Published in final edited form as:

Annu Rev Pathol. 2012 ; 7: 267–282. doi:10.1146/annurev-pathol-011811-120900.

Genetic Variation and Clinical Heterogeneity in Cystic Fibrosis

Mitchell L. Drumm^{1,2}, Assem G. Ziady^{1,3}, and Pamela B. Davis^{1,3,4}

Mitchell L. Drumm: mitchell.drumm@case.edu

¹Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

²Department of Genetics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

³Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

⁴Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Abstract

Cystic fibrosis (CF), a lethal genetic disease, is characterized by substantial clinical heterogeneity. Work over the past decade has established that much of the variation is genetically conferred, and recent studies have begun to identify chromosomal locations that identify specific genes as contributing to this variation. Transcriptomic and proteomic data, sampling hundreds and thousands of genes and their products, point to pathways that are altered in the cells and tissues of CF patients. Genetic studies have examined more than half a million polymorphic sites and have identified regions, and probably genes, that contribute to the clinical heterogeneity. The combination of these approaches has great potential because genetic profiling identifies putative disease-modifying processes, and transcript and protein profiling is shedding light on the biology involved. Such studies are providing new insights into the disease, such as altered apoptotic responses, oxidative stress dysregulation, and neuronal involvement, all of which may open new therapeutic avenues to exploration.

Keywords

polymorphism; ion channel; genetic and protein profiling

CYSTIC FIBROSIS

When cystic fibrosis (CF) was first described in 1938, it was clearly recognized as an inherited disorder. Although the parents of the affected children were not affected, siblings or cousins often were. Even before the responsible gene was identified, the inheritance

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pattern was determined to be autosomal recessive (1). In 1989, the gene causing CF was identified (2–4), and all or nearly all patients with CF have errors in both copies of a single gene that encodes a cyclic AMP (cAMP)-regulated chloride channel known as the cystic fibrosis transmembrane conductance regulator (CFTR). The gene that encodes CFTR resides on the long arm of chromosome 7 at q31.2, consists of 27 exons, is approximately 190 kb in size, and shows some level of conservation across species from frogs, pufferfish, and even *Caenorhabditis elegans* (5) to humans. This gene encodes a cAMP-activated anion channel, which conducts chloride (6) and other anions such as bicarbonate (7). Although the protein contains two membrane-spanning domains, two ATP-binding domains, and an intracellular regulatory (R) domain, and although ATP binding and hydrolysis must occur for the channel to open, CFTR only transports the chloride ion down the electrochemical gradient (2–4, 8). Thus, in the sweat duct, CFTR conducts chloride into the epithelial cell (and ultimately into the interstitium) from the duct, but in the gut, CFTR secretes chloride from the epithelial cell into the lumen, and in the airway, CFTR can play either a secretory or absorptive role, depending on the direction of the gradient. Marked reduction of CFTR function gives rise to disease that is ultimately fatal.

When CF was first described, malnutrition due to pancreatic insufficiency (which arises from plugging of the pancreatic ducts and failure to secrete pancreatic enzymes into the gut) was a major cause of death, but currently, more than 90% of the deaths due to CF are due to lung disease (1). Such lung disease arises from the failure to transport chloride ions and, therefore, water into the bronchi. The periciliary fluid layer is too shallow, and cilia cannot clear the mucus and the environmental agents trapped by it. Thus, inhaled bacteria are not cleared properly and establish chronic infection with consequent inflammation, which leads to bronchiectasis and eventual respiratory failure (1). Nearly 2,000 disease-causing mutations have been observed in the *CFTR* gene; these mutations have a range of effects on the protein product (see <http://www.genet.sickkids.on.ca/Home.html>). The number of mutations, coupled with CFTR expression in respiratory epithelia (sinuses and airways), pancreatic duct epithelia, intestinal epithelium, the epithelium lining the vas deferens and the cervix, the liver, the sweat ducts, and nonepithelial cells, probably explains the varying severity of protean clinical manifestations of CF in different organ systems in different patients.

VARIATION IN *CFTR* AND ITS EFFECT ON CLINICAL PHENOTYPE

The population and molecular genetics of CF are somewhat unique. There are nearly 2,000 disease-associated mutations documented for *CFTR* see <http://www.genet.sickkids.on.ca/Home.html>). Among them is a single common mutation, a three-nucleotide deletion that causes the absence of amino acid 508 of the normally 1,480–amino acid protein. This mutation, which lacks a single phenylalanine codon and is commonly referred to as F508 (c.1521_1523delCTT for the DNA mutation and F508del for the mutant protein, by current standard nomenclature), accounts for approximately 70% of the CF alleles in the population. The remaining 30% of alleles comprise the other 2,000 different mutations, each of which, individually, has a very low frequency. More recent molecular investigations suggest that some disease-causing alleles do not carry simply a single functional change but rather are

complex alleles that contain multiple changes with additive effects that reduce function to a disease-causing level (9).

