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ASTHMA CANDIDATE GENES IN THE FINNISH POPULATION

Paula Kauppi

Academic Dissertation

To be publicly discussed with the permission of the Faculty of Medicine, University of Helsinki, in the lecture hall 2 of Meilahti Hospital, Haartmaninkatu 4, Helsinki, on June 11th, 2001, at 12 noon.

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I Kauppi P., Laitinen L.A., Laitinen H., Kere J., and Laitinen T. Verification of self-reported asthma and allergy in subjects and their family members volunteering for gene mapping studies. *Respir. Med.* 1998, **92**; 1281-1288.

II Laitinen T., Kauppi P., Ignatius J., Ruotsalainen T., Daly M.J., Kääriäinen H., Kruglyak L., Laitinen H., de la Chapelle A., Lander E.S., Laitinen L.A., and Kere J. Genetic control of serum IgE levels and asthma: linkage and linkage disequilibrium studies in an isolated population. *Hum. Mol. Genet.* 1997, **6**; 2069-2076.

III Kauppi P., Laitinen T., Ollikainen V., Mannila H., Laitinen L.A., and Kere J. IL9R region contribution in asthma is supported by genetic association in an isolated population. *Eur. J Hum. Genet.* 2000, **8**; 788-792.

IV Laitinen T., Ollikainen V., Lázaro C., Kauppi P., de Cid R., Antó J.M., Estivill X., Lokki H., Mannila H., Laitinen L.A., and Kere J. Association study of the chromosomal region containing the FCER2 gene suggests it has a regulatory role in atopic disorders. *Am. J. Respir. Crit. Care Med.* 2000, **161**; 700-706.

V Kauppi P., Lindblad-Toh K., Sevon P., Toivonen H.T.T., Rioux J.D., Villapakkam A., Laitinen L.A., Hudson T.J., Kere J. and Laitinen T. A second-generation association study on the 5q31 cytokine gene cluster and IL4RA gene in asthma (submitted).

Some additional unpublished data are presented.

ABBREVIATIONS

A	Alanine
ADBR2	β2-adrenoreceptor
bp	Base pair
BHR	Bronchial hyperreactivity
BRCA1	Breast cancer, type 1
BRCA2	Breast cancer, type 2
C	Cysteine
CBAVD	Congenital bilateral absence of the vas deferens
CC16	Clara cell secretory protein 16
CD14	Monocyte differentation antigen
cNOS	Constitutive nitric oxide synthase
COAG	Consortium on Asthma Genetics
CSGA	The Collaborative Study on the Genetics of Asthma
cSNP	single nucleotide polymorphism on coding region
CFTR	Cystic fibrosis transmembrane conductance regulator
cM	CentiMorgan
COPD	Chronic obstructive pulmonary disease
DNA	Deoxyribonucleid acid
DP	Prostaglandin receptor
E	Glutamic acid
ECP	Eosinophil cationic protein
FCER1B	Encoding gene for the β chain of the high-affinity receptor for IgE
FcεRIβ	β chain of the high-affinity receptor for IgE
FCER2	Encoding gene of the low-affinity receptor for IgE
FcεRII	Low-affinity receptor for IgE
FEV1	Forced expiratory volume in one second
FGFA	Fibroblast growth factor acidic
G	Glutamine
GCR	Glucocorticoid receptor
GM-CSF	Granulocyte-macrophage colony stimulating factor
HLA	Human leucocyte antigen
HPM	Haplotype Pattern Mining
IgE	Immunoglobulin E
I	Isoleucine
IL3	Interleukin 3
IL4	Interleukin 4
IL4Rα	Interleukin 4 receptor alpha chain
IL4RA	Interleukin 4 receptor alpha chain encoding gene
IL5	Interleukin 5
IL5R	Interleukin 5 receptor
IL9	Interleukin 9
IL9Rα	Interleukin 9 receptor alpha chain
IL9RA	Interleukin 9 receptor alpha chain encoding gene

IL10	Interleukin 10
IL13	Interleukin 13
IFN-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
IRF-1	Interferon regulatory factor 1
L	Leucine
LD	Linkage disequilibrium
LM-SBE	Length-multiplexed single-base extension
LTC4S	Leucotrien C4 synthase
M	Methionine
MCH class II	Major histocompatibility complex class II
mRNA	Messenger ribonucleid acid
OVA	Ovalbumin
P	Proline
PAR	Pseudoautosomal region
PEF	Peak expiratory flow
PGD2	Prostaglandin D2
Q	Glutamine
R	Arginine
S	Serine
SII	Social Insurance Institution
SNP	Single nucleotide polymorphism
T	Threonine
TDT	Transmission disequilibrium test
TGF-β	Transforming growth factor β
Th_0	T-helper-0-type
Th_2	T-helper-2-type
V	Valine

ABSTRACT

Based on Finnish twin studies, genetic effect on asthma and atopy has been estimated to be 35 % - 87 %. For a genetic study on atopy and asthma, self-reported asthma patients and their family members (1015 individuals) were ascertained in the Kainuu province, representing a Finnish founder population. Founder populations like this have been suggested as ideal when studying genetic susceptibility to complex disorders. Phenotyping was done with questionnaires, interviews, evaluation of the medical records with patients' permission, and serum total IgE and allergy screening test measurements. 401 self-reported asthma patients were confirmed to have asthma according to information obtained in medical records and granted reimbursement for anti-asthmatic medication by the Social Insurance Institution. Family members were screened for atopic and asthmatic symptoms with questionnaires and serum total IgE and allergy screening test measurements. The self-reported allergic nasal symptoms and self-reported physician diagnosed rhinitis were then compared with allergy screening test results. Sensitivity and specificity of both self-reported symptoms and diagnosed rhinitis remained poor and an objective verification of allergy (high serum total IgE level or positive allergy screening test) was concluded to be better than self-reported data for the allergic phenotype.

Chromosomal regions 5q31-q33, IL4RA, 19p13 and Xq/YqPAR were studied using linkage and association analyses. All the linkage analyses remained negative, yet on the chromosome 19p13, around the FCER2 gene, a six-marker haplotype was found to be associated with high serum total IgE level (>100 kU/l) and on the chromosome Xq, a two-marker haplotype was shown to be associated with asthma. In the first analysis on the chromosome 5q with microsatellites, no significant haplotype association was found, although there was a clustering of haplotypes around the IL9 gene. When the 5q31-q33 was reanalyzed using SNPs and the Haplotype Pattern Mining method, IL13ex4.1 was found to be associated with high serum total IgE level. Also, in the IL4RA gene, S411L was shown to be associated with asthma and, respectively, C406R with high IgE level. In addition, the CFTR gene was screened for two Finnish major mutations, but because of the low frequency of the mutations, no conclusions could be made on the association between the CFTR mutation carriership and asthma. To conclude, we found suggestive

evidence for the IL4RA gene and the IL13 gene and the FCER2 gene region contribution to atopy as well as for the IL9RA gene region and the IL4RA gene contribution to asthma.

INTRODUCTION

In recent years, the prevalence rates for asthma and atopy have increased in Finland (Haahtela et al. 1990; Pallasaho et al. 1999; Rimpelä et al. 1995) as well as in other Western countries (Åberg et al. 1995). In the 1970's, 5 % of the Finnish population was estimated to have hay fever and 1 % asthma, while the respective numbers in the 1990's were 15 % and 3-5 % (Haahtela et al. 1990). Although the prevalence of asthma has risen in Finland, it still is somewhat lower than in some Anglo-Saxon countries (Burr et al. 1994; Duffy et al. 1990). The prevalence of hay fever has already been reported to be higher than, for instance, in Germany (Huovinen et al. 1999; Varjonen et al. 1992; von Mutius et al. 1994). Vaccinations and lower rates of infectious diseases, such as hepatitis A or tuberculosis, as a part of higher standard of living and better hygiene, have been suggested as possible explanations for the increase in atopic diseases (Matricardi et al. 1997; von Hertzen et al. 1999). The concept of genetic susceptibility to asthma is based on the epidemiological twin and family studies, which have offered evidence for familial aggregation of asthma and atopy. Among the Finnish twins, estimates of genetic effect on asthma have varied from 35 % to 87 % (Laitinen et al. 1998; Nieminen et al. 1991), and on hay fever from 74% to 82 % (Räsänen et al. 1998).

Both a candidate gene based strategy and a genomewide search have been introduced as methods for studying genetic susceptibility to asthma and also to other multifactorial diseases. The candidate gene strategy can be used to test a certain hypothesis: Does this particular genetic variant contribute to genetic susceptibility for asthma or atopy? This strategy is based on the previous knowledge of biological mechanisms in asthma (functional candidates) and in asthma-related traits, such as high serum total IgE level. Another way of studying is a genomewide screening which offers information on chromosomal regions with previously unknown but possibly important genes (positional candidates). For asthma and atopy, the inheritance pattern is not known but since they are common disorders, the susceptibility alleles are accordingly expected to be common in a given population. However, not all of those with the susceptibility alleles become affected (reduced penetrance) and not everyone of the affected individuals has the same

set of susceptibility alleles (genetic heterogeneity or phenocopies) hence making the gene studies more complex than when studying diseases with Mendelian inheritance.

Until recent years, both functional and positional candidate genes have been studied using microsatellite markers. The large-scale discovery of single nucleotide polymorphisms (SNPs) has changed this, because unlike the polymorphic markers, they may also be located on the coding region of the gene (cSNP), and thus they may change the gene product and be true disease causing alleles. To find out these DNA variations is one of the results of the systematic sequencing of the human genome (Human Genome Project). However, because of the many SNPs needed in the studies, both large-scale genotyping and statistical methods are required to analyze the results. Also electronical databases have been developed, since the amount of knowledge and studies of genetics on asthma and asthma related traits has enormously increased, such as the Asthma & Allergy Gene Database (Wjst and Immervoll, 1998; http://cooke.gsf.de/) and the Database of Single Nucleotide Polymorphisms (http://www.ncbi.nlm.nih.gov/SNP/). The ambitious aim of the asthma gene projects worldwide is to get more information of the pathology of asthma and, in future, also to utilize the susceptibility genes in diagnostic and therapeutic applications.

The studies on asthma and atopy susceptibility genes in the Finnish population are presented here. First, a reliable method of phenotyping the patients for the genetic study was required. Thus, the value of self-reported physician-diagnosed asthma and the value of self-reported respiratory allergic symptoms are discussed when they are compared to the retrospectively collected information in medical records and to the allergy screening test. Secondly, four studies are shown with linkage and association analyses of candidate regions in asthma and atopy among Finnish asthma families. The functional candidate regions include the chromosomal region 5q31-q33 with the cytokine gene cluster, the IL9RA gene in the pseudoautosomal region on the sex chromosomes, the IL4RA gene on the chromosome 16 and the FCER2 gene region on the chromosome 19.

1. REVIEW OF THE LITERATURE

1.1. Definition of asthma

Asthma is characterized as a reversible obstruction of airways causing cough, wheezing, dyspnea and mucus production. A typical but not specific finding in asthma is the eosinophilic inflammation in the bronchial mucosa (Laitinen et al. 1985), and often associating features are bronhcial hyperreactivity (Higgins et al. 1992), sputum eosinophilia, blood eosinophilia (Bousquet et al. 1990) and a high serum total IgE level (Burrows et al. 1989). The diagnosis of asthma is based on self-reported symptoms, the assessment of the patient's medical history, a clinical examination and a lung function testing (peak expiratory flow recording, spirometry and unspecific bronchial challenge testing) to verify reversible airway obstruction. In the diagnosis, skin prick tests, serum total IgE measurements and blood eosinophilia can be used as additional criteria. The difficulty with an asthma diagnosis is that there is no single test that would be both sensitive and specific for asthma (Siersted et al. 1996). Wheezing by auscultation can be regarded as a sign of bronchial obstruction but it is not sufficient for the diagnosis in adults. Sputum eosinophilia, or eosinophil cationic protein in sputum (ECP) as well as bronchial hyperreactivity and detection of exhaled nitric oxide measure bronchial inflammation (Henriksen et al. 2000, Pizzichini et al. 1997). The study of histological findings in bronchial biopsies is an invasive method of showing bronchial inflammation and is not used in a clinical diagnosis, but it may be used for research purposes. In Finland, the diagnostic procedure of asthma follows international guidelines (Official Statement of the American Thoracic Society 1987), and a national effort has also been made to provide all Finnish pulmonologists and primary care physicians with uniform instructions for diagnosis and treatment (Report of a Working Group 1996).

Although the main principle of the diagnosis, reversible bronchial obstruction, is simple, the wide spectrum in the difficulty of symptoms and findings still show the diversity of the disease. The clinical course of asthma varies between individuals and in the same individual during the time both spontaneously and depending on anti-inflammatory

treatment. Asthma can also be divided into an intrinsic and extrinsic form of the disease, the extrinsic being atopic and the intrinsic being the non-atopic asthma. However, mechanisms of the intrinsic asthma are not well understood.

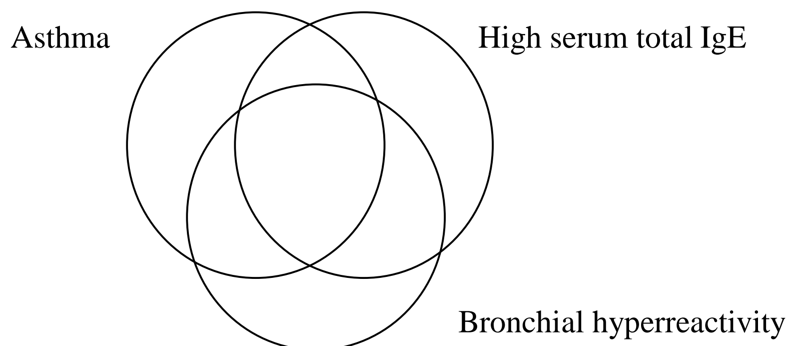
Asthma is aggregated in families which has raised the concept of inheritant factors contributing to the disease. Evidence for the genetic effect on asthma has been obtained with twin studies. The heretability of asthma has been estimated at 87 % in the Finnish twin study (Laitinen et al. 1998), 75 % in the Norwegian twin study (Harris et al. 1997) and 60 % in the Australian study (Duffy et al. 1990). Another way of estimating the genetic effect on asthma is to use a λs value which is the relative risk for the disease in the siblings of the proband divided by the relative risk for the disease in the general population. For asthma, the λs value is rather low, 2-5 (Barnes and Marsh 1998; Sandford et al. 1996) when compared e.g. to the sibling risk (λs) of the cystic fibrosis, which is 500 (Lander and Schork 1994). Among the Finnish twins, the λs is estimated to be 5,4 (Laitinen et al. 1998).

However, also environmental factors together with inheritance or even environmental exposure alone (occupational asthma) are the necessary conditions to get the disease. In addition to atopy, smoking and infections have been linked with asthma. Maternal smoking is a known risk factor for asthma in a child (Martinez et al. 1995), and respiratory infections cause exacerbations of asthma (Grünberg and Sterk, 1999). Yet, the avoidance of serious infectious diseases through better hygiene and vaccinations has been suggested as a basis for the increase in atopic diseases (Matricardi et al. 1997; von Hertzen et al. 1999). Moreover, in young children wheezing with colds is rather common, and only a part (40 %) of the " wheezing children" have asthma later in the childhood (over 6 years of age) (Martinez et al. 1995), which shows that wheezing is not specific for asthma, or that the course of the disease varies in the same individual in the course of time.

