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Original scientific article

INVESTIGATION OF THE CC CHEMOKINE RECEPTOR 5 (CCR5) DELTA32 AND GENE VARIANTS IN HIV INFECTED PATIENTS

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CCR5 is an important CC chemokine receptor for the normal entry of Human Immunodeficiency Virus (HIV) in host cells. Mutations in this gene have been linked with delayed HIV infection, therefore, the current case-control study was conducted to identify genetic polymorphism in CCR5 gene in Pakistani population and to evaluate its association with resistance for HIV infections. All regulations of MOOSE and Helsinki Declaration were strictly followed during the entire study. DNA was extracted from the blood samples of HIV positive and HIV negative patients. Extracted DNA was amplified for CCR5 gene by PCR. Amplified product was sequenced to screen out polymorphism mutation, including most prevalent 32 base-pairs deletion through Bioedit. Novel SNP in 5'UTR region was identified and examined by CRYP-SKIP server. None of the studied samples demonstrated any previously reported polymorphisms. CRYP-SKYP server predicted that this polymorphism has no effect on splicing or transcription of CCR5 gene. Genetic Polymorphism of delta32 mutation in CCR5 gene was found in Pakistani individuals. To the best of our knowledge, this is the first report from this region. However, large scale studies should be conducted for extensive view of the association of delta32 mutation in CCR5 gene and resistance for HIV infection.

Keywords: Human Immunodeficiency Virus, delta32 mutation, Genetic Polymorphism, Pakistan, CCR5 gene

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INTRODUCTION

Human Immunodeficiency Virus (HIV) is a virus that bouts the immune system of human body. If the host's immune system is not strong enough or if this virus is not exterminated at the early stages, then this virus establishes large number of viral reservoirs which ultimately prompts severe chronic inflammation and a chronic, potentially deadly disease, known as, acquired immunodeficiency syndrome (AIDS). AIDS is termed as life- threatening condition as it severely affects host's immune system, deteriorating its capability of fighting with infections and diseases (KLEIN, 2018).

AIDS becoming more and more prevalent with the passage of time, according to the recent report of World Health Organization (WHO), 37 million individuals are affected with this hazardous infection, out of which 1.5 million have been identified in 2020; this clearly shows the growing trends of HIV infection. Just like the other diseases, HIV is also quite prevalent in developing World; however, in the second and third World countries, the exact number of infections is not reported precisely. One example of such countries is Pakistan, where the estimated occurrence of HIV infection is <0.1% (AHMED *et al*., 2019). The major reasons of HIV infections in Pakistani patients include; drug Injections, sex with same gender and Prostitution (DAR *et al.,* 2017).

There are two distinct types of HIV virus namely; HIV-1 and HIV-2, out of which former one is responsible for approximately 95% cases of HIV infection and its occurrence has been reported all over the Globe. Contrary to type 1, HIV-2 is predominantly limited to communities that are socioeconomically associated with West Africa. Furthermore, HIV-2 is reported to be less infectious and less progressive in nature, therefore, linked with fewer deaths (ESBJÖRNSSON *et al*., 2019).

Although the exact mechanism of pathogenesis of AIDS is unclear, however, it is clear that HIV destroys CD4+ T cells which ultimately lead to immunodeficiency within the affected individual (CHUN and FAUCI, 2012). HIV gains entry within the monocytes and dendritic cells through CD4+ protein and certain chemokine receptor. CC motif Chemokine Receptor five (CCR5) is a chemokine receptors, mutations in which are reported to be associated with decreased susceptibility to HIV infection in Caucasians and American region. This chemokine receptor act as co-receptors for almost all types of HIV variants (ZHENG *et al*., 2017). For example, Polymorphisms in promoter region (-2459) and a 32- nucleotide deletion have been linked with delayed progression of AIDS (SALKOWITZ *et al.*, 2003; ALKHATIB *et al*., 1996). Although mutations in CCR5 gene have been reported to be associated with delayed progression of AIDS, yet no study has been conducted to identify the Polymorphisms in CCR5 gene in Pakistani population, therefore, the current study was conducted to screen out polymorphisms in CCR5 gene of AIDS patients with Pakistani ethnicity.

