Genotype/Phenotype Association in Cystic Fibrosis: Analyses of the $\Delta F508$, R553X, and 3905insT Mutations

SABINA LIECHTI-GALLATI, IRENE BONSALE, NASEEM MALIK, VERENA SCHNEIDER,
LILLIANE G. KRAEMER, ANNA RUEDEBERG, HANS MOSER, AND RICHARD KRAEMER

Medical Genetics Unit [S.L.G., V.S., H.M.] and Paediatric Respiratory Research Group [I.B., L.G.K., A.R., R.K.],
Department of Paediatrics, Inselspital, University of Berne, 3010 Berne, Switzerland; and Department of Genetics
[N.M.], University Children's Hospital, 4058 Basel, Switzerland

ABSTRACT. A striking clinical phenomenon of cystic fibrosis is the heterogeneous disease expression. It must therefore be assumed that the nature of the mutations associated with cystic fibrosis might partly determine the phenotypic manifestations. The relation between the cystic fibrosis mutations $\Delta F508$, R553X, and 3905insT and clinical parameters such as sweat test electrolytes, age at chronic *Pseudomonas aeruginosa* colonization, Crispin-Norman x-ray scores, and relative underweight have been investigated in 45 patients homozygous for $\Delta F508$ ($\Delta F2$), in 12 compound heterozygotes for $\Delta F508$ /R553X ($\Delta F1$ /RX1), in three R553X homozygotes (RX2), and in 13 patients compound heterozygous for $\Delta F508$ /3905insT ($\Delta F16$). We have found significant differences between the genetically defined subgroups concerning the mean age at onset and the cumulative incidence of chronic *P. aeruginosa* colonization and Crispin-Norman x-ray scores. The significant results as well as some trends regarding the relative underweight demonstrate a milder clinical course in R553X heterozygotes and more severe disease in the $\Delta F16$ group compared to $\Delta F508$ homozygotes. The three patients homozygous for R553X presented with a two-stage course showing mild progression before *P. aeruginosa* infection and as severe a course as the $\Delta F16$ patients after *P. aeruginosa* colonization at the age of 12 y. The findings presented here indicate that specific mutations can influence the severity and progression of the disease, implicating the importance of mutation and haplotype analyses. However, wide variations within the genetically homogeneous subgroups illustrate that other determinants of the clinical status do exist. (*Pediatr Res* 32: 175–178, 1992)

Abbreviations

CF, cystic fibrosis

CFTR, cystic fibrosis transmembrane conductance regulator

NBF, nuclear binding fold

of exocrine gland epithelia leading to abnormal secretions that cause obstruction of organ ducts (1). CF is clinically characterized by chronic airway infection and bronchiectasis, pancreatic insufficiency with malabsorption and impaired growth, abnormalities of the intestine (meconium ileus) and the reproductive organs (azoospermia, low female fertility), and elevated sweat chloride concentrations (2). The disease process is generally slow, but incessant, so that the median survival of patients receiving optimum treatment is 25–30 y (3). Although the majority of patients follow a classic clinical course, the manifestations and severity of CF vary considerably, and the genetic basis for the clinical heterogeneity is still unknown.

A series of reports published in 1985 demonstrated mapping of the CF gene to the long arm of chromosome 7 (4–6). The region containing the gene was defined by cloning CpG-rich islands of DNA, as well as by chromosome jumping and walking (7, 8). In 1989, the gene was finally cloned spanning about 250 kb of genomic DNA. It encodes a protein of 1480 amino acids called the CFTR. The functional regions of the CFTR protein are two NBF, a regulatory domain containing many possible phosphorylation sites, and two hydrophobic regions that interact with cell membranes. The specific role of CFTR in Cl transport remains unrevealed. However, recent data show that expression of intact CFTR, but not of a mutant form, corrects the CF defect, demonstrating a causal relationship between mutations in the CFTR gene and defective Cl transport and enabling further investigations on the function of the protein (9, 10). The most common mutation has been found to be $\Delta F508$, a 3-bp deletion in exon 10, which results in the loss of a phenylalanine residue at position 508 in the putative first NBF (11–13). More than a hundred mutations have been detected on CF chromosomes so far. Some of them that have recently been published demonstrate association with milder disease phenotypes (14–19). We previously reported on DNA marker haplotypes of restriction fragment length polymorphisms flanking the CF gene as well as on $\Delta F508$ and R553X frequencies in CF families of Switzerland (20, 21). Therefore, we present here the mutation and haplotype relation to some clinical manifestations of our CF patients to provide further support to a greater understanding of the nature of the mutations associated with CF that might influence the phenotypic expression of the disease. Clinical parameters such as onset of chronic *Pseudomonas aeruginosa* infection, lung involvement evaluated by chest x-ray scores, weight, and sweat electrolyte levels were examined.