1.2. The role of phenotyping in complex diseases

The main initiative of phenotyping in complex disorders is to group similarly affected individuals together so that the underlying set of susceptibility genes would also be similar. In asthma, as in other common multifactorial diseases, the phenotype is a broad one. The spectrum is a continuation from asymptomatic bronchial hyperresponsiveness to mild asthma-like symptoms, advancing from seasonal to chronic and severe asthma. Often associating but not totally overlapping intermediate phenotyps are bronchial hyperresponsiveness (Burrows et al. 1992) and high serum total IgE level (Burrows et al. 1989) which both have widely been used in genetic studies. Narrowing the phenotype down to an intermediate phenotype has been used to reduce the number of genes underlying the disease and thus to make the analysis of susceptibility genes to common diseases more simple (Lander and Schork 1994). Also, focusing on those with an early onset of the disease or on those with the most severe disease may be an useful approach to limit the phenotype when susceptibility genes are mapped.

Figure 1. Corrrelation of asthma, high serum total IgE and bronchial hyperreactivity.



For genetic studies on atopy and asthma, lung function testing (especially bronchial hyperreactivity) of the probands has been widely used (Marsh et al. 1994; Meyers et al.

1994; Postma et al. 1995) to verify the asthma diagnosis as well as to offer quantitative traits for phenotyping. However, performing the lung function testing on hundreds of probands within a genetic study is a financially demanding task, and the use of questionnaires for a diagnosis would be beneficial. Also, it is known that bronchial hyperresponsiveness as well as other lung function test results vary in the course of the time and they are affected by anti-asthmatic (anti-inflammatory) medication (Hopp et al. 1994; Redline et al. 1989; Siersted et al. 1996). While questionnaires for epidemiologic studies were developed and validated, self-reported physician diagnosed asthma was found to be more specific than self-reported asthma (Toren et al. 1993), and this results has accordingly been used to avoid false positives. Discordance between self-reported questionnaire data and objective test results favoring false positives has been reported (Kesten et al. 1997), thus leading to an emphasis on the need for lung function testing. However, contradictory results have also been presented (Barnes et al. 1999) suggesting the use of questionnaire data alone for phenotyping.

1.3.Studying inheritance in asthma

1.3.1. Modelling the inheritance of asthma and related traits

The inheritance model of asthma is not known, but it has been studied using the segregation analysis method where different inheritance models are matched with the data (Martinez et al. 1997). There is some evidence for inheritance of asthma being either oligogenic and including a recessive component or being polygenic (Holberg et al. 1996), but also other inheritance patterns have been suggested (Los et al. 1999). The inheritance of lung function (FEV1) has been reported to be different in families with asthmatic family members from that in families without asthmatic individuals. In asthma families, the polygenic inheritance with a weak recessive component or with common environmental factors has been suggested, whereas in families without asthma polygenic inheritance has been considered (Holberg et al. 1998). A maternal effect, either a maternal genetic effect or environmentally mediated maternal influence, has also been shown in asthma families. Segregation analyses have also been made on serum total IgE

levels leading to contradictory results. Recessive inheritance of low levels as well as of high levels have been proposed, codominant and polygenic inheritance has also been suggested and codominant autosomal inheritance of high IgE level (Los et al. 1999; Martinez et al. 1994).

These segregation analyses clearly demonstrate the complexity of the genetic effect on multifactorial diseases. The same phenotype may result from a different set of genes (genetic heterogeneity) or from environmental exposure (phenocopies). Also, only a part of those with the susceptibility alleles get the disease (reduced penetrance). This is contradictory to mendelially inherited diseases where the cut-off point for the affected phenotype usually is more clear and the underlying genotype can be more easily predicted than in complex diseases.

1.3.2. Mapping susceptibility genes

Two strategies have been used when susceptibility genes of asthma have been mapped: A candidate gene approach and genomewide searches. In the first one, previous knowledge of molecules involved in asthma has been used to study appropriate candidate gene regions. The second one is a method where the whole genome is scanned with an evenly distributed set of markers. In both strategies, linkage is measured by coinheritance of alleles at a marker locus with a disease in pedigrees. Linkage can be analyzed either as non-parametric without a specific inheritance model or as parametric, using the best-fitting model of segregation analysis. Also, the affected sib pair method can be used where the proportion of shared gene alleles between sibs is calculated when the expected shared proportion is 50 %. All these methods need family data, but susceptibility genes can also be studied by an association analysis using only cases and controls. However, the association can also be analyzed in family data using disease-associated and control chromosomes. Another method of using families is the transmission disequilibrium test (TDT) representing a method which is a combination of association and linkage analysis. The TDT studies a transmission distortion of alleles that are transmitted to an affected child from unaffected parents when compared with untransmitted alleles.

Linkage disequilibrium is a transmission distortion between two chromosomal loci. In isolated young populations, the intervals of linkage disequilibrium are longer than in mixed populations, which has been considered an advantage when disease genes are mapped. Thus, the linkage between the disease and the underlying gene region can be distinguished with a more sparse marker map, and also in haplotype association analyses the haplotype spanning the searched gene extends further in isolated populations than in mixed ones.

Gene mapping studies have previously been made with microsatellite markers but recently more attention has been given to single nucleotide polymorphisms (SNPs). SNPs occur in the DNA approximately at frequency of 1 in 350 base pairs, and some of the SNPs have been hypothesized to act as the predisposing genetic variations to common diseases (Cargill et al. 1999). SNPs may be located on coding gene regions, and they may intrinsically represent the predisposing factor or they may be in linkage disequilibrium with the actual predisposing gene allele. Microsatellite markers are either intronically situated or positioned on other uncoding genomic region, and they are in linkage disequilibirium with the predisposing gene alleles. Another factor favouring the use of SNPs is the improved technology for genotyping that makes large-scale screening possible (length-multiplex single-base extension, LM-SBE) (Lindblad-Toh et al. 2000). A Japanese study offers an example of screening of SNPs in multiple candidate genes in asthma (Unoki et al. 2000): 29 genes were screened for sequence variations, 33 SNPs were found in 14 genes, other genes being non-polymorphic, and the tromboxane A2 receptor gene was reported to associate with asthma. Also, the study showed the large variation in the density of SNPs, since there were several genes without alterations, and in genes, where the SNPs were found, the densities varied from 1/73 bp to 1/1842 bp.

1.3.3. Animal models and the use of monogenic diseases

In addition to the study of linkage or association in humans, it is also possible to carry out candidate gene studies and genome screens in mice, and because of the great homology of the human and murine genomes the results of animal studies can be utilized in human research. Inbred mice strains have been used to study genetically determined

characteristics such as bronhcial hyperresponsivenss (Nicolaides et al. 1997), and transgenic and knock-out murine models have been especially useful for clarifying the gain of function (or loss of function) of a single gene. One example is a prostaglandin receptor (DP) deficient mice model which offers evidence for the importance of the prostaglandin pathway in asthma. Prostaglandin D2 is produced by mast cells, and it is involved in bronchial smooth muscle constriction, mediating its effect through the prostaglandin receptor. The DP deficient mice have been reported to have similar serum IgE levels as the wild-type mice (Matsuoka et al. 2000). However, the DP deficient mice do not to develop airway hyperreactivity and eosinophilia in response to antigen challenge, and they also have lower levels of Th_2 type cytokines such as IL4, IL5 and IL13 in response to an antigen challenge than the wild-type mice. The IL4 and IL4Rα, IL5 and FcεRII deficient mice have also been studied as experimental asthma models: The airway responsiveness of the IL4 deficient and the IL5 deficient mice after the OVA sensitization and challenge was lower than in the sensitized wild-type mice and comparable to the non-sensitized wild-type mice (Hamelmann et. al 2000). In addition, IL13 and IL4Rα have been found to contribute to bronchial hyperreactivity according to Grünig et al. (1998) as well as IL9 (Nicolaides et al. 1997). FcεRII have been found to have a role in the IgE production, since the FcεRII deficient mice have been reported to have higher IgE levels after immunization than the wild-type mice (Yu et al. 1994).

Lastly, monogenic diseases may offer information of molecular mechanisms involved as well in complex disorders. Autosomally dominantly inherited familial eosinophilia has been mapped to the chromosomal region 5q31-q33 (Rioux et al. 1998). This could be considered as a suggestive finding also for other eosinophilic disorders such as asthma, which is associated with blood eosinophilia and eosinophilic inflammation in bronchial mucosa. In the same year, Martinez et al. reported a linkage between circulating eosinophils and the chromosomal region 5q31-q33 (1998). Likewise, hyper-IgE syndrome (Job's syndrome) has been applied as a simplified model of other IgE defects such as atopy. However, findings of the IL4RA SNP (Q576R) in hyper-IgE are contradictory and thus only of limited use in the study of atopy (Grimbacher et al. 1998, Hersey et al. 1997).

1.4. Candidate genes - the immunologic basis and previous genetic studies

The search for the candidate genes of asthma is based on the knowledge of biological mechanisms (functional candidate genes) underlying and correlating to asthma such as inflammation on bronchial mucosa (Laitinen et al. 1985), bronchial hyperresponsiveness and high serum total immunoglobulin E (IgE) level. Even though many molecules and biological pathways are known to be important in allergic inflammation, it is not known whether there are variations in corresponding genes that would predispose to asthma. For example, the accumulation of eosinophils on bronchial mucosa is a typical for asthma, and especially IL5 stimulates differentation and proliferation of eosinophils, which has made the IL5 signaling important in asthma. Other T-helper2-type cytokines, such as IL4 and IL13, activate B-cells to produce antigen-specific IgE which is then bound by high affinity receptors (FcεRI) on mast cells. This binding of IgE by FcεRI receptors leads to the degranulation of mast cells and to the release of histamine which is known to cause bronchial obstruction and mucus production and finally leading to symptoms of asthma. Genes for these molecules among others involved in allergic inflammation have been studied as candidate genes for asthma and atopy.

The chromosomal regions 5q and 11q were the first two regions to be studied as candidate gene regions in asthma and atopy (Cookson et al. 1992; Marsh et al. 1994; Meyers et al. 1994). The chromosome 5q includes an interleukin gene cluster (IL3, IL4, IL5, IL9, IL13), a granulocyte-macrophage colony stimulating factor (GM-CSF) gene, an interferon regulatory factor (IRF1) gene, a fibroblast growth factor acidic (FGFA) gene and a β2-adrenergic receptor gene, and the chromosome 11q includes e.g. a gene for the high affinity receptor for IgE (FCER1), all of which are considered to have an effect on the asthmatic phenotype. The 5q region has been studied in respect to different phenotypes such as asthma, bronchial hyperresponsiveness (BHR) (Postma et al. 1995), high serum total IgE level (Meyers et al. 1994, Marsh et al. 1994), circulating eosinophils (Martinez et al. 1998) and using different populations. Populations have varied from isolated Caucasians to populations with a different ethnic origin such as Japanese and African Americans (Noguchi et al. 1997, Hizawa et al. 1998). In addition to the mapping

of circulating eosinophil levels (sib pairs concordant for low levels of eosinophils, ≤2 %) to the chromosomal region 5q, autosomally dominantly inherited familial eosinophilia has also been mapped on 5q31-q33 (Martinez et al. 1998; Rioux et al. 1998) in a genomewide search. Both the association (Doull et al. 1996) and the linkage analysis (Marsh et al. 1994) have been used, as well as the SNPs (Hopes et al. 1998), in addition to microsatellite markers. The majority of the studies have yielded some evidence for the region 5q having asthma and atopy susceptibility genes, while only few have reported contradictory results (Kamitani et al. 1997) (Table 1). One of the latest 5q31-q33 studies is a retrospective analysis of the region using 11 study samples by the Consortium on Asthma Genetics (COAG) (Lonjou et al. 2000). In this retrospective collaboration study, evidence was found for susceptibility genes for asthma but not for atopy.

1.4.1. IL4 and IL4RA genes

Interleukin 4 is probably the strongest candidate of the 5q cytokines for the contribution to asthma and atopy. Interleukin 4 (IL4) is needed in the differentation from T-helper-0 (Th_0) cells into T-helper-2-type (Th_2) which are capable of producing more cytokines, finally leading to an allergic inflammation of the bronchial epithelium. IL4 is also known to stimulate differentation and proliferation of the B cells, IgE production, MCH class II antigen expression, proliferation of human mast cells and expression of FcεRI and FcεRII (Chomarat and Banchereau 1997). The effect of IL4 is mediated through its receptor which is a heterodimer consisting of two chains: A common γ-chain which is shared by several interleukin receptors (IL2, IL4, IL7, IL9 and IL13) and an IL4Rα chain which binds the IL4. The IL4Rα chain is a transmembrane protein of 140 kDa (Chomarat and Banchereau 1997) and also able to transduct signals of IL13 by a heterodimer formed by an IL4Rα and an IL13Rα chain (Izuhara and Shirakawa 1999). Strong evidence for the importance of the IL4 pathway in asthma has been obtained from an animal model where the administration of either IL4 or IL13 induced the asthma phenotype to wild type mice (Grünig et al. 1998). However, neither of the cytokines was able to induce the asthma phenotype to the IL4Rα deficient mice which demonstrates the importance of the IL4Rα in the signalling by IL4 (and IL13). Eight SNPs leading to an altered gene product have been reported in the IL4RA (Deichmann et al. 1997; Hershey et al. 1997; Ober et al.

Table 1. Candidate gene studies of the chromosomal region 5q on atopy and asthma. While all of the several studied phenotypes are not associated with/linked to the region, those in bold indicate the positively associated/linked phenotype. BHR= bronchial hyperreactivity, LTC4S = leucotrien C4 synthase, ADBR2 = B2-adrenoreceptor, SPT= skin prick tests, FEV1=forced expiratory flow volume in 1 second.

