

Screening of the FcεRI-β-Gene in a Swiss Population of Asthmatic Children: No Association with E237G and Identification of New Sequence Variations

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ABSTRACT: BACKGROUND: The gene of the beta subunit of the high affinity receptor for IgE (FcεRI-β) encoded on chromosome 11q13 has recently been identified as a candidate gene for asthma and atopy. Two coding variations, E237G and I181L have been described as being associated with asthma and atopy. Our aim was to investigate a Swiss population of atopic and asthmatic children for variations in this gene.

METHODS: We screened all 7 exons of the FcεRI-β-gene in 224 atopic/asthmatic, 68 relatives and 159 control subjects using exon amplification by PCR and single strand conformation polymorphism (SSCP) analysis followed by fluorescence based DNA sequencing.

RESULTS: The sequence variant E237G was found in 3.7% in atopics and in 2.6% in the control population. None of the samples carried the I181L mutation. In addition, we characterised nine novel mutations (1 nonsense mutation, 2 missense mutations, mutation, 2 silent mutations, 4 intronic mutations).

CONCLUSIONS: Our results suggest that the E237G does not have a primary effect on the development of atopy and asthma, and thus excludes the FcεRI-β locus from being a candidate gene directly involved in these diseases.

KEYWORDS: 11q13, atopy, BHR

INTRODUCTION

Atopic diseases such as allergic asthma, rhinitis and eczema are the most common chronic diseases encountered by paediatricians. They are characterised by genetic predisposition to produce an increased immunoglobulin E (IgE) response after exposure to common environmental allergens [1,2]. In addition to genetic factors there is evidence for strong environmental influence. Atopy is one of the greatest risk factors in the development of asthma [3], as identified by measurements of airway obstruction and bronchial hyperresponsiveness (BHR).

In 1989, Cookson was the first to describe linkage between IgE level and D11S97, a DNA marker sequence on chromosome 11q, in seven large, multiply affected, extended British families [4]. Transmission analysis revealed significant sharing of maternal alleles [5]. Further investigations resulted in the identification of the beta chain of the high affinity receptor for IgE (FcεRI-β), localised on chromosome 11q13, as a candidate gene for atopy [6]. Two variants of the FcεRI-β receptor gene, the I181L in exon 6 and

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the E237G in exon 7, have been reported to be associated with atopy and asthma [7,8,9].

The tetrameric high affinity receptor ($\alpha\beta\gamma_2$) for IgE [10] plays a critical role in the allergic inflammation process [11,12]. The cross linking by allergen of IgE bound to the receptor on mast cells, basophils and Langerhans cells [13,14,15] leads to the release of a variety of mediators and cytokines and to the manifestation of characteristic clinical symptoms. The receptor exerts positive feedback by regulation of the IgE production through Il-4 production by mast cells [16,17]. The beta chain, although without autonomous cell activation capacity, acts as an amplifier of antigen-mediated cell activation through phosphorylation of the Immunoreceptor Tyrosine-based Activation Motif (ITAM) [18].

One model used to explain the effect of mutations within the FcεRI-β is the modification of the signal transduction activity of the receptor either by producing a more sensitive receptor or by an exaggerated Il-4 release. The reported E237G, adjacent to the ITAM segment of FcεRI-β changes the hydrophilic nature of the C-terminus of the beta chain and may alter the signalling capacity [8].

Several independent studies have been unable to reproduce the original linkage [reviewed in 19] or to detect the I181L polymorphism [20,21]. This negative findings either due to false-negative PCR results [22], to variability's in

phenotype definition or to complex interactions between susceptible genes and environmental factors involved in the pathogenesis of asthma [23].

Our study was designed to investigate the association of the previously reported coding variations, I181L and E237G, in a well phenotyped Swiss atopic and asthmatic population. In addition we looked for further structural changes in the FcεRI-β-gene by screening the seven exons of the gene using single strand conformation polymorphism (SSCP) and direct sequencing of variants.

