

Single nucleotide polymorphism screening and association analysis – exclusion of integrin $\beta 7$ and vitamin D receptor (chromosome 12q) as candidate genes for asthma

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Summary

Background The human genes coding for integrin $\beta 7$ (*ITGB7*) and vitamin D receptor (*VDR*) are two of the several candidate genes for asthma and related phenotypes found in a promising candidate region on chromosome 12q that has been identified in multiple genomewide screens and candidate gene approaches.

Methods All exons, including parts of the neighbouring introns, and the predicted promoter region of the *ITGB7* gene were screened for common polymorphisms in 32 independent asthmatic and healthy probands, resulting in the detection of two single nucleotide polymorphisms (SNPs) unknown so far. In addition to these SNPs, five already described SNPs of the *ITGB7* and one in the human *VDR* gene were analysed in a Caucasian sib pair study of 176 families with at least two affected children, using matrix assisted laser desorption/ionization time of flight mass spectrometry. All confirmed SNPs were tested for linkage/association with asthma and related traits (total serum IgE level, eosinophil cell count and slope of the dose–response curve after bronchial challenge).

Results Two new variations in the *ITGB7* gene were identified. The coding SNP in exon 4 causes a substitution of the amino acid GLU by VAL, whereas the other variation is non-coding (intron 3). None of the eight analysed SNPs, of either the *ITGB7* or the *VDR* genes, showed significant linkage/association with asthma or related phenotypes in the family study.

Conclusions These findings indicate that neither the human *ITGB7* nor the *VDR* gene seem to be associated with the pathogenesis of asthma or the expression of related allergic phenotypes such as eosinophilia and changes in total IgE level.

Keywords association analysis, asthma, complex disease, *ITGB7*, MALDI-TOF mass spectrometry, SNP, *VDR*

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Introduction

Asthma is the most common chronic disease in children, affecting more than 155 million individuals mainly in industrial nations [1, 2]. For a long time it has been recognized as a typical complex disease with environmental as well as genetic factors involved in its aetiology. Several genomewide screens and fine-mapping approaches for asthma and related phenotypes have indicated a promising candidate

region on chromosome 12q [3–9] (for a review see <http://cooke.gsf.de/asthagen>). Recent fine-mapping approaches verified evidence for linkage on chromosome 12q13–24 for asthma and bronchial hyper-reactivity [10, 11]. This region contains numerous candidate genes for asthma including the genes coding for IFN- γ [12–14], mast cell growth factor 1 (*MGFI*) [15], leukotriene A(4) hydrolase (*LTA₄H*) [16], insulin-like growth factor (*IGFI*) [3], signal transducer and activator of transcription 6 (*STAT6*) [13, 17] and neuronal nitric oxide synthase (*NOS1*) [3, 14, 18] as well as the human genes coding for integrin $\beta 7$ (*ITGB7*) [19, 20] and vitamin D receptor (*VDR*) [21].

A key aspect of leucocyte function is its ability to migrate from the vascular space into extracellular tissue. This occurs by interaction of surface receptors of leucocytes such as integrins and their ligands on the endothelial cell surface. In asthma, much emphasis has been placed on endothelial adhesion receptors since their expression is increased because of the stimulation with cytokines such as IL-1 α and TNF- α . Several integrins such as the $\alpha 4 \beta 1$ integrin very late antigen

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(VLA)-4 and the $\alpha 4 \beta 2$ integrin Mac-1, which are expressed by eosinophils or neutrophils, respectively, are known to bind on vascular cell adhesion molecule (VCAM)-1 on the surface of IL-1-stimulated endothelium, while expression of VCAM-1 on endothelial cells and its ligand VLA-4 on lymphocytes and eosinophils seems to be a specific event in atopic asthma [22]. In addition, especially *ITGB7* is suspected of playing a role in adhesive interactions of leucocytes [23, 24]. Both members of the $\beta 7$ integrin subfamily are expressed primarily by leucocytes, including activated macrophages, subsets of lymphocytes, natural killer cells, mast cells and eosinophils [23–25]. Integrin $\alpha 4 \beta 7$ (LPAM-1) facilitates lymphocyte homing by mediating T cell adhesion to endothelial cell adhesion receptors, such as the mucosal vascular addressin MAdCAM-1, VCAM-1 and ECM molecules [26–28], while integrin $\alpha E \beta 7$ (M290) mediates in particular interaction of T cells with epithelial cells at mucosal surfaces by recognizing E-cadherin [29]. This interaction is mostly important in retention, migration and proliferation of intra-epithelial T cells.

Additionally, $\beta 7$ integrins seem to play a crucial role in other inflammatory diseases such as inflammatory bowel disease [30], Crohn's disease [31] and spondylarthropathies such as reactive arthritis, psoriatic arthritis and ankylosing spondylitis [32].

Therefore, following likely positive linkage results in a previous genome scan, the *ITGB7* gene was investigated in detail in the present asthma linkage study as there was subsequently reported a 25.7-fold ($P = 0.001$) up-regulation of mRNA expression of *ITGB7* in allergy-inducing T helper (Th)2 cells compared with Th1 cells [33]. These data predicted a preferential homing of Th2 cells to mucosal sites [33]. An imbalance in Th1 and Th2 responses can cause chronic inflammatory diseases and allergies respectively [34]. The differentiation process, initiated by triggering of the antigen receptor, is directed by cytokines present at the time of priming [35]. IFN- γ -producing Th1 cells develop in the presence of IL-12, whereas IL-4 induces Th2 cells, which generally secrete IL-4, IL-5 and IL-13 [36].

