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CONFIRMING FALSE HOMOZYGOSITY IN PRE-TRANSPLANT HLA TESTING

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ABSTRACT: The HLA gene complex is located on chromosome 6p, and this article examines a patient with acute myeloid leukemia (AML) who has the incorrect HLA homozygosity due to loss of heterozygosity (LOH). Successful hematopoietic stem cell transplant matching relies on precise HLA compatibility testing. Confusion in clinical conditions could arise if LOH is not detected. Oral swabs and peripheral blood samples were used for HLA molecular typing using methodologies such as sequence-based approaches, next-generation sequencing technologies, and chromosomal microarray analysis (CMA). After a bone marrow exam, it was determined that a 68-year-old man suffering from low platelets and anemia had acute myeloid leukemia (AML). Although the karyotype was normal, the blast crisis HLA testing of peripheral blood revealed that all three HLA loci—A, -B, and -C—were likely homozygous (46, XY[20]). Additional testing confirmed the previous results. Heterozygosity at each locus was determined by HLA typing using DNA from oral swabs. Afterwards, a substantial copy-neutral LOH region spanning from 6p25.3 to 6p21.1 and measuring approximately 43.5 Mb in length was discovered by CMA. By providing a reason for the divergent outcomes, this demonstrated the significance of checking for unexpected homozygosity in non-hematopoietic organs.

Keywords: *Loss of heterozygosity (LOH); HLA genotyping; Chromosome 6p; Copy-neutral LOH; Acute myeloid leukemia (AML); Hematopoietic stem cell transplantation; Chromosomal microarray analysis (CMA); False homozygosity; Buccal swab typing; Blast crisis.*

1. INTRODUCTION

Acute myeloid leukemia (AML) is a blood disease characterized by the fast proliferation of aberrant myeloid progenitors that disrupt normal hematopoiesis. One major method to treat AML is hematopoietic stem cell transplantation (HSCT). When it comes to hematopoietic stem cell transplantation (HSCT), the level of compatibility between the donor and recipient in terms of human leukocyte antigen (HLA) is crucial. Most people believe that using a related donor, a matched relative, or an unrelated registry donor will provide the best results when it comes to getting identical HLA matching. Donor selection, transplant success, and post-transplant complications can all be improved by HLA testing.

Cancer cells in hematologic tumors often undergo a genetic mutation known as copy-neutral loss of heterozygosity (CN-LOH), which allows them to evade the immune system. When chromosome 6p, which contains the major histocompatibility complex (MHC), experiences loss of heterozygosity (CN-LOH), it might lead to inaccurate HLA typing. The potential

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mismatch of patients with donors poses a serious risk if the item cannot be located. While CN-LOH is not typically detected upon diagnosis, it is typically discovered during HSCT or upon cancer recurrence.

We describe a case where further laboratory testing confirmed that CN-LOH was the true culprit behind the HLA homozygosity detected in the patient's blood during the diagnosis of AML. In this review, we examine previous instances of blood cancer diagnosis that included CN-LOH. Because of this, it is critical to think of CN-LOH as a variable that can alter the interpretation of HLA and the efficacy of existing detection approaches.

2. MATERIALS AND METHODS

Sources of DNA

We extracted DNA from buccal swab (BS) and peripheral blood (PB) samples using a Qiagen extraction kit (QIAGEN, Hilden, Germany) for molecular HLA testing. By measuring absorbance at 260 and 280 nm using the NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), we were able to determine the DNA's purity and concentration. The samples were deemed sufficiently clean since their A260/A280 rates ranged from 1.8 to 2.0.

HLA typing

To undertake high-resolution typing of the identified HLA loci, we employed a commercial sequence-based typing (SBT) kit from Biowithus (AVITA plus HLA SBT kits, Seoul, Republic of Korea). Analysis of nucleotide sequences generated by an ABI 3500 Dx Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) was carried out using the BIOWITHUS SBT Analyzer (Biowithus).

Next-generation sequencing (NGS)

The next-generation sequencing data corroborated the high-resolution typing findings. The HLA-A, -B, -C, -DRB1, and -DQB1 loci were replicated from genomic DNA that was free of contaminants using the AllType reagent kit (One Lambda, Inc., Canoga Park, CA, USA). The library was constructed using the Ion 520TM and 530TM ExT kits from One Lambda, Inc. Further, the data were sequenced using an Ion Torrent S5 sequencer and an ION S5-520 chip. The sequencing data was analyzed using TypeStream Visual 3.0 NGS Analysis, developed by One Lambda, Inc.

