Estudi de la correlació genotip- fenotip

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RESEARCH ARTICLE

Missense Mutation R1066C in the Second Transmembrane Domain of CFTR Causes a Severe Cystic Fibrosis Phenotype: Study of 19 Heterozygous and 2 Homozygous Patients

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We report the clinical features of 21 unrelated cystic fibrosis (CF) patients from Portugal and Spain, who carry the mutation R1066C in the *CFTR* gene. The current age of the patients was higher in the R1066C/any mutation group ($P < 0.01$), as compared to the ΔF508/ΔF508 group. Poor values for lung radiological involvement (Chrispin-Norman) and general status (Shwachman-Kulcycki) were observed in the R1066C/any mutation group ($P < 0.005$ and $P < 0.0004$). A slightly, but not significantly worse lung function was found in the R1066C/any mutation group when compared with the ΔF508/ΔF508 patients. No significant differences were detected regarding the age at diagnosis, sweat Cl-values, or percentiles of height and weight between the two groups. Neither were significant differences observed regarding sex, meconium ileus (4.7% vs. 11.1%), dehydration (10.5% vs. 14.7%), or pancreatic insufficiency (PI) (100% vs. 97.8%). The proportion of patients with lung colonization by bacterial pathogens was slightly, but not significantly higher in the R1066C/any mutation group (70.0%), as compared with the ΔF508/ΔF508 group (57.5%). Other clinical complications were significantly more frequent in the R1066C/any mutation patients ($P < 0.02$) than in the ΔF508/ΔF508 group. The two homozygous R1066C/R1066C patients died at the ages of 3 months and 7 years. The data presented in this study clearly demonstrate that the R1066C mutation is responsible for a severe phenotype similar to that observed in homozygous ΔF508 patients. The poor clinical scores and complications of patients with the R1066C mutation are probably related to their slightly longer survival. Hum Mutat 10:387–392, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: **cystic fibrosis; R1066C mutation; genotype/phenotype correlation**

INTRODUCTION: R1066C MUTATION STUDY

Cystic fibrosis (CF) is a multisystemic disorder with a wide clinical presentation involving the pulmonary, digestive, and reproductive systems (Welsh et al., 1995). The identification of the CF transmembrane

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conductance regulator (*CFTR*) gene (Rommens et al., 1989) responsible for CF has permitted the characterization of >600 mutations. Of these mutations, 42% are missense, 23% are frameshift, 16% are splice-site, 15% are nonsense changes, and 4% are other defects, including amino acid deletions and large DNA deletions (CF Genetic Analysis Consortium; http://www.genet.sickkids.on.ca).

The combination of the different types of mutations in the individual and the evaluation of the clinical features observed in the patients allow us to establish correlations between genotype and phenotype. The most common *CFTR* genotype in CF patients is homozygosity for the deletion of phenylalanine at position 508 (Kerem et al., 1989). This genotype is associated with the classical severe form of the disease with chronic obstruction and infection of the respiratory tract, exocrine pancreatic insufficiency (PI), and elevated levels of electrolytes in sweat (Kerem et al., 1990; Santis et al., 1990; Johansen et al., 1991). In the majority of cases, the absence of CFTR due to the combination of nonsense and/or frameshift mutations gives a severe phenotype, indistinguishable from that of the ΔF508/ΔF508 subjects (CF genotype-phenotype Consortium 1993). Several studies have shown that most mutations that alter the splicing of the *CFTR* gene lead to an abnormal or absent protein (Hull et al., 1993; Zielenski et al., 1993). However, some splicing mutations produce low amounts of the normal *CFTR* mRNA, together with the mutant *CFTR* mRNA, leading to different clinical situations that range from CF with PI to a normal phenotype. This is the case of mutation 3849+10kbC→T, which activates a cryptic exon (Highsmith et al, 1994), or mutation IVS8-6(5T), a sequence of five thymines (also named allele 5T) in the polypyrimidine tract of intron 8 that causes the skipping of exon 9 in the *CFTR* mRNA (Chillón et al., 1995).

The group of missense mutations comprises the largest number of *CFTR* defects (>250 mutations). Functional studies suggested that missense mutations in the nucleotide binding domains (NBD) or in the regulatory (R) domain have a severe effect when combined in *trans* with a severe mutation. In contrast, defects in the membrane spanning domains (MSD) were predicted to cause a mild CF phenotype (Welsh and Smith, 1993). However, the clinical data obtained for some mutations show that the domain location does not absolutely determine the severity of a mutation, that it also depends on the nature of the amino acid substitution, and that often the clinical presentation is variable (Estivill et al., 1995; Vázquez et al., 1996).

