HLA-A, -C, -B, AND -DRB1 ALLELIC AND HAPLOTYPIC DIVERSITY IN BONE MARROW VOLUNTEER DONORS FROM NORTHERN PORTUGAL

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Keywords - Haplotype frequency, human leukocyte antigen alleles, marrow donor registry, Portugal

Received September 27th, 2012, accepted January 31st, 2013

Summary - Analysis of the HLA allelic and haplotype frequency data in different populations helps to shed light on the evolutionary factors that result in genetic polymorphism and the biological relationships among different ethnic groups. It is important to analyse HLA allele and haplotype frequencies in different populations to find compatible marrow transplantation donors from unrelated individuals.

The aim of this study was to investigate the distribution of HLA-A, -C, -B and DRB1 alleles and haplotypes in Northern Portugal.

The HLA-A, -C, -B, and -DRB1 allele frequencies were determined by direct counting. The haplotype frequencies were calculated using the expectation-maximisation algorithm in Arlequin v3 software. The Hardy-Weinberg equilibrium was verified using the Guo and Thompson method.

The most frequent (> 10%) HLA-A alleles (A*02, A*01, A*03, and A*24), HLA-B alleles (B*44, and B*35) and HLA-C alleles (C*07, and C*04) found in this study frequently occur in many other Caucasian populations.

Of the class II HLA alleles at the HLA-DRB1* locus, the allelic groups HLA-DRB1*07 and -DRB1*13 occur most frequently (> 15%) in the Portuguese population, as previously reported by others.

The HLA-A*01-C*07-B*08-DRB1*03 and HLA-A*29-C*16-B*44-DRB1*07 haplotypes, described as being of Pan European and western European origin, respectively, were the most frequent haplotypes found in our sample, and they are very frequent in Caucasian Brazilian, German, Italian, Spanish and the previously described Portuguese populations.

These data represent an important contribution to future anthropological and disease association studies involving the Portuguese population.

Introduction

The human leucocyte antigen (HLA) system, located on the short arm of chromosome 6, comprises the most polymorphic loci in the human genome (1). The HLA system consists of class I, II, and III non-overlapping segments that encode cell-surface heterodimeric glycoproteins. Genetic variation at these loci plays an important role in the immune response and in haematopoietic stem cell transplantation (2-4). HLA class I and II genes play an essential role in histocompatibility and disease susceptibility, in the presentation of peptides to T-lymphocytes and in self/non-self recognition (5). The HLA system determines immune function, is involved in the susceptibility to a remarkably large number of complex diseases, determines the outcome of tissue transplantation, and is used in forensic science (6-8).

Examination of genetic data from the HLA region has proved valuable for the study of population affinities and

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histories (9-12). Genetic distances are calculated from the frequency of the HLA alleles, which facilitates the characterisation of a population (13). Genetic distances and correspondence analyses have revealed that class I and class II allele loci and haplotype distribution are racially and geographically restricted (14). The high levels of linkage disequilibrium (LD) in the HLA region provide the opportunity to examine population divergence using haplotypic data in addition to single loci. At the same time, the study of these extended haplotypes in long-established populations may allow the selective processes at work in the HLA region to become more apparent (9).

HLA population studies are of particular interest not only for anthropological and disease association studies but also for the implementation of haematopoietic stem cell transplantation networking through unrelated donor recruitment from the national registries (15,16). Analysis of the allele and haplotype frequency data in different populations sheds more light on the evolutionary factors that result in genetic polymorphism and the biological relationships among different ethnic groups. It is important to analyse HLA allele and haplotype frequencies in different populations to locate compatible marrow transplantation donors from unrelated individuals (17). Knowledge of HLA haplotype frequencies has numerous applications in clinical medicine and forensic sciences. In particular, HLA haplotype frequencies are important for predicting the outcome of unrelated donor searches for individual patients in need of hematopoietic stem cell transplantation and to optimise donor search strategies (18-21).

