Frontiers in Health Informatics
ISSN-Online: 2676-7104
www.healthinformaticsjournal.com
2024; Vol 13: Issue 8
Open Access

IL-6 (−174G>C) and Sickle Cell Disease; An Association Study.

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Cite this paper as: Sri Veda Mylamala*, Varun Chaithanya Gurram*, Sudhakar Godi (2024), IL-6 (−174G>C) and Sickle Cell Disease; An Association Study.. *Frontiers in Health Informatics*, *13(8) 4725-4733*

Abstract:
Aim: This study investigates the relationship between IL-6 (−174G>C) gene polymorphisms and sickle cell disease (SCD) in Indian patients, exploring whether these polymorphisms influence SCD progression. Given the limited existing research on the association between IL-6 (−174G>C) polymorphisms and SCD phenotypes, this study aims to contribute to a better understanding of the role of cytokines in disease progression.
Methods:150 patients with SCD and 150 control subjects participated in this study. Genotyping of IL-6 (−174G>C) polymorphisms was performed by genomic DNA extraction followed by PCR-RFLP. The analysis of the data was done using SYSTAT (Version 13, SPSS Inc, Chicago, IL, USA).
Results: The G allele is significantly associated with the SCD. The chi-square test results gave a significant p-value of (0.000084). Statistically significant increase has been observed in GG genotypes of SCD patients when compared to controls (p= 0.00001). In a study on Egyptian SCD patients it was found that IL-6 –174 G>C polymorphism is associated with recurrent and severe attacks of vascular occlusion in SCD patients. In a study from north east Brazil on SCD patients it was observed that that the IL6 174 G>C polymorphism is associated with a risk of stroke in patients with SCD. In another study from Brazil no association was found between IL-6 and SCD.
Conclusion: The IL-6 (−174G>C) genetic polymorphism are significantly linked to SCD progression. Further research with larger patient groups is needed to fully elucidate the role of IL-6 (−174G>C) in SCD.

Key Words: sickle cell disease, polymorphism, PCR-RFLP.

Introduction:

Globin gene abnormalities can lead to a variety of hemoglobinopathies. However, sickle cell disease (SCD) stands out as the most common worldwide. This genetic disorder is particularly prevalent in populations of African, Arabian and Indian heritage. Within India, SCD and other hemoglobinopathies represent a major category of inherited illnesses [1]. Mutations in the structural region of the globin gene alter the amino acid sequence. These changes result in sickle cell disorders [2]. Sickle cell disease (HbSS) and sickle cell trait (HbSA) are the two most common conditions in this group. They are caused by a point mutation in the β-globin gene (chromosome 11), where valine replaces glutamate at the sixth amino acid position (GAG to GTG). Autosomal recessive inheritance is the mode of inheritance [3]. The November 1910 issue of the Archives of Internal Medicine featured an article by James B. Herrick of Chicago, detailing "peculiar elongated and sickle-shaped corpuscles" found in a patient with severe anemia. This publication marked the introduction of what would become known as sickle cell anemia to the medical community [4]. In 1952, Lehmann and

Cutbush published a report detailing the first documented case of Hemoglobin S (Hb S) in individuals belonging to a tribal population in southern India. This discovery was significant as it expanded the known geographic distribution of this genetic variant [5]. Five distinct Hb S gene haplotypes are recognized, each prevalent in specific regions. Benin, Senegal, Burundi and Cameroon are of African origin. The fifth, the Saudi Arabia/Indian haplotype, is found in both these two regions. Despite sharing this haplotype across India, the clinical severity of SCD varies significantly between different regions across India [6]. To understand the variable clinical course of SCD, researchers have investigated the factors influencing patient outcomes. SCD is characterized by hemolysis, increased infection risk and recurrent painful vaso-occlusive crises (VOCs), all of which can contribute to chronic organ damage [7].

