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Mutational Analysis of *CTLA4* Gene in Pakistani SLE patients

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***Abstract*—**The main aim is to perform mutational analysis of *CTLA4* gene Exon 1 in SLE patients. A total of 61 SLE patients fulfilling "American College of Rheumatology (ACR) criteria" and 61 controls were enrolled in this study. The region of *CTLA4* gene exon 1 was amplified by using Step-down PCR technique. Extracted DNA of band 354 bp was sequenced to analyze mutations in the exon-1 of *CTLA-4* gene. Further, protein sequences were identified from nucleotide sequences of *CTLA4* Exon 1 by using Expasy software and through Blast P software it was found that *CTLA4* protein sequences of Pakistani SLE patients were similar to that of Chinese SLE population. No variations were found after patients sequences were compared with that of the control sequence. Furthermore it was found that *CTLA4* protein sequences of Pakistani SLE patients were similar to that of Chinese SLE population. Thus *CTLA4* gene may not be responsible for an autoimmune disease SLE.

***Keywords*—**American College of Rheumatology criteria, autoimmune disease, Cytotoxic T Lymphocyte Antigen-4, Polymerase Chain Reaction, Systemic Lupus Erythematosus

I. Introduction

MHC region genes are known as a prominent genetic factor of autoimmune diseases in both animal models and in humans. In humans, number of MHC genes has been identified and one of the most studied is Cytotoxic T lymphocyte associated antigen 4 gene (*CTLA4*), which is located on chromosome 2. This gene consists of four exons and the size spans to 6,175bases. It is identified as the fourth cDNA that are particularly expressed in Cytotoxic T Lymphocytes. This molecule is very much similar to CD28 molecule. CD28 as well as *CTLA4* play a dynamic role in autoimmune disorders [1]. So the main objective of this study is to perform mutational analysis of *CTLA4* gene Exon 1 in SLE patients.

II. Subjects And Materials

The human studies reported in this manuscript were approved by the "Ethical Committee of the School of Biological Sciences", University of the Punjab, Lahore, Pakistan. Samples described in this work are part of a larger study on lupus patients coming to different hospitals of Lahore for the treatment. The study population covered different areas of Punjab; mainly Lahore, Gujranwala, Sialkot, Raheem-Yar Khan, Gujrat and district Kasur. Of the 61 SLE patients, fifty-five (90.16%) were females and six (9.83%) were males. The female versus male ratio was 9.16:1. Mean age at diagnosis was 30.163 years (Range: 11-68 years). Mucocutaneous involvement was found in lupus patients such as malar rash 6 (9.84%), discoid rash 11 (18.03%), photosensitivity 11 (18.03%). Various syndromes were overlapping with SLE and the most common one was Rheumatoid arthritis (19 patients: 31.14 %) but cases of Sjogren's syndrome (10 patients: 16.39 %), Scleroderma (2 patients: 3.27%), Secondary Antiphospholipid syndrome (2 patients: 3.27%), and of Budd-Chiari Syndrome (1 patients: 1.63%) were also found along with SLE. Renal involvement was found in 32.78% patients characterized by proteinuria and red cell cast. Blood samples drawn by venepuncture from each patient and control were collected in a EDTA vial. An informed written consent was taken from all subjects recruited in the study as well as from the matched controls. Genomic DNA (Fig. 1) was purified from peripheral blood by using Genomic DNA purification kit (Fermentas, Cat # K0512-Germany). The concentration of DNA was estimated by taking A_{260}/A_{280} ratio with the help of spectrophotometer (Cecil, CE 7200). Absorbance ratio A_{260}/A_{280} 1.8 or above was taken as pure preparation.

Fig. 1 Genomic DNA Purification

PCR was performed by Step-down PCR technique in order to get rid of non-specific bands and achieved the band of our interest that is of 354 bp (Fig. 2).

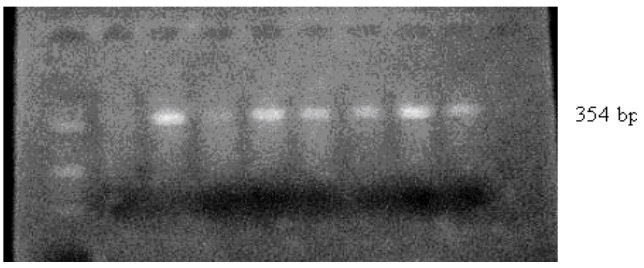

Fig. 2 PCR Amplified Products

Desired PCR amplified product with the band size of 354 bp (Well #: 3, 4, 5, 6, 7, 8, 9) for the study of Mutational analysis of CTLA4 Exon 1 in SLE patients. Ladder was loaded in well 1 (O-GeneRuler™ DNA Ladder, Low Range, Fermentas Cat No. R0631).

