BRIEF REPORT

CYSTIC FIBROSIS GENE MUTATION IN TWO SISTERS WITH MILD DISEASE AND NORMAL SWEAT ELECTROLYTE LEVELS

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CYSTIC fibrosis is the most common fatal recessive disease in whites. In the past the diagnosis has been based on the presence of lung disease, pancreatic exocrine insufficiency, and increased sweat electrolyte concentrations.[1] Patients with cystic fibrosis have well-defined abnormalities in epithelial tissue, including defective cyclic AMP–mediated regulation of chloride channels and an increased transepithelial potential difference, which reflects increased sodium absorption and decreased chloride permeability.[2-6] The raised sweat chloride values that result from impaired reabsorption of salt in the sweat ducts have been exploited in the pilocarpine iontophoresis test, the cornerstone of diagnosis since 1959.[7] Although the vast majority of patients with cystic fibrosis have elevated sweat electrolyte concentrations, some patients have symptoms[8] and epithelial bioelectric properties[9] consistent with cystic fibrosis, but normal sweat electrolyte concentrations. Until recently, it was impossible to determine whether these patients had a mild form of cystic fibrosis or a genetically distinct disease.

The cloning of the gene responsible for cystic fibrosis, termed the cystic fibrosis transmembrane conductance regulator (CFTR),[10,11] now permits molecular analysis of affected patients. The genetic evidence implicating the CFTR gene in cystic fibrosis was confirmed with the successful correction of the chloride-channel defect in pancreatic[12] and airway[13] cells by gene transfer. Analysis of the nucleotide sequence of the CFTR gene predicts a membrane-associated protein of 1480 amino acids whose structure is similar to that of the family of ATP-binding transport proteins.[14] The protein structure consists of two transmembrane domains, each followed by a nucleotide-binding fold. Unlike other members of this family, CFTR has an additional hydrophilic domain that may have a regulatory function. Whether CFTR transports chloride directly,[15,16] transports a substance that influences chloride transport,[17] or does both remains to be determined.

The severity of cystic fibrosis can be correlated to some extent with genotype.[18] The most common mutation responsible for the disease is the deletion of a single amino acid in a highly conserved region of the first nucleotide-binding fold, ΔF_{508}.[19] This mutation accounts for approximately 70 percent of cystic fibrosis chromosomes, and patients homozygous for the ΔF_{508} mutation have severe disease, including pancreatic insufficiency.[18,19] The identification of new mutations and studies correlating them with the severity of cystic fibrosis should provide insight into the structure and function of CFTR. We describe two siblings with mild pulmonary disease and normal sweat electrolyte concentrations who were found to be homozygous for the substitution of a serine for glycine at amino acid 551 in CFTR exon 11. The identification of a mutation in the CFTR gene in these patients suggests that the heterogeneity in phenotype may be broader than previously thought and that mutational analysis of the CFTR gene may be necessary in some clinical situations for accurate diagnosis.

CASE REPORT

The propositus was a 50-year-old white woman who was evaluated for a chronic cough and production of purulent sputum. She had been hospitalized several times for pneumonia beginning at the age of 3 years, and after the age of 12 she received oral antibiotic treatment for pneumonia once or twice a year. At the age of 33, she had recurrent hemoptysis. Bronchoscopy revealed purulent secretions, and a bronchogram demonstrated blunting of the distal bronchi in the lingula and right middle lobe. No bacterial pathogens were isolated from sputum or bronchial specimens. Tests of pulmonary function revealed mild obstruction of air flow (ratio of forced expiratory volume to forced vital capacity, 78 percent). An increase in daily sputum production prompted additional evaluation at the age of 37, at which time the patient had a moderate reduction in air flow at lower lung volumes and normal 48-hour excretion of fat in stools. Her sweat sodium concentrations were 61, 69, 69, and 69 mmol per liter (borderline normal levels) on four consecutive measurements. Fiberoptic bronchoscopy revealed purulent secretions; no bacterial pathogen was isolated from cultures of the secretions.