IL-6, a cytokine pivotal in inflammatory and immune responses, is implicated in aggravating SCD severity. Elevated IL-6 levels contribute to heightened inflammation, vascular dysfunction and potential impairment of RBC production, leading to worsened symptoms and complications like pain crises and vascular occlusions. A deeper understanding of IL-6's role in SCD may offer possibilities for developing targeted therapies to mitigate disease severity. IL-6, a multifaceted cytokine with both pro- and anti-inflammatory properties, plays a strategic role in the body's defence mechanisms. Beyond stimulating the activation and proliferation of B and T cells, IL-6 is involved in hematopoiesis, the process of blood cell formation. Notably, numerous studies have revealed elevated levels of various cytokines, including IL-6, in individuals with SCA, even during periods of apparent clinical stability [8]. The IL-6 cytokine, strongly associated with acute inflammation, is consistently elevated in SCD patients, both during acute events and periods of relative stability. This persistent elevation likely reflects underlying, subclinical inflammation that is characteristic of the disease [9].

Objectives:

This study investigates the relationship between IL-6 -174G > C gene polymorphisms and SCD in Indian patients. It also explores whether these polymorphisms influence SCD progression. Given the limited existing research in connection between IL-6 -174G > C polymorphisms and SCD, this study aims to contribute to a better understanding of how cytokines affect disease progression.

Materials and Methods:

This case-control study included 300 participants: 150 SCD patients and 150 controls. Data was collected via a detailed questionnaire and all participants provided written informed consent. The study was approved by the Institutional Ethics Committee, Andhra University, Vishakhapatnam (IEC No:29).

Inclusion Criteria: We included SCD patients in the study only if they were in a "steady state" which means they were not having a painful crisis. Specifically, they had to be free of any painful crises for at least four weeks (one month) before they joined the study so that their condition is relatively stable. This helps us understand the disease better in its more typical state, without the complications that come with a crisis.

Exclusion Criteria: Subjects were excluded if they had an infection or inflammatory condition at the time of sample collection, or if they had received a blood transfusion within three months prior to the study.

Control participants were unrelated, healthy individuals matched to the patient group by age and gender and residing in the same geographic area. Individuals with chronic conditions were excluded from the control group.

Two ml of venous blood were collected in EDTA tubes. Genomic DNA was then extracted using the Machery Nagel kit (Nucleo Spin Blood method), following the manufacturer's protocol.

DNA concentration was measured using a spectrophotometer at 260nm. The quality of the extracted DNA was assessed by agarose gel electrophoresis (0.8% agarose in 1X TEB buffer) and visualized under a UV trans illuminator. The primer sets and restriction enzymes (RE) used throughout the experimental procedures in this study are shown in table no:1. PCR amplification was carried out at a total volume of 25µl. The details of the reaction master mixture and amplification conditions used are as follows. PCR Buffer: 1.0µl, MgCl2: 1.5µl, DNTP: 0.3 µl, Forward: 0.5µl, Reverse: 0.5µl, Taq: 0.2µl, DNA: 0.2µl and Molecular water: 22.15µl. The cycling conditions are as follows initial denaturation for 5 minutes at 95° C, followed by denaturation: 35 cycles at 95 °C for 30 s, annealing: at 52 °C for 30 s, extension: at 72 °C for 45 s and final extension: at 72 °C for 5 min [10]. PCR products were digested using restriction enzymes (RE) from New England Biolabs (USA), following the manufacturer's protocols. The digested products and the original amplified products were then run on a 2% agarose gel stained with ethidium bromide. For IL-6(−174G>C) polymorphisms, a band of 186 and 30 bp indicates homozygous GG genotype, bands of 119 and 49 bp represent homozygous CC genotype and bands of 168,119 and 49 bp represent heterozygous GC genotype. To assess association between IL-6 (−174G>C) polymorphisms with SCD patients, the statistical methodology employed to analyze involved chi-square tests to evaluate associations between alleles, genotypes and disease status. For allelic analysis, a chi-square test for independence was used to compare the frequencies of individual alleles between control and patient groups. For genotypic analysis, the chi-square test assessed differences in genotype distributions across groups. In cases where significant associations were detected, post hoc pairwise chi-square tests were performed to identify specific genotypes contributing to the overall significance. Yate's continuity correction was applied to improve accuracy. This approach ensured a comprehensive evaluation of both allelic and genotypic associations with disease, identifying statistically significant patterns while controlling for potential biases. The analysis of the data was done using SYSTAT (Version 13, SPSS Inc, Chicago, IL, USA). Probability values <0.05 was considered statistically significant.

