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The role of KIR2DL3/HLA-C*0802 in Brazilian patients with rheumatoid vasculitis

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OBJECTIVES: Rheumatoid arthritis is a polygenically controlled systemic autoimmune disease. Rheumatoid vasculitis is an important extra-articular phenotype of rheumatoid arthritis that can result in deep cutaneous ulcers. The objective of this study was to establish a correlation between the frequency of major histocompatibility complex class I/II alleles and killer immunoglobulin-like receptor genotypes in patients with cutaneous rheumatoid vasculitis.

METHODS: Using the Scott & Bacon 1984 criteria to diagnose rheumatoid vasculitis and after excluding any other causes such as diabetes, atherosclerosis, adverse drug reactions, infection, and smoking, patients who met the criteria were selected. All of the selected rheumatoid vasculitis patients presented deep cutaneous ulcers. Identification of the major histocompatibility complex class I/II and killer immunoglobulin-like receptor genotypes was performed by polymerase chain reaction assays of samples collected from the 23 rheumatoid vasculitis patients as well as from 80 controls (40 non-rheumatoid vasculitis RA control patients and 40 healthy volunteers).

RESULTS: An association between the presence of the HLA-DRB1*1402 and HLA-DRB1*0101 alleles and cutaneous lesions in rheumatoid vasculitis patients and a correlation between the inhibitor KIR2DL3 and the HLA-C*0802 ligand in rheumatoid vasculitis patients were found.

CONCLUSION: An association was found between the presence of the HLA-DRB1*1402 and HLA-DRB1*0101 alleles and the development of cutaneous lesions in rheumatoid vasculitis patients. Additionally, the HLA-C*0802 ligand protects these individuals from developing cutaneous lesions.

KEYWORDS: Rheumatoid arthritis; Receptors, KIR; Rheumatoid vasculitis; HLA-C antigens/HLA-DRB1 chains.

■ INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by inflammatory polyarthritis (1,2). RA is polygenically controlled, with contribution from the major histocompatibility complex (MHC) (2–6). MHC class I molecules are expressed on most nucleated human cells and comprise the principal human leukocyte antigens (HLA) A, B and C (3). MHC class II molecules are expressed on antigen-presenting cells (APCs; B lymphocytes, macrophages, and dendritic cells) and include mainly HLA-DR and HLA-DQ (3). MHC class I molecule expression results in direct cytotoxicity from CD4$^+$CD28$^-$ T cells or natural killer (NK) cells, and MHC class II molecule expression results in cytotoxicity via APCs (3). The polygenic characteristics of RA, which result in different phenotypic expressions, determine the heterogeneous presentation of articular destruction and extra-articular manifestations (1,7). An important and potentially life-threatening extra-articular manifestation of RA is rheumatoid vasculitis (RV), which affects the small- and medium-sized arterial vessels of the upper and lower extremities, peripheral nerves and occasionally other organs (8,10,11). RV is a rare complication that may affect approximately 1–5% of RA patients and that is diagnosed by ruling out other possible clinical conditions such as diabetes, atherosclerosis, adverse drug reactions, infections and neoplasms (1,9). The HLA-DRB1 allele (B1*0401 homozygotes, in particular) is more common in RV patients (12,13). In

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addition to genetic factors, evidence of RA biomarkers such as $CD4^+CD28^-$ T cells, which are uncommon in healthy subjects, has been reported (14). $CD4^+CD28^-$ T cells are increased in RV patients, and the role of these cells has been reported in arterial damage caused by inflammatory injury (15,16). Because $CD4^+CD28^-$ T cells do not express the CD40 ligand, they develop direct cytotoxic abilities and work in conjunction with the CD57 expressed on NK cells in RV patients, suggesting that both cells are involved in vascular endothelial damage (17,18). Regarding NK cells, the development of vascular damage is intrinsically dependent on the interaction of these cells with MHC class I molecules in RA patients (11,19). NK cells express activating and inhibiting receptors on their surface, which interact with the MHC antigens of vascular endothelial cells, resulting in NK cell activation or inhibition. On the NK cell surface, killer immunoglobulin-like receptors (KIRs), which recognize MHC class I molecules, are present (20). Increased cell surface expression of HLA-C ligands has been shown in RV patients. These ligands interact with KIR2DS2, which is present on $CD4^+CD28^-$ T cells, confirming the similarity of $CD4^+CD28^-$ T cell characteristics with NK cells, as both cell types are derived from the same hematopoietic stem cell (3,11,21).

