International Journal of Community Medicine and Public Health
Reddy PP et al. Int J Community Med Public Health. 2025 Feb;12(2):767-771
http://www.ijcmph.com
pISSN 2394-6032 | eISSN 2394-6040

DOI: https://dx.doi.org/10.18203/2394-6040.ijcmph20250307

Original Research Article

A study on IL-35 gene polymorphism and mRNA expression in patients with rheumatic heart disease

Penagaluru Pardhanandana Reddy[1,2], Shehnaz Sultana[2], Shiva Kumar Yerra[3], Pranay Krishna Penagaluru[4]

[1]Department of Genetics, Bhagwan Mahavir Medical Research Centre, AC Guards, Hyderabad, Telangana, India
[2]MAA Research Foundation, Jubilee Hills, Hyderabad, Telangana, India
[3]Department of Cardiology, Bhagwan Mahavir Medical Research Centre, AC Guards, Hyderabad, Telangana, India
[4]Department of Emergency Medicine, ASRAM, Eluru, Andhra Pradesh, India

Received: 13 November 2024
Revised: 06 January 2025
Accepted: 07 January 2025

***Correspondence:**
Dr. Penagaluru Pardhanandana Reddy,
E-mail: pardhananda.reddy@gmail.com

ABSTRACT

Background: Rheumatic heart disease (RHD) is a chronic heart valve condition affecting around 40 million people globally. Inflammatory cytokines, acting as messengers, play a role in immune responses in various rheumatic diseases. Anti-inflammatory cytokines, produced by B cells and T cells, act as regulatory molecules controlling pro-inflammatory responses. Among these, Interleukin-35 (IL-35) stands out as a promising target for potential therapies in autoimmune and inflammatory diseases. Genetic variations and expression levels of IL-35 genes might have an impact on individuals with RHD.
Methods: This study is aimed to explore the connection between gene polymorphisms and mRNA expression levels in both RHD patients and control subjects. The investigation involved 135 RHD patients and 140 control subjects, utilizing RT-PCR as the methodology.
Results: The findings revealed a significant association between RHD and heterozygous variant (GC) (OR-1.77, 95% CI 0.3-3.04) and minor allele (C) (OR-0.60, 95% CI 0.39-0.92) of the EBi3 (IL-35) gene. In addition, increased mRNA expressions were observed, with a mean of 2.23±1.14 for EBi3 and 2.50±1.53 for IL-12A, indicating a noteworthy association with RHD patients compared to the control subjects.
Conclusions: The current study propose that gene polymorphisms could impact IL-35 expression levels in RHD patients. However, further analysis involving a larger number of cases is needed to draw conclusive results.

Keywords: Cytokines, Interleukin-35, mRNA expression, Polymorphisms, Rheumatic heart disease

INTRODUCTION

Rheumatic heart disease (RHD) is a chronic heart valve condition affecting approximately 40 million people globally as reported by Peters et al, in 2020.[1] The disease stems from an infection caused by the bacterium Group Streptococcus-A, also known as Streptococcus pyogenes, leading to an autoimmune response like acute rheumatic fever (ARF). In the realm of rheumatic diseases, inflammatory cytokines have been identified as crucial mediators of the immune response.[2] A noteworthy player in this context is Interleukin 35 (IL-35), a recently identified anti-inflammatory cytokine belonging to the IL-12 family. Comprising an α chain (p35) and a β chain (EBI3), IL-35 emerges as a potential target for novel therapies in autoimmune and inflammatory diseases.[3]

Secreted primarily by Treg cells, IL-35 counters the pro-inflammatory actions of other IL-12 family members in response to stimuli like IFN-γ or TLR.[4]

Although various cell types, including minimal B cells, endothelial cells, smooth muscle cells, DCs and monocytes, produce IL-35, tregs cells exhibit a higher production. IL-35 plays an important role in regulating the differentiation and function of T and B cells, macrophages and dendritic cells by binding to its receptors and activating subsequent signaling pathways. Recent studies highlighted abnormal IL-35 expression in inflammatory autoimmune diseases.[5-8]

However, limited research has explored into IL-35 gene polymorphisms and expressions to assess susceptibility to RHD. Therefore, our study aims to understand the association between IL-35 gene polymorphism and expression in the context of rheumatic heart disease.

