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ORIGINAL INVESTIGATION

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# Association of the *TNF*-α-308 (G→A) polymorphism with self-reported history of childhood asthma

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**Abstract** Asthma is a complex disease involving genetic and environmental aetiology. The tumour necrosis factor-alpha (*TNF*-α) and angiotensin-converting enzyme (*ACE*) genes have been implicated in asthma pathogenesis. This study investigated the association of a G-308A variant of *TNF*-α and an insertion/deletion (I/D) variant of *ACE* with a self-reported history of childhood asthma, in two population groups. At Northwick Park Hospital, London, 1,811 pregnant women attending for antenatal care were recruited. Participants with a self-reported history of childhood asthma, determined by a researcher-administered questionnaire, and controls with no personal or family history of asthma, of UK/Irish (cases $n$=20; controls $n$=416) and South Asian (cases $n$=6; controls $n$=275) origin were used in this study. Participants were genotyped for the *TNF*-α-308 and *ACE* I/D variants by a PCR-RFLP and PCR approach. The *TNF*-α-308 allele 2 (-308A) was significantly associated with self-reported childhood asthma in the UK/Irish (Odds ratios (OR): 2.6; 95% confidence intervals (CI): 1.1–6.2; $P$=0.03) but not in the South Asian population. The *ACE* DD genotype was not associated with childhood asthma in either population group. Gametic phase disequilibrium between the *TNF*-α-308 and *ACE* I/D variants was significantly different from zero in UK/Irish cases (Δ=0.09; $P$=0.034). The *TNF*-α-308 allele 2 or a linked major histocompatibility complex (MHC) variant may be a genetic risk factor for childhood asthma in the UK/Irish sample.

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## Introduction

Asthma is an inflammatory disease of the airways characterized by intermittent and reversible airflow obstruction causing wheeze, cough, and breathlessness. Chronic inflammation leads to bronchial hyperresponsiveness (BHR), airway remodelling and permanent airway constriction (reviewed in Cookson 1999). Asthma is phenotypically heterogeneous with a wide spectrum of severity, and it is likely that it consists of a collection of different disorders (Moffatt and Cookson 1999). Most childhood asthma (95%) is associated with atopy, which is the predisposition to generate an immunoglobulin E (IgE)-mediated inflammatory response to environmental allergens. Adult-onset asthma is poorly defined and many cases are caused by non-atopic mechanisms (Cookson 1999).

Asthma is a common, multifactorial disease (Sandford and Paré 2000) which has been shown, from family and twin studies, to have a major genetic component (heritability between 0.6 and 1) (reviewed in Sandford et al. 1996). Genome-wide screening for susceptibility loci suggests that the genetic basis involves interactions between multiple genes of moderate and major effect which are modulated by interacting environmental factors. Genetic heterogeneity is likely and may explain the highly variable disease manifestation (reviewed in Barnes 1999, Sandford and Paré 2000).

The tumour necrosis factor-α (*TNF*-α) and the angiotensin-converting enzyme (*ACE*) genes have been implicated in the pathogenesis of asthma based on the function of their protein products (Benessiano et al. 1997, Shah et al. 1995). TNF-α is a potent proinflammatory cytokine that is believed to have a central role in airway inflammation and increased bronchial responsiveness (Campbell et al. 1996, Hakonarson et al. 1996). Elevated levels of TNF-α have been reported in asthmatic airways (Bradding et al. 1994, Broide et al. 1992, Virchow et al. 1995). The *TNF*-α gene is located on chromosome 6p within the class III region of the major histocompatibility complex (MHC) (Carrol et al. 1987) which has been

linked to asthma in several genome screens (reviewed in Moffat and Cookson 1999). A G→A polymorphism at position -308 of the *TNF*-α gene promoter (Wilson et al. 1992) has been associated with increased TNF-α production in vitro (Bouma et al. 1996, Braun et al. 1996) and increased *TNF*-α transcription (Kroeger et al. 1997, Wilson et al. 1997). The functional importance of this promoter variant is unclear however, because other studies have not replicated these findings (Brinkman et al. 1996, Pociot et al. 1993, Stuber et al. 1996, Wilson et al. 1995).

Several studies have reported an association of the *TNF*-α-308 allele 2 (-308 A) with asthma (Campbell et al. 1996, Chagani et al. 1999, Li Kam Wa et al. 1999, Moffat and Cookson 1997, Moffat et al. 1999). In contrast, Albuquerque and colleagues (1998) found an association between the *TNF*-α -308 allele 1 (-308 G) and childhood asthma in a Caucasian population. Tan and colleagues (1999) found no association between asthma and *TNF*-α-308 genotype in Chinese and Malaysian subjects, and Malerba and colleagues (1999) found no association in an Italian population.