The patient maintained a stable weight and exercise tolerance with postural drainage and intermittent oral antibiotic therapy. She had two normal pregnancies and deliveries and raised these children while working as a truck inspector. Suppurative sinus disease developed when the patient was in her 40s, *Pseudomonas aeruginosa* (mucoid) was isolated occasionally from her sputum cultures, and she required hospitalization once or twice a year for parenteral antibiotic therapy. By 1990, at the age of 49, she had had a decline in her sense of well-being and in exercise tolerance

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that was associated with an increase in sputum production, up to 50 ml per day.

During an evaluation at North Carolina Memorial Hospital in 1991, she had posterior bibasilar rales, a few rhonchi, and mild end-expiratory wheezes. A chest radiograph revealed diffuse interstitial infiltrates with bibasilar predominance. Pulmonary-function tests revealed moderate obstructive impairment (forced vital capacity, 2.06 liters, 73 percent of the predicted value; forced expiratory volume, 1.29 liters, 54 percent of the predicted value; and mean midexpiratory flow, 0.73 liter, 27 percent of the predicted value). Sputum cultures revealed mucoid *P. aeruginosa* and *Xanthomonas maltophilia*. Consecutive sweat chloride measurements by pilocarpine iontophoresis[7] were normal (49, 53, 51, and 54 mmol per liter).[20] A series of studies were conducted to characterize the patient's nasal and sweat-gland epithelial function.

The patient's parents were second cousins of French and English origin. The patient had nonidentical twin sisters a year younger, one of whom had died of respiratory failure at the age of 48. This sister delivered four healthy children without difficulty, had no evidence of malabsorption, and was in good health until the age of 23, when she had an episode of hemoptysis. At that time she was reported to have clubbing and bronchiectasis on chest roentgenography. At the age of 36 she was evaluated for recurrent lung infections and hemoptysis. The results of several sweat tests performed at that time were normal; the sweat sodium concentration ranged from 32 to 37 mmol per liter. Sputum cultures yielded only hemophilus species. Cystic fibrosis was suspected but could not be diagnosed with certainty because of these unusual features and normal sweat tests.

Methods

Subjects

The experimental protocol was approved by the committees for the protection of human subjects of the University of Michigan and the University of North Carolina, and informed consent was obtained. DNA was obtained from the leukocytes of the patient, her family (including the affected sister before her death), and normal subjects. Samples of DNA from patients with cystic fibrosis and their parents were obtained from the Hospital for Sick Children in Toronto.[19]

Physiologic Studies

The nasal transepithelial potential difference and response to the perfusion of drugs and chloride-free solutions (replacement with gluconate) were measured by an established technique.[4,5] The degree of inhibition induced by 10^{-4} M amiloride, a sodium-channel blocker, was calculated as the percent change from base line. The chloride-diffusion potential difference (an index of chloride permeability) is the change in voltage that occurs during nasal perfusion with a chloride-free solution of amiloride. The change in the potential difference after the addition of 10^{-5} M isoproterenol to the perfusate is correlated with the increase in chloride permeability in response to the beta-agonist; a negative change indicates hyperpolarization.

The degree of chloride secretion by sweat acini induced by cyclic AMP was determined by measuring the rate of sweat formation after the intradermal injection of a mixture of isoproterenol, aminophylline, and atropine.[21,22] Samples were collected under oil, and the volume was measured in constant-bore calibration pipettes. Eleven patients with cystic fibrosis did not sweat in response to this injection.

Sweat chloride concentrations were measured by pilocarpine iontophoresis.[7] The sweat-duct potential difference was measured with a glass electrode filled with agar containing saturated potassium chloride and referenced to a subcutaneous electrode.[23,24] Values for each subject were calculated as the mean concentration in five to nine glands.

Detection of Mutation by Chemical Mismatch Cleavage

Exon 11 of the CFTR gene was amplified from genomic DNA by the polymerase chain reaction (PCR),[25] according to standard methods.[26] Primers for the PCR were selected from the flanking intron sequence and were modified by the addition of a *Bam*HI restriction-enzyme site, as follows: 5′ATACGGATCCCAACTGTGGTTAAAGCAATAGTGT3′ and 5′ATACGGATCCGCACAGATTCTGAGTAACCATAAT3′. Reactions were subjected to 35 cycles of denaturing at 94°C for 1 minute, annealing at 55°C for 1 minute, and polymerase extension at 72°C for 2½ minutes. PCR products were precipitated with ethanol and resuspended in 10 μl of water. For probe generation, wild-type PCR product was purified from a nondenaturing polyacrylamide gel by electroelution and ethanol precipitation. End-labeling of the wild-type fragment was performed as described,[27] and labeled DNA was resuspended in a final volume of 20 μl.