METHODS

Study design

The study enrolled 135 confirmed Rheumatic Heart Disease (RHD) patients with documented 2D Echo reports from Bhagwan Mahavir Medical Research Centre, Hyderabad of Telangana state. An age-matched group of 140 healthy controls without any history of autoimmune diseases was recruited from the same location.

Comprehensive clinical and physical examinations, along with information on medical history, lifestyle factors and family history of RHD, were collected using a standardized questionnaire. The period of the study was March 2019 to March 2022. The study type was original research.

Inclusion criteria

The inclusion criteria comprised patients with confirmed heart valve damage and a history of rheumatic fever in the age group of 15 to 60 years, without congenital heart disease.

Exclusion criteria

Exclusion criteria included individuals with other autoimmune diseases, hypertension and additional cardiac conditions. The Institutional Ethics Committee (IEC) approved this study and informed consent was obtained from both patients and control subjects.

Sample collection

Blood samples of 5 ml were collected from 135 patients and 140 control subjects in EDTA vacutainers. Each sample was assigned a unique identification number and stored for subsequent analysis.

Genotyping

Genotyping was conducted using the allelic discrimination method with the RT-PCR Step One Plus system from applied biosystems. Genomic DNA extracted from whole blood using the Qiagen Kit method was stored at -20°C for future analysis.

The genotyping of IL-35, specifically IL-12A-rs2243115 and Ebi3-rs428253, was performed using established RS (Reference Standards) numbers of primers with Taqman probes. For genotyping, specific probes for different alleles were labeled at their 5' end with the fluorescence reporter dyes FAM and VIC, respectively.

The universal genotyping master mix from applied biosystems was used, with 100 pmol of each primer and 1 pmol of probes added to the master mix to prepare the reaction mixture. Subsequently, 5 µl of the mixture was added to each well, containing 50 ng of DNA template.

The plates were sealed, subjected to a short spin and loaded into the RT-PCR machine. All samples were processed in duplicate. The PCR cycling conditions included an initial step at 50°C for 2 min, followed by 95°C for 10 min. This was succeeded by 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 1 min. Fluorescence signals were collected during amplification and the data was analyzed.

Gene expressions

Gene expression studies were conducted using the RT-PCR Step One Plus system from Applied Biosystems. RNA was extracted from whole blood using the Qiagen kit method and its purity was assessed using a Bio-photometer. Reverse transcription of RNA to cDNA was achieved using the Thermo Scientific reverse transcriptase kit. The SYBR Green labeling method was employed in the RT-PCR system for IL-35 gene expression studies and all samples were analyzed in duplicates.

The primer sequences used were as follows; Ebi3-forward: 5' GCC ACGTCCTTCATCCTCAG 3', reverse: 5' GCCGCCACTTGGACGTAGTA 3'. IL 12 p35 forward: 5' GCAAAGCTTCTGATGGATCCT 3', reverse: 5' GCCTGCATC AGCTCATCAAT 3'. The PCR conditions were set as follows: an initial step at 95˚C for 2 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 30 sec and extension at 70˚C for 30 sec.

The quantification of gene expression levels in the samples was determined by measuring the fractional cycle number at which the expression reached a fixed threshold (CT). Relative gene expression levels were calculated using the 2 ΔCT method, normalized to the endogenous control GAPDH.[9]

Statistical analysis

For continuous variables, we expressed the data as mean±SD with interquartile ranges. A normality test was employed to assess the distribution of the data. In cases of normal distribution, Student's t-test was utilized for comparison.

The association between single nucleotide polymorphisms (SNPs) in IL-35 (IL-12A, EBi3) and susceptibility to RHD was analyzed using the chi-square test ($\chi 2$), with odds ratio and 95% confidence interval (CI) calculated from a 2×2 contingency table. Fold levels of gene expressions were determined using mean±SD values. All statistical analyses were conducted using Graphpad Prism and MedCalc software. A P value less than 0.05 was considered statistically significant.