ACE, an enzyme of the renin-angiotensin system (RAS), converts angiotensin I into the vasoactive protein angiotensin II which also causes bronchoconstriction (Erdos 1990, Millar et al 1994). The proinflammatory mediators bradykinin and substance P, which are involved in inflammation in asthma (Barnes 1989, Kharitonov et al. 1999), are inactivated by ACE (Kuoppala et al. 2000). Thus ACE activity could either contribute to or alleviate the airway inflammation in asthma.

A polymorphism caused by an insertion or deletion (I/D) of a 287-bp *Alu* sequence in intron 16 of the *ACE* gene on chromosome 17q23 (Hubert et al. 1991) has been associated with higher circulating and cellular ACE levels (Costerousse et al. 1993, Rigat et al. 1990). Two studies have reported an association of the DD genotype with asthma (Benessiano et al. 1997, Hollá et al. 1999), whereas other studies have not replicated this finding (Chagani et al. 1999, Gao et al. 1998, Nakahama et al. 1999, Tomita et al. 1998).

In this study we investigated the association of the *TNF*-α-308 and the *ACE* I/D variants with a self-reported history of childhood asthma in women from two population groups, UK/Irish and South Asian (India, Pakistan and Bangladesh).

## Materials and methods

### Study sample

At Northwick Park Hospital (Northwest London Hospitals NHS Trust) 1,811 women attending the antenatal clinic for a routine 18-week anomaly ultrasound scan were recruited between 1996 and 1999. A researcher provided an information leaflet and a verbal description of the research project before verbal consent was obtained from each participant. A questionnaire about personal and family history of common diseases was administered by a researcher. Questions about the country of origin of parents, grandparents and any distant ancestry were included, in order to define the participants' gene pool of origin. The sample was divided into population groups based on all four grandparents originating from the same country, with no distant ancestry from anywhere else. The two main population groups were UK/Irish ($n$=649) and South Asian (India, Pakistan and Bangladesh; $n$=399). Each participant was asked about a personal or family history of asthma in childhood, adulthood or both, but severity was not recorded. Participants who reported a history of childhood asthma were selected as cases. There were 20 cases in the UK/Irish group and six cases in the South Asian group. The controls were individuals who reported no personal history of asthma of any kind (childhood or adulthood) and no family history of childhood asthma in first and second-degree relatives. In the UK/Irish there were 416 controls and 213 participants with a family history of asthma who were excluded from the study. In the South Asian group there were 275 controls and 118 participants with a family history of asthma who were excluded.

Mouthwash samples were collected in 10 ml of sterile 0.9% saline solution for extraction of genomic DNA. Ethics approval was obtained from Harrow Research Ethics committee, Northwick Park Hospital.

### Genotyping

DNA was extracted from 0.9% saline mouthwash samples using the ELUCIGENE CF12 kit protocol (ZENECA Diagnostics). PCR primers for amplification of the *TNF*-α -308 and *ACE* I/D polymorphisms were those described by Wilson et al. (1992) and Rigat et al. (1992) respectively. Reactions were carried out in a final volume of 12.5 μl containing mouthwash DNA, 2.5 mM $MgCl_2$, 1.25 units of *Taq* DNA polymerase (Promega), 1 μM of each primer, 250 μM dNTPs mixed (Promega) and 1X PCR buffer (Promega). For the *ACE* I/D polymorphism 5% DMSO was included in the reaction mixture to overcome the problem of preferential amplification of the deletion allele (Odawara et al. 1996). Reactions were carried out in 96-well plates with two blank controls in an MJ thermal cycler. PCR amplification conditions were 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s with a final extension for 5 min at 72 °C.

PCR products for the *TNF*-α-308 variant were digested with the *Nco*I restriction enzyme and the products were separated by electrophoresis through a 4% agarose gel. Allele 1, -308 G, (restriction site present) was detected as an 87 and a 20 bp fragment and allele 2, -308 A, (restriction site absent) was detected as a 107 bp fragment. PCR products for the *ACE* I/D polymorphism (insertion (I) allele 490 bp and deletion (D) allele 190 bp) were size-fractionated by electrophoresis through a 2.5% agarose gel. Genotypes were scored independently by two individuals and any ambiguous genotypes were repeated or omitted.

### Statistical analysis

Genotype distributions were compared with those expected for samples from populations in Hardy-Weinberg equilibrium using a $\chi^2$ test (1df). Genotype distributions were compared between the two population groups by contingency $\chi^2$ (2df) analysis. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated to test association between genotype and childhood asthma. The significance of the OR was calculated by a 2×2 contingency $\chi^2$ test (1df). Statistical significance was taken as $P<0.05$.

