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Original Article

Susceptibility to Hansen's Disease is Associated with TLR1 Polymorphisms and Down Regulation of TLR1 mRNA Expression

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TLR1 as a heterodimer recognizes peptidoglycan and triacyl lipoproteins in combination of TLR2. It was confirmed that through the mediation of TLR1-TLR2 heterodimers the strongest cell activation was achieved by killed *Mycobacterium leprae*. Single nucleotide polymorphisms that are associated with disease in either of these genes will influence the surface expression. SNPs G743A, T1805G of TLR1 are not well studied in leprosy. Hence, we aimed to evaluate these polymorphisms on expression of TLR1 which might impact an individual susceptibility or resistant to *M. leprae* infection. A total of 628 individuals (230 leprosy patients, 230 healthy controls and 168 house hold contacts) were screened for both the polymorphisms using PCR-RFLP method. TLR1 mRNA expression and IL-10, IFN-γ levels with respect to genotypes at G743A position was analyzed by RT-PCR and ELISA. Statistical analysis was performed using Chi-Square test and t-tests. Homozygous GG and heterozygous GA genotypes at 743 position were associated with leprosy. The association was still significant across the spectrum of the disease even after Ridley-Jopling classification thus confirming the real association of these genotypes with leprosy. Leprosy patients with GG genotype at 743 position were found to be expressing significantly low TLR1 mRNA and high anti-inflammatory IL-10 cytokine which might be a reason for susceptibility to leprosy. However, additional functional, whole genome studies in leprosy cases across the spectrum are necessary to confirm the role of each single nucleotide polymorphism in pathogenesis of leprosy.

Key words : TLR1, PCR-RFLP, RT-PCR, mRNA expression, Leprosy

Introduction

TLR1 recognises gram positive bacteria in combination with TLR2, also TLR1 as a heterodimer recognizes peptidoglycan and triacyl lipoprotiens. Krutzik et al (2003) reported strong cell activation by killed *Mycobacterium leprae* which was mediated by TLR1-TLR2 heterodimers (Krutzik et al 2003). This activation of heterodimeric

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complex is beneficial sometimes, various studies have identified that varied alleles at different SNP positions inhibiting TLR1 activity and reduce the risk of infectious diseases such as leprosy, tuberculosis and sepsis (Wurfel et al 2008, Pino-Yanes et al 2010)

In contrary, Alexopoulou et al provided the first evidence that polymorphisms within the *TLR1* gene influence surface expression and are associated with infectious diseases (Alexopoulou et al 2002). Moreover TLR1 expression at the cell surface, NF-κB signalling and pro-inflammatory cytokine production was impaired due to the presence of G allele of the I602S (T1805G) polymorphism which was associated with protection against leprosy (Johnson et al 2007, Misch et al 2008). However, in diseased population from Bangladesh the polymorphism I602S was not associated. The G allele frequency varied across the populations, as it was observed to be a major allele in Europeans while it was entirely absent in Africans and Asians (Wong et al 2010). Whereas two independent studies have shown that G allele play protective role against leprosy per se (Johnson et al 2007, Wong et al 2010). Another study illustrated that polymorphism N248S (G743A) located in extracellular domain of TLR1 is responsible for defining specificity toward different lipopeptide agonists (Bell et al 2003, Omueti et al 2005) and the G allele is associated with risk for leprosy (Schuring et al 2009).

Since single nucleotide polymorphisms (G743A, T1805G) of *TLR1* gene are non-synonymous polymorphisms and no functional differences were noted between different alleles/genotypes studied. However, other studies reported that enhanced expression of TLR1 was only in patients with self-healing, growth-resistant tuberculoid form but not in those with the lepromatous form of leprosy (Krutzik et al 2003, Krutzik and Modlin 2004). Besides these arguments recently published data from India has suggested that heterozygous G743A is conferring resistance to tuberculosis (Sinha et al 2014). Therefore in this study, we evaluated the association and influence of the single nucleotide polymorphisms in TLR1 gene with leprosy.

Materials and Methods

Subjects

A total of 628 individuals were enrolled for this study within the same geographical area (Hyderabad, Telangana, India). An equal number of age and sex matched healthy controls (n=230) to that of leprosy patients (n=230) were recruited. Additionally genetically related household contacts (n=168) were also included in this study.

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 were grouped as having multibacillary leprosy (MB) (http://www.who.int/lep/classification/en/index.html). Out of 230 patients, 78 were paucibacillary and 152 were multibacillary. Also according to Ridley Jopling classification 97 were TT/BT and 133 were BL/LL. After confirmation, at least one of the family members was enrolled as household contacts. The healthy control group was enrolled with exclusion criteria for HIV, TB and family history of leprosy. All subjects gave written informed consent to participate in the study. The protocol was approved by the IEC of BPHRC-LEPRA India.

