

Study of Class I and II HLA alleles in 30 Ecuadorian patients with rheumatoid arthritis compared with alleles from healthy and affected subjects with other rheumatic diseases

María Verónica Aguirre Arias¹, Emilia Vázquez Domingues², Rodrigo Barquera Lozano³, Carlos Vallejo Flores⁴, Marilú Mestanza Peralta⁴, Camilo Zurita Salinas⁵

ABSTRACT

Introduction: Rheumatoid arthritis (RA) is a chronic systemic inflammatory autoimmune disease that originates from a disabling disorder. To date, the etiology of RA is unknown. However, the existence of genetically susceptible individuals was considered. Many studies have been performed worldwide, for example, in Poland, Argentina, Chile, Mexico, Brazil, and Colombia, among others, regarding the influence between HLA-DR alleles and disease, but not in Ecuador. **Objective:** The aim of this study was to determine the involvement of Class I and II HLA alleles in patients with RA. **Patients and Methods:** This study was conducted in 30 adult patients with RA previously diagnosed, according to the classification criteria of the American College of Rheumatology (ACR, 1987) and 28 controls. For Class I and II HLA typing, we adopted the PCR-SSP, and statistical significances were evaluated by Chi-Square. **Results:** HLA-DR4 is present in 76.7% of patients, with an allele frequency of 45%, while only 21% of control subjects presented it. The chi-square confirms that HLA-DR4 and RA variables are highly bound ($X^2 = 11.38$, $P = 0.00074$). **Conclusion:** There is increased frequency of HLA-DR4 and HLA-DR14. The results are similar to those found in other studies. But it would be desirable to increase the sample size in order to find a greater number of genetic profiles and alleles involved.

Keywords: rheumatoid arthritis, HLA-DR4, allele, PCR-SSP.

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic, autoimmune inflammatory disease with a progressive character, and its inheritance is considered complex and polygenic. The prevalence of this disease in Latin America is about 0.4%, with a rate of 0.24% -1.0%.¹ Although the etiology of RA is unknown, several risk factors have been considered and it is reported the presence of genetically susceptible individuals, with the influence of mood and cell factors that lead to a process of immune activation.²

The Human Leukocyte Antigen (HLA) encoded by the Major Histocompatibility Complex (MHC) is associated with RA since 1976. Both the nature and incidence of this association vary according to ethnic groups and have been well documented in several studies. In Europe, a study by Plosker *et al.*, in Poles and Lithuanians patients, HLA-DR1 and HLA-DR4 were associated with vulnerability.³ Turesson *et al.* reported that one type of HLA-DR4 was present in 105 of 151 patients with extra-articular RA,⁴ while Citta *et al.* found the same presence in 70 of 140 patients studied.⁵ The aim of this study was to determine the involvement

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Molecular Biology Department of Zurita & Zurita Laboratorios; coordinated by Biomedical Research Center (Reumaceb).

1. Biotechnology Engineer, Army Polytechnic School, with graduation thesis at Reumaceb - Center for Biomedical Studies Zurita & Zurita Laboratorios.

2. Biotechnology engineer in charge of the Molecular Biology area of Zurita & Zurita Laboratorios. Quito, Ecuador.

3. Chemical and Biological Pharmacist of Laboratory of Molecular Genetics, National School of Anthropology and History of UNAM. Mexico DF, Mexico.

4. Rheumatologist, Centre for Biomedical Studies, Reumaceb, Quito, Ecuador.

5. Immunologist, investigator of Zurita & Zurita Laboratorios. Quito, Ecuador.

Correspondence to: María Verónica Aguirre Arias. Ambato, Ecuador. Phone/Fax: (593-3) 2841-055. E-mail: maveagar@yahoo.com.

of Class I and II HLA alleles on susceptibility to RA in Ecuadorian patients.

