

Surface Expression and Genetic Variants of Toll-like Receptor 2 in Pulmonary Tuberculosis Patients

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Innate immunity plays an important role in the host defense against *Mycobacterium tuberculosis* (*M. tuberculosis*). Toll-like receptor-2 (TLR2) is the main receptor and one of central components in initiation of innate immunity against *M. tuberculosis*. The study aimed to evaluate TLR2 surface expression on peripheral blood monocytes and study its association with variants in TLR2 597T/C single nucleotide polymorphisms (SNPs) in patients with pulmonary tuberculosis. Fifty pulmonary tuberculosis patients and 50 healthy controls were investigated for TLR2 expression on CD14+ monocytes by flow cytometry and TLR2 597T/C genotyping by Tetra primer amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR). A significant increase ($P<0.001$) in TLR 2 expression was detected in tuberculosis patients compared to controls. TLR2 597 TC and CC genotypes were higher in pulmonary tuberculosis patients (OR =2.79, 95% CI =1.02-7.95 and OR =4.26, 95% CI =0.40-214.00) respectively. Genotype TT was associated with reduced risk of being a case of pulmonary tuberculosis. There was no association of TLR2 surface expression on monocytes in pulmonary tuberculosis patients with different TLR2 597T/C genotypes. It is concluded that elevated expression of TLR2 on CD14+ monocytes in pulmonary tuberculosis patients confirms the role of TLR2 in host defense against *M. tuberculosis*. 597T/C polymorphism of TLR 2 gene may be a risk factor for susceptibility to pulmonary tuberculosis in a sample Egyptian population.

Tuberculosis is considered a major worldwide health problem. More than one-third of the world's population has been infected with the *M. tuberculosis* which is an intracellular pathogen capable of infecting and surviving within the host's mononuclear cells particularly macrophages. (Van Crevel *et al.*, 2002). Only 10% of those who are infected advance to clinical diseases, such as pulmonary tuberculosis (Barry *et al.*, 2009).

The reason why some infected individuals develop active disease while others do not has not been entirely understood. Multiple factors have been reported to affect the outcome of *M. tuberculosis* infection, including age, gender, ethnicity, etc. The host genetic factors are assumed to play a critical role in tuberculosis pathogenesis. These effects include impact on the gene expression of cytokines and chemokines implicated in the host immune response. Multiple susceptibility gene research studies have identified the

association of gene variants and pulmonary tuberculosis susceptibility, such as genes of toll-like receptors (TLRs), interleukin 10 (IL10), interferon γ (INF- γ), and human leukocyte antigen (HLA)(Ma *et al.*, 2007; Ben-Selma *et al.*, 2012; Magira *et al.*, 2012).

Mammalian TLRs comprise a large family consisting of at least 11 members. TLRs are expressed on macrophages as recognition receptors and recognize common structures of foreign substances (Medzhitov & Janeway, 2002). Each receptor recognizes different foreign substances. Of the different members of the TLR family, TLR2 has been shown to be a principal mediator of macrophage activation in response to *M. tuberculosis* which leads to direct bactericidal effect by suppressing the proliferation of *M. tuberculosis* or induction of apoptosis of infected macrophages (Noss *et al.*, 2001, Yoshida *et al.*, 2009).

TLR2 recognizes various proteins or lipids such as phosphatidylinositol mannoside and

19 kDa lipoprotein of *M. tuberculosis*. Activation of TLR2 by 19 kDa lipoprotein is indicated to increase nitric oxide (NO) production and induce killing of intracellular *M. tuberculosis* 48 hours after infection in both NO-dependent and -independent pathways in mice and human monocytes and alveolar macrophages (Thoma-Uszynski *et al.*, 2001).

Defective TLR2 genes have been associated with suppressed macrophages response to mycobacteria in both animal and human studies. Expression of mutant TLR2 in murine macrophages inhibited tumor necrosis- α production in response to both virulent and a virulent mycobacteria (Biswas *et al.*, 2009).

Human TLR2 gene is located on chromosome 4q32 and is composed of 2 non-coding exons and 1 coding exon (Haehnel *et al.*, 2002). To date, more than 175 single nucleotide polymorphisms (SNPs) or dinucleotide polymorphisms for the human TLR2 gene have been reported. Several studies investigating polymorphic variants of human TLR2 have been performed in patients with mycobacterial diseases such as leprosy and tuberculosis (Ogus *et al.*, 2004).