The objective of this study was to establish a correlation between the frequency of MHC class I/II and KIR genotypes and cutaneous RV.

◼ METHODS AND MATERIALS

Patient selection

We evaluated patients diagnosed with RA who were followed for a period of 2 years (2012–2013) in the Rheumatology Department at the University of Campinas Teaching Hospital (HC-UNICAMP), a tertiary referral hospital located in Campinas, Sao Paulo State, Brazil. The clinical data of patients, who were all ethically unrelated, were obtained through a review of their records. The study was approved by the Ethics Committee, and the patients provided informed consent. The RA diagnosis was based on the American College of Rheumatology (ACR) 1987 Rheumatoid Arthritis classification criteria (22). Patients under 16 years of age or with overlap syndrome, diabetes, atherosclerosis, a history of adverse drug reactions, infection, or a history of smoking were excluded. RV was clinically diagnosed by the presence of deep cutaneous ulcers (DCU) on the lower limbs, by localized pain in areas of healthy skin that was suggestive of neuropathic involvement of small sensory fibers or by digital infarction of the toes (9). The diagnosis was based on the Scott & Bacon 1984 clinical criteria and required 1 of the following 3 characteristics: DCU, symptoms suggestive of mononeuritis multiplex (MNM), or digital infarction (9,23,24). All patients were evaluated for rheumatoid factor (RF) and citrullinated protein antibody (anti-CCP).

Control population

Patients diagnosed with RA from the Rheumatology Department at the HC-UNICAMP who were selected during the period that the RV cases were analyzed were included as controls (22). Blood donors from HC-UNICAMP were also included as healthy patient (HP) controls. Using the RV diagnostic criteria, some of the patients exhibiting evidence of the condition were excluded from the control groups. In the comparative analysis of case and control groups, factors such as gender and age were evenly matched across all 3 groups.

Deoxyribonucleic acid (DNA) extraction methodology

Whole blood samples were collected and subjected to DNA extraction using the GE Healthcare Illustra Blood GenomicPrep Mini Spin Kit® in accordance with the manufacturer's guidelines.

MHC Class I/II Genotyping and Reading Methodology

DNA samples were processed using the high-resolution SSO/Luminex®. The SSO/DNA method used oligonucleotide sequence-specific probes immobilized on fluorescently encoded microspheres. HLA alleles were identified in DNA samples amplified by polymerase chain reaction (PCR) using hybridization probes and flow cytometry analysis. The manufacturer's guidelines were strictly followed for all methodological parameters.

KIR genotyping and reading methodology

Three reactions were analyzed for KIR genotyping to evaluate the genetic composition of exons 3–4 (group 1), exon 5 (group 2), and exons 7–9 (group 3). DNA amplification was performed by PCR with specific primer sequences, and the samples were aligned using the Luminex® system. All steps were performed in accordance with the manufacturer's guidelines.

Statistical analysis

In addition to the use of graphs and one-dimensional scatter plots, all data collected were first descriptively analyzed by calculating various measurements and summaries, including the mean, median, minimum and maximum values, standard deviations, and absolute and relative frequency percentages. Pearson's chi-squared (χ^2) test or an extension of Fisher's exact test was used to compare group profiles according to gender, class I and II allelic frequencies, and the presence of diverse KIR genotypes (25). Analysis of variance (ANOVA) with one fixed factor was used to compare the age profiles of the groups (26). Because the study was exploratory, sample calculations were not performed due to the lack of data in the literature comparing the allelic frequencies in all 3 groups of interest. In all conclusions made through inferential analysis, the level of α significance was equal to 5%. The data were compiled into a Windows Excel 2010 table to adequately manage the information. Statistical analyses were performed using R statistical software version 2.15.2.

Ethical standards

This study was approved by the ethics committee of the University of Campinas (UNICAMP) and was conducted in accordance with the Declaration of Helsinki of 1975, which was revised in 1983. All 103 subjects agreed to participate in this study by reading and signing the Informed Consent Form that was approved by the same committee.

◼ RESULTS

A total of 700 RA patients were evaluated for RV over a 2-year period. Forty patients with cutaneous ulcers were selected. None of the patients had a clinical diagnosis of systemic vasculitis. Of the 40 patients with cutaneous ulcers, 23 were classified as having RV, including 20 (87%) women

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Table 1 - General characteristics of the individuals in each group.

