Single step multiple genotyping by MALDI-TOF mass spectrometry, for evaluation of minor histocompatibility antigens in patients submitted to allogeneic stem cell transplantation from HLA-matched related and unrelated donor

Federica Cattina,1 Simona Bernardi,1 Vilma Mantovani,2 Eleonora Toffoletti,3 Alessandra Santoro,4 Domenico Pastore,5 Bruno Martino,6 Giuseppe Console,7 Giovanni Martinelli,8 Michele Malagola1

1Unit of Blood Disease and Stem Cell Transplantation, Department of Clinical and Experimental Sciences, University of Brescia, ASST Spedali Civili of Brescia; 2Center for Applied Biomedical Research (CRBA), St. Orsola-Malpighi University Hospital, Bologna; 3Division of Hematology and Bone Marrow Transplantation, Department of Experimental and Clinical Medical Sciences, Azienda Ospedaliero-Universitaria di Udine; 4Division of Hematology and Bone Marrow Transplantation, Ospedali Riuniti Villa Sofia-Cervello, Palermo; 5Hematology and Bone Marrow Transplantation Unit, Department of Emergency and Organ Transplantation, University of Bari; 6Hematology Division, AO BMM, Reggio Calabria; 7Hematology and Bone Marrow Transplant Unit, AO BMM, Reggio Calabria; 8Department of Experimental, Diagnostic and Specialty Medicine, Hematology “L.e.A. Seragnoli”, University of Bologna, Italy

Introduction

Allogeneic stem cell transplantation (allo-SCT) may be the only cure for patients affected by acute myeloid or lymphoid leukemia, or other hematological diseases such as lymphomas or multiple myeloma.1 The curative effects of allo-SCT are closely related to graft versus host disease (GVHD). However the severity of the graft versus host disease (GVHD) may override the GVl benefit and worsen the outcome of allotransplanted patients.1-3 Despite a full major HLA antigens (MHAs) compatibility, minor histocompatibility antigens (mHAgs) can also play a pivotal role in conditioning both GVl and GVHD response in HLA full-matched allo-SCT. Evidence from experimental and clinical studies on HLA-identical allo-SCT suggest that GVl and GVHD may be driven by donor T cell responses against disparate mHAgs.4-9 Indeed, mHAgs are polymorphic HLA-bound peptides derived from cellular proteins that can induce powerful alloreactive T cell responses. The mHAgs recipient-donor disparity may arise from a genomic variation in the coding region of the gene that leads to differences in the amino acid sequence of the homologous protein and, in most cases, it may depend on a non-synonymous single nucleotide polymorphism (nsSNP) or on a deletion.7,10,11 Recent advances in the molecular identification of mHAgs have significantly expanded our knowledge to a total of 23 autosomal-coded mHAgs and 10 Y-chromosome coded mHAgs, leading to an increased interest in the clinical application of mHAgs typing. Although several mHAgs, including Y-chromosome encoded mHAgs, are ubiquitously expressed, an increasing number of autosomal-encoded mHAgs is being identified as expressed exclusively by hematopoietic cells or by their malignant counterparts.12-21

About this, ACC-1, ACC-2 and HA-2 have been correlated with the beneficial GvL effects, while some mHAgs disparities, CD31, HA-5, HA-8 and UGT2B17, have been found to be involved in the induction of GVHD.8,22-28 The molecular identification of GVHD- and GVl-associated mHAgs could allow the evaluation of the clinical impact of mHAgs mismatches and their specific T cell responses triggered by allo-SCT. Several studies in HLA-matched allo-SCT reported an association between mHAgs mismatches and the clinical outcome,29-33 but other studies have not confirmed these observations.7,24

The heterogeneity of techniques suitable for mHAgs typing (SSP-PCR and Luminex) as well as the complexity of integrating mHAgs typing data and clinical information are likely the main reasons that do not facilitate the routine evaluation of mHAgs in clinics.34,36 In our study, we set up a new method for mHAgs genotyping based on Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF)
mass spectrometry (MS) and we tested it in a training set of donor-recipient pairs with the aim to propose a simple and standardizable methodology able to overcome the limits of the conventional methods and to make mHAgs genotyping suitable for clinical application.37-38

