



SHORT COMMUNICATION

# Possible association of a distinct combined Glutathione-S-transferase members with allergic asthma patients in Pakistan



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Received 25 November 2016; accepted 5 January 2017

Available online 17 January 2017

## KEYWORDS

Allergic asthma;  
Genetic  
polymorphism;  
GSTM;  
GSTT;  
GST deletions;  
Mutations

**Abstract** Allergic asthma is a diverse chronic respiratory disease characterized by the inflammation of the lower airway disease affecting many people around the world with rising morbidity and mortality. Association between asthma and certain demographic features was studied in relation to genotype from 244 allergic individuals of local population. Skin prick test was used to confirm asthma. Genetic polymorphism in Glutathione-S-transferases (GSTs) was studied using multiplex PCR based method and IgE level by ELISA. Pollen and dust were the major causative aeroallergens (26%), which were associated to higher IgE levels ( $P < 0.05$ ). Smoking was found to be significantly associated with asthma in only males ( $P = 0.004$ ). A low prevalence of null genotype of both GSTM1 and GSTT1 genes was observed in the patients (4.34%) compared to control group (14%). No association of combined GSTM1 and GSTT1 null genotype was found with the asthma in local population. GSTM1<sup>+</sup> and GSTT<sup>-</sup> genotype had higher risk (OR = 1.3681,  $P = 0.001$ ) for development of asthma. There was a significant association of asthma with combined genotype of GSTM1<sup>+</sup> and GSTT<sup>-</sup> when data was analyzed on gender basis in males ( $P = 0.006$ ) and highly significant in age range of 26–40 years ( $P = 0.001$ ). Combined GSTM<sup>+</sup> and GSTT<sup>-</sup> genotype was found to be risk factor for asthma in addition to family history in male patients. However a data with large patient size and different ethnic distribution may reveal the exact etiology.

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Peer review under responsibility of Chongqing Medical University.

## Introduction

Asthma is a disease characterized by the airway hyper responsiveness, inflammation along with fundamental structural changes to airways<sup>1</sup> which can lead to irreversible airway remodeling as well as intractable limitation.<sup>2</sup> Bronchial asthma is a severe multifactorial disease.<sup>3</sup> Prevalence of asthma has increased in the past few decades resulting in a significant socioeconomic impact.<sup>4</sup> Asthma is known to affect 35% of the children and 10% of the adults worldwide, therefore it is considered to be an increasing public health concern.<sup>5</sup> According to the Global Burden of Asthma report (2012) by Global Initiative for Asthma – GINA, 4.3% of the total Pakistani population (over six million people), are affected by asthma. The statistics also revealed that among asthmatic patients 32% children and 51% adults experience rigorous attacks of wheeze, cough, tightness or breathlessness.<sup>6</sup>

Atopy is a genetic predisposition for the development of an immunoglobulin E (IgE) induced response to the allergens. Genetic or host susceptibility to various allergens or atopy may lead to the development of asthma, and various studies show a significant relationship between allergens and asthma.<sup>5</sup> Atopic asthma is generally coupled with raised circulating levels of IgE.<sup>7</sup> But the increased level of total serum IgE is considered to be a risk factor for asthma even in nonallergic individuals. IgE molecules have been found to play a crucial role in allergic respiratory diseases and possibly cause chronic airway inflammation in asthma through activation of effector cells via high-affinity (FcεRI) or low-affinity (FcεRII) IgE receptors.<sup>8</sup>

It is beyond any doubt that environmental as well as genetic factors are the major contributors of allergic disease.<sup>9</sup> It is evident from many studies that the immune system's ability to interact with the environment is influenced by genetic predisposition.<sup>10</sup> In asthma a genetic predisposition may also change the capability of the respiratory airway from protecting itself against the inhaled allergens or toxic substances from the environment. This may lead to diminishing the barrier function of the airway by airway inflammation and airway epithelium damage.<sup>11</sup>

