Identification of Epitopes and Immunodominant Regions on the MICA Protein Defined by Alloantibodies From Kidney Transplant Patients

Beatriz Suárez-Álvarez,[1] Rebeca Alonso-Arias,[1] Cristina Bravo-Mendoza,[1] Antonio López-Vázquez,[1] Teresa Ortega,[2] Jose Maria Baltar,[3] Eliecer Coto,[4,5] Francisco Ortega,[3,5] and Carlos López-Larrea[1,5,6]

Background. Several reports showed a contribution of anti-MICA (major histocompatibility complex class I chain-related molecule A) antibodies (Abs) to the development of acute and chronic rejection. Identification of the epitopes to which the Abs bind may help to determine immunoreactive regions essential for the major histocompatibility complex compatibility between donor and recipients, leading to the best outcome of the transplant.

Methods. Sera from 284 kidney transplant patients were screened for anti-MICA Abs by Luminex assay. MICA allele typing of the recipients was determined. The epitopes of MICA were mapped by screening a synthesized library of overlapping peptides from the extracellular domains of the protein against the sera from kidney transplant patients with anti-MICA Abs.

Results. Antibodies against MICA were detected in 50 of 284 patients (17.6%) and correlated with the development of acute rejection. Nine antigenic regions were immunoreactive with anti-MICA Abs in the sera samples. Four of these continuous epitopes mapped to polymorphic amino acids (aa). Five antigenic regions were shared epitopes found in all the MICA alleles. The polymorphic residues, 173 (E/K), 175 (S/G), and 181 (R/T), had determined allele-specific epitopes (reactivity patterns 1 and 2). In contrast, the aa 208Y and 213T were implicated in the cross-reactivity among alleles.

Conclusions. The presence of anti-MICA Abs could be an important marker for diagnosis because of their contribution to the outcome of the graft, regardless of presence of anti-HLA Abs. Additionally, the identification of epitopes revealed the in vivo antigens of the transplant and is spurring the development of new matching strategies to reduce the incidence of acute and chronic rejection.

Keywords: Kidney transplantation, Epitope mapping, HLA, MICA, Acute and chronic rejection.

(*Transplantation* 2009;88: S68–S77)

The incidence of antibody-mediated rejection is increased in patients with a history of sensitization to human leukocyte antigens (HLAs). Antibodies (Abs) reactive against molecules expressed on endothelial cells have also been associated with graft loss. The major histocompatibility complex class I-related chain A (MICA) molecule is a cell surface glycoprotein, which shows homology with classical HLA-class I, but plays no role in antigen presentation. MICA is a polymorphic protein with more than 64 recognizable alleles (http://hla.alleles.org/classo.html) and is mainly expressed on endothelial cells, epithelial cells, fibroblasts, and on activated monocytes (*1*). MICA expression is induced by stress and is upregulated in infection and tumor transformation (*2*, *3*). This molecule is a ligand for natural killer (NK) and $CD8^+$ T cells, which express NKG2D, a common activating NK-cell receptor (*4*). The NKG2D receptor acts as an activating immunoreceptor in NK cells and as a co-stimulatory signal in $CD8^+$ T cells, which complements T-cell receptor-mediated antigen recognition on target cells (*5*).

Several reports showed that the MICA molecule contributes to the pathogenesis of acute and chronic allograft rejection due to its expression on endothelial cells and its capacity to induce Abs capable of complement-dependent cytotoxicity (*6–8*). In fact, renal and pancreatic grafts with evidence of both acute and chronic rejection have a remarkably high MICA protein expression (*9*), and anti-MICA Abs have been identified in the serum of these patients (*10*, *11*). Some studies showed that preformed anti-MICA Abs can provoke acute humoral rejection immediately after transplantation in the absence of anti-HLA donor-specific Abs (*12*, *13*). Anti-MICA Abs can develop after kidney transplantation. The presence of both anti-HLA and anti-MICA Abs in transplanted recipients was correlated with greater deterioration of renal function than those in transplanted recipients with exclusively anti-HLA or anti-MICA Abs (*14*, *15*). We have also demonstrated that anti-MICA Abs are directly correlated with an increased risk of acute rejection in heart transplantation (*16*). These results suggest that anti-MICA Abs might contribute to the outcome of the graft in organ transplantation. Whether MICA antigens play an essential

This work was supported by the Red de Investigación Renal (REDinREN) grant, FIS PI080566 from Instituto "Carlos III" grant, FICYT PC-06/010 grant, and "Fundación Mútua Madrileña 2007–2009" grant.

[1] Department of Immunology, Hospital Universitario Central de Asturias, Oviedo, Spain.
[2] Healthy Outcomes Research Unit, Department of Nephrology, Hospital Universitario Central de Asturias, Oviedo, Spain.
[3] Department of Nephrology, Hospital Universitario Central de Asturias, Oviedo, Spain.
[4] Department of Molecular Genetics, Hospital Universitario Central de Asturias, Oviedo, Spain.
[5] Fundación Renal "Íñigo Álvarez de Toledo," Madrid, Spain.
[6] Address correspondence to: Carlos López-Larrea, Ph.D., Department of Immunology, Hospital Universitario Central de Asturias, 33006 Oviedo, Spain.
E-mail: inmuno@hca.es

ISSN 0041-1337/09/8803S-68
DOI: 10.1097/TP.0b013e3181afeb7a

role in the rejection of organ allograft and full compatibility would improve graft acceptance cannot yet be definitively established.

To examine this issue, it is necessary to identify and characterize the antigenic regions or epitopes that are capable of immunoreacting with the specific Abs induced in the serum of transplant patients. Recent studies based on the analysis of polymorphic residues and patterns of reactivity of sera have deduced important amino acids (aa) involved in the binding with Abs (*17–19*). The aim of this study was to identify the immunodominant sites on the MICA protein by PepSets Peptide Libraries using chemically synthesized peptides spanning the primary structure of MICA protein (*20*). We analyzed the presence of anti-MICA Abs in the sera of 284 kidney transplant patients by Luminex assay and determined their allelic specificity. The presence of anti-MICA Abs was detected in 17.6% of transplant patients and was associated with the development of acute rejection. Nine antigenic regions on the MICA protein were recognized by sera from kidney transplant patients. These sites contain key aa capable of reacting with Abs and could explain the cross-reactivity among alleles during the development of the transplant.

