

# Human Leukocyte Antigen (HLA) Class II Association with Rheumatic Heart Disease in Pakistan

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**Background and aim of the study:** Rheumatic heart disease (RHD) is widespread in Pakistan. Specific alleles of the human leukocyte antigen (HLA) system are associated with RHD in various world populations. The study aim was to investigate the involvement of HLA class II alleles in genetic susceptibility to RHD in patients with relatively homogeneous clinical manifestations, in Pakistan.

**Methods:** Blood samples were collected from 114 unrelated patients (94 females, 20 males) with rheumatic mitral valve disease, predominantly mitral stenosis, as assessed by echocardiography. The control group comprised 109 unrelated, ethnically matched, healthy individuals (60 females, 49 males) with normal echocardiograms. Genomic DNA was extracted from venous blood using a standard phenol/chloroform extraction procedure. HLA-DRB, -DQA1, and -DQB1 alleles were typed using polymerase chain reaction with sequence-specific primers. HLA allele and haplotypes frequencies were then calculated.

Rheumatic fever (RF) and rheumatic heart disease (RHD) are among the leading causes of premature death and disability in the developing world (1). Recent studies have shown a high prevalence of RHD in both urban (22 per 1,000 population) and rural (5.7 per 1,000) areas of Pakistan (2,3).

Rheumatic fever occurs following throat infection by group A *Streptococcus pyogenes*, while RHD usually develops at four to eight weeks after streptococcal infection in approximately 30% of individuals with RF (4). Although the pathogenic mechanisms involved are

**Results:** A significantly higher frequency of DRB1\*07 was observed in patients as compared to controls (one-way parametric analysis of variance,  $F = 4.84$ ,  $p = 0.028$ ; OR = 1.76,  $p = 0.039$ ). No alleles for the HLA-DQA1 or -DQB1 loci were associated with the disease. HLA-DRB1\*07-DQA1\*0501-DQB1\*02, the only haplotype that differed significantly between patients and controls (one-way parametric Anova,  $F = 4.866$ ,  $p = 0.028$ ; OR = 7.33,  $p = 0.06$ ), did not exhibit significant linkage disequilibrium.

**Conclusion:** These results show that HLA-DRB1\*07, associated with RHD in various world populations, is also associated with RHD in the Pakistani population. The validation of HLA associations with RHD, which is observed in different world populations, may lead to the development of a cost-effective strategy in the primary prevention of this disease.

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still not completely understood, the immune system plays an important role in the development of RF and RHD. Molecular similarity between the M protein of streptococci and human heart tissue acts as a triggering factor, leading to autoimmunity in individuals with a genetic predisposition (5).

The results of several studies have shown that a genetic susceptibility to RF and RHD is linked to the human leukocyte antigen (HLA) system, particularly the class II loci (6-15). The study aim was to determine the role of the HLA class II alleles in Pakistani RHD patients.

## Materials and methods

### Patients

A total of 114 RHD patients (94 females, 20 males; mean age  $29 \pm 13.5$  years) attending clinics for secondary prophylaxis at two tertiary healthcare institutes,

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Table I: Clinical details of patients with rheumatic heart disease (RHD) (n = 114).

Age at RHD onset (years)	Gender	Severity of mitral stenosis			Total
		Mild	Moderate	Severe	
≤25	Male	6	2	7	15
	Female	14	10	48	72
>25	Male	0	1	4	5
	Female	4	6	12	22
Total		24	19	71	114

the Pakistan Institute of Medical Sciences (PIMS) Islamabad (which is a World Health Organization focal point for the prevention of rheumatic fever; n = 89), and the Armed Forces Institute of Cardiology, Rawalpindi (n = 25), were included in the study. In order to analyze a clinically homogeneous group, only those RHD patients who fulfilled the two-dimensional M-mode and Doppler echocardiography criteria (16) for mitral valve disease were recruited. Among these patients, 26 had mixed mitral stenosis and mitral regurgitation; the clinical details are listed in Table I.

