

# A Novel Mutation Eliminates GATA-1 and RUNX1-Mediated Promoter Activity in Galactosyltransferase Gene

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## Keywords

B deletion · ABO subgroup · Mutation · Promoter · Transcription factor

## Abstract

**Introduction:** Mutations in the promoter region and exons of ABO gene may cause changes in the expression of blood group antigens, often showing a weak ABO phenotype. Here, we identified a novel weak ABO subgroup allele that caused B<sub>el</sub> phenotype and explored its mechanisms. **Methods:** The ABO phenotype of subjects (Chinese Han nationality) was classified by serological method. The plasma activity of erythrocyte glycosyltransferase was detected by the phosphate coupling method. ABO subtype genotyping was performed by PCR-SSP and exon sequencing. The activity of the promoter was evaluated by a dual-luciferase reporter assay. **Results:** We identified a mutation exon 1 c.15\_16insTGTTG of the B allele in a B<sub>el</sub> subject. Genealogical investigation showed that the mutation was inherited from her mother. The mutation was located in the promoter region of the ABO gene. The dual-luciferase reporter assay showed that the mutation inactivated GATA-1 and RUNX1-mediated activity of the ABO gene promoter, leading to a decrease in the expression and activity of B glycosyltransferase. **Conclusion:** A novel B<sub>var</sub> ABO subgroup allele was identified. The novel mutation can reduce the promoter activity that activated by GATA-1 and RUNX1, subsequently causing the B<sub>el</sub> phenotype.

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## Introduction

The ABO blood group system includes four antigens, A, B, AB, and A1, and the corresponding antibodies, anti-A and anti-B [1]. A and B antigens have a common precursor substance-H antigen. Type A individuals have N-acetylgalactosyltransferase (A enzyme), and N-acetylgalactose is added to the fucose end of H antigen to produce A antigen; type B individuals have galactosyltransferase (B enzyme), galactose is added to the end of the H antigen glycosyl to produce B antigen; type O individuals do not have A enzyme and B enzyme, and cannot produce A or B antigen, so there is only H antigen on the cell [2]. ABH antigen is not directly encoded by the ABO gene but is an antigen formed by encoding glycosyltransferase, which then binds specific chains to the cell membrane surface [3]. The ABO gene is located on chromosome 9 and contains 7 exons. Exons 6 and 7 account for 77% of the gene sequence. They are the structural regions encoding ABO glycosyltransferases. When the ABO gene is mutated, including mutations in the promoter region and exons, it may cause changes in the expression of blood group antigens, which are manifested through serological reactions, often showing a weak expression pattern. In addition to promoter region, studies have shown that intron 1 of the ABO gene also contains the region that regulates promoter activity [4]. Therefore, when a suspected subtype is found in the routine ABO typing test using serological experiments, sequencing, and cell biology experiments can be used to discover new mutation sites and new subtype formation mechanisms.

In this study, we found a case of blood donor of sub-type B who had extremely weak B antigen and low serum glycosyltransferase activity. A new insertion mutation in the exon 1 of the ABO gene was identified by exon sequencing. This mutation reduced the promoter activity of the ABO gene that could be activated by transcription factors GATA-1 and RUNX1, leading to the B<sub>el</sub> phenotype.

## Materials and Methods

### Sample Collection

The whole blood samples (5 mL) with EDTA-K2 anticoagulant were collected from the proband volunteer blood donor, her parents and sister in the Blood Center of Loudi City, China. The proband was found with ABO blood group discrepant results between the serologic forward and reverse typing. Samples collected from healthy random donors ( $n = 2$ ) were used as normal controls. The ethnicity of the investigated individuals was Chinese Han nationality.

### Serologic Typing

ABO phenotypes were determined by agglutination and adsorption-elution tests, and serologic diagnostic classification according to standard methods and procedures as documented in the AABB Technical Manual [5]. The ABO microcolumn gel card was purchased from Changchun BoXun Co. (Changchun, China); monoclonal anti-A, anti-B, anti-AB, and anti-H antibodies and standard red blood cells were purchased from SHPBC Co. (Shanghai, China).

