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Brief report

Identification of the novel c.300C>G variation on the *ABO*A1.02* allele associated with an $A_{weak}B$ phenotype

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INTRODUCTION

The ABO system is the most important blood system in transfusion medicine[1]. The *ABO* gene is located on chromosome 9q34.1-q34.2 and is approximately 19.5 kb long containing a 1062 bp coding region with seven exons and several regulatory elements[2]. There are five common *ABO* alleles, including *ABO*A1.01*, *ABO*A1.02*, *ABO*B.01*, *ABO*O.01.01* and *ABO*O.01.02*, in the Chinese Han population[3]. ABO antigens on the red blood cells are biosynthesized by A or B glycosyltransferases (GTs) encoded by the *A* and *B* genes[4]. So genetic changes in the *ABO* gene, including missense mutations, insertions, or deletions in the coding regions, splicing sites, or regulatory elements could impair the catalysis capability of GTs to cause weak ABO subgroups[5-10]. ABO subgroup prevalence is rare, with a rate of approximately 0.015% in China[11]. Until now, over 300 *ABO* subgroup alleles have been reported worldwide[12-14], including A_{weak}, B_{weak}, CisAB, and B(A) subgroups.

Weak A subgroups are infrequently encountered and are usually recognized by apparent discrepancies between red cell and serum or plasma grouping. Multiple previous studies have reported explanations for the weakened expression of certain A or B variants. In this study, we identified one novel SNV *ABO*A* c.300C>G (p.F100L) in donor with $A_{weak}B$ phenotype and explored the possible mechanisms which caused the weak subgroup.

MATERIALS AND METHODS

Subjects and phenotype

The EDTA-anticoagulation peripheral venous blood samples were collected from a pregnant woman experiencing miscarrying because of infection. The ABO phenotype of the subject was first determined by the IH1000 system (Bio-Rad, Roanne, France). If the results determined by Bio-Rad were discrepant (forward and reverse typing), the results were deepened by manual tube method and adsorption-elution test according to standard methods and procedures as documented in the AABB technical manual[15]. In the tube method, monoclonal anti-A, anti-B, anti-A_1, anti-H (SHPBC, Shanghai, China), polyclonal anti-A, anti-B, anti-AB (SHPBC) and ABO red blood cell kit (SHPBC) were used for blood group typing. Samples were collected from apparently healthy random Chinese donors (No.=120) as normal controls.

The flow cytometry analysis was carried out by utilizing a flow cytometer and employing a phycoerythrin (PE)-conjugated murine IgG1 anti-A monoclonal antibody. To serve as reference points, red blood cells (RBCs) with confirmed with A_2 and O phenotypes were included as positive and negative controls, respectively. A weak phenotype carrying

the SNVs c.426C>G and c.467C>T has been used as a weak-positive control for comparison purposes. And 8,000 red blood cells were counted in the flow cytometry analysis. Median fluorescent intensity (MFI) was utilized as the parameter for data interpretation and analysis. The sample analyzed in this study is summarized in **Figure 1**.

ABO gene amplification and sequencing

Genomic DNA was extracted from EDTA-anticoagulation peripheral blood sample using a blood DNA kit (Tiangen, Beijing, China). All the seven exons of the *ABO* gene were amplified and the primers for polymerase chain reaction were designed as previously described[16,17]. The polymerase chain reaction products were purified from agarose gel using a gel extraction kit (QIAquick, Qiagen GmbH, Hilde, Germany) and subsequently sequenced by a sequencer (ABI 377, Applied Biosystems, Foster City, CA, USA). The haplotypes were confirmed by cloning and sequencing the gel-purified products containing the variant site.

Serum transferase activity assay

To assay the activity of A- and B- transferase, we followed the modified methods of Nagai and colleagues[18]. Initially, we washed group O red blood cells (RBCs) with 0.9 percent sodium chloride solution, repeating this process three times. Then, we took 10 microliters of the washed group O RBCs and combined it with 100 microliters of a reaction mixture. The reaction mixture consisted of imidazole buffer (pH 6.5) at a concentration of 0.05 mol per L, MnCl2 at a concentration of 25 mmol per L, NaCl at a concentration of 0.15 mol per L, UDP-galactose or N-acetylgalactose at a concentration of 0.5 mmol per L, bovine serum albumin at a concentration of 0.5 percent (wt/vol), and 50 microliters of plasma. Once the RBC suspension was prepared, it was incubated for 4 hours at a temperature of 37°C. To measure the A or B substance, artificially added to the surface of the group O RBCs, 10 microliters of the reaction suspension were added to each dilution of monoclonal anti-A or anti-B, up to a 256-fold dilution. We recorded the agglutination results. The transferase activity in the reaction mixture was expressed as the highest dilution factor of agglutinin that caused detectable agglutination. We included group A_1, B, and O plasma samples as controls in the experiment.

