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ORIGINAL ARTICLE

Determination of HLA-A, -B, and -DRB1 Allele and Haplotype Frequencies in the Croatian Population Based on a Family Study

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Abstract In the present study, HLA allele and haplotype frequencies among Croatian families were investigated to evaluate valuable information about HLA genotypes and to compare them with data from the Croatian Bone Marrow Donor Registry (CBMDR). A total of 350 families which have been typed for the purpose of HSCT were included. All individuals were tested using PCR-SSO or PCR-SSP methods for HLA-A, -B, and -DRB1 alleles. The HLA-A-B-DRB1 haplotypes were determined by segregation and directly counted. A total of 30 HLA-A, 54 HLA-B, and 38 HLA-DRB1 alleles and 716 different HLA-A-B-DRB1 haplotypes were identified. Of these, the three most frequent alleles at HLA-A, -B, and -DRB1 loci, respectively, were A*02:01 (30.39%), A*11:01 (13.37%), A*24:02 (10.91%); B*51:01 (12.48%), B*18:01 (8.35%), B*08:01 (8.06%); DRB1*03:01 (11.20%), DRB1*01:01 (9.84%), DRB1*16:01 (9.63%). The following HLA alleles were detected only once: A*02:09, A*24:03, A*24:04, A*24:07; B*07:04, B*15:07, B*15:08, B*39:04, B*39:10, B*39:24, B*40:04; DRB1*08:03, DRB1*11:06, DRB1*13:32, DRB1*14:05. Five most frequent haplotypes were: A*01:01-B*08:01-DRB1*03:01 (5.34%), A*02:01-B*18:01-DRB1*11:04 (1.57%), A*02:01-B*27:02-DRB1*16:01 (1.50%), A*02:01-B*27:05-DRB1*01:01 (1.42%) and A*02:01-B*44:02:01G-DRB1*16:01 (1.28%). The haplotype frequencies based on the family study were compared with the frequencies from CBMDR, and similar results were obtained for all except for the HLA-A*26:01-B*38:01-DRB1*04:02 haplotype. A significantly higher frequency ($P = 0.0017$) of this haplotype was observed among family individuals. Nine haplotypes were unique and data about their frequencies do not exist in current databases. The data obtained in this study could be useful for anthropology, transplantation and disease association studies.

Keywords HLA · Allele and haplotype frequencies · Family study · Croatian population

Introduction

The human leukocyte antigens (HLA) system is characterised as the most polymorphic genetic system in humans with more than 14,000 alleles known so far, and it is characterised by strong linkage disequilibrium (LD) between alleles at different HLA loci (Shiina et al. 2009). Positive selection is considered as one of the causes of such a huge number of HLA alleles and LD between them (Hughes and Nei 1988). This selection results in different "HLA profiles" of different human populations due to the exposure to various pathogens, as well as the influence of other populations following a migration. An additional force to preserve a large number of HLA alleles is a balancing selection that maintains a pool of functionally divergent haplotypes and alleles as suggested by Sanchez-Mazas et al. (2011). Because HLA allele and haplotype frequencies differ extensively among various populations, such an analysis can be used to follow the migration patterns of a given population as well as its relationship with other populations (Bronson et al. 2013; Fernandez Viña et al. 2012).

The knowledge of HLA allele and haplotype distribution is, on the other hand, very useful in the field of

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haematopoietic stem cell transplantation (HSCT). Namely, an HLA match between the donor and the recipient improves the success of unrelated HSCT. It is a well known fact that the lack of availability of HLA matched unrelated donors (MUD) represents one of the major obstacles to the success of HSCT and largely depends on the representation of patients' HLA alleles and haplotypes (Shaw et al. 2010). A similarly recognized fact is that HLA matching between a patient and a MUD lowers the risk of graft-versus-host disease, as well as the risk of graft rejection (Furst et al. 2013).

For all of the above stated reasons, the aims of the present family study were to perform an analysis of the HLA-A, -B, -DRB1 allele and haplotype frequencies, to obtain data about common, rare and very rare HLA haplotypes in a sample of family derived haplotypes, to compare the obtained results with the Croatian Bone Marrow Donor Registry (CBMDR) data, as well as with data from other population studies.

Materials and Methods

A total of 700 parents (from 350 families) of patients who have undergone the search for a related donor for HSCT were included. Families originated from all regions of Croatia.