For adsorption-acid elution testing, 1 mL of red cells to be tested was washed three times with saline, added with 1 mL reagent anti-B, and then incubated at 4°C for 1 h. After centrifuging, supernatant reagent was removed. The red cells were transferred to a clean test tube and washed with cold saline eight times. The antibody on the red cells was eluted by glycine-HCL solution (0.1 mol/L, pH = 1.5) and neutralized with trimethylol aminomethane solution (1 mol/L). After centrifuging at 1,000 g/min for 1 min, the supernatant was collected and used to test anti-B with B red cells. The agglutination was examined after immediate centrifugation.

### Saliva Testing with Anti-H, Anti-A, and Anti-B

The saliva (5 mL) was obtained from subjects and centrifuged at 1,000 g/min for 10 min. The supernatant was boiled for 10 min and centrifuged at 1,000 g/min for 10 min. One drop of standardized antiserum (anti-H, anti-A, or anti-B) and 1 drop of saliva were mixed well in each tube and incubated at room temperature for 30 min. Then, 1 drop of 5% red blood cell (O, A or B) was added into each tube and incubated at room temperature for 1 h. After centrifugation at 1,200 g/min for 15 s, the results were obtained. Saline was used as a negative control, and the known secretory and non-secretory types were used as positive controls. If anti-H, anti-A or anti-B failure to agglutinate with O, A or B red cells, respectively, after incubation with saliva, indicated that the saliva contains the corresponding antigen, otherwise the saliva did not contain the corresponding antigen.

### ABO Genotyping and Sequencing

The whole blood samples from subjects were centrifuged at 1,000 g/min for 3 min to obtain the red blood cells and white blood cells. A human red blood cell ABO blood group genotyping kit (Tianjin Super Biotechnology Developing Co., Ltd, Tianjin, Chi-

na) was used for ABO genotyping according to the manufacturer's instructions [6]. Genomic DNAs were extracted from the white blood cells of proband and her mother using a QIAamp Blood Kit (Qiagen, Germany) according to the manufacturer's instruction. The exons 1–7 of ABO gene were amplified using 2 × Taq Master Mix (Vazyme, P111-02) according to the manufacturer's instructions. The primers of exons amplification were provided by Tianjin Super Biotechnology Developing Co., Ltd (Tianjin, China). PCR products were purified by using an agarose gel DNA purification kit (TaKaRa) and ABO gene exon sequencing was performed by Thermo Fisher Corporation. The resulting sequence data were compared with GenBank Accession Number NG\_006669 using Chromas pro and DNAMAN V8.0 software.

### Measurement of the Glycosyltransferase B Activity

A glycosyltransferase activity kit (Catalog # EA001, R&D Systems, Inc. Minneapolis, MN, USA) was used to measure the glycosyltransferase B activity according to the manufacturer's instruction [7].

### Western Blotting

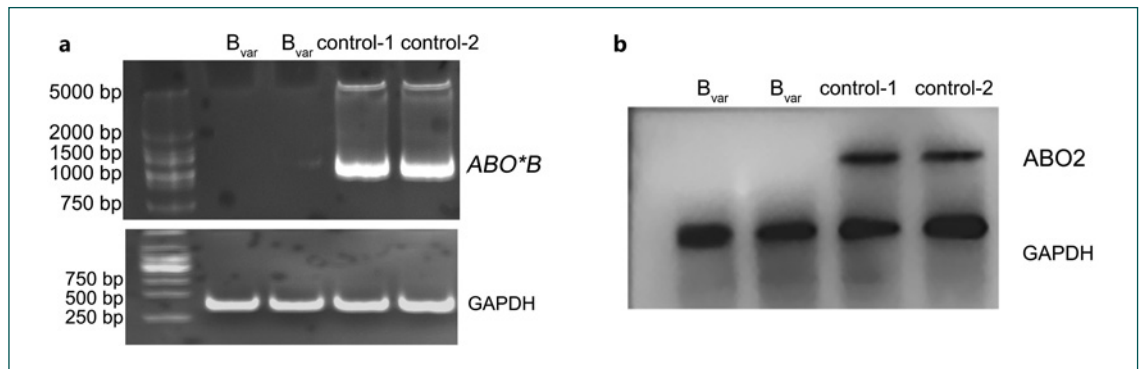
Total protein was extracted using RIPA lysis buffer premixed with protease inhibitor (Thermo), and quantified with a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein (25 µg) were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with 5% fresh nonfat milk in TBST at room temperature for 1 h, then incubated with antibodies against B antigen (1:1000, Beijing Kinghawk Pharmaceutical Co. Ltd., Beijing, China) and GAPDH (1:1000, cat no. A00227-1, Boster, Wuhan, China) overnight at 4°C. After three times of washing with TBST, membranes were incubated with the secondary antibody (HRP Conjugated AffiniPure Mouse Anti-Rabbit IgG (H + L), cat no. BM2006, Boster, Wuhan, China) for 1 h at room temperature. Then, membranes were visualized using the LI-COR Odyssey Scanning Imager.

