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# The effects of an insertion in the 5′UTR of the AMCase on gene expression and pulmonary functions

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**Summary**
*Background:* Studies regarding the physiological role of acidic mammalian chitinase (AMCase) and the effects of its genetic variants on asthma have produced conflicting results.
*Objectives:* We aimed to determine the genetic variants in the AMCase gene that could regulate the gene expression and thus influence disease severity.
*Methods:* Genetic variants of the AMCase gene were determined by sequencing of asthmatics and healthy controls in up to −1 kb in the promoter region and exon 1 and 2. In an association study, a population of asthmatic ($n = 504$) and healthy Turkish children ($n = 188$) were genotyped for the observed SNPs. A replication study was performed in a North American adult population of patients with mild ($n = 317$) and severe ($n = 145$) asthma. The functional properties of the insertion were determined by promoter reporter assay, electromobility shift assay and transcription factor ELISA experiments.
*Results:* Of the identified SNPs, only a ten base pair insertion (CAATCTAGGC) in the 5′UTR region of exon 2 was significantly associated with lower $FEV_1$ ($\beta = -14.63$ SE $= 6.241$, $P = 0.019$) in Turkish children with asthma. However, in the adult population, the same insertion showed a trend toward higher $FEV_1$. The insertion was shown to have enhancer activity and the mutant probe possessing the insertion had higher binding affinity for the nuclear extracts.

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*Conclusion*: Our study shows that a ten base pair insertion in the 5′UTR region of AMCase gene may modify gene expression and thus may affect the severity of asthma. However, its effects appear to be different in different populations.


## Introduction

Chitin is the second most abundant polysaccharide in nature after cellulose. It is found as a structural component of many species such as exoskeleton of crustaceans, parasitic nematodes and walls of fungi. It protects these organisms from harsh conditions.[1–4] Chitinases are enzymes that hydrolyze chitin and are part of the anti-parasitic response against chitin containing organisms in lower life forms. Although there is no chitin in humans, recent studies have identified functional mammalian chitinase genes such as chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase, CHIA) and several chitinase-like genes such as YKL-40 (CHI3L1).[5–9]

AMCase is one of the enzymes with true chitinase activity.[10] The gene is located on chromosome 1q13.1—21.3 and contains 12 exons which transcribes into a 50 kD protein. The gene also encodes various splice forms. The enzyme is acid stable and mostly expressed in the gastrointestinal tract and in the asthmatic lung.[10] In their pioneering study, Zhu et al. showed that AMCase is expressed in the epithelial cells and alveolar macrophages in patients with asthma.[11] In a murine model of asthma, they showed that AMCase acts downstream of IL-13 driven cytokine response. In support of this observation, Chupp et al. have shown that serum levels of a chitinase-like protein, YKL-40, were higher in asthmatics than control subjects and that they correlated positively with the severity of asthma and inversely with $FEV_1$%.[12] The same group also showed that a SNP in the promoter region of the gene encoding YKL-40 was associated with elevated serum YKL-40 levels, asthma, bronchial hyper-responsiveness, and measures of pulmonary functions.[13]

Even though many studies have suggested that AMCase is a positive mediator of Th2 response,[14] more recent studies have challenged these findings. For example, Reese et al. showed that in a mouse model, AMCase inhibits chitin induced eosinophil and basophil recruitment to the lungs suggesting that AMCase can prevent allergic response induced by chitin.[15] Similarly, in a human study, Seibold et al. measured chitinase activity in bronchoalveolar lavage (BAL) samples of asthmatics, habitual smokers and healthy controls and found that chitinase activity in human BAL samples was mostly related to chitotriosidase (CHIT1) but not to AMCase.[16] Furthermore, they showed that chitinase activity was not higher, but actually lower in BAL fluids of asthmatics than control subjects and was increased in habitual smokers.gr1

Similar to the conflicting findings regarding its physiological role in asthma, the studies on the effect of genetic variants on disease and disease phenotypes have produced some contradictory findings, as well. Bierbaum et al. identified six SNPs in the promoter and three SNPs in the exonic region of AMCase and reported that a non-synonymous SNP, Lys17Arg, and possibly a promoter SNP (rs12033184), are associated with pediatric asthma in a German population.[17] In contrast, Seibold et al. could not detect the Lys17Arg SNP in a population composed of adult African American, Puerto Rican, and Mexicans.[18] They genotyped this population for 8 SNPs in the coding region and reported that some haplotypes composed of these SNPs are protective against asthma and are in fact associated with increased levels of chitinase activity *in-vitro*. Chatterjee et al. repeated Bierbaum's study in an Indian population by including a wider region of promoter and 3′UTR region.[19] They reported quite a different set of SNPs with only four being common in both reports. In this report one promoter SNP, rs3806448, was associated with both asthma and IgE; and another one, rs10494132, was associated with IgE only. In a more recent study, however, neither of these SNPs nor any of the 24 SNPs analyzed was found to be associated with asthma or asthma related phenotypes.[20]

