Molecular matching for Rh and K reduces red blood cell alloimmunisation in patients with myelodysplastic syndrome

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Background. Matching for Rh and K antigens has been used in an attempt to reduce antibody formation in patients receiving chronic transfusions but an extended phenotype matching including Fy^a and Jk^a antigens has also been recommended. The aim of this study was to identify an efficient transfusion protocol of genotype matching for patients with myelodysplastic syndrome (MDS) or chronic myelomonocytic leukaemia. We also examined a possible association of HLA class II alleles with red blood cell (RBC) alloimmunisation.

Materials and methods. We evaluated 43 patients with MDS undergoing transfusion therapy with and without antibody formation. We investigated antigen-matched RBC units for ABO, D, C, c, E, e, K, Fy^a, Fy^b, Jk^a, Jk^b, S, s, Do^a, Do^b and Di^a on the patients' samples and on the donor units serologically matched for them based on their ABO, Rh and K phenotypes and presence of antibodies. We also determined the frequencies of *HLA-DRB1* alleles in the alloimmunised and non-alloimmunised patients.

Results. Seventeen of the 43 patients had discrepancies or mismatches for multiple antigens between their genotype-predicted profile and the antigen profile of the units of blood serologically matched for them. We verified that 36.8% of patients had more than one RBC alloantibody and 10.5% of patients had autoantibodies. Although we were able to find a better match for the patients in our extended genotyped/phenotyped units, we verified that matching for Rh and K would be sufficient for most of the patients. We also observed an over-representation of the *HLA-DRB1*13* allele in the non-alloimmunised group of patients with MDS.

Discussion. In our population molecular matching for C, c, E, e, K was able to reduce RBC alloimmunisation in MDS patients. An association of *HLA-DRB1*13* and protection from RBC alloimmunisation should be confirmed.

Keywords: blood group antigen, HLA, molecular matching, myelodysplastic syndrome, RBC alloimmunisation.

Introduction

The myelodysplastic syndrome (MDS), including chronic myelomonocytic leukaemia (CMML), is a group of heterogeneous clonal disorders caused by an intrinsic stem cell defect with a inherent propensity to bone marrow failure and ultimately transformation to acute myeloid leukaemia. The bone marrow failure results in the transfusion dependence and neutropenic infections that characterise MDS[1]. Anaemia is present in more than 80% of these patients[2] and treatment is often restricted to lifelong supportive therapy with red blood cell (RBC) transfusions[3]. Although blood transfusion is generally safe, many patients are at risk of transfusion-related complications such as iron overload and RBC alloimmunisation. This latter problem often makes finding compatible RBC products difficult and can be associated with delayed haemolytic transfusion reactions and autoantibody formation[4-6]. Matching for Rh and K antigens has been used in an attempt to reduce antibody formation in patients receiving chronic transfusions but an extended phenotype matching to include Fy^a, Jk^a and S antigens has also been advocated by some authors[7,8]. Molecular DNA-based methods, including array-based RBC genotyping platforms, have provided excellent tools to perform large-scale testing on numerous antigens simultaneously. This allows for efficient extended phenotype matching of donors and patients[9-11]. Such technology has been shown to improve transfusion therapy in patients with sickle cell disease by decreasing the risk of transfusion reactions, especially delayed transfusion reactions to existing alloantibodies, and preventing alloimmunisation. However, few studies are being performed in patients with MDS[12-14].

An interesting fact is that not all patients develop alloantibodies following exposure to transfused RBC[15]. The

results of a recent mathematical modelling study supported the hypothesis that alloimmunised patients represent a genetically distinct group with increased susceptibility to RBC sensitisation[16]. Knowledge of clinical conditions that predispose to alloimmunisation is important because it may influence the management of a patient. If a certain category of patients has a high risk of alloimmunisation, the consequence could be more extensive antigen typing and matching[17]. Polymorphisms in MHC-type, CD4+ cells, T cells, B cells and cytokines can all contribute to the presentation and recognition of foreign antigens resulting in antibody production[18]. Studies have identified possible relationships between HLA-DRB-restriction sites and alloimmunisation to RBC antigens. The HLA class II genotype has been shown to be a key predictor of an individual's response to RBC antigens and is likely to influence predisposition to RBC antibody responder status[19-22].

In order to identify an efficient transfusion protocol of genotype matching for patients with MDS, we retrospectively analysed RBC alloantibodies detected and identified in serum from patients with MDS and evaluated the usefulness of blood group antigen genotyping to provide a means to genotype/phenotype match donor blood units precisely to the antigen-negative profile of the patients. We also examined a possible association of HLA class II alleles with susceptibility to or protection against RBC alloimmunisation in patients with MDS.

