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Mannose Binding Lectin Gene Haplotype in Iranian Patients with Hepatitis C Infection

Mohammad H. Somi [1*], Sara Farhang [2], Mohammad Asgharzadeh [3], Rasoul Estakhry [4], Ali A. Pouri [2]

[1] Associate Professor of Gastroenterology, Liver and Gastrointestinal Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

[2] General Practitioner, Liver and Gastrointestinal Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

[3] Assistant Professor of Molecular Biology, Liver and Gastrointestinal Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

[4] Assistant Professor of Anatomical and Clinical Pathology, Liver and Gastrointestinal Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

Background and Aims: **Persistent infection with hepatitis C virus (HCV) leads to liver cirrhosis (LC) and often to liver cancer. Mannose binding lectin (MBL) is a C-type serum lectin, which plays an important role in innate immunity by activating the classical complement pathway. Variants of the MBL have been shown to be associated with low serum concentrations of the protein and to predispose to bacterial, fungal and viral infections. This study was undertaken to investigate the association between polymorphisms of MBL gene and hepatitis C virus infection.**

Methods: **We determined genotypes of two promoters and three exon 1 SNPs in mbl2 by SSP-PSR and grouped these genotypes according to related amount of functional MBL production in 100 patients infected with hepatitis C virus and 100 healthy blood donors in Iranian population. MBL gene mutations were determined by means of polymerase chain reaction and restriction fragment length polymorphism analyses.**

Results: **genotypes XA/O or O/O were significantly more frequent among patients infected with hepatitis C virus, where YA/YA genotype was more common among donors. Frequency of alleles X, Y, H and L did not have a significant difference between the two groups as well as alleles HYA, LYA nor LXA.**

Conclusions: **MBL may be one of the factors that influence the course of HCV infection. Additional study on subjects at a high risk for infection with hepatitis C may clarify the role of carriage for the variant allele of mbl2 in a life-long risk of infection.**

Keywords: **Mannose Binding Lectin, Haplotype, Hepatitis C**

Introduction

Hepatitis C is one of the major infectious diseases and according to WHO report, an estimated 170 million persons are chronically infected with HCV and 3 to 4 million persons are newly infected each year. Hepatitis C is the most common cause of chronic hepatitis. About 80% of newly infected patients progress to develop chronic infection which may step forward to cirrhosis and hepatocellular carcinoma with a high morbidity and mortality. The complications of cirrhosis due to chronic hepatitis C are the leading indications for liver transplantation [1, 2] while viremia persists after transplantation in nearly all patients due to their special conditions and commonly results in

**** Correspondence:***
Mohammad Hossein Somi MD, Associate Professor of Gastroenterology, Liver and Gastrointestinal Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Tel/Fax: *+98 411 3367499*

E-mail: *mhosseinsina@yahoo.com*

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recurrent liver injury [3]. It is likely that a combination of viral-related factors and host-related factors play a role in the natural history of HCV.

Mannose binding lectin is a calcium-dependent C-type lectin which is secreted by the liver as a part of acute phase response [4] and plays an important role in first line host defense [5].

MBL binds to an array of carbohydrate structures on microbial surfaces. MBL functions as an opsonin [6], and the biological effect is mediated by direct killing via complement [7] through the lytic membrane attack complex or by promoting phagocytosis either by the MBL lectin pathway of complement or by direct binding to one or more cell surface receptors [8].

In human, one of the three structural mutations found within exon 1 of the mbl2 gene on chromosome 10, which encodes MBL, results in low functional serum levels [9, 10].

These single nucleotide polymorphisms (SNPs) at codons 52, 54, and 57 resulting in aminoacid replacement, are believed to interfere with the stability of the protein [11, 12].

Deficiency of human mannose-binding lectin (MBL) caused by mutations in the coding part of the mbl2 gene is associated with increased risk and severity of infections and autoimmunity in children and adults [13, 14].

MBL concentrations are also dependent on SNPs and the promoter region of the mbl2. Several studies have demonstrated a relation between the promoter region haplotypes caused by a SNP at position -221 with serum MBL levels [15, 16].