Predicted protein stability changes upon the SNV

The site directed mutator (SDM) field in DUET was used to calculate the difference in free energy of the variant: ddG (ΔΔG) based on the GTA enzymes (PDB code, 4C2S) to examine the effect of the variant on protein stability. The predicted results are expressed as the variation in ΔΔG and the negative values denote destabilizing mutations[19].

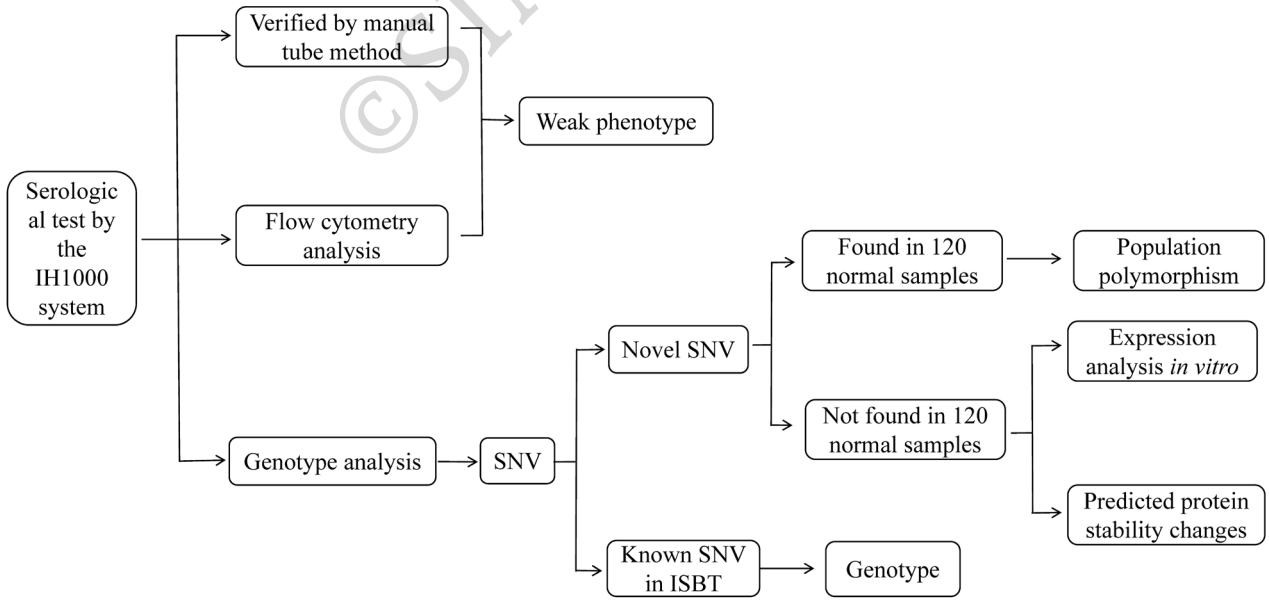

Figure 1 - Flow chart of the sample included in this study

The PolyPhen-2 was applied to predict impact of the variant on the structure and function of GTA. Two pairs of datasets, HumDiv and HumVar, were used to train and test PolyPhen-2 prediction models. The outcome can be one of probably damaging, possibly damaging, or benign based on the scores.

RESULT

Blood group phenotype characterization

The results of the ABO phenotype displayed an inconsistency between the forward and reverse typing. The red blood cells (RBCs) of subject 1 exhibited significant agglutination with both anti-B and anti-AB serum, as well as with anti-H in the tube tests. However, there was mixed field agglutination observed with anti-A. Conversely, the serum of subject 1 did not elicit a reaction with B cells or A_1 cells. Based on these findings, subject 1 is suspected to belong to the $A_{weak}B$ subgroup (**Table I**). Flow cytometry analysis revealed that the expression of the A antigen on the surface of the red blood cells from the subject was significantly lower compared to A_2 type cells. However, it was slightly higher than the expression seen in O type cells. Additionally, the A antigen expression was found to be comparable to that observed in known weak type A red blood cells. The RBCs of the subject display weak A patterns with MFI values of 0.7% of the wild-type RBCs (**Figure 2**).