Gametic phase disequilibrium, the non-random association of alleles at two loci, was estimated using the approach devised by Weir and Cockerham (1989) which included a test for statistical significance. This approach allows the inclusion of double heterozygotes without assuming the phase, and does not require that the loci under comparison be linked. The bounds of each disequilibrium were calculated (Weir and Cockerham 1989, reviewed by Haviland et al. 1991).

**Table 1** Genotype distribution for the *TNF*-α-308 and *ACE* I/D variants in a UK/Irish and South Asian population (*TNF-α* tumour necrosis factor-alpha, *ACE* angiotensin-converting enzyme, *I/D* insertion/deletion)

| | $n$[a] | | | | $P$[b] | Allele frequencies |
|---|---|---|---|---|---|---|
| *TNF*-α-308 | | 11 | 12 | 22 | | |
| UK/Irish | 649 | 369 | 162 | 22 | $1.6\times10^{-10}$ | 0.81/0.19 |
| S. Asian | 399 | 289 | 39 | 4 | | 0.93/0.07 |
| *ACE* I/D | | II | I/D | DD | | |
| UK/Irish | 649 | 132 | 289 | 197 | $1.2\times10^{-9}$ | 0.45/0.55 |
| S. Asian | 399 | 138 | 167 | 63 | | 0.6/0.4 |

[a]*TNF*-α genotypes were not available for 96 UK/Irish and 67 S. Asian participants. *ACE* I/D genotypes were not available for 31 UK/Irish and 31 S. Asian participants

[b]*P* value: the statistical significance for the comparison of genotype distributions for the two populations ($\chi^2$ 2df)

## Results

Genotype distributions for the *TNF*-α-308 and *ACE* I/D variants for each population did not differ significantly from those expected under conditions of Hardy-Weinberg equilibrium (data not shown). The *TNF*-α-308 and *ACE* I/D allele frequencies in the UK/Irish population are consistent with previous reports (Cambien et al. 1992, Walston et al. 1999). The *ACE* I/D allele frequency in this South Asian population is consistent with a previous report in an Indian population (Saha et al. 1996). There are no previous reports of the *TNF*-α-308 frequencies for South Asians. Genotype distributions differed significantly between the population groups (Table 1). The frequency of the *TNF*-α-308 allele 2 and the *ACE* D allele is lower in South Asians compared with UK/Irish.

Table 2 shows the genotype distributions and OR analysis for the *TNF*-α -308 polymorphism in cases and controls. The genotype distributions in cases and controls did not differ significantly from those expected under conditions of Hardy-Weinberg equilibrium (data not shown). To test association between the *TNF*-α-308 allele 2 and childhood asthma, we combined the subjects with 12 and 22 genotypes and compared them with subjects with a 11 genotype in cases and controls, using OR. This suggested that the presence of either one or two copies of allele 2 is associated with asthma in UK/Irish (OR: 2.6; 95% CI 1.1–6.2; $P$=0.03). There was not a significant excess of allele 2 in cases compared with controls for South Asians (OR: 3.32; 95% CI. 0.64–17.1; $P$=0.15) although the genotype frequency in the cases showed a visible trend toward this.

Table 3 shows the genotype distributions and OR analysis for the *ACE* I/D polymorphism in cases and con-

**Table 2** Genotype distributions and odds ratio (*OR*) of the *TNF*-α-308 variant in childhood asthma and controls in the UK/Irish and South Asian populations (*TNF-α* tumour necrosis factor-alpha, *CI* confidence intervals)

| | n | 11 | 12 | 22 | Odds ratio[b] (95% CI) | $\chi^2$(1df) | P |
|---|---|---|---|---|---|---|---|
| UK/Irish | | | | | | | |
| Childhood asthma | 20 | 9 | 9 | 2 | 2.6 (1.1–6.2) | 4.57 | 0.03 |
| Controls | 416[a] | 283 | 116 | 17 | | | |
| S. Asian | | | | | | | |
| Childhood asthma | 6 | 4 | 2 | 0 | 3.32 (0.64–17.1) | 2.06 | 0.15 |
| Controls | 275[a] | 239 | 33 | 3 | | | |

[a]For UK/Irish 416 out of the 629 participants (after cases removed) were used as controls. The remaining 213 participants had a family history of asthma in first- and/or second-degree relatives. For S. Asians 275 out of the 393 participants (after cases removed) were used as controls. The remaining 118 participants had a family history of asthma in first- and/or second-degree relatives

