Research Brief

Susceptibility of CTLA-4 –1661A/G polymorphism towards severity of rheumatic heart disease

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Aim: Genetic contribution in acute rheumatic fever (ARF)/rheumatic heart disease (RHD) has been suggested but not according to severity of the valve involvement. This study attempts to identify the relevance of CTLA-4 polymorphism with severity of the disease.

Methods: In a case-control design, 291 healthy controls and 83 patients were genotyped for association between RHD and single-nucleotide polymorphisms – 1661A/G of CTLA-4.

Results: Segregation of patients on the basis of severity i.e., MVL (Mitral Valve Lesion) and CVL (Combined Valve Lesion) revealed that the frequency of 1661A allele increased significantly in the CVL group and in females.

Conclusion: Our study suggests the risk associated with decreased frequency of CTLA-4 – 1661A allele in the CVL group and in females.

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1. Introduction

Acute rheumatic fever (ARF) is a post-infectious, non-suppurative, multisystemic inflammatory disease that contributes to typical clinical characteristics such as arthritis, chorea and carditis/valvulitis.1–4 These characteristics lead to a most serious complication called Rheumatic heart disease (RHD) that occurs in about 30–45% of RF patients and that further leads to chronic valvular lesions.5,6 The pathogenesis of RHD is quite intricate that involves both genetic and environmental factors.5 ARF and RHD are post-infectious diseases that involve an inflammatory reaction in addition to T and B cells, hence several SNPs in genes that code for inflammatory molecules and contribute to predisposition and manifestation of the disease have been investigated.7,8,9,10 However, interestingly, literature is scant on the role of these genes in the susceptibility of the valves and severity of the disease. Among the several genes that have been investigated in RHD, the Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) has attracted much attention.

CTLA-4 gene is co-localized on q33 of human chromosome 2. It is a co-stimulatory molecule and is expressed on the surface of activated T-cells, and plays a pivotal role in the inhibition of T-cell activation and peripheral tolerance.11,12 It is a negative regulator of T-cell activation and alteration of its expression may have a notable effect in immune-mediated diseases.13 In a multifactorial immune checkpoint system, known as peripheral tolerance, CTLA-4 immune checkpoint pathway regulates the activation of T cells during an immune response to prevent autoimmunity. CD28 molecules on the T cells binds to B7 molecules on antigen presenting cells producing a stimulatory signal. Thus, the activation/inhibition of a T cell depends upon the binding between CD28: B7 and CTLA-4: B7. The upregulation of CTLA-4 on the cell surface is induced as a result of stimulatory signals from T-cell receptor and CD28: B7 binding. The increased binding of CTLA-4: B7 results in a negative signal limiting IL-2 production and proliferation and thus, limiting T cell survival.14,15,16

Two SNPs –318C/T and +49A/G of CTLA-4 have been studied with respect to RHD17–19; whereas, a third SNP –1661A/G has been less characterized, although it has been associated with type 1 diabetes mellitus,20 systemic sclerosis,21 multiple sclerosis22 and...
oral squamous cell carcinoma thus indicating a correlation between this SNP and autoimmune diseases.

2. Materials and methods

2.1. Study participants and clinical evaluation

The human ethics committees of the institutes approved the study protocols and consent form. A total of 83 RHD patients (34 males and 49 females, mean age 36.8 ± 10.2 years), diagnosed on the basis of echocardiography, were included. The control group consisted of 291 healthy unrelated North-Indians (239 males and 52 females, mean age 31.4 ± 7.2 years) without any history of ARF or clinical or echocardiographic evidence of RHD. All the participants were aged between 20 and 60 years. Comprehensive history was recorded, followed by clinical examination and detailed 2D echocardiography. Five ml of blood was drawn in acid citrate dextrose (ACD) anticoagulant in supine position from the subjects after overnight fasting. Plasma was isolated and peripheral blood leukocytes were used for DNA extraction by modified salting out protocol. Plasma was stored at −80 °C and DNA samples at −20 °C, if not used immediately.

