

Genetic studies of cystic fibrosis and atopic asthma: current approaches and future aspects

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Since the early 1980s, several major developments in human genetics research have resulted in an explosion of the discovery of genes responsible for inherited diseases, especially those that follow simple Mendelian patterns of inheritance. By using positional cloning, nearly 50 inherited human disease genes have been identified (1). However, these single-gene, "simple" diseases are typically rare (<0.1% prevalence) and are frequently fatal. Much less is known about the more common yet complex diseases, such as asthma and atopic allergy.

Cystic fibrosis (CF) is a well-characterized monogenic disease and the most common simple disease affecting Caucasians of European descent (2); it affects as many as 1:2500 births among certain Caucasian groups (3). The molecular mechanisms of the CF mutation have been well described and divided into five classes for which a strong genotype-phenotype correlation can be observed. There is, however, tremendous allelic heterogeneity, resulting in a continuum of disease expression from very mild to very severe. Recently, evidence has emerged which suggests the existence of a modifier gene that potentially impacts CF severity (4). This finding will potentially force the scientific community to redefine monogenic diseases. The study of CF affords us an opportunity to establish a paradigm for the dissection of genetic diseases in general, and highlights the importance of the clinical phenotype in understanding the genetic mechanisms involved in disease expression.

Asthma has been described as a complex trait for which genetic predisposition is fundamental in disease etiology, but poorly understood, largely due to the myriad of potential phenotypes. Unraveling the genetic etiology of asthma is further complicated by the multifactorial nature of asthma - that is, there is

tremendous influence of the environment over expression of the disease. A strong case has been made for an IgE-mediated causation of asthma (5, 6), and environmental studies have demonstrated a causal relationship between exposure to specific levels of allergen (e.g., house dust mite) and allergic airway disease (7, 8). In keeping with the notion that asthma is a complex trait, we hypothesize that several genes are contributing to the susceptibility and subsequent manifestation of asthma. Much of our work and the work of others has focused on IL-4 and other related cytokines and factors believed to be central in both antigen-specific and nonspecific mechanisms of atopic disease (9–11). The following is a discussion of the theoretical basis for the study of genetic diseases, using CF and asthma as models for "monogenic" and "polygenic" diseases, respectively, and an overview of the genetic regions implicated thus far in these diseases.

Current hypotheses and approaches

Genetic mapping, the method by which "one compares the inheritance pattern of a trait with the inheritance patterns of chromosomal regions" (12) was revolutionized with the advent of highly polymorphic, genetic markers. These "flags" or "signposts", which indicate the approximate location of the genes suspected of contributing to a particular disease, have aided in the isolation of polymorphisms, or variants within a given allele, that play a role in differential expressions of a particular phenotype, or disease. With the development of positional cloning (13), a process by which linkage is established between the presence of the disease/disease-associated phenotype(s) and highly polymorphic genetic markers, geneticists could isolate a gene of in-

terest without knowledge of its biochemical function. To date most of the successes in positional cloning have been with the monogenic diseases, and CF was one of the first genetic diseases to be successfully mapped by this method (1).

A greater challenge lies in the dissection of complex diseases, such as asthma. Indeed, the search for sequence variations over vast numbers of DNA segments is underway; for example, a great deal of attention has been focused on defining genes that determine the overall propensity to synthesize IgE, and to identify asthma-specific genes. To date, however, the primary method by which researchers have undertaken the genetic analysis of atopic disease is the candidate gene approach. With the candidate gene approach, an educated guess as to which region one should be focused is made based on partial functional information about the disease (e.g., the role of IL-4 in atopic disease). After making the educated guess, candidate genes in the region of interest are selected for molecular analysis, and a process that allows for the visualization of potential functional polymorphisms involved in the candidate is commenced.

