Two novel mutations p. L319V and p. L91P in ABO glycosyltransferases lead to A_{el} and B_{el} phenotypes

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Background - Mutations of the ABO gene may cause the dysfunction of ABO glycosyltransferase (GT) that can result in weak ABO phenotypes. Here, we identified two novel weak ABO subgroup alleles and explored their mechanisms that caused A_{el} and B_{el} phenotypes.

Materials and methods - The ABO phenotyping and genotyping were performed by serological studies and direct DNA sequencing of the ABO gene. The role of the novel mutations were evaluated by a three-dimensional model, predicting protein structure changes, and *in vitro* expression assay. The total glycosyltransferase transfer capacity in supernatant of transfected cells was examined.

Results - We identified a mutation c. 955C>G (p. L319V) of *A* allele in an A_{el} subject and a mutation c. 272T>C (p. L91P) of *B* allele in a B_{el} subject. *In silico* analysis showed that the mutation p. L319V of the *A* allele and p. L91P of the *B* allele may change the local conformation of GT and impair the catalysis of H to A or B antigen conversion. *In vitro* expression study showed that mutation p. L319V impaired H to A antigen conversion, although it did not affect the expression of glycosyltransferase A.

Conclusions - Two novel "el"-type ABO subgroup alleles were identified. Both of the two novel mutations can change the local conformation of GTs and reduce protein stability. GTA mutation p. L319V can impair the conversion from H to A antigen and causes the A_{el} phenotype.

Keywords: *ABO, subgroup, mutation, novel allele.*

INTRODUCTION

The ABO system is the most important blood system in transfusion medicine[1]. The ABO blood groups are determined by the presence and absence of antigen A or B on the surface of red blood cells (RBC) and anti-A, anti-B antibodies in sera. The *ABO* gene is located on chromosome 9q34. 1-q34.2 and is approximately 19.5 kb long containing a 1,062 bp coding region with seven exons and several regulatory elements[2]. ABO antigens on the RBC are biosynthesised by A or B glycosyltransferases (GTs) encoded by the *A* and *B* genes[3]. Both GTA and GTB are type II transmembrane proteins with short cytoplasmic and transmembrane domains, a stem region, and a catalytic domain[4,5]. The GTB differs by only four amino-acid substitutions[2] (p. R176G, p. G235S, p. L266M,

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p. G268A) from GTA. Of those four amino acids, only p. L266M and p. G268A participate in the recognition of donor sugar[6], while p. G235S has been shown to affect acceptor binding, and p. R176G can influence interloop ordering[7].

Advances in molecular biology have allowed the detection of many subgroups with weak expression of A or B antigens on the RBC. For example, the phenotypes of A_3, A_x, A_{el}, cis-AB, B_3, B_x, B_{el} and B(A) have been defined[8] and over 300 *ABO* subgroup alleles have been reported worldwide[9,10]. Most weak ABO subgroups are caused by genetic changes in the *ABO* gene, including missense mutations, insertions, or deletions in the coding regions, splicing sites, or regulatory elements[11-16]. Most gene mutations of the weak ABO subgroups can impair the catalytic capability of GTA or GTB through reducing the expression or conformation changes and dysfunction of enzyme. However, the structural and molecular mechanism of GT mutants are still not clear. In this report, we identified two novel mutations *ABO*A* c. 955C>G (p. L319V) and *ABO*B* c. 272T>C (p. L91P) in two donors with A_{el} and B_{el} phenotypes and explored the possible mechanisms which may have caused these weak subgroups.

MATERIALS AND METHODS

Subjects and phenotype

The EDTA-anticoagulation peripheral venous blood samples used in the study were collected from blood donors of Shanghai Blood Center. The subjects' ABO phenotypes were first determined by the Galileo (Immucor, Norcross, GA, USA). If the results determined by Galileo were discrepant (forward and reverse typing), the results were further explored by manual tube method and adsorption-elution test according to standard methods and procedures, as documented in the AABB Technical Manual[17]. 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, Shanghai, China) and ABO red blood cell kit (SHPBC, Shanghai, China) were used for blood group typing. Samples were collected from apparently healthy random Chinese donors (n=120) as normal controls.

