Systemic Lupus Erythematosus Progression and role of Genetic Variation in IL-22 and FOXP3 gene in population of Lahore, Pakistan

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Abstract

Systemic Lupus Erythematosus (SLE) is one of autoimmune disorders. It is thought that the deregulation in the inflammatory markers is due to problem in Forkhead box family member (FOXP3) which is involved in tolerance mechanism. One cannot ignore the role of cytokine-mediated signaling pathways like IL-22. This study was done in the Lahore. Pakistan. The main objective of the study was to monitor the patients of SLE. The purpose was to check the alliance of *FoxP3* and *IL-22* gene polymorphism. Sixty samples (n = 60) were collected from different hospitals of Lahore. DNA was extracted from EDTA anticoagulated blood of SLE patients. After DNA extraction, *IL-22* and *FoxP3* genes were polymerized through PCR and further sequenced through Sanger Sequencing method. The *FOXP3* exon 2 and three SNPs in *IL-2* i.e. rs2227491, rs2227485 and rs2227513 which were already identified were confirmed by Chromas 2.6. The mutations were checked with the help of Nucleotide Blast. Our observation showed that there are nine mutations in studied genotyped samples. The frequency of mutation was 27.27%. Allele T in rs2227485 and, allele C in rs2227513 and rs2227491 was identified in the study predominantly. These 9 mutations were found in case of *IL-22* gene. No mutation was observed in Exon 2 of *FOXP3* gene in SLE patients. It is concluded that that there may be any association between *IL-22* gene polymorphism and SLE but *FOXP3* gene was not tangled in the progression of SLE in Lahore population.

1. Introduction

Systemic Lupus Erythematosus (SLE) is complex and prodigious disorder. It is serious inflammatory disease. It involves various organs and tissues of the body. There is a variation in SLE prevalence worldwide. In North America, it is about 241/100,000. In Asian countries, the prevalence of the disease is 30–50 in 100,000 populations [1]. If we talk about Pakistan, it is 7% of the rheumatologic burden [2]. It usually affects the child bearing women (age ranging from 15–45 years). It can also affect the old people and children. Women are 10 times more spiked as compared to men in case of SLE. This suggests that there may be any role of X chromosome in the pathophysiology of SLE [3].

Exact pathogenesis of SLE is still anonymous. The environmental factors could be involved to influence the autoimmune reactions. In the year of 1971 by Grumet *et al* [4] it was reported that Major Histocompatibility Complex (MHC) is associated with SLE. A case control study was done in India which indicates that there is an association between SLE and *IL-6* polymorphism [5]. Toll Like Receptors (TLR-5, TLR-9) and Programmed Cell Death-1 (PCD-1) have shown tendency with the activity of SLE [6]. A lot of research has done on SLE and the molecular machinery involved in the pathophysiology has not been identified in organized manner yet. The most valuable model of it is consists on cytokine-mediated signaling pathways and 182 genes in combination to it [7].

There is an important role of cytokines in the administration of systemic inflammation, tissue damage and immunomodulation. *IL-22* is bearded on cytokine family IL-10 and responsibility of its production is of T helper cells type 22 (Th22). *IL-22* was discovered in 2000. It is relatively a native type of gene. It was called as two-headed cytokine" and as it strength and enhance the inflammatory reactions so it is also

known as “a sheep in wolf’s clothing” [8]. In physiological state, the reconstruction of epithelial cells is done by them if the degradation of them occurs by apoptosis or any type of injuries. Similar function has done in case of some diseases i.e. malignancy and psoriasis. IL-22 receptor belongs to the family of IL-10. If we talk about the types of cytokines receptors it is of type 2. It consists of 2 subunits IL-22R1 and IL-10R2 and in nature it is heterodimer. In studies, it was found that it has great affinity for the IL-22R1 but no affinity was found for IL-10R2. These types of signaling pathways are involved in the progression of SLE [9]