Author	Year	Phenotype	Gene or region	Method	Outcome	Population
Reihsaus et al.	1993	Asthma, **steroid dependence**	B2-adrenoreceptor	Association	Positive	American
Marsh et al.	1994	Total IgE	5q31-q33	Linkage	Positive	Caucasian Amish
Meyers et al.	1994	Total IgE	5q31-q33	Linkage	Positive	Dutch
Bleecker et. al	1995	Total IgE, BHR	5q31-q34	Linkage	Positive	Dutch
Hall et al.	1995	BHR	B2-adrenoreceptor	Association	Positive	British
Postma et al.	1995	BHR	D5S436	Linkage	Positive	Dutch
Rosenwasser et al.	1995	Asthma	5q31-q33	Association	Positive	Not specified
Turki	1995	Nocturnal asthma	B2-adrenoreceptor	Association	Positive	Mixed
Xu et al.	1995	Total IgE	5q31-q33	Linkage	Positive	Dutch
Blumenthal et al	1996	Total IgE	5q	Linkage	Negative	American
Doull et al	1996	**Total IgE**, BHR	IL9	Association Linkage	Positive Negative	British
Dewar et al.	1997	Asthma, atopy, **total IgE**	B2-adrenoreceptor	Association	Positive	Caucasian
Kamitani et al.	1997	Asthma, BHR, atopy	D5S399	Linkage	Negative	Australian
Martinez et al.	1997	β-agonist desensitation	B2-adrenoreceptor	Association	Positive	American, mixed
Noguchi et al.	1997	Asthma, atopy	5q31-q33	Linkage	Positive	Japanese
Sanak et al.	1997	ASA intolerant asthma	LTC4S promoter	Association	Positive	Not specified
Tan et al.	1997	B-agonist desensitation	B2-adrenoreceptor	Association	Positive	British
Ulbrecht et al.	1997	Total IgE, **specific IgE**	IL9	Association	Weak positive	German
D'amato et al.	1998	BHR	B2-adrenoreceptor	Association	Positive	Italian
Dewar et. al	1998	Total IgE, SPT, **atopy**, BHR, Asthma, wheezing	B2-adrenoreceptor	Association	Positive	British
Hizawa et al.	1998	SPT, specific IgE	5q31-q33	Linkage	Positive	Afro-American
Hopes et al.	1998	Asthma	B2-adrenoreceptor	Association	Positive	British
Mansur et al.	1998	Total IgE, BHR	5q31-q33	Association	Weak positive	British
Martinez et al.	1998	Eosinophils	5q31-q33	Linkage	Positive	American
Noguchi et al.	1998	**Asthma**, total IgE	IL4 promoter	Association Linkage	Negative Positive	Japanese

Author	Year	Phenotype	Gene or region	Method	Outcome	Population
Palmer et. al	1998	**Total IgE, specific IgE** BHR, **eosinophils**	D5S393, D5S399	Linkage	Positive	Caucasian Australian
Pereira et al	1998	Asthma	IL5	Association	Negative	Australian
Rioux et al.	1998	Familial eosinophilia	IL3, IL5, GM-CSF	Association*	Positive	American
Weir et al.	1998	Asthma (mild/moderate)	B2-adrenoreceptor	Association	Positive	Caucasian
Baldini et al.	1999	Total IgE	CD14	Association	Positive	American
Burchard et al.	1999	**Reduced FEV1**, total IgE	IL4 promoter	Association	Positive	Caucasian
					Negative	Afro-American
Chouchane	1999	Asthma	IL4	Association	Positive	Tunisian
Deichmann	1999	Total IgE, specific IgE	D5S210	Linkage	Negative	German
			ADBR2	Association	Negative	German
Dizier et al.	1999	Total IgE	5q31-q33	Linkage	Positive	Caucasian Australian
Hook et al.	1999	Atopy and asthma	IL4 promoter	Association	Negative	Not specified
Kotani et al.	1999	Asthma	B2-adrenoreceptor	Association	Positive	Japanese
Ramsay et al.	1999	Asthma, **BHR**, SPT, total IgE Specific IgE, **wheeze with cold**	B2-adrenoreceptor	Association	Positive	Australian
Rohrbach et al.	1999	Asthma	GM-CSF	Association	Positive	Swiss
Graves et al.	2000	Total IgE	IL13	Association	Positive	Caucasian
Heinzmann et al.	2000	Asthma	IL13	Association	Positive	British
Hijazi et al.	2000	Asthma, atopy	IL4	Association	Negative	Kuwaiti Arab
Holloway et al.	2000	Asthma severity	B2-adrenoreceptor	Association	Positive	Not specified
Israel et al.	2000	PEF	B2-adrenoreceptor	Association	Positive	American
Liu et al.	2000	Total IgE, atopic dermatitis	IL13	Association	Positive	German
Mansur et al.	2000	Total IgE, BHR	5q23-33	Linkage	Negative	Scottish
Sandford et al.	2000	Asthma severity	IL4 promoter	Association	Positive	Mixed
Summerhill et al.	2000	Asthma, BHR, **FEV1**	B2-adrenoreceptor	Association	Positive	Hutterite
Takabyashi et al.	2000	Atopy	IL4 promoter	Association	Negative	Japanese
Ulbrecht et al.	2000	BHR	B2-adrenoreceptor	Association	Positive	German
Unoki et al.	2000	Asthma	LTC4S	Association	Negative	Japanese
Van Sambeek et al.	2000	ASA intolerant asthma	LTC4S promoter	Association	Negative	Mixed
Zhu et al.	2000	Atopy, asthma, rhinitis	IL4	Association	Positive	Caucasian

*The original finding of familial eosinophilia was done by Rioux et al. in a genome wide search using linkage analysis.

2000), of which Q576R and I50V have been reported to associate with atopy (Hershey et al. 1997; Mitsuyasu et al. 1998). Using an *in vitro* model, the I50 variant of the IL4Rα was shown to upregulate the receptor response to the IL4 in the cell lines, thus providing supporting data on functional effects of the polymorphism. Although Mitsuyasu et al. found evidence for IL4RA gene contribution to atopy in Japanese, in another study among the Japanese population, no association between the IL4RA (Q576R) and atopy or asthma was found (Noguchi et al. 1999).

1.4.2. IL9 and IL9RA genes

Interleukin 9 is one of the 5q cytokines and known to affect differentation and proliferation of mast cells, proliferation of T cells, IgE production by B cells (together with IL4) and mRNA expression of the α chain of the FcεRI (Demoulin and Renauld 1998). Indeed, a reduced expression of the IL9 gene has been found in the lung tissue of a hyporesponsive mouse strain (Nicolaides et al. 1997). In humans, the expression of IL9 mRNA is increased in asthmatic individuals compared to atopic and non-atopic control individuals (Shimbara et al. 2000), and the IL9R immunoreactivity is also higher in asthmatic than in control individuals. The other part of the IL9 pathway is IL9R, which is composed of two chains: A ligand specific IL9Rα and a common γ-chain. The IL9RA gene, which encodes the α chain, is located on the pseudoautosomal region (320 kb) in the long arms of the X and Y chromosomes (Kermouni et al. 1995) and also expressed by both chromosomes (Vermeesch et al. 1997). Holroyd et al. found linkage between this pseudoautosomal region and asthma or bronchial hyperreactivity in Italians (Holroyd et al. 1998).

1.4.3. Studies of other genes on 5q31-q33

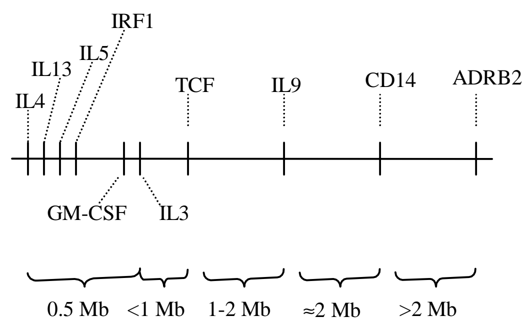
Although the cytokine gene cluster has been an obvious candidate through the effect of the cytokines on IgE and eosinophil production, the β2-adrenoreceptor (ADBR2) gene and the leucotrien C4 synthase (LTC4S) gene (Table 1), locating telomeric from the cytokine gene cluster, have also been studied on the chromosome five. The ADBR2 gene is an important candidate gene in asthma, since the receptor is involved in smooth muscle relaxation which is caused by endogenous or exogenous agonists. Four coding

polymorphisms (R16G, Q27E, V34M and T164I) and linkage disequilibrium between the first two ones have been reported in the gene. The first two amino acid variations are located extracellularly and the last two are found in the transmembrane region of the receptor (Liggett 1997; Reihsaus et al. 1993). The similar distribution of polymorphisms reported in asthma and control groups exclude the SNPs as a cause for asthma (Reihsaus et al. 1993). However, the SNPs have been suggested to explain variations in asthma phenotype, such as the response to bronchodilators (Martinez et al. 1997) or nocturnal asthma (Turki et al. 1995). Moreover, *in vitro* studies have shown enhanced downregulation of the G16 variant and resistance to the downregulation of the E27 variant of the receptor (Liggett 1997).

Other candidate genes on 5q31-q33 include genes for IL5, GM-CSF, CD14 and IRF1 of which the cytokine IL5 is known to act on the growth and differentiation of eosinophils. In an animal model, the IL5 deficient mice did not develop increased bronchial hyperreactivity in response to sensitization and an airway challenge with ovalbumin (Hamelmann et al. 2000). In addition, there was no accumulation of eosinophils in the bronchial mucosa in the IL5 deficient mice, which demonstrates the importance of IL5 both in aggregation of eosinophils and in bronchial hyperreactivity. Still, as 30 atopic and 30 control individuals were screened for sequence variations in the IL5 gene and in the IL5RA gene, no polymorphisms were detected in the coding regions or in the promoter of the IL5 gene, which suggests that the alterations in the IL5/ IL5RA genes would not explain the genetic susceptibility to asthma or atopy in humans (Pereira et al. 1998). GM-CSF has been explained to have an effect on migration of eosinophils into the tissues. As variations were searched for in the GM-CSF gene, an I117T polymorphism was found to be associated with asthma and bronchial hyperreactivity in a Swiss asthma study (Rohrbach et al. 1999). Another two genes on 5q31-q33 that affect the Th1/Th2 balance are the genes for CD14 and IRF1. CD14 functions as a receptor on monocytes, macrophages and is also found as a soluble form, and it binds bacterial antigens favouring differentation of lymphocytes into Th1 type. An SNP in the promoter of the CD14 gene has been reported to associate with serum total IgE level (Baldini et al. 1999). Also a study on knock-out mice of IRF1 gene showed an altered cytokine production with higher

levels of Th2 type cytokines such as IL3, IL4, IL5 and IL6, and respectively, lower levels of IFNγ and IL2 emphasizing the role of IRF1 in the polarization of Th_1/Th_2 cells and thus in atopic disorders (McElligott et al. 1997). However, in a Japanese study, the IRF1 gene was screened for polymorphisms, but no coding variants were found, and no association of the three found non-coding polymorphisms with asthma or atopy was detected (Noguchi et al. 2000).

Figure 1. Map of the chromosomal region 5q31-q33



1.4.4. High and low affinity receptor for Immunoglobulin E

Th_2 type cytokines lead to the production of Immunoglobulin E, which then functions via high and low affinity receptors, the former being located on mast cells and on basophils, the latter being located on B- and T-lymphocytes, eosinophils, Langerhans cells etc. The immediate allergic response is mediated through high affinity receptors (FcεRI) which leads to the degranulation of mediators such as histamine. The high-affinity receptor for IgE is constructed of a ligand-specific α-chain and a transmembrane β-chain. Low affinity receptor (FcεRII) for IgE has been found as a single chain transmembrane protein in two forms (T-cell- and B-cell-derived) and also as a soluble factor (Delespesse et al. 1989). The FcεRII is involved in IgE production and it especially seems to be important in negative feedback of IgE formation, since the IgE levels were twofold higher in the FcεRII deficient mice than in controls (Yu et al. 1994). The gene for β-subunit of FcεRI is located on the chromosome 11q and the gene for FcεRII on the 19p, and these both can

be considered as candidate genes for susceptibility to asthma and atopy. Cookson et al. found an association between the maternal inheritance of FCER1B gene alleles and the IgE responsiveness and later particularly with the I181L polymorphism of the gene (Cookson et al. 1992; Shirakawa et al. 1994). However, contradictory results exist (Amelung et al. 1998).

1.4.5. CFTR carriership and asthma

The carriership of mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene has been reported to associate with asthma (Dahl et al. 1998) but, conversely, Schroeder et al suggested that the ΔF508 mutation might protect against asthma (Schroeder et al. 1995). The CFTR is a chloride channel and found e.g. in the bronchial epithelium in addition to sweat glands; an individual homozygous for a CFTR gene mutation has either cystic fibrosis with emphysema and bronchiectasiae and pancreas insufficiency or congenital bilateral absence of the vas deferens (CBAVD). Not only mutations but also the polymorphisms of the gene (M470V) have been reported to affect the function of the protein: the M470 variation has been indicated to have a greater chloride channel activity than the V470 variation (Cuppens et al. 1998). Furthermore, the number of thymidines (5, 7 or 9) in the intron 8 of the CFTR gene has an effect on the exon 9 splicing which in turn has an effect on protein function. This 5T allele has been associated with asthma-like symptoms in addition to CBAVD (Kerem et al. 1997). A combination of a CFTR mutation and the M470 allele has also been suggested to have influence on asthma susceptibility (Lazaro et al. 1999)

1.4.6. Other functional candidate genes

Other possible candidate genes include the HLA-area on the chromosome 6p, since the HLA class II antigens are important in (exogenous) antigen presentation and antigen specific IgE production (cognate IgE production). Data of the study concerning soybean epidemic asthma patients in Spain support the hypothesis that the specific HLA alleles would predispose to asthma (Soriano et al. 1997). This Spanish study population offers a possibility to examine if genes as susceptibility factors together with specific environmental exposure can cause a disease.

Anti-inflammatory cytokines such as interleukin 10 (IL10) and transforming growth factor β (TGF-β) have also been connected with asthma and atopy. IL10 is known to inhibit the IL4 and IL5 production by Th_2 cells, but contradictorily, IL10 stimulates B cell proliferation and immunoglobulin synthesis. The TGF-β deactivates macrophages, inhibits mast cell proliferation and induces an apoptosis of eosinophils. However, in the presence of allergic inflammation, the TGF-β has been considered to stimulate the formation of extracellular matrix and fibrosis, and thus the TGF-β might be involved in the remodelling of airways in chronic asthma. Promoter polymorphisms of the IL10 gene (chromosome 1q32) and of the TGF-β gene (chromosome 19q13) have been reported to associate with high serum total IgE level in the Jewish population (Hobbs et al. 1998).

The IFN-γ gene is located on the chromosomal region 12q15-q24 and is also one of the obvious candidate genes since the IFN-γ inhibits the IL4 production by Th_2 cells, and thus it also has an inhibitory effect on the Th_2 cell differentation and on the IgE synthesis by B cells. *In vitro* studies have shown that the expression of the IFN-γ mRNA in stimulated cell cultures is negatively correlated to serum total IgE levels of atopic and control individuals (Teramoto et al. 1998). Barnes et al. reported linkage between asthma and 12q15-q24 as well as linkage between serum total IgE level and the same region (1996). However, the results were not obtained with the intragenic marker of the IFN-γ gene but with other markers further off the gene. Furthermore, when the IFN-γ gene exons were screened for polymorphisms in two populations (265 Australian and Venezuelan individuals), no sequence variations were found (Hayden et al. 1997). Another candidate on the chromosome 12 is the nitric oxide synthase 1 (NOS1) gene. An increased synthesis of nitric oxide has been connected with asthmatic inflammation on bronchial mucosa, and non-invasive methods for measuring the amount of exhaled nitric oxide have been developed. Nitric oxide is formed by constitutive and inducible nitric oxide synthase (cNOS and iNOS, respectively) and of these the cNOS is considered to be involved in vasodilatation and bronchodilatation (Barnes and Liew 1995). Proinflammatory cytokines, such as IFN-γ, can induce the production of NO by the iNOS in macrophages and epithelial cells. Interestingly, the IRF1 gene knock-out mice were not

able to produce any NO in response to IFN-γ or lipopolysaccharide stimulation. Two IRF1 response elements were identified in the iNOS promoter in mice (Kamijo et al. 1994), which emphasizes the role of IRF1 in the iNOS gene induction.