In this context the human *VDR* gene was also further investigated in the present study as another promising asthma candidate gene on 12q13–14, since the nutritional intake of vitamin D starting in pregnancy and early infancy is suspected of influencing the development of allergic diseases by affecting the Th1/Th2 -balance [37, 38, 8]. Interestingly, calcitriol (1.25(OH) $_2$ D $_3$), one of the principal metabolites of vitamin D, inhibits the secretion of IL-12 [38, 39]. Furthermore, Lemire et al. [37] demonstrated the immunosuppressive action of vitamin D by cholecalciferol-induced inhibition of IFN- γ secretion by a Th1 clone in a dose-dependent manner. Thus, polymorphisms in the *VDR* gene could possibly influence the binding capacity or signal transduction of the vitamin D. The manifold immuno-modulatory effects, which vitamin D exerts with respect to other inflammatory diseases have been reported for diabetes [40, 41], renal diseases [42] and psoriasis [43]. The *VDR* gene structure has been extensively investigated, leading to the identification of an abundance of intronic or untranslated polymorphisms as well as three silent coding ones [44, 45]. Interestingly, the *VDR* gene has two potential translation initiation sites. A T-to-C polymorphism has been noted in the first ATG, abolishing the first translation initiation site and resulting in a peptide

lacking the first three amino acids [46]. This sole protein-modifying *VDR* polymorphism Fok I had been shown to be associated with abnormal glucose metabolism and insulin sensitivity [40]. Moreover, it was found that Fok I was associated with a higher sensitivity to changes in the extracellular ionized calcium and thereby influences the parathyroid function in patients with end-stage renal disease [42]. The physiological *VDR* ligand, 1- α ,25-dihydroxyvitamin D $_3$, acts upon a wide variety of tissues and cells, both related and unrelated to calcium and phosphate homeostasis. The potent anti-proliferative, pro-differentiative and immuno-modulatory activities of 1- α ,25-dihydroxyvitamin D $_3$ in various systems have suggested a wide clinical application of *VDR* ligands in such diverse disease states as inflammation (rheumatoid arthritis, psoriatic arthritis), dermatological indications (psoriasis, photo-ageing and skin rejuvenation), osteoporosis, cancers (breast, prostate, colon, leukaemia and myelodysplastic syndrome) and autoimmune diseases (multiple sclerosis, type I diabetes and systemic lupus erythematosus) [47].

In this study, the predicted promoter region and all exons, including parts of the neighbouring introns, of the *ITGB7* gene were screened for common polymorphisms in 32 independent (16 asthmatic and 16 healthy) probands of a Caucasian population. Subsequently, two thereby newly discovered variations of the human *ITGB7* gene, five already described single nucleotide polymorphisms (SNPs) of the *ITGB7* gene [30] and the sole protein-modifying SNP of the *VDR* gene (Fok I) [46] were analysed for linkage/association with asthma and related traits (total serum IgE level, eosinophil cell count and slope of the dose-response curve after bronchial challenge) in a Caucasian sib pair study of 176 families with at least two affected children (352 parents and 411 children).

Materials and methods

Study probands

SNP screening and genotyping were carried out with DNA from our asthma family study. Caucasian asthma sib pair families have been recruited since the beginning of 1994 as described elsewhere [48]. In addition to these 97 families used in a genomewide scan [8] and a subsequent fine-mapping approach [10], another set of 90 sib pair families was collected and added for candidate gene testing. All 830 participants in the study gave informed written consent. The family structure chosen included at least two asthmatic sibs and both parents, and sometimes further non-affected sibs. All the children were at least 6 years old. Prematurity as well as other severe pulmonary diseases were exclusion criteria for the study. The phenotyping procedure for all 830 participants (children and parents) comprised a detailed interview, skin prick test, blood withdrawal for DNA isolation, total serum IgE, RAST and total cell counts, house dust collection and measurement of lung function as described previously in detail [8, 17].

The SNP screening panel included 32 (16 asthmatic and 16 healthy) independent adult probands. The genotyping panel included 743 individuals: 172 families with up to four children and with a mean family size of 4.3 individuals (range of 4–6), consisting of 344 parents and 399 children. Some descriptive statistics on the phenotype outcomes of the genotyped participants in the study are shown in Table 1.

Table 1. Descriptive statistics on the phenotype outcomes of the genotyped participants in the asthma family study

Phenotype	Children		Parents	
	Asthmatics	Non-asthmatics	Asthmatics	Non-asthmatics
Age	10.7 ± 3.8 <i>n</i> = 355	11.5 ± 4.3 <i>n</i> = 44	39.9 ± 5.8 <i>n</i> = 78	39.9 ± 6.0 <i>n</i> = 266
Sex	Female: <i>n</i> = 153 Male: <i>n</i> = 202	Female: <i>n</i> = 27 Male: <i>n</i> = 17	Female: <i>n</i> = 41 Male: <i>n</i> = 37	Female: <i>n</i> = 131 Male: <i>n</i> = 135
Total IgE	487.6 ± 566.4 <i>n</i> = 249	261.3 ± 446.6 <i>n</i> = 29	212.5 ± 283.5 <i>n</i> = 50	182.3 ± 347.8 <i>n</i> = 195
Eosinophil cell counts	0.47 ± 0.38 <i>n</i> = 326	0.27 ± 0.23 <i>n</i> = 43	0.24 ± 0.18 <i>n</i> = 70	0.19 ± 0.16 <i>n</i> = 244
Slope	0.07 ± 0.05 <i>n</i> = 183	0.04 ± 0.03 <i>n</i> = 25	0.06 ± 0.04 <i>n</i> = 36	0.02 ± 0.03 <i>n</i> = 155

Approval

The collection of serum and DNA material and the experiments themselves were approved by the ethics commission of 'Nordrhein-Westfalen', Germany. Written informed consent was given by all participants or their parents.

