INTRODUCTION

GvHD and graft rejection are the main limiting factors for successful allogeneic hematopoietic SCT (allo-HSCT) to cure malignancy or inborn genetic disorders of the hematopoietic system. The improvement of HLA molecular typing has allowed the definition of better criteria for compatibility and donor selection. A more accurate HLA class I and II matching, together with improved graft preparations, patient conditioning and targeted prophylaxis, has been essential to decrease the incidence of GvHD, especially in the context of malignant diseases. In addition, recent studies in large cohorts of non-malignant patients transplanted with unrelated allo-HSCT have revealed the relevance of allelic and antigenic HLA matching for post-transplant graft failure and rejection; nonetheless, a high rate of both complications persists especially in the non-malignant setting.1–5

In the last decade, the potential role of polymorphisms in genes encoding for non-HLA antigens in predicting HSCT complications has been widely investigated in an attempt to identify novel independent risk factors that may permit a more accurate prediction of transplant-related complications and help in designing individualized prophylaxis. Single-nucleotide polymorphisms (SNPs) of cytokines, such as TNF-α or IL-10, cytokine receptors, or genes associated with innate immunity (that is, killer-cell Ig-like receptors) have been associated with allo-HSCT outcomes in patients transplanted with hematopoietic stem cells from related or unrelated HLA-identical donors.5–9

To date, allo-HSCT represents the only curative treatment for β-Thalassemic patients, 10 who still have a lower median life expectancy compared to healthy individuals, despite regular blood transfusions and supportive care. In these patients the incidence of GvHD after transplant is typically low, thanks to the optimization of transplant composition, conditioning and prophylaxis in both related and unrelated transplants.11 However, the success of allo-HSCT for the cure of β-Thalassemia is still hampered by the rate of graft rejection, which is significantly higher as compared to patients transplanted for malignancies. Several factors have been evoked to explain this difference. Recipient allo-immunization due to massive pre-transplant exposure to blood products and absence of chemotherapy treatments prior to conditioning contribute to better preserve or even enhance immune reactivity in β-Thalassemic patients.12 However, a comprehensive characterization of the immunogenetic factors that influence graft rejection in this patient population, as in other non-malignant hematologic diseases, is still missing.

In this study, we performed a whole-genome association analysis using Affymetrix gene chip array technology (Affymetrix, 948B, Biomedicine, Pula (CA), Italy; 2IRGB, CNR, Monserrato (CA), Italy; 3Laboratory of Immunogenetics and Transplant Biology, IME Foundation, Polyclinic of Tor Vergata University, Rome, Italy; 4International Institute, Milan, Italy; 5San Raffaele Telethon Institute for Gene Therapy, IRCCS San Raffaele Ospedale, Milan, Italy; 6Centro Trapianti di Midollo Osseo, P.O. “R. Binagghi”, Cagliari, Italy; 7Department of Hematology, University of Cagliari, Cagliari, Italy; 8Unit of Molecular and Functional Immunogenetics, Division of Hematology, Stem Cells and Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy; 9San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells, and Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy; 10Università Vita-Salute, IRCCS San Raffaele Scientific Institute, Milan, Italy; 11Bioflag Srl, Pula (CA), Italy. Correspondence: Dr R Bacchetta, San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells, and Gene Therapy, IRCCS San Raffaele Scientific Institute, Via Olgettina 58, Milan 20132, Italy. E-mail: rosa.bacchetta@hsr.it

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The genetic background of donor and recipient is an important factor determining the outcome of allogeneic hematopoietic SCT (allo-HSCT). We applied whole-genome analysis to investigate genetic variants—other than HLA class I and II—associated with negative outcome after HLA-identical sibling allo-HSCT in a cohort of 110 β-Thalassemic patients. We identified two single-nucleotide polymorphisms (SNPs) in BAT2 (A/G) and BAT3 (T/C) genes, SNP rs11538264 and SNP rs10484558, both located in the HLA class III region, in strong linkage disequilibrium between each other ($R^2 = 0.92$). When considered as single SNP, none of them reached a significant association with graft rejection (nominal $P < 0.00001$ for BAT2 SNP rs11538264, and $P < 0.0001$ for BAT3 SNP rs10484558), whereas the BAT2/BAT3 A/C haplotype was present at significantly higher frequency in patients who rejected as compared to those with functional graft (30.0% vs 2.6%, nominal $P = 1.15 \times 10^{-8}$; and adjusted $P = 0.0071$). The BAT2/BAT3 polymorphisms and specifically the A/C haplotype may represent a novel immunogenetic factor associated with graft rejection in patients undergoing allo-HSCT.