RESULTS

Tables 1-3 present the analysis of demographic and molecular studies conducted on individuals with RHD and control subjects. The data reveal a notable gender distribution difference, with 94 (69.63%) females and 41 (30.37%) males among the study subjects, while 93 (66.43%) females and 47 (33.57%) males in the control group. The patient group displayed a higher incidence of females and the average age of patients was 38.8±13.09, slightly higher than the control group's mean age of 38.2±9.2 years (Table 1). Significant variations were observed in alcohol consumption, hypertension, family history between RHD patients and control subjects. The investigation focused on IL-35 and IL-12 TC polymorphisms and EBI3 GC genes, utilizing the Taqman assay for allelic discrimination.

RT-PCR with specific fluorescent probes was employed for allele study, where red indicated homozygous major alleles, green represented heterozygous alleles and blue indicated homozygous minor alleles.

The analysis did not reveal any significant difference in rs2243115 (IL-12A) genotypes between RHD patients and control subjects. However, the genotyping results statistically associated the GC genotype and minor allele 'C' of the EBi3 gene with RHD patients compared to control subjects.

The mRNA expressions of IL-12A and EBi3 were higher in RHD patients (2.50 and 2.23, respectively) than in control subjects (1.83 and 1.27). Statistical analysis indicated significantly elevated expression levels of both genes in RHD patients compared to control subjects (Table 3).

Table 1: Demographic and clinical data of the RHD patients and control subjects.

Parameters	RHD (n=135)	Control subjects (n=140)
Age in years (mean±SD)	38.8±13.09	38.2±9.2
Sex		
Males	41 (30.37)	47 (33.57)
Females	94 (69.63)	93 (66.43)
Smoking status		
Smokers	29 (21.48)	32 (22.86)
Non-Smokers	106 (78.52)	108 (77.14)
Alcohol consumption		
Yes	12 (8.89)	31 (22.14)
No	123 (91.11)	109 (77.86)
Socio economic status (Income/month)		
<5000	82 (60.74)	35 (25.0)
5000-10000	35 (25.93)	69 (49.29)
>10000	18(13.33)	36 (25.71)
Population		
Rural	84 (62.22)	90 (64.29)
Urban population	51 (37.78)	50 (35.71)
BMI		
BMI (kg/m^2)	24.65±3.26	24.77±3.27
Hypertension		
Yes	88 (65.19)	31 (22.14)
No	47 (34.81)	109 (77.86)
Family history of RHD		
Yes	69 (51.11)	9 (6.43)
No	66 (48.89)	131 (93.57)

Table 2: Genotyping of IL-35 (IL-12A, EBI3) gene in RHD patients and control subjects.

Parameter	RHD patients n=135	Controls n=140	OR (95% CI)	P value
(IL-12A) rs2243115 TT	90 (66.67)	99 (70.71)	1	Reference
TG	31 (22.96)	35 (25)	0.89 (0.51-1.56)	0.691
GG	14 (10.37)	6 (4.29)	2.58 (0.96-6.94)	0.059
T	211 (78.15)	233 (83.21)	0.72 (0.47-1.10)	0.133
G	59 (21.85)	47 (16.79)		
(EBI3) rs428253 GG	81 (60)	103 (73.57)	1	Reference
GC	44 (32.59)	30 (21.43)	1.77 (1.03-3.04)	0.038
CC	10 (7.41)	7 (5.0)	1.52(0.56-4.116)	0.410
G	206 (76.30)	236 (84.29)	1	Reference
C	64 (23.70)	44 (31.43)	0.60(0.39-0.92)	0.019

Table 3: mRNA expressions of IL-35 (12A, EBI3) gene in RHD patients and control subjects.