2.2. Genotyping

SNPs were selected on the basis of their location, clinical and functional relevance with respect to the other SNPs. The specificity of all the primers was evaluated using nucleotide BLAST (Basic Local Alignment Search Tool). The SNP of CTLA-4 –1661 A/G was screened using SNaPshot ddNTP Primer Extension PCR method (Applied Biosystems, Foster City, USA) in the two groups.

2.3. Statistical analysis

SPSS version 16.0 and EPIINFO version 6.0 software were used for statistical analyses. Genotype and allelic distributions of CTLA-4 was compared by multivariate logistic regression analysis; the covariates taken into consideration were age and gender. The HWE (Hardy–Weinberg equilibrium) was scrutinized using a χ2 goodness-of-fit test. The OR (Odds ratio) and 95% CI (Confidence interval) were calculated using multivariate logistic regression analysis and were used to measure the strength for the association of genotypes and their combinations with the disease. Correction for multiple comparisons was performed by false discovery rate (FDR) correction (BenjaminiHochberg.xlxs calculator). The power of the sample size to detect the association at α = 0.05 was calculated using an online tool “OSSE-An Online Sample Size Estimator” (link: http://osse.bia.a-star.edu.sg/calculation2.php). A p value of ≤0.05 was considered statistically significant after FDR correction.

3. Results

3.1. Population characteristics

The clinical characteristics and echocardiographic findings of the patients are depicted in Table 1. Among the 83 patients, 45% were affected with mitral valve lesion (MVL), while 55% were affected with combined valve lesion (CVL). Also, 25% were diagnosed with tricuspid regurgitation while 23% and 17% were diagnosed with mitral and aortic regurgitation, respectively, in moderate to severe cases.

| Parameter                          | Control (n = 291) | Patients (n = 83) |
|------------------------------------|------------------|------------------|
| Age, years                         | 31.4 ± 7.1       | 36.8 ± 10.2      |
| Gender                             |                  |                  |
| Male                               | 239 (82%)        | 34 (41%)         |
| Female                             | 52 (18%)         | 49 (59%)         |
| Valvular lesion                    |                  |                  |
| Mitral stenosis                    | Mild             | 5 (6%)           |
|                                   | Moderate-Severe  | 78 (94%)         |
| Mitral valve lesion                | –                | 37 (45%)         |
| Combined Valvular Lesion           | –                | 46 (55%)         |
| Mitral regurgitation               | Mild             | 51 (77%)         |
|                                   | Moderate-Severe  | 15 (23%)         |
| Aortic regurgitation               | –                | 38 (83%)         |
| Tricuspid regurgitation            | Mild             | 61 (75%)         |
|                                   | Moderate-Severe  | 20 (25%)         |

n, number of samples. The age is represented as mean ± standard deviation (SD). Each column under controls and patients represents number of samples (% distribution). p-value was calculated using the Epi Info® software version 6. Significance was maintained at p ≤ 0.05.

3.2. Single locus association analysis

We analyzed our genotype data for single locus association of CTLA-4 –1661 A/G by applying 6 genetic models. Under a dominant model, carrying G allele would increase the risk of RHD, where AG and GG are pooled (AA versus AG and GG). While under a recessive model for G allele, AA and AG would be pooled (AA and AG versus GG). An over-dominant model would assume that the heterozygote AG would be of the strongest impact versus AA and GG. The additive model evaluated the impact of A and G alleles individually towards the disease. The co-dominant model evaluated the disease risk associated with AG individuals, the heterozygotes.

Plausible association and the respective genotype and allele distributions of SNP –1661A/G of CTLA-4 was investigated and is represented in Table 2. In our pursuit we first screened these genes for understanding the role in the disease and followed with the valvular association.