MATERIALS AND METHODS

Subjects

292 individuals (224 patients and 68 relatives) were recruited from the Paediatric Pulmonary Out-patient Clinic of the University Children's Hospital of Berne, Switzerland. The age of the patients ranged from 2–16 years (mean age 8.3). In total, we ascertained 45 unrelated families, 14 of these were nuclear in structure (affected child with both parents; or patient, one parent and at least one sibling), 14 sib-pairs and 179 patients without any relatives.

Informed written consent was obtained from all parents. The study was approved by the

Table 1
Association between E237G and atopy and asthma

Proband	E237G % (n)	χ^2 ^a	<i>p</i> ^a
control (n = 159)	2.6% (8)		
probands total (n = 224)	3.7% (17)	1.118	0.29
non atopic (n = 88)	5.2% (8)	1.45	0.23
atopics (n = 136)	2.9% (8)	1.34	0.71
very high IgE (> 1000 kU/l) (n = 29)	0.0% (0)		
with BHR ^c (n = 47)	4.2% (4)	2.37	0.12
no BHR (n = 54)	3.7% (4)	1.12	0.29
with Airway obstruction (n = 126)	3.9% (10)	0.00	0.99
no airway obstruction (n = 60)	3.3% (4)		

^a χ^2 : Chisquare *p*: *p* value (Jandel, Sigmastat Version 2.0, computerprogram)

^bImmunglobulin E

^cBronchial hyperresponsiveness

Ethical Committee of the University of Berne. All affected children had to answer an oral questionnaire on atopic and asthmatic symptoms as well as a family history questionnaire. Clinical examination of patients, performed by two independent physicians, included lung function tests, measurement of airway responsiveness to inhaled carbachol (if practicable), measurement of serum total IgE level and IgE specific antibodies for the most common allergens (birch, lye grass, house dust mite, cat and dog dander). 159 age matched randomly selected individuals of the same local population were used as a control group.

Classification of subjects

We looked for an association between variants in the *FcεRI-β*-gene and the following two phenotypes: atopy and asthma. Atopy is defined as the presence of a specific IgE against one or more tested allergens, a high total serum IgE level, or a combination of these two factors [7] as well as the existence of one clinical parameter (rhinitis, eczema, allergen induced asthma, family history of atopy). Serum total IgE (kU/l) was measured using the CAP system fluorimmunoassay (Diagnostics CAP FEIA, Kabi Pharmacia, Sweden) and IgE for the most common allergen was determined by RAST (Diagnostics CAP FEIA, Kabi Pharmacia, Sweden). For Serum IgE, subjects were divided into two groups based on the measurements of IgE values. A high IgE level was defined as being greater than the published normal values for children [24]. 136 atopic individuals were registered (Table 1).

The asthmatic phenotype was defined by the presence of significant reversible obstruction of airways and/or bronchial hyperresponsiveness (BHR) to inhaled carbachol as well as by documented history of recurrent wheeze and cough [25].

Lung function testing was performed by whole-body plethysmography [26,27], using an air conditioned, constant volume, variable pressure whole-body plethysmograph (Jaeger, Würzburg, Germany). The thoracic gas volume

(TGV) was taken as an estimate of degree of pulmonary hyperinflation. Lung function data were expressed as a percent of predicted values [28]. As an estimate of bronchial obstruction the airway resistance (R_{aw}), measured during quiet breathing, the forced expiratory resistance volume (flow-volume curves) in one second (FEV_1) and the maximal expired flow at 50% of vital capacity (MEF_{50}) (flow-volume curves) were taken. Bronchial obstruction was defined as $R_{aw} > 130\%$ or $MEF_{50} < 80\%$ [26]. Bronchial responsiveness was assessed by bronchial challenge test with carbachol chloride (Bronchoscreen, Jaeger, Würzburg, Germany). BHR was presumed to be present when the cumulative provocation dose of carbachol induces a rise of $R_{aw} > 165\%$ of initial value (PD_{65}) was $< 480 \mu\text{g}$ [29,30]. Patients were divided into four groups, relative to BHR and bronchial obstruction (Table 1).