Materials and Methods

Patients and transplant procedures

For this study, we tested the MALDI-TOF iPLEX Gold method on a cohort of Ph+ CML and Ph+ ALL patients who underwent allo-SCT at six Italian Centres from 1990 to 2011. To this purpose, we retrospectively selected 46 donor-recipient pairs fully HLA compatible for HLA-A, -B, -C, -DRB1 and -DQB1 alleles, according to SSP-PCR high resolution molecular methods. Out of the 46 selected cases, 29 were Ph+ CML and 17 were Ph+ ALL patients who underwent allo-SCT by sibling (29 cases, 63%) or MUD (17 cases, 37%).

GvHD effects, either acute or chronic, were defined according to the Glucksberg scale and NHI criteria, respectively, and they were reported as cumulative incidence. Relapse free survival (RFS) was calculated using Kaplan-Meier method and it was assumed as an indicator of GVL effect.39-40 All patients provided informed consent according to the policy of each participating Centre. Patients and transplant features are reported in Table 1.

mHAgs’s biological characteristics and definitions

The HLA matched donor-recipient pairs evaluated for this study were genotyped for a panel of 23 mHAgs (and causal SNPs). The biological characteristics of each mHAg (gene, locus, SNP reference number, nucleotide switch and HLA restriction) are detailed in Table 2. We specify that CD31 exists in two isoforms (CD31125 and CD313563) because it results from two different SNPs (rs668 and rs12953, respectively). We genotyped both SNPs, but we considered the two isoforms together during the analysis because of the strong linkage between the two SNPs. On the contrary, the SNP rs2289702 determine two different mHAgs, ACC-4 and ACC-5, according to the HLA molecule that present them.

For the purpose of this study, immunogenic mHAg difference was defined when within a given donor/recipient pair, only one individual had an immunogenic phenotype of a particular mHAg accompanied by the appropriate HLA restriction molecule. Genomic mHAg difference was identified when mHAg genotypes in donor and recipient were different, but phenotypically they were either the same or the mHAg immunogenic phenotype was not accompanied by the appropriate HLA restriction molecule. Both genomic and immunogenic mHAgs disparities were included in the analysis. This is due to an incomplete knowledge of mHAgs because the epitope-prediction strategy often makes it hard to confirm the immunogenicity of the predicted putative mHAgs and there is currently no controlled way of isolating mHAgs-specific T cells directed against mHAgs.

Table 1. Patients and HSCT characteristics.

| Characteristic | N. | % |
|---------------|----|---|
| Age, mean (range) | 36.5 | 17-67 |
| Male | 27 | 59 |
| Male-female sex mismatch | 9 | 19 |
| Matched sibling donor | 29 | 63 |
| Matched unrelated donor | 17 | 37 |
| Ph + CML | 29 | 63 |
| CP | 23 | 79 |
| AP/BP | 6 | 21 |
| Ph + ALL | 17 | 37 |
| 1st CR | 12 | 71 |
| 2nd CR | 2 | 12 |
| Relapse | 3 | 18 |
| Stem cell source | | |
| Mobilized peripheral blood | 24 | 52 |
| CD34 ×10^6/kg, median (range) | 5.07 | 2.2-8 |
| Bone marrow, n (%) | 19 | 48 |
| CD34 ×10^6/kg, median (range) | 3.2 | 2.8-4 |
| CD3 ×10^6/kg, median (range) | 23.5 | 20-40 |
| Interval between diagnosis and SCT | | |
| ≤1 year | 24 | 52 |
| >1 year | 17 | 37 |
| Not available | 5 | 11 |
| Date of SCT | | |
| 1990-1999 | 19 | 41 |
| 2000-2012 | 27 | 59 |
| Conditioning regimens | | |
| Busulfan based | 27 | 59 |
| TBI based | 17 | 37 |
| Others | 2 | 4 |
| GVHD prophylaxis | | |
| Cyclosporine/MTX | 46 | 100 |
| Gratwohl score | | |
| 1 | 6 | 13 |
| 2 | 12 | 26 |
| 3 | 11 | 24 |
| 4 | 6 | 13 |
| 5 | 4 | 9 |
| ≥6 | 1 | 2 |
| ND | 6 | 13 |