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 (PCR) were designed as previously described[18,19]. The PCR 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 gel-purified products containing the mutation sites were cloned and then sequenced to confirm the haplotypes.

Modelling of the three-dimensional structure of GTs

Unliganded X-ray structure of GTA (PDB code, 4C2S) and GTB (PDB code, 3SXE) were used as the template to construct the initial molecular model. The *in silico* mutations were modeled by the Chimera software (v.1. 11.2. University of California, San Francisco, CA, USA)[20]. The structural figures were also generated in Chimera or prepared by visual molecular dynamics[21].

Predicted effect of the mutation on protein stability

To examine the effect of the mutation on protein stability, the empirical protein design FoldX force field in SNPeffect was used to calculate the difference in free energy of the mutation: ddG (ΔΔG) based on the GTA enzymes (PDB code, 4C2S) and the GTB enzymes (PDB code, 3SXE). If the mutation destabilises the structure, ΔΔG is increased, whereas stabilising decrease the ΔΔG. Since the FoldX error margin is around 0. 5 kcal/mol, changes in this range were considered insignificant.

In vitro expression of GTA mutant p. L319V

The full length *ABO*A 1.01* cDNA was cloned into expression vector pcDNA3.1 containing the *1X FLAG* gene. The mutation p. L319V was introduced by site-directed mutagenesis (Quick-Chang mutagenesis kit, Stratagene, Germany). HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum. HeLa cells (5×10^5) were then transfected with 1ug wild-type or mutant type expression vector by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 1ug expression vector without ABO cDNA was transfected as negative control. After 48 hours, the transfected HeLa cells and supernatant were collected.

Total GTA transfer capacity in supernatant of transfected HeLa cells was determined according to a previous report[16]. The agglutination was scored by standard serological tube method.

Western blot

The transfected cells were collected and lysed. The proteins of cells lysates were measured by the BCA method according to the manufacturer's instructions. Cell lysates were then tested by western blotting with rabbit anti-FLAG antibody (Sigma-Aldrich, St Louis, MO, USA). The β-actin protein in the transfected cells was used as control. The protein bands were detected by chemiluminescence reagents (Thermo Fisher Scientific, Rockford, IL, USA).

RESULTS

Blood group phenotype characterisation

The serological results of ABO phenotype showed a discrepancy between the forward and reverse typing (**Table I**). RBCs of subject S575 did not react with anti-A, anti-B, anti-AB serum and anti-A_1 in the tube tests, even when incubated at 4 °C. However, the RBCs of subject S575 exhibited a strong agglutination with anti-H; the absorption-elution test showed the presence of a weak A antigen on the RBCs. Subject S575 was suspected to be the A_{el} subgroup, since a blood group chimera could not be excluded. The serological results of subject S425 were similar to those of subject S575 and were suspected to be an indication of the B_{el} subgroup (**Table I**).

ABO gene sequence analysis

According to the sequence analysis, two novel mutations were detected (**Table II**) which were not found in 120 random healthy Chinese individuals with common ABO blood groups. A novel heterozygous mutation c. 955C>G (p. L319V) was detected in the *A* allele in subject S575 (Genbank No. KJ130041) and mutation c. 272T>C (p. L91P) in the *B* allele in subject S425 (Genbank No. KF956525). The novel mutations have been submitted for ISBT allele designation. Combined with the gene analysis results, we identified these two variant phenotypes as A_{el} and B_{el}.

Three-dimensional structural analysis of the mutants

The complete structure of the unliganded wild-type GTA and GTB were mainly built from the crystal structure 4C2S and 3SXE (**Figure 1A**). The overall structures of the mutant GTs are predicted to be similar to that of the wild-type GTs. In the wild-type GTA (**Figure 1B**, left), a hydrogen bond framework was formed by L232. Hα-L319. Oα, G230. Oα-L319. Hα, V95. Oα-S320. Hα and V95. Oα-H of hydroxyl S320. In the GTA p. 319V mutant (**Figure 1B**, right), the hydrogen bonds formed by G230. Oα-L319. Hα and V95. Oα-S320. Hα disappeared. In the wild-type GTB (**Figure 1C**, left), a strong hydrogen-bond framework was formed by L91. Hα-T88. Oα, A92. Oα-T88. Hα and Oxygen on the side chain of T88-A92. Hα, while in the mutation GTB (**Figure 1C**, right), the former hydrogen bond framework disappeared. Thus, the p. L319V and p. L91P mutation can change the local conformation of GTs by damaging the hydrogen bonds.

