Original Article

ARL15 Gene Variant rs255758 Provides Susceptibility to Rheumatoid Arthritis in Northwest Indian Population

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Abstract

Introduction: Rheumatoid arthritis (RA) is a systemic, chronic, and inflammatory autoimmune disease with a strong genetic component. *ARL15* gene variant rs255758 has been reported as a candidate for RA susceptibility. A replication study was performed on this variant by taking 188 RA cases and 310 healthy non-RA controls from northwest India in a case–control association study design. **Materials and Methods:** DNA isolated from collected blood samples was analyzed by genotyping of the variant on real-time polymerase chain reaction using TaqMan Allele Discrimination Assay and statistically analyzed. **Results:** The variant was found to follow Hardy–Weinberg Equilibrium (P = 0.079) in the control group. The variant was significantly associated with RA susceptibility in the present studied population cohort (P = 0.024) with C as a risk allele and increased risk in the recessive model (CC vs. $CA + AA$; P = 0.004). **Conclusion:** The present study corroborates the earlier findings on the role of *ARL15* gene variant rs255758 in RA and further contributes to its genetic etiology.

Keywords: *Autoimmune diseases, genetic susceptibility, genotyping, single nucleotide polymorphism, TaqMan assay*

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory autoimmune disease, which first attacks the synovial tissues. When the condition is not treated, it causes significant impairment and untimely mortality. It affects approximately 0.8% of people globally. It is three times more common in women, and also manifests earlier in females, typically during the reproductive years.[1] Among the Indian population prevalence of RA is 0.7%.[2] In RA, B-cells, T-cells, dendritic cells, and macrophages often form distinct microarchitectures in synovial inflammatory infiltrates.[3] An early RA diagnosis allows for preliminary therapy with disease-modifying antirheumatic drugs with methotrexate as the first line of treatment for RA.[4]

Genetic components are the main determinants of susceptibility to RA, however, environmental factors are also known to play a role in its occurrence.[5] To date, more than 150 RA risk loci have been identified, though with the dominance of HLA gene associations.[6] RA risk loci which are not part of the major histocompatibility complex explain only 4.7% and 5.5% heritability among the Asian and European populations, respectively.[7] Among the Indian population, the role of HLA locus to RA is already established but there is very little data available on non-HLA loci.[8-10] Some Indian population-specific studies were done to find an association between non-HLA gene *PTPN22* polymorphisms and RA but found no significant association,[11,12] but *ARL15* variant rs255758 was found to be significantly associated with north Indian population-specific genome-wide association study (GWAS).[13] The link between homozygous variant genotype (CC) in *ARL15* intronic SNP rs255758 and RA patients was supported by this study.[13] *ARL15* (ADP Ribosylation Factor Like GTPase 15) gene in humans (5q11.2) codes for small GTP-binding protein (204 aa)-ADP-ribosylation factor (ARF)-like protein 15.[14] The ARL (ARF-like GTPase) family of proteins is categorized as an ARF subfamily of the RAS superfamily. The GTP binding proteins, ARLs with a low molecular weight (21–24 kDa) are distinct from ARFs.[15] This family regulates

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intracellular vesicle trafficking and is also implicated in insulin signaling. *ARL15* gene variants are significantly associated with many diseases such as RA, coronary artery disease, and Type II diabetes in various GWASs.[14] *ARL15* gene also encodes for an uncharacterized GTPase, which is associated with RA and also with other metabolic disorders.[16] Another study on the north Indian population has shown that RA patients with homozygous variant genotype (*CC*) of *ARL15* SNP rs255758 have higher levels of ARL15 protein which suggests the functional significance of this intronic SNP.[15]

The objective of the present study was to perform a replication case–control-based association study of variant rs255758, to determine its association with RA, in an independent cohort from the population of northwest India.

Materials and Methods

Sample collection

The study design involves a case-control study. For the present study, participants between the age group of 18–80 years were included. The study population consisted of two groups – RA cases and healthy controls from the same geographical area. The average age of cases was 48.43 ± 12.08 and controls were 52.87 ± 12.28. Controls were included on priority of relatively higher age to exclude false-negative controls (NTCs). Cases and controls were included in the study in a ratio of 1:1.5. The study was initiated after the due approval by the institutional ethics committee. After getting written informed consent from the participants, a venous blood sample of 2 mL was obtained and transferred to ethylenediaminetetraacetic acid vials to avoid coagulation. The samples were transported to the laboratory, in an icebox and kept at −20°C until the DNA had been extracted. For the present study, 188 RA cases who were coming for treatment at various rheumatology clinics in the northwest state of India were selected. Out of these cases around 78% (146/188) were females. All cases fulfilled the American College of Rheumatology/European League Against Rheumatism classification criteria.[17,18] Unrelated 310 healthy controls with no autoimmune disorder history were selected from a similar geographical area for this population-based case–control study.

