Third-generation sequencing detects a novel variant in the regulatory RUNX1 motif of the *ABO* gene causing mixed-field agglutination in an AB individual

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Background: Mixed-field agglutination in ABO phenotyping (A_3/B_3) has been linked to rare variants in *ABO* exon 7 and regulatory regions. Incomplete knowledge about regulatory regions, however, hinders the discovery of genetic causes of unexplained cryptic ABO phenotypes. Long-read sequencing has great potential in this regard by enabling complete gene haplotype sequencing. As proof of concept, we used nanopore sequencing to resolve a case of mixed-field agglutination in ABO forward typing in a blood donor.

Methods: The ABO phenotype was determined by standard serological methods**,** including anti-A1 and anti-H specific agglutination. Commercially available PCR-SSP kits (inno-train, Germany) were used for *ABO* genotyping. Expression of A-, B-, and H-antigen was measured by flow cytometry. We excluded potential presence of chimerism by digital PCR (STILLA, France). The entire *ABO* gene was amplified by two overlapping long-range PCR fragments of ~13 kb each. PCR-products were sequenced with newest sequencing technology of Oxford Nanopore Technologies (ONT). Results were confirmed using Sanger sequencing. In addition to the blood donor, we also analysed her mother and brother.

Results: We observed a mixed-field reaction with anti-A in a blood donor genotyped as AB. Agglutination with anti-H was weak and absent with anti-A_1. In concordance, we found ~20% erythrocytes expressing A and B while ~80% had only B antigen. Nanopore sequencing revealed a novel heterozygous g.10924C>A variant on the A-allele in a known transcription factor binding site for RUNX1 in intron 1 (+5.8 kb site). Inheritance of the novel SNV was proven by the donor's mother, who was genotyped AO_1 and shared the anti-A specific mixed-field agglutination (~35% of A-expressing cells). As expected, the novel variant was absent in the donor's brother, who had phenotype O and genotype O_1O_1.

Conclusion: We discovered an unknown SNV causing an A_3 phenotype. The SNV falls into the 8-bp RUNX1 motif located in the large intron 1, which is rarely sequenced. Our finding extends current knowledge of four other variants affecting this motif, all leading to A_3/B_3 or A_m/B_m phenotypes. Our strategy of ONT-sequencing of long-range PCRs allowed haplotype generation of the entire *ABO* gene. This simplifies the crucial assessment of known and unknown regulatory regions in cases of complex antigen expression.

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