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Unveiling the Impactof HLA-DRB1*0401 Allele inRheumatic Heart Disease: An Insightful Investigation

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Abstract
Rheumatic heart disease (RHD), a chronic condition, results from rheumatic fever—an inflammatorydisease caused byuntreatedstreptococcalinfections. The human leukocyte antigen (HLA) system, specifically the HLA DRB1 gene, has been strongly associated with the development and progression of RHD. Certain HLA DRB1 alleles, such as HLA-DRB1*01 and
*04, have been identified as major genetic risk factors for RHD. These alleles playa crucialrole in the activation of the immune systemand the generation of auto antibodies, contributing to the pathogenesis ofRHD. Understanding the connectionbetweenHLADRB1 and RHD canprovide insights into disease mechanisms and potentially guide personalized treatment approaches. This research employed a case-control study design to investigate the significance of HLA DRB1 in rheumaticheartdisease(RHD).Atotalof40participantswereincluded inthestudy,consistingof
30 female and 10 male individuals. The participantswererecruited froma specialized cardiology
clinic,basedontheirdiagnosisofrheumatoidarthritis(RA)withorwithoutRHD.Genomic DNA was extracted from peripheral blood samples collected from each participant. The genotyping of
HLADRB1alleleswasperformedusingpolymerasechainreaction(PCR)amplificationfollowed by sequence-specific oligonucleotide (SSO) hybridization or allele-specific PCR. These techniques allowed for the identification of specific HLA DRB1 alleles in each participant. The values were

analyzed with SIGMAPLOT 13 using SYSTAT software, USA using One way ANOVA.TheresultswereexpressedasmeanSEMandthevalueswithP<0.05wereconsidered statistically significant. A comprehensive investigation conducted on both preoperative and postoperative patients revealed a robust correlation between the disease-associated HLA-DRB1 allele combination and the clinical presentation. The study findings not only demonstrated the involvement of this allele combination in the disease but also highlighted its potential in determining the severity of the condition.
Keywords:Autoantibodies,HLADRB1,Polymerasechainreaction,Rheumatic heartdisease.

Introduction

Rheumatic heart disease, a global affliction, impacts a staggering 40 million individuals worldwide. Withadevastatingtollofover300,000 lives lost eachyear, it constitutes nearly2%of all cardiovascular disease-related deaths, solidifying its position as the leading cause of mortality ona globalscale. The initialcause leading to autoimmunityin cases ofRheumatic fever (RF) and Rheumatic heart disease (RHD) is believed to be molecular mimicry between streptococcal and human proteins (Dajani et al., 1992). Mutations in various genes, including those responsible for components of the immune system such as ficolin, have been linked to ARF and RHD. Dysfunction or abnormal expression of ficolins can significantly influence the etiology of autoimmune illnesses (Catarino et al., 2021). While several studies have associated HLA class II alleles with genetic vulnerability to RF and RHD, there is some confusion regarding the specific types of susceptibility and protective alleles (Valdastanevicha et al., 2003). This confusion may arise fromthe use of less precise serological HLA typing techniques, leading to inaccurate results and failure to differentiate between allelic subgroups (Carapetis et al., 2005 & Wilson, 1954).

Moreover, racial variations in the distribution ofHLA alleles and the contribution ofother genes with distinct linkage disequilibrium patterns may contribute to these apparent disparities in different populations (Hunte et al., 2000) Another confounding factor in several studies is the inability to distinguish between subgroups of RHD or segregate RF patients with and without carditis, which hampers the investigation of genetic predisposition to RHD. Many studies have not attempted to analyze the HLA class II connections with specific clinical manifestations of RHD . Consequently, it remains unclear whether a connection exists between class II allotypeand RHD and, ifso, whether it varies among patients ofdifferent racialor ethnic backgrounds or persists across all patients with the same pattern of valve lesions (Dasgupta et al., 2009 &Mirabel et al., 2012). The rationale for this study is to explore the potential role of HLA DRB1 alleles in the context of rheumatic heart disease, aiming to advance our understanding of the genetic basis of RHD and potentially provide valuable insights for clinical management and interventions in patients with rheumatoid arthritis. Moreover, the existing research has shown a strong link betweenthehumanleukocyte antigen(HLA)system, particularly HLADRB1alleles, and the susceptibilityto rheumatoid arthritis (Andreu et al.,1999). Since bothRAand RHD have anautoimmune component, it isplausible to investigate whether specific HLADRB1 allelesplay a role in the development of rheumatic heart disease. This study aims to identify the presence of the HLA-DRB1*0401 allele in patients diagnosed with RHD.