Prediction of mutant protein stability

Protein stability is believed to be affected by the alterations in the number of hydrogen bonds, disruption

Table I - *ABO subgroup phenotypes for the two blood donors*

Subject n.	Forward typing				Reverse typing				Absorption-elution		
	Anti-A	Anti-A_1	Anti-B	Anti-AB	Anti-H	A_1	B	O	A_1	B	O
S575	-	-	-	-	3+	-/1+*	4+	-/-*	1+	nd	-
S425	-	-	-	-	4+	4+	1+w	-	nd	1+	-

*The reactions were observed both at room temperature (left) and 4 °C (right).
A_1 : red blood cells with A_1 phenotype; B: red blood cells with B phenotype; O: red blood cells with O phenotype; - : negative reaction; w : weak reaction; nd: not determined.

Table II - *The two novel ABO subgroup alleles in this study*

Subject n.	Allele	Critical nt change	Corresponding aa change	Phenotype	Genotype	Genbank n.
S575	*AEL-var*	c. 955C>G	p. L319V	A_{el}	*AEL-var /O. 01*	KJ130041
S425	*BEL-var*	c. 272T>C	p. L91P	B_{el}	*BEL-var /O. 01*	KF956525

aa: amino acid.; nt: nucleotide.

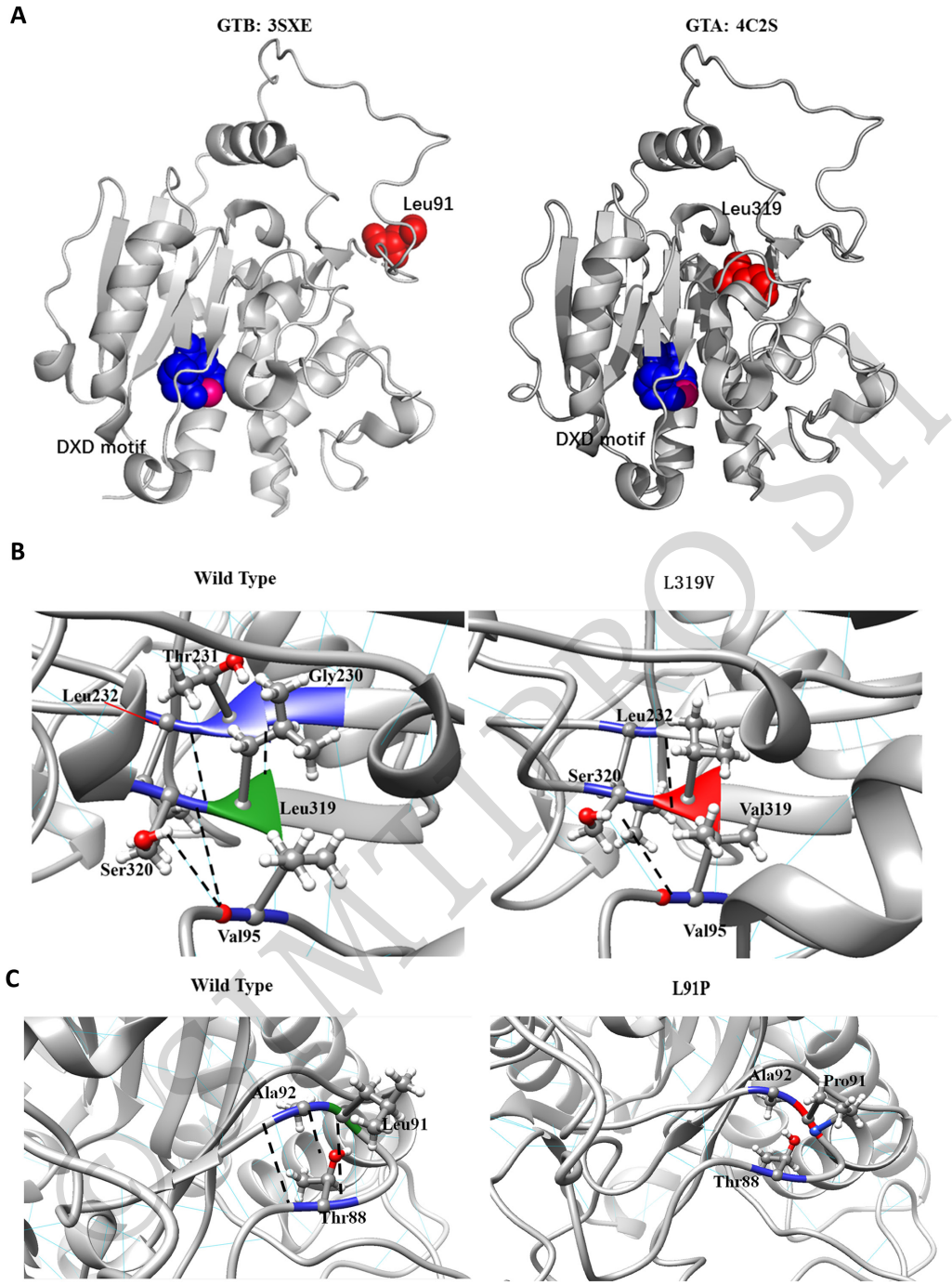

Figure 1 - The different conformations and hydrogen-bond framework in wild-type and mutant glycosyltransferase (GT)
Black dotted line indicates a hydrogen-bond. (A) The overall structure of the wild-type GTs. The sides of L319 in GTA and L91 in GTB in red. (B) Local ribbon drawing of wild-type and mutant GTA. Main chain of L319 in green and V319 in red; main chains of L232, T231, G230, S320 and V95 in blue. Other elements of the amino acids were coloured by elements in ball and sticks method. O, C and H atoms represented by red, grey and white balls. Black dotted line indicates a hydrogen bond. In the wild-type GTA (left), a hydrogen bond framework was formed by L232. Hα-L319. Oα, G230. Oα-L319. Hα, V95. Oα-S320. Hα and V95. Oα-H of hydroxyl S320. In the GTA p. 319V mutant (right), the hydrogen bonds formed by G230. Oα-L319. Hα and V95. Oα-S320. Hα disappeared. Figures were