SNPs of 597T/C of the TLR-2 gene have been identified by Naderia *et al.* (2013) that impaired the macrophage response to *M. tuberculosis* in humans.

Owing to the central role of TLR2 in the recognition of tubercle bacilli (TB), our study aimed to evaluate TLR2 surface expression on peripheral blood monocytes and study its association with variants in TLR2 597T/C SNPs in patients with pulmonary tuberculosis.

Patients and Methods

Patients and controls

This study was conducted on a sample of Egyptian cohort population consisting of 50 pulmonary tuberculosis patients attending Benha University Hospital and Benha Chest Hospital and 50 clinically

and radiologically healthy subjects matched with patients' age and sex as controls. Healthy controls were previously vaccinated by BCG with positive tuberculin test.

The patients and controls were subjected to: Full Clinical evaluation including a full clinical and family history, physical examination, and routine laboratory tests, plain chest x-ray P.A. and lateral view, sputum analysis for acid fast bacilli by Ziehl-Neelsen stain (Z.N), tuberculin skin test (TST) using Mantoux technique and sputum culture on conventional Lowenstein-Jensen Medium (LJ). The diagnosis of tuberculosis patients was based on sputum positive microscopy and positive LJ culture.

Patients on antituberculosis regimens and those with history of recent viral infections, long use of corticosteroid or cytotoxic drugs, patients with diabetes mellitus, renal failure or liver cell failure were excluded.

The study was approved by the local ethics committee of Benha University Hospitals and written consent was taken from each participant.

Samples collection

- Sputum samples

Morning sputum samples were collected for 3 days by asking the patient to cough deeply into a sterile screw cap cups. The specimens were kept at 4 °C prior to liquefaction and decontamination with NaOH, centrifugation and neutralization of the deposit with HCl. Sputum samples were subjected to Z.N stain and culture on LJ media.

- Blood samples

Three ml of venous blood were collected by venepuncture under complete aseptic technique from all patients and controls in an ethylene diamine tetra-acetic acid (EDTA) containing blood collection tube. The samples were divided into two parts. The first part was immediately processed for flow cytometry analysis. The second part was stored at -70 till used in DNA extraction and amplification.

- Flow Cytometric Analysis

Hundred μ L of anticoagulated blood was incubated with 10 μ L phycoerythrin (PE) conjugated anti-human CD14 (Mouse IgG1 isotype, catalog Number: FAB3832P, R&D system, USA), and 10 μ L Peridinin chlorophyll Protein Complex (PerCP) conjugated antihuman TLR-2 (Mouse IgG2B isotype, catalog Number: FAB2616C, R&D system, USA) for 20 minutes in the dark at 4°C. Erythrocytes were lysed with lysing buffer (0.155M ammonium chloride, 0.01M

potassium bicarbonate and 0.127 M EDTA). Cells were washed with PBS 1% and resuspended in cold PBS.

PE Mouse IgG1 (Catalog Number: IC002P, R&D system, USA) and PerCP Mouse IgG2B (Catalog Number: IC0041C, R&D system, USA) isotypes were used as negative controls to detect non-specific staining.

The positive and negative populations overlap and a negative control was needed to estimate the fraction positive by subtraction of isotype control from the positive sample.

The immunostained cells were fixed with 0.5% paraformaldehyde. Labeled cells were analyzed on Fluorescent activated cytometric (FACS)-caliber using Cell Quest software (Becton Dickinson San Jose, CA, USA).

Live gating of monocytes using the size (forward scatter - FSC) and cell granularity (side scatter - SSC) as parameters was performed. The second gating was performed using SSC versus CD14- PE fluorescence, and the CD14- PE -tagged cells population was selected. Another CD14- PE -tagged cells versus TLR2- PerCP gating was set. Ten thousand events were measured for each sample. The percentage of TLR2 expressing cells and the level of expression per cell was calculated: $\Delta \text{MFI} = \text{MFI}_{\text{TLR2}} - \text{MFI}_{\text{isotype}}$.