Four thousand volunteer unrelated donors from CBMDR were chosen for the comparison of the allele and haplotype frequencies with those obtained from the family study (Grubic et al. 2014a). These donors are also from all regions of Croatia and they form a representative sample of our population.

Methods

Genomic DNA from whole blood containing EDTA was extracted using a commercial kit (MagNA Pure LC DNA; Roche Diagnostics GmbH, Manheim, Germany).

HLA-A, HLA-B and HLA-DRB1 typing was performed using the PCR-LABType SSO method (Immucor Transplant Diagnostics Inc., Stamford, CT, USA) (Dalva and Beksac 2007). The software used for the generation of the HLA data was QuickType for Lifecodes, version 2.5. This method uses kits for HLA-A and HLA-B, which cover exons 2 and 3, while kits for HLA-DRB1 cover exon 2. The 5′ ends of upstream primers (included in kits) were labelled with biotin, and each PCR product was hybridized with 72 probes for HLA-A, 92 probes for HLA-B and 68 probes for HLA-DRB1, complementary to the polymorphic sequences. After hybridization, amplicons were labelled with streptavidin-*R*-phycoerythrin which is a specific fluorescent ligand of biotin and quantified on the Luminex LABScanTM 100 flow analyzer (Luminex Corporation, Austin, TX, USA). Additional typing was performed using PCR-SSP high resolution typing kits for each allelic group (GenoVision Inc, West Chester, PA, USA) (Olerup and Zettequist 1992).

Statistical Analysis

Allele frequencies were calculated using the GeneRate program (http://geneva.unige.ch/ahpd/). The haplotypes were determined by segregation and directly counted. The significance of differences in allele and haplotype frequencies between the family study and CBMDR, as well as in comparison to the other populations, was evaluated using the χ^2 test, while Fisher's exact test was used if any of the values in 2×2 tables was <5.

Results

A total of 30 HLA-A, 54 HLA-B, and 38 HLA-DRB1 alleles were identified. Table 1 presents the list of the HLA-A, -B, and -DRB1 alleles with frequencies >5%. The

Table 1 Most frequent ($f > 5\%$) HLA-A, -B and -DRB1 alleles observed in the Croatian families

	Family study, *f*	CBMDR, *f*
HLA-A*		
01:01	0.1371	0.1244
02:01	0.3031	0.2916
03:01	0.1034	0.1184
11:01	0.0670	0.0691
24:02	0.1091	0.1098
HLA-B*		
07:02	0.0721	0.0681
08:01	0.0799	0.0778
18:01	0.0827	0.0816
35:01	0.0606	0.0611
44:02:01G	0.0642	0.0589
51:01	0.1248	0.1113
HLA-DRB1*		
01:01	0.0986	0.0979
03:01	0.1113	0.1001
07:01	0.0820	0.0978
11:01	0.0764	0.0788
11:04	0.0770	0.0766
13:01	0.0649	0.0644
15:01	0.0777	0.0871
16:01	0.0963	0.0941

CBMDR Croatian Bone Marrow Donor Registry, *f* allele frequency

frequency of HLA alleles at tested loci in the present study was similar to that reported for volunteer donors from CBMDR, as well as for the Croatian population (Grubic et al. 2014a, b). A total of 22 HLA alleles were observed only once in this sample (5 at HLA-A locus, 12 at HLA-B locus and 5 at HLA-DRB1 locus; Table 2). Five of them (A*24:04, A*02:09, B*15:07, B*52:02, and DRB1*13:32) were detected for the first time in our population. All those HLA alleles belong in the category of common or well documented alleles according to current databases. The only exception is the DRB1*13:32 allele which belongs to the group of not defined alleles (Gonzales-Galarza et al. 2015; IMGT/HLA Database 2016). There are currently no sufficient data about the frequencies of these five alleles except for the B*15:07 allele which has been reported for more than 50 different populations all over the world so far (Gonzales-Galarza et al. 2015). The A*02:09 allele is frequent among Asian populations, but has thus far been found in only two European populations (Italian and Czech) (Gonzales-Galarza et al. 2015). The A*24:04 allele was observed among European populations only in a study from German Bone Marrow Donor Centre (DKMS) for the Bosnian and Herzegovinian minority, the B*52:02 allele is detected in the Greek population, while for the DRB1*13:32 allele frequency (0.03%) the only available data are for the Austrian minority in DKMS (Gonzales-Galarza et al. 2015).