There are several studies about MBL mutations, risk of infection and course of the viral hepatitis in different ethnic groups, specially hepatitis B. We studied MBL gene mutations in Iranian patients with chronic hepatitis C against healthy controls. Our aim was to investigate the possible relation between MBL mutations and susceptibility to be infected by hepatitis C virus.

Materials and Methods

HCV infected group and control population

One hundred continuous patients with chronic hepatitis C were recruited from hepatitis clinic, Tabriz University of Medical Sciences, which is the referral clinic of East Azarbaijan province, northwestern Iran. The mean age (±SD) of the patients was 42.5±11.1 years, all had Azeri ethnic background, and 57 % were male. The patients were informed about the purpose of the study and were enrolled with their consent.

A group of 100 healthy individuals were randomly selected from Red Crescent blood donor center of this province (mean age = 39.5±10.5 years, 80 % male, all Azeri) to demonstrate the general population. Age and sex of the control group and the HCV infected patients were not significantly different.

DNA extraction, Genotyping and Haplotyping of mbl2:

DNA was extracted from peripheral blood mononuclear cells by the modified proteinase K, SDS (sodium dodecyl sylfate) and CTAB (N- cetyl - N, N, N- trimethylammonium bromide).

We analyzed SNPs in the promoter (-221 X/Y and 550 H/L) and codons 52, 54 and 57 in the exon one of the MBL gene using previously described PCR- based methods. DNA was amplified by Polymerase Chain Reaction (PCR) and sequence-specific primes (SSP- PCR) which is able to determine whether the promoter and structural alleles are in *cis* or *trans* [17, 18, 19].

PCR reactions were performed in a volume of 20 µl that contained 500 ng of the genomic DNA , 0.5 µM of each specific primer (described in Table 1) in the presence of 1.5 mM $MgCl_2$, 100 mM each deoxynocleotide, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, and 2.5 U recombinant DNA polymerase (Fermentas).

All PCR procedures were initiated by a 4-min denaturizing 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 for 40 sec at 94°C, annealing temperature for 40 sec and 72°C for 55 sec. Annealing temperatures are described in table 1.

Statistical analysis

Statistical analysis was performed using SPSS software, version 13.5. The difference of age and gender of the two groups was examined with student's t and chi-square test, respectively. Frequencies for each SNP were observed by simple gene counting and goodness of fit between the observed and expected genotypes according to Hardy-Weinberg equilibrium was determined by the means of chi-square test (fisher's exact where necessary).

MBL genotypes frequencies were compared by contingency table analysis using the 2 test. When a significant difference was obtained, logistic regression was used to calculate odds ratio (OR) with 95% confidence intervals (95% CI). A P value of 0.05 was considered to be significant.

Table 1. Oligonucleotides used in human MBL genotyping

Primer name		Sequences (5'→3')	Annealing temperature °C
Codon 57 (wild type)	Forward	GAG GCT TAG ACC TAT GGG GCT AG	60
	Reverse	TAC CTG GTT CCC CCT TTT CTC	
Codon 57 (mutant)	Forward	GAG GCT TAG ACC TAT GGG GCT AG	63
	Reverse	TAC CTG GTT CCC CCT TTT CTT	
Codon 54 (wild type)	Forward	GAG GCT TAG ACC TAT GGG GCT AG	63
	Reverse	CCC CTT TTC TCC CTT GGT GC	
Codon 54 (mutant)	Forward	GAG GCT TAG ACC TAT GGG GCT AG	62
	Reverse	CCC CTT TTC TCC CTT GGT GT	
Codon 52 (wild type)	Forward	CTT CCC AGG CAA AGA TGG GC	66
	Reverse	CAG GCA GTT TCC TCT GGA AGG	
Codon 52 (mutant)	Forward	CTT CCC AGG CAA AGA TGG GT	63
	Reverse	CAG GCA GTT TCC TCT GGA AGG	
Haplotype HY	Forward	GCT TAC CCA GGC AAG CCT GTG	67
	Reverse	GGA AGA CTA TAA ACA TGC TTT CC	
Haplotype LY	Forward	GCT TAC CCA GGC AAG CCT GTC	67
	Reverse	GGA AGA CTA TAA ACA TGC TTT CC	
Haplotype LX	Forward	GCT TAC CCA GGC AAG CCT GTC	65
	Reverse	GGA AGA CTA TAA ACA TGC TTT CG	
Haplotype HX	Forward	GCT TAC CCA GGC AAG CCT GTG	65
	Reverse	GGA AGA CTA TAA ACA TGC TTT CG	

