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A novel ABO allele with weak A expression

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Running title:
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A novel ABO allele with weak A expression

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Abstract

Objectives

To carry out genetic and serological analysis of a Swiss blood donor and a Danish patient carrying an aberrant ABO phenotype with weak A expression.

Background

ABO is the clinically most important blood group system but also one of the most complex. The system antigens are determined by carbohydrate structures generated by A and B glycosyltransferases encoded by the ABO gene. Genetic variants of ABO may encode a glycosyltransferase with reduced activity leading to weak expression of A antigen.

Methods

Samples from two individuals were examined with genetic testing and extended immunohaematological evaluation including standard serological methods, flow cytometry and analysis of plasma glycosyltransferase activity.

Results

Both individuals were serologically determined to be A<sub>weak</sub>B. Genetic testing revealed that both were heterozygous for a novel ABO*A1.01-like allele with an in-frame duplication of 21 nucleotides in exon 7 (c.543_563dup) leading to the insertion of seven amino acids (QDVSMRR). Flow cytometric testing of native RBCs showed very weak A antigen expression. This was in accordance with the enzyme activity test.

Conclusion

A novel ABO allele with weak A expression
A novel *ABO* allele with weak A expression

In summary, we describe a novel A allele with a duplication of 21 nucleotides in exon 7 that significantly decreases the enzyme activity and leads to very weak expression of A antigen. (200 words)
A novel ABO allele with weak A expression

Introduction

ABO was the first blood group system recognized and can be divided into 4 groups: A, B, O and AB depending on the presence of antigens. The A and B antigens are carbohydrate structures generated by A and B glycosyltransferases adding N-acetyl-D-galactosamine or D-galactose, respectively, to erythrocyte surface glycoproteins and glycolipids. ABO glycosyltransferases are encoded by alleles of the ABO gene on chromosome 9. Genetic alterations that affect the enzyme’s activity, specificity or its subcellular location, may lead to weakened expression of either antigen, resulting in a weak A or weak B phenotype [1]. Close to 70 different weak A alleles have been recorded by the International Society of Blood Transfusion (ISBT) [2, 3] but many more have now been identified. In this report, we describe a novel weak A genotype detected in two unrelated individuals, a Swiss blood donor and a Danish patient, with similar ABO phenotypes.

Material and Methods

Subjects studied

Blood samples from two individuals (Danish and Swiss) were examined regarding their aberrant ABO phenotypes detected during routine testing. Serological and genomic testing was performed at the Department of Clinical Immunology, Odense University Hospital (OUH) for the Danish individual. This was a seventy-year old male submitted for a routine surgical procedure. He had never been transfused. He gave written informed consent to access to and extraction of health-related and laboratory data. The Swiss sample was from a healthy blood donor and serological evaluation was performed by the Swiss Blood Transfusion Service, Bern, Switzerland. Genetic testing was done at the Nordic Reference Laboratory for
A novel ABO allele with weak A expression

Blood Group Genomic Typing, Lund, Sweden, where also extended serological evaluation and glycosyltransferase activity analysis by flow cytometry was performed for both samples.

Serology

Odense

Primary ABO grouping was carried out using Ortho Vision Analyzer with Ortho BioVue Anti-A/Anti-B/anti-D supplemented with ABD confirmation cassettes (Ortho Clinical Diagnostics, Raritan, NJ). Further serological investigation was carried out using manual methods; column agglutination technique using Bio-Rad ID-Card, DiaClon ABO/D + Reverse Grouping (Bio-Rad, Cressier, Switzerland), saline test-tube using Bio-Rad Anti-A DiaClon, Bio-Rad Anti-H-Lectin (Ulex europaeus [Bio-Rad Cressier, Switzerland]) Anti-A Seraclone and Anti-A1 Seraclone (Bio-Rad, Dreieich, Germany).

Bern

Serology was performed by column agglutination technique with both human polyclonal antibodies (ID-50031) and monoclonal antibodies (A: A5, B:G1/2, AB: ES131 [ES15]+Birma-1+ES-4,ID-Card, Bio-Rad), anti-A, anti-B and anti-AB. Reverse testing was performed in tubes with A₁, A₂, B and O red blood cells (RBCs). Additional testing with ABO reagents in test tubes (anti-A clone A003, anti-B clone B005 and anti-AB clone BS63+BS85) was performed to verify the results and this testing also included anti-A(1) lectin and anti-H Immuclone (Immucor, Dreieich, Germany).

Flow cytometry
A novel ABO allele with weak A expression

Semi-quantification of ABO antigen density on RBCs was assessed by flow cytometry. Testing was performed with monoclonal ABO reagents as previously described [4]. Controls with defined positive and negative phenotypes were included in the assay (A1, A2, B, O and Bombay/Oh). In addition, genetically verified controls, (ABO*AW.30, ABO*BW.03) were used as weakly positive controls to ensure sensitivity in assay.