DNA preparation

DNA was partly extracted from leucocytes as described previously [8], partly by using the PuregeneTM DNA Isolation Kit (Gentra Systems, Plymouth, MN, USA) according to the manufacturer's recommendation.

Single nucleotide polymorphism screening

For polymorphism detection in the human *ITGB7* gene sequence analysis of defined PCR products of the promoter region and all exons was performed. Two overlap PCR products covered the promoter region from 462 to 1162 bp (according to AF 257803 of the NCBI database), which was predicted by the software PromoterInspector (Genomatix Software GmbH, Munich, Germany). All other PCR fragments contained one or two entire exons plus up to 200 bp of the neighbouring introns. All PCR and sequencing primers (Table 2) were designed using the Vector NTI suite 6.0 (www.informaxinc.com). The screening panel included 16 asthmatic and 16 healthy independent adult probands.

PCR was carried out in a volume of 50 µL (20 µL) containing 80 ng (30 ng) of DNA template, 25 pmol (5 pmol) of each PCR primer, 1.5 mM (2.5 mM) MgCl₂, 0.2 mM (0.2 mM) of each dNTP, 0.5 U Taq-polymerase (0.1 U Thermo Start Taq-polymerase), (Abgene, Hamburg, Germany) and the buffer recommended by the supplier (all from MBI Fermentas, Vilnius, Lithuania). Thermocycling started with a single denaturation step for 5 min (15 min) at 95 °C, followed by 35 cycles of denaturation for 1 min at 95 °C, annealing for 1 min and extension at 72 °C for 1 min. One final extension step was added for 10 min at 72 °C. Purification of the PCR products was performed manually either using the QIAquick PCR purification kit according to the manufacturer's protocol (Qiagen, Hilden, Germany), the MontageTM PCR 96 Clean up kit according to the manufacturer's protocol (Millipore, Schwalbach, Germany), or on the BIOMEK 2000 robotic system (Beckman Coulter Inc., Fullerton, CA, USA) using the Nucleo-Spin Robot 96-

B-Extract-Kit (Macherey-Nagel GmbH & Co. KG, Dueren, Germany). Samples were eluted with 50 µL elution buffer (Macherey-Nagel GmbH & Co. KG). Sequencing reaction and purification of the reaction products were performed using the ABI PRISM[®] Big DyeTM v 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the MontageTM SEQ96 Sequencing Reaction Cleanup Kit according to the manufacturer's protocol (Millipore).

Cycle sequencing of the purified PCR products was performed partly by a third party (MWG, Ebersberg, Germany), partly on the Capillary Sequencer CEQ 2000 (Beckmann Coulter) or the ABI prism 3100 Genetic Analyser following a standardized protocol (Applied Biosystems). All sequences were analysed using the alignment program of the Vector NTI database (Vector NTI suite 6.0, www.informaxinc.com).

Single nucleotide polymorphism genotyping

For polymorphism analysis DNA was subjected to PCR and homogenous MassEXTEND reaction (hMETM, Sequenom Inc., San Diego, CA, USA). Genotyping was performed using MALDI-TOF MS (matrix-assisted laser desorption/ionization time of flight mass spectrometry) with little modification according to Buetow et al. [49] (Biflex mass spectrometer, Bruker Daltonik GmbH, Bremen, Germany; MassArrayTM, Sequenom Inc.). The genomic DNA of each subject was tested individually.

PCR was performed under standard conditions for all assays. Genomic DNA (2.5 ng) was amplified in a 5 µL reaction including 0.1 U of Hot Star Taq Polymerase (Qiagen), 5 nmol MgCl₂, 200 µmol of dNTP (both from MBI Fermentas), 2 pmol of each PCR primer (Table 3), using 384-well microtitre plates. Thermocycling started with an initial denaturation step at 95 °C for 15 min followed by 45 cycles of 95 °C for 20 s, annealing for 30 s (Table 3), and extension at 72 °C for 1 min. One final extension step was added for 3 min at 72 °C.

Two microlitres of 15% shrimp alkaline phosphatase (SAP) (USB Corporation, Cleveland, OH, USA) solution was added to the PCR products to dephosphorylate non-incorporated dNTPs. The temperature conditions were 37 °C for 10 min followed by the inactivation of SAP at 85 °C for 5 min. Subsequently, homogenous MassEXTEND reactions (hMETM) were performed by adding a mixture of dideoxy- and deoxynucleotides according to the assay design (dACG/