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The region of "*CTLA4* gene exon 1" that was amplified is as follow:

GCCTTCTGTGTGTGCACATGTGTAATACATATCTGGGATCAAAGCT
ATCTATATAAAGTCCTTGATTCTGTGTGGGTTCAAACACATTTCAA
AGCTTCAGGATCCTGAAAGGTTTTGCTCTACTTCCTGAAGACCTGA
ACACCGCTCCCATAAAGCCATGGCTTGCCTTGGATTTCAGCGGCAC
AAGGCTCAGCTGAACCTGGCTACCAGGACCTGGCCCTGCACTCTCC
TGTTTTTTCTTCTCTTCATCCCTGTCTTCTGCAAAG

Procedure of Step-down PCR

The PCR reaction was performed in a total volume of 50 µl using 250 ng of genomic DNA, 10 pmol of each of the primer mixture and 2X PCR mixture. *CTLA-4* gene exon 1 was amplified with the primers designed by entering the selected portion of *CTLA-4* exon 1 in Primer 3 Software. Step-down PCR conditions consisted of an initial 95°C denaturation for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, then annealing step and finally elongation at 72°C for 30 seconds followed by final extension at 72°C for 3 minutes. The difference lies in the annealing temperature; in the beginning annealing temperature was higher than the melting temperature of the primers that was 60°C; then there was fewer and steeper decline in the PCR annealing temperature that was up to 52°C. Genomic DNA was subjected to 35 cycles of amplification according to "Step-down PCR" protocol in a peQLab advance primus 96 thermal cycler, USA. The DNA fragment of 352 bp was analyzed on a 2% agarose gel. Low-range DNA ladder (Fermentas Cat # R0631) was used to verify results.

III. Results

Extracted DNA of band 354 bp was sequenced to analyze mutations in the exon-1 of *CTLA-4* gene. "Chromas" software was used to convert the instrumental data in to a nucleotide base order and "Edit Seq" software to analyze the resulting nucleotide sequence. Particularly, we were interested in *CTLA4* gene polymorphism of exon 1 at +49. According to "Genbank", the standard sequence of *CTLA4* gene includes adenine (A) at +49. If there is any A to G polymorphism at position +49 in *CTLA4* Exon 1 then there should be threonine to alanine substitution in the leader peptide. But no variations were found after patients sequences (TABLE 1) were compared with that of the control sequence by using Basic Local Alignment Search Tool (BLAST).

TABLE I
ACCESSION NUMBER GIVEN TO SEQUENCES

Sample #	Accession #	Patient description
Sample 02- P7	HM545121	SLE
Sample 59-P8	HM545122	SLE + Budd-chiari syndrome
Sample 24-P14	HM545123	SLE + Lupus nephritis
Sample 03-P16	HM545124	SLE + Skin involvement
Sample 26-P30	HM545125	SLE overlapping Scleroderma
Sample 05-P36	HM545126	SLE + Antiphospholipid syndrome

Patient sequences were also compared with sequence submitted to "GenBank" by Celera and identical sequences submitted by "Human Genome Organization" were found. Thus all case as well as control sequences contained adenine (A) at +49. Further, protein sequences were identified from nucleotide sequences of *CTLA4* Exon 1 by using "Expasy software" and through "Blast P" software it was found that *CTLA4* protein sequences of Pakistani SLE patients were similar to that of Chinese SLE population.

IV. Discussion

Number of polymorphisms in the *CTLA4* gene has been identified that are genetically linked to several autoimmune disorders, namely systemic lupus erythematosus, grave's disease, rheumatoid arthritis, type 1 diabetes, celiac disease, autoimmune thyroid disease, and multiple sclerosis [2]. There are more than 100 known single nucleotide polymorphisms in the *CTLA4* gene. In humans, *CTLA4* gene has been proposed to play a vital role in autoimmune pathogenesis [3]. SLE patients have been genotyped for allelic determinants at four polymorphic sites in the CTLA-4 gene. Three polymorphisms were in the promoter region while one is within the exon 1 at position +49 (A/G). *CTLA4* exon 1 49G allele coding for position 17 Ala has been shown to be significantly associated with autoimmune diseases. In Chinese population, no statistical difference was found in both exon 1 and promoter gene polymorphism between SLE patients and controls [4]. In the present study, adenine deletion at 49bp could not be considered because the peak was quite wide (Fig. 3) so the experiment was repeated to confirm results but no such mutations were found.