**Enzyme activity testing in plasma**

A 10 percent suspension of washed group O RBCs mixed with allogeneic plasma from the Danish patient or from genetically defined control samples with the following genotypes: ABO*A1.01/O.01.01, ABO*A2.01/O.01.02 or ABO*O.01.01/O.01.02. Mixtures were incubated at 37°C with 0.35 mmol per L final concentration of UDP-GalNAc donor substrate (Sigma-Aldrich, Stockholm, Sweden) and 10 mmol per L MnCl₂ (Sigma-Aldrich) for 24 and 48 hours in a total volume of 250 μL as previously described [5]. The treated RBCs were washed after incubation and subjected to flow cytometric analysis of A and H antigen expression as described above.

**Molecular genetic methods**

Odense

DNA was extracted and all 7 exons and the adjacent 20 bp of the introns in the ABO gene were amplified and sequenced in 2019 with BigDye Terminator v.3.1 and run on an ABI3500Dx (Life Technologies, Denmark) (for details see [6]). For separation of the two alleles, the PCR product of exon 7 carrying the
A novel ABO allele with weak A expression
duplication was cloned using TOPO TA cloning kit (Invitrogen, Denmark). DNA from 8 colonies was
extracted and sequenced as described above.

Lund

Genotyping was performed with in-house PCR-RFLP and PCR-ASP routine analysis [7-10]. Exons 1 through 7 of the ABO gene were examined by Sanger sequencing in 2014 using BigDye Terminator v.3.1 and run on an ABI3500Dx. Allele-specific primers were used to verify that the duplication was present on the ABO*A1.01-like allele. Testing for the upstream polymorphic CCAAT-binding factor/nuclear factor Y (CBF/NF-Y)-binding enhancer was done performed as previously reported [11].

Three-dimensional modelling

The crystal structure of A glycosyltransferase [PDB ID 1LZ0 (12)] was visualized by Cn3D v.4.3.1 (produced by the National Center for Biotechnology Information and available from www.ncbi.nlm.nih.org) and important amino acid sequences in the structure in relation to the 21-bp duplication highlighted in yellow using the sequence/alignment viewer function in the software.

Results

Serology

Both samples showed (+) to 1+ reaction with all anti-A reagents tested, although a 2+ reaction was seen with the anti-A of the Ortho ABD confirmation cassette (Danish patient), and reactivity was completely absent with anti-A of the Bio-Rad DiaClon ABO/D + Reverse Grouping gel card (Danish patient). Clearly
A novel ABO allele with weak A expression

positive reactions with anti-B were seen in both samples and with anti-A,B (Swiss donor), see Table 1 for details. An anti-A1 was detected in both individuals at 20°C but not at 37°C.

**Flow cytometry**

The flow cytometric pattern for samples from both individuals showed a severely lowered A antigen expression that was slightly weaker than the included weakly positive control (ABO*AW.30, which gives rise to the classical A, phenotype) (Figure 1A, B). A clearly positive reaction was detected with anti-B whilst the H antigen levels were equivalent to those seen on normal group B RBCs.

**Genetic testing**

The molecular genetic investigation showed that both individuals were heterozygous for ABO*B.01 and a new A allele of consensus ABO*A1.01-derived sequence but also carrying a duplication of 21 nucleotides in exon 7 (c.543_563dup) leading to an insertion of seven amino acids (QDVSMRR). This nucleotide sequence has been deposited to GenBank under the Accession Number MN401412 (submitted on March 19, 2020).

Since this insertion remains in frame, this in turn results in the duplication of seven amino acids (p.182_183dup7) in the conserved “disordered loop” adjacent to the active site of the glycosyltransferase, amino acids 179-194 [12]. It is likely that this change interferes with the movement of this loop and thereby hampers the enzyme’s ability to synthesize A antigen (Figure 2). For the Swiss donor the number of repeats in the upstream enhancer region was as expected for the ABO*A1.01/B.01 genotype, one and four, respectively.

**Enzyme activity in plasma**

After incubation for 48 h with the appropriate reagents and group A plasma from different genetically and serologically defined A phenotypes (A1, A2) used as controls, different levels of A antigen were clearly
A novel ABO allele with weak A expression
detectable on the converted group O RBCs by flow cytometry (Figure 1C). As expected, no A activity could be detected when group O plasma was applied. When using plasma from the Danish individual with the 21-bp duplication in exon 7 of the ABO gene, no clear glycosyltransferase activity could be detected, although an extremely small shift of the histogram curve was observed (Figure 1C). This suggests that the aberration to the ABO gene and hence the change in glycosyltransferase structure diminishes the enzyme activity dramatically and that it is virtually as inefficient as group O plasma to transform group O RBCs to group A in this setting.

