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Original Research

STAT6 variants and non-atopic asthma in Pakistani population

Ayesha Riaz[1], Muhammad Ahsan Riaz[2], Luqman Khan[3], Muhammad Shareef Masoud[4], Ghulam Hussain[5], Muhammad Iqbal[6], Muhammad Qasim[4*]

[1] Department of Zoology, Government College Women University Faisalabad, Pakistan
[2] Department of Environmental Sciences and Engineering, Government College University Faisalabad, Pakistan
[3] Graduate School of Life Sciences, Tohoku University, Sendai, Japan
[4] Department of Bioinformatics and Biotechnology, Government College University Faisalabad, Pakistan
[5] Department of Physiology, Government College University Faisalabad, Pakistan
[6] Pakistan Council of Scientific and Industrial Research (PCSIR) Islamabad. Pakistan

Correspondence to: qasemawan@gmail.com

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Abstract: Asthma a chronic airway inflammatory disease mainly characterized by airways obstruction. Airway hyper responsiveness particularly in eosinophils and inflammatory mediators affect the bronchial mucosa. Genetic association studies show the association of single nucleotide polymorphisms (SNPs) in the STAT6 gene with asthma risk. Role of Signal transducer and activator of transcription 6 (STAT6) is acute for T-helper 2 (Th2) mediated responses during allergic airway diseases. Objective was to investigate whether the two single nucleotide polymorphism (rs4559 and rs324011) in STAT6 gene are associated with non-atopic asthma risk in Pakistani population. One hundred (100) asthma patients with a positive family history with at least one-first degree asthma affected relative were enrolled. Normal healthy individuals (n=100) were also included as control subjects in the current study. STAT6 SNPs rs4559 and rs324011 were genotyped using SNaPSHOT mini-sequencing assay and the obtained data was statistically analyzed by online SHEsis software. A case-control study for association of STAT6 polymorphisms rs4559 and rs324011 with asthma risk was performed. The SNP rs4559 was found statistically significantly associated with increased susceptibility of developing non-atopic asthma in Pakistani individuals. The SNP rs324011 polymorphism in intron 2 of STAT6 gene may be associated with increased susceptibility of the development of non-atopic asthma as a strong statistically significant difference in allele frequency and genotype was observed between asthmatics and controls showing association with non-atopic asthma in Pakistani individuals. rs4559 and rs324011SNPs in STAT6 found associated with non-atopic asthma risk. We observed the statistically significant association between STAT6 polymorphisms with intrinsic (non-atopic) asthma in Pakistani population.

Key words: Asthma; STAT6; Minisequencing; Gene polymorphism; SNPs.

Introduction

Asthma is clinically described by an extremely variable and revocable obstruction of accompanying airways and related symptoms (1). Traditionally, two forms of asthma have been defined in the clinic (2).

Intrinsic (non-atopic) asthma is the most common chronic childhood disease affecting millions of people worldwide (3). STAT6 represents one of the most promising candidate genes which belong to the STAT-family of transcription factors (4, 5). The Common single nucleotide polymorphisms (SNPs) of the human STAT6 suggest strong evidence of eukaryotic *STAT6*gene involvement in the regulation of translation of the mRNA that can contribute to the pathogenesis of several human diseases (6). Experimentation in the asthma field suggest *STAT6* role in elevated production of IgE and airways obstruction due to enhanced bronchial reactivity in the asthmatic response (7-10). As asthma is multifactorial diseases, a high genetic heterogeneity has been observed among different ethnic groups in relation to the influence of STAT6 on asthma and related traits that differs in different populations (11, 12). Asthma has become a highly prevalent chronic disease of children and young adults in relevance of asthma is contributed both by gene-gene as well as gene-environment interactions (13, 14). Environmental factors, including changing patterns of early childhood are candidate mechanisms for the rapid rise of asthma (15-18) but genetic factors are also important in occurrence of the disease.

The Current study was performed to delineate the association of two *STAT6*polymorphisms (rs4559 and rs324011) with the risk of asthma based on case-control studies. This study is the first genetic analysis to identify the association between these STAT6 gene polymorphisms and the risk of asthma in Pakistani population. We genotyped already reported polymorphisms in this gene and studied possible association of these polymorphisms with asthma in patients with at least one first degree affected relative.