In summary, similar to studies assessing associations with physiological outcomes, the results of studies evaluating genotypic associations of AMCase gene with asthma have produced conflicting results. We aimed to search for genetic variants in the AMCase gene that could regulate the gene expression and thus may influence disease expression in a case-control study involving Turkish children. For this purpose, we sequenced the promoter region up to −1 kB and also exon 1 and exon 2 as several different transcripts of the gene have been shown to include different exons. Here, we report our findings on the association of several SNPs and a 10 base pair insertion in the 5′UTR region of exon 2. We also attempted to replicate our findings with the 10 base pair insertion in a population composed of adult asthmatics from the United States; and also determine the functional properties of this insertion.

## Materials and methods

### Study population

*Children with asthma*: The patients in the asthma group have been detailed previously.[21–23] Briefly, we enrolled children aged 6—18 years who were diagnosed with asthma at the Pediatric Allergy and Asthma Unit of Hacettepe University, School of Medicine, Ankara, Turkey between 2002 and 2009. Asthma diagnosis was confirmed by the examining pediatric allergist according to GINA guidelines.[24] All children had evidence of reversible airway obstruction as defined by at least a 12% improvement in $FEV_1$% following bronchodilator administration, therapeutic response to anti-asthma treatment, or an abnormal result in methacholine bronchoprovocation test ($PC20 < 8$ mg/dl). Spirometric measurements, total IgE, and eosinophil counts were obtained. Skin testing was performed as detailed

previously.[23] The study was approved by the Ethics committee of Hacettepe University and the parents have provided written informed consent for the study.

*Healthy Children*: The patients in the control group have been detailed previously.[23] Briefly, the control group was composed of Turkish school children who presented between 2005 and 2006 to the outpatient department of the same hospital. They presented for reasons such as minor trauma or for their regular follow-up. They responded negatively to an established and validated asthma questionnaire (ISAAC),[25] never had any diagnosis of asthma or allergic bronchitis by a physician, and never had any history of wheezing. They all had normal pulmonary function tests. All children underwent skin prick testing and had their total IgE measured in serum.

*Adults with asthma*: To test whether the findings observed in children with asthma could be replicated in an adult asthma population, we genotyped adult asthmatics from the USA composed of patients with mild and severe asthma. 462 subjects classified as either mild or severe asthmatics by the NAEPP guidelines were identified from the Brigham and Women's Hospital's (BWH) Asthma Research Center's database. These patients were recruited between 2001 and 2008, and each had signed a written informed consent agreeing to exploratory genetic analysis related to asthma, which was approved by the BHW institutional review board. Baseline demographic information was available for each subject and baseline spirometry was performed on the day of the blood draw. All the patients in the adult population were non-smokers, defined as less than or equal to 10 pack year smoking history and none in the last 12 months. DNA was stored at the Channing Laboratory where genotyping was performed for the 10 base pair insertion (CAATCTAGGC) identified in the 5′UTR region of exon 2, as described below.

**Identification of genetic variants**

DNA was extracted from whole blood by standard techniques. To determine the polymorphisms in the promoter region and 5′UTR region of the transcript including exon 1 and exon 2 of AMCase gene, DNA samples from 20 children with asthma and 20 healthy children were sequenced with Big Dye Terminator cycle sequencing kit (3.2 version) from ABI Prism (Foster City, CA, USA). Sequencing of the promoter and the first 2 exons revealed the presence of 10 SNPs, 8 in the promoter, 2 in exon 1 and a ten base pair insertion in the 5′UTR of Exon 2. According to the initial analysis, 8 of the 10 SNPs, six SNPs in the promoter region and two SNPs in Exon 1, seemed to be in linkage disequilibrium. To prove this, 100 individuals were further sequenced in the promoter region.

**Genotyping of the promoter region**

Sequencing of 100 samples showed that eight of the promoters' SNPs were in perfect linkage disequilibrium. Therefore, we chose one of them, rs4442363 SNP, to determine the genotype by restriction fragment length polymorphism (RFLP) analysis. A 613 base pair of the promoter region was amplified using the following primer pairs: forward 5′-CGGACACTGGACTTAAGTTGT-3′ and reverse 5′-GAAGCTTTGGCACCGTCT-3′. The amplicon was digested with *Hinf I* (New England BioLabs, MA, USA) and the products were fractionated on a 2% agarose gel. Digestion occurs only in the presence of the T allele and produces a 214 and 399 base pair products; whereas the undigested C allele is visualized as a single 613 base pair band. 10% of samples randomly selected but including from all genotypes were re-genotyped for quality control.