IL-22 cytokine is encoded by a special gene known as *IL-22.* In case of human, it is found on 12q15 chromosome. It modulates many autoimmune disorder and processes like Rheumatoid Arthritis (RA), SLE, crohn’s disease, Multiple Sclerosis (MS), and psoriasis. In case of Behcet’s disease expression of this gene is high of. So, mRNA plays a major role in the progression of this disease [10]. In case of increased level of CCR6 + Th22 cells destruction of skin and renal tissues takes place in SLE. Level of *IL-22* is raised in muscular tissues in case of Dermatomyositis (DM) and Polymyositis (PM). This indicates that it has a correlation in myositic activity. Variant of the gene *IL-22* has expressed tendency to the thyroid disease which is autoimmune in nature (27). It is described in a recent Chinese research that the level of *IL-*2 in case SLE is decreased and rs2227513 polymorphism occur along with it [11]. So, *IL-22* polymorphism might possible tendency to SLE. This study would be a good contribution to scientific literature. Recently, a research is done in Iran. This research forecast the connotation of promoter polymorphism of gene *Interleukin 10* along with the action of SLE [12].

There are certain susceptible genes which trigger the SLE. Forkhead box P3 (*FOXP3*) is the gene which is reported to be involved in the T regulatory cells (Tregs) production and regulation. The cytogenetic location is represented as Xp11.23 [13]. Mutation in *FOXP3* causes the dysfunctionality of Tregs and trigger autoimmune disorders like Diabetes type 1, Polyendocrinopathy, X linked syndrome (IPEX), and Systemic Lupus Erythematous (SLE). Expression of *FOXP3* is stimulated by $CD4^{+}CD25^{-}$ *FOXP3* cells. The suppressive activity is seemed to be highly conflicted. Suppressive activity of CD25 + and CD4 + run in a parallel fashion [14]. This suppressive activity is an ectopic version of *FOXP3.* That’s why we call the *FOXP3* a marker for tracking Treg cells and can be used as in therapeutics of autoimmune diseases [15].

In human, there are two isoforms of *FOXP3* shown with alternative splicing, one wild type and one lacking exon 2. The *In-vitro* experiments showed that isoforms of *FOXP3* in mice show that get damage in immune system, while *In-vivo* functions are not yet cleared. The role of these isoforms in humans is also still unknown [16]. In this study, *FOXP3* gene which is marker for track Treg cell and *IL-22* gene are evaluated that whether they are involved in causing SLE in humans or not.

2. Methodology

The current research of *IL-22* genetic variants i.e. rs2227485, rs2227491 and rs2227513 were investigated entirely in the SLE patients enlisted from Rheumatology departments of three hospitals of Lahore, Pakistan i.e. Sheikh Zaid Hospital, Fatima Memorial Hospital and Mayo Hospital. All patients

were informed before sample collection. Patients' clinical profile and individual information were collected by filling questionnaire or by inspecting hospital record file. Patient's consent form and questionnaire were signed. EDTA vials were used and sixty samples were collected. Those SLE patients were selected who at least fulfilled 4 out of 11 revised American College of Rheumatology (ACR) Criteria [17]

2.1 Undertaking:

I confirm that all the experiments and work was done under the guidelines and under the permission of institution or licensing institute.

The approval of this research is taken by "Institute of review board Federal post Graduate Medical Institute Sheikh Zayed medical complex National Health Research Complex Lahore, by Institute of Review board Fatima Memorial Hospital College of Medicine and Dentistry Lahore and by ethical committee of Institute of Microbiology and Molecular Genetics University of the Punjab Lahore, Pakistan.
All these institute have capability to approve studies on Human beings. The informed consent was taken from all the patients. The patients and their guardians were informed about the purpose of sample collection.

The informed consent form was taken from the guardians of minors.
The samples were collected after the informed approval of the patient or their guardian. The approval certificates are provided here as well.

