

## Association of Cytokine Gene Polymorphism with Susceptibility and Clinical Types of Leprosy

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### Abstract:

**Background:** Both pro- and anti-inflammatory cytokines are implicated in development and prognosis of leprosy so the genetic regulation of such cytokines could play an important role.

**Objective:** This study was planned for testing the association of cytokine gene polymorphisms with susceptibility and clinical types of leprosy among Egyptian cases.

**Subjects:** This study included 47 cases (29 men, 18 females, mean age = 46.3 years) with leprosy in addition to 98 healthy unrelated controls (52 males, 46 females, mean age = 44.9 years). Cases were recruited from Leprosy Clinics, Delta region of Egypt. Cases were classified into paucibacillary (PB) (n = 17; 10 males, 7 females; mean age 42.6 years) and multibacillary (MB) (n = 29; 19 males, 10 females; mean age 43.9 years).

**Methods:** For all cases and controls, DNA was extracted and amplified using polymerase chain reaction with sequence specific primers (PCR-SSP) for detection of single nucleotide polymorphisms (SNPs) in the promoter regions of cytokine genes, TNF- $\alpha$ -308 (G/A), IL-10-1082(G/A), IL-6-174(G/C) as well as IL-1RaVNTR in intron 2 of the gene.

**Results:** Compared to controls, all cases have shown increased frequency of homozygous genotypes : IL-10-1082 (GG) (Odds ratio 6.6, P <0.05), homozygous TNF- $\alpha$ -308 (GG) (Odds ratio = 3.23), and homozygous IL-1Ra (11) (Odds ratio = 3.6, P<0.05) with increased frequency of IL10 G and ILRa 1 alleles (P<0.05). BP subgroup showed increased frequency of homozygous IL-10-1082 (GG) (Odds ratio = 18.6, P<0.05) with increased frequency of IL10 G allele (P<0.05). On the other hand, MB subgroup showed increased frequency of homozygous TNF- $\alpha$ -308 (GG) (Odds ratio = 5.84, P<0.05) and homozygous IL-1Ra (11) (Odds ratio = 4, P<0.05) with increased frequency of IL-1Ra 1 allele (P<0.05). There is predominance for heterozygous IL-6-174 (G/C) polymorphism in all studies patient subgroups as well as controls with no significant difference among them.

**Conclusion:** Genetic polymorphisms related to TNF- $\alpha$ -308 and IL-10-1082 and IL-1Ra may be used as genetic markers for susceptibility and clinical outcome of leprosy among Egyptian cases from the Nile Delta.

**Abbreviations:** TNF; tumor necrosis factor, IL; interleukine, IL-1Ra; IL-1 receptor antagonist, PCR; polymerase chain reaction, SNP; single nucleotide polymorphism, VNTR; variable number tandem repeat.

**Key words:** Leprosy, Cytokines, Gene polymorphism, Egypt

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## Introduction

Leprosy or Hansen's disease is a chronic debilitating disease caused by obligate intracellular pathogen; *Mycobacterium leprae* (M. Leprae) that annually affects more than 700,000 new individuals.<sup>(1)</sup> Leprosy is characterized by a large spectrum of clinical manifestations depending upon host immunity<sup>(2)</sup>; at one pole tuberculoid leprosy (TL) in which patients manifest a strong cellular immunity resulting in few, localized, often self-healing skin and nerve granulomas. At the opposite pole, lepromatous leprosy (LL) patients have a limited cellular immune response leading to a disseminated disease with diffuse infiltration of skin and nerves and bacilli-loaded foamy macrophages in spite of having also high serum antibody levels specific to the bacilli. Between two poles the majority of patients are borderline classified as borderline tuberculoid (BT), mid-borderline (BB), or borderline lepromatous (BL).<sup>(3,4)</sup>

Factors influencing type of leprosy are not well understood, yet several studies support role of genetics in susceptibility to leprosy as evidenced by the familial clustering and concordance in monozygotic than dizygotic twins.<sup>(5, 6)</sup> Evidences of associations to HLA (HLA-DR2 in tuberculoid and HLA-DQ1 in lepromatous)<sup>(7)</sup> as well as to non-MHC genes e.g. natural resistance-associated macrophage protein 1 (NRAMP1)<sup>(8)</sup>, vitamin D receptor (VDR)<sup>(9)</sup> and more recently, the intracellular domain of Toll like receptors 2 (hTLR2) confirm the genetic role but still not sufficiently explaining the clinical diversity in leprosy.<sup>(10)</sup>