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 of TLR1 G743A (rs4833095) and T1805G (rs5743618) polymorphisms:

Genotypes were determined by using the following primers forward 5'-TGACCCTGTAG CTTCACGTTT-3' and reverse 5'-CAAACAAATCC AAAGTTATCTA-3' with an annealing temp-erature of 59^{o}c for G743A polymorphism, forward 5'-CT TGATCTTCACAGCAATAAAATAAAGAGCATTCC-3' and reverse 5'-GGCCATGATACACTAGAACACA CATCACT-3' with an annealing temperature of 60^{0}c for T1805G polymorphism respectively.

Restriction fragment length polymorphism:

Restriction digestion was performed using restriction enzyme Bfa-I for G743A and Pst-I for T1805G polymorphisms respectively. The restriction enzyme was designed using NEB cutter (version 2.1) freely available online software (http://tools.neb.com/NEBcutter2/).

The amplicon size for G743A position was 137bp, when digested with BfaI enzyme at 37^{0}c for 3hours resulted the homozygous GG genotype band with 137bp, heterozygous GA with 137bp, 115bp and 22bp bands, homozygous AA genotype with 115bp and 22bp bands (Fig 1).

The amplicon size for T1805G position was 1334bp, when digested with Pst-I enzyme at 37^{0}c for 3 hours resulted the homozygous TT genotype with 1334bp, heterozygous TG genotype with three bands with 1334bp, 876bp, 458bp and homozygous GG genotype with 876bp, 458bp (Fig 2).

Cell cultures for stimulation study:

PBMCs were isolated by differential centri-fugation. Blood and Histopaque (Sigma, Ayrshire, UK) are diluted in 1:3 ratio and centrifuged at 1800 RPM (363g - REMI Centrifuge) for 30 min. White interface of cells were collected and washed twice with 1xHBSS (Sigma, Paiseley, UK). Cells were re-suspended in RPMI 1640 (Sigma, Ayrshire, UK) complete medium with 10% Human AB serum (Invitrogen, California, USA), L-gluta-mine and Penicillin/Streptomycin (Sigma, St. Louis, USA). Viability and cell count were checked with Trypan blue (Sigma, St. Louis, USA) and 2x10^{6}

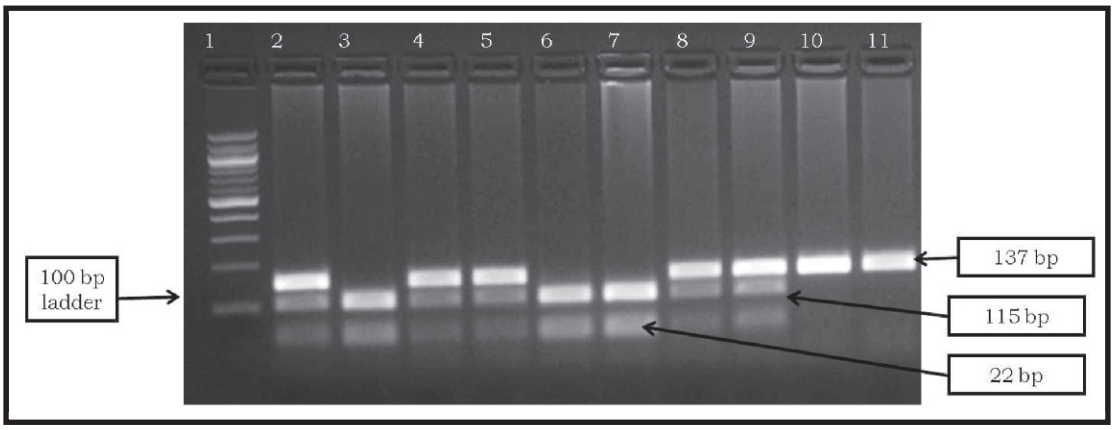

Fig 1 : Gel picture representing TLR1 G743A (N248S-rs4833095) polymorphism Restriction digestion. 3% agarose gel containing 100 bp ladder in lane 1, lane 2, 4, 5, 8, 9 containing heterozygous GA genotype, lane 10 and 11 homozygous GG genotype, lane 3, 6, 7 AA genotype.