- **TLR2 597T/C genotyping**

TLR2 597T/C genotyping was done by Tetra primer amplification refractory mutation system -PCR (T-ARMS-PCR) as described by (Naderi *et al.*, 2013):

T-ARMS-PCR has been proved to be rapid, simple and economical allowing simultaneous amplification of both alleles in a single tube without the need for costly and often difficult post-PCR manipulation (Collins & Ke, 2012).

Principle: In T-ARMS-PCR, 2 pairs of primers in a single PCR tube, can simultaneously amplify both alleles and allow amplification of an internal DNA control. The common outer primers amplify a large control band of the target gene, irrespective of its genotype. The SNPs points were positioned asymmetrically with respect to the common (outer) primers. Each inner primer combines with a particular opposite outer primer to generate smaller allele-specific amplicons, which are of different sizes and can easily be discriminated on gel electrophoresis either as homozygous or heterozygous (Ye *et al.*, 2001).

Genomic DNA was extracted from the whole blood using Gene JET™ genomic DNA purification kit

supplied by Fermentas, Germany, according to the manufacturer's instructions. The extracted DNA concentration was detected through measurement by UV spectrophotometer. Readings were taken at wave lengths of 260 and 280 nm according to that reported by Al Hussein *et al.* (2010).

Amplification was done using two common outer primers (control band) and two inner primers (allele specific primers) as shown in Table1.

The reaction was performed in a final volume of 50µL consisting of 25µL of Dream Green Taq PCR Master Mix (2x) (Fermentas, Germany), five µL of template DNA, 0.5µM concentration of each primer (Fermentas, Germany), water (nuclease free) to a final volume of 50 µL. All reagents were prior vortexed, and finally 25 µL of mineral oil were added to the reaction mixture.

Reaction was carried out in Thermal Cycler (Biometra, Germany) with the following steps: Initial denaturation step at 95°C for 3 minutes, forty repeated cycles of: denaturation at 95°C for 30 seconds, annealing at 49°C for 30 seconds and extension at 72°C for 1 minute followed by final extension step at 72°C for 15 minutes then hold at 4 °C.

Ten µL of each amplified DNA & 100 bp ladder (molecular weight marker) (Fermentas, Germany) were separated by electrophoresis using 2% agarose gel containing 0.3 µg/ml of ethidium bromide. The bands were visualized using UV transilluminator (254nm).

The size of DNA fragments amplified with these four primers of TLR2 597 SNPs are 349 bp control band, 228 bp for T allele and 173 bp for C allele. The control band is detected in all DNA samples. Individuals with 228 bp band are TT homozygous, individuals with 173 bp band are CC homozygous and individuals with both bands are TC heterozygous.

Quality assurance was confirmed through:

- Internal positive amplification control to amplify 349 bp for two outer primers (control band) (Table 1).

- Negative control reaction was confirmed by inserting a tube containing all components needed for amplification except DNA template in each amplification run.

Specificity of allele specific primer is conferred to the 3' end corresponding to the different alleles, in addition to mismatched bases deliberately added to position 2 from the 3' end of the primer to improve allele specificity (Ye *et al.*, 2001).

Primers are designed by the original software on the website: <http://cedar.genetics.soton.ac.uk>. 'BLAST' program at <http://www.ncbi.nlm.nih.gov/blast> are used to check for the specificity of the primers.

Statistical Analysis

The collected data were summarized in terms of mean±SD for quantitative data and frequencies and percentages for qualitative data. Comparisons between the proportions of different genotypes and allele distributions in the studied groups were carried out using the Chi-squared test (χ^2) and the Fisher's exact test as appropriate and the corresponding Odd ratio and

95% CI (OR; 95%CI) was calculated. The differences in the mean levels of TLR-2 expression between patients and controls were tested using the Student's t-test. The Analysis of Variance (F) was used to compare the mean levels of TLR-2 expression between the different genotypes followed by multiple comparisons using the Bonferroni method to detect differences in pairs. A *P*-value for the calculated tests statistics was obtained. A *P*-value < 0.05 was considered statistically significant. The statistical analysis was conducted using STATA version 11 (STATA corporation, College Station, Texas).

Table 1. Primers used for determination of TLR-2 597T/C polymorphism (Naderi *et al.*, 2013).

