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Original Article

Genetics Section

Association of Human Leukocyte Antigen Gene Variants rs13192471 and rs6457617 with Rheumatoid Arthritis Susceptibility: A Case-control Study from North-western India

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ABSTRACT

Introduction: Rheumatoid Arthritis (RA) is a systemic, chronic, inflammatory, and autoimmune disease which is characterised by the progressive ruination of joint structures. The Human Leukocyte Antigens (HLA) genes: HLA-DRB1 and HLA-DQB1, belonging to HLA family, presented on human chromosome six, are involved in the immune system. Various studies involving Genome-wide Association Studies (GWAS), meta-analysis, and replication studies have shown the association of HLA-DRB1 variant rs13192471 and HLA-DQB1 variant rs6457617 with RA susceptibility in different population groups.

Aim: To perform a replication case-control based association study of variants rs13192471 and rs6457617, in order to determine their association with RA, in an independent cohort from the population of North-western India.

Materials and Methods: The Case-control study conducted at Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar, Punjab, India, duration of the study was, from May 2019 to June 2022. In this study, Deoxyribonucleic Acid (DNA) was isolated from 188 RA cases and 310 healthy controls, followed by qualitative and quantitative analyses. The genotyping was performed on a Real Time-polymerase Chain Reaction (RT-PCR) using TaqMan Allele Discrimination Assay. Statistical power of the study was estimated using PS: Power and sample size calculator version 3.1.

Results: The statistical analysis of the genotyping data showed a significant association (p=0.005) of variant rs13192471 with RA susceptibility, whereas the variant rs6457617 did not show an association (p=0.275) with RA in the studied population cohort.

Conclusion: The present study successfully replicated the association of HLA-DRB1 variant rs13192471 in the population group of North-western India. It is pertinent to screen these variants for RA susceptibility in other population groups of India before their use as potential genetic biomarkers in the Indian population.

Keywords: Autoimmune diseases, Genome wide association study, Genotyping, TaqMan assay

INTRODUCTION

The rheumatoid arthritis is a chronic systemic autoimmune disease with immunologically arbitrated inflammation of synovia-lined joints that can significantly disrupt joint construction and task [1]. RA is an exemplar of an intricate genetic state with undetermined genetic diversity and origin, and it almost certainly involves several genes [2]. A persistent inflammatory joint illness RA is known to be caused by reactions of autoimmunity to Anti-citrullinated Peptide Antibody (ACPA) and Immunoglobulin G (IgG), frequently known to be Rheumatoid Factor (RF) [3]. Besides RF, RA patients often exhibit antibodies peculiar to other proteins, such as type II collagen (COL-II) and Human Cartilage glycoprotein 39 (HC gp-39) [4]. Regular demonstration of RA is generally polyarthritis with firmness, ache, and protuberance of multiple joints in a symmetric pattern [5].

The human Major Histocompatibility Complex (MHC) system genes, also known as the HLA family, are one of the most polymorphic genetic systems and control effector immunity units [6]. The heritability of RA is approximately 60%, to which HLA contributes around 11-37% [7]. *HLA-DRB1* and *HLA-DQB1* genes are located on chromosome 6, play an essential role in the immune system, and are well known for playing a role in RA pathogenesis [8]. A strong connection between RA and HLA alleles has long been reported. The DR and DQ loci of the *HLA* gene have been reported in strong linkage disequilibrium and associated with RA in many studies of Caucasian and East asian populations [9]. GWAS of RA also exhibited remarkable associations with various Single Nucleotide Polymorphisms (SNPs) near the *HLA-DRB1* gene with RA in European, Korean and Japanese populations. The significant SNPs with this association are rs7765379, rs6457617, rs660895 and rs13192471 [10-12]. HLA variant rs6457617 was also associated with RA in Spanish and Han Chinese populations [13,14]. HLA SNP rs6457617 was associated with RA susceptibility, while SNP rs13192471 showed no association with RA in Tunisian [12]. Indian populations are highly diverse. A study from North-eastern India showed an association of SNP rs13192471 in susceptibility and severity of RA, whereas SNP rs6457617 did not [15]. However, another study showed that both HLA SNPs, rs13192471 and rs6457617, are associated with a significant risk for RA in the Northern Indian population [16] which indicate genetic heterogeneity in RA.

As there is significant genetic heterogeneity in RA among different world populations, replication analysis studies to find the association of various susceptibility loci with RA in multiple populations is essential, making screening in the North-western Indian population critical. With this objective, the present study was performed in a case-control association designed to screen

HLA variants rs13192471 and rs6457617 in the North-western Indian population cohort.

MATERIALS AND METHODS

The present case-control study was conducted at Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar in association with Sri Mata Vaishno Devi University, Katra, Jammu and Kashmir, duration of the study was, from May 2019 to June 2022. The study was initiated after the due approval by the Institutional Ethics Committee (Ref no. Patho 339/19.dated 06/04/2019). After getting the written informed consent of the participants, a venous blood sample of 2 mL was obtained and transferred to Ethylenediaminetetraacetic Acid (EDTA) vials to avoid coagulation. The samples were transported to a laboratory, in an icebox and kept at-20ºC until the Deoxyribonucleic Acid (DNA) had been extracted. Participants included 188 ACR/EULAR (American College of Rheumatology/European League Against Rheumatism) Criterion confirmed RA cases, and 310 age and sex matched healthy controls.

