

Screening of *IL-22* first and second introns and *FOXP3* second exon for SNPs and mutations with potential role in the susceptibility of SLE in selected population

N. HUSSAIN¹, S. ANEELA¹, N. AOSA¹, A. SAEED², T. AZIZ³, A. MAJID⁴, S.A. ABDULHAKEEM⁴, N.S. AL-ABBAS⁵, N.A. SHAER⁶, A.A. BARQAWI⁶, S. ALGHAMDI⁷, A. SHAKOORI⁷, S.A. ALSHAREEF⁸

¹Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan

²Rheumatology Department, Fatima Memorial Hospital Lahore, Lahore, Pakistan

³Department of Agriculture, University of Ioannina, Arta, Greece

⁴Department of Clinical Laboratory Sciences, The Faculty of Applied Medical Sciences, Taif University, Taif, Saudi Arabia

⁵Department of Biology, Jamoum University College, Umm Al-Qura University, Makkah, Saudi Arabia

⁶Department of Chemistry, Al-Leith University College, Umm Al-Qura University, Makkah, Saudi Arabia

⁷Laboratory Medicine Department, Faculty of Applied Medical Sciences, Umm Al-Qura University, Makkah, Saudi Arabia

⁸Department of Biology, College of Science and Arts at Khulis, University of Jeddah, Jeddah, Saudi Arabia

Abstract. – OBJECTIVE: The main objective of performing this study was the mutational analysis of *Forkhead* box family member (*FoxP3*) and Interleukin-22 (*IL-22*) genes and their associations with systemic lupus erythematosus (SLE).

MATERIALS AND METHODS: A total of sixty blood samples were collected from SLE patients from different hospitals in Lahore. Proforma was based on American College of Rheumatology (ACR) criteria. The total time for this research was one year (2018-2019). DNA was extracted, and *FoxP3* and *IL-22* genes were polymerized through PCR and further sequenced through the Sanger Sequencing method. Chromas version 2.6.6 was used for the similarity index of sequences. NG_060763 and NG_007392.1 were used as Reference Sequences of *IL-22* and *FoxP3* genes, respectively.

RESULTS: Three already identified Single Nucleotide Polymorphisms (SNPs) in the *IL-22* gene i.e., rs2227491, rs2227485, and rs2227513, were confirmed in the sequencing results of SLE patients. Results showed that there were nine novel mutations (27.27%) in the case of the *IL-22* gene in the studied genotyped samples. These SNPs had remarkably increased allele T frequency in rs2227485 and allele C frequency in rs2227491 and rs2227513. On the other hand, in the case of *FoxP3* gene exon 2, there was an addition of T at position 10 in the intronic portion, thus not involved in the progression of the disease.

CONCLUSIONS: The importance of cytokine-mediated signaling pathways, such as the *IL-22* gene, is thus established. Novel variants in the *IL-22* gene likely contributed significantly to the development of this autoimmune disorder. The current study found that the dysregulation of the inflammatory markers in SLE is not related to the *FoxP3* gene, even though *FoxP3* is implicated in the tolerance process.

Key Words:

SLE, *IL-22*, *FoxP3* gene, Polymorphism, Genotype.

Introduction

Systemic lupus erythematosus (SLE) is a severe inflammatory disorder, which affects multiple organs and systems. The estimated prevalence rate in Asian communities is 30-50 per 100,000 people¹. Women of childbearing age are disproportionately affected by the condition, and it can also strike young children and the elderly². Women are 10 times more vulnerable to this disease when compared to men. This suggests that X-chromosomal genes may have a role in SLE etiology³. The pathophysiology of SLE is not fully understood; however, environmental and immunogenetic variables have been hypothesized⁴,

but a definitive answer remains unknown. The dysregulation of cytokines is thought to contribute significantly to disease vulnerability⁵.

The cytokine IL-22 is a member of the Interleukin-10 family. It is made by innate lymphoid cells (ILCs), T cells, NKT cells, and T-helper (Th)-17 cells⁶. Multiple tissues, including the skin, thymus, pancreas, and intestines, have been found to produce and secrete IL-22. IL-22 promotes cell proliferation and has a role in tissue regeneration, mainly in non-hematopoietic epithelial and stromal cells⁷. Rheumatoid arthritis (RA), Crohn's illness, multiple sclerosis (MS), systemic lupus erythematosus (SLE), psoriasis, etc., have all been linked to IL-22 dysregulation^{8,9}. Muscle tissues in people with dermatomyositis (DM) and polymyositis (PM) have abnormally high levels of IL-22. This also links to myositic action. Thyroid illness is another autoimmune disorder that has been linked to a variation of the IL-22 gene¹⁰. The rs2227513 polymorphism is associated with a decrease in IL-22 levels in the Chinese SLE population^{11,12}. Recently, studies¹³ were conducted in Iran that determined the relationship between a polymorphism in the IL-22 gene's promoter and the development of systemic lupus erythematosus. The IL-22 gene sits on chromosome 12 at position 15q. There are 5 exons and 4 introns in total. In diverse populations, several polymorphisms have been linked to the onset of SLE; however, data is limited. No research on the population of Pakistan has been conducted yet.