PATIENTS AND METHODS

All participants received an explanation about the study process and voluntarily signed the informed consent, without compromising the doctor-patient relationship or the treatment being followed. The study, in all its principles and methodologies, had the approval of the Ecuadorian Bioethics Committee.

Patients

We analyzed 30 Ecuadorian patients aged over 18 years, diagnosed with RA according to the American College of Rheumatology criteria (ACR, 1987). Patients without a confirmed diagnosis or complete medical records were not evaluated. The age of patients was between 25 and 54 years, with an average of 43.3 years (25 females and five males).

Controls

In total, 28 adults over 18 years of age without a diagnosis of RA, distributed among healthy individuals ($n = 12$), seronegative spondyloarthritis ($n = 13$), and osteoarthritis ($n = 3$). The age of controls ranged from 26 to 65, with an average of 45.3 years. Among these controls, 11 were male (three healthy, eight with seronegative spondyloarthritis) and 17 were female (nine healthy, five with seronegative spondyloarthritis, and three with osteoarthritis).

Immunogenetic study

For all patients and controls in the study, there was a collection of blood 5 mL by phlebotomy in a tube with anticoagulant K3EDTA. From each of these samples, DNA was extracted by the salting-out method (Miller S *et al.*, 1988). HLA class I and II alleles were determined by PCR-SSP (Pel-Freez SSP HLA-A/B/DR/DQ UniTray®, BrownDeer, Wisconsin, USA). This method is based on the PCR technique, which measures low and high resolution levels of different types of HLA Class I and II. The thermal tray of 96 vessels includes formulation of several primers, a reaction buffer in which DNA is incorporated at a concentration of 100 ng/uL, and Taq polymerase. The mixture of these three components will join the tray, followed by sealing and subsequent thermal cycle as follows: step 1) 1 minute at 96 °C; step 2) five cycles of 96 °C for 25 seconds, 70 °C for 50 seconds, 72 °C for 45

seconds; step 3) 21 cycles of 96 °C for 25 seconds, 65 °C for 50 seconds, 72 °C for 45 seconds; step 4) four cycles of 96 °C for 25 seconds, 55 °C for 60 seconds, 72 °C for 120 seconds, and maintained at 4 °C, until its loading on agarose gel in 2% adding ethidium bromide. Finally, electrophoresis and photo documentation of agarose gels were made and we performed the interpretation of results.

Allele frequency

Allele frequency (AF) is defined as the percentage by which a specific allele is presented in relation to other subtypes found, which may also occupy the same locus and should be distinguished from simple percentage of individuals who have a particular allele. The first parameter is calculated by counting the number of times an allele appears in a population, divided by the total number of alleles and multiplied by 100.¹⁶ The second parameter indicates only the percentage of individuals that, homozygous or heterozygous (without discrimination), have the allele, and therefore, their values are different from the first.

Where:

AF = allele frequency (percentage).

a = number of times an allele appears in the study population (double count in case of homozygosis).

N = total number of alleles that are present in the population.

Statistics and experimental design

The results were evaluated by Chi-square test (Q_{hi}^2), with statistical correction for multiple contrast comparisons by Bonferroni's method, to determine the association of qualitative HLA and RA variables. We measured allele frequency (AF) to quantify the proportion of each allele regarding the totality of existing alleles.

RESULTS

HLA-DR4

The highest percentage of allele found in patients was HLA-DR4, present in 23 of 30 individuals diagnosed with rheumatoid arthritis (Figure 1). In four of these patients, the allele appears in homozygous and, in five, it is combined with -DR1. The allele frequency corresponds to 45%. This same allele was found in control subjects with a frequency of 21%. Table 1 shows all the allele frequencies found.