Materials and methods

Patients

We evaluated 43 patients self-identified as Caucasians with MDS undergoing transfusion therapy with and without RBC alloimmunisation. The diagnosis of MDS was made according to the World Health Organisation 2008 classification of myeloid neoplasm[23]. The patients agreed to participate in this study by signing an Institute Review Board-approved informed consent form. All patients' DNA samples were genotyped for RBC and HLA genes.

Controls

The control group consisted of 151 DNA samples from blood donors from a blood bank inventory previously genotyped for RBC blood group antigen alleles and 427 blood donors from a bone marrow bank previously genotyped for HLA genes. These controls were used to determine the genotype frequencies in the population. Control subjects were healthy and randomly selected. There were no significant regional or ethnic differences between the patients and control groups. The donors agreed to participate in this study by signing an Institute Review Board-approved informed consent form.

DNA preparation

Genomic DNA was extracted from leucocytes by manual spin column separation (QIAamp, Qiagen, Valencia, CA, USA), according to the manufacturer's instructions and eluted into 100 µl of water molecular grade. The DNA concentrations were measured in a spectrophotometer at 260 nm and the purity was determined by 260/280 nm. An 8µL aliquot of approximately 10 ng of genomic DNA was transferred for the polymerase chain reactions (PCR).

Red blood cell genotyping

DNA array analysis. RBC genotyping on samples of DNA from patients and donors was performed using the Human Erythrocyte Antigen BeadChip™ (BioArray Solutions, Immucor, Norcross, GA, USA) containing probes directed to polymorphic sites in *RHCE, FY* (including *FY-GATA* and *FY265*), *DO* (including *HY* and *JO*), *CO, DI, SC, GYPA, GYPB* (including markers permitting the identification of U-negative and U-variant types), *LU, KEL, JK,* and *LW* for all samples from controls, donors, and patients. The Human Erythrocyte Antigen BeadChip™ assay was performed in accordance with the manufacturer's instructions.

RHD genotyping. All donors' and patients' samples were analysed for the presence of *RHD* in both intron 4 and exon 10, as previously reported[24]. The other assays used were a PCR system involving sequence-specific primers which detects the common weak D types[25] and a multiplex PCR that detects hybrid alleles of the *RHD* gene[26].

Molecular matching

We performed molecular matching for D, C, c, E, e, K, Fy^a, Fy^b, Jk^a, Jk^b, S, s, Do^a, Do^b and Di^a on the patients' samples and on the donor units serologically matched for them based on their ABO, Rh and K phenotypes and presence of antibodies. Matches were first identified by ABO and RhD, then by C, c, E, e, K and then by the other antigens. The non-Rh antigens prioritised were Fy^a, Jk^a, S and Di^a. Using specific software developed in our laboratory, an electronic link was established between the extended genotyped/phenotyped donor units and the patients, allowing automatic identification of the most compatible blood.

HLA genotyping

The HLA class I and II alleles were typed using the reverse sequence-specific oligonucleotide technique (rSSO; One Lambda Inc., Canoga Park, CA, USA) with Luminex x Map technology (Luminex Corporation, Austin, Texas, USA). The *DRB1*13* group of alleles was also typed with the High Definition Class II DRB1 Typing Test, for definition of alleles (rSSO; One Lambda Inc.), according to the manufacturer's instructions.

Statistical analysis

The genotype and allele frequencies were determined by direct counting on Excel spreadsheets (Microsoft® Office Excel 2003) and compared between patients and controls. The comparison was performed using the chi-square test or Fisher's exact test, when appropriate, using a 2×2 contingency table. Significant p values were corrected by the number of alleles studied in the *locus minus* one (p_c; Bonferroni's correction). Odds ratios with 95% confidence intervals (CI) were also calculated. The Arlequin computer programme version 3.1 (available at http://cmpg.unibe.ch/software/arlequin3/) was used to see whether the distributions of genes and alleles were in Hardy-Weinberg equilibrium[27].

Results

Patients

We examined 43 clinical records of patients with MDS undergoing transfusion therapy phenotype-matched for ABO, Rh (D, C, E, c, e) and K. The median age of the patients was 64 years (range, 22-85); 23 were females and 20 were males.

Among the patients, 63% (27/43) were chronically transfused and had received six or more RBC units in the preceding 3 months, while 37% (16/43) were episodically transfused and had received one to six RBC units in the preceding 3 months. Nine of the 27 chronically transfused patients and seven of the 16 episodically transfused patients had received at least one transfusion prior to initiation of Rh and K matching.