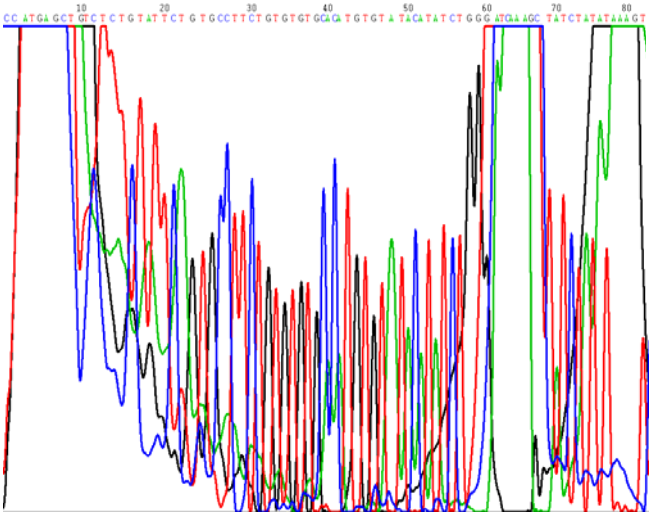

Fig. 3 Sequence representing 49 A/G region

In the present study, *CTLA4* gene Exon 1 was studied in SLE patients. Amplification of 356 bp fragment from *CTLA4* gene supposed to be carried 49 A/G polymorphism but no such SNP was found in *CTLA4* gene Exon 1 of SLE patients thus the results matched with Chinese popuation. After sequencing, alignment of six different sequences was done by the use of multiple sequence alignment tool "Seaview". These nucleotide sequences were not only similar to *CTLA4* gene Exon 1 but also to Eukaryota, Metazoa, Chordata, Craniata, Vertebrata, Euteleostomi, Mammalia, Eutheria, Euarchontoglires, Primates, Haplorrhini.

Further, protein sequences were identified from nucleotide sequences of *CTLA4* Exon 1 by using "Expasy software" and through "Blast P" software it was found that *CTLA4* protein sequences of Pakistani SLE patients were similar to that of Chinese SLE population. HM545121 was a linear DNA of 216 bp, its protein sequence was similar to Cytotoxic T lymphocyte associated protein 4 and its product was "MACLGFQRHKAQLNLATRTWP". HM545122 and HM545123 contained frameshifts relative to similar CTLA4 protein. In case of HM545122, coding region contain frameshifts that were present around nucleotides 168, 175 and 195 while in HM545123 frameshift was present around nucleotide 164 that result in different translations from similar proteins in the database. Therefore in both cases the CDS was converted in to misc_features and the translation was removed. HM545124 was a linear DNA of 190 bp and after translation the product was "MACLGFQRHKAQLNLATRTWPCT". HM545125 and HM545126 had a linear DNA of 216 bp and 230 bp respectively and their translated products were "MACLGFQRHKAQLNLATRTWP" and MACLGFQRHKAQLNLATRTWPCTL" (TABLE 2).

TABLE II
NUCLEOTIDE SEQUENCES TRANSLATED TO PROTEIN PRODUCT SIMILAR TO CTLA4 PROTEIN

Accession #	**Protein product**
HM545121	MACLGFQRHKAQLNLATRTWP
HM545122	Frameshifts similar to CTLA4 protein
HM545123	Frameshifts similar to CTLA4 protein
HM545124	MACLGFQRHKAQLNLATRTWPCT
HM545125	MACLGFQRHKAQLNLATRTWP
HM545126	MACLGFQRHKAQLNLATRTWPCTL

V. Conclusion

No CTLA-4 gene Exon 1 mutations were found in Pakistani SLE patients with "Sjogren's syndrome" or SLE patients with "Budd-Chiari syndrome" and "Scleroderma". One of the major reasons is that SLE is a complex genetic disorder and multiple genes are involved so the effects of this gene may be weak and thus go undetected experimentally. Another reason for the conflicting results with other populations may include differences in the ethnic groups and the age at the onset of the disease. Thus *CTLA4* gene is not responsible for SLE.

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