PATIENTS AND METHODS

Patients and Sera

Retrospectively, we examined the sera posttransplant of 284 patients who had received a kidney transplant graft from deceased donors between 1992 and 2003 at University Hospital of Asturias (Spain), and all patients gave written informed consent. One serum sample for each patient was taken in December 2005 and tested for anti-MICA and anti-HLA Abs. Aliquots of sera were stored at −20°C. Clinical characteristics of the patients are summarized in the Table 1. Because we are interested in the contribution of MICA Abs to the chronic rejection, we followed up the patients up to 3 years after the detection of anti-MICA Ab.

Luminex Assay

HLA and MICA Abs were determined using LABScreen assay by Luminex technology, according to the manufacturer's specifications (One Lambda, Inc., Canoga Park, CA). The fluorescent signal for each MICA-allele coated bead was measured using LABScan 100 flow cytometry and analyzed by HLA-Visual software (One lambda, Inc.). Serum samples of kidney transplant patients were tested against MICA alleles *001, *002, *004, *007, *009, *012, *017, *018, *019, and *027. Because the MICA alleles *027 and *008 share the same aa sequence in the extracellular domains, we denoted this sequence as *008.

HLA and MICA Typing

DNA samples were obtained from recipients and stored at −20°C until use. MICA gene polymorphisms were typed by polymerase chain reaction-sequence-specific primer as previously described (*13*) and confirmed by LABType SSO MICA (One lambda Inc.), based on the manufacturer's instructions.

Epitope Mapping

To identify linear B-cell epitopes of MICA, we screened a synthetic peptide library. A set of biotinylated

TABLE 1. Clinical characteristics of the kidney transplant patients

	MICA-positive patients n = 30 (%)	MICA-negative patients n = 131 (%)
Recipient age (yr)	62.8±10	59.2±12.4
Donor age (yr)	46.9±15.6	42.9±17.8
Recipient sex (male:female)	18:12	81:50
Donor sex (male/female)	21:9	92:39
Cold-ischemia time (hr)	18±4.8	16.7±4.55
Transplant number		
First transplant	23/28 (82.2)	109/131 (83.2)
Subsequent transplants	5/28 (17.9)	18/131 (13.7)
Posttransplant years		
<5 yr	11/27 (40.7)	60/131 (45.8)
5–10 yr	9/27 (33.3)	44/131 (33.6)
>10 yr	7/27 (25.9)	27/131 (20.6)
HLA-A+B+DR mismatches		
0–3	5/27 (18.5)	25/121 (20.6)
4–6	22/27 (81.5)	96/121 (79.3)
Acute rejection		
Yes	8/27 (29.6)*	13/98 (13.3)
No	19/27 (70.4)	85/98 (86.7)
Baseline diagnosis		
Vascular nephropathy	7/27 (25.9)	12/111 (10.8)
Diabetes mellitus	3/27 (11.1)	11/111 (9.9)
Glomerulonephritis/ vasculitis	7/27 (25.9)	31/111 (27.9)
Intersticial nephropathy	1/27 (3.7)	13/111 (11.7)
Policystis kidney disease	6/27 (22.2)	22/111 (19.8)
Others	3/27 (11.1)	22/111 (19.8)
Immunosupression		
Cyclosporine+MMF	5/17 (29.4)	45/91 (49.5)
Cyclosporine+Aza	5/17 (29.4)	18/91 (19.8)
Tacrolimus+MMF	7/17 (41.2)	26/91 (28.6)
Tacrolimus+Aza	0	2/91 (2.2)
Corticosteroid use		
Corticosteroids	17/26 (65.4)	78/131 (59.5)
No corticosteroids	9/26 (34.6)	53/131 (40.5)
Creatinine levels (mg/dL)		
<1.5	15/29 (51.7)	80/123 (65)
1.5–2	9/29 (31)	32/123 (26)
>2	5/29 (17.3)[a]	11/123 (8.9)

Percentages were calculated for the number of patients whose clinical data were available.

*$P<0.05$.

[a] When we studied the combined effect of anti-MICA and anti-HLA antibodies, 23% of the patients have creatinine serum levels more than 2 compared with the negative patients, $P=0.05$.

MMF, mycophenolate mophetil; Aza, azathioprine; MICA, major histocompatibility complex class I chain-related molecule A; HLA, human leukocyte antigen.

cleaved peptides spanning the extracellular domains $\alpha 1$–$\alpha 3$ (aa 1–274) of the MICA protein were synthesized by Mimotopes Ltd. (Victoria, Australia). The peptides were 12 aa in length and had a sequential overlap of seven aa. The final PepSets Peptide Library consisted of a total of 58 peptides plus two control peptides. A second set of biotinylated peptides were synthesized spanning polymorphic aa residues of several MICA alleles. The peptides were dissolved in 200 μL of an 80% (vol/vol) dimethyl sulfoxide to a 20% (vol/vol) water mixture and stored at −20°C. For maximum sensitivity, a four-residue spacer sequence (seryl-glycyl-seryl-glycyl) between the biotin and the peptide was inserted, and the format of each peptide was biotin-SGSG-peptide-amide.

ELISA

The reactivity of serum samples containing anti-MICA Abs with the peptide library was detected by enzyme immunoassay. Briefly, 96 well plates (Nunc, Sigma-Aldrich, St. Louis, MI) were coated with streptoavidin (5 μg/mL) at 37°C overnight and blocked with 1% bovine serum albumin (BSA; 100 μL/well) for 1 hr at room temperature. The biotinylated peptides (10 μg/mL) diluted with phosphate-buffered saline (PBS) and 1% BSA, pH 7.5, were added, respectively, to each well and incubated at room temperature for 4 to 5 hr. The microplate was incubated with human sera (dilution 1/50 or 1/100) overnight at 4°C and bound Abs were detected with peroxidase-conjugated goat anti-human IgG at 37°C for 1 hr. PBS washing were performed before each addition. Dilutions of sera and conjugates were prepared in PBS-T containing 1% BSA. The reaction was visualized by addition of the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich) and read at 450 nm. The readings obtained for peptides incubated with secondary conjugates were then deducted from the corresponding readings obtained with the serum samples. Samples with OD values twice the negative control were judged as a positive reaction. Sera from 20 MICA-negative healthy blood donors were used as negative control.