CP, chronic phase; AP/BP, accelerate phase/blastic phase; CR, complete remission; TBI, total body irradiation; MTX, methotrexate.
discriminate the two variants of an SNP in a very efficient way, so it was considered suitable for the aim of the study. The MassARRAY Assay Design software was used to design 3 different multiplex reactions to investigate the 23 SNPs. Genotyping was performed using iPLEX Gold technology and MassARRAY high-throughput DNA analysis with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS [Agena Bioscience Inc., San Diego, CA], according to the manufacturer’s protocol.\textsuperscript{41} Multiplex design and primer sequences are shown in Table 3.

The multiple-genotyping assay was validated using intra- and extra-run controls. Firstly, a DNA sample (NA10589) from the CEPH (Centre d’Etude du Polymorphisme Humain CEPH, Paris, France) panel was genotyped simultaneously in every single run. Six mHAgs (ACC-1, ACC-2, ACC-6, HA-8, HB-1 and LB-ADIR-1R) were reported. Then, the genotype of each polymorphism was validated in 10 randomly selected samples by amplification with PCR and subsequent direct Sanger Sequencing (ABI Prism 3730, Applied Biosystems, Foster City, CA) as gold standard.

### Statistical analysis

For continuous factors, the median and ranges were calculated. The \( \chi^2 \)-test was used to compare differences in percentage, and Mann-Whitney U test was used to compare continuous values. The probability of GvHD (acute and chronic) was estimated as cumulative incidence. In GvHD analysis, competing risks were relapse or death before the onset of GvHD. Probabilities for RFS were calculated using the Kaplan-Meier method.\textsuperscript{42} RFS was calculated from the date of allo-SCT until the date of relapse or death, whichever occurred first. Death in remission was considered as a competing risk in the relapse analysis. Differences in RFS were evaluated by log-rank testing in univariate analysis. Multivariate analyses were performed using the Fine and Gray regression model. The Cox proportional hazard regression model was used for multivariate analyses of variables affecting RFS. The following patient- and transplant-related variables were analyzed: CML or ALL diagnosis and type of bcr-abl transcript, immunogenic/genomic mHAgs mismatches between donor and recipient, patient age at SCT, type of donor, patient gender and sex mismatch between donor and recipient, graft source, time from diagnosis to HSCT, conditioning regimen, GVHD prophylaxis and development of GvHD. All P-values were 2-sided and \( P<0.05 \) was considered statistically significant. Each SNP was tested for departures from the Hardy-Weinberg equilibrium (HWE).

### Results

#### SNPs genotyping by MALDI-TOF iPLEX Gold technology

The MALDI-TOF iPLEX Gold technology method was used on a training
mHAgs mismatches, patients’ clinical features and correlation with GVHD/GvL effects

The analysis of immunogenic mismatches showed that sibling pairs had a lesser number of mismatches compared to MUD pairs (median 1 vs. 3; t-test with P<0.003). The evaluation of genomic mismatches point out that sibling pairs have higher identity than MUD pairs (t-test, P<0.0001). In fact, the median number of genomic differences was 8 (range 0-15) in sibling pairs and 13 (range 11-17) in MUD pairs (t-test with P<0.05). Only one sibling pair showed a perfect genomic mHAgs match.

We also tried to correlate if some mHAgs mismatches could be involved in GvHD development. DPH1 genomic mismatch resulted to be correlated with the risk of grade ≥2 aGvHD development (multivariate analysis HR 2.2, P=0.04, Table 5), while no mHAgs mismatches were found to be correlated with an increased risk of cGvHD (Table 5).