In this sample 716 different HLA-A-B-DRB1 haplotypes were determined, 239 (33.38%) were found ≥2×, while 477 (66.62%) were present only once. Six (0.84%) different HLA-A-B-DRB1 haplotypes demonstrated a frequency higher than 1.00%, 116 (16.20%) haplotypes were detected with a frequency of 0.2–1%, while 594 (82.96%) haplotypes were present with a frequency <0.20%. Haplotype distribution regarding the number of occurrences in this family study is shown in Fig. 1.

The HLA-A-B-DRB1 haplotypes with a frequency ≥0.5% are listed in Table 3. The most frequent one was HLA-A*01:01-B*08:01-DRB1*03:01 (5.34%), followed by HLA-A*02:01-B*18:01-DRB1*11:04 (1.57%) and HLA-A*02:01-B*27:02-DRB1*16:01 (1.50%).

The haplotype rank based on the family study was compared with the rank from CBMDR and similar results were obtained except for three haplotypes. The HLA-A*25:01-B*18:01-DRB1*15:01 haplotype and the HLA-A*33:01-B*14:02-DRB1*01:02 haplotype in the present study rank among the first ten haplotypes (7 and 10, respectively), but differences are not statistically significant. The only exception was found for the HLA-A*26:01-B*38:01-DRB1*04:02 haplotype which was observed with

Table 2 Pattern of occurrences for the observed HLA-A, -B and -DRB1 alleles in a family study

	N	*n* (%)	Alleles observed only once
HLA-A*	30	5 (16.67)	02:09; 02:35; 24:03; 24:04; 24:07
HLA-B*	54	12 (22.22)	07:04; 15:07; 15:08; 39:04; 39:10; 39:24; 40:04; 48:01; 52:02; 54:01; 57:02; 57:03
HLA-DRB1*	31	5 (16.13)	08:03; 11:06; 13:15; 13:32; 14:05

Underline indicates alleles observed only in this study not among donors from Croatian Bone Marrow Donor Registry

N total number of alleles observed at tested HLA locus, *n* number of alleles observed only once in this family study

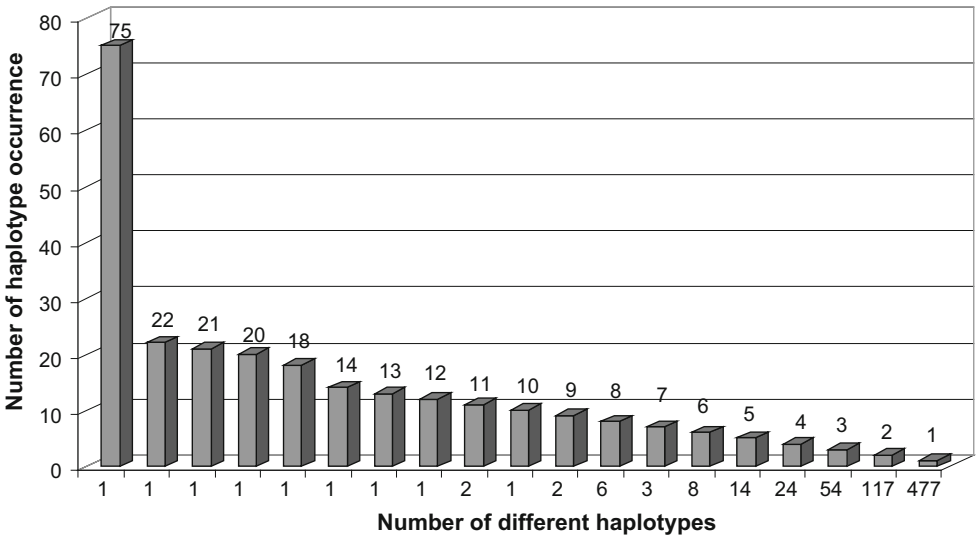

Fig. 1 The number of different haplotypes and their number of occurrences among family studied individuals

Table 3 The list HLA-A-B-DRB1 haplotypes with frequency >0.5% in the family study and their rank and frequency among donors from CBMDR