### Reverse Transcription Polymerase Chain Reaction (RT-PCR)

One ml whole blood was added to 3 mL red blood cell lysis buffer (Solarbio, Beijing, China) and incubated for 10 min. After centrifuging at 12,000 g/min for 3 min, the supernatant was discarded, and the peripheral blood mononuclear cell pellet was added to 1 mL Trizol to extract total RNA. Total RNA was reverse-transcribed into cDNA with HiScript II Q RT SuperMix for qPCR (Vazyme, R223). cDNA amplification was performed using 2 × Taq Master Mix (Vazyme, P111-02) according to the manufacturer's instructions on a QuantStudio real-time PCR system. The condition for PCR: 98°C for 5 min; 30 cycles of 98°C for 10 s, 68°C for 10 s, and 72°C for 90 s; 72°C for 8 min; stored at 4°C. The primers used for RT-PCR were as follows: forward (5'-3'), GCATGGCTGTTAGGGAACCT, and reverse (5'-3') ACTTGTTTCAGGTGGCTCTCG. The PCR products (5 µL) were electrophoresed in agarose gel (1%).

### Reporter Plasmids Construction and Transfection

The ABO gene promoter (–117 to c.31), the cis-acting element sequence that is indispensable for the regulatory activity of the erythroid cell-specific regulatory element 5.8 kb site (+5,653 to +6,154 from the ATG translation start site of exon 1) [8, 9], and the mutant type promoter (B<sub>var</sub>, c.15\_16ins TGTTG) were directly synthesized by Songon Biotech Corporation (Shanghai, China). The wild-type and mutated sequences built into the plasmids is shown in the online supplementary file (for all online suppl. material, see [www.karger.com/doi/10.1159/000524632](http://www.karger.com/doi/10.1159/000524632)). The ABO gene promoter and 5.8 kb site fragment were inserted into the pGL4.10 [luc2] plasmid (Promega Corp., Madison, WI, USA) to construct the re-



**Fig. 1.** The expression of B antigen in the proband blood sample. **a** PCR analysis for the mRNA levels of galactosyltransferase (B enzyme, ABO\*B). **b** Western blot analysis for the protein level of B antigen (ABO2). B<sub>var</sub>, the proband's samples with two duplicates; control, the healthy donors with B blood group.

**Table 1.** ABO subgroup phenotypes for the proband

Forward typing					Reverse typing			
anti-A	anti-A <sub>1</sub>	anti-B	anti-AB	anti-H	A <sub>1</sub>	B	O	Self-control
0	0	0	0	4+	3+/4+*	w/1+*	0/0*	0/0*

A<sub>1</sub>, red blood cells with A<sub>1</sub> phenotype; B, red blood cells with B phenotype; O, red blood cells with O phenotype; Self-control, the proband's red blood cells; 0, negative reaction; w, weak reaction. \* The reactions were observed both at room temperature (left) and 4°C (right).

combinant reporter vectors, wild-type and B<sub>var</sub> type. The nucleotide sequences of all recombinant plasmids were verified by DNA sequence analysis. The plasmids expressing SP1, GATA1, and RUNX1 were purchased from OriGene Technologies (cat no. SC101137, RC224854, RC223809, Beijing, China). 293T cells were co-transfected with recombinant reporter vectors and plasmids expressing transcriptional factors (SP1, GATA1, and RUNX1), combined with pTK-RL using Lipofectamine 3000 (Thermo Fish Scientific., Carlsbad, CA, USA) according to the manufacturer's instruction. The cells transfected with recombinant reporter vectors and pTK-RL vectors were used as a control. The activities of firefly and Renilla luciferase were determined by the dual-luciferase reporter assay system (Promega Corp., Madison, WI, USA) using an absorption spectrophotometer (GLOMAX-96 microplate luminometer, Promega Corp.).