The control group comprised 109 healthy individuals (60 females, 49 males) with normal echocardiograms. Both, patients and controls were from a similar ethnic background, and belonged to various castes and tribes from northern Punjab and the North West Frontier Province in Pakistan.

Venous blood samples were obtained with the informed consent of the individuals, or their legal guardians. The study protocol received the approval of the institutional bioethics committee in March 2003.

#### Genetic studies

Genomic DNA was extracted from blood samples using standard organic extraction protocols (17). HLA typing for class II alleles (DRB1, DRB3, DRB4, DRB5, DQA1 and DQB1) was carried out using the polymerase chain reaction with sequence-specific primers (18,19).

#### Data analysis

Hardy-Weinberg equilibrium, maximum likelihood estimate of allele and three-locus haplotype frequencies for the HLA class II loci, were calculated using Arlequin version 3.1 software (<http://cmpg.unibe.ch/>

software/arlequin) (20). Linkage disequilibrium and its significance were calculated as described by Imanishi et al. (21). The one-way parametric analysis of variance option of the Statistical Package for Social Sciences (SPSS) was used to calculate significant variation between the class II allelic frequencies and three-locus haplotypes in the patients and controls (22). The samples in which only one allele was detected at any of the three loci were considered to be homozygous for that locus. Odds ratios (OR) with 95% confidence intervals (CI) were estimated using the Calculator for Confidence Intervals for Odds Ratio (<http://www.hutchon.net/ConfidOR.htm>) (23). The significance of the OR was calculated by the 2x2 chi-square contingency test with Yates' correction for continuity (<http://faculty.vassar.edu/lowry/VassarStats.html>).

#### Results

Deviation from Hardy-Weinberg equilibrium, due to a deficiency of heterozygotes, was observed for HLA-DQA1 and HLA-DQB1 loci in both patients and controls (Table II). Although the samples belonged to unrelated individuals, this deviation from equilibrium could be the result of inbreeding following many generations of consanguineous marriages in Pakistan. It is also possible that a higher resolution sequence-based HLA typing may resolve the observed shift from Hardy-Weinberg equilibrium.

The frequencies of the HLA class II alleles in patients and controls, the results of the analysis of variance and OR are listed in Table III. Although the DRB1\*07 allele was present at a significantly higher frequency in patients as compared to the controls (p = 0.028), its

Table II: Hardy-Weinberg equilibrium in patients and controls.

HLA	Heterozygosity in patients		p-value	Heterozygosity in controls		p-value
	Observed	Expected		Observed	Expected	
DRB1	0.851	0.875	0.206	0.835	0.871	0.456
DQA1	0.614	0.841	0.000	0.771	0.829	0.017
DQB1	0.588	0.866	0.000	0.541	0.856	0.000

Table III: HLA class II allele frequencies in patients and controls, with results of analysis of variance (F-test value and significance) and odds ratios (OR) (95% CI).