Primers	597T/C (rs3804099)
Forward inner (T allele specific)	5'- CCAAAAAGTTTGAAGTCAATTCAGCAT- 3'
Reverse inner (C allele specific)	5'- TCATATGAAGGATCAGATGACTTCCG -3'
Common outer primer (forward)	5'- ATTGCAAATCCTGAGAGTGGGAA -3'
Common outer primer (reverse)	5'- CAACTTTCATCGGTGATTTTACA -3'

Results

This study was conducted on 50 pulmonary tuberculosis patients (46 males and 4 females) with mean age of 53.0±13.8 years attending Benha University Hospital and Benha Chest hospital and 50 (46 males and 4 females with mean age 51.6±13.7 years) clinically and radiologically healthy subjects as controls. No significant difference was detected between patients and controls groups as regard to age and sex.

In this study, cell surface expression of TLR2 on peripheral blood monocyte was evaluated by measuring the percentage of TLR2 positive CD14+ cells and their MFI. Both parameters showed high significant elevation (*P*<0.001) in patients than controls (Table 2), Figure 1, 2.

Genotype and allele frequencies of TLR2 597 T/C SNPs in patient and control groups were summarized in Table 3, Figure 3.

Table 2. TLR-2 expression in patients and controls.

Monocytes	Patients (n=50)		Controls (n=50)		<i>P</i> value
	Mean ± SD	Range	Mean ± SD	Range	
%	35.44±19.21	16.6-70.2	11.34±6.70	3.6-23.8	<0.001
MFI	7622.4±3520.1	2831-14324	3230.5±1454.83	1103-5216	<0.001

There were highly significant differences (*P*<0.001) in the mean levels of TLR-2 expression between patients and controls. *P*<0.05 is significant.

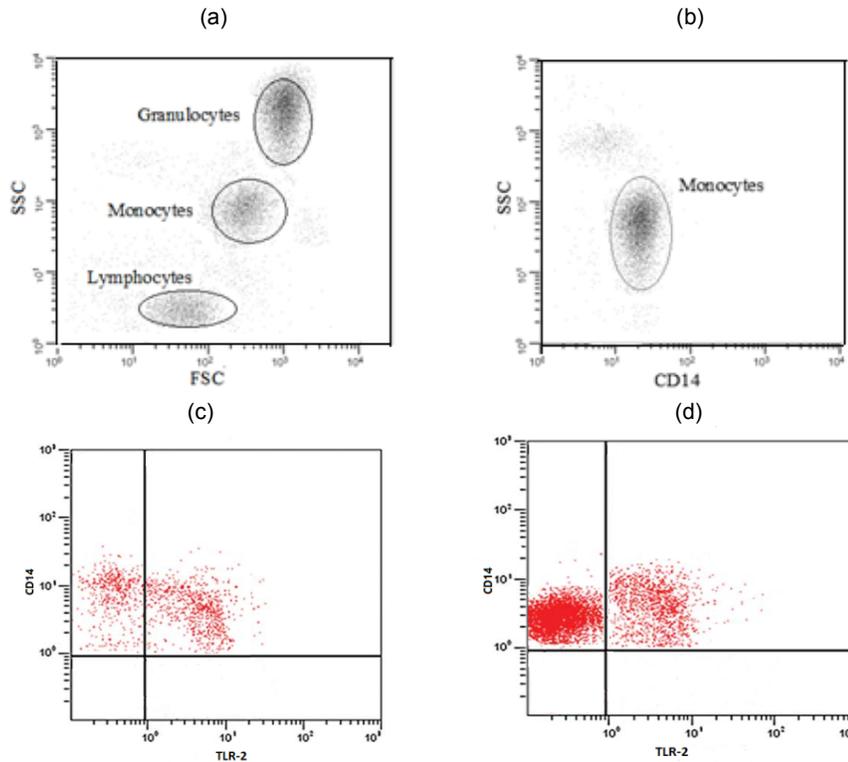


Figure 1. Flow cytometric dot-plot analysis of Monocytes. Monocytes were identified by a combination of FSC/SSC gating (fig.1-a), additional gating on the CD14 population by SSC versus PE-CD14 staining (fig.1-b). CD14 versus TLR2 dot plot: (fig.1-c) a healthy control, (fig.1-d) a tuberculosis patient (13% and 27.01 %) respectively.

