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Original Paper

Single Nucleotide Polymorphisms (SNPs) and Asthma: A Population-Based Risk Association Study in Pakistan

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Abstract

Objective: Asthma is characterized by recurring symptoms of reversible airflow obstruction, with episodes of wheezing, coughing, chest tightness and shortness of breath. This study aimed to determine possible associations between asthma and single nucleotide polymorphisms (SNPs) in patients living in the heavily polluted Faisalabad and Multan regions of Pakistan. ***Methods*:** In this case-control study, we conducted an association analysis on rs2280090, rs2787094 (*ADAM33*), rs3816470 (*IKZF3*), rs7216389 (*GSDMB*), rs3859192 (*GSDMA*) and rs1695 (*GSTP1*) in a Pakistani cohort of 275 patients with asthma and 400 healthy control subjects. Association was determined by basic allelic and genotypic models, and results were adjusted by logistic regression analysis using Graph Pad Prism7 software. ***Results*:** Significant differences in the genotype and allele frequencies for rs2787094, rs3816470, rs1695 and rs7216389 were found among patients and controls ($p < 0.05$). SNPs rs3859192 and rs2280090 indicated a protective role, while the wild-type forms suggested an increased susceptibility to the disease. SNP rs2280090 showed a significant protective association in the heterozygous model. Results were evaluated based on three patient groups determined by age of asthma onset, as follows:< age five (n = 3); between ages 5 – 18 (n = 117), and > age 18 (n = 155). ***Conclusion*:** This study provides ample evidence supporting the role of the *ADAM33, IKZF3* and *GSDMB* genes as asthma susceptibility genes in a Pakistani population exposed to heavy air pollution.

Kew words: Asthma, Single nucleotide polymorphisms, Genes, GWAS, ADAM33, IKZF3, GSDMB, GSDMA ,GSTP1

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INTRODUCTION

Asthma is a complex obstructive airway disease caused by interactions of multiple genes and environmental factors [1,2,3]. According to the Centers for Disease Control and Prevention (CDC),7.7% of adults in the United States≥age 18 currently have asthma, as do 7.5% of children < age 18[4]. A European study in 10 cities determined that 14% of all cases of children with asthma were attributable to nearby traffic-related pollutants, and the percentage of all aggravations of childhood asthma with a causal relationship with these pollutants equaled

15% [5]. In Pakistan, nationwide surveys are rare, but limited epidemiological studies suggest a high burden of this disease. It has been estimated that 7.5 million Pakistani adults and 15 million children suffer from asthma.[6].

Both heritable and environmental factors play a crucial role, with a major contribution from genetic factors in asthma pathogenesis and severity. To date, most of the gene-environment interactions have suggested few candidate genes. Further investigations by genome-wide association studies (GWAS) of genome-wide interactions could provide a better understanding of the role of candidate genes in asthma. The correlative approach to genome-wide linkage studies and candidate gene analysis has uncovered several genetic loci of asthma. For our study, we selected a set of asthma associated/susceptibility genes and evaluated their candidate role in pathogenesis of the disease. They include: *ADAM33*(rs2280090, rs2787094) [7,8], *IKZF3* (rs3816470)[9], *GSDMA* (rs3859192) [10], *GSDMB* (7216389) [10] and *GSTP1* (rs1695) [11,12].

*ADAM33*is a disintegrin and metalloprotease that is normally expressed in human lungs, heart and brain tissues. It was the first asthma susceptibility gene to be identified in Caucasians[13]. Subsequent association studies revealed rs2280090 and rs2787094 SNPs within the exonic regions of *ADAM33,* indicating a robust association with asthma [7, 14].

IKZF3 plays a vital role in the regulation of B and T cells, which influence allergic response [15], but despite numerous datasets linking *IKZF3* to asthma, its biological role in lungs and asthma pathogenesis remains unknown [16]. Shahid et al reported on the role of SNP 3816470, an intronic variant of *IKZF3,* in association with asthma and environmental exposure [17], but further studies are needed to clarify*IKZF3's* role

GSDM proteins have mainly been examined in gastrointestinal epithelium. Studies on mouse and human *GSDM* family genes, and the location of *GSDMA* and *GSDMB* genes suggest that these two may play a role in susceptibility for asthma or asthma-related phenotypes. GSDMA polymorphisms have been previously reported to have an important role in the pathology of asthma [18]. In addition, SNP rs7216389 in *GSDMB* is one of the known functional risk variants for asthma [10].

Glutathione *S*-transferase P1 (*GSTP1*), an isoform of glutathione *S*-transferases (GSTs) in lung epitheliumis key to providing cellular protection against oxidative stress and exposure to toxic environmental chemicals. Polymorphisms in *GSTP1*are associated with asthma and related phenotypes. For example, rs1695 has shown a strong disease causing role in patients with bronchial asthma [12].

In this present study, we performed genetic association research on six SNPs in a cohort of patients with asthma from two representative Pakistani regions experiencing urbanization and industrialization. We sought to investigate and correlate the impact of environmental pollutants on severity and susceptibility to asthma.

MATERIALS AND METHODS

STUDY SUBJECTS

This case-control association study included 275patients diagnosed with asthma, and 400 ethnic-, age-, and gender-matched controls, sampled from Faisalabad and Multan in Pakistan. None of the controls had a history of asthma or any other respiratory disease. All adult patients with moderate to severe chronic persistent asthma were recruited from outpatient units of hospitals from both cities. The clinical diagnosis was based on the Global Initiative for Asthma (GINA) guidelines. Patients with known histories of allergies prior to exposure to polluted environment were excluded. Also, patients who had viral infections, or pets or animals at home, were excluded from the study, as pets are a known source of allergic reaction.