Parameter	Controls ΔCt (Mean±SD)	RHD patients ΔCt (Mean±SD)	P value
Il-35 (IL-12A) rs2243115	1.83±1.31	2.50±1.53	0.0001
IL-35 (EBi3) rs428253	1.27±1.16	2.23±1.14	<0.0001

DISCUSSION

Cytokines play a crucial role in the pathogenesis of autoimmune diseases by influencing various processes, including the regulation of inflammation and angiogenesis.[10] They are essential for defending the body against infections and serve as fundamental mediators of immune responses.[11] Researchers have been investigating the pathogenesis and mechanisms of autoimmune diseases like rheumatic heart disease, but there remain significant gaps in understanding the exact processes involved.[12]

IL-35, a recently discovered anti-inflammatory cytokine, is derived from the IL-12 and IL-27 super-families. It exhibits immunosuppressive activity in both infectious and autoimmune diseases.[13] IL-35 is a heterodimeric cytokine consisting of two polypeptide chains, namely "α" and "β." These chains contribute to the formation of two or more cytokines for instance, the α-chain p35 is shared by IL-12A and the β-chain EBi3 is shared by IL-27.[14] Understanding the roles and interactions of IL-35 in autoimmune diseases, such as rheumatic heart disease, sheds light on potential therapeutic targets and aids in understanding the intricate mechanisms involved in these conditions.

IL-35, composed of IL-12A (p35) and EBi3, is recognized for its immunosuppressive role in conditions like cancer and chronic infectious diseases. However, it plays a crucial role in preventing autoimmune complications. Various studies have explored the relationship between gene polymorphisms in interleukin 35 (IL-35) encoding genes (IL-12A and EBi3) and susceptibility to different autoimmune diseases.[15] In our study, we conducted genotyping and mRNA expression analyses of IL-35 (IL-12A and EBi3) genes in Rheumatic Heart Disease (RHD) patients and control subjects. The heterozygous variant GC and minor allele 'C' of the EBi3 (IL-35) gene were associated with RHD patients. Additionally, mRNA expression levels of IL-12A and EBi3 genes were significantly elevated in RHD patients compared to control subjects. Lin et al, (2018) investigated IL-35 gene polymorphisms in coronary heart disease (CHD) patients in the Chinese population, revealing an association of the CC genotype of EBi3 with CHD, while IL-12A genotypes did not show significant differences compared to control subjects.[16]

Zhang et al reported that EBi3 gene polymorphism contributed to the susceptibility of Aortic Regurgitation in the Chinese population.[17] Hamidinia et al, found elevated expression levels of IL-35 mRNA in early breast cancer patients, while Wu et al noted higher levels of IL-35 mRNA expressions in laryngeal squamous cell carcinoma patients.[18,19] Our results also indicated a significant increase in peripheral blood IL-35 mRNA levels in RHD patients compared to control subjects. These findings collectively contribute to understanding the role of IL-35 in RHD and underscore its potential as a biomarker or therapeutic target.

While research on IL-35 has yet to reach conclusive findings, it is increasingly recognized as a promising drug target. However, significant efforts targeting IL-35 for cancer therapies have not been reported thus far. This lack of attention may be attributed to the relatively recent discovery of IL-35 and its complex nature. IL-35's pleiotropic effects, multiple inducing molecules and the presence of more than one subunit with independent transduction pathways contribute to its challenging targeting. Despite these hurdles, the potential benefits of overcoming these challenges could be substantial. Immune evasion remains a formidable obstacle in cancer therapy and targeting IL-35 represents a potential avenue for addressing this challenge.

CONCLUSION

A notable connection was found between the heterozygous variant GC and the minor allele 'C' of EBi3 (IL-35) in RHD patients. Additionally, there were significantly increased expression levels of both IL-12A and EBi3 in RHD patients compared to the control subjects. These findings collectively suggest that the IL-35 gene may indeed play a significant role in the pathogenesis of RHD.

Funding: Indian Council of Medical Research (ICMR), New Delhi
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Reddy PP, Sultana S, Yerra SK, Penagaluru PK. A study on IL-35 gene polymorphism and mRNA expression in patients with rheumatic heart disease. Int J Community Med Public Health 2025;12:767-71.