T cells with suppressive/regulatory capacity (Treg) are best identified by the *Forkhead* family transcription factor *FoxP3*. It is located at Xp11.23¹⁴ on the chromosome. The X-linked syndrome (IPEX), SLE, and type 1 diabetes are just a few of the disorders connected to *FoxP3* dysregulation. Increased *FoxP3* expression causes Treg dysfunction, which in turn triggers autoimmune illnesses like SLE, and maybe a disease activity marker. SLE patients' haplotypes of *FoxP3* genetic variations are linked to susceptibility, autoantibodies, and TGF-1¹⁵.

The aim of conducting this research is to identify SNPs and mutations in the second exon of the *FoxP3* gene and the first and second introns of the *IL-22* gene in 33 Pakistani SLE patients.

Materials And Methods

Sixty patients (7.13%) were pretested for the questionnaire and confirmed by their doctors in the Rheumatology Departments of various hospitals.

Patients with SLE were selected if they met at least four of the eleven criteria established by the American College of Rheumatology (ACR)¹⁶. Patients were diagnosed with SLE after testing positive for both Anti-Nuclear Antibody (ANA) and anti-dsDNA autoantibodies. This process took a full calendar year (2018-2019) to complete. The sample size was calculated by using online software available at <https://www.calculator.net/sample-size-calculator.html>.

Ethics Approval

This study was approved by the Institutional Review Boards at both the Fatima Memorial Hospital College of Medicine and Dentistry in Lahore (IRB-FMH-09-2018-IRB-494) and the Sheikh Zayed Medical Complex National Health Research Complex in Lahore (IRB-1593). Literate participants provided written informed consent. Participants who were illiterate provided informed consent over the phone before the study began. Patients under the age of 18 who participated in the study gave their legal guardians or parents written informed permission.

Inclusion Criteria

All SLE patients fulfilling ACR criteria were included in this study.

Exclusion Criteria

SLE patients overlapping with various syndromes were excluded from sequencing analysis.

DNA isolation and Polymerase Chain Reaction (PCR)

A controlled DNA isolation process was used for the DNA extraction. Primer sequences were retrieved from Wang et al¹⁶ further validated and checked by performing primer blasting and in silico PCR in University of California Santa Cruz (UCSC) genome browser.

Primer Location for rs2227485¹⁶. The primer amplified 166 bases of the first intronic region i.e., 4656-4798 of the *IL-22* gene.

GCCCGGAGGGTATTTTACAGACAaatccca-gaaattttctaaaccctacatatttttcaagaacacaattgtttgtct-tagtagagttcagatttagtataaatgttttgatctcctatagtggtga-gtaagcattttGGTCACGGACTCATTTTCCTACCA

Primer Location for rs222749¹⁶. The primer amplified 159 bases of the second intronic region i.e., 5821-5958 of the *IL-22* gene.

TCTGCAGGTGGAAGGGAAACAGaaagggtca-taaggataagacctaaccatcatcaccaccacccaagtaccca-tggacggcacacggccctgttcgtcacaactgtagcttacactgactcc-gtgaacagttTCTCCCCAATGAGACGAACGTC

Primer Location for rs2227513¹⁶. The primer amplified 274 bases of the first intronic region i.e., 4883-5135 of the *IL-22* gene.

CAACTGGTGACTGGGGAAGGAGaacctg-gtcgaagacaacgtgaaggaacaaaattgtcaagaagtatttca-tcaactaaccaattgtaccaagttgccgaacgcttacgtgtct-gaagattctgctgtgacggggaaggtagaagctgctgtttata-gccccagatactgacttttttaattgtgaaaaatatgacatca-gcaattatctaatttccagtactgtcttctgagaacgacCTAAC-CACCACAGGAGCCCCACA

In total, 50 µl was used for the PCR reaction. Forward and reverse Primers (2.5 µl each), Nuclease-free water (15 µl), genomic DNA (5 µl), and PCR master mix (25 µl) made up the PCR mixture. Initial denaturation of DNA in the PCR reaction was carried out for 5 minutes at 95°C, followed by 30 seconds of denaturation at 95°C and annealing at 59.4°C for rs227485 (*IL-22* gene) and rs2227491 (*IL-22* gene), and 40 seconds at 60.7°C for rs2227513 (*IL-22* gene). The first 45 seconds of the extension were performed at 72°C, and the following final extension was also performed at 72°C for 5 minutes. The reaction was set for 30 cycles. Electrophoresis on a 1.3% agarose gel revealed that the rs2227485, rs2227491, and rs2227513 products had lengths of 166bp, 159bp, and 274bp, respectively⁴. The *FoxP3* gene's exon 2 primers (Table I) were self-designed using Primer 3 software (<https://primer3.ut.ee>) (Table I). Additionally, exon 2 of the *FoxP3* gene was amplified via polymerase chain reaction (PCR) with an annealing temperature of 60.4°C.