ABO gene sequence analysis

According to the directed sequence and cloning analysis, there were *A1.02* allele with a novel heterozygous SNV c.300C>G (p. F100L) and *B.01* allele in the subject 1 (GenBank No. OQ822174.1). The novel SNV had been submitted for the ISBT alleles designation and not found in 120 random healthy Chinese individuals with common ABO blood groups.

Table I - *ABO subgroup phenotypes for the blood sample*

No.	Forward typing					Reverse typing			Plasma
	Anti-A	Anti-A_1	Anti-B	Anti-AB	Anti-H	A_1	B	O	GTA transfer capacity (titre)[a]
1	3+mf	-	4+	4+	3+	-	-	-	<1

A_1 indicates red blood cells with A_1 phenotype; B indicates red blood cells with B phenotype; O indicates red blood cells with O phenotype. "-" indicates negative reaction, "mf" indicates mixed filed. [a] : The titre values showed the highest dilution factor of anti-A antibodies causing detectable agglutination.

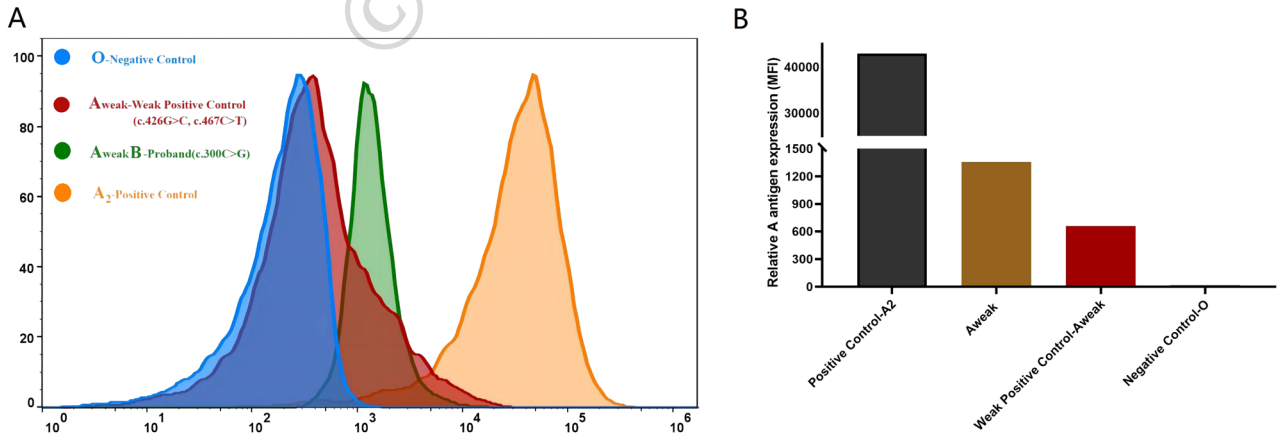

Figure 2 - A antigen expression on RBCs of the $A_{weak}B$ subject detected through the flow cytometry analysis
The proband (green), positive control (group A_2) (orange), weak-positive control (red) (A_{weak} with SNV c.426G>C, c.467C>T) and negative control (blue) (group O) are included. **A**) Histograms of FACS analysis of subjects' RBCs, as well as A_2 cells, A_{weak} and O cells. **B**) Median fluorescent intensity (MFI) of RBC from wild-type (group A_2), proband, weak positive and negative (group O).

Serum transferase activity assay of GTA with p. F100L

The catalytic ability of transferase from group A_1 and B plasma samples to convert O type red cell into A type red cell was still at high dilutions (1:128), while the catalytic ability of transferase from the subject 1 did not be detected (**Table I**). Thus, the weak A antigen expression on RBCs may be caused by the dysfunction of the p. F100L variant.

Prediction of the stability of the variant protein

The complete structure of the unliganded wild-type GTA and F100L variant protein was mainly built from the crystal structure 4C2S. There was no significant change in the overall structure between the wild-type and variant (**Figure 3A**). The results of Chimera analysis showed that the hydrogen bond structure around the 100 site did not change significantly between the wild-type and variant GTA (**Figure 3B**). Thus, the weak phenotype was not caused by changing the local conformation of GTs. To assess the impact of the variant on GTA stability, the thermodynamic stability changes of the protein variant were calculated. Homology models were constructed using the 4C2S by DUET. The variant at position 100, changing from Phe to Leu, resulted in a ΔΔG of -1.841 kcal/mol, indicating a reduction in protein stability. According to the PolyPhen-2 analysis, this variant was predicted to be potentially damaging with a score of 0.999 based on HumDiv and possibly damaging with a score of 0.942 based on HumVar (**Figure 3C**). Thus, the p. F100L variant could impair the stability of GTA.