Another example of other candidates is the gene for the Clara cell scretory protein (CC16, previously CC10) on the chromosome 11q13. The CC16 is a product of nonciliated respiratory tract cells (Singh et al. 1988) and it can be detected in the bronchoalveolar lavage fluid as well as in the plasma. The CC16 downregulates IFN-γ production and activity (Dierynck et al. 1995) and thus has anti-inflammatory properties. A polymorphism of the CC16 gene (38A→G) has been reported to be associated with asthma and lower plasma CC16 levels (Laing et al. 2000), although the functional effects of the 38A→G are not clear since the polymorphism is located on the untranslated region of the CC16 gene. In another study, no association was found between the CC16 gene and atopy or asthma (Campbell DA et al. 1996).

1.4. Genomewide searches

In addition to the candidate gene strategy, genomewide searches have been used to study asthma and atopy susceptibility genes. With this method, using markers evenly spaced across the whole genome, novel loci involved in the genetic susceptibility of the disease can be identified (positional candidate gene regions). It has been considered that a chromosomal location or a SNP associated with a trait in more than one study population would strengthen the results: A region associating with a disease in several studies would include a susceptibility gene more likely than if a locus was found to be associated in only one population. Several genome scans have been published, the first one by Daniels et al. (1996). These scans have been made using several asthma associated phenotypes such as serum total IgE level, BHR, eosinophil count, specifig IgE responsiveness, PEF variation, symptoms and mite-sensitive atopic asthma; and several chromosomal loci have been reported to be linked with these phenotypes (Daniels et al. 1996; CSGA 1997; Malerba et al. 1999; Wjst et al. 1999, Ober et al. 2000) (Tables 2 and 3). As in the

Table 2. Reported linkages between the asthma-related phenotypes and the chromosomal locations in eight published genome scans.

Chr.	Daniels	CSGA African Americans	CSGA Caucasians	CSGA Hispanics	Wjst	Ober	Malerba	Dizier	Xu	Yokouchi
1p					IgE, Eos.	AB, Specif. IgE				
1q								SPT, BHR		MS-asthma
2p					AB, BHR, IgE; RAST					
2q				AB	IgE	Specific IgE		BHR, IgE, eos	IgE	
3p						AB				MS-asthma
3q						Specif. IgE		AB, IgE, SPT	IgE	MS-asthma
4					RAST					
4q	BHR									MS-asthma
5					PEF					
5p		AB				BHR		BHR		
5q						AB			IgE	MS-asthma
6p	Eos, atopy				AB, IgE, RAST, Eos.	Specif. IgE			IgE	MS-asthma
7p	IgE, BHR				RAST			Eos		
7q								Eos, SPT, AB, BHR	IgE	MS-asthma
8p						AB, Specif. IgE		AB, SPT		
8q								AB, SPT		
9					AB, IgE, RAST	Asthma symptoms				
9p					BHR	Asthma symptoms				
9q					AB					MS-asthma
10					PEF					
10p								Eos		
11						Specif. IgE				
11p			AB					SPT, IgE		
11q	IgE				Eos.			IgE		
12q			AB				AB	Eos, AB, BHR	IgE	MS-asthma
13						Asthma symptoms				
13p									IgE	
13q	Atopy		AB					Eos		MS-asthma
14q			AB			AB	BHR			

Chr.	Daniels	CSGA African Americans	CSGA Caucasians	CSGA Hispanics	Wjst	Ober	Malerba	Dizier	Xu	Yokouchi
15q					IgE, PEF			Eos, BHR		
16p						AB, Specif. IgE				
16q	IgE					Specif. IgE				
17p		AB								
17q		AB						AB, SPT		MS-asthma
19q			AB				Atopy			
21q				AB				SPT		
Xq					IgE					

AB= Asthma bronchiale, BHR= bronchial hyperreactivity, PEF= peak expiratory flow recording, Specific. IgE= positive reaction to one or more of the studied allergens by skin prick tests, IgE= serum total IgE level, MS-asthma=mite sensitive atopic asthma.

Table 3. Characteristics of the eight published genome scans of asthma and associated phenotypes.

Study	Year	Population	Number of studied	Number of markers	Marker interval
Daniels et. al.	1996	Australians	364 individuals	269	
CSGA	1997	African-Americans	117 affected	360	10 cM
		Caucasians	215 affected	360	10 cM
		Hispanics	48 affected	360	10 cM
Wjst et. al.	1999	Caucasians	415 individuals		10,7 cM
Malebra	1999	North-East Italians	420 (-560) individuals*	300	
Ober et al.	2000	Hutterites	693 individuals	386+177	9,1 cM
Dizier et al.	2000	French	493 individuals	254	13 cM
Xu et al.	2000	Dutch	200 families	344	10 cM
Ykouchi et al.	2000	Japanese	375 individuals	391	

*560 studied individuals for the chromosome 12 and 14, 420 individuals for the chromosome 19.
**386 markers microsatellites at 9.1 cM intervals and 177 additional markers including SNPs

candidate gene studies, the results have varied in different populations reflecting the heterogeneity of the studied populations, the complexity of the disease and the difference between used phenotypes. Also, the most studied candidate gene region, the 5q, has been identified in three of the genomewide searches with phenotypes for asthma, mite-sensitive atopic asthma and logarithm of serum total IgE (Ober et al. 2000, Yokouchi et al. 2000 and Xu et al. 2000). The chromosomal region 16p has been reported with one scan (Ober et al. 2000), the region Xq with one (Wjst et al. 1999), and the region 19p has not been found in any of the genome scans. The most often found regions (5/8) in the genomewide searches are 2q, 6p (including e.g. the HLA area) and 12q (including e.g. the IFN-γ gene).

1.6. Advantages of a founder population in gene mapping studies

As previously explained, the power of the genetic study of complex diseases can be increased by limiting the phenotype. Another factor affecting the power of the study is the appropriate study population. Founder populations and small stable populations, inbred ones, and geographically localized populations have been suggested as an optimal choice (Wright et al. 1999). A more consistent environment as well as more uniform genetic backround underlying the disease make isolated populations ideal for studying multifactorial diseases. Hence, the number of loci/genes affecting the disease is expected to be lower (reduced genetic heterogeneity). In addition, longer segments of chromosomes around the disease gene appear identical in disease associated chromosomes (linkage disequilibrium) in isolated gene pools than in mixed populations. Indeed, the Finnish population has been used for studies of many multifactorial diseases, e.g., familial combined hyperlipidemia, schizophrenia, multiple sclerosis and psoriasis (Asumalahti et al. 2000; Hovatta et al. 1999; Kuokkanen et al. 1997; Pajukanta et al. 1998). In breast and ovarian cancer (BRCA1 and BRCA2 carriers), the extent of LD has been shown to be 1.6-15.5 cM in the conserved haplotypes of the carriers of the BRCA1 and BRCA2 gene mutations.

Also, the Finnish population as a whole forms an isolate, which has been confirmed by molecular genetic studies. Sajantila et el. (1996) have shown the Finns to have significantly less diversity in the Y chromosomal haplotypes compared to the Saami, Swedes and Estonians indicating that the number of male founders of the Finns has been small. The frequency of the “Finnish disease heritage” is also in agreement with the population history, over 30 rare Mendelian inherited disorders that are typical to Finland and seldom met in other countries fave been found (de la Chapelle 1993; Peltonen et al. 1999). Likewise, a few other inherited diseases, such as cystic fibrosis, are considerably less frequently met in Finland than in other countries (Kere et al. 1994), which also indicates an isolated gene pool of the Finns. Linkage disequilibrium mapping has been successfully used when the disease genes of the “Finnish heritage” have been mapped. Already in 22 of the 35 diseases, the disease causing gene has been identified (Peltonen et al. 1999, Varilo et al. 2000).

In Finland, the population living in the Kainuu province can be regarded as an isolated subpopulation of the Finns. Kainuu was settled in the sixteenth century mainly by people from South Savo and the estimated number of founders was low, up to few hundreds families (Koskinen et al. 1994, de la Chapelle 1993; Peltonen 2000). The castle of Kajaani was built during the years of 1604-1619, and the town of Kajaani (capital of the Kainuu province) was founded in 1651 around the castle. After the initial settlement the immigration rate remained low. Also, the growth of the population started rather slowly, and it was not until in the 19th century than the total number of the Finnish population reached the limit of 1,000 000. Now, approximately 5,100 000 inhabitants live in Finland with 1.8 % of the population in Kainuu (Finnish Statistics on Medicines, 1996). The incidence of the congenital chloride diarrhoea being higher in Kainuu than elsewhere in Finland provides evidence for the isolated character of this subpopulation (Höglund et al. 1995). Also, when congenital chloride diarrhoea was mapped, haplotype analysis revealed a linkage disequilibrium spanning for 12 cM (Höglund et al. 1995) in the disease gene carrying chromosomes. Thus, both the molecular genetic studies and the data of the population history support the designation of the Kainuu population as a founder population.

Other isolated populations have also been used for genetic and epidemiologic studies on asthma and atopy. The Pennsylvania Old Order Amish and Hutterites are religious inbred isolates living in the USA; the members of both groups live mainly on communal farms (Marsh et al. 1994, Ober et al. 2000). Only two major mutations of cystic fibrosis have been found in the Hutterites. The populations on Barbados in the Caribbean Sea and on Tristan da Cunha in the South Atlantic Ocean are examples of geographical isolates. The population on Tristan da Cunha is very small (only 300) but with a very high frequency of asthma (23 % of the population has definite asthma) (Zamel et al. 1996). On the island of Barbados, the asthma prevalence is 13 % and the size of the population is 250 000. With this Afro-Caribbean population, a linkage between asthma and total serum IgE and chromosomal region 12q15-q24 has been reported (Barnes et al. 1996).

1.7. Optimizing the likelihood for finding a susceptibility gene in complex disorders

Since the inheritant component of multifactorial diseases is complex and difficult to determine, the power for gene-mapping study can be optimized by focusing on the appropriate phenotype, by choosing an optimal study population and by creating suitable family structures (Lander and Schork 1994). In multifactorial diseases the phenotype is typically a broad one, and by limiting the phenotype to those with an early onset of the disease or to those with the most severe disease, the underlying genetic effect is considered to be stronger than among the individuals with a late-onset or mild disease. Also by narrowing the phenotype down to those with some intermediate phenotype, the number of susceptibility genes can be restricted. A young isolate may often be regarded as an optimal study population and not only because of the wider intervals of linkage disequilibrium and less genetic heterogeneity but also because of a more uniform environment and culture (Peltonen et al. 2000). What kind of family structures are best suitable for the study depends on the preferred statistical approach. If linkage analysis is used, large pedigrees with several affected individuals are optimal and if association analysis is used, multiple nuclear families are favored.

2. AIMS OF THE PRESENT STUDY

The aims of the present studies were to study the possibility if selected biological candidate gene regions would act as genetic regulators, increasing the susceptibility to asthma and atopy in the Finnish population, as well as to find reliable methods for phenotyping a large number of patients for genetic studies on atopy and asthma.

The specific aims were:

2.1. In the linkage and association analyses, the aim was to study the possibility whether the following chromosomal regions contribute to asthma and atopy among the Finnish asthma families:

5q31-q33
IL4RA gene on 16p12
IL9RA gene region on Xq/Yq PAR
FCER2 gene region on 19p13.

2.2. To study the supposition whether self-reported physician-diagnosed asthma could be used as an inclusion criterium, and to study the possibility if self-reported respiratory allergic symptoms could be used alone without allergy testing when phenotyping individuals for a genetic study.

3. MATERIAL AND METHODS

3.1. Study population

Study families were ascertained through media in Central Eastern Finland (the Kainuu province) in two batches in November 1994 and in November 1996. The selection criteria for a proband were: the self-reported asthma diagnosed by a physician, the parents and/ or grandparents born in Kainuu and the nuclear family willing to participate (proband - spouse - at least one child, or proband - mother - father, or proband - one of the parents - at least one sibling). Additional affected family members (uncles, aunts, grandparents, cousins, further sibs) were included when available. All participants signed an informed consent where they gave a permission to use their blood samples for a scientific study. The asthma patients were also inquired in which hospitals they had been diagnosed and treated, and they were asked for a permission to study their hospital files concerning asthma and allergy. The National Board of Health confirmed the permission to attain the access to these patients' medical records. The study was approved by the ethical committees of the Department of Medical Genetics, University of Helsinki and the Kainuu Central Hospital.

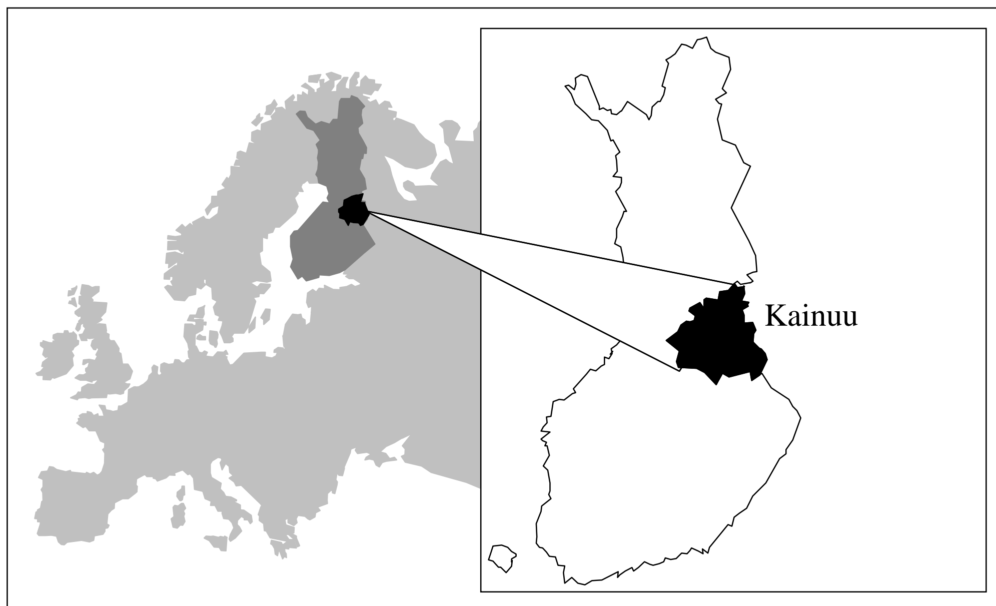


Figure 1. The Kainuu province’s geographical reference to Finland and Europe

Figure 2. Municipalities of the Kainuu province.