By these evidences, we investigated any correlation between mHAgs mismatches and RFS as a clinical surrogate of GvL effect. Despite some clinical factors affecting the RFS (i.e. the underlying disease, b3a2 transcript isoform and chronic GvHD development), in multivariate analysis we observed that only LB-ADIR-1R, with genomic mismatch on graft versus host direction (HR 0.3, P=0.03, Table 5) was positively correlated with a better RFS.

Discussion

The study aimed to set up a new laboratory assay for genotyping minor histocompatibility antigens which are thought to play a key role in the allo-immune responses in fully HLA-matched stem cell transplantations.

The MALDI-TOF iPLEX gold approach was used to overcome the limits of conventional methods, such as SSP-PCR and Luminex, and to make mHAgs genotyping analysis suitable for clinical application. PCR-SSP and Luminex are commonly used for HLA typing, but both methods have several limitations. Complex primer design and identification of the annealing temperature are critical for the PCR-SSP test; while biotinylated DNA probes, beads and streptavidin-phycoerythrin binding are critical steps for Luminex.34,35 MALDI-TOF was used effectively for KIR (killer-cell immunoglobulin-like receptor) and platelet antigens genotyping and, due to the expected advantages in terms of rapidity, simplicity and high throughput capability, it was

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### Table 3. Primers using for MALDI-TOF assays are listed; PCR primers tags are in bold, no-template bases are reported in lower case letters.

| Multiplex | mHAg | SNP | Amplification Primer | Extension Primer |
|-----------|------|-----|---------------------|-----------------|
| 1         | ACC-1 | rs113857 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 2         | ACC-2 | rs653007 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 3         | ACC-3 | rs694594 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 4         | C19or48 | rs3745526 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 5         | DRN7 | rs1365776 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 6         | HA-1 | rs1801294 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 7         | HA-2 | rs6739531 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 8         | HA-3 | rs273964 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 9         | HB-1 | rs161557 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 10        | HB-2 | rs8958311 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 11        | HB-3 | rs2275687 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 12        | LB-MTHFD1 | rs2236225 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 13        | PBX7 | rs705811 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |
| 14        | UB-1 | rs216807 | AGCTTGAGATGGAGACTTTGACCTGCACGTTGTT | GTGTGATTTAAGAGGAGAG |

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identified as a potential new method for mHAGs genotyping.\textsuperscript{36,37} From a technical point of view, one of the main advantages of SNPs genotyping by MS system consists in the direct measurement of the mass of the molecules of interest without using any surrogate, such as fluorescence. MS genotyping has shown high accuracy; moreover, this methodology is rapid and highly automated, with a genotyping throughput of up to 128 matched pairs (256 samples) per run. The MS approach presents other advantages: it requires only a small amount of DNA, it is highly reproducible, and, furthermore, it works on multiplex and the design of each multiplex is made directly by the instrument software. The only drawbacks of this method are that it does not allow the genotyping of mHAGs resulting from deletions and can be used only if both the polymorphism and the polymorphism’s flanking region are known.\textsuperscript{36} The use of designed primers for SNPs of interest and the MS protocol in this training set allowed us to genotype 100% of the SNPs (2116 genotypes of a predicted total number of 2116) and mHAGs. Intra- and extra-run controls demonstrated the reliability of this method. Analyzing the data obtained by genotyping the mHAGs of this set of donor/recipient pairs with their clinical features, particularly GvHD development and RFS, some interesting suggestions have emerged.

Sibling pairs have fewer mHAGs disparities despite the pairs with HLA-matched unrelated donor (\textit{P}<0.0001). This data may appear obvious, but from a biological point of view no study has clearly shown that until now. This means that the genomic compatibility of HLA full matched MUD pairs will never be greater than full HLA sibling pairs.