HLA-A*-B*-DRB1* halotype	Family study		CBMDR		*P* value
	Rank	%	Rank	%	
01:01-08:01-03:01	1	5.35	1	4.15	
02:01-18:01-11:04	2	1.57	2	1.48	
02:01-27:02-16:01	3	1.50	7	0.76	
02:01-27:05-01:01	4	1.43	4	0.85	
02:01-44:02:01G-16:01	5	1.28	8	0.72	
26:01-38:01-04:02	6	1.00	23	0.35	0.0017
25:01-18:01-15:01	7	0.93	13	0.53	
03:01-07:02-15:01	8	0.86	3	1.2	
11:01-35:01-01:01	9	0.78	10	0.67	
33:01-14:02-01:02	10	0.77	16	0.44	
01:01-57:01-07:01	11	0.71	14	0.52	
02:01-51:01-01:01	12–13	0.64	18	0.42	
02:01-51:01-13:01		0.64	19	0.42	
02:01-07:02-15:01	14–19	0.57	9	0.68	
02:01-51:01-04:02		0.57	21	0.37	
02:01-51:01-08:01		0.57	29	0.31	
02:01-51:01-16:01		0.57	11	0.63	
68:01-39:01-16:01		0.57	38	0.25	
02:01-13:02-07:01		0.57	6	0.76	
11:01-18:01-11:04	20–22	0.5	15	0.49	
23:01-44:03-07:01		0.5	12	0.62	
24:02-07:02-15:01		0.5	22	0.35	

CBMDR Croatian Bone Marrow Donor Registry, % haplotype frequency, *LD* relative linkage disequilibrium value

a significantly higher frequency ($P = 0.0017$) among family individuals (Grubic et al. 2014a).

The next aim of our study was to compare our data with the haplotype ranking reported for the DKMS, as well as for "European Caucasian" in the National Marrow Donor Program (NMDP) (Gragert et al. 2013; Muller et al. 2003; Schmidt et al. 2009). Ranks of ten most frequent haplotypes in this study, DKMS and NMDP are presented in Table 4. Only two haplotypes, HLA-A*01:01-B*08:01-DRB1*03:01 and HLA-A*03:01-B*07:02-DRB1*15:01, were in the group of the ten most frequent haplotypes in all three analyses, while for all the other eight haplotypes the ranks were quite different. One of the largest disparities in ranking was again pointed out for the HLA-A*29:02-B*44:03-DRB1*07:01 haplotype which is common in these two studies and is ranked in position 7 in DKMS and in position 5 in NMDP (Gragert et al. 2013; Muller et al. 2003; Schmidt et al. 2009). This haplotype in our family sample with three (0.21%) occurrences was not placed among frequent HLA-A-B-DRB1 haplotypes in our population.

Table 4 Comparison of HLA-A-B-DRB1 haplotypes ranking in the Croatian family study with data from DKMS and NMDP

HLA-A*-B*-DRB1*	Family study Rank	DKMS Rank	NMDP Rank
01:01-08:01-03:01	1	1	1
02:01-18:01-11:04	2	28	32
02:01-27:02-16:01	3	49	99
02:01-27:05-01:01	4	60	30
02:01-44:02:01G-16:01	5	95	79
26:01-38:01-04:02	6	149	17
25:01-18:01-15:01	7	20	20
03:01-07:02-15:01	8	2	2
11:01-35:01-01:01	9	23	18
33:01-14:02-01:02	10	21	23

DKMS Deutsche Knochenmarkspenderdatei, *NMDP* National Marrow Donor Program

In the group of HLA-A-B-DRB1 haplotypes which included an allele present only once in this family data analysis, eleven haplotypes observed only once (11/477; 2.31%) were unique and data about their frequencies do not exist in current databases (Table 5). One of these unique haplotypes was comprised of two alleles observed only once (HLA-A*02:09-44:02-13:15) in this family analysis.

Discussion

The comparison of HLA-A allele frequencies between the family sample and neighbouring populations did not reveal any significant difference (Gonzales-Galarza et al. 2015). This study supports the data from our previous investigation carried out among unrelated donors from CBMDR, which showed that B*51:01 is the most frequent among HLA-B alleles, as well as among some other populations on the European South. The HLA-B*18:01 allele, placed in the second position, is also present with a similar frequency among several other European populations (Albanians, Bosnians, Bulgarians, Czechs, Italians, Portuguese, Romanians, Serbians), while in some other (Macedonians, Greeks and North Italians), its frequency is higher (around 15%). The third most frequent allele, B*08:01, showed a similar frequency in numerous European descent populations all over the world (Gonzales-Galarza et al. 2015).

The present family study showed again that DRB1*11:04 (7.70%) is present with a similar frequency as DRB1*11:01 (7.64%) in our population and this fits in with the decreasing frequency of DRB1*11:04 allele from the south to the north of Europe (Gonzales-Galarza et al. 2015).