METHODOLOGY

Study Population

This study was conducted by following the rules and regulations of MOOSE reporting guidelines and the Helsinki Declaration. To ensure the willingness of participants, verbal and written consent was taken from each patient before collection of blood samples. The whole research was conducted in the influenza laboratory of the Department of Microbiology,

University of Veterinary and Animal Sciences (UVAS), and the Institute of Public Health, Lahore. This study was approved by the ethical committee, University of Veterinary and Animal Sciences Lahore, Pakistan.

Blood samples of registered HIV positive patients were collected from the Punjab AIDS Control Program (PACP) of the Primary and Secondary Health Care Department (PSHCD), Lahore, Pakistan. The selection of samples was based on the clinical confirmation of HIV through HIV-1 Rapid Testing kit®. HIV negative samples, validated on the same testing kit, were also collected from the same department and considered as the control group. As all of the individuals were registered in the PSHCD, therefore, there demographic data was taken from the department to get the information of age, gender and residency of the studied individuals.

Sequencing of CCR5 Gene

To identify novel polymorphisms and delta32 mutation in CCR5 gene, DNA was extracted from the blood samples of both studied groups i.e. HIV positive (n=50) and HIV negative (n=30) patients through QIAamp®. Qualitative and quantitative analysis of extracted DNA was confirmed on 0.8% agarose gel and Nanodrop method respectively. Isolated DNA was then amplified for CCR5 gene by Polymerase Chain Reaction (PCR) amplification. Three different sets of primers were used to amplify the entire region of CCR gene (including 5'UTR region), all of which were designed by Primer3 plus online tool (UNTERGASSER *et al*., 2007) These primer sets were named as Primer 1 (F: GATACGGGGAGAGTGGAGA and R: AGCTAACTAACAGGCCAAGC), Primer 2 (F: AAAGGGTCACAGTTTGGAAT and R: CCACCACAGATGAATGTCA) and Primer 3 (F: AGGGGTGAGGTGAGAGGATT and R: CAGAAGGGGACAGTAAGAAGGA) with the product size of 610bp, 579bp and 737 base pairs. The first step of PCR amplification was initial denaturation, which was optimized at 95°C for 5 minutes for both Primer 1 and 2, whereas, 94.5°C (for 5 minutes) was found to be the optimal temperature for the initial denaturation of primer 3. This step was followed by 35 amplification cycles of denaturation for 0.5 minutes (Primer 1 and 2; 94°C and Primer 3; 94.5°C), annealing for 0.5 minutes (Primer 1; 62°C, Primer 2; 58°C and Primer 3; 60°C), and extension for 0.5 minutes at 94°C for all three primer sets. The reaction was then terminated with the final extension at 72°C for 15 minutes. Amplified DNA was then analyzed qualitatively on 1.5% agarose gel. Amplified amplicons were then sequenced bi-directionally through ABI 3130XL genetic analyzer. Sequencing results were inspected with "Bio-Edit" software (HALL et al., 2010) that aligned the query sequences with the normal sequence, to investigate some already reported or novel mutations in CCR5 gene.

In-Silico Assessment of Identified Polymorphism

Effects of identified genetic variations were explored by "Variant Effector Predictor (VEP)" server (MCLAREN *et al*., 2016) of "Ensemble" online tool. For novel mutations in 5ÚTR, CRYP-SKIP online tool (DIVINA *et al*., 2012) was used to predict its effects on splicing and transcription.

Statistical analysis

SPSS 18.0 software was adopted for statistical analysis (12). Data was calculated for means and standard deviations. T test was applied and p-values < 0.05 were considered as significant.