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ORIGINAL ARTICLE

BAT2 and BAT3 polymorphisms as novel genetic risk factors for rejection after HLA-related SCT

This article has been corrected since Advance Online Publication and a corrigendum is also printed in this issue.
Inc., Santa Clara, CA, USA), which allows to genotype more than 900,000 SNPs. We investigated whether specific genetic variants are associated with graft rejection after HLA-related HSCT in a cohort of β-Thalassemic patients treated with fairly homogenous transplant protocols.

MATERIALS AND METHODS

Patients

The present study includes 110 patients who underwent HSCT between 2004 and 2010 from a genetically HLA-identical 12/12 allele-level matched sibling. All patients were affected by β-Thalassemia Major and received a myeloablative-conditioning regimen followed by the infusion of un-manipulated BM cells. Patients with β-Thalassemia in class 1 or 2 (according to the Pesaro classification) were given protocol PC6, a conditioning regimen based on 14 mg/kg BU and 200 mg/kg CY, or PC6.1, 14 mg/kg BU, 200 mg/kg CY and 10 mg/kg Thiothepta (TT). β-Thalassemic patients in class 3 (according to the Pesaro classification) were conditioned with PC26 consisting of a pre-transplant treatment starting at day – 45 with 3 mg/kg Azathioprine (A2z), on day – 17 with 30 mg/kg of hydroxyurea (HU) and from day – 16 to day – 12 with 30 mg/m² Fludarabine (Flu), followed by 14 mg/kg BU and a reduced dose of CY (160 mg/kg). PC26 MOD was equal to PC26, with the addition of 10 mg/kg TT (Table 1). IV. CSA was started at 5 mg/kg from day – 2 to day +5, and later reduced to 3 mg/kg until post-transplant day 60 when it was tapered off 5% per week and discontinued at 1 year. The desired plasma range was 150–250 ng/mL. IV. methylprednisolone (MP) was started at 0.5 mg/kg on day 1 and discontinued at 1 year. The desired plasma range was 150–250 ng/mL. We investigated whether specific genetic variants are associated with graft rejection after HLA-related HSCT in a cohort of β-Thalassemic patients treated with fairly homogenous transplant protocols.

Table 1. Characteristics of HLA-related HSC transplanted β-Thalassemic patients

| Patients | n (%) |
|----------|-------|
| Male     | 59 (53.6%) |
| Female   | 51 (46.4%) |
| Age transplantation; years; median (range) | 10.5 (2–27) |

| Conditioning protocols | |
|------------------------|-------|
| PC 26                  | 46 (41.8%) |
| PC 26MOD               | 24 (21.8%) |
| PC 6                   | 30 (27.3%) |
| PC 6.1                 | 10 (9.1%) |

| Pesaro class risk | |
|-------------------|-------|
| 1                 | 6 (5.4%) |
| 2                 | 36 (32.7%) |
| 3                 | 68 (61.8%) |

| Transplantation outcome | |
|-------------------------|-------|
| Graft rejection (GR)    | 15 (13.6%) |
| Persistent mixed chimerism (PMC, FG) | 10 (9.1%) |
| Complete chimerism (CC, FG) | 85 (77.3%) |

Abbreviations: FG = functional graft; HSC = haematopoietic stem cell. *PC26, protocol 26, was a conditioning regimen for β-Thalassemic patients in class 3 (according to the Pesaro classification, PC) consisting of a pre-transplant treatment starting on day – 45 with 3 mg/kg Azathioprine (A2z), on day – 17 with 30 mg/kg of hydroxyurea (HU) and from day – 16 to day – 12 with 30 mg/m² Fludarabine (Flu), followed by 14 mg/kg BU and a reduced dose of CY (160 mg/kg). PC26 MOD, protocol 26 modified, was a conditioning regimen for β-Thalassemic patients in class 3 (according to the Pesaro classification, PC) and was equal to PC26, with the addition of 10 mg/kg TT. PC6, protocol 6, was a conditioning regimen for β-Thalassemic patients in class 1 or 2 (according to the Pesaro classification) based on 14 mg/kg BU and 200 mg/kg CY. PC6.1, protocol 6.1, was a conditioning regimen for β-Thalassemic patients in class 1 or 2 (according to the Pesaro classification) based on 14 mg/kg BU, 200 mg/kg CY and 10 mg/kg Thiothepta (TT).

