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Original article

Genetic and mechanistic evaluation of an individual with para-Bombay phenotype associated with a compound heterozygote comprising two novel *FUT1* variants

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Background - As is well documented, the para-Bombay phenotype is typically characterized by the reduction or absence of ABH antigens on red blood cells but the presence of corresponding antigens in saliva. Herein, the underlying molecular mechanism of an individual with para-Bombay AB phenotype combined with two novel variants of the *FUT1* gene was investigated.

Materials and methods - ABH antigens and antibodies were detected in the serum of the proband using conventional serological methods. The coding region nucleotides of the *ABO*, *FUT1*, and *FUT2* genes were directly sequenced by polymerase chain reaction. Moreover, the *FUT1* haploid type in the proband was analyzed by TA clone sequencing. The 3D structure of wild-type and mutant fucosyltransferases were simulated and analyzed using Phyre2 and Pymol software. Lastly, the effect of missense substitution on the function of fucosyltransferase was predicted by the Polymorphism Phenotyping algorithm (PolyPhen-2) and MutationTaster.

Results - ABH antigens were noted to be absent on the surface of red blood cells of the proband. The ABO genotype was *ABO*A1.02/ABO*B.01*, while the FUT2 genotype was *FUT2*01/FUT2*c.357T*. Interestingly, two novel missense variants (c.289G>A, p.Ala97Thr and c.575G>C, p.Arg192Pro) and one synonymous SNP (c.840G>A) were identified in the *FUT1* gene. Furthermore, c.289G>A was detected in one haploid type, whereas c.575G>C and c.840G>A were discovered in another haploid type. Meanwhile, in silico analysis revealed that amino acid substitution caused by missense variants altered the partial spatial structure of the α-helices where residues 97 and 298 were located using 3D homology modeling software. Finally, both missense variants were defined as probably damaging based on PolyPhen-2 prediction.

Discussion - Two novel *FUT1* variants were identified in a Chinese individual with para-Bombay AB phenotype, which can expand our understanding of the molecular mechanism underlying the para-Bombay phenotype and contribute to improving the safety of blood transfusion.

Keywords: *para-Bombay, FUT1, variant, molecular mechanism.*

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INTRODUCTION

Human red blood group H antigens serve as the precursor substance for A and B antigens of the ABO blood group[1]. Therefore, the antigens of the ABO blood group can be directly impacted by variations in substance H, which is encoded by α-(l,2)-fucosyltransferase genes, *FUT1* and *FUT2*[2]. Indeed, variations in the *FUT1* affect α2-fucosyltransferase (α2FucT1) activity and result in deficient or absent H antigen synthesis in red blood cells (RBCs), whilst variations in the *FUT2* (encode α2-fucosyltransferase, α2FucT2) determine H antigen expression in secretions[3-5]. Total absence or weak expression levels of the H, A, and B antigens is referred to as Bombay and para-Bombay phenotype, respectively[6-8]. Prior studies have established that the molecular mechanisms of these phenotypes vary in different populations[2,9-11]. Notably, the incidence of para-Bombay individuals was estimated to be approximately 1:8,000 in Taiwan, 1:8,500 in the Fujiang province, and 1:15,620 in Hong Kong[12-14]. This phenotype is frequently accompanied by a significant decrease in the function of α2FucT1 and a normal function of α2FucT2. To date, more than 70 *FUT1* variants associated with H antigen deficiency have been cataloged by the International Society of Blood Transfusion (ISBT) Red Cell Immunogenetics and Blood Group Terminology Working Party. This study aimed to analyze the molecular mechanism of a Chinese individual with para-Bombay phenotype associated with two novel *FUT1* variants.

MATERIALS AND METHODS

Study specimen

The proband was a 22-year-old female blood donor. Owing to discrepancies in red blood cell and serum grouping and a negative reaction with anti-H, the specimen was suspected to be a para-Bombay phenotype, and blood samples were sent to the Blood Center of Zhejiang Province for further testing. The individual provided the informed consent form, and the research was approved by the ethics committee of the Blood Center of Zhejiang Province. A total of 5 mL venous blood was collected from the proband. Genomic DNA was extracted from blood cells using a commercial DNA isolation kit (RBC Bioscience Corporation, Taiwan) according to the manufacturer's instructions.