Structural Analysis of Antigenic Regions

The secondary structure, solvent accessibility, and physical-chemical properties were analyzed using the amino acid sequence with the biocomputing software program POLYVIEW-2D (*21*). The crystalline structure of MICA (*22*), code 1B3J, was obtained from the RCSB Protein Data Bank Database (http://www.pdb.org). Structural analysis was performed with Swiss-Pdbviewer and WeLab Viewer Lite software.

Statistical Analysis

Descriptive data are presented as the mean±standard deviation. The statistical significance of mean differences was determined by Student's *t* test. Significance between frequencies was determined by Fisher's exact test or chi-square analysis. *P* value less than 0.05 was considered significant.

RESULTS

Detection of Anti-MICA Abs in Kidney Transplant Patients

Antibodies against MICA alleles were detected in 50 of 284 (17.6%) kidney transplant patients and 10.6% (n=30) of them had only anti-MICA Abs (without HLA class I or II Abs). To study the effect of MICA Abs on graft survival, we excluded the patients with both classes of Abs (Table 1). In this study, 29.6% of recipients who developed acute rejection had anti-MICA Abs compared with the 13.3% without Abs ($P=0.047$). Thus, the presence of MICA Abs post-transplant was significantly associated with kidney allograft acute rejection.

Monitoring the renal graft function of these patients for 3 years after the detection of MICA Abs revealed that 17.3% had serum creatinine levels greater than 2 compared with 8.9% of the patients with no detectable MICA- or HLA-specific Abs. Although this difference had not reached a significant level, 23% of the patients with Abs of both specificities (MICA+HLA) had serum creatinine levels more than 2 versus 8.9% of the patients without Abs ($P=0.05$; data not shown). These data suggest that the correlation between the presence of anti-MICA Abs and reduced kidney allograft survival was influenced by the simultaneous presence of MICA and HLA Abs. There were no statistically significant differences in the frequency of MICA Abs in relation to the other parameters analyzed.

Specific Reactivity of Anti-MICA Abs

Table 2 depicts a summary of MICA allele-specific Abs and the receptor MICA typing in the transplant patients with MICA Abs. Comparing the reactivity patterns, we observed that 37 of 50 patients (74%) recognized three or more alleles of MICA, suggesting cross-reactive among alleles. The most frequent specificity of the MICA Abs were against MICA alleles *018 (76%) followed by *019 (70%), *001 and *007 (68%), *002 (62%), *012 (58%), *017 (22%), and *008 (16%). Antibodies against the MICA alleles *004 and *009 were not detected in these samples. It is interesting to remark that sera from six patients recognized only the MICA allele *019.

To determine the allospecificity of the immune response in these patients, we performed sequence-based typing of the MICA alleles in 41 of 50 recipients with MICA Abs. Analysis of the reactivity pattern of each serum combined with MICA typing showed that nine (21.95%) of the patients had Abs against their own alleles. All sera were polyspecific, so the development of increased specificities over time and the cross-reactivity among alleles could have led to induction of these autoantibodies. The polyspecific sera reacted predominantly against a group of alleles defined by MICA alleles *001, *002, *007, *012, *017, *018, and *019. Further analysis of the amino acid alignment in the extracellular domain of MICA indicated that these alleles (except MICA allele *019) were similar and suggested the possibility that MICA alloantibodies induced to mismatched MICA alleles may react against other alleles with shared epitopes. This group of alleles was renamed as "reactivity pattern 1". Studies based on MICA typing showed that these alleles were poorly represented in the patients, except the MICA allele *002 which had a frequency of 8.5% in these patients. MICA alleles *004, *008, and *009, were named "reactivity pattern 2" and were the most prevalent alleles in these patients, occurring in 62% of the patients. Only Abs against MICA *008 were detected.

TABLE 2. Relationship between MICA allele-specific antibodies detected by Luminex assay and MICA allele typing from recipients in 50 kidney transplant patients

Patient number	MICA allele-specific antibodies									MICA allele typing	
	*001	*002	*004	*007	*008	*012	*017	*018	*019		
1										008	008
2										**002**	**018**
3										001	016
4										008	008
5										**001**	004
6										–	–
7										008	027
8										008	009
9										**008**	016
10										**008**	011
11										004	009
12										–	–
13										009	032
14										004	004
15										–	–
16										004	005
17										008	009
18										–	–
19										004	008
20										002	017
21										008	008
22										–	–
23										008	021
24										**002**	**002**
25										004	008
26										016	017
27										015	016
28										009	016
29										004	009
30										008	017
31										002	008
32										004	008
33										027	027
34										–	–
35										009	011
36										006	008
37										–	–
38										**001**	008
39										004	008
40										016	027
41										001	008
42										009	038
43										–	–
44										**008**	**008**
45										**008**	027
46										–	–
47										008	008
48										**009**	015
49										**002**	004
50										002	011

Positive anti-MICA antibodies against several alleles were assayed using Luminex technology. Antibody specificities are shown by shaded boxes (black: score 8 and gray: score 6). Right columns show the MICA allele typing of the recipients by LABType SSO MICA. In bold are the patients who developed anti-MICA antibodies against own alleles. (–): nontyped receptor.