generated by Chimera software. (C) The local ribbon drawing of wild-type and mutant GTB. Main chain of L91 in green and P91 in red; main chains of A92 and T88 in blue; O, C and H atoms represented by red, grey and white balls. Black dotted line indicates a hydrogen bond. In the wild-type GTB (left), a strong hydrogen-bond framework was formed by L91. Hα-T88. Oα, A92. Oα-T88. Hα and Oxygen on side chain of T88-A92. Hα. In the GTB p. L91P mutant (right), the former hydrogen bond framework disappeared.

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of salt bridges, or other changes in protein folding. Based on a three-dimensional (3D) structure analysis, hydrogen bands may disappear in the presence of the GTB mutation p. L91P and GTA mutation p. L319V. Thus, GTA and GTB stability may be affected by the mutations. To evaluate the effect of the mutations on GT stability, we calculated the protein thermodynamic stability changes of the mutants. We built homology models based on 4C2S PDB and 3XSE PDB by FoldX.

The mutation from LEU to PRO at position 91 of GTB resulted in a ΔΔG of 3.81 kcal/mol. The mutation from LEU to VAL at position 319 results in a ΔΔG of 4.96 kcal/mol. These imply that both of the mutations can reduce protein stability, which is consistent with the findings in the 3D modeling analysis that the two novel mutations can destroy the local conformation.

***In vitro* expression of GTA and mutant p. L319V in HeLa cells**

The catalytic ability of supernatant from HeLa cells transfected with wild-type GTA to convert O type RBC into A type was still at high dilutions (1:512), while the p. L319V mutant GTA could only reach 1:64 (**Figure 2A**). Western blot showed the synthesis of GTA protein was not significantly affected by the p. L319V mutation (**Figure 2B**). Thus, the weak A antigen expression on RBCs may be caused by the dysfunction of the p. L319V mutant.