There is substantial clinical variation among CF patients with the same *CFTR* genotype (10), which indicates non-*CFTR* sources of variations, but variation in *CFTR* itself is a strong contributor to clinical heterogeneity. With so many different mutations, as well as combinations of changes in many cases, classification is a challenging task. The most commonly used scheme is to classify disease-causing mutations by their molecular effects on *CFTR*, incorporating mutations that produce truncated proteins, misprocessed proteins, channels that fail to open, mutations that conduct ions poorly, and splice mutants that fail to produce functional *CFTR*. In this classification system, class 1 mutations result in no functional *CFTR* produced, class 2 mutations cause misprocessed/misfolded proteins, class 3 mutations exhibit impaired activation or channel gating, class 4 mutations show reduced ion conduction through the channel pore, and class 5 mutations are quantitative ones that reduce the amount of *CFTR* in the cell (11). Figure 1 demonstrates the effects of different classes of mutations of *CFTR*, exemplified by the predominant mutation of each class.

Class 1 mutations are defined as loss of function, and the disease phenotypes associated with them are relatively severe and consistent. However, mutations in the other groups display a continuum of effects on *CFTR* both quantitatively and qualitatively, which reduces the clarity of the relationship between mutation classification and clinical outcome. For example, patients who display exocrine pancreatic sufficiency are unlikely to carry two class 1 mutations; rather, they have at least one copy of a class 2, 3, 4, or 5 mutation, as these alleles may exhibit some residual *CFTR* function and preserve some exocrine pancreatic function. The converse, however, is not always true. That is, mutations in any of the other classes display a range of effects, so one cannot infer the clinical presentation of a patient simply on the basis of the class of mutation they carry. This point can be demonstrated by numerous examples. Take, for instance, codon 551, which normally encodes a glycine (G551). Two different amino acid–substitution mutations have been identified in this codon: one for aspartic acid (G551D) and one for serine (G551S). The clinical consequences are quite different for these two changes, as the G551D variant has virtually no detectable activity, and consequently a classic, severe phenotype is associated. G551S, however, has reduced but clearly detectable function and is associated with a much milder presentation of CF (13).

Another example involves the amino acid substitution R117H, in which an arginine is replaced with a histidine. This mutant *CFTR* has a reduced ability to conduct chloride (class 4), but it clearly has function. The R117H mutation, however, can be found on two different “backgrounds” of *CFTR* with respect to sequence variation in intron 8 (14). This variant, a string of thymidines (T), can be found in three varieties, 5T, 7T, and 9T, and it affects the ability of the *CFTR* messenger RNA (mRNA) to be spliced correctly. The 5T variant makes the smallest proportion of normally spliced mRNA, 7T an intermediate proportion, and 9T the largest proportion. The R117H mutation exists on both the 5T and 7T backgrounds and, when on the 7T background, often has minimal associated clinical features. The 5T background, in contrast, reduces the quantity of partially functional *CFTR* below a threshold that results in CF, usually of a pancreatic-sufficient form.

In a further demonstration of the effects of CFTR heterogeneity, these same intron 8 T-tract variants, in the absence of other known functional changes, can impart some physiological effects. Nearly all male patients with CF, of whatever severity or genotype, have congenital bilateral absence of the vas deferens (CBAVD) (15). Homozygosity of the 5T allele in males can be sufficient to cause CBAVD, but without evident clinical consequences in females.

CFTR-RELATED DISORDERS

Although mortality is usually due to pulmonary complications, similar variations in effects occur in other tissues as well. Nearly all CF patients have either sinusitis or maldevelopment of sinuses (16). In contrast, severe loss of function of CFTR causes early manifestation of pancreatic insufficiency, but patients with so-called mild mutations (that are, in general, thought to be those associated with small residual CFTR function; these cases constitute as much as 15% of the CF population) develop pancreatic failure later in life (17), which is sometimes marked by recurrent episodes of pancreatitis. However, other clinical manifestations that occur in a minority of patients, such as hepatic failure and cirrhosis (severe in up to 10% of patients), meconium ileus (neonatal intestinal obstruction, which announces the disease in 15% to 30% of CF newborns), and distal intestinal obstruction syndrome (a blockage, usually near the ileocecal junction, caused by accumulated solid or semisolid feces), cannot be unambiguously linked to the CF genotype per se.