Analysis of donor chimerism after HSCT

Recipient and donor DNA samples, extracted by QiAamp DNA Blood mini Kit (Qiagen, Valencia, CA, USA) were typed by STR and amelogenin locus using the AmpFISTR Profiler Plus kit (Applied, Foster City, CA, USA). Multiplex reactions were carried out using 1–2 ng of input DNA following the manufacturer’s recommendations. PCR products were run on an ABI Prism 3130xl Genetic Analyzer (Applied). Informative loci for post-transplant samples were screened for quantification of the donor cell percentage in mixed chimerism (MC). Quantitative determination of engraftment was performed using fluorescent PCR primers for human identity markers based on the ratio between peak areas of donor and recipient alleles. The mean value obtained after performing calculations for each informative STR was considered as the percentage of MC.

SNP genotyping

PBMC from β-Thalassemic patients transplanted with HLA-related HSCT and donors were genotyped with the Affymetrix Genome-Wide Human SNP 6.0 Array (Affymetrix, Inc.), including probes for more than 906,000 SNPs and for the detection of copy number variants (CNVs). According to the manufacturer’s instructions. Briefly, total genomic DNA (500 ng) was digested with NsiI and StyI enzymes, ligated to adaptors and amplified using a primer that recognizes the adapter sequence. The amplified DNA was then fragmented, labelled and hybridized to oligonucleotide probes attached to the surface of an array in a GeneChip Hybridization Oven 640 (Affymetrix, Inc.), followed by washing and staining procedures performed on a GeneChip Fluidics Station 450 (Affymetrix, Inc.). Arrays were scanned using the GeneChip Scanner 3000 7 G (Affymetrix, Inc.).

Sequencing

Two different pairs of primers that amplify the DNA fragments containing the rs11538268 and the rs10484558 variants were designed to provide 222- and 263-bp fragments, respectively. The template DNA was amplified in a total volume of 25 μL standard PCR procedures using an annealing primer-specific temperature of 56°C for rs11538268 and 54°C for rs10484558. The two specific amplification primer pairs were (a) 5’–CTGCTTTGGGTGGGAGGAGA-3’ and 5’–AGCAATCTTCCCCACAGAATC-3’, (b) 5’–CTGCCACTCTTCTGCTCCCA-3’ and 5’–CTGCCCTCGAAGGAGTA-3’, respectively. PCR products were purified using the Agencourt Ampure XP (Beckman Coulter, Inc., Beverly, MA, USA) system following the manufacturer’s instructions. Sequencing reactions were performed using 10 μL of purified PCR products, forward or reverse primers used in PCR reaction and ABI Bigdye terminator cycle sequencing kits performed on a GeneChip Fluidics Station 450 (Affymetrix, Inc.). Arrays were scanned using the GeneChip Scanner 3000 7 G (Affymetrix, Inc.).

