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ORIGINAL ARTICLE

DISTRIBUTION OF MINOR HISTOCOMPATIBILITY ANTIGENS HA-1, HA-2 AND HA-8 IN THE CROATIAN POPULATION

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Abstract: Minor histocompatibility antigens (mHAgs) are polymorphic, endogenously synthetized products recognized by alloreactive T cells in the context of major histocompatibility complex molecules. Recipients of allogeneic bone marrow grafts run the risk of graft-versus-host disease (GvHD), even when the donor is an HLA-identical sibling. This may be caused by disparities in mHAgs between the donor and the recipient, with the antigen present in the recipient and not in the donor. In such cases, T cells in the transplanted donor marrow respond to the recipient's mHAgs. We determined the allele, genotype and phenotype frequencies for mHAgs HA-1, HA-2 and HA-8 in 102 healthy, unrelated individuals previously typed for HLA-A, HLA-B and HLA-DR. We compared the results with existing studies in other populations and found no significant differences between allele, genotype and phenotype frequencies in the Croatian population and frequencies reported for Caucasian population. The results presented will be used for further studies investigating the role of mHAgs in hematopoietic stem cell transplantation.

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INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is an effective therapy for many patients with hematological malignancies. Transplantation of allogeneic hematopoietic cells is followed by reciprocal immunological reactions of the graft against the host which can lead to graft versus host disease (GvHD), and the host against the graft which can lead to graft rejection. Human leukocyte antigen (HLA) is crucial for the development of these reactions. HLA molecules are fundamental in T-cell activation since they bind peptides and present them to T cells.[1]

In HLA-matched HSCT, GvHD develops as a consequence of donor T cell response to host peptides bound to HLA molecules. These peptides are derived from a wide range of endogenous proteins which possess genetic polymorphism between individuals and are called minor histocompatibility antigens (mHAgs).[2] Minor histocompatibility antigens are immunogenic peptides encoded by non-HLA genes that can induce a specific T cell reaction in individuals lacking the antigen if presented in the context of class I or II HLA molecules.[3] They are polymorphic peptides consisting of 9-12 amino acids. After being presented on cell surface by their class I or class II HLA molecules, they can be recognized by T cells. Therefore, the occurrence of mHAgs is restricted by the presence of specific HLA antigens.[4]

It is well-documented that HSCT between HLA-matched related donor-recipient pairs shows lower rates of GvHD and higher rates of engraftment compared to HLA-matched unrelated donor-recipient pairs. However, even HLA-identical sibling pairs still exhibit GvHD, which confirms the role of mHAgs in HSCT outcome.[3]

HA-1, HA-2, HA-8 and other mHAgs can be recognized on leukemic precursors by specific T-cell clones. T cell-mediated specific immunological

responses against mHAgs of the leukemia cells improve positive graft versus leukemia effect[2] making minor histocompatibility antigens interesting to explore in terms of improving patient outcomes.

The HA-1 peptide consists of 9 amino acids and is coded by the KIAA0223 gene on chromosome 19, expressed only on hematopoietic cells. Two alleles (dominant HA-1H and recessive HA-1R) differ in 2 positions of cDNA sequence resulting in one amino acid polymorphism.[5]

The HA-2 peptide consists of 9 amino acids and is coded by the MYO1G gene on chromosome 7 which is expressed on hematopoietic precursor cells, including leukemia cells. The two alleles are HA-2V and HA-2M, with HA-2V being dominant.[6]

HA-8 is also a peptide constructed of 9 amino acids, coded by the KIAA0020 gene on chromosome 9. This gene has at least two alleles (HA-8R and HA-8P), of which only HA-8R can lead to specific cytotoxic T cell recognition.[7]

For immune recognition, HA-1, HA-2[8] and HA-8[9] antigens must be presented to cytotoxic T cells by the major histocompatibility antigen HLA-A2.