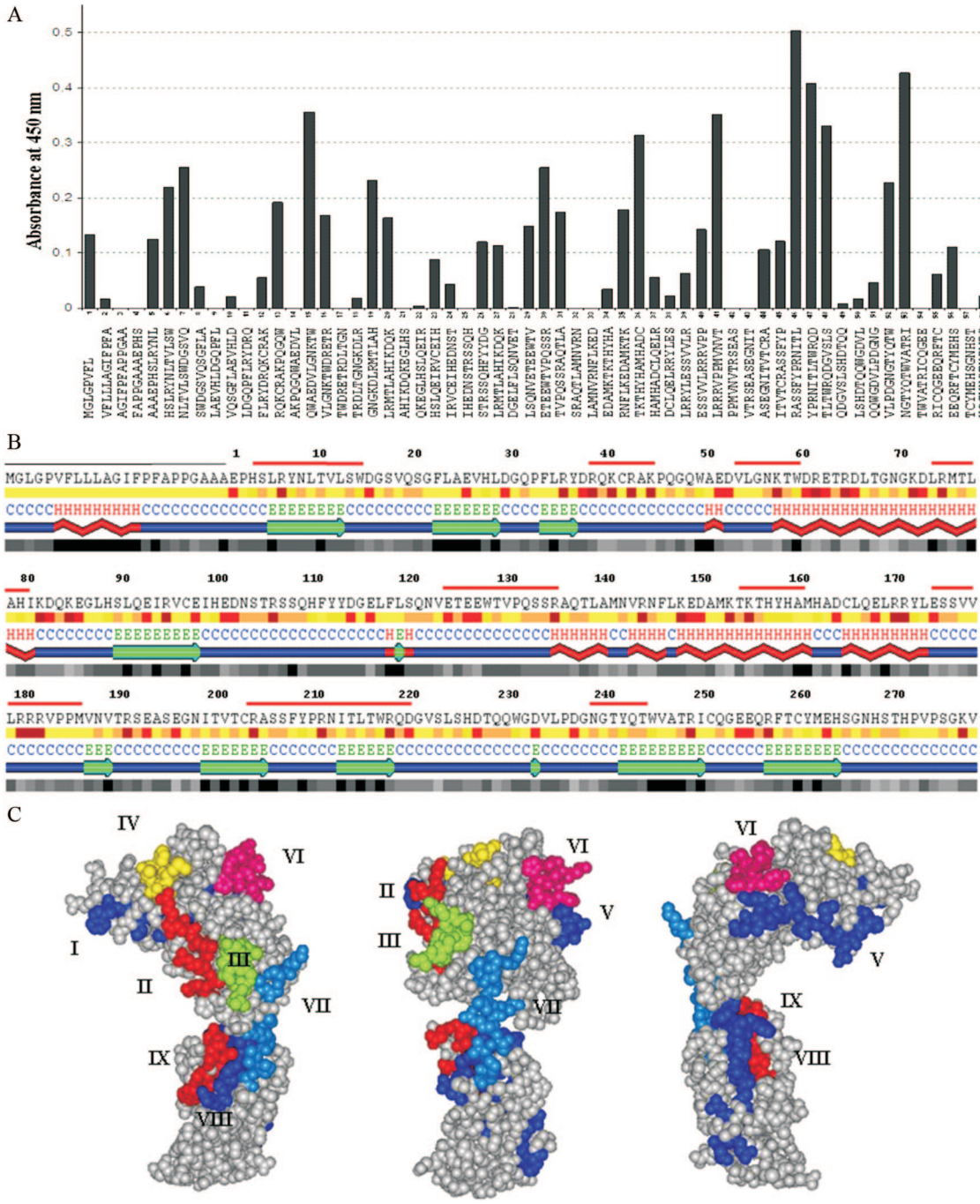

FIGURE 1. Epitope mapping of anti-MICA Abs and structural analysis. (A) Mapping of the immunodominant regions on the major histocompatibility complex class I chain-related molecule A (MICA) protein by PepSets analysis with biotinylated overlapping peptides libraries. A set of 58 biotinylated peptides that cover the extracellular domains of the MICA protein were synthesized. The peptides were 12 aa in length and have a sequential overlap of seven aa. Sera (dilution 1/50 and 1/100) from 50 kidney transplant patients and 20 healthy blood donors were tested by ELISA. Sera were considered positive when OD values were twice the negative control (healthy donors). (B) Analysis of antigenic regions of MICA protein by computer algorithms was performed using the POLYVIER-2D software. Secondary structure is identified by α-helix (*zigzag red lines*), β-sheet (*green arrow*), and coils (*blue line*). Solvent accessibility is represented by gray scale from hidden aa (*black square*) to surface-exposed aa (*white square*). Physical-chemical properties of the aa are identified by the scale ; the colors from *yellow* to *dark red* represent hydrophobic, amphipathic, polar, negative-charged, and positive-charged aa, respectively. (C) 3D-Structural model of the nine epitopes is determined by the screening of the peptide library. Regions I to IX are identified by different colors. This model is based on the crystalline structure of MICA allele *001 (PDB code 1B3J) and created by WebLab Viewer Lite software.

TABLE 3. Antigenic regions of MICA detected by anti-MICA antibodies from kidney transplant patients

Region	Peptide number	Peptide	Immunoreactivity (OD-450 nm)	Minimun sequence of epitope	Aminoacidic position	Allelic variability
I	5	AAAEPHSLRYNL	0.124	HSLRYNLTVLSW	3–14	Variable
	6	HSLRYNLTVLSW	0.219			
	7	NLTVLSWDGSVQ	0.256			
II	12	FLRYDRQKCRAK	0.05	RQKCRAK	38–44	Constant
	13	RQKCRAKPQGQW	0.193			
III	15	QWAEDVLGNKTW	0.356	VLGNKTW	53–59	Constant
	16	VLGNKTWDRETR	0.168			
IV	19	GNGKDLRMTLAH	0.232	LRMTLAH	73–79	Constant
	20	LRMTLAHIKDQK	0.164			
V	29	LSQNVETEEWTV	0.149	ETEEWTVPQSSR	123–134	Variable
	30	ETEEWTVPQSSR	0.256			
	31	TVPQSSRAQTLA	0.174			
VI	35	EDAMKTKTHYHA	0.179	TKTHYHA	153–159	Constant
	36	TKTHYHAMHADC	0.314			
VII	39	LRRYLESSVVLR	0.063	ESSVVLRRRVPP	173–184	Variable
	40	ESSVVLRRRVPP	0.143			
	41	LRRRVPPMVNVT	0.351			
VIII	45	ITVTCRASSFYP	0.122	RASSFYPRNITLTWRQD	203–219	Variable
	46	RASSFYPRNITL	0.503			
	47	YPRNITLTWRQD	0.407			
	48	TLTWRQDGVSLS	0.331			
IX	52	VLPDGNGTYQTW	0.229	NGTYQTW	238–244	Constant
	53	NGTYQTWVATRI	0.427			

The underlined amino acids indicate polymorphic residues among alleles. The minimum sequence of epitope was determined by overlapping of the positive peptides. Right column shows the presence of variable or polymorphic amino acids among the MICA alleles.