Data analysis

The obtained intensity files were analysed with the Affymetrix Power Tools package (APT version 1.12.0). Quality control was performed using the Contrast QC algorithm (CCQ), carried out with the executable APT-GENO-QC, using the default settings. Eight individuals showing QC values < 0.4 were removed from the data set. Genotypes used for association analysis were called using the Birdseed v2 algorithm implemented in the executable APT-PROBESET-GENOTYPE, using defaults settings. All samples with a call rate > 90.00 were retained. The call rate of the remaining samples was 98.88 ± 1.031, with a maximum of 99.804 and a minimum of 94.277. Finally, the adopted algorithm successfully called 905,556 SNPs, located on autosomes and sex chromosomes. Output files were converted in PLINK format, and annotated with Affymetrix release 31, corresponding to Build GRCh37. In order to create an informative data set of common autosomal markers, to avoid detection of false-positive signals of association and to remove SNPs incorrectly genotyped, we excluded from the 905,556 called markers SNPs without map references, SNPs with call rates < 95%, MAF < 0.05 and out of Hardy–Weinberg equilibrium (P < 10^-3). A data set of 617,049 informative autosomal SNPs was obtained. All filtering procedures were performed with PLINK software. Association tests were performed between patients with rejection (GR) and patients with functional grafts (FG). At the SNP level, we used the function –fisher of PLINK software, and we corrected results for multiple tests with the Bonferroni method. We also used a second cutoff of
to detect potential associations. At the haplotype level, we used the \(\text{--hap-assoc}\) function to estimate haplotype frequencies with the EM algorithm\cite{15} and to compute \(\chi^2\)-test between cases and controls (Affymetrix, Inc.). With the aim of associating candidate SNPs with known and predicted regulatory elements, we used the Regulome database.\cite{16} Finally, the regression analysis to investigate the influences of covariants on transplant outcome (conditioning regimens, Pesaro class risk, and age at transplantation) was performed using the function \text{gml} as implemented in the R package.\cite{17}

RESULTS

Identification of SNPs differentially expressed in β-Thalassemic patients who reject HLA-identical HSCT graft

A total of 110 β-Thalassemic patients transplanted from genotypically HLA-identical 12/12 allele-matched siblings were studied. To define genetic risk factors associated with rejection, β-Thalassemia patients who rejected transplants (graft rejection, GR, \(n=15\)) were compared to patients who developed either complete chimerism (CC, \(n=85\)) or persistent mixed chimerism, in which donor and recipient cells stably co-exist (PMC, \(n=10\)) (Table 1). Patients belonging to those two last groups are henceforth referred to as functional graft (FG, \(n=95\)). Unlike patients who reject the graft, patients who develop CC or PMC are indeed disease free and transfusion independent.\cite{18}

Initially, SNPs were genotyped and analysed in a first group of 45 transplanted patients (GR, \(n=9\) vs FG, \(n=36\)) using the Affymetrix 6.0 array, allowing the analysis of a total of 617,049 informative SNPs located on autosomes. After Bonferroni correction for multiple tests (correction for 617,049 test, cutoff \(P\)-value = \(8.1 \times 10^{-5}\)), no SNPs showed a statistically significant difference between GR and FG patients. We then chose a nominal \(P\)-value of \(< 1 \times 10^{-3}\) as cutoff to detect potential differences in allele frequency between GR and FG patients. Using this criterion, we found six SNPs located in chromosomes 1, 2, 6, and 11 (Table 2, and Supplementary Figure 15). In addition to these SNPs, we found also 37 SNPs with a nominal \(P\)-value \(< 1 \times 10^{-4}\) (Supplementary Table 15).

SNPs in BAT2 (A/G) and BAT3 (T/C) genes are predominant in β-Thalassemic patients who rejected HLA-identical HSCT graft

All but one SNPs with potential differences in allele frequency between GR and FG patients were located either in intergenic regions, in genes with unknown function, or not directly associated to immunological functions (Table 2). We therefore characterized this unique SNP—rs11538264 (\(P=4.26 \times 10^{-5}\)—located in the BAT2 gene encoding for proline-rich coiled-coil 2A protein (PRRC2A) and in proximity of the TNF-α and TNF-β loci of chromosome 6 in HLA class III region.\cite{19} Interestingly, these SNPs is in linkage disequilibrium (LD) (\(R^2=0.86\)) with the SNP (rs10484558) located in BAT3 gene, at 12.3 kb downstream of the BAT2 gene, encoding for a large proline-rich protein (BAG6), that showed a nominal \(P\)-value of 3.01 \times 10^{-5} (Supplementary Table S1).