Haematopoietic stem cell transplantation (SCT) can be an effective treatment for certain malignant diseases involving the bone marrow, such as leukaemia, lymphoma, and myeloma. The success of haematopoietic SCT is determined largely by the degree of HLA matching between donor and recipient (19,22-25). The HLA system plays a major role in allograft rejection. Since the early days of haematopoietic stem cell transplantation, a genotypically HLA-identical sibling has been the donor of choice. Approximately 30% of patients have an HLA-identical sibling, 30-50% find a fully matched voluntary unrelated donor, whereas for 20-40% of patients, the search is unsuccessful (19). HLA molecules expressed by transplanted organs are strongly immunogenic, and if they are not matched with the donor HLA, they are recognised as non-self and initiate T-cell proliferation and destruction of the transplanted organ, which in association with other cellular and antibody responses, may lead to graft rejection. Therefore, to predict the probability of finding compatible donors for unrelated bone marrow transplantations, it is valuable to have reliable estimates of HLA allele and haplotype frequencies (18,26,27). HLA matching at the haplotype level may have a higher likelihood of matching at other loci within the HLA region than merely matching at the allele level (24).

The aim of this study was to investigate the distribution of HLA-A, -C, -B, and DRB1 alleles and haplotypes in Northern Portugal. We comprehensively documented the background HLA allelic representation and respective haplotype profiles in a Portuguese population. This information should provide useful data for HLA matching in transplantations and for disease associations in the Portuguese population.

Material and methods

Subjects

Our data set consisted of 37,993 unrelated donors from the Northern Portuguese bone marrow voluntary donor registry who were recruited between January 2010 and December 2011, as previously described (28). Donors were not recruited from projects that focused on the specific recruitment of donors with rare HLA phenotypes. The donors were typed for HLA-A, -C, -B, and -DRB1 at intermediate resolution. On recruitment, the donors were asked to indicate their ethnicity, and only 47 (0.01%) declared that they were not Caucasian. The mean age of the donors at recruitment was 31.72 (± 7.5) years and 14,408 (37.9%) of the donors were male.

The donors signed an informed consent form.

HLA typing

The intermediate-resolution HLA typing was carried out at the American Society for Histocompatibility and Immunogenetics – accredited laboratory of Histogenetics, Inc. (Ossining, NY). Sequence-based typing (SBT) was used for typing HLA class I (HLA-A, -B, -C) and class II (DRB1) genes. Sequencing templates were produced by locus- or group-specific pairs of oligonucleotide primers from genomic DNA by polymerase chain reaction (PCR). A total of 40 locus- and group- specific primers were used to amplify the target sequences. Sanger cycle sequencing was carried out using BigDye V3.1 (Applied Biosystems) chemistry and ABI 3730xl capillary sequencer for base calling. The PCR product was treated with Exonuclease, and ethanol precipitation was used to clean up post-sequencing reaction extension products. Class I sequencing primers were those locus-specific sequences for each locus in the intron/exon boundary regions to sequence the entire exons. All Class II PCR primers were tailed with M13 forward and T7 reverse sequences. Sequencing was performed on both strands.

When further resolution was required for the ambiguous combinations that were not distinguished by these groups, additional sequencing of group-specific amplifications or group-specific sequencing primers were used.

Generally, the alleles were identified using the National Marrow Donor Program codification system and then grouped according to their corresponding low resolution typing (two digits (29)).

A peripheral whole-blood sample of each volunteer bone marrow donor was collected in acid citrate dextrose (ACD) anticoagulant. ACD whole blood was stored at 4-8ºC until genomic DNA was extracted. The blood was collected by the *Centro de Histocompatibilidade do Norte*, the reference histocompatibility laboratory in Northern Portugal (28).

Statistical analysis

The HLA antigen typing results were obtained at an intermediate resolution. All statistical analyses were performed at the two digit-allele family level.

The HLA-A, -C, -B, and -DRB1 allele frequencies (AF) were determined for each allele in the donors using the following formula: AF(%) = (n/2N) x 100, where n indicates the sum of a particular allele and N indicates the total number of individuals. The haplotype frequencies and verification of the Hardy-Weinberg equilibrium were calculated using Arlequin v3 software (30). The expectation-maximisation algorithm was used to determine the haplotype frequencies, as described by Excoffier and Slatkin (31). This method allows the estimation of the random haplotype frequencies of the sample. The Hardy-Weinberg equilibrium was verified using the method described by Guo and Thompson (32). The linkage disequilibrium between all pairs of loci was also tested (33) using Arlequin v3.