Inclusion criteria: The minimum age for cases to include in this study was 18 years to avoid inclusion of juvenile RA cases in this study with no upper limit of age for the inclusion of cases. Same age criteria were kept for controls with disease free status related to any autoimmune disease, and any disease related to bone, articular cartilage, or endocrine system.

Exclusion criteria: For both cases and controls, individuals with conditions like cancer, diabetes mellitus, neurological disorders, thyroid dysfunction, infectious disease, arthritis other than RA for cases and females with pregnancy and nursing mothers were excluded.

Study Procedure

The isolation of DNA is done using a MagGenome® XpressDNA blood kit. Then agarose gel electrophoresis was performed to determine the quality of isolated DNA samples. The concentration of the DNA samples was determined using NanoDrop™ 2000/2000c (Thermo Scientific). The 50 µL dilutions (10 ng/µL) of DNA samples were prepared by adding nuclease-free water.

Genotyping of variant rs13192471 and rs6457617: Using the TaqMan allele discrimination test, the SNP genotyping of variants was assessed in the studied population group. Taqman® SNP genotyping assay from Applied Biosystems (Thermo Fischer Scientific, Pleasanton, CA, USA) was used for genotyping the variants rs13192471 and rs6457617 using Mx3005p Agilent RT-PCR (Stratagene Agilent Technologies, Waldbronn, Germany). The Taqman genotyping assay was diluted using TE (Tris EDTA) from 40X to 20X as directed by the manufacturer. The genotyping was performed using a 96-well plate with 93 samples and three Non Template Controls (NTC). The overall amount of the PCR reaction mix was 10 µL, including 3 µL of DNA concentration of 10 ng/µL. Rest of the constituents included 1µL 1X PCR buffer, 0.25 µL 0.5X of TaqMan assay, 0.2 µL 0.2 mM of dNTPs, 0.1 µL 0.3 U of Taq Polymerase, 5.45 µL of distilled water was used to make up the volume of the reaction. The cycling conditions consist of a 4-minute hold at 95°C, 40 cycles at 15-second intervals at 95°C, and one minute at 60°C with endpoint detection. The RT-PCR post-PCR detection equipment was used to assess the allele-specific fluorescence. Reruns were performed to determine final inclusion for samples that failed genotyping or had ambiguous genotypes.

STATISTICAL ANALYSIS

Statistical power of the study was estimated using PS: Power and sample size calculator version 3.1. The analysis of genotyping data was performed in PLINK version 1.09. An Odds Ratio (OR) was calculated to determine the chance of an outcome in response to exposure (allele or genotype). The value and 95% Confidence Interval (CI), which gives the relationship or assesses the likelihood of an outcome, were computed together. The OR indicates how frequently the patients are more at risk than controls. The Hardy Weinberg Equilibrium (HWE) was also calculated using the Chi-square test.

RESULTS

Most of the RA cases in this study cohort were females (146/188, 77.65%). For enrolled subjects, the average age was 48.43±12.08 years. The average age of onset of disease was 44.49±10.96 years. Of 188 RA cases, 68.61% of the patients were seropositive for RF. This study group's average Body Mass Index (BMI) was 24.72±3.78. The middle Waist-Hip Ratio (WHR) was 0.88±0.072. Only 3.19% of patients were smokers, and 5.85% took alcohol regularly. Around 62% of the patients were taking a purely vegetarian diet.

SNP Analyses

Genotypic analyses of single nucleotide polymorphism (SNP) rs6457617: The allelic distribution of variant rs13192471 was evaluated. The C allele was observed as a minor allele with a frequency of 0.18 (18%) in cases and 0.11 (11%) in controls in the studied cohort. The variant rs13192471 showed a significant association (p-value 0.005) with RA susceptibility and the T allele as a risk allele for RA with the OR of 1.77, 95% CI (1.18-2.64), in the present studied cohort. The allelic distribution of variant rs13192471 of *HLADRB1* was in not in accordance with HWE in both the cases as well as controls.

In order to further evaluate and increase the power of the study and overcome confounding factors effects, logistic regression analyses were adopted with the recessive (CT+TT *vs* CC) & dominant model (CT+CC *vs* TT), where TT is wild homozygous genotype, CT is heterozygous genotype and CC is mutant homozygous genotype. In the dominant model, the OR obtained was 1.96 (1.23-3.14 at 95% CI), p=0.004, adjusted with age, gender, BMI and ethnicity. The results showed a significant association of variant rs13192471 with RA in the population belonging to Northern India. The frequency of TT, CT and CC genotype was found to be 69.18% *vs* 81.51%, 25.34% *vs* 14.72% and 5.48% *vs* 3.77% in the RA cases and controls respectively. A Chi-square test of independence was performed to examine the relation between three genotypes between cases and controls. The result came to be significant with the p-value 0.016 [Table/Fig-1].