ABH antigen detection on RBCs by serological method

Routine ABO phenotyping was carried out with conventional tube test[15]. Monoclonal anti-A, anti-B, anti-A, B, and anti-H (IgM) reagents (Shanghai Blood Biotechnology Co., Shanghai, China) were used for ABO forward blood grouping by the tube test. A1, B, and O cells were used to determine the antibodies in the serum.

ABO genotyping by PCR-SBT method

The sequence in the full coding region, including exons 1 to 7 of the *ABO* gene, was amplified and bidirectionally sequenced using polymerase chain reaction sequencing-based typing (PCR-SBT) according to our previous reports[16,17]. Sequencing data was assembled by the software Seqscape 2.5 (Applied Biosystems, Foster City, CA, USA). The *ABO* genotype of the proband was determined according to the nucleotide polymorphism compared with the reference sequence of the *ABO*A1.01* allele (NG_006669.1).

Sequencing of *FUT1* and *FUT2* genes

The DNA fragments of the *FUT1* and *FUT2* genes encompassing the full coding region and partial intron sequence were amplified, respectively. The primer sequences and amplification conditions were reported in our previous study[18]. The amplicons were clean with enzymatic (exonuclease and shrimp alkaline phosphatase [Exo-SAP]) digestion and then directly sequenced. Next, the acquired sequences were analyzed by Seqscape 2.5 software (Applied Biosystems). *FUT1* and *FUT2* genotypes were identified based on the nucleotide polymorphism compared with the standard reference of the *FUT1* and *FUT2* gene (NG_007510.1 and NG_007511.1, respectively).

Haplotype analysis of the novel FUT1 alleles

To investigate the novel *FUT1* alleles, the haploid was analyzed by TA clone sequencing according to our previous article[19]. Briefly, the PCR amplicon of the *FUT1* gene was directly subcloned within the pCR4-TOPO TA vector and transformed into TOP-10 *Escherichia coli* (Invitrogen, Carlsbad, CA, USA). Thereafter, the positive recombinant colonies were randomly selected and cultured in LB after successful transformation. Plasmid DNA was extracted and purified from each colony by a spin kit (3S spin Plasmid MiniPrep Kit V3·1, Shenergy Biocolor, Shanghai, China) and subsequently sequenced. Finally, the haplotype of the *FUT1* gene was assigned based on the retrieved sequencing data.

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Table I - *Serological analysis of ABH antigens and antibodies in saliva*

	Forward grouping				Reverse grouping			
	Anti-A	Anti-B	Anti-A,B	Anti-H	Ac	Bc	Oc	Auto-cell
Proband	0	0	0	0	1+	1+	2+	0

Ac: A cells; Bc: B cells; Oc: O cells; 0: no agglutination.

3D model of mutant fucosyltransferase in silico

The initial molecular model of wild-type and mutant fucosyltransferases were constructed according to the unliganded X-ray structure template of fucosyltransferase (PDB code, 2HLH) through homology modeling using Phyre2[20]. The aberrant protein structure was aligned with wild-type and analyzed via the Pymol software[21] (Schrödinger Inc, New York, NY, USA).

Prediction of the damaging effect of the variant on fucosyltransferase function

The putative deleterious effect of *FUT1* missense variants was predicted on the biological function of fucosyltransferase using bioinformatics software PolyPhen-2 (*http://Genetics.bwh.harvard.edu/pph2/*)[22] and MutationTaster (*https://www. mutationtaster.org/*)[23].

RESULTS

Blood group serological results

The serological results of the ABO blood group and H antigen of the proband are listed in **Table I**. Discrepancies were observed in the ABO phenotyping results between the forward and reverse typing. RBCs were non-reactive with anti-A, anti-B, and anti-A, B in the forward typing. Notably, the H antigen was also negative on the surface of RBCs. A weak agglutination reaction was found in the serum with Ac, Bc, and Oc, respectively (**Table I**), indicating the presence of antibodies in the proband. According to the serological characteristics, the proband was preliminarily assigned as para-Bombay phenotype.