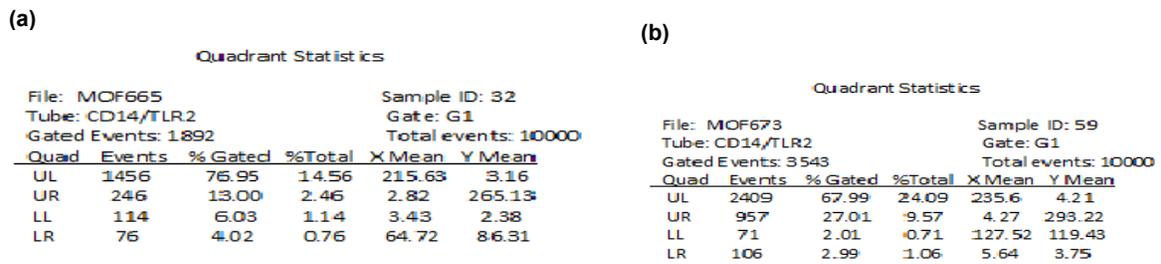


Figure 2. Quadrant statistics for a healthy control (a), a tuberculosis patient (b).

Table 3. Genotype and allele distributions of TLR2 597T/C SNPs among patients and controls.

Polymorphisms	Patients (N=50)		Controls (N=50)		OR (95% CI)	P value
	N	(%)	N	(%)		
Genotypes	TT	5	(10.0)	18	(36.0)	0.20 (0.05-0.64)
	TC	41	(82.0)	31	(62.0)	2.79 (1.02-7.95)
	CC	4	(8.0)	1	(2.0)	4.26 (0.40-214.00)
Allele	T allele	51	(51.0)	67	(67.0)	0.51 (0.28-0.94)
	C allele	49	(49.0)	33	(33.0)	

*Obtained using the Fisher's exact test; **Obtained by X² test, P<0.05 is significant.

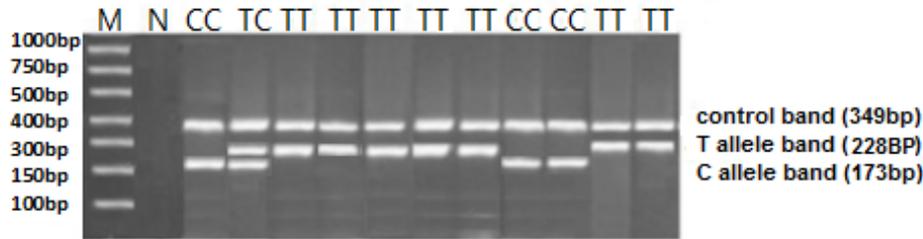


Figure 3. Gel electrophoresis of amplified T-ARMS-PCR products of TLR2 597 SNPs. Lane M shows DNA ladder. Lane N shows negative control. Other lanes show PCR products of different genotypes.

There was a significant difference ($P=0.003$) in the frequencies of T/C genotypes between patient and control groups. Patients were more likely to have TC genotype (82% vs. 62%, OR =2.79, 95% CI =1.02-7.95) and CC genotype (8% vs. 2%, OR =4.26, 95% CI =0.40-214.00) as compared to controls. However, genotype TT was associated with reduced risk of being a case of pulmonary tuberculosis.

There were significant differences in the

TLR2 597 T and C alleles frequencies between patients and controls (OR=0.51, 95% CI= 0.28-0.94 & $P=0.02$). The frequency of the C allele was higher in patients than controls (49% vs. 33%).

There was no association between the TLR2 genotypes and cell surface expression of TLR2 including percent of TLR2 expressing monocytes and MFI ($P= 0.19$ and 0.68 respectively), Table 4, Figure 4, 5.

Table 4. Association of TLR2 expression on monocytes with different TLR2 597T/C genotypes in pulmonary tuberculosis patients.