Epitope Mapping of MICA Protein

To determine the antigenic sites on the MICA molecule, we used a set of 58 overlapping peptides spanning the extracellular domains (α1–α3) of the protein. Each of these peptides contained 12 aa with seven residues overlapping with the adjacent peptides. A serum pool from 50 transplant patients with anti-MICA Abs was assayed by ELISA to characterize the immunogenicity of MICA protein. PepSets analysis of these peptides revealed that the MICA protein contained nine immunodominant regions recognized by the Abs in the sera samples (Fig. 1A), which were designated as regions I to IX. As negative controls, sera from 20 healthy donors showed no significant binding to the peptides.

Table 3 summarized the nine antigenic regions identified in the MICA molecule. Four of these regions (I, V, VII, and VIII) mapped to variable sites in the molecule with polymorphic aa among the different alleles of MICA. Five epitopes were located in constant regions and present in all MICA alleles. Screening of the peptide library and analysis of the amino acid sequences of MICA alleles had led to the definition of three main sites of high amino acid variability, regions V, VII, and VIII. First site (region V) included the aa 122 (V/L), 125 (K/E), and 129 (M/V), whereas the residues 173 (K/E), 175 (G/S), and 181 (T/R) defined the second polymorphic site (region VII). Both regions V and VII were localized in domain α2. The third area (region VIII) was composed of the aa 206 (G/S), 210 (W/R), 213 (T/I), and 215 (S/T). These polymorphic aa permit discrimination of the MICA alleles into two groups (1 and 2), corresponding to the aforementioned reactivity patterns.

Furthermore, analysis based on the secondary structure and three-dimensional (3D) conformation of the MICA protein exposed the accessibility of these regions on the molecule surface (Fig. 1B and C). Region I has only one variable amino acid in position 14 (W/G). This region (3) HSLRYNLTVLSW (14) is located in a β-strand and the polymorphic amino acid formed the beginning of a coil loop as highly exposed. Region V, which was established by the peptides 29 to 31, mapped between the aa (123) ETEWTVPQSSR (134), and formed a highly exposed loop. The polymorphic aa 125 and 129 are exposed on the surface, whereas the amino acid 122 is situated inside the molecule. The binding between the domains α1–α2 and α3 is formed by the immunogenic Region VII extending from (173) ESSVVLRRRVPP (184). This Region VII epitope included three variable residues with only the amino acid 173 shown on the surface. The peptides with the strongest reactivity, 40 and 41, identified a sequence containing three consecutive arginines (RRR). The RRR motif defined a positively charged region that is highly antigenic. The peptides 45 to 48 mapped to the third variability site and defined Region VIII (203) RASSFYPRNITLTWRQD (219). The polymorphic aa 206, 213, and 215 were buried inside the MICA molecule and, therefore, were unlikely to be recognized by Abs. In contrast, the aa 210 is a highly surface-exposed residue. Region VIII induced the strongest immunoreactivity by MICA-specific Abs present in transplant patients.

TABLE 4. Reactivity of polymorphic amino acids with anti-MICA antibodies from kidney transplant patients

Region	Peptide number	Peptide	Immunoreactivity (OD-450 nm)	Polymorphic aminoacid	MICA allele
V	29	LSQNVETEEWTV	0.149	V(122)T(124)E(125)V(129)	*004,*009 *008,*019: **L(122)**
	30	ETEEWTVPQSSR	0.256		
	31	TVPQSSRAQTLA	0.174		
	65	LSQNLETKEWTM	0.147	L(122)T(124)K(125)M(129)	*001
	67	ETKEWTMPQSSR	0.31		
	69	TMPQSSRAQTLA	0.146		
	66	LSQNLETEEWTM	0.17	L(122)T(124)E(125)M(129)	*002,*007,*012, *017,*018
	68	ETEEWTMPQSSR	0.4		
	69	TMPQSSRAQTLA	0.146		
VII	38	DCLQELRRYLES	0.022	E(173)S(175)V(176)R(181)	*004 *009: **T(181)**, *019: **T(181)**
	39	LRRYLESSVVLR	0.063		
	40	ESSVVLRRRVPP	0.143		
	41	LRRRVPPMVNVT	0.351		
	70	DCLQELRRYLKS	0.519	K(173)G(175)V(176)T(181)	*001,*002,*007,*012, *017,*018 *008: **E (173),**
	71	LRRYLKSGVVLR	0.312		
	72	KSGVVLRRTVPP	0.176		
	73	LRRTVPPMVNVT	0.034		
VIII	46	RASSFYPRNITL	0.503	S(206)Y(208)R(210)T(213)T(215)	*004,*019 *008,*009: **I(213)**
	47	YPRNITLTWRQD	0.407		
	48	TLTWRQDGVSLS	0.331		
	74	RASGFYPWNITL	0.38	G(206)Y(208)W(210)T(213)S(215)	*001,*002,*007,*012, *017,*018
	75	YPWNITLSWRQD	0.313		
	76	TLSWRQDGVSLS	0.101		

The underlined amino acids indicate polymorphic residues among alleles. The shaded residues show polymorphic amino acids essential in the formation of allele-specific epitopes (reactivity patterns #1 and #2) or in the cross-reactivity among MICA alleles. Right column shows the group of alleles representative of the variable amino acid sequence. Variations in residues inside the same group of alleles are marked in bold.

The other five antigenic regions mapped to conserved aa are present in all alleles of MICA. Region II (38) RQKCRAK (44) is composed of four positively charged aa and formed a very highly exposed coil loop, indicating a potentially highly antigenic area. Analyzing the 3D structure, region II was found near region III (53) VLGNKTW (59), and composed of 53V and 57K residues that appeared highly exposed to the solvent. Two constant regions were identified in the upper area of the MICA molecule, region IV (73) LRMTLAH (79) and region VI (153) TKTHYHA (159). Both epitopes contained aa situated on the molecular surface in an α-helical structure. Finally, the peptides 52 and 53 defined region IX that overlapped the sequence (238) NGTYQTW(244) and formed a loop near to the polymorphic region VIII.

Briefly, these constant sites were shorter and had less surface exposure than the aforementioned variable immunogenic regions. Generally, the polymorphic regions consisted of coils mainly found in highly accessible loops. From a structural viewpoint, these findings further support the evidence of antigenic regions being highly surface-exposed sites and loop regions, which have more flexibility and access to the Abs.

