A Missense Cystic Fibrosis Transmembrane Conductance Regulator Mutation With Variable Phenotype

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ABSTRACT. Objective. Cystic fibrosis (CF) has variable clinical presentation. Disease severity is partially associated with the type of mutation. The aim of this study was to report genotype-phenotype analysis of the G85E mutation.

Patients. The phenotype of 12 patients (8 were from the same extended family, and 5 of them were siblings from 2 families) carrying at least one copy of the G85E mutation was evaluated and compared with the phenotype of 40 patients carrying the two severe mutations, W1282X and/or ΔF508 (group 1), and with 20 patients carrying the splicing mutation, 3849+10kb C>T, which was found to be associated with milder disease (group 2).

Results. A high phenotypic variability was found among the patients carrying the G85E mutation. This variability was found among patients carrying the same genotype and among siblings. All the studied chromosomes carrying the G85E mutation had the 7T variant in the polythymidine tract at the branch/acceptor site in intron 8. Of the G85E patients, 25% had pancreatic sufficiency and none had meconium ileus, compared with 0% and 32%, respectively, of patients from group 1, and 80% and 0%, respectively, from group 2. Two patients carrying the G85E mutation had sweat chloride levels <60 mmol/L whereas all the others had typically elevated levels >80 mmol/L. Compared with group 2, patients carrying the G85E mutation were diagnosed at an earlier age and had higher sweat chloride levels, with mean values similar to group 1 but significantly more variable. Forced expiratory volume in 1 second (FEV1) was similar values similar to group 1 but significantly more variable.

Conclusions. The G85E mutation shows variable clinical presentation in all clinical parameters. This variability could be seen among patients carrying the same chromosome the same CFTR mutation, and also among siblings. This variability is not associated with the level of exon 9 skipping. Thus, the G85E mutation cannot be classified either as a severe or as a mild mutation.

Cystic fibrosis (CF), the most common lethal autosomal recessive disease among whites, is caused by defects in the CF transmembrane conductance regulator (CFTR) gene, which encodes a chloride channel regulated by cyclic adenosine monophosphate protein. Defects in the CFTR cause abnormal chloride concentration across the apical membrane of epithelial cells in the airways, pancreas, intestine, sweat gland and duct, and in the male genital system. It therefore results in progressive lung disease, pancreatic and intestinal dysfunction, elevated sweat electrolytes, and male infertility. CF is characterized by a wide variability of clinical expression. The cloning of the CFTR gene and the identification of mutations in the gene, has promoted extensive research into the association between genotype and phenotype, which has contributed to our understanding the mechanisms of the remarkable clinical heterogeneity of CF. Previous studies analyzed the genotype-phenotype correlation in several mutations for which a large enough number of patients was available. These studies have shown that genetic factors influence the severity of the disease, and that there are two groups of mutations. One group is associated with pancreatic insufficiency (PI) (>95% of cases) and a young age at diagnosis (usually <1 year of age), high sweat chloride levels (>80 meq/L), and meconium ileus (20% to 30% of the cases). The other group of mutations is associated with a high rate of pancreatic insufficiency (PS) (70% to 80%), and a later age at diagnosis (usually >10 years of age), lower sweat chloride levels, and no meconium ileus. Severity of lung disease varies considerably among both groups of phenotypes. Pancreatic status was suggested to be the best parameter in differentiating between the two groups.
ing from a substitution of glutamic acid for glycine at amino acid 85. The result is a relatively major change replacing a polar amino acid with a negatively-charged one within the first membrane spanning domain of CFTR. It might be expected to have a significant effect on the protein. Two patients, 1 with PI and the other with PS and exceptionally mild lung disease, were previously reported.18,19 Thus, for further determination of the correlation between the G85E mutation and disease phenotype a larger cohort of patients was required. We undertook this study to analyze the genotype-phenotype correlation of this mutation among our CF patient population.