Table 1
Allele frequencies in patients and controls: Values are expressed as percentages

Alleles	Pacientes	Controles	Alleles	Pacientes	Controles	Alleles	Pacientes	Controles	Alleles	Pacientes	Controles	Alleles	Pacientes	Controles
A2	36.67	21.67	B35	31.67	16.67	DR4	45.00	21.67	DRB4	60.00	33.33	DQ8 (3)	36.67	25.00
A24 (9)	30.00	20.00	B65 (14)	3.33	1.67	DR103	3.33	5.00	DRB3	25.00	30.00	DQ7 (3)	33.33	21.67
A68 (28)	10.00	3.33	B61 (40)	13.33	10.00	DR1	10.00	6.67	Null	8.33	20.00	DQ5 (1)	10.00	16.67
A11	6.67	13.33	B39 (16)	15.00	13.33	DR11 (5)	5.00	13.33	DRB5	6.67	16.67	DQ6 (1)	6.67	15.00
A1	3.33	3.33	B62 (15)	3.33	0.00	DR14 (6)	18.33	3.33				DQ2	0.00	11.67
A23 (19)	0.00	3.33	B7	6.67	8.33	DR9	8.33	6.67				DQ9 (3)	11.67	6.67
A33 (19)	1.67	3.33	B60 (40)	3.33	0.00	DR15 (2)	3.33	13.33				DQ4	1.67	1.67
A26 (10)	1.67	0.00	B44 (12)	1.67	1.67	DR16 (2)	3.33	5.00				DQX	0.00	1.67
A43	1.67	0.00	BX	3.33	0.00	DR10	1.67	1.67						
A29 (19)	1.67	3.33	B37	3.33	0.00	DR8	1.67	6.67						
A30 (19)	1.67	5.00	B51 (5)	3.33	3.33	DR17 (3)	0.00	5.00						
A31 (19)	1.67	5.00	B50 (21)	1.67	0.00	DR13 (6)	0.00	6.67						
A32 (19)	1.67	5.00	B53	1.67	1.67	DRX	0.00	1.67						
A3	1.67	13.33	B8	1.67	0.00	DR7	0.00	3.33						
			B56 (22)	1.67	3.33									
			B27	1.67	15.00									
			B48	1.67	3.33									
			B57 (17)	1.67	0.00									
			B18	0.00	11.67									
			B41	0.00	3.33									
			B45 (12)	0.00	1.67									
			B13	0.00	1.67									
			B47	0.00	1.67									
			B40	0.00	1.67									

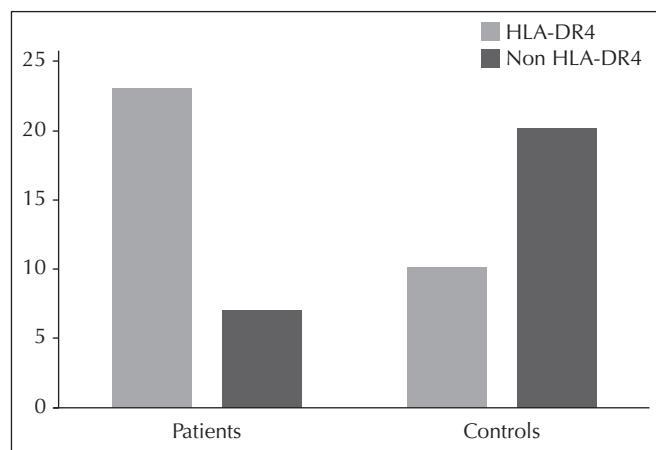


Figure 1
Distribution of HLA-DR4 in patients and controls (blue column). Class DR alleles other than DR4 appear in red column.

In control group, this allele was found in 4 of 12 healthy individuals and in 5 of 13 seronegative spondyloarthritis patients. However, in patients with osteoarthritis this allele was not found.

HLA-DR14

The HLA-DR14 appeared in eight patients with RA (AF = 18%), with two patients presenting HLA-DR14 in combination with-DR4. In the control group, this allele was found in two individuals, one healthy and one with seronegative spondyloarthritis.