A signed informed consent explaining the purpose of the study was obtained from all participants or from guardians on behalf of the children enrolled in the study. This study was approved by the institutional research ethics committee at the National Institute for Biotechnology and Genetic Engineering (NIBGE) in Faisalabad, Pakistan.

SNP Selection

Six potential risk associated SNPs—rs2280090, rs3816470, rs2787094, rs7216389, rs1695 and rs3859192—were selected as they are top hits of previously conducted GWAS studies on asthma in

other populations [11, 17-19]. Data on these SNPs in Asian populations are limited. *Genotyping*

Genomic DNA was extracted from peripheral blood samples according to the manufacturer's protocol [19]. Specific primers were designed for polymerase chain reaction (PCR) amplification of respective SNPs for genotyping using Restriction Fragment Length Polymorphism (RFLP), as described in Table S1. For genotyping, PCR was performed using 20 ng of genomic DNA as a template in a 15 µl reaction volume, with ampli-Taq DNA polymerase (VWR Life Science), as described in the manufacturer's instructions. Thermal cycling conditions are composed of initial denaturation at 96°C for three minutes, followed by 40 cycles of 96°C for 15 seconds, 55°C for 30 seconds, 72°C for 45seconds, and a final extension for five minutes at 72°C. Amplification was confirmed by running samples in 1% agarose gel, with subsequently amplified PCR products digested at 37°C with appropriate restriction enzymes. Finally, digested products were resolved on 2% agarose gel for genotyping. To verify the RFLP results, a few randomly selected PCR products from each analyzed SNPs were sequenced using Big Dye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems) in an ABI 3130*xl*genetic analyzer (Applied Biosystems).

Statistical Analysis

All six SNPs were evaluated by Hardy-Weinberg equilibrium (HWE) for both cases and controls. Chi-square analysis was used to compare observed and expected frequencies of the genotypes. Both allelic and genotypic frequencies were distributed and compared in different groups by chi-square test with 2×2 contingency tables, using a major genotype or allele as a reference. Multiple comparisons were controlled by Bonferroni correction. Odds ratio (OR) was calculated with a 95% confidence interval (CI) for disease susceptibility. Haplotype and linkage disequilibrium (LD) analyses was performed with SHEsis software. All statistical analyses were plotted using Graph Pad Prism 7(Graph Pad Software, USA).

To evaluate potential LD between SNPs on chromosome 17 (rs3816470, rs7216389, and rs3859192) and chromosome 20 (rs2280090, and rs277094), the pairwise normalized coefficient of disequilibrium (D 0 values) was calculated at 95% confidence interval using SHEsis.

Results

A total of 166 male and 109 female patients were studied against a control population of 267 males and 133 females to determine any associations between the six analyzed SNPs and asthma in patients exposed to environmental pollutants. Patients and controls< 18 years of age comprised 22.2% and 16%of the study population respectively, while the remaining subjects were adults. Age of onset was also taken into consideration in determining the environmental effect. It was found that the disease started at< five years of age in only 0.95% patients, 42.5% of onset occurred between 5-18 years, and in 56.55% of the patients, disease onset was > 18 years of age (Table S2).

Allelic and genotypic frequencies and HWE*p* values for all six SNPs in patient and control groups were calculated (Table1). Bonferroni correction was applied to *p* values to assess statistical significance of the results and to account for any interference due to statistical tests being performed simultaneously on a single data set. Association studies were also performed for the various subgroups based on gender and age of disease onset.

Allelic and genotypic frequencies and ORs for rs2787094, rs3816470 rs7216389, and rs1695 revealed a significant disease association ($p<0.0001$, Table 1). SNPs rs2280090 and rs3859192 showed a significant protective association, both in the minor homozygous and heterozygous genotypes (Table 1).

Similar results were obtained in almost all the subgroups, except for males with age of onset <five years of age, where no association was found with any of the SNPs studied. No significant association of rs1695 with disease was observed in the females with age of onset between 5-18 years. Results are summarized in Tables S3 and S4.

DISCUSSION

Asthma is rapidly increasing in developed as well as developing countries due to greater exposure to polluted environments and rising urbanization [20]. In our study, we highlighted the important sets of genes that are either directly involved in aggravation of asthma or are regulators of susceptibility based on their polymorphism status in the Pakistani population. We found that the SNPs in ADAM33, IKZF3, GSDMB, and GSTP1genes were associated with increased risk

of asthma compared to controls in two highly polluted areas of Pakistan. Both groups with age of asthma onset between 5-18 years and >18 years showed similar results. However, no association was found with any of the SNPs in the group with age of onset < five years old.

SNPs in GSDMA genes were shown to play a protective role in disease progression, whereas wild-type forms of this gene demonstrated the opposite effect. Importantly, the heterozygous model of SNP in ADAM33 has demonstrated a protective role in asthmatic individuals exposed to environmental pollution. Genotypic and allelic frequencies and ORs for rs2787094, rs3816470 rs7216389, and rs1695 revealed a significant disease association ($p<0.0001$). SNPs rs2280090 and rs3859192 also showed a significant protective association, both in the minor homozygous and heterozygous genotypes.