Sweat chloride concentration is markedly elevated in patients with CF compared with healthy controls, and this concentration differs enough between CF and non-CF individuals to serve as the diagnostic test for the disease. However, patients with mutations that allow some residual CFTR function often have lower (although still abnormal) sweat chloride concentrations than do those with severe mutations; rarely, the concentration may be normal (18). Lung disease appears to be less severe in patients with mutations that either allow some CFTR to arrive at the membrane or permit a small amount of normal CFTR to be produced—that is, patients who have some residual CFTR function (19, 20)—but it is difficult to be certain whether improved pulmonary outcome arises from residual CFTR function in the airway itself or from better nutrition due to retained pancreatic function. Nevertheless, CF genotype is associated with pulmonary disease phenotype.

Some studies indicate that even a single abnormal copy of CFTR can pose a risk factor for disease. Abnormalities in CFTR are highly prevalent in patients with CBAVD, even in the absence of CF (21). The vas deferens appears to be exquisitely sensitive to reduced function of CFTR. Abnormalities in CFTR have also been associated with recurrent or chronic pancreatitis, including pancreas divisus (22, 23). Abnormalities in CFTR probably predispose patients to sinusitis (24, 25). Some evidence suggests that abnormalities in a single copy of CFTR may predispose patients to non-CF bronchiectasis (26), chronic obstructive pulmonary disease (COPD), (27), or asthma (28), but such associations have been difficult to confirm and may occur in some populations but not in others.

However, even for siblings or unrelated patients with the same CF genotype, severity of the lung disease varies widely. Other factors, including environmental factors and adventitious

infections, may influence the outcome, but other, non-CFTR genetic variations, termed modifier genes, probably affect the severity of lung disease as well as other aspects of CF.

MOLECULAR PHENOTYPES THAT VARY WITH DISEASE OR DISEASE SEVERITY

Technological advancements have enabled the simultaneous measurement of hundreds and thousands of analytes from individual samples. Such measurements have resulted in large data sets of mRNA, proteins, and metabolites and are referred to as transcriptomics, proteomics, and metabolomics, respectively.

Numerous studies have reported that CF tissues, both mouse and human, exhibit differential expression of many mRNA transcripts and protein (29–36). Xu and colleagues (30) measured significant differences in the expression of numerous genes in the lungs of murine *Cftr* knockout mice compared with those of non-CF controls. The study employed gut-corrected mice that expressed human CFTR under the control of the fatty acid-binding protein promoter. Significant differences (29 increased and 25 decreased) were identified in the expression of genes involved in signal transduction, transcriptional activation, glycolysis, cAMP biosynthesis, and inflammatory response in the lung. These changes were observed even in the absence of gross disease pathology, which suggests that there are innate differences in CF cells and tissues and that these changes are not simply due to immune responses, remodeling, and other secondary consequences of tissue injury. These authors (30) concluded that some of the observed differences, such as increases in the transcription factor CCAAT enhancer-binding protein and in interleukin (IL)-1 β (both of which regulate CFTR expression), indicate an adaptive response to the loss of *Cftr* (30). Interestingly, even in the absence of lung infection, elevations in genes encoding calgranulin-S100 family members, IL-1 β , and IL-4 were observed, which indicates a predisposition to inflammatory signaling in CF lungs. In a follow-up study by Xu et al. (29), transcriptome analyses were carried out on murine respiratory epithelia of *Cftr* knockout mice carrying transgenes that express either normal or F508 human CFTR. The expression of F508 in respiratory epithelia resulted in the altered expression of numerous genes in specific pathways, including a significant increase in the expression of pro-oxidant genes and a decrease in antioxidant genes in mice that express F508 (29). This finding may indicate that CF lungs are unable to properly regulate redox balance.

To study the effects in human cells, Wright and colleagues (34) examined gene expression in samples collected by nasal curettage. These authors collected airway epithelial cells of the nose, well away from the diseased area of the lung, from homozygous F508 CF patients and compared them with cells from age-matched non-CF control subjects. The authors stratified their data by severity of CF lung disease on the basis of the lowest (severe) or highest (mild) twentieth percentile of predicted forced expiratory volume in 1 s (FEV₁) corrected for age (FEV₁% predicted). This study identified significant differences in the expression of genes between CF patients and non-CF controls as well as between severity groups of CF patients (34). In results similar to those obtained in studies conducted with F508 mice (29), these authors reported the highest number of significant increases in genes that influence oxidoreductase activity, followed by those that influence lipid metabolism,