DISCUSSION

In this study, we identified two separate ABO mutations in A_{el} and in B_{el} phenotypes, both of which can impair the function and stability of GTs and significantly down-regulate the expression of A or B antigens on the RBCs.

A_{el} is a type of ABO subgroup that expresses the least amount of A antigens: approximately 1/1,000 of the antigen detected as A_1. The RBCs with A_{el} blood type shows no agglutination by anti-A sera. The A type antigen on the RBCs can be detected by adsorption-elution test. Until 2012, nine *Ael* alleles had been identified and reported[15,22-26]. Because we have found more than twenty ABO blood group chimera in healthy Chinese individuals from SBC and Ruijin Hospital with very similar serological characteristics with "el" phenotype (*L Hang and C Xiaohong, 2019, unpublished data*), A_{el} subgroup was not confirmed until a mutation c. 955C>G (p. L319V) was detected in *A* allele in subject S575. It is noteworthy that, although the limitation of DNA meant that we could only use direct sequencing, the conventional SSP-PCR is

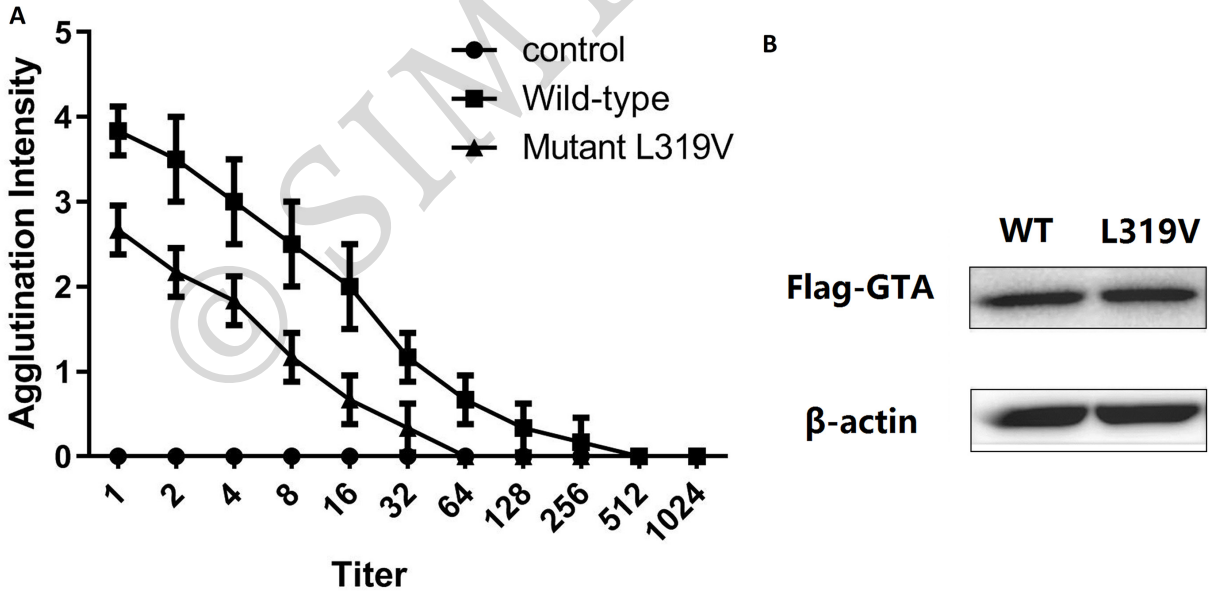

Figure 2 - p. L319V did not affect glycosyltransferase A (GTA) expression but did affect its function
(A) The dots in the plot represent the mean agglutination intensity±standard deviation of three independent experiments using a serial dilution of monoclonal anti-A antibody up to 1,024-fold. p<0.01 by one-way ANOVA. (B) Western blot analysis of GTs produced by HeLa cells transfected with wild-type (WT) and mutant constructs. β-actin was used as loading control.

also very useful for 'el' subtypes, and can usually provide information faster. Serological results in S575 showed that the mutant GTA can significantly reduce the expression of A antigens on RBCs. However, western blot analysis shows the synthesis and expression of GTA protein in HeLa cells are not affected by mutation p. L319V. The total GTA transfer capacity test showed the mutant GTA has lost the capability to convert O type into A type cells. Thus, the expression of GTA on the HeLa cells transfected with the c. 955C>G (p. L319V) mutation is not affected, and the low expression of A antigens on the RBCs may be caused by the dysfunction of the mutation GTA.