HLA-B27

This allele had a frequency of 1.67% in patients and 15% in controls, which means that 13 control subjects have

seronegative spondyloarthritis, a disease widely associated with the presence of this allele.¹

Table 2 lists the most statistically important values, which were obtained in HLA:-DR4,-DR14, the -DR4-DR14, -DR9, -DQ8, -DQ7, and -B27 combination. In the other alleles found, the significance was not relevant.

Susceptibility to RA

We performed a chi-square, with a confidence interval of 95%, based on contingency tables with Bonferroni statistical correction and a p-value threshold of 0.05. With the above mentioned statistical analysis, we conclude that HLA-DR4 is significantly associated with RA susceptibility ($Qui^2 = 11.38$, $P = 0.00074$), as well as HLA - DR14 ($Qui^2 = 6.40$ and $P = 0.0113$). Table 2 shows the most important statistical significances obtained.

We also compared separately the statistics of DR4 and DR14 alleles, which were the most important alleles among patients and healthy controls, resulting in for -DR4 values $Qui^2 = 12.81$, $P = 0.0003$, which confirms the association of RA with this allele. For DR14, no association was found important, as described in Table 3.

DISCUSSION

In Ecuador, within the limits of our knowledge, this is the first research on the association between HLA and RA. The most important statistical significance found in this group of Ecuadorian patients corresponds to DR4 allele that was present in 23 of 30 patients. Similar values have been shown previously in studies conducted in other countries, such as the study by Citera *et al.* in Argentina (2001), in which 70 of 140 patients had this allele.⁵

Table 2

Allele frequencies in rheumatoid arthritis versus control: Qui^2 values and statistical significance

Alleles found	Qui^2	P
HLA-DR4	11.38	0.00074
HLA-DR14	6.40	0.0113
HLA-DR4 + HLA-DR14	6.80×10^{-5}	0.9934
HLA-DR9	0.1307	0.7176
HLA-DQ8	1.6685	0.1964
HLA-DQ7	0.2777	0.5981
HLA-B27	0.0113	0.9153

Table 3

Allele frequencies in percentages of patients with rheumatoid arthritis in contrast to healthy individuals grouped by gender

Alleles	Allele Frequency in Patients (n = 30)	Allele Frequency in healthy controls (n = 12)	Qui^2	P
-DR4	45%	5%	12.81	0.0003
-DR14	18%	1.67%	1.71	0.1908

In another study of a Peruvian population, 28 of 52 patients (53.8%) had HLA-DR4,⁶ while Turesson *et al.* reported in another study the positive indicator of the aforementioned allele in 69.5% of patients with extra-articular RA.⁴ Therefore, there is a need to assess the involvement of this allele in somatic manifestations of disease and in patterns characteristic of serological diagnosis.

Unlike the previous study, the discovery of this allele is much lower in a healthy control population. In this study, it was found in 4 of 12 healthy individuals (33.3%), while in a study by Kythera it was found in 23.3% of 202 surveyed patients and in 29.7% of 81 controls in a Peruvian study.^{5,6} As for the remaining control population, of the 13 patients with seronegative spondyloarthropathy, 38.5% of them had DR4.

HLA-DR14 allele had statistical significance in the group of studied patients. Further study with a larger sample of patients is necessary in order to categorically dismiss or affirm this allele association with the disease. It is known that the subtype HLA-DRB1*1402 is prevalent among Native Americans, among which RA is considered a common disease,⁶ although they are genetically different from the Ecuadorian population.

A2 and A24⁹ show significant allele frequency (36.6% and 30%) but have no relevance to disease. Its presence was confirmed in a Mexican mestizo population (32.2% and 16.4%, respectively),⁷ as well as in the Spanish Murcia and Peruvians. Similarly, HLA-B35 and-B39 alleles,¹⁶ whose frequency is at 31.7% and 16.7%, respectively, occur in Mexican mestizo population, Nahuatl, Quechua, Mixteques, Peruvians, and inhabitants of Central Europe.⁸

As stated in the results, HLA B27 had an important presence in the control subjects (15% allele frequency), unlike the group of patients who showed an allele frequency of 1.67%. The frequency in controls is justified because B27 is a gene strongly associated with seronegative spondyloarthropathy, a characteristic that has been described frequently in literature throughout the world. Therefore, it should not be interpreted as an allele that has protective role.