The relative linkage disequilibrium values (LD) D'_{ij} of alleles i and j were calculated as described elsewhere (7). The 20 strongest positive relative LD were identified for each analysed haplotype. To focus on relevant LDs, only haplotypes with allele frequencies of at least 0.1% for both alleles were considered (34).

Results

None of the loci tested showed any significant departure from the Hardy-Weinberg equilibrium; all of the p values for the exact test exceeded 0.05 (Table 1). Statistical significance was reached when overall linkage equilibrium was tested between all pairs of loci (Table 2).

The frequencies of HLA-A, -C, -B, and -DRB1 alleles in our Portuguese samples are summarised in Table 3. Different HLA-A alleles, 20 in total, were detected; A*02, with a frequency of 27.5%, was the most frequent allele, followed by A*01 (10.9%), A*03 (10.4%), A*24 (10.3%) and A*11 (6.5%). At the HLA-C locus, 14 different alleles were detected. The most common allele was C*07 (23.1%), followed by C*04 (15.5%), C*06 (8.6%), C*05 (7.7%) and C*08 (6.8%). At the HLA-B locus, 34 different alleles were observed. The most frequent allele was B*44 (15%), followed by B*35 (11.8%), B*51 (10.7%), B*14 (7.6%) and B*08 (6.7%). A total of 13 different DRB1 alleles were detected. DRB1*07 was the most common allele, with a frequency of 17.7%. Other frequent alleles were DRB1*13 (15.1%), DRB1*04 (13.0%), DRB1*01 (12.2%) and DRB1*03 (10.2%). The alleles corresponding to HLA-B*46, B*54, B*67, B*73, B*81 and B*82 were detected at frequencies lower than 0.05%.

The 20 most frequent HLA-A-C, HLA-C-B, and HLA-B-DRB1 haplotypes are summarised in Table 4. The negative linkage disequilibria reported are primarily an epiphenomenon of the positive disequilibria. For example, the negative disequilibria found for haplotypes HLA-A*02-C*07, HLA-A*02-C*04, HLA-A*02-C*15, HLA-A*02-C*16 and HLA-A*02-C*12 are just the immediate consequence of the strong positive disequilibria between HLA-A*02-C*14, HLA-A*02-C*05 and HLA-A*02-C*01.

In total, 239 HLA-A-C, 224 HLA-C-B, 336 HLA-B-DRB1, 1,299 HLA-A-C-B and 4,416 HLA-A-C-B-DRB1 distinct haplotypes were identified. The most common two-locus HLA haplotypes, with a haplotype frequency > 5%, were as follows: A*01-C*07 (5.7%), A*02-C*07 (5.6%), C*04-B*35 (10.5%), C*08-B*14 (6.7%), C*07-B*08 (6.6%), C*05-B*44 (5.5%), and C*07-B*07 (5.4%). Table 5 shows the 20 strongest relative LDs for the HLA-A-C, HLA-C-B, and HLA-B-DRB1 haplotypes. As expected, the strongest LD was observed for the HLA-C-B haplotype, reflecting the small spatial distance between these HLA genes, followed by HLA-B-DRB1. The most common three-locus haplotypes, with a haplotype frequency > 2%, were as follows: A*01-C*07-B*08 (4.1%), A*02-C*05-B*44 (3.5%), A*29-C*16-B*44 (2.9%), A*33-C*08-B*14 (2.4%), A*03-C*04-B*35 (2.2%), A*03-C*07-B*07 (2.1%), and A*02-C*04-B*35 (2.1%) (data not shown).

The most common four-locus haplotypes, with a haplotype frequency > 1% (Table 6), were as follows: A*01-C*07-B*08-DRB1*03 (3.1%), A*29-C*16-B*44-DRB1*07 (2.1%), A*33-C*08-B*14-DRB1*01 (1.4%), A*03-C*07-B*07-DRB1*15 (1.2%), A*02-C*05-B*44-DRB1*04 (1.1%), and A*23-C*04-B*44-DRB1*07 (1.0%).