**Genotyping of the insertion at 5′UTR region of exon 2**

The genotyping for the 10 base pair insertion (CAATCTAGGC) was again done by PCR-RFLP analysis. A 451 base pair segment in the 5′UTR region of exon 2 was amplified using the following primers: Forward 5′-CTGACCACAGTATCTAAACAG-3′, reverse 5′-GGGATGTAGTATGAAGACCACT-3′. The amplicon was digested with *BfaI* (New England Biolabs) and the product was fractionated on an agarose gel which resulted in three fragments 308, 94 and 59 base pairs in the presence of insertion; while two fragments of 392 and 59 base pairs were obtained in the absence of the insertion. 10% of samples randomly selected but including from all genotypes were re-genotyped for quality control.

**Transient transfection analysis with reporter constructs**

Genomic DNA from asthmatic individuals with and without the 10 base pair insertion was amplified to include 200 base pairs upstream from the start codon of exon 2. Purified PCR products were cloned into two different types of vectors: *i*. a pGL3 basic vector without a promoter to check for the promoter activity of the inserted sequence *ii*. a pGL3 control vector possessing a SV40 promoter region (Promega, Madison, WI, USA) at *Kpn I* and *Xho I* to check for the enhancer activity of inserted sequence.

A549 bronchial type II alveolar cells (DSMZ, Braunschweig, Germany) were used for the transfection assays. A549 cells were grown to 90% confluence in 24-well plates, transiently transfected with either promoter reporter constructs or empty vectors and co-transfected with a pRL-TK vector ($n = 4$) (Promega, Madison, WI, USA) containing Renilla Luciferase gene using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. 48 h after the transfection, cells were lysed with reporter lysis buffer and luciferase activity of each lysate was measured using Dual Luciferase Assay kit (Promega). Firefly luciferase activity was normalized to renilla luciferase activity to control for differences in transfection efficiency.

**Electrophoretic mobility shift assay (EMSA)**

Nuclear extracts were prepared from A549 cells either without any stimulation or after stimulation with IL-4 (25 ng/ml for 24 h) (Biosource, Camarillo, CA, USA) and/or IL-13 (25 ng/ml for 24 h) (PeproTech Inc., Rocky Hill, NJ, USA) using NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL, USA). Protein concentrations of nuclear extracts were measured using Bradford Assay (Bio-Rad, Munich, Germany). Double-stranded, biotin labeled oligonucleotides (IDT, Coralville, IA, USA) with and without the insertion were synthesized: without the insertion 5′-AGAACATATAAAAAGCTCTGCGGGACTGGT-3′; with the insertion 5′-TATAAAAAGCCAATCTAGGCTCTGCGGG- AC-3′. Protein—DNA binding reactions were performed with chemiluminescent LightShift EMSA kit (Pierce Biotechnology). Briefly, 2 μg of nuclear protein and 2 pmol of labeled

probes were incubated in binding mix containing 10 mM Tris, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/$\mu$l Poly(dI.dC), 0.05% NP-40 at room temperature for 20 min. Protein–DNA complexes were resolved on a 6% non-denaturing polyacrylamide gel in a Tris–borate–EDTA buffer in a cold room. Complexes were transferred onto a Biodyne Nylon membrane and cross-linked with UV. Biotin labeled free oligos and oligo-protein complexes were detected using streptavidin–horseradish peroxidase system. Gel bands were visualized using the CDD camera system (Vilber Lourmat, Marne-la-Vallée, France).

Intensity of binding bands was measured by image analyzer (Syngene Gene Genius, Cambridge, UK) by using Gene Tools Analaysis Software 3.02.00 (Synoptecs software). Intensity of band which represents the binding of wild type oligos to unstimulated extract was defined as 1 and intensity of other conditions calculated relatively according to this one's.

### Transcription factor ELISA

Since the insertion sequence contained a CAAT motif for C/EBP, a custom designed transcription factor ELISA (Active Motif, Carlsbad, CA, USA) was performed to show the binding of insertion to C/EBP protein. In this method, the plates were initially coated with biotin labeled double stranded oligonucleotides with and without the insertion; followed by incubation with 5 $\mu$g of nuclear extract for 1 h. The wells were then washed and incubated with a C/EBP alpha or beta antibody. The reaction was developed with an anti-IgG HRP conjugate and the colorimetric reaction was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm. The experiment was performed in quadruplicate for each condition.