Cytokines play a critical role in triggering host-pathogen interaction in leprosy; TT onset correlates with cytokines produced by Th1 cells whereas, LL was associated with a Th2 response mainly through IL-4 secretion. Besides IFN- $\gamma$  and IL-4, other cytokines produced by T cells such as tumor necrosis factor-alpha (TNF- $\alpha$ ), IL-6 and IL-10 and by macrophages such as IL-1 family also regulate M. leprae cell mediated and humoral immune responses.<sup>(11, 12, 13, 14, 15)</sup>

TNF- $\alpha$ , IL-1 and IL-6 are early pro-inflammatory mediators of innate response to bacterial challenge whereas, IL-10 and *IL-1Ra* are anti-inflammatory cytokines that terminate the immune response and limit the pathology via suppression of Th1 and macrophage function.<sup>(16, 17, 18)</sup>

Two gene polymorphisms at positions -308 and -238 have been described within the promoter region of TNF- $\alpha$  gene on chromosome 6 and are associated with susceptibility or resistance to different diseases including leprosy but yet results are controversial.<sup>(19,20,21)</sup> In this sense, several single-base pair substitutions spanning promoter region of IL-10 on chromosome 1 have also been identified, including positions -1082 (G-A), -819 (C-T), and -592 (C-A) with a growing body of evidence suggesting the role of these polymorphisms in protection, induction and/or maintenance of energy in patients with leprosy.<sup>(22)</sup>

Interleukin-1 on chromosome 2q12-2q14 harbors various promising candidate genes for inflammatory diseases, of which *IL-1Ra* gene that has a variable number tandem repeat (VNTR) in intron 2, with up to five variants depending on number of repeats of 86-base pair (bp) fragment. Allele 2; with two repeats has been associated with increased *IL-1Ra* production *in vitro*; some studies found an influence *IL-1Ra* genotype on tuberculosis phenotype.<sup>(23, 24, 25)</sup> Similarly, allele G of IL-6 gene on chromosome 7 at position -174 showed a higher plasma IL-6 level compared to allele C.<sup>(26)</sup>

The goal of current study is to check for association of cytokine gene polymorphisms with leprosy susceptibility and progression in Egyptian cases recruited from the Nile Delta. Candidate genes selected for the study included 2 genes of pro-inflammatory cytokines, TNF- $\alpha$  at position -308 (G/A) and IL-6 at position -174 (G/C) in addition to another 2 genes of anti-inflammatory cytokines; *IL-1Ra* VNTR and IL-10 at position -1082 (G/A).

## Methods

Subjects included 47 leprosy cases recruited from Leprosy Clinics linked to University Hospitals resident in the Nile Delta region of Egypt. They presented with leprosy or its complications as a main medical problem. In these cases, diagnosis of leprosy was established according to standard procedures, including histological examination of skin biopsy samples.<sup>(27)</sup> Patients' age range was 18-59 years (mean  $\pm$  SD = 46.3  $\pm$  7.3), they included 29 men and 18 women. Using Ridley and Toppling classification<sup>(28)</sup>, patients were classified clinico-pathologically into paucibacillary (PB) or tuberculoid (TL) (n = 17; 10 males, 7 females; mean age 42.6  $\pm$  8.1) and multibacillary

(MB) or lepromatous (LL) (n = 29; 19 males, 10 females; mean age  $43.9 \pm 5.4$ ). Diagnosis of LL (MB) required presence of generalized skin lesions with a large number of acid-fast bacilli on smear testing but that of TL (PB) showed a small number or smear negative, well defined dry skin lesions.<sup>(29)</sup>

To test for associations between genetic polymorphism and leprosy, patients were compared to 98 control adult healthy unrelated volunteers attending Blood Bank at the same locality. They were age and sex matched (age mean  $44.9 \pm 6.7$ ; 52 males, 46 females).