**Table 3** Genotype distributions and odds ratio (*OR*) of the angiotensin-converting enzyme insertion/deletion (*ACE I/D*) variation in childhood asthma and controls in the UK/Irish and South Asian populations

| | n | II | ID | DD | Odds ratio[b] (95% CI) | $\chi^2$ (1df) | P |
|---|---|---|---|---|---|---|---|
| UK/Irish | | | | | | | |
| Childhood asthma | 20 | 3 | 11 | 6 | 0.9 (0.34–2.4) | 0.04 | 0.84 |
| Controls | 416[a] | 89 | 193 | 134 | | | |
| S. Asians | | | | | | | |
| Childhood asthma | 6 | 4 | 2 | 0 | NC[c] | | |
| Controls | 275[a] | 105 | 128 | 42 | | | |

[a]For UK/Irish, 416 out of the 629 participants (after cases removed) were used as controls. The remaining 213 participants had a family history of asthma in first- and/or second-degree relatives. For South Asians, 275 out of the 393 participants (after cases removed) were used as controls. The remaining 118 participants had a family history of asthma in first- and/or second-degree relatives

[b]DD versus I/D, II

[c]Not calculated: the OR could not be calculated because there were no DD genotypes in S. Asian cases

**Table 4** Gametic phase disequilibrium between *TNF*-α-308 and *ACE* I/D variants in UK/Irish childhood asthma cases. $P$= 0.034; $\Delta$=0.09; $\Delta_{max}$=0.574 (*TNF-α* tumour necrosis factor-alpha, *ACE* angiotensin-converting enzyme, *I/D* insertion/deletion)

| | TNF-α | 11 | 12 | 22 |
|---|---|---|---|---|
| ACE | DD | 4 | 2 | 0 |
| | ID | 5 | 5 | 1 |
| | II | 0 | 2 | 1 |

**Table 5** Gametic phase disequilibrium analysis between *TNF*-α-308 and *ACE* I/D variants in UK/Irish controls. $P$=0.58; $\Delta$ = –0.006; $\Delta_{min}$ = –0.161 (*TNF-α* tumour necrosis factor-alpha, *ACE* angiotensin-converting enzyme, *I/D* insertion/deletion)

| | TNF-α | 11 | 12 | 22 |
|---|---|---|---|---|
| ACE | DD | 85 | 44 | 5 |
| | ID | 136 | 51 | 6 |
| | II | 62 | 21 | 6 |

trols. The genotype distributions in cases and controls were not significantly different from those expected under conditions of Hardy-Weinberg equilibrium (data not shown). The frequency of the DD genotype was compared with the frequency of the combined II and I/D genotypes in cases and controls to test for an association of the DD genotype with childhood asthma. The prevalence of the DD genotype was not significantly increased in the case group in either population.

The results from the gametic phase disequilibrium analysis for the UK/Irish population are shown in Tables 4 and 5. Gametic phase disequilibrium between alleles of the *TNF*-α-308 and *ACE* I/D polymorphisms was significantly different from zero for the UK/Irish childhood asthma group (Table 4; $P$=0.034) but not for the control group (Table 5; $P$=0.58). The gametic phase disequilibrium for the childhood asthma cases was not at its maximum, given the allele frequencies, indicating that the small numbers were not distorting the disequilibrium to its bounds (reviewed in Haviland et al. 1991). Gametic phase disequilibrium can be observed if the genotypes are deviating from expectation under conditions of Hardy-Weinberg (HW) equilibrium. However in the UK/Irish case and control groups the genotype distributions were not significantly different from those expected under HW equilibrium. This analysis therefore suggests that there is a non-random association between the alleles of the *TNF*-α-308 and *ACE* I/D polymorphisms in UK/Irish cases with self-reported childhood asthma. It indicates that the *TNF*-α-308 allele 2 is associated with the *ACE* I allele and the *TNF*-α-308 allele 1 is associated with the *ACE* D allele more often than would be expected, given the allele frequencies. Gametic phase disequilibrium between these polymorphisms was not significant in either the case or the control group from the South Asian population (data not shown).

## Discussion

In this study we found a significant association between the *TNF*-α-308 allele 2 and self-reported history of childhood asthma in the UK/Irish population. This supports previously reported associations of allele 2 with bronchial hyperreactivity in asthma (Campbell at al. 1996, Li Kam Wa et al. 1999) and with asthma in white Caucasian populations (Chagani et al. 1999, Moffatt and Cookson 1997, Moffatt et al. 1999). This result does not replicate the study by Albuquerque and colleagues, (1998) which found an association between the *TNF*-α-308 allele 1 and childhood asthma.