ABO and H blood group genotyping results

The *ABO* genotype of the proband was a normal *ABO*A1.02/ABO*B.01* heterozygote according to the full coding region sequence of the *ABO* gene. Three variants were identified at the heterozygous state in exon 4 of the *FUT1* gene, resulting in an ambiguous genotype (**Figure 1**). A heterozygous single nucleotide variation (SNV) c.357C>T synonymous variant (p.Asn119) was detected in the *FUT2* gene, implying that the proband was secretor. The sequencing results of the *ABO*, *FUT1*, and *FUT2* genes are summarized in **Table II**.

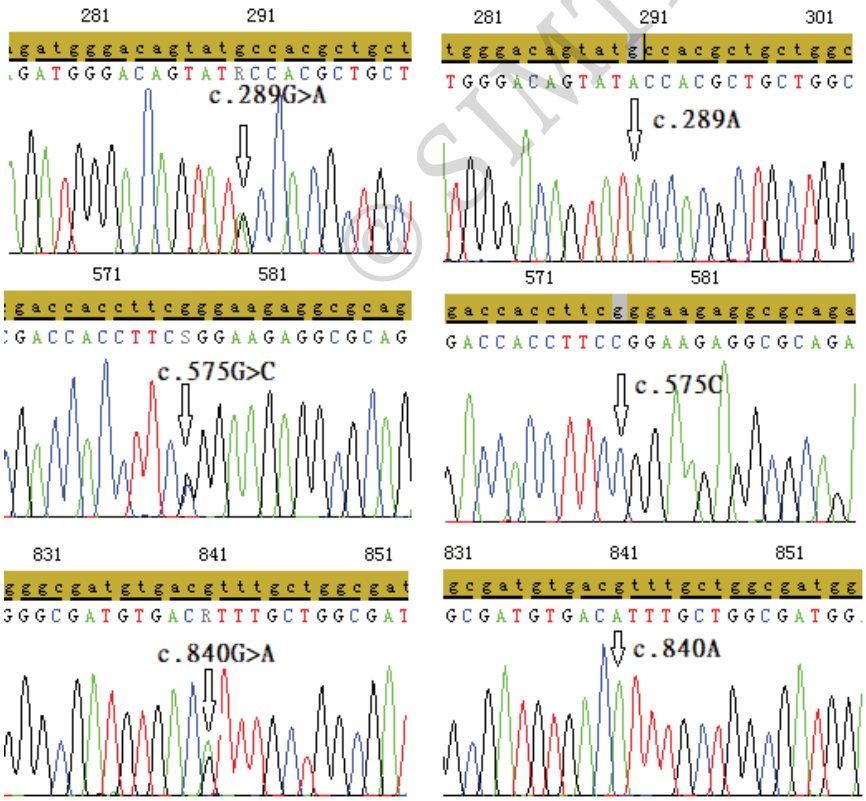

Figure 1 - DNA sequencing and TA clone sequencing
The results of DNA sequencing are displayed on the left. Three heterozygous variants, c.289G>A, c.575G>C, and c.840G>A, were identified. The results of TA cloning are illustrated on the right. The haplotypes of the *FUT1* alleles were confirmed.

Table II - *Genotype results of ABO, FUT1, and FUT2 in the proband*

	ABO		FUT1				FUT2	
	Nucleotide change	Genotype		Nucleotide change	Amino acid change	Accession No.	Nucleotide change	Genotype
Proband	c.297A>G; c.467C>T; c.526C>G; c.657C>T; c.703G>A; c.796C>A; c.803G>C; c.930G>A	*ABO*A1.02/ ABO*B.01*	*FUT1*01N.41*	c.289G>A	p.Ala97Thr	MT925649	c.357C/T	*FUT2*01/ FUT2*c.357T*
			*FUT1*01N.42*	c.575G>C; c.840G>A	p.Arg192Pro	MT925650		

Haplotype analysis of FUT1

Two novel FUT1 alleles were detected after clone sequencing. One allele was a missense variant of c.289G>A (NC_000019.10:g.48750993C>T), encoding p.Ala97Thr amino acid substitution of the fucosyltransferase. The other one was c.575G>C (NC_000019.10:g.48750707C>G) variant and c.840G>A (NC_000019.10:g.48750442C>T, rs568411797) combination, resulting in p.Arg192Pro substitution only, because c.840G>A was a synonymous single nucleotide polymorphism (SNP). The nucleotide sequences of the two FUT1 novel alleles were submitted to the GenBank Database and were assigned the accession numbers MT925649 and MT925650, respectively. The novel alleles have been nominated as the FUT1*01N.41 and FUT1*01N.42 by the red cell immunogenetics and blood group terminology group in ISBT. The characteristics of the two novel FUT1 alleles are presented in **Table II**, and the sequences are illustrated in **Figure 1**.