HLA	Patients (n = 114)	Controls (n = 109)	Analysis of variance		OR (95% CI)
			F-test value	p-value	
<b>DRB1*</b>					
*01	0.070	0.023	2.993	0.084	2.20 (0.90-5.39)
*03	0.114	0.147	0.779	0.378	0.78 (0.45-1.35)
*04	0.079	0.096	0.421	0.517	0.80 (0.42-1.55)
*07	0.210	0.128	4.840	0.028	1.76 (1.06-2.86)**
*08	0.009	0.009	0.002	0.964	0.96 (0.13-6.83)
*09	0.004	0.028	3.878	0.050	0.22 (0.05-1.00)
*1001	0.061	0.041	0.920	0.338	1.51 (0.65-3.49)
*11	0.066	0.092	1.036	0.309	0.70 (0.35-1.39)
*12	0.013	0.018	0.194	0.660	0.72 (0.16-3.18)
*13	0.101	0.078	0.245	0.621	1.17 (0.62-2.22)
*1302	0.018	0.023	0.005	0.943	0.96 (0.27-3.34)
*14	0.053	0.055	0.013	0.910	0.95 (0.42-2.17)
*15	0.197	0.252	1.359	0.244	0.77 (0.49-1.20)
*16	0.004	0.009	0.376	0.540	0.96 (0.13-6.83)
<b>DQB1</b>					
*02	0.259	0.266	0.004	0.948	0.99 (0.65-1.50)
*0301	0.114	0.133	0.374	0.541	0.84 (0.47-1.48)
*0302	0.031	0.028	0.209	0.648	1.28 (0.44-3.71)
*030302	0.031	0.000	2.894	0.090	7.13 (0.74-68.98)
*0305	0.000	0.005	1.046	0.307	0.13 (0.00-6.52)
*04	0.009	0.005	0.291	0.590	1.87 (0.19-18.06)
*0501	0.114	0.069	2.278	0.132	1.65 (0.86-3.15)
*0502	0.040	0.064	0.296	0.587	0.79 (0.34-1.86)
*0503	0.066	0.083	0.720	0.396	0.74 (0.37-1.49)
*0601	0.118	0.170	2.389	0.123	0.66 (0.39-1.12)
*0602	0.048	0.023	1.500	0.221	1.90 (0.68-5.32)
*0603	0.145	0.115	1.198	0.274	1.36 (0.78-2.38)
*0604	0.026	0.041	0.766	0.382	0.63 (0.23-1.77)
<b>DQA1</b>					
*0101	0.044	0.018	2.414	0.121	2.50 (0.90-7.00)
*0102	0.149	0.142	0.008	0.929	1.05 (0.63-1.77)
*0103	0.241	0.238	0.042	0.837	1.07 (0.69-1.65)
*0104	0.118	0.110	0.017	0.895	1.04 (0.58-1.87)
*0201	0.132	0.124	0.068	0.794	1.11 (0.64-1.93)
*0301	0.092	0.115	0.588	0.444	0.82 (0.45-1.51)
*0401	0.018	0.009	0.595	0.441	2.28 (0.51-10.13)
*0501	0.202	0.238	0.832	0.362	0.83 (0.53-1.30)
*0601	0.004	0.005	0.001	0.997	0.96 (0.06-15.34)

\*\*Significant OR value (p <0.05).

frequency did not vary significantly with the gender, age of onset, or severity of the disease in the patients. An OR of 1.76 was obtained for the DRB1\*07 allele, which was statistically significant (p = 0.039). The OR for all other HLA class II alleles observed in the samples were not statistically significant (Table II). The high OR of 7.13 obtained for the DQB1\*030302 allele was most likely due to its absence in the control sam-

ples and its presence in only three patients.

The three locus haplotype frequencies for the HLA loci are listed in Table IV. DRB1\*07-DQA1\*0501-DQB1\*02 was the only haplotype to show any significant difference in frequency between the two experimental groups (p = 0.028). However, this haplotype did not exhibit significant linkage disequilibrium.

Table IV: Three-locus haplotype frequencies in RHD patients and controls. Only haplotypes with frequency greater than 2% in either patients or controls are shown.

DRB1-DQA1-DQB	Patients		Controls		Analysis of variance		Odds ratio	
	HF (%)	LD (×100)	HF (%)	LD (×100)	F-test value	p-value	(95% CI)	p-value
07 0201 02	9.2	8.5	7.3	6.9	0.511	0.475	1.31 (0.64-2.67)	0.476
03 0501 02	8.8	8.2	13.8	12.8	2.792	0.095	0.56 (0.30-1.06)	0.079
13 0103 0603	8.7	8.3	7.8	7.6	0.138	0.710	1.15 (0.57-2.34)	0.722
15 0103 0601	7.5	7	11.2	10.2	1.305	0.254	0.65 (0.33-1.30)	0.294
11 0501 0301	6.1	6	9.2	8.9	1.455	0.228	0.62 (0.30-1.31)	0.263
1001 0104 0501	4.8	4.7	3.2	3.2	0.747	0.388	1.56 (0.58-4.17)	0.464
14 0104 0503	3.9	3.9	4.6	4.5	0.111	0.739	0.85 (0.33-2.18)	0.821
15 0102 0602	3.1	2.9	1.4	1.3	1.457	0.228	2.31 (0.58-9.18)	0.330
01 0101 0501	2.6	2.6	0.9	0.9	1.858	0.174	2.97 (0.59-15.06)	0.281
04 0301 0302	2.2	2.2	2.3	2.3	0.005	0.943	0.95 (0.27-3.39)	1.000
07 0501 02**	2.2	1.1	-	-	4.866	0.028	7.33 (1.25-43.02)	0.060
15 0102 0502	2.2	2.1	4.1	3.9	1.371	0.242	0.51 (0.17-1.57)	0.277
15 0102 0601	2.1	1.8	3.9	3.3	1.965	0.162	0.45 (0.15-1.37)	0.186
04 0301 02	1.8	1.6	2.8	2.5	0.505	0.478	0.62 (0.17-2.28)	0.531