Identification of Specific Epitopes of MICA Alleles

To determine whether anti-MICA Abs detected in the sera of transplant patients recognized a common epitope present in all MICA alleles or allele-specific regions, overlapping peptides spanning all the polymorphic amino acid combinations present in regions V, VII, and VIII were synthesized. We tested their reactivity to a pool of sera (n=20) from patients with anti-MICA Abs of reactivity pattern 1, which was the most frequent reactivity pattern in the analyzed population. We did not test serum with Abs of reactivity pattern 2 because of its absence in these patients.

The reactivity of the sera to these peptides was compared to determine the effects of polymorphic aa on the specificity or the cross-reactivity among alleles (Table 4). Three different polymorphic sequences defined by the overlapping peptide numbers 29 to 31, 65 to 69, and 66 to 69 were recognized with a similar reactivity by the sera sample. This finding suggested that the combination of the variable aa 122 (V/L), 125 (E/K), and 129 (V/M) in the immunogenic region V was not discriminated by the Abs induced in transplant patients. Within region VII, two possible groups of alleles can be grouped by their sequence. Although both sequences were reactive with this serum, the alleles with 173K and 175G were recognized with higher reactivity than the alleles with 173E and 175S. These two residues could be essential for differentiation between reactivity patterns 1 and 2. By contrast, the amino acid 181R in peptide 41 was more reactive (OD=0.351) than residue 181T in the peptide 73 (OD=0.034), creating a highly exposed area formed by three basic aa (RRR). These samples reacted similarly with the two allelic combinations within region VIII. Comparing the sequences, peptides 46, 47, 74, and 75 had the highest reactivity which showed that 208Y and 213T could be immunodominant aa and contribute to the cross-reactivity among alleles.

In summary, we observed that the residues 173 (E/K), 175 (S/G), 181 (R/T) contributed to the conformation of spe-

cific epitopes of each reactivity pattern, whereas the residues 208Y and 213T were implicated in the cross-reactivity among alleles (groups 1 and 2).

Analysis of Immunodominant Regions of the MICA Protein in Kidney Transplant Patients

Based on the above results, these nine antigenic regions were assayed independently against a bank of 30 sera from patients with anti-MICA Abs (Fig. 2A). All sera yielded similar antibody-binding profiles against the peptides and confirmed the presence of nine immunoreactive sites within the MICA sequence tested. The antigenic regions I, II, VII, and VIII reacted with more than 75% of the sera, suggesting that these sites could be immunodominant regions in the MICA protein.

To confirm the specificity of binding of the Abs with the epitopes on the MICA protein, the peptides that cover each of the antigenic regions were mixed and tested for their reactivity with a pool of sera from 10 transplanted patients with MICA Abs. As control, we used 10 sera from transplanted patients without Abs and 10 sera from transplanted patients with anti-HLA class I and II Abs. As shown in Figure 2(B), all the regions were highly reactive with the pool from patients with MICA Abs. In contrast, control pools from transplant patients without MICA Abs had only weak reactivity with all antigenic sites except for region II. The sequence mapped by the peptides 12 and 13 determined a highly antigenic epitope composed of positively charged aa that may exhibit cross-reactivity with other proteins because of the elevated reactivity with Abs in the serum of MICA-negative transplant patients.

Antibodies against the MICA allele *019 were found in 70% of the positive-MICA patients. Six of these patients had Abs only against this allele. Because this allele was poorly represented in the white population, we studied the specificity of its reactivity with the antigenic regions described. A mixture of six serum samples positive for this allele was tested against the nine antigenic regions previously determined, and eight of the nine regions were reactive. Moreover, when these sera were analyzed by flow cytometry against several MICA alleles transfected in a human B-lymphoblastoid cell line (*23*), no reactivity was observed (data not show).

DISCUSSION

Numerous groups have demonstrated the presence of MICA Abs posttransplant and its contribution to a shorter graft survival. In this study, the exclusive presence of anti-MICA Abs was significantly associated with the frequency of acute rejection independently of the presence of anti-HLA Abs. Furthermore, the combined effect of both Abs (MICA+HLA) correlated with worse graft survival than the group with no anti-MICA or HLA Abs. This confirmed the observation made by previous studies (*14*, *24*), which showed the impact of anti-MICA Abs, regardless of anti-HLA Abs, on the frequency of acute rejection and graft survival. Thus, anti-MICA Abs could be important markers for diagnosis because of their involvement in the outcome of the allograft.

It was difficult to confirm whether the anti-MICA alloantibodies were donor specific because of few typing studies of MICA alleles that have been made previously. Our results demonstrated that although MICA Abs could be induced against donor alleles, other allele-specific Abs were also present such as Abs which recognizes receptor alleles. Our study demonstrated that these Abs were polyspecific in most of the patients. In accordance to Zou et al. (*17*), we described two groups of reactivity with MICA. One group that recognizes the MICA alleles *001, *002, *007, *012, *017, and *018 (group 1), which comprised the more frequently developed MICA Abs. A second group composed of MICA alleles *004, *008, and *009 (group 2), whose Abs were poorly represented or absent in the patients. Although it had been suggested that polymorphic aa could be responsible of these two patterns of reactivity, we demonstrated that the most common pattern 1 recognized the polymorphic aa present in linear polymorphic sequences of the group 2, corroborating that cross-reactivity among alleles can occur. The differences that exist in the immunoreactivity indicate that several polymorphic aa contribute to an immunodominant epitope(s) and were essential in forming a discontinuous epitope.

Surprisingly, Abs against the MICA allele *019 were present in a high number of patients, and some of these patients only had this allele, even though this allele has a low prevalence in our population. Based on flow cytometric analyses, several groups proposed that antigens coated on the surface of beads may be structurally different from the membrane-expressed antigens and some "unnatural" antigens may detect irrelevant Abs (*25*, *26*). Our studies revealed that Abs against the MICA allele *019 detected by Luminex assay may recognize a linear epitope due to a misfolding of the molecule on the surface of the beads. In this way, these Abs would have induced against linear epitopes due to a misfolding of the molecule in these patients.