Sample processing and DNA isolation

The DNA samples were isolated using Mag genome xpressDNA blood kit (250 rxn). Then, agarose gel electrophoresis was done to determine the quality of isolated DNA samples. The concentrations of DNA samples were determined using Nanodrop (USA) at specific wavelengths. The DNA samples isolated were diluted by adding nuclease-free water (10 ng/µL).

Genotyping of variant rs255758

TaqMan® SNP genotyping assay from Applied Biosystems (Thermo Fischer Scientific, Pleasanton, CA, USA) was used to genotype the variants rs255758 of *ARL-15*; using MX 3005p Agilent real-time polymerase chain reaction (RT PCR) (Stratagene Agilent Technologies, Waldbronn, Germany). The TaqMan genotyping assay was diluted using TE from ×40 to ×20 as directed by the manufacturer. In a 96-well plate format that included 93 samples and three NTCs, all samples were genotyped [Figure 1].

The overall amount of the PCR reaction mix was 10 µL, including 3 µL of DNA concentration of 10 ng/µL. The rest of the constituents included 1 µL 1X PCR buffer, 25 µL 0.5X of TaqMan assay, 0.2 µL 0.2 mM of dNTPs, 0.1 µL 0.3 *U* of Taq Polymerase, and 5.45 µL of distilled water was used to make up the volume of the reaction. The cycling conditions consist of a 4-min hold at 95°C, 40 cycles at 15-s intervals at 95°C, and 1 min at 60°C with endpoint detection. The RT PCR post-PCR detection protocol was used to assess the allele-specific fluorescence. Reruns were performed to determine final inclusion for samples that failed genotyping or had ambiguous genotypes.

Data analyses

The information was entered into MS Excel along with the sample IDs, demographic data, and genotyping data. The statistical power of the study was estimated using PS: power and sample size calculator version 3.1 (https://biostat.app.vumc.org/wiki/Main/PowerSampleSize). PLINK (Population Linkage) was used to produce the Pedigree File and MAP file for analysis. Hardy–Weinberg Equilibrium (HWE) was calculated using the Chi-square test. An odds ratio (OR) was used to determine the chance of an outcome in response to an 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 cases are more at risk than controls. PLINK v1.07, a statistical analysis, was used for all calculations. The gene–gene interaction analysis of ARL15 gene was performed using Cytoscape v3.10.1 (Institute for Systems Biology, Seattle, USA).

Results

Demographical and clinical profile of the enrolled rheumatoid arthritis cases and controls

Around 78% (146/188) of the RA cases in this study cohort were female, for controls this number stands at 67% (206/310). The average age of onset of the disease was 44.49 ± 10.96 years. Around 69% (129/188) of the cases were seropositive for rheumatoid factor. The average age for cases was 48.43 ± 12.08 years and in controls average age was found to be 52.87 ± 12.28 showing

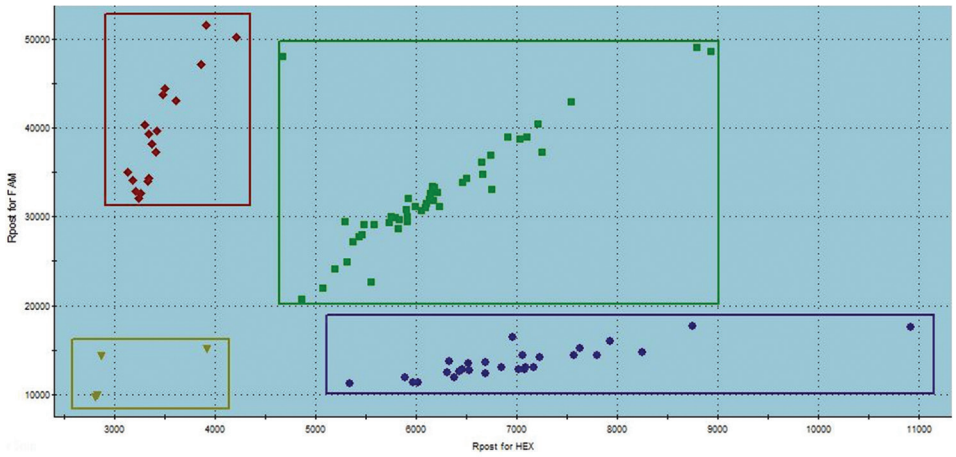

Figure 1: Representative cluster plot obtained after genotyping by real-time polymerase chain reaction. The red and blue clusters represent the homozygous genotype calls (HOMO), while the heterozygous genotype calls are represented by the green cluster (HET). Negative controls (NTCs) are represented by the yellow cluster (NTC)