Materials and Methods

Recruitment of proband

We selected 100 non-atopic asthma patients diagnosed according to the Global Initiative for Asthma (GINA) criteria. In addition, normal healthy Subjects (n=100) as controls for association analysis of STAT6 polymorphism rs4559 and rs324011 with asthma risk also included in study. Current study is single centered

study based on non-atopic asthma patients presented within single year (2012) to outpatient Asthma Clinic Gulab Devi Hospital Lahore, Pakistan. After the analysis of clinical data by using a standard questionnaire based studies the persistent bronchial asthmatic (n=100 individuals; median age 49± 24.5 years) were recruited for studies. Written consent was taken from all recruited Proband. All the recruited patients were clinically stable. While collecting samples from all patients' geographic origin and migration status of Proband and controls was also observed.

Collection of blood samples and DNA extraction

Whole peripheral blood samples were collected from asthmatics as well as controls for DNA isolation by Phenol chloroform DNA extraction method (Sambrook and Green, 2012). 5ml whole blood were collected from all investigated subject in sterile (EDTA)-containing tubes for DNA extraction The DNA was quantified by measuring absorbance at 260 nm and at 280 nm before storage at − 20 °C till the time of further use.

Polymerase chain reaction amplification of primers

We designed PCR primers for 120 bp product size and to obtain a melting temperature of 60°C. Specificity of designed primers checked for binding sites in BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat). Homology of designed primer pairs to other primers checked using Primer 3 version 0.2 (http://hpc.ilri.cgiar.org/cgi-bin/primer3_www.cgi).

PCR amplification of polymorphic regions

We performed Multiplex PCR by using a total reaction volume of 20 µl in a Thermocycler ABI GeneAmp 2700. 2.0 µl of genomic DNA (10 ng/µl), 2.4 µl of 25 mM $MgCl_2$,2.0 µl of PCR buffer, 2.4 µl of dNTPs mix (2.5 mM each dNTPs), 0.4 µl of each of forward and reverse primer (10 µM) (Primers Sequence presented in Table1) and 2.0 µl of Taq DNA polymerase (2U/µl) and 6.8 µl of deionized water. DNA was incubated at 94°C for 5 min, 95°C for 30 secs, 57°C for 50 sec and extension at 72°C for 30 secs for 35 cycles of amplification and final extension at 72°C for 7 min for PCR reaction.

SNaPSHOT minisequencing assay

For SNaPSHOT minisequencing assay 5.0 µl of amplified PCR product, 1.66 µl of Shrimp Alkaline Phosphatase (SAP: 1U/ µl) and 1 µl of Exonuclease1 (1U/ µl) were incubated at 37°C for 60 min and 80°C for 15 min. 2.5 µl of SNaPSHOT Ready Reaction Mix (ABI PRISM SNaPSHOT Multiplex Kit), 1.5 µl of PCR (SAP, Exo1 treated), 0.5 µl of single base extension primer (6 µM) (Primers Sequence presented in table I) was incubated at 96 °C for 10 sec, 56 °C for 5 sec, 60 °C for 30 sec. for 25 cycles of amplification for Single Base Extension PCR. 0.5 µl of SAP (0.5U/ µl) was also added and were incubated at 37 °C for 60 min. and 80 °C for 15 min. After the SNaPSHOT minisequencing was done on ABI PRISM™ 3730 Genetic Analyzer (Applied Biosystems).

Statistical analysis of data

Allele frequencies and Genotypes for the rs4559 and rs324011 in *STAT6* gene was determined by using counting method and online SHEsis software. Hardy–Weinberg equilibrium (HWE), linkage disequilibrium also analyzed by using online SHEsis software facility for studying significance of differences between asthmatics and control groups(19).

Ethical approval

Current study was performed after ethical approval from Ethical Review Board of Gulab Devi hospital Lahore and Centre for Applied Molecular Biology Ministry of Science and Technology Lahore.

Results

A case control study performed for association analysis of *STAT6* polymorphisms rs4559 and rs324011 with non-atopic asthma risk among Pakistani individuals. The SNP rs4559in *STAT6* genotyped and found statistically significantly related to non-atopic asthma in Pakistani population (Data presented in Table 2). The distribution of T allele was significantly associated with asthma patients as compared to allele C with Odd Ratio 0.54, at 95% confidence interval. Similarly, genotype T/T was significantly associated with increased susceptibility of developing non atopic asthma as compared to C/T or C/C genotypes in case of rs4559 polymorphism (data presented in Table 2). The data showed a significant association of the*STAT6* rs4559 polymorphism with asthma among study group in Pakistani population.

The allelic frequencies and genotype of STAT6 gene polymorphism rs324011(in intron 2 of *STAT6*) investigated for association analysis with asthma phenotypes in non-atopic asthmatics and healthy control subjects are demonstrated in table 2. We found a highly significant association between rs324011 SNP and non-atopic asthma in our population where higher frequency of allele G was observed among asthma patients as compared to allele A at 95% confidence interval. When testing for genotypes the genotype G/G was more prevalent among asthmatics as compared to A/G or A/A geno-

Table 1. List of selected SNP loci and their primers.

