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α-1,3-N-acetylgalactose aminotransferase gene 539G>C mutation leads to the A_2B isoform

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ABSTRACT. In this study, the phenotypic identification and molecular mechanism of one case of an A_2B subtype pedigree was investigated. ABO blood groupings were identified by serological methods and sequence amplification was performed by polymerase chain reaction (PCR) using TA cloning and DNA sequencing analysis to identify the pedigree and the ABO gene haploid of the proband. There were both A and B antigens on the proband's red blood cells, and anti-A_1 antibodies were found in the serum. Direct sequencing of the 6th and 7th exons of the ABO gene showed the A208/B101 genotype, and haploid determination revealed the A208 and B101 alleles. Compared with the A102 allele sequence, the A208 allele was mutated at the 539 G>C site. Pedigree analysis showed that the ABO blood phenotypes of the proband's father, mother, husband, and daughter were A2, B, AB, and A_2B, respectively, and their genotypes were A208/O02, B101/B101, A102/B101, and A208/B101, respectively. The father of the proband had anti-A_1 antibodies and the A208 allele of the proband was inherited from her father, which can be passed on to her daughter. The α-1, 3-N-acetylgalactose aminotransferase gene 539G>C mutation

resulted in A_2B phenotype generation, and individual serum contained the anti-A_1 antibody.

Key words: A_2B subtypes; anti-A_1 antibody; ABO gene; Sequencing

INTRODUCTION

The ABO blood group system is a type of human erythrocyte blood group system that has important clinical significance, involving the clinical safety of blood transfusion, organ transplantation, and forensic identification. Under normal circumstances, when A or B antigens are present, there will be a lack of corresponding antibodies; therefore, positive and negative stereotyping can accurately identify the ABO blood group type. Molecular cloning studies have shown that the ABO gene locus comprises three major alleles including A101 (or the α-1,3-N-acetylgalactosamine aminotransferase allele), B101 (or the α-1, 3-galactosyltransferase enzyme allele), and O01. The A101 and B101 alleles contain 7 exons, and the full length of the transcribed mRNA is 1065 bp, encoding a protein containing 354 amino acid residues. The encoded protein has glycosyltransferase enzyme activity, which facilitates the binding of the corresponding sugar to the fucosyltransferase-1 (FUT1) gene-associated precursor H, thereby forming the A or B antigens (Reid and Lomas-Francis, 2004). A101 and B101 differ in seven nucleotides (nucleotide positions 297, 526, 657, 703, 796, 803, and 930), but only four of these nucleotide differences (526, 703, 796, and 803) lead to the substitution of four amino acids (297, 657, and 930 site substitution silencers), in which the 235, 266, and 268 amino acid sites play a decisive role in the specificity of the enzyme. The O allele causes a frameshift mutation due to a 261 cDNA single nucleotide G deletion, resulting in the formation of a polypeptide chain with sugar-free-transferase activity (Yamamoto et al., 1990). In addition to the three major alleles, more and more alleles related with ABO subtypes are continuously being identified in different races and individuals (Chester and Olsson, 2001; Yip, 2002; Lin et al., 2003; El-Zawahri and Luqmani, 2008; Zhen et al., 2011). Although their frequencies are relatively low, these alternate alleles serologically manifest mainly as differentially expressed ABO blood group antigens and by phenomena such as the weakening of antigen strength, mixed-field-agglutination, and inconsistent positive and negative stereotypes in blood typing (Issitt and Anstee, 1998). Furthermore, molecular diagnostic techniques have also been used to clarify the molecular mechanisms of these partial ABO subtypes (Seltsam et al., 2003; Roubinet et al., 2004; Sun et al., 2006; Zhu et al., 2010). We found one case of the A_2B subtype in clinical blood typing, and here report its molecular mechanism and results of the pedigree analysis.