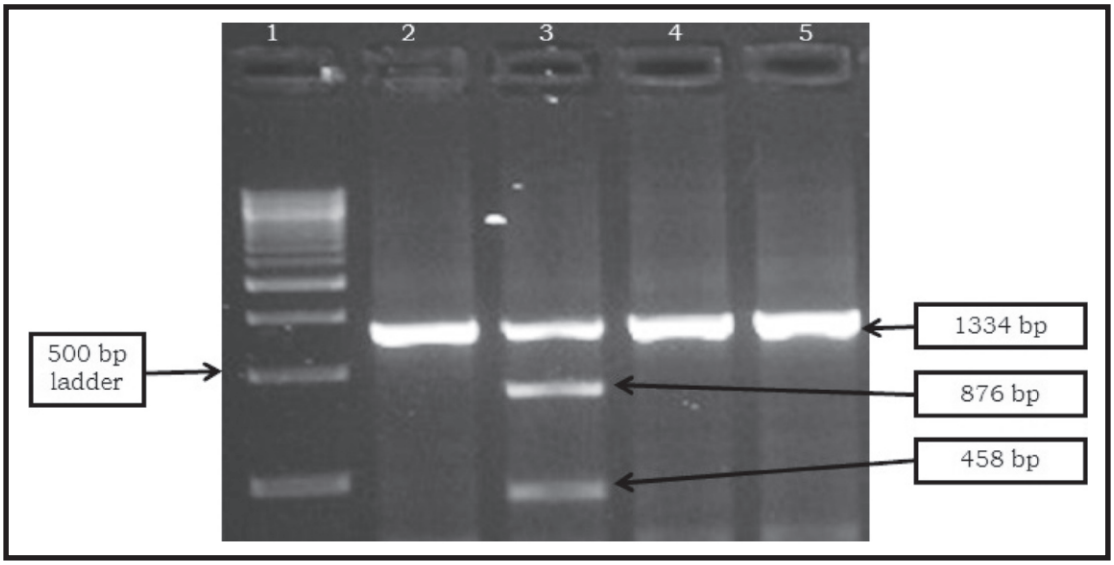

Fig 2 : Gel picture representing TLR1 T1805G (S602I - rs5743618) polymorphism Restriction digestion. Lane 1 is 500bp ladder; lane 2, 4, 5 containing homozygous TT genotype and lane 3 containing heterozygous GT genotype.

cells/ml cell suspension was made for stimulation assay. PBMCs were cultured with *M. leprae* Soluble Antigen (MLSA). Duplicate wells were maintained with MLSA (10 ug/ml) and un-stimulated wells, the plate was incubated at 37^{0}C with 5% CO2 and humidified air for 5 days for optimal proliferation of the cells.

RNA isolation & real time-PCR:

Total RNA was extracted from cultured cells using Tri-reagent (Sigma, St. Louis, USA). Total RNA content was measured in a spectrophotometer at 260 nm (Nanodrop-1000, Thermo, Wilmington, DE, USA). Total RNA was reverse transcribed using Superscript-III first strand synthesis system (Invitrogen, California, USA). 500 ng of total RNA was transformed into cDNA in a final volume of 20 uL. The expression of TLR2 gene transcript was amplified on Mini Opticon detector for Real Time PCR (CFB-3120, Bio-Rad Laboratories Inc., USA.) using the SYBR Green PCR kit (Qiagen, Hilden, Germany). The forward and reverse primers for TLR1 were 5'-CAATGCTGCTGTTCAGCTCTTC-3' and 5'-GCCCAATATGCCTTTGTTATCC-3' respectively, amplicon size was 156 bp. Primers for GAPDH were forward, 5'-GCCATCAATGACCCCTTCATT-3'; and reverse, 5'-TTGA CGGTGCCATGGAATTT-3'. PCR was performed in duplicates with the conditions 94 ^{0}C for 10 min, and 45 cycles of 94 ^{0}C for 15 s, 60 ^{0}C for 60 s, 72 ^{0}C for 30 s, followed by a melting curve starting with 60 ^{0}C rising to 94 ^{0}C at 0.5 per 2 s. All samples were normalized to the amount of GAPDH transcript present in each sample. Relative expression was calculated as 2ΔCT stimulated 2ΔCT control where ΔCT = CTTLR1–CTGAPDH.

IL-10 and IFN-γ assay:

The cytokine levels were measured in culture supernatants by sandwich ELISA using commercial kits (eBioscience Inc., San Diego, CA, USA Human IFN-γ ELISA Ready-SET-Go! Cat no: 88-7316-88, Human IL-10 Ready- SET-Go! Cat no: 88-7106-88). To each well, 100 lL of capture

antibody (1x) diluted in coating buffer was added and incubated overnight at 4 ^{0}C. The wells were blocked with 200 lL/well of 1xAssay Diluent and incubated for 1 h at room temperature (RT). The plate was incubated for 2 h at Room Temperature after addition of 100 lL/well of supernatants and standards. Detection antibody (100 lL/well) diluted in 1x assay diluent was added and incubated for 1 h at RT. After addition of 100 lL/well of avidin-HRP diluted in 1x assay diluent and incubation for 30 min at RT, the wells were incubated with the substrate (100 lL/well) for 15 min at RT. Each of the above steps was interspersed by 5 - 7 washes. Stop solution (50 lL/well) was added and absorbance was measured at 450 nm and subtracted with the absorbance values at 540 nm. The concentrations were calculated using MPM software version 6.1.

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^2 test was done for comparing genotype frequencies. A p value of $\leq$ 0.05 was considered to be statistically significant.