2.2 DNA isolation and PCR:

DNA was extracted through standardized DNA isolation protocol. Reported primers were taken [18] and, confirmed by UCSC In-Silico PCR and primer blast as well. The reaction volume of the PCR was 50 µl. The polymerization of gene *IL-22* gene was done by using the process of PCR. In it both forward and reverse primers were added and the quantity of each of them was about 2.5 µl. Water (nuclease free) was 15 µl. 5 µl of genomic DNA and the quantity of master mix was 25 µl. In PCR reaction first of all denaturation of DNA was done for time period of 5 minutes at 95°C, denaturation at 95°C for 30 seconds, annealing at 59.4°C for rs227485 and rs2227491, while 60.7°C for rs2227513 for 40 seconds. Initial extension done for the time period of 45 seconds at temperature 72°C and final extension was done at temperature 72°C for time period of 5 minutes. This PCR was set for 30 cycles. Samples were stored at 4°C. The product lengths were 166bp, 159bp and 274bp for rs2227485, rs2227491 and rs2227513 respectively and these were analyzed on 1.3 % gel by agarose gel electrophoresis [37]. Primers of Exon 2 of *FOXP3* gene were designed by using Primer 3 Software. The target for which the primers were designed was *FOXP3* gene exon 2. The primers designed were shown in Table-1. PCR reaction carried out to amplify the *FOXP3* gene's Exon 2 at 60.4°C.

2.3 Sequencing:

PCR products were purified and later genotyped by Sanger sequencing. Thirty-three samples were sequenced from ABI international company. The sequencing results were analyzed using Chromas

version 2.6.6. The similarity of sequences was checked by NCBI Blast after obtaining contigues via CAP3 software. Reference Sequence of IL-22 i.e. NG_060763 was used as control for checking SNPs in these sequences.

3. Results

Sixty SLE patients recruited in the present study. Six (10%) male participants were included in the study while fifty-four (90%) were recruited in the study. The percentage of male and female theme was demonstrated through gender distribution graph. Average age group of all participants was 30.24 ± 1.29 years. They were subdivided in three major age groups. Group A includes from 11 to30 years age, group B consists of patients having age from 31 to 50 years and group C from 51 to 60 years. Group A consists of 40 patients, group B comprises of 16 patients and group C comprised 4 patients. After DNA extraction and PCR, samples were further processed for sequencing. Mutation was found at position 10 of their reverse complement strand; there was an addition of T [Figure 1]. The mutations did not lie in Exon 2 region; they were before Exon 2; in intronic part. As the introns, do not encode proteins, they are non-coding regions, so mutation in them does not count for mutating the genome. Hence, no mutation was observed in Exon 2 of the gene *FOXP3* in patients of SLE in population of Lahore-Pakistan.

3.1 Screening of *IL-22 gene* plymorphisms:

Samples collected from population of Lahore, Pakistan were screened and investigation for mutations in *IL-22 gene* was done. Results showed that 9 out of 60 patients has mutations in *IL-22 gene*.

3.1.1 Mutation A > C at position 4672 in contrast to the reference sequence:

Mutation was observed which was A > C in sample 04-A. chromatogram showed change in the nucleotide at the position 127 i.e. C was replaced by A as compared to the reference sequence. [Figure 2a]

3.1.2 Mutation T > A at position 4674 in contrast to the reference sequence:

Mutation was observed which was T > A in two samples i.e. 65-A and 66-A [figure 2b and 2c]. Chromatogram showed change in the nucleotide at the position 129 i.e. A was replaced with T as compared to the reference sequence.

This showed that two types of SNPs were there in rs2227485.

3.1.3 Mutation C > G at position 5877 in constrast to the reference sequence:

Mutation was observed which was C > G three samples i.e. 31-B, 02-B and 42-B [figure 2d, 2e and 2f] respectively. Chromatogram showed change in the nucleotide at the position 85 and 86 (42-B) i.e. G was replaced by C as compared to the reference sequence.

3.1.4 Mutation C > T at position 5928 in contrast to the reference sequence:

Mutation was observed which was C > T in one sample i.e 67-B [figure 2g]. Chromatogram showed change in the nucleotide at the position 33.

3.1.5 Mutation C > A at position 4947 and 4932 in contrast to the reference sequence:

Mutation was observed which was C > A. It was in two samples i.e 65-C and F17-C [figure 2h and 2i] repectively. Chromatogram showed change in the nucleotide at the position of 205 and 191 correspondingly.

