PRETRANSPLANT HLA TYPING REVEALED LOSS OF HETEROZYGOSITY IN THE MAJOR HISTOCOMPATIBILITY COMPLEX IN A PATIENT WITH ACUTE MYELOID LEUKEMIA.

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Keywords: HLA, hematological malignancies, microarray analysis, loss of heterozygosity, hematopoietic stem cells.

Abbreviations:

CMA, chromosomal microarray analysis;

SNP, single nucleotide polymorphism;

LOH, loss of heterozygosity;

CN-LOH, copy-neutral loss of heterozygosity;

UPD, uniparental disomy;

HCT, hematopoietic stem cell transplantation;

GVHD, graft vs. host disease;

MHC, major histocompatibility complex;

AML, acute myeloid leukemia;

STREGA, Strengthening the reporting of Genetic Association studies;

PB peripheral blood;

BS, buccal swabs;

L/MR, low/medium resolution;

NGS, next generation sequencing;

CN, copy number;

BM, bone marrow;

The informed consent was obtained.

Declarations of interest: none.

Abstract

Introduction. Chromosomal abnormalities are frequent events in hematological malignancies. The degree of HLA compatibility between donor and recipient in hematopoietic stem cell transplantation is critical.

Purpose of the study. In this report, we describe an acute myeloid leukemia case with loss of heterozygosity (LOH) encompassing the entire HLA.

Materials and methods. HLA molecular typing was performed on peripheral blood (PB) and buccal swabs (BS). Chromosomal microarray analysis (CMA) was performed using a whole genome platform

Results. Typing results on PB sample collected during blast crisis demonstrated homozygosity at the -A, -B, -C, -DR, and -DQ loci. A BS sample demonstrated heterozygosity at all loci. A subsequent PB sample drawn after count recovery confirmed heterozygosity. The CMA performed on PB samples collected during and after blast crisis revealed a large terminal region of copy-neutral LOH involving chromosome region 6p25.3p21.31, spanning approximately 35.9 Mb. The results of the CMA assay on sample collected after count recovery did not demonstrate LOH.

Conclusions. LOH at the HLA gene locus may significantly influence the donor search resulting in mistakenly choosing homozygous donors. We recommend confirming the HLA typing of recipients with hematological malignancies when homozygosity is detected at any locus by using BS samples, or alternatively from PB when remission is achieved.

1. Introduction.

Chromosomal abnormalities are frequent events in many diseases including solid tumors, hematological malignancies and aplastic anemia [1-6]. Single nucleotide polymorphism (SNP)

and chromosomal microarray analysis (CMA) are the types of DNA microarrays that allow for detection of both copy number changes and loss of heterozygosity (LOH), both of which are observed in many neoplasms [3, 7, 8, 9]. Copy-neutral loss of heterozygosity (CN-LOH), in a broad sense, means that a locus for which a cell is heterozygous becomes homozygous without concurrent changes in the gene copy number. The main mechanism of CN-LOH in cancer is somatically acquired uniparental disomy (UPD), where a locus that is heterozygous in a normal cell becomes homozygous in a cancerous cell derived from the normal cell through somatic recombination [11, 12, 13]. This mechanism can result in a selective advantage when tumor cell acquires potentially pathogenic homozygous gene mutations. Other mechanisms of CN-LOH include deletions or other chromosomal rearrangements, but the mechanism explained above is the most important [5, 14].

Allogeneic hematopoietic stem cell transplantation (HCT) is a potentially curative treatment for many malignant and nonmalignant diseases. Although recipients receive high-doses of chemotherapy/irradiation preparative regimens, residual recipient immune cells may attack donor stem cells causing graft failure or rejection. More commonly, donor immunocompetent cells contained in the graft recognize tissues of the recipient causing graft vs. host disease (GVHD). In this context, the degree of compatibility between donor and recipient human leukocyte antigens (HLA) is a critical determinant. The HLA gene family encodes the major histocompatibility complex (MHC) proteins and is localized in humans to a region of approximately 4 megabases at chromosome 6p21.3. LOH at MHC (6p) has been reported by many groups [7, 14-18]. One established method to identify LOH is short tandem repeat (STR) analysis, which is a powerful and broadly applicable technique due to the simplicity of the assay and the high degree of polymorphism in the MHC region [19]. In most transplant center, matching donor and recipient at the HLA-A, -B, -C, -DRB1 and DQB1 loci is the goal whenever possible. In this report, we

describe an acute myeloid leukemia (AML) case with LOH of chromosome 6p encompassing the entire HLA gene family detected in peripheral blood DNA samples.