Red blood cell alloimmunisation

Among 43 transfused patients, 12 of 27 receiving chronic transfusions (44%) and 7 of 16 receiving episodic transfusions (44%) were alloimmunised (Table I). Twenty-four patients (56%) were not alloimmunised. The non-alloimmunised patients had received a median of 50 RBC units (range, 5-255 units) and the alloimmunised patients had received a median of 65 units (range, 4-604 units) (p>0.05).

The antibodies identified in the serum of the alloimmunised patients are shown in Table II. There were ten alloantibodies, occurring alone or in combinations, detected among the 19 alloimmunised patients with MDS. Forty-seven percent of the patients had one antibody against Rh antigens. Two or more antibodies were found in 37% of patients in association with at least one Rh antibody. Three patients, who received Rh and K matched units only, had other antibody specificities (one anti-S, one anti-Di^a and one1 anti-Lu^a). Six (32%) of the alloimmunised patients and four (17%) of the non-alloimmunised patients had autoantibodies.

Table I - Chronic and episodic transfusions in 43 MDS patients.

	Chronic transfusion n (f)	Episodic transfusion n (f)	p	Median RBC units
Alloimmunised	12 (0.44)	7 (0.44)	ns	65
Not alloimmunised	15 (0.56)	9 (0.56)	ns	50
Total	27	16		

n: number of patients; f: frequency; p: p value; ns: not significant.

Table II - Alloantibodies present in 19 multi-transfused MDS patients.

	Alloantibody	n	f
One antibody	Anti-C	2	0.11
	Anti-E	6	0.32
	Anti-C^w	1	0.05
	Anti-S	1	0.05
	Anti-Di^a	1	0.05
	Anti-Lu^a	1	0.05
Two antibodies	Anti-C, -E	1	0.05
	Anti-C, -e	1	0.05
	Anti-c, -E	1	0.05
	Anti-c, -Jk^a	1	0.05
	Anti-E, -K	1	0.05
Multiple antibodies	Anti-C, -K, -Di^a	1	0.05
	Anti-E, -Jk^a, -Di^a	1	0.05
Total		19	1

n: number of patients; f: frequency.

Red blood cell genotyping

The RBC blood group antigen allele frequencies were compared between the two groups (MDS patients and donors) and no significant differences were observed. The most common extended phenotype predicted from the genotype found in the patients was D+C+c+E–e+ (R_1r), K–, Fy(a+b+), Jk(a+b+), M+N+S–s+.

We found four patients with *RH* variants (one *RHD*weak D type 2*, one *RHD*weak D type 4.0*, one *RHD*DV.1* and one *RHD*DVI.1*).

Molecular matching

Of the 43 MDS patients studied, 17 had discrepancies or mismatches for multiple antigens between their genotype profile and the antigen profile of the blood units serologically matched for them. The main discrepancies or mismatches occurred in the RH, FY, JK and MNS systems. We found discrepancies between the previous phenotype and genotype-derived phenotype in four chronically transfused alloimmunised patients whose *in vivo* RBC survival was not good, as assessed by decreases in their haemoglobin levels and increased

frequency of transfusions (Table III). The patients who had discrepancies between phenotype and genotype had been receiving transfusions more frequently than those who did not have discrepancies, and were the only ones who had received transfusion in the last 3 months or less. We found mismatches for multiple antigens in 13 patients receiving blood units matched for ABO, Rh and K.

HLA genotyping

HLA alleles frequencies were compared between the two groups (MDS patients and donors) and no significant differences were observed, which means that no HLA allele was associated with MDS in the group of subjects studied.

We observed that the *HLA-DRB1*13* allele was present in 15% of non-alloimmunised patients and in 0% of alloimmunised patients (p=0.0137; OR=0.1583; 95% CI=0.01859-1.348) (Table IV). However, the p value lost significance after Bonferroni's correction, probably due to the small sample size. To determine which *DRB1*13* allele could be associated with a possible protection against immunisation, *HLA-DRB1*13* genotypes were determined at the allelic level. We found three *HLA-DRB1*13:01,* two *HLA-DRB1*13:02* and two *HLA-DRB1*13:03.*

Table III - Phenotype and genotype discrepancies found in samples from four patients.

Genotype	Phenotype			Antibody
	RhE+e−	RhE+e+	RhE−e+	
*RHCE*Ee*	0	0	2	Anti-E
	Jk(a+b−)	Jk(a+b+)	Jk(a-b+)	
*JK*B/JK*B*	0	1	0	Anti-Jk^a
	S+s−	S+s+	S−s+	
*GYPB*ss*	0	1	0	Anti-S

Discussion

We report here our experience with MDS patients undergoing transfusion therapy phenotype-matched for Rh (D, C, c, E, e) and K. Few studies have characterized alloimmunisation in such patients and in our study, 44% of the patients were alloimmunised to RBC antigens. The main alloantibodies detected were against Rh and Kell antigens due to previous transfusions not matched for Rh and K or RhD variants. The most prevalent antibody found in those patients was anti-E (32%), which is consistent with other studies in which a high prevalence of anti-E was also found in patients with MDS[3,28]. The high prevalence of anti-E in our population of patients is explained by the lack of the E antigen in the majority of patients.