RESULTS

The current study was conducted on 80 individuals out of which 50 were HIV positive patients and other 30 were HIV negative patients. The mean age of patient's and control group was found to be 44 ± 15 and 51 ± 12, respectively. Gender figures depicted that 47% patients were male and remaining 53% patients were females with an average age of 48 ± 18 and 40 ± 10, correspondingly. Contrary to this, 53% individuals from the control group were males, while the remaining 47% subjects were female.

Initially, thirty-two base pair deletion in CCR5 gene was screened in all sequencing results of this study; however, none of the studied sample depicted such deletion (figure1). We then looked into the other previously reported variations in CCR5 gene. Again, neither any normal sample nor any HIV positive sample demonstrated any of these variants in their sequencing results. Followed by this step, we figured out any novel genetic variation in CCR5 gene of studied individuals. One novel SNP, 46370768C/T, was found in 5'UTR region (location was confirmed by Ensemble) of CCR5 in samples of both control and HIV positive group. Table 1 represents the occurrence of 46370768C/T variation in HIV infected population. As this SNP was found in 5'UTR region, we used "CrypSkyp" online tool to predict whether this novel variant effects splicing during transcription or not. Results of this online software demonstrated that the identified SNP has no effect on splicing of CCR5, as it doesn't result in the generation of new donor splice site (figure 2). Furthermore, statistical analysis confirmed that there is no significant effect of identified SNP in HIV positive and negative individuals (Table 1).

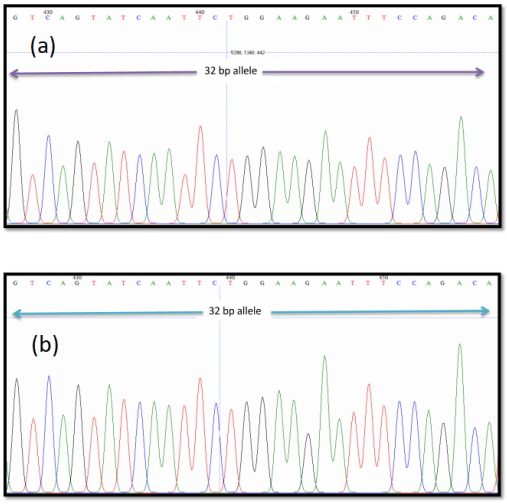

Figure 1. Electropherogram of thirty two base pair allele from position 46373456-46373487 in (a) HIV negative and (b) HIV positive Samples

Table 1. Distribution of identified SNP in HIV infected population

SNP ID	Chromosomal Location	Nucleotide Change		Transition or Transversion	Chi^2 (<0.05)	HWE*	Allele Frequency	
		Wild	Mutant				Allele A	Allele B
CCR5.1	46370768	C	T	Transition	0.0371	S	0.7613	0.2387

Figure 2. Screenshot of the results of CRYP-SKIP analysis, where actual donor splice site is shown with red vertical marker while actual acceptor splice site is shown by blue vertical markers. Green arrow in the above figure shows Cytosine at 46370768 position, while in the lower image same arrow represents the Tyrosine at the same position. It can clearly be seen that the SNP 46370768C/T doesn't create any new donor splice site, showing that this SNP has no effect on transcription of CCR5 gene

DISCUSSION

CC chemokine receptor 5 (CCR5) is among the foremost protein co-receptors which, along with a surface envelope glycoprotein gp120, are responsible for the appropriate entry of Human Immuno-Deficiency Virus (HIV) in the human immune cells including; macrophages and CD4+ T lymphocytes (DENG *et al*., 1996). This entrance, if not treated at the early stages of viral infection, eventually prompts the endemic condition of Acquired immuno-deficiency syndrome (AIDS). Genetic variations in CCR5 lead to defective CCR5 protein structure, as a result of which HIV becomes incapable to gain entry in the immune cells.

