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Short communication

Polymorphisms in mannose-binding lectin (*MBL*) gene and their association with *MBL* protein levels in serum in the Hu sheep

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ABSTRACT

Mannose-binding lectin (*MBL*) is the archetypical pathogen recognition protein of the innate immune defence. In humans, three frequently occurring single nucleotide polymorphisms (SNPs) in the coding region of *MBL* gene are associated with the abnormal polymerization, decreased serum concentration and strongly impaired function of *MBL* protein. To understand whether or not SNPs in *MBL* gene are associated with serum concentration of *MBL* in sheep, we investigated 105 individuals of the Hu sheep by PCR single-strand conformation polymorphism (SSCP) analysis, DNA sequencing, and enzyme-linked immunosorbent assay. SSCP analyses of PCR amplicons from a 194-bp section of the exon-I region of the *MBL* gene revealed four patterns: A, B, C and D. In comparison with the sequences of the full-length *MBL* gene of sheep (GenBank accession numbers FJ977629 and AM933378; reference sequence hereafter), pattern A has a 3-bp deletion, a 6-bp deletion and 42 SNPs. Pattern B has 3 SNPs, pattern C has 2 SNPs, whereas pattern D is identical to the reference sequence. Twenty-four of the 47 SNPs of the four patters are synonymous whereas the other 23 SNPs are non-synonymous. The two deletions in the pattern A result in deletions of amino acids but there are no frame shifts in the putative *MBL* protein. The concentration of *MBL* protein in serum ranges from 1571 to 3657 μg/L in the Hu sheep. Our statistic analyses showed that patterns A and B are associated with reduced *MBL* protein level in serum, whereas pattern C is associated with increased *MBL* protein level in serum ($P < 0.05$) in the Hu sheep.

1. Introduction

Mannose-binding lectin (*MBL*) is a calcium-dependent collagenous lectin produced in the liver. *MBL* is present in serum and plays an important role in the host immune defence against a wide range of pathogens (Super and Ezekowitz, 1992). The high-molecular-weight oligomeric form of *MBL* binds to carbohydrates on the surface of bacteria (Emmerik et al., 1994), fungi (Neth et al., 2000), and parasites such as *Leishmania spp.* (Peters et al., 1997; Green et al., 1994), *Trypanosoma cruzi* (Kahn et al., 1996), *Schistosoma mansoni* (Klabunde et al., 2000) and *Plasmodium falciparum* (Klabunde et al., 2002). After binding to carbohydrates, *MBL* mediates the activation of the complement cascade through *MBL*-associated serine proteases (*MASP*)-1 and -2; this results in the destruction of pathological microorganisms by opsonization and direct complement-mediated death (Turner, 2003).

Among the complement deficiencies identified to date, deficiencies of *MBL* are the most common in human populations (Roos et al., 2006). A number of single nucleotide polymorphisms (SNPs) have been identified in humans in both the coding and the non-coding regions of the *MBL*

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gene; these SNPs have been shown to be associated with the production and function of *MBL in vivo* (Thiel and Gadjeva, 2009). For instances, three SNPs in the exon I of the human *MBL* gene, known as the D-allele (Arg52Cys), B-allele (Gly54Asp) and C-allele (Gly57Glu), interfere with the formation of high *MBL* oligomers, leading to alterations in circulating *MBL* levels (Madsen et al., 1994; Sumiya et al., 1991; Lipscombe et al., 1995). These variant alleles in the exon I of the human *MBL* gene cause changes in the structure of *MBL*, impairing the polymerization and the function of this protein. Furthermore, these variant alleles are associated with reduced serum concentrations of *MBL* (Super et al., 1992; Lipscombe et al., 1995; Garred et al., 2003; Larsen et al., 2004; Roos et al., 2004). It has been reported that low concentrations of *MBL* can cause defects in opsonization and phagocytosis; these defects are associated with recurrent infections in immune-compromised patients and in patients with other underlying diseases (Eisen and Minchinton, 2003). It is not known whether there are polymorphisms in the *MBL* gene in sheep and if there are, whether the polymorphisms are associated with *MBL* protein levels in serum. We investigated these two issues in the Hu sheep in this study.

2. Materials and methods

2.1. Collection of sheep blood

Whole blood was collected from 105 healthy individuals of the Hu sheep from different sheep farms in the ninth agricultural unit of the Xinjiang Production and Construction Corps, Tacheng, China. Fresh blood samples were mixed immediately with EDTA buffer. Sera were separated by centrifugation at $3000 \times g$ for 10 min, and were then transferred to 1.5-ml Eppendorf tubes and stored at −80 °C.