	STAT6 Polymorphisms	Primer Sequence
1	rs4559	F: CGTGTATAGCTGTGTGAACGTG R: TGTCACGTAGGCAAAAGCAG SBE: TTTTTTTTTTTTTTTTTTTTTCGGTCCAGCCCCCA
2	rs324011	F: CCAAGAAACTTGGCCTATCTCC R: CACCCCTGTGTCTATCACTGAA SBE:TTTTTTTTTTTTTTTTTTTTTTATGAGTGGTGGTGGGGAC

Table 2. *STAT6* polymorphisms frequency and genotype distribution in asthma and control group.

SNP rs4559							
	Allele Frequency		**Genotype Frequency**			**P-Value**	**HWE**
	C(Allele)	**T (Allele)**	**C/C (Freq)**	**C/T(Freq)**	**T/T(Freq)**		
Asthma	24(0.120)	176(0.880)	7(0.070)	10(0.100)	83(0.830)	0.029	Yes
Controls	40(0.200)	160(0.800)	11(0.110)	18(0.180)	71(0.710)		
SNP rs324011							
	Allele Frequency		**Genotype Frequency**			**P-Value**	**HWE**
	A (Freq)	**G (Freq)**	**A/A (Freq)**	**A/G(Freq)**	**G/G(Freq)**		
Asthma	11(0.055)	189(0.945)	0(0.000)	11(0.110)	89(0.890)	0.00077	Yes
Controls	0(0.000)	200(1.000)	0(0.000)	0(0.000)	100(1.000)		

types showing a significant association with asthma. Statistically significant difference in allele frequency and genotype was observed between asthmatics and controls in case of rs324011 polymorphism.

Discussion

Signal transducer and activator of transcription 6 (STAT6) may promote the development of asthma by facilitating airway hyperresponsiveness and increasing serum IgE level (20). Susceptibility of genetic variants in *STAT6* gene to asthma predisposition and/or IgE levels has been reported in different populations, although discrepancies have also been observed with inconsistent results (21, 22). Current study aimed to investigate the likelihood of different STAT6 variants for developing non atopic asthma. Our study demonstrated that STAT6 polymorphisms contributes significantly to the susceptibility of non-atopic asthma in Pakistani population. We investigated the role of two *STAT6* single nucleotide polymorphisms (rs4559 and rs324011) in causing non atopic asthma. In our data SNPs (rs4559 and rs324011) were found significantly associated with non-atopic asthma risk in Pakistani population. Previous studies shown trends to increased bronchial hyper responsiveness and atopic asthma association with STAT6 gene (21, 22). a significant interaction between *STAT6* and risk of asthma development in the Chinese population (23). Expression and activation of *STAT6* SNP rs4559 in asthmatic individuals has been investigated in other studies (21). The studies have implicated the association of rs4559 SNP in the pathogenesis of IgE dysregulation (24, 25). However, in our study we have observed a significant association of rs4559 SNP with non-atopic asthma in Pakistani population.

Similarly our data was in consistence with results of showing that Peripheral blood lymphocytes from asthmatic patients displayed significant differences in the level of STAT6 polymorphism rs324011 relative to healthy controls (26). A rs324011 a common polymorphism (SNP) in intron 2 of STAT6 and found none of the linkage to the occurrence of the asthma phenotype (27), however the rs324011 was found statistically significantly associated with total IgE level (25, 28). The rs324011 was also found in association with IgE level rather than asthma risk in Finnish population(29). Studies demonstrated an association between the wild type allele (C) of rs324011 polymorphism with allergy phenotypes (30, 31). This disparity could be explained by the facts points possibly to individual differences in SNPs distribution inside the examined populations as well as to different patient group size in the studies. Following the obtained data polymorphisms in STAT6 gene can be connected for further investigations of functional consequences of STAT6 gene that might be of greater impact for possibility of targeting therapy for non-atopic asthma.

Genetic variations in the *STAT6* gene may be associated with a predisposition for developing non atopic asthma in Pakistani population. The allele T of rs4559 and the corresponding genotype T/T was significantly associated with asthma predisposition in asthmatic patients from Pakistan; STAT6 rs324011 G/G genotype was also significantly associated with developing non atopic asthma, where allele G was more common in asthma patients relative to control group. Increasing the prevalence of asthma in recent years demands the urgent need for studies on specific genetic susceptibilities to asthma. Identification of SNPs that are associated with asthma, and other asthma related phenotypes may be helpful to find a cure for genetic asthma in order to devise effective and better drug against disease.

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