**DNA extraction and purification:** After obtaining informed consent from all cases and controls, venous blood samples (3 ml) were collected in EDTA (ethylenediamine tetraacetate) containing tubes, DNA was extracted promptly using DNA extraction and purification kit (Gentra systems, USA) according to manufacturer's instructions and then stored at  $-20^{\circ}\text{C}$  till used.

**PCR amplification;** Four single nucleotide polymorphisms (SNPs) were analyzed including promoter sites TNF- $\alpha$ -308(G/A), IL-10-1082(G/A) and IL-6-174(G/C) as well as IL-1Ra intron 2 VNTR using PCR with sequence-specific primers (PCR-SSP) in two PCR-SSP reactions employing a common forward and 2 reverse primers for TNF- $\alpha$ , IL-6 and IL-10 promoter polymorphisms, and a single PCR reaction employing a forward and a reverse primers for IL-1Ra VNTR polymorphism. All primers, Taq polymerase, dNTP, and MgCl were purchased from QiaGene (QiaGene, USA). The assay was performed in Techne-Genius thermal-cycler (England). Briefly, 100-500 ng of genomic DNA was added to 25  $\mu\text{l}$  of reaction mixture containing 1  $\mu\text{M}$  of each comm-on/specific primer, 200  $\mu\text{M}$  of each dNTP, and 1 U of Taq DNA polymerase. Primers sequence, MgCl<sub>2</sub> concentrations, and PCR conditions optimal for each reaction were listed in Table 1.<sup>(25,30-32).</sup>

**Detection of amplified products:** The entire reaction volume plus 5  $\mu\text{l}$  of bromophenol blue track dye were loaded into 2% agarose gel (Boehringer Mannheim) containing ethidium bromide. Gels were electrophoresed for 20 minutes at 200 V, photographed under UV light (320 nm) and then scored for the presence or absence of an allele specific band with reference to PCR control band (Figure 1).

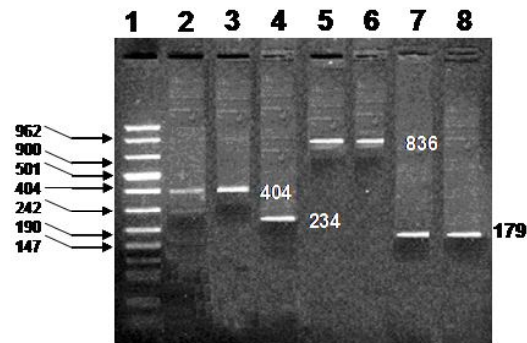


Fig. (1). TNF- $\alpha$  -308, IL-10 -1082, IL-6 and IL-1Ra PCR products. Lane 1: Show size marker VIII. Lanes 2, 3: amplified product of IL1 Ra (VNTR) with 2 alleles of size 404, 242 bp (lane 2) and one allele of size 404 bp (lane 3). Lane 4: amplified product of IL-6 shows band size 234 bp. Lanes 5, 6: amplified product of TNF- $\alpha$  band size 836 bp. Lanes 7, 8: amplified product of IL-10 band size 179bp.

**Statistical analysis:** Data were processed and analyzed using the Statistical package of social science version 10.0. The frequency of studied allelic polymorphisms among cases was compared to that of controls describing number and percent of each and tested for positive association using Fisher's exact test (modified chi square test) and Odds ratio with a minimum level of significance of  $<0.05$ .

## Results

Compared to controls, total cases have shown increased frequency of homozygous IL-10-1082 (G/G)(26.2% Vs 5.1%, Odds ratio = 6.6,  $P < 0.05$ ), homozygous TNF alpha-308 (G/G) (17.3% Vs 6.1% with Odds ratio 3.23), and homozygous IL-1Ra (1/1) (84.1% Vs 58.8%, Odds ratio = 3.6,  $P < 0.05$ ) with increased frequency of IL10 G and ILRa 1 alleles ( $P < 0.05$ ) (Tables 2,3,5).

BP subgroup showed increased frequency of homozygous IL10 GG (Odds ratio = 18.6,  $P < 0.05$ ) with increased frequency of IL10 G allele ( $P < 0.05$ ) (Table 3).

