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The M142T Mutation Causes B_3 Phenotype: Three Cases and an *in vitro* Expression Study

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The B_3 phenotype is the most common B subtype in Korea. The *B305* allele (425 T>C, M142T) was first reported in 2 Chinese individuals; however, it has not yet been reported in the Koreans, and the impact of the M142T mutation on the expression of the B3 phenotype has also not been studied. To resolve an ABO discrepancy between a group O neonate and her group O father and A_1B_3 mother, blood samples from these individuals and other family members were referred to our laboratory for *ABO* gene analysis. The *B305* allele was discovered in the neonate (*B305/O01*), her mother (*A102/B305*), and her maternal aunt (*B305/O02*), while her father was typed as *O01/O02*. Transient transfection experiments were performed in HeLa cells using the *B305* allele synthesized by site-directed mutagenesis; flow cytometric analysis revealed that this transfect expressed 35.5% of the total B antigen produced by the *B101* allele transfect. For comparison, *Bx01* allele transfects were also created, and they expressed 11.4% of the total B antigen expressed on the surface of *B101* transfects. These experiments demonstrate that the M142T (425 T>C) mutation is responsible for the B subtype phenotype produced by the *B305* allele. *(Korean J Lab Med 2010;30:65-9)*

Key Words : *ABO Blood Group System, Gene Expression, B305*

INTRODUCTION

The human ABO blood group is determined by the *ABO* gene located on chromosome 9. This gene contains 7 exons, of which the last 2 (6 and 7) comprise nearly 80% of the coding sequence of the gene and encode the catalytic domain [1, 2]. The genetic underpinnings of several B subtype phenotypes have been reported [3-12]. Although it is considerably rarely observed in Korea and probably the entire Asia, the B_3 phenotype is the most common B subtype phenotype [6, 7]. *B*[3] alleles are heterogeneous and contain single nucleotide polymorphisms (SNPs) scattered throughout both the coding and intronic DNA sequences; however, some individuals with the B_3 phenotype had no unexpected SNPs relative to the *B101* allele [8].

Recently, blood samples were referred to our laboratory because of an ABO typing discrepancy between a neonate and her parents; a routine pretransfusion testing revealed that the neonate and her father were group O, while her mother had the A_1B_3 phenotype. A genetic investigation revealed that both the neonate and her mother contained

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the *B305* allele. Although the *B305* allele was first reported in 2 Chinese individuals, it has not yet been reported in the Koreans, and the impact of the M142T mutation in exon 7 of the *ABO* gene on the activity of B transferase has also not been studied. To confirm that the *B305* allele was responsible for the weak B phenotype in the mother, we transfected HeLa cells with a recombinant *B305* allele and studied its expression characteristics using flow cytometry. We then compared the expression of the *B305* transfects to that of the HeLa cells transfected with a normal *B101* allele and another representative *B* subtype allele, *Bx01* (871G>A, D291N).

CASE REPORT

Five blood samples from the family of a 7-day-old female neonate were sent to our laboratory for the resolution of an ABO discrepancy between the neonate and her parents. Written informed consent for the genotyping was obtained from all family members and from the parents on behalf of the neonate. Forward typing was performed by the manual tube method using murine monoclonal anti-A, anti-B, anti-H, and anti-AB antisera (Biotest AG, Dreieich, Germany) according to standard protocols [13]. Reverse typing was also performed by the tube method using A1 and B RBCs (Diamed, Cressier sur Morat, Switzerland) [13]. The serological findings are presented in Fig. 1. The neonate's plasma did not react with A_1 or B reagent red cells. Adsorption and elution tests could not be performed on her RBCs due to the small sample volume. The RBCs from both the neonate's mother (II-3) and aunt (II-1) demonstrated 1+ mixed-field agglutination with anti-B reagents (B_3 phenotype), and anti-B was not detected in either of their reverse typings.

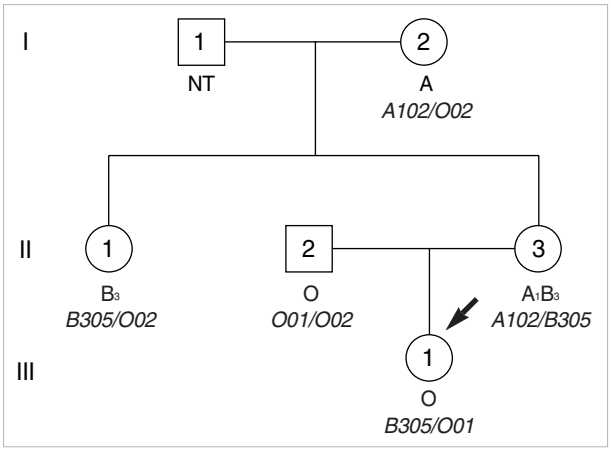

Fig. 1. The phenotype and *ABO* genotype of the family members included in this study. The arrow indicates the propositus, a 7-day-old neonate. NT=not available for testing.

