# Genotypes Coding for Mannose-Binding Lectin Deficiency Correlated With Cryptococcal Meningitis in HIV-Uninfected Chinese Patients

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***Background.*** There is increasing evidence that mannose-binding lectin (MBL) has a complex role in many diseases, particularly in infectious diseases. However, the relationship between MBL deficiency and cryptococcal meningitis has not been clarified. The purpose of this study was to investigate the correlation between MBL polymorphism and non-HIV cryptococcal meningitis.

***Methods.*** A case-controlled genetic association study was conducted. Patients with cryptococcal meningitis and control subjects were genotyped for 6 alleles of MBL2 gene (H/L, Y/X, P/Q, A/D, A/B, and A/C). The distributions in allele frequency, genotypes, haplotypes, and genotype groups were compared between patients and control subjects.

***Results.*** Study participants included 103 HIV-uninfected patients with cryptococcal meningitis and 208 healthy control subjects, all of Chinese Han ethnicity. The homozygous mutative genotypes (O/O) of the coding region were associated with cryptococcal meningitis ($P = .023$; odds ratio [OR], 4.29; 95% confidence interval [CI], 1.11–19.88), the correlation more overt in immunocompetent patients ($P = .005$; OR, 6.65; 95% CI, 1.49–33.05). MBL-deficient participant group was associated with cryptococcal meningitis ($P = .039$; OR, 2.09; 95% CI, .96–4.51), particularly in immunocompetent patients ($P = .028$; OR, 2.51; 95% CI, .96–6.22).

***Conclusions.*** This is the first to show genotypes coding for MBL deficiency are associated with cryptococcal meningitis in nonimmunocompromised hosts.

Cryptococcal meningitis is usually caused by *Cryptococcus neoformans*. It most commonly occurs in immunocompromised patients. The incidence of HIV-associated cryptococcal meningitis has been decreasing in recent years owing to the advent of highly active antiretroviral therapy (ART), whereas cryptococcal meningitis in previously healthy patients has emerged as an issue of increasing interest. Thus, it raises the question of whether the so-called healthy hosts are, in fact, accompanied with other immunocompromising conditions that have not been identified.

Mannose-binding lectin (MBL), an important member of pathogen recognition receptors (PRRs), binds with a broad range of microorganisms and activates the lectin-complement pathway of innate immunity. MBL genetic mutations may result in malformation or reducing of the encoded protein and increase the susceptibility to a number of infections. The expression of functional MBL protein is largely genetically determined, with 3 common functional single-nucleotide polymorphisms (SNPs) and 3 other mutations. The 3 common nonsynonymous SNPs are found in the codons 52, 54, and 57, which cause amino acid replacements, arginine-cysteine, glycine-aspartic acid, and glycine-glutamic acid, respectively, in the collagen domain altering its ability to oligomerize (the mutant forms). The 3 mutations are called *D*, *B*, and *C* alleles, respectively. The presence of any of these coding mutations is represented by *O*. Two of the other 3 mutations are located in the MBL promoter region, including SNPs at positions −550 (*H/L* variant) and −221 (*Y/X* variant), both G to C nucleotide substitutions. The

third SNP is located at position +4 of the 5′-untranslated region (5′-UTR) of the MBL gene (P/Q variant, C→T).

Over the past 2 decades, both human epidemiological data and mouse models of MBL deficiency have been widely investigated. In many human populations, MBL deficiency is relatively common and there have been a large number of studies attempting to link the deficiency state with certain clinical presentations. There is increasing evidence that MBL has a complex role in many diseases, particularly in infectious diseases. Deficiency of the protein has been shown to be associated with increased susceptibility to many infectious diseases [1–5]. MBL recognizes mannose, a major component of fungal cell wall, and binds a variety of fungi, including *Candida albicans*, *Aspergillus fumigatus,* and unencapsulated mutants of *C. neoformans*. Reduced vaginal MBL levels and an increased occurrence of the polymorphism in codon 54 of *MBL* gene were present in women with recurrent vulvovaginal candidiasis [6]. The susceptibility of *MBL-A* and *MBL-C* knockout mice to systemic infection with *C. albicans* was increased [7]. MBL-deficient genotypes were associated with chronic necrotizing pulmonary aspergillosis [8] and acute invasive aspergillosis in immunocompromised patients [9]. Eisen et al [10] compared the proportion of *MBL* genotypes *LAX/O* and *O/O* (associated with deficient plasma MBL levels) between 25 central nervous system cryptococcosis and 11 patients without central nervous system cryptococcosis, showing a difference with marginal significance (7/25 vs 0/11; *P* = .06). To date, the relationship between MBL deficiency and cryptococcal meningitis has not yet been clarified. Here, we performed this study to investigate the correlation between *MBL* gene and cryptococcal meningitis in HIV-uninfected patients.