Chromosomal microarray (CMA)

We examined genomic DNA from peripheral blood using a chromosomal microarray and the Affymetrix CytoScan Dx Assay (Affymetrix, Santa Clara, CA, USA), all in accordance with the instructions provided by the manufacturer. With a resolution of approximately 100 kb, this whole-genome tool is equipped with approximately 550,000 copy-number markers and 200,000 SNP markers. We examined the data using Affymetrix's Chromosome Analysis Suite program to determine their significance. We aligned and labeled using the GRCh37/hg19 reference sequence, which is from the human genome and was released in February 2009.

Samples collected from the patient and tests performed

An EDTA tube was used to store a peripheral blood sample collected on August 26, 2022. The entire DNA extraction process took place in a single day. An analysis was conducted on this data using CMA. We completed DNA extraction on September 2, 2022, the same day we

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took the second peripheral blood sample. The task of DNA extraction was finished once they received the third cheek swab sample on September 8, 2022.

Literature review

In order to identify cases of deceptive HLA homozygosity that have been associated with hematologic malignancies, researchers conducted a comprehensive search of the PubMed database. The following search phrases were utilized to locate case reports and clinical research concerning hematologic cancer patients with incorrect HLA homozygosity: "false homozygosity," "HLA," "LOH," "leukemia," and "pretransplant."

3. RESULTS

Case description

An HLA typing was performed on a 68-year-old Asian man with acute myeloid leukemia (AML) to determine the potential benefits of allogeneic hematopoietic stem cell transfusion. Acute Myeloid Leukemia (AML) was diagnosed after a bone marrow sample taken on August 25, 2022, revealed that 27% of the cells were blasts. The cytogenetic analysis revealed a typical karyotype (46,XY). On the same day, a complete blood count revealed...

Hemoglobin was 75 g/L, platelets were 58.0 × 10^2/L, and white blood cells (WBCs) were 5.53 × 10^2/L, with 26% of them being blasts. Mutations in NPM1, KRAS, NRAS, PTPN11, and KMT2A were discovered by next-generation sequencing. But the fusion gene testing failed. Three units of packed red blood cells that had been leukocyte-filtered were administered to the patient before storage due to their anemia.

For the purpose of HLA typing, we received peripheral blood sample #1 on August 26, 2022. In the whole blood count, 5.87 × 10^2/L of white blood cells, 98 g/L of hemoglobin, 61.0 × 10^2/L of platelets, and 24% of blasts were detected.

The start of chemotherapy induction was on August 30, 2022. Idarubicin (12 mg/m^2 daily for three days) and cytarabine (100 mg/m^2 daily for seven days) were both administered. September 2, 2022, was the date of the peripheral blood sample collection (#2). The white blood cell count was 2.22 x 10□/L, hemoglobin was 95 g/L, and platelet count was 47.0 x 10^9/L, according to the total blood count. None of the responses were particularly intriguing.

The third sample, an oral swab, was collected six days subsequent to the second. That day, there were no explosions. The hemoglobin level was 83 g/L, the platelet number was 24.0 x 10^4/L, and the white blood cell number was 1.41 x 10□/L. Another bone marrow test performed 30 days after diagnosis confirmed the absence of leukemic blasts and low cell count in the patient's marrow.

HLA typing

The HLA typing data for each patient group is displayed in Table 1. The results of the sequence-based typing of Sample #1 demonstrated that the HLA-A, -B, and -C loci were homozygotes, while the HLA-DRB1 locus was heterozygotes. Chromatograms still revealed a few tiny secondary spots, therefore heterozygosity could not be excluded. The second sample, taken seven days following therapy, was the peripheral blood sample. The results were identical to example #1. Sample #3, a buccal swab collected six days subsequent to sample #2, nevertheless, demonstrated heterozygosity at all loci examined by SBT analysis.

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We repeated the process on sample #1 using next-generation sequencing to determine the source of the discrepancy in the results. Since the afflicted loci only contained a single allele, the NGS coverage graph demonstrated homozygosity (Figure 2).