Results

Single nucleotide polymorphism of *TLR1* gene at position G743A:

Genotypes: The homozygous genotype GG frequency was found to be 77.39%, 65.21% and 76.19% in leprosy patients, Healthy controls and House Hold Contacts respectively. While the heterozygous genotype GA was observed to be 19.56%, 31.73% and 21.42% in leprosy patients, Healthy controls and House Hold Contacts respectively, whereas homozygous genotype AA frequency was nearly equal in all the three groups.

When compared with healthy control the frequency of GG genotype was significantly high in leprosy patients (p=0.003) suggesting a positive association of the homozygous GG genotype with leprosy. On the other hand, when compared with healthy control group the heterozygous GA genotype was significantly low in leprosy patients (p=0.002), indicating negative association of GA genotype with leprosy (Table 1). While none of the genotype have association with household contacts group.

Table 1 : Distribution of Genotype frequency for TLR1 gene at position G743A in patients with leprosy (LP), healthy controls (HC) and household contacts (HHC)

	Study Groups			LP vs HC		LP vs HHC	
Genotype	LP n=230 (%)	HC n=230(%)	HHC n=168 (%)	p-Value	OR (CI 95%)	p-Value	OR (CI 95%)
GG	178 (77.39)	150 (65.21)	128 (76.19)	0.003	1.86 (1.21-2.75)	0.77	1.07 (0.66-1.71)
GA	45 (19.56)	73 (31.73)	36 (21.42)	0.002	0.51 (0.34-0.80)	0.64	0.89 (0.54-1.45)
AA	7 (3.04)	7 (3.04)	4 (2.38)	0.99	1 (0.34-2.89)	0.69	1.28 (0.37-4.46)

In above table 'n' represents sample number and % for frequency distribution; p-value is calculated by chi-square and value $\leq$0.05 was considered significant, OR: Odds Ratio, CI: Class intervals.

Alleles: The major allele G frequency was 87.17%, 81.08% and 83.92% while the minor allele frequency was 12.82%, 18.91% and 13.09% in leprosy patients, Healthy controls and House Hold Contacts respectively.

When compared with healthy control the frequency of G allele was significantly high (p=0.01) in leprosy patients group indicating a positive association of the C allele with leprosy. On the contrary, the frequency of A allele was significantly low (p-0.01) in leprosy patient group demonstrating a negative association of A allele with leprosy (Table 2). Similarly as in genotypic results none of the allele was found to be associated with household contacts group.

Table 2 : Distribution of Allele frequency TLR1 gene at position G743A in patients with leprosy (LP), healthy controls (HC) and household contacts (HHC)

	Study Groups			LP vs HC		LP vs HHC	
ALLELE	LP n= 460 (%)	HC n= 460 (%)	HHC n= 336 (%)	p-Value	OR (CI 95%)	p-Value	OR (CI 95%)
G	401 (87.17)	373 (81.08)	282 (83.92)	0.01	1.58 (1.10-2.27)	0.78	1.06 (0.69-1.61)
A	59 (12.82)	87 (18.91)	44 (13.09)	0.01	0.63 (0.44-0.90)	0.78	0.9 (0.62-1.43)

In above table 'n' represents sample number and % for frequency distribution; p-value is calculated by chi-square and value ≤ 0.05 was considered significant, OR: Odds Ratio, CI: Class intervals.

Table 3 : Genotype and allelic distribution of TLR1 gene at position G743A in Paucibacillary (PB), Multibacillary (MB) leprosy patients and healthy controls

Genotype	MB n=152 (%)	PB n=78 (%)	HC n=230 (%)	MB vs HC p value	OR (95% CI)	PB vs HC p value	OR (95% CI)
GG	118 (77.63)	60 (76.92)	150 (65.21)	0.009	1.85 (1.15-2.95)	0.05	1.77 (0.95-3.21)
GA	28 (18.42)	17 (21.79)	73 (31.73)	0.003	0.48 (0.29-0.79)	0.09	0.59 (0.32-1.09)
AA	6 (3.94)	1 (1.28)	7 (3.04)	0.63	1.3 (0.43-3.97)	0.39	0.41 (0.05-3.41)
Allele	MB n=304 (%)	PB n=156 (%)	HC n=460 (%)				
G	264 (86.84)	137 (87.82)	373 (81.08)	0.03	1.53 (1.02-2.31)	0.05	1.68 (0.98-2.86)
A	40 (13.16)	19 (12.18)	87 (18.92)	0.03	0.64 (0.43-0.97)	0.05	0.59 (0.34-1.01)

In above table 'n' represents sample number and % for frequency distribution; p-value is calculated by chi-square and value ≤ 0.05 was considered significant, OR: Odds Ratio, CI: Class intervals.