## METHODS

### Study Population
The study population included 103 Chinese patients who were referred to Huashan Hospital, Fudan University, China, from 2003 through 2010, for diagnosis and treatment of cryptococcal meningitis, and 218 volunteers. This study was approved by the Local Medical Ethics Committee, and informed consent was obtained from each participant. A definite diagnosis of cryptococcal meningitis was made if the patient met at least 1 of the following criteria: (1) culture positive for *C. neoformans* from cerebrospinal fluid (CSF), (2) positive result of CSF India ink smear of centrifuged sediment for *Cryptococcus*, and (3) compatible histopathological findings (5–10-µm encapsulated yeasts observed in brain tissue). Probable cryptococcal meningitis was diagnosed in patients who presented with the clinical syndrome of meningitis and positive cryptococcal antigen titer in CSF, albeit without microbiological or pathological documentation. A positive result of testing of undiluted CSF with use of

the latex-cryptococcus antigen detection system (Immuno-Mycologics) was considered to be diagnostic. The API 20C AUX system (bioMérieux Shanghai) was used for identification of isolates. $CD4^+$ T lymphocyte count <300 cells/mm$^3$ with no immunocompromising conditions was considered to be idiopathic $CD4^+$ T lymphocytopenia. Predisposed hosts were defined as patients with immunocompromising diseases (cirrhosis, chronic kidney diseases, autoimmune diseases, diabetes mellitus, solid malignancies, hematologic malignancies, splenectomy, and solid organ transplantation), corticosteroid, other immunosuppressive medications, or idiopathic $CD4^+$ T lymphocytopenia. Patients without identifiable risk factors were considered to be immunocompetent.

### DNA Extraction
From each patient, 3 mL of venous blood was obtained by venopuncture. Genomic DNA was extracted using the QIAamp DNA kit (Qiagen) according to the manufacturer's instructions.

### Analysis of SNPs in the *MBL2* Gene
Six SNPs in the *MBL2* gene, including -550 G/C (rs11003125), -221 C/G (rs7096206), 4 C/T (rs7095891), codon 52 CGT/TGT (rs5030737), codon 54 GGC/GAC (rs1800450), and codon 57 GGA/GAA (rs1800451) were analyzed using a sequencing-based typing method. In brief, a 926-bp fragment encompassing from the promoter to the end of exon 1 of *MBL2* was obtained by polymerase chain reaction (PCR) amplification using the sense 5′-GGGGAATTCCTGCCAGAAAGT-3′ and antisense 5′-CATA-TCCCCAGGCAGTTTCCTC-3′ primers [11] and the GeneAmp PCR System 9700 (GeneAmp ABI9700; Applied Biosystems). The reactions were performed with 2 µL of DNA in a total volume of 25 µL containing 2 × Master (containing 3 mmol/L MgCl$_2$, .2 mmol/L dNTPs, .1 U/µL proof-reading DNA polymerase, and 2 × buffer; SK2072; Sangon Biotech) and 10 µmol/L of each primer. The cycling conditions were 95°C for 5 min, 35 cycles of 94°C for 30 s, 61°C for 30 s, 72°C for 30 s, and 72°C for 5 min. Five microliters of the resulting PCR were treated with Fluorescence Chemiluminescence and Visible Imaging System (Tanon-2500) and then subjected to direct sequencing.

### MBL Genotype Groups
According to previous studies that have documented that the ability of MBL production is determined by MBL genotypes, participants were classified into the following 3 groups based on their genotypes: high producing, low producing, and deficient [12, 13]. Mutation homozygosity and compound heterozygosity (*O/O*) are associated with MBL deficiency [12].

### Determination of MBL Concentration in Plasma
MBL levels were measured in 58 patients and 58 control subjects with use of a mannan-coated commercial enzyme-linked immunosorbent assay kit with an anti-MBL antibody recognizing only functional oligomers (HK232; Hycult

Biotechnology). The purpose was to validate the correlation between genotyping groups and serum MBL levels. Insufficient samples were available to test correlation of MBL levels with cryptococcal meningitis. The assay was performed according to instructions provided by the manufacturer. Eight standards with MBL concentrations of 0, .41, 1.02, 2.56, 6.4, 16, 40, and 100 ng/mL were prepared by dilutions of a human MBL standard (474 ng/mL) provided with the kit and assayed simultaneously.