Methodology

This research is a case-control study designed to investigate the significance of HLA DRB1 in rheumatic heart disease (RHD). The study was initiated after obtaining permission from the institutional ethics committee, which is referenced with a specific number and date (VMKVMC&H/IEC/21/008).TheinclusioncriteriaareindividualsdiagnosedwithRheumatic Heart Disease (RHD), known history of Rheumatic fever (RF) and individuals who possess the HLA-DRB1*0401allele.TheexclusioncriteriaareRHDpatientswithcoexistingconditionssuch asdiabetesorrheumatoidarthritisandindividualswhoareunableorunwillingtoprovideinformed consent for participation in the study. A total of 40 participants were included in the study, consistingof30femaleand10maleindividuals.Theparticipantswererecruitedfromaspecialized cardiologyclinic,basedontheirdiagnosisofrheumatoidarthritis(RA)withorwithoutRHD.The sample size of 40 participants was determined based on practical considerations and the availabilityofeligible participants withinthe studytimeframe. While a larger sample sizewould

have been ideal, the selected sample size aimed to provide preliminary insights into the significance of HLA DRB1 in rheumatic heart disease. Future studies with larger cohorts could build upon these findings.

All of these individuals had documented histories of Rheumatic fever (RF) and subsequently developed Rheumatic heart disease (RHD). Among them, 32 individuals developed mitral valve lesions, two individuals developed aortic valve lesions, and six individuals developed multiple valvular diseases. Since both the innate and adaptive immune responses play a role in the development ofRFandRHD, it is crucialto identifythegene(s)responsible forthepathogenesis of the disease. Genetic testing will assist us in gaining a better understanding of how the disease progresses (Walsh et al., 1991 & Alexseyev et al., 1998). The study details were clearly explained, and written consent was obtained from all the subjects. Genomic DNA was extracted from peripheral blood samples collected from each participant. The genotyping of HLA DRB1 alleles was performed using polymerase chain reaction (PCR) amplification followed by sequence-specific oligonucleotide (SSO) hybridization or allele-specific PCR (Olerup, 1992 & Sallakci et al., 2005). These techniques allowed for the identification of specific HLA DRB1 alleles in each participant

Approximately5 ml of blood was collected in EDTA tubes fromthe median cubital vein. Blood samples were obtained from both preoperative and postoperative RHD populations, which were further categorized into two groups. This studywas conducted at Dhanyaa Cardiac and Diabetic Centre inSalem. The reports werereviewed bya cardiologist, and genetic testing was conducted at Bright Care Molecular Centre. Samples were collected from M/s. Dhanyaa Cardiac Center,and the serum was separated before sending the samples to the laboratory for analysis. All the results have been documented. A total of forty samples were collected from individuals of variousagegroups,including boththosewho underwent surgeryandthosewho did not.Toavoid misleadingpositiveresults, RHDpatientswithcomplicationsofdiabetesandrheumatoidarthritis wereexcludedfromthestudy. DNAsampleswereobtained frombloodusing aDNAisolationkit (Qiagen, Germany) and analyzed to determine the presence of the HLA-DRB101 allele group. Commercially available kits (ProTrans GmbH, Ketschau, Germany) were used for the typing process. The HLA-DRB10401-specific primer sequence, as mentioned in Table 1, was used for SingleSpecificPrimer(SSP)binding.TheannealingtemperatureforPCRprimerswasestablished byconductingagradientPCRreactionwithDNAsamples,usingthefirstexonoftheHLA-DRB1

geneasaninternalcontrol.ThePCR-SSPstechniquewascarriedoututilizing2xPCRMasterMix Solution. The resulting PCR product was then loaded into wells of a 2.3% agarose gel, and a separate ladder (100 bp) (ThermoFisher Scientific, USA) was used to determine the size of the PCR product. To visualize the DNA, ethidium bromide (1.5µl) was added to the buffer, and the gelwasexaminedunderanultraviolet transilluminatorequippedwith ageldocumentationsystem (BioRad, USA) (Erlich et al., 1991 & Hasegawa et al., 1985).

All the statistical analyses were done in SPSS software version 12 using descriptive Statistics to summarize the characteristics of the DNA samples, including sample size, mean, standard deviation, and frequency distribution. And chi-Square Test was conducted to evaluate the independencebetweenthepresenceoftheHLA-DRB101allelegroupandtheDNAsamples.This testhelpeddetermine iftherewasasignificantassociationorcorrelationbetweenthevariables. A predetermined significance level (e.g., $p < 0.05$) is typically used to determine statistical significance (Librado & Rozas, 2009 & Kumar, 2016).

