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LIMITATIONS OF HUMAN LEUKOCYTE ANTIGEN MOLECULAR TYPING METHODS FOR PATIENTS WITH ACUTE MYELOID LEUKEMIA RELAPSE AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Aim. The work purposed to compare molecular typing methods with different resolutions in clinical cases without loss of heterozygosity (LOH) in HLA genes.

Methods. HLA typing was performed using molecular techniques based on sequence-specific primers (SSP) or high-resolution sequencing (NGS and Sanger sequencing).

Results. the presence of an extra allelic variant in testing DNA samples was determined only with the low-resolution SSP method. At the same time, any type of sequencing did not allow for distinguishing donors' alleles from patients.

Conclusion. Despite the high sensitivity of NGS and Sanger sequencing, the use of these methods is associated with difficulties in accurately determining the absence of HLA-LOH.

Keywords: HLA-LOH, HLA typing, hematopoietic stem cell transplantation, sequencing.

Human leukocyte antigen (HLA) typing plays a key role in hematopoietic stem cell transplantation (HSCT) by ensuring the selection of the most genetically compatible donor and, as a result, reducing the risk of graft-versus-host disease. Molecular genetic methods, including Next-Generation Sequencing (NGS) and Sanger sequencing-based typing (SBT), improved the accuracy of HLA gene determination and decoding [Geo, 2024]. However, their application for patients who have been diagnosed with relapse of acute myeloid leukemia (AML) after HSCT shows significant limitations. Loss of heterozygosity (LOH) at HLA loci and the presence of mixed chimerism can complicate accurate genotyping and affect the interpretation of results [Lee, 2024]. Additionally, standard methodologies may fail to distinguish between donor-derived and residual malignant cells, leading to diagnostic challenges [Arnold, 2022; Lozac'hmeur, 2024]. Understanding these constraints is essential for the optimization of post-transplant monitoring and improving treatment strategies.

Our study aimed to compare molecular HLA typing methods with different resolutions in clinical cases without loss of heterozygosity of HLA genes in patients with hematological disease relapse after HSCT.

Methods. DNA was extracted from peripheral blood using the "Genomic DNA from Blood Extraction Kit" (Macherey-Nalel, Germany). The concentration and purity of samples were assessed

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using NanoDrop (Thermo Fisher Scientific, USA). Sequence-specific primers method (SSP) was performed using Micro SSP Generic typing kits for Class I (One Lambda, USA) according to the manufacturer's protocol. SBT SeCore Kit (One Lambda, USA) was used for the SBT method. PCR amplification was followed by sequencing using a 3500xL Dx Genetic analyzer (Thermo Fisher Scientific, USA). Sequence alignment and allele identification were performed using Fusion 4.6.1 and Fusion 6.0 software (One Lambda, USA). NGS was performed using AllType FASTplex 11 Loci Kit (One Lambda, USA). Data analysis was performed using TSV 3.1 software (One Lambda, USA).

Results and Discussion. We used DNA staples of patients who were diagnosed with relapsed AML after allo-HCST. To demonstrate the influence of HLA-A locus-related alleles belonging to the same split group on typing results performed by different methods, from the archive of the Tissue Typing Department of NSCH "OKHMATDYT" were selected DNA samples, in which allelic variants of the HLA-A genes have been previously determined by NGS method.

Two patients with AML received bone marrow allo-HSCT. On day +106 and day +396, the patients were diagnosed with central disease relapse. Post-transplant monitoring showed a partial chimerism without any confirmed HLA-LOH. SSP confirmed parallelism but was not observed using NGS or SBT.

Due to the limitations of genotyping methodologies, the presence of true LOH cannot be entirely neither confirmed nor denied. This is especially important to consider for alleles with minimal nucleotide sequence differences. The experimental part of our study demonstrated that extra alleles present in a patient's peripheral blood cells, which may belong to both the patient and the bone marrow donor, and at the same time differ by 15 or fewer nucleotides, may not be detected using high-resolution molecular genotyping methods.

Two experimental DNA samples simulated a clinical situation where a patient can receive allo-HSCT from a haploidentical donor with a mismatch at the HLA-A locus containing allelic variants belonging to the same split group. The first mixed sample consisted of *HLA-A*33:01* and *HLA-A*68:01* + *HLA-A*29:01* and *HLA-A*68:01* with a 1:4 ratio, while the second experimental sample included *HLA-A*25:01* and *HLA-A**26:01 + *HLA-A*26:01, HLA-A**66:01 with 1:1 ratio. SBT results analysis demonstrated the presence of three nucleotides at some positions that matched the HLA-Fusion 6.1 software database references. Still, the sequencing result remained indeterminate even after the step-by-step substitution of nucleotides at each doubtful position manually by the operator (Figure).

Figure. **Sanger sequencing results of a mixed sample, fusion data analysis. Red color refers to T nucleotide, green color refers to A nucleotide, blue color refers to C nucleotide and black color refers to G nucleotide**

Testing of these samples by NGS also did not reveal the presence of an extra allelic variant, which may be due to a number of reasons, such as the belonging of selected alleles to the same split group and the ratio of the samples, which reflects a low percentage of partial chimerism.

Conclusion. Thus, despite the high sensitivity of NGS and Sanger sequencing, the use of these methods is associated with difficulties in accurately determining the absence of HLA-LOH.

Authors' contribution

LOS carried out SBT, data analysis, and writing original draft; IIT carried out library preparations for NGS, NAT, and AOM and performed NGS and data analysis; AVK performed SSP method; VVA performed DNA extraction, AOM conceptualization, data curation, writing original draft YAH conceptualization, supervision.

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