The aim of this study was to examine allele, genotype and phenotype frequencies of minor histocompatibility antigens HA-1, HA-2 and HA-8 since they are restricted by the HLA-A2 antigen, the most frequent antigen in the Croatian population (26.9%)[10], making them most clinically relevant.

MATERIAL AND METHODS

Polymorphism of minor histocompatibility antigens HA-1, HA-2 and HA-8 was investigated in 102 healthy, unrelated individuals (57 female, 45 male) who originate from different regions of Croatia and form a representative sample of the Croatian population. The study was conducted in agreement with the principles of the Declaration of Helsinki as revised in 2013.

All individuals included in the study were previously typed for HLA-A, HLA-B, HLA-DR loci, and their allele frequencies were in line with allele frequencies published for the Croatian population[10], confirming that the group is a true representation of the Croatian population.

Furthermore, we examined HA-1, HA-2 and HA-8 polymorphism within a subgroup of 52 samples positive for HLA-A*02 (30 female and 22 male) and compared them with the polymorphism found in the main group. Genomic DNA was obtained by extraction from peripheral blood samples using the NucleoSpin® Blood kit (Macherey Nagel) and subsequently typed using polymerase chain reaction with sequence-specific primers (PCR-SSP) (Dynal *All*Set^{+TM} Minor Histocompatibility Antigen (mHA) kit; Invitrogen). The commercial kit used in the study is designed to detect alleles of 11 different minor histocompatibility antigens, including HA-1, HA-2 and HA-8. The typing kit consists of primer mixes which contain one or more allele- and/or group-specific primer pairs, as well as a control primer pair matching non-allelic sequences in the sample. Control primer functions as an internal control to verify the efficiency of PCR amplifications. Amplicons were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide (Figure 1).

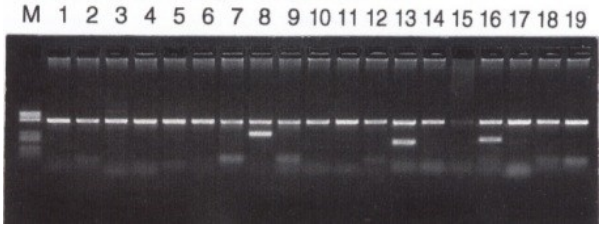

Figure 1. Example of a PCR-SSP typing result: image of a 2% agarose gel stained with ethidium bromide. Line M contains specific bands of molecular size marker. All lines (1 to 19) contain a specific control band which is the product of specific amplification of control primers. Control band in line 15 is faint, indicating poor efficiency of the PCR reaction. Lines 8, 13 and 16 show a positive reaction because they contain additional band indicating that allele-specific amplification occurred.

Allele assignment consisted of determining whether a specific band was present, i.e. whether allele-specific amplification occurred. A specific amplification in a lane indicates that the DNA sample contains the allele defined by the primer pair in that PCR reaction. Approximate sizes of expected specific PCR products were used in order to distinguish between a true positive amplification and primer oligomer artefacts (primer dimers), carryover from adjacent wells and non-specific amplification (Table 1).

Table 1. Part of the interpretation table supplied with the used commercial kit, containing the molecular size of specific amplification bands (in base pairs, b.p.) for each reaction tube. A positive band of the appropriate size in any reaction tube corresponds to the presence of a related specific allele (polymorphism).

Tube No.	Minor	b.p.	Polymorphism
1	HA-1	190	H
2		190	R
3	HA-2	271	V
4		271	M
7	HA-8	186	R
8		186	P

Legend: Tube No. - PCR reaction tube, b.p. - molecular size of the amplification band in base pairs

We determined sample genotypes based on assigned alleles and subsequently assigned a positive phenotype to samples which were heterozygous or homozygous for the dominant allele.

Fisher's exact test was applied for comparisons between the main group and the HLA-A*02 positive subgroup, as well as between the Croatian population and other studied populations, and the calculated

differences were considered statistically significant if the P-value was less than 0.05.