## Statistical analyses

Statistical analyses were done with SPSS 15 for Windows (Chicago, IL, USA), Prism 5 for Windows (GraphPad Software, Inc. CA, USA) and SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Comparison of quantitative variables such as age, eosinophil counts, IgE levels and $FEV_1$% were done with parametric or non-parametric tests depending on the normalcy of distribution. In case of non-normal distribution, non-parametric tests were used only if various transformation methods failed to normalize the data. Chi square or Fisher's exact test was used for comparison of categorical variables as appropriate. As expected, there was a significant difference in the gender distribution between asthmatic children and healthy controls.[26] Therefore, all data are stratified to account for this gender difference. For all analyses, a p value $<0.05$ was considered significant.

We performed linear regression analysis to establish the factors that were associated with $FEV_1$% and eosinophil counts. With this regression model, we calculated the residuals which are the difference between observed values minus values predicted in the regression. Then, we constructed a box plot as well as a histogram to see whether residual distributions are skewed. Both graphs showed that the distribution of the residuals was not skewed. In this regression model, our primary endpoint was the effect of genotype on $FEV_1$% levels or eosinophil counts. We considered the following as covariates in the regression model: age, gender, age of onset, skin test positivity and plasma total IgE levels. The model was constructed using backward elimination. In order to ensure that the assumptions for linear regression analysis are met, we checked for the normality of residuals by Q–Q plots and Shapiro–Wilk test and in addition we constructed a scatter plot of residuals versus predicted values.

## Results

Five hundred and four children with asthma and 188 healthy controls were included in the study. Characteristics of study population are summarized in Table 1. Asthma and atopy–related findings including IgE, skin prick tests, $FEV_1$%, eosinophil counts and family history of allergic diseases were significantly different between the 2 groups.

### Sequencing results

We sequenced 20 asthmatic and 20 healthy controls up to −1 kB in the promoter region and Exon 1 and 2. We identified 10 SNPs in the promoter and 5′UTR region and a 10 base pair insertion (CAATCTAGGC) in the 5′UTR region of exon 2.

Of the 10 SNPs, eight (rs12033184, rs35042265, rs4546919, rs4554721, rs4442363, rs11102235, rs34698010, rs12026825) were in complete linkage disequilibrium as shown by sequencing of 100 DNA samples from each of asthmatic children and healthy controls. The study population was genotyped for the presence of only one of these SNPs, rs4442363 polymorphism, in order to investigate the association between this block of SNPs and asthma and asthma phenotypes.

Of the remaining two SNPs in the promoter region, sequencing studies of 63 healthy and 80 asthmatic children showed that there was no genotypic variance in rs11102234; and that the genotypic distribution of the rs12023459 was very similar between children with asthma and healthy controls and failed to show any association with asthma and atopic phenotypes. Therefore, these two SNPs were not pursued any further.

### Association studies between the promoter SNPs, asthma diagnosis and asthma phenotypes

Promoter SNPs were analyzed by PCR-RFLP in a subpopulation of the whole cohort involving 352 children with asthma and 172 healthy controls. There was no difference in the genotype distribution of the promoter SNPs between children with asthma and healthy controls (Table 2). There was no association between the reported SNPs and asthma phenotypes such as atopy, $FEV_1$%, eosinophil numbers, either.

### Association studies between the 5′UTR insertion (CAATCTAGGC), asthma diagnosis and asthma phenotypes

504 children with asthma and 188 healthy controls were genotyped for the insertion at 5′UTR of exon 2 by PCR-RFLP

**Table 1** Characteristic of study population.

| | Healthy controls $n = 188$ | Children with asthma $n = 504$ | $p$ |
|---|---|---|---|
| Age (years)[a] | 10.8 (8.0–13.0) | 10.0 (7.8–12.7) | >0.05 |
| Gender | | | |
| Male (%) | 90 (47.6) | 324 (64.5) | <0.001 |
| Female (%) | 99 (52.4) | 179 (35.5) | |
| $FEV_1$ (% predicted)[a] | 101 (91–108) | 92 (79–102) | <0.001 |
| Eosinophil count/$mm^3$[a] | 150 (98.9–280.8) | 300 (170–500) | <0.001 |
| Skin test positivity (%) | 29 (15.4) | 295 (59.4) | <0.001 |
| IgE (kU/L)[a] | 35 (17–72) | 178 (55–454) | <0.001 |
| Family history of atopic diseases (%) | 22 (11.8) | 162 (32.3) | <0.001 |
| Pet ownership (%) | 25 (13.4) | 34 (6.8) | >0.05 |
| Smoke exposure | 106 (57.0) | 202 (40.2) | >0.05 |

[a] Median (Interquartile range).

analysis. There was no difference in the genotype frequencies between children with asthma and healthy controls (Table 3) showing that this insertion is not associated with asthma.