Overall 40–60% asthma risk is contributed by genetic factors. Many candidate genes have been identified which are related to the occurrence of atopy and asthma. The glutathione transferases (GSTs) are ubiquitously found in nature. The human GST family can be grouped into 7 distinct classes of catalytically active enzymes namely Alpha, Mu, Theta, Pi, Omega, Sigma, and Zeta.<sup>12</sup> It is possible that genetic alterations of GST enzymes alter the ability of the airways to deal with toxic substances and manipulate airway inflammation and lung development. Thus, GSTM1 and GSTT1 genes have been suggested as candidate genes for asthma.<sup>13</sup> The purpose of this work is to determine the possible association between a) IgE status with polymorphism of Glutathione-S-transferase (GSTM1 and GSTT1) gene in Pakistani population and 2) demographic and clinical parameters with polymorphism of GSTM1 and GSTT1 genes in the blood of asthmatic and allergic patients.

## Materials and methods

### Study subjects

Study was approved from the ethical committees of university and hospital. Informed consent was signed from all the participating individuals. Healthy individuals with no history of any sort of allergy or chronic illness with negative skin prick test were randomly selected as controls (n = 232). The study included 244 unrelated asthmatic patients from twin cities (Islamabad/Rawalpindi) of Pakistan that were confirmed by specialist doctors. All patients were recruited from Allergy Centre of National Institute of Health (NIH), Islamabad. Blood samples were taken from clinically stable asthmatic patients with positive SPT. Patients having food allergies were excluded from the data. None of the participants had received antihistamine and corticosteroids in 3 weeks prior to clinical evaluation. Questionnaires were filled for each patient as well as control group for the analysis of the data of various demographic features. This data was used to determine the association of Glutathione-S-transferase M1 and T1 gene polymorphisms with atopic asthma.

Genomic DNA from human blood samples was extracted in two consecutive days by phenol-chloroform method described by Masood et al.<sup>14</sup> The IgE levels of controls and patients were determined from serum by using commercial kit method (MicroLISA, AMGENIX Intl. Inc., USA), a value of  $\geq 100$  IU/ml was considered as positive value for allergic asthma.

Multiplex PCR for GSTT1 (F: 5' GGCGAGAGCAA-GACTCAG 3'; R: 5' GGCAGCATAAGCAGGACTTC 3') and GSTM1 (F: 5' GAACTCCCTGAAAAGCTAAAGC 3'; R: 5' GTTGGGCTCAAATATACGGTGG 3') was carried out to amplify fragments of 385 bp and 219 bp respectively.  $\beta$ -globin was used as an internal control (F: 5' CAATTCATC-CACGTTCCACC 3'; R: 5' GAAGAGCCAAGGACAGGTAC 3'). Multiplex PCR was performed by using 50 ng of the genomic DNA in 20  $\mu$ l reaction mixture and ready-to-use Mastermix (Solisbiodyne FIRE Pol 5x). The contents were mixed and placed in a thermocycler with conditions as follows; First of all, preheating of template DNA at 95 °C for 5 min which was followed by 35 cycles of amplification. Each cycle consisted of 3 steps: denaturation at 95 °C for 1 min, primer hybridization at 57.5 °C for 50 s and extension at 72 °C for 1 min. Then, the last step was of final extension at 72 °C for 10 min. Amplified products were subjected to 2% agarose gel electrophoresis stained with ethidium bromide and visualized in gel document analyser.

The collected data was coded and typed onto computer files using SPSS Statistics version 19.0. Descriptive statistics including frequency, percentages, arithmetic mean (X), standard deviation (SD) were used to describe asthma patients and healthy individuals. The study also used analytic measures such as student *t*-test, Chi-square test ( $\chi^2$  test), and non-parametric tests. The level of significance selected for this study was 0.05. Odd ratios were also calculated through online available calculator (Med Calc. Version 13.0.6.0).

