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 high 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 in the three groups, with no differences in the slope or in age-adjusted mean values of FEV1. The levels of transcripts lacking exon 9 transcribed from the G85E allele measured in 3 patients were 55%, 49%, and 35% and their FEV1 values were 82%, 83%, and 50% predicted, respectively.

Conclusions. The G85E mutation shows variable clinical presentation in all clinical parameters. This variability could be seen among patients carrying the other 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.
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.\textsuperscript{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**

Detection of the Polypyrimidine 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 9i-5 and 9i-3 using previously described methodology.\textsuperscript{22} Nested PCR was subsequently performed with outer primers TT-5 (5’CTGGCTGTTGCTGTTTCTT3’) and TT-3 (5’CTGGCTCTTCTATCTTGTTT3’). The PCR conditions were: 94°C 60”, 54°C 30”, 72°C 1 minute followed by 35 cycles of 94°C 30”, 54°C 30”, 72°C 1 minute. 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 \( \mu L \) 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 \( \mu L \) diethylpyrocarbonate-treated RNase-free double-distilled water. RNA was extracted from the polyp biopsies of the patients by the guanidium thiocyanate method. The RNA was purified by centrifugation through a CsCl cushion.\textsuperscript{26} cDNA was synthesized using 2.5 \( \mu M \) random hexamer mix (Pharmacia Fine Chemicals, Piscataway, NJ), 5 mM MgCl\textsubscript{2}, 1 mM dNTP mix (Pharmacia), 2.5 units of Moloney murine leukemia 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 the nucleic acid was subjected to the dideoxy-chain termination sequencing method essentially as described,\textsuperscript{23} using the US Biochemicals Sequencing Kit (Biotek Laboratories, IN) with either one of the 15 primers 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.\textsuperscript{24}

Nondifferential PCR 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 exons 3 and 4 of the CFTR gene was performed by analysis of available family members. The genomic region flanking the polythymidine tract was amplified by polymerase chain reaction (PCR)\textsuperscript{20,21} with oligonucleotide primers. Amplification of the region between exon 3 and 8 of the CFTR gene was performed by PCR with oligonucleotide primers 3Ri5 5’GGATAGAGAGCTGGCTTCAAAGAAA3’ and 8/9Ri3 5’AAATAATCTTCCCCAATTTTTGCTTTT3’. The genomic region flanking the polythymidine tract was amplified by PCR using the primers 9i-5 and 9i-3 using previously described methodology.\textsuperscript{22} Nested PCR was subsequently performed with outer primers TT-5 (5’CTGGCTGTTGCTGTTTCTT3’) and TT-3 (5’CTGGCTCTTCTATCTTGTTT3’). The PCR conditions were: 94°C 60”, 54°C 30”, 72°C 1 minute followed by 35 cycles of 94°C 30”, 54°C 30”, 72°C 1 minute. 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.

**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 \( \Delta F 508 \), \( W 1282 X \) or the 3849\( +10 k b \) C\( \rightarrow \)T mutation. 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 variability in the age at diagnosis among patients carrying the G85E. Although 7 patients were symptomatic and diagnosed before 1 year of age, 3 patients were diagnosed after 5 years of age. Forced expiratory volume in 1 second (FEV\textsubscript{1}) was measured and expressed as a percentage of predicted normal values for height and sex, using previously described standard-pulmonary equations.\textsuperscript{27} Current height and weight percentiles were computed using the tables of Tanner.\textsuperscript{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 \( W 1282 X \) and \( \Delta F 508 \) mutations, both associated with severe disease presentation.\textsuperscript{23} In the second group were 20 patients carrying the 3849\( +10 k b \) C\( \rightarrow \)T mutation, which is associated with higher frequency of PS and a milder phenotype.\textsuperscript{74}

**Hybridization to RT-PCR Products**

50 \( \mu L \) of each differential RT-PCR reaction were subjected to electrophoresis and were subsequently blotted and hybridized to the exon 3 oligonucleotide G85E-N 5’TGTTCATGGAAATCTT 3’ that identified the normal sequence, washed at 42°C, and to G85E-M 5’TGTTCATGAATCTT 3’ that identified the G85E mutation, washed at 40°C. The intensity of the RT-PCR products was measured by phosphorimagery.