CMA

In Sample #1, chromosomal microarray analysis revealed a large region of copy-neutral loss of heterozygosity, spanning approximately 43.5 Mb and included chromosomes 6p25.3-p21.1 (184,718-43,635,395) (Figure 3). Here you might find every single HLA gene. An out-of-the-ordinary increase of approximately 26.7 Mb was noted between 11p15.5 and p14.2 (230,615 to 26,929,451).

Table 1. Result of HLA typing.

	Sample #1 (PB)		Sample #2 (PB)	Sample #3 (BS)
DATE	26/08/2022		02/09/2022	08/09/2022
CBC (blast%)	5870-9.8-61K (26%)		2220-9.5-47K (0%)	1410-8.3-24K (0%)
HLA typing loci	SBT	NGS	SBT	SBT
A	*24:02	*24:02	*24:02	*24:02
	*24:02	*24:02	*24:02	*02:01
B	*52:01	*52:01	*52:01	*52:01
	*52:01	*52:01	*52:01	*48:01
C	*12:02	*12:02	*12:02	*12:02
	*12:02	*12:02	*12:02	*08:01
DRB1	*09:01	*09:01	*09:01	*09:01
	*15:02	*15:02	*15:02	*15:02

PB, peripheral blood; BS, buccal swab; SBT, sequence based typing; NGS, next-generation sequencing.

Literature review of loss of HLA heterozygosity in hematologic malignancies at diagnosis

The median percentage of leukemic blasts in peripheral blood was 86% when loss of HLA heterozygosity (LOH) was discovered at diagnosis. The patient's arm or hand was used as a blood sample for all HLA tests that were documented. Bone marrow karyotyping came out normal in every single case. The determination of HLA homozygosity was accomplished using several molecular typing techniques. Among these methods were next-generation sequencing (NGS), sequence-based typing (SBT), sequence-specific priming (SSP), and single-specific oligonucleotide (SSO) typing. Oral swabs, alternative typing methods, and chromosomal microarray analysis (CMA) were among the new test materials used to confirm LOH following therapy. A mutation in either NPM1 or FLT3 was found in almost 50% of the cases. Of all the patients that tested positive for LOH at diagnosis, these accounted for 64 percent.

4. DISCUSSION

This case study describes a patient with acute myeloid leukemia (AML) and a history of HLA copy-neutral loss of heterozygosity (CN-LOH). Solid tumors are known to evade the immune system through loss of HLA heterozygosity (LOH). Between ten percent and fifty-five percent of tumor types are susceptible to this. However, hematologic malignancies do not present with HLA LOH as frequently. According to the results, 1.27% of patients with

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cytogenetically normal AML developed uniparental disomy at 6p21, while 0.44% of HSCT patients lost heterozygosity in the HLA area. These analyses focus on certain points in time, such as when the disease returns or following a transplant, to determine the severity of the condition. This being said, the majority of LOH patients are discovered following relapses or haploidentical transplants. Because of this, LOH discovered during diagnosis is quite rare. Since CN-LOH may not be detectable in the first diagnostic test, the HLA typing results may be misinterpreted.

Few cases have been recorded that affected the HLA area at the time of diagnosis, making CN-LOH far less common.