SNPs rs11538264 (BAT2) leads to the A or G alleles, whereas SNP rs10484558 (BAT3) leads to the C or T alleles, with the G allele for BAT2 and the T allele for BAT3 being the most represented in human populations, with frequencies > 80% in the populations analysed.\cite{20} The A allele of SNP rs11538264 (BAT2) and the C allele of SNP rs10484558 (BAT3) were significantly more frequent in the GR (\(n=9\)) than the FG (\(n=36\)) patient group (43.8 vs 0.00%, and 43.8 vs 1.8%, respectively). This result is even more significant considering that the frequency of the allele A (rs11538264, BAT2) and of the C allele (rs10484558, BAT3) was 4.2% in the parents of transplanted β-Thalassemic patients, and 5% in a small (\(n=10\)) cohort of independent individuals from the same ethnic group, analysed in parallel. The latter findings are in line with the frequencies of the A allele (rs11538264, BAT2) and of the C allele (rs10484558, BAT3) reported in databases obtained in wide populations (Supplementary Table S2).\cite{21}

We validated the presence of SNPs rs11538264 and rs10484558 by means of Sanger sequencing in 25 patients previously genotyped by microarray, and we extended the analysis to an additional 65 β-Thalassemic patients who underwent HLA-identical HSCT (\(n=6\), GR and \(n=59\), FG patients). The frequency of the A allele (rs11538264, BAT2) and of the C allele (rs10484558, BAT3) in this latter population analysed was higher in the GR compared to the FG patient group, but it did not reach statistical significance, possibly because the number of patients was insufficient (data not shown). Since these additional transplanted β-Thalassemic patients were identical to those analysed in microarrays in terms of disease, ethnicity and treatment, we then established the allele and genotype frequencies of the total samples (\(n=15\), GR and \(n=95\), FG patients). Overall, GR patients (\(n=15\)) carried significantly higher frequencies of the A allele of rs11538264 (BAT2) (9/30 (30%) vs 5/190 (2.6%); \(P=6.34 \times 10^{-5}\); OR = 15.86), and of the C allele of rs10484558 (BAT3) (9/30 (30%) vs 6/190 (3.1%); \(P=1.44 \times 10^{-5}\); OR = 13.1) compared to FG patients (\(n=95\)) (Table 3). Furthermore, the respective genotype frequencies A/A, A/G and G/G of BAT2, SNP rs11538264, and C/C, C/T and T/T of BAT3, SNP rs10484558, were differentially represented in the GR compared to the FG patient group, with the genotype A/A (BAT2 , SNP rs11538264) and C/C (BAT3 , SNP rs10484558) being never detected in the FG patients (Table 2).

The BAT2/BAT3 A/C haplotype is associated with graft rejection after allo-HSCT for β-Thalassemia

SNP rs11538264 (BAT2) and SNP rs10484558 (BAT3), separated by 12.3 kb, are in strong LD (\(R^2=0.92\) considering the total sample). Haplotype estimates by EM algorithm revealed only three (AC, GC and GT) of the four possible haplotypes. Haplotype AT was not found in the present patient cohort and haplotype GC showed a frequency < 1%. In line with the allele frequency observations, haplotype AC had a significantly higher frequency (9/30 (30%) vs 5/190 (2.6%); \(P=6.34 \times 10^{-5}\); OR = 15.86), and of the allele C (rs10484558, BAT3) in 80% in the populations (Supplementary Table S2).\cite{21}

**Table 2.** Allele frequency differences between GR and FG patients of SNPs with nominal \(P\)-value \(< 1 \times 10^{-5}\)

| Chr | SNP | Location (bp) | Allelic variants | \(P\)-value | Adjusted \(P\)-value | Gene | Left gene | Right gene |
|-----|-----|---------------|------------------|-------------|-------------------|------|-----------|------------|
| 1   | rs1831870 | 57627203 | G/A | \(9.3 \times 10^{-7}\) | 0.574 | DAB1 | C88 | LOC729423 |
| 6   | rs10899030 | 57628701 | T/A | \(2.88 \times 10^{-6}\) | 1.000 | DAB1 | C88 | LOC729423 |
| 11  | rs11538264 | 31603189 | G/A | \(4.26 \times 10^{-6}\) | 1.000 | BAT2 | SNORA38 | BAT3 |
| 11  | rs108991245 | 111167792 | T/G | \(6.29 \times 10^{-6}\) | 1.000 | FLJ45803 | C11orf53 | LOC120376 |
| 11  | rs12792445 | 111176351 | C/T | \(6.29 \times 10^{-6}\) | 1.000 | LOC120376 | FLJ45803 | POU2AF1 |
| 2   | rs983970 | 126140847 | A/G | \(6.59 \times 10^{-6}\) | 1.000 | – | CNTNAP5 | LOC150554 |

