



Leukemia specific loss of heterozygosity of MHC in a CLL patient: Disease state impacts timing of confirmatory typing

Myra Coppage^{a,*}, Anwar Iqbal^a, Ausaf Ahmad^a, Michael W. Becker^b

^a Department of Pathology and Laboratory Medicine, University of Rochester Medical Center and the Wilmot Cancer Center, Rochester, NY, United States

^b Department of Medicine Hematology/Oncology, University of Rochester Medical Center and the Wilmot Cancer Center, Rochester, NY, United States

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ABSTRACT

A 63 year old white male with refractory B-CLL presented for allogeneic HSCT evaluation; HLA typing was performed on PBL at time of WBC = 53 K, ALC = 47 K and revealed homozygosity at Class I locus and heterozygosity at Class II locus. Two siblings were full mismatches with the recipient and an unrelated search initiated. The patient was treated with Fludarabine and Rituxan complicated by aplastic anemia and bacteremia. Prior to transplant, confirmatory typing performed on PB revealed two full haplotypes at Class I and II. Sample identification error and the presence of third party lymphocyte engraftment as a result of prior red cell or granulocyte transfusion(s) were ruled out by STR analysis of 8 loci of all samples, T and B cells from cryopreserved PB at blast crisis were HLA typed independently. T cell typing yielded both complete haplotypes (genotype verified by offspring HLA typing); B cells typed for homozygous haplotype indicating loss of heterozygosity of MHC locus. Microarray based comparative genomic hybridization of tumor cells confirmed LOH at 6p.

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1. Introduction

Chromosomal abnormalities are common among patients with hematologic malignancies and may be useful in tailoring therapy. In chronic lymphocytic leukemia several, such as del 13q14, del 17p and trisomy 12 are of prognostic significance [1]. Fluorescent in situ hybridization (FISH) testing is used routinely to identify such aberrations, but detection is limited to the probes used in the panel [2]. Thus other, potentially important genetic changes may be undetected. We describe a CLL case with undetected genetic aberration including loss of heterozygosity of MHC genes which impacted recipient-donor matching for hematopoietic stem cell transplant.

2. Case

The patient is a 63 year old Caucasian male diagnosed with CLL in 1985 when he presented with generalized lymphadenopathy. Lymph node biopsy and bone marrow aspirate and biopsy were consistent with diagnosis of chronic lymphocytic leukemia. He was observed through July 2009 when his white blood cell count was found to be 83,000 and 79% lymphocytes. He received 2 cycles Fludarabine and Rituxan, but within 3 weeks presented with fever and was found to be pancytopenic. He was subsequently referred

to our institution for consultation. At time of evaluation his WBC was 87,100 with 99% lymphocytes, hematocrit was 34% and platelets count was 27,000. Peripheral blood flow cytometry revealed a kappa-restricted CD10[−], CD20⁺, CD5⁺, CD23⁺, CD19⁺, FMC7[−] CD38[−] clonal population of B cells. A bone marrow aspirate and biopsy demonstrated diffuse involvement of the marrow by CLL. FISH on the marrow revealed 13q14.3 (D13S319) deletion detected in 200/200 cells as well as a 17p13 (p53) deletion detected in 200/200 cells. Routine G-banding was not performed. He underwent HLA typing for consideration of allogeneic transplant using a peripheral blood sample obtained at the time of evaluation. In addition, he consented to participate in an IRB approved protocol in which peripheral blood and marrow samples are prospectively collected in patients with hematologic malignancies to be used in research studies.

Following a cycle of Fludarabine, Cyclophosphamide and Rituxan, he was switched to Campath chemotherapy for two cycles but developed pancytopenia with transfusion dependence. His neutropenia was refractory to filgrastim.

3. Methods

3.1. Histocompatibility

Recipient HLA typing was performed by molecular method. DNA was extracted from white blood cells using a manual blood kit (Qiagen). Low resolution typing for HLA-A, B, C and DQ and high

* Corresponding author. Fax: +1 585 275 2436.

E-mail address: myra_coppage@urmc.rochester.edu (M. Coppage).

resolution typing for HLA-DRB1 was performed in a bead based assay using reverse sequence specific oligonucleotide probes (rSSO), Labtype® (One Lambda, Canoga Park CA). High resolution typing for HLA-A, B, C, and DQB1 used sequence specific primers (SSP) and gel electrophoresis (Unitray; Invitrogen). Related donors were screened by serological HLA typing (Gentrak and Transtype); those that are potential matches proceed to molecular typing. T and B subsets are obtained from peripheral blood by Dynal immunomagnetic bead method for CD8+ and CD19+ cells respectively (Invitrogen; Carlsbad, CA).