The second most frequent allele, DRB1*16:01, is also present with a similar frequency among several other

Table 5 HLA-A-B-DRB1 haplotypes of alleles observed only once and their frequencies in other populations

HLA-A*-B*-DRB1* haplotype	Population data
02:09-44:02-13:15[a]	No data
02:35-44:02-11:01	Poland DKMS
24:03-51:01-11:04	Germany DKMS-HRV, BIH, TUR; Poland DKMS
24:04-51:01-13:01	Germany DKMS-BIH
24:07-35:01-01:01	Germany DKMS-HRV, BIH; USA—Asian population
11:01-15:07-04:04[a]	No data
02:01-39:04-12:01[a]	No data
03:02-39:10-03:01[a]	No data
02:01-39:24-11:04[a]	No data
01:01-40:04-14:04[a]	No data
02:11-52:02-15:02[a]	No data
24:02-54:01-14:05	Germany DKMS-HRV, TUR, CHN; USA—Asian population; China; Japan
23:01-57:02-01:02	Germany DKMS-HRV, BIH
32:01-57:03-01:01[a]	No data
02:01-07:05-08:03[a]	No data
02:11-52:01-11:06[a]	No data
02:01-15:01-13:32[a]	No data

Underline indicates HLA alleles observed only once in our sample

DKMS Deutsche Knochenmarkspenderdatei, *BIH* Bosnia and Herzegovina minority, *CHN* China minority, *HRV* Croatia minority, *TUR* Turkey minority

[a] No population data available on the Allele Frequency Net Database

neighbouring populations and its observed frequency supports the increasing frequency of this allele from the north to the south of Europe (Gonzales-Galarza et al. 2015). The haplotype analysis demonstrated more than 700 different HLA-A-B-DRB1 haplotypes and approximately 20% of Croatians carry five most frequent haplotypes which demonstrated strong LD.

The frequencies of ten most frequent haplotypes are similar to those in the neighbouring populations (Gonzales-Galarza et al. 2015). For some of those haplotypes (A*02:01-B*18:01-DRB1*11:04, A*02:01-B*27:02-DRB1*16:01, A*02:01-B*27:05-DRB1*01:01, and A*02:01-B*44:02:01G-DRB1*16:01) existing differences in comparison to DKMS and NMDP data suggest a benefit of establishing national bone marrow donor registries which can contribute the specificities of each population to the development of Bone Marrow Donor Worldwide (BMDW). Namely, the large, already existing number of donors in BMDW is not enough to provide a MUD for all patients in the program of unrelated HSCT.

A statistically significant higher frequency ($P = 0.0017$) of the HLA-A*26:01-B*38:01-DRB1*04:02 haplotype was found among families than among unrelated donors from CBMDR and it is the highest so far reported (Gonzales-Galarza et al. 2015). This finding should be proved on a larger cohort of families because the frequency of this haplotype in Europeans ranges from 0.06% for the Greek minority recruited in the German registry to 0.68% for the Bosnian and Herzegovinian minority in the same registry. In the same registry, the reported frequency for the Croatian minority was 0.53%.

The haplotype analysis also helps in understanding the history of human migrations, as well as the history of each population. This study demonstrated that the "HLA haplotype population profile" has been impacted by various influences such as migrations, admixture with other populations, but also the present position which is specific on the Balkan Peninsula, known as a crossroad of many different migration paths. Namely, the history of the Croatian population is very complex; it includes migrations on the Balkan Peninsula and an admixture of Croatians with autochthonous populations (Illyrians, Thracians), the influence of Turks in the period of the Ottoman Empire, but also an admixture of neighbouring populations (Italians, Germans, Hungarians, Austrians).

In summary, the data obtained in this family study could be useful in the field of transplantation, especially in developing a mismatch strategy in our department because patients with at least one frequent HLA haplotype, as well as with conserved HLA haplotypes have a higher probability of finding a 10/10 MUD than those carrying rare haplotypes or HLA haplotypes with a low frequency (Balas et al. 2010; Jöris et al. 2013). The knowledge about the distribution of common and conserved HLA haplotypes but also non-frequent HLA haplotypes can be implemented in the search protocols for MUDs and the data obtained in this study could be used as a tool for elucidating the presence of uncommon HLA haplotypes in worldwide populations.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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