2. Materials, methods and sampling.

The manuscript is in compliance with the guidelines for Strengthening the reporting of Genetic Association studies (STREGA)

2.1. Sources of DNA.

Molecular typing of the patient was performed using DNA extracted from peripheral blood (PB) and buccal swabs (BS) (Puritan Hydraflock, Fisher Scientific, Chicago, IL, USA) using a Qiagen kit (QIAGEN, Hilden, Germany). The concentration and purity of DNA were determined with a spectrometer using the ratio of absorbance at 260 nm and 280 nm wavelengths (NanoDrop One Spectrophotometer, ThermoFisher Scientific, Wilmington, DE, USA). In all cases, the A260/A280 ratio was within the range of 1.8-2.0.

2.2. HLA typing.

Low/medium resolution (L/MR) typing at the HLA-A, -B, -C, -DRB1, and –DQB1 loci was performed by a polymerase chain reaction (PCR) and sequence specific oligonucleotide probes (SSOP) genotyping assay on a Luminex LABScan3D platform (One Lambda, Inc., Canoga Park, CA, USA). The HLA Fusion v4.2 software was used for data analysis.

High resolution (four digits) typing at the indicated loci was performed by the Sanger dideoxy sequencing method using a Genetic Analyzer 3500xL (Applied Biosystems, HITACHI, Japan). Sequencing reagents were obtained from GENDX (Utrecht, The Netherlands) and One Lambda. The SBTengine v3.18 software was used for data analysis.

2.3. Next generation sequencing (NGS)

NGS was performed to verify the high resolution typing results according to vendor recommendations. Briefly, HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 loci were amplified from purified sample DNA using the NXType reagent kit (One Lambda, Inc, Canoga Park, CA,

USA). Following amplification, sample libraries were prepared using ION-XLIB and IONCHEF-EXT kits (One Lambda, Inc, Canoga Park, CA, USA). The final prepared libraries were sequenced using an IonTorrent S5 sequencer (One Lambda, Inc., Canoga Park, CA, USA) on an IONS5-530C4 chip (One Lambda, Inc,). One Lambda's TypeStream Visual software was used to analyze the data.

2.4. CMA.

CMA was performed on genomic DNA extracted from PB using a whole genome platform that included both copy number (CN) and SNP probes on an Affymetrix CytoScan HD Array (Affymetrix, Santa Clara, CA, USA) according to manufacturer's protocols. The data were analyzed with the ChAS software (Affymetrix) using the following filtering criteria: deletions >25 kb (a minimum of 25 markers), duplications >50 kb (a minimum of 50 markers), and LOH > 3000 kb (a minimum of 50 markers).

a. Samples collected from the patient and tests performed.

Two samples of PB were collected in EDTA tubes on December 18, 2017. DNA was extracted from one tube on December 19, 2017 (sample #1) and from the other tube on December 21, 2017 (sample #3). The BS sample (sample #2) was received on December 21, 2017, and DNA was isolated on the same day. In addition, another PB sample (sample #4) was obtained on February 2, 2018 upon completion of a course of chemotherapy in blast-crisis-free period. This sample was also tested with the CMA assay. The tests performed on the samples specified above are summarized in Table 1.

3. Case description

A 65-year-old Caucasian woman with AML was HLA typed as a candidate for allogeneic HCT. A bone marrow (BM) biopsy performed on December 18 2017 was consistent with the diagnosis of AML, with blasts accounting for 89% of total cellularity, and cytogenetic analysis revealed a normal karyotype (46, XX.) A PB sample was drawn on the same date for HLA typing. A

complete blood cell count on the same day also showed that the platelet count was 16.0 x $10^3/mm^3$ and the white blood cell count was 15.3 x $10^3/mm^3$, of which 89% were blasts. The patient received chemotherapy, which consisted of 7 days of cytarabine continuously administered at 100 mg/m^2 and idarubicin given in 3 daily doses at 12 mg/m^2. Twenty-one days after the start of induction chemotherapy, a repeat BM biopsy demonstrated aplasia with clearance of blasts, and a PB (sample #3, see Table 1) had a platelet count of 94.0 x $10^3/mm^3$ and white blood cell count of 0.4 x $10^3/mm^3$ with no circulating blasts. Two potential sibling donors were also HLA typed, and the results are presented in Table 2.