Sanger Sequencing and Bioinformatics Analysis

After PCR products were purified, they were sequenced using Sanger technology. Sanger sequencing was performed on a total of 33 samples. The sequencing data were analyzed with Chromas 2.6.6. After accumulating repeats with the use of CAP3 software (<https://droua.prabi.fr/software/cap3>), we ran them through NCBI Blast to check their degree of similarity. SNPs in these sequences were compared to the reference sequences NG_060763 for *IL-22* and NG_007392.1 for *FoxP3*.

Statistical Analysis

Univariate analysis was performed on the results using GraphPad Prism version 13 (<https://www.graphpad.com/features>). At a 95% confidence level, a *p*-value lower than 0.05 is considered statistically significant.

Results

Sixty (n = 60) systemic lupus erythematosus (SLE) patients were included in this analysis. There were 54 female participants (90%) and only 6 male individuals (10%). Altogether, the participant's average age was 30.24 ± 1.29 years. Patients with SLE tend to be between 11 and 30 years old. Five individuals (8.33%) had a family history of autoimmune illnesses, while the remaining 55 (91.66%) did not show any history of autoimmune disease. There were 34 patients with fever (56.6%), and 37 with photosensitivity (61.66%). Among the 38 patients, arthritic pain was reported by 63.3%. Thirty patients, or 50%, exhibited skin rashes. Despite the prevalence of renal involvement (23 individuals, or 38.33%), only 10 (16.60%) were diagnosed with lupus nephritis. Seven patients (11.66%) were diagnosed with pleurisy. Twenty-five patients (41.66%) were found to have ulcers, and twenty-one (35 percent) showed Raynaud's phenomenon at some point in their illness. Twenty-five individuals (41.66%) were diagnosed with blood coagulation disorders. Only five patients (8.33%) were found to have multiple infections at the same time.

Only 9 (15%) patients had overlap syndrome. Fatigue was the dominant symptom among general manifestations. Depression, along with arthritis, was the prevalent clinical feature observed in these patients, as 38 (63.3%) patients were recorded for arthritis and depression. Only 2 (3.33%) patients presented with psychosis. Figure 1 shows the distribution of different clinical features among SLE patients. Blood samples of SLE patients were processed for DNA extraction PCR, and 33 patients were sequenced to rule out three SNPs (rs2227485, rs2227491, rs2227513).

Table I. *FoxP3* Exon 2 primers description.

Oligon Name	Sequence (5'-3')	Length	MW	TM	EC	nmol	µg	A260 Units
Exon-2F	cgtgtgactcctttccccta	20.0	5,995.0	57.50	185.40	56.60	339.5	10.50
Exon-2R	Acagtaaagtcggcacctg	20.0	6,151.0	57.50	224.50	50.60	311.0	11.35

MW=Molecular weight, TM=Melting temperature, EC=Electrical conductivity.