Within the asthmatic population, in a model where the presence of the insertion behaves as the recessive allele [i.e., wild type + heterozygotes vs. mutant (insertion present in both alleles] the insertion was significantly associated with lower $FEV_1$% and lower eosinophil counts. Multiple linear regression analysis showed that the homozygous presence of the insertion was significantly and independently associated with lower $FEV_1$% values ($\beta = -14.63$ SE = 6.241, $P = 0.019$). In this model, eosinophil counts had a small but significant effect, as well ($\beta = -0.008$ SE = 0.003, $P = 0.003$).

## The effect of the 10 base pair insertion in the 5′UTR of exon 2 on the expression of AMCase gene

Due to the observed association between insertion and $FEV_1$%, we attempted to determine the function of the mutation and tried to establish whether it affects the expression of AMCase gene using A549 alveolar cell line. In order to see whether this insertion independently increases the promoter activity or whether it behaves as an enhancer, we designed two types of reporter constructs: promoter type and enhancer type. Cloning of the 200 bp region of 5′UTR into a basic vector from both the wild and mutant genotypes failed to show any difference between the genotypes ($p > 0.05$; data not shown). However, cloning of the same region into a control vector just in front of the SV40 promoter as an enhancer element showed that the sequence with the insertion displayed significantly higher luciferase activity compared to the wild type ($p < 0.05$) (Fig. 1). Stimulation of A549 cells with IL-4, IL-13 or both further increased the enhancer activity conferred by the presence of the insertion (Fig. 1). The results of this experiment suggested that the studied insertion behaves as an enhancer element for the AMCase gene and that this enhancer activity increases in the presence of IL-4.

## The effect of the 10 base pair insertion in the 5′UTR on nuclear protein avidity and specific binding of C/EBP

Electrophoretic mobility shift assay (EMSA) was performed with nuclear extracts obtained from A549 cells with and without stimulation with IL-4 and IL-13. The EMSA assay showed more intensive bands with the mutant probe possessing the insertion suggesting stronger binding affinity of the mutant sequence with the insertion to the nuclear extracts (Fig. 2).

In order to identify the transcription factor that is responsible for higher binding avidity of the mutant probe, we performed supershift experiments with antibodies to various transcription factors. Our main candidate was the transcription factor C/EBP (CAAT enhancer binding protein) as the inserted sequence contains a CAAT motif for C/EBP binding. However, antibodies to C/EBP failed to induce any supershift (Fig. 2). We were unable to induce any supershift with antibodies to other transcription factors such as Oct-1 and GATA-3 (data not shown).

Since EMSA assay failed to show whether C/EBP is the transcription factor responsible for the specific binding, a more sensitive TransAM transcription factor ELISA was performed. Plates were covered with biotin labeled double stranded wild type and mutant type oligonucleotides; incubated with nuclear extracts and C/EBP $\alpha$ or $\beta$ antibodies were

**Table 2** Genotype frequencies of the promoter polymorphism rs4442363 in the AMCase gene.

| Genotype | Healthy Controls $n = 172$ $n$ (%) | Children with asthma $n = 352$ $n$ (%) | $P$ |
|---|---|---|---|
| CC | 59 (34.3) | 97 (27.6) | |
| CT | 78 (45.3) | 190 (54.0) | |
| TT | 35 (20.3) | 65 (18.5) | >0.05 |

**Table 3** Genotype frequencies of 10 the base pair insertion in the 5′UTR region of exon 2

| Genotype | Healthy controls $n = 188$ $n$ (%) | Children with asthma $n = 504$ $n$ (%) | $p$ |
|---|---|---|---|
| Wild type (no insertion) | 153 (84.1) | 409 (81.2) | |
| Heterozygote | 33 (17.6) | 87 (17.3) | |
| Mutant (insertion present in both alleles) | 2 (1.1) | 8 (1.6) | >0.05 |

used as a primary antibody. Data revealed that antibodies to C/EBP β but not to α showed specific binding. Fig. 3 shows that stimulation with IL-4 significantly reduced binding of C/EBP β to nuclear extracts ($p = 0.002$) but there was no difference in the binding avidity between the oligonucleotides with or without the insertion. Further experiments with GATA family transcription factors including GATA-1, 2 and 3 failed to show any difference between the two genotypes.