Our work adds to the major research efforts that have elucidated several variants in multiple candidate genes associated with asthma in specific populations [21]. Although genetic risk factors of asthma show a discrepancy within and among populations [22], they are often reported as major components in the etiology of asthma. The presence of genetic polymorphism implicates inflammation and protection, which may manipulate the response to air pollutants, and exert an oxidative stress on airways. Notably, a study by Schurman et al demonstrated the relationship between pollution and genetic polymorphisms in asthma risk [23]. Other studies have highlighted the contribution of environmental factors towards the dramatic rise in asthma prevalence in developed countries over the past five decades [20,24,25]. Work by Simpson et al showed that environmental exposures to bacterial products can negatively regulate asthma and allergic conditions [26].Elevated levels of outdoor air pollutants, namely nitrogen dioxide (NO_2), ozone (O_3), and reactive oxygen species (ROS) in urban areas, have been associated with higher risk of asthma[27,28].

In our study, we selected a set of asthma genes, *ADAM33* and *IKZF3*, along with potential candidate genes on chromosome 17, *GSDMA*, *GSDMB*, and *GSTP1*.Importantly, a systematic review of longitudinal studies reported the relationship between the presence of perinatal pets and asthma risk [29]. Based on that study, it is more likely that the presence of various pets with individuals having a familial history of asthma could be a confounding factor for our study. Hence, we excluded those individuals who have pets at home. Additional studies are needed to evaluate the variables associated with pets.

Abnormal iso forms, such as those found in ADAM33, may affect signaling of molecules, ultimately resulting in airway obstruction and accelerate deterioration of lung function in patients with asthma [30]. A number of studies have reported a rare prevalence of AA genotype in the Asian population [31], and our patient cohort followed this same geographic trend. SNP rs2280090 showed a complete absence of homozygous mutant genotype "AA" in both the patient group and in controls. However, a significant negative relationship was observed in heterozygous genotype "AG" and environmental pollution in patients with asthma ($p <0.001$ and OR= 0.29, CI=0.22-0.42). This probability and OR highlighted the protective role of this SNP in environmentally exposed asthmatic cases. These results align with the meta-analysis conducted in the Chinese Han population, as reported by Li *et al* [14]. Research by Ghani *et al*, [9] and Li *et al*, [8] found no correlation in environmentally exposed asthmatic patients with this SNP, but contrasting results were observed in the Iranian population, which suggested the disease-causing role of this SNP [32].

SNP rs2787094 was found to be significantly associated with environmental pollutants in asthma susceptibility, as observed in allelic (OR=1.82, 95% CI =1.45-2.29, $p<0.0001$) and genotypic frequency ($p<0.0001$, OR = 3.5, CI = 2.08-5.09). A similar frequency pattern has been previously reported in a number of populations, including China and Germany [33, 34]. These studies indicate that rs2787094 is a highly disease-causing polymorphism, yet its penetrance may vary among different ethnic groups. Male and female populations showed a similar association (OR= 4.61, 95% CI =1.23-17.34, $p <0.001$) and (OR= 1.79, 95% CI =0.29-10.95, $p = 0.03$) respectively).

The rs3816470 is an in tronic SNP of *IKZF3* gene. This marker appeared to be strongly associated with disease status in our studied population (Table1). The

mutant genotype showed a disease-causing association of this SNP (OR=3.55, CI=1.89-6.54, p <0.0001), which concurs with previous work [17].

SNP rs3859192 is an intronic variant of the *GSDMA* gene. Our study revealed its significant negative association with asthma (p<0.0001; OR =0.08; CI= [0.02-0.33]). In our studied population, this variant was found to be protective, which strongly contrasts with findings of a previous study conducted on the Lahore population of Pakistan [17], in which this marker showed a strong positive association with the disease.

Several GWAS studies have reported SNP rs7216389 in the *GSDMB* gene as associated with asthma pathogenesis [10, 35]. Environmental pollutant-mediated genetic variants of this gene cause dysregulation of sphingolipids biosynthesis, which subsequently results in cell membrane inflammation and mucous formation [28]. Our results showed a significant disease association between a polluted environment in homozygous "TT" (OR=5.98, CI=3.08-11.61, p<0.0001) (Table1) and heterozygous "CT" status (OR=1.76, CI=1.02-3.09). This finding is consistent with previously reported studies by Liu *et al.*[36] and Shi *et al*[37] in several Asian and Caucasian populations, namely Jordanians, Netherlanders, Japanese, Chinese, Russians, and Americans.

In our study, the patient's age was considered a variable, and major populations were either 5-18 years or > 18 years of age. Disease frequency was observed to be highest in patients > 18 years of age (56.5%), followed by teenagers (42.5%) and children <five years of age (1%). Our findings aligned with a previous study demonstrating that age of asthma onset can affect asthma-related outcomes, and among adults, the onset of asthma and subsequent-outcomes are associated with aggravation of the disease [38].

The gender of the patient was studied as an important variable. Male and female populations demonstrated similar correlation with rs2280090, rs2787094, rs3816470, rs7216389, and rs3859192.In a group-wise category, a similar association was observed in males with age of asthma onset >18 years, and 5-18 years for all the SNPs in studied genes (Table 4a and 4b), whereas no positive association was found in the group with age of onset< age 5. (Table4c). In the case of females, a similar association was observed for all the SNPs with age of onset >18 years, and for the SNPs rs2280090, rs2787094, rs3816470 in the age of onset group 5-18 years (Table5a). However, no association was observed for SNPs rs7216389, rs385919 in females associated with the age of onset group 5-18 years (Table 5b). Age of onset group <five years in females showed no associations with reported SNPs (Table5c). Collectively, the data showed that gender is not a confounding variable for the SNPs included in our study nor in its relationship with asthma occurrence in Pakistani populations exposed to environmental pollution. Surprisingly, we did not observe any significant association of SNPs with asthma in the age group <five years, which may be due to this group's small population size.