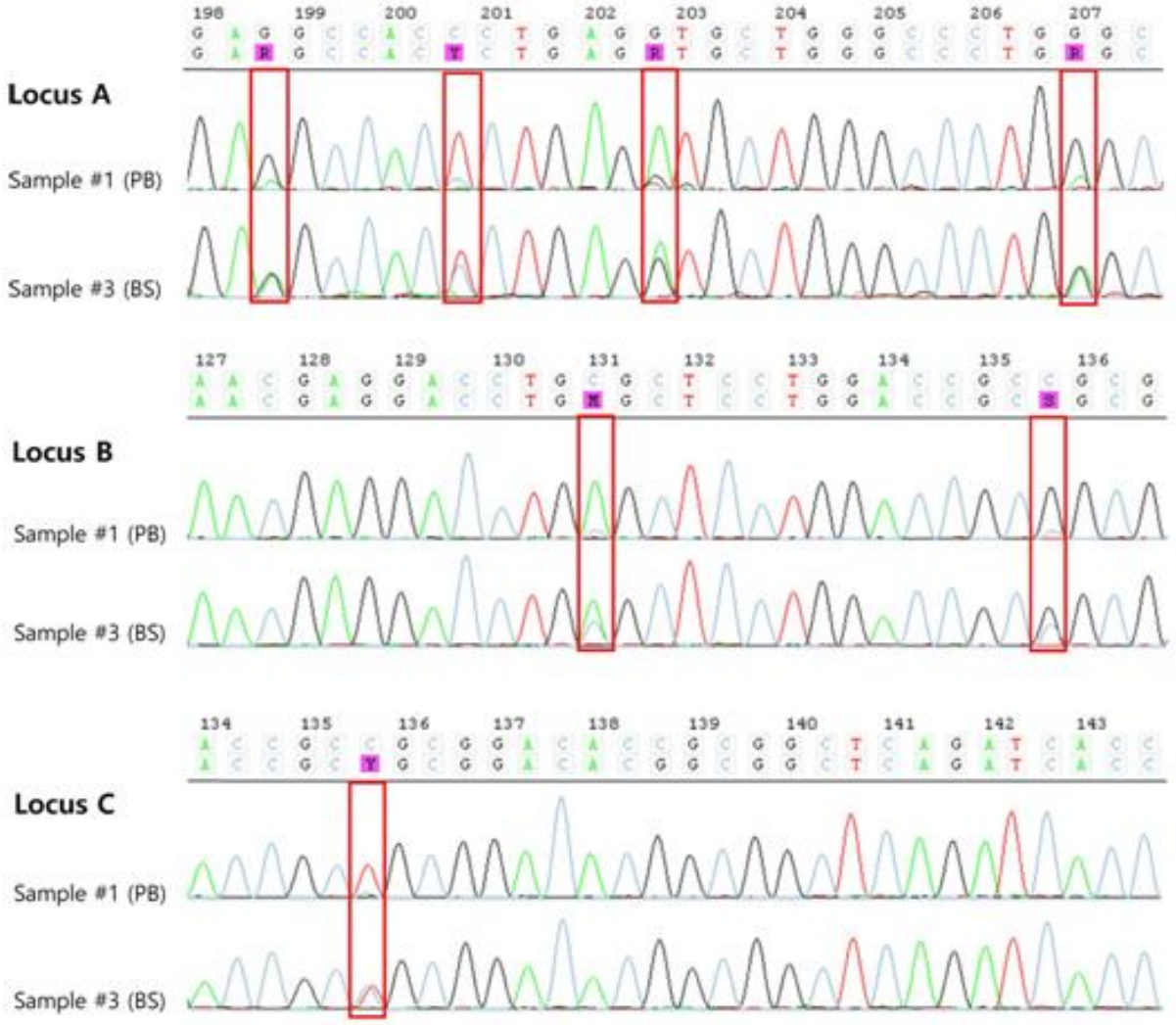


Figure 1: Gene types A, B, and C as determined by PB and BS sequences (sample #1 and #3, respectively). Potentially representing heterozygosity or just background noise, the little dots in the chromatogram data of SBT Sample #1 are worth considering. A red box indicates that Sample 3 does, in fact, display heterozygosity.