## Statistical Analysis

$\chi^2$ Analyses were used for each individual SNP to test for deviation from the Hardy-Weinberg equilibrium. Differences in gene polymorphism distributions between patients and control subjects in background characteristics were analyzed with $\chi^2$ or Fisher's 2-tailed exact tests. Odds ratios (ORs) and 95% confidence intervals (CIs) were also determined. Differences in MBL plasma concentration among the high, low, and deficient-producing MBL groups were analyzed using 1-way analysis of variance (ANOVA). All reported $P$ values are 2-sided, and a significance level of $\alpha = .05$ was used. Stata software, version 7.0 (Stata) was used for statistical analysis.

## RESULTS

### Demographic and Clinical Information

A total of 103 HIV-uninfected patients with cryptococcal meningitis were included in the study—87 with proven diagnosis and 16 with probable diagnosis. Sixty-two patients were male. The age range of patients was 14–78 years (median, 44 years). Ten of 218 volunteers were excluded from the analysis because of their predisposing diseases (diabetes mellitus in 6 and solid malignancies in 4). One hundred nineteen of 208 control subjects were male, and the age range was 12–79 years (median, 43 years). The 208 healthy control subjects did not have predisposing conditions, such as diabetes mellitus, solid malignancies, or corticosteroid usag, but CD4$^+$ T lymphocyte counts were unchecked. All patients and control subjects were of Chinese Han ethnicity. Demographic characteristics, predisposing factors, and clinical information of the 103 patients are summarized in Table 1.

### Allele and Haplotype Frequencies in MBL2 Gene

The distribution of genotypes, carriage rate of allele, and haplotype frequencies for the 6 polymorphisms were tested. The frequencies of all alleles in the control group were verified to be in accordance with those expected by the Hardy-Weinberg equilibrium. In 103 patients with cryptococcal meningitis, gene frequencies of exon 1 variants were 78.6% (162 of 206 alleles) for $A$, 19.9% (41 of 206) for $B$, and 1.5% (3 of 206) for $D$, with no $C$ allele identified. Frequencies of promoter −550 site were 49.5% (102 of 206) for $H$ and 50.5%

**Table 1. Demographic Characteristics, Predisposing Factors, and Clinical Features of 103 HIV-Uninfected Chinese Patients With Cryptococcal Meningitis**

| Characteristic | Value |
|---|---|
| Male sex | 62 (60.2) |
| Age, years | 44 (14–78) |
| Confirmed cases | 87 (84.5) |
| Predisposing factors | 51 (49.5) |
| Immunocompromising conditions[a] | 42 (40.8) |
| Idiopathic CD4+ T lymphocytopenia[b] | 9 (8.7) |
| Extraneural complications | 38 (36.9) |
| Pulmonary cryptococcosis | 29 (28.2) |
| Cryptococcemia | 5 (4.9) |
| Pulmonary cryptococcosis and Cryptococcemia | 4 (3.9) |
| Severe crytococcal meningitis[c] | 32 (31.1) |
| Coma | 28 (27.2) |
| Cerebral herniation | 5 (4.9) |
| Ventricular drainage | 7 (6.8) |

**NOTE.** Data are no. (%) of participants or median (range).

[a] Immunocompromising conditions including autoimmune diseases in 18, corticosteroid in 18, cirrhosis in 11, diabetes mellitus in 10, immunosuppression in 7, chronic kidney diseases in 4, solid malignancies in one, hematologic malignancies in one, splenectomy in one, and kidney transplantation in one patient.

[b] Idiopathic CD4$^+$ T lymphocytopenia was defined as less than 300 CD4$^+$CD3$^+$ cells/μL.

[c] Patients with one or more manifestation such as coma, cerebral herniation, and ventricular drainage were classified as severe cases.

(104 of 206) for $L$, of promoter −221 site were 84.5% (174 of 206) for $Y$ and 15.5% (32 of 206) for $X$, and of 5′-UTR were 90.8% (187 of 206) for $P$ and 9.2% (19 of 206) for $Q$. The frequencies of $HYPA$, $LYQA$, $LYPA$, $LXPA$, $LYPB$, and $HYPD$ haplotypes were 47.6% (98 of 206), 9.2% (19 of 206), 6.3% (13 of 206), 15.5% (32 of 206), 19.9% (41 of 206), and 1.5% (3 of 206), respectively, with no $LYQC$ haplotype identified. On the other hand, among 208 control subjects, gene frequencies of exon 1 variants were 84.4% and 15.6% for the $A$ and $B$ alleles, respectively, with no $C$ and $D$ allele identified; gene frequencies of upstream variants were 50.2%, 49.8%, 84.9%, 15.1%, 87.7%, and 12.3% for the $H$, $L$, $Y$, $X$, $P$, and $Q$ alleles, respectively; and the frequencies of $HYPA$, $LYQA$, $LYPA$, $LXPA$, and $LYPB$ haplotypes were 50.5%, 12.3%, 6.5%, 15.1%, and 15.6%, respectively.