MATERIALS AND METHODS

DNA Sequence Determination and Mutation Analysis

DNA sequences spanning individual exons of the CF gene were amplified by polymerase chain reaction (PCR)20,21 with oligonucleotide primers located in the respective flanking introns of the CF gene.22 The amplified genomic DNA fragments eluted from 5% polyacrylamide gels were extracted with phenol/chloroform and were subjected to the dideoxy-chain termination sequencing method essentially as described,21 using the 5' Biochemicals Sequencing Kit (Bethesda, MD) with either one of the 5' primer or internal oligonucleotides as sequencing primers. After the identification of a specific mutation in an individual, the entire studied CF population was detected for this mutation using previously described methods.24

Detection of the Polyipyrimidine Tract Length Variants at the Acceptor/Branch Site of Exon 9

The genomic region flanking the polythymidine tract was amplified by PCR using the primers 9-i-5 and 9-i-3 using previously described methodology.25 Nested PCR was subsequently performed with primers TT-i-5 (5'TCTGCTGTGCTGTCGTTT 3) and TT-i-3 (5'CCTGCTCTTCTTTATCTTG 3). The PCR conditions were: 94°C 6' followed by 35 cycles of 94°C 30', 54°C 30', 74°C 40', and 74°C 6'. The PCR products were visualized on 12% nondenaturing polyacrylamide gel (sequencing format) that were electrophoresed at room temperature, at 600 V, for 18 hours and subsequently silver-stained. Assignment of the splice variant alleles was performed by analysis of available family members.

RNA Extraction and Single-strand cDNA Synthesis

Nasal epithelial cells were scraped from 3 individuals. The scraped cells were suspended in 300 μL RNAzol B buffer (BIOTEC Laboratories, Inc, Houston, TX). Total RNA was extracted from the scraped nasal cells using the acid-phenol-chloroform method according to the manufacturer’s instructions. RNA was solubilized in 10 μL diethylpyrocarbonate-treated RNase-free double-distilled water. RNA was extracted from the polyp biopsies of the control individuals by the guanidinium thiocyanate method. The RNA was purified by centrifugation through a CsCl cushion.26 cDNA was synthesized using 2.5 μM random hexamer mix (Pharmacia Fine Chemicals, Piscataway, NJ), 5 mM MgCl2, 1 mM dNTP mix (Pharmacia), 2.5 units of Moloney murine leukaemia virus reverse transcriptase (RT) (BRL, Gaithersburg, MD), and 4 units of RNase inhibitor (Boehringer, Indianapolis, IN). The tubes were incubated at room temperature for 10 minutes, at 42°C for 20 minutes, at 99°C for 5 minutes, and at 4°C for 5 minutes. Each cDNA synthesis experiment included a control sample in which all reagents except RNA were present.

Nondifferential PCRs of cDNA Products

Nondifferential RT-PCR reactions in which the normally spliced transcripts, containing exon 9, and the aberrantly spliced transcripts, lacking exon 9, produce products of the same size were designed. Amplification of the region between exon 3 and the junction of either exons 8/9 or 8/10 using the oligonucleotide primers 3Ri5 5' GGATAGAGAGCTGGCTTCAAAG AAA 3', 8/9Ri5 5' AAATAATTCCCCAAATCCCTCCTCC 3', and 8/10Ri5 5' CATCATTAGAAGTGAAGTCTCCTCC 3', respectively.

The cDNA samples were heated at 94°C for 3 minutes and then subjected to 35 cycles of denaturation at 94°C for 60 seconds, primer annealing for 30 seconds at 55°C for the RT-PCR 1 and 4, 60°C for the RT-PCR 2, and 51°C for the RT-PCR 3. In RT-PCR 1, 2, and 3, the 4 extension was performed at 65°C for 60 seconds, in RT-PCR 2 at 60°C for 120 seconds, followed by a final extension of 7 minutes at 65°C. RNA-less samples were used as controls. Semi-quantitative PCR conditions, which reflect the initial relative amounts of the normal and aberrantly spliced transcripts, were established by serial dilutions (1:3) of the cDNA products before the nondifferential PCR. Only RT-PCR products in the linear phase were analyzed.

