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Intriguing outcomes from Nanopore sequencing of two cryptic A_3 samples: a case of blood group mosaicism and a novel regulatory variant in the ABO system

G. A. Thun[1], M. Gueuning[1], C. Engström[2], E. Gourri[1,3], S. Sigurdardottir[3], L. Schneider[3], E. Meyer[3], J. Sigle[4], B. M. Frey[1,2,3], S. Meyer[3], M. P. Mattle-Greminger[1]

[1] Blood Transfusion Service Zurich (SRC), Department of Research and Development, Schlieren, Switzerland
[2] Blood Transfusion Service Zurich (SRC), Department of Immunohematology, Schlieren, Switzerland
[3] Blood Transfusion Service Zurich (SRC), Department of Molecular Diagnostics and Cytometry, Schlieren, Switzerland
[4] Blood Transfusion Centre Aargau-Solothurn (SRC), Aarau, Switzerland

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Background: Mixed-field agglutination (MFA) in ABO phenotyping (A_3,B_3), i.e. when two separable cell populations are present in agglutination tests with anti-A or anti-B, is mostly linked to rare variants in *ABO* exon 7 and regulatory regions. Alternatively, MFA could be observed because of chimerism (cells derived from different zygotes, e.g. due to bone marrow transplantation) or, in exceedingly rare cases, true mosaicism (genetically different cell lines derived from the same zygote, e.g. due to somatic mutations in stem cells). Elucidating the genetic cause of MFA is technologically challenging. Incomplete knowledge about regulatory regions further hinders resolving unexplained cryptic ABO phenotypes. Latest long-read sequencing has great potential in this regard by enabling complete gene haplotype sequencing to a high read depth, even allowing the detection of subclonal variants at low variant allele frequencies (VAFs).

Aims: We used long-read sequencing by Oxford Nanopore Technologies (ONT) to resolve two cases with cryptic ABO phenotypes (A_3 and A_3B, respectively).

Methods: ABO phenotypes were determined by standard serological methods**,** including anti-A1 and anti-H specific agglutination. PCR-SSP kits (inno-train, Germany) were used for *ABO* genotyping. Expression of A-, B-, and H-antigen was measured by flow cytometry. The entire *ABO* gene was amplified by two overlapping long-range PCR fragments of ~13 kb each. PCR-products were sequenced with ONT on a MinION flow cell to a read depth >1000x. Result confirmation was derived from multiple lines of evidence, including analysis of presence of chimerism and mosaicism by digital PCR (STILLA, France), Sanger sequencing of the region of interest, and family analysis where applicable.

Results: Blood cells of the first case, genotyped AO_1 and presenting A-antigen on ~80% of erythrocytes, showed strong reactions with anti-A_1 and anti-H, pointing to very distinct cell populations. Nanopore sequencing depicted a subclonal 3-bp insertion in exon 6 (VAF ~10%). The variant was only present on *ABO*A* haplotypes and translated into a frameshift (Tyr126Ilefs*69) causing a null phenotype in the affected cells. Allele-specific Sanger sequencing confirmed the insertion and no chimerism was detected. Instead, we suggest the uncommon case of mosaicism as a digital PCR approach quantified VAF at ~14% and a blood sample donated 10 months later pointed to a robustly affected cell proportion (VAF ~17%). The second case, a A_3B sample, showed weak and absent agglutination with anti-H and anti-A_1, respectively. Almost 60% of erythrocytes carried only B antigen. Nanopore sequencing revealed a novel heterozygous g.10924C>A variant phased to the

*ABO*A*-allele in a known transcription factor binding site for RUNX1 in intron 1 (+5.8 kb site). Sanger sequencing confirmed this variant, and its inheritance was proven by analysing the donor's mother, who shared the anti-A MFA.

Conclusions: Here, we disclose an exceptional case of genetic ABO mosaicism presumably arising from a somatic indel mutation in a hematopoietic stem cell before its division into myeloid and lymphoid progenitor cells. The second reported case of MFA was resolved by the discovery of a regulatory variant in the 8-bp RUNX1 motif of *ABO*, extending current knowledge of four other variants affecting the same motif and also leading to A_3 or B_3 phenotypes. Overall, long-range PCR combined with ONT sequencing proved powerful for the resolution of both ABO MFA cases.