Genotyping

Genomic DNA was isolated according to standard protocols and quantified by spectrophotometry. PCR amplifications were carried out in 50 μl reaction volumes (Perkin Elmer, Thermal cycler 2400, Oak Brook, IL) containing 100–200 ng of genomic DNA, 10 pmol of each primer, 50 mM KCl, 1.5 M MgCl₂, 10 mM Tris-HCl pH 8.3, 10 mM dNTP and 2.5 U *Taq* polymerase Gold (Perkin Elmer). The samples were denatured for 12 min at 95 °C, followed by 28 cycles each of 15 s 95 °C denaturation, 15 s 58 °C annealing, 45 s 72 °C extension. Final extension was at 72 °C for 7 min. Primers used to amplify the 7 exons of the *FcεRI-β*-gene are listed in Table 2.

2 μl of PCR products were denatured at 95 °C for 10 min with 3 μl SSCP loading buffer (0.2 M NaOH, 0.25%w/v each bromphenol blue and xylene cyanol in formamide) and analysed on a polyacrylamide gel containing glycerol using a two-buffer system [39]. DNA was visualised by silver staining. PCR products producing band shifts in either single (SSCP) or double strands (heteroduplex formation) were purified using PCR purification columns (High Pure™ PCR

Table 2
Amplification Primer sequences (Hill, 1996) for the amplification of the 7 exons of the *FcεRI-β*-gene

Exon	Proximal primer 5'→3'	Distal primer 5'→3'	PCR fragment (bp)	SSCP (%)
1	GCCTGTTGATCTTAATCAGC	AGTTTCATCTCCTAAGCACC	323	8
2	TCTGTCTGTCGAGAATGTTG	GACCTAACACCTCTCATGAA	309	10
3	CGCAGTTTCTCATGTTTGGC	CTGGTTAGATCTGAGAAAGAG	322	8
4	GTAACCTTATCGAGTACCCC	GCAAAAAGCCTTAGGACACA	321	8
5	CCAGCCCTGAAATGAAGATA	AGGATGGTGTTCATGGAACA	329	8
6	GGAGATGAAAACAGGAGATG	GTTTCAGAAAACCCAGGCC	242	8
7	GATGAGGTAAGTCTCTTGAG	CAAAACCTTGGCCTTCTGG	239	10

All primers were synthesised by Microsynth (Balgach, Switzerland)

Product Purification Kit, Boehringer) according to the suppliers recommendations, followed by fluorescence based sequencing (ABI373, Perkin Elmer). Sequences were analysed by SeqEd program (Applied Biosystem) and compared to the *FcεRI-β*-gene sequence (GenBank, M89796).

Reverse transcription PCR

Total RNA was extracted from blood cells according to Chomczynski and Sacchi [31]. Reverse transcription was performed using commercial reagents (Boehringer Mannheim, Expand™ Reverse Transcriptase) with initial incubation of template RNA and primer FEE7b (5'-CAAAACCTTGGCCTTCTGG-3', [8]) for 10 min at 65 °C, followed by 60 min at 42 °C for cDNA synthesis. The cDNA was amplified in a 50 µl reaction volume using a primer set flanking the nonsense mutation (exon 4-exon 7): proximal primer 5'-AGGAGAAATGCAACATATCTG-3' (newly designed) and distal primer 5'-CAAAACCTTGGCCTTCTGG-3'. PCR conditions are as described above. PCR products were analysed on 12% SSCP gels and sequenced as described above.

Restriction digest

RT-PCR products enclosing exon 4-exon 7 were digested with *MaeIII* according to the manufacturer's instructions (Boehringer Mannheim, 822230). cDNA was incubated for 1 h at 55 °C with 2x *MaeIII* buffer and 1 Unit

restriction enzyme, *MaeIII*. The resulting products were separated on 12% SSCP gels.