The negative association between asthma and SNPs rs2280090, rs3859192, and rs1695 in our samples perhaps reveals disease heterogeneity prejudiced by ethnic differences. It is also possible that the relatively small size of our study cohort is the reason for this non-significant result.

Small cohort size is the major limitation of the study, resulting in direct correlation of genetic variation with environmental pollutants remaining both unknown and difficult to confirm. A future study that can accommodate asthmatic and non-asthmatic cohorts from non-polluted areas of the same ethnicity as cohorts from polluted areas, and large sample size for all age groups might impart further validation to our study outcomes. Confounding factors such as food patterns, smoking habits, or medication allergies should be considered in future studies.

Overall, the study uncovered the role of SNPs in asthma risk in the Pakistani population exposed to environmental pollution. Although we did not establish a direct relationship between the genetic variations and environmental pollution, which are known risk factors for asthma, our study demonstrated the potential genotypic variants involved in asthma risk and prevention in Pakistani populations exposed to environmental pollution. The sample size of our studied population, while limited, remains the first of its kind in terms of selecting a study population to establish the direct/indirect relationship between two important factors for asthma risk.

There are numerous GWAS studies conducted in developed countries; however, studies establishing a relationship between gene environment interaction and aggravation of asthma are rare in developing countries with high pollution rates. Hence, it is of utmost importance to study this relationship in patients with asthma who reside in those areas. Once multiple studies have been conducted in underdeveloped countries, a greater understanding of the actual genetic pathway should emerge, enabling the design of DNA targeted drugs to treat or possibly cure this disease.

CONCLUSION

Our study showed a positive disease association for SNPs rs2787094 (*ADAM33* gene), rs3816470 (*IKZF3* gene), rs7216389 (*GSDMB* gene), and rs1695 (*GSTP*1 gene) in individuals exposed to industrial and other environmental pollutants. The rs3859192 (*GSDMA*) polymorphism revealed a protective role in the disease, while the wild-type forms caused increased susceptibility to the disease. The heterozygous model of the SNP rs2280090 (*ADAM33*) polymorphism showed a protective role in individuals exposed to environmental pollutants. No association was found with any of the SNPs in the age group with onset <five years of age.

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SNP	Genotype/ Allele	Patients	Controls	p for Association	Corrected p value	Odds Ratio	Confidence Interval	p for Hardy Weinberg Equilibrium (HWE)
rs2280090	GG	226(0.82)	229(0.58)	R*	R	1		0.91 P, 0.78 C
	GA	49(0.18)	171(0.42)	<0.0001	<0.0001	0.29	0.22-0.42	
	AA	0	0	0	0	0		
	G	0.91	0.79	R		1		
	A	0.09	0.21	<0.0001	<0.0001	0.0263	0.0114-0.0607	
rs2787094	CC	24(0.08)	77(0.19)	R	R	1		0.98 P, 0.97C
	CG	121(0.44)	204(0.51)	0.0135	0.00225	1.9	1.142-3.17	
	GG	130(0.472)	119(0.30)	<0.0001	<0.0001	3.5	2.08-5.90	
	C	0.31	0.45	R	R	1		
	G	0.69	0.55	<0.0001	<o.ooo1	1.82	1.45 –2.29	
rs3816470	TT	91(0.33)	271(0.68)	R	R	1		0.81 P, 0.83C
	CT	159(0.58)	108(0.27)	<0.0001	<0.0001	4.38	3.12-6.16	
	CC	25(0.09)	21(0.05)	0.0003	0.00005	3.55	1.89.-6.64	
	T	0.62	0.81	R	R	1		
	C	0.38	0.19	0.0034	0.0006	2.61	1.37-4.96	
rs7216389	CC	19(0.07)	56(0.14)	R	R	1		0.76 P, 0.78C
	CT	187(0.68)	310(0.77)	0.039	0.0065	1.78	1.02-3.09	
	TT	69(0.25)	34(0.09)	<0.0001	<0.0001	5.98	3.08-11.61	
	C	0.41	0.53	R	R	1		
	T	0.59	0.47	<0.0001	<0.0001	1.62	1.3 - 2.01	
rs3859192	CC	15(0.05)	2(0.005)	R	R	1		0.64 P, 0.97 C
	CT	69(0.25)	60(0.15)	0.006	0.001	0.15	0.03-0.7	
	TT	191(0.7)	338(0.845)	<0.0001	<0.0001	0.08	0.02-0.33	
	C	0.18	0.08	R		1		
	T	0.82	0.92	0.04	0.006	0.017	0.0065-0.0441	
rs1695	AA	227(0.83)	281(0.70)	R		1		
	AG	36(0.13)	84(0.21)	0.014	0.002	0.59	0.38-0.9	
	GG	12(0.04)	35(0.09)	0.019	0.003	3.29	1.14-9.46	
	A	0.9	0.8	R	R			
	G	0.1	0.2	<0.0001	<0.0001	0.44	0.32-0.62	