Abbreviations: FG = functional graft; GR = graft rejection; SNP = single-nucleotide polymorphism. SNPs with putative association (\(P<10^{-5}\)) in GR patients as compared to FG patients. Nominal \(P\)-value from association study and adjusted \(P\)-values obtained with Bonferroni correction (correction for 617 049 test, cutoff \(P\)-value = \(8.1 \times 10^{-5}\)) are shown.
The haplotype containing the predominant alleles (GT) had a prevalence of 70.0% (21/30) in GR patients and 96.8% (184/190) in FG patients (nominal P = 5.94 × 10^{-8}, adjusted P = 0.037, Table 3). To associate the identified SNP rs11538264 (BAT2) and SNP rs10484558 (BAT3) with known and predicted regulatory DNA elements, we used the RegulomeDB database.16 Results indicate SNP rs11538264 as an eQTL (expression quantitative trait loci) influencing the expression level of non-classical HLA-C gene, as reported by Zeller et al.17 (GHSdatabase: http://genecanvas.ecgene.net/uploads/ForReview/ghs Probe_Express030510.zip).

With the aim of testing the influence of additional risk factors for rejection, we performed regression analysis considering as independent variables conditioning regimens, Pesaro risk class, and age, by comparing patients with different SNPs. Results were not significant for conditioning protocols, age at transplantation and the Pesaro risk class 3, to which the majority of the analysed patients belonged (n = 68, Table 1). Notably, risk class often shows a high association with post-transplantation complications and mortality, but in the regression analyses previously performed rejection was never associated with Pesaro risk class 3.15 Based on these analyses, we concluded that patients carrying different BAT2 and BAT3 SNPs are similar with respect to other risk factors of rejection after allo-HSCT.

**DISCUSSION**

By genome-wide analysis we have identified two SNPs (rs11538264 and rs10484558) in the BAT2 and BAT3 genes, within the HLA class III region, in strong LD, that are statistically associated, at the haplotype level (adjusted P = 0.0071), with graft rejection after HLA-identical HSCT. For the first time, we indeed detected an association between the haplotype AC of BAT2 and BAT3 genes, and rejection after HLA-identical sibling HSCT in ß-Thalassemia patients. In conclusion, by genome-wide analysis we have identified two novel SNPs associated with rejection after HLA-identical sibling HSCT. Notably, the SNP rs11538264 (A/G) of BAT2 described here is a non-synonymous variation, causing a VAL1774MET substitution that may impact on the biological function of the protein. Our results are strengthened by the evidence that the SNP rs11538264 could be involved in regulation of expression levels of HLA-C in monocytes.21

BAT3 has recently been shown to be critically involved in regulating HLA class II expression:22 IFN-γ induces BAT3 expression that facilitates the nuclear import of the class II transactivator (CIITA), with subsequent activation of HLA class II gene expression in APCs. Moreover, BAT3 is primarily expressed by T helper (Th) 1 cells and protects them from cell death.23 Interestingly, BAT2 and BAT3 polymorphisms have been associated with increased incidence of type 1 diabetes and have been hypothesized to be involved in the inflammatory process of pancreatic beta-cell destruction.30,31 In addition, it has been shown that several isoforms of BAT3 exist and that their expression is cell-specific and may contribute to a specific activity in a given cells.32 These findings, together with the described role of BAT3 as chaperone in regulating the pattern of HLA class II gene expression in APCs, suggest that BAT3 may be critically involved in modulating effector T cell responses.

Although we could not test in our ß-Thalassemia patients whether the allelic variant of BAT3 resulted in an altered protein and function, sequence analysis of the genetic variants and bioinformatic analyses with RegRNA33 and ESEFinder34 predicted the disruption of an exon splicing enhancer, binding site for the splicing factor Srp20, caused by the C allele of the rs10484558 SNP (BAT3),35 and the introduction of two novel binding sites for the SRSF1 and SRSF5 splicing factors, respectively. It can thus be hypothesized that alteration of the putative enhancer results in an inefficient inclusion of the seventh exon of the BAT3 transcript encoding the first of the two proline-rich regions of the protein, and may limit the protein–protein interactions. Future studies are warranted in order to elucidate whether, for example, the BAT3 C allele encodes a protein with modulated chaperone activity, which may lead to increased HLA class II expression in APCs and/or to enhanced Th1 cell activation in the context of allo-HSCT rejection.