Some authors reported that the risk of alloimmunisation increases with an increasing number of transfusions[17,29-31]. In this study the number of RBC units transfused in the alloimmunised and non-alloimmunised MDS patients was basically the same, demonstrating that in our patients the number of units transfused did not affect RBC alloimmunisation. Indeed, the *HLA-DRB1*13* allele was identified as a possible protective factor against RBC alloimmunisation as this allele was

Table IV - Comparison of HLA-DRB1 allele frequencies in alloimmunised and non-alloimmunised MDS patients.

HLA-DRB1 alleles	Alloimmunised patients (n=19)		Non-alloimmunised patients (n=24)		p	OR	95% CI
	Number of alleles	f	Number of alleles	f			
*DRB1*01*	1	0.03	4	0.08	ns	-	-
*DRB1*03*	3	0.08	5	0.10	ns	-	-
*DRB1*04*	7	0.18	4	0.08	ns	-	-
*DRB1*07*	8	0.21	5	0.10	ns	-	-
*DRB1*08*	5	0.13	2	0.04	ns	-	-
*DRB1*09*	2	0.05	2	0.04	ns	-	-
*DRB1*10*	2	0.05	0	0.00	ns	-	-
*DRB1*11*	3	0.08	7	0.15	ns	-	-
*DRB1*12*	1	0.03	2	0.04	ns	-	-
*DRB1*13*	0	0.00	7	0.15	0.0137	0.1583	0.01859-1.348
*DRB1*14*	2	0.05	1	0.02	ns	-	-
*DRB1*15*	2	0.05	6	0.13	ns	-	-
*DRB1*16*	2	0.05	3	0.06	ns	-	-
Total	38	1	48	1			

n: number of alleles; f: allele frequency; p: p value; OR: odds ratio; 95% CI: confidence interval; ns: not significant.

present in 15% of non-alloimmunised MDS patients and in no alloimmunised patient. However, high resolution HLA genotyping revealed that there was not a predominance of a specific *HLA-DRB1*13* allele in the non-alloimmunised patients.

Several studies have associated RBC alloimmunisation for specific antigens with different *HLA-DRB1* alleles. Chiaroni and Cols showed that *HLA-DRB1*11* and *HLA-DRB1*13* were associated with the risk of anti-K alloimmunisation[32]. In contrast, we observed that *HLA-DRB1*13* could be associated with protection against alloimmunisation to different RBC antigens in patients with MDS. Another study related numerous HLA-DRB1 molecules with anti-K alloimmunisation, but not with a specific allele[22]. In patients alloimmunised to Fy^a antigen *HLA-DRB1*04* and *DRB1*15:01* were shown to be overrepresented[22,33] and *HLA-DRB1*01:01*, *DRB1*01:02*, and *DRB1*10:01*, appeared to be overrepresented in Jk^a immunised patients[20]. These data suggest that beyond the direct link between HLA class II and antigen specificity, HLA alleles may also modulate alloimmunisation at a non-antigen specific level[18].

One limitation of our study is the very small number of subjects studied. Larger studies are necessary to identify possible risk factors to RBC alloimmunisation in patients with MDS and to corroborate our findings about an association of HLA alleles with alloimmunisation.

In this study, we performed Rh and K genotyping instead of RBC phenotyping because many MDS patients had already been transfused when chronic transfusion-dependence was first defined. When we applied molecular-matching to the 43 MDS patients we found discrepancies or mismatches for multiple antigens between their genotype profile and the antigen profile of the blood units serologically matched for them. Molecular testing indicated that mistyping by haemagglutination in recently transfused patients was the source of the discrepancies. We were also able to identify *RH* variants that would never have been identified by serology in four patients and distinguished between alloantibodies and autoantibodies. In fact, two patients who were alloimmunised to Rh antigens had discrepancies between their phenotype and genotype and two patients had partial D. Although we were able to find a better match for the patients in our extended genotype-typed units, molecular matching for Rh and K would have prevented antibody formation in 68% of the alloimmunised patients. This observation is consistent with that by Sanz *et al*. in a study on RBC alloimmunisation in transfused patients with MDS/chronic myelomonocytic leukaemia, in which 62% of the alloimmunised patients formed antibodies against Rh and K[34].

In conclusion, this study provides data to support the utility of a limited genotype matching (Rh and K) in patients with MDS to prevent RBC alloimmunisation.

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The Authors declare that they have no conflicts of interest.

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