\*\* Not significant LD ( $p = 0.071$ ). All other haplotypes show significant LD ( $p < 0.01$ ).  
CI: Confidence interval; HF: Haplotype frequency; LD: Linkage disequilibrium.

## Discussion

The identification of host genetic factors that confer protection or increase the risk of RHD is an important first step in understanding the mechanism of progression of this disease. More importantly, it would aid in the design of a strategy for the control and prevention of the condition.

This association of the DRB1\*07 allele with RHD in Pakistani patients is concordant with the findings of other studies carried out in several countries, including Brazil (8,9), Egypt (10), Latvia (12), and Turkey (14,15). Functionally, it has been shown that the DRB1\*07-DR53-positive RHD patients present the M5 region of the streptococcal antigen to T cells during infection. These stimulated T cells then migrate to the heart and damage heart tissue due to cross-reactivity with several heart proteins, including vimentin, cardiac myosin and other valve-derived proteins (24).

A comparison of the three-locus haplotype frequencies for HLA -DRB1-DQA1-DQB1 showed that the only haplotype (DRB1\*07-DQA1\*0501-DQB1\*02) to show significant variation between the two groups was not in significant linkage disequilibrium, and the difference observed could most likely be attributed to the HLA-DRB1\*07 allele. The high OR of 7.33 obtained for this haplotype was also insignificant ( $p = 0.06$ ). The haplotypes DRB1\*07-DQA1\*0201-DQB1\*02 and DRB1\*15-DQA1\*0103-DQB1\*0601 associated with predisposition and resistance to RHD, respectively, in a

study from Egypt (10), were not associated with RHD in Pakistan. Similarly, the haplotype DRB1\*13-DQA1\*0103-DQB1\*0603, which was absent in the patient group of the same study, was present at comparable frequencies in both patients and controls in Pakistan.

These results corroborate the findings of previous studies that have demonstrated an association of HLA-DRB1\* 07 with a predisposition to RHD. Recent data have shown the potential of genetic testing in providing a cost-effective strategy for the primary prevention of rheumatic fever and its sequelae (25). It is hoped that a knowledge of individual susceptibility will prove helpful in the development of effective prophylactic strategies for this disease in Pakistan.

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## References

1. Eisenberg MJ. Rheumatic heart disease in the developing world: Prevalence, prevention and control. *Eur Heart J* 1993;14:122-128