rs13192471	Frequency in cases (%)	Frequency in controls (%)	OR (95% CI)	p-value
Genotype				
Homozygous wild TT	69.18	81.51		0.016
Heterozygous variant CT	25.34	14.72		
Homozygous variant CC	5.48	3.77		
Allele frequency in decimal				
T allele	0.82	0.89	1.77 (1.18-2.64)	0.005
C allele	0.18	0.11		
Genetic model				
Dominant Model (CT+CC vs TT)			1.96 (1.23-3.14)	0.004
Recessive Model (CT+TT vs CC)			1.48 (0.57-3.83)	0.418

[Table/Fig-1]: Genotype and allele frequencies of rs13192471 polymorphism in RA patients and controls.
CI: Confidence level; OR: Odds ratio; p-value<0.05 is considered as significant

Genotypic analyses of single nucleotide polymorphism rs6457617: The allelic distribution of variant rs6457617 was evaluated. The T allele was observed as a minor allele with a

frequency of 0.38 (38%) in cases and 0.34 (34%) in controls in the studied cohort. The variant rs6457617 showed a non significant association (p-value 0.275) with RA susceptibility with an OR of 1.18, 95% CI (0.87-1.60), in the present studied cohort. The allelic distribution of variant rs6457617 of *HLADQB1* was in not in accordance with HWE in both the cases as well as controls. In order to further evaluate and increase the power of the study and overcome confounding factors effects, logistic regression analyses were adopted with dominant model (CT+TT *vs* CC) and recessive models (CT+CC *vs* TT), where CC is homozygous wild genotype, CT is heterozygous genotype and TT is homozygous mutant genotype. The results showed no significant association of variant rs6457617 with RA in the population belonging to Northern India (p>0.05). The frequency of CC, CT and TT genotype was found to be 44.12% *vs* 48%, 36.03% *vs* 36% and 19.85% *vs* 16% in the RA cases and controls respectively. A Chi-square test of independence was performed to examine the relation between these three genotypes in cases and controls. The result came to be non significant with the p-value 0.584 [Table/Fig-2].

rs6457617	Frequency in cases (%)	Frequency in controls (%)	OR (95% CI)	p-value
Genotype				
Homozygous wild CC	44.12	48		0.584
Heterozygous variant CT	36.03	36		
Homozygous variant TT	19.85	16		
Allele Frequency in Decimal				
C allele	0.62	0.66	1.18 (0.87-1.60)	0.275
T allele	0.38	0.34		
Genetic Model				
Dominant Model (CT+TT *vs* CC)			1.17 (0.77-1.77)	0.457
Recessive Model (CT+CC *vs* TT)			1.30 (0.77-2.21)	0.330

[Table/Fig-2]: Genotype and allele frequencies of rs6457617 polymorphism in RA patients and controls.
CI: Confidence level; OR: Odds ratio; p-value<0.05 is considered as significant

DISCUSSION

The RA is a long-term autoimmune disorder with a world prevalence of approximately 0.46% [17]. India's majority distribution is found to vary among various geographical regions and ranges from 0.28% to 0.75% [18]. Various GWAS and meta-analyses in Asian populations came out with results which link multiple genes/loci of known immune function with RA susceptibility [19]. The disease has been explored for its genetic susceptibility to various genes and its SNPs across diverse populations using multiple study designs. Findings from these studies signify genetic heterogeneity in RA susceptibility across different populations [16]. The involvement of genes from the HLA region in RA susceptibility and severity has been well-known [9], especially the HLA class II genes which include *DQA1, DQB1* and *DRB1* [20]. The SNPs from the HLA region, rs13192471 and rs6457617, were most widely reported across various populations across the world with heterogeneity. Thus, the variants were chosen to study the association with RA in a population cohort from North-western India.

Interestingly, the variant rs13192471 was found to be significantly associated with risk (p-value=0.005) in present study population. At the same time, the variant rs6457617 was not significantly associated with RA susceptibility (p-value=0.275) in this population cohort. Our findings showed similar results as observed in the North-eastern Indian population [15] and partial overlap with the study from populations corresponding to Northern India [16] and the European population [10-12] but contrasting to results from Tunisian, Spanish and Han Chinese populations, where they established a significant association of rs6457617 [12-14]. A peculiar finding of deviation in the genotype frequencies for both the variants from the HWE was observed in the study. This could be attributed to the polymorphic nature of the HLA locus and another possibility that the region might be under natural selection and with bias in allele frequencies of the variants, thereby not following HWE.

Limitation(s)

The study group comprised of participants from North-western India only. As Indian population is very diverse, replication and exploration in other population cohorts is critically needed.

CONCLUSION(S)

This is the first replication study evaluating the association of *HLA-DRB1* variant rs13192471 and *HLA-DQB1* variant rs6457617 in the population group of North-western India. The present study highlights the need to replicate the study with increased sample size and investigate other genes in the HLA region with RA susceptibility. Moreover, with high genetic diversity and thus anticipated genetic heterogeneity, such replication studies are warranted in different population groups of India. It will strengthen the identification of potential genetic biomarkers for the various Indian populations and contribute towards futuristic goals of polygenic risks and personalised medicine.

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