RESULTS

All 7 exons of the *FcεRI-β*-gene were screened for sequence variations using PCR amplification including the intron/exon boundaries. The published sequence of Küster and Kinet [32] was used as reference sequence. 451 individuals were screened for sequence variations using single strand conformation polymorphism (SSCP)

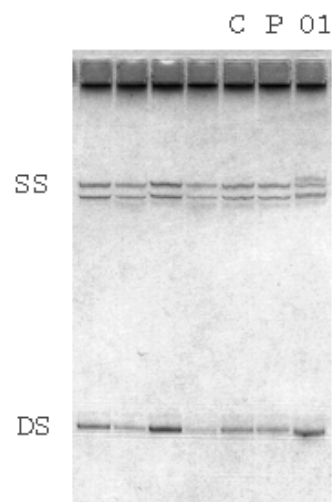


Fig. 1a. SSCP gel analysis of PCR product exon 6. SS stands for single strands, DS for double strands, C for PCR positive control, and P for wildtype control. 01 shows a band migration in the SS and DS and carries the nonsense mutation, G192X.

Table 3
FcεRI-β variants identified in 224 (448 chromosomes) patients and 159 (318 chromosomes) controls

Nucleotide change	Aminoacid change	Exon	frequency %	control frequency %
457T→G	M1R	1	0.2	0
2130A→G	none	3	0.2	0
5676G→T	G192X	6	0.2	0
5691G→A	V197M	6	0	1.3
7256C→T	none	7	0.45	0
7297A→G	E237G	7	3.7	2.6

Table 4
Intronic sequence variants

Nucleotide change	Intron	frequency %	control frequency %
Exon 4 - 87 T to G	3	31.6	28.5
Exon 4 + 11 C to T	4	0.2	0
Exon 4 + 37 A to G	4	1.7	1.3
Exon 6 - 12 G to A	5	0.4	0

analysis (Figure 1a and 4). PCR products with irregular band migration were subjected to sequencing.

The substitution of glutamic acid for glycine at residue 237 (E237G), was recently reported in 5.3% and 6% in an Australian [8] and Japanese [9] population, respectively and described as being strongly associated with asthma and atopy. We found the E237G variant in 17 out of 448 chromosomes (3.7%) of our selected Swiss asthma and atopy population and in 8 out of 318 control chromosomes (2.6%) (Table 3). No homozygous individual for E237G was found in a total of 451 persons screened. Among 136 atopic patients 2.9% showed the E237G variant, whereas 5.2% of non-atopic patients carried the mutation (Table 1). Compared to the control group there was no association of E237G with atopy (IgE < 1000 kU/l) ($\chi^2 = 1.34$; $p = 0.71$), with very high IgE levels (IgE > 1000 kU/l) ($\chi^2 = 1.00$; $p = 0.317$) (Table 1) or with one of the tested specific allergens, as previously reported [7,8]. No significant relation between E237G and the asthmatic phenotype was observed (BHR: $\chi^2 = 0.02$; $p = 0.866$ / Airway obstruction: $\chi^2 = 0.00$; $p = 0.99$) (Table 1). Of fourteen atopic or asthmatic sibpairs only one sibling carried the E237G. Inheritance of FcεRI-β-alleles through the maternal lines were reported to increase the

genetic risk for atopy and BHR [5,33]. Controversial, transmission analysis of the E237G allele in 4 affected nuclear Swiss families indicated no maternal effect. All 3 children who had inherited the allele E237G paternal were atopic including elevated IgE, rhinitis and asthma. In contrast the E237G allele of a healthy mother was not transmitted to her affected son. Thus, a predominant inheritance through maternal line can be excluded.

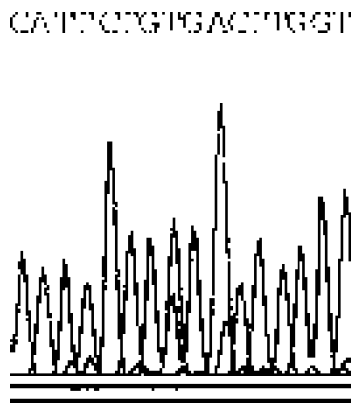
Using both SSCP and direct sequencing of purified PCR fragments enclosing exon 6 we were not able to detect the I181L polymorphism described by Shirakawa et al. [7].

