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Association of Single Nucleotide Polymorphism in the Upstream Region of Tumor Necrosis Factor Alpha Gene with Asthma

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MA executed the experimental work and data analysis. SZ supervised the work and helped in manuscript preparation. AA helped in experimental work and data analysis. MEB helped in preparation of manuscript.

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ABSTRACT

Asthma is an obstructive airway inflammatory disease, which causes narrowing of airway, airflow obstruction and produces wheezing sounds. Tumor necrosis factor α polymorphism leads to inflammation and provoke asthma which lead to morbidity and mortality in the population. The objective of the study was to look into single nucleotide polymorphism in upstream region of -238TNF-α and -308 by ARMS PCR technique in asthmatic and control group. It was a prospective study constituting 17 clinical diagnosed asthmatic patients (Diseased) and 19 non-asthmatic patients (Control). The female was 6 (31.6%) and males were 13 (68.4%) in control group while females were 9 (52.9%) and male were 8 (47.1%) in asthmatic group. Mean age (49.59±15.82 year) and BMI (21.96±06.67 kg/m^2) were recorded for asthmatic group while mean age and BMI for control group were 32.82±12.50 year and 18.87±05.17kg/m^2, respectively. PCR amplification of TNF-α gene was done in a thermocycler for -238 and -308 genotype analysis by the ARMS technique and statistical analysis showed that there was association between asthma and TNF-α polymorphism at -238 G>A ($p < 0.0001$, OR 0.48) and -308 G>A ($p < 0.0036$, OR 0.26).

INTRODUCTION

Asthma is a reversible obstructive airway disease that causes obstruction of airway which limits air flow to airways and produce symptoms of wheezing sounds, dyspnea, bronchospasm, chest tightness and coughing. Peak inspiratory flow is found reduced and obstructive airway pattern in the spirometry test report during early diagnosis (Baur *et al*., 2012). Asthmatic patients with repeated acute exacerbation may require intensive care monitoring, mechanical ventilation, medication, bronchodilator, steroids and prolong hospitalization which increase economic cost burden (Afessa *et al*., 2001; Papiris *et al*., 2002).

Asthma affect approximately three hundred million individuals worldwide and this figure will increase up to four hundred million till 2025 and can cause approximately 250,000 deaths per year in world (Masoli *et al*., 2004). CDC report 2018 showed that asthma occurred among children (8.4%) and adults (7.7%). Asthma prevalence was higher among black persons (10.1%) as compared to white persons (8.1%) in United Sate of America (CDC, 2018). Prevalence of asthma (according to reversibility in FEV1) was found to be 11.3% in Pakistan in 2015 (Shama et al., 2018) and 19% in 2018 (Sabar *et al*., 2018).

Genetic asthma is inherited in family and causes mortality but there is need of medicine throughout life. Lung functions decrease with aging and asthma worse in severity in adult as compared to children. Asthmatic parents have more tendencies of asthmatic children than non-asthmatic children (Karakurt and Ceyhan, 2001).

Genetic variation causes 40-60% risk of asthma among individuals. Studies have highlighted that there are about 25 genes involved in asthma development (Bijanzadeh *et al*., 2011). Asthma susceptible genes are located mostly on 5q31. 5q32, 5q33, 6p21 and 12q13 to 12q24 loci in different population (Ober, 2016). Single nucleotide polymorphism has been reported in tumor necrosis factor α, Interleukin 4, ADAM 33, Chemokine receptor 5 genes, ORMI1 like protein 3 gene and Glutathione S Transferase P1 (GSTP1) (Kirkbride *et al*., 2001; Masoli *et al*., 2004).

TNF-α gene is located on chromosome 6 and is involved in inflammatory events (Thomas, 2001). SNPs in the upstream region of transcription initiation site of TNF-α promotor are associated with increased level of

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TNF-α which leads to increased risk of asthma (Berry *et al*., 2006; Elahi *et al*., 2019). TNF-α is increased in bronchial lavage fluids of asthmatic patients (Sharma *et al*., 2006). Polymorphisms in promotor region TNF-α increased bronchial hyper-responsiveness (Berry *et al*., 2006). TNF-α polymorphism causes asthma in United Kingdom and Irish population (Winchester *et al*., 2001). Asthma developed due to SNPs in -308 TNFα(G>A) (Kang *et al*., 2018) and -238 TNF-α (G>A) (Jones *et al.,* 2013). The polymorphism in TNF-α gene -308 and -238 may affect production of cytokines and cause atopic diseases (Mekinian *et al*., 2011). So that this study was aimed to investigate association of single nucleotide polymorphism in the upstream region of tumor necrosis factor alpha gene at -308 and -238 site with asthma.

MATERIALS AND METHODS

Study population

This study was case control single center study, consisting of two group cases; (asthmatic) and control (non-asthmatic). Case group contained n=17 asthmatic patients who were clinically diagnosed with asthma, by physician in pulmonary clinic and control group comprised of 19 non-asthmatic individuals. The subjects were counseled well to participate in study and were agreed.