Many mutations in CCR5 gene have been reported previously, that are associated with resistance towards HIV infection. 32 base pair deletions (delta 32) is most widely reported mutation in CCR5 gene. It was observed that this variation is far more common in European populations with an overall frequency of Δ32 allele was 2.5% (MARTINSON *et al.*, 1997; NOVEMBRE *et al*., 2005). However, this prevalence decreases from Northern to Southern Europe (NOVEMBRE *et al*., 2005) and becomes entirely absent as move further towards Asian countries, many previous studies have reported no or very little occurrence of this polymorphism. SOLLOCH *et al*. (2017) conducted an extensive study to estimate the frequency of Δ32 mutation in different ethnic groups, however, they didn't find this mutation in any of the studied individuals of India and China. Similarly many other Asian studies have reported no association of this mutation with HIV infection, for example, the recent study conducted in Nepal in 2020 reported no Δ32 mutation in CCR5 gene in all of the studied samples (SHRESTHA et al., 2020). In another study, conducted on the Iranian populations, no association between Δ32 mutations was found with delays in HIV infection (TAJBAKHSH *et al*., 2019).

In the current study, 15 HIV positive patients and the same number of HIV negative patients were considered for the assessment of previously reported genetic polymorphisms, including Δ32, or any other novel mutations in CCR5 gene. However, neither Δ32 nor any other already reported variations were found in the gene of interest. Subsequently, we screened the sequencing data to identify any novel mutation and we found one novel SNP 46370768C/T in 5'UTR region. As this mutation was present in 5'UTR region, therefore, we suspected that it could affect the splicing/transcription of CCR5 gene. Consequently, we used CRYP-SKIP a bioinformatics tool that can predict the effects of genetic mutations in UTR regions; however, this software estimated that this SNP imparts no effect on the transcription of CCR5 gene. This software has been used previously in many studies to predict the exon skipping effects of genetic variations (SÁNCHEZ-ALCUDIA *et al*., 2011; DIVINA *et al*., 2009).

It was concluded that the lack of common genetic polymorhism in the CCR5 gene associated with resistance to HIV infection suggests that genetic variation in CCR5 is not a siginicant modulator of HIV infection risk and progression in the Pakistanian population.

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ISTRAŽIVANJE CC HEMOKINSKOG RECEPTORA 5 (CCR5) DELTA32 I VARIJANTE GENA KOD BOLESNIKA ZARAŽENIH HIV-om

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Izvod

CCR5 je važan CC hemokinski receptor za normalan ulazak virusa humane imunodeficijencije (HIV) u ćelije domaćina. Mutacije ovog gena su povezane sa odloženom HIV infekcijom, stoga je ovo proučavanje sprovedeno da bi se identifikovao genetski polimorfizam u CCR5 genu u pakistanskoj populaciji i da bi se procenila njegova povezanost sa otpornošću na HIV infekcije. Svi propisi MOOSE i Helsinške deklaracije su striktno poštovani tokom čitave studije. DNK je ekstrahovan iz uzoraka krvi HIV pozitivnih i HIV negativnih pacijenata. Ekstrahovana DNK je amplifikovana za CCR5 gen pomoću PCR-a. Amplifikovani proizvod je sekvencioniran da bi se izdvojila mutacija polimorfizma, uključujući najčešću deleciju 32 para baza putem Bioedit-a. CRYP-SKIP server je identifikovao i ispitao novi SNP u 5'UTR regionu. Nijedan od proučavanih uzoraka nije pokazao ranije prijavljeni polimorfizam. CRYP-SKIP server je predvideo da ovaj polimorfizam nema efekta na spajanje ili transkripciju gena CCR5. Genetski polimorfizam delta32 mutacije u genu CCR5 pronađen je kod pakistanskih pojedinaca. Prema našim saznanjima, ovo je prvi izveštaj sa ovog područja. Međutim, treba sprovesti velike studije da bi se opširno sagledalo povezanost mutacije delta32 u genu CCR5 i otpornosti na HIV infekciju.

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