a statistically significant difference between the two groups ($P = 1 \times 10^{-4}$). There was no statistically significant difference between male ($P = 0.73$) and female ($P = 0.88$) adiposity in cases and controls. Other demographic features such as average body mass index (BMI), smoking status, alcohol usage, and diet preferences also showed no statistically significant difference [Table 1].

SNP analyses

The allelic distribution of variant rs255758 was evaluated. The *C* allele was observed as a minor allele with a frequency of 0.26 in cases and 0.19 in controls in the studied cohort. In the present study cohort, the variant rs255758 showed a significant association ($P = 0.024$) with RA susceptibility and the *C* allele as a risk allele for RA with the OR of 1.44, 95% CI (1.04–1.99) [Table 2].

The allelic distribution of variant rs255758 of *ARL15* was in accordance with HWE ($P = 0.079$) in controls. To further evaluate and increase the power of the study and overcome the confounding factors effect, logistic regression analyses were adopted with a recessive model (*CA* + *AA* vs. *CC*). The OR obtained was 2.54 (1.31–4.94 at 95% CI), $P = 0.004$, adjusted with age, gender, BMI, and ethnicity [Table 3].

The results showed a significant association of variant rs255758 with RA in the population of northwest Indians. The frequency of *AA*, *CA*, and *CC* genotypes was found to be 62.20 versus 67.23%, 24.39 versus 27.03%, and 13.41 versus 5.74% in the RA cases and controls, respectively. A Chi-square test of independence was performed to examine the relationship between three genotypes in cases and controls. The result came to be significant with a $P = 0.018$ [Table 4].

The interaction of ARL15 with other genes (*ZNF333, CNNM2, B4GALT3, GRIPAP1, UBE2K, FBXO10, CNNM4,* and *TMEM120B*) has been represented in the Figure 2.

Table 1: Clinical and demographic characteristics of rheumatoid arthritis cases and controls

Characteristics	Cases	Controls	*P*
Average age[@] ± SD (years)	48.43±12.08	52.87±12.28	1×10^{-4}
BMI[@] ± SD (kg/m^2)	24.73±3.78	24.57±4.14	0.67
Adiposity^			
Males (%)	73	69	0.73
Females (%)	89	91	0.88
Smoking^			
Smokers (%)	96	97	0.94
Nonsmokers (%)	4	3	0.71
Alcohol^			
Yes (%)	6	13	0.11
No (%)	94	87	0.60
Diet^			
Vegetarian (%)	62	67	0.66
Nonvegetarian (%)	38	33	0.55

[@]*P*-value calculated using *t*-test; ^*P*-value calculated using Chi-square. SD: Standard deviation; BMI: Body mass index

The gene–gene interaction depicts the strong physical interaction of 80.38% of the *ARL15* gene with *ZNF333, CNNM2, B4GALT3, GRIPAP1, UBE2K, FBXO10, CNNM4*, and *TMEM120B*. Coexpression between *CNNM2*, *FBXO10* and *TMEM120B* was around 8.22%. it was found a similar level of coexpression of around 8.22% between *ARL15* and *XRP1*, *CNNM4*, *UBE2K* genes. While *ARL15* was observed to be a part of colocalization with *TMEM74B* and *XPR1* with 3.70%. Only *B4GALT3* was found to show the genetic interaction with the ARL15 gene.

Discussion

RA is a serious medical condition that can be life-threatening if left untreated for a long time.[19] Adiponectin is an adipokine found in the synovial fluid and synovial membrane of RA patients, in high amounts.[20,21] An *ARL15* variant (rs4311394) is associated with increased levels of

Table 2: Allelic frequency distribution among cases and controls

Gene/SNP	Risk allele	Cases	Controls	Allelic OR	*P*
ARL15/rs255758	*C*	0.26	0.19	1.44 (1.04–1.99)	0.024

OR: Odds ratio; SNP: Single-nucleotide polymorphism

Table 3: Various genetic models and rheumatoid arthritis risk

Genetic model	Genotype	OR	95% CI	*P*
Dominant	*CA* + *CC* versus AA	1.25	0.84–1.86	0.276
Recessive	*CA* + *AA* versus CC	2.54	1.31–4.94	0.004