RESULTS

We determined the allele, genotype and phenotype frequencies for HA-1, HA-2 and HA-8 loci in the group of 102 healthy, unrelated individuals and afterwards in the subgroup of 52 HLA-A*02 positive samples from the main group (Table 2). Comparison of allele, genotype and phenotype frequencies of studied mHAgs between the main group and HLA-A*02 positive subgroup showed no statistically significant differences. The HA-1 antigen was present in the test population with allele frequency of 41.7% for HA-1H and 58.3% for HA-1R. Genotype frequencies were 17.7% for HH, 48.0% for HR and 34.3% for RR, and 65.7% of samples had an HA-1 positive phenotype. The HLA-A*02 positive subgroup showed an allele frequency of 46.2% for HA-1H and 53.8% for HA-1R. Genotype frequencies in the subgroup were 23.1% for HH, 46.1% for HR and 30.8% for RR, resulting in 69.2% of samples in the subgroup having an HA-1 positive phenotype (Figure 2).
Allele frequencies for the HA-2 locus in the main group were 76.5% for HA-2V and 23.5% for HA-2M. Genotype frequencies were 58.8% for VV, 35.3% for VM and 5.9% for MM. Among the test group, 94.1% of samples showed HA-2 positive phenotype. HLA-A*02 positive samples showed an allele frequency of 72.1% for HA-2V and 27.9% for HA-2M. Genotype frequencies were 53.9% for VV, 36.5% for VM and 9.6% for MM. Positive phenotype was present in 90.4% of the HLA-A*02 positive samples (Figure 3).
The frequency of HA-8R allele in the main group was 39.1% and it was 60.9% for HA-8P. Genotype frequencies were: 15.7% for RR, 47.0% for RP and 37.3% for PP. HA-8 positive phenotype was found in

Table 2. Allele, genotype and phenotype frequencies of HA-1, HA-2 and HA-8 loci in the main group of 102 healthy, unrelated individuals (57 female, 45 male) and in the subgroup of 52 HLA-A*02 positive individuals (30 female and 22 male)

locus	polymorphism		Healthy individuals (N=102)	HLA-A*02 positive (N=52)
			n (%)	n (%)
HA-1	allele	H	85 (41.7)	48 (46.2)
		R	119 (58.3)	56 (53.8)
	genotype	HH	18 (17.7)	12 (23.1)
		HR	49 (48.0)	24 (46.1)
		RR	35 (34.3)	16 (30.8)
	phenotype	+	67 (65.7)	36 (69.2)
		–	35 (34.3)	16 (30.8)
HA-2	allele	V	156 (76.5)	75 (72.1)
		M	48 (23.5)	29 (27.9)
	genotype	VV	60 (58.8)	28 (53.9)
		VM	36 (35.3)	19 (36.5)
		MM	6 (5.9)	5 (9.6)
	phenotype	+	96 (94.1)	47 (90.4)
		–	6 (5.9)	5 (9.6)
HA-8	allele	R	80 (39.1)	45 (43.3)
		P	124 (60.9)	59 (56.7)
	genotype	RR	16 (15.7)	9 (17.3)
		RP	48 (47.0)	27 (51.9)
		PP	38 (37.3)	16 (30.8)
	phenotype	+	64 (62.7)	36 (69.2)
		–	38 (37.3)	16 (30.8)

Legend: "+" - positive phenotype, mHAg present on cell surface,
"–" - negative phenotype, mHAg not present on cell surface

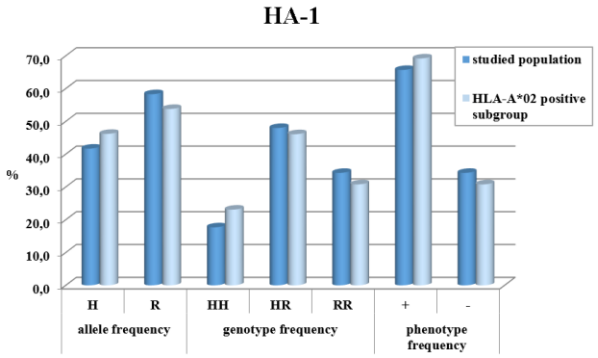

Figure 2. Allele, genotype and phenotype frequencies for the HA-1 locus in 102 healthy individuals and the subgroup of 52 HLA-A*02 positive samples

