the protein and alter the efficiency of the immune response to an infectious disease. A total of 132 subjects, patients 68 and controls 64 were studied. Biological sample (Blood) was collected with informed consent. DNA was isolated using Qiagen DNA extraction kit. Primers were designed using Primer3 software for PCR amplification of the *TLR4* A896G (rs4986790) gene. PCR followed by Restriction Fragment Length Polymorphism (RFLP) with restriction enzyme BCC-I was performed to confirm the genotype. The genotype distribution in cases and controls were similar ( $p=0.6959$ ,  $OR=1.214(0.4587- 3.213)$ ). The frequency AA (44.6%, 40.9%) genotype was predominately high compared to AG (6.8%, 6.8%) and GG (0%, 0.7%) genotypes frequency. Unlike *TLR2*, *TLR4* gene (A896G) polymorphism had no significant association with leprosy, did not affect the disease outcome. The results presented are from early stage of project. Further if the sample size is increased the outcome may vary.

**Keywords:** *Mycobacterium leprae*, Leprosy, PCR, RFLP

### **INTRODUCTION**

*Mycobacterium leprae* is the etiological agent of human leprosy; human infection with *M. leprae* offers a unique opportunity to link innate and adaptive immune responses to specific host genes. Identifying and functionally exploring the genetic and immunological factors that underlie human susceptibility to leprosy have yielded important insights into *M. leprae* pathogenesis. TLR's play a crucial role in identification of pathogens.

*TLR4* is a limiting factor in lipopolysaccharide (LPS) signal transduction. Genetic variations of *TLR4* may change the function of the protein and alter the efficiency of the immune response to an infectious disease. The susceptibility or resistance to leprosy of SNPs in toll-like receptor (TLR) genes were studied in a number of genetic association studies which are still debated. It is known by accumulated evidences that, polymorphisms in TLRs influence the pathogenesis of leprosy, a disease whose manifestations depend on host immune responses.

Since polymorphism in *TLR4* gene modulates immunologic effects such as inflammatory processes, hence we have screened G896A polymorphism in *TLR4* gene which may influence overall susceptibility or resistance to leprosy. For example Bochud et al. have reported that altered responses were observed in monocytes when stimulated with *Mycobacterium leprae* due to G896A polymorphism of *TLR4* gene.

A number of genome wide association studies (GWAS) using family-based designs and case-control designs have identified some innate immune genes that cause susceptibility to leprosy (Zhang *et al.*, 2009; Wong *et al.*, 2010; Wong *et al.*, 2010).

Genetic variants in human hosts, mainly single nucleotide polymorphisms (SNPs) can predict susceptibility to leprosy per se and can also explain clinical variation in disease.

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### MATERIALS AND METHODS

#### Methodology

**Subjects:** A total of 132 subjects, patients 68 and controls 64 were studied. Biological sample (Blood) was collected with informed consent. Leprosy patients were bacteriologically confirmed by slit skin smears staining (SSS) at Blue Peter Health and Research Center (BPHRC) clinic. According to WHO classification, patients showing negative smears at all sites are grouped as paucibacillary leprosy (PB), while those showing positive smears at any site are grouped as having multibacillary leprosy (MB) (<http://www.who.int/lep/classification/en/index.html>).

**DNA Isolation:** DNA was extracted from whole blood using Flexigene DNA kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). DNA concentrations were determined using ND-1000 spectrophotometer (Thermoscientifics, Wilmington, DE, USA).

**Genotyping:** Primers were designed using Primer3 software for PCR amplification of the TLR4 A896G (rs4986790) gene. PCR followed by Restriction Fragment Length Polymorphism (RLFP) with restriction enzyme BCC-I was performed to confirm the genotype. To determine the genotype TLR4 D299G (rs4986790), PCR was performed with the following primer sequences: forward 5'-AGTCCATCGTTTGGTTCTGG-3' and reverse 5'-CACACTCACCAGGGAAAATG 3'. Using standardized conditions: 95°C for 1 minute followed by 35 cycles of 94°C for 45 seconds, the appropriate annealing temperature 58°C for 45 seconds, 72°C for 1 minute, and then one cycle at 72°C for 10 minutes PCR was performed. The PCR products were resolved on 2% agarose gel by ethidium bromide staining and viewed under UV transilluminator (Bio-Rad, Hercules, CA, USA). The reaction mixture included primers (forward and reverse) DNA template, dNTP's, MgCl<sub>2</sub> and Taq polymerase of standard concentrations.