4. Discussion

In total lupus cases SLE describes about 70%. Dut to this reason it is a common type of lupus. “Lupus Foundation of America” evaluated that it effect a large number of people in America i.e 1.5 million and worldwide about 5 millions people. It is chronic disease. It is difficult to diagnose it at early stages. The reason behind the difficult dignosis is that it has the characteres of other autoimmune disorders. this it reason it is known as “the Great Imitator” [19]. SLE is serious inflammatory disease which can attack about each and every part of the body. Its pathophysiology is unknown yet. In case of homozygous twins, different etiological factors of SLE i.e. hormonal, environmental, genetic components, and infections are the influencing ones which distinctly reinforce the incidence of SLE i.e. >35% [20]. In this study, 13.3% were suffering from the severe typical butterfly rash along with the discoid rash. They had the problem of hair loss and seizures. The condition of patient reached to high intensity at some times and calmed down at other times. It indicates that the autoimmunity can hit in childhood with severity and passes with you as life pass. Immunosuppressive medications lower the severity but the disease bounces back at any time.

The progression of autoimmune diseases is carried out by Inflammatory cytokines. When T helper cells are triggered, they generate the cytokines. These cytokines are proved to contribute in the pathophysiology of SLE [21]. Principally IL-22 is generated by TH 22 [22]. Moreover, in synovial fibroblast receptor activator of the nuclear factor kappa B-Ligand (RANKL) is triggered by IL-22. This phenomenon is proved to cause the condition of osteoclastogenesis in case of RA [23]. So, these studies proved the role of IL-22 in the disease which is autoimmune in nature such as in SLE. Noticeably the receptors of IL-22 (IL-22R1) are usually present on the surface of non-immune cells i.e. pancreatic, epithelial and renal tubular cells. IL-22 cannot trigger the immune cells, rather the inflammatory mechanism is being stimulated and expressions of the cytokines like IL-1, IL-6 is controlled [24]. So, it indicates that there is a need of more studies to investigate the role of this disease in this pathological problem.

“Genome Wide Association study (GWAS)” discovered association of more than about 100 loci along SLE at the level of significance i.e. $<5x10^{-8}$ and it is recognized in Asian and communities of Europe. If we consider these loci a lot of objective genes and SNPs are recognized in it. Surprisingly, the number of SNPs in the coding sequence that effect the structure of protein is very low in number. Many of the SNPs are present in the non-coding region of gene. These SNPs fluctuate the expression of post transcriptional modifications and epigenetics [25]. Recent study is done to study the association of polymorphism of *IL-22 gene* i.e. rs2227485, rs2227491 and rs2227513 with the patiients of SLE. The results showed that there were 9 locations were mutations were found in total thirtythree (33) samples. The frequency of 27.27 %. All the detected SNPs detected were seen that they were homozygous. This was proved because

only one peak arised in all mutations. All of these SNPs were intrinsic. Remarkably, it was observed that the frequency of allele T is high in rs2227485, the frequency of allele C is high in the rs2227491 and in rs2227513. Present study was designed by following the foot steps of Chinese study. In this study, an association of rs2227513 with the SLE was proved [26]. In another study the association of *IL-22 gene* polymorphism was seen with the Papillary Thyroid Carcinoma (PTC). Significant frequency of association of allele T was seen with PTC [27].

Another research has proved the notable association of HIV infection with rs2227513 in women. However, another study was done that proved that there is no association of ulcerative colitis with *IL-22 gene* polymorphism [28]. Intronic varients added the importance in this era of time. They play most important in the modulation of gene expressions i.e it is described by Qaddourah *et al* that there is an association intronic varient of *IL-10* with frequent miscarriages in case of women [29]. Xu *et al* explained that certain specific proteins for SNP that contain G act as the energetic binding partner. Any changing that occur from G to A incapacitate the binding type of protein and promotes the bouncing of exon and ultimately they result in the expression of gene which is altered [30].