protein processing, and ubiquitin conjugation activity in severe CF patients. Genes with increased expression in individuals with mild CF were the most numerous for pathways that regulate lipid metabolism, ion transport, and cell signaling. Significant decreases in gene expression in CF patients versus non-CF controls occurred in pathways that control growth, apoptosis, and immune tolerance. Although most differences in specific gene-expression profiles vary between animal models and patients, the cellular pathways affected by differential gene expression in CF, as a whole, are strikingly similar. This finding indicates that in addition to the effects of disease pathology, both the loss of CFTR function and the presence of mutant proteins (especially F508) elicit significant gene-expression differences that are related to secondary defects associated with the disease, such as increased inflammatory and redox signaling and decreased growth.

Transcriptome analyses identify changes in gene expression that reflect perturbations in regulatory networks (transcription factors, epigenetic modifications, etc.) but do not necessarily predict protein levels or biochemical effects on the cells involved. Thus, an important additional assessment is how protein quantity or function changes. Accordingly, numerous studies have demonstrated that differential protein expression plays a significant role in CF pathophysiology. Much of the current understanding of the inflammatory processes altered in CF has come from measuring protein levels in the airway surface liquid of CF patients via bronchoscopy and bronchoalveolar lavage. Differences in protein expression contribute to the inflammatory response in CF on many levels. Inflammatory mediators such as IL-8 (37) and IL-9 (38) are overexpressed in the airways of CF patients. Significant increases in proinflammatory cytokine levels have been detected in CF airways, even in the absence of detectable infection (39). In animal models and cultured cells, anti-inflammatory cytokines such as IL-10 (40) are decreased in CF. Furthermore, numerous transcriptional activators such as signal transducer and activator of transcription 1 (STAT1) (41) and nuclear factor κ B (NF- κ B) (42, 43), as well as the proteins that regulate them, are differentially expressed or activated in such a way as to enhance the inflammatory response or diminish host defenses. In the case of STAT1, although its overexpression is observed in CF, increased activation of protein inhibitor of activated STAT1 interferes with STAT1 activity, resulting in a significant decrease in the expression of various proteins regulated by STAT1, including nitric oxide synthase 2. This decrease reduces nitric oxide production, thereby reducing host defense against bacterial infection. The expression of antioxidant proteins in CF, including thioredoxin 1; peroxiredoxins 1, 3, and 6; and glutathione-S-transferase 1 is decreased in CF, whereas levels of superoxide dismutase 2 are elevated compared with those in non-CF controls (32). This alteration in the expression of redox-related protein hinges on the dysfunction of an important transcription factor, nuclear factor E2-related factor 2 (32).

The advent of mass spectrometric techniques for the high-throughput study of differential protein expression has allowed investigators to conduct wide-scale comparisons between CF and non-CF samples and to identify cellular signaling pathways whose components differ quantitatively or qualitatively. Importantly, these analyses have been confined mostly to cell culture models, both immortalized and primary, and to animal models; therefore, the degree to which they reflect changes in humans in vivo has yet to be determined. Proteomic analyses have identified significant increases in proteins that induce or respond to oxidative

stress, proinflammatory signaling, and increased metabolism (e.g., glycolysis) (8, 9). Significant decreases in protein expression occurred in antioxidant proteins (32) and in numerous transcription factors (35). Table 1 summarizes studies in humans and model systems of differential gene and protein expression in CF versus non-CF controls. Taken together, proteomic studies suggest alterations in pathways that are similar to those observed in gene-array studies. However, whereas some specific differences match well with those observed in gene-expression studies, most protein differences are not identified as such at the message level. Pathways that are heavily affected in transcriptomic and proteomic screens predict further alterations in the homeostasis of CF cells. For example, on the basis of data gathered in laboratory cell and animal models and in severe-CF patients, increased levels of, and a decreased ability to resolve, oxidative stress is predicted for CF cells. This prediction is supported by findings that levels of glutathione are decreased (44), whereas levels of nitrates and nitrotyrosine (oxidized protein) (45) are elevated in the airways of CF patients. In the lung, the influx of inflammatory cells, which produce oxidants to kill bacteria, introduces a heavy oxidative challenge, which would not be well resolved on the basis of transcriptomic and proteomic data and may therefore exacerbate activation of redoxsensitive cascades, such as the NF- κ B pathway (46). Furthermore, effects of protein oxidation, including altered function, reduce enzymatic activity and enhance susceptibility to proteolytic degradation (47), a pathway that is elevated in CF at both the gene- (2, 7) and protein-expression levels (35). Similar inferences, based on screening studies, can be made for other secondary defects that contribute to CF pathology, such as inflammation and metabolism problems.