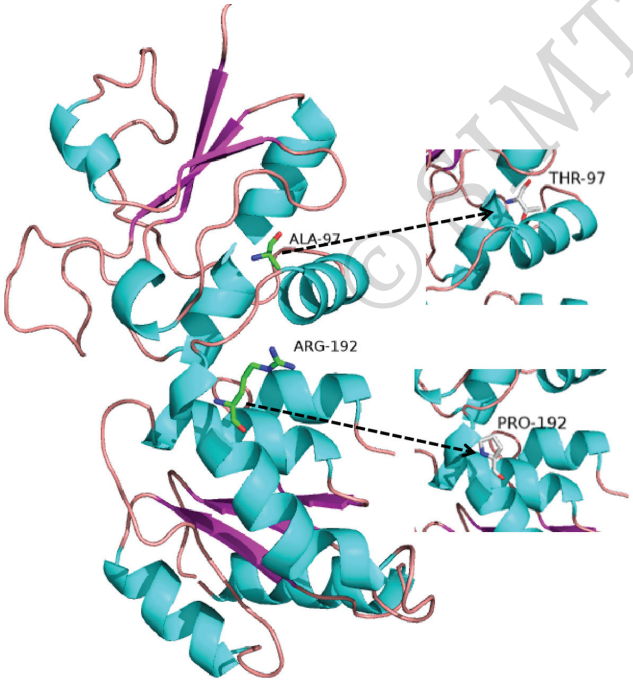

Figure 2 - The overall structure of the wild-type FUT1 enzyme and the side chain of p.Ala97Thr and p.Arg192Pro
Residues 97 and 192 are represented by sticks. The black arrow indicates amino acid substitution.

3D structure simulation and analysis results of the mutant protein

The overall structure of the mutant fucosyltransferases was predicted, which was similar to that of the wild-type protein model. In the 3D structural model, both amino acid substitutions caused by the two missense variants were located in the α-helix region. p.Ala97Thr, caused by the c.289G>A variant, was located in the α-helix spanning Phe89-Asn106, whereas p.Arg192Pro, caused by c.575G>C variant, occupied the α-helix spanning Asp189-Arg206 (**Figure 2**). Differences in the α-helix structure between wild-type and mutant proteins (residue 97 change) are depicted in **Figure 3A**. In the abrupt structure, residue Thr97 increased a hydrogen bond connection with Leu177 in the adjacent α-helix structure, along with a positional displacement of Gln104, which dramatically formed a seemingly closed region in its spatial structure (**Figure 3B** *vs* **3C**). Meanwhile, p.Ala97Thr affected the shift in amino acid side chains of Gln104 and Leu99, with distances of 3.9Å and 2.2Å (**Figure 3A**), respectively. The sequence alignment diagram of the α-helix structure between Arg192 wild-type and Pro192 mutant proteins is displayed in **Figure 4A**. In the wild-type structure, the long side chain of the Arginine pointed inward to the helical structure, whereas the aromatic ring of the mutant Pro192 faced outward (**Figure 4B** *vs* **4C**). Based on the distal C atom of