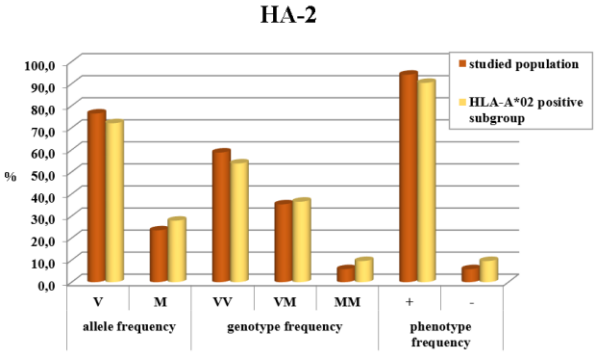

Figure 3. Allele, genotype and phenotype frequencies for the HA-2 locus in 102 healthy individuals and the subgroup of 52 HLA-A*02 positive samples

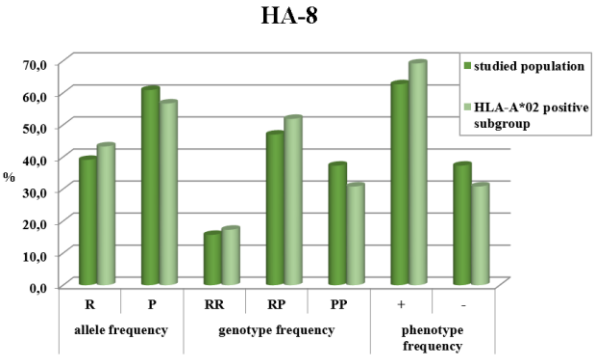

Figure 4. Allele, genotype and phenotype frequencies for the HA-8 locus in 102 healthy individuals and the subgroup of 52 HLA-A*02 positive samples

62.7% of the samples. The HLA-A*02 positive subgroup showed following allele frequencies: 43.3% for HA-8R and 56.7% for HA-8P. Genotype frequencies were 17.3% for RR, 51.9% for RP and 30.8% for PP, and 69.2% samples had HA-8 positive phenotype (Figure 4).

No statistically significant difference was found between the Croatian population and Caucasian population when comparing allele, genotype and phenotype frequencies.[3, 11]

DISCUSSION

This study was performed as part of "The Minor Histocompatibility Workshop", within the "14th International HLA and Immunogenetics Workshop 2005" which included 33 laboratories from different countries worldwide (the USA, Mexico, Brazil, Australia, South Africa, Taiwan, Japan, India, Croatia, Italy, France, Austria, the Netherlands, Belgium, Germany, England, Ireland, Poland, Finland and Turkey). The workshop objectives were to investigate minor histocompatibility antigen frequencies in healthy populations and determine the significance of mHAgs matching and mismatching in GvHD onset and severity, giving insight into the role of mHAgs in hematopoietic stem cell transplantation.[11] Participation in this workshop provided us with the opportunity to investigate mHAg frequencies in the Croatian population for the first time.

HA-1, HA-2 and HA-8 antigens need to be presented to cytotoxic T cells by major histocompatibility antigen HLA-A2[8, 9], which is the most frequent antigen in the Caucasian population. Allele frequency of HLA-A*02 in the Croatian population is 26.9%,[10] which makes HLA-A2 positive samples most informative for the investigation of mHAgs significance. Therefore, we investigated allele, genotype and phenotype frequencies of these mHAgs in a subgroup of 52 HLA-A*02 positive samples and compared the results with frequencies found in the main population group. No statistically significant difference in allele, genotype or phenotype frequency was found between the subgroup and the main group for any of the three studied mHAgs, as shown in Figure 2, Figure 3 and Figure 4.

