An angiotensin I-converting enzyme insertion/deletion polymorphism is associated with Pakistani asthmatic cases and controls

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Asthma is a chronic disease due to inflammation of the airways of lungs that is clinically characterized by variable symptoms including wheezing, coughing and shortness of breath. Angiotensin I-converting enzyme (ACE) plays a major role in fibrous tissue formation and is highly expressed in lungs. The main aim of this research work was to study the role of ACE insertion/deletion (I/D) polymorphism, rs4646994, in asthma in Pakistani patients. A total of 854 subjects, including 333 asthma patients and 521 ethnically matched controls, were studied. The ACE (I/D) polymorphism was genotyped using polymerase chain reaction (PCR). Chi-square, Fisher's exact and Hardy–Weinberg equilibrium tests were used to compare groups. Homozygous insertion genotype II ($p<0.0001$, OR=3.38) and insertion allele (I) was significantly more frequent in Pakistani asthmatics than in healthy controls ($p=0.0007$, OR=1.40). The ID genotype ($p<0.0001$, OR=0.43) and the deletion allele (D) were associated with protection of disease in Pakistani patients ($p=0.0007$, OR=0.71). These data suggest the involvement of ACE I/D polymorphism in asthma risk in the Pakistani population. This marker may be an important indication in the molecular mechanism of asthma and can become a useful tool in risk assessment and help in designing strategy to combat disease.

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1. Introduction

Asthma is a chronic inflammatory disorder of the airways. It is recognized as one of the most chronic diseases which affects more than 300 million people all around the world, causing approximately 255,000 premature deaths annually. This accounts for about one out of 250 deaths and it is estimated that the death rate may increase by 20% in the coming 10 years if no necessary action is taken to control asthma. Asthma is common worldwide but more than 80% deaths due to asthma occur in low- and middle-income countries (Masoli *et al.* 2004). Asthma prevalence is dramatically increasing in Pakistan, with an estimated 5% increase annually. Approximately 5–8% of the adult Pakistani population is already suffering from this disease. Recent data revealed 19% prevalence in children and 5% prevalence in adults (Pak PRwire 2009).

Both environmental and genetic factors are known to contribute towards the development of asthma. It is a multigenic disorder in which its development is due to different genetic factors, most of them influence immune responses (Martinez 2007). Immune system linked genes, e.g. human leukocyte antigen (HLA) class II genes, cytokines and the genes indirectly implicating inflammatory reactions in lungs like β-adrenergic receptor gene (angiotensin-converting enzyme, *ACE*) are reported in asthma pathogenesis (Malerba and Pignatti 2005). Polymorphisms in the *ADRB2, NOS3, ADCY9, ARG1, SLC24A4* (Ortega *et al.* 2015) and *ACE* gene may also predict the response to asthma therapy and a variant of *ACE* insertion/deletion (I/D) polymorphism is found to be associated with asthma in different populations (Eryüksel *et al.* 2009).

The *ACE* gene encodes two isozymes: expression of the somatic isozyme is high in different tissues, primarily

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in the lung, including vascular endothelial cells, testicular Leydig cells and epithelial kidney cells, whereas expression of the germinal is mainly in sperm. The *ACE* gene has functional insertion/deletion (ID) polymorphism of a 287 bp Alu sequence within intron 16 (Rigat *et al.* 1990). *ACE* DD carriers have higher levels of ACE than those with the ID and II genotypes (Rigat *et al.* 1990; Tiret *et al.* 1992). The angiotensin I-converting enzyme (ACE) inactivates bradykinins and tachykinins, such as neurokinin A and substance P, which play important roles in asthma. Patients with asthma reportedly have bronchial hyper-reactivity to bradykinin compared to control groups (Fuller *et al.* 1987). The *ACE* polymorphism rs4646994 at chromosome 17 (position 61565900) is actually not a single nucleotide polymorphism but an insertion/deletion of an Alu repetitive element in an intron of the *ACE* gene. The allele containing the insertion is called 'I' allele, and the other without insertion is called 'D' allele. It has therefore been suggested that the *ACE* genes insertion/deletion (I/D) polymorphism in intron 16 is associated with response to asthma therapy (Wang *et al.* 2008; Eryüksel *et al.* 2009). Because no studies of this polymorphism have been conducted in Pakistani asthmatic patients, the present study of *ACE* (ID) polymorphism in asthma development was carried out in a local Pakistani population. *ACE* converts angiotensin (Ang) I into the potent vasoconstrictor Ang II, which has been proven to play a pro-inflammatory role (Ehlers and Riordan 1989). It also inactivates bradykinin (Yang *et al.* 1970), which has been suggested to play a role in the pathogenesis of inflammatory disorders (Schremmer *et al.* 1999).