4. Results

4.1. HLA typing.

The results of HLA typing of the samples taken from the patient are shown in Table 3 and Figure 1. Low resolution typing on sample #1 demonstrated homozygosity at the -A, -B, -C, -DR, and -DQ loci, which was confirmed by NGS performed in a reference laboratory (results obtained on April 05, 2018, not on December 18, 2017 when it was drawn). We were unable to reach typing at DR locus using SBT. A buccal swab (BS) sample (Sample #2) obtained from the patient 3 days later for confirmation of HLA typing, however, demonstrated heterozygosity at all loci tested with both low and high resolution assays including SBT and NGS. Notably, low resolution and NGS typings of sample #3 revealed heterozygosity at all loci (Table 3). Finally, a subsequent PB sample (sample #4) drawn after count recovery and attainment of remission 46 days after induction also confirmed heterozygosity when low and high (SBT and NGS) resolution typing methods were applied. NGS histogram results showed that, although heterozygous calls were observed for both samples #2 and #3, coverage of one allele was significantly lower than coverage of the other in HLA-A, -B, -C, -DRB1 and –DQB1 loci (Fig.1 b, c). Furthermore, only one allele coverage was shown in sample #1 when NGS test was performed (Fig. 1a)

4.2. CMA.

The CMA performed on sample #1 (Fig. 2 a, c) and sample 3 (Fig. 2 b, d), both drawn before remission was achieved (during blast crisis), revealed a large terminal region of copy-neutral LOH involving chromosome region 6p25.3p21.31 (184,718 – 36,084.672), spanning approximately 35.9 megabases in size. This region of LOH encompassed the entire HLA gene family locus (Fig. 3). The results of the CMA assay on sample #4 (after count recovery) did not demonstrate any clinically significant CN changes or regions with LOH (Fig. 4).

5. Discussion

Chromosomal abnormalities are frequent in various malignancies [2, 3, 6, 8, 10, 17, 20]. Karyotypic analyses of neoplastic cells have revealed a steadily increasing number of recurrent chromosomal rearrangements that are found to be associated with particular diseases or disease subgroups, including net loss of chromosomal material (i.e. deletions, monosomies), net gains of chromosomal material (duplications, trisomies) and structural rearrangements producing fusion genes (inversions, insertions and translocations). The short arm of chromosome 6, where HLA genes are located, is often reported to be altered [2, 6, 16, 17, 21-24]. Among these alterations, absence of heterozygosity or LOH is a common event [12, 15, 17]. These genetic variations may affect one or several HLA loci [2, 7, 23]. The recent studies suggest that HLA LOH is an immune escape mechanism conferring a selective advantage to tumor cells [25, 26]. Furthermore, correlation between HLA-I expression and the number of tumor infiltrating T lymphocytes was demonstrated [27].

In humans, the HLA complex consists of numerous genes located close together on chromosome 6p that play critical roles in tumor, transplant and infectious immunology. Graft outcomes are strictly dependent on compatibility of the donor and recipient HLA genotype. HLA compatibility at both haplotypes is generally preferred whenever possible in the setting of allogeneic HCT due to the risk graft versus host disease.

Hemizygosity or LOH at the HLA locus (deceptively indicating homozygosity) in recipient cells affects correct donor identification and can result in deleterious or even fatal consequences if unrecognized. In this report, we describe an AML patient with LOH affecting the HLA-A, -B, -C, -DR, and – DQ loci when genotyping was performed using DNA extracted from PB at the time of diagnosis (sample #1) (89% of blasts in PB). The results of low and high (NGS) resolution typing of sample #1 demonstrated LOH at the aforementioned loci, whereas the results of typing of sample #3 (drawn at the same time) revealed heterozygosity at all loci (both samples confirmed by a reference laboratory by NGS). In this respect, it is important to note that most true heterozygous calls range from ratios of 20:80 to 50:50 and HLA alleles present below 10% are typically not called. While the true depth of coverage is critical for minimizing false allelic calls, reliable genotyping is obtained as long as there are enough reads for each allele. To rule out sample integrity issues, low resolution genotyping on sample #3 was performed in our laboratory, and heterozygosity was detected at all loci (see Table 3). This discrepancy may potentially be explained by an increased number of tumor cells in sample #1 compared to sample #3. The CMA results for samples #1 and #3 disagree with the results of the NGS and low resolution typing tests performed on the same samples. Namely, the NGS test results demonstrated heterozygosity in sample #3, whereas the CMA results showed LOH. This discrepancy is most likely due to the increased level of sensitivity for mosaicism by NGS-based methods compared to CMA. Since there were normal cells present at a low level in the blood sample, and these cells harbor a heterozygous genotype at the HLA loci, sequencing with a high read depth would generate a small number of reads with the alternate allele. CMA, however, is much less sensitive to mosaicism and would be unlikely to detect the normal heterozygous genotype below approximately 10% of the total cell population. In our case, the CMA revealed LOH for chromosome 6p in samples #1 and #3 (PB drawn during blast crisis), while NGS demonstrated homozygosity for this region in sample #1 but not sample #3. Review of the CMA