## Results

According to the data, age group distribution was 15–55 years in patients and 15–72 years in controls. The average age of asthma patients was 30.62 ( $\pm 9.809$ ) years, whereas controls was 31.06  $\pm$  11.845 years.

Comparison of log IgE levels in case and healthy individuals showed a significantly increased ( $P \leq 0.05$ ) log IgE levels in allergic patients as compared to controls. Genotypically all subjects were categorized into four groups on the basis of combination of genotypes: (1) T<sup>+/+</sup>M<sup>+/+</sup> genotype; (2) T<sup>-/-</sup>M<sup>+/+</sup> genotype; (3) T<sup>+/+</sup>M<sup>-/-</sup> genotype; and (4) T<sup>-/-</sup>M<sup>-/-</sup> genotype. In patients with combined T<sup>+/+</sup>M<sup>-/-</sup> genotype with reference to wild type genotype, had significant association as compared to the other genotypes. While in healthy control individuals, there was no association of IgE levels using T<sup>+/+</sup>M<sup>+/+</sup> as reference to any genotypes (Table 1).

Among patients of asthma, 64% did not have any sort of family history of the allergic diseases, while around 36% of the patients had a family member having allergic problems either first degree or second (Table 2). Statistical analysis of the data revealed that family history was significantly associated with asthma ( $P < 0.05$ ) and even after stratification of data on the basis of gender, it was found to be a significant variable (Table 3). Stratification on the basis of age groups showed that family history has a significant association ( $P < 0.05$ ) with asthma only in age group of patients  $>40$  years (Table 3).

Smoking is known to be another modifiable risk factor affecting asthmatic patients. Many studies have reported an association of smoking and respiratory diseases. It was found that 77% subjects had no history of smoking (active or passive), while 23% patients amongst the total 244 subjects

were active smokers. Same trend was observed in the control group with 72% of the non-smokers and only 28% were smokers. Overall, smoking was found to be associated to asthma ( $P = 0.006$ ) (Table 2). While stratification of data on gender basis disclosed significance of smoking as a risk factor in male asthmatic patients ( $P = 0.004$ ), while it was insignificant in female patients (Table 3).

Type of allergens is an important factor affecting the atopic asthma outcome in patients. Five different types of allergens were used to check for the hypersensitivity test on asthmatic patients by skin prick test. Extract of five local allergens (dust, pollen, paper mulberry, raw cotton and thresher) were applied for 20 min, subjects having wheal of diameter  $\geq 3$  mm on the prick area were considered positive for allergy. Patients were predominately sensitive to dust and pollen (26%). Combination of dust + pollen + paper mulberry was the second highest causative agent in 13.15% patients. Statistical analysis shows that allergen types are significantly associated with asthma pathogenesis (Tables 2 and 3). Among these allergic patients seasonal allergies were more frequent than perennial allergies.

Among 244 total patients of asthma, the majority of the patients belonged to group 1, i.e. T<sup>+/+</sup>M<sup>+/+</sup> with the total percentage of 38%. Group 2 with genotype T<sup>-/-</sup>M<sup>+/+</sup> + were 20% of the patients, group 3 with genotype T<sup>+/+</sup>M<sup>-/-</sup> were 27% and finally group 4 with both null genotypes had least number of patients (15%). Almost similar trend was observed in the control in various groups. Most prevalent genotype was group 3, which were 42% followed by group 2 and 1. Group 4 with both null genotypes had the lowest percentage among the controls (Fig. 1) Among studied patients, combined null genotype of T<sup>-/-</sup> and M<sup>-/-</sup> although had more risk of developing asthma (OR = 1.6521, 95% CI 0.9399 to 2.9039) compared group

**Table 1** Comparison of IgE levels with combined null genotype of GSTT1 and GSTM1 in allergic asthma patients and healthy controls of the study.