Among the alleles found in the study group, the allele frequency of HLA-DQ8 and HLA-DQ7 has no association with the disease. They were previously reported in several ethnic groups: Asians, Blacks, and Caucasians; and this information was found according to studies conducted in Brazilian, Mexican, Scandinavian, Indian, Japanese, and Koreans.⁹⁻¹²

In Latin America, there are important studies in Brazil and Colombia. In Brazil, it is important to note the research done by Louzada, Junior, and Donadi, published in 2008. In this study, HLA typing was performed by high resolution in 140 patients and 161 controls, among whites, blacks, and mulattos, finding several subtypes of HLA -DR4. The most important allele frequencies are *0401, with 7.5%; *0404, with 7.5%, and *0405, with 4.7%; all of which are type DR4. Furthermore, and overwhelmingly, DR β *0101 allele showed frequency of 12.1% in patients and 4.35% in control, with a confidence interval of 95%. Our study shows similar values of 10% and 6.67%, respectively, although in a smaller sample, but without important statistical significance ($P > 0.05$). All this speaks in favor of further studies of high resolution and with larger sample sizes to identify the subtypes of DR4.

In Colombia, where miscegenation is similar to Ecuador, we highlight the research conducted by Juan Manuel Anaya in 83 RA female, with 39 of them DR4-positive (46.7%).¹⁴ In reviewing our data, 19 out of 26 women who participated in the study are carriers of this allele, representing 73% of the female group. In another HLA study conducted by Correa and Anaya, in 100 unrelated healthy subjects from the northwestern region of that country, the DR4 allele is present in 8.5%.¹⁵ Among our healthy controls ($n = 12$), the allele frequency is 4.1%, 95%, but this percentage in both healthy patients and affected female may vary with the inclusion of new subjects.

The results obtained in patients surveyed demonstrated that HLA-DR4, in particular, has statistical significance and is likely to be associated with rheumatoid arthritis, without discarding that -DR14, -B35, -DR9, and other alleles may also be associated with the disease. Nevertheless, as explained before, the statistical significance of HLA-DR4 found in this study – combined with strong development of the disease – is confirmed by the results of previous studies. Anyway, it would be desirable to verify the presence of these alleles and their statistical significance of association in a larger number of patients, also involving the clinical characteristics and sera and radiological assessment of the disease itself.

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in a larger number of patients, involving also the clinical features and serological/radiological parameters of this disease assessment.

The alleles associated with the disease found in this and other studies in Latin America, HLA-DRB1*04 e -DRB1*14, subtypes *1402 and *1406*, coincide with those found as prevalent in several native or mixed American populations, which reveals the existence of an ethnic factor among components that predispose to RA.

This kind of study contributes to a better understanding of the frequency and distribution of the associated variants throughout the region, and offers more opportunities to clarify its mechanism, providing data on the prevalence of alleles, strength of the association, and other genetic and environmental components shared or unique to populations.

Likewise, other gene systems inside or outside the HLA region must be investigated to complete the construction of genotypes that influence the development and prognosis of disease, for example, polymorphisms in TNF- α 1 and PTPN22 generating region,¹⁶ whose association with physiopathogenesis has been demonstrated.

CONCLUSION

There are similar studies well documented in scientific literature worldwide, but we also find that within the researched group, HLA DR4 is strongly associated with RA disease. Within the group analyzed, we also found statistical significance of HLA DR14, but its involvement in disease has not yet been accepted nor rejected by a broader sample study.

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