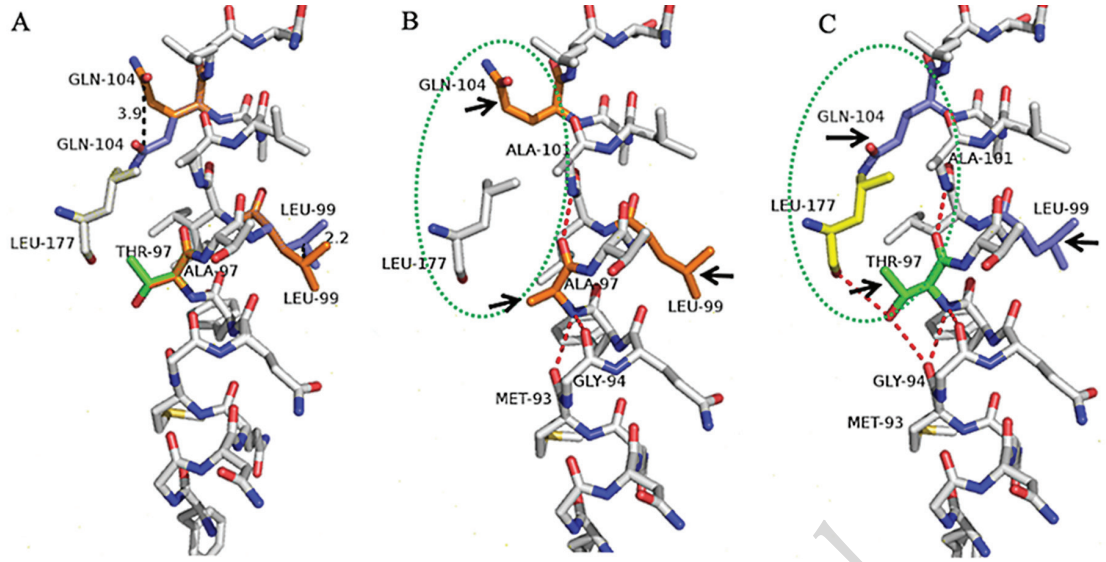

Figure 3 - Differences in the α-helix structure between wild-type and mutant protein (change in amino acid residue 97)
A) Sequence alignment diagram of α-helix structure spanning 89Phe-106Asn. **B**) Sequence of the α-helix structure in the wild-type protein. In the wild-type structure, Ala97 was only connected to Met93 and Gly94 by hydrogen bonds and was not associated with Leu177. **C**) The sequence of α-helix structure changes in the mutant protein. In the mutant protein structure, residue Thr97 increased the hydrogen bond with Leu177 in the adjacent α-helix structure, along with the positional displacement of Gln104, which dramatically formed a seemingly closed region in its spatial structure. The amino acid substitution in residue 97 is represented by the green color. The green dotted circles represent major differences in the α-helix structure between wild-type and mutant protein where the amino acid 97 was located. The golden yellow amino acid corresponding to the purple amino acid denotes the shift in the other amino acids in wild-type and mutant proteins. The black arrows point toward amino acids with major alterations in the spatial structure. The short dashed lines in the protein structure represent hydrogen bonds between amino acids, and the corresponding number represents the distances in shift.