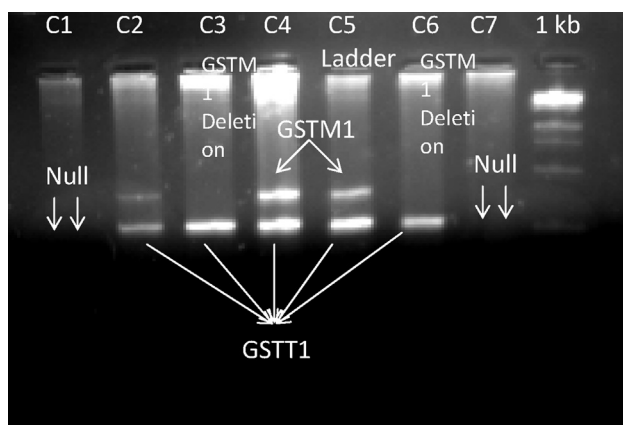
Genotype	Patients			Controls		
	Mean log IgE (IU/ml)	Paired t-test ( <i>P</i> -value)	95% CI (Lower–Upper)	Mean IgE (IU/ml)	Paired t-test ( <i>P</i> -value)	95% CI (Lower–Upper)
T <sup>+/+</sup> and M <sup>+/+</sup>	1.84	–	–	1.41	–	–
T <sup>-/-</sup> and M <sup>+/+</sup>	2.16	0.23	–0.7–0.2	0.00	–	–
T <sup>+/+</sup> and M <sup>-/-</sup>	2.25	$<0.05$	–0.7–0.06	1.657	0.14	0.5–0.8
T <sup>-/-</sup> and M <sup>-/-</sup>	2.31	0.06	–0.9–0.02	1.000	0.25	0.3–1.1

**Table 2** Odd ratio of for interaction of family history, smoking, allergens and frequency of GSTT1 and GSTM1 genotype among allergic asthma patients.

Variables	Odd Ratio	Confidence Interval (95%)	z- statistic	<i>P</i> -value	$\chi^2$ Test <i>P</i> -value
Family History	–	–	–	–	0.000
Smoking	0.7445	0.4074 to 1.3604	0.959	0.3374	0.006
Allergens	–	–	–	–	0.000
T <sup>+/+</sup> and M <sup>+/+</sup>	0.8276	0.5732 to 1.1949	1.010	0.3126	0.018
T <sup>-/-</sup> and M <sup>+/+</sup>	1.3681	0.8519 to 2.1971	1.297	0.1947	0.001
T <sup>+/+</sup> and M <sup>-/-</sup>	0.7770	0.5246 to 1.1507	1.259	0.2079	0.012
T <sup>-/-</sup> and M <sup>-/-</sup>	1.6521	0.9399 to 2.9039	1.745	0.0810	0.200

**Table 3** Association of various features and combine null genotype with allergic asthma after stratification on basis of gender and age group showing *P* values.

Variables	Males	Females	15–25 years	26–40 years	>40 years
Family History	0.000	0.000	0.125	0.591	0.000
Smoking	0.004	0.451	0.303	0.223	0.009
Allergens	0.000	0.000	0.497	0.009	0.015
T <sup>+/+</sup> and M <sup>+/+</sup>	0.135	0.054	0.144	0.018	0.965
T <sup>-/-</sup> and M <sup>+/+</sup>	0.006	0.069	0.056	0.001	0.096
T <sup>+/+</sup> and M <sup>-/-</sup>	0.069	0.079	0.053	0.012	0.850
T <sup>-/-</sup> and M <sup>-/-</sup>	0.625	0.217	0.416	0.464	0.579



**Fig. 1** Ethidium bromide stained 2% agarose gel image of multiplex PCR with 1 Kb ladder (Thermoscientific). GSTM1 bands are visible as upper bands and lower bands are GSTT1.

T<sup>-/-</sup> and M<sup>+/+</sup> genotype (OR = 1.368, 95% CI 0.8519–201971) but statistically GSTT<sup>-/-</sup>-M<sup>+/+</sup> genotype was significant (Table 2).