SNP data for each case reveals an increased spread of the b-allele frequency plot in sample #3 (Fig. 2d) compared to sample #1 (Fig. 2c), which is suggestive of low-level mosaicism near the limit of detection for this assay. We propose that the most likely mechanism of LOH in our case is somatic recombination as the CN has not changed. Similar results have been reported by Coppage M et al. [16]. These investigators observed LOH at the HLA –A, -B, -C, - DRB1 and -DQB1 loci when DNA extracted from the PB B-cells was used in the CMA in a chronic lymphocytic lymphoma patient. However, the same assay did not detect LOH when DNA isolated from the PB T-cells and BS epithelium was used for testing. The likely mechanism of LOH in their case is partial deletion as they detected CN loss as well.

Alternatively, the observed discrepancy may be due to the high sensitivity of the NGS technology compared to other molecular assays. That is, this case may be an example of the minor allele of the HLA gene being amplified in an imbalanced manner due to its underrepresentation in the sample. If so, this would be characteristic of an instance when even 5% of the total reads derived from a particular gene is reliable enough to confirm the presence of a minor allele. While this study utilized a SNP microarray to identify the region of LOH in this patient, STR analysis could be used in future cases where LOH is suspected due to its high sensitivity and lower cost.

In summary, while LOH is not a rare event in solid tumor cells, it is also observed in patients with hematological malignancies requiring stem cell transplantation. LOH at the HLA gene locus may significantly influence the donor search resulting in mistakenly choosing homozygous (and hence mismatched) donors at aforementioned genes. We recommend confirming the HLA typing of recipients with hematological malignancies when homozygosity is detected at any locus by using DNA extracted from BS when typing is done at the time of diagnosis when active disease is present, or alternatively from PB when remission is achieved if typing was not done earlier.

Author's contribution: AL carried out testing design, data analysis, writing and preparation of the manuscript, discussion of results; MK-S, RB, CL and CC carried out HLA testing, SS participated in testing design and carried out HLA testing; ZK reviewing the manuscript, discussion of results; RM carried out NGS testing, participated in discussion of the results; AB and GV carried out CMA testing, participated in discussion of the results, manuscript preparation; SF preparation and reviewing the manuscript, discussion of the results.

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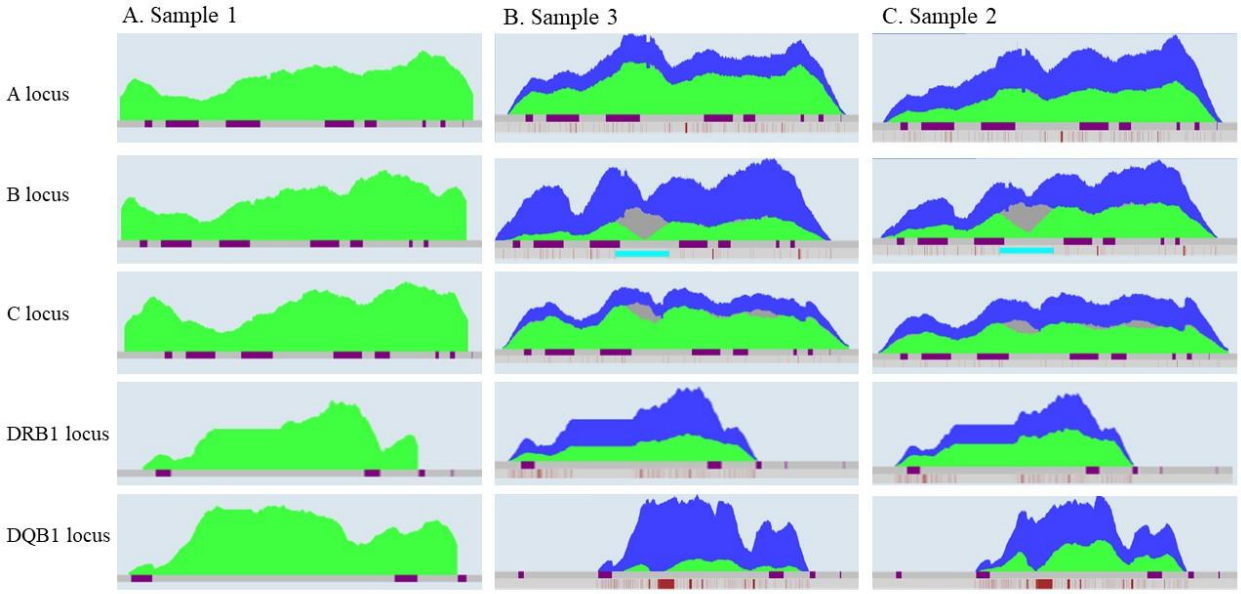