PATIENTS AND METHODS

Clinical data and blood were collected from 73 CF patients, 36 females (mean age 14.4 y; range = 3.4–28.0 y) and 37 males (mean age 16.9 y; range = 2.6–30.0 y), attending regularly the

CF is the most common severe autosomal recessive genetic disorder in Caucasian populations, with an incidence of about 1 in 2000 live births, implying a carrier frequency of about 1 in 22. The fundamental defect in CF appears to be a dysfunction

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Correspondence: Prof. Dr. R. Kraemer, Pediatric Pulmonary Division, Department of Pediatrics (Inselspital), CH-3010 Berne, Switzerland.

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CF center in Berne. All patients are native Swiss from Central, West, North, and East Switzerland, mostly of Celtic and Germanic origin.

The diagnosis had been previously confirmed in each patient by typical clinical symptoms and/or family history of CF together with at least two abnormal sweat tests measuring Na as well as Cl concentrations. Pulmonary involvement was biannually established by chest x-rays evaluated by the Chrispin and Norman score (22). Onset of chronic *P. aeruginosa* infection was defined as continuous culture of the bacteria from sputum for 6 mo. All patients required pancreatic enzyme supplementation since the date of diagnosis. To evaluate the effect of or the resistance to the enzyme treatment, the patients' weight and height were determined twice a year and the relative underweight was calculated (23).

DNA extraction from whole blood, restriction enzyme digestion, Southern blotting, and hybridization using oligo-labeled probes were performed as described before (20).

Restriction fragment length polymorphisms were determined for the XV2c/*TaqI*, KM19/*PstI*, MP6d9/*MspI*, and J3.11/*MspI* probes, and haplotypes were constructed using the CF patients' genotypings to establish phase. The larger allele detected by each probe was denoted as 1, the smaller as 2. The haplotypes for XV2c-KM19-MP6d9-J3.11 are 1 = 1221, 2 = 1222, 3 = 1122, 4 = 1112, 5 = 2112, 6 = 2111, 7 = 2221, and 8 = 2222.

The $\Delta F508$ mutation defined by a three-base deletion resulting in the loss of phenylalanine residue 508 in exon 10 was identified by amplification of genomic DNA by polymerase chain reaction followed by vertical electrophoresis in a 12% polyacrylamide gel and ethidium bromide staining (21). The presence of R553X and 3905insT was tested by amplification of exons 11 and 20 followed either by digestion of the amplification product by the restriction enzyme *HincII* or by sequencing of the single-stranded polymerase chain reaction product.

Tables 1 and 2 present the course of Chrispin-Norman x-ray

Table 1. Chrispin-Norman scores of CF patients with different genotypes

Age (y)	$\Delta F2$	RX2	$\Delta F1/RX1$	$\Delta F16$
1				
Mean	3.78	3.50	3.50	7.00
SD	± 2.94	± 0.71	± 1.00	± 3.16
n	18	2	3	7
Range	0-11	3-4	3-5	2-10
4				
Mean	6.04	6.00	6.20	8.37
SD	± 3.81	± 0.00	± 2.77	± 3.16
n	26	2	5	8
Range	0-13	6-6	3-10	5-12
8				
Mean	11.82	6.50	7.14	11.20
SD	± 5.25	± 0.71	± 4.52	± 1.79
n	27	2	5	5
Range	3-28	6-7	3-14	9-13
12				
Mean	14.28	17.00	9.50	17.75
SD	± 5.32	± 2.83	± 4.93	± 5.44
n	18	2	6	4
Range	8-29	15-19	2-15	12-25
16				
Mean	15.47	22.00	13.86	17.25
SD	± 4.02	± 2.83	± 3.34	± 5.12
n	15	2	7	4
Range	8-23	20-24	10-18	12-24
20				
Mean	15.80	18.50	12.80	18.75
SD	± 3.36	± 3.54	± 6.18	± 1.50
n	10	2	5	4
Range	10-21	16-21	4-21	17-20