2. Methods

2.1 *Patient population and study design*

This study was approved by the ethical review committees of KRL Hospital (ERC-08-01) and Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi. Written informed consent was obtained from all participants or from their guardians. 854 blood samples, including 333 asthma cases and 521 healthy controls, were collected for the present study. Cases of asthma were recruited from the clinics in Rawalpindi, Islamabad and Lahore. The inclusion criteria for asthma cases was asthmatics of both genders and not less than 10 years of age. Asthma was confirmed by chest specialists based on clinical examination. Each participant was asked about the family history of asthma but the severity of disease was not recorded. Control subjects were healthy individuals void of asthma, selected from general population of the same area, same age and gender as cases. Characteristics of cases and controls are given in table 1.

Table 1. Characteristics of asthma patients and controls

	Asthma Patients	Controls
Sample size	333	521
Mean age (years±SE)	40±0.93	37±0.77
Males	148 (44.5%)	316 (60.6%)
Females	185 (55.5%)	205 (39.3%)

2.2 *Blood sample collection, DNA extraction and genotyping*

Blood sample was taken from each individual, and genomic DNA was extracted by using standard phenol chloroform extraction protocol (Sambrook and Russel 2001). The *ACE* (I/D) polymorphism (rs4646994) was studied using primers sequences: forward primer 5′-CTGGAGACCACTCCCATCCTTTCT-3′ and reverse primer 5′-GATGTGGCCATCACATTCGTCAGAT-3′. A 15 µL volume was used for each PCR reaction, each reaction contained 1 µM of both primer, 1x of 10xPCR buffer, 0.45 µM of $MgCl_2$, 200 µM of dNTPS and 1 U of Taq polymerase (Fermentas EU) and 30 ng of DNA as final concentration per reaction. PCR cycling parameters were, 1 cycle 94°C for 3 min, following 35 cycles of 94°C for 45 s, 58°C for 1 min, and 72°C for 45 s and of 1 cycle of 72°C for 10 min (Batzer *et al.* 1996).

Amplimers were separated on 2% agarose gels. The 190 bp fragment represent D allele (for deletion) while the I (for insertion) represented by a band of 490 bp. ID heterozygotes are represented by both bands.

2.3 *Statistical analysis*

Phenotype characteristics are described as the means ± standard error (SE). The results are presented in the form of odds ratio (OR) with 95% confidence intervals (CI), as calculated by

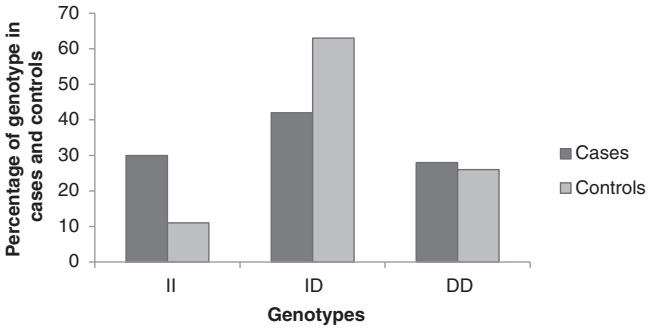

Figure 1. Percentage of genotypes of ACE I/D polymorphism in asthma cases and controls in Pakistani population.

Table 2. Genotype and allele counts (frequencies), chi (χ^2) and odds ratio (OR) of ACE I/D polymorphism in asthma cases and controls

Genotypes	Asthma cases n=333	Controls n=521	χ^2	OR (95%CI)	p-Value
II	99 (30%)	58 (11%)	46.83	3.38 (2.35–4.84)	<0.0001
ID	140 (42%)	326 (63%)	34.54	0.43 (0.33–0.57)	<0.0001
DD	94 (28%)	137 (26%)	0.38	1.11 (0.81–1.5)	0.54
Alleles	Asthma cases n=333	Controls n=521	χ2	OR (95%CI)	p-Value
I	338 (53%)	442 (42%)	11.37	1.40 (1.15–1.70)	0.0007
D	328 (47%)	600 (58%)	11.37	0.71 (1.59–0.87)	0.0007

Odds Ratios (*http://www.hutchon.net/ConfidOR.htm*) (Bland and Altman 2000). Significance was assessed by using chi-square contingency-test with Yates' correction for continuity (*http://faculty.vassar.edu/lowry/VassarStats.html*).