2.2. DNA extraction, primer design and PCR amplification

Genomic DNA was extracted from EDTA anticoagulated blood samples using phenol/chloroform method as described in Sambrook and Russell (2001). Two primers, MBLF (5′-CGCTGTTTACATCACTTCCT-3′) and MBLR (5′-CACTGTACCTGGTTCTCCCT-3′), were designed with Primer 5.0 from the sequences of the *MBL* gene of sheep available in GenBank (accession numbers FJ977629 and AM933378). Primers were synthesized at Sangon Biological Engineering Technology Company (SBETC, Shanghai, China) and were used in a 25-μL PCR reaction to amplify a 194-bp section of the exon-I region of the *MBL* gene. A 25-μL PCR reaction contains 1 μL (∼50 ng) of genomic DNA extracted from an individual Hu sheep, 2.5 μL 10× PCR buffer, 1 μL (5 mM) of each primer, 2.5 μL dNTPs (2.5 mM), 1.5 μL $MgCl_2$ (15 mM), 0.6 μL (1.5 units) Taq DNA polymerase, and 14.9 μL MilliQ H_2O. The PCR reagents were supplied by the SBETC. The conditions for PCR reactions are 94 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 45 s at 58 °C, 30 s at 72 °C, and a final extension at 72 °C for 10 min. PCR products were electrophoresed on 1% agarose-gel using 0.5× TBE buffer; the agarose gel was stained with ethidium bromide.

2.3. PCR single-strand conformation polymorphism analysis

PCR products were analysed for single-strand conformation polymorphisms (SSCP), following protocols described in Xianyong et al. (2007) and Lan et al. (2007). Aliquots of 2 μL PCR products were mixed with 8 μL denaturing solution (98% formamide, 25 mM EDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), were incubated at 98 °C for 10 min and were then chilled on ice for 10 min. Denatured PCR products were electrophoresed on 12% PAGE gel (80 mm × 73 mm × 0.75 mm) in 0.5× TBE buffer at 140 V and 12 °C for 20 h. The gel was stained with 0.1% silver nitrate solution.

2.4. Cloning of PCR products and DNA sequencing

PCR products representative of different SSCP patterns in the Hu sheep were cloned using pGEM-T Easy Vector System (Promega) and competent *Escherichia coli* cells following the manufacturer's instruction. Six to 12 colonies were selected for each SSCP pattern and cultured overnight in Terrific Broth medium that contained 50 mg/mL ampicillin. To isolate plasmids, a 50-mL aliquot of the overnight culture was centrifuged at 13,000 rpm for 2 min; the supernatant was discarded. The pellet was mixed with 30 mL (10×) TE buffer, was boiled for 10 min, and was then centrifuged at 13,000 rpm for 2 min. 1 μL of the supernatant was used in a PCR with primers MBLF and MBLR (see above for primer sequences). The PCR products from isolated plasmids were electrophoresed on 12% PAGE gels under the same conditions described above for the PCR products from the genomic DNA. The PCR products with MBLF and MBLR from both isolated plasmids and genomic DNA were sequenced at BGI (Beijing, China; http://www.genomics.cn).

2.5. Measurement of MBL protein levels in serum

Serum samples from the Hu sheep were stored at −80 °C. *MBL* levels in serum samples were measured using the MBL Oligomer ELISA Kit (ADL, America), which contains a 96-well test plate, standards of known *MBL* concentrations, wash buffers, a *MBL* antigen and a biotinylated monoclonal antibody specific to *MBL*, an enzyme (streptavidin-peroxidase) and a substrate solution. Serum samples from the Hu sheep and standards of known *MBL* concentrations were loaded into the wells on the test plate: 50 μL of each serum sample or standard per well. The *MBL* antigen and the biotinylated monoclonal antibody specific to *MBL* were added to each well and were incubated at 37 °C for 60 min. The wells were washed and the enzyme, streptavidin-peroxidase, was added. After incubation at 37 °C for 30 min, the wells were washed to remove unbound enzymes; the substrate solution, which reacted with the bound enzyme to induce a colour, was added. The intensity of the colour was proportional to the concentration of *MBL* protein present in the serum samples. The intensity of the colour was measured with an ELISA reader at 450 nm and was then converted into *MBL* concentration