3.2. VNTR and aCGH-SNP array and analysis

The fluorescently-labeled PCR products targeting 8 loci (D11S554, D12S391, D16S539, FGA, Penta E, SE33, THO, VWF-B) were resolved using capillary electrophoresis with the relative quantity of each product measured by its fluorescence. Microarray CGH experiment was carried out on custom-designed Agilent's SurePrint G3 CGH-SNP 4 × 180 K cancer targeted platform (Agilent Technologies, Santa Clara, CA, USA) containing approximately 120,000 CGH probes covering over 500 cancer genes and more than 130 cancer-associated genomic regions and 60,000 SNP probes. Genotypes on this array are measured using one SNP probe per SNP, providing ~5–10 Mb resolution for LOH/UPD detection across the entire genome. Test DNA was referenced against same-sex control DNA. Patient DNA was isolated from peripheral blood and the control DNA (NA12891) was obtained from the Coriell Cell Repository (Camden, NJ, USA). Briefly, patient's DNA and control DNA (1.0 µg) were digested with restriction enzymes, Alu1 and Rsa1, and enzymatically labeled with dyes Cyanine-5dUTP and Cyanine-3dUTP respectively by using Agilent's Enzymatic Labeling Kit (Cat #5190–0449) as per the manufacturer's recommendations. The labeled DNA was hybridized as per the Agilent's recommendations at 65 °C for 40 h. Following washing, the slides were scanned in Agilent's high resolution scanner (Model #G2505C) at 3 µm resolution. The output files were processed using Feature Extraction software v10.10 and analyzed by Genomic Workbench Software v6.5 (Agilent Technologies, Santa Clara, CA, USA), using an ADM-1 algorithm for aberration analysis and visualization, with the threshold set at 6.7. To control small variations appearing in the data analysis, we used an extra aberration filter, defining the minimum number of probes that should be present in an aberrant region as 3 (gain or loss), with the minimum absolute level of average log2 ratio of 0.25. The centralization was on with threshold and bin size set at 6.0 and 10, respectively, and GC correction and fuzzy zero were also included in the analysis. For the detection of SNP and LOH, 95% confidence level and thresholds 6.0 were set respectively.

4. Results

Results of HLA typing are listed in Table 1. Both low and high resolution typing was performed using DNA from the initial blood draw as his post therapy ANC was 0. Verification typing was ultimately performed on a buccal swab, and the presence of a second Class I HLA haplotype was observed. Three scenarios were considered (1) lab error, (2) blood product contamination, and (3) loss of heterozygosity in tumor cells typed in the primary sample.

The first consideration was that the patient's first sample had been switched with another person whose sample was processed in the laboratory on the same day. Results from all of the samples tested within two days of receipt of the patient were examined (or retested if typing could not be confirmed); none matched the HLA typing obtained in the verification sample. Error occurring within the HLA laboratory was excluded.

Table 1

HLA typing results from patient at initial evaluation, two potential sibling donors, the whole blood sample received for verification and the T and B lymphocytes separated from a cryopreserved tumor containing peripheral blood sample. Antigens in bold type represent the haplotype lost in tumor cells.

	Class I	Class II
Initial blood sample (low and high resolution)	A*01:01, B*08:01, C*07:01	DRB1*03:01, DQB1*02:01, DRB1*04:01; DQB1* 03:01
Sibling 1	A3, 11; B51, 55	NT
Sibling 2	A3, 11; B51, 55	NT
Verification sample (buccal)	A*01:01, B*08:01, C*07:01 A*02:01; B*44:02; C*05:01	DRB1*03:01, DQB1*02:01, DRB1*04:01; DQB1* 03:01
Peripheral T cells	A*01:01, B*08:01, C*07:01 A*02:01; B*44:02; C*05:01	DRB1*03:01, DQB1*02:01, DRB1*04:01; DQB1* 03:01
Peripheral B cells "tumor blasts"	A*01:01, B*08:01, C*07:01	DRB1*03:01, DQB1*02:01

A second possibility was the presence of transient lymphocytes from blood products. The recipient had become aplastic as a result of therapy and was transfusion dependent. During a 4 month period he had received more than 100 units of RBCs, platelets and granulocytes at our institution, most of which were leukoreduced and irradiated. To rule out the presence of blood product contaminants or a sample mix-up occurring outside the HLA lab, DNA from multiple samples from the recipient were examined including the initial and verification samples from the HLA lab and a specimen from the research bank. Eight distinct loci were examined using VNTR analyses; the included loci comprise our standard chimerism panel used to distinguish DNA from separate individuals. Results supported that the all of the DNA samples were obtained from the same individual (data not shown). These results indicate that all HLA typings were obtained from the same individuals, that is, samples were not mixed up and no contaminating cells were present in the verification sample.