B_{el} is a subgroup of the B blood group in which RBCs are not agglutinated by anti-B or antiA_1B. No B glycosyltransferase can be detected in the serum of B_{el} subjects. A weak anti-B might be present in the serum of subjects with this subgroup. At present, this extremely rare phenotype can only be determined by adsorption and elution analysis of anti-B, and B_{el} phenotype is usually misidentified as O phenotype or A phenotype (AB_{el}). Ogasawara *et al.* first described a molecular genetic analysis of the B_{el} phenotype, and two B_{el} alleles were identified in Japanese individuals[22] and nine B_{el} alleles have been identified and reported. The nine alleles have one additional non-synonymous substitution on the *B.01* haplotype background, as listed: c. 502C>T (p. R168W), c. 641T>G (p. M214R) and c. 669G>T (p. E223D)[27].

The results of subject S425 were similar to those of subject S575, and were identified as B_{el} subgroup when mutation c. 272T>C (p. L91P) was detected in the *B* allele. Neither of the two mutations (p. L319V and p. L91P) had been found previously in weak ABO subgroup individuals. Leucine and Valine are the two of the three amino acids with a branched hydrocarbon side chain, and are hydrophobic and generally buried in folded proteins, while Proline is the only cyclic amino acid which is formally an amino acid without a hydrogen on the α amino group. The 3D structural modelling studies showed that a hydrogen-bond framework may be destroyed in the presence of the mutant p. L319V and that it disappears in the p. L91P mutant. We calculated the thermodynamic stability changes of the mutant GTs, and found 3.81 kcal/mol in p. L91P mutation and 4.96 kcal/mol in p. L319V mutation. These findings suggest that GTA L319 and GTB L91 are essential to the function of the protein, and the mutations may cause the inadequate conversion of H antigen and lead to a weak phenotype.

CONCLUSIONS

In conclusion, two novel "el"-type ABO subgroup alleles were identified**.** GTA mutation p. L319V and GTB mutation p. L91P can change the local conformation of GTs and reduce protein stability, resulting in the "el"-type phenotype.

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AUTHORSHIP CONTRIBUTIONS

HL and ZW contributed equally to this work.

XC, DX and XW are responsible for experiment design and development. HL, ZW and YW are responsible for phenotype identification. HL and ZW are responsible for carrying out and analysing the *in vitro* experiments. XC is responsible for carrying out and analysing the molecular modelling and MD simulations. HL and XC are responsible for writing and editing the manuscript.

The Authors declare no conflict of interests.