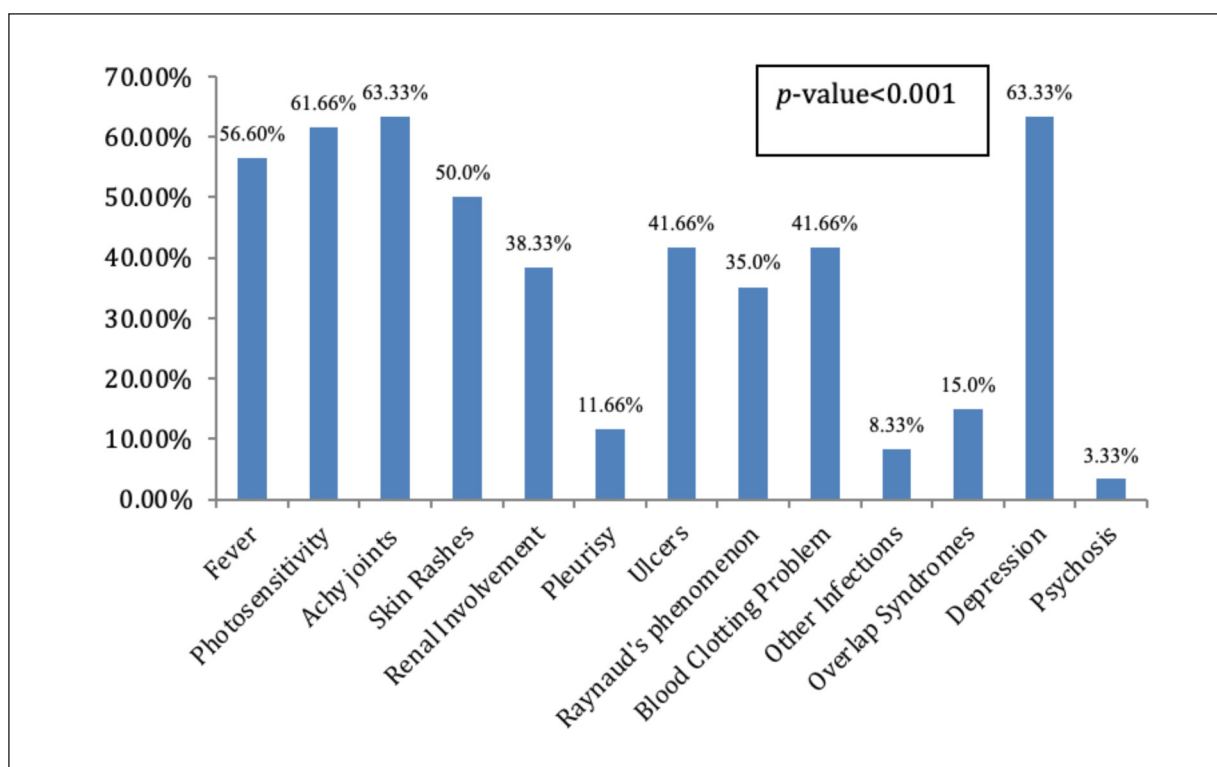


Figure 1. Distribution of different clinical features among SLE patients (n = 60.0).

in the *IL-22* gene and to screen and investigate mutations in the *FoxP3* gene. Other subjects were showing syndromes overlapping with SLE, so we sequenced only SLE-specific patients to get a clear picture of mutations in the target genes.

Analysis of Polymorphisms in the FoxP3 Gene

At base 10 on the reverse strand, the T allele was inserted into the *FoxP3* gene (Figure 2). The mutations were found in the intronic region of the

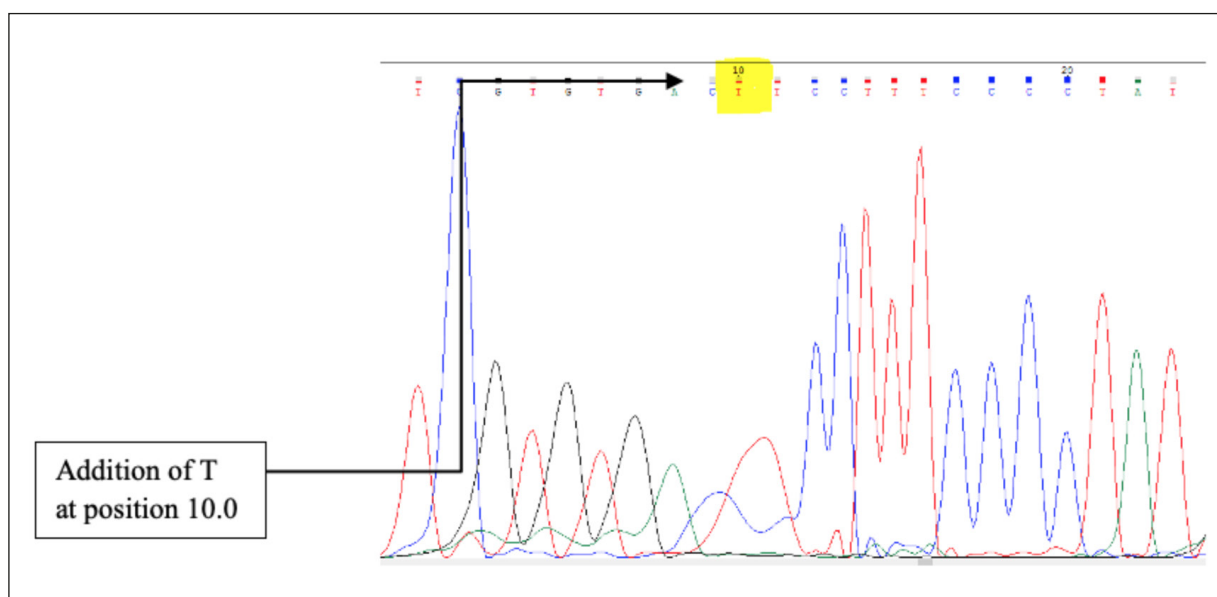


Figure 2. Chromatogram of Addition of T at position 10.0 in reverse complement strand exon 2 of *FoxP3* gene.

FoxP3 gene rather than in exon 2. A mutation in an intron does not count as a mutation of the genome since introns are non-coding areas that do not produce proteins. Exon 2 of the *FoxP3* gene was found to be 100% identical to the reference sequence in SLE patients. Homo sapiens forkhead box P3 (*FoxP3*), RefSeq Gene NG_007392.1 was used. On the other hand, the reported SNPs that were looking for in the *IL-22* gene of lupus patients were not found rather, we found novel mutations listed below (Table II):

A>C transition at position 4672 in the *IL-22* gene differs from the reference sequence.

A>C mutation in the *IL-22* gene (rs2227485) was found in sample 04-A. Chromatographical analysis revealed a nucleotide substitution, C for A, at position 127 when compared to the standard sequence (Figure 3A).

T>A substitution at position 4674 in the *IL-22* gene, in contrast with the reference sequence.

Two samples, 65-A and 66-A had a T>A mutation in the *IL-22* gene (rs2227485; Figure 3 B-C). The chromatogram demonstrated a change in the nucleotide at position 129, with A being replaced with T. As a result, it was established that rs2227485 had two distinct SNPs.