Moreover, we identified heterozygosity for 9 different mutations: one nonsense, one start codon, one missense, two silent mutations and four intronic polymorphisms (Table 3 and 4).

A start codon mutation at nt 457T→G was found in one atopic patient (Table 3). It was the only mutation identified in the coding region of exon 1. The two silent mutations were found in exon 3 (2130A→G) and in exon 7 (7256C→T), in one and two patients, respectively (Table 3).

A single nonsense mutation located at nt 5676G→T (G192X) in exon 6 was detected in two relatives (atopic patient and atopic father) (Table 3, Figure 1a and 1b). This mutation creates a new restriction site for *Mae*III. Exon

A



B

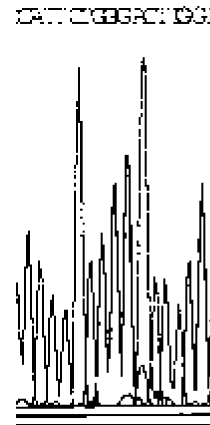


Fig. 1b. Automated sequence analysis, ABI373, Perkin Elmer, of a purified exon 6 PCR product. A: shows heterozygosity for a G to T substitution (G192X). B: shows the sequence of a wildtype sequence of exon 6.

skipping of exon 6 was excluded by RT-PCR amplifying exon 4-exon 7 and direct sequencing of the corresponding cDNA (Figure 2). RT-PCR from RNA extracted from blood lymphocytes of these two individuals followed by MaeIII restriction of the corresponding cDNA showed no additional restriction product (Figure 3). Thus, the mutation was not detectable on mRNA level.

We tested all exons in at least 318 control chromosomes under the same PCR and SSCP conditions (Table 3). Only the previously reported sequence variation E237G and a missense mutation in exon 6 were identified in control chromosomes (Table 3).

We found four intronic polymorphisms (Table 4) concentrated in three regions: intron 3, intron 4 and intron 5. The frequent exon 4-87 T to G polymorphism (Figure 4) is located near the acceptor splice site of exon 4, but it is unlikely to affect the splicing efficiency. 31% of patients and 28.5% of controls are homozygous for this variant, whereas only 16% and 17%, respectively, were homozygous for the published wildtype T. The heterozygous proportion is about 53% in both populations. The Hardy Weinberg equilibrium is fulfilled with this distribution (patients: $p = 0.34$; $q = 0.18$ /controlgroup: $p = 0.30$; $q = 0.19$). No significant association between atopy or BHR and one of the

three genotypes was detected. The remaining three intronic polymorphisms (intron 3, 4, 5) are very rare, 0.2%–1.7% (Table 4), and none of them influence splicing elements.

DISCUSSION

The tetrameric ($\alpha\beta\gamma_2$) high affinity receptor for IgE, FcεRI, plays a central role in the process of IgE-dependent allergic inflammation [11]. A sequence variant, E237G, in the gene for the beta subunit of the high affinity receptor for IgE (FcεRI-β-gene) has recently been reported to be associated with BHR and high IgE levels in an Australian and Japanese population [8,9].

The aim of our study was to screen 242 unrelated patients, 68 relatives and 159 controls for the complete coding sequence of the FcεRI-β-gene, which encodes a 244 amino acid beta subunit of the tetrameric high affinity receptor for IgE, using SSCP. The E237G mutation was identified in 3.7% of our selected Swiss atopy/asthma population and in 2.6% of the control group (Table 3). Statistical evaluation showed no evidence of higher prevalence of E237G in atopics or asthmatics compared to the control population and non asthmatics, respectively (Table 1). Transmission analysis of E237G was performed in order to take account of