In addition to these methodological issues, the genetic study of complex traits, and even the study of monogenic traits such as CF, are made more difficult because of different degrees or levels of genetic heterogeneity. Certain diseases are genetically heterogeneous because many distinct genes, or groups of genes, lead to similar clinical phenotypes. In CF, for example, different mutations of a single gene lead to different disease phenotypes. In allergic disease, it is increasingly apparent that different groups of genes could be associated with different types of disease manifestation (e.g., asthma, allergic rhinitis, eczema), or, different groups of genes may be associated with the differential expression of certain characteristics of the overall phenotype (e.g., IgE levels). Lastly, many complex diseases, and notably atopic disease, are multifactorial, in that disease expression is influenced by interactions between multiple major and minor genes, and modulated by interacting non-genetic factors, such as the environment.

Cystic fibrosis: a "simple" disease?

Cystic fibrosis is typically referred to as a "simple disease" because, like other simple diseases, it obeys the classical Mendelian law of inheritance; in this case, the inheritance pattern is autosomal recessive. Furthermore, CF is relatively uncommon, although prevalence varies considerably depending on ethnicity and geography. Lastly, CF follows the usual pattern of simple diseases in that morbidity and even mortality are quite substantial, although survival has increased dramatically in the last several

decades, with an increase in median survival from one year during the 1940s to 30 years during the 1990s (14).

CF is caused by a single-gene defect, or mutation, of the CF transmembrane conductance regulator (*CFTR*). It is located on human chromosome 7q31.3, is ~250 kb in length, and consists of 27 exons ranging in sizes from 38 to 724 bp (15–21). *CFTR* encodes a cAMP-regulated chloride channel found almost exclusively in the secretory epithelial cells (19, 22–24) and encodes a 6.5-kb mRNA that is detectable (by Northern blot) in many tissues, including lung, sweat glands, pancreas, liver, salivary gland, colon, and nasal polyps (24). The functional pathology is increased viscosity of mucous gland secretions, resulting in obstruction of many organs, and leading to a myriad of life-threatening conditions, including chronic pulmonary obstruction, pancreatic enzyme insufficiency, focal biliary fibrosis, reduced fertility in males, and elevated sweat electrolytes.

A curious feature of CF, and one that separates it from other simple diseases, is the heterogeneous nature of the gene responsible for expression of the disease. That is, different mutations of the *CFTR* gene lead to different disease phenotypes (disease expression). In fact, there are more than 550 possible mutations of the *CFTR* gene which vary according to ethnic group and/or geography and can lead to conditions ranging from almost normal to mild-to-severe disease (2, 25).

The most common mutation by far, accounting for ~66% of CF chromosomes, is a deletion of phenylalanine at position 508 in exon 10, referred to as $\Delta F508$ (23, 25). The relative frequency of $\Delta F508$ varies substantially according to ethnicity and geography (26), ranging from 30–35% among Ashkenazi Jews (27) and Hutterites (28) to 87% in Denmark (29). There is a gradient of frequency observed in Europe from a low in the southeast to a high in the northwest (30). It is believed that all $\Delta F508$ chromosomes evolved from a single mutational event some 52,000 years ago, spreading during the Neolithic Age and coinciding with the migration of farmers out of the Middle East into northwest Europe (31–33). Given the very high prevalence of the $\Delta F508$ mutation among Caucasians of northern European ancestry, it has been argued that, if this mutation did not exist, the frequency of CF among Caucasians would be no higher than among other racial populations (34). Indeed, even G542X, the mutation that follows $\Delta F508$ in order of population frequency, only occurs in about 2.4% of CF patients (25).

The many possible *CFTR* mutations result in different degrees of expression of the disease. In fact, some sequence variations in the *CFTR* gene are benign (35) or may confer only mildly abnormal

CFTR function (e.g., congenital bilateral absence of vas deferens [CBAVD] only; 36). *CFTR* mutations are divided into five classes according to the molecular basis of their dysfunction (see review [3]). For example, pancreatic insufficiency occurs in subjects who are homozygotes or compound heterozygotes for two severe mutations associated with Classes I, II, or III, whereas CF patients with normal pancreatic function have at least one "mild" allele associated with Classes IV or V. In short, the variability, or continuum, of a single phenotype (e.g., pancreatic insufficiency) is dependent upon different combinations (e.g., classes I-V) of mutations, complicating the understanding of genotype-phenotype correlations (2, 37, 38).

