ORIGINAL ARTICLE

SINGLE NUCLEOTIDE POLYMORPHISMS OF TRANSFORMING GROWTH FACTOR-B1 GENE AS POTENTIAL ASTHMA SUSCEPTIBLE VARIANTS IN PUNJABI POPULATION OF PAKISTAN

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Background: Candidate gene approach based on case-control model is a valuable strategy to determined disease related genetic variants. Two single nucleotide polymorphisms rs1800469 and rs2241715 in TGF-β1gene have been reported to affect the asthmatic status in different populations. The main focus of this research was to find any relationship between these SNPs and asthma in Pakistani population. **Methods**: Using case-control model, a total of 108 individuals including 52 asthma patients and 56 healthy controls were screened to find asthma susceptibility of variants rs1800469 and rs2241715. These SNPs were genotyped using SNaPshot minisequencing assay followed by capillary electrophoresis using ABI 3130xl genetic analyzer platform. The statistical analysis of genetic data was performed by using SPSS 21, SHEsis online platform and SNPStats online web software. **Results:** No association with asthma was seen in allelic model for both SNPs but genotypes analyzed under codominant, dominant, over dominant and recessive models of inheritance revealed that SNP rs2241715 is strongly associated with asthma under genotypic model. **Conclusion:** rs2241715 was found to be a genetic risk factor for asthma in Pakistani population.

Keywords: Asthma; Genetics; inflammation; Pakistan; SNPs

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INTRODUCTION

Asthma is a long term chronic respiratory condition that causes inflammation and narrows the airways resulting in the recurring periods of coughing, wheezing, shortness of breath, and chest tightness.[1] It is characterized by bronchial hyper-responsiveness, enhanced infiltration of different inflammatory cells including eosinophils into the airways[2], hypertrophy of smooth cells[3], constriction and mucous hypersecretion resulting in the airways obstruction and airway hyperresponsiveness[4]. Currently 334 million of global population is affected by asthma[5] and it is expected to have 100 million more asthma patients by 2025.[1] Pakistan is one of the most populous country and almost 15.8% of its population is suffering from chronic asthma.[6,7] Asthma is considered to be a complex genetic syndrome resulting from the interplay of various genes and environmental factors.[6] During last ten years different studies have been performed to identify association of different genes with atopy and asthma related phenotypes globally across various populations[8–15] which reveal that more than 100 genes are responsible for asthma pathogenesis[16]. Transforming growth factor-β (TGF-β) superfamily is an important group of ligands responsible for alveolarization, recruitment of immune cells, endothelial and epithelial barrier functions, platelet aggregation, cell proliferation and differentiation, apoptosis and other biological pathways within the airway.[17] TGF-β1 is more frequently expressed in the mammalian tissues and its expression varied significantly in the airway of asthmatics both in animals and humans.[18] Enhanced level of TGF-β1 is involved in airway remodelling through different mechanisms that may lead to asthma development.[19,20] Many studies have been conducted to investigate SNPs present on TGF-β1 gene and their association with asthma. Studies on Chinese population have shown that the SNPs rs2241715 and rs1800469 in TGF-β1 gene are strongly associated with asthma.[21,22] Similarly different strategies have been adopted to find association of SNPs rs1800469 and rs2241715 with asthma among different ethnic populations and the results showed amazing variations of marginal, strong and no association of these SNPs with asthma.[23–26] The contradictory results of previous studies suggest the need of screening of other populations for these variants to understand the molecular mechanism of the disease in a better way.[27,28] The objective of the current study is to investigate any association of polymorphisms rs1800469 and rs2241715 in TGF-β1 with asthma in Pakistani population.

MATERIAL AND METHODS

A questionnaire was designed and the patients were assessed based on taking to account different genetic and environmental factors. Fifty-four asthma patients and fifty-eight healthy controls having age from 4–15 years were enrolled for this study. The participants

(patients/controls) were randomly enrolled for study without gender discrimination. Blood samples were taken from patients at "The Children's Hospital and Institute of Child Health Lahore, Pakistan". Individuals with no history of asthma, allergy or some other serious diseases were selected for controls from the general population. The physician diagnosed asthma patients were selected on the basis of recurrent breathlessness along with chest tightness and variability in forced expiratory flow rate. Patients with the habit of smoking and patients with illness like emphysema, chronic bronchitis, pneumonia and tuberculosis were excluded from the study. Ethical approval of the study was taken from ethical review board of ''The Children Hospital & Institute of Child Health'' and institutional approval was taken from ''Centre for Applied Molecular Biology''. A written consent form was got signed from all healthy controls and patients or their guardians in case of minors. For the extraction of genomic DNA organic method (using phenol, chloroform and isoamylalcohol) of DNA extraction was used.[29] For the qualitative and quantitative analysis of the extracted genomic DNA, 1% agarose gel was used. A standard DNA was also used for the quantitative analysis of the extracted genomic DNA.