Wild-type–patient heteroduplexes and wild-type–wild-type control homoduplexes were formed as previously described,[28] with the following modifications. The total annealing volume was 30 μl. The samples were boiled for five minutes and then allowed to cool to room temperature for two hours. Heteroduplexes were redissolved in 12 μl of water. The reagents and volumes used for chemical modification and cleavage reactions were those described by Cotton et al.,[29] with the following changes. Osmium tetroxide and hydroxylamine reactions were performed for each heteroduplex at 37°C for 30 minutes with 5 μl of redissolved heteroduplex mixture. After treatment with piperidine, DNA was vacuum-dried and resuspended in 5 μl of loading buffer (95 percent formamide, 20 mmol of EDTA per liter, 0.05 percent bromophenol blue, and 0.05 percent xylene cyanol FF). The fragments were analyzed by electrophoresis in a 6 percent denaturing polyacrylamide gel and subsequent autoradiography.

Nucleotide Sequencing of CFTR Exon 11

Exon 11 was amplified from DNA of the patient by PCR as described above. The PCR products were purified on a 1 percent agarose, 1.5 percent NuSieve gel, digested with *Bam*HI, and subcloned in M13mp18 DNA. Single-stranded DNA was isolated and sequenced with the dideoxy-chain–termination method.[30,31] Four independent clones were sequenced.

Allele-Specific Oligonucleotide Screening

A total of 20 μl of amplified DNA was denatured by the addition of an equal amount of 0.5 mol of sodium hydroxide per liter and 1.5 mol of sodium chloride per liter for 15 minutes. The DNA was then blotted onto a nylon membrane. After blotting, the membrane was neutralized for 15 minutes in 0.5 mol of TRIS per liter and 1.5 mol of sodium chloride per liter. Oligonucleotides (50 ng) representing the normal (5′GAGTGGAGGTCAACG3′) and mutant (5′GAGTGGAAGTCAACG3′) sequences were end-labeled with kinase in a 20-μl reaction containing 70 mmol of TRIS per liter, 10 mmol of magnesium chloride, 5 mmol of dithiothreitol per liter, 10 mCi of [γ^{32}P]ATP, and 10 U of polynucleotide kinase at 37°C for 30 minutes. The labeled oligonucleotides were hybridized to the blot in a buffer of 40 mmol of sodium phosphate per liter (pH 7), 1 mmol of EDTA per liter, and 7 percent sodium dodecyl sulfate at 42°C for 12 to 24 hours.[32] The filters were washed for 15 minutes in a solution of 40 mmol of sodium phosphate per liter, 1 mmol of EDTA per liter, and 3 percent sodium dodecyl sulfate, for 30 minutes in the same solution except that it contained 1 percent sodium dodecyl sulfate, and for 15 minutes in the same solution except that it contained 0.5 percent sodium dodecyl sulfate. All washes were done at 42°C. After washing, the blots were exposed to film with intensifying screens for two to four hours at −70°C.

Results

The results of physiologic studies in the propositus are summarized in Table 1. Her nasal bioelectric properties were characteristic of cystic fibrosis: the transepithelial potential difference was elevated, there was marked inhibition of the potential difference with superfusion of 10^{-4} M amiloride, and the chloride-diffusion potential difference, which is an index of chloride permeability, was low. The increase in chloride diffusion in response to superfusion with 10^{-5} M isoproterenol was less than normal, albeit greater than

in most patients with cystic fibrosis. The absence of a sweat response to intradermal injection of isoproterenol is consistent with the cyclic AMP–mediated defect in chloride secretion in the sweat acinar cells of patients with classic cystic fibrosis.

In contrast to the cystic fibrosis–like epithelial dysfunction found in the nasal and sweat acinar epithelium of the propositus, the sweat chloride values were normal. This reflects normal ductal reabsorption of sodium chloride and implies normal chloride permeability. The sweat-duct potential difference was also normal, consistent with normal chloride permeability of the sweat ducts.