Discussion

The HLA loci are the most polymorphic gene clusters in humans, playing a key role in peptide presentation to T-lymphocytes and self/non-self recognition. Since its discovery, the most important clinical application of the HLA system has been the selection of suitable donors for transplantation or transfusion (7,35). In spite of the expansion of unrelated donor registries, allelic HLA matching remains a problem for many patients because of the large

Locus	# Genotypes	Observed Heterozygosity	Expected Heterozygosity	P-value
A	37993	0.8737	0.8739	0.098
C	37993	0.8811	0.8829	0.732
B	37993	0.9273	0.9250	0.280
DRB1	37993	0.8817	0.8828	0.157

HW – Hardy-Weinberg; HLA – human leucocyte antigen.

TABLE 1 - HW equilibrium test of HLA-A. -C. -B and -DRB1 genotype frequencies.

	HLA-A	HLA-C	HLA-B	HLA-DRB1
HLA-A	-	P<0.00001	P<0.00001	P<0.00001
HLA-C	-	-	P<0.00001	P<0.00001
HLA-B	-	-	-	P<0.00001

HLA – human leucocyte antigen.

TABLE 2 - P-values for pairwise linkage equilibrium test.

HLA-A	No. of allele	frequency (%)	HLA-C	No. of allele	frequency (%)	HLA-B	No. of allele	frequency (%)	HLA-DRB1	No. of allele	frequency (%)
01	8278	10,9%	01	1859	2.45%	07	4573	6.0%	01	9262	12.2%
02	20899	27.5%	02	4819	6.34%	08	5097	6.7%	03	7757	10.2%
03	7874	10.4%	03	5129	6.75%	13	1168	1.5%	04	9866	13.0%
11	4925	6.5%	04	11758	15.47%	14	5753	7.6%	07	13448	17.7%
23	3427	4.5%	05	5829	7.67%	15	4291	5.6%	08	3375	4.4%
24	7814	10.3%	06	6570	8.65%	18	3624	4.8%	09	514	0.7%
25	1220	1.6%	07	17524	23.06%	27	2337	3.1%	10	1294	1.7%
26	2501	3.3%	08	5195	6.84%	35	8946	11.8%	11	7639	10.1%
29	4068	5.4%	12	5110	6.72%	37	994	1.3%	12	1169	1.5%
30	2147	2.8%	14	2224	2.93%	38	1818	2.4%	13	11470	15.1%
31	1914	2.5%	15	4031	5.30%	39	1318	1.7%	14	1953	2.6%
32	2977	3.9%	16	5081	6.69%	40	2633	3.5%	15	6384	8.4%
33	2668	3.5%	17	803	1.06%	41	859	1.1%	16	1855	2.4%
34	337	0.4%	18	54	0.07%	42	89	0.1%	Total	**75986**	100.0%
36	45	0.1%	Total	**75986**	100%	44	11426	15.0%			
66	695	0.9%				45	1001	1.3%			
68	3798	5.0%				46	4	0.0%			
69	255	0.3%				47	204	0.3%			
74	85	0.1%				48	64	0.1%			
80	59	0.1%				49	2579	3.4%			
Total	**75986**	100%				50	2851	3.8%			
						51	8122	10.7%			
						52	560	0.7%			
						53	813	1.1%			
						54	4	0.0%			
						55	832	1.1%			
						56	291	0.4%			
						57	2173	2.9%			
						58	1422	1.9%			
						67	23	0.0%			
						73	28	0.0%			
						78	82	0.1%			
						81	6	0.0%			
						82	1	0.0%			
						Total	**75986**	100%			

HLA – human leucocyte antigen.

TABLE 3 - Distribution of HLA-A -C, -B, and DRB1 allele frequencies in the Northern Portuguese population (n = 37993).