Table : 1. Primer Sequences of IL-6 (−174G>C) Polymorphisms

Primer	Sequence	Restriction Enzyme
Forward	F: 5'-TGA CTT CAG CTT TAC TCT TTG-3'	*NlaIII*
Reverse	R: 5'-CTG ATT GGA AAC CTT ATT AAG-3'	

Results:

Table No: 2 Allelic distribution of IL-6 (−174G>C) Polymorphisms in SCD patients and controls.

A l	Control (n=150)	Patients (n=150)	C h	P -

lele	Count	Frequency (%)	Count	Frequency (%)	iSquare(X^2) Statistic after Yate's Correction	value
G	113	37.67	162	54.00	15.47	**0.000084**
C	187	62.33	138	46.0		

				0		

Fig:1 Graphical representation of IL-6 (−174G>C) allelic frequencies

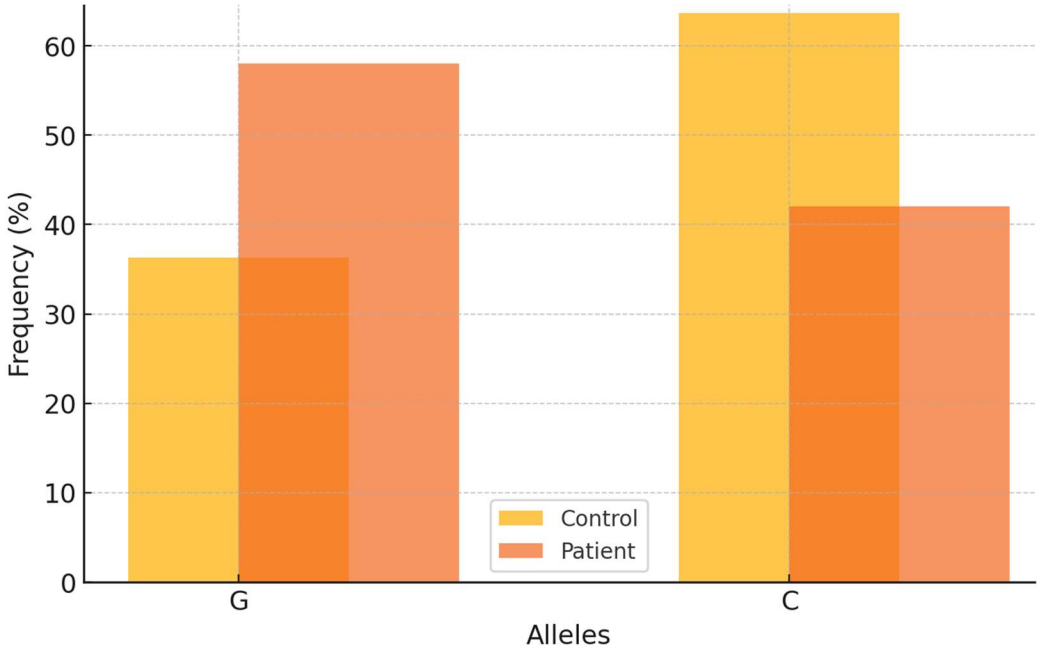
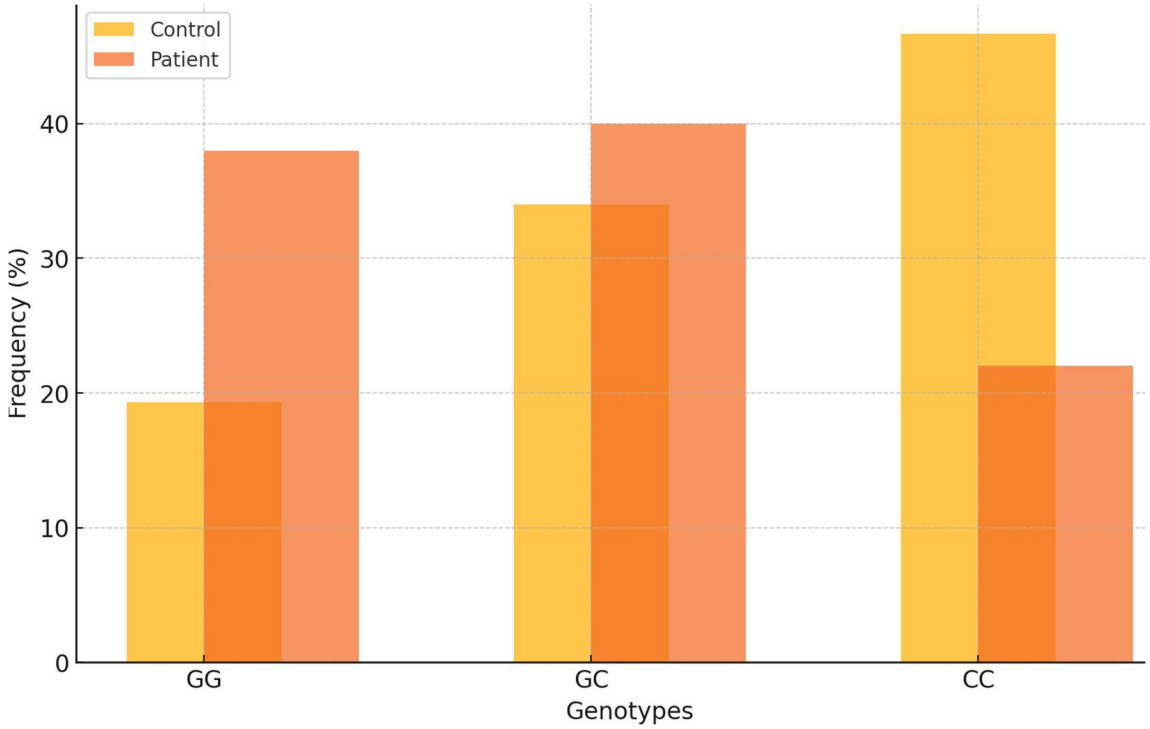