On the other hand, MB subgroup showed increased frequency of homozygous TNF -308 GG (Odds ratio = 5.84,  $P < 0.05$ ) and homozygous IL-1Ra A1A1 (Odds ratio = 4,  $P < 0.05$ ) with increased frequency of ILRa A1 allele ( $P < 0.05$ ) (Tables 2,5).

There is predominance for heterozygous IL-6-174 GC polymorphism in all studies patient subgroups as well as controls with no significant difference among them, denoting that this polymorphism is a feature of Egyptian population (Table 4).

**Table (1). Primer sequences and PCR conditions of the different studied cytokines**

Cytokine	Primer sequences 5'-3'	PCR product size (bp)	MgCl <sub>2</sub> conc/ mM	PCR conditions
<b>TNF-<math>\alpha</math> -308</b>	F: CTG CAT CCC CGT CTT TCT CC R1: ATA GGT TTT GAG GGG CAT CG R2: ATA GGT TTT GAG GGG CAT CA	<b>836</b>	<b>2.5</b>	3 minutes at 96°C; 30 cycles: 96°C(45 sec), 55°C (80 sec) and 72°C 2 min); 72°C (3 min).
<b>IL-10-1028</b>	F: AGC AAC ACT CCT CGT CGC AAC R1: CCT ATC CCT ACT TCC CCC R2: CCT ATC CCT ACT TCC CCT	<b>179</b>	<b>1.5</b>	30 cycles: 94°C (30 sec), 60°C (60 sec), 72°C (60 sec) then 72°C (7 min).
<b>IL-6-174</b>	F: GAG CTT CTC TTT CGT TCC R1: CCT AGT TGT GTC TTG CC R2: CCC TAG TTG TGT CTT GCG	<b>234</b>	<b>1.5</b>	30 cycles: 94°C (30 sec), 54°C (60 sec), 72°C (60 sec) then 72°C (7 min).
<b>IL1RaVNTR</b>	F: TCC TGG TCT GCA GGTAA R: CTC AGC AAC ACT CCT AT	<b>Variable 404, 242</b>	<b>2</b>	96°C (1 min); 35 cycles: 94°C (1 min), 60°C (1 min), 70°C (1 min), then 70°C (5min).

**Table (2). Genotype and allelic frequency of TNF  $\alpha$ -308 (G/A) polymorphism in control compared to Cases groups**

Subject group	<u>Genotypes Frequency</u>			<u>Allele Frequency</u>	
	GG	GA	AA	G	A
<b><u>CONTROLS (n=98)</u></b>					
N (%)	6 (6.1)	81 (82.7)	11 (4.2)	93 (47.4)	103 (52.5)
<b><u>Total Cases (n=46)</u></b>					
N (%)	8 (17.3)	37 (80.4)	1 (2.2)	53 (57.6)	39 (42.3)
OR (95% CI)	3.23(1.05, 9.9)	0.86(0.35, 2.1)	0.18(0.02, 1.4)	1.51(0.9, 2.5)	0.66(0.4, 1.1)
<b><u>Paucibacillary (BP) (n=17)</u></b>					
N (%)	0 (0.0)	17 (100)	0 (0.0)	17(50)	17(50)
OR (95% CI)	0.41(0.02, 8.03)	7.52(0.4, 131)	0.22(0.01, 3.9)	1.11(0.5, 2.3)	0.9(0.4, 1.9)
<b><u>Multibacillary (MB) (n=29)</u></b>					
N (%)	8 (27.5)*	20 (68.9)	1 (3.4)	36 (62)	22 (37.9)
OR (95% CI)	5.84(1.8, 18.6)	0.47(0.19, 1.3)	0.28(0.04, 2.3)	1.81(1, 3.3)	0.55(0.3, 1)

OR: Odds ratio., 95% CI : 95% Confidence interval \* Statistically significant when  $P < 0.05$