Table 2. Primer for *ITGB7* mutation screening

Region	Primer sequence	Annealing temperature (°C)	Length of PCR product in bp
Promoter (–572 to –142)	5'-AAGAATATAAAACGTTGCTGGGC-3' 5'-AACCCCTGTCATGTTGAAC-3'	60	431
Promoter (–239), exon 1	5'-TCTGTAAAATTGGGGCTTGG-3' 5'-TATCCAGGTCAGAGCCCTTTC-3'	60	404
Exon 2	5'-CCTGCAGAGAGTTCTTGTGG-3' 5'-ATTTACCGAGATCCCAAGCC-3'	60	345
Exon 3	5'-GAGGACTGAGAGTTTGAGAG-3' 5'-GACCATAGGCCCATCTTTAC-3'	56	288
Exon 4	5'-GGAGTTCAAGGGTAGCTGTGG-3' 5'-CATTGGAACGCCAGCCTAGAC-3'	60	421
Exon 5	5'-GTCTAGGCTGGCGTTCCAATG-3' 5'-GGGCAGTGGTTGAATAGGCAAG-3'	60	281
Exon 6	5'-CTCATGGGTCCTCTGTTGCTG-3' 5'-CCATAAGGGGCAGTCCTGTTC-3'	59	370
Exon 7	5'-CCTACCACCCCACTGTAAC-3' 5'-GAGGCAGGGTGACAATAGGG-3'	60	209
Exons 8, 9	5'-CCTATTCCATAGCAAGCCTCC-3' 5'-CTTCCACCTTTCCCTAGTGC-3'	57	538
Exon 10	5'-TGGGGTTGGAGTCCTTTTCTG-3' 5'-CCTGCCTGCTTAATTTCCAC-3'	56	272
Exon 11	5'-GGATGATTGGTGAGTTGGGG-3' 5'-TGGTTGAAGGAAGAGGAGG-3'	56	293
Exon 12	5'-TTGGGTTTAGAGGTTCTGG-3' 5'-CTAACCCATACACCGGAATC-3'	63	424
Exon 13	5'-GATTGGATGCGTGGGCTTGTC-3' 5'-GCTCCAAGATGCAAGACCTG-3'	56	337
Exon 14	5'-CTCTCCGTCCTGCTGTAGTTG-3' 5'-GAGACCCTGCCCATCAAATTC-3'	59	341
Exon 15	5'-ATGCTCCAGCTCCCAAGTG-3' 5'-GAGGAATCAGGGCTGGTCTTG-3'	59	271
Exon 16	5'-CTGATTCCTCCCACTGGGTTTC-3' 5'-GGCAAGAGGAGGATGGACAAG-3'	55	571

bp; base pairs.

ddTTP, dACT/ddGTP, and dCGT/ddATP, respectively (Table 3)). The reaction volume was 10 µL including 1 U of Thermosequenase (Amersham Pharmacia, Freiburg, Germany), 50 µmol of the respective termination mix (Table 3), and 10 pmol of test-specific MassEXTEND primer (Table 3), complementary to the template at a region directly adjacent to the SNP site. A universal temperature program was applied (95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 52 °C for 5 s and 72 °C for 5 s). Primer extension proceeded until a first dideoxynucleotide was incorporated and the reaction terminated. As the termination point and number of nucleotides were sequence specific, the size of the extension products generated was used to identify the SNP variants. Fifteen nanolitre of desalted (SpectroCLEAN resin, Sequenom Inc., San Diego, CA, USA) hME products were finally spotted onto a 384 SpectroCHIP microarray (Sequenom Inc.) for fully automated mass spectrometry analysis. (Software: XACQ 4.0, Bruker Daltonik GmbH, MassArray™, Sequenom Inc., San Diego, CA, USA).

Statistical analysis

Mendelian inheritance was tested by the SIB-PAIR program release 0.996 [Duffy 2000 (<http://www.qimr.edu.au/davidD/>

<http://www.qimr.edu.au/davidD/davidd.html>)]. For qualitative traits (cut-offs: asthma: yes/no; eosinophil cell count: 20th percentile; IgE: 200 kU/L; slope of the dose–response curve of the methacholine challenge: 0.7) the Reconstruction combined-transmission/disequilibrium test (RC-TDT) [50] was used to test for linkage in the presence of association within families. When parental genotypes could not be determined, the RC-TDT used genotype data of sibships with both affected and unaffected offspring to reconstruct missing parental genotypes and corrected for the bias resulting from reconstruction [50]. Exact *P*-values were given. The quantitative traits (logarithmed total serum IgE, eosinophil cell count and slope of the dose–response curve after methacholine challenge) were analysed using the quantitative transmission/disequilibrium test (Q-TDT) on an extended orthogonal decomposition model [51] using genotypes of all family members and incorporating age and gender as covariates. To test for linkage disequilibrium (LD), empirical *P*-values to the likelihood ratio statistic were obtained conducting 1000 permutations. The LD chart of the *ITGB7* region shown in Fig. 2 was produced using the GOLD 1.0 program [52]. The statistical power of the TDT for complex disease genes was estimated using the TDT Power Calculator Version 1.2.1. [53].

Table 3. PCR and MassEXTEND primer for SNPs in the *ITGB7* and *VDR* genes

SNP name	Position	PCR primer sequence	Annealing temperature (°C)	Length of PCR product (bp)	MassEXTEND primer sequence	HME stop Mix
<i>ITGB7</i> rs1554753	Promoter	5'-ACGTTGGATGACTTCAATGAGACTCCTTCCC-3' 5'-ACGTTGGATGTTTCAGAGGTCTGTAAGCCTG-3'	62	104	5'-TGGACTCCTTCCCAGAGAA-3'	ACT
<i>ITGB7</i> rs4919732	Promoter	5'-ACGTTGGATGCTTACTCCACATCCTCAAC-3' 5'-ACGTTGGATGCTGCTTCCCTACCTTGTTC-3'	66	100	5'-TAACCACAGGTCAACCAATGAAGA-3'	ACT
<i>ITGB7</i> 241 [T/A]	Promoter	5'-ACGTTGGATGAGATTGGGCTTTTGGAGAG-3' 5'-ACGTTGGATGCTTCAAGATCCCTGAC-3'	58	100	5'-GGTACTGTGAACACTGGAGG-3'	CGT
<i>ITGB7</i> 9278 [C/T]	Intron 3	5'-ACGTTGGATGCTGCTCAGCAAGAAAAAGGC-3' 5'-ACGTTGGATGGGTTTGACGGGTGTTTTTGG-3'	67	100	5'-GGACCCGGTCTGTCATAG-3'	ACT
<i>ITGB7</i> 9410 [A/T]	Exon 4	5'-ACGTTGGATGAGCAGAACTTCAACCGCTCG-3' 5'-ACGTTGGATGCTCGAGCACCTCCTGCTG-3'	71	147	5'-GCCCGCTGGAGAGAGCTGG-3'	CGT
<i>ITGB7</i> 11546 [T/C]	Intron 7	5'-ACGTTGGATGATATTGCTGCAGAGAGGGC-3' 5'-ACGTTGGATGAGGGCACCTTGCATGTTGTG-3'	68	134	5'-CAGAAAGGTAGTCTCTGGG-3'	ACT
<i>ITGB7</i> rs2272300	Intron 11	5'-ACGTTGGATGCAATGTGGTGTATGCAGGTG-3' 5'-ACGTTGGATGCCAGGGTTGGTTGTTG-3'	54	93	5'-GCCTCCTCTTCCCTTCA-3'	CGT
<i>VDR</i> 117 [C/T]	Exon 2	5'-ACGTTGGATGTGGCCTGCTGCTGTTCTTA-3' 5'-ACGTTGGATGTTCCGGTCAAGTCTCCAGG-3'	68	101	5'-CTTGCTGTTCTTACAGGG-3'	ACG