	CC		TC		TT		P value
	Mean± SD	Range	Mean± SD	Range	Mean± SD	Range	
%	18.73±7.05	11.2-28.6	14.7±9.2	3.3-32.4	12.39±6.3	5.1-19.9	NS
MFI	2967± 963.13	1706-4448	2863.2± 1076.58	1599-4579	2619.7± 609.9	1943-3939	NS

There were no significant differences in the mean levels of TLR-2 expression between the different TLR2 genotypes. $P>0.05$ is not significant (NS).

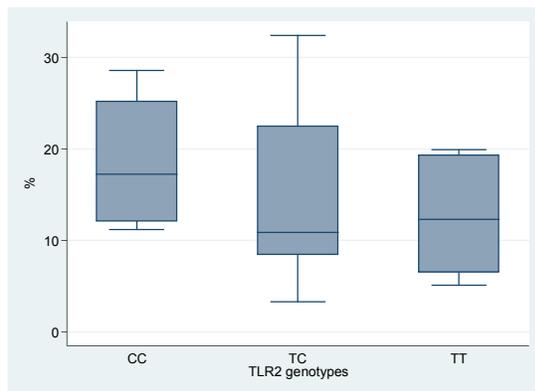


Figure 4. Percentage of TLR2 expressing monocytes in tuberculosis patients with different TLR2 597T/C genotypes.

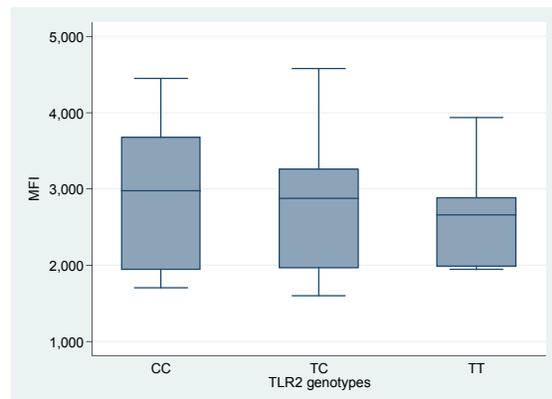


Figure 5. MFI of TLR2 expression among tuberculosis patients with different TLR2 597T/C genotypes.

Discussion

Tuberculosis remains one of the leading causes of death from a single infectious agent worldwide (Mortaz *et al.*, 2014). Kawamura (2006) clearly indicated that TLRs play an essential role in the recognition of *M. tuberculosis* components, resulting in not only activation of innate immunity but also development of antigen-specific adaptive immunity.

Changes in TLR expression and/or their down-stream activation state might represent useful markers of the immunological status of tuberculous patients and their contacts (Rook *et al.*, 2005). Owing to the central role of TLR2 in the recognition of TB, our study aimed to evaluate TLR2 surface expression on peripheral blood monocytes and study its association with variants in TLR2 597T/C SNPs in patients with pulmonary tuberculosis.

The present study revealed increase the percentage of TLR2 expressing CD14⁺ monocytes and MFI for this receptor in pulmonary tuberculosis patients compared to healthy controls ($P < 0.001$). These results are supported by findings of De Oliveira *et al.* (2014) who demonstrated an up-regulation in the expression of TLR2 in monocytes isolated from patients with tuberculosis.

Our results could provide supporting evidence of the role of TLR2 in host defense against infection with *M. tuberculosis*. TLR2 is believed to be important in the initiation of innate host defense through its stimulatory effects on TNF α and IL-12 production in macrophages (Bafica *et al.*, 2005, Pompei *et al.*, 2007). Drennan *et al.* (2004) reported that TLR2^{-/-} mice show defective granuloma formation, and when infected with high doses of *M. tuberculosis*, they have a greatly enhanced susceptibility to infection compared to the wild type mice. In addition, TLR2^{-/-} mice display defects in controlling chronic infection with *M. tuberculosis*. *In vitro* study has revealed that TLR2 activation directly

leads to intracellular killing of *M. tuberculosis* by alveolar macrophages (Thoma *et al.*, 2001).

High susceptibility to *M. tuberculosis* infection in TLR2-deficient mice suggested that mutations affecting TLR2 expression may impair host response to this pathogen (Drennan *et al.*, 2004; Reiling *et al.*, 2002). Chodisetti *et al.* (2015) observed reduced lung pathology upon administration of TLR-2 agonist in the chronic infection model of tuberculosis.