The final scenario investigated was that the HLA typing at evaluation was performed using tumor cells that had undergone a loss of heterozygosity within the major histocompatibility complex. This possibility was supported by a notation in the sample accession that a very large buffy coat (WBC fraction) was observed. Cryopreserved peripheral blood mononuclear cells from the patient were collected prior to therapy based on a University IRB approved protocol. T and B lymphocytes were isolated from the pre-therapy sample using cell-type specific immunomagnetic beads. DNA from each cell population was HLA typed independently (Table 1). DNA from the T cells demonstrated the two full haplotypes observed in the buccal swab sample (verification) while the B cell DNA exhibited a single haplotype for both Class I and II. This finding is consistent with the presence of mosaic in the original HLA typing of peripheral blood containing both tumor blasts and non-B cells. Interestingly the rSSO typing of the malignant cells did detect heterozygosity at Class II. The methods used may be a confounding factor. In this case the Class II rSSO typing achieved high resolution so no second method was required. A single exon 2 amplification is used in this method; it is possible that had SSP or SBT been performed the deletion would have been observed as it was in Class I. On retrospective analysis of the Class I SSO, very weak hybridization was actually observed for the Class I allele dropouts, but the amplification using SSP was clear negative. Confirmation of the LOH was achieved by genomic microarray analysis (Fig. 1). The sample contains 97% tumor cells as calculated by Agilent Genomic Workbench and approximately 36 Mb hemizygous

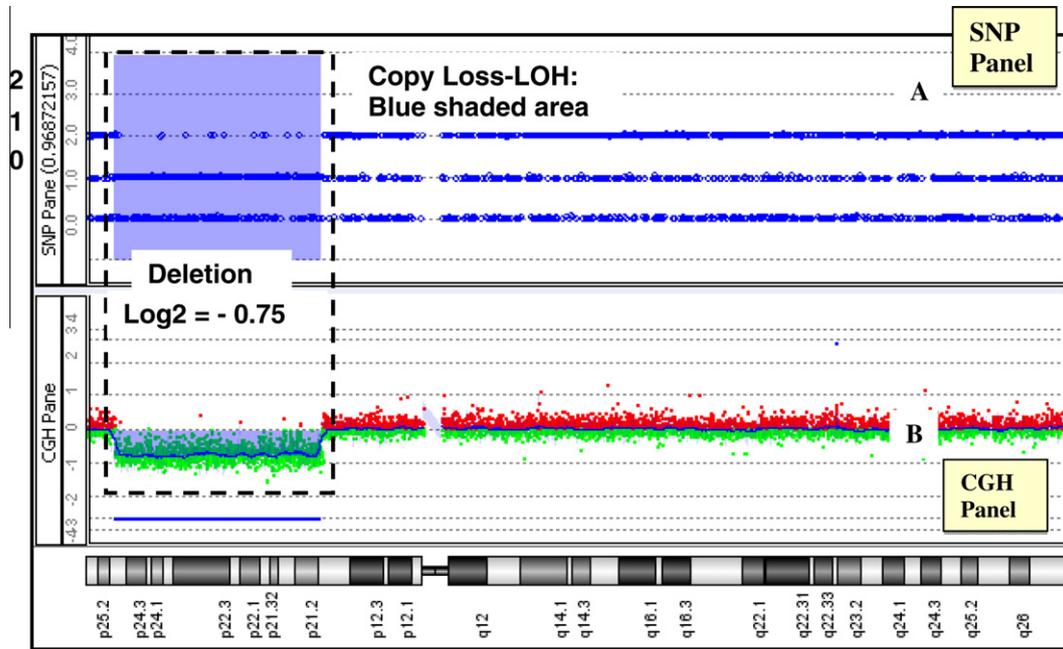


Fig. 1. Panels showing the combined aCGH – SNP arrays data of Chromosome 6 in the patient DNA. *Panel A:* LOH showing the hemizygous deletion of HLA allele. There are three SNP states with 0, 1 or 2 intact (AA, AB and BB) allelic copies (Panel A, blue lines). SNP data also indicated the hemizygous deletion of short arm (p) of chromosome 6 (0, 1 allelic copies i.e. A, B). *Panel B:* Copy number loss of approximately 36 Mb deletion in the chromosome 6p25.1–p21.1 region (4847853–40962142 bp).

deletion in the chromosome 6p25.1–p21.1 region (4847853–40962142 bp).