## Results

### Serological Testing

Serological testing of the ABO phenotype found that the proband's blood group showed a discrepancy between the forward and reverse typing. The red blood cells of the proband reacted negatively with anti-A, anti-B, and anti-AB, and the reaction with anti-H was strong agglutination (4+); the serum reacted with A<sub>1</sub> cells at room temperature as 3 + agglutination, and it

**Table 2.** Saliva testing with anti-H, anti-A, and anti-B for the proband

A <sub>1</sub>	B	O	Saline
2+	2+	1+	2+

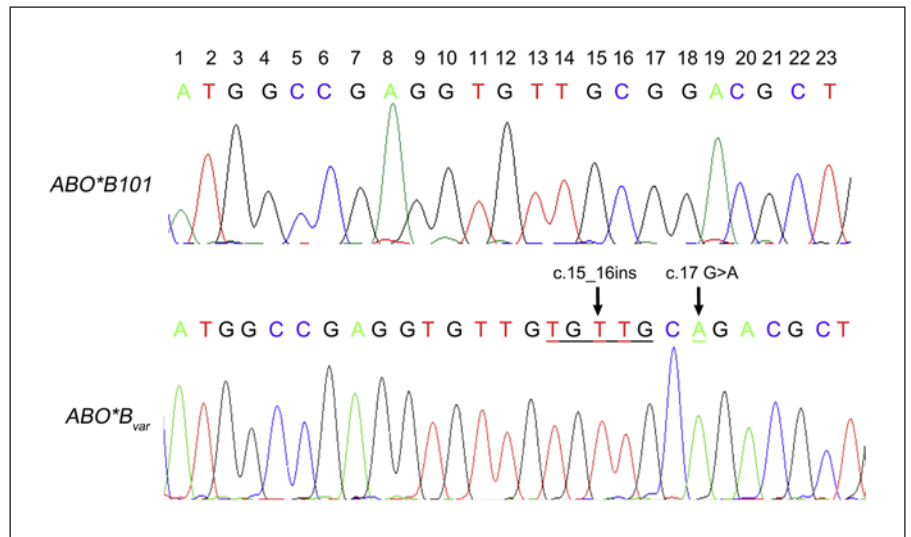
A<sub>1</sub>, red blood cells with A<sub>1</sub> phenotype; B, red blood cells with B phenotype; O, red blood cells with O phenotype; saline was used as a control.

**Table 3.** Absorption-acid elution test for the proband

A <sub>1</sub>	B	O
0	4+	0

A<sub>1</sub>, red blood cells with A<sub>1</sub> phenotype; B, red blood cells with B phenotype; O, red blood cells with O phenotype; 0, negative reaction.

reacted with B cell as w + agglutination, and it did not aggregate with O cells; after incubation at 4°, the reaction of serum with A<sub>1</sub> and B cells is enhanced (Table 1). The results of the saliva experiment showed that the proband's saliva contained only weak substance H but



**Fig. 2.** Exon sequencing analysis reveals the novel inserted mutation in exon 1 (c.15\_16insTGTTG) and single-base substitution mutation c.17 G>A of ABO gene.

**Table 4.** The novel ABO subgroup alleles in this study

Allele	nt mutant	aa change	Phenotype	Genotype	Genbank no.
B <sub>var</sub>	Exon 1 c.15_16insTGTTG	No change	B <sub>el</sub>	ABO*B <sub>var</sub> /O.02	MT745886

had no B antigen (Table 2). In addition, RT-PCR and western blot analysis could not detect ABO\*B mRNA and ABO2 antigen in the proband's whole blood samples (shown in Fig. 1). However, the results of absorption-acid elution testing with anti-B showed that the proband's red blood cells had extremely weak B antigen expression (Table 3). The above results suggest that the proband's ABO blood type (B<sub>var</sub>) is very likely to be the B<sub>el</sub> subtype.

#### ABO Gene Sequence Analysis

In order to further explore the cause of the B<sub>el</sub> subtype in the proband, we obtained the whole blood of the proband to extract DNA for exon sequencing. Aligning to an ABO\*B101 consensus sequence AF134414.1 and analyzing the sequencing results (Gene bank No. MT745886), we found a novel insertion mutation in exon 1 (c.15\_16insTGTTG) and a base substitution c.17 G>A (shown in Fig. 2, Table 4). We wanted to know whether this novel insertion mutation came from inheritance. We obtained the peripheral whole blood of the proband's parents and sister and performed serological testing, ABO genotyping, and DNA sequencing. The results showed that her father and sister were both normal blood type O. However, her mother had the novel insertion mutation, showing weak expression of B antigen (shown in Fig. 3), suggesting that the mutation was inherited from her mother.