SNP	Genotype/ Allele	Patients	Controls	p for Association	Corrected p value	Odds Ratio	Confidence Interval	p for Hardy Weinberg Equilibrium (HWE)
rs2280090	GG	226(0.82)	229(0.58)	R*	R	1		0.91 P, 0.78 C
	GA	49(0.18)	171(0.42)	<0.0001	<0.0001	0.29	0.22-0.42	
	AA	0	0	0	0	0		
	G	0.91	0.79	R		1		
	A	0.09	0.21	<0.0001	<0.0001	0.0263	0.0114-0.0607	
rs2787094	CC	24(0.08)	77(0.19)	R	R	1		0.98 P, 0.97C
	CG	121(0.44)	204(0.51)	0.0135	0.00225	1.9	1.142-3.17	
	GG	130(0.472)	119(0.30)	<0.0001	<0.0001	3.5	2.08-5.90	
	C	0.31	0.45	R	R	1		
	G	0.69	0.55	<0.0001	<o.ooo1	1.82	1.45 –2.29	
rs3816470	TT	91(0.33)	271(0.68)	R	R	1		0.81 P, 0.83C
	CT	159(0.58)	108(0.27)	<0.0001	<0.0001	4.38	3.12-6.16	
	CC	25(0.09)	21(0.05)	0.0003	0.00005	3.55	1.89.-6.64	
	T	0.62	0.81	R	R	1		
	C	0.38	0.19	0.0034	0.0006	2.61	1.37-4.96	
rs7216389	CC	19(0.07)	56(0.14)	R	R	1		0.76 P, 0.78C
	CT	187(0.68)	310(0.77)	0.039	0.0065	1.78	1.02-3.09	
	TT	69(0.25)	34(0.09)	<0.0001	<0.0001	5.98	3.08-11.61	
	C	0.41	0.53	R	R	1		
	T	0.59	0.47	<0.0001	<0.0001	1.62	1.3 - 2.01	
rs3859192	CC	15(0.05)	2(0.005)	R	R	1		0.64 P, 0.97 C
	CT	69(0.25)	60(0.15)	0.006	0.001	0.15	0.03-0.7	
	TT	191(0.7)	338(0.845)	<0.0001	<0.0001	0.08	0.02-0.33	
	C	0.18	0.08	R		1		
	T	0.82	0.92	0.04	0.006	0.017	0.0065-0.0441	
rs1695	AA	227(0.83)	281(0.70)	R		1		
	AG	36(0.13)	84(0.21)	0.014	0.002	0.59	0.38-0.9	
	GG	12(0.04)	35(0.09)	0.019	0.003	3.29	1.14-9.46	
	A	0.9	0.8	R	R			
	G	0.1	0.2	<0.0001	<0.0001	0.44	0.32-0.62	

Table 1.: Allelic and Genotypic Association of Patients vs. Controls

R= The Reference/Wild type against which mutant P value and corrected P value is calculated.

The numbers mentioned in parentheses are the ratio of the said genotype to the total no. of cases/controls.

Supplementary Tables

Table S1: Primers sequence and restriction enzymes used for genotyping of SNPs

Gene	SNP	Primer Forward	Primer Reverse	Product Size	RestrictionEnzyme
ADAM33	rs2280090	GAGGCTTTGAATCCAGGTCC	CTCAGTAAACGCAGAACTCCC	472bp	HpyCH4III
ADAM33	rs2787094	AAATGGTTCCCTCTGTCCCC	CCCAGAAGCAAAGGTCACAC	400bp	PSTI
GSDMB	rs3816470	GTCTGCACACTACGGCTCAC	CGAACCCTGGGCTTATTTC	312bp	BsaHI
GSDMA	rs7216389	CATCTCTACCAAATTAGTCGGGG	CTCCCCTGTAGGTTCCCATC	394bp	NsiI
IKZF3	rs3859192	ATGCAAACCTTCCCTCCTG	GGATGACCTTCTCCTCTCCTG	459bp	NcoI
GSTP1	rs1695	GGTGTCAGGTGAGCTCTGAG	ATGACCCGTTACTTGGCTGG	500bp	HpyCH4IV

Table S2: Gender wise distribution of patients based on age of onset

Age of onset	Males	Females	Total
<5	3	0	3 (0.95%)
5-18	67	50	117 (42.5%)
>18	96	59	155 (56.55)
Patients With Asthma	166	109	275
Controls	267	133	400

Table S3: Allelic and genotypic association in male patients against male controls based on age of onset

Table 3(a): Association in males with age of onset >18 years (n = 96)

SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio (OR)	Confidence Interval (CI)
rs2280090	GG	88 (0.89)	147 (0.55)	R	R	1	-
	GA	9 (0.091)	120 (0.45)	<0.0001	<0.0001	0.13	0.06-0.26
	AA	0	0	-	-	-	-
	G	0.95	0.78	R	R	1	-

SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio (OR)	Confidence Interval (CI)
	A	0.045	0.22	<0.0001	<0.0001	0.17	0.15-0.19
rs2787094	CC	8 (0.08)	51(0.19)	R	R	1	-
	CG	47 (0.48)	134(0.50)	0.139	0.023	2.19	0.97-4.95
	GG	42(0.44)	82(0.31)	0.0706	0.012	3.27	142-7.51
	C	0.32	0.44	R	R	1	-
	G	0.38	0.56	<0.0001	<0.0001	1.66	1.57-1.76
rs3816470	TT	23 (0.24)	179 (0.67)	R	R	1	-
	CT	68(0.70)	76 (0.28)	<0.0001	<0.0001	6.86	3.98-11.83
	CC	6(0.06)	12 (0.05)	0.0083	0.001	3.89	1.33-11.37
	T	0.59	0.81	R	R	1	-
	C	0.41	0.19	<0.0001	<0.0001	3.03	2.84-3.23
rs7216389	CC	6(0.06)	33(0.12)	R	R	1	-
	CT	59(0.60)	210(0.79)	0.37	0.06	1.52	0.61-3.8
	TT	32 (0.34)	24(0.09)	<0.0001	<0.0001	7.33	2.65-20.3
	C	0.36	0.52	R	R	1	-
	T	0.64	0.48	0.023	0.004	1.93	1.09-3.39
rs3859192	CC	5 (0.05)	1(0.004)	R	R	1	-
	CT	28 (0.28)	42 (0.16)	0.036	0.006	0.13	0.01-1.16
	TT	64 (0.67)	224(0.84)	0.0005	0.00008	0.06	0.01-0.05
	C	0.19	0.08	RE	R	1	-
	T	0.81	0.92	0.027	0.0045	0.37	0.15-0.89
rs1695	AA	80(0.84)	206(0.77)	R	R	1	-
	AG	12 (0.12)	58(0.22)	0.06	0.01	0.53	0.27-1.04
	GG	4(0.04)	3(0.01)	0.09	0.015	3.43	0.75-15.68
		0.9	0.88	R	R	1	-
		0.1	0.12	0.65	0.11	1.23	0.5-2.99

SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio (OR)	Confidence Interval (CI)
S 3 (b): Association in males with age of onset 5-18 years (n = 67)							
SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio (OR)	Confidence Interval (CI)
rs2280090	GG	57 (0.85)	147 (0.55)	R	R	1	-
	GA	10(0.15)	120 (0.45)	<0.0001	<0.0001	0.21	0.11-0.44
	AA	0	0	-	-	-	-
	G	0.92	0.78	R	R	1	-
	AA	0.08	0.22	0.0076	0.0013	0.31	0.13-0.73
rs2787094	CC	7 (0.10)	51(0.19)	R	R	1	-
	CG	32(0.48)	134(0.50)	0.213	0.035	1.74	0.72-4.19
	GG	28(0.42)	82(0.31)	0.042	0.007	2.49	1.01-6.11
	C	0.34	0.44	R	R	1	-
	G	0.66	0.56	0.14	0.025	1.52	0.86-2.7
rs3816470	TT	23 (0.35)	179 (0.67)	R	R	1	-
	CT	37(0.55)	76 (0.28)	<0.0001	<0.0001	3.79	2.11-6.8
	CC	7(0.10)	12 (0.05)	0.0083	0.001	4.54	1.62-12.69
	T	0.62	0.81	R	R	1	-
	C	0.38	0.19	0.0034	0.0006	2.61	1.37-4.97
rs7216389	CC	5 (0.07)	33(0.12)	R	R	1	-
	CT	51(0.77)	210(0.79)	0.37	0.06	1.6	0.6-4.31
	TT	11(0.16)	24(0.09)	0.059	0.009	3.03	0.93-9.85
	C	0.45	0.52	R	R	1	-
	T	0.55	0.48	0.32	0.05	1.32	0.76-2.3
rs3859192	CC	4)(0.06)	1(0.004)	R	R	1	-
	CT	16(0.24)	42 (0.16)	0.016	0.005	0.1	0.01-0.92
	TT	47(0.70)	224(0.84)	0.00035	0.00006	0.05	0.01-0.48

SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio (OR)	Confidence Interval (CI)
	C	0.18	0.08	R	R	1	-
	T	0.82	0.92	0.04	0.007	0.39	0.16-0.96
rs1695	AA	57(0.85)	206(0.77)	R	R	0	-
	AG	6(0.09)	58(0.22)	0.025	0.004	0.37	0.15-0.91
	GG	4(0.06)	3(0.01)	0.027	0.0045	4.82	1.05-22.15
		0.9	0.88	R	R	1	-
		0.1	0.12	0.65	0.11	1.23	0.5-2.99

* The numbers mentioned in parentheses are the ratio of the said genotype to the total no. of cases/controls.

R= The Reference/Wild type against which mutant P value and corrected P value is calculated.

S 3 (c): Association in males with age of onset <5 years (n = 3)							
SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio (OR)	Confidence Interval (CI)
rs2280090	GG	2 (0.67)	147 (0.55)	R	R	1	-
	GA	1(0.33)	120 (0.45)	0.689	0.11	0.61	0.05-6.84
	AA	0	0	-	-	-	-
	G	0.83	0.78	R	R	1	-
	A	0.17	0.22	0.37	0.62	0.72	0.359-1.45
rs2787094	CC	1(0.33)	51(0.19)	R	R	1	-
	CG	2 (0.67)	134(0.50)	0.82	0.14	0.76	0.07-8.58
	GG	0	82(0.31)	0.2	0.035	0	-
	C	67	0.44	R	R	1	-
	G	0.33	0.56	0.0019	0.0003	0.403	0.227-0.715
rs3816470	TT	2(0.67	179 (0.67)	R	R	1	-
	CT	1(0.33)	76 (0.28)	0.89	0.15	1.18	0.11-13.18