The amplification primers were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) and Primer-BLAST online tools for the amplification of flanking regions of the desired SNPs. The primer sequences used for this purpose along with their product size are given in Table 1. For both SNPs in this study (rs2241715, rs1800469); a multiplex PCR reaction was optimized for 20µl reaction volume. DreamTaq DNA Polymerase (ThermoFisher Scientific Cat # EP0701) 0.1µl with conc (1U/µl), 10X buffer 2µl, dNTP's (ThermoFisher Scientific Cat # R0191) 1µl, Primers Mix (8 pmol/µl) 1.5µl, genomic DNA 1.5µl and nuclease free water 13.8µl were used in the reaction. Thermal cycler conditions were hot start at 95ºC for 5min and final extension at 72ºC for 10 min with 30 cycles of denaturation at 95ºC for 45 sec, annealing at60ºC for 45 sec, extension at72ºCfor 90 sec. Quality of amplicons was checked by using 2% agarose gel and quantity was determined by comparison of bands with 100bp DNA ladder (Thermofisher Gene Ruler 100bp Plus DNA Ladder cat # SM0321). Amplified PCR products were purified by Exonuclease I and shrimp alkaline phosphatase enzymes both from (Thermo Scientific) and purified products were processed in SNaPshot reaction using ABI Prism SNaPshot SBE multiplex kit (Applied Biosystems, Foster City, CA) as described by Sabar and coworkers.[13] A list of primers used in SNaPshot assay is given in Table 2. Poly GACT tails of different lengths were added at the 5′ end to differentiate the products of SNaPshot assay by their relative size. The SNaPshot products were purified and pooled with Hi-Di formamide and Liz-120 size standard and then subjected to the ABI PRISM 3130XL Genetic Analyzer for genotyping. The GeneMapper IDX software was used for the analysis of the fluorescence and size of the extended product. Statistical analysis was performed using SHEsis online platform and IBM-SPSS Statistics 21 software. The demographic data of cases and controls was compared applying χ^2 test and independent sample t-test using IBM-SPSS Statistics 21. Distribution of allele frequencies among asthma patients and controls were determined by using SHEsis online platform, however genotypic association under different inheritance models (codominant, dominant, recessive and over dominant) was determined with SNPStats online web software (https://www.snpstats.net/start.htm).

RESULTS

One hundred and twelve participants (112; 58 Controls and 54 patients) were enrolled for the current case-control based study from local population of Pakistan. Among these 58controls and 54 patients, 2 controls and 2 patients were excluded from study on the basis of low genotyping call rates (<95%). Rest of the 108 samples were subjected to statistical analysis to find association of rs1800469 and rs2241715 with asthma. Hardy-Weinberg Equilibrium (HWE) value for SNPs rs2241715 and rs1800469 was 0.10 and 0.882 respectively which is greater than cut off value (0.001) set for the exclusion criteria.

The demographic variables and their characteristics are shown in table-3. It was observed that the difference between cases and controls was non-significant in terms of gender and living environment while the difference between their ages is significant (*p*-value 0.03). Genotypes for both the genetic variants of TGF-β1 were determined using SNaPshot minisequencing technique (Figure-1) and then further subjected to statistical analysis for genotype and allele frequency distribution among controls and asthma patients using SHEsis online platform and SNPStats online web software. The goodness-of-fit test for HWE was also applied tors2241715 and rs1800469 in controls showing their genotype frequencies were in Hardy-Weinberg equilibrium with *p*-values 0.10 and 0.882 respectively (>0.001). Allele frequencies for both SNPs in TGF-β1 are listed in Table-4. The allele frequencies distribution for rs2241715 revealing that the minor allele T marginally more prevalent in asthma patients suggesting its trend towards asthma association (*p*-value 0.064 approximately near to the significant value). However, rs2241715 was significantly associated under codominant (p=0.02), dominant (p=0.023) and over dominant (p=0.0095) genotypic models, over dominant model is best fit model due to lowest AIC value (146.8) (Table-5). In case of rs1800469 a non-significant association was observed in genotype distribution and allele frequencies between cases and controls (Table-4 &6).