## Genotyping of the adult population

In order to confirm our findings in another population, we genotyped 317 patients with mild and 145 patients with severe asthma for the presence of the insertion (Table 4). The severe asthmatics were older and had lower lung function than the mild asthmatics. There was no difference in the frequency of the insertion between the two groups. In contrast to our observation in the pediatric population, adults with the homozygous form of insertion had showed a trend toward higher $FEV_1$% values that approached significance [90.4 ± 4.6% in mutant (insertion present in both alleles) vs 81.2 ± 0.9 in wild type + heterozygotes, $p = 0.06$]. Because of our adult population composed of different ethnic backgrounds population stratification was assessed, but not significant differences was found between groups.

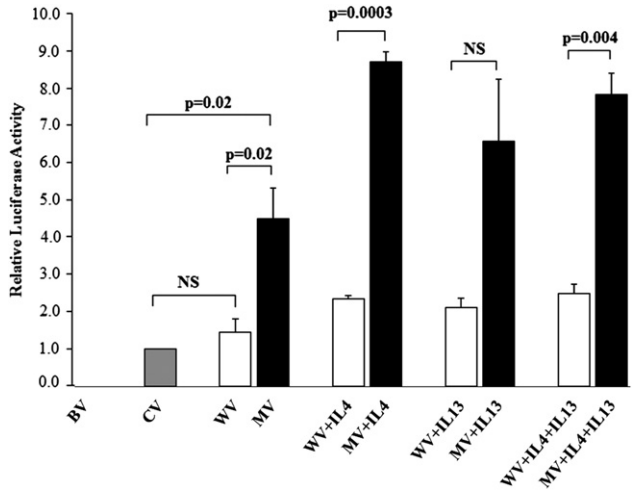


**Figure 1** Promoter reporter assay. A549 cells were transiently transfected with wild type reporter vector (WV) or mutant type reporter vector (MV). Reporter activity was normalized relative to the activity of pGL3 control vector. No promoter activity was observed after transfection with the basic vector, therefore only the results of transfection with the control vector are shown. Reporter activity was significantly higher in A549 cells transiently transfected with control reporter vector containing the ten base pair insertion than the constructs bearing the wild type allele. (BV = pGL3 Basic vector without a promoter, CV = pGL3 control vector with a SV40 promoter).

## Discussion

There is a great deal of controversy surrounding the physiological effects of chitinase proteins as well as the effects of the variants of the encoding genes in asthma. In this report, we show that a genetic variant in the second exon of the AMCase gene may modify the function of the gene and the same variant may have different and even opposite effects in two different populations.

In their initial report, Zhu et al. have shown that AMCase is expressed in the asthmatic lung and is found in the BAL fluid of asthmatics.[11] They have also shown that it acts downstream of IL-13 in the inflammatory pathway. These results were challenged in a mouse model where AMCase was shown to inhibit chitin induced eosinophil and basophil recruitment to the lungs suggesting that AMCase can prevent allergic response induced by chitin. In a human study Seibold et al.[16] showed that chitinase activity in BAL samples was mostly related to chitotriosidase (CHIT1) but not to AMCase. More importantly, they showed that chitinase activity was not higher, but actually lower in BAL fluids of asthmatics than control subjects and was increased in habitual smokers. Similar to the controversies regarding the function of chitinase, there have been conflicting reports on the effect of AMCase genotypes on asthma and asthma related phenotypes, as well.

We attempted to search for the genetic variants of the AMCase gene that may modify the disease expression and thus may affect the severity of asthma. We found that an insertion containing a CAAT motif (CAATCTAGGC) in the 5′UTR region of exon 2 was associated with lower $FEV_1$% in children with asthma. In a reporter gene system, we also showed that this variant enhances the promoter activity of the gene especially in the presence of IL-4. Further to this observation, EMSA studies revealed that the sequence with the insertion binds the nuclear extracts containing the regulatory proteins with higher avidity compared to the wild type oligonucleotides that does not carry the insertion. Although C/EBP beta binds to the region containing the inserted sequence, the nuclear factor ELISA study showed that the presence or absence of the insertion does not change the binding, suggesting that C/EBP is not the transcription factor responsible for the enhancer activity of the insertion sequence. Interestingly, in contrast to our observation in the reporter system, IL-4 decreased the binding of C/EBP beta in the ELISA experiment. Collectively, these data implicate that the differential binding of other transcription factor (or factors) may be responsible for the action of this inserted 10 base pair sequence. These factors


| Oligonucleotide | W* | M† | W | M | W | M | W | M | W | M |
|---|---|---|---|---|---|---|---|---|---|---|
| Nuclear extracts | | | US‡ | US | US | US | 4/13§ | 4/13 | 4/13 | 4/13 |
| Antibody to C/EBP-β | | | | | + | + | | | + | + |

