Strategies for Identifying Modifier Genes in Cystic Fibrosis

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Even in patients with cystic fibrosis (CF) with identical CFTR genotypes, there is a wide range in the severity of lung disease, with some individuals facing death or lung transplantation early in life and others demonstrating mild lung disease well into adulthood. Although numerous environmental factors have been identified that influence CF pulmonary phenotype, there is now growing evidence that polymorphic variants in genes besides CFTR play an important role in determining severity of CF lung disease. This article reviews the most recent findings regarding genetic modifiers in CF and also discusses in detail the strategies currently being used to identify novel modifiers of CF pulmonary phenotype. These include single- and multicenter studies, twin and sib studies, microarray approaches, and whole genome association studies.

Keywords: cystic fibrosis; genotype; modifier; phenotype; transforming growth factor β

Although the DNA sequence in two unrelated individuals is about 99.9% identical, the remaining 0.1% is characterized by normal variations that have the potential to alter protein structure and function (1). Classically, these polymorphic variations are differences in single nucleotide polymorphisms (SNPs), although some polymorphisms may include deletion or insertion of several nucleotides (2). When polymorphisms present in the exon region of a gene result in variability in the amino acid sequence of the resulting protein, they have the greatest potential to influence function. Other polymorphisms may not change amino acid sequence but still influence protein expression or regulation. Polymorphisms play a particularly important role when they occur in genes that modify the phenotypic presentation of a disease (2). These modifier genes may be involved in the underlying pathophysiology of a disease, as part of the body's response to the disease, or in a pathway involving a treatment for a disease.

Cystic fibrosis (CF) is the most common lethal autosomal-recessive disorder in whites, affecting approximately 30,000 individuals in the United States. In 1989, it was found that CF is caused by the presence of mutations in both copies of CFTR, a 230-kb gene located at chromosome 7q31.3 (3). CFTR encodes a 1,480-amino acid protein that acts as a cAMP-dependent chloride channel in the apical membrane of cells lining the lungs, sinuses, pancreas, intestines, vas deferens, and sweat ducts. CFTR has been demonstrated to play a significant role in these tissues by influencing chloride transport and sodium and water balance (4). Classically, individuals with CF develop a disease characterized by progressive bronchiectatic lung disease, pancreatic insufficiency, chronic sinusitis, and male infertility. Although CF involves multiple organ systems, it is the severe lung disease that results in the shortened median survival of only 36.8 yr (5). There is a wide range seen in the severity of CF lung disease and survival, however, with some individuals with CF facing death or lung transplantation while in their teens and other individuals with CF having very mild disease well into adulthood.

This dramatic variability in severity of lung disease has long been recognized, and when CFTR was identified as the causative gene for CF, it was assumed that different mutations in CFTR would form the basis for the variability. It was quickly demonstrated, however, that although certain aspects of the CF phenotype, such as pancreatic insufficiency, are determined by CFTR genotype, many other aspects of the disease are not (6). In fact, although more than 1,500 different CFTR mutations have been identified (7), over 50% of all individuals with CF carry the following same CFTR genotype: homozygous for ΔF508/ΔF508 mutations (5). Even within this large group of ΔF508 homozygotes, a full range in severity of pulmonary disease is seen, making clear that CFTR genotype alone does not determine severity of disease (8).

The basis for the variability in severity of CF lung disease is still not fully understood. Certain mild CFTR mutations have been identified, which result in preservation of CFTR function and milder lung disease (9), although they do not help in explaining the variability seen in most individuals with pancreatic-insufficient CF. Numerous infectious, nutritional, and environmental influences have also been identified that influence severity of CF lung disease, although none begin to fully explain the degree of variability seen (10–15).

MODIFIER GENES IN CF

There has been growing interest in trying to identify non-CFTR genes that act as modifiers of CF lung disease phenotype. This interest has been stimulated by a few observations. One is that the European CF Twin and Sibling Study compared phenotypes for a cohort of 41 sets of twins with identical CFTR genotypes and found that monozygous (MZ) twins had a significantly higher concordance in severity of lung disease than did dizygous (DZ) twins (suggesting that even when CFTR genotype is identical, differences in other genes can influence phenotype) (16). Second, studies in CFTR-knockout mice have identified a clear genetic modifier for intestinal manifestations of CF (17).