Established that HLA differences between donor and recipient are the major predictor of GvHD, we investigated a possible role of mHAGs on GvHD development and relapse incidence in a training set of Ph-positive CML and ALL allotransplanted patients. These patients were chosen because representative of chronic and acute leukemias sharing a unique cytogenetic alteration: t(9;22). The only observation is that genomic DPH1 mismatch appeared to be related to an increased risk of grade ≥2 aGvHD development. This possible correlation between DPH1 and aGvHD is supported by the fact that DPH1 is expressed by a broad range of non-hematopoietic tissues. The role of DPH1 on extramedullary toxicity has already been described by Warren, who pointed out that pulmonary toxicity was observed with infusion of DPH1-specific T

| Table 4. Hardy-Weinberg equilibrium. |
|-------------------------------------|
| **Dominant** | **Recessive** | **HWE** | **Failed, HWE** | **Allele** | **aH** | **Aa** | **aa** | **Failed, HWE** |
|-----------------|-----------------|--------|-----------------|-----------|--------|--------|--------|-----------------|
| ACC-2 | rs3826007 | G | A | 0.438 | ... | 5 | 1.2 | 0.198 | 63 | 29.6 | 7.4 |
| ACC-4/5 | rs2289702 | C | T | 0.568 | ... | 0 | 0 | 0.296 | 79.3 | 20.7 | 0 |
| C19orf48 | rs3745526 | A | T | 0.796 | ... | 6.2 | 1.2 | 0.737 | 59.3 | 34.6 | 6.1 |
| rs12953 | G | A | 0.391 | ... | 24.7 | 1.2 | 0.295 | 35.8 | 43.2 | 21 |
| DPH1 | rs35394823 | G | C | 0.185 | ... | 0 | 0 | 0.365 | 81.7 | 18.3 | 0 |
| DRN7 | rs1365776 | A | G | 1 | ... | 9.9 | 1.2 | 0.03 | 38.3 | 56.6 | 11.6 |
| HA-2 | rs61739531 | C | T | 0.167 | ... | 7.5 | 1.2 | 0.318 | 67.9 | 30.9 | 11.6 |
| HEATR-1 | rs2275687 | C | T | 0.206 | ... | 12.2 | 1.2 | 0.82 | 36.6 | 48.8 | 14.6 |
| LB-ADIR-1R | rs2296377 | G | A | 0.043 | ... | 5 | 1.2 | 0.493 | 44.4 | 46.9 | 8.7 |
| LB-PDK2B | rs751019 | C | A | 0.356 | ... | 18.3 | 1.2 | 0.494 | 25.6 | 53.1 | 12.3 |
| UT1A-1 | rs2166807 | G | A | 0.53 | ... | 1.2 | 1.2 | 0.228 | 65.4 | 33.3 | 10.2 |

AA, homozygous dominant allele; aa, homozygous recessive allele; Aa, heterozygous genotype.
cells. On the contrary, leukemic blasts were poorly recognized by DPH1-specific T cells.\textsuperscript{43} Conversely, we found that genomic mismatch of LB-ADIR-1R on graft versus host direction was related to a better RFS. Our findings on LB-ADIR-1R mismatch are consistent with previous data from van Bergen, showing that LB-ADIR-1R specific T cells perform wide-reaching antitumor activity with a limited recognition of non-activated tissues. Indeed, LB-ADIR-1R specific T cell recognize cell lines from haematological tumours, while generally mesenchymal and biliary epithelial cells are recognized to be GvHD target tissues.\textsuperscript{14}

### Conclusions

This work prove that MS may be a simple, effective, and accurate method for mHAgs genotyping. The method requires a small amount of gDNA that can be easily extracted also from cryopreserved cells. Furthermore, MS is able to genotype all mHAgs in a single work session, thus saving a lot of time.

Data analysis of our patients training set lead us to say that despite the full major HLA match, the minor-HLA genomic and immunogenic compatibility between a patient and his unrelated donor is always lower compared to the genomic and immunogenic compatibility of a patient and his sibling donor. In fact, sibling pairs had a lesser number of mHAgs mismatches compared to MUD pairs (P=0.003). Of 23 mHAgs evaluated, only 2, DPH1 and LB-ADIR-1R, proved to be correlated with the GvHD and GvL effect respectively, and these results confirm the previous reports. Our study suggests that MS would be used and useful for mHAgs genotyping. A larger and prospective trial would be warranted to validate this method.

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