The proband was a 25-year-old female belonging to the Han nationality who came to our hospital for prenatal care. She had no history of blood transfusion, and the prenatal blood test results were as follows: red blood cell (RBC) count = 4.13×10^{12}/L, hemoglobin (Hb) = 116.0 g/L, hematocrit (HCT) = 0.34, platelet (PLT) count = 150×10^{9}/L. Due to the inconsistent positive and negative stereotypes of conventional ABO blood group testing, further inspection and analysis was required. The family was surveyed, and blood samples were collected from her parents, husband, and daughter. This study was conducted in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of Wenzhou Medical College. Written informed consent was obtained from all participants.

Serological testing of ABO blood groups

Positive and negative stereotypes were all assessed using the test tube method. The monoclonal reagents anti-A, anti-B, anti-H, and anti-A_1 blood grouping were purchased from the Changchun Boulder Biological Products Co., Ltd. (China) and the Shanghai Blood Biopharmaceutical Co., Ltd (China). The A, B, and O anti-stereotype cells were made in our laboratory, which are mixed cells of more than 3 ABO blood types.

Polymerase chain reaction (PCR) amplification of the 6th and 7th exons of the ABO gene

Genomic DNA was extracted using the QIAamp DNA Mini Tip pillar extraction kit (Qiagen; Germany), the primer sequence for PCR amplification was based on the ABO gene sequence; the upstream primer E67F sequence was 5'-CTCAAGGGGCTGTTCTGAAG-3' and the downstream primer E67R5 sequence was 5'-GCGATTGCGTGTCTGTGTAT-3' (synthesized by Shanghai Shen Neng Bo Cai Biotechnology Co., Ltd.). The total length of the amplification product was approximately 2749 bp, including the 6th exon, the 6th intron, and the full-length 7th exon, as well as the partial sequence of the 5th exon and its 3' non-coding region. The total amplification reaction volume was 25 μL including 2.5 μL 10X PCR buffer, 50-100 ng specimen DNA, 0.9 U Taq DNA polymerase (TaKaRa Co.; Dalian, China), 0.2 mM dNTP, 1.5 mM $MgCl_2$, and 0.5 μM primers. The amplification conditions were 94°C denaturation for 3 min, 94°C denaturation for 30 s, 62.3°C annealing for 30 s, and 72°C extension for 3 min for 30 cycles, followed by a 72°C extension for 10 min, and 10°C insulation.

PCR products were successfully identified by 2% agarose gel electrophoresis for 30 min, amplified under ultraviolet transillumination, and then the targeted DNA fragments were cut from the gel to which 10 U exonuclease (TaKaRa) and 2 U shrimp alkaline phosphatase (Promega Corporation) were added. Enzyme digestion was performed on the ABI 9700 PCR amplification machine at 37°C for 30 min, cooled to 4°C, and then 80°C for 15 min.

Sequencing analysis

The PCR product was enzyme-digested and purified as a DNA template with the BigDye Sequencing Kit (ABI) for the sequencing reaction, and the primers were based on previous reports (Chen et al., 2005). The sodium acetate/ethanol method was used to purify the sequencing reaction product, and the purified product was subjected to thermal denaturation, quickly cooled, underwent polyacrylamide gel electrophoresis (PAGE) on an ABI PRISM3730 sequencing instrument, and the results were analyzed using sequence analysis software. The A101 allele sequence (GenBank No. AF134412) was used as a template to analyze and record the mutation status at each nucleotide position.

The PCR amplification product of the ABO gene was cloned using the TOPO TA cloning technique strictly following manufacturer (Invitrogen, USA) instructions. First, the PCR product was connected to the PCR4_AT_TOPO plasmid vector to transfect *E. coli*, which were inoculated on Luria-Bertani (LB) agar plates and cultured overnight at 37°C. Numerous clones were selected and cultured in LB liquid medium for proliferation, and then the plasmid DNA was extracted. Recombinant plasmid DNA was used as a template and the sequencing reaction was performed according to instructions of the BigDye Sequencing Kit (ABI; USA).

RESULTS

Serology of ABO blood grouping

The positive and negative stereotype results of the proband's ABO blood group suggested that there were both A and B antigens expressed on red blood cells, and that the A expression was weaker than the B expression. Furthermore, there were anti-A_1 antibodies found in her serum, which did not agglutinate with the red blood cells. Therefore, she was determined to have the A_2B subtype (Table 1).