Table 4 : Genotype and allelic distribution of leprosy patients according to Ridley-Jopling classification and healthy controls for polymorphism in TLR1 gene at position G743A

Genotype	BL/LL n=133 (%)	TT/BT n=97(%)	HC n=230(%)	BL/LL vs HC p-Value	OR	TT/BT vs HC p-Value	OR
GG	107 (80.4)	77 (79.3)	150 (65.21)	0.001	2.19 (1.32-3.64)	0.01	2.05 (1.71-3.6)
GA	23 (17.2)	19 (19.5)	73 (31.73)	0.002	0.44 (0.26-0.76)	0.02	0.52 (0.29-0.92)
AA	3 (2.2)	1 (1.0)	7 (3.04)	0.65	0.73 (0.18-2.89)	0.28	0.33 (0.04-2.73)
Allele	BL/LL n=266	TT/BT n=194	HC n=460				
G	237 (89)	173 (88.5)	373 (81.8)	0.004	1.9 (1.21-2.99)	0.01	1.9 (1.15-3.19)
A	29 (11)	21 (11.5)	87 (18.2)	0.004	0.52 (0.33-0.82)	0.01	0.52 (0.31-0.86)

In above table 'n' represents sample number and % for frequency distribution; p-value is calculated by chi-square and value ≤0.05 was considered significant, OR: Odds Ratio, CI: Class intervals.

Genotype and *Allele* distribution in leprosy patients after WHO classification: The genotype GG frequency in the MB group was 77.63% while it was 76.92% in the PB group. The GA genotype frequency was 18.42% in the MB group while it was 21.79% in the PB group. The AA genotype frequency was 3.94% in MB group whereas it was 1.28% in PB group (Table 3). Homozygous GG genotype was observed to be significantly high (p=0.009) while the heterozygous GA genotype was significantly low (p=0.003) in the MB leprosy group compared to healthy controls. Even though he frequency distribution of homozygous GG and heterozygous GA genotypes in the PB leprosy group varied to that of healthy control group, only a borderline significance was observed. While both the alleles (G and A) were found to be associated with MB and PB leprosy patient groups compared to healthy controls.

Genotype and *Allele* distribution in leprosy patients after Ridley-Jopling classification: For the convenience of analysis BT&TT patients were grouped into one while BL, LL patients were other group. The genotype GG frequency in the BL/LL group was 80.4% while it was 79.3% in the BT/TT group. The GA genotype frequency was 17.2% in the BL/LL group while it was 19.5% in the BT/TT group. The AA genotype frequency was 2.2% in BL/LL group whereas it was 1.0% in BT/TT group (Table 4). Homozygous GG genotype was observed to be significantly high (p=0.001, 0.01) while the heterozygous GA genotype was significantly low (p=0.002, 0.02) in both BL/LL & BT/TT groups respectively compared to healthy controls. Also both the alleles (G and A) were found to be associated with BL/LL and BT/TT leprosy patient groups compared to healthy controls.

Table 5 : Distribution of Genotype frequency in TLR1 gene at position T1805G in patients with leprosy (LP), healthy controls (HC) and household contacts (HHC)

	Study Groups			LP vs HC		LP vs HHC	
Genotype	LP n=230 (%)	HC n=230(%)	HHC n=168 (%)	p-Value	OR (CI 95%)	p-Value	OR (CI 95%)
TT	195 (84.7)	189 (82.1)	145 (86.3)	0.45	1.2 (0.73-1.98)	0.66	0.88 (0.50-1.56)
GT	35 (15.2)	38 (16.5)	23 (13.6)	0.7	0.7 (0.54-1.49)	0.66	1.13 (0.64-1.99)
GG	0	3 (1.3)	0	0.13	0.14 (0.007-2.74)	0.87	0.73 (0.01-37.02)

In above table 'n' represents sample number and % for frequency distribution; p-value is calculated by chi-square and value ≤ 0.05 was considered significant, OR: Odds Ratio, CI: Class intervals.

Table 6 : Distribution of Allele frequency in TLR1 gene at position T1805G in patients with leprosy (LP), healthy controls (HC) and household contacts (HHC)

	Study Groups			LP vs HC		LP vs HHC	
ALLELE	LP n= 460 (%)	HC n= 460 (%)	HHC n= 336 (%)	p-Value	OR (CI 95%)	p-Value	OR (CI 95%)
T	425 (92.39)	416 (90.43)	313 (93.15)	0.29	1.28 (0.80-2.04)	0.68	0.89 (0.51-1.54)
G	35 (7.60)	44 (9.56)	23 (6.84)	0.29	0.77 (0.48-1.23)	0.68	1.21 (0.64-1.93)

In above table 'n' represents sample number and % for frequency distribution; p-value is calculated by chi-square and value ≤ 0.05 was considered significant, OR: Odds Ratio, CI: Class intervals.