A significant association was not observed in the South Asian population, which could be because of the lower frequency of the *TNF*-α-308 allele 2 and the small numbers. The significant association in the UK/Irish population is encouraging because the small numbers of cases and the reliance on self-reported history of childhood asthma is likely to reduce the power to detect significant associations.

The association between *TNF*-α-308 and childhood asthma may be due to a direct functional effect of this variant on TNF-α expression. Functional studies have shown the *TNF*-α-308 allele 2 to be a much stronger transcriptional activator than the wild-type allele in vitro (Kroeger et al. 1997, Wilson et al. 1997), which may lead to increased TNF-α secretion and explain the association of the 22 genotype with higher levels of TNF-α production in vitro (Bouma et al. 1996, Braun et al. 1996). Thus this variant could be responsible for the increased production of TNF-α that has been observed in asthmatic airways, and could contribute to asthma pathogenesis (Bradding et al. 1994, Broide et al. 1992, Gosset et al. 1991). However, other studies have not found enhanced transcriptional activity with this variant (Brinkman et al. 1996, Stuber et al. 1996) or a significant association between allele 2 and TNF-α levels (Pociot et al. 1993, Wilson et al. 1995).

The *TNF*-α-308 allele 2 may be in linkage disequilibrium with a functional variant that alters TNF-α expression, either within the *TNF*-α gene or another gene within the MHC. For example, variation in the HLA-DRB1 locus has been associated with increased TNF-α secretion (Jacob et al. 1990), and other *TNF*-α promoter variants may alter TNF-α secretion (reviewed in Skoog et al. 1999).

The *TNF*-α gene is located in tandem with the Lymphotoxin ß (*LTß*) and *LT*α genes within the class III region of the 3.6 Mb MHC region on chromosome 6p21.31 (Carrol et al. 1987). The TNF gene superfamily (*LT ß*, *TNF*-α, *LT*α) is part of a set of more than seven genes involved in inflammation, which has been referred to as the class IV region of the MHC (The MHC sequencing consortium 1999). There is strong linkage disequilibrium across the MHC, and the *TNF*-α-308 allele 2 could be in linkage disequilibrium with a functional variant of a gene from the cluster of inflammation genes.

HLA-DR genotypes have been associated with atopic responses to common allergens (Young et al. 1994). An

extended haplotype containing the *TNF*-α-308 2 allele and the HLA-DRB1 locus in class II of the MHC (*LT*α *Nco*I*1/*TNF*-308*2/HLA-DRB1*02) has been shown to be strongly associated with asthma and bronchial hyperresponsiveness (Moffatt et al. 1999). It is difficult to differentiate between the effects of these alleles in this haplotype, but a functional polymorphism contributing to asthma susceptibility is likely to be embedded within this haplotype (Moffatt et al. 1999).

In this study, there was no significant association between the *ACE* DD genotype and self-reported history of childhood asthma in either population. This supports some previous studies in a British and Japanese population (Gao et al. 1998, Nakahama et al. 1999, Tomita et al. 1998) and disagrees with other studies in which associations between asthma and the *ACE* I/D variant have been observed in a French and a Czech population (Benessiano et al. 1997, Hollá et al. 1999). The conflicting results do not exclude the *ACE* I/D polymorphism as a risk factor for asthma, and may reflect the genetic heterogeneity underlying asthma aetiology.

Gametic phase disequilibrium is the non-random distribution of alleles at two loci into gametes, either because they are genetically linked or because they interact to "influence fitness" (Weir and Cockerham 1989). Gametic phase disequilibrium between the *TNF*-α-308 and *ACE* I/D loci for the UK/Irish case group but not for the control group was significantly different from zero. Small sample size and departures from HW equilibrium can distort gametic phase disequilibrium, but these factors were not influencing the disequilibrium in this sample (see results). This suggests a non-random association of alleles at these unlinked loci in individuals with childhood asthma, with the *TNF*-α-308 allele 2 being inherited with the *ACE* I allele and the *TNF*-α-308 allele 1 being inherited with the *ACE* D allele more often than expected, given the allele frequencies.

It is difficult to draw any conclusions from this finding since there is no known biological relationship between ACE and TNF-α, and in this study the *ACE* I/D variant was not associated with self-reported childhood asthma. This finding warrants further investigation in a larger sample.

In summary, this study provides evidence for a role of the *TNF*-α-308 variant or a linked MHC gene in the genetic susceptibility to childhood asthma in a UK/Irish sample. There may also be a genetic risk factor within this region on chromosome 6p in South Asians, but further studies with larger numbers are required to confirm this.

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