Table 1. Serology results of the proband's ABO blood group.

Temperature	Positive form				Negative form				
	anti-A	anti-A_1	anti-B	anti-H	A_1 cells	A_2 cells	B cells	O cells	Self cells
4°C	3+	0	4+	3+	3+	0	0	0	0
Room temperature	2+	0	4+	3+	3+	0	0	0	0
37°C	1+	0	4+	3+	1+	0	0	0	0

ABO gene sequencing analysis

The direct sequencing analysis of the 6th and 7th exons of the proband's ABO gene revealed that the 6th exon was 261 G/G, the 7th exon was 297 A/G, 467 C/T, 526 C/G, 539 C/G, 657 C/T, 703 G/A, 796 C/A, 803 C/G, 930 G/A. Referring to the Blood Group Antigen Gene Mutation Database and related sequence information (Blumenfeld and Patnaik, 2004), and according to the polymorphism sites, the genotype of the specimen was determined to be A208/B101. Further cloning and sequencing confirmed that the two alleles were A208 and B101, respectively. Compared with the A102 sequence, the A208 allele was mutated at the 539G>C site (Figure 1), leading to an arginine > proline change at the 180th amino.

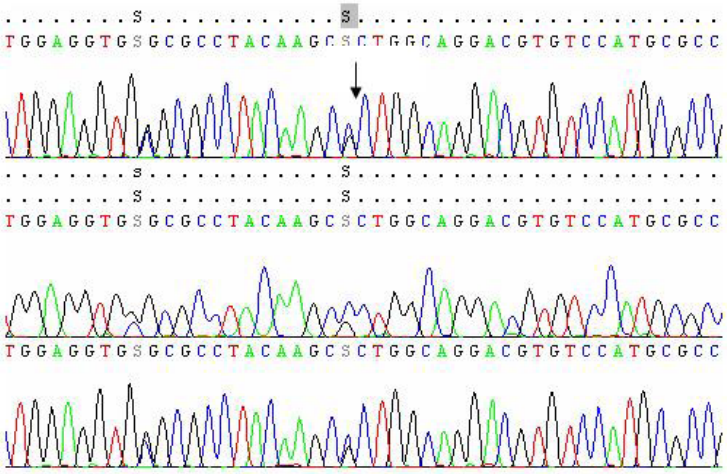

Figure 1. Partial sequence of the direct sequencing of the 7th exon of proband's ABO gene. Arrow indicates 539C/G heterozygous.

ABO blood grouping and genotype analysis of family members

Pedigree analysis revealed that the blood grouping phenotypes of the proband's father, mother, husband, and daughter were A_2, B, AB, and A_2B, respectively, and their genotypes were A208/O02, B101/B101, A102/B101, and A208/B101. The serum of the proband's father and daughter contained anti-A_1 antibodies. Family survey results showed that the proband's A208 allele was obtained from her father and could be passed on to her daughter.

DISCUSSION

The ABO blood group system contains certain subtypes, of which the A2 subtype is the most common, and most of the A2 alleles are found in Asian people (Yamamoto et al., 1990). The A2 subtype accounts for 0.41% of the A type, and the A_2B subtype accounts for 3.8% of the AB type in the Chinese Han ethnic population (Ying et al., 2013). A_2B individuals show weakened agglutination with the corresponding antibody, a mixed-field type of the agglutination reaction, a decrease in the antibody titer, and inconsistent positive and negative stereotypes. Clinically, attention should be paid to the phenomena of inconsistent positive and negative stereotypes and changes in the antibody titer, and, if necessary, molecular biology techniques should be used to complement characteristics revealed by the serological experiments in order to clarify the patient's blood type. The specimens of this proband were obtained in the course of regular prenatal care, and were treated to genotype analysis because of inconsistent positive and negative stereotypes.