**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 (FEV\textsubscript{1}) was measured and expressed as a percentage of predicted values for height and sex, using previously described standardized pulmonary equations.\textsuperscript{27} Current height and weight percentiles were computed using the tables of Tanner.\textsuperscript{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 \( W 1282 X \) and \( \Delta F 508 \) mutations, both associated with severe disease presentation.\textsuperscript{23} In the second group were 20 patients carrying the 3849\( +10 k b \) C\( \rightarrow \)T mutation, which is associated with higher frequency of PS and a milder phenotype.\textsuperscript{74}
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 a relatively 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 was similar to group 1, and significantly higher than 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>
<thead>
<tr>
<th>Patient No.</th>
<th>Genotype</th>
<th>Sex</th>
<th>Age at Diagnosis</th>
<th>Sweat Chloride meq/L</th>
<th>Mode of Presentation</th>
<th>Current Age</th>
<th>Pancreatic Status</th>
<th>FEV1 % Predicted</th>
<th>Weight Percentile</th>
<th>Spumon Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>G85E/G85E</td>
<td>F</td>
<td>9 mo</td>
<td>143</td>
<td>Respir + Gl</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>184</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>54</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/ΔF508</td>
<td>F</td>
<td>6 mo</td>
<td>157</td>
<td>Respir + Gl</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/ΔF508</td>
<td>F</td>
<td>2 mo</td>
<td>90</td>
<td>Respir + Gl 4 mo</td>
<td>PI</td>
<td>NA</td>
<td>&lt;3 H influenzae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>G85E/G85E</td>
<td>F</td>
<td>3 mo</td>
<td>97</td>
<td>Respir + Gl 6 yr</td>
<td>PS</td>
<td>36</td>
<td>35</td>
<td>+ Staph</td>
<td></td>
</tr>
<tr>
<td>7†</td>
<td>G85E/ΔF508</td>
<td>M</td>
<td>6 mo</td>
<td>158</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>108</td>
<td>Screening 12 y</td>
<td>PS</td>
<td>83</td>
<td>80</td>
<td>Staph</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>G85E/ΔF508</td>
<td>M</td>
<td>5 mo</td>
<td>NA</td>
<td>Respir + Gl 18 y</td>
<td>PI</td>
<td>50</td>
<td>&lt;3 P aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>G85E/W1282X</td>
<td>F</td>
<td>7 mo</td>
<td>117</td>
<td>Respir 20 y</td>
<td>PI</td>
<td>65</td>
<td>50</td>
<td>P aeruginosa</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>G85E/W1282X</td>
<td>F</td>
<td>19 y</td>
<td>82</td>
<td>Pancreatitis 34 y</td>
<td>PI</td>
<td>22</td>
<td>50</td>
<td>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 10 y</td>
<td>PI</td>
<td>65</td>
<td>97</td>
<td>P aeruginosa</td>
<td></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>
<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>79</td>
<td></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 expired 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 x2 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%) (\(P < .001\); 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 (0.05).

As shown in Table 2, FEV\(_1\) 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\(_1\). There were no differences in the slope or in age-adjusted mean values of FEV\(_1\), 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,\(^{2,10,15}\) 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\(_1\) was 82, 83, and 50% predicated, respectively. The patient with the lowest FEV\(_1\) 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,\(^{2,11}\) W1282X,\(^{3,6}\) G542X,\(^{6}\) N1303K,\(^{4,6}\) and R533X\(^{5,6}\) in which >95% of the patients had PI 2–10kb C>T,\(^{7,8}\) showing that the spread of the values are typical. Severe: ΔF508/ΔF508, W1282X/W1282X, ΔF508/W1282X. Mild: 3849+10kb C->T,\(^{7,8}\) A455E\(^{15}\)

![Fig 1. FEV\(_1\) versus age for the three groups showing similar distribution with wide scatter of the values in all groups. The superimposed lines are the reference values and 1 and 2 standard deviations around the reference line of patients from Toronto homozygous for ΔF508, showing that the spread of the values are typical. Severe: ΔF508/ΔF508, W1282X/W1282X, ΔF508/W1282X. Mild: 3849+10kb C->T.](image-url)
R117H\(^6\) 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.7,8,13 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, contains five, seven, or nine thymidines (the 5T, 7T, and 9T alleles, respectively). Chu et al31 showed that the G85E mutation is 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


A Missense Cystic Fibrosis Transmembrane Conductance Regulator Mutation With Variable Phenotype

Eitan Kerem, Malka Nissim-Rafinia, Zvi Argaman, Arie Augarten, Lea Bentur, Aharon Klar, Yaacov Yahav, Amir Szeinberg, Ornit Hiba, David Branski, Mary Corey and Batsheva Kerem

Pediatrics 1997;100;e5
DOI: 10.1542/peds.100.3.e5

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