Table No:3 Genotypic distribution of IL-6 (−174G>C) polymorphisms in SCD patients and controls.

Genotype	Control (n=150)		Patients (n=150)		Chi Square (X^2) Statistic	P-value
	Count	Frequency (%)	Count	Frequency (%)		
GG	29	19.33	57	38.00	23.14	**<0.000**

GC	51	34.0	60	40.000		**0.001**
CC	70	46.67	33	22.000		

Fig:2 Graphical representation of IL-6 (−174G>C) genotypic frequencies



The G allele appears to be significantly associated with the SCD supported by the fact that G allele has a higher frequency of 54.00% in patients when compared to 37.67% in controls. The chi-square test results gave a significant p-value of (**0.000084**). Thus, individuals with the G allele have a stronger association with SCD. When the genotypes are considered in patients 57 have GG genotype (38.00), 60 have GC genotype (40.0) and 33 have CC genotype (22.00) (frequencies in parenthesis). Among the controls 29 have GG genotype (19.33), 51 have GC genotype (34.0) and 70 have CC genotype (46.67) (frequencies in parenthesis). Statistically significant increase has been observed in GG genotypes of SCD patients when compared to controls (**p= 0.00001**). The statistically significant values are shown in bold.

After conducting a post hoc analysis to determine which genotypes are significantly associated with the disease the following results were obtained.

Table No: 4 Post hoc Test Results for Genotypes		
Comparison of Genotypes	Chi-Square (X^2)	P-value
GG vs GC	2.55	0.11
GG vs CC	24.09	0.000000917
GC vs CC	11.90	0.00056

The post hoc test used here is the pairwise chi-square test with Yate's continuity correction which is performed between pairs of genotypes (GG vs GC, GG vs CC, GC vs CC) to identify significant differences in their distributions between the control and patient groups. Higher frequency of GG Genotype in patients (38.00%) compared to controls (19.33%) suggests that the GG genotype may be associated with an increased risk of SCD. Slightly higher frequency GC Genotype in patients (40.00%) than controls (34.00%) may also contribute to disease risk, but less compared than GG. Lower frequency of CC Genotype in patients (22.00%) compared to controls (46.67%) suggests a possible protective effect against SCD. This analysis supports the conclusion that the G allele is linked to disease susceptibility and GG genotypes are significantly more frequent in patients than in controls.