It is necessary to remark that this epitope-mapping technique is limited to the identification of linear epitopes defined by continuous aa residues in the primary structure of the protein. Alternatively, other conformational epitopes composed of residues that are discontinuous and brought together by folding may be involved in the immunogenic regions of MICA. Furthermore, MICA is a highly glycosylated protein, so the carbohydrate portion could provide antigenic determinants that cannot be identified by this methodology or mask otherwise immunogenic regions. Mapping the epitopes of MICA by human sera from transplanted patients defined nine different regions in the primary structure of the three extracellular domains of the molecule. These results indicate that the MICA protein contains multiple linear antigenic sites that are capable of inducing a site-specific antibody response. As mentioned earlier, some polymorphic regions (V and VIII) were recognized by all the alleles, whereas the region VII (aa 203–219) contained variable aa that defined allele-specific epitopes (reactivity patterns 1 and 2). Currently, all studies that define MICA epitopes are based on the distribution of polymorphic residues. Interestingly, this study identified five regions common to all MICA alleles. These regions were determined to have shorter epitopes and less surface exposure of the molecule than the variable regions. It could indicate that these epitopes contain immunodominant aa that could participate in the configuration of a conformational epitope when they are carried together in the 3D structure of the molecule.

The identification of antibody-binding sites in conserved regions of MICA may seem like a paradox. However,

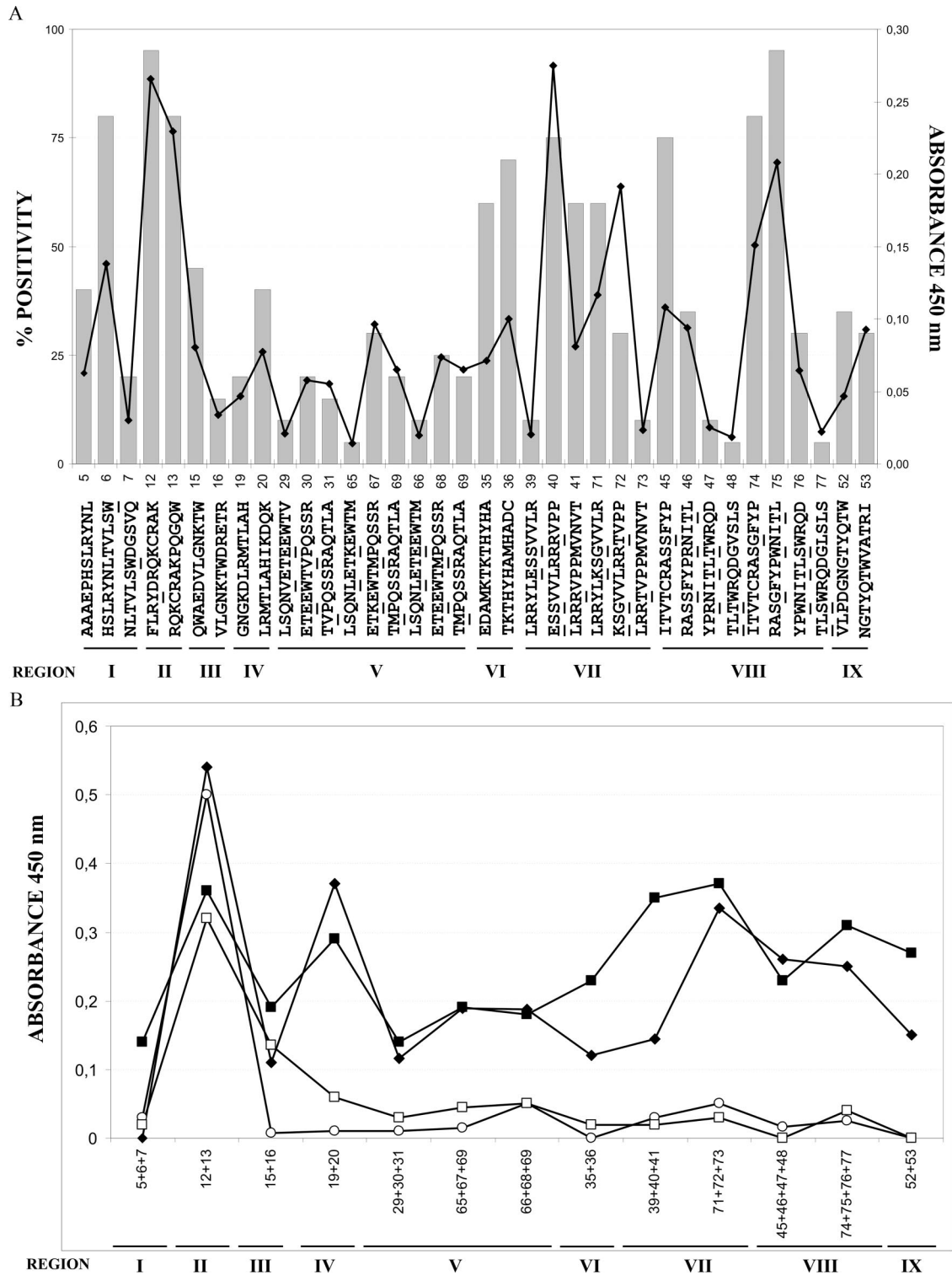

FIGURE 2. Specificity of epitopes of major histocompatibility complex class I chain-related molecule A (MICA) is recognized by anti-MICA Abs present in kidney transplant patients. (A) Distribution of immunodominant regions of the MICA protein by Abs from 30 patients with positive-MICA sera. Percentage of positivity (*gray columns*) indicates frequency of binding to each peptide. Absorbance (*black line*) shows the reactivity to each peptide. (B) Specificity of binding of anti-MICA Abs. The antibody activity in patients with reactivity pattern 1 (*filled squares*). The antibody activity in mixed sera from six patients with Abs only against MICA allele *019 (*filled diamonds*). The reactivity from transplant patients without anti-MICA Abs but with Abs against human leukocyte antigens classes I and II (dotted circles), and the reactivity of sera from transplant patients without Abs to MICA and human leukocyte antigens (dotted squares). The peptides tested are shown along the horizontal axis. The y-axis shows the absorbance at 450 nm. Sera were tested at dilutions 1/50.

there are several potential explanations. First, the conserved regions may be less immunogenic than the more variable regions of MICA. Development of Abs directed against these conserved regions would be less frequent and result in a delay in developing immunity. Second, the binding surface to whole antibody may be composed of both conserved and variable regions. The variability of a few residues would be sufficient to disrupt the binding or differentiate the reactivity patterns into the two groups observed in this study.