Gel electrophoresis results: PCR and RFLP products were resolved on 2% and 3% gels respectively the amplicon size prior to restriction digestion was 137bp's. After digestion the homozygous GG genotype specific band was 137bp, while the heterozygous GA genotype specific bands were 137bp, 115bp, 22bp and the homozygous AA genotype were 115bp, 22bp bands (Fig. 1).

Single nucleotide polymorphism of *TLR1* gene at position T1805G:

Genotypes: The homozygous genotype TT frequency was 84.7%, 82.1% and 86.3% in leprosy patients, Healthy controls and House hold contacts respectively. While the heterozygous GT genotype was 15.2%, 16.5% and 13.6% in leprosy patients, Healthy controls and House hold contacts respectively (Table 5). Since the genotype frequencies were more or less similar in all the three groups studied none of the genotypes were found to be significant.

Alleles: The major allele T frequency was 92.39%, 90.43% and 93.15% while the minor allele G frequency was 7.60%, 9.56% and 6.84% in leprosy patients, Healthy controls and Household contacts respectively (Table 6). Either of the alleles was found to be significant due to similar frequencies in all the three groups.

Table 7 : Genotype and allelic distribution in TLR1 gene at position T1805G in Paucibacillary (PB), Multibacillary (MB) leprosy patients and healthy controls

Genotype	MB n=152 (%)	PB n=78 (%)	HC n=230 (%)	MB vs HC p value	OR (95% CI)	PB vs HC p value	OR (95% CI)
TT	127 (83.55)	68 (87.17)	189 (82.1)	0.72	1.1 (0.63-1.90)	0.3	1.47 (0.70-3.10)
GT	25 (16.45)	10 (12.83)	38 (16.5)	0.98	0.99 (0.37-1.72)	0.53	0.74 (0.35-1.57)
GG	0	0	3 (1.3)	0.26	0.21 (0.01-4.15)	0.54	0.41 (0.02-8.10)
Allele	MB n=304 (%)	PB n=156 (%)	HC n=460 (%)				
T	279 (91.77)	146 (93.58)	416 (90.43)	0.52	1.18 (0.70-1.97)	0.22	1.54 (0.75-3.14)
G	25 (8.22)	10 (6.42)	44 (9.57)	0.52	0.84 (0.52-1.41)	0.22	0.64 (0.31-1.32)

In above table 'n' represents sample number and % for frequency distribution; p-value is calculated by chi-square and value $\leq$0.05 was considered significant, OR: Odds Ratio, CI: Class intervals.

Genotype and allele distribution in leprosy patients after WHO classification: The homozygous TT genotype frequency in the MB group was 83.55% while it was 87.17% in the PB group. The GT genotype frequency was 16.45% in the MB group while it was 12.83% in the PB group (Table 7).

On the other hand the major allele T frequency was 91.77% and 93.58% while the minor allele G frequency was 8.22% and 6.42% in MB and PB leprosy groups respectively.

Genotypes or alleles are not associated with MB and PB leprosy groups when compared with healthy controls, since the genotype and allele frequencies of MB and PB leprosy groups were similar to that of healthy controls.

Genotype and allele distribution in leprosy patients after Ridley-Jopling classification: None of the genotypes were significantly correlated with either of the spectrum of leprosy. While Allele T was significantly high in BL/LL group conferring protection, on the other hand allele G was significantly low conferring susceptibility to BL/LL form of leprosy compared to healthy controls (Table 8).

TLR1 mRNA expression:

Relative *TLR1* mRNA expression in PBMCs stimulated with MLSA was almost similar in GG genotype (mean ± SEM = 2.224 ± 0.5275, n=8) and GA genotype (mean ± SEM = 2.576 ± 0.7352, n=8) of healthy controls. While *TLR1* mRNA expression in PBMCs stimulated with MLSA was significantly low in leprosy patients with GG genotype (mean ± SEM = 1.268 ± 0.2050, n=8) (p=0.01) compared to that of GA genotype (mean ± SEM = 2.653 ± 0.4292, n=8). No significant difference of *TLR1* mRNA expression was observed with respect to genotypes between leprosy patients and healthy controls (Fig 3). All the patients included in mRNA expression study are BL/LL.

Table 8 : Genotype and allelic distribution of leprosy patients according to Ridley-Jopling classification and healthy controls for T1805G polymorphism

Genotype	BL/LL n=133 (%)	TT/BT n=97(%)	HC n=230(%)	BL/LL vs HC p-Value	OR	TT/BT vs HC p-Value	OR
TT	119 (89.4)	83 (85.5)	189 (82.1)	0.06	1.84 (0.96-3.52)	0.45	1.28 (0.66-2.48
GT	14 (10.5)	14 (14.4)	38 (16.5)	0.11	0.59 (0.30-1.14)	0.63	0.85 (0.43-1.65)
GG	0	0	3 (1.4)	0.31	0.24 (0.01-4.79)	0.44	0.33 (0.01-6.51)
Allele	BL/LL n=266	TT/BT n=194	HC n=460				
T	252 (95)	180 (93)	416 (91)	0.03	1.9 (1.02-3.54)	0.33	1.36 (0.72-2.54)
G	14 (5)	14 (7)	44 (9)	0.03	0.52 (0.28-0.97)	0.33	0.73 (0.39-1.37)

In above table 'n' represents sample number and % for frequency distribution; p-value is calculated by chi-square and value ≤0.05 was considered significant, OR: Odds Ratio, CI: Class intervals.