Table 2. Relative underweight of CF patients with different genotypes

Age (y)	$\Delta F2$	RX2	$\Delta F1/RX1$	$\Delta F16$
1				
Mean	2.22		0.00	6.62
SD	± 4.51		± 0.00	± 7.87
n	18		4	8
Range	0-16		0-0	0-20
4				
Mean	5.68	0.00	2.60	6.44
SD	± 5.94	± 0.00	± 4.34	± 8.46
n	37	3	5	9
Range	0-20	0-0	0-10	0-19
8				
Mean	8.53		7.20	14.86
SD	± 7.67		± 7.26	± 7.40
n	32		5	7
Range	0-24		0-15	0-22
12				
Mean	12.43	13.67	11.25	24.00
SD	± 8.02	± 9.24	± 6.70	± 9.27
n	21	3	4	5
Range	0-26	3-19	5-18	9-33
16				
Mean	11.87	20.75	16.50	17.25
SD	± 9.36	± 3.95	± 13.10	± 13.20
n	16	3	4	4
Range	0-30	16-24	0-32	0-29
20				
Mean	6.80	28.67	14.00	18.50
SD	± 9.98	± 6.11	± 14.14	± 10.61
n	5	3	2	2
Range	0-23	22-34	4-24	11-26

scores and relative underweight during a period of 20 y. Mean values and SD are calculated from all available data evaluated at the age of 1, 4, 8, 12, 16, and 20 y.

The two-tailed Mann-Whitney U test was used to compare continuous data, allowing for nonnormal distributions and small subgroup sizes. The Spearman rank correlation coefficient was defined for correlation analysis. Regression was calculated for simple linear and polynomial regression. The cumulative incidence of chronic *P. aeruginosa* infection was calculated according to Pedersen *et al.* (24).

RESULTS

DNA from 73 patients was analyzed, involving 146 chromosomes. Forty-five patients were found to be homozygous for the $\Delta F508$ deletion ($\Delta F2$). The new detected 3905insT mutation of exon 20 (manuscript in preparation) and the R553X mutation have turned out to be the second and third most common mutations in Switzerland, showing strong linkage to haplotype 6 and 3, respectively. Three patients presenting with genotype 33 were homozygous for R553X (RX2), 12 demonstrated heterozygosity for $\Delta F508$ and R553X ($\Delta F1/RX1$), and 13 patients were compound heterozygous for $\Delta F508$ and 3905insT related to haplotype 6 ($\Delta F16$).

The following data and figures present the relation between parameters of the clinical course and the specific CF mutations $\Delta F508$, R553X, and 3905insT. The number of patients with available data are given in parentheses.

The sweat test mean values of the $\Delta F2$ ($n = 26$), $\Delta F1/RX1$ ($n = 7$), and $\Delta F16$ ($n = 7$) groups do not significantly differ from each other for Na or for Cl concentrations, but the $\Delta F16$ group shows the highest mean values for Na (93.3 mmol/L) and Cl (123.6 mmol/L) concentrations when compared with $\Delta F1/RX1$ (Na 82.83 mmol/L; Cl 116.67 mmol/L) and $\Delta F2$ (Na 86.81 mmol/L; Cl 114.50 mmol/L).