3.3. CTLA-4 –1661A/G SNP inclines toward disease susceptibility

The CTLA-4 –1661A/G SNP revealed a statistically significant association for AG and GG genotype between the two groups (p = 0.015) with RHD. Also, on comparing the additive model, G allele revealed a significant association among the two groups (p = 0.012). The heterozygotes of CTLA-4 –1661A/G differed between the two groups (p = 0.064) as can be seen from Table 3. Importantly, however, the dominant model A/G significantly associated with the CVL subgroup (p = 0.043) but marginally with MVL subgroup (p = 0.070). The additive model revealed that the G allele distribution was significantly depleted in CVL (p = 0.036) and marginally depleted in MVL (p = 0.059) when compared with the distribution in controls (Table 3).

In a comparison of age and gender, the –1661 A/G heterozygotes were depleted in all the age group patients, however the middle age group i.e., 31–45 years as more affected (p = 0.062). The dominant model for –1661 A/G was significantly associated with RHD in the age group 31–45 as compared to the same age group controls (p = 0.046). The additive model with depleted distribution of G allele in the middle age group further strengthened the risk association (p = 0.041) (Table 4). The gender-based analysis was
**Table 2**

Genotype and allele distribution of the CTLA-4 – 1661 A/G gene polymorphisms in healthy controls and RHD patients.

| Gene/SNPs       | Genetic Model | Genotype/allele | Controls (n = 291) | Patients (n = 83) | χ2  | p-value | OR (95%CI) | p-value and odds ratio (OR) were calculated by performing multivariate logistic regression analysis after adjustment for age and gender by using SPSS version 16.0. Significance was maintained at p < 0.05 after FDR correction. The p values written in bold are statistically significant values. |
|-----------------|---------------|-----------------|--------------------|-------------------|-----|---------|------------|-------------------------------------------------|
| CTLA-4 – 1661A/G (rs4553808) | Co-dominant   | -1661AA         | 224 (77%)          | 73 (88%)          | –   | –       | Reference  |                                                                                     |
|                 |               | -1661AG         | 63 (22%)           | 10 (12%)          | 4.95| 0.026  | 0.39 (0.17–0.90) |
|                 |               | -1661GG         | 4 (1%)             | 0 (0%)            | –   | –       | –          |                                                                                     |
|                 | Dominant      | -1661AA         | 224 (77%)          | 73 (88%)          | –   | –       | Reference  |                                                                                     |
|                 |               | -1661AG + GG    | 67 (23%)           | 10 (12%)          | 5.89| 0.015  | 0.36 (0.16–0.82) |
|                 | Recessive     | -1661AA + AG    | 287 (99%)          | 83 (100%)         | –   | –       | Reference  |                                                                                     |
|                 |               | -1661GG         | 4 (1%)             | 0 (0%)            | –   | –       | –          |                                                                                     |
|                 | Over dominant | -1661AA + GG    | 228 (78%)          | 73 (88%)          | –   | –       | Reference  |                                                                                     |
|                 | Additive      | -1661AA         | 511 (88%)          | 156 (94%)         | –   | –       | Reference  |                                                                                     |
|                 |               | -1661GG         | 71 (12%)           | 10 (6%)           | 6.38| 0.012  | 0.40 (0.18–0.92) |

**n**, number of samples (% distribution); SNP, Single nucleotide polymorphism; χ2, Chi-square; OR, odds ratio; CI, confidence interval; CTLA-4, Cytotoxic T-lymphocyte associated protein-4.

**Table 3**

Single locus association analysis of CTLA-4 – 1661 A/G SNP based on MVL and CVL subgroups.