	HP (n=40)		RA (n=40)		RV (n=23)		Total (n=103)		p-value
Gender									
Female	31	77.5%	31	77.5%	20	87.0%	82	79.6%	0.611[a]
Male	9	22.5%	9	22.5%	3	13.0%	21	20.4%	
Age (years)									0.927[b]
Average	54.3		54.8		55.5		54.8		
Median	56.0		56.0		56.0		56.0		
Minimum-maximum	25.0–77.0		17.0–78.0		32.0–71.0		17.0–78.0		
Standard deviation	12.3		12.9		9.5		11.9		

[a] Pearson's chi-squared test, [b] ANOVA with 1 fixed factor

Table 2 - Distribution of the HLA-A/B/C allelic frequencies among the patient groups.

		HP (n=40)		RA (n=40)		RV (n=23)		Total (n=103)		p-value
A*2601	-	40	100.0%	38	95.0%	19	82.6%	97	94.2%	0.017[c]
	+	0	0%	2	5.0%	4	17.4%	6	5.8%	
B*1402	-	36	90.0%	40	100.0%	20	87.0%	96	93.2%	0.057[c]
	+	4	10.0%	0	0%	3	13.0%	7	6.8%	
B*1501	-	40	100.0%	35	87.5%	22	95.7%	97	94.2%	0.048[c]
	+	0	0%	5	12.5%	1	4.3%	6	5.8%	
B*3906	-	40	100.0%	40	100.0%	21	91.3%	101	98.1%	0.048[c]
	+	0	0%	0	0%	2	8.7%	2	1.9%	
B*5001	-	35	87.5%	40	100.0%	23	100.0%	98	95.1%	0.024[c]
	+	5	12.5%	0	0%	0	0%	5	4.9%	
C*0501	-	37	92.5%	33	82.5%	23	100.0%	93	90.3%	0.056[c]
	+	3	7.5%	7	17.5%	0	0%	10	9.7%	
C*0602	-	27	67.5%	38	95.0%	22	95.7%	87	84.5%	0.001[a]
	+	13	32.5%	2	5.0%	1	4.3%	16	15.5%	
C*0802	-	35	87.5%	40	100.0%	19	82.6%	94	91.3%	0.019[c]
	+	5	12.5%	0	0%	4	17.4%	9	8.7%	
C*1502	-	38	95.0%	31	77.5%	22	95.7%	91	88.3%	0.027[c]
	+	2	5.0%	9	22.5%	1	4.3%	12	11.7%	

[a] Pearson's chi-squared test, [c] extension of Fisher's exact test, absent (-), present (+)

and 3 (13%) men. Active ulcer cutaneous biopsies were performed in 3/23 case patients and demonstrated the standard histopathology of vessel wall fibrinoid necrosis with inflammatory cell infiltration and areas of occlusion with recanalization. All 23 case patients were undergoing treatment with Disease Modifying Anti-Rheumatic Drugs (DMARDs) and had also used high-dose corticosteroids at some point during the course of RV. Therapeutic doses of antiplatelet agents were introduced in cases of toe digital infarctions. The 40 RA control patients were also treated with DMARDs and occasional low doses of corticosteroids.

Of the 103 patients studied, 40 (38.8%) were in the HP control group, 40 (38.8%) were in the RA control group, and 23 (22.4%) were in the RV case group. The age and gender distributions of the groups are displayed in Table 1.

Notably, when comparing the gender and age profiles of the 3 groups, these groups are statistically equivalent with respect to gender (p=0.611) and age (p=0.927).

The frequency of class I alleles varied between 0.0% and 47.5% in the HP group, between 0.0% and 45.0% in the RA group, and between 0.0% and 43.5% in the RV group. The high frequencies of expression in all 3 groups can be explained by the presence of the A*0201 allele (p=0.949), which was observed in all evaluated patient groups.

The alleles with significantly different A*2601 (p=0.017) and B*3906 (p=0.048) frequencies were more commonly observed in the RV group than in the HP and RA groups. The B*5001 (p=0.024) and C*0602 (p=0.001) alleles were more frequently observed in the HP group than in the RA and RV

groups. The B*1501 (p=0.048) and C*1502 (p=0.027) alleles were more frequently observed in the RA group than in the other two groups. With respect to the C*0802 (p=0.019) allele, it was less frequently observed in the RA group than in the HP and RV groups. In the HP and RV groups, there was a trend towards more frequent expression of the B*1402 allele (p=0.057) and less frequent expression of the C*0501 allele (p=0.056) compared with the RA group. The other alleles were observed equally in all 3 groups, without significant differences. These data are displayed in Table 2.