### MBL Genotypes Groups and Plasma Concentrations

MBL plasma concentrations of 116 participants were measured, and the mean level was 1890.22 ng/mL (range, 468.47–4293.87 ng/mL) in the high-producing $MBL$ genotypes group, 366.22 ng/mL (range, 32.96–1776.08 ng/mL) in the low-producing group, and 2.71 ng/mL (range, .00–36.04 ng/mL) in the MBL-deficient genotype group. There were statistically significant differences among the 3 groups (1-way ANOVA, $P < .001$), which verified that $MBL2$ genotypes were consistent with the MBL plasma concentration.

**MBL Genotypes and Cryptococcal Meningitis**

Homozygous mutative genotypes (O/O) of the coding region were detected in 8 (7.8%) of 103 patients, 6 (11.5%) of 52 immunocompetent patients, and 4 (1.9%) of 208 control subjects (Table 2). The O/O genotype was associated with cryptococcal meningitis (7.8% in patients vs 1.9% in control subjects; $P = .023$; OR, 4.29; 95% CI, 1.11–19.88). The correlation was more overt in immunocompetent patients (11.5% in patients vs 1.9% in control subjects; $P = .005$; OR, 6.65; 95% CI, 1.49–33.05).

In the control group, the proportions of *MBL* genotypes correlated with high, low, and deficient MBL levels were 69.2%, 22.1%, and 8.7%, respectively. The proportions of *MBL* genotypes associated with high, low, and deficient plasma MBL levels were 63.1% (65 of 103), 20.4% (21 of 103), and 16.5% (17 of 103), respectively, in patients with cryptococcal meningitis, and 65.4% (34 of 52), 15.4% (8 of 52), and 19.2% (10 of 52), respectively, in immunocompetent patients. Although we found no differences in the distribution of high and low MBL-producing groups between patients and control subjects, the deficient MBL-producing genotypes were associated with cryptococcal meningitis ($P = .039$; OR, 2.09; 95% CI, .96–4.51), particularly in patients without immunocompromising underlying diseases ($P = .028$; OR, 2.51; 95% CI, .96–6.22) (Table 2).

## DISCUSSION

*MBL* polymorphism distribution has been investigated in Chinese populations. Our study showed that the distribution of *MBL* genotypes was consistent with that in other studies about the Chinese Han population [14, 15]. We found that there was no C allele and only 3 persons with a D allele identified in our study of 321 individuals; this finding was comparable with reports of the C allele in 1.0%–1.2% and the D allele in 4.7%–6.0%

of white populations. It was indicated that the proportion of *MBL* genotypes that were associated with deficient plasma MBL levels in white populations (7.0%–16.1%) might be higher than that in the Chinese Han population (8.8%) [16–21].

The proportion of previously healthy patients with cryptococcal meningitis has varied greatly from region to region among HIV-uninfected patient populations. Data from America, France, and Thailand series all showed a relatively low proportion of previously healthy hosts (range, 17%–32%),with the majority of patients having some underlying conditions [1,22–28]. However, a high percentage of previously healthy hosts among HIV-uninfected patients with cryptococcal meningitis have been reported from other regions [29–32]. Our recent epidemiological study revealed that up to 54.4% of patients without AIDS with cryptococcal meningitis were without predisposing factors [33]. Although the role of ethnic factors in the susceptibility of this disease is still unclear; the previously healthy patients with cryptococcosis in our country renders an opportunity for us to study the genetic susceptible factors in this disease. Our results showed that the homozygous variant genotype O/O was associated with the risk of cryptococcal meningitis, and the proportion of deficient MBL-producing genotypes in patients with cryptococcal meningitis was significantly higher than that in the control group, indicating that MBL deficiency may be one of the predisposing factors of cryptococcal meningitis that has not been reported. Admittedly, this predisposition was present in only 7.8% of HIV-uninfected patient with cryptococcosis. The difference was also present in the subgroup of patients (11.5%) without immunocompromising factors. Therefore, the genetic defect in MBL may provide one of the answers to the mystery why apparently immunocompetent patients develop cryptococcal meningitis. Eisen et al [10] found no statistically significant difference between the proportion of *MBL* genotypes *LAX/O* and *O/O* (associated with