#### Restriction Fragment Length Polymorphism

Restriction digestion was performed using Bcc-I restriction enzyme. The restriction enzyme was designed using NEB cutter (version 2.1) freely available online software <http://tools.neb.com/NEBcutter2/>. The 217bp amplified product was digested with Bcc-I enzyme (1U; New England Biolabs Inc, Ipswich, MA, USA) at 37°C for 3 hours resulting in products with 217 bp for homozygous AA, 167 bp and 50 bp for homozygous GG and 217 bp, 167 bp and 50 bp for heterozygous GA genotype (Figure 1).

#### Statistical Analysis

Statistical analysis of the first study group was performed using Open Source Epidemiologic Statistics for public Health OPEN EPI: (Version 2.2.1. Emory University & Rollins Schools of Public Health, Atlanta, GA, USA). The 2x2 cross-tabulation method was used to determine OR with a CI of 95%. The x<sup>2</sup> test was done for comparing genotype frequencies. A p value of  $\leq 0.05$  was considered to be statistically significant.

### RESULTS AND DISCUSSION

#### Results

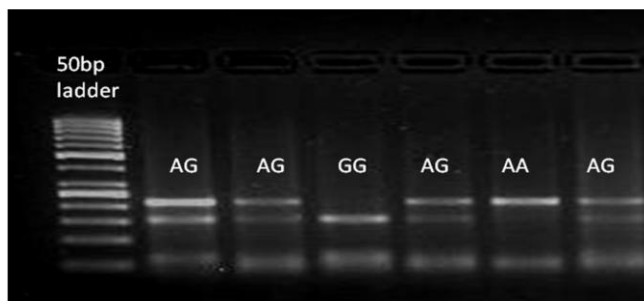


Figure 2: Agarose gel with 50bp marker in lane 1

AG genotype = 213bp+150bp+63bp (heterozygous), AA genotype = 213bp (homozygous for major allele), GG genotype = 150bp+63bp (homozygous) Restriction enzyme is BCC-I (NEB) Site: GGTAGxG

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The genotype distribution in cases and controls were similar ( $p = 0.6959$ ,  $OR = 1.214(0.4587-3.213)$ ). The frequency AA (86%, 84%) genotype was predominately high compared to AG (13%, 14%) and GG (0%, 1.5%) genotypes frequency.

**Table1: Represents the genotype distribution where n is number of genotypes and Odds ratio (OR) with confidence interval (CI). p values calculated by  $\chi^2$  and value  $\leq 0.05$  was considered significant**

TLR4 genotype distribution		AA vs others		
	%(n)	P value	OR	(CI 95%)
CONTROLS	84(54)	.	.	.
Leprosy CASES	86(59)	0.69	1.21	(0.45-3.21)
%(n)				
		AG vs others		
	%(n)	P value	OR	(CI 95%)
CONTROLS	14(9)	.	.	.
Leprosy CASES	13(9)	0.88	0.93	(0.34-2.51)
%(n)				
		GG vs others		
	%(n)	P value	OR	(CI 95%)
	1.5(1)	.	.	.
	0	0.45	0.3	(0.01-7.72)

**Discussion**

While innate immune responses to both *M. leprae* and *M. tuberculosis* rely heavily on TLR1/2 stimulation by various cellwall components, TLR4 has also been shown to play a role in detecting mycobacteria. The classical ligand for TLR4 is lipopolysaccharide (LPS), derived from the outer cell membrane of Gram-negative bacteria. Despite the fact that LPS is absent from mycobacterial membranes, studies in transfected cells and murine macrophages have shown that *M. leprae* and *M. tuberculosis* are both recognized by TLR4 (Means *et al.*, 1999; Means *et al.*, 1999). Fitness *et al.*, (2004) found no association of the G896A polymorphism with leprosy but heterozygosity of rare G allele at 299 position was reported to be high in controls and associated with hypo-responsiveness to inhaled LPS (Fitness *et al.*, 2004). Moreover the protective nature of heterozygous GA genotype was confirmed in other diseases like the development (but not the severity) of rheumatoid arthritis, Crohn's disease and ulcerative colitis (Radstake *et al.*, 2004; Franchimont *et al.*, 2004; Torok *et al.*, 2004). Heterozygosity is also associated with hypo-responsiveness to inhaled LPS.31 Studies in HIV/ TB co-infected patients indicate that G896A is a risk factor for active tuberculosis in Mediterranean Caucasians (Fitness *et al.*, 2004; Ferwerda *et al.*, 2007).

**Conclusion**

Unlike TLR2, TLR4 gene (A896G) polymorphism had no significant association with leprosy, did not affect the disease outcome. The results presented are from early stage of project. Further if the sample size is increased the outcome may vary.

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**Competing interests:** None

**Ethical approval:** This study was approved by IEC-LEPRA India (Blue Peter Public Health & Research Centre).

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