SNP, single nucleotide polymorphism; bp, base pairs; ITGβ7, integrin β7.

Statistical analysis was performed with SAS release 8.1 (data handling, basic descriptions, RC-TDT), the QTD package [51] for analysis of the quantitative traits, J. H. Zhao 2-locus LD calculator, version 1.00, for the analysis of pairwise LD and SIB-PAIR release 0.996 (Mendel check).

Results

Single nucleotide polymorphism screening and single nucleotide polymorphism discovery

To discover common variants in the human *ITGB7* gene, sequencing was carried out of the predicted promoter region (701 bp from 462–1162 according to NCBI database AF 257803, respectively, from base pair – 572 up to exon 1) and all exons 1–16, including 200 bp of the neighbouring introns of the gene in 16 unrelated healthy and 16 asthmatic probands of a Caucasian population. Using this approach, two new SNPs in the *ITGB7* gene were identified, as shown in Fig. 1, together with 11 additional SNPs already known from a public SNP database (www.ncbi.nlm.nih.gov/SNP) and a publication [30]. One of the novel variants is non-coding: 9278[C/T] in intron 3; whereas the SNP 9410[A/T] in exon 4 causes a substitution of the amino acid GLU by VAL.

Single nucleotide polymorphism genotyping, allelic frequencies and linkage disequilibrium

For further association analysis, both variations of the *ITGB7* gene found by sequence analysis, plus five already described polymorphisms of the *ITGB7* gene [30], three of them in the predicted extended promoter region (Fig. 1) and one already known SNP (Fok I) in the human *VDR* gene [46], were genotyped within the sib pair asthma study (176 families with 352 parents and 411 children) using MALDI-TOF mass spectrometry, and allele frequencies were determined. All alleles showed Mendelian inheritance (data not shown).

Genotyping revealed the following parental allele frequencies of the coding SNP 9410[A/T]: 97.4%/2.6% ($n = 646$ examined) and of the non-coding SNPs 11546[T/C] (intron 7, alias SNP7 in Van Heel et al. [30]): 92.9%/7.1% ($n = 666$ examined) and rs2272300[A/C] (intron 11): 90.1%/9.9% ($n = 624$ examined) of the *ITGB7* gene. The silent intron 3 polymorphism 9278[C/T] of the *ITGB7* gene, determined with 3% frequency of the T-allele in the initial screening population, was not polymorphic in the asthma family parents ($n = 648$). For this reason, no association analysis was performed with regard to this polymorphism.

The extended promoter region polymorphisms of the *ITGB7* gene showed the following parental allele frequencies SNP rs1554753[T/C], 79.5%/20.5% ($n = 668$ examined) and of the SNP 241[T/A] (alias SNP18 in Van Heel et al. [30]), 91.0%/9.0% ($n = 630$ examined). The public database SNP rs4919732[G/A] was not polymorphic in the asthma family study ($n = 705$). Therefore, no association analysis was performed with regard to this polymorphism.

For the SNP 117[C/T] (Fok I) in the human *VDR* gene, a parental allele frequency was determined of [C/T], 65.7%/34.3% in our Caucasian asthma family study ($n = 664$), which strongly varies from the allele frequency of [C/T], 48.1%/51.9%, found in a Japanese population by Arai et al. [46].