OR: Odds ratio; CI: Confidence interval

Table 4: Genotype frequencies of rs255758 polymorphism in rheumatoid arthritis cases and controls

rs255758 (reference ID)	Frequency in cases	Frequency in controls	*P*
Homozygous wild *AA*	62.20	67.23	0.018
Heterozygous variant *CA*	24.39	27.03	
Homozygous variant *CC*	13.41	5.74	

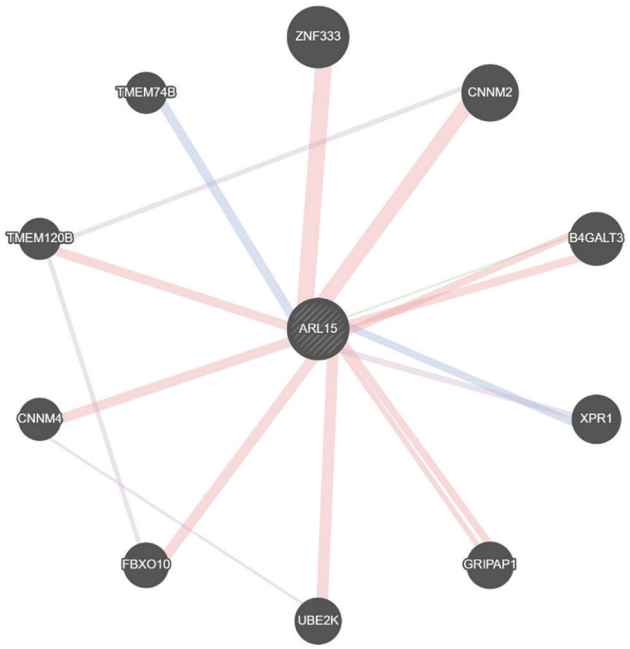

Figure 2: Gene–Gene interaction analysis of *ARL15* using Cytoscape tool

adiponectin in subjects with Type 2 diabetes mellitus and coronary heart disease with European ancestry.[22,23] There was a significant difference ($P \leq 0.0001$) found between adiponectin levels in RA patients with wild *AA* genotype and homozygous variant *CC* genotype of *ARL* variant rs255758 polymorphism in the north Indian population.[13] The raised levels of adiponectin are found to be correlated with the severity of RA.[21,24] This italicizes the significance of adiponectin in RA pathogenesis and as a potential therapeutic target.[25] A north Indian population study has confirmed that by inhibiting the ARL protein family, inflammation, and overall arthritis can be improved and it can be used as a potential therapeutic target.[26] Another study showed that ARL15 protein can be established as a potential novel drug target to treat RA by exploring its structural and functional attributes.[16] The strong background of *ARL15's* role in RA drives us to investigate the role of this genetic locus in our study population. Results from the present study are found to be analogous to the earlier study done on the north Indian population[13] and showed the statistical significance of *ARL15* polymorphism rs255758 with RA in the northwest Indian population ($P = 0.024$). Another study on the same polymorphism in RA patients of the Han Chinese population shows a statistically significant difference between genotype frequencies between cases and controls but no statistical difference was found in allelic frequencies. In this Han Chinese population study, under the dominant model (*AA* vs. *AC* + *CC*), the *AA* genotype of the polymorphism rs255758 was found to decrease the risk of RA.[27] Our study shows that under the recessive model (*CC* vs. *CA* + *AA*), the *CC* genotype increases the risk of getting RA (OR = 2.55, 95% CI = [1.31–4.94], $P = 0.004$) in the northwest Indian population. This difference in allele distributions between Han Chinese and northwest Indian populations can be attributed to the genetic heterogeneity underlying the RA.[28] Furthermore, the endogamous nature of the Indian population can be a potential reason for this difference. In addition, gene–gene interaction analysis further highlights the importance of screening the genes that were strongly interacting with the *ARL15* gene. These genes might also provide a cumulative heightened risk for RA in the population which needs to be further validated.

Conclusion

As of now, most of the RA-associated genes/loci findings are related to the Caucasian population, so Indian population-specific genetic studies are the need of the hour. The variant in the present study was found to be associated with RA in the present population cohort and replicates findings from other Indian population studies, suggesting the potential of this SNP as a common biomarker for RA in Indian populations.

Ethical clearance

The study was approved by institutional Ethics Committee of Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar vide letter no. Patho339/19 dated 06/04/2019.

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Conflicts of interest

There are no conflicts of interest.

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