## Discussion

Age, family history, smoking history, atopic status, viral infections, and particularly airway obstruction are important parameters, which influence the airway reactivity.<sup>15</sup> Ahmed et al<sup>6</sup> reported an apparent seasonal pattern in the patients with higher number of admissions in early spring and the winter season particularly in the female adults with the age-group above 55 years. Our data revealed a continual pattern of allergy throughout the year, but still high number of effected individuals in the cited time period. Hence, majority of the patients in present study had seasonal allergies rather than perennial allergies which can affect any time of the year. Significant differences were not observed between asthma patients and healthy individuals for age distribution, sex ratio, or other characteristics analyzed in earlier work of Piacentini et al<sup>16</sup> Ahmed et al<sup>6</sup> showed age and sex-specific rates are marked to gender especially females (65%) and patients above 55 years of age were vulnerable groups. This study is based on age group of 11–60 years patients, with high prevalence in patients belonging to 25–35 years of age. In this present study, the male percentage was higher than

the female subjects. But this might be due to more males visiting the allergy centre. Family history had a highly significant association with asthma according to our analysis.

Smoking is considered as one of the leading cause of respiratory diseases. It is itself main cause of inflammation. Such inflammation can become chronic. Piacentini et al<sup>16</sup> reported significant association of smoking with asthma. Gender based stratification revealed a significant association of male asthma patients with smoking (*P* = 0.004). However, Mak et al<sup>17</sup> reported that asthmatic smokers had significantly higher levels of null GST polymorphism compared with non-smokers, while smoking did not affect the GST activity in healthy subjects. It is possible that in healthy subjects the antioxidant defense is efficient and protects against oxidant stress. In asthma, the disease process itself is associated with oxidative stress and cigarette smoking increases free oxygen radicals, while GST activity may increase as a compensatory mechanism. Our study reveals that smoking does not have any significant association with GSTM1 or GSTT1 activity or their null genotype.

Aeroallergens were found to cause asthma in 78% of population aged 15–30 years with elevated serum IgE antibodies and in many instance this nasal allergy lead to more severe disease allergic rhinitis.<sup>16</sup> Allergens mostly affecting the patients (26%) in the present study were pollen and dust. Pollen allergies are very common in the region of Islamabad and Rawalpindi from where majority of our patients belonged. Findings of Kabesch et al<sup>11</sup> suggest that the strongest effect on the development of asthma and asthma symptoms occurs in GSTM1 and GSTT1 deficient individuals exposed to smoke, on the contrary in present work most of these patients were non-smokers. According to Elshaer et al<sup>18</sup> workers of textile industry had bronchial asthma (28%), 18% who were involved in general public services followed by 16% worked in chemical industry. In the present study, individuals affected by raw cotton were low in number.

In the present study, GSTM1 and GSTT1 null genotype was not found to be associated with the occurrence of asthma and allergic rhinitis in local population. A significant association was found between GSTT1 and asthma when the data was sorted on gender basis. These results are related to the study by Holla et al<sup>19</sup> where the distribution of null genotype in GSTs did not differ significantly between patients within allergic asthma individuals and healthy controls. However, Elshaer et al<sup>18</sup> found in Egyptian population, an association between combined GSTM1/T1 null-genotype and asthma phenotype within 20% of workers with bronchial asthma compared to only 6% of the controls.

In conclusion, this study shows no association of GSTM1 and GSTT1 in pathogenesis of these allergy diseases. However, combined GSTM<sup>+</sup> and GSTT<sup>-</sup> genotype could be a possible candidate for development of allergic beside family history. In addition, this disease develops from interplay of environment and gene, therefore, there is need to expand this work to have concrete evidence for these genes and environmental features.

## Conflict of interest

All authors declare that there are no conflicts of interest in this manuscript.

## Acknowledgment

All authors would like to thank the participants of the study and COMSATS Institute of IT, Islamabad for lab support. All the hospitals and staff are also acknowledged for their contributions.

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