ResultsandDiscussion

White blood cells contain genetic abnormalities known as human leukocyte antigen (HLA)-DRB10401alleles.Thepresenceoftheseallelesincreasesthelikelihoodofdevelopingrheumatoid arthritis (RA) (Walsh et al., 1992). The DR4 serogroup, which includes various MOD frequency alleles, is widely distributed across different populations and has been associated with several clinicaldisorders (Mehta et al., 1985). HLA-DR4 is a member ofthe HLAcomplexgene family, which plays a crucial role in the immune system's ability to distinguish between the body's own proteins and those produced by pathogens like viruses and bacteria. The separation of PCR products in Figure 5 demonstrates the presence of the DRB10401 allele (indicated by an arrow) in lanes 2, 5, and 6, while lanes 3 and 4 show the absence of the DRB10401 allele. Similarly, in Figure 4,the separationofPCRproducts in Lanes1 to 7 confirmsthe presence ofthe DRB10401 allele with a 260 bp amplified product. Among the 40 samples from different categories, 10 samplesexhibitedpositiveresultswith variations in gene expression levels, as depicted in a chart. The age group above 40 showed a higher percentage of gene expression, while the age group below 40 showed a lower percentage. Failure to receive proper treatment in individuals below 40 years of age can lead to secondary complications such as Sydenham's chorea and an increased risk of mitral and multivalvular lesions (Hernández-Pacheco et al., 2003 & Bodis, 2018). Prophylactic treatment helps reduce these secondarycomplications. Inthe post- operative group, genetic testing should be conducted a few months after valve replacement, while echocardiography indicates normal functioning of the replaced valve. In cases where the genetic

test yields positive results in the post-operative group, regular monitoring through echocardiographyandrepeatinggenetictestscanhelpmitigatefurthercomplicationsandincrease awareness among cardiologists.

In this study, we conducted a comparison of gene expression based on gender. Male patients exhibited a mean deviation of 2.22+0.82 in gene expression, while female patients had a mean deviation of 2.30+0.75 in gene expression levels. Although the difference was not significant, female patients showed a slightly higher level ofgene expression. The statistical data illustrating this comparison is presented in Figure 2. Additionally, Table 3 presents a comparison of gene expression levels in Rheumatic Heart Disease (RHD) patients with valvular lesions, divided into two groups: preoperative and postoperative patients. Interestingly, postoperative patients demonstrated ahigher levelofgene expressioncompared to preoperativepatients, withap-value of0.052ns.Furthermore,wecategorizedthepatientsbasedonageintotwogroups:above40years andbelow40years.Amongthesegroups,weobservedthatamajorityofthepostoperativepatients were above 40 years, with a standard mean value of 44.25+6.50. Conversely, most of the preoperative patients fell under the category of below 40 years, with a standard deviation of 33.50+19.72. The statistical analysis indicated a non-significant p-value of 0.331 NS.

The given RHD population displayed a genetic analysis result of 25.0%. The summary table provides an overview of the genetic diversity analysis conducted on the study population. The individualsinthestudypopulationwerecategorizedintocontrol,unoperated,andoperatedgroups. Thegeneexpressionlevels variedamongtheindividuals. Genetic variationwasobserved inboth unoperatedandoperatedRHDpatients.Thepresentfindingsindicateahighmutationrateof2.80 ±0.60%intheoperatedsubjects.ItispossibleforunoperatedRHDpatientstodevelop

cardiomyopathyas aconsequenceofRHD,resulting inpermanentvalvedamageandtheapparent induction ofthe disease in them. In a thorough examination encompassing both preoperative and postoperative patients, a strong associationwasobserved betweenthe disease-linkedcombination ofHLA-DRB1allelesandtheclinicalmanifestations. Thestudycategorizedpatientsintotwo age groups, below 40 and above 40, with those above 40 exhibiting a greater likelihood of elevated gene expression levels. Additionally, female patients showed a higher level of gene expression. Notably, postoperative cases displayed a mutation rate of 2.80 ± 0.60 %.