*: Wild type oligonucleotide that does not include the ten base pair insertion sequence
†: Mutant type oligonuclotide carrying the ten base pair insertion sequence
‡: Nuclear extracts obtained from unstimulated (US) A549 cells
§: Nuclear extracts obtained from A549 cells stimulated with IL-4 and IL-13

**Figure 2** EMSA experiment. Nuclear proteins from A549 cells, unstimulated or stimulated with IL-4 and IL-13, bind to the probes carrying the insertion sequence (M) with more avidity than those probes without the insertion (W). Antibody to C/EBP-β failed to induce a supershift.

remain to be determined. It should also be noted that the functional analyses are carried out in A549 cells which belong to an alveolar cell line. Even though A549 cells have been used in numerous studies to investigate various pathological and physiological aspects of lung diseases,[27] they do not truly represent the cells of the airways which are the major players in lung function. Therefore, even though we describe functions related to this insertion, we do not know whether expression of this gene is actually related to the phenotype of lung function.

In addition to our study, four studies have previously investigated the association between the genotypic variant of the AMCase gene and asthma. The promoter SNPs that we report here have previously been reported by Bierbaum et al.[17] They also failed to show any association between these SNPs and asthma phenotypes in German children. Chatterjee et al.[19] on the other hand, have reported significant associations with 2 SNPs in the part of the promoter that goes 1000 bp further up to the 5′ region that was selected in our study. One of these SNPs rs3806448 is associated both with asthma and IgE in the Indo-Aryan population. This particular SNP was in linkage disequilibrium with the SNPs used in our study. Therefore, one can assume that we would have failed to find an association with this SNP even if we had done our analysis to include that far in the 5′ of the promoter. To further support the discrepancy, Wu et al.[20] also failed to find any association with rs3806448 in a North American population of asthmatic children. Similarly, rs10494132 was found to be associated with IgE by Chatterjee et al. but not by Wu et al. In fact, Wu et al., in a comprehensive analysis, failed to show any association between asthma and any of the 24 SNPs in the AMCase gene that they studied.

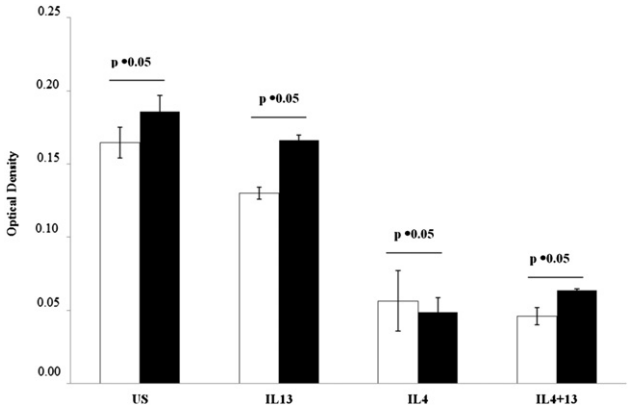


**Figure 3** Custom designed C/EBP transcription factor ELISA. Plates are initially coated with biotin labeled double stranded oligos with and without the insertion followed by incubation nuclear extracts obtained from A549 cells. Optical density is measured after incubation with C/EBP beta antibody. Empty bars represent wild type probes without the insertion and black bars represent mutant probes with the insertion.

The insertion that we report in our study was only detected in a recent study by Seibold et al.[18] in a population of African American, Puerto Rican, and Mexicans. However, they did not report any association with this particular SNP. In our study, we report two discrepant findings in two different populations. In a population of Turkish asthmatic children we found that the presence of insertion was associated with lower $FEV_1$%, a finding which was supported by the results of the logistic regression analysis that showed that the insertion was in fact significantly and independently associated with lower $FEV_1$%. In a North American population composed of mild and severe asthmatics, the same analysis has showed that the same insertion was associated with higher $FEV_1$%.