3.2. Questionnaires and medical records

Both the asthmatics and their family members filled out a health questionnaire. The medical records of asthmatics were reviewed separately by two pulmonary physicians (P.K. and T.L.). The descriptive remarks of the clinical condition and lung function test results were collected in a structured form. The significance of reversible airway obstruction was evaluated according to the ATS criteria (Official statement of the American Thoracic Society 1987) (Study I, Table 1). Those patients, whose lung function tests had remained nondiagnostic or were insufficiently done or documented at the time of the diagnosis, were followed through hospital records to get evidence either against or for an asthma diagnosis. At the same time, other diseases as a possible cause for the patients’ dyspnea were evaluated.

3.3. Allergy screening and IgE measurements

All participants donated their blood samples. The IgE measurements were made of serum samples which were stored at - 70 °C and analyzed in two batches in the same laboratory. From each sample the following parameters were measured: Serum total IgE level (Diagnostics CAP FEIA, Kabi Pharmacia, Sweden); an allergy screening test with birch, mugwort, timothy, horse, cat, dog, home dust mite (*Dermatophagoides pteronyssinus*), and Cladosporium herbarum mould (Phadiatop®, CAP FEIA, Kabi Pharmacia, Sweden) (Gleeson et al. 1996; Haahtela et al. 1980; Haahtela and Jaakonmäki 1981; Varjonen et al. 1992). If the allergy screening was >0.3 kU/L, the levels of specific IgE antibodies were measured.

3.4. Genotyping

3.4.1. Markers and maps

Both the microsatellite markers and the single nucleotide polymorphisms in coding and non-coding DNA were used as markers (Table 4). The markers were organized by different published genetic and physical maps: Marshfield Center for Medical Genetics (http://research.marshfieldclinic.org/genetics/), Genethon (http://www.genethon.fr/genethon), the Human Resources in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genome/guide/human/), the Cooperative Human Linkage Center (http://lpg.nci.nih.gov/CHLC/), Genome Data Base (http://gdbwww.gdb.org/); and Lawrence Berkeley National Laboratory (http://www-gsd.lbl.gov/) and Whitehead Institute (http://www-genome.wi.mit.edu/). A physical map was constructed for fine mapping regions by radiation hybrid mapping when there was no accurate information of the order of the markers. In the haplotype association analyses it is essential that the order of the closely located markers is correct and the published maps usually are rather sparse. In Studies II and IV, the GeneBridge 4 and the Stanford panel (Research Genetics, Inc., USA) were used for physical mapping, and the scoring results were analyzed by software (http://www-genome.wi.mit.edu/ or http://www-shgc.stanford.edu/). Physical maps were then used as a basis for genetic maps, 0,9 Mb corresponding to roughly 1 cM, although the recombinations do not occur evenly throughout the genome.

Table 4. Genotyped markers are based on published maps from Marshfield Center for Medical Genetics (http://research.marshfieldclinic.org/genetics/), Geneton (http://www.genethon.fr/genethon), the Cooperative Human Linkage Center (http://lpg.nci.nih.gov/CHLC/) and Genome Data Base (http://gdbwww.gdb.org/); and Kvaloy et al. 1994, Freije et al. 1992, and Marsh et al. 1994.

Chromosome 5q	**Chromosome 5q**	**Chromosome 16p**	**Chromosome 19p**	**Xq/Yq**
Microsatellites	*SNPs*	*SNPs*	*Microsatellites*	*Microsatellites*
D5S393	IL4ex1	I50V	D19S120	sDF1
D5S399	IL13ex4.2	E375A	D19S216	sDF2
D5S404	IL13ex4.1(R129Q)	C406R	D19S534	
D5S413	IL5pro	S411L	D19S536	**CFTR/7q**
D5S434	IRF1pro	S761P	D19S567	*Mutations*
D5S436	CSFex4	Sil676	D19S884	del394TT
D5S490	CSFenh2	Sil1114	D19S922	Δ508
D5S500	CSFenh1	Sil1417	FCER2	
D5S622	IL3 (P27S)			
D5S642	TCF7			
D5S816	IL9int4			
D5S1995	IL9ex5(T113M)			
D5S2115				
IL4				
IL9				
IRF1				

3.4.2. Genotyping of microsatellite markers

In these studies, the DNA was extracted from blood leukocytes by a standard non-enzymatic method. The DNA segment under study was amplified exponentially by polymerase chain reaction (PCR) in the PCR assays containing 50 ng of genomic DNA, 0.2 mM of each primer, 0.3 U of the DNA polymerase (Dynazyme, Finnzymes, Finland) and 0.2-0.4 mM of each dNTP in a total volume of 20 µl. The PCR products were electrophoresed on denaturating 7 M urea/ 6 % polyacrylamide gels where the alleles were distinguished by size and visualized by silver staining.

3.4.3. Genotyping of SNPs by restriction enzyme digestion

In Study V, four SNPs (I50V, E375A, C406A and S761P) of the IL4RA gene were genotyped with the restriction enzyme digestion method. Each of the polymorphic sites was amplified by PCR in a total volume of 10 µl with 50 ng of the dried DNA in each. The amount of the polymerase enzyme (AmpliTaq Gold, Perkin Elmer, New Jersey) varied from 0.5U (E375A and C406R) to 1.0U (I50V and S761P) per reaction. The polymorphic sites were cut by restriction enzyme digestion using *Aci I* (E375A), *Tsp45 I* (C406R), *Msl I* (I50V) or *Dde I* (S761P) (BioLabs, New England, MA). Finally, the samples were electrophoresed on an agarose gel where alleles were distinguished by size and visualized by UV illumination.

3.4.4. Genotyping of SNPs by length-multiplexed single-base extension

The length-multiplexed single-base extension method (LM-SBE) includes the multiplying of the SNPs by PCR using a primer with a tail of different length, single-base extension with fluorescently labelled dideoxynucleotide (also called mini sequencing) to locate and mark the SNP and finally, electrophoresis (Lindblad-Toh et al. 2000). The SNP alleles can be distinguished by size and different colours of fluorescent dyes. 12 SNPs on the 5q31 (IL4ex1, IL13ex4.1, IL13ex4.2, IL5pro, IRF1pro, CSFenh1, CSFenh2, CSFex4, IL3, TCF, IL9int4, IL9ex5) and 4 SNPs in the IL4RA gene (S411L, Sil676C/T, Sil1114T/C, Sil1417G/T) were genotyped with the LM-SBE method. The PCR primers

were designed as close as possible to the SNP (maximum length 150 bp). The primer pairs were checked for homology to all amplicons and sorted into pools. In the first round, 10 ng of the genomic DNA was amplified using a pool of primer pairs (0.1 uM) and 2.5 units of Amplitaq Gold (Perkin Elmer, NJ). In the second round, a 3 µl aliquot of the primary amplification product was amplified with biotinylated-T7 and biotinylated-T3 primers. A 7 µl aliquot of this secondary amplification product was purified from the unincorporated dNTPs using streptavidin-coated Dynabeads (Dynal, Norway). A multiplex SBE reaction was then carried out on the purified product using SNP-specific primers, JOE-ddATP (0.12 M),TAMRA-ddCTP (0.12 M), FAM-ddGTP (0.12 M), ROX-ddUTP (0.60 M; NEN DuPont) and Thermosequenase (0.5 U; Amersham). The excess ddNTPs were removed from the SBE products using 96-well gel filtration blocks (Edge Biosystems) prior to the electrophoresis on the ABI 377 sequencers. The LM-SBE gels were analyzed using an in-house computer program at the Whitehead Institute/MIT Center for Genome Research (Lindlad-Toh et al. 2000).

3.4.5. Screening for polymorphisms in the IL9 and FCER2 genes

The IL9 and FCER2 genes were sequenced in selected individuals using the genomic DNA. The amplicons covered all exons (exons 1-5 in IL9 and exons 1-11 in FCER2 gene) and the exon-intron boundaries. The sequencing was performed with a dye terminator chemistry using an ABI 373A sequencer (PE Biosystems, Foster City, CA). The screening for the T113M variant of the IL9 gene was done with the enzyme restriction digestion *Nco I* (BioLabs, New England, MA), then the samples were electrophoresed on an agarose gel and visualized by UV illumination.

3.4.6. Mutation screening

For the del394TT and the Δ508 mutation screening of the CFTR gene, the PCR assays were carried out with 50 ng of genomic DNA, 0.2 mM of each primer, 0.3 U of DNA polymerase (Dynazyme, Finnzymes, Finland) and 0.2 mM of each of dNTP in a total volume of 20 µl. The primer pairs were CTG GAG CCT TCA GAG GGT AAA AT and CAT GCT TTG ATG ACG CTT CTG TA for the Δ508 mutation, and CCT GGG TTA ATC TCC TTG GA and ATT CAC CAG ATT TCG TAG TC for the del394TT

mutation. The enzyme restriction digestion was applied using *Hinf I* (BioLabs, New England, MA), then the samples were electrophoresed on polyacrylamide gels and visualized by silver staining.

3. 5. Statistical analyses and power estimations

3.5.1. Linkage analysis

The linkage analyses are used when coinheritance of alleles at a marker locus with a disease is studied in a pedigree using families with two or more affected individuals and a cytogenetic map. The linkage analyses can be made either as two-point (with one marker) or as multipoint analyses (using several markers simultaneously). Also, the linkage can be studied either as non-parametric where no genetic modelling is needed, or as parametric with a supposed inheritance model. Here, the non-parametric linkage analyses were made as multipoint (studies II, IV and V) and as two-point (study III) using the computer package GENEHUNTER (Kruglyak et al. 1996; Daly et al. 1998). The qualitative traits were used for phenotypes: asthma/ unaffected, high (>100 kU/L)/ low serum total IgE level (Burrows et al. 1989; Postma et al. 1995), and positive/ negative allergy screening test result. Serum total IgE level was also analyzed as a quantitative trait with the MAPMAKER/SIBS (studies II and IV).

3.5.2. Transmission disequilibrium test

In families with heterozygotic unaffected parents and an affected child the transmission disequilibrium test (TDT) was performed. In the TDT, the transmitted alleles/chromosomes to the affected child are compared with the untransmitted alleles/chromosomes (Ewens and Spielman 1995) of the parents. For the study III, the TDT was also analyzed separately for paternal and maternal transmissions to sons and daughters because of the different inheritance patterns of the two studied markers.

3.5.3. Allele and haplotype association analyses

In allele and haplotype association studies a chromosome was marked as 'trait-associated' if it occurred in any of the affected family members and as a 'control' if it

occurred only in unaffected individuals. Every chromosome in each family was counted only once, and the unaffected family members formed the pool for control chromosomes (Thomson 1995). In the study IV, another method for marking trait-associated chromosomes was used to analyze dominant inheritance. A chromosome was marked as 'trait-associated' if it occurred in at least two affected family members, and again, as a 'control' if it occurred only in unaffected individuals. The haplotypes were constructed by hand for the studies II, III and IV and by data mining for the study V. The significance of the associations was analyzed first by a chi-square ($\chi2$) test and then by a permutation test. The chi-square ($\chi2$) test was applied in comparison with the size of two different groups, except when the expected number in a single cell in a 2x2 contigency table was less than five, Fisher's exact test was applied. When a $\chi2$-test was used several times independently, the nominal P values were multiplied by the number of tests (Bonferroni correction).

3.5.4. Haplotype Pattern Mining method

In the Haplotype Pattern Mining (HPM) all trios with one or two affected individuals were chosen for haplotype analyses. The trios were formed of larger pedigrees with an in-house computer program which excluded trios having a member with an unknown phenotype or an unknown genotype. The chromosomes were divided into 'trait-associated' or into 'controls' as explained previously (see allele and haplotype association) and in case of ambiguities, the alleles were zeroed out. Then, using algorithms, the specific haplotype patterns associated with the trait were searched for. The association was studied with a $\chi2$ test and a number of qualified haplotype patterns spanning across the marker and exceeding the set threshold for disease-association was counted (a marker-wise score). The method relies on the assumption that there is more linkage disequilibrium in the set of disease-associated chromosomes than in the set of non-associated chromosomes in the proximity of a disease susceptibility allele. The greater the difference in the levels of the linkage disequilibrium, the more significantly disease associated patterns will be found.

In addition to the association threshold, the maximum length of a pattern and the number of gaps (missing data or genotyping errors) are also set as parameters. A haplotype pattern matches the chromosome, if all of its non-gap alleles agree with the respective alleles in the chromosome. The scores of different markers are not directly comparable, since the marker densities and their information content vary. To compensate for this, HPM uses randomization to obtain marker-wise P values that are comparable with each other. At each randomization cycle, the disease association status of each haplotype is assigned at random, keeping the total number of affected and unaffected haplotypes constant, and the scores are recalculated. The P value for a given marker is the proportion of iterations at which a score larger than or equivalent to the experimental pattern is obtained. The disease susceptibility gene is most likely to lie in the proximity of the marker with the lowest P value (Toivonen et al. 2000).

3.5.5. Homozygosity testing

If an autosomal or X-chromosomal allele is associated with a disease, the homozygosity for the allele should associate with the disease even more strongly, which can be tested by simulations. In the study III, the simulations for homozygosity testing (P_{simul2}) of the X-chromosomal marker alleles were carried out with females only. All unrelated affected females were considered, and their observed alleles were used for simulations. Then, in each of 100,000 iterations, random pairs of these alleles were formed, and the number of simulated homozygous chromosome pairs was counted. The iterations provided a distribution for the number of expected homozygotes under the null hypothesis of no excess homozygosity, treating the number of alleles as fixed. Finally, the observed number of homozygotes was compared with this distribution to obtain the P_{simul2} value.

3.5.6. Statistical significance estimations using a permutation test

To estimate the significance of overall observations ($\chi 2$ test performed several times) of the association analysis the permutation test was done by simulating the results (P_{simul1}): The observed genotypes were used with the same number of trait-associated and control chromosomes as in the real data, but the affectus status of the chromosomes was randomized. This randomization was iterated 100 – 10.000, times and the permutation

results were then compared to the observed results. The comparison was done with Pχ2 values of the most extreme association in actual and simulated data.

3.5.7. Power estimations

The power estimations were based on simulations and the estimations (Simul3) were done by choosing a haplotype present at a frequency F (5%, 10%, 15% and 20%) among the affected chromosomes. The association was then simulated with 10 000 iterations for each F, and the power to detect association was measured by the fraction of the replicates in which the haplotype association showed Pχ2=0.05.