The chronic *P. aeruginosa* colonization starts at the age of 6.7 y (range = 0.2–17.4 y; $n = 35$; $p < 0.02$) in CF patients with two $\Delta F508$ deletions. $\Delta F508$ heterozygotes demonstrate dependency on their second mutation: Chronic *P. aeruginosa* infection is detected at the mean age of 7.0 y (range = 2.3–10.8 y; $n = 6$) and 4.3 y (range = 0.4–15.7; $n = 10$) in $\Delta F1/RX1$ and $\Delta F16$ patients, respectively, whereas the mean age of patients without *P. aeruginosa* is 14 y (range = 8.2–25.5; $n = 5$) in the $\Delta F1/RX1$ group and 3.4 y (range = 3.3–3.5; $n = 2$; $p = 0.05$) in $\Delta F16$ patients. Homozygosity for R553X means later onset of infection (12.0 y; range 6.2–17.3 y; $n = 3$) than homozygosity for $\Delta F508$ (6.7 y; range 0.2–17.4 y; $n = 35$).

The cumulative incidence of *P. aeruginosa* infection is higher in the $\Delta F2$ group than in $\Delta F1/RX1$ patients (Fig. 1A). In the $\Delta F16$ group, the prevalence of infection is higher than in $\Delta F2$

patients until the age of 9 y; afterwards it is about the same in both groups (Fig. 1B). The difference is highest between $\Delta F1/RX1$ and $\Delta F16$ patients in all age groups, with much lower percentages (0–50%) demonstrated in the $\Delta F1/RX1$ group than in patients presenting with haplotype 6 (18–100%) (Fig. 1C).

There was a significant positive correlation between Chrispin-Norman score and age in all patient groups ($\Delta F2$, $r = 0.672$; $RX2$, $r = 0.786$; $\Delta F1/RX1$, $r = 0.657$; $\Delta F16$, $r = 0.786$; $p < 0.001$). Regression analysis demonstrated nonlinear regressions that did not obviously differ between the genetic subgroups. However, the mean values of x-ray scores present some differences and trends depending on the genetic background (Table 1): The $\Delta F16$ patients started at a higher score ($p = 0.03$) than all the other patients. The $\Delta F1/RX1$ group presented with lower values than the $\Delta F508$ homozygotes at the ages of 8 ($p = 0.04$) and 12 y (NS), reaching nearly the same scores as the $\Delta F2$ group at the ages of 16 and 20 y (Table 1). Patients with the 3905insT mutation showed higher scores (NS) than $\Delta F2$ patients in all age groups except at the age of 8 y, when they were the same in both patient groups (Table 1). Comparison between the compound heterozygotes ($\Delta F1/RX1$ and $\Delta F16$) demonstrates higher scores in $\Delta F16$ patients in all age groups, with the difference reaching significance at the age of 12 y ($p = 0.03$) (Table 1). The three $RX2$ patients seemed to be in good condition up to the age of 8 y, but showed impressive progress of lung disease from the age of 12 y, presenting with higher scores (16 y $p = 0.04$) than did $\Delta F2$ patients (Table 1).

Underweight did not express significant differences between the genetically defined subgroups because of very large standard deviations. However, the tendencies are the same as for x-ray scores: $\Delta F16$ patients are the most underweight, and R553X patients develop mild underweight during the 1st decade, but thereafter dramatically increase the underweight up to 28.7% at the age of 20 y (Table 2).

Sex differences have been tested for each of the four clinical parameters and no statistical significance has been found.

DISCUSSION

Progression in lung disease and gastrointestinal complications are determinants of the course in CF patients that leads to death in adolescence or early adulthood in the majority of these patients. CF presents a high degree of clinical heterogeneity, which may be caused, in part, by different mutations. Therefore, clinical characterization of each combination of compound heterozygotes as well as of homozygotes for any mutation is of great importance.