In addition to yielding useful information about altered pathways in disease, transcriptomic and proteomic studies can in themselves be an indicator of modifier genes of CF disease, discussed in more detail below. Comparisons such as the one undertaken by Wright et al. (34) can reveal novel genes associated with the severity of disease. For example, this study found that the expression of the genes for statherin, a protein that protects against infection, and for adiponectin, a protein that can act as an inhibitor of inflammation, were elevated in mild-CF patients. Similar findings were observed for genes that modulate inflammatory signaling, metabolism, oxidative stress, and protein degradation. However, it is difficult to discern whether these differences contribute to the phenotypes of mild or severe CF or whether underlying differences in disease pathology cause these disparities. Therefore, for such exploration of modifier genes, extensive validation and examination of the individual genes' sequence for single nucleotide polymorphisms (SNPs) must be performed. Nevertheless, significant differences observed at the gene- and protein-expression levels shed light on both the pathways that are influenced and implicated in the disease and the functional outcomes of modulation of such pathways. In the context of modifier genes, information about the pathways that are associated with and produce the variously severe forms of CF provides a focus for investigators on the potential role of identified modifier genes (discussed in the following section), allowing for the characterization of their impact on molecular phenotypes that define and affect the progression of disease in patients.

The relationship between modifier genes and broad changes in genes and proteins is not yet clear, but it is reasonable to expect one to exist. The exploration of modifiers of CF disease is relatively new, and a full understanding of candidates that significantly contribute to CF

disease severity has yet to be realized. Nevertheless, alterations in modifiers that are associated with poorer prognoses in CF patients may be related to the differential expression of genes and proteins (molecular phenotypes) that contribute to disease pathology. Interactions between modifier genes and molecular phenotypes may be direct, such as would be the case if the modifier gene were a transcription factor or a modulator of transcription factor activity. Indirect interactions may include the effects of products of modifier genes (e.g., metabolites) on the expression of molecular phenotypes.

Such studies provide important correlative data that suggest relationships between molecules and processes and are useful for hypothesis generation. In some cases, a correlation may be so strong that one is tempted to infer causal relationships, but descriptive analyses do not provide causal relationships. That is, when multiple phenotypes track together, one cannot conclude that one causes another; rather, they may be independent consequences of the same or different causes. To determine causation, one must employ other approaches. These methods typically involve prospective studies in which a variable (e.g., a drug) can be manipulated to determine its effect on outcome, or genetic studies, which identify outcomes that track with genetic variation.

GENETIC HETEROGENEITY AND MODIFIER GENES

Among individuals with CF, the heterogeneity of clinical outcomes is broad. Even in patients with the same mutant *CFTR* genotype, variation in features such as lung function can be dramatic (48, 49). The human genome contains a large amount of variation; there are more than 3 million SNPs (50), along and hundreds, perhaps thousands, of copy number variants (51) in which a region of DNA is duplicated or deleted. Although the functional consequences of these variants is largely unknown, virtually all of the ~25,000 genes in our genome have variants. In situations where the effects of such variants have been examined, they can be neutral, but many influence gene products quantitatively or qualitatively. Thus, they provide a mechanism to explain much of the interindividual phenotypic variation observed in the human population.

From a clinical perspective, identification of genomic variants that cause differences in disease outcome has significant implications. The products of these genes, or the pathways in which they act, may become potential therapeutic targets. That is, nature has provided evidence that a particular target can modify disease, so drugs that can mimic the effects of the advantageous version of the gene are likely to be effective therapeutically. For example, enzymes or receptors with deleterious functions could be targeted with inhibitors or antagonists, respectively. Conversely, enzymes or receptors with beneficial or protective effects could be activated or stimulated. An extension of this concept is that the gene product conferring the phenotypic effect may not be a readily “druggable” target, but understanding the pathway in which it works would probably present more feasible targets.

GENES THAT MODIFY CYSTIC FIBROSIS LUNG DISEASE

The search for genetic causes of clinical variation has been approached in various ways. The generic recipe for such studies is to examine the relationship between variants in a gene or position in the genome (known as a locus) and a clinical trait that varies among patients. The

trait may be continuous, such as pulmonary function indices, or discrete, such as intestinal obstruction. Because mortality is closely associated with lung disease, pulmonary phenotypes have been the most intensely studied in this regard.