Hybridization to RT-PCR Products

50 μL of each differential RT-PCR reaction were subjected to electrophoresis and were subsequently blotted and hybridized to the exon 3 oligonucleotide G85E-N 5'GTTCTATGGAATTCTT 3' that identified the normal sequence, washed at 42°C, and to G85E-M 5'GTTCTATGGAATTCTT 3' that identified the G85E mutation, washed at 40°C. The intensity of the RT-PCR products was measured by phosphorimager.

Assessment of Disease Severity

The clinical phenotype was assessed by the following parameters: age at diagnosis and at assessment, sex, mode of presentation, history of meconium ileus, diagnostic sweat chloride levels, most recent sputum cultures, and pancreatic function as determined by a 3-day fecal fat collection. Patients in whom fecal fat loss exceeded 7% of dietary intake were considered to have PI. The patients who died before the study did not have stool fat analysis but presented with severe malabsorption responding to enzyme supplementation. Pulmonary function was assessed in all CF patients >6 years of age. Forced expiratory volume in 1 second (FEV1) was measured and expressed as a percentage of predicted values for height and sex, using previously described standardized pulmonary equations.27 Current height and weight percentiles were computed using the tables of Tanner.28 Data was collected throughout 10 years and the most recent values were considered.

To assess the severity of disease presentation of the patients carrying the G85E mutation, we compared their clinical parameters with those of two groups of patients. The first was comprised of 40 patients homozygous or compound heterozygous for the W1282X and ΔF508 mutations, both associated with severe disease presentation.29 In the second group were 20 patients carrying the 3849+10kb C→T mutation, which is associated with higher frequency of PS and a milder phenotype.30

Statistical Analysis

Mean values of continuous variables were compared using analysis of variance and Student’s t test. The F statistic was used to assess the assumption of equal variances in comparison groups. x2 analysis was used to compare proportions and frequency distributions. In which expected values were small, Fisher’s exact test was used to compare proportions.

RESULTS

Twelve CF patients were found to carry the G85E mutation, 9 were Arab, 8 from the same extended family (Table 1, patients 1 to 4 and 6 to 9), and 3 were Jews from Turkish origin (patients 10 to 12). Six patients were homozygous for the G85E mutation and 6 were compound heterozygote for the G85E and the ΔF508, W1282X or the 3849+10kb C→T mutations. All the studied chromosomes carrying the G85E mutation had the 7T variant in the polythymidine tract at the branch/acceptor site in intron 8.

The clinical parameters of the patients carrying the G85E mutation are presented in Table 1. It shows a high prevalence in the age at diagnosis among patients carrying the G85E. Although 7 patients were symptomatic and diagnosed before 1 year of age, 3
were diagnosed after 10 years of age with atypical presentation. One patient (No. 8) was diagnosed in a carrier screening performed in his school. Likewise, sweat chloride levels varied among the patients; 2 had sweat chloride levels <60 meq/L, and diagnosis of CF was confirmed only after genotype analysis. There were two families in our study. One had 3 children homozygous for the G85E mutation (Table 1, patients 1 to 3). The first 2 presented before 1 year of age with typical CF disease, had severe course, and died at an early age. Their subsequent brother presented with liver disease at age 12 with no signs of pancreatic or respiratory involvement, and with sweat chloride levels of <60 mmol/L. The diagnosis of CF was made by genotyping only. The second family had 2 children compound heterozygous for G85E and ΔF508 mutations (Table 1, patients 7 and 8). The first child was diagnosed at an early age with typical severe course and died at an early age, whereas his subsequent brother was diagnosed at age 10 during CF screening. After the diagnosis he was further assessed and was found to have PS, elevated sweat chloride levels, minimal bronchiectasis, and normal pulmonary function.