HLA-A-C				HLA-C-B				HLA-B-DRB1			
A	C	Hap freq	Rel LD	C	B	Hap freq	Rel LD	B	DRB1	Hap freq	Rel LD
01	07	5.71%	0.38	04	35	10.52%	0.87	44	07	5.79%	0.25
02	07	5.55%	-0.12	08	14	6.70%	0.98	08	03	5.05%	0.73
02	05	4.06%	0.35	07	08	6.59%	0.98	14	01	3.92%	0.45
02	04	3.36%	-0.21	05	44	5.52%	0.67	35	01	3.03%	0.15
29	16	3.08%	0.54	07	07	5.42%	0.87	07	15	2.86%	0.43
03	07	3.04%	0.08	16	44	4.81%	0.67	44	04	2.43%	0.04
02	06	2.97%	0.09	15	51	3.89%	0.70	44	13	2.19%	-0.03
24	07	2.44%	0.01	07	49	3.34%	0.98	35	11	2.02%	0.09
03	04	2.42%	0.09	06	50	2.97%	0.77	51	13	1.80%	0.02
33	08	2.37%	0.65	03	15	2.96%	0.49	51	04	1.77%	0.04
11	04	2.21%	0.22	04	44	2.88%	0.04	51	11	1.74%	0.07
02	02	2.00%	0.06	14	51	2.65%	0.89	57	07	1.68%	0.50
02	03	1.87%	0.00	12	38	2.23%	0.93	15	13	1.67%	0.17
23	04	1.84%	0.30	03	40	2.06%	0.57	50	07	1.62%	0.31
01	06	1.65%	0.09	02	27	1.61%	0.49	14	07	1.49%	0.02
02	14	1.64%	0.40	07	58	1.55%	0.78	35	04	1.45%	-0.05
24	04	1.64%	0.01	05	18	1.53%	0.26	35	13	1.33%	-0.25
02	15	1.31%	-0.10	06	13	1.49%	0.97	51	08	1.27%	0.20
02	16	1.30%	-0.30	07	18	1.45%	0.09	18	03	1.19%	0.16
02	12	1.26%	-0.32	06	57	1.41%	0.44	51	07	1.16%	-0.39

HLA – human leucocyte antigen; Hap freq – haplotype frequency; Rel LD – relative linkage disequilibrium.

TABLE 4 - The 20 most frequent haplotypes for HLA-A-C, HLA-C-B and HLA-B-DRB1.

HLA-A-C				HLA-C-B				HLA-B-DRB1			
A	C	Hap freq	Rel LD	C	B	Hap freq	Rel LD	B	DRB1	Hap freq	Rel LD
33	08	2.37%	0.65	17	42	0.12%	1.00	78	13	0.09%	0.78
34	07	0.30%	0.59	16	78	0.11%	0.99	08	03	5.05%	0.73
29	16	3.08%	0.54	07	49	3.34%	0.98	52	15	0.53%	0.69
74	02	0.05%	0.40	08	14	6.70%	0.98	13	07	1.12%	0.67
25	12	0.71%	0.40	06	37	1.28%	0.98	42	03	0.07%	0.53
02	14	1.64%	0.40	07	08	6.59%	0.98	57	07	1.68%	0.50
01	07	5.71%	0.38	06	13	1.49%	0.97	14	01	3.92%	0.45
66	17	0.35%	0.37	12	52	0.71%	0.95	07	15	2.86%	0.43
02	05	4.06%	0.35	01	56	0.36%	0.95	53	13	0.55%	0.42
23	04	1.84%	0.30	12	38	2.23%	0.93	41	13	0.55%	0.39
26	12	1.10%	0.29	04	53	0.98%	0.90	38	13	1.02%	0.33
69	12	0.10%	0.26	14	51	2.65%	0.89	50	07	1.62%	0.31
11	04	2.21%	0.22	04	35	10.52%	0.87	47	13	0.11%	0.30
30	05	0.77%	0.21	17	41	0.92%	0.87	44	12	0.61%	0.29
31	15	0.60%	0.20	07	07	5.42%	0.87	56	01	0.14%	0.28
25	03	0.36%	0.17	03	55	0.93%	0.84	37	10	0.38%	0.28
02	01	0.96%	0.16	07	58	1.55%	0.78	44	07	5.79%	0.25
69	03	0.07%	0.15	06	50	2.97%	0.77	35	14	0.81%	0.22
74	04	0.03%	0.14	15	51	3.89%	0.70	56	08	0.10%	0.22
30	06	0.59%	0.13	16	44	4.81%	0.67	49	04	1.08%	0.22

HLA – human leucocyte antigen; Hap freq – haplotype frequency; Rel LD – relative linkage disequilibrium.