4. RESULTS

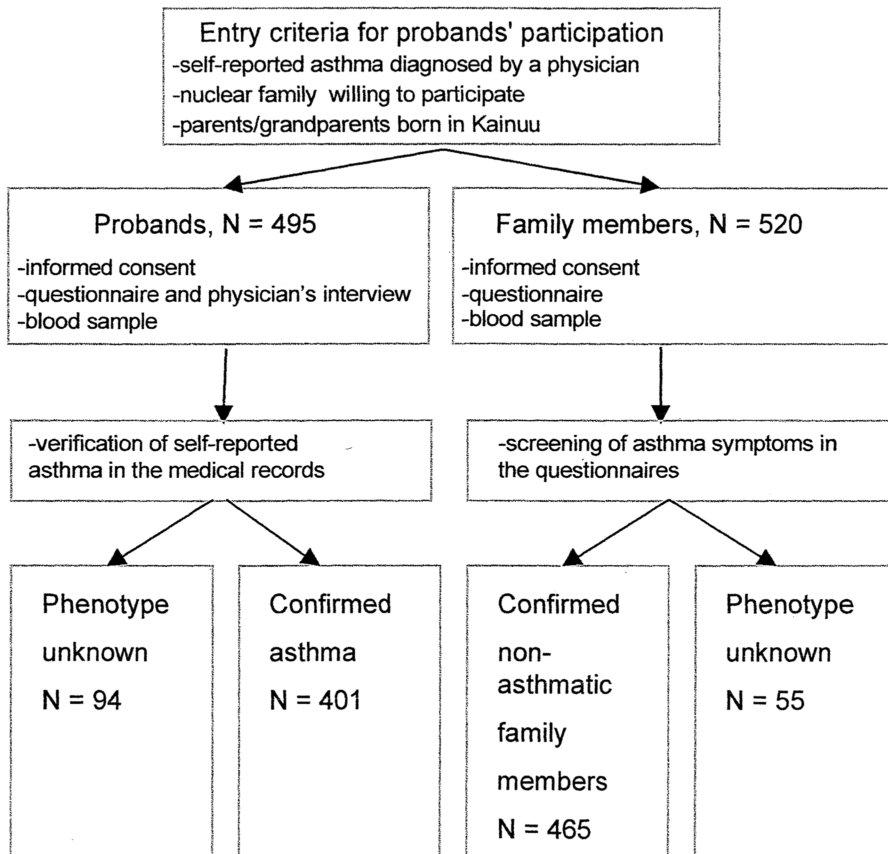
4.1 Phenotyping results

4.1.1. Verification of self-reported asthma diagnosis

Altogether 1015 individuals took part in the study, 401 of these were confirmed to have asthma (132 males and 269/67 % females), and 465 individuals were grouped as non-asthmatic family members (239 males, 236 females) (Figure 3). 149 individuals (94 self-reported asthma patients and 55 family members) could not be classified into either group, and they were handled as having an unknown phenotype. The study population comprised 253 families, of which 111 were multiplex families with two or more affected family members. The population included also 139 sib pairs which could be divided into 50 sib pairs concordant with asthma and 28 control sib pairs, the rest being discordant pairs (Figure 4). The verification of self-reported physician-diagnosed asthma was based both on medical history and diagnostic lung function tests showing either reversible airway obstruction or BHR or both using the criteria of the Social Insurance Institution for reversible airway obstruction (Study I, Table 1). All the individuals were finally categorized into asthma patients, controls (unaffected family members) or those with unknown phenotype (Figure 3).

After the ascertainment, the obtained study population was carefully analysed to see if self-reported physician-diagnosed asthma could be used as an inclusion criterium for genetic studies. Self-reported asthma patients (N = 495) were divided into six diagnostic categories (confirmed asthma, probable asthma, possible asthma, chronic obstructive pulmonary disease, other causes for dyspnea) based on a review of medical records, and those with insufficient data for classification formed the seventh group. In 364 patients (73,5 %), self-reported asthma was confirmed, the results being based both on medical history and diagnostic lung function tests showing either reversible airway obstruction, BHR or both or who had reimbursement for anti-asthmatic medication granted by the Social Insurance Institution (Table 5). The long-term smokers had been excluded from this group. The group of probable asthma patients (N = 37; 7 %) included all the patients

Figure 3. Classification of the study individuals into asthma patients and non-asthmatic family members.



who reported recurrent asthmatic symptoms but who did not have a reimbursement for medication. 54 % of these patients had a reversible airway obstruction shown in lung function tests but either the disease history was too short for the reimbursement or the data for the reimbursement was not found in medical records. 16 % of the patients with probable asthma had bronchial hyperreactivity and had been auscultated with wheezings but had not had diagnostic findings in spirometry, in PEF recording or in exercise test. 30 % of the patients with probable asthma had developed asthma in childhood and their lung function tests were either insufficiently done or the documentation of the testing was poor or inaccessible, or the results were not diagnostic according to our criteria. However, both the group of confirmed and probable asthma patients represented those who reported a constant need for medication and a positive response to medication. The groups of confirmed asthma and probable asthma were included in our genetic studies with the verified phenotype of asthma.

In the group of possible asthma patients (N = 26), some asthma symptoms and reversible airway obstruction/BHR were found, but during the follow-up period found in the medical records, neither recurrent symptoms nor a need for medication was developed. In the group of patients with chronic obstructive pulmonary disease (COPD, N = 28), those were included who had been smoking for over twenty years. The main component of their disease was the irreversible obstruction of the airways developed at later age than in verified asthma patients. Five patients had other causes of pulmonary insufficiency. All these patients had dyspnea, but no clinical evidence of asthma. Seven patients had been diagnosed with occupational asthma and were not considered suitable for a genetic study. In 28 patients the medical records were either insufficient or not available for classification. The groups of possible asthma and COPD patients as well as those with other causes of pulmonary insufficiency or insufficient data for classification were labelled as phenotype unknown (non-classified) in our genetic studies. Those with occupational asthma were also labelled as phenotype unknown. In addition, those family members that reported having asthma symptoms (dyspnea or wheezing) and having used anti-asthmatic medication (N = 55) were classified as phenotype unknown.

Table 5. Characteristics and lung function test results of the self-reported asthma patients either with (A) or without (B) reimbursement for medication and who were verified to have asthma. Those who were excluded from the asthma patients were classified as having an unknown phenotype (C) and consisted of patients with mild asthma like symptoms but no diagnostic finding in lung function tests, patients with COPD, patients with other causes for dyspnea, patients with occupational asthma and patients with insufficient data.

	Verified asthma patients		**Phenotype unknown**
	A. Reimbursement for medication *N = 364 (%)*	B. No reimbursement for medication *N = 37 (%)*	C. *N = 94 (%)*
Male	120 (33)	12 (32)	32 (34)
Age, average (years)	43 *#	32 £	47
S - IgE, average (kU/L)	237	389	331
Smoking regularly > 10 y	59 (16) #	3 (8) £	39 (41)
Wheezing by auscultation	227 (62) *#	20 (54)	29 (31)
Average age at diagnosis (y)	35 #	28 £	46
Diagnosis made in childhood	15 (4)*	11 (30) £	2 (2)
Spirometry			
Information available	307 (84) *#	21 (57)	55 (59)
Diagnostic results	174 (48)	11 (30)	36 (38)
PEF recording			
Information available	312 (86) *#	26 (70) £	47 (50)
Diagnostic results	197 (54)	14 (38)	30 (32)
Exercise testing			
Information available	103 (29) #	10 (27)	15 (16)
Diagnostic results	29 (8)	3 (8)	2 (2)
Histamine or methacholine challenge			
Information available	229 (63) #	19 (51)	37 (39)
Diagnostic results	176 (48) #	16 (43)	22 (23)
Any of the above tests			
Information available	325 (89) *#	27 (73)	58 (62)
Diagnostic results	280 (77)	20 (54)	46 (49)

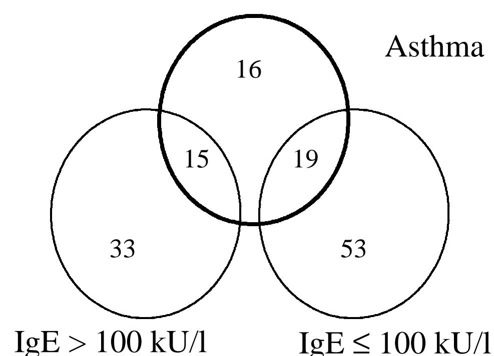
* Statistically different results between the groups A and B.
# Statistically different results between the groups A and C.
£ Statistically different results between the groups B and C.

4.1.2. Allergy screening, serum total IgE and self-reported allergic symptoms

The serum total IgE level was measured of 1008 samples, and the cut-of-point for high serum total IgE level was set to 100 ku/L (Burrows et al. 1989; Postma et al. 1995), although in the clinical medicine the reference value of serum IgE for the Finnish adults is <110 kU/L (Björksten et al 1987). There were 362 individuals (47% males) with a high serum total IgE level and 646 (39% males) with a low IgE level, and the difference between sexes was significant ($p<0.02$). 49% of the high responders had confirmed asthma, while the respective number for the low responders was 35% ($P<0.00002$). Smoking habits did not differ between the two groups. Those with a high serum total IgE level, were 39.5 years old on average and those with a low level were significantly older, 46 years on average.

To see how well phenotypes of sib pairs correlated to each other, the serum total IgE values of concordant sib pairs with asthma and the unaffected phenotype were compared. The correlation for the IgE among the sib pairs concordant with asthma was 0.43 (95% CI ± 0.17) and among sib pairs concordant with an unaffected asthma status 0.32 (95% CI ± 0.23) (Figure 5).

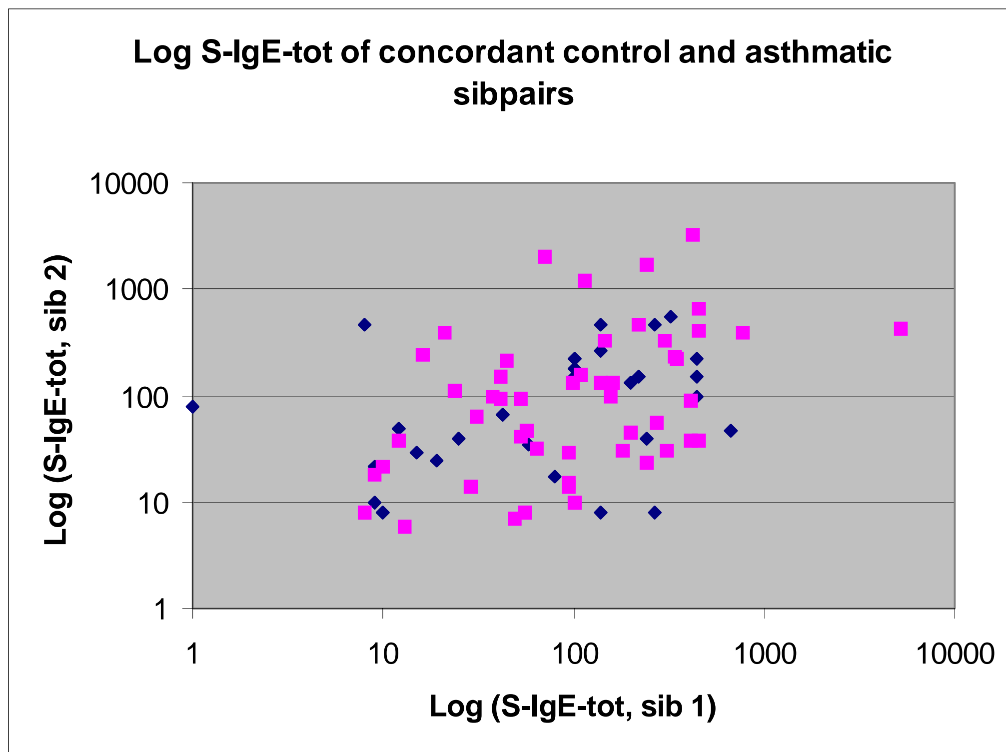
Figure 4. Number of concordant sib pairs with asthma, with a low serum total IgE and with a high serum total IgE level.



When the characteristics of the allergy screening test positive (N=334) and negative (N=672) individuals were studied, their sex did not have a significant effect (males 46 % vs. 40 %, respectively) but the smoking habits were significantly different between the positive and negative individuals (smoking for over 10 years, 16 % and 23 % respectively), which was different from the high and low serum total IgE groups. As expected, the allergy screening test positive individuals were significantly younger (33 years vs. 49 years, respectively), and the serum total IgE level was higher (420 kU/L vs. 72 kU/L, respectively) as well as the percentage of asthma patients was higher (52 % vs. 34 %, respectively) among the screening test positive individuals.

The distribution of specific IgE antibodies was also studied with the following observation among all the study individuals: 17.5 % had specific IgE antibodies for cat, 17.0 for % timothy, 16.8 % for dog, 13.6 % for house dust mite, 12.6 % for birch, 11.6 % for horse, 7.2 % for mugwort, and 3.9 % for mould Cladosporium herbarum. The respective numbers for the verified asthma patients were: 28.4 % had specific IgE antibodies for cat, 28.4 % for dog, 27.2 % for timothy, 20.4 % for horse, 19.2 % for birch, 17.2 % for house dust mite, 10.2 % for mugwort, and 6.5 % for mould cladosporium herbarum. The spectrum of allergy antibodies among the asthma patients did not differ from that found among the family members, but the screening positive asthma patients had more frequently two or more specific antibodies elevated compared to their family members.

Figure 5. Logarithm of total serum IgE values in sib pairs concordant with the asthma status: Asthma (□, gray) and non-asthmatic sib pairs (◊, black).



For the whole study population, the screening results for IgE-mediated allergy were compared to the self-reported nasal allergic symptoms and self-reported physician diagnosed allergic rhinitis among the asthma patients and among their family members. Neither of the questions reached a high sensitivity, specificity nor positive/ negative predictive value. Among the self-reported asthma patients, 93 % of the allergy screening positive but also 74 % of the screening negative, reported symptoms of allergic rhinitis (Table 6). The corresponding figures among their family members were 61 % and 40 %, respectively. Self-reported allergic symptoms showed a better sensitivity in both groups (93 % for the asthma patients and 61 % for the family members) than a physician diagnosed allergic rhinitis (68 % and 29 %, respectively), whereas a diagnosed allergic rhinitis showed a better specificity (54 % among the asthma patients and 91 % among the family members) than self-reported allergic symptoms (26 % and 60 %, respectively) in both of the groups.

Table 6. Self-reported allergic nasal symptoms and self-reported physician diagnosed allergic rhinitis among asthma patients and their unaffected family members compared to the allergy screening results. The screening results of 9 individuals are missing for technical reasons. Calculations for sensitivity, specificity, positive and negative predictive values are adjusted for missing data (= N.d.).

		Asthma patients				**Family members**		
		Allergy screening				*Allergy screening*		
		Positive	Negative	Total		Positive	Negative	Total
		N=169	*N=226*	*N=395*		*N=104*	*N=354*	*N=458*
Self-reported allergic nasal symptoms	Yes	157	162	319	Yes	62	133	195
	No	11	56	67	No	39	203	242
	N.d.	1	8	9	N.d.	3	18	21
Sensitivity	157/168 = 93%				62/101 = 61 %			
Specificity	56/218 = 26 %				203/336 = 60 %			
Positive predictive value	157/319 = 49 %				62/195 = 32 %			
Negative predictive value	56/67 = 84 %				203/242 = 84 %			
Physician diagnosed allergic rhinitis	Yes	106	81	187	Yes	27	24	51
	No	51	95	146	No	65	256	321
	N.d.	12	50	62	N.d.	12	74	86
Sensitivity	106/157 = 68 %				27/92 = 29 %			
Specificity	95/176 = 54 %				256/280 = 91 %			
Positive predictive value	106/187 = 57 %				27/51 = 53 %			
Negative predictive value	95/146 = 65 %				256/321 = 80 %			

4.2. Genotyping results according to chromosomal locations

4.2.1. Chromosomal region 5q31-q33 (Studies II and V)

A large chromosomal area on chromosome 5q including a cytokine gene cluster has been suggested to be involved in IgE production, eosinophilia and bronchial hyperractivity. In Study II, 16 polymorphic markers were genotyped on the chromosome 5q31-q33 spanning 30 cM. The markers were genotyped in 313 individuals forming 157 families with 73 sib pairs and 41 cousing pairs. Two phenotypes were analyzed as qualitative traits (asthma vs. unaffected, and serum IgE >100 kU/L vs. serum IgE ≤100 kU/L), and no linkage between asthma or high serum total IgE level was found. The NPL scores were negative across the studied region. In Study V, the 5q31 region was reanalyzed in 79 families for asthma and in 45 families for high serum total IgE level using 12 SNPs in addition to microsatellites. Again, asthma and high serum total IgE level were used as phenotypes and analyzed as qualitative traits. The non-parametric linkage score (NPL) was negative throughout the studied region for both phenotypes (information content 35%-69%).