The coding and flanking intronic sequences of *ABO* exons 6 and 7 of the neonate and 4 other family members were amplified, cloned, sequenced, and analyzed, as described previously [6, 14-16]. The *B305* allele (425T>C, M142T) was detected in the neonate, her mother, and her aunt. The remaining 5 exons and flanking intronic regions were identical in the individuals with the *B305* allele, as revealed by sequencing analysis. The *ABO* genotypes of the family members are presented in Fig. 1. To verify that 425C was associated with the *B* allele in the neonate's mother, we cloned and sequenced the *ABO* alleles of the mother. The results are presented in Fig. 2.

Next, *in vitro* expression studies were performed to investigate the pattern of B antigen expression produced by the *B101* and *B305* alleles, as well as by another subtype allele (*Bx01*). Full-length *B101* cDNA was obtained by reverse-transcriptase polymerase chain reaction (PCR) using the mRNA obtained from the buffy coat of a homozygous *B101* laboratory volunteer. The volunteer provided an informed consent for the genotyping. The coding region of the *ABO* gene was amplified using the primer

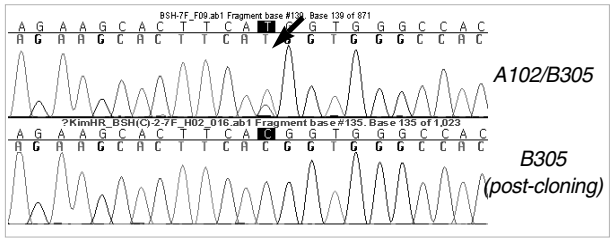

Fig. 2. The DNA sequence of the region around nucleotide 425 (arrow) in exon 7 of the *ABO* gene in the neonate's mother. The upper chromatogram demonstrates the heterozygous sequence (C and T) at nt. 425. The lower chromatogram demonstrates the nucleotide sequence of the region around nt. 425C in the setting of a *B305* allele after cloning (*B* allele-specific polymorphisms are not shown; the *A102* and *B305* genes are otherwise identical in this area).

pair 5′-GAAGAATTCGCCATGGCCGAGGTGTTGCGGA-CG-3′ and 5′-CTCCTCGAGTCACGGGTTCCGGACCGC-CTG-3′, which were modified from Seltsam and Blasczyk [17]. The resulting 1,086-bp cDNA was digested with *Eco*RI and *Xho*I and then cloned into the pBluescript SK II (Stratagen, La Jolla, CA, USA) linearized with these enzymes. The plasmid containing the insert was then designated pBS-ABO. Site-directed mutagenesis of the *B101* gene was performed by *Pfu* polymerase-based amplification using 0.3 pmol/μL oligonucleotide primers and the pBS-ABO plasmid as the template. The following primers were used to create the *B305* and *Bx01* alleles-specific expression vectors: for the mutation 871 G>A (D291N), Bx01-F: 5′-GCCATGATGGTCAACCAGGCCAACG-3′ and Bx01-R: 5′ CGTTGGCCTGGTTGACCATCATGGC 3′; for the mutation 425 T>C (M142T), B305-F: 5′-AGAAGCACTTCACGGTGGGCCACC-3′ and B305-R: 5′-GGTGGCCCACCGTGAAGTGCTTCT-3′.

The thermocycler (Perkin Elmer, Norwalk, CT, USA) settings were as follows: initial denaturation at 95℃, followed by 20 cycles of denaturation at 95℃ for 30 sec, annealing at 55℃ for 30 sec, and extension at 68℃ for 8 min. The amplified products were first treated with *Dpn*I to digest the template DNA and then transformed into *Escherichia coli* DH5α. The accuracy of the site-directed mutagenesis was confirmed by sequencing using the ABI 3130xl Genetic Analyzer (Hitachi, Tokyo, Japan). To express the *B* alleles, we digested the respective cDNAs with *Eco*RI and *Xho*I and cloned them into the corresponding sites of the expression vector pIRES2-EGFP (BD Biosciences Clonetech, Palo Alto, CA, USA). Plasmids containing the cDNA inserts were designated ABO-pIRES2-EGFP. HeLa cells were used for the transfection experiments because they show surface expression of H antigen and are homozygous for the human *O02* allele. These cells were separately transfected with the ABO-pIRES2-EGFP plasmids-*B101*, *B305*, and *Bx01*-using Effectene reagent (Qiagen, Hiden, Germany) according to the manufacturer's instructions. For the mock control, the pIRES2-EGFP plasmid without a cDNA insert was transfected into HeLa cells. Briefly, 1×10^6 cells were transfected with 1 μg of plasmid DNA, 8 μL of Enhancer, and 25 μL of Effectene transfection reagent. After the HeLa cells with the plasmids containing cDNA from the various *B* alleles were incubated at 37℃ for 15 hr at 5% CO_2, they expressed the corresponding B glycosyltransferase, and the resulting phenotypes could be detected by flow cytometry.