In this study, the heterogeneity of the CF phenotype was assessed in quantitative terms such as the age of chronic *P. aeruginosa* infections, x-ray scores, relative underweight, and sweat test values to allow statistical testing. The sweat test provides the most reliable basis for diagnosing CF, whose characteristics are elevated Na and Cl concentrations due to defective ion absorption. No significant differences were detected in the electrolyte concentrations between the genetically defined subgroups (25). Our data demonstrate that patients carrying two R553X mutations present with the latest onset of *P. aeruginosa* infection of all genetic subgroups and that $\Delta F508/R553X$ compound heterozygotes show significantly later onset than do $\Delta F508$ homozygotes (Fig. 1A). Moreover, the 3905insT mutation, localized in the second NBF of the CFTR and causing the termination of the translation six codons downstream, presents with an extremely early onset (Fig. 1B and C), high Chrispin-Norman scores (Table 1) already at the age of 1 y, and the greatest relative underweight (Table 2) and results in a more severe clinical form than all the other mutations. Although the role of *P. aeruginosa* colonization in the pathogenesis of lung disease in CF is still not clear (26), we found some relationship between the age at *P. aeruginosa* onset and a significant increase of x-ray scores in $\Delta F508$ as well as R553X homozygous patients (Table 1). These

Fig. 1. Cumulative percentage of CF patients with chronic *P. aeruginosa* infection vs age at onset. A, $\Delta F508$ homozygotes ($\Delta F2$; ■) and compound heterozygotes for $\Delta F508$ and R553X ($\Delta F1/RX1$; ●); B, $\Delta F508$ homozygotes ($\Delta F2$; ■) and compound heterozygotes for $\Delta F508$ and 3905insT ($\Delta F16$; ▲); and C, compound heterozygotes for $\Delta F508$ and R553X ($\Delta F1/RX1$; ●) and compound heterozygotes for $\Delta F508$ and 3905insT ($\Delta F16$; ▲).

results give some evidence for more rapid progression of lung disease after *P. aeruginosa* infection in homozygous patients.

Despite pancreatic supplementation, intestinal absorption can only be partly corrected in most of the CF patients. As initially shown (23), relative underweight (Table 2) is in accordance with the x-ray score (Table 1), confirming the experience of Neijens *et al.* (2) that lung involvement is often linked with a decrease in body weight.

We conclude from our findings that the 3905insT mutation associated with haplotype 6 predicts a more severe clinical course than the $\Delta F508$ deletion. The hypothesis that the presence of an altered CFTR protein may be more deleterious than its total absence (15, 27) leads to the speculative suggestion that the combination of one $\Delta F508$ deletion and one 3905insT mutation will probably result in two mutant CFTR proteins trapped in the endoplasmic reticulum and therefore acting at incorrect cellular locations (27). The nonsense mutation R553X seems to decrease the influence of the $\Delta F508$ deletion in $\Delta F508/R553X$ heterozygotes, causing a milder course and slowed progression (Fig. 1A, Tables 1 and 2). The two compound heterozygous groups $\Delta F1/RX1$ and $\Delta F16$ indicate dominant effects of both the R553X (Fig. 1B, Table 1) and 3905insT (Fig. 1C, Table 1) mutations compared with $\Delta F508$. Several patients with both CF chromosomes carrying nonsense mutations were reported to have mild lung disease based on the argument that truncation or total absence of CFTR might be associated with nearly normal lung function (15, 28). In their 1st decade, the three patients homozygous for R553X confirm this hypothesis. However, once affected by chronic *P. aeruginosa* infection, they develop rapid progression of lung disease, illustrating either that lack of the gene product may suddenly turn out to be deleterious or that homozygosity, as a consequence of doubling the same defect, may result in a more severe course than compound heterozygosity after *P. aeruginosa* infection.

The wide variations in each patient group, even in the $\Delta F508$ homozygotes, indicates that the severity and progression of the disease may also be determined by environmental and other genetic factors within and/or outside the CFTR gene (25, 29). Therefore, caution is advisable in interpreting mutation analysis and in predicting the prognosis of individual patients.

On the other hand, the finding of only a few significant differences between the genetically defined subgroups may be due to the small number of patients with identical genotypes. To prove any further correlations between genotype and phenotype, we require larger patient populations—a need that is hindered by low frequencies of the remaining mutations and their markedly different geographical and/or ethnic distributions.

Although the work presented here does not permit many definitive conclusions, it is considered to be a valuable pilot study awaiting larger studies on a worldwide scale.

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