Higher allelic frequencies were observed for HLA-DRB1*0701 (p=0.428) and DQB1*0302 (p=0.740) in the HP group, DQB1*0301 (p=0.752) in the RA group, and DQB1*0501 (p=0.480) in the HP and RA groups. The comparison of class II allelic frequencies between the groups suggests that almost all alleles were observed equally across the groups. The only exception was the DRB1*1402 allele (p=0.048), which was found more frequently in the RV group than in the HP and RA groups. A trend towards a higher frequency of the DRB1*0101 (p=0.053) allele was found in the RV group compared with the HP and RA groups, as well as a higher frequency of the DRB1*1503 (p=0.075) allele in the HP group compared with the RA and RV groups. The other alleles were equally observed in the 3 groups, without significant differences. These data are displayed in Table 3.

Higher frequencies were observed for the KIR2DL2 genotype (p=0.529) in the RA and RV groups, as well as for the KIR3DL2 and 3DL3 genotypes in all 3 groups (a p-value could not be calculated). Higher frequencies were

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Table 3 - Distribution of HLA-DRB1 allelic frequencies among the patient groups.

		HP (n=40)		RA (n=40)		RV (n=23)		Total (n=103)		p-value
*0101	-	37	92.5%	34	85.0%	16	69.6%	87	84.5%	0.053[a]
	+	3	7.5%	6	15.0%	7	30.4%	16	15.5%	
*0102	-	36	90.0%	40	100.0%	20	87.0%	96	93.2%	0.057[c]
	+	4	10.0%	0	0%	3	13.0%	7	6.8%	
*1402	-	40	100.0%	40	100.0%	21	91.3%	101	98.1%	0.048[c]
	+	0	0%	0	0%	2	8.7%	2	1.9%	
*1503	-	36	90.0%	40	100.0%	23	100%	99	96.1%	0.075[c]
	+	4	10.0%	0	0%	0	0%	4	3.9%	

[a] Pearson's chi-squared test, [c] extension of Fisher's exact test, absent (-), present (+)

Table 4 - Distribution of KIR genotypes among the patient groups.

		HP (n=40)		RA (n=40)		RV (n=23)		Total (n=103)		p-value
2DL3	-	5	12.5%	12	30.0%	3	13.0%	20	19.4%	0.096[a]
	+	35	87.5%	28	70.0%	20	87.0%	83	80.6%	
2DL5	-	11	27.5%	20	50.0%	12	52.2%	43	41.7%	0.064[a]
	+	29	72.5%	20	50.0%	11	47.8%	60	58.3%	
3DL1	-	7	17.5%	1	2.5%	1	4.3%	9	8.7%	0.060[c]
	+	33	82.5%	39	97.5%	22	95.7%	94	91.3%	

[a] Pearson's chi-squared test, [c] extension of Fisher's exact test, absent (-), present (+)

observed for the KIR3DP1 genotype ($p>0.999$) only in the RV group. The various KIR genotypes showed comparable frequencies in all 3 groups; however, some trends suggested a higher frequency of 2DL3 in the HP and RV groups than in the RA group ($p=0.096$). A trend towards more frequent expression of 2DL5 was observed in the HP group compared with the RA and RV groups ($p=0.064$). Finally, a trend indicating higher frequencies of 3DL1 was observed in the HP group compared with the RA and RV groups ($p=0.060$). The other genotypes were equally observed in the 3 groups, without significant differences. These data are displayed in Table 4.

■ DISCUSSION

Our study evaluated the frequencies of HLA class I and II alleles in 23 RV patients compared with HPs and RA patients without RV. KIR genotyping was performed in these groups, which was correlated with HLA class I antigens. This is the first work to perform correlation genotyping of HLA class I/KIR antigens in Brazilian RV patients. Studies concerning HLA class I/KIR in RV patients are relevant from an etiopathogenic standpoint as an effort to identify patients with a severe disease.