The formation of ABO isoforms results from different molecular mechanisms, including nucleotide deletions, insertions, replacements, splicing point mutations, and allele hybridization, among others. Common alleles of ABO blood groups are A101, A102, B101, O01, and O02 (Zhu et al., 2010). To date, approximately 200 ABO alleles have been identified (Blumenfeld and Patnaik, 2004). Since Yamamoto (Zhang et al., 2012) first reported the A2 subtype, several more A2 allele subtypes have been reported, including: A201, A202, A203, A204, A205, A206, A207, A208, A209, A210, A211, A212, A213, A214, A215, A216, A217, A218, A219, A220, A221, and A222 (http://www.ncbi.nlm.nih.gov/gv/rbc/xslcgi.fcgi?cmd=bgmut/systems_alleles&system=abo). Of these alleles, A201, A206, A209, A212, A214, A215, and A216 all have a C missing at site 1061, the A204 allele may be the result of genetic recombination, whereas the other alleles are all point mutations (Yip, 2000; Roubinet et al., 2002; Seltsam et al., 2002; Blumenfeld and Patnaik, 2004; Yan et al., 2005; Shastry and Bhat, 2010; Zhang et al., 2012). A Chinese survey (Ying et al., 2013) found that A205 was the most common A2 subtype, accounting for 80.48%, followed by A201, accounting for 12.20%, and other types were found in less than 1% of the population. The proband of this case carried the A2B 539G>C mutation, and according to the polymorphic loci, the genotype was determined to comprise the A208 allele (Chen et al., 2005). In the A2 alleles that had been identified to date, most of the nucleotide mutations causing changes in enzyme activity were located at the 3' end of exon 7 (encoding the C-terminal of the polypeptide chain), such as 1061delC of A201 and A206, 1054C>T of A202, 1054C>G of A203, and 1009A>G of A205. Therefore, amino acid variations of the C-terminal of the polypeptide chain of the A2 subtype are thought to lead to glycosyltransferase activity changes. However, the mutation sites of the A207 and A208 alleles are all close to the 5' end (539G>C) of exon 7, causing the Pro156Leu and Arg-180Pro replacements, and this replacement is far away from the active enzyme center, which

is composed of the key amino acids 235Gly, 266Leu, and 268Gly that determine the enzyme's spatial structure. Compared with A102, A208 has only the 539G>C substitution, which leads to replacement of the arginine at position 180 of the amino acid polypeptide chain with a proline, which can cause an activity change of α-1, 3-N-acetylgalactosamine aminotransferase (A gene coding), thus forming the A2 subtype (Chen et al., 2005). This suggests that the genetic background of the A2 subtype might not be as limited as originally thought. The same situation is also found in the nucleotide variations of the A304 and Bw05 alleles at the 539G locus, although in this case, it is a 539G > A conversion (Olsson et al., 2001; Svensson et al., 2005). In the current study, the A208 allele resulted from a 539G> C transversion, although both may be occurring on different alleles with independent nucleotide mutations, but at exactly the same point. In this study, the proband's A208 allele was inherited from her father, and could be passed on to her daughter, and the family member results showed that the A208 allele was stably passed on for three generations. The ABO gene 539G>C mutation is believed to be the molecular mechanism causing the A_2B phenotype of this pedigree.

A_2B is an ABO blood group subtype, accounting for approximately 0.78-8.17% of the AB population. Approximately 22-35% of the serum of A_2B subtypes have the anti-A_1 antibody; however, the antibodies mostly have no clinical significance, except for the responsive anti-A_1 antibody at 37°C. The specimen in this case was the A_2B subtype, the proband's serum had anti-A_1 antibodies, and *in vitro* experiments showed that the antibody had appropriate biological activity at 37°C, which can cause the discrepancies of RBC-positive and reverse blood A_2B typing and mismatched cross-match tests. With regards to the A_2B subtype subject as the recipient, based on existing serological characteristics and the molecular mechanisms perspective, this subject should be given A_2B blood infusions or O-washed erythrocytes in order to ensure the safety of blood transfusion. In this case, according to the probands own conditions, we carried out 400 mL prenatal autologous blood storage and transfusion. The feedback did not cause side reactions, and a discharge review of the blood showed that RBC = 3.69 x 10^{12}/L, Hb = 103.0 g/L, HCT = 0.30, and PLT = 183 x 10^9/L, which indicated that the desired effect was achieved.

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