Primary research

Simultaneous identification of GSTP1 Ile105→Val105 and Ala114→Val114 substitutions using an amplification refractory mutation system polymerase chain reaction assay: studies in patients with asthma

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Introduction

Polymorphisms in members of the GST supergene family have been associated with individual susceptibility to lung diseases [1]. In the context of asthma GSTP1 – the predominant GST expressed in human lung [2] – is a candidate because this enzyme has a role in cellular protection against oxidative stress [3]. Thus, GSTP1 catalyzes the detoxification of byproducts of lipid and DNA oxidation [1]. Asthma is characterized by airway inflammation [4]. Indeed, BHR reflects the presence of inflammation, and is exhibited by virtually all asthmatic patients. Atopic individuals (as defined by serum IgE levels and skin prick tests)
are very likely to have increased airway responsiveness [4]. Thus, studies designed to identify susceptibility genes for asthma must consider the possible interrelationship of BHR and atopy in the expression of the asthma phenotype.

We previously showed that the Ile105 → Val105 substitution in GSTP1 is strongly associated with severity of BHR [5]. A further polymorphism is present at amino acid 114 (Ala114 → Val114), however, indicating that unequivocal identification of GSTP1 alleles requires consideration of both substitutions. These polymorphisms give rise to wild-type GSTP1*A (Ile105 → Ala114), GSTP1*B (Val105 → Ala114), GSTP1*C (Val105 → Val114) and GSTP1*D (Ile105 → Val114) [6–9]. Although the Ile105 variant has a higher catalytic efficiency for 1-chloro-2,4-dinitrobenzene than does the Val105 variant [6], the Val105 variant appears to confer higher catalytic efficiency for polycyclic aromatic hydrocarbon diol epoxides [7–11]. The effect of the Ala114 → Val114 substitution is unclear, although it may enhance the effect of the Ile105 → Val105 substitution [11].

Because the substitution at amino acid 114 may modify the association of GSTP1 Ile105 → Val105 with asthma phenotypes, we developed an ARMS assay in order to identify unambiguously those genotypes that result from the A, B, C and D alleles. This approach is necessary, because presently described assays do not differentiate AC and BD genotypes. We also determined the frequencies of these genotypes in atopic nonasthmatic, atopic asthmatic and nonatopic nonasthmatic healthy persons.

**Materials and methods**

**Patients**

Unrelated Northern European nonatopic nonasthmatic, atopic nonasthmatic and atopic asthmatic persons (n = 191) were recruited in North Staffordshire, UK [5]. They were stratified by degree of airway reactivity/obstruction as follows (Table 1): group 1, BHR negative (normal), with forced expiratory volume in 1 s (FEV1) greater than 80% predicted and PC20 greater than 16 mg/ml metacholine; group 2, borderline BHR (mild) with FEV1 greater than 80% predicted and PC20 positive with 8–16 mg/ml metacholine; group 3, BHR positive (moderate) with FEV1 greater than 80% predicted and provoking concentration of an inhaled substance that causes a 20% reduction in FEV1 (PC20) positive with 0.03–8 mg/ml metacholine; and group 4, severe airway dysfunction, with FEV1 of 80% or less than predicted, which was not challenged with metacholine for ethical reasons. A positive skin reaction (mean wheal diameter of at least 3 mm more than with saline control) in response to at least one of a panel of seven common aeroallergens (house dust mite, house dust, grass mix, tree pollen, cat fur, dog fur, feathers) and serum IgE levels greater than 100 IU/ml were used to define atopic status, together with personal history. The local Ethics Committee approved the study, and all participants provided written informed consent.

**Determination of GSTP1 genotypes**

Genotyping was performed using leucocyte DNA. The Ile105 → Val105 substitution was identified using primers to exon 5 as previously described [7,12]. The Ala114 → Val114 substitution was identified as described
by Board et al [13], with an annealing temperature of 65°C. However, GSTP1 AC and BD gave heterozygous patterns using both of the above assays. An ARMS assay was therefore developed to differentiate between these genotypes. This included a forward primer upstream of the codon 105 substitution (5′-ACCCCAGGGCTTATGGGAA-3′) and two reverse primers (primer A [Ala114 specific], 5′-TCACATAGTCATCCTTGCCGG-3′; and primer B [Val114 specific], 5′-TCACATAGTCATCCTTGCCGA-3′).

For each DNA sample two polymerase chain reactions (PCRs) were performed, amplifying a 998 base pair fragment. PCRs were carried out in 50 µl containing forward primer, reverse primer A or B (2 × 0.25 µmol/l), Taq polymerase (1 U), dNTP (4 × 200 µmol/l), 1 x polymerase buffer (10 mmol/l Tris-HCl pH 9.0, 50 mmol/l KCl, 0.1% [vol/vol] Triton X-100, 1.5 mmol/l MgCl2), and target DNA (approximately 0.5 µg). Conditions were as follows: 94°C for 4 min, 30 cycles of denaturation (94°C, 1 min), primer annealing (62°C, 1 min) and elongation (72°C, 2 min).