DNA from the propositus was studied by the technique of chemical mismatch cleavage[30] to identify a mutation in her CFTR gene. This technique detects mismatches between patient and wild-type DNA by chemical modification of mispaired nucleotides in heteroduplexes generated between the patient's DNA and radiolabeled wild-type DNA. The modified base is then cleaved with piperidine, and the abnormal cleavage product is detected by acrylamide-gel electrophoresis. When wild-type–patient heteroduplex DNA from CFTR exon 11 was chemically modified with hydroxylamine and cleaved with piperidine, a 241-base-pair (bp) cleavage product was observed (Fig. 1A). The band was not seen in wild-type–wild-type homoduplex DNA. This result indicated the presence of a mutation in exon 11.

To confirm the presence of a mutation, DNA from the propositus was cloned and the sequence was determined. The sequence demonstrated a nucleotide change of G to A at base pair 1783 (Fig. 1B). This change results in the substitution of a serine for a glycine residue at the highly conserved position of amino acid 551 (G551S). This mutation was found in all four clones from the patient. The presence of this missense mutation in all the clones sequenced suggested that the patient might be homozygous for this mutation. To confirm this, DNA from the patient's family was amplified and screened with allele-specific oligonucleotides corresponding to the mutant and normal sequences (Fig. 2). The propositus (V-1) and her affected sister (V-3) both had two copies of the G551S mutation, whereas an unaffected sister (V-2) was a

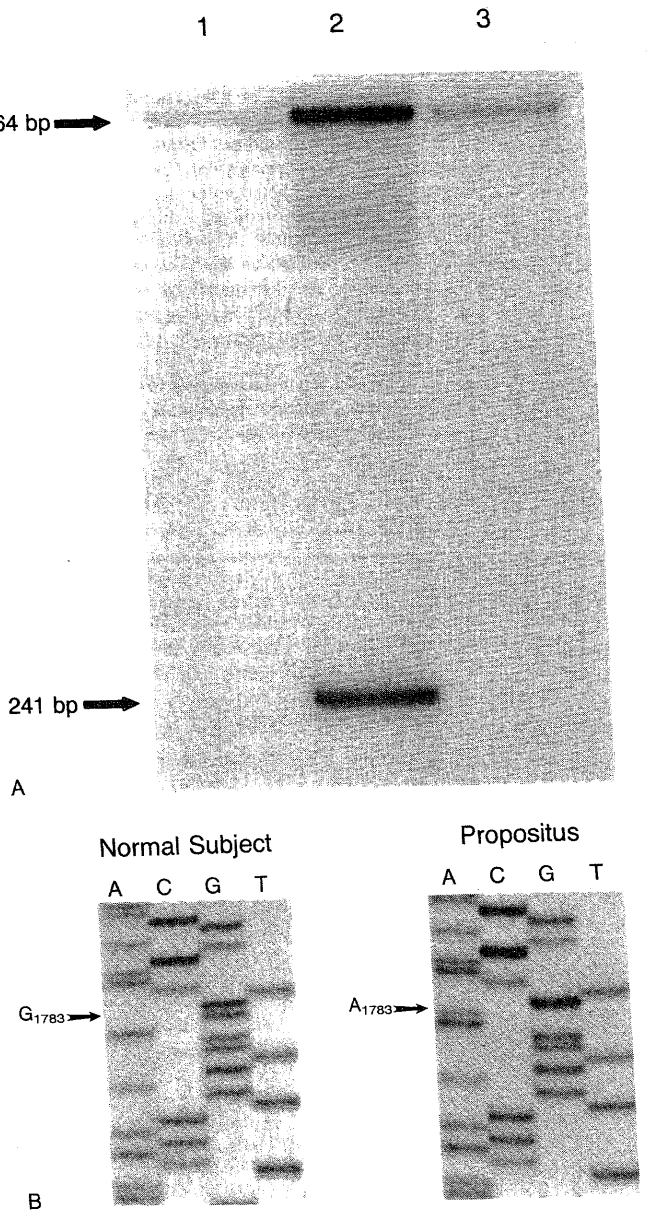

Figure 1. Detection of the G551S Mutation by Chemical Mismatch Cleavage (Panel A) and DNA Sequence Analysis (Panel B).
In Panel A, lanes 1 through 3, heteroduplexes between radiolabeled wild-type probe and amplified DNA from three patients with cystic fibrosis were modified with hydroxylamine and cleaved by piperidine. Lanes 1 and 3 show DNA from a patient with cystic fibrosis with no mutations in exon 11, and lane 2 shows DNA from the propositus. Because the cleavage was partial, the 464-bp heteroduplex is present in all lanes. Panel B shows the nucleotide sequence of a portion of exon 11 from a normal subject and the propositus. The G-to-A transition at base pair 1783 in the propositus is shown by the arrow.