C>G mutation at position 5877 in the *IL-22* gene in contrast to the reference sequence.

Three samples, 31-B, 02-B, and 42-B, had the *IL-22* gene (rs2227491) mutation, which was C>G (Figures 3D, 4A-B, respectively). The chromatogram's nucleotide at positions 85 and 86 (position 42-B) changed from G to C.

C>T mutation at position 5928 in the *IL-22* gene in contrast to the reference sequence.

One sample, 67-B, had the *IL-22* gene (rs2227491) mutation that was C>T (Figure 4C). The nucleotide at position 33 changed, as shown by the chromatogram.

C>A mutations at positions 4947 and 4932 in the *IL-22* gene in contrast to the reference sequence.

The C>A *IL-22* gene mutation (rs2227513) was discovered. It was present in 65-C and F17-C, respectively (Figures 4D and 5). A change in the nucleotide was visible in the chromatogram at positions 205 and 191, respectively.

Discussion

A recent study shows that 90% of SLE cases are in women, and the female-to-male ratio is 9:1. Fatigue was the most prevalent general symptom among SLE patients (86.6%). In these SLE patients, arthritis was a definite clinical characteristic (63.3%). These results are comparable to those of Pakistani research where 91.4% of females had lupus, whereas 79.03% and 78.1%, respectively, reported weariness and arthritis¹⁷. The patients' average age was 30.25 ± 1.3 years, which is consistent with findings from a different Pakistani study where the average age of SLE patients was 31.6 ± 10 years¹⁸. In the current study, oral ulcers and photosensitivity were found in 61.66% and 41.66%, respectively, of the SLE patients. These results are better than those of Rasheed et al¹⁹, who found that 49.12% and 31.35% of SLE patients, respectively, reported mouth ulcers and photosensitivity. Even though only 33% of patients had renal involvement noted by Rabbani et al²⁰ 38.33% of patients had

Table II. Allele frequencies in *IL-22* SNPs (rs2227485, rs2227491, rs2227513) among SLE patients.

SNPs	Allele	Position	Pathological manifestations	p-value
rs2227485	A>C	4,672.0	Fever (50.0%)	0.0081
	T>A	4,674.0	Malar rash (90.0%) Renal Involvement (80.0%) Ache joints (72.0%) Photosensitivity (100.0%)	
rs2227491	C>G	5,877.0	Fever (50.0%)	0.0001
	C>T	5,928.0	Malar rash (91.0%) Renal Involvement (50.0%) Ache joints (67.0%) Photosensitivity (100.0%)	
rs2227513	C>A	4,932.0	Fever (50.0%) Malar rash (61.0%) Renal Involvement (80.0%) Ache joints (50.0%) Photosensitivity (100.0%)	0.0023

$p < 0.05$ significant, IL=Interleukin, SNP=Single Nucleotide Polymorphism, SLE=Systemic Lupus Erythematosus.