Discussion: IL-6, a cytokine pivotal in inflammatory and immune responses, is implicated in aggravating SCD severity. Elevated IL-6 levels contribute to heightened inflammation, vascular dysfunction and potential impairment of RBC production, leading to worsened symptoms and complications like pain crises and vascular occlusions. A deeper understanding of IL-6's role in SCD may offer possibilities for developing targeted therapies to mitigate disease severity. IL-6, a multifaceted cytokine with both pro- and anti-inflammatory properties, plays a strategic role in the body's defence mechanisms. Beyond stimulating the activation and proliferation of B and T cells, IL-6 is involved in hematopoiesis, the process of blood cell formation. Notably, numerous studies have revealed elevated levels of various cytokines, including IL-6, in individuals with SCA, even during periods of apparent clinical stability [11]. The IL-6 cytokine, strongly associated with acute inflammation, is consistently elevated in SCD patients, both during acute events and periods of relative stability. This persistent elevation likely reflects underlying, subclinical inflammation that is characteristic of the disease [9].

The -174 polymorphism in the IL-6 gene is associated with variations in IL-6 gene transcription and subsequent circulating levels. This genetic marker has been widely used as a tool to investigate the link between causality in various diseases and elevated IL-6 levels [12]. A comparative analysis of the genotypic and allelic frequencies of the IL-6 -174G>C polymorphism between individuals with SCD and a healthy control group in this study revealed statistically significant differences. This finding suggests that this particular genetic variation does play a role in the development or progression of SCD. Therefore, the -174G>C polymorphism in the IL-6 gene is a major genetic factor contributing to the susceptibility or severity of SCD. The observed frequencies of the IL-6 -174G>C polymorphism among the SCD patient and control groups are comparable to those reported in various other studies conducted across diverse ethnic populations. In a study on Egyptian SCD patients it was found that IL-6 –174 G>C polymorphism is associated with recurrent and severe attacks of vascular occlusion in SCD patients [13]. In a study from north east Brazil on SCD patients it was observed that that the IL6 174 G>C polymorphism is associated with a risk of stroke in patients with SCD [14]. In another study from Brazil no association was found between IL-6 and SCD [15].

The IL-6 -174G>C polymorphism, a genetic variation in the interleukin-6 gene, has been

studied in relation to various autoimmune diseases. Researchers have investigated its association with type 2 diabetes in German population [16], systemic lupus erythematosus in Malaysian patients [17], systemic sclerosis [18] and multiple sclerosis in Polish patients [19]. While some studies have suggested a positive correlation between this polymorphism and these diseases, further research is necessary to definitively establish its role in disease susceptibility. While previous studies have linked the IL-6 -174G>C polymorphism to an increased risk of type 2 diabetes, a recent study conducted in the Isfahan population did not find a significant correlation between this genetic variation and the disease. This discrepancy in findings highlights the complex nature of genetic influences on disease susceptibility and the importance of considering population-specific factors [10].

Conclusions: In SCD patient's G allele of IL-6(-174G>C) is significantly high in patients than in controls. This suggest that G allele is associated with SCD. GG genotype of IL-6 (-174G>C) polymorphism is a high risk factor for SCD. Statistically significant increase has been observed in GG genotypes G alleles of SCD patients when compared to controls. While the findings of this preliminary study offer valuable insights into the genetic factors associated with SCD across diverse ethnicities, further investigation is necessary to solidify these conclusions. A larger-scale study encompassing a broader spectrum of disease severity is required to validate these results and delve deeper into the complex genetic underpinnings of SCD.

Acknowledgement: The authors express their gratitude to the Department of Human Genetics, Andhra University, Visakhapatnam for the essential resources and support that made this research possible.

Conflict of interest: The authors declare no conflict of interest.

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