In the allele association analysis where SNPs were also used (study V), no allele association with asthma or high serum total IgE level could be detected. In the haplotype association analysis (Study II) with microsatellites, some clustering of haplotypes around the IL9 marker was found, but according to the permutation test the clustering was not significant. Because of the concentration of the haplotypes around the IL9 marker in the first study on 5q, the IL9 gene was sequenced in four individuals. A polymorphism (338C→T, T113M) was found, but it was not associated with asthma or high serum total IgE level.

The haplotype reanalysis of the 5q31 region with SNPs added to the microsatellites was done with 299 disease-associated and 245 control chromosomes for asthma, and with 231 trait-associated and 177 control chromosomes for high serum total IgE level. The SNP

haplotypes were made using an in-house computer program, and the same haplotypes were used as an input for the Haplotype Pattern Mining. 30 % missing data but no errors were allowed in the haplotype patterns. The majority of the asthma 5q31 haplotype patterns spanned across the marker CSFenh2, but no pattern around the CSFenh2 was significantly disease-associated. For high IgE, haplotype patterns around the SNP IL13ex4.1 were found, but the chi square values were rather low.

The HPM analysis of the 5q31 cytokine cluster was done by setting the maximum length of pattern for 5 markers, allowing one gap in the pattern (for missing data and possible errors) and using the $\chi 2$ threshold 3.0 for association. The best obtained P value for asthma was 0.3 (IL13ex4.1), 0.015 for high serum total IgE (IL13ex4.1) (Study V, Figure 1). To analyze the significance of the results, a permutation test with 1000 randomisation of the affectus status of the chromosomes was performed. The permutation showed that the best observed P values were between the 0.05 and 0.001 levels of the permutated data. However, the marker-wise P values need a correction for multiple testing even after the permutation. This correction is dependent on the number of studied phenotypes, the number of studied markers and the number of studied HPM settings. The two correlated phenotypes and the two different loci that have been studied form a correction factor of 2.6. Although the final number of the independent tests is difficult to determine, the best obtained marker-wise P values do not tolerate much further correction.

4.2.2. The IL4RA gene on 16p12 (Study V)

On the chromosome 16p12 is located the IL4RA gene which codes the α chain of the interleukin 4 receptor. In the linkage analysis of the study V, 79 families were analyzed for asthma phenotype and 45 families for high serum total IgE level using 7 SNPs in the IL4RA gene. No evidence for linkage was found in the IL4RA region for either of the phenotypes with the NPL score from –1.4 to 0.1 (information content 53%-62%).

No allele association was found with asthma or high serum total IgE level with IL4RA SNP alleles, when one affected individual was randomly selected from each family and

compared to family based controls. The haplotype association was studied with 320 disease-associated and 260 control chromosomes for asthma and 257 trait-associated and 195 control chromosomes for high IgE level. Again, the haplotyping was done with the in-house computer program. Both asthma- and high IgE -associated haplotype patterns clustered around markers Sil676 and Sil1114, but the found associations were weak.

The HPM analysis of the IL4RA gene SNPs was done with the same settings as for 5q (the maximum length of pattern for 5 markers, one gap in the pattern and the $\chi 2$ threshold 3.0). The best obtained P value in the IL4RA was 0.012 for asthma (S411L) and 0.019 for high serum total IgE (C406R) (study V, Figure 1). The permutation test with 1000 randomisation showed that the best observed P values were between the 0.05 and 0.001 levels of the permutated data. According to the HPM results, the IL4RA as an asthma and atopy susceptibility gene cannot be excluded in this population.

The allele combinations in IL4/IL4RA and IL13/IL4RA genes of each asthma patient with a 2x2 contingency table were also studied. To avoid an ascertainment bias, only one affected family member was randomly selected from each family. Since attained sample size did not have statistical power to evaluate the significance of very rare combinations, affected individuals who were homozygous for the minor alleles in both marker loci were excluded. The only observation for asthma that differed from what was expected was the distribution between IL13ex4.2 and Sil676 alleles (P<0.03). For high IgE, the distribution between IL13ex4.2 and Sil676 alleles showed a similar weak association (P<0.04, data not shown). However, the correction for multiple testing rendered these weak associations insignificant.

4.2.3. The FCER2 gene region on 19p13 (Study IV)

The FCER2 gene for the low affinity receptor for IgE on chromosome 19 has been suggested to be involved in IgE production by a negative feed-back. In the chromosomal region 19p13 spanning a 10-cM region, 8 markers were screened for 124 families with 57 sibpairs and 35 cousin pairs. Linkage analysis was done with 51 pedigrees for asthma,

with 44 pedigrees for high serum total IgE level and for the phenotype of positive allergy-screening result with 40 pedigrees. For asthma, the NPL score remained negative. For the phenotypes of high serum total IgE level and positive allergy-screening test, the NPL score was positive but not significant (<1) (information content 72-74%). The serum total IgE level was also used as a quantitative trait for linkage analyses, but again, no linkage was found.

In the association analyses, the allele D19S922*8 was associated with asthma in a $\chi 2$ test (70/285 disease associated chromosomes and 38/226 control chromosomes, P<0.03) but after the correction for multiple testing, the association was not significant. When the haplotypes were studied, the haplotype D19S567*16-FCER2*2-D19S534*7-D19S922*8 showed a 5-fold risk for asthma with 18/316 (5.7 %) disease-associated and 3/244 (1.2 %) control chromosomes. However, the permutation test showed no significance for the results (in 1 163 permutations out of 10 000 the association results exceeded the observed, P=0.12). The haplotype D19S120*3-D19S536*4-D19S216*4-D19S567*16-FCER2*3-D19S534*7 was detected for the phenotype of high serum total IgE level in 11/255 (4.3 %) trait-associated and in 3/352 (0.9 %) control chromosomes, and for positive allergy-screening in 15/246 (6.0 %) trait-associated and in 7/370 (1.9 %) control chromosomes (relative risk 5.2 and 4.9, respectively). With the permutation test, however, the haplotype association reached significance only for high serum total IgE level, P_{simul1}=0.038 (for the positive allergy-screening P_{simul1}=0.19).

When the haplotype association analysis was done by limiting the disease-associated chromosomes into those that were found at least in two affected individuals in a family, the haplotype D19S567*16-FCER2*2-D19S534*7 was found in 12/49 (24.5 %) of the disease-associated chromosomes for asthma. In the previous analysis the respective numbers were 18/316 (5.7 %), P=8.3e10^{-6}. The same haplotype was present in 3/244 (1.2 %) of the control chromosomes (P=1.1e10^{-11}). Using the same method for the phenotype of high IgE, the haplotype D19S216*4-D19S567*16-FCER2*3-D19S534*7 was not significantly enriched in the trait-associated chromosomes 6/48 (12.5 %). In the previous analysis the respective numbers were 24/255 (9.4 %, P=0.2) for the trait-associated

chromosomes and 12/352 (3.4 %) for the control chromosomes. For the phenotype of the positive allergy-screening, none of the haplotypes was enriched.

The FCER2 gene was sequenced in four affected individuals including a homozygote for the D19S567*16-FCER2*2-D19S534*7-D19S922*8 haplotype, and also in one control individual, but no coding polymorphisms were found.

4.2.4. IL9RA region on Xq/Yq PAR (Study III)

Near the IL9RA gene region, one pseudoautosomal marker and one X-chromosomal marker were genotyped in the area of 400kb. The linkage was studied as a non-parametric two-point in 84 families including 80 sib pairs and 62 cousin pairs for asthma and high serum total IgE level. The NPL score remained negative for both markers and for both phenotypes with an information content of 34%-46%, but it did not allow exclusion.

The TDT was first analyzed in all the families with an affected child and heterozygous parents and then separately for paternal and maternal transmissions to sons and daughters. The allele sDF2*10 was transmitted to 34 and untransmitted to 16 asthmatic offspring ($P\chi^2 = 0.01$). The allele sDF2*10 originated mainly from two X-chromosomal haplotypes: sDF2*10-sDF1*6 (11 transmitted vs. 3 chromosomes untransmitted, $P\chi^2$= 0.03) and sDF2*10-sDF1*1 (5 transmitted vs. 0 chromosomes untransmitted, $P\chi^2$= 0.03), while none of the Y chromosomes showed a transmission distortion. When studying maternal transmission to affected daughters, the haplotype sDF2*10-sDF1*6 was transmitted (5) more frequently to affected daughters than untransmitted (0), ($P\chi^2$= 0.03). For high serum IgE, the TDT results were not significant.

In the allele association analysis, the X-chromosomal allele sDF2*10 was found more frequently in disease-associated (asthma) than in control chromosomes (28% vs. 20%, $P\chi^2$=0.05). In 176 permutations out of 1,000, the most extreme allele association found in simulated data was stronger than in actual data (P_{simul1}=0.18). The allele frequencies of

the marker sDF1, present both in X and Y chromosomes, were equally distributed in trait-associated and control chromosomes. The haplotype association analysis was performed on X chromosomes and for asthma, the haplotype sDF2*10-sDF1*6 was found more frequently in disease-associated chromosomes than in control chromosomes (40 vs. 7, $P\chi^2<0.005$). The finding remained significant in the permutation test. In only 40 out of 1 000 iterations, the found haplotype association was more extreme than in actual data ($P_{simul1}=0.04$). Analyzed separately for male and female X chromosomes, the association was significant for females (34 affected haplotypes and 6 controls, $P\chi^2<$ 0.05). For high IgE level, no allele or haplotype associations were found.

If an excess of homozygotes could be found, was next tested. The testing for the sDF2 alleles was done with unrelated females only in 100 000 simulations. In the testing, an excess of homozygosity was found for the sDF2*10 allele and asthma phenotype but not for other alleles and not for the marker sDF1. Among the asthma patients, 13 sDF2*10 homozygous individuals were observed, whereas the average, as expected from the simulation, was 7.7 ($P_{simul2}=0.009$). For the controls, the number of homozygous individuals was only 3. The overall observed homozygosity for both markers did not differ from the expected homozygosity.

In this data set, our power to show haplotype association for high total serum IgE level was high, if the frequency of a susceptibility haplotype among trait-associated chromosomes (F) was between 0.15 and 0.20 (99% and 93%, respectively). When F=0.10 or F=0.05, the corresponding power to detect the association gradually decreases (67%, and 18%, respectively). Our power to detect a haplotype association for the asthma phenotype was similar: 97% (F=0.20), 81% (F=0.15), 49% (F=0.10), and 6% (F=0.05).

4.2.5. Cystic fibrosis mutations carriers and asthma (unpublished data)

The CFTR mutation carriership has both been reported to predispose individuals to asthma (Dahl et al. 1998) and to protect them from asthma (Schroeder et al. 1995). Because only two major CFTR gene mutations (del394TT and ΔF508) have been

detected in Finland (Kere et al. 1994), the possibility to screen for CFTR mutation carriers in asthma families was unique. All unrelated individuals were selected from the family data and screened for the del394TT and the ΔF508 (208 individuals) mutations. Of the 166 asthma patients, four were carriers of the del394TT mutation whereas none of the 42 control individuals was a carrier (P not significant). No carriers of the ΔF508 were found. The carrier frequency of the del394TT mutation was 1,9 % in this population which was selected through a proband with asthma and which is located in the area with a known clustering of Finnish cystic fibrosis patients.

5. DISCUSSION

Our study population was designed to find major genetic regulators that would increase the risk of the disease 3-5 fold. The simulations showed that in the haplotype association analyses we had a good power to detect an excess of 20-15 % of the susceptibility haplotypes. In these studies, we found evidence of the IL13 and IL4RA genes containing susceptibility alleles as well as the FCER2 gene region and the IL9R region including genetic asthma and atopy determinants in the Finnish population. In the previous studies on candidate genes worldwide, the results have been controversial, and none of the genes has been shown to be common for all studied populations. However, the 5q31 cytokine gene cluster and especially the IL4 pathway with its receptor, have probably been the most apparent finding, and our results are in agreement with others. Next, we shall represent some aspects that have to be taken into consideration when our studies are estimated.

5.1. Aspects of phenotyping

We confirmed the self-reported asthma diagnosis using retrospective data on medical records with previously done lung function testing and clinical examination. This was possible, because the diagnostic criteria for asthma in Finland are based on the recommendation of the American Thoracic Society (ATS, 1987) and the criteria are uniform in the whole country. Also, the reimbursement policy of the Finnish Social Insurance Institution favours uniform diagnostic procedures: The reimbursement for anti-asthmatic medication is granted only if the exact criteria are met. This procedure is to guarantee that especially those patients who need regular medication are carefully diagnosed, including the basic lung function testing before the medication is started. The third beneficial factor was that most of the asthma patients had undergone the clinical examination for asthma in the same clinic (Kainuu Central Hospital) which has employed only few pulmonologists over the years. Thus, although we were not able to examine the patients under the current study protocol, we were familiar with the used diagnostic criteria, and we were able to trace back the diagnostic procedures in the medical records

in most cases. Since then, diagnosing asthma on the basis of questionnaire data was studied, and it was demonstrated that the questionnaire-based asthma diagnoses agree well with those based on interviews, and that the pulmonary function testing added little to questionnaires and interviews (Barnes et al. 1999). Also, a good agreement between questionnaire data and medical records of other chronic diseases, such as cardiovascular diseases and diabetes, has been shown in the Finnish study by Haapanen et al. (1997). Since we verified the asthma diagnoses in medical records neither used the self-reported data alone nor accepted those with long smoking history, we also rejected individuals with other obstructive lung diseases and thus false positives (Los et al. 1999).

We used three phenotypes in these studies (asthma, high serum total IgE level and positive allergy screening), two of them being especially overlapping. Bronchial hyperreactivity (BHR) would have been an interesting subphenotype to use, but because of the time and the method differences (both histamine and methacholine challenge had been used) in the previously done bronchial hyperreactivity testing we found the BHR problematic to study. Also, Finnish asthma patients are normally treated with inhaled corticosteroids as soon as the diagnosis is obtained, and since inhaled corticosteroids are known to reduce the BHR (Laitinen et al. 1991), the number of hyperreactive individuals would probably been lower than during the diagnostic testing. However, it would have been interesting to have a clinical examination of the probands to evaluate a more detailed course of their disease. Also, if bronhial hyperreactivity should be used as a phenotype in this population, retesting would be advisable both to avoid the previously mentioned time and methodological differences and to offer a quantitative trait for linkage analysis. Also, 11 % of the self-reported asthma patients were excluded because of either insufficient data or negative test results. By having included a retesting of lung functions in our study protocol, we might have been able to classify some of those as confirmed asthma patients and thus to get more affected ones for our analyses.

