TWO PATIENTS WITH CYSTIC FIBROSIS, NONSENSE MUTATIONS IN EACH CYSTIC FIBROSIS GENE, AND MILD PULMONARY DISEASE

Garry R. Cutting, M.D., Laura M. Kasch, B.S., Beryl J. Rosenstein, M.D., Lap-Chee Tsui, Ph.D., Haig H. Kazazian, Jr., M.D., and Stylianos E. Antonarakis, M.D.

CYSTIC fibrosis is the most common lethal inherited disorder in the white population. It is manifested by viscous secretions in the lungs and pancreas and abnormal electrolyte composition of sweat.[1] Epithelial cells from patients with cystic fibrosis have abnormal conductance of chloride ions across apical membranes due to defective regulation of a particular chloride channel.[2-4] Inadequate secretion of chloride is believed to cause the insufficient hydration of mucus in the airways and pancreatic ducts.[1] The cystic fibrosis gene has recently been identified, and it is predicted to encode a 1480-amino-acid protein termed the cystic fibrosis transmembrane conductance regulator (CFTR).[5,6] The deduced amino acid sequence of CFTR suggests that this protein has several functionally important regions, including two ATP-binding domains, two areas that interact with cell membranes, and a highly charged domain with several potential sites for phosphorylation by protein kinases. The structure of CFTR is similar to the multidrug-resistance proteins and several other membrane-associated ATP-binding proteins.[6] The latter proteins are known to transport small molecules (drugs, carbohydrates, and amino acids) across cell membranes in a process that appears to be coupled with ATP hydrolysis.[7,8] Structural similarities between CFTR and this family of proteins raise the possibility that CFTR may be involved in the regulation of secretory chloride channels.[6,9]

The identification of naturally occurring deleterious gene mutations has provided insight into the functionally important regions of several proteins.[10,11] The most common mutation of the cystic fibrosis gene, ΔF_{508}, leads to the omission of a phenylalanine residue in the putative first ATP-binding domain of CFTR, indicating that this region is crucial for function.[6-12]

A number of missense and nonsense mutations have been discovered in the first and second ATP-binding domains, confirming the importance of these regions.[13,14] In this report, we describe two unrelated American black patients with nonsense mutations in

each cystic fibrosis chromosome. Nonsense mutations frequently cause severe illness, since premature termination of translation creates truncated proteins that are usually unstable. Each patient had severe pancreatic disease but mild pulmonary illness. This discrepancy is a remarkable finding and indicates that truncation or even the absence of CFTR is not lethal and may have a greater effect on pancreatic function than on lung function.

METHODS

Patients

Seventeen American black patients and 25 American white patients with cystic fibrosis, carrying 23 and 29 unknown cystic fibrosis mutations, respectively, were available for study. All patients had two positive sweat chloride tests (>60 mmol per liter) and clinical features consistent with the diagnosis of cystic fibrosis. Exons 10, 11, 20, and 21 were investigated in these patients as part of a systematic search for mutations in the proposed functionally important regions of CFTR.

Nucleotide Sequencing of Cystic Fibrosis Gene Exons

Exons 20 and 21 in the second putative ATP-binding domain were amplified from the genomic DNA of each patient with the polymerase chain reaction (PCR).[15] Primers for the PCR were selected from the flanking intron sequence of each exon as follows: 20i-5 (5'GGTCAGGATTGAAAGTGTGCA3') and 20i-3 (5'CTATGAGAAAACTGCACTGGA3') for exon 20, and 2Ii-5 (5'AATGTTCACAAGGACTCCA3') and 2Ii-3 (5'CAAAGTAC-CTGTTGCTCCA3') for exon 21. The PCR was performed in a volume of 100 µl containing 500 to 1000 ng of genomic DNA, 2.5 units of Taq polymerase (Cetus), 0.02 µmol of each 2'-deoxynucleotide 5'-triphosphate (Pharmacia), and 2 µl of a 10 µM solution of the 5' and 3' primers for each exon in Taq polymerase buffer (50 mM potassium chloride; 10 mM TRIS, pH 8.3; 1.5 mM magnesium chloride; and 0.01 percent [wt/vol] gelatin). The genomic DNA was amplified by denaturation at 94°C for 6 minutes, followed by 30 cycles consisting of annealing at 58°C for 30 seconds, extension at 72°C for 1 minute, denaturation at 94°C for 30 seconds, and a final extension at 72°C for 10 minutes. Amplification of genomic DNA with primers 20i-5 and 20i-3 produces a fragment of 472 base pairs (bp), whereas primers 2Ii-5 and 2Ii-3 produce a fragment of 475 bp. The nucleotide sequence of each exon was determined by sequencing the PCR-amplified DNA with a third primer selected from an intron sequence 40 bp from the 5' splice site of each exon. Sequencing primers 20s-5 (5'TAT-TTATGGCATGGTACC3') for exon 20 and 21s-5 (5'TTAC-AATACAATAAGGGA3') for exon 21 were end-labeled with [32P]ATP and T4 kinase, as described elsewhere.[12] Direct sequencing of double-stranded DNA amplified by PCR was performed as previously described.[16,17]