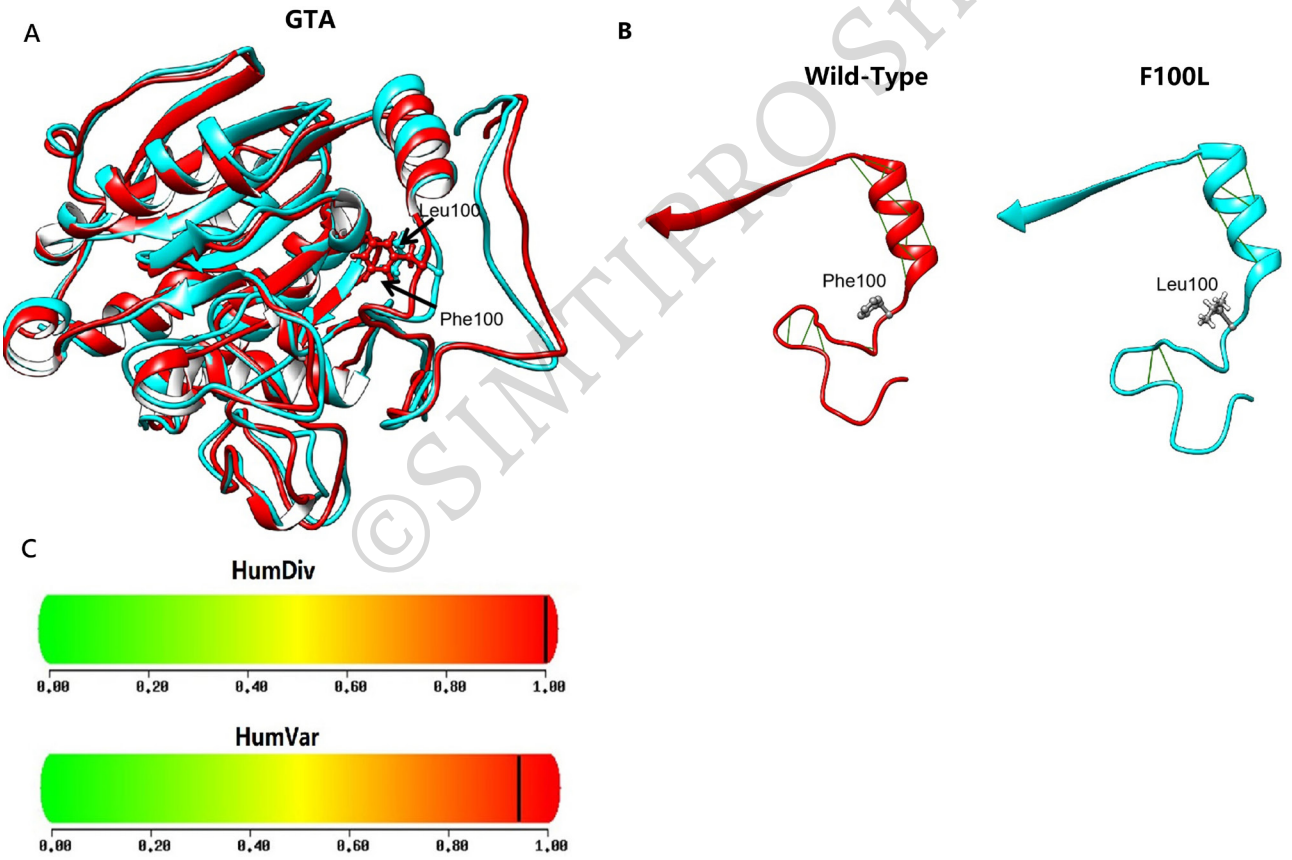

Figure 3 - 3D structure of the wild-type GTA and the variant
The amino acids were colored by elements in a ball and sticks method. The green line indicates a hydrogen-bond. **A**) The comparative of overall structural wild-type and variant GTA. The wild type was colored in red, and the variant was in azure. The side of Leu100 was colored in azure and the side of Phe100 was colored in red and indicated by arrow. **B**) The local ribbon drawing from Cys80 to Phe121 of wild type and variant GTA. The green line indicates a hydrogen bond. Fewer hydrogen bonds were found in variant GTA (colored in azure, right) compared with wild-type (colored in red, left). The figures were generated by Chimera software. **C**) Impact of the variant on the structure and function of GTA. This variant was predicted to be potentially damaging with a score of 0.999 based on HumDiv (top) and possibly damaging with a score of 0.942 based on HumVar (bottom).