DNA isolation and primer designing

Disposable syringes (3 ml) were used to draw venous blood samples. The blood was collected in vacuum tubes containing EDTA and samples were stored in refrigerator at -20 °C. DNA from blood samples was extracted by using organic technique (Sambrook and Russel, 2001).

Primer 1 software (http://primer1.soton.ac.uk/primer1.html) was used to design primer for -308 TNFα and -238. Blast search of primers was done on NCBI websites to analyze nonspecific binding. The tetra primer sequences were optimized and designed for each of the SNPs in the TNF-α promoter region.

Amplification of TNF-α *gene*

All DNA samples were amplified with tetra primers (TNF forward inner, TNF forward outer and TNF reverse inner, TNF reverse outer) using ARMS PCR technique. The total reaction was prepared in 25 µL PCR tube for each SNP. The reaction mixture comprised of 3 µL of sample DNA, 2.5 µL $MgCl_2$, 2.5 µL dNTP, 0.7 Taq polymerase, 9.3 µL H_2O, 1.5 µL forward outer primer, 1.5 µL reverse outer primer, 1 µ forward inner primer and 1 µL reverse inner primer for SNP-308 and -238 TNF-α. ARMS PCR protocol was used and denaturation was at 95°C for 5 minutes. Total 35 cycles were used, each cycle consisting of 95°C for 30 seconds, 65°C to 55°C for 30 seconds with decrease of 1°C per cycle and 72°C for 40 seconds. The final extension was done at 72°C for 10 min. After gel electrophoresis, SNP band viewed/analyzed under ultraviolet light.

RESULTS AND DISCUSSION

A total of n=17 asthmatic patients (with confirm clinical history of asthma) and n=19 non-asthmatic (control) were included from Jinnah Hospital of Lahore, Pakistan.

Present study was done to investigate SNPs in -308 and -238 TNF-α gene in the asthmatic patients of Pakistan. Female were 6 (31.6%) and male were 13 (68.4%) in non-asthmatic group while females were 9 (52.9%) and males were 8 (47.1%) in asthmatic group. Mean age was 49.59±15.82 year and BMI 21.96±06.67 kg/m^2 in asthmatic and mean age was 32.82±12.50 year and BMI 18.87±05.17 kg/m^2 in control group (Table I).

Sanger sequencing was done commercially from BGI and results were read by Bio-Edit alignment editing software. Sequence data was analyzed by using NCBI BLAST Tool. By using Hardy Weinberg Theorem, genotype frequency for -308 and -238 TNF-α was calculated. The genotype frequencies of wild Type (GG) was 8 in asthmatic group while in control group, it was 19 in -238 TNFα (Table II). The heterozygous mutant (GA) frequencies were seven and zero in control group. The allelic frequencies of homozygous mutant (AA) was 2 in asthmatic group. Genotype frequencies of wild Type (GG) was 11 in asthmatic group while in control group it was 19 in -308 TNFα. The Heterozygous mutant (GA) genotype frequencies were 5 and homozygous mutant (AA) were 1 in asthmatic group (Table II).

Table I. Descriptive statistics of asthmatic and non-asthmatic patients.

	Control (n= 19)		Asthmatic (n= 17)	
	Male	**Female**	**Male**	**Female**
Frequency (f)	13 (68%)	6 (32%)	8(47%)	9 (53%)
Age (year)	32.82±12.50		49.59±15.82	
BMI (kg/m^2)	5.17±5.17		21.96±6.67	

The present study showed that association exist between polymorphism (G>A) in -308 and -238 TNFα gene and asthma. Allele G alters into A in -308 TNFα and -238 and causes a risk to develop asthma. Shin *et al*. (2004) showed that TNFα-308 was associated with increased risk of asthma ($p<0.0007$). The development of asthma and its severity was caused by polymorphism in

-308 TNF-α G >A factor (Zedan *et al*., 2008). Literature also showed association of TNFα-238 polymorphism G>A with asthma (Li *et al*., 2018). Another study showed that the asthmatic individuals which had genotype AA, AG and GG, were more likely to develop asthma (p value <0.001). Polymorphism in TNF-α that alters nucleotide sequence was found to be associated with increased risk of asthma occurrence in adults (p value < 0.001) and children (p value < 0.001) (Huang *et al*., 2014). Study of Mukhopadhyay *et al*. (2006) revealed that polymorphism in TNF-α gene was one of the causes of different types of pulmonary pathological discords including chronic obstructive airway disease, cystic fibrosis lung disease, acute respiratory/lung disease and bronchitis.

Table II. Genotype and allelic frequency of TNFα-238 and TNFα-308.