Current study is indicating that the distibution of SLE in females (90%) is predominent as that of male i.e 9:1 respectively. 86.6% patients were facing the problem of fatique which was a predominant general sign in the SLE patients. 63.3% patients showed arthiritis as a clinical feature. These findings were compared with a study done in Pakistan where 91.4% females were facing the problem of lupus while probability of fatigue and Arthritis was 79.03% and 78.1% respectively [31]. The calculated mean age of the personnels in this study was 30.25 ± 1.3 years while in another pakistani study this was 31.6 ± 10years [32]. Problem of photosensitivity and oral ulcers were also observed in this study in SLE patients and their probability was 61.66% and 41.66% respectively. The value of these results is high as compared to the findings done by Rasheed *et al* where problem of the Photosensitivity were 49.12% and complain of oral ulcers was 31.35% [33]. Renal involvement was also noticed in this study which was seen in the 38.33% patients which seems parallel to results of Rabbani *et al.* In that study only 33% of renal invovement was found [34]. The results of ANA were 98.3% and that of Anti-ds DNA were 96.6%. In an Arabian research these numbers were 99.7% in case of ANA and 80.1% in case of Anti ds DNA. Overlap syndrome was studied as well in case of 16.66% patients. A research done by Ahsan *et al* where these the presence of these syndrome cases was 0.5% [35].

First time in the history of Pakistan this study has described the *IL-22 gene* polymorphism in SLE patients The mutations which are detected are very urgent to notice them. Further studies sould be needed to programme for the detection that how intronic varient *IL-22* is responsible for modulation of the expression of gene in patients of SLE.

Forkhead box P3 *(FOXP3)* gene is the production, regulation house of Treg cells. It efficiently control the transcriptional activities of CD4 + CD25 + cells in our body. A minute deregulation in these Tregs lead to autoimmunity [36]. Since it was known that *FOXP3* is involved in SLE, it was thought to find out the exact region of the gene which get mutated and causes SLE. A number of researches were done on *FOXP3*

related to SLE, but typical region is still unknown. In the present study Exon 2 of *FOXP3* was selected to check mutation in SLE patients. The region of *FOXP3* Exon 2 lies at 11,305 – 11,536, which is only 231bp long.

When the results were analyzed, it was noticed that 11, 281 is the intronic part, not the exonic region. As the exonic region falls under 11,305 – 11,536, and 11,281 is out of that exonic region. The primers also amplified the introns along-with exons. As the mutation in introns does not consider a mutation because introns are non-coding regions and they don't form proteins. Mutation in introns doesn't affect any mechanism of immune dysregulation. So, no FOXP3 Exon 2 was found to be involved in progression of disease in SLE patients. The Exon 2 of patients showed 100% similarity index with the reference sequence.

5. Conclusion

The present research indicates that there may be a posible association of polymorphism of IL-22 gene with SLE but not of FOXP3 gene.

Declarations

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CONFLICT OF INTEREST:

The Authors declare that there is no conflict of interest.

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Table

Table 1: *FOXP3* Exon 2 Primers Description

Olign Name	Sequence (5’-3’)	Length	MW	TM	EC	nmols	µg	A260 Units
Exon-2F	cgtgtgactcctttccccta	20	5995	57.5	185.4	56.6	339.5	10.50
Exon-2R	Acagtaaaggtcggcacctg	20	6151	57.5	224.5	50.6	311.0	11.35

Figures

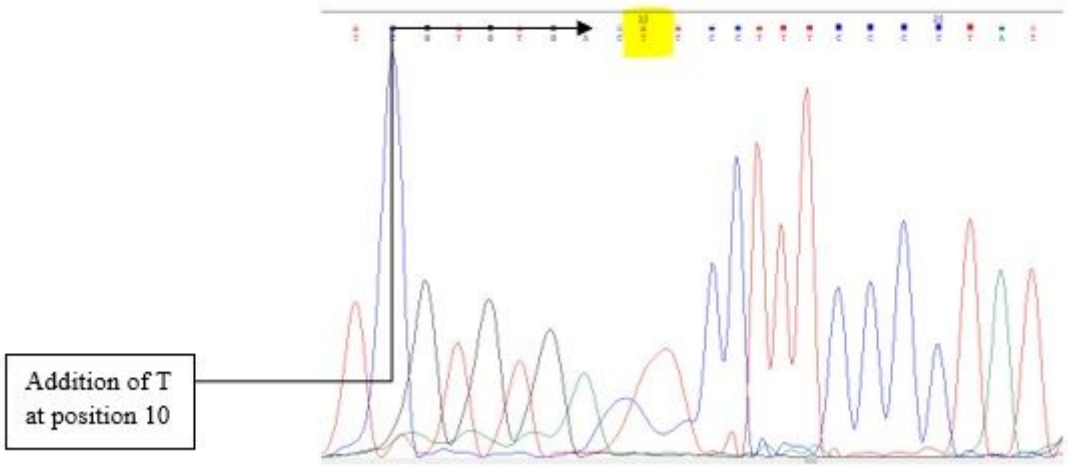

Figure 1

Chromatogram of Addition of T at position 10 in reverse complement strand exon 2 of Fox P3 gene