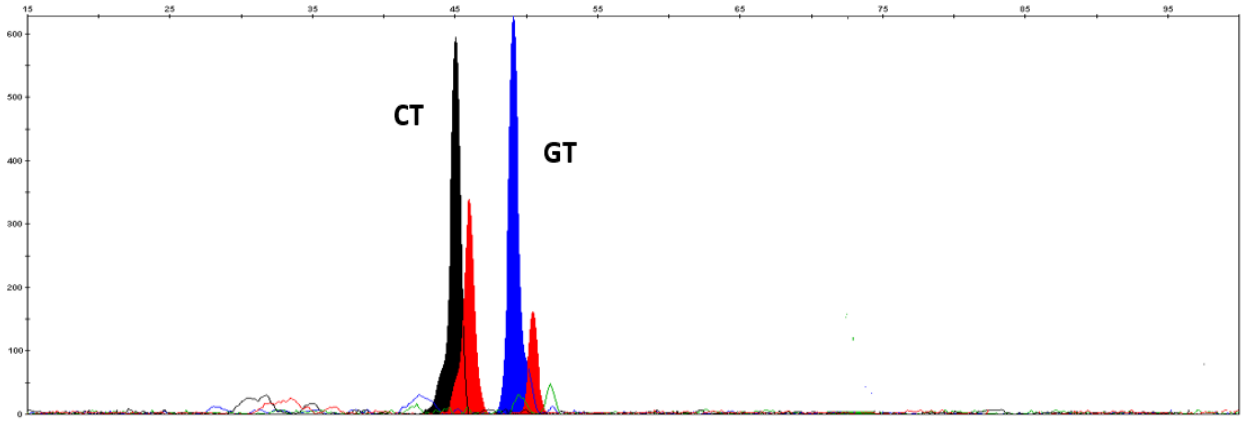

Figure-1: Peak view on GeneMapper IDX for rs1800469 and rs2241715 respectively

X-Axis represents allele size and Y-Axis represents relative fluorescence units (peak heights)

Table-1: Primers for amplification PCR

SNP	Primer ID	Primer	Primer Sequence 5´–3´	Length (bp)	Amplicon Size (bp)
rs2241715	M17-1F	Forward (F)	TGGGTCTCCTGGTTTTTGTC	20	210
	M17-1R	Reverse (R)	TACTCAGCAAACCCCAAAGG	20	
rs1800469	M17-2F	Forward (F)	TTGAGTGACAGGAGGCTGCTTA	22	248
	M17-2R	Reverse (R)	CTTAATCCGGGGGATGAGAC	20	

Table-2: SBE Probes for SNaPshot Minisequencing (SBE) reactions

Target SNP	Primers Orientation	SNP	SBE Primers with GACT tail	Primer Length (bp)
rs1800469	Forward	C/T	GACTGACTGACTGACTGACTCCTCCTGACCCTTCCATCC	39
rs2241715	Forward	G/T	TGACTGACTGACTGACTGACTGACTTTTTTCTCCTCCACGGTCC	44

Table-3: Demographic Data of cases and controls

	Controls	Cases	*p*-value*
Total no. of participants	58	54	
Individual shaving genotyping rate of 95%	56	52	
Gender, %(Male)	28(50.0%)	32(61.5%)	0.25
Age (Mean±SD) years	6.07±3.53	9.25±2.66	0.03
Living Environment, %(Urban)	41(73.2%)	28(53.8%)	0.103

* *p*-value of less than 0.05 is considered as a level of significance

Table-4: Allele frequencies of rs2241715 and rs1800469 for asthmatic patients and controls calculated by SHEsis

SNP	Allele/Genotype	Frequency		*p*-value
		Controls	Asthmatic Patients	
rs2241715	G	0.946	0.875	0.064
	T	0.053	0.125	
rs1800469	C	0.892	0.836	0.225
	T	0.107	0.163	

Table-5: Genotype analysis under different inheritance models for rs2241715

Percentage of typed samples: 108/108 (100%)							
Association of rs2241715 with response Affection (n=108, crude analysis)							
Model	**Genotype**	**Cases**	**Controls**	OR (95% CI)	***p*-value**	**AIC**	**BIC**
Codominant	G/G	39 (75%)	51 (91.1%)	1.00	0.02	147.7	155.8
	G/T	13 (25%)	4 (7.1%)	0.24 (0.07-0.78)			
	T/T	0 (0%)	1 (1.8%)	NA (0.00-NA)			
Dominant	G/G	39 (75%)	51 (91.1%)	1.00	0.023	148.4	153.8
	G/T-T/T	13 (25%)	5 (8.9%)	0.29 (0.10-0.89)			
Recessive	G/G-G/T	52 (100%)	55 (98.2%)	1.00	0.25	152.2	157.6
	T/T	0 (0%)	1 (1.8%)	NA (0.00-NA)			
Over dominant	G/G-T/T	39 (75%)	52 (92.9%)	1.00	0.0095	146.8	152.2
	G/T	13 (25%)	4 (7.1%)	0.23 (0.07-0.76)			