Table 1. Results of Physiologic Studies in the Propositus as Compared with Data on Normal Subjects and Patients with Cystic Fibrosis.*

VARIABLE	PROPOSITUS	NORMAL SUBJECTS	PATIENTS WITH CYSTIC FIBROSIS
Nasal bioelectric measure			
Mean potential difference (mV)	−49.6	−10 to −40	−40 to −60
Maximal potential difference (mV)	−77	−10 to −50	−50 to −80
Amiloride inhibition (%)	83	<70	>70
Chloride-diffusion potential difference (mV)	−5.5	−15 to −40	0 to −12
Isoproterenol augmentation (mV)	−9.5	−11 to −28	0 to −6
Sweat acinar			
Sweat response to isoproterenol (nl/min/gland)	0.0	0.2 to 3.0	0.0
Sweat duct			
Sweat chloride (mmol/liter)	49,53,51,54†	<60	>70
Sweat-duct potential difference (mV)‡	−21	−10 to −45	−45 to −80

*The ranges of values for normal subjects and patients with cystic fibrosis are from previous[4,5,21-24] publications, and are shown only for the purpose of general comparison.

†Serial measurements were made.

‡ The sweat-duct potential difference in 9 normal subjects ranged from −16.2 to −39.4 mV (mean, −27.8), and in 10 patients with cystic fibrosis it ranged from −46.3 to −80.0 mV (mean, −62.9).

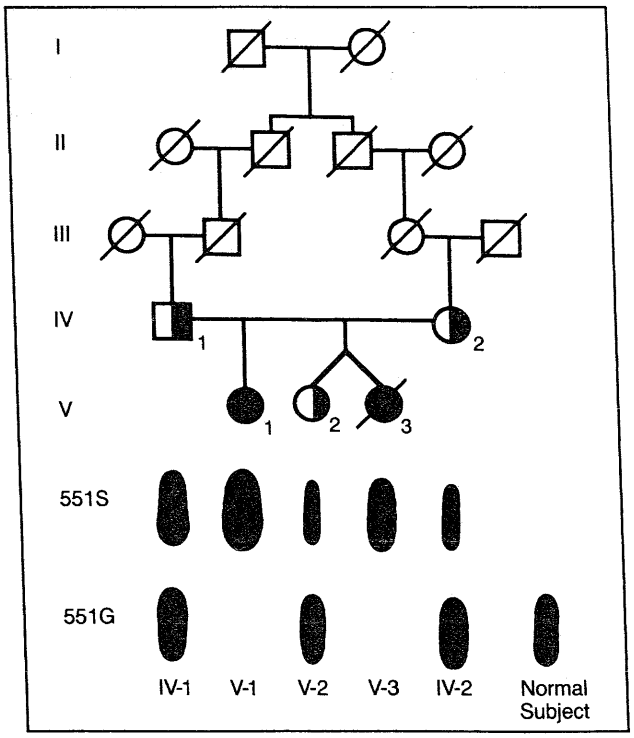

Figure 2. Detection of the G551S Mutation by Allele-Specific Oligonucleotide Screening.

Allele-specific oligonucleotide screening of the family demonstrated that the propositus, V-1, and her affected sibling, V-3, were homozygous for the G551S mutation. The nonidentical twin sister of V-3 was found to be a carrier for this mutation. Both parents of V-1 and V-3 had the G551S mutation. Squares denote male family members, circles female family members, slashes deceased members, half-solid symbols carriers, and solid symbols affected members. The 551S row shows below the symbol for each person his or her DNA hybridized to the radiolabeled oligonucleotide representing the mutant sequence. The 551G row shows each person's DNA hybridized to a radiolabeled oligonucleotide representing the normal sequence.

carrier for this mutation. Both parents were heterozygous for the G551S change. Analysis of the family history revealed that the parents were second cousins. The G551S mutation was not detected in 727 ΔF_{508} chromosomes, in 363 non-ΔF_{508} cystic fibrosis chromosomes, or in 63 normal chromosomes screened with allele-specific oligonucleotides.