Genotypes in the case and control groups were compared by Fisher's exact test.

3. Results

The percentage frequency of genotypes and alleles of *ACE* I/D in the asthma cases and controls are presented in figure 1. Our results indicate that homozygosity for the insertion (II) is associated with a significantly increased risk of asthma (OR=3.38, 95% CI=2.35–4.84) compared with homozygosity for the deletion allele. Heterozygotes (ID) have reduced risk compared with homozygotes for the deletion allele (OR=0.43, 95%CI=0.33–0.57 and p≤0.0001).

ACE I/D alleles were also analysed separately in cases and controls and checked for their association with asthma in the studied Pakistani asthmatic cases. As presented in table 2, these results have shown that I allele is strongly associated with asthma risk in the studied population (OR=1.4, 95% CI=1.15–1.7, p=0.0007), whereas deletion allele D showed a positive trend to asthma protection (OR=0.71, 95% CI=0.59–0.87, p=0.0007).

In this study there were 185 asthmatic and 205 control female patients, and 148 asthmatic and 316 control male patients. These were compared on the basis of gender and there was no difference in the results on the basis of gender. The genotypic frequency of II genotype was found significantly (p<0.0001) high (tables 3 and 4). There was no difference with respect to association of polymorphism between males and females (figures 2 and 3).

4. Discussion

ACE polymorphism (I/D) has been implicated in susceptibility to asthma as it affects serum ACE levels. Variable results have been reported in various studies and most of them are inconclusive. Our results report association of II genotype of *ACE* with asthma which is in contrast to previous results reported in France where the DD genotype of *ACE* was higher (Benessiano *et al.* 1997). ACE DD genotype has also been reported with severe disease presentation in some populations (Gao *et al.* 2000; Ortega *et al.* 2015). However, there are some studies on Japanese and Americans

Table 3. Genotypic and allelic frequencies, chi (χ^2) and odds ratio (OR) of ACE I/D polymorphism in female asthma cases and female controls

Genotypes	Asthma cases n=185	Controls n=205	χ2	OR (95%CI)	p-Value
II	60 (32.4%)	27 (13.2%)	20.82	3.16 (1.90–5.26)	<0.0001
ID	76 (41.1%)	130(63.4%)	19.46	0.40 (0.27–0.61)	<0.0001
DD	49 (26.5%)	48 (23.4%)	0.49	1.78 (0.74–1.87)	0.48
Alleles	Asthma cases n=185	Controls n=205	χ2	OR(95%CI)	p-Value
I	196 (53%)	184 (45%)	5.1	1.38 (1.04–1.83)	0.02
D	174 (47%)	226 (55%)	5.1	0.72 (0.54–0.96)	0.02

Table 4. Genotypic and allelic frequencies, chi (χ^2) and odds ratio (OR) of ACE I/D polymorphism in male asthma cases and male controls

Genotypes	Asthma cases n=148	Controls n=316	χ2	OR (95%CI)	p-Value
II	39 (26.4%)	31 (9.8%)	21.53	3.29 (1.95–5.54)	<0.0001
ID	64 (43.3%)	196(62.1%)	14.43	0.47 (0.31–0.69)	0.0001
DD	45 (30.1%)	89 (28.1%)	0.25	1.11 (0.73–1.71)	0.62
Alleles	Asthma cases n=148	Controls n=316	χ2	OR (95%CI)	p-Value
I	142 (48%)	258 (41%)	4.2	1.34 (1.01–1.76)	0.04
D	154 (52%)	374 (59%)	4.2	0.75 (0.57–0.99)	0.04

populations which did not find association with any *ACE* genotype and asthma or asthma severity (Chagani *et al.* 1999; Nakahama *et al.* 1999; Lee *et al.* 2000). Our study is the first one from Pakistan exploring the relationship between *ACE* (I/D) genotypes and asthma risk.