We considered the granted reimbursement for anti-asthmatic medication as an objective verification for asthma if long-term smoking was excluded and if there was no other respiratory diseases. However, in 11 % of those with confirmed asthma and

reimbursement, the lung function test results were inaccessible for us. Thus, also in this group the possibility to include the lung function testing in our study protocol would have been valuable. The clinical course of asthma varies both between individuals and in the same individual by time. The variation is both spontaneus and is affected by anti-asthmatic treatment making the asthma diagnosis problematic. Having included lung function testing in our study protocol would not have faded these problems but would have offered current lung function test results for the diagnosis.

The unaffected family members did not undergo a clinical testing. Again, we chose to accept the questionnaire information of asthmatic and allergic symptoms (or no symptoms). Although allergic rhinitis and high serum total IgE are risk factors for asthma in a population level (Burrows et al. 1989; Huovinen et al. 1999), there still are no tests available that would point out those individuals actually getting symptomatic asthma. But this is a general problem concerning studies of diseases with an additional adult-onset phenotype. Also, if there are affected individuals among the unaffected ones, the results of association analysis are diluted rather than strengthened. However, also in this study group, the possibility to do lung function tests might have been beneficial, since 11 % of the family members were excluded because of self-reported asthma symptoms. Some of these might have been included into affected ones with lung function testing.

When taken together, we considered self-reported physician diagnosed asthma as an appropriate inclusion criterium for a genetic study when combined with the information in the medical records and the granted reimbursement for the anti-asthmatic medication by the Social Insurance Institution. Furthermore, self-reported allergic nasal symptoms and self-reported physician diagnosed rhinitis had both a poor specificity and a poor sensitivity, and these were not used without a verification of allergy, such as serum total IgE level or allergy screening test.

5.2 Choice of population

Isolated populations have been considered to be more advantegous than outbred populations when studying multifactorial disorders because of the smaller number of susceptibility alleles/genes as well as because of longer genetic intervals of linkage disequilibrium. However, the number of founders or the genetic bottleneck needs to be small (Kruglyak 1999) when using linkage disequilibrium based methods. In Kainuu, the number of unrelated founding chromosomes has been estimated to be only a few hundred. Also, during the first 200-250 years, the population remained small (a long and narrow genetic bottleneck), after that the population rapidly increased to reach the current size (Koskinen et al. 1994). Within the Kainuu population, the LD of those individuals with a rare inherited disorder has previously been shown to extend up to 12 cM (Höglund et al. 1995). Kruglyak et al. estimated the LD extent by simulations to be up to 3 kb in the general population and that the extent of LD in isolated populations would not differ significantly from that unless the number of founders was very low.

In other multifactorial diseases studied in Finland, promising results have been obtained: Familial combined hyperlipidemia has been linked to the chromosomal area 1q21-q23 with a lod score of 5.9, but no specific haplotype was found (a study population ascertained from several places across the country). For the psoriasis, a haplotype spanning across two genes in the HLA area was found to be associated with the disease (study population from the Kainuu region). In a genomewide search for the schizophrenia, four loci with lod scores from 1.95 to 3.82 were detected (Kuusamo, another isolated Finnish subpopulation) and in a genome scan of multiple sclerosis the chromosomal area 17q22-q24 was found with a lod score of 2.8 (study population mainly from the province of Vaasa). In the studies of the backround LD in a random Finnish population and in a subpopulation of Finland (Kuusamo) Varilo et al. found only little more LD in the Kuusamo population than in the general Finnish population (Varilo et al. 2000). This, however, does not mean that LD would not exist also in common diseases in the disease-associated chromosomes around the disease causing gene, as shown in the study of BRCA1 and BRCA2 carriers (Sarantaus et al 2000). Besides, as stated by Varilo et al., a more uniform environment with the same culture and genome backround makes

isolates better when phenotype-genotype correlations are assessed. Thus, we found the Kainuu population optimal for susceptibility gene studies for asthma and atopy, although the Finnish population as a whole would already have been too diverse for this study design.

Although we considered that an isolated population would offer advantages in gene mapping, it still may as well provide disadvantage. A susceptibility gene found in an isolate may not be so important for genetic susceptibility in a more outbred population. And even more probable is that not all those susceptibility genes found in a mixed population will also be present in a founder population. Large populations likewise include more affected individuals than small populations, which makes it easier to achieve an adequate number of affected individuals for a genetic study. Additionally, it is more difficult to obtain another population sample for the replication of the analysis in an isolated and rather small population than in a large mixed population. A replication study may be used to test if the found association between an allele/haplotype and a trait is achieved by a chance or if it is a true association.

5.3. Power for linkage

The linkage analysis measures coinheritance of alleles at a marker locus with a disease in families. It is measured by recombinations between a disease locus and a studied marker, using a logarithm of odds score. In this type of analyses, large pedigrees with many affected individuals are optimal. The linkage analysis is an especially suitable method for rare inherited diseases where the inheritance pattern is known, the phenotype has a clear cut-off point, the susceptibility alleles are rare in the common population and the number of needed pedigrees is relatively small. Having negative linkage results despite of the positive association results for the IL13, IL4RA, the IL9R gene region and the FCER2 gene region can be explained by a study population consisting of mainly nuclear families, phenocopies in the same pedigree, low penetrance of the studied allele and genetic heterogeneity (same phenotype may result from different genotypes) (Lander and Schork 1994). Even if genetic homogeneity could be achieved by choosing a founder for the

study population, nevertheless, the low penetrance cannot be excluded as a possible explanation for the negative linkage results.

The transmission disequilibrium test measures the possibility if a specific allele or haplotype is more often transmitted to an affected individual than to an unaffected individual in a family (transmission distortion). Thus, it is a combination of the linkage and allele association approach. With trios, where one of the parents could also be affected, our study population was designed for the haplotype association, and out of this reason the sample size for the TDT was rather low, and it was not an optimal statistical method for analyzing the results.

5.4. Association studies

The association analysis is a method for studying an association between a disorder and a specific allele or a haplotype. An allele association analysis can be made either by using a case-control approach or by using a family-based population strategy. The allele association studies represented here are made by the latter method which is optimal, when false positive results raising from the population stratification are wished to be avoided (Lander and Schork 1994). The probability to find an association is also affected by the dilution of control individuals with affected individuals, which would, however, rather weaken than strengthen the results. Still, when association analyses have been compared to linkage analyses, it was estimated that mapping complex diseases using association based methods might require considerably less families compared with the method of linkage analyses (Risch and Merikangas 1996).

The allele and haplotype associations, however, are not sufficient without a permutation test which is used to correct the results for multiple testing. If the finished tests are independent of each other, a simple Bonferroni correction may be used by multiplying the obtained P-value with the number of accomplished tests. In association studies, the number of tests that have been carried out is dependent on the number of tested loci, on the number of used markers and on the number of obtained alleles/haplotypes as well as

on the number of tested phenotypes. Since many of these variables are correlated with each other, the effective number of independent tests is often difficult to determine. Thus, in these situations, a permutation test may be more valid than a Bonferroni correction of the results for multiple testing. A permutation test is done by randomizing the results: For example, the real genotyping results are used with the same number of affected and control chromosomes as in the real data, but the affectus status of the chromosomes is randomized. A randomization of 100 – 10.000 times can provide information for results that can be expected by chance and for results that might be considered significant.

Our study population with mainly nuclear families was designed to have an optimal power for haplotype analysis, and we found evidence for the FCER2 gene region and for the IL9RA gene region associating with high serum total IgE level and asthma. In the FCER2 gene region, the haplotype D19S120*3-D19S536*4-D19S212*4-D19S567*16-FCER2*3-D19S534*7 associated with high serum total IgE level (P_{simul1}=0.038) and in the IL9RA gene region, the haplotype sDF2*10-sDF1*6 associated with asthma. However, for both the FCER2 and the IL9RA gene regions, the simulated P values showed only borderline significance which raises the question of the biological significance of the results. The weak allele associations (D19S922*8 and sDF2*10) did not remain significant after permutation tests. Furthermore, the sequencing of the FCER2 gene did not reveal any polymorphisms. For the FCER2 gene region, some other still unknown gene or regulatory element affecting the expression or function of the FCER2 gene might explain the positive haplotype association results, since no variations were found. For the Xq/YqPAR region, the IL9RA gene is the obvious candidate because of the immediate localization, but since we have neither used an intragenic marker nor sequenced the gene, our evidence for the association to IL9RA is only partial. This is, however, supported by the results of another study reporting linkage between the region and bronchial hyperreactivity (Holroyd et al. 1998).

The carriers of the mutations of CFTR gene 1,9 % did not differ significantly from what was expected to be the frequency of carriers, calculated by the incidence of cystic fibrosis in the whole of Finland, although the mutations were enriched in Kainuu. Also, as

expected by the previous studies on the geographical distribution of the two Finnish main mutations, all four found carriers had the del394TT mutation and none the ΔF508 mutation (Kere et al. 1994). Because of the low frequency of the CFTR mutation carriers in the study population, the results of the CFTR mutation carriers and asthma were not statistically significant. However, being very rare, the CFTR mutation cannot be a major genetic regulator for asthma in Finland.

In the reanalysis of the 5q31-q33 with SNPs in addition to microsatellites, we used the Haplotype Pattern Mining which is an another method for haplotype association analysis and an application of data mining methods (Toivonen et al. 2000). By allowing gaps in haplotype patterns, it is also more robust in case of genotyping errors and missing data than the haplotype analysis we have used in our other studies. Also, the HPM parameters can be optimized for each data set by studying the individual features of genotype data such as marker information, marker density, and missing genotypes. With the HPM method, we obtained evidence for the IL13 gene contribution to atopy (the best P value 0.015 for high serum total IgE with IL13ex4.1) and also for the IL4RA gene contribution to asthma (the best P value with S411L was 0.012 for asthma, and with C406R 0.019 for high serum total IgE). The association of IL13 gene polymorphisms with serum total IgE level has previously been detected (Heinzmann et al. 2000, Graves et al. 2000) as well as the association of IL4RA polymorphisms with asthma and atopy (Hershey et al. 1997; Shirakawa et al. 1994; Ober et al. 2000). The evidence for these and many other studies on the IL4 pathway contribution to atopy has been so convincing that it has led to clinical studies with a soluble IL4 receptor (Borish et al. 1999). Still, more studies are needed to get more experience from applications of the HPM method in haplotype and allele analyses as well. Also, in these studies we have not tested functional consequences of the IL13ex4.1 polymorphism, although the IL13 has previously been shown to be an important molecule in the animal model of asthma (Grünig et al. 1998). In the IL4RA gene, the polymorphisms associating with asthma and atopy were different (S411L, C406R) and as far as these polymorphisms in this population are concerned, evidence for functional changes is still missing.

6. Summary and conclusions

Asthma is the third most common chronic disease in Finland considering the number of individuals entitled to the reimbursement for medication or where the costs of refunded medicines are considered (Finnish Statistics on Medicines, 1996). During recent decades, the prevalence of atopy and asthma has increased, thus setting more people into an urgent requirement for medication, although the clinical picture of the disorders has simultaneously changed from severe into mild-moderate (Report of a Working Group 1996). Lower rates for infectious diseases and other environmental factors have been suggested as the cause for the increasing prevalence.

According to the twin and family studies, there is a clear familial aggregation of atopic disorders and estimations of genetic effect on asthma have varied from 35% to 87%. With this study, we wished to investigate the candidate gene regions in asthma and atopy in the Finnish population. This population was chosen because of the population history and because of the previous molecular genetic findings that have revealed the isolated character of the population. When susceptibility genes in complex disorders are mapped, these kind of founder populations have been considered to be optimal (Wright et al. 1999).

The phenotyping was carried out with questionnaires, interviews, an evaluation of the medical records with patients' permission and serum total IgE and allergy screening test measurements. The unaffected family-members were screened for atopic and asthmatic symptoms with questionnaires and serum total IgE and allergy screening test measurements. Self-reported physician-diagnosed asthma was compared to the data obtained in medical records and self-reported allergic symptoms were compared to allergy screening test results. We obtained the most accurate results in phenotyping by combining the self-reported physician diagnosed asthma with the information in the medical records and the granted reimbursement for the anti-asthmatic medication by the Social Insurance Institution. The questionnaire based data has also been confirmed as satisfactory for genetic studies by others (Barnes et al. 1999). However, if bronchial

hyperreactivity should be used as a phenotype, retesting would be beneficial to achieve current and accurate quantitative data. As to the allergies, because of the poor sensitivity and specificity of the self-reported allergic nasal symptoms and of the physician-diagnosed allergic rhinitis, we found objective verification of allergy (high serum total IgE level or positive allergy screening test) necessary.

The genetic susceptibility to asthma and atopy was studied on chromosomal regions 5q31-q33, 16p12 (the IL4RA gene), 19p13 (the FCER2 gene region) and Xq/YqPAR (the IL9RA gene region) using linkage and association analyses. Also the CFTR gene was screened for two Finnish major mutations. All the linkage analyses remained negative, but evidence was found for the FCER2 gene region and Xq/YqPAR contribution to atopy and asthma with haplotype association analyses. On the chromosome 19p13, a six-marker haplotype was found to be associated with high serum total IgE level and on chromosome Xq, a two-marker haplotype was shown to be associated with asthma. Moreover, with the Haplotype Pattern Mining method, the SNP IL13ex4.1 was shown to be associated with high serum total IgE level, and in the IL4RA gene, S411L was shown to be associated with asthma and, respectively, C406R with high IgE level. Because of the low frequency of the CFTR mutations, no conclusions could be made for the association between the CFTR mutation carriership and asthma.

The IL13 and IL4RA gene contribution in asthma and atopy has also been suggested in other studies on both humans (Heinzmann et al. 2000; Ober et al. 2000) and mice (Grünig et al. 1998), and our findings are in agreement with others and thus emphasized. Also, the Xq/YqPAR with the candidate gene IL9RA has previously been linked to asthma (Holroyd et al. 1998), and our findings of the halotype association with asthma support the former results again underlining the importance of the IL9 pathway. The association of the FCER2 region with high serum total IgE level was a novel discovery, but since the sequencing of the FCER2 gene did not reveal any variations, the chromosomal region needs to be studied further.

Although several candidate gene studies on atopy and asthma as well as eight genomewide searches have been done in different human populations during the recent years, there still is no functional and well-established evidence for any of the genes affecting genetic susceptibility to atopy and asthma. However, some molecules have been demonstrated to be especially important in IgE mediated disorders, and these results have led to clinical studies. Future studies will show whether the molecules of the IL4, IL9 or IL13 pathway or antagonists for the low affinity receptor of IgE or any other studied molecules are applicable for diagnostic use or for medication.

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