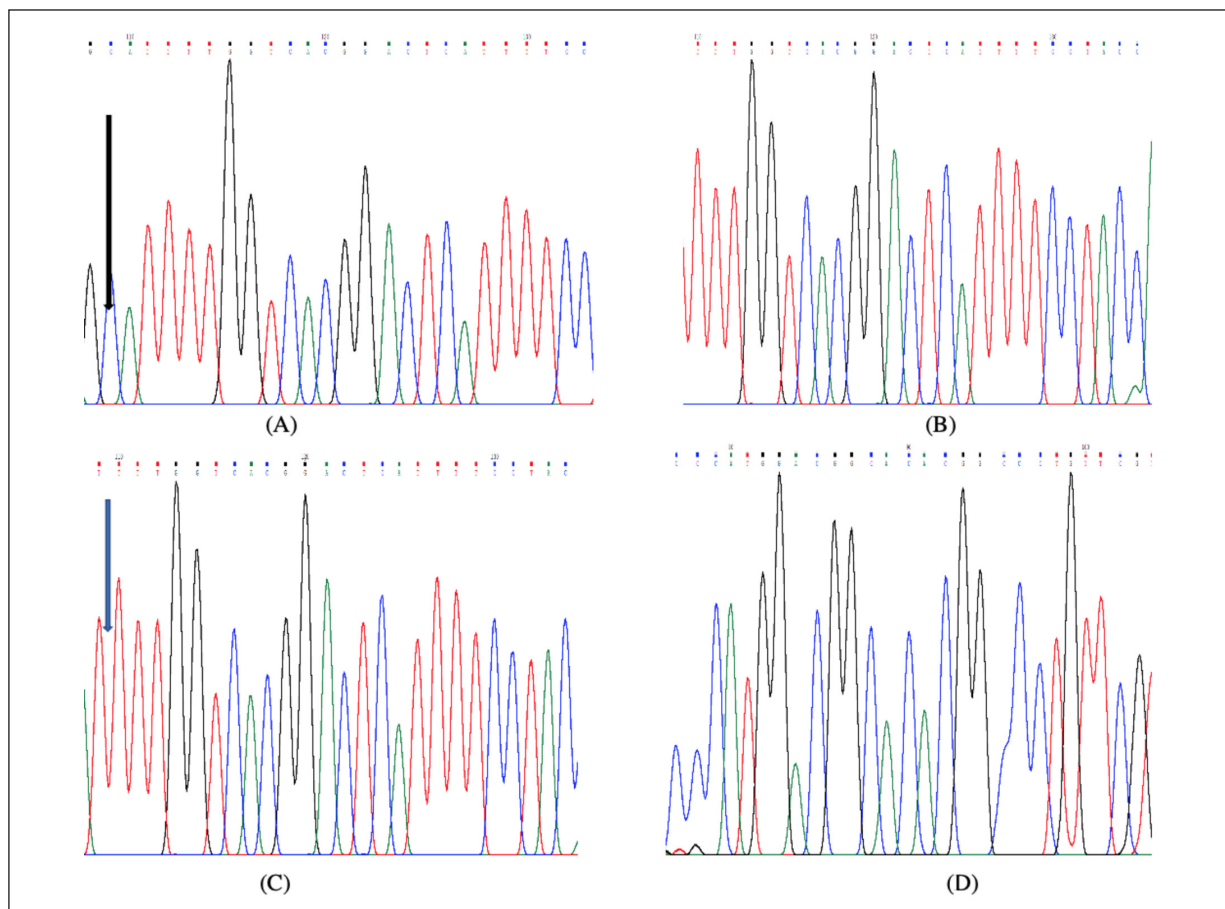


Figure 3. **A,** Chromatogram showing mutation of A>C at 4,672.0 in RefSeq, **(B)** Chromatogram showing mutation of T>A at 4,674.0 in RefSeq **(C)** Chromatogram showing mutation of T>A at 4,674.0 in RefSeq. **D,** Chromatogram showing mutation of C>G at 5,877.0 in Ref Seq.

renal involvement. Our patients ANA and Anti-ds DNA values remained at 98.3% and 96.6%, respectively. These findings closely matched those of Arabian research, where ANA and Anti-ds DNA results were 99.7% and 80.1%, respectively²¹. Compared to Ahsan et al²² findings, where only 0.5% of instances were recorded, overlap syndrome was diagnosed in 16.66% of patients.

It has been established that the cytokines produced by activated T helper cells play a role in the pathogenesis of SLE²³. The higher levels of Th-1 cytokines including TNF, interferon (IFN), and IL-12 found in SLE are supporting this concept. Th-22 is primarily in charge of *IL-22* production. However, the elevated expression of *IL-22* in transgenic mice causes striking hypo granularity and acanthosis of skin phenotype that mimic psoriasis-like modifications²⁴. Critically located on epithelial, pancreatic ductal, and renal tubular cells are *IL-22* (*IL-22R1*) receptors. *IL-22* does not directly activate immune cells; instead, it stimulates the inflammatory response

by modulating the expression of other cytokines, such as IL-1 and IL-6. SLE's precise pathophysiology is yet unknown. Therefore, it is essential to investigate the cause of the disease to develop new therapeutic and diagnostic agents. Environmental factors may exacerbate abnormal autoimmune reactions in people with a hereditary susceptibility. Genes have thus been linked to a person's susceptibility to SLE for a very long time.

Major Histocompatibility Complex (MHC) and SLE were first linked by Grumet et al²⁵. The association between the incidence of SLE and *IL-6* polymorphism has been demonstrated by a case-control Indian study²⁶. Similarly, recent Iranian research²⁷ predicted that Interleukin-10 gene promoter polymorphism and SLE activity are related. Programmed Cell Death-1 (PCD-1) and Toll-Like Receptors (TLR-5, TLR-9) have been described as associated with SLE activity^{28,29}. Despite extensive research into SLE, it is still unclear what molecular system oversees the disease's pathophysiology. Six functional modules that are responsible for the