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REFERENCES

1. Yamamoto F. Molecular genetics of ABO. Vox Sang 2000; **78** (Suppl 2): 91-103.
2. Yamamoto F, Clausen H, White T, et al. Molecular genetic basis of the histo-blood group ABO system. Nature 1990; **345**: 229-33.
3. Morgan WT, Watkins WM. Unravelling the biochemical basis of blood group ABO and Lewis antigenic specificity. Glycoconj J 2000; **17**: 501-30.
4. Paulson JC, Colley KJ. Glycosyltransferases. Structure, localization, and control of cell type-specific glycosylation. J Biol Chem 1989; **264**: 17615-8.
5. Yamamoto M, Cid E, Yamamoto F. Crosstalk between ABO and Forssman (FORS) blood group systems: FORS1 antigen synthesis by ABO gene-encoded glycosyltransferases. Sci Rep 2017; **7**: 41632.
6. Patenaude SI, Seto NO, Borisova SN, et al. The structural basis for specificity in human ABO(H) blood group biosynthesis. Nat Struct Biol 2002; **9**: 685-90.
7. Gagnon SML, Legg MSG, Polakowski R, et al. Conserved residues Arg188 and Asp302 are critical for active site organization and catalysis in human ABO(H) blood group A and B glycosyltransferases. Glycobiology 2018; **28**: 624-36.
8. Chester MA, Olsson ML. The ABO blood group gene: a locus of considerable genetic diversity. Transfus Med Rev 2001; **15**: 177-200.
9. Moller M, Joud M, Storry JR, Olsson ML. Erythrogene: a database for in-depth analysis of the extensive variation in 36 blood group systems in the 1000 Genomes Project. Blood Adv 2016; **1**: 240-9.
10. Ying Y, Chen S, Ma K, Hong X, Zhu F. Identification of a novel B allele with missense mutation (c. 98G > C) in the ABO gene. Transfusion 2017; **57**: 219-20.
11. Hosseini-Maaf B, Letts JA, Persson M, et al. Structural basis for red cell phenotypic changes in newly identified, naturally occurring subgroup mutants of the human blood group B glycosyltransferase. Transfusion 2007; **47**: 864-75.
12. Seltsam A, Gruger D, Just B, et al. Aberrant intracellular trafficking of a variant B glycosyltransferase. Transfusion 2008; **48**: 1898-905.
13. Hult AK, Yazer MH, Jorgensen R, et al. Weak A phenotypes associated with novel ABO alleles carrying the A2-related 1061C deletion and various missense substitutions. Transfusion 2010; **50**: 1471-86.
14. Sano R, Nakajima T, Takahashi K, et al. Expression of ABO blood-group genes is dependent upon an erythroid cell-specific regulatory element that is deleted in persons with the B(m) phenotype. Blood 2012; **119**: 5301-10.
15. Cai X, Jin S, Liu X, et al. Molecular genetic analysis of ABO blood group variations reveals 29 novel ABO subgroup alleles. Transfusion 2013; **53**: 2910-6.
16. Cai X, Qian C, Wu W, et al. An exonic missense mutation c. 28G>A is associated with weak B blood group by affecting RNA splicing of the ABO gene. Transfusion 2017; **57**: 2140-9.
17. Fung MK, Eder AF, Spitalnik SL, Westhoff CM. *AABB Technical Manual*. 19th Ed. Bethesda (MD): AABB, 2017: 777-778.
18. Cai XH, Jin S, Liu X, et al. Molecular genetic analysis for the B subgroup revealing two novel alleles in the ABO gene. Transfusion 2008; **48**: 2442-7.
19. Olsson ML, Irshaid NM, Hosseini-Maaf B, et al. Genomic analysis of clinical samples with serologic ABO blood grouping discrepancies: identification of 15 novel A and B subgroup alleles. Blood 2001; **98**: 1585-93.
20. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem 2004; **25**: 1605-12.
21. Humphrey W, Dalke A, Schulten K. VMD visual molecular dynamics. pdf. Journal of Molecular Graphics 1996; **14**: 33-8.
22. Ogasawara K, Yabe R, Uchikawa M, et al. Molecular genetic analysis of variant phenotypes of the ABO blood group system. Blood 1996; **88**: 2732-7.
23. Yu Q, Deng ZH, Wu GG, et al. Molecular genetic analysis for a novel Ael allele of the ABO blood group system. J Hum Genet 2005; **50**: 671-3.
24. Wu GG, Yu Q, Su YQ, et al. Novel ABO blood group allele with a 767T>C substitution in three generations of a Chinese family. Transfusion 2005; **45**: 645-6.
25. Sun CF, Chen DP, Tseng CP, et al. Identification of a novel A1v-O1v hybrid allele with G829A mutation in a chimeric individual of AelBel phenotype. Transfusion 2006; **46**: 780-9.
26. Olsson ML, Thuresson B, Chester MA. An Ael allele-specific nucleotide insertion at the blood group ABO locus and its detection using a sequence-specific polymerase chain reaction. Biochem Biophys Res Commun 1995; **216**: 642-7.
27. Sousa N, Anicchino-Bizzacchi JM, Leite EM, et al. Association of ABO gene mutations resulting in a rare B subgroup. Vox Sanguinis 2005; **88**: 31-4.