Results

Individual SNPs and haplotypes

The genotype distribution of mbl2 polymorphisms at codons 54 (variant B), 57 (variant C) and 52 (variant D) and promoter haplotypes are presented in Table 2.

No significant deviation from Hardy-Weinberg equilibrium was seen in the observed genotypes in both groups.

Genotype A/A was more frequent among healthy donors (P<0.0001, OR: 0.272) while genotype A/O was significantly more frequent among patients with hepatitis C (P =0.0004, OR: 2.90). Genotype O/O was also more frequent among patients but did not reach the significance. Frequency of alleles X, Y, H and L did not have significant different between the two groups.

Relationship between the haplotypes associated with MBL serum levels

Previous studies have shown that when in a *cis* with a normal coding region (A) the promoter haplotype HY (HYA), LY (LYA) and LX (LXA) are associated with high, intermediate and low levels of MBL respectively [16, 17].

Using these data, the study population was divided to three groups and also according to the presence of a normal coding (A) or coding mutation (O) on the other chromosome. Among the genotypes HYA, LYA and LXA presenting with a normal coding on the other chromosome genotypes HYA/A and LYA/A were significantly more frequent among healthy donors (p= 0.0039, OR: 0.364 and p= 0.0003, OR: 0.250, respectively).

Only individuals homozygote for HYA (HYA/HYA) were significantly less frequent among

Table 2. *mbl2* Genotype in Iranian HCV infected patients and controls

Coding genotype†	%Patients (n=100)	%Donors (n=100)
A/A	29	60
A/B	45	23
A/C	5	3
A/D	11	10
Total A/O	61	36
B/B	6	2
B/C	1	1
B/D	2	1
C/C	1	0
Total O/O	10	4
Total with coding mutations(A/O or O/O)	71	40
Promoter genotypes‡		
X/X	5	5
X/Y	24	24
Y/Y	71	71
H/H	15	18
H/L	28	38
L/L	57	44
Promoter haplotypes		
HYA/A	17	36
HYA/O	19	15
Total HYA	36	51
LYA/A	11	33
LYA/O	24	12
Total LYA	35	45
LXA/A	12	21
LXA/O	14	6
Total LXA	26	27
HXA/A	1	0

†A: wild type coding region, B: mutation at codon 54, C: mutation at codon 57, D: mutation at codon 52, O: any mutation on structural gene
‡ X/Y: promoter alleles at -221 H/L: promoter alleles at -550

hepatitis C patients (P=0.023, OR: 0.256) and difference in other stratifications (HYA/LYA, HYA/LXA, LXA/LYA, LYA/LYA, LXA/LXA, LXA/HXA) did not reach the significance.

When presenting with a structural mutation on the other chromosome, genotype LYA/O was observed significantly more frequent among patients (p= 0.042, OR= 2.315) while the difference in genotypes LXA/O and HYA/O did not reach the significance.

The frequencies of described haplotypes are compared between patients with chronic hepatitis C and donors in table 3. The haplotype HYA was significantly more frequent among healthy blood donors. The haplotype LXA was seen more frequently among patients but did not reach the significance.

Table 3. Frequency of haplotypes corresondidng to functional MBL concentrations in Iranian patients with hepatitis C and donors

MBL levels (haplotypes) †	Frequency in		OR	P
	Patients	Donors		
High (HYA)	22.5	32.5	0.603	0.154
Intermediate (LYA)	20	28.5	0.627	0.216
Low (LXA)	16.5	17	0.964	0.920

† A: wild type coding region, X/Y: promoter alleles at -221

Several studies have verified a relationship between MBL levels and mbl2 genotype regardless of the mutations of allele H\L [20, 21]. Individuals homozygous for YA have the highest levels, whereas allele X on one chromosome and a structural mutation on the other, or homozygosis for O have the lowest level. Study population divided by these previously established data is shown in table 4. Those subjects with the XA/O or O/O genotype were significantly more frequent among patients infected with hepatitis C virus, where YA/YA genotype was more frequent among controls.