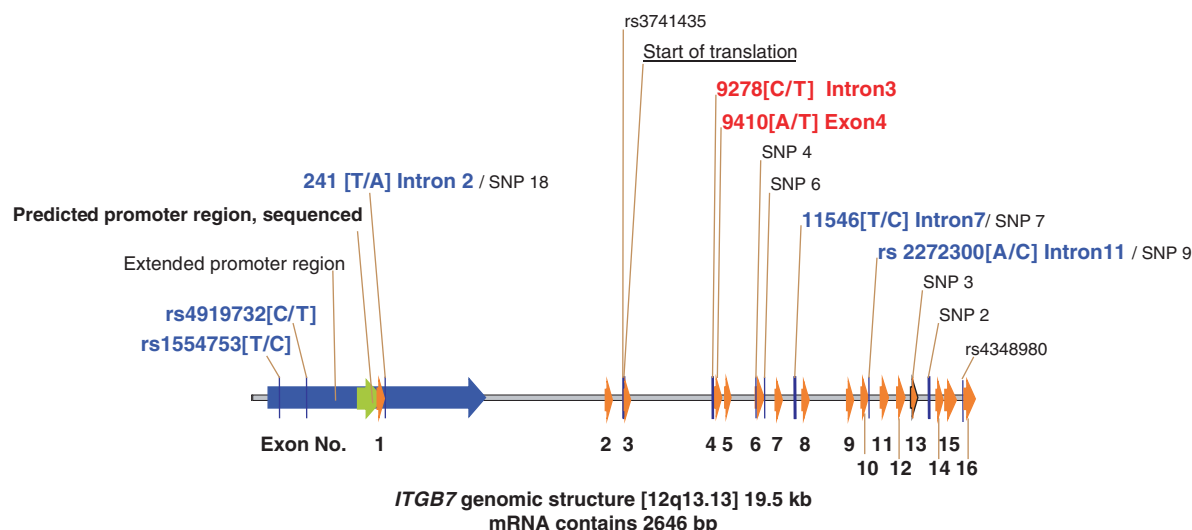


Fig. 1. Gene structure of the human integrin $\beta 7$ (*ITGB7*) gene on chromosome 12q13.13: recently discovered single nucleotide polymorphisms (SNPs) are highlighted in red, additionally genotyped SNPs are highlighted in blue, further SNPs from the NCBI databank (by July 2003) are typed in black, SNP Nos. 2, 3, 4, 6, 7, 9 and 18 refer to Van Heel et al [30].

Table 4. Pairwise linkage disequilibrium between the SNPs of the *ITGB7* gene

D' (standard deviation)	rs1554753[T/C]	241[T/A]	9410[A/T]	11546[T/C]	rs2272300[A/C]
rs1554753[T/C]	–	0.900 (0.045)	0.343 (0.153)	0.394 (0.090)	0.362 (0.078)
241[T/A]		–	0.008 (0.079)	0.562 (0.323)	0.332 (0.326)
9410[A/T]			–	1.000 (no value)	1.000 (no value)
11546[T/C]				–	0.945 (0.037)
rs2272300[A/C]					–

Disequilibrium coefficients D' and standard deviations (in parentheses) are given.

The results of the pairwise LD analysis between the SNPs of the *ITGB7* gene are given in Table 4 and illustrated in Fig. 2. Two distinct blocks of strong LD were discovered, between the two SNPs 11546[T/C] and rs227320[A/C], and between the SNPs 9410[A/T], rs1554753[T/C] and 241[T/A] of the *ITGB7* gene. Remarkably, the only coding SNP 9410[A/T] is in strong LD with the latter block. Due to a haplotype frequency estimated as zero, the analysis program estimates perfect LD ($D' = 1$) between the coding SNP 9410[A/T] and the SNPs rs1554753[T/C] and 241[T/A], respectively. However, all the minor allele frequencies involved are rather low.

Linkage analysis and association results

Linkage and association analysis of asthma and related phenotypes were tested for the polymorphisms rs1554753[T/C], 241[T/A], 9410[A/T], 11546[T/C] and rs2273200[A/C] of the *ITGB7* gene, as well as for the SNP 117[C/T] of the human *VDR* gene. Table 5 represents the P -values of the linkage and association analysis. The following numbers of families with up to four children were analysed by RC-TDT for association between the SNP genotypes and different phenotypes (with respect to the SNPs rs1554753[T/C], 241[T/A], 9410[A/T], 11546[T/C], rs2273200[A/C] and SNP117[C/T]): Asthma (161, 142, 153, 161, 139 and 152), IgE (82, 76, 77, 82, 73 and 76), eosinophil cell count (45, 41, 42, 45, 39 and 43) and slope of the dose–response curve of the methacholine challenge (60, 53, 55, 59, 51 and 57). The RC-TDT for

qualitative traits (cut-offs: asthma: yes/no; eosinophil cell count: 20th percentile; IgE: 200 kU/L; slope of the dose–response curve of the methacholine challenge: 0.7) revealed no linkage in the presence of association within the families. These findings were confirmed by Q-TDT for quantitative traits as logarithmed total serum IgE (numbers of analysed families with respect to rs1554753[T/C], 241[T/A], 9410[A/T], 11546[T/C], rs2273200[A/C] and SNP117[C/T], respectively, 121, 120, 122, 122, 119 and 120), eosinophil cell count (164, 162, 165, 165, 161 and 163, respectively) and slope of the dose–response curve of the methacholine challenge (103, 104, 105, 104, 103 and 103, respectively). Because of the multiple testing problem, the P -value of 0.02 for the association between SNP 241[T/A] and slope of the dose–response curve of the methacholine challenge must not be overestimated.

To summarize, this family study did not show evident linkage or association between any of the SNPs and asthma, total serum IgE levels, eosinophil cell count or slope of the dose–response curve after methacholine challenge.

Discussion

The aim of this study was to investigate a possible association between asthma or related phenotypes, and the human genes coding for *ITGB7* and *VDR*, which are two of several genes in a promising candidate region on chromosome 12q13–24 [8, 10, 33, 38]. These two genes were chosen because they