In conclusion, by genome-wide analysis we have identified two polymorphisms (rs11538264 and rs10484558) in the BAT2 and BAT3 genes within the HLA class III region, in strong LD. The haplotype containing the A allele of SNP rs11538264 (BAT2) and the C allele of SNP rs10484558 (BAT3) is associated with graft

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**Table 3.** Allele and genotype frequencies of rs11538264 (BAT2) and rs10484558 (BAT3) genes in HLA-related HSC transplanted ß-Thalassemic patients with different outcomes

| Gene   | Number (frequency %) of patients | Nominal P-value | Adjusted P-value |
|--------|----------------------------------|-----------------|------------------|
|        | Graft rejection                  | Functional graft|                  |
| BAT2   | Allele                           |                 |                  |
|        | A                                | 9/30 (30.0)     | 5/190 (2.6)      | 6.34 × 10^{-6}  | 1.000 |
|        | G                                | 21/30 (70.0)    | 185/190 (97.4)   |                  |      |
|        | Genotype                         |                 |                  |
|        | A/A                              | 1/15 (6.7)      | 0/95 (0.0)       |                  |      |
|        | A/G                              | 7/15 (46.7)     | 5/95 (5.3)       |                  |      |
|        | G/G                              | 6/15 (40.0)     | 90/95 (94.7)     |                  |      |
| BAT3   | Allele                           |                 |                  |
|        | C                                | 9/30 (30.0)     | 6/190 (3.2)      | 1.44 × 10^{-5}   | 1.000 |
|        | T                                | 21/30 (70.0)    | 184/190 (96.8)   |                  |      |
|        | Genotype                         |                 |                  |
|        | C/C                              | 1/15 (6.7)      | 0/95 (0.0)       |                  |      |
|        | C/T                              | 7/15 (46.7)     | 6/95 (6.3)       |                  |      |
|        | T/T                              | 6/15 (40.0)     | 89/95 (93.7)     |                  |      |
| BAT2/BAT3 | Haplotypes                  |                 |                  |
|        | AC                               | 9/30 (30.0)     | 5/190 (2.6)      | 1.15 × 10^{-8}   | 0.007 |
|        | GC                               | 0/30 (0.0)      | 0/95 (0.0)       | 0.690            | 1.000 |
|        | GT                               | 21/30 (70.0)    | 184/190 (96.8)   | 5.94 × 10^{-8}   | 0.037 |

The allele, genotype and haplotype frequencies for BAT2 and BAT3 polymorphisms in 110 ß-Thalassemic transplanted patients (n = 15, GR and n = 95, FG patients). Nominal P-value from association study and adjusted P-values obtained with Bonferroni correction (for 617 049 test, cutoff P-value = 8.1 × 10^{-8}) are shown.
rejection after HLA-identical sibling HSCT in β-Thalassemia patients. In addition to providing a potentially useful indication for individualized prophylaxis against rejection in β-Thalassemic patients carrying the relevant polymorphisms, the identified haplotype for the BAT genes may represent a new molecular marker for risk assessment in allo-HSCT for other non-malignant haematologic diseases and, possibly, organ transplantation. An important question to be addressed in future studies is whether these polymorphisms are specifically associated with graft rejection after allo-HSCT in β-Thalassemia or can also be relevant in other clinical settings of allo-HSCT.

Therefore, the general relevance of these polymorphisms as predictive markers of rejection beyond the present context of allo-HSCT in β-Thalassemia has to be addressed in future studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

ISP and AA performed the research and contributed to the preparation of the manuscript; MA provided the clinical samples and information, supervised the project and prepared the manuscript; MT performed HLA typing; MF performed protein prediction analysis; GL, SM, FC and GLN provided the clinical samples; KF and MGR provided scientific advices; AB designed the study, supervised the research and edited the manuscript; SG and RB designed the study, coordinated and supervised the project, and prepared the manuscript.

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