The use of animal disease models and gene knockout is usually one of the most powerful tools available for testing candidate modifiers of disease phenotype. Unfortunately, this strategy is not available for identifying or testing candidate modifiers of CF lung disease because no rigorous animal model of CF lung disease exists. Although CFTR-knockout mice develop gastrointestinal complications, they do not demonstrate pulmonary disease representative of that seen in humans, perhaps due to the presence of alternative chloride channels (18). This has forced investigators to use human studies to investigate modifier genes in CF.

Challenges of Human Modifier Gene Studies

Although human studies may sound ideal for determining the effect of modifier genes in CF, numerous challenges exist. One

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of the most basic challenges is deciding how best to quantify severity of lung disease. Although FEV_1% predicted is commonly accepted as reflecting severity of CF lung disease, it is essential that the FEV_1% be age-adjusted to allow comparison among groups. Even this correction can be problematic, because the typical % predicted correction is based on decline in lung volume with age in non-CF control subjects. Different approaches to correcting lung function for age in CF have been developed, including a correction based on expected decline in all individuals with CF (19) and another based on lung function in ΔF508 homozygotes (20). A separate challenge in human studies is controlling for nongenetic influences that may strongly alter lung phenotype, including socioeconomic status (14), variability in care between centers (21), and exposure to *Burkholderia cenocepacia* infection (15). Finally, cross-sectional studies must be designed to ensure that they are not overly influenced by dropout in the adult population of disadvantageous polymorphisms that lead to early death.

Single-/Few-Center Studies

The general approach to most human modifier gene studies in CF has been to compare phenotypic characteristics of CF populations segregated by modifier gene polymorphism type. Usually, these have been single-center studies, which have the advantage of ready access to detailed phenotypic data on participants and less variability in medical care, but often have too small a sample size to be conclusive. Some examples of previous single-center studies in CF include those involving MBL2 (22, 23) and TGFB1 (24).

Mannose binding lectin (MBL), a product of gene MBL2, aids in the phagocytosis of bacteria by activating the classical complement pathway after recognizing and binding to mannose or N-acetylgalactosamine on the surface of bacteria (25). Three separate single–base pair substitutions at codons 52, 54, and 57 result in significant decrease in circulating functional MBL levels, and these variant alleles are collectively named MBL2-O (26). In a single-center study over 10 yr, Garred and coworkers (22) studied 149 individuals with CF and chronic *Pseudomonas* infection and found that those carrying MBL-insufficient alleles had significantly lower FEV_1 (64 vs. 52% predicted, $p = 0.005$) and FVC (85 vs. 72%, $p < 0.001$). They also demonstrated a significantly higher risk of death or lung transplantation (proportional risk ratio, 3.0). A subsequent single-center study by Davies and coworkers (23) involved 566 individuals with CF (298 adults and 268 children). They determined that, in adults, both FEV_1 and FVC were significantly lower in individuals homozygous for MBL2-O. Furthermore, over the 12-yr time period studied, subjects with the O/O genotype also had the fastest annual rate of decline in FEV_1 (2.2 ± 0.5% of baseline vs. 1.3 ± 0.1% in the A/A group and 0.7 ± 0.1% in the A/O group, $p = 0.001$).

Transforming growth factor β (TGF-β) is a cytokine with both proinflammatory and antiinflammatory properties. In airway epithelium, TGF-β modulates fibroblast proliferation and deposition of collagen (27, 28). TGF-β is also known to be a direct inhibitor of CFTR function (29). Functional polymorphisms have been identified in gene TGFB1 in both the promoter (−509) and at position +869 (codon 10), which influence circulating levels of TGF-β (30, 31). In a study of 171 ΔF508 homozygous individuals with CF (24), Arkwright and coworkers found that the TT allele in codon 10 was associated with nearly a doubling of the risk of rapid progression to $FEV_1 < 50$% and FVC < 70% predicted (hazard ratio, 1.95; 95% confidence interval, 1.24–3.06; $p < 0.005$). They cited studies that suggested the TT allele was associated with elevated expression of TGF-β (*see* Reference 32) and termed it a "high-producer genotype." However, in a subsequent study of 261 patients with CF by the same research group, the association between the 10 TT allele and severe CF lung disease was not confirmed (33). In addition, other promoter studies suggested that it may not be the codon 10 TT allele but codon 10 CC that results in the highest levels of TGF-β expression (30).

Multiple other candidate modifier genes have been studied in CF with single- or few-center investigations. These have included, among many, α_1-antitrypsin (SERPINA1) (34), β_2-adrenergic receptor (ADRB2) (35), glutathione S-transferase (GSTM1) (36), nitric oxide synthase 3 (NOS3) (37), tumor necrosis factor (TNF) (38), and gluatamate-cysteine-ligase (GCLC) (39). All have been identified in these studies as potentially modifying pulmonary phenotype in CF.