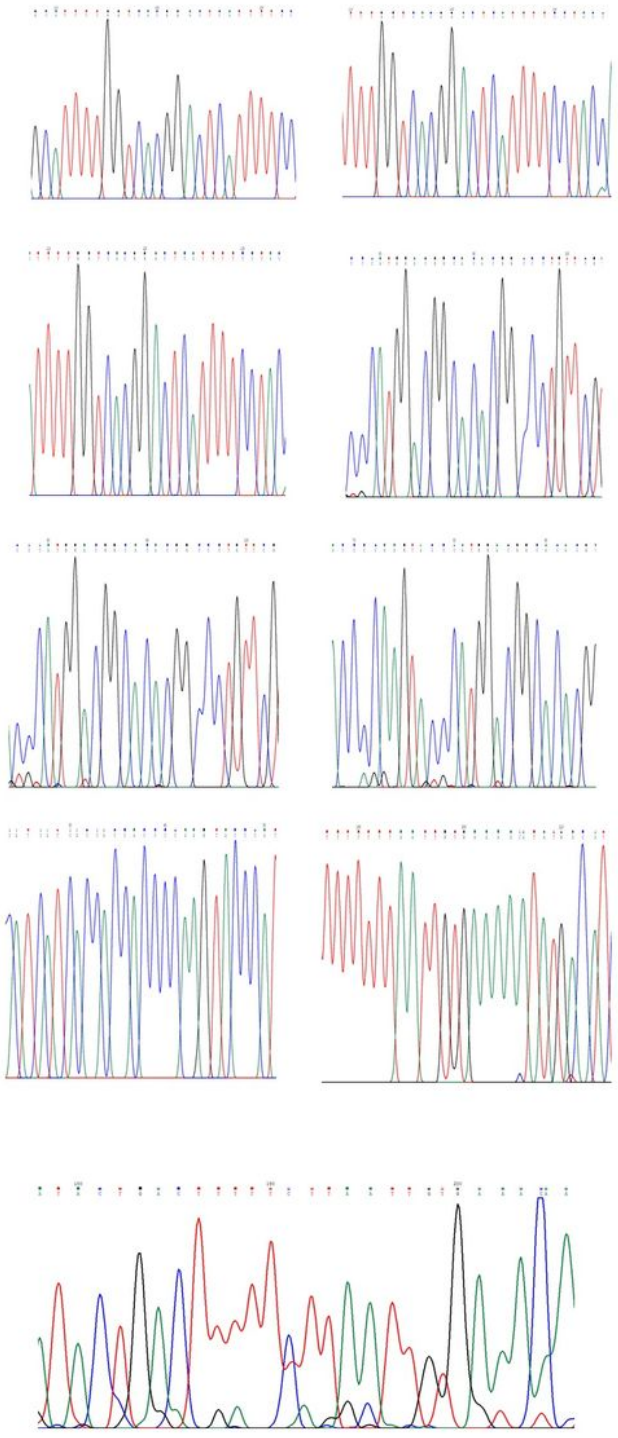

Figure 2

a: Chromatogram showing mutation of A>C at 4672 in RefSeq. b: Chromatogram showing mutation of T>A at 4674 in RefSeq. c: Chromatogram showing mutation of T>A at 4674 in RefSeq. d Chromatogram showing mutation of C>G at 5877 in Ref Seq. e: Chromatogram showing mutation of C>G at 5877 in Ref Seq. f: Chromatogram showing mutation of C>G at 5877 in Ref Seq. g: Chromatogram showing mutation

of C>T at 5928 in Referance Sequence. h: Chromatogram showing mutation of C>A at 4932. i: Chromatogram showing mutation of C>A at 4947