There is yet another confounding characteristic of this so-called simple disease. Curiously, there are a number of conditions that are associated with the *CFTR* gene – that is, pathologies that are typically expressed in the event of a *CFTR* mutation – but these diseases are not always caused by *CFTR* mutation. For example, male CF patients typically suffer from obstructive azoospermia as a result of bilateral absence of vas deferens (39), but in some cases (15–20%) CBAVD is manifested as a separate autosomal recessive disease in which there are practically no CF manifestations (3, 40, 41). Because certain CF disease manifestations overlap with diseases that are not caused by a *CFTR* mutation, it becomes particularly difficult to tease out what is uniquely CF and what is caused by other unrelated genetic factors. Moreover, the presence of shared manifestations between atypical diseases and CF depend very much on the specific *CFTR* mutation, or class of mutation.

Most recently, the classification of CF as a simple disease has been further threatened with the apparent existence of a modifier gene located on mouse chromosome 7, which corresponds to human 19q13 (4). In backcross experiments with mice having the class III mutation, the group found that the prolonged survival of homozygous mutant mice depended on this modifier locus. It has been proposed that this gene may modify disease severity in human CF, depending upon the genetic background; such a finding would explain, for example, why some CF patients who are homozygotes for "severe" mutations express a milder form of the disease than others with the same genotype (42).

In summary, the multitude of different mutations (~550) clustering among many different ethnic groups, for a disease in which different combinations of the genotype (five classes of mutations) result in a multitude of phenotypes (normal to severe disease), compounded by the fact that several of the disease expressions overlap with other genetic entities, in addition to the recent finding of a potential modifier gene associated with expression of CF, il-

lustrates the complex nature of even simple genetic diseases. We can use CF as a model for refining our approach of the dissection of complex traits (e.g., asthma), with consideration of the almost infinite number of possibilities for types and degrees of disease expression and manifestation in the event that a disease is genetically heterogeneous.

Atopic asthma: a complex disease

Historical overview – Although several hypotheses have been put forth as to the possible advantage that might be conferred by the $\Delta F508$ mutation (43–45), the selective pressure under which the *CFTR* mutation could have evolved is not entirely understood. In contrast, the origin of IgE, the proponent of atopic disease, is generally accepted to be a defense against extracellular parasites (46–48). However, the inflammatory response is misdirected in the allergic diseases, especially asthma.

Potentially, the symbiotic relationship of mammals and parasites spans nearly 200 million years, since the first appearance of mammals toward the end of the Triassic period of the Mesozoic era. We assume, given this long symbiotic history, that the mammalian inflammatory network has evolved mechanisms, both unique and redundant, that function to maximize host well-being in the event of parasitic infestation. There are redundancies, for example, within the genes encoding the complement cytokines or cell-adhesion molecules and their receptors (49–51). We assume that mutations have occurred but that their effects are modulated by the interactive nature of the complex system. Thus, we accept *a priori* that there will be multiple mutations of multiple genes controlling the inflammatory response (including regulatory elements of these genes), and that these mutations will have variable and interdependent effects on the expression of the different allergic disease phenotypes. The expression of these "inflammation genes" will also vary according to the environmental context.

In view of these considerations, it is not surprising that genetic studies of the allergic diseases have met with limited success (52, 53). After all, in studying the genetics of asthma, we clearly do not have the advantages that we do in studying a monogenic disease such as CF, in which pathogenic mutations are evident within a single gene (although potentially a modifying gene is playing a role in gene expression), and disease expression is not compounded by multifactorial effects (e.g., environment).

Numerous studies in which the concordance for atopic disease was greater between monozygotic (MZ) twins compared to dizygotic (DZ) twins provided some of the first evidence that allergic disease

had a familial basis (54–57). However, a concordance of only 50–60% for atopy (58) suggests that, in addition to the role of environmental influences in the expression of disease, which could account for some of the disparity of disease expression, gene mutations and/or rearrangements are contributing to the complex nature of asthma. This is particularly pertinent in view of the findings that MZ twins raised in presumably the same environmental setting frequently exhibit similar total serum IgE levels, but disparate specific IgE Ab responsiveness (58, 59).