| SNPs               | Genetic Model | Genotype/allele | Control (n = 291) | Patients (n = 83) | p-value OR (95%CI) | p-value and odds ratio (OR) were calculated by performing multivariate logistic regression analysis after adjustment for age and gender by using SPSS version 16.0. Significance was maintained at p < 0.05 after FDR correction. The p values written in bold are statistically significant values. |
|--------------------|---------------|-----------------|-------------------|-------------------|-------------------|-------------------------------------------------|
| CTLA-4 – 1661A/G   | Co-dominant   | AA              | 224 (77%)         | 41 (89%)          | 0.010            | 0.064  | 0.844 |
|                    |               | AG              | 63 (22%)          | 5 (11%)           | 0.040 (0.14–1.20)| 0.37 (0.13–1.06) | 0.87 (0.22–3.44) |
|                    |               | GG              | 4 (1%)            | 0 (0%)            | –                | –       | –     |
|                    | Dominant      | AA              | 224 (77%)         | 41 (89%)          | 0.007            | 0.043  | 0.844 |
|                    |               | AG + GG         | 67 (23%)          | 5 (11%)           | 0.036 (0.12–1.08)| 0.33 (0.12–0.97) | 0.87 (0.22–3.44) |
|                    | Recessive     | AA + AG         | 287 (98%)         | 41 (89%)          | 0.114            | 0.071  | 0.844 |
|                    |               | AG              | 63 (22%)          | 5 (11%)           | 0.042 (0.14–1.23)| 0.38 (0.13–1.09) | 0.87 (0.22–3.44) |
|                    | Overdominant  | AA + GG         | 228 (78%)         | 41 (89%)          | 0.059            | 0.036  | 0.850 |
|                    | Additive      | A               | 511 (88%)         | 87 (95%)          | 0.37 (0.13–1.03)| 0.34 (0.12–0.93) | 0.88 (0.23–3.31) |
|                    |               | G               | 71 (12%)          | 5 (5%)            | –                | –       | –     |

**n**, number of samples (% distribution); MVL-Mitral valve lesion; CVL-Combined valve lesion; SNP, Single nucleotide polymorphism; χ2, Chi-square; OR, odds ratio; CI, confidence interval; CTLA-4, Cytotoxic T-lymphocyte associated protein-4.

**Table 4**

Age-based single locus association analysis of CTLA-4 – 1661 A/G SNP.

| SNPs               | Genetic Model | Genotype/allele | 20–30 years Patients (n = 160) | 31–45 years Patients (n = 39) | 46–60 years Patients (n = 15) | p-value OR (95%CI) | p-value and odds ratio (OR) were calculated by performing multivariate logistic regression analysis after adjustment for age and gender by using SPSS version 16.0. Significance was maintained at p < 0.05 after FDR correction. The p values written in bold are statistically significant values. |
|--------------------|---------------|-----------------|-------------------------------|-------------------------------|-------------------------------|-------------------|-------------------------------------------------|
| CTLA-4 – 1661A/G   | Co-dominant   | AA              | 122 (76%)                     | 95 (78%)                      | 70 (70%)                     | 0.062            | 3 (30%)  | 13 (87%)  | 2.72)  |
|                    |               | AG              | 35 (22%)                      | 25 (21%)                      | 5 (5%)                       | 0.32 (0.10        | –        | –        | –     |
|                    | Dominant      | AA              | 3 (2%)                        | 1 (1%)                        | 0 (0%)                       | –                | –        | –        | –     |
|                    |               | AG + GG         | 122 (76%)                     | 27 (22%)                      | 2 (2%)                       | 0.046 (0.09        | –        | –        | –     |
|                    | Recessive     | AA + AG         | 157 (98%)                     | 22 (20%)                      | 0 (0%)                       | 0.29 (0.09        | 3 (30%)  | 2 (13%)  | –     |
|                    |               | AG              | 35 (22%)                      | 10 (10%)                      | 2 (2%)                       | 0.21 (0.02        | –        | –        | –     |
|                    | Overdominant  | AA + GG         | 125 (78%)                     | 26 (21%)                      | 0 (0%)                       | 0.07 (0.03        | 2 (2%)   | –        | –     |
|                    | Additive      | A               | 279 (87%)                     | 216 (89%)                     | 7 (7%)                       | 0.041 (0.31        | 3 (35%)  | 2 (7%)   | –     |
|                    |               | G               | 41 (13%)                      | 28 (11%)                      | 4 (4%)                       | 0.26 (0.03        | –        | –        | –     |

