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Mannose-Binding Lectin Gene and Promoter Polymorphism in Visceral Leishmaniasis Caused by *Leishmania infantum*

[1]Mohammad Asgharzadeh, [2]Abdolsamad Mazloumi,
[3]Hossein Samadi Kafil and [4]Ardavan Ghazanchaei
[1]Department of Biochemistry and Laboratory Sciences, Faculty of Medicine,
Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Infectious and Tropical Disease Research Center,
Tabriz University of Medical Sciences, Tabriz, Iran
[3]Department of Microbiology, Faculty of Medicine,
Tarbiat Modares University, Tehran, Iran
[4]Department of Immunoparasitology, Faculty of Medicine,
Tabriz University of Medical Sciences, Tabriz, Iran

Abstract: Visceral leishmaniasis is an infectious disease caused by various species of Leishmania and *Leishmania infantum* is known to be associated with VL in Iran. Different factors can consider risk factors for VL that some remain unknown. The aim of present study is to determine the distribution of the alleles of mannose-binding lectin gene codon 52, 54, 57 and promoter variants H/L, X/Y, P and Q in confirmed VL patients while compares then with normal controls and seek correlation between these variants and confirmed VL patients. Fifty eight confirmed VL patients blood samples were compared with one hundred and twenty normal controls from Azarbaijan population of Iran. MBL genotypes were investigated by polymerase chain reaction and restriction fragment length polymorphism. Allelic and genotypic frequency of the polymorphism at promoters and genes didn't show statistical differences in patients and normal controls, but frequency of alleles with high MBL concentration in VL patients was higher than controls ($p = 0.03$). We can conclude that normal alleles with high MBL serum level are risk factor for VL and defective alleles have protective role in VL.

Key words: *Leishmania infantum*, MBL, polymorphism, RFLP, SSP-PCR, visceral leishmaniasis

INTRODUCTION

Visceral leishmaniasis is an infectious disease transmitted by sand flies and caused by various species of leishmania parasites. It is known as kala-azar, which is a fatal infection if not treated successfully. Leishmania infantum is known to be associated with VL in Iran (Mohebali *et al*., 2005).

It is estimated that the annual occupance of human VL cases worldwide is 500,000 and is one of the world most serious parasitic disease (WHO, 1999), young age, malnutrition and human immunodeficiency virus infection are risk factors for VL (Alvar, 1994), but other host susceptibility factors remain unknown.

Mannose-binding lectin is a member of the collection family of proteins found in serum (Presanis *et al*., 2003), it binds to mannose and N-acetylglucosamine residues, while presented in the orientations and densities commonly found on microorganisms (Jack *et al*., 2001b; Mass *et al*., 1998). On binding, it activates the complement system independent to antibodies via two associated serin protease, MBL-associated serin protease 1 and 2 (Thiel *et al*., 1997). Several studies have shown direct interaction of MBL with phagocytic cells, resulting in enhanced phagocytosis and modification of cellular activation (Jack *et al*., 2001a; Jack and Turner, 2003) thus MBL plays an important role in the innate immunity of the immune system.

Human MBL is derived from a single gene on chromosome 10 (Sastry *et al*., 1989), the normal structural MBL allele is named A, while the common designation for the three variant structural allele B (mutation in

Corresponding Author: Mohammad Asgharzadeh, Department of Biochemistry and Laboratory Sciences, Faculty of Medicine, Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
Tel: +98-411-3357126 Fax: +98-411-3364666

codon 54, Gly to ASP), C (mutation in codon 57, Gly to Glu) and D (mutation in codon 52, Arg to Cys) are O (Hegele *et al*., 1999; Neth *et al*., 2001). In general, individuals with a normal genotype (A/A) have MBL concentration in serum that are 6-8 time higher than those in individuals heterozygous for one of the variant alleles (A/O: A/B, A/C or A/D), while individuals with a defective genotypes (2 alleles B/B, C/C, D/D, B/C, B/D or C/D) have almost undetectable MBL serum levels (Garred *et al*., 1999; Graudal *et al*., 2000). Moreover, MBL expression is influenced by polymorphic sites in the upstream part of the MBL gene (Crosdale *et al*., 2001; Ip *et al*., 1998). The promoter region of the MBL gene has nucleotides substitution at position-550, -221 and +4 give rise to H/L, X/Y and P/Q respectively, that makes different haplotypes, while LX haplotype is associated with low MBL serum level (Santos *et al*., 2001; Soborg *et al*., 2003).

The aim of present study is to determine the frequency of the MBL gene and promoter variants and seeks correlation between MBL alleles and developing of visceral lishmaniasis.

MATERIALS AND METHODS

Samples: Blood samples were obtained from 58 confirmed VL patients detected by clinical signs and direct smear and direct agglotination test and 120 normal control from Azarbaijan population of Iran. DNA was isolated from either granulocytes and mononuclear cells by the modified proteinase K, SDS and CTAB.