Unfortunately, the challenge for nearly all single-center studies is including a sufficient number of study subjects to avoid being underpowered. Being underpowered increases the risk of both false-negative and false-positive results. Statistical analyses have demonstrated that at least 500 individuals must be genotyped to detect with confidence SNP–phenotype associations that account for a 5% variability in a phenotypic trait (40). Analyses have also demonstrated that SNP–phenotype association studies of smaller than 500 participants with positive findings have low repeatability (37). The limitations of small modifier studies have been dramatically demonstrated in CF, because, recently, the positive results of many of the small studies described above have failed to be confirmed in a large multicenter study (41). In particular, the association results from the studies of MBL2 and TGFB1 have been called into question by conflicting results in the large study (*see below*).

Large Multicenter Studies

In response to the recognized need for larger, more conclusive studies of candidate modifier genes in CF, several multicenter investigator groups have been established. The advantage of these groups is the dramatic increase in the number of potential study subjects. The disadvantages are the challenge in acquiring sufficiently detailed phenotypic data and the introduction of the potential variability of differences in center care practices.

In late 2005, the results of one of the first large multicenter studies were published. Drumm and coworkers collected genotype and phenotype data on 808 ΔF508 homozygotes with extreme phenotypes from over 60 different CF centers (41). Limiting the study to only ΔF508 homozygotes minimized variability at the CFTR locus, which might contribute to phenotypic differences. The participants with CF were only included if they clearly fell into a "severe" lung disease group or a "mild" lung disease group. To qualify for a group, they had to meet FEV_1 criteria that would place them in the most severe or most mild 20th percentile of lung disease for their age. Using these criteria, 263 severe and 545 mild ΔF508 homozygotes were identified. The investigators then genotyped numerous genetic polymorphisms, including 16 polymorphisms from 10 genes previously identified to be candidate CF modifiers in single-center studies. They then determined their prevalence in each severity group.

Only 2 of the 16 reported polymorphisms demonstrated significant association with severity of lung disease, both from TGFB1 (29). High-expressing promoter genotype (−509 TT) and codon 10 CC were found to be more than twice as prevalent in individuals in the severe lung disease group (−509 TT: 12.3 vs. 5.9%, $p = 0.006$; codon 10 CC: 19.7 vs. 10.1%, $p = 0.0008$). To confirm their findings, the investigators studied an additional 498 patients with various CFTR genotypes who were dichotomized for severity of lung disease by FEV_1 at age 20. The high-expressing codon 10 CC allele was again found to be significantly more prevalent in the severe lung disease group (23.6 vs. 11.1%, $p = 0.0002$).

The results from this study highlight the importance of having large populations for association studies. Not only were the MBL2 results from previous smaller studies not confirmed but the identified codon 10 CC allele in TGFB1 associated with more severe CF lung disease contradicted the 10 TT allele previously suggested (24).

Even with its large study size and a replication study in a separate population, the study by Drumm and coworkers it is not without potential for error. Although the phenotype groups were divided according to FEV_1, FEV_1 may be closely linked to other phenotypic features, such as body mass index (BMI). BMI was noted to be significantly different between the two study groups ($p < 0.001$) (41). The observed association between TGFB1 and CF phenotype might be explained not just by an effect of TGF-β on lung disease but instead or in part by an effect on BMI.

The multicenter Canadian Modifier Gene Project has also been collecting genotype and phenotype data on a large number of individuals with CF and family members (42). They have collected samples from over 5,000 individuals representing over 2,000 families. An initial screen of 77 candidate genes identified polymorphisms in TGFB1 and TNF as being associated with severity of CF lung disease (31). Full results should be available in the upcoming year.

Twin/Sib Studies

Another strategy that is being used to help determine the role of modifier genes in influencing CF phenotype is analysis of CF twin and sib pairs. One of the particular advantages of this strategy is that it permits analysis of the contribution of both genetic and environmental influences to differences in phenotype (43). There are currently CF twin and sib collections in both North America and Europe. A study in the European twins demonstrated that MZ twins had a significantly higher concordance in severity of lung disease than did DZ twins, suggesting a strong genetic contribution to variability in severity of CF lung disease (16). An ongoing analysis in the United States' twins also found that MZ twins have a significantly higher concordance in severity of lung disease when compared with DZ twins. This was found to be true, however, only during the period the twins were living together, suggesting a significant role for both genetic and environmental contribution to CF pulmonary phenotype (44).