From these studies as well as the work by Burrows and colleagues (5), we hypothesize that there are, perhaps, three main categories of genes controlling the expression of atopic disease. First, there are immune response (*Ir*) genes, which determine the ability of the individual to make specific immune responses. This cognate response is dictated in part by environmental factors, especially the level of the person's exposure to indoor allergens. A second category of atopic genes determines the overall propensity of the individual to synthesize IgE of any specificity, which we refer to as nonspecific, or non-cognate *Ir* genes. Lastly, we propose that there is a category of asthma-specific genes, which may be separable from those determining atopic disease overall.

Genetic control of the cognate immune response – Some of the early molecular studies of genes controlling immune responses focused on the HLA-D subregions (DR, DQ, DP) of the human MHC, located on chromosome 6p21.3 (60–65). This region was an obvious group of candidate "cognate" response genes because of their involvement in "Ag presentation". It was hypothesized that the cognate immune response involved the interaction of a single MHC class II molecule with a single major T-cell epitope of the Ag, or a group of closely related class II molecules. Based on this assumption, it was expected that unrelated subjects would share a role for *HLA-D* genes in cognate responses. Analyses were simplified by studying very restricted phenotypes: immune responsiveness to the major allergen of short ragweed, *Amb a 5* ($M_r = 5,000$).

Marsh et al (60) were able to establish that the class II restriction is limited to subjects carrying HLA-DR2 and -Dw2 (DR2.2) in 95% of the cases; however, atopic subjects not responding to *Amb a 5* demonstrated a frequency of DR2.2 similar to the Caucasian population at large (~22%). By using a combination of both DNA typing of *HLA-D* genes and detailed analyses using human *Amb a 5*-specific T-cell clones, the group subsequently established that two class II restriction elements, the DR α 1*1501 molecule in Caucasians and the

DR α 1*1502 molecule in Asians, are the functional gene products presenting *Amb a 5* to the T cell (65). These and other data clearly demonstrate a cause-and-effect relationship between the presence of the DR2.2 molecule in Caucasians, or the related DR2.12 molecule, which is common in Asian subjects, with specific IgE and IgG Ab responsiveness to *Amb a 5*.

Over the past several years, numerous studies have confirmed these findings, and the HLA-D association has been extended to other allergens, such as giant ragweed and perennial rye, and also the mite allergens *Der p 1* and *Der p 2* (60–62, 66–70). These results provided a clear demonstration of the role of HLA-D-encoded molecules in well-defined immune responses toward simple allergen molecules which appear to possess single major epitopes.

Genetic control of the noncognate immune response

– As discussed, we postulate that the total serum IgE concentration arises through the production of IgE through a combination of cognate and noncognate (non-antigen-directed) mechanisms. In addition to the current knowledge of the cellular mechanisms underlying the IgE response (10, 11), this postulate is based in large part on the landmark study by Burrows et al (5) in which they observed that the log of the risk for asthma is linearly related to the log[total serum IgE], after the log[total IgE] has been adjusted for age, sex, smoking habits and the subject's specific skin-test responsiveness to common inhalant allergens. The log of the risk for asthma was, however, much less well correlated with skin test (ST) responsiveness to common environmental allergens, and even in ST negative subjects, the presence of asthma was correlated with total IgE (5). These findings have been confirmed by others (6, 71, 72).

To date, at least three chromosome regions have been implicated in noncognate IgE responsiveness: the *IL4*-cytokine gene cluster and *ADRB2* as well as other loci in chromosome 5q31–33, supported by several independent studies and including both mixed and isolated populations (73–80); the β subunit of the IgE high-affinity receptor (*FCER1B*) in chromosome 11q13, found in both nuclear and multiplex families in the U.K. (81, 82); and a locus in the *TCRA/D* region in chromosome 14 in nuclear and multiplex British and Australian families (83).

The *IL4* gene cluster was a particularly attractive candidate region for the genetic analysis of noncognate IgE responsiveness. The cytokine IL-4 is known to be important in immunoglobulin class-switching to IgE and in the differentiation of naive T cells into Th2/Th0 cells (9–11, 84). In addition to *IL4*, the cytokine genes *IL3*, *IL5*, *IL13*, and *CSF2* (*GMCSF*), which also play important interactive

roles in the allergic inflammatory response, are part of the clustered family in this region (human chromosome 5q31.1). The genetic analysis of this region affords the opportunity to test for linkage not just to *IL4*, but to a group of genes important in the expression of the allergic diseases.