* *p*-value of less than 0.05 is considered as a level of significance

Table-6: Genotype analysis under different inheritance models for rs1800469

Percentage of typed samples: 108/108 (100%)							
Association of rs1800469 with response Affection (n=108, crude analysis)							
Model	**Genotype**	Cases	Controls	OR (95% CI)	*p*-value	AIC	BIC
Codominant	C/C	35 (67.3%)	45 (80.4%)	1.00	0.11	151.2	159.3
	C/T	17 (32.7%)	10 (17.9%)	0.46 (0.19-1.12)			
	T/T	0 (0%)	1 (1.8%)	NA (0.00-NA)			
Dominant	C/C	35 (67.3%)	45 (80.4%)	1.00	0.12	151.2	156.5
	C/T-T/T	17 (32.7%)	11 (19.6%)	0.50 (0.21-1.21)			
Recessive	C/C-C/T	52 (100%)	55 (98.2%)	1.00	0.25	152.2	157.6
	T/T	0 (0%)	1 (1.8%)	NA (0.00-NA)			
Over dominant	C/C-T/T	35 (67.3%)	46 (82.1%)	1.00	0.074	150.4	155.7
	C/T	17 (32.7%)	10 (17.9%)	0.45 (0.18-1.10)			

* *p*-value of less than 0.05 is considered as a level of significance

DISCUSSION

TGF-β1 is a protein that belongs to the TGF-β superfamily involved in different biological processes in the airway.[17] TGF-β1 gene was selected as a possible candidate gene because it is an important mediator that plays a pivotal role in airway remodelling and inflammation and considered to be involved in asthma development.[30] Any change in the promoter region of DNA may lead to change in binding rates by altering transcription factor binding sites. So, a SNP present in promoter region can affect gene expression by changing the binding rates of transcription factors responsible for regulation of gene expression[31]as in case of rs1800469 (a promoter region SNP of TGF-β1).It was observed that the change in allele from C to T is associated with the variation in concentration level of TGF-β1 in plasma.[32] rs1800469 is a functional genetic variant of TGF-β1 that has been studied as a potent asthma susceptible variant in many populations.[23,24,33,34] rs2241715 is located in the intronic region of TGF-β1 that has been evaluated as a potential risk factor for asthma in various populations.[21,25,22] Current study is the first effort to evaluate the association pattern of these SNPs with asthma in Pakistani population. There is slight variation in allele frequency distribution of SNP rs2241715 for controls and cases showing marginal association (*p*-value 0.064) at allelic model. Further, heterozygous genotype ''GT'' of rs2241715 was found to be more prevalent in asthma patients as compared to both homozygous genotypes ''GG'' and ''TT'' and showing its strong association with asthma under codominant, dominant and over dominant models (*p*-value 0.02, 0.023 and 0.0095 respectively) as shown in Table5.The results of rs2241715 are in accordance to the other studies conducted in different populations. A genome wide association study in Mexican population[25] Chinese population[21] support our findings, i.e., SNP rs2241715 is associated with clinically diagnosed asthma. The results of the SNP rs1800469 in current study showed that there is no significant variation in allele frequencies and genotype distribution of controls and cases. The genotypic and allelic models of SNP rs1800469 did not show any significant association with asthma as shown in Table4 and Table-6. Our finding regarding SNP rs1800469 is also in concordance with various studies conducted in different ethnic populations.[24,33] In contrast to our findings, some studies have shown the significant association of SNP rs1800469 with asthma.[23,34]

These dissimilarities in results might be owing to various factors like ethnicity, geographical locations, life style and environmental factors. Sample size is another important factor that can affect the outcome of case- control based population studies. In the current study we have studied only two SNPs of TGF-β1 gene due to funding constraints. So, for the better understanding of genetic association of TGF-β1 gene with asthma, inclusion of more SNPs of this gene with larger sample size is suggested.

CONCLUSION

The outcomes of the current study suggests that heterozygous "GT" genotype for the SNP rs2241715 of TGF-β1 is more prevalent in asthma patients. Analysis of genotypes under different inheritance models reveal that rs2241715 associated with asthma in genotypic model and this SNP also shows trend towards asthma association in allelic model while the SNP rs1800469 shows no significant association with asthma in Pakistani population. It can be concluded from the findings of this work that SNPs of TGF-β1 gene may be suggested as potential asthma susceptible genetic variants for asthma development in Pakistani population but the findings of current study require further validation by addition of more SNPs from TGF-β1 gene along with considerably larger sample size.

AUTHORS' CONTRIBUTION

MA: Conceptualization and study design, data analysis, data interpretation, write-up. MFS: Conception and design of the work, data acquisition, data analysis, drafting, literature review. IB: Conceptualization, data interpretation. MUG: Conceptualization, data analysis, data interpretation, revision. MS: Data analysis, data interpretation, revision.

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