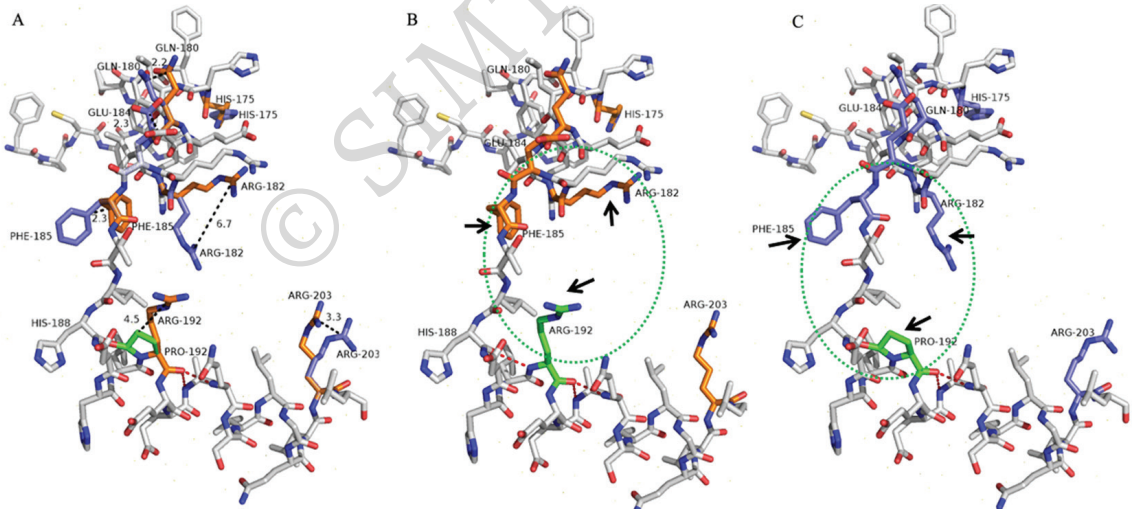

Figure 4 - Differences in the α-helix structure between wild-type and mutant proteins (change in amino acid residue 192)
A) Sequence alignment diagram of α-helix structure spanning 189Asp-206Arg. **B**) Sequence of α-helix structure in the wild-type protein. In the wild-type structure, the long side chain of Arginine pointed inward to the helical structure. **C**) The sequence of α-helix structure changes in the mutant protein. Amino acid substitution at residue 192 is represented by the green color. In the mutant protein structure, the aromatic ring of the Pro192 faced outward. The displacement distance between the two groups was 4.5Å based on the distal C atom of the side chain group. The green dotted circles represent major differences in the α-helix structure between wild-type and mutant protein where the amino acid 192 was located. The golden yellow amino acid corresponding to the purple amino acid denotes the shift in the other amino acids in the wild-type and mutant proteins (His175, Gln180, Glu184, Arg182, Phe185, and Arg203). The black arrows point toward amino acids that have undergone major spatial changes. The short dashed lines shown in the protein structure represent the hydrogen bonds between amino acids, and the corresponding number represents the distances in shift.

the side chain group, the displacement distance between the two groups was 4.5Å. Furthermore, the substitution of amino acid at position 192 led to the displacement of the side chains of other amino acids in the α-helix structure, including Gln180 (2.2Å), Glu184 (2.3Å), Phe185 (2.3Å), Arg182 (6.7Å) and Arg203 (3.3Å) (**Figure 4A**).

Functional impact prediction of protein mutations

In silico analysis uncovered that both p.Ala97Thr and p.Arg192Pro were probably damaging variants with HumDiv and HumVar scores of 1.000/1.000 (score 0.000~0.452 = benign, 0.453~0.956 = possibly damaging, 0.957~1.000 = probably damaging). MutationTaster evaluated that the value was medium, which was indicated to damage the protein function.

DISCUSSION

The para-Bombay phenotype is a rare blood phenotype in China, which congenitally impairs the generation of the H antigen associated with the variants in the *FUT1* and/or *FUT2* genes. Substance H is a key precursor of A and B antigens[1]. Therefore, individuals with the para-Bombay phenotype not only affect the A/B antigens, but may also develop anti-H and/or anti-HI in addition to naturally occurring anti-A and anti-B[24]. Antigenic alteration and antibody formation can potentially result in complicate pre-transfusion testing[25,26]. Numerous novel *FUT1* alleles have been previously reported in the Chinese population[18,19]. In this study, two novel *FUT1* variants were identified in an individual with para-Bombay AB phenotype.

The para-Bombay phenotype is predominantly caused by several mutations in the *FUT1* gene, including missense, deletion, or insertion mutations[3]. Previous studies have demonstrated significant regional differences in the molecular basis among populations around the world[9-11]. The majority of individuals with the para-Bombay phenotype are homozygous in one variant, while few are heterozygous with two different variants[27-29]. In the Chinese population, the phenotype in heterozygotes with para-Bombay is usually accompanied by a null allele, such as *FUT1*01N06* (c.551_552delAG) or *FUT1*01N.13* (c.881_882delTT), which is the most common type[12]. In this study, the proband was a heterozygote of two novel alleles *FUT1*01N.41* and *FUT1*01N.42*, containing two missense variants and one synonymous variant. Moreover, two missense variants identified in the proband were not been previously reported, which was an extremely rare combination. Regrettably, investigation could not be performed on the family members of the proband. These novel variants can enrich the genetic database of the para-Bombay phenotype and yield a better understanding of the molecular mechanism underlying this phenotype in the Chinese population.