In humans, the association of TLR polymorphisms with susceptibility to tuberculosis remains to be confirmed (Songane *et al.*, 2012). Different polymorphisms in the human TLR2 gene were reported to associate with increased susceptibility to tuberculosis in some studies (Velez *et al.*, 2010; Zhang *et al.*, 2013) but not others (Selvaraj *et al.*, 2010; Xue *et al.*, 2010).

Our study demonstrated that patients were more likely to have TC and CC genotypes (82% vs. 62% and 8% vs. 2%) as compared to controls. However, genotype TT was associated with reduced risk of being a case of pulmonary tuberculosis. The frequency of C allele was significantly higher in patients than controls (49% vs. 33%, $P = 0.02$).

These results agree with Caws *et al.* (2008) who found that individuals with 597 C allele of TLR-2 were more likely to have tuberculosis than other individuals. Also Thuong *et al.* (2007) found that 597 CC genotype was associated with tuberculosis in Vietnam and demonstrated a strong association with tuberculous meningitis and miliary tuberculosis indicating that TLR2 influences the dissemination of *M. tuberculosis*.

Our findings are also in consistent with Naderi *et al.* (2013) who detected significant difference between case and control groups regarding 597T/C polymorphisms ($\chi^2 = 12.21$, $P = 0.002$). They found that TC and CC

genotypes were associated with the risk of pulmonary tuberculosis (OR = 2.13, 95% CI = 1.25–3.62, $P = 0.005$ and OR = 4.88, 95% CI = 1.56–15.26, $P = 0.007$) respectively.

In contrast to our finding Arji *et al.* (2014) reported that TLR2 597 TC heterozygous genotype was found to be more prevalent in controls than in patients in Moroccan population (60% vs. 49%, respectively; $P=0.012$). These conflicting results can be explained by genetic heterogeneity among the studied population samples or by potential difference in the involved *M. tuberculosis* strains. Khor *et al.* (2007) reported that discrepancy might be due to a dynamic host-pathogen interplay between genetic and pathogen phenotypes.

Bochud *et al.* (2003) reported that polymorphism of the TLR2 gene caused severe impairment of the macrophage response to *M. leprae* and *M. tuberculosis*. It was also shown that a mutation in mouse TLR2 acts as a dominant negative inhibitor of TLR2 signaling.

As regard the association of TLR2 surface expression levels on peripheral blood monocytes of tuberculosis patients with different TLR2 597T/C genotypes, our results revealed insignificant role of TLR2 597T/C SNPs in TLR2 surface expression. This finding indicates that the observed increase in TLR2 expression on monocytes of tuberculosis patients is not due to 597T/C SNPs in TLR2 gene. It can be explained by the possibility of ethnic differences in linkage of these polymorphisms with other TLR2 polymorphisms modulating TLR2 expression as well as other possible genetic and environmental factors.

The molecular mechanism by which the TLR2 597 T/C could influence the susceptibility or the resistance to pulmonary tuberculosis is still unknown. As this variant is a synonymous mutation, it could merely reflect another, yet-to-be-identified marker in

close linkage disequilibrium. In this context, a microsatellite region within the TLR2 intron-2 (GT repeat) affecting gene regulation was previously described with significant frequency variations across populations (Yim *et al.*, 2004; Yim *et al.*, 2006).

Since linkage disequilibrium patterns can vary among populations, and may cause differences in disease associations of this SNP in different populations. Thus, it is possible that the linkage disequilibrium between the causal mutation and TLR2 597 T/C may differ among different population (Arji *et al.*, 2014).

Therefore, TLR2 gene polymorphism may be one of the factors influencing disease susceptibility, and other factors or defects in different steps of the immune response (even other polymorphisms of TLR2 or other TLRs) might also be responsible for an inability to prevent progression of TB infection to disease (Dupuis *et al.*, 2001).

From this study we conclude that elevated expression of TLR2 on CD14⁺ monocytes in pulmonary tuberculosis patients confirms the role of TLR2 in host defense against *M. tuberculosis*. TLR2 597T/C polymorphism may be a risk factor for susceptibility to pulmonary tuberculosis with no associated role in higher TLR2 expression in pulmonary tuberculosis patients.

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