Recent meta-analyses in Europeans and Asians have suggested that the *ACE* I/D polymorphism is a risk factor for asthma (Zhang *et al.* 2011; Ding *et al.* 2012). Results of both meta-analyses indicated that DD homozygote carriers had almost 59% more risk of asthma compared to individuals with the II genotype and that this risk is more evident in Asians especially in children compared to adults. In contrast, two studies conducted on adult Turkish population (Urhan *et al.* 2004; Yildiz *et al.* 2004), one on 49 cases and controls and other on 100 cases and 88 controls, reported that *ACE* gene I/D polymorphisms are not an important determinant of asthma susceptibility. In another study of Egyptian asthmatic adults (30 cases and 30 controls), it was found that the *ACE* gene polymorphism was not significantly associated with the disease (El-Shafei *et al.* 2012). Conversely, a significant association of the *ACE* I/D polymorphism with asthma was reported in asthma patients from the Czech Republic in 231 cases and 141 controls (Holla *et al.* 1999). *ACE* II polymorphism was also reported in Pakistani samples with rheumatic heart disease (Rehman *et al.* 2015) and in psoriasis (Munir *et al.* 2016).

Case-control studies may have some biases, which may result in false-positive or false-negative interpretations. We have matched our patient and control groups for the main parameters (age, ethnicity and gender) to avoid this incidence. *ACE* is a strong candidate gene that is probably involved in vascular complications of lungs. On the other hand, a major study on asthmatic families will be necessary to confirm the role of the *ACE* locus. The frequency of II genotype was not significantly different in asthmatic patients when males and females were separately analysed. This led to conclusion that the *ACE* insertion polymorphism is a risk factor for asthma patients due to its involvement in the growth of epithelial tissues of lung. *ACE* gene polymorphisms might be playing some important role in the pathological processes that result in heart diseases and some other related diseases (Panahloo *et al.* 1995; Mathew *et al.* 2001; Ismail *et al.* 2004). Data compiled from different geographical regions confirmed the importance of this factor (Mathew *et al.* 2001). Therefore, ethnic basis must be given priority in

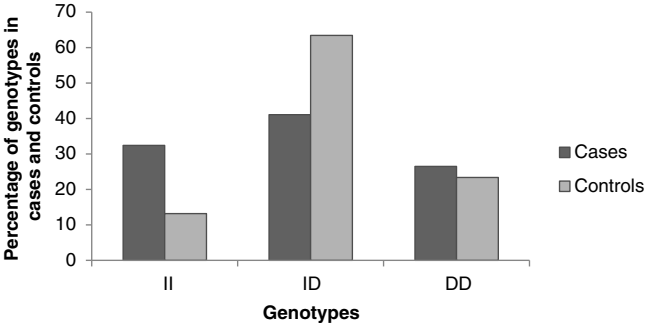

Figure 2. Percentage of genotypes of ACE I/D polymorphism in female asthma cases and controls in Pakistani population.

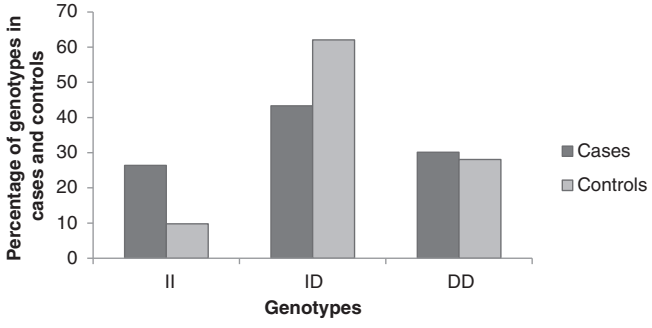

Figure 3. Percentage of genotypes of ACE I/D polymorphism in male asthma cases and controls in Pakistani population.

case of association studies like *ACE* I/D with etiology of disease and results of one ethnic group cannot be applied to the other group.

Deviation from Hardy–Weinberg equilibrium (HWE) due to presence of heterozygotes was observed for *ACE* in both patients and controls. Although the samples belonged to unrelated individuals, this deviation could be the result of inbreeding following many generations of consanguineous marriages in Pakistan. It is also possible that a large number of samples may resolve the observed shift from the HWE.

Different results from the literature about *ACE* (I/D) polymorphisms may be explained by the genetic differences and multifactorial reasons of asthma. The present study concludes that there is a significant difference in the frequencies of *ACE* alleles; genotype II and I allele are strongly associated with asthma among Pakistani patients and controls. Studies with a large number of cases are required to confirm these results in our population. Single polymorphism in the *ACE* gene did not represent comprehensive coverage of this gene; thus, it is possible to get more striking association with disease if more markers are added.

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