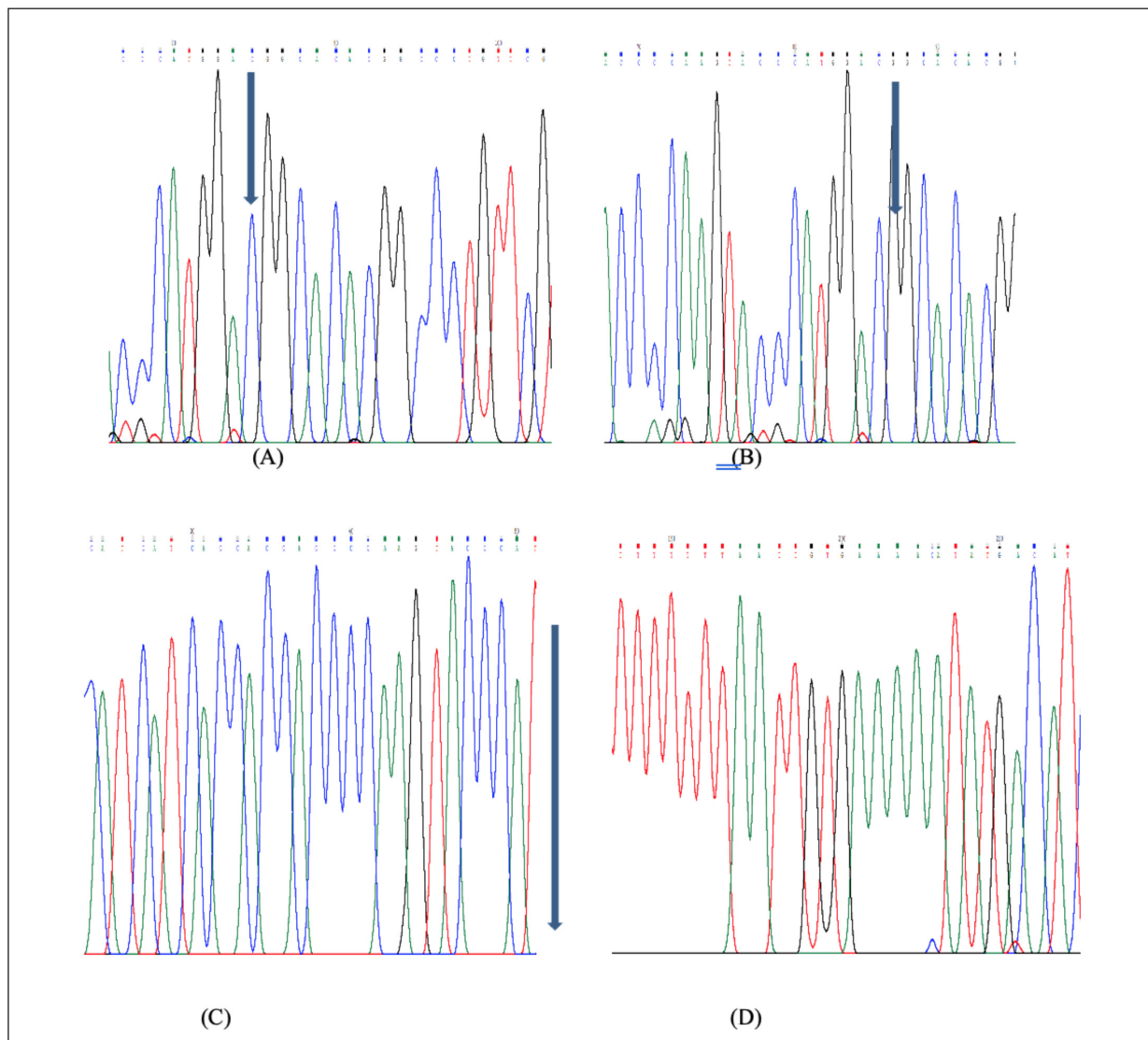


Figure 4. A, Chromatogram showing mutation of C>G at 5,877.0 in Ref Seq (B). Chromatogram showing mutation of C>G at 5,877.0 in Ref Seq (C). Chromatogram showing mutation of C>T at 5,928.0 in Reference Sequence (D). Chromatogram showing mutation of C>A at 4,932.0.

pathophysiology of SLE have been categorized. The most valuable module contains 182 genes, including those involved in signaling pathways regulated by cytokines³⁰. More than 100 strong loci have been linked to SLE at a substantial level (5×10^{-8}), according to a genome-wide association study (GWAS). These loci are primarily found in European and Asian populations. In these loci, numerous SNPs or objective genes have been found. Surprisingly, only a small portion of matching SNPs are found in coding regions that have an impact on the behavior of the proteins they express. The remaining SNPs are found in the non-coding region, where they impact gene

expression through epigenetic, transcriptional, or post-transcriptional changes³¹. The main objective of the current study was to identify three *IL-22* gene SNPs, namely rs2227485, rs2227491, and rs2227513, in SLE patients seeking follow-up care at several hospitals in Lahore's rheumatology department. The results showed 9 mutations in 33 SLE patients, with the C allele and T allele having substantially higher frequencies than the A allele. Since only one peak was observed for each mutation, all SNPs that were found were homozygous. All these SNPs are intronic, and our findings suggested that SLE may be associated with *IL-22* gene SNPs.