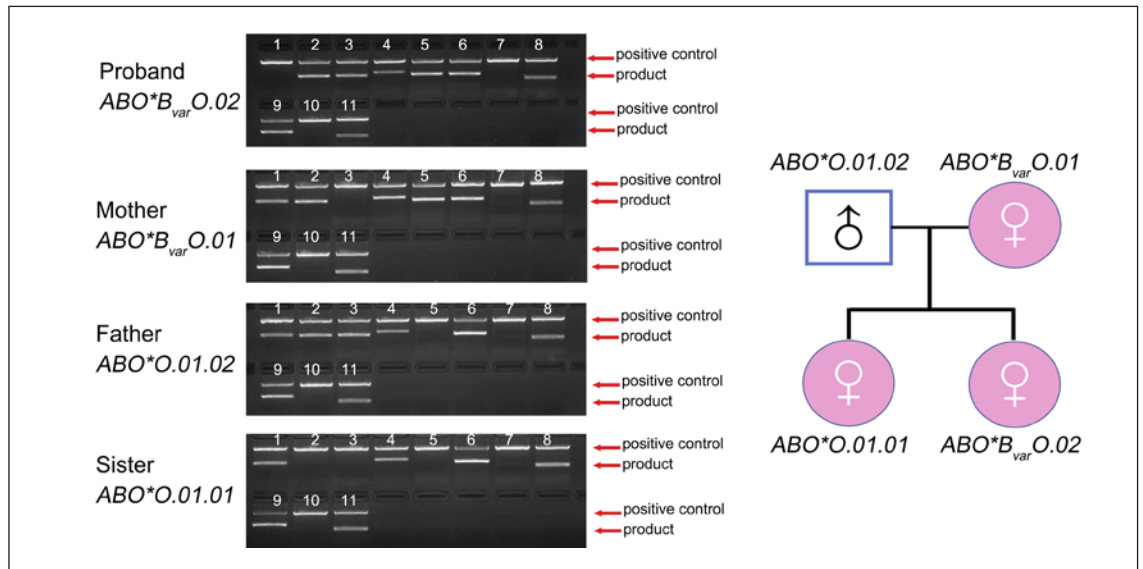
**Table 5.** The activity of glycosyltransferase B in this study