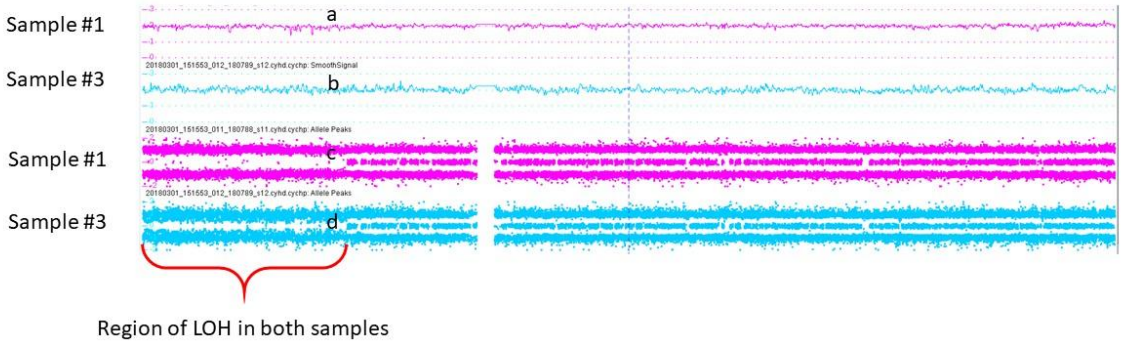

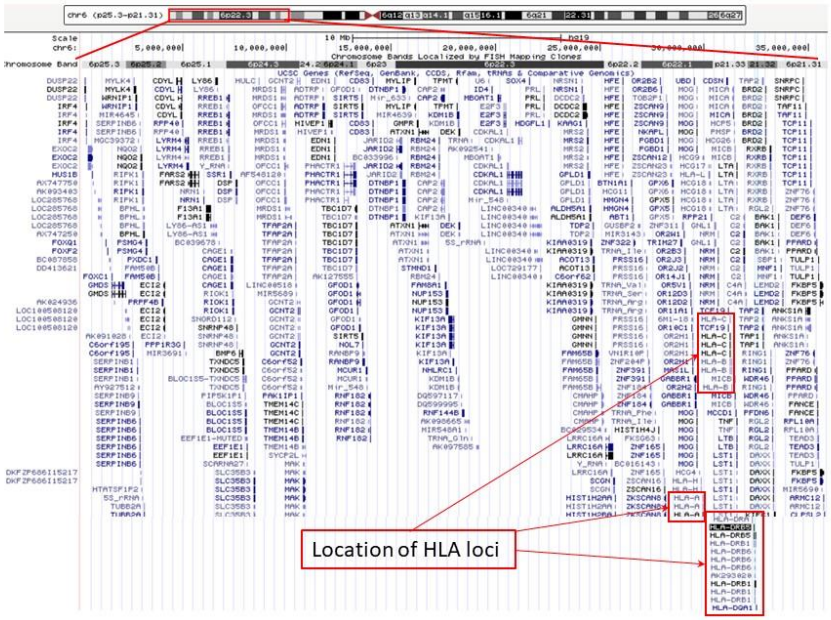

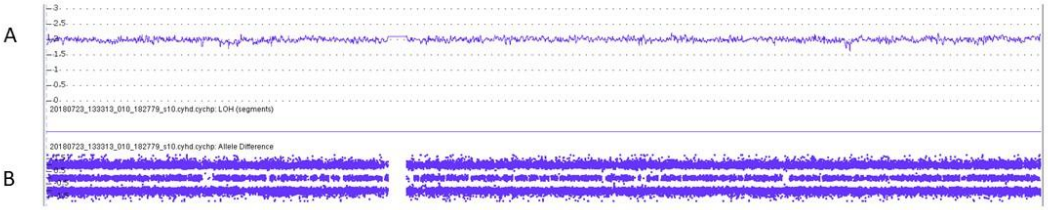

Table 1. Tests performed on the samples taken from the patient.