Early modifier studies revolved around one or a few candidate genes, focusing on those genes that fit into a pathway or process believed to contribute to CF disease pathophysiology (Figure 2); such pathways include ion channels (particularly chloride and sodium channels, etc.) that could compensate for CFTR's absence (52, 53), innate defense (54–57) and inflammatory pathway components (58, 59) that could modulate the response to pulmonary pathogens, and so on. For the most part, these studies were carried out on relatively small numbers of patients and were dictated by the small number of variants known for most genes, the genotyping technologies available, and the cost of using the technology. As genome information and technology have progressed, and as multicenter approaches have provided larger patient and family cohorts, large-scale genotyping has been made more technologically and economically feasible and possible. Consequently, increased sample sizes are providing better statistical power to detect associations, and scans encompassing the whole genome are allowing one to probe nearly every gene and region of the genome, which may permit the discovery of effects of loci not previously considered in CF.

Although this approach has resulted in many published associations, few of these have been replicated. Replication is important for the validation of modifiers, as the small sample size and the testing of multiple genes and polymorphisms make these studies prone to spurious associations. The first CF modifier study to use large patient cohorts and replication identified SNPs in the 5' region of the *TGFB1* gene that are associated with lung function in CF patients. The initial association was found in CF subjects who were homozygous for the F508 mutation and in the upper and lower quartiles for lung function; the replicating population included other CF genotypes (48). *TGFB1* encodes transforming growth factor β 1, a signaling molecule made by many cell types involved in multiple processes, such as inflammation and wound healing. The same region of *TGFB1* also associates similarly with COPD in smokers (60), whereas the opposite association was found for asthma (61).

Using a different approach, in which patient samples with similar disease severity were pooled for genotyping, Gu et al. (62) identified allele frequency differences in the interferon-related developmental regulator 1 (*IFRD1*) gene between severely and mildly affected CF patients. The *IFRD1* SNPs were examined in a second cohort of patients and revealed skewed frequencies that tracked with pulmonary disease severity; they were also examined in a mouse model in which the gene was expressed predominantly in neutrophils.

The neutrophil chemoattractant IL-8 is elevated in the airways of CF patients and is believed to be largely responsible for the neutrophilia that is characteristic of CF lungs. Variants in IL-8 that associate with other pulmonary conditions (63) were examined and found to associate with pulmonary function in two independent cohorts of CF subjects. Molecular studies indicated that the adverse allele expressed nearly three times more IL-8 mRNA than did the protective allele (58).

From a list of genes proposed to be involved in various aspects of airway physiology, alleles of SNPs in the endothelin receptor A gene (*EDNRA*) were associated with pulmonary function in the same cohort used to assess *TGFB1*, and these findings were replicated in cohorts originating from the Seattle, Washington, area as well as from Ireland (64). Endothelin stimulation of this receptor causes contraction and proliferation of smooth muscle. The variants associated with pulmonary function were also associated with mRNA-expression differences; the adverse variant showed greater *EDNRA* mRNA levels in airway smooth muscle than did the protective variant.

The ability to replicate associations adds confidence to their validity and supports the idea that such associations are the consequences of biologically based mechanisms. These associations and molecular investigations support models such as that depicted in Figure 3, in which inflammation is deleterious to the airways. However, they also suggest that we may have underestimated the role of tissue remodeling (*TGFB1* and *EDNRA*) and airway mechanics (*EDNRA*) in the disease process.

MODIFIERS OF OTHER CYSTIC FIBROSIS TRAITS

Although the most attention has been paid to pulmonary disease in the hunt for modifiers, it is by no means the only relevant phenotype to the disease. For example, meconium ileus and other gastrointestinal manifestations affect a large proportion of CF patients. Genetic influences on intestinal obstruction were identified in mouse studies (65), and those observations appear to hold true in human CF patients as well (66, 67).

CF-related diabetes (CFRD) is a prominent comorbidity of CF that affects nearly half of CF patients at some point in their lifetime. The etiology of CFRD is unclear, and it has features that are similar to those of type 1 and type 2 diabetes. The type 1 similarities appear to revolve around the loss of insulin-producing islet cells, but there is no evidence for an autoimmune mechanism. However, having a relative with type 2 diabetes increases the risk of CFRD, which suggests that there are mechanistic similarities between the two. To explore this hypothesis, Blackman et al. (68) examined SNPs in genes reported to be susceptibility genes for type 2 diabetes for their association with CFRD; they found that one of the most significant type 2 diabetes genes, an islet cell transcription factor named transcription factor 7-like 2, also associated with CFRD and did so reproducibly in several cohorts of subjects.

These studies provide important principles and insight into CF pathogenesis. The genetics of intestinal obstruction suggest that mouse models may be powerful tools to examine in vivo mechanisms of obstruction and perhaps a resource for testing therapeutics designed to alleviate or prevent obstruction.