Comparison of the patients carrying the G85E mutation with patients carrying the ΔF508 and/or W1282X mutations previously associated with PI and the more severe disease (group 1) or with patients carrying the 3849+10kb C→T previously associated with higher frequency of PS and a milder disease (group 2), revealed that the mean age at diagnosis and age at assessment in the group of patients carrying the G85E mutation were not significantly different from those in group 1, but significantly lower than in group 2 (P = .001 for age of diagnosis, P = .04 for age at assessment; Table 2). Likewise, mean sweat chloride levels in patients carrying the G85E mutation were similar to group 1, and significantly different from those in group 2 (P = .001; Table 2). Thus, it seems that patients carrying the G85E mutation have similarly severe disease as patients carrying the ΔF508 and/or W1282X mutations. However, the F test of equal variance was significantly different between these groups: the range of age at diagnosis and sweat chloride levels were much broader for the G85E group than for group 1 (P < .0001), and sweat chloride was more variable in the G85E mutation than in group 2 (P = .002).

**TABLE 1.** Clinical Data of CF Patients Carrying the G85E Mutation

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Genotype</th>
<th>Sex</th>
<th>Age at Diagnosis</th>
<th>Mode of Presentation</th>
<th>Current Age</th>
<th>Pancreatic Status</th>
<th>FEV₁, % Predicted</th>
<th>Weight Percentile</th>
<th>Sputum Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>G85E/G85E</td>
<td>F</td>
<td>9 mo</td>
<td>Respir + GI</td>
<td>Died 13 y</td>
<td>PI</td>
<td>NA</td>
<td>&lt;3 Klebsiela</td>
<td></td>
</tr>
<tr>
<td>2*</td>
<td>G85E/G85E</td>
<td>M</td>
<td>2 mo</td>
<td>Respir</td>
<td>Died 10 y</td>
<td>PI</td>
<td>NA</td>
<td>&lt;3 H influenzae</td>
<td></td>
</tr>
<tr>
<td>3*</td>
<td>G85E/G85E</td>
<td>M</td>
<td>12 y</td>
<td>Liver</td>
<td>13 y</td>
<td>PS</td>
<td>82</td>
<td>&lt;3 H influenzae</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>G85E/G85E</td>
<td>F</td>
<td>6 mo</td>
<td>Respir + GI</td>
<td>Died 6 y</td>
<td>PI</td>
<td>20</td>
<td>&lt;3 P aeruginosa</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>G85E/G85E</td>
<td>F</td>
<td>2 mo</td>
<td>Respir + GI</td>
<td>4 mo</td>
<td>PI</td>
<td>NA</td>
<td>&lt;3 H influenzae</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>G85E/G85E</td>
<td>F</td>
<td>3 mo</td>
<td>Respir + GI</td>
<td>6 yr</td>
<td>PS</td>
<td>86</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>7†</td>
<td>G85E/ΔF508</td>
<td>M</td>
<td>6 mo</td>
<td>GI</td>
<td>Died 11 y</td>
<td>PI</td>
<td>NA</td>
<td>3 NA</td>
<td></td>
</tr>
<tr>
<td>8†</td>
<td>G85E/ΔF508</td>
<td>M</td>
<td>10 y</td>
<td>Screening</td>
<td>12 y</td>
<td>PS</td>
<td>83</td>
<td>80 Staph</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>G85E/ΔF508</td>
<td>M</td>
<td>5 mo</td>
<td>Respir + GI</td>
<td>18 y</td>
<td>PI</td>
<td>50</td>
<td>&lt;3 P aeruginosa</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>G85E/W1282X</td>
<td>F</td>
<td>7 mo</td>
<td>Respir</td>
<td>20 y</td>
<td>PI</td>
<td>65</td>
<td>50 P aeruginosa</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>G85E/W1282X</td>
<td>F</td>
<td>19 y</td>
<td>Pancreatits + Respir</td>
<td>34 y</td>
<td>PI</td>
<td>22</td>
<td>50 P aeruginosa</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>G85E/3849 + 10KB</td>
<td>M</td>
<td>5 mo</td>
<td>54</td>
<td>Respir</td>
<td>10 y</td>
<td>PI</td>
<td>65</td>
<td>97 P aeruginosa</td>
</tr>
</tbody>
</table>

* and † Patients are siblings, respectively.

Abbreviations: F, female; M, male; Respir, respiratory symptoms; GI, steatorrhea and/or failure to thrive; PI, pancreatic insufficiency; PS, pancreatic sufficiency; NA, not available.