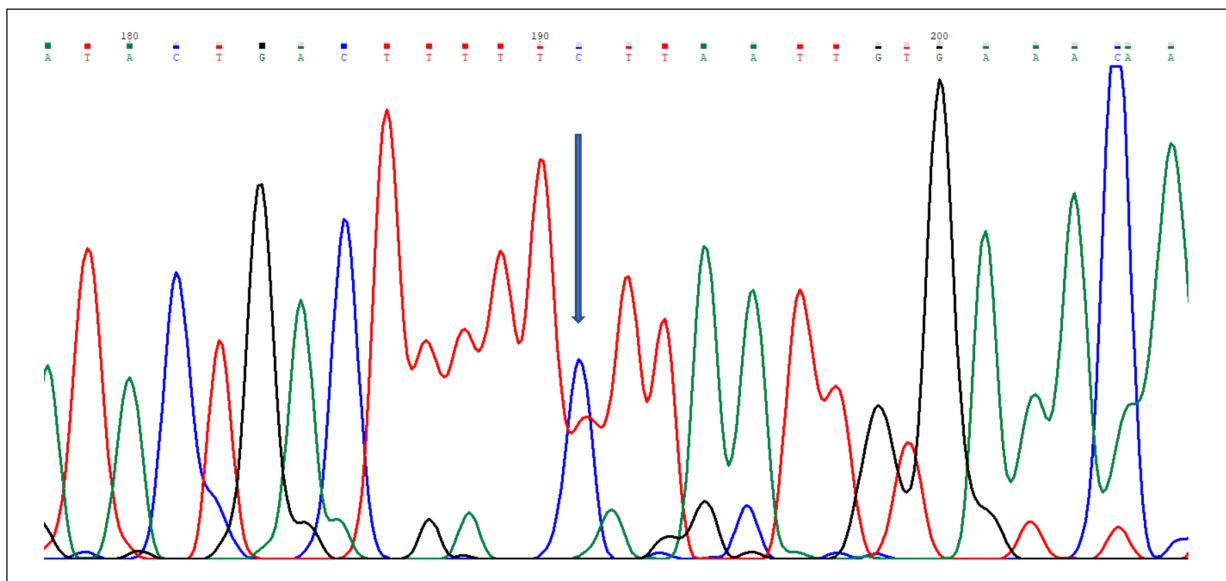


Figure 5. Chromatogram showing mutation of C>A at 4,947.0.

Using clinical traits from several groups, the rs2227485 minor allele has already been linked to autoimmune, cardiovascular, and pulmonary conditions. Mohammadi et al³¹ demonstrated a link between the minor allele and increased *IL-22* expression. Its presence causes a variation in the factor's ability to bind to transcription. Our study validates this minor allele in Pakistani SLE patients with noticeably higher allele T frequency in rs2227485. The current research builds on a prior investigation in which a relationship between the gene rs2227513 and SLE was established³²⁻³⁴. *IL-22* gene polymorphism rs2227485, which was demonstrated to be associated with papillary thyroid carcinoma (PTC), by Eun et al³⁵. Additionally, they demonstrated a significant correlation between allele T frequency and PTC33. Another study³⁴ has shown a striking correlation between rs2227513 and female HIV infection. These outcomes unquestionably support the current research. Yamamoto et al³⁶ found no evidence linking the *IL-22* gene polymorphism to ulcerative colitis. SLE patients from Pakistan demonstrated a striking rise in allele C frequency in rs2227491 and rs2227513.

The *FoxP3* gene controls and regulates Treg cell production. It successfully regulates the transcriptional processes that take place in our body's CD4+CD25+ cells. These Tregs have some degree of autoimmunity-causing dysregulation³⁷. Knowing that *FoxP3* contributes to SLE, it was expected to identify the precise area of the gene that mutates to produce SLE. *FoxP3* has

previously been the subject of various studies about SLE, although the typical location is still unknown. The current investigation examined SLE patients' exon 2 of *FoxP3* for mutations. Because the addition of the T at position 10 did not affect the entire framework, it was a synonymous substitution, and the region of *FoxP3* Exon 2 – which is only 231 bp long – showed 100% similarity with the reference, which is a normal healthy individual. As a result, this one is quite close to the exon's beginning and may be important for regulation. The population of SLE should be divided into groups according to ethnicity because the Lahore population is relatively homogeneous because of cousin marriages, indicating that the genome must be extremely distinctive³⁸⁻⁴¹.

Conclusions

Altogether 3 known and 6 new substitutions were found within the Pakistani patients with SLE. Some of the substitutions could have a role in the susceptibility to SLE. A study of a healthy cohort would establish their precise role.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Availability of Data and Materials

All the data generated in this research study has been included in the manuscript.

Authors' Contributions

Nageen, and Aqsa.; methodology, Aneela.; software, Saeed.; validation, Saad A and Afnan S.; formal analysis, Al-Abbass N.S; investigation, Tariq; resources, Aqsa; data curation, Barqawi A.A; writing—original draft preparation, Al-Abbass N.S, and Shaer NA.,; writing-review and editing, Barqawi; Afnan and Saad; visualization, Alshareef SA; supervision, Tariq.; project administration, Nageen; funding acquisition, Abdulhakeem SA and Majid A.

ORCID ID

Aqsa Bashir: 0000-0003-0436-9588

Tariq Aziz: 0000-0003-0905-8076

Ethics Approval

This study was approved by the Institutional Review Boards at both the Fatima Memorial Hospital College of Medicine and Dentistry in Lahore (IRB-FMH-09-2018-IRB-494) and the Sheikh Zayed Medical Complex National Health Research Complex in Lahore (IRB-1593).

Informed Consent

A written consent form was signed by the participants in this study.

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