Blood samples revealed the presence of mutations in autoimmune response-related cells, particularly in macrophages, T and B lymphocyte cells. Self-reactive T cells were observed to migrate from peripheral circulation to the heart and undergo multiplication in the valves in responsetospecificcytokines'activation.TheelevationoftheT cellactivationmarker,HLA-DR, is a known characteristic of autoimmune diseases (Donadi et al., 2000). Valda Stanevicha et al. suggested that HLA DRB0401 alleles were a risk factor for acquiring rheumatic fever (RF) and rheumatic heart disease (RHD) (Kaplan, 1964). Focusing on RHD patients with multivalvular involvement, there is a likelihood of carditis during their RF attacks. Yajaira Guedez et al. reported that most patients never experienced carditis during RF, but HLA class II association with RHD was more evident (Guilherme et al., 2000). Similarly, Fatouballa Wade observed genetic differences in MT-CYB between preoperative and postoperative populations, with mutations being neutral in operated patients and positive in unoperated patients (Valda et al 2003). Our study confirms that HLA DRB0401 is positive even in unoperated patients. Yitian Zhou, Kristi Krebs, et al. stated that frequencies of HLA alleles and haplotypes varyconsiderablyamongracesandethnicgroups(Fatouet al., 2019),whereasT.Koyanagietal.found no statistically significant association with HLA DRB1 alleles based on age, gender, and the presence of combined aortic valvular diseases (Yitian & Kristi, 2020). Our study suggests that females below the age of 40 are more affected by RHD than males(Koyanagi et al., 1996). Inour examination of two sets of RHD patients, preoperative and postoperative, we observed positive bands after surgery in those with mitral and multiple valve dysfunction. The risk of the DRB1 0401 mutation was more prevalent in mitral valvular lesions.Patientsabovetheageof40 displayed highlevelsofgene expression,while those under 40 displayed neutral to low levels of gene expression. Additionally, our study revealed that women are more likely to be impacted based on their gender. Nevertheless, diverse research has

been conducted in different locations, but there is a lack of reported data on Salem populations. Therefore, we conducted a pilot study on HLA class type II to explore the relationship between RHD and allelesbydividing patientsinto potentiallymore homogeneoussubgroups, asobserved in Salem groups of RHD patients.

Despite the limited sample size, this study suggests that specific HLA class II genetic variations were a risk factor for developing RHD in the Salem community. This study confirms the relationship between RHD and HLA DRB 1004 that has been reported in prior studies. Additionally, this studyoffered the hypothesis that inthe Salempopulation, HLADRB1 ISTHE RiskforRHD.Immunecellstargetproteins invalvularcellsthatresemblethoseofStreptococcus pyogenes (Chand et al., 2020). The mechanism underpinning autoimmunity would be correctedif the pathology's sources were eliminated, and the cells engaged in the body's defense against Streptococcus of Group A and autoimmune would resume normal function in the following generation. According to selection analysis, mutations in both operated and unoperated individuals are being positively selected for. These results are consistent with earlier research; HLA DRB 1*0401 mutations frequentlyoccur in patients who have not had surgery, and people who have had surgery also exhibit good results with lower levels of gene expression. These variations have a harmful effect onthe protein, and an increase in their frequency would worsen the condition while also causing more protein damage. Since these mutations were ultimately found inoperatedpatients,theamount ofgeneexpressionissignificantlylower inthosewho still have them following valve replacement. The awareness of Rheumatic fever will be helpful in prevention of RHD and various complications. Giving penicillin in the early stage of RF may prevent thecomplication RHD.Thus, repetition of genetic text will be helpful to prevent further complications. This study will be extended in future.

Conclusion

In conclusion, our investigation on the impact of HLA-DRB10401 allele in Rheumatic Heart Disease(RHD)hasprovidedvaluableinsights.Weobservedthepresenceofgeneticabnormalities inwhitebloodcells,specificallytheHLA-DRB10401allele,whichhasbeenlinkedtoanincreased risk of developing RHD. The association of the DR4 serogroup with various clinical disorders further emphasizes the importance of HLA-DRB1*0401 in disease pathogenesis. Our study comparedgeneexpressionlevelsbasedongender,revealingslightlyhigherlevelsinfemale

patients compared to male patients, although the difference was not significant. Moreover, we found that postoperative RHD patients exhibited higher gene expression levels compared to preoperative patients with valvular lesions, indicating potential changes in gene regulation following valve replacement surgery. Additionally, age played a role in the study outcomes, as most postoperative patients were above 40 years of age, while preoperative patients were predominantly below 40 years. These findings suggest that age and surgical intervention may influence gene expression patterns in RHD patients. It is important to note that further research with larger sample sizes and comprehensive genetic analyses are needed to fully understand the implications of HLA-DRB1*0401 allele and gene expression in RHD. Nevertheless, our study contributestothegrowingbodyofknowledgesurroundingthegeneticfactorsandgeneexpression profilesassociatedwithRHD,whichcanpotentiallyaidinthedevelopmentoftargetedtreatments and personalized interventions for affected individuals.