Detection of Exon 11 Mutations

The previously described nonsense mutations, replacing a glycine at codon 542 (G542Stop) and an arginine at codon 553 (R553Stop) were detected in each family by direct sequencing of DNA amplified by PCR from exon 11 of the CFTR gene.[13,14] The presence of the G542Stop mutation was confirmed by allele-specific oligonucleotide hybridization as described elsewhere.[14] The R553Stop mutation causes the loss of a HincII site in exon 11.[13] The lack of digestion of PCR-amplified exon 11 DNA with HincII confirmed the presence of this mutation.

RESULTS

Nucleotide sequencing of exons 20 and 21 from the cystic fibrosis genes from 17 blacks and 25 whites

revealed two nonsense mutations. One mutation ($C_{3896} \rightarrow A$) changing serine to a stop at codon 1255 (S1255Stop) was present in exon 20 in a black patient (Patient 271) (Fig. 1, left panel). This mutation creates a HindIII recognition site in exon 20. Digestion of the 472-bp DNA fragment amplified from exon 20 with HindIII produced fragments of 214 and 258 bp when the $C_{3896} \rightarrow A$ mutation was present (Fig. 2A). The mutation was inherited from the patient's father (Fig. 2A) and was associated with the XV2c/KM19 DNA polymorphism haplotype D.[18] The discovery of a nonsense mutation in exon 20 in this patient was surprising, since he inherited another nonsense mutation, G542Stop, from his mother (Fig. 2A). Detailed description of the G542Stop mutation has been presented elsewhere.[14] This patient was an 11-year-old boy with serious pancreatic disease but only mild pulmonary involvement (Table 1). With the exception of minimal digital clubbing, his physical examination was unremarkable. He is fully active and has never been hospitalized for treatment of a pulmonary infection.

A second black patient (Patient 246) had a substitution of G for A at nucleotide 4079 in exon 21, replacing the tryptophan residue at codon 1316 with a termination signal (W1316Stop) (Fig. 1, right panel). This mutation appears to have been inherited from the patient's father, although he was not available for study. The presence of the mutation in the patient was confirmed by allele-specific oligonucleotide hybridization (Fig. 2B).[13] The patient also carried the exon 11 nonsense mutation R553Stop on her other cystic fibrosis chromosome, which was inherited from her mother (Fig. 2B). Like Patient 271, this 21-year-old woman had substantial pancreatic disease but mild pulmonary involvement (Table 1). She is fully active and has never been hospitalized for treatment of a

pulmonary infection. With the exception of minimal digital clubbing, her physical examination was unremarkable.

DISCUSSION

Rapid advances in our understanding of the basic defect in cystic fibrosis have occurred in the past five years. Altered chloride conduction in epithelial cells has been reported, and this abnormality is thought to be due to a failure of protein kinases to activate chloride channels in the apical membrane.[3,4,21,22] Furthermore, the gene responsible for cystic fibrosis was identified by a reverse genetic approach.[5,6] Although the role of the putative protein (CFTR) in chloride secretion is unknown, the identification of naturally occurring deleterious mutations and the characterization of the associated phenotypes have provided some insight into the function of CFTR.[13,23,24]