Category	n	Genotype			Allelic frequency	
		GG	GA	AA	G	A
TNFα-238						
Asthmatic	17	8	7	2	23	11
Control	19	19	0	0	38	0
Total		27	7	2	61	11
TNFα-308						
Asthmatic	17	11	6	0	28	6
Control	19	19	0	0	34	0
Total		30	6	0	62	6

Another study on -308 TNFα described odd ratio of 1.05 and confidence interval of 0.68-1.63 for frequency of GG and odd ratio was 0.99 with confidence interval 0.64-1.53 for frequency of GA contents. There was no difference between frequency of GG and GA contents in polymorphism of -308 TNF-α. The genotype frequency AA was present in only one case control group. This study finally concluded that polymorphism in -308 TNFα was not related to asthma occurrence (Saba *et al*., 2015). Another research conducted on n=236 asthmatic patients and n=275 non-asthmatic (control) subjects showed that polymorphism in -308 TNFα was linked with asthma. The adjusted odds ratio was 1.86 for G to A alteration at site −308 that was associated with asthma (Witte *et al*., 2002). Aoki *et al*. (2006) conducted study on n=2477 asthmatic and n=3217 control subjects that revealed that the -308 TNFα G>A polymorphism was associated with asthma. The present study shows that SNPs in -238 (G>A), -308 TNF-α, are causes to develop of asthma in an individual (Figs. 1 and 2).

SNPs in -308 TNFα G>A was related to asthma into North Indian population (Choi *et al*., 2005; Gao *et al*., 2006), Punjabi population (Kumar *et al*., 2008) and Iranian population (Movahedi *et al*., 2008). The frequency of SNPs in -308 TNF-α G>A was compared in n=100 asthmatic children, wheezy infants and n=100 control individuals. The frequency of SNP in -308 TNF-α in asthmatic group was 60% (Shaker *et al*., 2013).

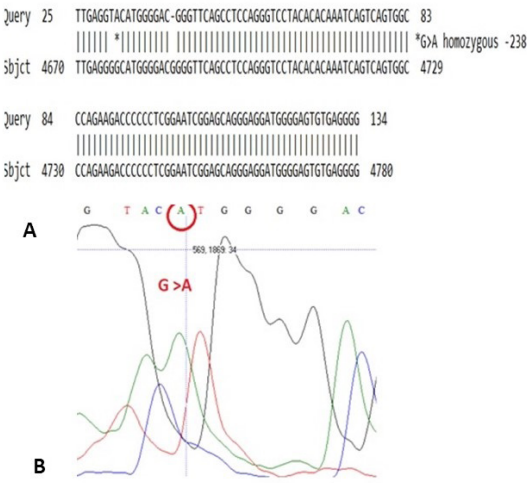

Fig. 1. Blast results of sample for TNF α -238 (A), chromatogram of sample showing SNP in -238 TNFα (B).

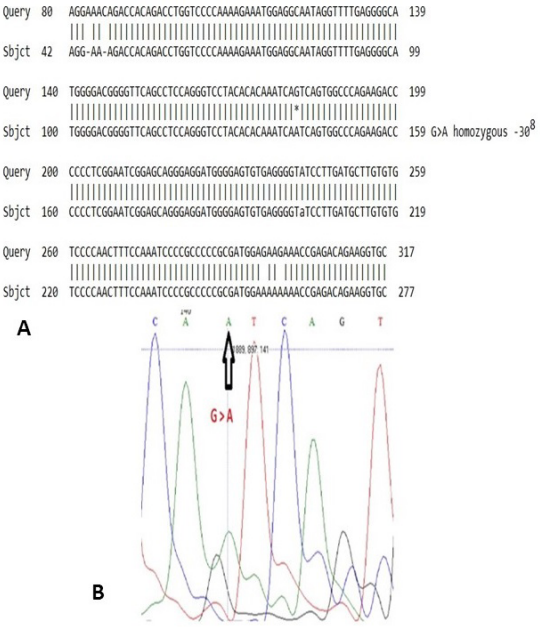

Fig. 2. Blast result of sample for -308 TNF α (A), Chromatogram of sample showing SNP in -308 TNF α (B).

The single nucleotide polymorphism (G>A) in -308 TNFα (p = 0.0036, OR= 0.26) and -238 TNFα (p = 0.0001, OR= 0.48) in present study were found to be associated

with asthma that lead to increased prevalence of asthma in population. Therefore, there is need to use TNF-α polymorphism as biomarker to manage asthma in Pakistan.

CONCLUSION

Single nucleotide polymorphism (G>A) in -238 TNFα (p value < 0.0001) and for -308 TNFα (p value < 0.0036 was associated with asthma as compared to control group and ARMS PCR technique was found to a faster tool to asses' polymorphism in a population.

TNF-α variation G>A at -238 and -308 sites can be used as a biomarker for detection of asthma severity in population after large sample cohort study. It will help to spread awareness among clinicians and the general public that not only suffers from asthma but everyone should get themselves tested for single nucleotide polymorphism in -308 TNF-α and -238 to prevent asthma and to avoid worsening symptoms of asthma.

Statement of conflict of interest

The authors have declared no conflict of interest.

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