Sample ID	Date of DNA extraction	Date of testing	Tests performed		
			CMA	L/MR	SBT NGS
Reference lab			IU	IU	IU
Sample #1 (PB)[a] +	12/18/2017	12/19/2017	+	+	+
Sample #2 (BS) +	12/21/2017	12/22/2017	NT	+	+
Sample #3 (PB)[a] +[c]	12/21/2017	01/08/2018	+	+	NT
Sample #4 (PB)[b] +	02/02/2018	02/05/2018	+	+	+

NT, not tested; NA, not applicable; CMA, chromosomal microarray; L/MR, low/medium resolution HLA typing;

SBT, sequence based typing; NGS, next-generation sequencing; IU, Indiana University;

PB, peripheral blood;

BS, buccal swab.

[a] - Sample #1 and Sample #3 were collected on 12/18/2017 during blast crisis.

[b] - Sample #4 was collected on 02/02/2018 after count recovery and attainment of remission (after blast crisis).

[c] – NGS testing on sample #3 was performed on 04/05/2018

Table 2. HLA typing results from the patient at initial evaluation and two potential sibling donors.

	Patient (BS)	Sibling 1 (PB)
	Sibling 2 (PB)	
	a/c	a/c
	c/b	
	SBT[b]	PCR-SSOP
	PCR-SSOP	
HLA-A1	*24:02	*24
	*24	
HLA-A2	*29:02	*29
	*32	
HLA-B1	*44:03	*44
	*44	
HLA-B2	*51:01	*51
	*44	
HLA-C1	*02:02	*02
	*05	
HLA-C2	*16:02	*16
	*16	
HLA-DR1	*07:01	*07:01[a]
	*07	
HLA-DR2	*13:01	*13:01
	*12	
HLA-DQ1	*06:03	*06:03
	*03	
HLA-DQ2	*02:02	*02:02
	*02	

[a] – class 2 high resolution was performed by SBT

[b] – BS epithelium was used as a source of DNA

Table 3. Results of HLA typing at the time of diagnosis on PB, BS and at follow up after 2 months on PB.

Specimen	Sample #1 (PB)			Sample #2 (BS)			
Sample #3 (PB)				Sample #4 (PB)			
Date tested	12/19/2017[b]			12/22/2017			
01/08/2018[b]				02/05/2018[c]			
	PCR-SSOP	SBT	NGS[a]	PCR-SSOP	SBT	NGS	
PCR-SSOP	SBT	NGS					
HLA-A1	*24	*24:02	*24:02	*24	*24:02	*24:02	*24
NT	*24:02	*24	*24:02	*24:02			
HLA-A2	lost	lost	lost	*29	*29:02	*29:02	*29
NT	*29:02	*29	*29:02	*29:02			
HLA-B1	lost	lost	lost	*44	*44:03	*44:03	*44
NT	*44:03	*44	*44:03	*44:03			
HLA-B2	*51	*51:01	*51:01	*51	*51:01	*51:01	*51
NT	*51:01	*51	*51:01	*51:01			
HLA-C1	*02	*02:02	*02:02	*02	*02:02	*02:02	*02
NT	*02:02	*02	*02:02	*02:02			
HLA-C2	lost	lost	lost	*16	*16:02	*16:02	*16
NT	*16:02	*16	*16:02	*16:02			
HLA-DR1	lost	*xx:xx[d]	lost	*07	*07:01	*07:01	*07
NT	*07:01	*07	*07:01	*07:01			
HLA-DR2	*13	*xx:xx[d]	*13:01	*13	*13:01	*13:01	*13
NT	*13:01	*13	*13:01	*13:01			
HLA-DQ1	*06	*06:03	*06:03	*06	*06:03	*06:03	*06
NT	*06:03	*06	*06:03	*06:03			
HLA-DQ2	lost	lost	lost	*02	*02:02	*02:02	*02
NT	*02:02	*02	*02:02	*02:02			

[a] – NGS typing of Sample #1 was performed on 04/05/2018 in reference laboratory
[b] – Sample #1 and Sample #3 were taken simultaneously during blast crisis. Dates indicate when testing was performed.
[c] – Sample #4 was taken after count recovery and attainment of remission (after blast crisis)
[d] – no matched four digits alleles were detected