Genomic PCR: PCR was performed in 20 to 100 μL volumes that contained 50 to 500 ng of genomic DNA, 0.5 μM of specific primers (Table 1) in the presence of 1.5 mM $MgCl_2$, 100 μM of each dNTP, 50 mM KCl, 20 mM Tris-Cl, pH 8.4 and 1 to 2.5 unit recombinant Taq DNA polymerase (Cinnagen, Iran). DNA was amplified by general PCR and SSP-PCR. All PCRs were initiated by a 4 min denaturation step at 94°C and completed by a 7 min extension step at 72°C. The temperature cycles for different types of PCRs were as follows: 32 cycles of 40 sec at 94°C, annealing temperatures for 40 sec and 72°C for 55 sec. Annealing temperatures which were used as follow: 60, 63, 63, 62, 66, 63, 66, 67, 64, 67, 67, 65, 65 and 66°C for codon 57 (wild type), 57 (mutant), codon 54 (wild type), 54 (mutant), codon 52 (wild type), 52 (mutant), allele H, L, P, Q, haplotype Hy, Ly, Lx and Hx amplification, respectively (Crosdale *et al*., 2000; Madsen *et al*., 1995; Sullivan *et al*., 1996).

PCR-RFLP: In addition to SSP-PCR, B and C alleles were detected by BanI and MboII restriction enzyme digestion of the 320 bp product amplified by the alleles P and Q primers, respectively (Table 1), followed by a 2.5% agarose gel electrophoresis. BanI cleaves the

Table 1: Oligonucleotides used in human MBL genotyping

AG	GCT	GGG	TAT	ACC	TAG	GCT	GAG	Forward	Codon 57 (wild type)
	CTC	TTT	CCT	CCC	GTT	CTG	TAC	Reverse	
AG	GCT	GGG	TAT	ACC	TAG	GCT	GAG	Forward	Codon 57 (mutant)
	CTT	TTT	CCT	CCC	GTT	CTG	TAC	Reverse	
AG	GCT	GGG	TAT	ACC	TAG	GCT	GAG	Forward	Codon 54 (wild type)
	GC	GGT	CTT	TCC	TTC	CTT	CCC	Reverse	
AG	GCT	GGG	TAT	ACC	TAG	GCT	GAG	Forward	Codon 54 (mutant)
	GT	GGT	CTT	TCC	TTC	CTT	CCC	Reverse	
	GC	TGG	AGA	CAA	AGG	CCC	CTT	Forward	Codon 52 (wild type)
	AGG	GGA	TCT	TCC	GTT	GCA	CAG	Reverse	
	GT	TGG	AGA	CAA	AGG	CCC	CTT	Forward	Codon 52 (mutant)
	AGG	GGA	TCT	TCC	GTT	GCA	CAG	Reverse	
	GTG	CCT	AAG	GGC	CCA	TAC	GCT	Forward	MBL allele H
	AGG	GGA	TCT	TCC	GTT	GCA	CAG	Reverse	
	GTC	CCT	AAG	GGC	CCA	TAC	GCT	Forward	MBL allele L
	AGG	GGA	TCT	TCC	GTT	GCA	CAG	Reverse	
	TC	TGC	GCA	AGG	CAG	GGA	GTA	Forward	MBL allele P
	AGG	GGA	TCT	TCC	GTT	GCA	CAG	Reverse	
	TT	TGC	GCA	AGG	CAG	GGA	GTA	Forward	MBL allele Q
	AGG	GGA	TCT	TCC	GTT	GCA	CAG	ReveRse	
	GTG	CCT	AAG	GGC	CCA	TAC	GCT	Forward	Haplotypes Hy
CC	TTT	TGC	ACA	TAA	CTA	AGA	GGA	Reverse	
	GTC	CCT	AAG	GGC	CCA	TAC	GCT	Forward	Haplotypes Ly
CC	TTT	TGC	ACA	TAA	CTA	AGA	GGA	Reverse	
	GTC	CCT	AAG	GGC	CCA	TAC	GCT	Forward	Haplotypes Lx
CG	TTT	TGC	ACA	TAA	CTA	AGA	GGA	Reverse	
	GTG	CTT	AAG	GGC	CCA	TAC	GCT	forward	Haplotypes Hx
CG	TTT	TGC	ACA	TAA	CTA	AGA	GGA	Reverse	

A allele into two fragments (245 and 83 bp) and leaves the B allele undigested, while MboII specifically cleaves the C allele into two fragments (266 and 62 bp) (Madsen *et al*., 1995).

Statistical analysis: The distribution of alleles and genotypes between groups were compared using chi-square (χ^2 test) and $p<0.05$ was considered significant.