5. Discussion

Chronic lymphocytic leukemia is associated with a wide variety of presentations and clinical courses; it is characterized by a variety of recurring cytogenetic aberrations frequently identified by routine G-banding and fluorescence in situ hybridization (FISH) which serve as a prognostic tool and for determining therapeutic approach. However, many genomic mutations are below the resolution of routine G-banding and FISH analysis does not evaluate genetic aberrations beyond those in the probe panel. Genome wide analyses using microarrays provide much more extensive information about the genetic mechanisms of transformation. Pfeiffer [2] examined 70 consecutive CLL cases using 10 k and 50 k Affymetrix SNP arrays. They observed chromosomal imbalance in 65% and 80% of cases respectively. In 14 cases, copy-neutral loss of heterozygosity was observed in regions of greater than 10 Mb in areas that would not have been detected by FISH. Afaible [3] reported SNP array-based karyotyping in aplastic anemia versus hypocellular myelodysplastic syndrome in 93 patients. Their intent was to improve diagnostic precision by a broader identification of defects. As part of the analysis the authors note that copy neutral LOH involving the HLA locus in 3 of 93 patients with AA.

This report describes a patient with loss of heterozygosity at the MHC in malignant CLL cells. While LOH is described in hematopoietic malignancy [4–8], especially in CLL, this case highlights the potential jeopardy for the HLA laboratory in typing such patients. Laboratories typically receive a blood sample early in the evaluation process for HLA typing should transplantation be a viable therapeutic option. In many cases a diagnosis is not available as cytogenetics and hematopathology are being performed concurrently to HLA typing so that the laboratory is not aware of the potential for malignant cells in the typing specimen. In the case presented here, the sample on which HLA typing was performed contained a large percentage of tumor cells, and because it was a

commonly observed phenotype in our patient population, the laboratory did not question the homozygosity of Class I antigens. The initial donor search was run on this erroneous typing, and the problem was not detected until the verification typing was performed, which was delayed as the recipient became pancytopenic. Understanding what had happened became critical and urgent. We quickly identified 3 possible scenarios, a sample mix-up, transient lymphocytes from transfusion, and presence of malignant cells, each of which required investigation and further testing. The first two hypotheses were disproved, but the presence of tumor cells in the typing sample was supported by a note in the accession record that the buffy coat was quite large. Unfortunately, as the cells had been frozen as a pellet there was no way to separate DNA from normal and malignant cells. Routine cytogenetics using FISH without g-banding was not informative. Only the fortunate presence of cryopreserved cells in the tumor bank allowed confirmation of LOH of malignant B cells via typing of separated T and B cells. The full deletion was later identified by array CGH.

The implications for stem cell transplant of an incorrectly or incompletely typed recipient are grave, including severe graft versus host disease and death. Since 2010, verification of HLA typing has been required by the NMDP, but may not be simple to obtain. One proposed algorithm [9] is to draw the verification sample at the evaluation visit, with the second sample drawn separately at least 60 minutes after the primary sample. In the case of the patient described here, this process would not have identified the deleted haplotype because the sample drawn at evaluation consisted primarily of tumor cells. Our laboratory has recently undertaken to get both blood and a buccal swab on all new stem cell transplant recipients. Given current understanding of the potential for allelic loss that may affect clinical laboratory studies (HLA typing [10] and engraftment studies [11]), the use of a temporally separated or other somatic cell sample should be used for verification. Additionally, the targeted use of a cytogenetics array for malignancies such as CLL known for genetic instability would be useful to interrogate immunologically relevant regions.

The mechanism of LOH in this case is unknown, but has immunological relevance. Vago et al. [12] and others [13] have reported

loss of mismatched HLA in relapsing AML after haploidentical stem cell transplantation with donor T lymphocyte infusion, resulting in a clonal escape. An autologous, T-cell mediated selection is plausible for an HLA molecule presenting a tumor associated antigen, and has been described for AML [14] but not for CLL. In addition, a potential role for NK cells in tumor control and progression has been reported [15,16]. Gonzalez-Rodriguez [17] reported that patients with leukemia presented with elevated NK and T cell populations, and that a higher CD8+ count was associated with longer survival. In our case the lost haplotype includes antigens HLA-B*44:02 and HLA-C*05:01, ligands for KIR receptors 3DL1 and 2DL1 respectively. These missing ligands would be expected to activate NK cells and target cell death, yet this patient's malignant cells escaped. The tumor cells did exhibit multiple cytogenetic aberrations in eleven chromosomes, including the common del(13)(q14.3) and del17p(p53), but none were noted for chromosome 19, location of the KIR genes. However, the patient was originally diagnosed with CLL in 1985 and was monitored without treatment for 24 years until presenting with symptomatic disease. At that time his WBC count was elevated, but was composed primarily of malignant B cells, and the NK cell subset was below normal (2%: range 4–26%). In addition, following treatment he became pancytopenic and developed multiple infections. We speculate that immune surveillance kept the malignancy in check for over 2 decades during which time multiple genetic aberrations accumulated, including loss of 6p21. The presence of multiple infections may have subsumed the innate response, and tumor cells escaped.

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