Discussion

It is important to determine the correct ABO blood type of both patients and donors to avoid unforeseen transfusion reactions. Weak reactions with anti-A can have many underlying reasons, one of them being the suggested presence of an A allele that results in weak expression of A antigen. Here, we describe a novel A allele carrying a duplication of 21 nucleotides (c.543_563dup) detected in two individuals very likely to be unrelated. The insertion leads to the duplication of seven amino acids (p.182_183dup7) adjacent to the active site of the enzyme [12]. Our data suggest that this duplication is likely to affect the conformation of this vital and moving part of the A glycosyltransferase which in turn would reduce the activity causing a weakened expression of A antigen (Figure 2). However, we cannot, exclude that the duplication in question changes the folding of the enzyme in a more profound way so that it is displaced from its original position in the Golgi apparatus, for instance due to misfolding-induced quality control or lack of chaperones to guide it to its place. The absence or extremely low levels of A enzyme activity in plasma from these two individuals indicates either near undetectable levels of activity in the enzyme present in plasma, or the absence of enzyme in plasma. The presence of ABO glycosyltransferase in plasma requires proteolytic
A novel ABO allele with weak A expression

cleavage to occur in the stem region of the molecule for the eventual release of the enzyme to the extracellular space. The duplication described here affects the globular domain and is relatively unlikely to affect the stem. However, if the protein misfolds and forms inclusion bodies, it would neither be available in Golgi or in plasma. Thus, we acknowledge that our data do not differentiate between the different possible underlying mechanisms that lead to the weakened phenotype.

We noted that both individuals carrying the novel A allele are group AB. It has been shown that weak ABO antigen expression can be enhanced by the allele in trans i.e. the allelic enhancement phenomenon first reported in 1976 [13] and nicely exemplified in a paper from 2005[14] where the ABO*AW.31.03 allele gave rise to weak antigen expression with non deletional alleles, ABO*O.02 and ABO*B.01, in trans but typed as group O in the presence of ABO*O.01. On the other hand, lower levels of antigen expression has been reported when there is competition from a fully functional glycosyltransferase as exemplified by Kim et al [15] where individuals carrying the ABO*AW.14 allele in combination with ABO*B.01 typed as normal group B, i.e. no A antigen detected, whereas with a deletional O allele (ABO*O.01) in trans the crippled A transferase resulted in weak A antigen expression. It is difficult to speculate which of these mechanisms would be at play here since we have only come across the reported weak A allele in combination with the ABO*B.01 allele. With an O allele in trans this phenotype could either become group O (absence of allelic enhancement) or normal, or at least stronger, group A (absence of allelic competition) or perhaps more likely, still display a weak A phenotype although at a different antigen level. An intriguing and (to our knowledge) still unanswered question is what causes a weak allele to be either helped or overthrown by the allele in trans. We have previously hypothesized that heterodimerization between a mutated A enzyme and a consensus full-length B enzyme could stabilize and enhance the weak enzyme [4, 14, 16]. Obviously, further elucidation of this complex phenomenon is beyond the scope of this study. In summary, we report a new A weak variant that caused discrepant typing result in routine blood grouping. Interestingly, the
A novel ABO allele with weak A expression

identification of this allele in two unrelated individuals of European descent suggests that this novel A allele is not a private variant, but probably present across the European population.

Acknowledgement

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Author contributions

MJ, AH, SC and ÅH performed the testing. MJ, US and AH wrote the manuscript. MLO interpreted data, made the 3D model figure and edited the manuscript.

References

A novel ABO allele with weak A expression


A novel ABO allele with weak A expression

Legends to the figure:

**Figure 1. Flow cytometric testing.** Native RBCs were tested (A-B) and control samples included are RBCs of the following phenotypes: A₁ (green), ABO*AW.30 (orange), and O (grey). The two samples (in red) tested show weak reactivity when tested with anti-A and are in both cases weaker than the included Aₓ (ABO*AW.30) control RBCs. (C) Enzyme activity testing after 48 hour incubation. Group O RBCs incubated with MnCl₂, UDP-GalNAc and plasma from individuals with different phenotypes. Peaks corresponding to addition of A₁ plasma (dark green), A₂ plasma (blue), O plasma (grey) or patient plasma (red) are shown. Clearly detectable A antigen is seen after incubation with A₁ and A₂ plasma whereas with the patient plasma no (or possibly an extremely low synthesis of) A antigen is detectable.

**Figure 2.** 3D-structural model of A glycosyltransferase based on crystal structure deposition with PDB ID 1LZ0 (12). The amino acids are indicated in yellow, just prior to or after the disordered loop, in which the seven-residue duplication occurs. In addition, the unique DVD motif coordinating the manganese ion that is involved in binding of the phosphate groups of the UDP-GalNAc is highlighted in the centre of the enzyme since this shows the approximate location of the catalytic centre.
Figure 1.
Table 1. Overview of the serological results

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*Varying results depending on the reagent used (see text).