The European sib collection has also been used recently to identify novel CF candidate modifiers: the gene for TNF receptor TNFRSF1A and the genes encoding the amiloride-sensitive channel ENaC (SCNN1G and SCNN1B) (45). Stanke and coworkers studied 37 ΔF508 homozygote sib pairs in which both sibs demonstrated markedly mild or severe lung disease (45). Genotyping revealed specific TNFRSF1A and SCNN1B haplotypes that are associated with severity of lung disease.

One particular advantage of twin and sib studies is that they allow a calculation of the heritability of a trait by comparing concordance in MZ and DZ twins. Recent calculations based on data from the United States' twin collection suggest that the heritable genetic contribution to severity of CF lung disease is approximately 0.6 to 0.7 (44). The biggest concern, however, about both the United States' and European twin studies is sample size. In its last published data, the United States' collection had only 38 MZ twins and 8 DZ twin pairs (44), although recent recruitment has since increased the size to approximately 70 MZ twin pairs and 25 DZ twin pairs (Dr. G.R. Cutting, personal communication). The European twin and sib collection is approximately half the size of that of the United States (46). Although the decrease in genetic variability between twins and sibs permits the use of smaller sample sizes, a greater number of twins in these studies will be required to increase the confidence in conclusions drawn about specific modifier genes.

Comparing Polymorphism Prevalence between Pediatric and Adult Populations

One unique strategy for identifying modifiers that may affect survival has been used by Buranawuti and coworkers (47). They took advantage of the fact that a modifier gene polymorphism that causes a significant survival disadvantage should be less prevalent in a cross-sectional sample of an adult population than in a cross-section of a pediatric population. This is because individuals carrying the disadvantageous polymorphism would be more likely to have died at an early age. It would also be expected that this disadvantageous polymorphism would be more prevalent in individuals who had died early in life or, in the case of CF, undergone lung transplantation early in life. They used this strategy to assess the role of the O/O variant of the MBL gene. The O/O variant is associated with significant decrease in functional MBL production. After genotyping 247 individuals with CF and 396 control subjects, they determined that individuals with the O/O genotype were much less prevalent in the adult CF group than in children with CF (1.8 vs. 9.0%, $p = 0.018$). The prevalence of the O/O genotype in the 34 adults who had died or undergone lung transplant was significantly higher than the surviving adult CF group (15 vs. 1.8%, $p = 0.008$). These data suggest that carrying the MBL O/O genotype may be associated with a long-term survival disadvantage. Although the strategy of comparing polymorphism prevalence in healthy survivors with CF with individuals dying or undergoing lung transplantation at an early age is a unique one that may help to determine modifier gene effect on survival, the results from this study have only been published in abstract form. Furthermore, the specific results found for MBL in this study would have to be independent of effect of FEV_1, because the large study by Drumm and coworkers did not find an effect of the MBL O/O genotype on severity of lung disease (41). Alternatively, the effect of the MBL O/O genotype may vary depending on airway microbiology (48).

Using Microarray Expression Profiles to Identify Novel Modifiers

A separate strategy used by Wright and coworkers focused on identifying completely novel potential modifiers of CF lung disease (49). This investigation used high-density oligonucleotide microarray analysis of nasal respiratory epithelium to investigate the molecular basis of phenotypic differences in severity of CF lung disease. They identified differences in gene expression between ΔF508 homozygotes with the most severe lung disease (bottom 20th percentile of FEV_1) and ΔF508 homozygotes with the most mild lung disease (top 20th percentile of FEV_1). The 12 participants with CF were matched for age as well as clinical characteristics designed to minimize environmental influences on severity of lung disease. A control group of 11 individuals without CF was also studied. A total of 652 of the 11,867 genes identified as present in 75% of the samples were significantly differentially expressed in one of the three disease phenotypes: 30 in non-CF, 53 in mild CF, and 569 in severe CF. An analysis of those genes differentially expressed by severity of CF lung disease demonstrated significant up-regulation in severe CF of genes involved in protein ubiquitination ($p < 0.04$), mitochondrial oxidoreductase activity ($p < 0.01$), and lipid metabolism ($p < 0.03$).