	RBC, pmol/min/μg	Serum, pmol/min/μg
B <sub>var</sub>	0	0
Wild type B	7,450	3,210

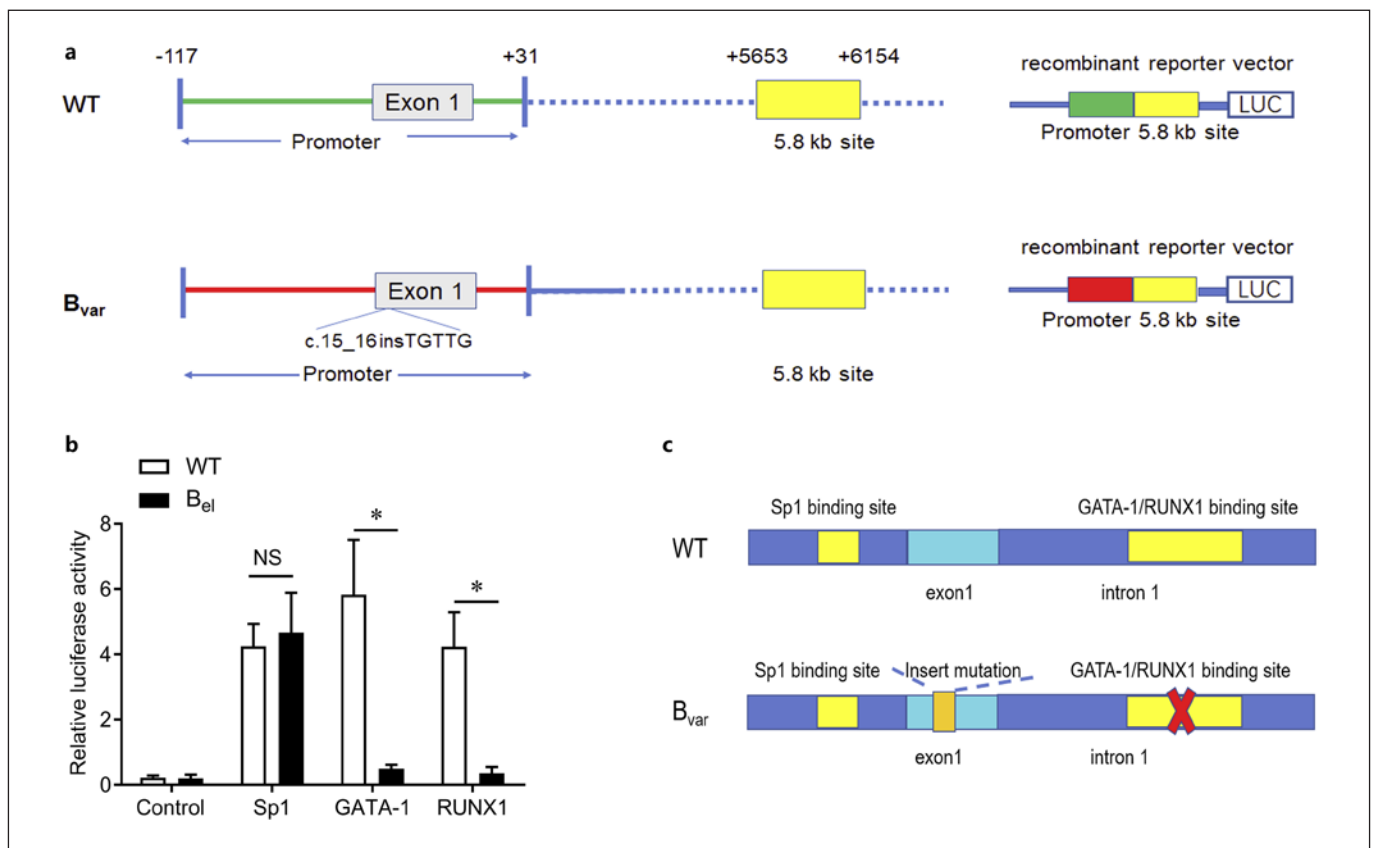
#### Effects of Novel Mutation on the Activity of ABO Gene Promoter

The transcription factor Sp1 binds to the region from -22 to c.14 of the ABO gene promoter and affects the activity of the ABO promoter [10, 11], and GATA-1 and RUNX1 are associated with ABO promoter activity by binding to the 5.8 kb site [4, 12]. Since the insertion was located in the promoter region of the ABO gene, we speculated that the mutation would have an impact on the promoter activity of the ABO gene. We subcloned the wild-type and B<sub>var</sub> promoters and the 5.8 kb regulatory element into the basic luciferase reporter plasmid (shown in Fig. 4a) and tested the promoter activity of the ABO gene by the dual luciferase reporter gene assay. We found that SP1 activated both wild-type and mutant ABO promoters, while GATA-1 and RUNX1 only activated the wild-type ABO promoter and had no obvious effects on the mutant ABO promoter (shown in Fig. 4b, c). In addition, compared with normal type B blood donors, this B<sub>var</sub>





**Fig. 3.** The ABO genotyping of proband's family. **a** The genotyping gel electrophoresis reveals the gene typing of proband's family. **b** Combined the exon sequencing analysis with genotyping gel electrophoresis indicates that the mutation inherited from her mother.



**Fig. 4.** The novel inserted mutation in exon 1 reduces the regulatory activity of GATA-1 and RUNX1 on the 5.8 kb site. **a** The ABO gene promoter (−117 to c.31), the erythroid cell-specific regulatory element 5.8 kb site (+5,653 to +6,154 and from the ATG translation start site of exon 1), and the mutant type promoter (B<sub>var</sub>, c.15\_16insTGTTG) were directly synthesized and constructed into upstream of luciferase reporter gene. **b** Dual luciferase reporter gene assay was performed. The results demonstrated that the novel inserted mutation in exon 1 reduced the regulatory activity of GATA-1 and RUNX1 on the 5.8 kb site. **c** Schematic diagram of the novel mutation on regulation of promoter activity. \**p* < 0.05. NS, no significant.

proband failed to detect the activity of glycosyltransferase in the serum and red blood cells (Table 5), suggesting that this mutation may lead to a reduction in promoter activity and subsequently decrease glycosyltransferase activity.