In this report we describe two patients who have nonsense mutations in each of their cystic fibrosis genes. Nonsense mutations in human genes have been associated with decreased levels of mutant messenger RNA.[25-28] The truncated protein produced by the nonsense mutation is difficult to isolate, presumably because of instability and rapid degradation.[29] This type of mutation is usually associated with more severe disease than that caused by missense mutations.[30,31] The presence of nonsense mutations in both cystic fibrosis genes might be expected to lead to a complete loss of the CFTR protein and the severest form of the disorder. Each patient had severe pancreatic disease, as evidenced by meconium ileus at birth in both patients and the development of diabetes mellitus in Patient 246. Each patient, however, had excellent nutritional status and mild pulmonary disease. Objective clinical scoring and laboratory testing
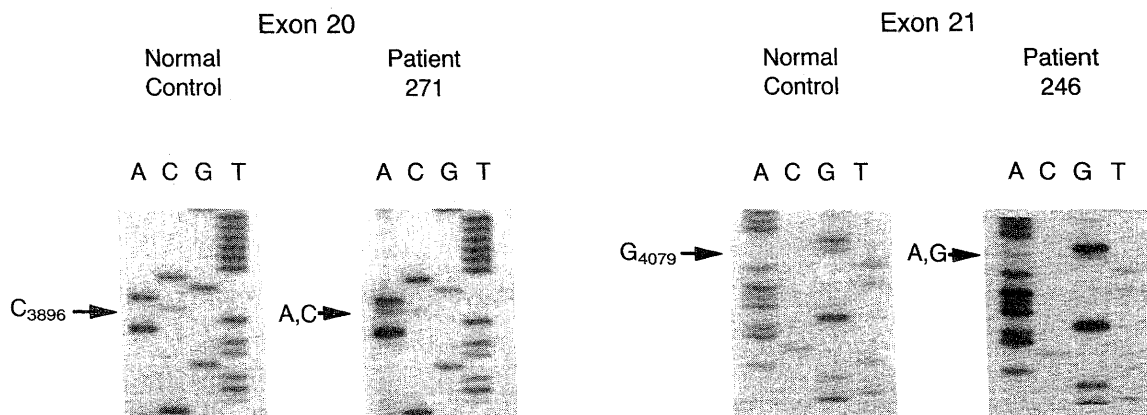

Figure 1. Nucleotide Sequencing of PCR-Amplified Genomic DNA from Exons 20 and 21 from Each CFTR Gene with Primers 20s-5 and 21s-5, Respectively.

The normal sequence extending from nucleotide positions 3887 to 3906 in exon 20 is shown beside the abnormal sequence in Patient 271. The normal sequence extending from nucleotide positions 4065 to 4083 in exon 21 is shown beside the abnormal sequence in Patient 246. Patient 271 had a C-to-A mutation at position 3896 in one of his cystic fibrosis genes, whereas Patient 246 had the substitution of A for G at position 4079 in one of her cystic fibrosis genes.
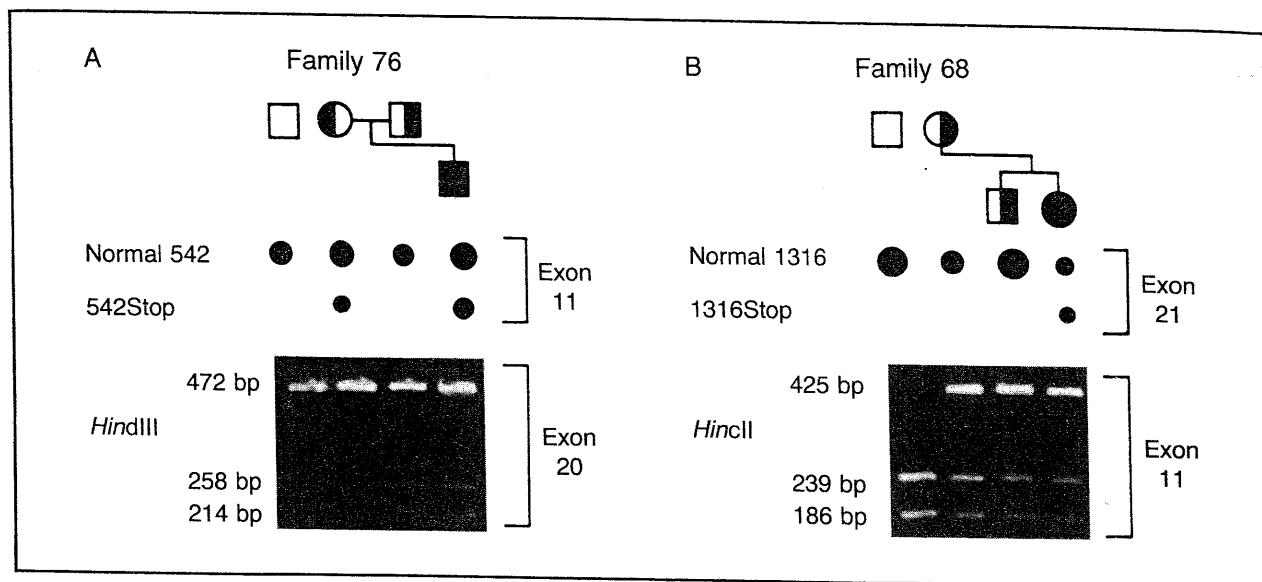
Figure 2. Detection of Nonsense Mutations in the Family (Family 76) of Patient 271 (Panel A) and Mutation Analysis of Patient 246 and Her Family (Family 68) (Panel B).