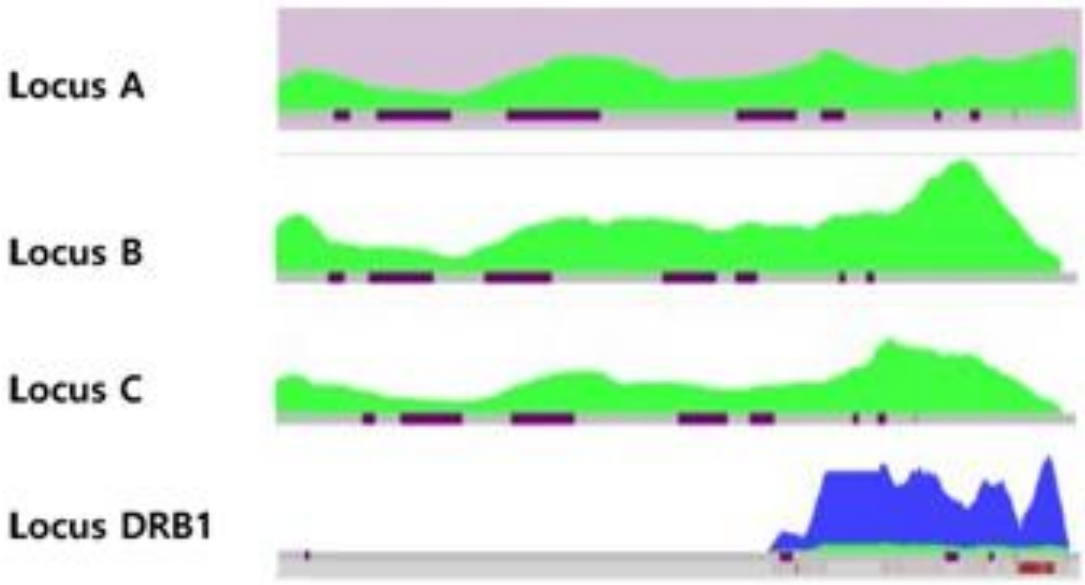


Figure 2. Plots of NGS results for the initial sample. The histograms display the allele rates discovered in the NGS data regarding coverage depth. In the blue region, you can see Allele 2, and in the green region, you can see Allele 1. Loss of heterozygosity (LOH) at the HLA-A, -B, and -C loci is indicated by the fact that the heat map for Sample #1 only displays one gene in green. Despite the fact that the heat map for the HLA-DRB1 gene displayed two distinct colors, there was insufficient coverage for Allele 1.

Table 2. As of the time of discovery, hematologic malignancies can be caused by a decrease of HLA heterozygosity.

Case #	Diagnosis	Blast % (PB/BM)	Specimen	Chromosome BM	Mutations	HLA typing	HLA loci with LOH	Methods for confirmation of LOH	LOH region
1[1]	AML	91%/ N/A	PB	Normal	N/A	SSO, SSP	-A, -B	SBT in remission	N/A
2[2]	AML M1	95%/ 96%	PB	Normal	*FLT3, WT1, NPM1*	SBT, SSO	-A, -B, -C, -DRB1, -DQB1	SSO, SSP and SBT after treatment	N/A
3[2]	AML M5b	96%/ N/A	PB	Normal	*FLT3, WT1*	SBT, SSO	-A, -B, -C, -DRB1	SSO, SSP and SBT after treatment	N/A
4[2]	AML M5a	85%/ N/A	PB	Normal	*FLT3 weak, NPM1*	SBT, SSO	A, -B, -C,	SSO, SSP and SBT after treatment	N/A
5[2]	AML M1	N/A/ N/A	PB	Normal	*FLT3*	SBT, SSO	-A, -B, -C	SSO, SSP and SBT after treatment	N/A
6[2]	AML M1	96%/ N/A	PB	Normal	*FLT3*	SBT, SSO	-A, -B, -C, -DRB1, -DQB1	SSO, SSP and SBT after treatment	N/A
7[2]	AML M4	27%/ N/A	PB	Y loss	*FLT3, NPM1*	SBT, SSO	-A	SSO, SSP and SBT after treatment	N/A
8[3]	AML	90%/ N/A	PB	Normal	*NPM1*	SSO, SSP	-A, -B, -C	HLA typing with BS; SSP and SBT after remission	N/A
9[3]	FL transformed into leukemic DLBCL	65%/ N/A	PB	t(14;18),+ i(14q),+18	N/A	SSO, SBT, SSP	-B, -C	SSP	N/A
10[3]	AML	79%/ N/A	PB	Normal	N/A	SSO, SBT, SSP	-A, -C	SSP	N/A
11[4]	AML	89%/ 89%	PB	Normal	N/A	SSO, SBT, NGS	-A, -B, -C, -DRB1, -DQB1	HLA typing with BS; SSO, SBT and NGS after CTx, CMA	6p25.3p21.31 (184,718–36084.672) (36Mb)
12[5]	T-ALL	87%/ 85%	PB	Normal	*SIL-TAL1, SH2B3, TCRβ,* and *TCRγ* rearrangement	SBT, NGS	-B, -C, -DRB1, -DPB1	NGS; SBT and NGS after CTx; CMA	6p25.3p21.31 (203877_33528424) (33.32Mb)
13[6]	Therapy-related AML	98%/ 93%	PB	Normal	*IDH1, NPM1*	NGS	-A	SSO and NGS after CTx; CMA	6p25.3p21.33(184,719_30,798,988) (30.6Mb)
14 *	AML	26%/ 27%	PB	Normal	*NPM1, KRAS, NRAS, PTPN11, KMT2A*	SBT, NGS	-A, -B, -C	NGS; HLA typing with BS; CMA	6p25.3p21.1(184,718–43,635,395) (43.5Mb)