Table 4. Iranian patients with hepatitis C and control divied by genotypes corresondidng to functional MBL concentrations

MBL levels (haplotypes) †	% of subjects		OR	P
	Patients	Donors		
High (YA/YA)	18	41	0.315	0.0006
Intermediate (YA/XA,YA/O, XA/XA)	54	49	1.221	0.571
Low or none (XA/O, O/O)	28	10	3.5	0.002

† A: wild type coding region, O: any mutation on structural gene, X/Y: promoter alleles at -221

Discussion

In this study we found a significantly low frequency of that genotype YA/YA in patients infected with hepatitis C virus compared to Iranian healthy blood donors while genotype XA/O and O/O were significantly more frequent in this group. In addition, the effect of variation on position -550 of promoter seems to be less dominant. The effect of the promoter variants seems to be influenced by the presence of mutant structural codons, a status which was more observed among HCV infected patients.

Because MBL is a well-characterized part of the immune defense system, identifying the mutations and its effect on the serum MBL levels can help to define susceptible individuals to infection. The major role of MBL is activation of complement; however, its role in the natural course of the viral hepatitis is not clear.

MBL variant alleles resulting in decreased serum MBL levels have been shown to be associated with increased risk and severity of viral hepatitis. *mbl2* genotypes correlating with low MBL levels have been shown to be associated with viral persistence in HBV infection [21]. In another study, the frequency of mbl2 polymorphism and serum MBL levels did not differ significantly in spontaneously recovered individuals from hepatitis B, non-progressed carriers and controls whereas low MBL level was associated with occurrence of cirrhosis and hepatocellular carcinoma in progressed carriers [22].

A few other published studies, all from Asia, have examined *mbl2* with respect to the course of HCV infection. Since the -221 promoter SNP was not observed, the YA/YA or XA/O genotypes were not analyzed, except in one which shows a higher frequency of haplotype LXPA and LYPB in interferon resistant patients and controls compared to interferon responsive HCV infected patients [23]. In the other study on fifty-two patients, no significant relationship was observed between MBL polymorphisms and levels of HCV RNA [24]. In another study, difference in the mutations rate and MBL levels between chronic hepatitis C patients and controls did not reach the significance level, but MBL levels in asymptomatic HCV carriers were significantly lower than those of the control population without codon 54 mutation [25]. In the present study, we found that homozygous variant alleles of carriers of *mbl2* may be more prone to HCV infection.

Both coding and promoter *mbl2* variants were shown to be significantly associated with MBL levels, but we observed only a significantly increased frequency of coding mutations in patients against donors. These results may need to be confirmed by studies larger in size of study population.

mbl2 polymorphism is frequent among general population but differences have been observed in different ethnic backgrounds [26, 15, 18]. To the best of our knowledge this is the first study from Middle East evaluating the mutations on *mbl2*, so we used a randomly selected group of blood donors from this region to perform a control group. The most frequent structural mutation was seen in Codon 54, followed by Codon 52 and 57 which is near to results from UK Caucasoids and Australian population [16, 18].

Characteristics of the control group are the major limitation of this study. Further studies comparing the mbl2 polymorphism between HCV infected patients and healthy subjects at a known risk for infection (e.g. undergoing hemodialysis or an immunosuppressive therapy) can be valuable specially in concordance of measuring functional MBL serum levels. Such studies can be improved by quantifying the viral load as well.

mbl2 polymorphisms and serum levels may not only be associated with susceptibility to the infection with HCV, but may play an important role in the further behavior of the disease. Determining the mbl2 genotype may have the advantage of predicting the response of MBL levels to therapy with triggers such as interferon.

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