**n**, number of samples (% distribution); p-value and odds ratio (OR) were calculated by performing multivariate logistic regression analysis after adjustment for gender by using SPSS version 16.0. Significance was maintained at p < 0.05 after FDR correction. The p values written in bold are statistically significant values.
another revelation with the frequency of G allele being significantly higher in females compared to their counterparts. A significant difference was observed in the frequencies of the dominant genotype model i.e. AA versus AG + GG of CTLA-4 – 1661A/G (p = 0.049) as well as the additive model’s G allele (p = 0.040) in the female subgroup; the same models although showed similar trend in males but it did not reach significance suggesting that females were at higher risk of RHD with the lower distribution of the G allele (Supplementary Table 1).

We next did extensive association study of this SNP – 1661A/G with the clinical parameters that were pre-specified into various sub-groups. Our observations were no doubt interesting; however, we could note that the smaller sample size due to distribution into several groups depleted the power.

4. Discussion

Population based studies on prevalence of RHD in Indian population and also the role of inflammation in this disease have been conducted, in contrast no concerted efforts have been made to understand the genetic contribution; whereas, predisposing genetic markers of RHD are anticipated to be strongly effective in the detection of susceptible individuals. This study is an attempt in this direction and hence, screened – 1661A/G SNP of CTLA-4 for association with RHD in North-Indians.

Of interest, a detailed evaluation for association with respect to MVL and CVL, gender, age and clinical parameters of RHD patients revealed encouraging results for the CTLA-4 – 1661A/G SNP; the distribution of G allele in controls and the subgroups of patients made the difference. While the AG and GG genotype of CTLA-4 – 1661A/G showed a marginal association with MVL, the association with CVL subgroup of patients was statistically significant suggesting that absence of G allele increased the susceptibility toward the disease. It was equally interesting to note that the G allele followed a trend that was visible with frequency further decreasing in the upper age group i.e., 46–60 years; however, it could not be taken into consideration due to poor number of subjects in both controls and patients. It nevertheless suggested that the risk of disease increased with increasing age. Similarly, in the gender-based analysis the females were at higher risk of RHD with the lower distribution of the G allele. We attribute the risk of RHD to G allele, however, to counteract the very statement it may be said that the G allele, because of its greater distribution in healthy subjects or controls associates with lesser risk of RHD.

We do realize that the – 1661A/G being a promoter SNP may play a relevant role in the gene regulation thereby in regulating the physiological outcome. Studies based on chromatin immunoprecipitation and electrophoretic mobility shift assays have reported that a transcription factor c/EBPβ binds specifically at position – 1661 of the CTLA-4 gene and regulates its expression in the presence of the G allele rather than the A allele. Factor c/EBPβ participates in the CD152 (a CTLA-4 receptor) transcription as its binding activity is instigated after activation of T cell when the membrane form of CD152 is distinctly increased. In addition, an allelic variant may also contribute epigenetically by regulating the gene expression quantitatively or qualitatively by altering transcription factor binding sites or other controlling domains.22,23 Hence, CTLA-4 – 1661A/G SNP may have a significant role in pathophysiology of RHD.

To conclude, to the best of our knowledge, this is the first study concerning association of the CTLA-4 – 1661A/G especially with the various clinical parameters (MVL and CVL) of RHD associating with the minor allele. Our findings are interesting and may have long-lasting consequences. Overall, the present study emerged relevant with CTLA-4 – 1661A/G SNP as a potent candidate for RHD susceptibility in North Indian population.

The limitation of the present study that it is a single gene and single SNP investigation, while several genes could contribute towards RHD progression. The outcome also needed further investigations in diversified global populations with a larger sample size.

Declaration of competing interest

All the authors state that they have no conflict interest to declare with respect to the present article titled “Susceptibility of CTLA-4 – 1661A/G polymorphism towards severity of Rheumatic Heart Disease”.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ihj.2021.05.002.

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