	CC	0	12 (0.05)	0.72	0.12	0	-
	T	0.93	0.81	R	R	1	-
	C	0.17	0.19	0.72	0.12	0.87	0.42-1.8
rs7216389	CC	1(0.33)	33(0.12)	R	R	1	-
	CT	2(0.67)	210(0.79)	0.33	0.055	0.31	0.03-3.56
	TT	0	24(0.09)	0.39	0.07	0	-
	C	0.67	0.52	R	R	1	-
	T	0.33	0.48	0.03	0.006	0.53	0.30-0.95
rs3859192	CC	0	1(0.004)	R	R	1	-
	CT	3(1)	42 (0.16)	0.79	0.13	Infinty	-
	TT	0	224(0.84)	infinity	Infinty	-	-
	C	0.5	0.08	R	R	1	-
	T	0.5	0.92	<0.0001	0.0001	0.087	0.038-0.19
rs1695	AA	3(1)	206(0.77)	R	R	0	-
	AG	0	58(0.22)	0.36	0.06	0	-
	GG	0	3(0.01)	0.84	0.16	0	-
		1	0.88	R	R	1	-
		0	0.12	0.02	0.003	0.035	0.021-0.6036

(d): Allelic and genotypic association among all male patients versus male controls (n = 166)

SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio (OR)	Confidence Interval (CI)
rs2280090	GG	134 (0.82)	147 (0.55)	R	R	1	-
	GA	29 (0.18)	120 (0.45)	<0.0001	<0.0001	0.27	0.17-1.42
	AA	0	0	-	-	-	-
	G	0.91	0.78	R	R	1	-
	A	0.09	0.22	0.14	0.002	0.35	0.15-0.80
rs2787094	CC	15 (0.009)	51(0.19)	R	R	1	-
	CG	80 (0.48)	134(0.50)	0.028	0.004	2.03	1.07-3.85
	GG	71 (0.43)	82(0.31)	0.001	0.0001	2.94	1.54-5.68

	C	0.33	0.44	R	R	1	-
	G	0.67	0.56	0.11	0.02	1.59	0.89-2.8
rs3816470	TT	57 (0.34)	179 (0.67)	R	R	1	-
	CT	96 (0.58)	76 (0.28)	<0.00001	<0.00001	3.97	2.6-6.06
	CC	13 (0.08)	12 (0.05)	0.003	0.0005	3.4	1.47-7.88
	T	0.63	0.81	R	R	1	-
	C	0.37	0.19	0.0052	0.0009	2.5	1.32-4.77
rs7216389	CC	12 (0.07)	33(0.12)	R	R	1	-
	CT	120 (0.73)	210(0.79)	0.201	0.03	1.57	0.78-3.16
	TT	34 (0.20)	24(0.09)	0.001	0.0001	3.9	1.68-9.05
	C	0.43	0.52	R	R	1	-
	T	0.57	0.48	0.2	0.03	1.43	0.82-2.5
rs3859192	CC	9 (0.05)	1(0.004)	R	R	1	-
	CT	46 (0.28)	42 (0.16)	0.023	0.004	0.12	0.001-1
	TT	111(0.67)	224(0.84)	0.0001	0.00001	0.06	0.01-0.44
	C	0.19	0.08	R	R	1	-
	T	0.81	0.92	0.027	0.004	0.37	0.15-0.89
rs1695	AA	139 (0.84)	206(0.77)	R	R	0	-
	AG	18 (0.11)	58(0.22)	0.06	0.01	0.61	0.36-1.04
	GG	9 (0.05)	3(0.01)	0.01	0.001	4.61	1.23-17.34
	A	0.9	0.88	R	R	1	-
	G	0.1	0.12	0.65	0.1	0.81	0.33-1.98

Table S4: Allelic and genotypic association in female patients versus female controls based on age of onset

Table S4(a): Association in females with age of onset >18 years (n = 59)

SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio	Confidence Interval (CI)
rs228009	GG	54 (0.92)	82 (0.62)	R	R	1	-

SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio	Confidence Interval (CI)
0	GA	5(0.08)	51(0.38)	0.00003	<0.00005	0.15	0.06-0.4
	AA	0	0	-	-	-	-
	G	0.96	0.81	R	R	1	-
	AA	0.04	0.19	0.0025	0.0004	0.18	0.05-0.55
rs2787094	CC	6(0.10)	26(0.19)	R	R	1	-
	CG	13 (0.22)	70(0.53)	0.69	0.115	0.8	0.28-2.34
	GG	40(0.68)	37 (0.28)	0.0014	0.0002	4.68	1.73-12.66
	C	0.21	0.46	R	R	1	-
	G	0.79	0.54	0.0002	0.00003	3.2	1.72-5.97
rs3816470	TT	28(0.48)	92 (0.69)	R	R	1	-
	CT	26(0.44)	32 (0.24)	0.0035	0.0006	2.67	1.37-5.21
	CC	5(0.08)	9 (0.07)	0.307	0.05	1.83	0.57-5.89
	T	0.69	0.89	R	R	1	-
	C	0.31	0.11	0.0008	0.0001	3.63	1.71-7.75
rs7216389	CC	1(0.02)	23 (0.17)	R	R	1	-
	CT	51(0.86)	100 (0.75)	0.003	0.00005	11.73	1.54-89.35
	TT	7 (0.12)	10 (0.08)	0.003	0.0005	16.1	1.74-148-68
	C	0.45	0.55	R	R	1	-
	T	0.55	0.45	0.16	0.025	1.49	0.86-2.60
rs3859192	CC	3(0.05)	1(0.01)	R	R	1	-
	CT	23(0.39)	18 (0.14)	0.47	0.08	0.43	0.04-4.45
	TT	33(0.56)	114 (0.85)	0.015	0.0025	0.1	0.01-0.96
	C	0.25	0.08	R	R	1	-

SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio	Confidence Interval (CI)
	T	0.75	0.92	0.002	0.0003	0.26	0.1112-0.62
rs1695	AA	51(0.86)	105 (0.79)	R	R	1	-
	AG	5(0.08)	26 (0.20)	0.06	0.01	0.4	0.14-1.09
	GG	3(0.05)	2(0.01)	0.2	0.03	3.09	0.5-19.06
	A	0.91	0.89	R	R	1	-
	G	0.09	0.11	0.048	0.008	0.27	0.07-0.99
S 4 (b): association in females with age of onset 5-18 years (n = 50)							
SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio	Confidence Interval (CI)
rs2280090	GG	43 (0.86)	82 (0.62)	R	R	1	-
	GA	7(0.14)	51(0.38)	0.0016	0.0003	0.26	0.11-0.63
	AA	0	0	-	-	-	-
	G	0.93	0.81	R	R	1	-
	A	0.07	0.19	0.015	0.002	0.32	0.13-0.80
rs2787094	CC	3 (0.06)	26(0.19)	R	R	1	-
	CG	25 (0.5)	70(0.53)	<0.00001	<0.00001	10.06	4.48-22.61
	GG	22 (0.44)	37 (0.28)	0.005	0.0008	5.11	1.43-18.26
	C	0.31	0.46	R	R	1	-
	G	0.69	0.54	0.0084	0.0014	5.15	1.4-19.03
rs3816470	TT	10 (0.2)	92 (0.69)	R	R	1	-
	CT	35 (0.7)	32 (0.24)	0.11	0.018	2.68	0.76-9.49
	CC	5(0.1)	9 (0.07)	0.0014	0.0002	9.2	2.12-39.89
	T	0.55	0.89	R	R	1	-
	C	0.45	0.11	<0.0001	<0.0001	6.62	3.16-13.88

SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio	Confidence Interval (CI)
rs7216389	CC	3 (0.06)	23 (0.17)	R	R	1	-
	CT	35 (0.7)	100 (0.75)	0.11	0.02	2.68	0.76-9.49
	TT	12 (0.24)	10 (0.08)	0.0011	0.0002	9.2	2.12-39.89
	C	0.41	0.55	R	R	1	-
	T	0.59	0.45	0.048	0.008	1.76	1.0-3.08
rs3859192	CC	3 (0.06)	1(0.01)	R	R	1	-
	CT	20(0.4)	18 (0.14)	0.55	0.09	0.77	0.32-1.84
	TT	27 (0.54)	114 (0.85)	0.37	0.06	0	0--
	C	0.26	0.08	R	R	1	-
	T	0.74	0.92	0.0013	0.0002	0.25	0.105-0.58
rs1695	AA	42 (0.84)	105 (0.79)	R	R	1	-
	AG	8 (0.16)	26 (0.20)	0.56	0.07	0.4	0.14-1.09
	GG	0	2(0.01)	0.37	0.06	0	0-0
	A	0.92	0.89	R	R	1	-
	G	0.08	0.11	0.47	0.06	0.7	0.27-1.83

S 4(c): Allelic and genotypic association among all female patients versus female controls (n = 109)

SNP	Genotype	Cases	Controls	p for Association	Corrected p	Odds ratio	Confidence Interval (CI)
rs2280090	GG	97 (0.89)	82 (0.62)	R	R	1	-
	GA	12 (0.11)	51(0.38)	0.0016	0.0003	0.26	0.11-0.63
	AA	0	0	-	-	-	-
	G	0.95	0.81	R	R	1	-
	A	0.05	0.19	0.015	0.002	0.32	0.13-0.80

rs2787094	CC	9(0.08)	26(0.19)	R	R	1	-
	CG	38(0.35)	70(0.53)	<0.00001	<0.00001	10.06	4.48-22.61
	GG	62(0.57)	37 (0.28)	0.005	0.0008	5.11	1.43-18.26
	C	0.26	0.46	R	R	1	-
	G	0.74	0.54	0.0084	0.0014	5.15	1.4-19.03
rs3816470	TT	38 (0.35)	92 (0.69)	R	R	1	-
	CT	61(0.56)	32 (0.24)	0.11	0.018	4.62	2.61-8.17
	CC	10(0.09)	9 (0.07)	0.0014	0.0002	2.69	1.01-7.14
	T	0.71	0.89	R	R	1	-
	C	0.29	0.11	<0.0001	<0.0001	6.62	3.16-13.88
rs7216389	CC	4(0.04)	23 (0.17)	R	R	1	-
	CT	86(0.79)	100 (0.75)	0.002	0.0003	4.95	1.65-14.86
	TT	19(0.17)	10 (0.08)	0.0012	0.0002	10.93	2.95-40.95
	C	0.43	0.55	R	R	1	-
	T	0.57	0.45	0.048	0.008	1.76	1.0-3.08
rs3859192	CC	6(0.05)	1(0.01)	R	R	1	-
	CT	43(0.39)	18 (0.14)	0.39	0.06	0.4	0.01-3.55
	TT	60(0.56)	114 (0.85)	0.005	0.0008	0.09	0.01-0.75
	C	0.25	0.08	R	R	1	-
	T	0.75	0.92	0.0013	0.0002	0.25	0.105-0.58
rs1695	AA	88(0.81)	105 (0.79)	R	R	1	-
	AG	18(0.16)	26 (0.20)	0.06	0.01	0.4	0.14-1.09
	GG	3(0.03)	2(0.01)	0.2	0.03	1.79	0.29-10.95
	A	0.88	0.89	R	R	1	-
	G	0.12	0.11	0.82	0.13	1.1	0.46-2.63