Further analysis identified several specific novel genes whose expression in nasal respiratory epithelium was associated with differences in severity of CF lung disease. These included statherin

(STATH) (49), which encodes a calcium-binding protein known to have antibacterial properties and is found in saliva, nasal secretions, and the upper airway (50). STATH was found to be up-regulated in the ΔF508 homozygotes with mild CF lung disease, and this increased expression was confirmed by reverse transcriptase–polymerase chain reaction in an additional 12 mild and severe ΔF508 homozygote samples (40). Statherin plays a key role in the development of the oral cavity biofilm, by mediating adhesion of bacteria, and was recently identified as being the most prominent protein in the saliva–air interface (51). Work will be needed before STATH can be further investigated as a CF modifier, however, as it has not been investigated for functional polymorphisms.

Adiponectin (ADIPOQ), which encodes a protein that potently inhibits inflammation and modulates insulin sensitivity, was also significantly up-regulated in ΔF508 homozygotes with mild disease (49). Adiponectin leads to leukocyte production of antiinflammatory mediators (52) and also modulates energy metabolism and glucose sensitivity (53). Functional polymorphisms have been identified that influence circulating adiponectin levels (54), and those polymorphisms resulting in higher levels of adiponectin in CF could lead to more mild disease by both suppression of CF-related inflammation and improved nutritional status.

Also identified in the study as potentially important areas for candidate modifiers of CF phenotype were ubiquitin-activating and ubiquitin-conjugating enzymes. The microarray results demonstrated a significant increase in activity of the ubiquitin system in individuals with severe CF. ΔF508 CFTR is known to be degraded by ubiquitination, so increased ubiquitination would result in less CFTR reaching the epithelial cell apical membrane. Among the numerous ubiquitin cycle genes identified to be up-regulated were the specific ubiquitin–activating enzyme UBA2, ubiquitin-like protein target NEDD8, and ubiquitin-conjugating enzyme HIP2 (E2–25K). HIP2 (E2–25K) identifies proteins for intracellular proteolysis by the 26S proteasome (55).

Using microarray analysis of tissues as a strategy for identifying novel modifiers of phenotype has significant limitations. Most important is the difficulty in distinguishing between those differences in gene expression that contribute to differences in phenotype and those that are the result of differences in phenotype. This is particularly true in CF for genes involved in inflammatory and immune responses. Also of concern is the inability to detect potentially important modifiers that occur in only a small percentage of the population. The statistics of microarray analysis will not identify as significant a marked decrease in expression in only a small percentage of the samples. Finally, microarray analysis only provides hypothetical leads: identification of functional polymorphisms in the genes and subsequent population-based studies to determine segregation by phenotype are still the only method for demonstrating association.

The Future: Identifying Novel CF Modifiers by Whole Genome Association

Most efforts to date in CF have concentrated on acquiring genotypic and phenotypic data on sufficient numbers of patients to determine the role of already suspected genetic modifiers. As large multicenter groups collect a growing number of CF patient DNA samples and detailed phenotypic data, however, focus is beginning to switch to using whole genome association techniques to identify completely novel modifiers. Whole genome association studies have the potential to be significantly more sensitive for identifying novel modifiers than standard family linkage studies. The International HapMap Consortium recently completed genotyping over 3.8 million SNPs in three different populations and found that certain SNPs tend to be transmitted together from generation to generation in haplotype blocks (56). By genotyping specific SNPs that characterize these blocks (tagSNPs) (57, 58), large stretches of chromosomes will be simultaneously evaluated for linkage disequilibrium between CF groups with contrasting severity of lung disease. This genotyping will be challenging and expensive, however, because segments of linkage disequilibrium are only tens of thousands of bases in length (rather than tens of millions for family linkage studies). This will require genotyping hundreds of thousands of tagSNPs in a very large group of patients to scan the whole genome (59). Two-stage genotyping designs, in which all markers are genotyped in an initial group and only promising markers are genotyped in a second group, have been proposed to optimize ability to detect meaningful associations and limit cost (60). Study design and statistical interpretation of results will require accounting for both the large-scale multiple-hypothesis testing inherent in the technique and the potential influence of population stratification (61). When specific chromosomal regions are identified that consistently segregate with severity of lung disease, further evaluation within these regions will lead to identification of previously unsuspected genes responsible for modifying CF phenotype. Although results are not yet available, whole genome approaches to identifying novel modifiers of CF are underway (62).

CONCLUSIONS

The approach to identifying genetic modifiers of CF phenotype is evolving. Small association studies are giving way to large multicenter association studies and focused twin/sib studies. The clinical specimens available from the large studies combined with emerging whole genome technologies will permit identification of novel modifiers of CF phenotype in the near future. Identification of these modifiers will result in increased ability to predict severity of CF lung disease in individuals and, most important, provide targets for future therapeutic intervention.

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