Only haplotypes with allele frequencies of at least 0.1% for both alleles were considered.

TABLE 5 - The 20 strongest relative linkage disequilibria for the haplotypes HLA-A-C, HLA-C-B and HLA-B-DRB1.

HLA-A-C-B-DRB1				
A	C	B	DRB1	Hap freq
01	07	08	03	3.1%
29	16	44	07	2.1%
33	08	14	01	1.4%
03	07	07	15	1.2%
02	05	44	04	1.1%
23	04	44	07	1.0%

HLA – human leucocyte antigen; Hap freq – haplotype frequency.

TABLE 6 - Haplotypes with a frequency higher than 1% for HLA-A-C-B-DRB1.

diversity of HLA alleles and haplotypes. Patients showing common HLA alleles on conserved haplotypes are more likely to find full-matched unrelated donors than those with rare alleles or haplotypes showing low frequencies. On the other hand, the probability of identifying a matched donor is higher when both patient and donor are of the same ethnic background (36,37).

HLA polymorphism in Portugal was analysed in previous studies using samples from less than 200 individuals from mainland Portugal (38), the Azores Islands (39), and the Madeira Islands (40,41). This study, using almost 38,000 volunteer bone marrow donors recruited from Northern Portugal, represents the first investigation of the HLA class I (HLA-A, -C, -B) and class II (HLA-DRB1) allele and haplotype frequencies in the Portuguese population.

The most frequent (> 10%) HLA-A alleles (A*02, A*01, A*03, and A*24), HLA-B alleles (B*44, and B*35) and HLA-C alleles (C*07, and C*04) found in this study have been shown to occur at a high frequency in many other Caucasian populations (38,42-45).

Of the class II HLA alleles at the HLA-DRB1 locus, the two most frequent (> 15%) allelic groups were HLA-DRB1*07 and -DRB1*13, as previously reported by others (38) for the Portuguese population.

HLA-A*01-C*07-B*08-DRB1*03 and HLA-A*29-C*16-B*44-DRB1*07, described as being of Pan European and Western European origin, respectively (44), were the most frequent haplotypes in our sample and occur at high frequencies in the Caucasian Brazilian (42), German (20), Italian (45), Spanish (44) and the previously described Portuguese population (38). The Iberian haplotype HLA-A*33-C*08-B*14-DRB1*01 (44) was the third most frequent haplotype in our sample and has been described previously in north Portugal (38), Spain (44), Italy (45) and Caucasian Cuba (43). Although HLA- A*03-C*07-B*07-DRB1*15, the fourth most frequent haplotype in our sample (western European origin (44)), has been described as a frequent haplotype in German (20), Italian (45), Brazilian (42), Caucasian Cuban (43), Spanish (44), Madeiran (11,40) and Portuguese from Azores (39) populations, it was not identified as a common haplotype in other mainland Portuguese populations (38). HLA-A*23-C*04-B*44-DRB1*04 was the sixth most frequent (>1%) haplotype in our sample and was described as a frequent haplotype in the German (20), Caucasian Brazilian (42), and other mainland Portuguese populations (38).

It is important to remember that the haplotypic frequencies presented in this study are estimates based on the frequencies of alleles; thus, they are not necessarily true. Information on the ancestors of each individual is required to identify the inherited haplotypes and thus determine the true distribution.

Conclusions

We have described the distribution of HLA-A, -C, -B, and -DRB1 alleles and haplotypes in volunteer bone marrow donors recruited in Northern Portugal. Our data show similarities between Portuguese and other western and southern European populations in terms of allele and haplotype frequencies.

The characterisation of the HLA composition in the Portuguese population is important for understanding several autoimmune diseases and most importantly, to organise registries of volunteer bone marrow donors.

These data will make an important contribution to future anthropological and disease association studies involving the Portuguese population.

Acknowledgment

The authors acknowledge the contributions of Dr Nezih Cereb from Histogenetics, Inc. (Ossining, NY) for invaluable advice and help with respect to the HLA typing methods described in this manuscript.

We are grateful to each bone marrow volunteer donor who has registered to offer a stranger a second chance at life.

The authors declare that they have no conflict of interest.

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