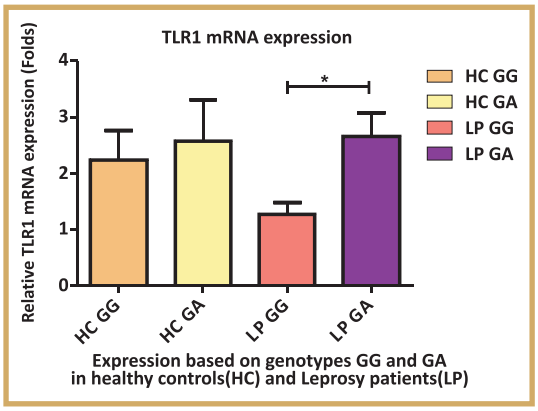

Fig 3 : Relative *TLR1* mRNA expression with respect to GG and GA genotype (G743A) in healthy controls and Leprosy patients.

Relative fold expression of *TLR1* mRNA, Horizontal line and error bars denote Mean ± SEM; p-value ≤ 0.05 is considered significant by Unpaired t-test, * denotes p≤0.05.

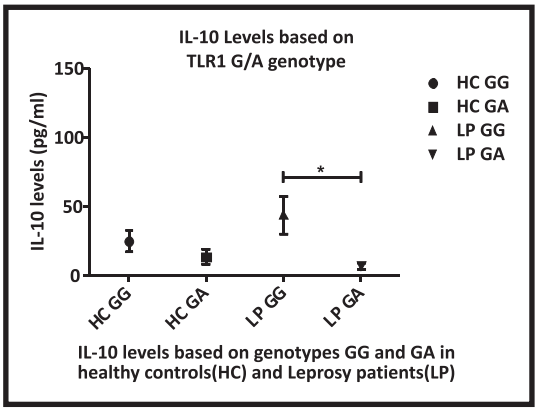

Fig 4 : IL-10 levels in leprosy patients and healthy controls with respect to GG and GA genotypes of TLR1 (G743A) polymorphism.

LP=leprosy patients, HC= healthy controls, Horizontal line and error bars denote Mean± SEM; p-value ≤ 0.05 is considered significant (Unpaired t-test); * denotes p≤0.05.

IL-10 levels in stimulated culture supernatants:

On co-relation of IL-10 levels based on genotypes in healthy controls and leprosy patients found that leprosy patients with GG genotype (mean ± SEM = 44.12 ± 13.63, n=8) produce significantly high (p=0.01) levels of IL-10 compared to that of GA genotype (mean ± SEM = 6.988 ± 2.001, n=8). On the other hand no such significance was observed in healthy controls with GG genotype (mean ± SEM = 25.10 ± 7.692, n=8) and GA genotype (mean ± SEM = 13.65 ± 5.398, n=8) (Fig 4).

IFN-γ levels in stimulated culture supernatants:

The mean±SEM for GG genotype and GA genotype of healthy controls was 37.66 ± 10.77, n=8 and 18.26 ± 2.569, n=8 respectively. While mean± SEM for GG genotype and GA genotype of leprosy patients was 37.20 ± 12.33, n=8 and 37.20 ± 12.33, n=8 respectively. There was no significant IFN-γ level with GG and GA genotypes in Leprosy patients and healthy controls (Fig. 5).

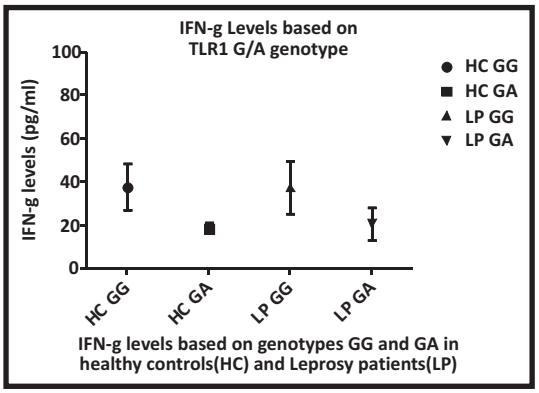

Fig 5 : IFN-γ levels in leprosy patients and healthy controls with respect to GG and GA genotypes of TLR1 (G743A) polymorphism.

LP=leprosy patients, HC= healthy controls, Horizontal line and error bars denote Mean± SEM; p-value ≤ 0.05 is considered significant (Unpaired t-test); * denotes p ≤ 0.05.