The cells were then detached by trypsin-ethylenediaminetetraacetic acid (EDTA), washed with 1× phosphate buffered saline (PBS) containing 1% bovine serum albumin (Sigma, St. Louis, MO, USA), and incubated for 30 min at room temperature with R-phycoerythrin-conjugated murine IgG1 anti-B monoclonal antibodies (9431PE BG-RL1, obtained from the International Blood Group Reference Laboratory, Bristol, England). The cells that had been successfully transfected with ABO-pIRES2-EGFP expressed the green fluorescent protein (GFP), facilitating their sorting by a flow cytometer (Epics XL; Beckman

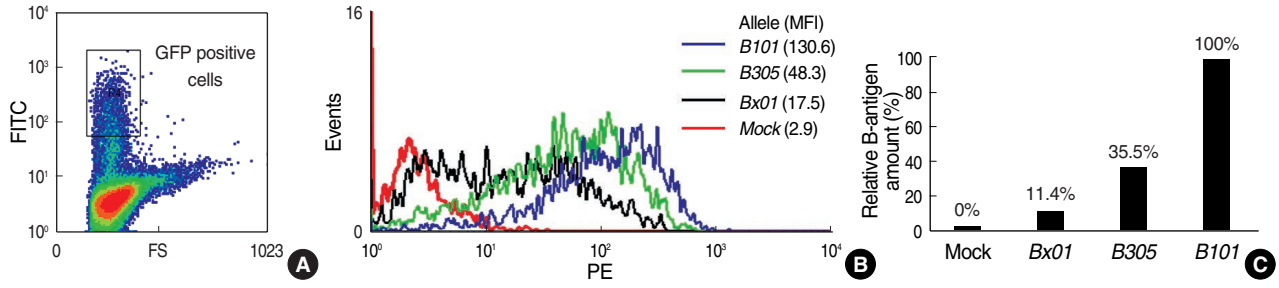

Fig. 3. (A) Flow cytometric histogram demonstrating the population of green fluorescent protein (GFP)-positive transfected HeLa cells. (B) Representative histograms from each of the 3 *B* allele constructs expressed in HeLa cells, and the mock transfect used as a control. Note the absence of a mixed-field pattern in the *B305* histogram and the weak expression of B antigen in the *Bx01* histogram. The mean fluorescence intensity (MFI) for each transfect is shown in parentheses. (C) The amount of *B* antigen produced by each of the 2 *B* subtype transfects relative to that produced by the *B101* transfect.

Coulter, Hialeah, FL, USA). Expression of the B antigens on the GFP-positive transfected cells (Fig. 3A) were then analyzed with WinMDI version 2.8 software.

A representative histogram from each of the different *B* allele transfects and their average Mean Fluorescence Intensity (MFI) is presented in Fig. 3B. MFI is a relative measure of the antigen expression on the cell surface. The relative amount of B antigen produced by the *B* allele subtype transfects compared to that produced by the normal *B101* allele was calculated using the formula: ([MFI of $B_{subtype}$-MFI of Mock]/[MFI of *B101*-MFI of Mock])×100%. The *B305* transfects expressed approximately 35.5% of the total B antigen produced by the *B101* transfects, while the *Bx01* transfects expressed only 11.4% of the B antigen produced by the control *B101* transfects (Fig. 3C).

DISCUSSION

The alleles associated with the B_3 phenotype demonstrate remarkable genetic heterogeneity and are geographically dispersed. Xu et al. [9] reported 2 unrelated Chinese individuals with the *B305* allele whose serology was consistent with the B_3 subgroup because their sera demonstrated mixed-field hemagglutination. In this study, the *B305* allele was found in a Korean family, suggesting that the 425T>C SNP is relatively common across Asians.

Among the *B*[3] alleles, the *B306* allele has also been found in some Korean blood donors [6]. This allele demonstrated allelic competition: it was expressed as a normal B phenotype in *B306/O01* heterozygous individuals, but as A_1B_3 phenotype in *A101/B306* or *A102/B306* heterozygotes. The *B305* allele does not demonstrate this serological phenomenon: the same mixed-field agglutination pattern was observed when the *B305* allele was inherited with an *O02* allele or with an *A102* allele. Surprisingly, the 7-day-old neonate's RBCs did not express enough B antigen that could be detected by routine serological techniques; this could be because of the very young age and the presence of a *B* subtype allele. It will be interesting to conduct a follow-up study to determine if the child develops the B_3 phenotype as predicted by her genotype.

Expression studies can be a useful tool for studying the phenotype produced by the *A* and *B* subtype alleles [17, 18]. In this study, to better understand the effects of the M142T mutation on the activity of B glycosyltransferase, we created an expression model using HeLa cells transfected with *B305* cDNA. The MFIs of the *B305* and *Bx01* subtype transfects were lesser than those of the *B101* transfects, and the MFIs of the former transfects were reasonably consistent with those of the reported RBC serological findings, although a mixed-field pattern was not observed with the *B305* transfect probably because of the differences in the antibodies used for pretransfusion testing and those used for flow cytometry experiments.

In summary, by conducting flow cytometric analysis of HeLa cells transfected with a recombinant *B305* allele, we confirmed that the M142T (425 T>C) mutation reduces the B-enzyme's activity and leads to a B subtype phenotype that is detectable in pretransfusion testing.

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