RESULTS

The genotype frequency of three MBL structural alleles between confirmed VL patients and normal controls were as follow: variant B (mutation in codon 54: 0.069 vs 0.142, p = 0.1584), variant C (mutation in codon 57: 0.017 vs 0.021, p = 0.7434), variant D (mutation in codon 52: 0.0345 vs 0.058, p = 0.496), variant A (wild type: 0.8793 vs 0.779, p = 0.097). Whereas, there was not statistical difference in the distribution of MBL between patients and controls. The frequency of gene variants of promoter in VL patients and Normal controls were: allele H (0.46 vs 0.383, p = 0.296), allele L (0.54 vs 0.617, p = 0.296), allele P (0.74 vs 0.725, p = 0.8174), allele Q (0.26 vs 0.275, p = 0.817) and haplotype variants included: Hy (0.448 vs 0.383, p = 0.408), Ly (0.336 vs 0.429 p = 0.212), Lx (0.207 vs 0.188 p = 0.8106) and Hx (only VL =0.009). There was not significant difference between patients and controls.

The distribution of haplotypes in confirmed VL patients were 42.28% HyPA, 24.13% LyQA, 18.32% LxPA, 6.9% LyPB, 3.45% HyPD, 1.72%. LyQC, 0.86% LyPA, 0.86% HxPA and in normal controls were, 31.66% HyPA, 25.4% LyQA, 19.15% LxPA, 13.75% LyPB, 5% HyPD, 2.5% LyQC and 2.5% LyPA and there were not any statistical difference. Table 2 shows the distribution of MBL structural and promoter alleles in VL patients and normal controls and as Table 2 showed frequency of alleles with high MBL serum level was higher in VL patients in comparing with controls (p = 0.03).

Table 2: Structural mannose-binding lectin gene and promoter alleles in VL patients and controls

Serum MBL levels	MBL structural alleles	Patients with VL	Control group
High			
	YA/YA	27 (46.55)	43 (35.81)
	YA/XA	12 (20.06)	19 (15.83)
	XA/XA	5 (8.62)	8 (6.66)
	YA/O	11 (18.96)	33 (27.47)
	Total	55 (94.82)	103 (85.77)
Low	XA/O	3 (5.14)	11 (9.25)
Deficient			
	O/O	0	6 (4.97)
	Total	58	120

Note: Data are No. (%) of patients or control group

DISCUSSION

We studied the association between exon1 and promoter haplotypes and outcome upon infection with *Leishmania infantum*, we have shown that genotypes with high MBL concentration were more frequency among patients infected with *Leishmania infantum* than healthy controls and on the contrary genotypes with low or deficient MBL serum levels were more frequent among healthy individuals in comparing with infected patients (p = 0.03), but in comparing promoter alleles between healthy individuals and VL patients we couldn't find any differences.

MBL have different roles in infections, in extracellular infections MBL deficiency may confer a life-long risk of infections (Summerfield *et al*., 1995). MBL acts as a complement activator to kill gram-negative organisms directly via the membrane-attack complex or to enhance complement-mediated phagocytosis through the increased deposition of opsonic C3 fragments (Jack *et al*., 2001a) as a extra cellular pathogen. In aspergillosis codon 52 mutation was particularly common that demonstrated MBL low serum level as a risk factor for chronic necrotizing pulmonary aspergillosis (Crosdale *et al*., 2001). In intracellular infections there are conversion and deficiency confers protection against these infections. In tuberculosis has been demonstrated that heterozygosity for MBL variant allele (XA/O), which encodes low serum MBL level is associated with protection against clinical turberculosis (Bellamy and Hill, 1998; Soborg *et al*., 2003) or a new study suggest a protective role for MBL deficiency against development of the most severe and multibacillary form of leprosy but not the tuberculoid form (Dornelles *et al*., 2006). It has proved lack of mannose-binding lectin enhances survival in a mouse model of acute septic peritonitis (Takahashi *et al*., 2002). Also in intracellular parasite like Leishmania increasing concentration of MBL cause increasing the release of TNF-α and interleukin 6 from monocytes contaminated with parasite (Jack *et al*., 2003). MBL binds to the parasite surface and most intense at base of the flagella in many parasites, on area of the plasma membrane that is the major site for exocytosis for these cells and contains a high concentration of surface antigen (Plmenta *et al*., 1991). MBL can promote the opsonization of microorganisms, thus intensify their attachment to phagocytic cells (Jack and Turner, 2003). *Leishmania infantum* is an intracellular pathogen. Phagocytosis is essential for the disease establishment. Thus, MBL binding to *Leishmania promastigotes* could provide an additional uptake mechanism of the parasites by

phagocytic cells. According to MBL role in the internalization of intracellular microorganism by phagocytic cells, MBL has been considered a candidate molecule for modifying the disease progression of intracellular pathogens (Ambrosio and De Messias-Reason, 2005; Dornelles *et al*., 2006). MBL modulate the function of Leishmania chagasi-infected cells and high levels of circulating MBL are directly correlated to the development of VL upon infection with *Leishmania chagasi* (Santos *et al*., 2001). Because MBL deficiency has observed frequently in the general population, it has been speculated how MBL deficiency could be advantageous to the host, it seems plausible that under certain circumstance, MBL binding to pathogens would lead to excessive activation of complement being harmful to the host.

In conclusion low-expression MBL genotypes can be associate with protection against VL caused by Leishmania infantum and wild type alleles with high MBL level can be consider as a risk factor for confirming visceral leishmaniasis.

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