**TABLE 2.** Comparison of Clinical Data Between CF Patients Carrying the G85E Mutations and Patients Homozygous or Compound Heterozygous for Two Severe Mutations (W1282X and ΔF508) and Those Carry a Milder Mutation (3849+10kb C→T)

<table>
<thead>
<tr>
<th>Variable</th>
<th>G85E</th>
<th>W1282X/W1282X</th>
<th>ΔF508/ΔF508</th>
<th>3849 + 10kb C→T</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>40</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis*</td>
<td>3.7 ± 6.3</td>
<td>0.7 ± 1.8]</td>
<td>13.2 ± 7.8</td>
<td></td>
</tr>
<tr>
<td>Sweat chloride (mmol/L)*</td>
<td>119 ± 38</td>
<td>111 ± 12]</td>
<td>71 ± 18[¶]</td>
<td></td>
</tr>
<tr>
<td>Died, n</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pancreatic sufficiency, %§</td>
<td>25</td>
<td>0</td>
<td>85</td>
<td>79</td>
</tr>
<tr>
<td>Meconium ileus, %§</td>
<td>12.8 ± 8.5</td>
<td>10.8 ± 7.0</td>
<td>19.8 ± 8.8</td>
<td></td>
</tr>
<tr>
<td>Current age, y†</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Weight (percentile)‡</td>
<td>28 ± 34</td>
<td>24 ± 24</td>
<td>50 ± 34</td>
<td></td>
</tr>
<tr>
<td>Forced expiratory volume in 1 second (% predicted)</td>
<td>55 ± 26 (n = 7)</td>
<td>66 ± 28 (n = 24)</td>
<td>55 ± 21 (n = 19)</td>
<td></td>
</tr>
</tbody>
</table>

* P < .0001, † P < .001, ‡ P < .01 analysis of variance for three means.
§ P < .002 χ² test of three populations.
¶ P < .001 F test of equal variance compared with G85E.
‡ P < .01 F test of equal variance compared with G85E.
Of the patients carrying the G85E mutation, 25% had PS significantly higher than in group 1 (0%), and significantly lower than in group 2 (80%) \( (P < .001; \text{Table 2}) \). Furthermore, similar to the patients in group 2, none of the patients carrying the G85E mutation had meconium ileus, whereas a third of the patients from group 1 had meconium ileus \( (P = .002) \). The nutritional status of patients carrying the G85E mutation was poor. As a group, they had similar mean weight percentile to group 1. However, 58% of the patients carrying the G85E mutation were under the third percentile of weight, compared with 25% of the group 1 patients \( (P = .02) \), and 20% of group 2 \( (P < .05) \).

As shown in Table 2, FEV\textsubscript{1} was similar in the three groups. Because pulmonary function is age-dependent, and ages in the three groups are different we performed analysis of covariance for FEV\textsubscript{1}. There were no differences in the slope or in age-adjusted mean values of FEV\textsubscript{1} in the three groups. However, the scatter of the values was very wide with typical spread of the points (Fig 1), similar to that seen in other studies\textsuperscript{2,10,15} and very large numbers would therefore be necessary to reveal possible group differences.

Levels of Correctly Spliced CFTR RNA Transcribed From the G85E Allele in Respiratory Epithelium

We next studied the possibility that the variability in pulmonary function found among our patients carrying the G85E mutation is associated with the level of exon 9 skipping that leads to the translation of a nonfunctional CFTR protein. The level of aberrantly spliced CFTR RNA transcribed in respiratory epithelial cells from the G85E allele was analyzed using semiquantitative nondifferential RT-PCR. The RT-PCR was performed on respiratory epithelial cells from 3 patients who carried at least one G85E allele (patients 3, 8, and 9). The reaction was repeated three times for each patient. The results showed that the level of transcripts lacking exon 9 transcribed from the G85E allele was 55 ± 3% for patient 3, 49 ± 2% for patient 8, and 35 ± 4% for patient 9. Their FEV\textsubscript{1} was 82, 83, and 50% predicted, respectively. The patient with the lowest FEV\textsubscript{1} had the lowest level of RNA transcripts lacking exon 9. Thus, the level of aberrantly spliced transcripts could not explain the variable pulmonary function among patients carrying the G85E mutation.