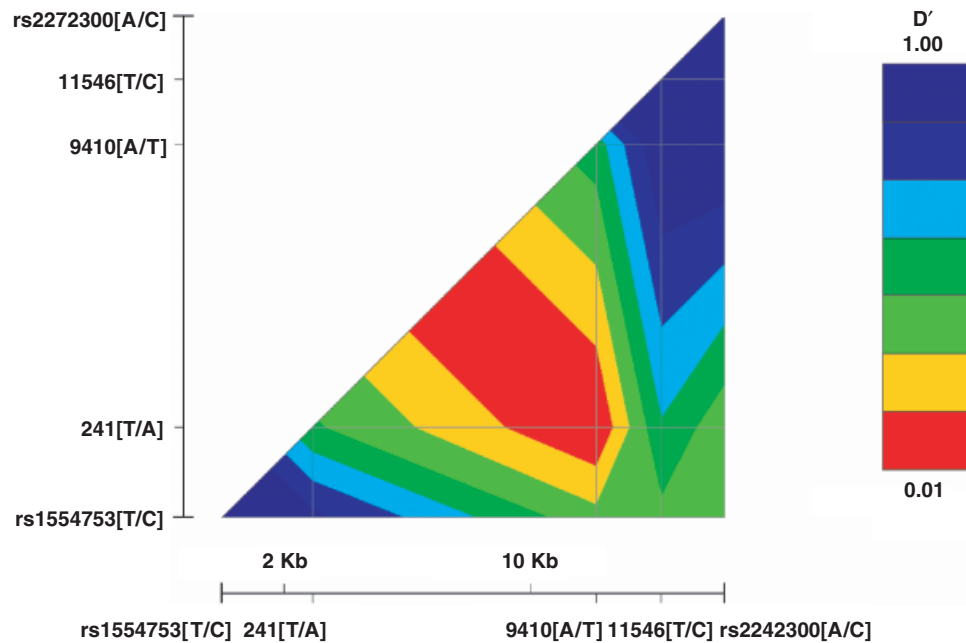


Fig. 2. Gold plot of pairwise linkage disequilibrium between the single nucleotide polymorphisms (SNPs) of the human integrin $\beta 7$ (*ITGB7*) gene. The colour-coded dimension units D' of LD are given in the legend.

Table 5. P -values of RC-TDT and Q-TDT for linkage/association between SNPs and asthma or related phenotypes

	<i>ITGB7</i>										<i>VDR</i>	
	9410[A/T]		11546[T/C]		rs1554753[T/C]		241[T/A]		rs2272300[T/G]		117[C/T]	
	RC-TDT	Q-TDT	RC-TDT	Q-TDT	RC-TDT	Q-TDT	RC-TDT	Q-TDT	RC-TDT	Q-TDT	RC-TDT	Q-TDT
Asthma	0.265		0.187		0.370		0.943		0.220		0.405	
Total IgE	0.791	>0.1	0.868	>0.1	0.456	>0.1	0.833	>0.1	0.875	>0.1	0.777	>0.1
Eosinophil cell counts	1.000	>0.1	0.804	>0.1	0.690	>0.1	1.000	>0.1	0.302	>0.1	1.000	>0.1
SLOPE	0.727	>0.1	1.000	>0.1	0.888	>0.1	0.074	0.02	0.829	>0.1	0.907	>0.1

Cutoffs of qualitative traits: asthma: yes/no; eosinophil cell count: 20th percentile; IgE: 200 kU/L; slope of the dose-response curve of the methacholine challenge: 0.7.

Q-TDT, quantitative transmission/disequilibrium test; RC-TDT, reconstruction combined transmission/disequilibrium test.

possibly play an important role in the development and exacerbation of the disease with particular respect to lymphocyte homing and immune modulation by influencing the Th1–Th2 homeostasis.

By screening the *ITGB7* gene for common polymorphisms (especially SNPs) in a panel of independent healthy and asthmatic probands of a Caucasian population, two further SNPs were discovered, one in a coding region (exon 7), causing an exchange of amino acid GLU by VAL, and one silent variation in intron 3. The coding variant was confirmed together with four already published SNPs in the extended promoter region and in introns of *ITGB7* and one additional SNP of the *VDR* gene within the Caucasian study sample ($n = 763$). Because of the high genotyping rates and robust assays, all the genotyping results were considered to be exceedingly accurate and genotyping errors very unlikely. As one result, rather low minor allele frequencies of the SNPs of the *ITGB7* gene ($< 10\%$) were obtained, except the C allele of the SNP rs1554753[T/C], which showed 20.5%. These findings agreed with those of Van Heel et al. [30] who reported predominantly allele frequencies less than 10% in a

Caucasian healthy control population with the exception of the 10% allele frequency (vs. 9% in this family study) of the minor allele of SNP 241[T/A].

In contrast, in the present study a more frequent minor allele frequency of the *VDR* polymorphism (34%) was determined. Because of the generally known ethnic variations [54], the allele frequencies detected in this Caucasian study population differed greatly from the one in Asians (48%) detected by Arai et al. [46].

Furthermore, by means of pairwise LD analysis, we detected strong LD over 2924 bp between the extended promoter SNP rs1554753[T/C] and SNP 241[T/A] in intron 2 as well as over 4180 bp between the SNPs 9410[A/T] (exon 4), 11546[T/C] (intron 7) and rs2272300[A/C] (intron 11) of the *ITGB7* gene. The analysis program estimated perfect LD ($D' = 1$) between the coding SNP 9410[A/T] and the SNPs rs1554753[T/C] and 241[T/A] respectively, because of a haplotype frequency estimated as zero. However, these estimates must be interpreted carefully, as the minor allele frequencies involved are very low. Nevertheless, the only coding SNP 9410[A/T] (exon 4) could be a phylogenetic

young variant, as its low allele frequency of only 2.6% would indicate, and those SNPs might lie in conserved haplotypes that extend across haplotype blocks [55].

In addition, these genotyped SNPs were tested for linkage and association with asthma, total serum IgE levels, eosinophil cell count, and slope from the resulting dose-response curve after methacholine challenge within the 176 Caucasian asthma families. Concerning study performance, high accuracy was obtained. For this reason, the wrong classification of healthy and asthmatic family members because of the age dependency of asthma outcome or differences in allergen exposure between affected and unaffected sibs, can be precluded. However, considering the multiple testing adjustment, none of the SNPs of the *ITGB7* or the *VDR* genes, showed significant linkage or association with asthma or related phenotypes in this family study. From these findings, it is concluded that the SNPs of both the human *ITGB7* and the *VDR* genes that were tested here do not seem to influence the pathogenesis of asthma or the expression of atopic asthmatic phenotypes such as eosinophilia and changes in total IgE levels in the present study population.