Allele, genotype and phenotype frequencies for HA-1, HA-2 and HA-8 found in the test group do not deviate significantly from frequencies reported for the Caucasian population within the workshop results[11] (Table 3).

We compared our results with scarcely available publications from similar population studies. We focused on HA-1, HA-2 and HA-8 since they are most investigated and most represented in literature. Our population showed a genotype frequency of 17.7% for the HA-1 HH genotype which correlates with data shown for Spanish (10.4%)[12], Tunisian (19%)[13] and Korean (12.9%)[14] populations but is significantly different from the genotype frequency of 8.9 % reported by Pietz and al. in their study on 259 healthy Caucasian blood donors[3] (P=0.026). Allele frequencies in our group were 41.7% for HA-1H and 58.3% for HA-1R, and they do not deviate significantly from findings reported for German (39% for HA-1H, 61% for HA-1R)[15], Dutch (44.3% for HA-1H, 56.7% for HA-1R)[16], Italian (29.3% for HA-1H, 70.7% for HA-1R)[17], Greek (46.5% for HA-1H, 53.5% for HA-1R)[18] and Spanish (32.8% for HA-1H, 67.2% for HA-1R)[12] populations.

Table 3. Comparison of allele and genotype frequencies of HA-1, HA-2 and HA-8 loci in 102 healthy, unrelated individuals in our study and 2011 Caucasian individuals included in the "14th International HLA and Immunogenetics Workshop 2005"[11] did not show statistically significant difference when using Fisher's exact test

locus	polymorphism		Healthy individuals N=102	Caucasian individuals N=2011[11]
			%	%
HA-1	allele	H	41.7	35.9
		R	58.3	64.1
	genotype	HH	17.7	13.0
		HR	48.0	45.8
		RR	34.3	41.2
HA-2	allele	V	76.5	75.6
		M	23.5	24.4
	genotype	VV	58.8	56.8
		VM	35.3	37.7
		MM	5.9	5.5
HA-8	allele	R	39.1	45.0
		P	60.9	55.0
	genotype	RR	15.7	19.7
		RP	47.0	50.4
		PP	37.3	29.8

HA-2 genotype frequencies in our group (Table 2) correlate with reports for the Caucasian population[3, 11], but the HA-2 VV genotype, which occurs with the frequency of 58.8% in our population, is significantly lower than the frequencies found in Tunisian[13] (71%, P=0.04) and Korean populations[14] (86.3%, P=0.0000014).

We compared allele, genotype and phenotype frequencies of HA-8 with the few available studies for other populations and found no significant difference. Our data correlates with genotype frequencies found in 139 healthy Korean individuals[14] and does not deviate from the frequencies present in the Caucasian group of the "The Minor Histocompatibility Workshop"[11] or from data provided by Pietz and al[3].

CONCLUSION

Our study on 102 healthy, unrelated individuals is the first study on distribution and polymorphism of allele, genotype and phenotype frequencies of minor histocompatibility antigens HA-1, HA-2 and HA-8 in the Croatian population.

No statistically significant deviations were found between allele, genotype and phenotype frequencies of studied minor histocompatibility antigens between our main study group and the HLA-A*02 positive subgroup, nor between our Croatian study group and Caucasian population. We reported some significant differences in allele and genotype frequencies compared to other populations. These differences could be geographically correlated or more randomly distributed.

It is well known that a minor histocompatibility antigen is clinically relevant only if it is immunogenic and when it is moderately frequent in the population. Therefore, this study was necessary to provide information on distribution of mHAgs in Croatian population, which allows further investigations on mHAgs' role in graft versus host disease and graft versus leukemia effect following hematopoietic stem cell transplantation in HLA identical donor-recipient pairs with mHAg disparities.

It would be interesting to explore if mHAg typing could be used as an additional criterion in HSCT donor selection.

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