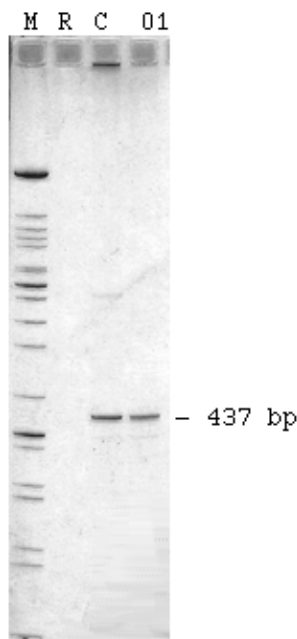


Fig. 2. Gel analysis of RT-PCR exon 4-exon 7. R = PCR reagents control, C = control person, and M = size marker. The size of PCR products without exon skipping is expected to be 437 bp, whereas exon skipping would produce a 338 bp fragment. PCR product of index patient 01, carrying one G192X, shows the same size as C. An exon skipping of exon 6 can be excluded.

the suggested increased atopy risk in the presence of maternal inheritance [5,33]. The phenotypic expression of an 11q13 locus did not depend on maternal inheritance in our Swiss asthma population. Thus, our results exclude E237G from having a primary effect on the development of atopy and asthma, however a secondary pathogenic influence in combination with another mutation cannot yet be excluded, since it is located adjacent to the Immunoreceptor Tyrosine-based Activation Motif (ITAM). The ITAM segment is involved in amplifying the tetrameric FcεRI receptors allergic cell activation [18] in human mast cells and basophils. A mutation located nearby the ITAM could influence the cell signalling by modifying the amplifier function. To evaluate the effect of a mutation within this region, a cultured cell model for human Fc receptor is under construction [34]

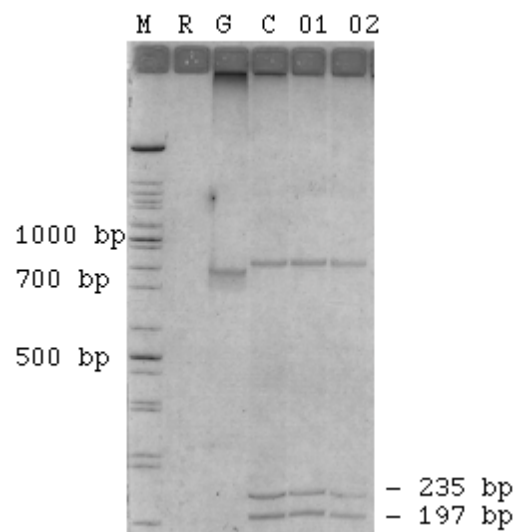


Fig. 3. Gel analysis of RT-PCR exon 4-exon 7 after digestion with *MaeIII* from the two relatives with the nonsense mutation, G192X (01, 02), and from a control (C). R = PCR reagents control, G = genomic DNA control, M = size marker (100 bp marker). Restriction with *MaeIII* creates two digestion products (197 and 235 bp) in wildtype mRNA and three products in mutated mRNA (20, 197 and 215 bp) are expected. Patients 01 and 02 show the same digestion pattern as the control.

and will give insights into functional consequences of sequence variations.

Another variant of the FcεRI-β-gene associated with atopy, I181L in exon 6, has been reported in a UK (17%) population [7]. We did not detect any I181L carrier in 902 screened chromosomes. This is in agreement with several other investigations [reviewed in 23]: 12 out of 15 investigations analysing different ethnic populations and using a variety of different methodologies, did not find the mutation within exon 6. Regarding our screening system, it is very unlikely that we missed the I181L due to methodological failure because: 1) By screening 902 chromosomes with a newly developed, highly sensitive SSCP technique [39] around exon 6 we were able to identify three rare polymorphisms, but not the I181L. Moreover, a portion of PCR products of exon 6 were directly