Watts et al. described an annual RV incidence of 12.5 per million people (95% CI=8.5–17.2), with 15.8 per million (95% CI=9.5–24.7) men and 9.4 per million (95% CI=4.8–16.4) women (27). Alternatively, Symmons et al. found an annual incidence of 140 per million men and 360 per million women with RV in a similar population (28). RV affects approximately 1–5% of RA patients, which emphasizes the relative rarity of RV (1,9). We found 23 RV patients in a population of 700 RA patients over a period of 2 years, which was equivalent to a rate of 3.3%. Our findings are similar to previously reported studies.

RV typically presents in patients who have suffered from rheumatoid arthritis for more than 10 years (1,9). In this study, the average age in the RV group was 55.5 years, which is comparable to the global general RA patient population. RV was more frequent in females (87.0%), but this gender predominance is not different from that of general RA patients without RV (1,9). In contrast to the findings of this study, gender has been reported to influence the disease phenotype, leading to more severe articular destruction and extra-articular manifestations, including RV, in men (29).

Cutaneous biopsies were performed on 3 patients with a confirmed RV diagnosis. In the remaining 20 patients, RV was diagnosed according to the Scott & Bacon clinical criteria because all patients had a previous RA diagnosis and all other causes of vasculopathy were excluded (9,22–24,30).

Before comparing significant allelic frequencies or analyzing statistical trends between groups for MHC class I and II antigens or KIRs, understanding the behavior of these alleles in the population studied was necessary. This was accomplished using the Allele Frequency Net Database (AFND) to evaluate whether any associations that existed between the alleles examined could under- or overestimate the frequency of each allele in a particular geographic region of the population studied (31). When the search tool on the AFND site was utilized, no data on class I alleles were found for the Campinas region in Sao Paulo, Brazil. For the class II alleles, the same search tool was used for the region of Campinas, and data were found for the DRB1*0101, DRB1*0102, DRB1*1402 and DRB1*1502 alleles. Similarly, no association of these alleles with other alleles was found in this geographic region.

After confirming the allelic frequencies, their correlations with KIR frequency findings were analyzed. KIR2DL3 is an inhibitor that interacts with HLA-C (C*01, C*03, C*07, C*08, C*12, C*13, C*14 and C*16) ligands (32). KIR2DL5 is also an inhibitor, but its interactions with MHC class I molecules remain controversial (32). Therefore, correlating the frequency of KIR2DL5 with the findings from this class was not possible. KIR3DL1 is an inhibitor that interacts with HLA-A and HLA-B (A*24, B*13, B*27, B*37, B*44, B*51, B*52, B*53, B*57 and B*58) ligands (32). Nonetheless, the interaction of KIR2DL3 with the C*0802 ligand can disable the missing-self response; thus, in our RV patient population, C*0802 could be a protective allele against the development of cutaneous lesions from direct cytotoxicity (NK cells or CD4$^+$CD28$^-$ T

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cells). This finding contradicts the results of other studies (11,19,21,33, 34).

Regarding the frequency results for class II alleles in the RV group, the frequency of DRB1*1402 (8.7%) was significantly different, and a statistical trend towards a difference in DRB1*0101 (30.4%) was observed compared with the other groups. Similarly to the findings of other studies (12,13), these findings suggest that these alleles are associated with the development of cutaneous lesions in our RV patient population because they are associated with APCs (B lymphocytes, macrophages and dendritic cells). For the DRB1*1503 allele (10.0%), a statistical trend towards a higher frequency was found in the HP group; this allele could be considered protective against RA and RV in our population.

An association exists between the presence of MHC class II alleles (HLA-DRB1*1402 and HLA-DRB1*0101) and the development of cutaneous lesions in RV patients. In other words, an association exists between APCs and vascular damage. The KIR2DL3 inhibitor was frequently found in RV patients, and its interaction with the HLA-C*0802 ligand suggests that this allele protects these individuals from cutaneous lesions caused by direct cytotoxicity. Our findings have not been previously described. Despite the rarity of RV, this study could be considered a baseline study for new genetic and multicenter descriptive studies to better our understanding of the phenotypic expression of this disease in Brazil.

■ ACKNOWLEDGMENTS

■ AUTHOR CONTRIBUTIONS

Nishimura WE was responsible for recruiting RV patients and collecting biological materials. Sachetto Z, Costallat LTL, Yazbek MA, Marques SB and Bertolo MB were responsible for guiding the study. Londe CS and Guariento EG were responsible for analyzing the biological materials from RA patients.

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