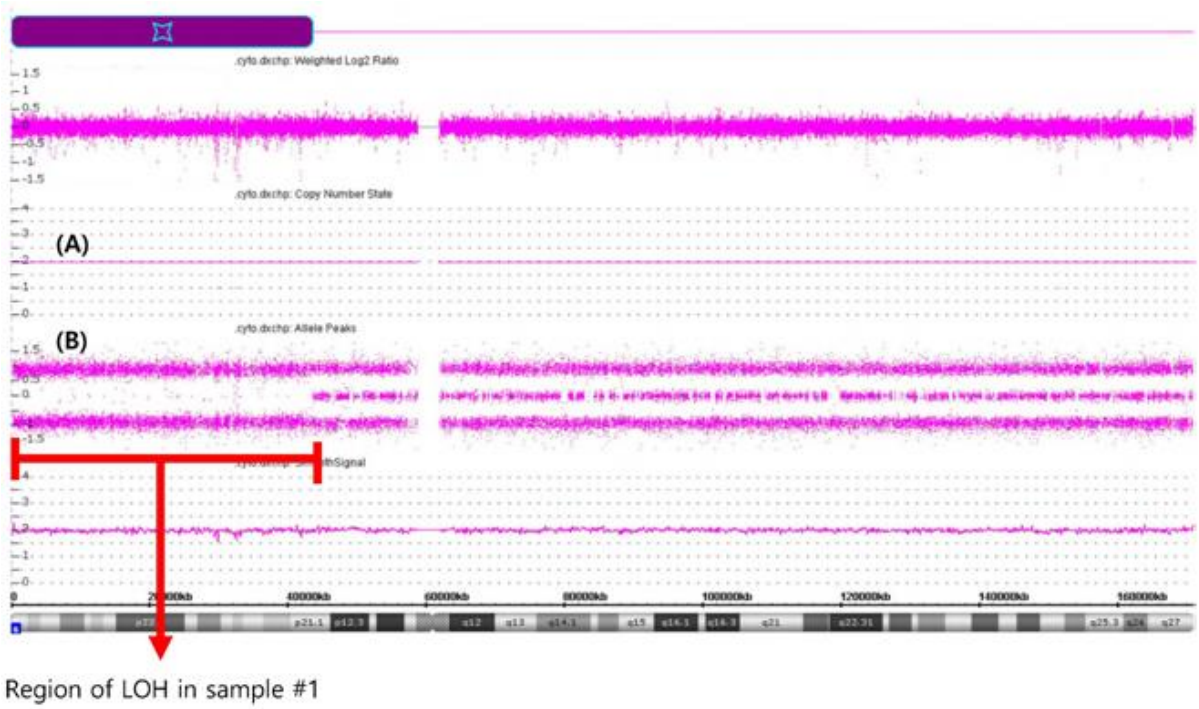


Figure 3. In order to examine the DNA in the PB of sample #1, we employed CMA. Panel (A) displays the CN condition as straight lines, indicating that CN remains constant. Panel (B) displays the SNP findings.

This rules out the possibility that chromosome 6's short arm (43.5 Mb) exists.

For hematologic malignancies without mutations on chromosome 6p or with a normal karyotype, the possibility of incorrect homozygosity while attempting to determine HLA homozygosity remains unclear. There may be significant implications for treatment planning, particularly in the selection of hematopoietic stem cell donors, if HLA loss of heterozygosity is detected early. Discovering LOH at this stage may further enhance prognostic outcomes by providing crucial details regarding the operation and progression of AML. The impact of HLA loss of heterozygosity on treatment outcomes and prognoses for patients with recurrent leukemia following hematopoietic stem cell transplants has been the subject of extensive research. One of the primary mechanisms by which leukemic cells evade the immune system and continue to proliferate following a donation is the loss of heterozygosity (LOH). This procedure decreases graft-versus-leukemia activity, which in turn increases recurrence risk and decreases therapy efficacy. In certain cases, new forms of immunotherapy have demonstrated potential, such as the use of CAR-NK cells and the blockage of immunological checkpoints. To determine the efficacy of these approaches for individuals with an HLA LOH diagnosis, further research is required.