The initial ARMS PCR was used to determine the Ala114→Val114 genotype. Thus, in persons with Ala114/Ala114 amplification occurred only with primer A, and only with primer B in persons with Val114/Val114; for Ala114/Val114 heterozygotes, amplification occurred with both primers. ARMS PCR products were then digested with Bsm Al to determine the cis/trans configuration (which variant at position 105 is paired with which allele at position 114) of the Ile105→Val105 encoding allele and resolved in 2% (vol/wt) agarose gels. Products gave fragments of 343, 322, 260 and 73 base pairs with Ile105/Ile105; 93, 250, 322, 260 and 73 base pairs with the Val105/Val105; and all six fragments in heterozygotes (Ile105/Val105).

Every run (including both restriction fragment length polymorphism and ARMS assays) included DNA samples of known genotype as positive controls and one negative control (no DNA). Samples with AA (Ala114/Ala114) and CC (Val114/Val114) were used to optimize the procedure, and as controls for the ARMS PCR. As an assessment of quality control, approximately 15% of DNA samples were reassayed at least once in order to confirm the assigned genotype. All results from the reassayed samples were consistent with the original genotype assignment.
The frequencies of GSTP1 genotypes are shown in Table 2. In 191 persons, none had BD, CD or DD, reflecting the rarity of GSTP1*D. The frequencies of GSTP1 genotypes achieved Hardy–Weinberg equilibrium. As reported previously [14], we found significant ($P < 0.0001$, $\chi^2 = 50.9$) linkage disequilibrium between Ile105 and Ala114 and between Val105 and Val114.

**Association of GSTP1 genotype with atopic indices**

Table 2 shows the association of GSTP1 genotypes with skin test positivity or IgE level. The frequencies of GSTP1 BB and BC were reduced in individuals with at least one positive skin test (2.5 and 1.7%, respectively) compared with individuals who were skin test negative (5.6 and 9.9%, respectively). Although the frequency of GSTP1 BB was not significantly lower in the persons with positive skin tests (odds ratio [OR] 0.44; $P = 0.297$) or with IgE levels of 100 IU/ml or less, GSTP1 BC was significantly associated with skin test negativity (OR 0.17; $P = 0.031$; Table 2). There were no significant associations between GSTP1 genotypes and IgE level, although all individuals with GSTP1*BD had IgE levels of 100 IU/ml or less.
Association of \(\text{GSTP1}\) genotypes with airway obstruction/reactivity

Table 2 shows the frequencies of \(\text{GSTP1}\) genotypes in relation to degree of airflow obstruction and BHR. The \(\text{GSTP1}\) AA frequency increased with severity of airway reactivity/obstruction, whereas BB and BC frequencies displayed a reverse trend. Compared with \(\text{GSTP1}\) AA, trend test analysis across the four groups revealed a significant decrease in frequency of \(\text{GSTP1} \) BC (and \(\text{GSTP1}\) BB, albeit not significant) with increasing airway reactivity/obstruction \((P=0.031)\), indicating a protective effect. This effect remained significant after correction for age and sex \((P=0.022)\) using ordered logistic regression analysis.

Association of \(\text{GSTP1}\) genotype with presence of asthma

We further examined the association of \(\text{GSTP1}\) genotypes with the clinical presence of asthma, as defined using the recognized cutoff of \(<8\) mg/ml methacholine [15] on bronchial challenge (groups 1 and 2 versus groups 3 and 4). The frequency of \(\text{GSTP1}\) AA was increased in groups 3 and 4 (44.9%) as compared with groups 1 and 4. The frequency of bronchial challenge (groups 1 and 2 versus groups 3 and 4) recognized cutoff of \(<8\) mg/ml methacholine [15] on bronchial challenge (groups 1 and 2 versus groups 3 and 4).

The data reported here also show that the frequencies of \(\text{GSTP1}\) BC and BB were reduced in asthmatic persons, thus indicating a protective effect. This supports the view that there is little additional effect on disease risk from the Ala114→Val114 substitution, although the number of individuals with some genotypes was small and larger numbers would be required to confirm this observation. The advantage with using this ARMS assay is that it is possible to discriminate between genotypes AC and BD. It relies on a relatively large fragment (998 base pairs), however, and is therefore not suitable for genotyping of archival DNA. Thus, this technique is applicable for studies into population genetics.

In conclusion, we described an ARMS assay to identify genotypes resulting from the A, B, C and D alleles of \(\text{GSTP1}\), and provided data on the effect of \(\text{GSTP1}\) genotype on asthma risk. Our findings suggest that \(\text{GSTP1}^B\) and \(\text{GSTP1}^C\) confer similar protective effects. However, large patient groups are required to identify differential effects between \(\text{GSTP1}^B, \text{GSTP1}^C\) and \(\text{GSTP1}^D\).

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