DISCUSSION

This study demonstrates high variability of clinical presentation among patients carrying the G85E mutation. Thus, the G85E mutation cannot be classified either as a severe or as a mild mutation. This variability was found also among patients carrying the same CFTR mutations on the other chromosomes, and among siblings. Therefore, no other CFTR mutation is expected. Several genotype-phenotype studies including the CF genotype-phenotype consortium have shown that there are mutations like the ΔF508\textsuperscript{2,6,11}, W1282X\textsuperscript{3,6}, G542X\textsuperscript{6}, N1303K\textsuperscript{4,6}, and R533X\textsuperscript{5,6} in which >95% of the patients had PI 2–10 whereas others like the 3849+10kb C→T\textsuperscript{7,8} and A455E\textsuperscript{15}
R117H in which >65% of the patients were PS. The incidence of PS among the patients carrying the G85E mutation (25%), suggests that it cannot be classified either as a PI- or PS-associated mutation. Slightly elevated, borderline, or even normal values of sweat chloride were previously reported among patients carrying at least one copy of the splicing mutation 3849+10kb C>T.7,8,15 Two of the patients in our study, carrying the G85E mutation, had borderline sweat chloride levels, whereas the others had typically high levels. Furthermore, as shown in Table 2, it seems that the G85E patients present to medical attention at a later age than the patients in the severe group. However, again this is attributable to the wide range of the age of diagnosis among the G85E patients, and not attributable to a milder disease course. As shown in Table 1, the patients were diagnosed either typically under 1 year of age, or at a significantly later age. Thus, the results of our analysis show that the G85E mutation is associated with variable disease presentation in all the studied parameters. Most of the patients have typically a severe course, although approximately 25% of the patients have an atypical course. Interestingly, pulmonary disease is similar in all types of mutations, thus the atypical course is related mainly to the gastrointestinal involvement.

The mechanism causing the variability seen among the patients carrying the G85E mutation is unknown. So far, such high variability among patients with the same genotype was found in lung disease only. Sequence variations within the CFTR gene that modulate the CF phenotype have been reported in 2 CF patients.29,30 However, in our study 8 patients were from the same extended family, carrying the same G85E allele, as evidenced by extended haplotype analysis using polymorphic markers in the CFTR locus (data not shown). These patients show the high variability of disease expression. Splicing variance of thymidine at the branch/acceptor site of intron 8, known as a polyT tract, varies among the patients carrying the G85E mutation (data not shown). Chu et al31 showed that the 5T, 7T, and 9T alleles, respectively. Chu et al31 showed that the 5T allele is associated with the low-normal levels of normal splicing of exon 9, and high levels of exon 9 skipping leading to a nonfunctional channel. Furthermore, the 5T allele was shown to affect the disease severity of patients carrying the mutation R117H.33 In patients carrying the R117H mutation when the allele also contained the 5T variant the phenotype was mild CF, whereas in patients carrying R117H together with the 7T variant the phenotype was male infertility only. In our study all the G85E chromosomes contained the 7T variant. In addition, the level of RNA transcripts lacking exon 9, transcribed from the G85E allele, varied among different individuals. However, the severity of the pulmonary disease could not be explained by the variable level of aberrantly spliced transcripts. Thus, the high phenotypic variability found among the patients in our study is not a result of another mutation in the CFTR locus, or attributable to different levels of thymidine at the branch/acceptor site of intron 8, and not associated with exon 9 skipping. Another cause for this variability might be different levels of the CFTR protein regulated by genes associated with the translation machinery. In addition, other genes coding for alternative ion channels or channel regulators, might modify the CF disease severity as was recently found in studies in knockout CF mice.34 Thus, other genetic and/or environmental factors modulate the severity of the disease expression. Further understanding the mechanisms underlying the disease variability found among patients carrying the G85E mutation will contribute to our understanding of the genotype-phenotype association and disease variability in CF.

REFERENCES

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