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Table1.Nucleotideprimersequencesofmoleculartargets		
GENE	FORWARDPRIMER	REVERSEPRIMER
HLA-DRB1 gene as a internal control	5'-AGCATCTCTGACCAGCAACT-3'	5'-AGGCCCCTTACACAAGTCTC-3'
HLA-DRB1*0401	5′-TACTTCCATAACCAGGAGGAGA-3'	5′-TGCAGTACTTGTCCACCCG-3′

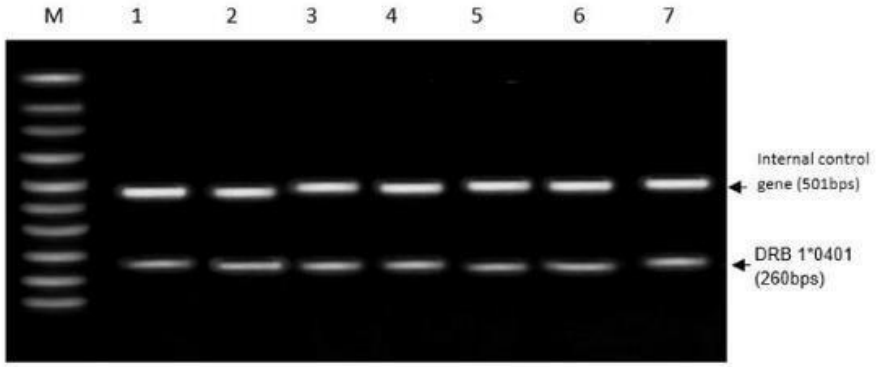
Table2positivegenderdistributionbetweenmaleandfemale

Variable	Positive Operative groups	F	Mean ±SD	T value	P value
Gene expression	Pre	6	1.90 ± 0.62	-2.277	**0.052 NS**
	Post	4	2.80 + 0.60		
Age	Pre	6	33.50 ± 19.72	-1.035	**0.331 NS**
	Post	4	44.25 ± 6.50		

Table3Meancomparisonforgender withgene expression

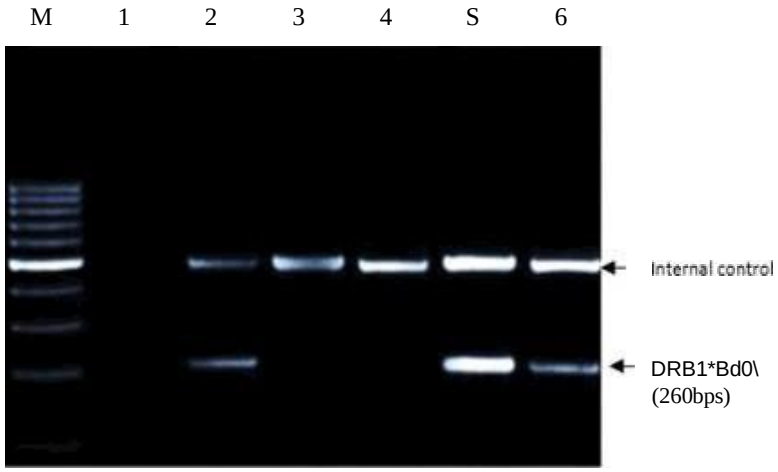
Variable	Gender	F	Mean ±SD	T value	P value
Gene expression	Male	5	2.22 ±0.82	-0.161	0.876 NS
	Female	5	2.30 ±0.75		
Age	Male	5	45.80 ± 17.99	1.731	0.122 NS
	Female	5	29.80 ± 10.18		

FIG 1: GENE EXPRESSION LEVELS OF (HLA) DRB 1*0401 BY PCR



Lane M- Marker lane (100-1000 bp); Lane 1 –Sample 1; Lane 2 – Sample 2; Lane 3 -Sample 3; Lane 4 –Sample 4; Lane 5 – Sample 5; Lane 6 – Sample 6; Lane 7 – Sample 7.

FIG2:GENEEXPRESSIONLEVELSOF(HLA)DRB1•0401BYPCR



LaneM-Marker lane(UO-1000bp);Lant1-Negativecontrol.Lane2 -Sample I,Lane3- Sample 2. Lane 4 -Sample 3: Lane S -Sample 4: Lane 6 - Sample 5.