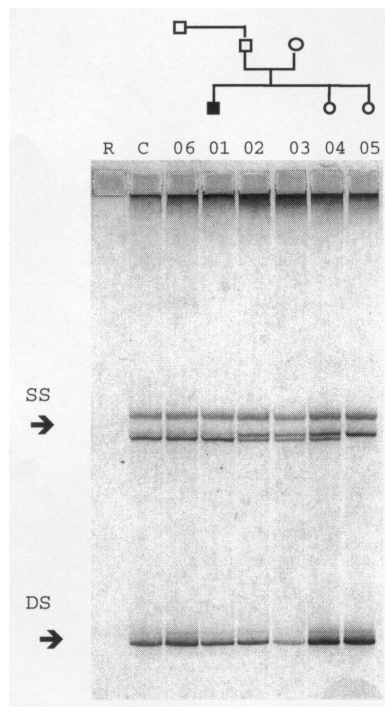


Fig. 4. SSCP analysis of *FcεRI-β* exon 4 variant (exon 4-87 T to G) in an atopic family (6 members), with index patient 01. SS = single strands, DS = double strands, R = PCR reagents control, C = PCR positive control. Family member 02,03,04 represent the heterozygous band migration in SS. Member 05 shows normal band migration, C, 01 and 06 represent homozygosity for G.

sequenced in order to exclude a SSCP failure. 2) A microsatellite repeat near to the start of exon 6, thought to be responsible for false-negative PCR products [22], was not enclosed in our exon 6 PCR amplification.

We suggest therefore that this I181L mutation is a rare polymorphism and an involvement in the development of asthma and atopy is rather unlikely.

In addition, we report here a nonsense mutation within exon 6, G192X, found in two relatives (Table 3, Figure 1a and 1b). Our results indicate instability of the corresponding mRNA because we could not detect this mutation on cDNA level, using restriction with MaeIII (Figure 3). This makes a protein truncation very unlikely. In any case, a nonsense mutation is expected to result in a loss of function or

reduction of the beta subunit of *FcεRI*. Since variations in the *FcεRI-β* promote the atopic state by enhanced release of inflammatory mediators by mast cells; a loss of function mutation would not explain an atopic phenotype. Even though the nonsense mutation is not responsible for atopy, it might be of importance for functional studies of the beta subunit.

Heterozygosity for the widespread intron 3 substitution (exon 4-87 T to G) (Table 3 and Figure 4) was found in 53 % of both patients and controls. The higher prevalence of homozygosity for G in both groups indicates that the G allele is more likely to be the wildtype sequence. There was no relation found between one of the three genotypes (G/G, G/T, T/T) and asthma or atopy. This frequent polymorphism may be useful for further linkage analysis studies, since association between IgE and BHR and the markers D11S527 and D11S534 telomeric to *FcεRI-β* has been reported [35].

We found two silent mutations (Table 3), located within the coding region of the *FcεRI-β*-gene. We did not investigate in the corresponding RNA, because synonymous changes are not supposed to be pathogenic. However, some caution must be kept in considering these synonymous mutations as neutral, because they might hide cryptic splice sites [36] and/or affect mRNA stability.

In summary, we characterised 9 novel variants and the previously reported E237G mutation within the gene for the beta subunit of the high affinity receptor for IgE (Table 3 and 4). None of them showed a significant association with either atopy or asthma phenotype giving no explanation for the strength of chromosome 11q13 linkage (Table 1), that has been revealed in two very recent genome-wide screens in families from different racial groups [37,38].

Our findings are not yet surprising since there is little doubt that the susceptibility of developing asthma or atopy is influenced not only by one, but by multiple genes that may interact with each other and with environmental factors determining the asthmatic or atopic phenotype. It is unlikely that a single cell type, mediator or cytokine involved in the allergic cascade accounts for all

of the features of allergic inflammation. Novel regions of interest have been identified using genome screening [37,38], which have to be characterised. Further investigations including gene-gene and gene-environment interactions, and a generally accepted phenotype definition will be necessary to understand the complex molecular mechanisms underlying asthma and atopy. The knowledge of these mechanisms will open a variety of potential diagnostic and therapeutic avenues.

Acknowledgments

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