**Table (3). Genotype and allelic frequency of IL 10 -1082 (G/A) polymorphism in control compared to Cases groups**

Subject group	<u>Genotypes Frequency</u>			<u>Allele Frequency</u>	
	GG	GA	AA	G	A
<b><u>CONTROL (n=98)</u></b>					
N (%)	5 (5.1)	85 (86.7)	8 (8.2)	95 (48.4)	101 (51.5)
<b><u>Total Cases (n=42)</u></b>					
N (%)	11(26.2)*	28 (66.7)*	3 (7.1)	50 (59.5)*	34 (40.4)
OR (95% CI)	6.6(2.1, 20.5)	0.31(0.12, 0.7)	0.87(0.22, 3.4)	1.56(0.9, 2.6)	0.64(0.4, 1.1)
<b><u>Paucibacillary (BP) (n=14)</u></b>					
N (%)	7 (50)**	6 (42.8)**	1 (7.2)	20 (71.4)*	8 (28.5)*
OR (95% CI)	18.6(4.7, 74)	0.12(0.03, 0.38)	0.87(0.1, 7.5)	2.66(1.1, 6.3)	0.38(0.2, 0.9)
<b><u>Multibacillary (MB) (n=28)</u></b>					
N (%)	5 (17.8)	21 (75)	2 (7.1)	31 (55.3)	25 (44.6)
OR (95% CI)	4.04(1.08, 15.2)	0.46(0.16, 1.29)	0.87(0.17, 4.33)	1.32(0.7, 2.4)	0.76(0.4, 1.4)

OR: Odds ratio., 95% CI : 95% Confidence interval \* Statistically significant when  $P < 0.05$ .

**Table (4). Genotype and allelic frequency of IL-6 -174 (G/C) polymorphism in control compared to cases**

Subject group	<u>Genotypes Frequency</u>			<u>Allele Frequency</u>	
	GG	GC	CC	G	C
<b><u>CONTROL (n=98)</u></b>					
N (%)	5 (5.1)	87 (66.6)	6 (6.1)	97 (47.5)	99 (48.5)
<b><u>Total Cases (n=46)</u></b>					
N (%)	3 (6.3)	40 (85.1)	4 (8.5)	46 (48.9)	48 (51)
OR (95% CI)	1.3(0.3, 5.7)	0.84(0.3, 2.4)	1.46(0.4, 5.4)	0.98(0.5, 1.5)	1.1(0.7, 1.8)
<b><u>Paucibacillary (BP) (n=17)</u></b>					
N (%)	1 (5.9)	14 (82.3)	2 (11.8)	17 (47.2)	19 (52.7)
OR (95% CI)	1.2(0.13, 10.6)	0.59(0.15, 2.4)	2.04(0.4, 11.1)	0.91(0.4, 1.9)	1.2(0.6, 2.5)
<b><u>Multibacillary (MB) (n=29)</u></b>					
N (%)	2 (3.4)	25 (68.2)	2 (6.9)	29 (50)	29 (50)
OR (95% CI)	1.4(0.3, 7.5)	0.79(0.2, 2.7)	1.14(0.2, 6)	1.02(0.6, 1.8)	0.98(0.55, 1.8)

OR: Odds ratio., 95% CI : 95% Confidence interval

**Table (5). Genotype and allelic frequency of IL1Ra polymorphism in control compared to cases**

Subject group	Genotypes Frequency		Allele Frequency	
	A1A1	A1A2	A1	A2
<b><u>CONTROL (n=98)</u></b>				
N (%)	58 (58.8)	40(41.2)	156 (79.6)	40 (20.4)
<b><u>Total Cases (n=44)</u></b>				
N (%)	37 (84.1)*	7 (15.9)*	81 (92)*	7 (8)*
OR(95% CI)	3.6(1.5, 9)	0.3(0.1, 0.7)	2.96(1.27, 6.92)	0.34(0.15, 0.8)
<b><u>Paucibacillary (BP) (n=17)</u></b>				
N (%)	14 (82.4)	3.(17.6)	31 (83.7)	3(16.2)
OR(95% CI)	3.2(0.87, 11.9)	.3(0.1, 1.2)	2.7(0.8, 9.1)	0.38(0.11, 1.3)
<b><u>Multibacillary (MB) (n=27)</u></b>				
N (%)	23 (85.2)*	4 (14.8)*	50 (86.2)*	4 (13.7)*
OR(95% CI)	4(1.3, 12.3)	0.3(0.08, 0.79)	3.2(1.1, 9.4)	0.31(0.1, 0.9)

OR: Odds ratio., 95% CI : 95% Confidence interval

It has to be stressed that when analyzing the frequency of these genotypes and alleles related to age and sex groups and parental consanguinity showed no significant difference between patients and controls and within patient subgroups as well (data not shown).