## Discussion

ABO blood group antigen is a clinically important antigen, expressing on the surface of human red blood cells and other tissue cells [13]. The B subtype is rare and usually found due to changes in the intensity of the reaction with anti-B and anti-AB reagents. In this study, we observed a case of heritable B subtype with weak B antigen (secretory B<sub>el</sub>) that was evaluated by the strength and type of reaction with anti-B, anti-AB, and anti-H reagents, absorption-elution test, and the saliva blood group substance test.

Frameshift, insertion, deletion, mutation, and base substitution of ABO gene may change the expression of ABO gene or glycosyltransferase activity, and eventually form different subtype phenotypes of blood group type [14, 15]. In addition, the transcription factors bind to the ABO gene promoter to regulate the expression and activity of glycosyltransferase, leading to subtype A or subtype B [10, 16]. Cai et al. [17] found that the reduced activity of the ABO gene promoter was associated with the ABO\*B<sub>el</sub> (B deletion). Sp1 binding on the ABO gene promoter (region from -22 to c.14) activates the activity of the ABO promoter [10]. Sano et al. [18] found that GATA-1 bound to an erythroid cell-specific regulatory element at a 5.8 kb site to enhance the activity of the ABO gene promoter [18–20]. In addition, Runt-related transcription factor 1 (RUNX 1) can also bind to a 5.8 kb site and control the A antigen expression [21]. The deletion of 5.8 kb site results in the B<sub>m</sub> subtype phenotype. The individuals with the B<sub>3</sub> phenotype might be caused by mutations at the 5.8 kb site [19]. The above studies indicate that ABH antigen in red blood cells is regulated by the transcription factors Sp1, GATA-1, and RUNX1.

The ABO gene promoter (-117 to c.34) contains a partial fragment of exon 1 [10]. In this study, we found that the c.15\_16insTGTTG mutation in exon 1 resulted in reduction of promoter activity regulated by GATA-1 and RUNX1, but not Sp1. It can be explained that the binding site of Sp1 (-22 to c.14) was located at the upstream of mutation and could not be changed by the mutation, while the binding site of GATA-1 and RUNX1 in the 5.8 kb site was located at the downstream of mutation, which might result in alterations in spatial conformation and function of downstream exon and intron. GATA-1 and RUNX1 therefore cannot activate

the transcription of the ABO gene, resulting in a decrease in the expression and activity of B glycosyltransferase (shown in Fig. 4c). However, since exon sequencing of the ABO gene was performed in this study, we cannot reveal mutations in nonexonic regions, such as the 5.8 kb site. Therefore, we cannot exclude additional mutations in the 5.8 kb site, potentially linked to the c.15\_16insTGTTG on the respective ABO\*B haplotype. Additionally, we could not exclude that the mutation caused a codon reading frameshift and resulted in early termination of translation, which can lead to nonsense mediated mRNA decay. Furthermore, variants of FUT1 and FUT2 have been reported to influence ABO subtype expression and are reported to be present at higher frequencies in the Asian population compared with others. Matzhold et al. [22] found that a case presented functional FUT2 alleles and showed an H-deficient secretor type O<sub>h</sub><sup>B</sup>-secretor. Thus, we also cannot exclude the influence of FUT1/2 mutation on the expression of B antigen.

## Conclusion

We identified a novel B<sub>var</sub> subtype caused by an inserted mutation. This mutation resulted in inactivation of GATA-1 and RUNX1 on regulation of the activity of the ABO gene promoter, leading to a decrease in the expression and activity of B glycosyltransferase. Combined serological, gene sequencing, cytological research, and genealogical investigation will help to further understand the formation mechanism of B subtypes.

## Statement of Ethics

This study was approved by the Ethics Committee of the Blood Center of Loudi and the Third Xiangya Hospital of Central South University (No. 2021-S124). Written informed consent was obtained from participants to participate in the study.

## Conflict of Interests

The authors declare no conflict of interests.

## Funding Sources

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## Author Contributions

Fengxia Liu, Guocai Li, Jian Li, Rong Gui, Yanwei Luo, and Ming Zhou conceived the study; Rong Gui, Yanwei Luo, and Ming Zhou participated in the study design, performance, coordination, and manuscript writing; Fengxia Liu, Guocai Li, and Jian Li performed the research. All authors have read and approved the final manuscript.

## Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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