Regarding CFRD, the loss of endocrine cells was historically thought to be a bystander effect of the disease in which the destruction of exocrine tissue spreads to the endocrine cells. The parallels with type 2 diabetes bring those models into question and will probably require revisiting those models as the pathogenesis of type 2 diabetes becomes clearer. As CF patients appear to be a diabetes-prone population, our understanding of type 2 diabetes may incorporate mechanistic information from CFRD.

WHOLE-GENOME STUDIES

As a consequence of the large number of small studies, consortia have evolved to provide the ability to replicate associations and to increase the power to detect them. The Gene Modifier Study (48), the CF Twin and Sib Study (69), and the Canadian Consortium for Genetic Studies (48, 56) formed a North American CF Gene Modifier Consortium. Combined, the groups surveyed 3,467 CF patients, and because of the various study designs used by each group, they were able to examine these subjects by case/control designs, as well as by linkage analysis, thereby capitalizing on the strengths of each approach.

The consolidation of cohorts presents logistical problems, particularly with regard to phenotype. Studies investigating related but different phenotypes, or phenotypes arrived at by different methods, may generate different results. To reduce the chances of such occurrences, investigators developed a phenotypic definition based on lung function, but corrected for survival and referenced to individuals with CF rather than the non-CF population, to characterize all the subjects in the study (70).

Another potential confounding feature for genetic studies is the effect of ancestry on associations. Individuals of common ancestry, such as racial and ethnic groups, are genetically more similar than are individuals from different backgrounds. As a consequence, phenotypes that are more common in one related group than in an other may display apparent genetic associations that are due to population structure rather than the gene or genes in question. To explore this possibility, investigators studied the structure of the population (71) and found it to be of predominantly Caucasian descent.

With a sample size of nearly 3,500 CF subjects to examine, more than 500,000 SNPs spread across the genome were genotyped and assessed for association with pulmonary function. Two genomic intervals surpassed the strict statistical criteria for multiple testing. One region, on chromosome 11 and residing between the Apaf-1-associated protein gene (*AP1P*) and the Ets-homologous factor gene (*EHF*), showed significant association ($P = 9.4 \times 10^{-9}$). A second region, on chromosome 20, was detected by linkage analysis [logarithm (base 10) of odds score of 5.033] in families, and when the region was examined for association in unrelated CF individuals, SNPs in the genes encoding melanocortin-3 receptor (*MC3R*) and cerebellin-like 4 (*CBLN4*) showed the most significant association ($P = 1.34 \times 10^{-4}$); combining linkage and association this region achieved a significance level of $P < 10^{-8}$ (74). Figure 3 shows a schematic of these two regions and the genes known to reside in those regions.

In addition to the associations depicted in Figure 3, there are other tantalizing loci that nearly meet the rigorous statistical thresholds of these types of associations. Such loci include the major histocompatibility complex genes on chromosome 6 and SNPs included in the angiotensin 2 type 2 receptor gene (*AGTR2*) on the X chromosome.

THE UTILITY OF VARIANTS AND VARIATION

Studying variation is a standard approach for understanding biology and, in this case, the biology underlying a disease. By understanding the variation that occurs between

individuals affected by CF compared with those not affected, we hope to obtain insight into the pathogenesis. By examining variation between CF patients and comparing it with their clinical outcome, we hope to identify variation that plays a causal role in modifying disease severity and thereby become enlightened about therapeutic approaches. To this end, the massive amounts of data that continue to be collected must be integrated in recognition of the complementary attributes of the data. The molecular and clinical data that have been collected in various venues for decades have provided us with extensive view of systemic effects of CF, but they do not typically inform us about causal relationships and where one might intervene. Historically, that has been left to the clinician to predict. Although this process has been effective by dramatically extending the life span of CF patients dramatically, we now have the opportunity to directly identify genetic factors that cause differences in outcome, as outlined above for modifier genes.

Thus, one of the next key challenges will be to integrate the massive amounts of molecular, genetic, and clinical data for these purposes. Genetic analyses will allow us to focus attention on a finite number of processes, whittling down the 20,000 to 30,000 genes of the genome to a handful that influence pathogenesis. The molecular characterizations should allow us to place those modifying genes into pathways, and comparison with clinical information should permit us to determine how to alter the disease itself. However, we are still in the early stages of tapping into this potential; the effects of genetic variation on the plethora of relevant traits have yet to be realized. Two fascinating questions that remain are: How does gender affect modifying influences? What are the genetics of drug metabolism in cystic fibrosis?