Furthermore, comparison of the sequences of each of these epitopes with a protein database suggests that these sites are unique to MICA, except the epitope RQKCRAK (region II) that could react with other Abs in the sera of transplant patients without anti-MICA Abs. Region II has four positively charged aa. Because charged polypeptides can elicit high-titer Abs, the presence of this epitope may have important implications in triggering cross-reactive Abs of high affinity.

Although various issues are still pending, resolution and further studies are necessary to establish whether a matching for MICA alleles between donor and receptor may lead to a better outcome of the organ. This study demonstrated that the presence of anti-MICA Abs posttransplant is associated with poorer graft outcome. Moreover, these Abs recognize common linear epitopes in the MICA protein and exhibited reactivity to different alleles and as a consequence of their cross-reactivity. The immunodominant regions shared by all the alleles can be used for immunizing animals to produce polyclonal or monoclonal Abs to this epitope, with neutralizing activity against MICA for using in therapy with transplanted patients or for generating Abs with increased activity to use in cancer therapy.

REFERENCES

1. Bahram S, Bresnahan M, Geraghty DE, et al. A second lineage of mammalian major histocompatibility complex class I genes. *Proc Natl Acad Sci USA* 1994; 91: 6259.
2. Groh V, Rhinehart R, Secrist H, et al. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc Natl Acad Sci USA* 1999; 96: 6879.
3. González S, López-Soto A, Suarez-Alvarez B, et al. NKG2D ligands: Key targets of the immune response. *Trends Immunol* 2008; 29: 397.
4. Wu J, Song Y, Bakker AB, et al. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* 1999; 285: 730.
5. Bauer S, Groh V, Wu J, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 1999; 285: 727.
6. Suárez-Alvarez B, López-Vázquez A, Baltar JM, et al. Potential role of NKG2D and its ligands in organ transplantation: New target for immunointervention. *Am J Transplant* 2009; 9: 251.
7. Zwirner NW, Marcos CY, Mirbaha F, et al. Identification of MICA as a new polymorphic alloantigen recognized by antibodies in sera of organ transplant recipients. *Hum Immunol* 2000; 61: 917.
8. Zou Y, Mirbaha F, Lazaro A, et al. MICA is a target for complement-dependent cytotoxicity with mouse monoclonal antibodies and human alloantibodies. *Hum Immunol* 2002; 63: 30.
9. Hankey KG, Drachenberg CB, Papadimitriou JC, et al. MIC expression in renal and pancreatic allografts. *Transplantation* 2002; 73: 304.
10. Mizutani K, Terasaki PI, Shih RN, et al. Frequency of MIC antibody in rejected renal transplant patients without HLA antibody. *Hum Immunol* 2006; 67: 223.
11. Zou Y, Heinemann FM, Grosse-Wilde H, et al. Detection of anti-MICA antibodies in patients awaiting kidney transplantation, during the post-transplant course, and in eluates from rejected kidney allografts by Luminex flow cytometry. *Hum Immunol* 2006; 67: 230.
12. Sumitran-Holgersson S, Wilczek HE, Holgersson J, et al. Identification of the nonclassical HLA molecules, mica, as targets for humoral immunity associated with irreversible rejection of kidney allografts. *Transplantation* 2002; 74: 268.
13. Terasaki PI, Ozawa M, Castro R. Four-year follow-up of a prospective trial of HLA and MICA antibodies on kidney graft survival. *Am J Transplant* 2007; 7: 408.
14. Mizutani K, Terasaki P, Rosen A, et al. Serial ten-year follow-up of HLA and MICA antibody production prior to kidney graft failure. *Am J Transplant* 2005; 5: 2265.
15. Zou Y, Stastny P, Susal C, et al. Antibodies against MICA antigens and kidney-transplant rejection. *N Engl J Med* 2007; 357: 1293.
16. Suarez-Alvarez B, Lopez-Vazquez A, Gonzalez MZ, et al. The relationship of anti-MICA antibodies and MICA expression with heart allograft rejection. *Am J Transplant* 2007; 7: 1842.
17. Zou Y, Qin Z, Silveus A, et al. Polymorphisms of MICA recognized by human alloantibodies. *Immunogenetics* 2009; 61: 91.
18. El-Awar N, Terasaki PI, Cai J, et al. Epitopes of the HLA-A, B, C, DR, DQ and MICA antigens [Review]. *Clin Transpl* 2007: 175.
19. Duquesnoy RJ, Mostecki J, Hariharan J, et al. Structurally based epitope analysis of major histocompatibility complex class I-related chain A (MICA) antibody specificity patterns. *Hum Immunol* 2008; 69: 826.
20. Tribbick G. Multipin peptide libraries for antibody and receptor epitope screening and characterization. *J Immunol Methods* 2002; 267: 27.
21. Porollo A, Meller J. Versatile annotation and publication quality visualization of protein complexes using POLYVIEW-3D. *BMC Bioinformatics* 2007; 8: 316.
22. Li P, Willie ST, Bauer S, et al. Crystal structure of the MHC class I homolog MIC-A, a gammadelta T cell ligand. *Immunity* 1999; 10: 577.
23. Suárez-Alvarez B, López-Vázquez A, Díaz-Peña R, et al. Post-transplant soluble MICA and MICA antibodies predict subsequent heart graft outcome. *Transpl Immunol* 2006; 17: 43.
24. Panigrahi A, Gupta N, Siddiqui JA, et al. Post transplant development of MICA and anti-HLA antibodies is associated with acute rejection episodes and renal allograft loss. *Hum Immunol* 2007; 68: 362.
25. Morales-Buenrostro LE, Terasaki PI, Marino-Vázquez LA, et al. "Natural" human leukocyte antigen antibodies found in nonalloimmunized healthy males. *Transplantation* 2008; 86: 1111.
26. Cai J, Terasaki PI. Post-transplantation antibody monitoring and HLA antibody epitope identification. *Curr Opin Immunol* 2008; 20: 602.