In Panel A, the patient's mother carries the exon 11 nonsense mutation G542Stop, as shown by hybridization of the 542Stop oligonucleotide to DNA amplified from exon 11. Digestion of PCR-amplified exon 20 DNA with *Hind*III revealed that the patient's father has the 214-bp and 258-bp fragments, indicating that he is heterozygous for the S1255Stop mutation.

In Panel B, the patient and her brother have inherited the exon 11 mutation R553Stop from their mother, as demonstrated by the presence of the 425-bp fragment after *Hinc*II digestion of exon 11 DNA. The father in this family was not available for study; however, the presence of the second nonsense mutation, W1316Stop, was confirmed by hybridization of the 1316Stop oligonucleotide to DNA amplified from exon 21. A normal male control (□) is shown in both panels.

confirmed the mildness of the lung involvement. The common cystic fibrosis mutation, ΔF_{508}, has been associated with pancreatic insufficiency and variable severity of pulmonary disease.[32] American black patients with cystic fibrosis who attend the Johns Hopkins Cystic Fibrosis Clinic and are homozygous for the ΔF_{508} mutation have clinical disease that is indistinguishable from that in white patients with the same genotype. Therefore, the strikingly similar phenotype observed in the two patients described here is probably the result of nonsense mutations in both cystic fibrosis genes of each patient and not due to differences in the genetic background of the black patients and white patients in North America.

Three hypotheses are presented to explain the unexpected mild pulmonary involvement in these patients. Alternative splicing of messenger RNA is a mechanism used by a cell to produce different forms of a protein (isoforms) from a single gene.[33-35] This process can occur in a tissue-specific manner.[36,37] The effect of a gene mutation can therefore be minimized if the mutation occurs in an exon that is not included in a mature transcript encoding a functional isoform. If a CFTR isoform lacking an amino acid sequence encoded by exon 11 or exons 20 and 21 is normally produced, then the patients described in this study could have some functional CFTR. Expression of this

CFTR isoform only in airway epithelia could explain the difference in the severity of pulmonary and pancreatic disease in these patients.

The nonsense mutations in exons 20 and 21 are

Table 1. Clinical and Laboratory Features of Patients with Cystic Fibrosis and Nonsense Mutations in Each Cystic Fibrosis Gene.

CHARACTERISTIC	PATIENT 271	PATIENT 246
Clinical features	Meconium ileus; several episodes of hyponatremic dehydration; mild pulmonary disease	Meconium ileus; diabetes mellitus; recurrent episodes of distal intestinal obstruction; mild pulmonary disease
Sweat chloride (mmol/liter)*	118, 111	103, 130
Height — cm (percentile)	140.3 (25–50)	154 (10)
Weight — kg (percentile)	32.2 (25–50)	53.9 (25)
Brasfield chest radiograph score†	23	23
Forced vital capacity — liters (% predicted)	1.82 (82)	2.43 (78)
Forced expiratory volume in 1 second — liters (% predicted)	1.48 (66)	2.09 (82)
Shwachman–Kulczycki clinical score‡	96	89

*Two measurements were made to confirm that the sweat chloride level was elevated.

†According to the scoring system of Brasfield et al.,[19] in which 25 is the best score.

‡According to the scoring system of Shwachman and Kulczycki,[20] in which 100 is the best score.

predicted to produce a protein missing only the second ATP-binding domain. This shortened protein would contain both transmembrane segments, the regulatory region, and the first ATP-binding domain. It is possible that this protein is not degraded and is integrated into the cell membrane. In this case, the truncated protein could be partially functional in certain tissues. Previous studies have shown that truncated receptors for low-density lipoprotein cholesterol are produced by genes with premature termination signals in the COOH-terminal cytoplasmic domain.[38] These proteins bind low-density lipoprotein cholesterol but cannot transport it into cells.