Glossary

CFTR	cystic fibrosis transmembrane conductance regulator
SNP	single nucleotide polymorphism

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SUMMARY POINTS

1. Much of the clinical variation among individuals with CF is conferred genetically.
2. Molecular technology has allowed massive amounts of information to be generated in the form of transcriptomic, proteomic, and genetic data.
3. Analyses of these data have begun to identify pathways not previously considered important to CF and to point out key players in pathways that were previously considered important.
4. The identification of these processes and their key components will provide novel therapeutic targets and insight into how to improve existing therapies.

FUTURE ISSUES

1. The identification of putative disease-modifying genes must be followed by biological studies to verify the modifying effects of these genes.
2. The proteins encoded by these modifying genes, as well as the pathways in which they act, may become potential therapeutic targets for cystic fibrosis. Thus, future work should aim to evaluate the modulation of these pathways for therapy.
3. Additional genotype/phenotype studies for associations with pharmacologic responses (pharmacogenetics) will be possible and may allow the optimization of CF pharmacology based on an individual's genetic profile.
4. The biology of CF certainly overlaps with that of other disorders; therefore, the modifying effects of genes identified in CF studies probably have effects in other disorders and will need to be examined.

Figure 1.

Effect of *CFTR* gene mutations on the cystic fibrosis transmembrane conductance regulator (CFTR) protein and function. (*Top*) The *CFTR* gene with examples of each of the mutation classes and their positions in the gene. The horizontal black arrow represents the transcriptional promoter, and the vertical lines represent exons. The 5' and 3' untranslated regions are designated by gray shading, and the mutations are designated by vertical arrows. (*Bottom*) The effects of the mutations on function; the mutation class (12) is circled. G542X, a class 1 mutation, creates a stop codon and causes no full-length, functional protein to be produced. F508del, a class 2 mutation, misfolds and is mostly degraded. If able to reach the plasma membrane, F508del does not activate as readily as normal CFTR; therefore, this mutation is a class 3 example. G551D, another class 3 example, is completely nonresponsive to activation. R117H, a class 4 mutation, resides in a transmembrane region of the protein

and impairs chloride transit (conduction) through the channel pore. The T-tract variants in intron 8 represent class 5 mutations in that they alter splicing efficiency. These variants do not eliminate functional CFTR, which differentiates them from class 1 mutants. Note that the original description of *CFTR* contained only 24 exons; later, exons 6, 14, and 17 were found to contain small introns. These exons were renumbered 6a and 6b, 14a and 14b, and 17a and 17b. Thus, what is referred to as exon 10, for example, is actually exon 11.

Figure 2.

Selection of candidate modifier genes. Cystic fibrosis (CF) pulmonary and airway disease has been modeled as a consequence of insufficient luminal hydration (including mucus), an inability to clear bacteria such as *Pseudomonas aeruginosa*, and an excessive inflammatory burden, either as a response to infection or inherent to CF epithelia. Candidate modifying genes were chosen for their potential to modulate these processes. Genes encoding epithelial ion channels were examined because they can influence airway luminal hydration. Genes encoding chemotactic signaling molecules, such as interleukin (IL)-1 β , IL-8, and tumor necrosis factor (TNF)- α , were examined because they can influence the level of immune cell recruitment, and genes involved in antimicrobial processes or immune cell function were investigated because they may exert modifying effects on airway disease by influencing the ability to clear or suppress infection.

Figure 3.

Location of single nucleotide polymorphisms (SNPs) identified by genome-wide association studies as associated with lung function. Clusters of associating SNPs are shown as gray boxes. The height of the box corresponds to the most significant SNP in the cluster. For chromosome 11, the most significant association lies between the genes *APIP* and *EHF*; less significant associations overlap both genes. The chromosome 20 association is most significant with SNPs 3' to *CBLN4*, but there is also a peak over *MC3R*.

Table 1

Messenger RNA and protein-expression differences in cellular pathways

Pathway(s)	Studies of gene expression	Studies of protein expression
Metabolism (increased in CF)	29, 30, 34, 73, 74	33, 36, 75
Oxidoreductase and stress (increased in CF)	29, 34	32, 36, 44–46, 75
Inflammation (increased in CF)	29, 30, 73, 74	32, 36–39, 42, 43, 75
Lipid synthesis (decreased in CF)	34	31, 36
Protein degradation (increased in CF)	34	33, 36, 75
Antioxidant, anti-inflammatory, and protective (decreased in CF)	34	32, 40, 41, 44, 47

Abbreviation: CF, cystic fibrosis.