2. Sadiq M, Islam K, Abid R, Latif F, Azhar M, Khan JS. Prevalence of rheumatic heart disease in school children of inner Lahore. *Proceedings, Symposium on Rheumatic Heart Disease* 2003;14
3. Rizvi SF, Khan MA, Kundi A, Marsh DR, Samad A, Pasha O. Status of rheumatic heart disease in rural Pakistan. *Heart* 2004;90:394-399
4. Gibofsky A, Khanna A, Suh E, Zabriskie JB. The genetics of rheumatic fever. Relationship to streptococcal infection and autoimmune disease. *J Rheumatol Suppl* 1991;30:1-5
5. Dale JB, Beachey EH. Multiple, heart-cross-reactive epitopes of Streptococcal M proteins. *J Exp Med* 1985;161:113-122
6. Ayoub EM, Barrette DJ, MacLaren NK, Krischen JP. Association of Class II histocompatibility leukocyte antigens with rheumatic fever. *J Clin Invest* 1986;77:2019-2026
7. Maharaj B, Hammond MG, Appadoo B, Leary WP, Pudifin DJ. HLA-A, B, DR and DQ antigens in black patients with severe chronic rheumatic heart disease. *Circulation* 1987;76:259-261
8. Guilherme L, Weidebach W, Kiss MH, Snitocowsky R, Kalil J. Association of human leukocyte class II antigens with rheumatic fever or rheumatic heart disease in a Brazilian population. *Circulation* 1991;83:1995-1998
9. Visentainer JE, Pereira FC, Dalalio MM, Tsuneto LT, Donadio PR, Moliterno RA. Association of HLA DR 7 with rheumatic fever in the Brazilian population. *J Rheumatol* 2000;27:1518-1520
10. Guedez Y, Kotby A, El Demellawy M, et al. HLA class II associations with rheumatic heart disease are more evident and consistent among clinically homogenous patients. *Circulation* 1999;99:2784-2790
11. Hernandez-Pacheco G, Aguilar-Garcia J, Flores-Dominguez C, et al. MHC class II alleles in Mexican patients with rheumatic heart disease. *Int J Cardiol* 2003;92:49-54
12. Stanevecehia V, Eglite J, Socheves A, Gardovska D, Zavadska D, Shantere R. HLA class II association with rheumatic heart disease among clinically homogenous patients in Latvia. *Arthritis Res Ther* 2003;5:340-346
13. Koyanagi T, Koga Y, Nishi H, et al. DNA typing of HLA class II genes in Japanese patients with rheumatic heart disease. *J Mol Cell Cardiol* 1996;28:1349-1353
14. Ozkan M, Carin M, Sonmez G, Senocak M, Ozdemir M, Yakut C. HLA antigens in Turkish race with rheumatic heart disease. *Circulation* 1993;87:1974-1978
15. Haydardedeoglu FE, Tutkak H, Kose K, Duzgun N. Genetic susceptibility to rheumatic heart disease and streptococcal pharyngitis: Association with HLA-DR alleles. *Tissue Antigens* 2006;68:293-296
16. Braunwald E, Zipes DP, Libby P. *Heart Disease: A Textbook of Cardiovascular Medicine*. 6th edn. WB Saunders Company, Philadelphia, 2001
17. Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual*. 3rd edn. Cold Spring Harbor Laboratory Press, New York, 2001
18. Bunce M, O'Neill CM, Barnardo MCNM, et al. Phototyping: Comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR SSP). *Tissue Antigens* 1995;46:355-367
19. Olerup O, Aldener A, Fogdell A. HLA- DQB1 and DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. *Tissue Antigens* 1993;41:119-134
20. Schneider S, Roessli D, Excoffier L. Arlequin: A software for population genetics data analysis Ver 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva, 2000
21. Imanishi T, Akaza T, Kimura A, et al. Estimation of allele and haplotype frequencies for HLA and complement loci. In: Tsuji K, Aizawa M, Sasazuki T (ed.). *HLA 1991. Proceedings of the Eleventh International Histocompatibility Workshop and Conference, Vol. I*. Oxford University Press, Oxford, 1992:76-79
22. Voelkl KE, Gerber SB. *Using SPSS for Windows: Data Analysis and Graphics*. Springer-Verlag, New York, 1999
23. Bland JM, Altman DG. *Statistics notes: The odds ratio*. *Br Med J* 2000;320:1468
24. Guilherme L, Oshiro SE, Fae KC, et al. T-cell reactivity against streptococcal antigens in the periphery mirrors reactivity of heart-infiltrating T lymphocytes in rheumatic heart disease patients. *Infect Immun* 2001;69:5341-5345
25. King CH, Fischler DF, Gerkin RD. Will genetic testing alter the management of disease caused by infectious agents? A cost-effectiveness analysis of gene-testing strategies for prevention of rheumatic fever. *Clin Infect Dis* 2002;34:1491-1499