**Table 2.** MBL-2 Genotypes in Cases of Cryptococcal Meningitis, Immunocompetent Patients, and Healthy Control

| Genotype | Cryptococcal meningitis | | | | | | Control subjects (n = 208) (%) |
|---|---|---|---|---|---|---|---|
| | Overall (n = 103) (%) | OR (95% CI) [a] | P[a] | Immunocompetent patients (n = 52) (%) | OR (95% CI) [b] | P[b] | |
| Coding genotype[c] | | | | | | | |
| A/A | 67 (65.1) | 0.75 (.44–1.29) | .273 | 34 (65.4) | 0.77 (.39–1.56) | .417 | 148 (71.2) |
| A/O[c] | 28 (27.2) | 1.01 (.51–1.77) | .961 | 12 (23.1) | 0.81 (.36–1.73) | .572 | 56 (26.9) |
| O/O | 8 (7.8) | 4.29 (1.11–19.88) | .023 | 6 (11.5) | 6.65 (1.49–33.05) | .005 | 4 (1.9) |
| Genotype group[d] | | | | | | | |
| high | 65 (63.1) | 0.76 (.45–1.29) | .279 | 34 (65.4) | 0.82 (.42–1.66) | .544 | 144 (69.2) |
| low | 21 (20.4) | 0.90 (.48–1.66) | .727 | 8 (15.4) | 0.64 (.24–1.51) | .285 | 46 (22.1) |
| deficient | 17 (16.5) | 2.09 (.96–4.51) | .039 | 10 (19.2) | 2.51 (.96–6.22) | .028 | 18 (8.7) |

**NOTE.** [a]Overall cases of cryptococcal meningitis vs healthy control subjects.

[b] Immunocompetent patients vs healthy control subjects.

[c] A/O including A/B, A/D; O/O including B/B, B/D.

[d] High including HYPA/HYPA, HYPA/LYQA, HYPA/LYPA, HYPA/LXPA, LYQA/LYQA, LYQA/LYPA, LYQA/LXPA, LYPA/LYPA, LYPA/LXPA; low including LXPA/LXPA, HYPA/LYPB, LYQA/LYPB, LYPA/LYPB, HYPA/HYPD, *LYQA/HYPD*; deficient including *LXPA/LYPB, LYPB/LYPB, LYPB/HYPD*.

deficient plasma MBL levels) and other MBL2 genotypes in non-compromised patients with cryptococcosis and normal control subjects (7 of 35 vs 38 of 236). In addition, the authors did not find an increased proportion of patient with cryptococcosis with plasma MBL levels <500 ng/mL (11 of 36 vs 69 of 236). Apart from the relatively small sample size, the difference in ethnic population (Australian versus Chinese) and *Cryptococcus* strains may explain the inconsistent results of the 2 studies, because patients with cryptococcal meningitis were infected with both *Cryptococcus gattii* and *C. neoformans* in Australia and predominantly *C. neoformans* in China [34, 35]. A validation study is needed to further examine the results of the current study.

MBL as one of the proteins of the complement system is an important constituent of the innate immune system. MBL can modify the efficiency of uptake and the expression of other phagocytic receptors. Researches over the past decade have indicated that MBL provides a distinct third pathway of complement activation by which MBL plays a role of host defense [36]. Pathogenic strains of the fungus *C. neoformans* are known to be resistant to MBL binding because of the protection by polysaccharide capsule, and soluble MBL binds acapsular *C. neoformans* only [37–39]. Panepinto et al [40] also demonstrated that MBL can bind only to *C. neoformans* mutant defective in cell integrity, but not to the encapsulated wild-type strain in vitro observations. Acapsular mutants of *C. neoformans* appear to have a surface MBL-binding mannoprotein that is not present on the encapsulared cell surface. However, it was suggested that solid phase MBL increased binding of encapsulated *C. neoformans* to monocytes, neutrophils, and monocyte-derived macrophages, although the biological significance of solid-phase MBL and its mechanism of improving *C. neoformans* binding to phagocytes remained unclear [38].

This study is the first, to our knowledge, to show that MBL deficiency is associated with cryptococcal meningitis, particularly in nonimmunocompromised hosts. Although MBL may play an important role in a small proportion of patients with cryptococcal meningitis, cryptococcosis is a complex disease and other genetic predisposing factors and environmental factors must also explain infection in previously healthy persons.

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