If the nonsense mutations in these patients produce unstable proteins that are rapidly degraded, then one can conclude that the complete CFTR protein is not essential for cell viability. The CFTR protein may be necessary for a highly specialized function only in epithelial cells. The mutation ΔF_{508} causes the omission of a single residue in the first ATP-binding domain. In cells homozygous for this mutation, the CFTR messenger RNA is present and is believed to produce an altered protein.[6] Since the majority of patients homozygous for the ΔF_{508} mutation have more severe pulmonary disease than the two patients described here,[32] the presence of an altered CFTR protein may be more deleterious to airway epithelial cells than its total absence. In osteogenesis imperfecta, the production of an abnormal pro–alpha collagen chain interferes with the triple-helix formation of Type I collagen, leading to extreme fragility of connective tissue.[39] Missense mutations allow an altered protein to be produced, causing severe disease (Type II), whereas nonsense mutations have a much milder phenotype (Type I).[39] In osteogenesis imperfecta, however, a nonsense mutation acts in an autosomal dominant fashion, whereas cystic fibrosis is an autosomal recessive disorder.

Studies with CFTR antibodies will determine the presence or absence of the CFTR protein on apical membranes of epithelial cells from these patients. These results coupled with detailed studies of chloride conductance should provide considerable insight into the function of the CFTR protein in different epithelial tissues and the pathophysiology of cystic fibrosis.

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CASE RECORDS

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CASE 50-1990

PRESENTATION OF CASE

A 65-year-old man was admitted to the hospital because of an obstructing nasopharyngeal mass and lymphadenopathy.

The patient was well until one month earlier, when a sore throat and dysphagia developed, and he became unable to breathe through his nose. He was seen by a physician, who prescribed penicillin, ciprofloxacin, and amoxicillin–clavulanate potassium in sequence, without improvement. The patient began to notice enlarging lumps in the submental region and in both groins, with thickening of the skin over his shoulders. One day before admission a computed tomographic (CT) scan of the head and neck, obtained elsewhere, showed a large nasopharyngeal mass and cervical lymphadenopathy (Fig. 1 and 2). Adrenocorticosteroid medication was administered by vein and by mouth, and he was admitted to another hospital.

The patient was a retired furnace worker of a glass factory. Previous surgical procedures included an operation for a ruptured appendix and a lumbar fusion. He had smoked two or three packs of cigarettes daily from the age of 13 years until one year before admis-
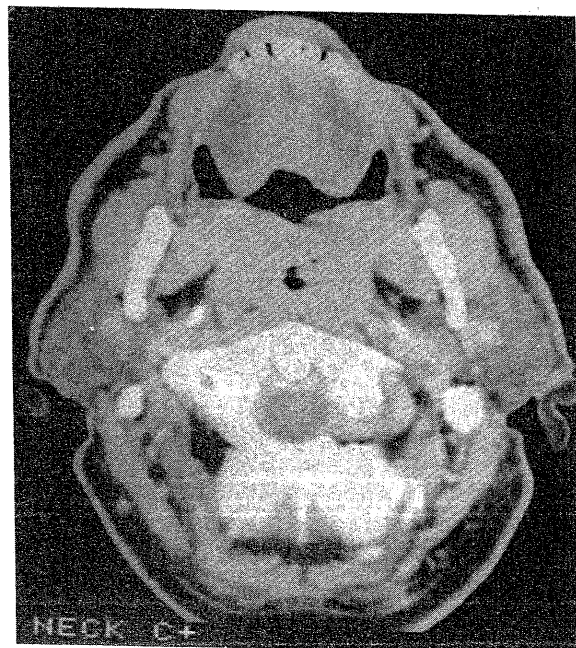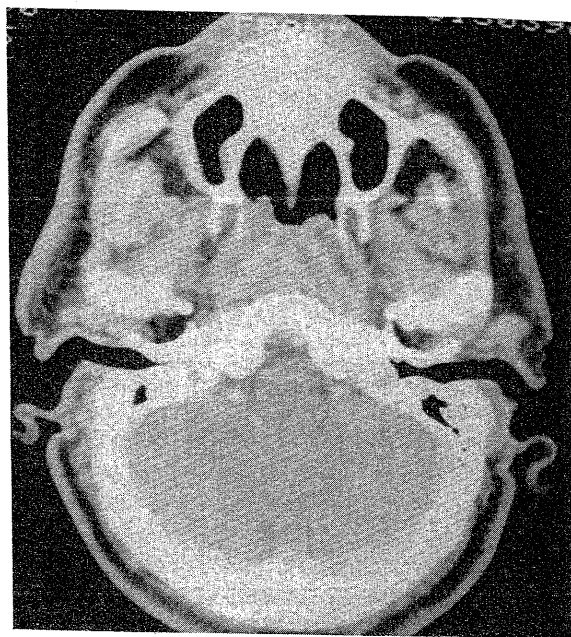

Figure 1. CT Scan of the Upper Neck, Showing Mass-like Thickening of the Nasopharynx (Top) and Tonsils (Bottom).