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## Short Communication

# Mutational analysis of *Forkhead box P3* gene in Pakistani Human Immunodeficient Virus Patients

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**The project was designed and supervised by NH. Sample collection and experimental work were done by AJ.**

**Key words**
**Human immunodeficiency virus, *FOXP3* gene, T regulatory cells, Gradient PCR, mutation**

## ABSTRACT

*Human immunodeficiency virus* (HIV) becomes one of the most severe endemic viruses that advance around the world in a deadly manner. It is a serious issue in Pakistan as well and so far 130,000 HIV cases in Pakistan have been identified by the National AIDS Control Programme. Nuclear transcription factor forkhead box P3 (*FOXP3)* gene is involved in tolerance mechanism so failure of tolerance can lead to mutations in this gene. The main objective of this project was to analyze the possible mutation especially in *FOXP3* gene exon 1 that may clarify the reason of reduction of T regulatory cells (Tregs) due to HIV/ AIDS. A total of 25 HIV patients were chosen from the Institute of Public Health on the basis of confirm HIV infection and 25 healthy controls as well. First genomic DNA was extracted from the peripheral blood and then amplified by using specific designed primers. Gradient PCR was performed and the product length was 197 bps which was further analyzed on 1% agarose gel. Sequencing was done through genetic analyzer (3500 ABI). No mutation was observed in *FOXP3* gene exon 1of Pakistani HIV patients.

*Human immunodeficiency virus* (HIV) becomes one of the most severe endemic viruses that advance around the world in a deadly manner. Currently, no well-known remedial therapy available that knock out virus from HIV patients; however, antiretroviral drugs act vital aspect in lessening the rate of fatality and also restraining the infection's development (Janahi *et al*., 2016). In 2005, overall deaths of AIDS patients was about to 2.3 million that shrink to 1.6 million in 2012. In 2012, an approximated 9.7 million people in middle income countries had initiated antiretroviral remedy (Maartens *et al*., 2014). In Pakistan, 130,000 HIV cases have been observed by the National AIDS Control Programme (Ahmed *et al*., 2016). HIV can occur in virtually any body fluid but its transference can happen principally via breast milk, rectal and vaginal fluids, semen and blood (Becerra *et al*., 2016). Mature HIV transmitted to host cell by binding of surface glycoproteins gp120 present on HIV to CD4 receptors on host cell surface. After entrance into the cell, reverse transcription process starts for the formation of provirus DNA. When HIV provirus will assimilate into host cellular DNA, both viral components and cellular DNA become mandatory to trigger the viral expression genes resulting in affecting the immune system (Hu *et al*., 2006).

Main well-known target of HIV are T helper cells (CD4+ T cells) and most well examined immune cells (Woodham *et al*., 2016). During primary infection of HIV disease, CD4 and CD8 T cells behave as central to control the viral point and manage the early viremia. For maintenance of early viremia, high levels of expression of nuclear transcription factor forkhead box P3 (*FOXP3*) and CD25 (chain of IL-2 receptor) was done by CD4 natural regulatory T cells (nTregs) (Chevalier *et al*., 2016). Overexpression of *FOXP3* gene enables to inhibit the HIV replication in CD4+ T cells during primary infection, preventing the infection of both FOXP3+ and FOXP3- cells (Selliah *et al*., 2008). Later on, Tregs become dysfunctional or decreased in AIDS patients (Suchard *et al*., 2009). Less count of CD4+ and greater amount of mobilized T cells have less number of FOXP3+CD4+CD25$^{hi}$ T cells found in HIV positive patients, implying dysregulation of Tregs in the course of HIV infection (Oswald-Richter *et al*., 2004) Thus, it was evaluated that the frequency of Tregs are decreased in HIV+ positive patients while in group of uninfected, no reduction of Tregs was observed. This clarifies that FOXP3 mRNA expression is reduced in HIV+ positive patients as compared to controls (Apoil *et al*., 2005). In agreement with other analysis, it was being noticed that number of Tregs are above the normal but function is extremely decreased with noticeable HIV-1 RNA in plasma in contrast to healthy controls (Tsunemi *et al*., 2005). *FOXP3* gene is mostly associated with

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autoimmune diseases, indirectly may disclose association of autoimmune disease with HIV/AIDS. Mutation in *FOXP3* gene particularly in exon 1 was observed in Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome (Oda *et al*., 2009). The main objective of this project was to analyze the possible mutation in *FOXP3* gene exon 1 that may clarify the reason of reduction of Tregs due to HIV/AIDS.

*Materials and methods*

The total sample size was 50 and the focal point of this study was different cities of the Punjab province of Pakistan. A total of 25 HIV patients were chosen from the Institute of Public Health on the basis of confirm HIV infection and 25 controls were selected. Completely covered Eppendorf's were further carefully stored at 4°C in an isolated rack of refrigerator before DNA isolation.