Discussion

The range of clinical manifestations in patients with cystic fibrosis is broad. Pancreatic exocrine insufficiency is characteristic of the disease, but approximately 15 percent of patients do not require enzyme supplements for normal digestion.[33] Occasional patients with mild lung disease or only some of the usual findings of cystic fibrosis have been described.[8,9,34] For patients such as these, analysis of the CFTR gene at the DNA level may provide information crucial for a correct diagnosis.

In this report, we describe two sisters with mild disease and normal sweat tests who were found to be homozygous for a missense mutation in the first nucleotide-binding fold of CFTR. These patients had many findings indicative of cystic fibrosis, including a lifelong history of productive cough, obstructive lung disease, bronchiectasis, and in the case of one sister, *P. aeruginosa* in sputum cultures. However, their disease course was unusually mild, with pancreatic sufficiency, unusual longevity, a late decline in lung function, and multiple pregnancies. Despite a demonstrated mutation in their CFTR gene, these siblings did not have elevated sweat electrolyte concentrations. Physiologic studies confirmed that the epithelial function of the sweat ducts was normal. By contrast, the propositus had abnormal nasal and sweat acinar epithelial function characteristic of cystic fibrosis. It is possible that the interaction of the CFTR protein with tissue-specific proteins accounts for the apparent variability in the physiologic effects of this mutation in different tissues. Alternatively, the mutant CFTR protein encoded by the patient's gene may have residual function that is adequate for effective ion transport in the pancreas and sweat duct but inadequate for normal function in the airway and sweat acinar epithelium.

DNA analysis of the CFTR gene in these siblings demonstrated the substitution of a serine for a glycine residue at amino acid 551. This residue is located in the first nucleotide-binding fold, a highly conserved region in the family of transport proteins of which CFTR is a member; and this glycine is particularly well conserved. Several other mutations have been described in this region,[35,36] including the substitution of aspartic acid for the glycine at the same position.[35] The highly conserved nature of this glycine residue, the correlation of mutations in the first nucleotide-binding fold of CFTR with disease, and the finding that this substitution was not detected on any of the normal chromosomes tested indicate that this substitution represents a disease-causing mutation. In addition, functional studies in a homologous bacterial transport protein are consistent with the hypothesis that this mutation is deleterious to the function of CFTR. The creation of this mutation in the homologous amino acid residue in the bacterial transport protein LivG resulted in reduced transport of the protein's substrate.[37]

The cloning of the gene responsible for cystic fibrosis now permits the association of phenotype with specific mutations in the gene and provides insight into the normal function of CFTR. The homozygous nature of the mutation in these patients provides information about the phenotype of this mutation without the complicating factor of a different mutation on the other chromosome. In addition to the siblings described here, we have studied two siblings who were homozygous for the aspartic acid substitution at amino acid 551 (G551D) described above (unpublished data). In contrast to the patients who were homozygous for the G551S mutation, these siblings had classic

cystic fibrosis, including high sweat sodium values (110 and 113 mmol per liter), severe lung disease, and pancreatic insufficiency. Since a change from glycine to aspartic acid results in an alteration in charge, whereas both glycine and serine are uncharged, the phenotypes of these patients suggest that the relatively conservative nature of the G551S mutation may produce a milder phenotype. However, since these mutations have not been detected in the homozygous state in a large number of patients, the full spectrum of the phenotype and the importance of additional contributing genetic and environmental factors remain to be determined.

Because of the mild disease and normal sweat electrolyte concentrations in the patients described here, the diagnosis of cystic fibrosis might not have been considered so strongly had these patients not been sisters with consanguineous parents. On the basis of DNA analysis of the CFTR gene, the disease in these patients can now be diagnosed with certainty. Knowledge of the normal sequence of this gene now permits confirmation of the diagnosis at the molecular level when clinical data are inconclusive. The case reported here indicates that these new criteria may result in a broadening of the definition of cystic fibrosis to include patients with mild disease that has previously defied precise diagnosis.

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