DISCUSSION

In this study, an $A_{weak}B$ subgroup was identified. The A weak subgroup is rare phenotype of the A blood group. According to the International Society of Blood Transfusion, there are 47 *AW* alleles known. In this study, we identified one novel *AW* allele containing a SNV (c.300C>G, p. F100L) on *A1.02* allele which can impair the stability of GTA and downregulate the expression of A antigens on the RBCs significantly resulting in $A_{weak}B$ phenotype.

The findings from the serologic and flow cytometry analysis indicated that subject 1 had a small quantity of A antigen on their red blood cells. Further testing, specifically the total GTA transfer capacity, revealed that the GTA in subject 1 had lost the ability to convert O type cells into A type cells. Thus, the GTA from subject 1 was able to significantly reduce the expression of A antigens on the red cells. Through directed sequence and clone analysis, there was a novel SNV c.300C>G on the *A1.02* allele, and the genotype of subject 1 was *ABO*AW/ABO*B.01*. This novel SNV encoding p.F100L, likely played a role in the low expression of A antigens on the red blood cells by impairing the function of GTA.

The impact of amino acid variants on protein structure, stability, substrate binding, and specificity has been extensively studied[20]. Phe and Leu are both hydrophobic amino acids with no charge. Therefore, substituting one for the other does not affect the charge or polarity of the protein. To evaluate the impact of the p.F100L variant on the protein, we employed 3D structural modeling and analyzed the changes caused by the variant. Overall, the mutation did not cause a significant alteration in the protein's overall structure. Due to the hydrophobic nature of both Phe and Leu, the hydrogen bond structure near the mutation site remained unchanged. However, the total number of hydrogen bonds in the protein decreased from 233 pairs to 178 pairs. Consequently, the amino acid substitution or change likely induces a conformational change in the protein's space, affecting its nearby hydrogen bond network.

To assess the thermodynamic stability of the protein, we performed experiments which revealed that substituting Phe with Leu at position 100 resulted in a ΔΔG value of –1.841 kcal/mol, indicating a decrease in protein stability. Additionally, analysis using PolyPhen predicted that the p.F100L variant would have a harmful effect on the protein's GTA. Consequently, we suggest that the amino acid substitution at position 100 leads to alterations in the hydrogen bond network within the protein's overall structure and reduces the stability of GTA.

The SNV that leads to a reduction in the formation of the A antigen presents a challenge in accurately and promptly determining the proband's blood type, thus hindering timely and accurate blood transfusion treatment. While homologous transfusion is the recommended strategy for ABO subgroups, the low occurrence of these subtypes makes it difficult to find compatible blood. It has been reported that the patient with A subgroup could produce anti-A_1 antibodies after transfusion with 4 units of red blood cells[21]. As a result, individuals with ABO subgroups are advised to receive washed red blood cells of O-type, along with platelets or plasma of AB-type, in order to ensure the safety of the blood transfusions. Blood type AB is not invariably the optimal choice for transfusing non-red blood cell components (platelets, plasma, and cryoprecipitate) in all subgroups of patients. This is not only due to the relatively scarce supply of AB type components but also due to the presence of strong ABO antibodies in the recipient's plasma, which can reduce the lifespan of transfused platelets. In such cases, the selection of non-RBC components should be guided by the results of reverse typing. It's noteworthy that in several instances, we have successfully transfused A type platelets to patients of the A_{el} subgroup without observing any hemolytic reactions post-transfusion (*data not shown*).

CONCLUSIONS

In conclusion, one novel "weak"-type ABO subgroup allele was identified. This aminoacid change p. F100L can change the local conformation of GTA and reduce the protein stability resulting in the "weak"-type phenotype.

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AUTHORSHIP CONTRIBUTIONS

XHC and HL contributed equally as co-last Authors. XHC and HL supervised the research and reviewed and edited the manuscript. YQS and JSG analysed the data and wrote the first draft of the manuscript. YYZ , NZS and LC performed the research. JML and HL designed the research study.

The Authors declare no conflict of interests.

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