The results obtained do not affirm the up-regulation of *ITGB7* mRNA expression in Th2 cells discovered by Rogge et al. [33]. Since, like Van Heel et al., we did not discover any variation in the promoter region causing the up-regulation of *ITGB7* mRNA expression in Th2 cells, this may mean that another gene than *ITGB7* is responsible for the imbalance in Th1 and Th2 in asthma. Nevertheless, the promoter region of the human *ITGB7* gene is highly conservative to the mouse *ITGB7* promoter sequence, which suggests an important function. In particular, the Genomatix program predicted two binding sites of interest for the mammalian caudal-related intestinal transcription factor *cdx2* and the special AT-rich sequence-binding protein 1 (STAB1) in the *ITGB7* promoter. As *cdx2* regulates proliferation and differentiation of epithelial gut cells, an *ITGB7* promoter SNP at the binding site of *cdx2* could be relevant in inflammatory bowel disease or Morbus Crohn, whereas STAB1, which binds at cell-specific nuclear matrix attachment regions, is mainly expressed in thymocytes. Again, here is a conceivable link between the *ITGB7* promoter and the imbalance between Th1 and Th2.

In addition to the possibility of no association existing between the candidate genes investigated and the outcome of asthma, there are several aspects that might have influenced the negative outcome. The design of an affected sib pair study was given by the precluding genome scan and fine-mapping analysis. A different design might be preferable for some aspects, as outlined below. Given the family structure, the RC-TDT was chosen to analyse the qualitative trait asthma using all the siblings to gain more statistical power, thus testing for linkage in contrast to the classical TDT, which selects trios to test for LD and thereby loses a great amount of information. The Q-TDT was also performed for quantitative traits, which is a test for association and linkage and also considers genotype information of all the children. In addition, possible confounding factors, such as age or sex, were implemented. However, neither qualitative nor quantitative statistics revealed any association or linkage between *ITGB7* or *VDR* and asthma or related traits. This could also

be because of the low number of informative families that participated in the tests. One reason for this might be the low frequency of the minor alleles.

Although a considerably large number (176) of affected sib pair families with 352 parents and 411 children was analysed, the question remains whether the sampling size provided enough power depending on the allele frequency of the SNPs. Presuming a multiplicative inheritance model, a prevalence of asthma of 7% and a genotypic relative risk of 1.8, a power of 98% was obtained for the 117[C/T] polymorphism of the *VDR* gene, 96% for the highly polymorphic *ITGB7* SNP rs1554753[T/C], 70% for 241[T/A], 74% for rs2272300[A/C], and 64% for the less frequent SNP 11546[T/C] of the *ITGB7* gene, whereby a strong (fourfold) Bonferroni-adjusted level of significance of $\alpha = 0.001$ was assumed. This was adapted to multiple testing analysis of up to 50 SNPs while only six were tested, with four phenotypes each. In contrast, because of its extremely low allele frequency in this population, the power of the rare coding SNP 9410[A/T] *ITGB7*-SNP accounted for only 16%. All power estimates were derived by the method and program of Chen and Deng [53].

On the other hand, as asthma is a complex disease influenced by many vague or unknown factors, these estimates are prone to be too optimistic. Firstly, the genotypic relative risk of a single locus might be lower than the 1.8 used in the power calculations, e.g. under the multiplicative model above but at a genotypic relative risk of 1.5 power estimates are 67% (117[C/T]), 53% (rs1554753[T/C]), 22% (rs2272300[A/C]), 19% (241[T/A]), 16% (11546[T/C]) and 3% (9410[A/T]). Moreover, the putative disease alleles might not act multiplicatively; under the recessive model, the power estimate even for the 117[C/T] polymorphism of the *VDR* gene is as low as 21%. Risch states that the power of multiplex affected sibships vs. singletons is diminished for traits with high background heritability [56]. If the SNPs are in LD with a putative disease locus and not directly involved in disease aetiology the power of the TDT decreases, even more so if the allele frequencies differ between disease and marker loci [57]. Environmental factors play an important role in the development of asthma. Guo investigates the influence of environmental factors on the performance of the TDT and suggests using affected sibs discordant for a strong environmental factor [58]. However, this suggestion seems problematical, as asthma is influenced by a high number of environmental factors. In the present study, the covariables sex and age were incorporated in the analysis of the quantitative traits, but the model might still be too simplistic. Thus, and because of the study size, the permutation procedure was employed for the Q-TDT to avoid bias and accept a slight loss of power [51]. For the analysis of the quantitative traits, the asthma study design has the disadvantage that these traits are correlated with the asthma affection status, while for quantitative traits the selection of extremely discordant sibs enhances power [59]. Nevertheless, higher power was expected when analysing these traits as quantitative variables compared with the TDT analysis of the dichotomized traits – where cut-off values were rather arbitrary and up to two-thirds of the samples were dropped from analysis.

Even though a causative contribution of these candidate genes to the pathology of asthma is unlikely in this Caucasian

population, it is possible that the influence of *ITGB7* and *VDR* on asthma and related traits varies in different populations, as asthma and allergy are multifactorial diseases, characterized by high genetic heterogeneity among different ethnic groups.

To fully elucidate the role of these candidate genes in the development of asthma and allergic diseases, additional genetic and epidemiological studies are required. Taken together, it does not seem reasonable to replicate the association studies presented here. The results lead us to suspect there are most likely other more promising candidate genes in the region on chromosome 12q13–14 such as *STAT6* and *NOS1*, to which the auspicious results of the genome scans could be traced back. Nevertheless, for the same reason it is considered useful to present these negative association results.

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