The allelic location of the novel variants was clarified by clone sequencing. The change from Alanine to Threonine at residue 97, predicted by the c.289G>A variant, was anticipated to adversely affect enzyme function. Amino acid residue 97 is located in the stem region of 2-α-fucosyltransferase. Storry et al. reported a substitution, p.Gly89Val, that occurred in close proximity to acid residue 97[30], whose serological profiling exposed that the α2FucT1 activity of the donor was decreased. It is worthwhile emphasizing that a mutation in the amino acid of the stem domain of vertebrate α1,3/1,4-fucosyltransferases can alter the specificity of the acceptor substrate[31]. Meanwhile, α2FucT1 displayed similar protein structure regions to α1,3/1,4-fucosyltransferases. Therefore, we speculate that the c.289G>A variant in the stem region has the same effect on the activity of α2FucT1 enzyme. Allele *FUT1*01N.42* was identified as the c.575G>C variant and c.840G>A SNP. Considering that c.840G>A is a synonymous SNP that does not result in alteration in amino acids, we hypothesize that the c.575G>C variant could affect the enzyme activity. Amino acid residue 192 (caused by c.575G>C) was located in the predicted active region of enzyme function, which is in the proximity of the highly conserved α-2 motif I of fucosyltransferase found in vertebrates[32]. Motif structure has been documented to play a pivotal role in transferase activity. Notably, several variants inducing enzyme deficiency were located in these domains[32]. Therefore, the c.289 G>A and c.575G>C variants may be responsible for the para-Bombay phenotype in the proband.

Homologous modeling and 3D structure comparison allow investigation of the effects of the variants on enzyme function. Structural or functional changes can occur through the introduction of amino acids with very different properties[33]. Regarding p.Ala97Thr and p.Arg192Pro substitutions found in this study, the

characteristics of wild-type and mutant amino acids were dramatically altered. Ala97 is a hydrophobic non-polar molecule, whereas Thr97 is a hydrophilic polar molecule. Similarly, residue 192 was replaced from a hydrophilic charged Arginine to a non-polar hydrophobic Proline. We inferred that the substitution of these different amino acids may lead to spatial changes in the helical structure by disrupting the connections between peptides. Besides, the present study noted that Gln104 was directly connected to Leu177 located at the neighboring helix in the p.Ala92Thr mutant protein, forming a semi-closed ring. Lei et al. described a displacement in mutant amino acids and a partial amplitude of turnover of loop spanning Ala115-Phe124 in a three-dimensional structural model of a p.Ala121Thr mutant[34]. Conjointly, these results signal that the composition and sequence of amino acids in the protein exert a decisive effect on the formation and stability of protein structure. In silico prediction of protein function also validated the disruptive effect of the mutations, with a score of 1.000 (maximum likelihood) by PolyPhen-2.

The effects of *FUT1* variants involve both the ABO and H blood group systems and thus lead to a complex serological phenotype, resulting in inappropriate transfusion and serious adverse transfusion reactions. In the clinical setting, further detection and analyses are necessitated in case of discrepancies in ABO serologic profiles for timely detection of the para-Bombay phenotype, because it may be easily misidentified as type O. Anti-H and O cell reagents can be employed whenever the Bombay/para-Bombay phenotype is suspected. Meanwhile, caution is warranted during blood transfusion in patients with the para-Bombay phenotype, especially in patients with reactive antibodies at 37°C. Otherwise, hemolytic transfusion reactions (HTR) or even adverse consequences may occur in case of incompatible transfusions. The transfusion strategies for patients with para-Bombay phenotype are different. In the individuals with weak and only reactive at low temperatures anti-H or absence of anti-H, transfusion with para-Bombay blood is not recommended owing to the limited blood supply. However, blood with para-Bombay or autologous blood transfusion may be considered if the anti-H antibody was reactive at 37°C. To sum up, additional research on the molecular mechanism underlying the para-Bombay phenotype will assist in the identification of the para-Bombay/Bombay phenotype and enable safe and effective blood transfusions.

CONCLUSIONS

In summary, this study revealed the underlying molecular mechanism of an individual with a rare AB-type para-Bombay phenotype. Amino acid substitutions caused by the missense variants c.289G>A and c.575G>C could change the structure of α2FucT1 and subsequently affect enzyme activity. The findings of this study can contribute to the growing database of the H blood group and help elucidate the genetic mechanism underlying the para-Bombay phenotype, which could further ensure the safety of blood transfusion.

ETHICAL CONSIDERATION

This study was approved by the Institutional Ethics Committee of the Blood Center of Zhejiang Province. Written informed consent was obtained from the patient for study participation and data publication.

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AUTHORS' CONTRIBUTIONS

YY performed the research, analyzed the data, and wrote the paper. XH, JZ and KM performed the research. XX collected the specimen. FZ designed the research.

The Authors declare no conflicts of interest.

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