Both controls and HIV positive Genomic DNA were extracted from peripheral blood using (Favorgen kit, Catalogue No. FABGK 001, 50 Preps). DNA bands extraction were allowed to check thorough agarose gel electrophoresis. Amplification of exon 1 of *FOXP3* gene was performed by using specific designed primers (Table I). The PCR reaction was executed in total volume 20ul using 250 ng of genomic DNA, 2X PCR mixtures and 1ul of each of primer mixture. Gradient PCR was performed and the product length was 197 bps which was analyzed on 1% agarose gel. Thermo Scientific GeneRuler 100bp plus DNA ladder Catalog (# SM0323) was used to identify the results.

Ethanol purification of sequencing PCR products (BigDye Terminator V3.1 Sequencing standard Kit) was done and analyzed through genetic analyzer (3500 ABI).

*Results and discussion*

Most of the HIV patients (n=25) lie within the age group of 21-40 years (Fig. 1). CD4 and CD8 counts/ul were very lowered as compared to reference range measured through Flow cytometry in Figure 2. DNA bands were extracted and Exon 1 of *FOXP3* gene was amplified at 57.8°C annealing temperature and the product size of 197bp was achieved (Fig. 3). Random purified HIV sequencing PCR products were analyzed, and it was observed that no mutation occurred in exon 1of *FOXP3* gene of HIV patients. Sequencing results are represented in Figure 4 and 5.

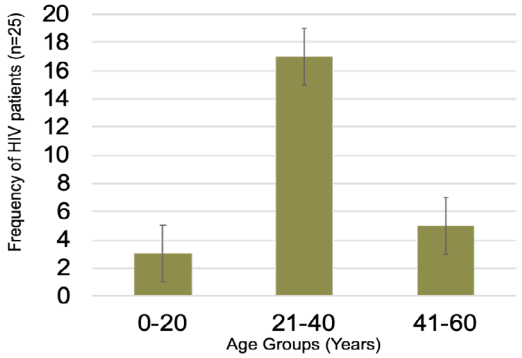


Fig. 1. Frequency distribution of HIV patients (n=25) with reference to age.

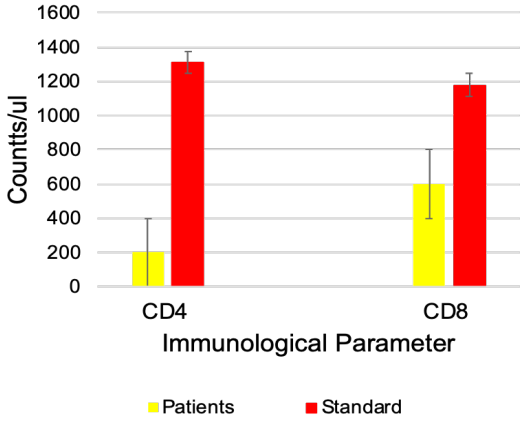


Fig. 2. Comparison of patients CD4 and CD8 counts/ul with standards.

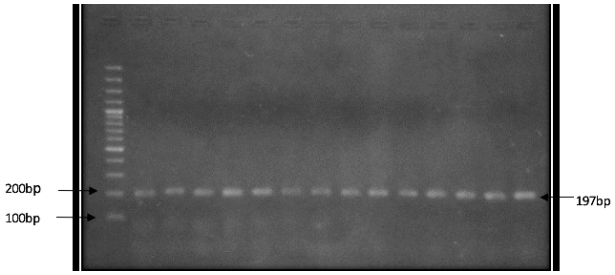


Fig. 3. Representation of PCR bands (197bp each) of exon 1 of FOXP 3 gene of both HIV and Control samples. HIV PCR bands from 2nd well to 7th well and control PCR bands from 8th.

**Table I.- Primers designed for exon 1 of *FOXP3* gene.**

| Oligo name | Sequence (5'-3') | Length (bp) | MW | TM (°C) | EM | nmols | µg | Area units |
|---|---|---|---|---|---|---|---|---|
| Forward Primer | CGCACACACTCATCGAAAAA | 20 | 6048 | 53.4 | 230.8 | 42.8 | 258.9 | 9.88 |
| Reverse Primer | CATCTGGTAGGGGAGAGCAG | 20 | 6247 | 59.5 | 228.8 | 45.5 | 284.2 | 10.41 |