In order to test the hypothesis that a gene(s) controlling noncognate IgE production is located in the region of the *IL4* gene cluster, Marsh et al (73) selected 11 multigenerational families from the Pennsylvania Old Order Amish, a religious isolate group that has several desirable attributes for genetic studies, including: a high degree of intermarriage; large family size, often with 10 or more children per family; negligible smoking, a well-known confounding factor in atopic disease; and a relatively uniform environment with respect to allergens. The selected families contained at least one child with IgE Abs to one or more common inhalant allergen. Eight genetic markers within or near the region of interest were selected for amplification from each subject's DNA sample by the PCR using radiolabelled primers, and was analyzed by acrylamide gel electrophoresis.

Segregation analysis was performed using the sib-pair method (85), in which the differences in the trait ($\log[\text{total IgE}]$) between two siblings are computed, and the squared differences are regressed against the proportion of the alleles of each marker found to be identical by descent (IBD) within each sib pair. If linkage with a particular marker is present, then sibs who carry the same marker allele will, on average, show a smaller difference in their $\log[\text{total IgE}]$ than sibs who do not carry the allele, and this is reflected as a significant negative slope. This was the case, in fact, for all five of the genetic markers within the 5q31.1 region; however, no evidence of linkage was observed for three markers lying outside of this region. No evidence for linkage between any of the 5q31.1 markers and specific IgE Ab levels was present.

In order to eliminate the influence of specific IgE antibody on the observed total IgE levels, Marsh et al. (73) deleted the sib pairs who had specific IgE antibody to a composite of 20 common inhaled allergens (Magic Lite, ALK, Copenhagen-CIBA-Corning, Medfield, MA). Subsequently they found even greater evidence for linkage to these markers, especially *IL4-R1* ($P = 4 \times 10^{-6}$), despite the reduction in the number of sib pairs from 349 sib pairs to 128. Again, markers mapping outside 5q31.1 showed no evidence for linkage to $\log[\text{total IgE}]$. Among these sib pairs, the total IgE level should more closely reflect noncognate IgE production.

Genetic control of atopic asthma – Few studies have focused on the asthma phenotype in the genetic dissection of atopic disease. De Sanctis et al (86) and

Ewart et al (87) reported the first linkage of airway hyperresponsiveness using the mouse model. Postma et al (76) found co-inheritance for elevated serum total IgE and bronchial hyperresponsiveness (BHR) among pairs of siblings, and concluded that a gene controlling BHR is located in the *IL4*-cytokine gene cluster. Doull et al (79) found evidence for linkage with BHR on chromosome 11q; however, linkage to the markers on 11q was not associated with *FCER1B*, the gene for which there was previous evidence for linkage to total IgE (81, 82). It is interesting that, in the study by Doull et al. (79), a marker at 11q13.5 was significantly associated with BHR but not $\log[\text{IgE}]$, whereas a marker in the same region, at 11q13, was significantly associated with $\log[\text{IgE}]$ but not BHR.

Conclusions

The findings thus far highlight the genetic heterogeneity of atopic disease. Several studies have concluded that *IL4*, or a nearby gene in 5q31.1, is involved in the regulation of IgE production in a nonantigen, or noncognate selective fashion. Basophils, in particular, may be implicated in this noncognate IgE production, although other FcεRI-positive cells may also be involved. Early findings of association between the *HLA-D* gene on chromosome 6p and the cognate *Ir* response to an array of major allergens, in addition to the landmark work by Burrows et al. (5), support our hypothesis that expression of noncognate and cognate IgE is driven by separate genes or groups of genes. Although evidence for “asthma genes” *per se* is insufficient, there is a great deal of focus on this area of research at this time. A better understanding of the role of modifier genes, such as the CF gene on mouse chromosome 7 (human 19q13; [4]), will enhance our understanding of genotype-phenotype correlations in terms of disease expression.

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