Our study may be criticized for attempting to replicate the findings from a pediatric population in a population composed of adults. Since the presence of insertion was associated with lower $FEV_1$ in the pediatric group, we

**Table 4** Characteristics of the adult population with asthma.

| | Mild asthma $n = 317$ $n$ (%) | Severe asthma $n = 145$ $n$ (%) | $P$ |
|---|---|---|---|
| Age (years)[a] | 30 (±0.57) | 44 (±1.12) | $< 0.0001$ |
| Gender | | | |
| Male | 114 (36) | 56 (39) | |
| Female | 203 (64) | 89 (61) | $>0.05$ |
| Race | | | |
| Caucasian | 215 (68) | 86 (59) | |
| African American | 65 (20) | 43 (30) | |
| Asian | 10 (3) | 7 (5) | |
| Other/Mixed | 27 (9) | 9 (6) | $>0.05$ |
| Ethnicity | | | |
| Hispanic | 25 (8) | 10 (7) | |
| Non-Hispanic | 292 (92) | 135 (93) | $>0.05$ |
| $FEV_1$ (% predicted)[a] | 90 (±0.80) | 64 (±1.33) | $< 0.0001$ |
| ICS use | 28 (9) | 145 (100) | $< 0.0001$ |
| Genotype[b] | | | |
| Wild | 237 (75) | 108 (75) | |
| Heterozygote | 68 (21) | 33 (23) | |
| Mutant | 12 (4) | 3 (2) | $>0.05$ |

[a] Mean (Standard error of the mean).
[b] Genotype frequencies of 10 the base pair insertion in the 5′UTR region of exon 2.

hypothesized that it may be associated with the severity of the disease as lower $FEV_1$ is a major determinant of asthma severity. However, severe asthma is quite rare in children. Therefore, in order to obtain a robust finding regarding the association of the SNP with asthma severity we chose an adult population containing a significant number of patients with severe asthma.

There are two major differences between our initial and replication cohorts: they are of different genetic backgrounds and they are of different age groups. There may be several reasons to account for the discrepant results reported in the literature and here. One explanation would be differences in the genetic backgrounds of the populations studied. The presence of polymorphisms in the other regions of the AMCase gene or variants in other genes causing epistatic effects may also be operative in underlying the differences.

In addition to age and ethnic differences, another factor could be the differences in the environmental exposures as several studies have shown that the response of the genes can vary significantly depending on the environmental exposures. In one of the best examples of the gene environment interaction, when the results of the asthma and endotoxin study were stratified according to the endotoxin exposure it was shown that the C allele of the CD14-159 C/T SNP was associated with a higher risk of allergic sensitization at low levels of endotoxin exposure and lower risk at high levels of exposure.[28,29] This observation was later substantiated by two independent studies.[30,31] In the *in-vitro* environment, we have shown that the two genotypes respond differently to increasing concentrations of endotoxin.[32] Therefore, there is reason to believe that the effects of the genetic variants may vary significantly depending on the environmental chitin exposure, as well. To make the issue even more complicated, a recent study by Wu et al. have shown that high mold exposure significantly modified the relation between 3 SNPs in *CHIT1* (rs2486953, rs4950936, rs1417149) and severe exacerbations, even though the same SNPs showed no association in their original study where the environmental exposures have not been taken into account.[33] This observation suggests that the association between the chitinase genes and environmental exposure can go even beyond environmental chitin exposure and may be influenced by mold exposure, as well.

A third factor to account for the discrepant results between the two populations of this study can be the age of the studied populations. Expression patterns of genes may be under epigenetic influences and thus may change with age. Recently, Munthe-Kaas et al. have shown that CD14 methylation increased significantly from age 2–10 years, and that the level of methylation was inversely correlated with sCD14 levels at 10 years.[34] They have also shown that even though 3 SNPs were associated with sCD14 levels within the first two years of life, only one was associated at 10 years. Therefore, the differences that we observed between the two populations, one pediatric and the other adult, can be partially due to the differences in the age of the study cohorts and be modified by epigenetic factors. Finally, as we have extensively discussed, the two populations are different with respect to many variables including their ethnic backgrounds and environmental exposures. Even though we have chosen an adult population of patients with severe asthma in order to obtain a robust finding regarding the association of the SNP with asthma severity, it would definitely be desirable and increase the strength of our findings if we could do the same comparison in a well matched pediatric population with severe asthma.

Our study has weaknesses. Firstly, it would be extremely helpful to have the measures of the environmental chitin exposure or, in light of recent studies, mold exposure levels of our population. Secondly, even though we have shown that the insertion increases the expression of the reporter gene, we do not have any evidence that it changes the

protein levels in the lungs of the asthmatics and that it is directly associated with measures of lung function. Similarly, we do not have evidence that it affects the tissue levels of infiltrating eosinophils.

To conclude, our study shows that a ten base pair insertion in the second exon in the 5′UTR region of the AMCase gene may modify the gene expression and thus may affect the severity of asthma. However, its effects may be different in different populations and may be modified by various environmental exposures and demographics of the population under study.

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## Conflict of interest

All the authors declare that there is no conflict of interest.

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