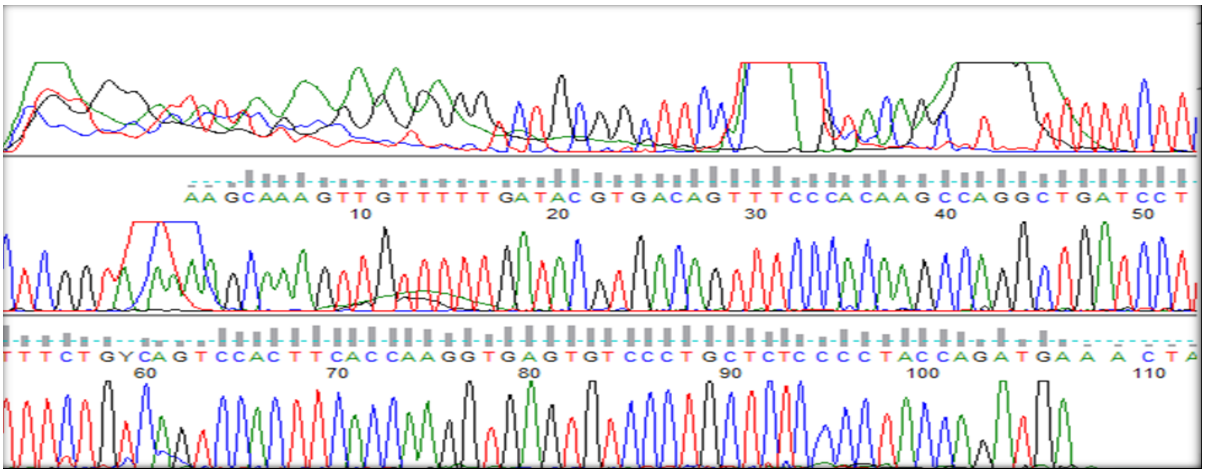

Fig. 4. Representation of sequencing of HIV sample 1 from Genetic Analyzer (3500 ABI) Software

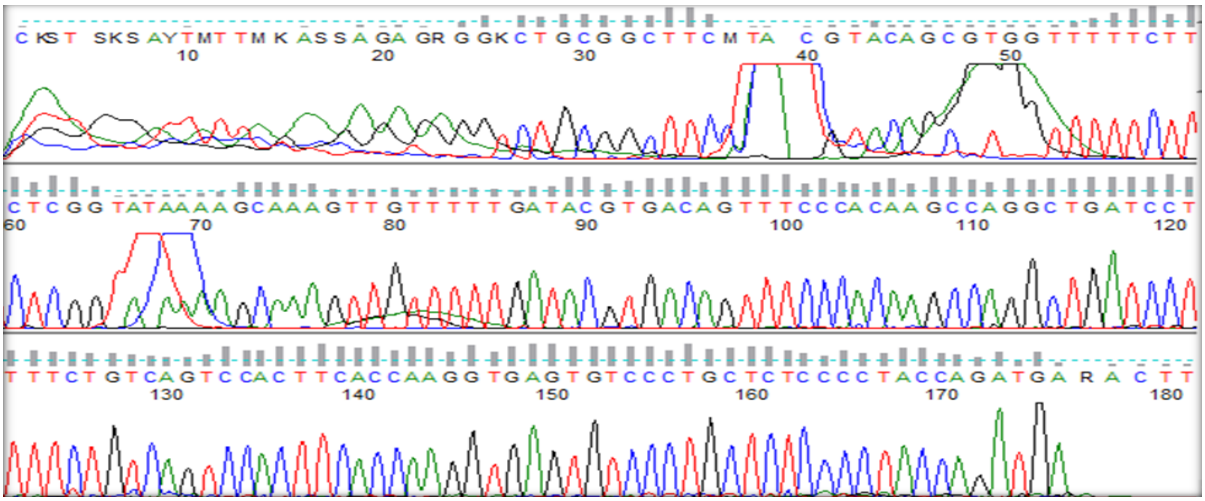

Fig. 5. Representation of sequencing of HIV sample 2 from Genetic Analyzer (3500 ABI) Software

*FOXP3* is a central controller gene responsible for the production and activity of Tregs cells are defected in IPEX syndrome indicated that mutation has occurred in exon 1 of FOXP3 gene. Deletion mutation was observed that included 1388bp consisted of 5 half exon-1 and segment of 1st intron. This caused the deficiency of Tregs due to low production of degraded mRNA transcribed from *FOXP3* gene in IPEX patients. Defective Tregs are present in HIV patients and can be cleared according to one scientific research, when Tregs from HIV patients did not suppress the function of polyclonal autologous CD8+T cells specifying lack of suppressive activity of Treg/HIV+ as compared to HIV negative controls (Oda *et al*., 2013) So this clarifies that genetic change in *FOXP3* gene cause defective production of Regulatory T cells. This point emphasizes to find out whether the mutations exist or not in *FOXP3* gene as the number and function of Tregs are abnormal in HIV patients.

In Pakistan, HIV is mostly prevalent because of the usage of poor management of blood transfusion systems. No health care working institution ponders over this bad and old management of transfusion systems and using constantly resulting most resistant HIV infection advancement. A survey conducted in Faisalabad, Pakistan proved the HIV infection was more in males as compared to females. In this study, the frequency of HIV infection was found to be highest in males (100%). CD4 T cells depletion is an important indicator of HIV infection. It was also confirmed that CD4:CD8 and CD4 counts are inversely related with proviral DNA and both lower values against reference values were confirmation of HIV infection. This study has proved below an average CD4:CD8 ratio

(0.3428) in HIV patients (Nikolova *et al*., 2017). In current scenario, exon 1 having product size of 197bp of *FOXP3* gene was amplified by using extracted genomic DNA of both 25 HIV patients and 25 HIV negative controls. PCR bands of HIV samples were sequenced through DNA sequencing Genetic Analyzer. In the present study, no mutation was observed in *FOXP3* gene exon 1 of HIV patients by using BLAST and Chromas Pro 2.4.1 software.

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*Conflicts of interest*

Authors declare no conflict of interest.

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