When HLA typing of peripheral blood (including blast cells) reveals conclusive homozygosity, loss of heterozygosity (LOH) must be considered, as demonstrated in previous examples. We investigated the likelihood of heterozygosity when we noticed tiny secondary spots in our SBT chromatograms. The absence of investigation into these faint peaks should not have been justified given their presence in all exons and their exact heterozygous forms (HLA-A*24:02/02:01, HLA-B52:01/48:01, and HLA-C12:02/*08:01). An incorrect homozygosity due to loss of heterozygosity (LOH), issues with blood transfers, or unclean materials was the initial theory. We can rule out contamination and transfer effects as

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potential reasons because the results of Sample #2 were consistent across several tests, suggesting that loss of heterozygosity (LOH) may have occurred. Examining samples taken from the mouth (oral swabs) finally verified the findings.

These findings highlight the need for caution when testing for HLA homozygosity in patients undergoing HSCT for blood malignancies. Checking SBT chromatograms for low-intensity spots for links to known alleles is of the utmost importance. Lab procedures may record generic peaks, but if allele-specific peaks are observed, it could indicate that homozygosity is incorrect. In such a case, it is recommended to examine the material once again following chemotherapy-induced blast clearing or to do confirmatory testing on many types of specimens. It is worth noting that following blast clearing, our patient had consistent homozygosity at the HLA-A, -B, and -C loci, in agreement with the diagnostic findings. As far as anyone can tell, this case is unique. Typically, samples with high blast rates will display loss of heterozygosity (LOH), but, the patient initially had just 26% blasts, and even without blasts, LOH was discernible in the samples. The exact number of blasts needed to alter HLA type in a LOH patient remains unknown. The abnormalities in the erythroid and myeloid lines prompted us to consider the likelihood that a past myelodysplastic tumor may have been the culprit. Acute myeloid leukemia (AML) with alterations associated with myelodysplasia could not be diagnosed because access to previous medical data was unavailable.

Research has demonstrated that the detection limits for minor alleles vary across HLA typing methods. A sensitivity threshold of 15% for the detection of heterozygosity was established by Dubois et al. using Luminex microbead assays. As a result, when blast rates exceed 80%, typos may occur. Researchers have found that approximately 20% of SBT cases involve heterozygosity, according to studies that combine DNA. For SSP, the sensitivity ranges from 2.5 to 17%, for reverse SSO, from 5 to 17%, and for SBT, from 17 to 33%. Accordingly, SBT may provide inaccurate homozygosity results at lower limits, but SSP may only provide inaccurate classification results at extremely large blast fractions (>95%). Although leukemic blasts do contain more DNA and larger nuclei than regular leukocytes, the limits of detection for DNA-mixing techniques are unrelated to blast percentages. Prolonged loss of heterozygosity (LOH) following blast clearance in acute myeloid leukemia (AML) is uncommon and the underlying mechanism is unclear. Factors contributing to clonal expansion in populations of non-blast leukocytes include the possibility of FLT3 and NPM1 mutations in multipotent hematopoietic progenitors.

Finally, HLA LOH can be present at the time of AML diagnosis, although it is more commonly associated with the immune system's ability to evade detection following a repetition. Therefore, for HLA typing in HSCT, it is best to use samples obtained after the blast if at all possible. To confirm the likelihood of CN-LOH, alternative samples or additional tests should be employed when HLA homozygosity persists after clearance. Separating real homozygosity from LOH-related artifacts requires a careful examination of low-intensity areas on SBT chromatograms.

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5. CONCLUSION

A very important safety measure in transplant immunology is checking for wrong homozygosity during HLA testing before the transfer. If homozygosity isn't checked, it can cause unexpected immune system problems, bad matching between donors and recipients, and eventually less-than-ideal transplant outcomes. Homozygosity can happen because of primer-binding mismatches, low-resolution typing methods, allele loss, poor DNA quality, or mistakes made in the lab. Without a full investigation, these results could be wrongly seen as proof that a gene is not present in the genome instead of a technical error.

As a result, labs have to do confirmation in a planned, orderly way. To do this, different amplification methods must be used, multiple tests must be run with improved DNA extraction, high-resolution typing methods like next-generation sequencing must be used, and findings must be confirmed using different methods. Family studies, serological associations, and analyses of past data can help clear up unclear meanings. To make decisions more consistent and lower differences between labs, it's important to have strict internal quality controls, staff competency reviews, and well-defined formulas.

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