Discussion

To explore the association between *TLR1* gene polymorphisms and leprosy we examined two polymorphisms i.e. TLR1 at G743A (N248S) and T1805G (S602I) that are been studied in various diseases. Recent studies suggest that polymorphisms in TLR1 regulate signaling pathways when stimulated with lipopeptide (Wurfel et al 2008, Johnson et al 2007, Misch et al 2008). Amino acid at 248 of TLR1 is located in the external ligand-binding site of the receptor, S248 i.e. A variant enables normal functioning of TLR1, whereas the N248 i.e. G variant diminishes the response of TLR1 to bacterial agonists (Omueti et al 2005). The same polymorphisms are also been associated with leprosy susceptibility and the onset of especially type 1 reaction of leprosy by impairing NFκ-B signaling in some populations (Johnson et al 2007, Schuring et al 2009). According to our data, at 743 position, the homozygous GG genotype is positively associated with leprosy, while the heterozygous GA genotype is offering protection from leprosy. In support to our data that heterozygous GA genotype was offering protection for leprosy, was also found to be true for tuberculosis (Sinha et al 2014). Similarly when the genotypes are segregated into alleles, the G allele was found to be positively associated while that of A allele was found to be protective for leprosy. After WHO classification of leprosy patients, the similar association was observed as that of leprosy *per se.* The allele frequency was almost similar before and after WHO classification, although there was a number difference the association still remained same in multibacillary and paucibacillary leprosy patients. In support to our results a study on Bangladesh population revealed similar association with leprosy that strengthens our data (Schuring et al 2009).

Krutzik et al showed that there was no difference

in the expression or function of TLR1 in the peripheral monocytes of patients with tuberculoid or lepromatous leprosy but there was difference in local expression of TLR1 that could be due to interleukins (Krutzik et al 2003). Our results of TLR1 mRNA expression were supported as we included only patients from BL/LL spectrum of disease. Based on genotype the expression of TLR1 in leprosy patients significantly differed, while other compounding factors such as environmental, gene - gene interactions and stage of pathogenesis of leprosy for low expression is to be determined yet. Leprosy patients with GG genotype expressed low levels compared to that of GA genotype. While in healthy controls no such significant difference was observed. Our results were supported by a recent study done on molecular dynamics of TLR1 N248S polymorphism that expression of TLR1 on PBMC is quite low in individuals with N allele compared to that of individuals with S allele (Marques et al 2013).

Further based on genotype this N248S polymorphism also influenced the immune responses. Interestingly the low TLR1 expressed group had significantly high levels of anti-inflammatory cytokine i.e IL-10 suggesting that change in genotype has a key biological role in leprosy. Our results were in concordance to that of marques et al molecular analysis, which adds to the existing data that a decreased immune response measured based on log (TNF/IL-10) could be associated with change (N248S) in the molecular structure of TLR1 (Marques et al 2013). This structural change may impact the extracellular ligand-binding domain or the intracellular TIR signaling domain that binds to adaptor proteins such as MyD88 and TIRAP/Mal. On the whole the homozygous GG genotype at 743 position of TLR1 is associated with low TLR1 mRNA expression and high IL-10 production that could be one of the reason for susceptibility to leprosy.

In addition to G753A polymorphism, we have also screened T1805G polymorphism in our study. The T1805G polymorphism frequency varied across various populations. For instance the T allele frequency is high in Vietnam and African-Americans, where as the frequency was less in European-Americans (Pierik et al 2006). While some molecular studies have stated that S allele of the I602S (T1805G) polymorphism has been associated with protection against leprosy and with impaired TLR1 expression at the cell surface, NF-κB signaling, and pro-inflammatory cytokine production (Johnson et al 2007, Misch et al 2008, Hawn et al 2007). Our results provided that there is no association for either of the allele with leprosy. In contrast to our results Johnson and colleagues, reported that one of the variant of T1805G is associated with leprosy in Turkish cohort (Johnson et al 2007). Other group in Nepal cohort has observed association of 1805G allele with lepromatous rather that tuberculoid leprosy (Hawn et al 2007). This association bias may be due to different frequencies across different ethnic groups, also Bryan et al noted interesting fact that, in areas such as India with highest incidences of endemic mycobacterial disease, the protective TLR1 602S variant exhibits only 1% in frequency i.e. which was lowest allele frequencies (Hart et al 2012). Moreover our results were supported by the data from nearby population i.e. Bangladesh that T1805G (I602S) is not associated with leprosy (Schuring et al 2009).

In summary, leprosy patients with GG genotype at 743 position are expressing significantly low TLR1 mRNA and high anti-inflammatory IL-10 cytokine which might be a reason for susceptibility to leprosy. However additional functional, whole genome studies in leprosy cases across the spectrum are also warranted to confirm the role of each single nucleotide polymorphism in pathogenesis of leprosy.

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