### Discussion

Identification of the most important host susceptibility/resistance genes will allow a better understanding of infectious disease pathogenesis and would likely facilitate development of new therapeutic strategies.

Leprosy is an example whereby cytokines could influence leprotic immune reaction possibly due to genetic variability in cytokine production and consequently influencing the clinical outcome.<sup>(19)</sup>

The present study highlighted for the first time the role of polymorphism of TNF- $\alpha$ -308, IL-10-1082, IL-6-174 and IL-1Ra VNTR on susceptibility to and prognosis of leprosy in Egyptian cases. In this study, TNF- $\alpha$ -308 polymorphism, showed that heterozygous predominance GA was found among patients groups as well as controls. However, homozygous TNF- $\alpha$ -308 GG was found significantly higher in patients compared to controls due to its higher frequency in MB subgroup. Therefore allele TNF- $\alpha$ -308 allele G

could be considered a genetic marker for development of disseminated leprosy in Egyptian patients. Other authors have reported that this allele is associated with both disease susceptibility and disease progression where it favour the development of tuberculoid leprosy. In addition, a significant correlation was found between TNF2 carriage in patients with PB leprosy and the extent of in vivo cutaneous lepromin response. Moreover, TNF- $\alpha$ -308 allele A has been found to increase TNF- $\alpha$  transcription.<sup>(19-21, 32-34)</sup>

In our study, homozygous IL-10-1082 GG was significantly more frequent in patients PB subgroup compared to controls due to the predominance of G allele suggesting an impact for allele G in leprosy susceptibility/protection as well as a role of A allele against disease dissemination. In vitro studies proved that this polymorphism interacting with that of TNF- $\alpha$  had more response to lipopolysaccharides thus giving immunity against wide range of infections including MB leprosy. Similar conclusion had been reported for IL-10 -819 proximal promoter region polymorphism and secretion and IL-10-1082 GA genotype has been implicated in modulation of IL-10 gene activity. Another study in the same locality revealed that TNF- $\alpha$  expression was significantly higher in PB than MB patients while the reverse with IL-10 expression.<sup>(22,35-37)</sup>

Thus, it could be concluded that TNF- $\alpha$ -308 and IL-10 -1082 polymorphisms may alter the level of gene transcription, stability of mRNA, or quantity and activity of resulting protein and thus altering host resistance to local infection by influencing production of TNF- $\alpha$  and IL-10 at infection site.<sup>(38, 39)</sup>

Cases have shown increased frequency of homozygous IL-1Ra A1A1 genotype with increased frequency of allele A1 especially among cases of MB subgroup. IL-1Ra polymorphism was also implicated in the pathogenesis of many chronic inflammatory diseases and they showed roles in disease susceptibility and severity. Furthermore, it had been proven that heterozygous individuals for IL-1Ra A1A2 produced higher levels of IL-1Ra than individuals of other genotypes, this could explain the predominance of MB disease in our study patients.<sup>(40-45)</sup>

Although IL-6 polymorphisms showed no significant difference in frequencies among cases and controls, we could observe gene to gene interaction between individual studied genes polymorphisms. Thus, subjects with the IL-6-174 GC genotype had about three times higher incidence of leprosy especially lepromatous type than subjects with other genotypes in the presence of TNF- $\alpha$ -308 A allele and IL-10-1082 GG genotype. The possibility of how these polymorphisms could interact with each other is that synthesis of IL-6 is tightly regulated and controlled by TNF- $\alpha$  and IL-6 has been suggested to negatively control TNF- $\alpha$  production, also TNF- $\alpha$  release had an influence on IL-10.<sup>(46-48)</sup>

In conclusion, our work could throw some light on the association of cytokine gene polymorphisms with the susceptibility and clinical severity of leprosy among Egyptian cases from the Nile Delta. This may be helpful in planning an effective control and management programs. Our results reveal that IL-10-1082, TNF-308 and IL-1Ra polymorphisms may influence development of leprosy in the Egyptian locality where this study has been conducted that could be used as genetic markers for the disease. IL-10-1082 polymorphism is associated with both susceptibility to leprosy and its clinical outcome, while TNF- $\alpha$  could control only the severity of the disease.

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