We present here a phenotype/genotype correlation analysis of 21 unrelated CF patients (from a total of 28 identified) with mutation R1066C in the second MSD of CFTR. The patients were from Portugal and Spain, where this mutation represents 5% and 1% of the CF chromosomes in the population, respectively (Pacheco et al., 1994; Casals, unpublished observation). The study clearly demonstrates that the R1066C mutation is responsible for a severe phenotype similar to that observed in the homozygous ΔF508 patients.

MATERIALS AND METHODS

Patients

More than 700 CF families from Portugal and Spain were investigated. The clinical information studied for each CF patient was the current age, age at diagnosis, sex, sweat Cl^- concentrations, pancreatic status, weight, height, Chrispin-Norman chest radiological score (from 0 to 38, with 0 being the best score), Shwachman-Kulcycki general status score (100 is the best score), history of meconium ileus, dehydration, lung colonization with bacterial pathogens, forced expiratory volume in 1 sec (FEV1), forced vital capacity (FVC), and other clinical complications of abnormalities.

Genetic Analysis

Genomic DNA was isolated from peripheral blood lymphocytes according to standard protocols. The DNA samples were analyzed for the common CF mutations detected in each population group. To study the rare CF mutations, SSCA (Chillón et al., 1994) and DGGE (Costes et al., 1993) were performed and the exons that gave abnormal patterns were sequenced (DyeDeoxy™ Terminator method on an ABI 373A sequencer or with manual sequencing using ^{35}S). This allowed the detection of mutation R1066C in the different samples. Also, the microsatellite haplotype for the repeats IVS8CA, IVS17bTA, and IVS17bCA was analyzed, which facilitated the identification of several cases of mutation R1066C, as described (Morral et al., 1993). The detected mutations were confirmed by sequencing.

The specific analysis of the R1066C mutation was performed by PCR amplification using primer 17bi5´ (Zielenski et al., 1991) and the mutagenesis primer 17bRx1: 5´ GCTGCCGTCCGAAGGCT*C 3´, that creates a restriction site for *TaqI* if the R1066C mutation is present (*indicates the modified nucleotide). PCR conditions were, denaturation for 3 min at 95°C, 35 cycles of 95°C for 30 sec, 54°C for 40 sec, and 74°C for 50 sec, with a final incubation at 74°C for 5 min.

Statistical Analysis

Current age, age at diagnosis, sweat Cl^- test, percentile of height, percentile of weight, Chrispin-

Norman score, Shwachman-Kulcycki score, FEV1 and FVC were compared between the R1066C/any mutation and the ΔF508/ΔF508 genotype groups using the Student unpaired *t*-test for continuous variables. Chi-square analysis was used to compare differences between groups for the following variables: sex, lung colonization with bacterial pathogens, meconium ileus, dehydration, pancreatic insufficiency (PI), and presence of other clinical complications.

RESULTS

Twenty-eight CF patients with mutation R1066C were detected in Portuguese (13 patients) and Spanish (15 patients) CF patient populations. Seventeen patients were R1066C/ΔF508 heterozygotes, five of them were deceased, two shortly after birth, one at 5 years of age (not included in Table 1), and two others at the ages of 7 and 24 years. Nine patients were heterozygous for five different mutations: R334W, G542X (1 sib deceased), 712-1G→T (1 sib deceased), 711+1G→T (2 sibs deceased), and 3905insT (only one patient for each one of these five mutations is included in Table 1). Finally, two patients were homozygous for R1066C mutation and died at the ages of 3 months and 7 years (Fig. 1, Table 2).

Table 1 shows the clinical values obtained for several parameters in the 21 patients for which clinical data were available. The data are presented as R1066C/any mutation (4 of which were deceased) and also includes the data obtained for 82 Spanish ΔF508/ΔF508 patients used as a control (Estivill et al., 1995). In six families, there was more than one affected child (5 families with 2 affected children and 1 family with 3), but only one is still alive in each family (Fig. 1).

Table 2 shows the clinical features of the two homozygous patients for the R1066C mutation, who died at the ages of 3 months and 7 years. The two families with the R1066C homozygous patients were consanguineous (Fig. 1).

The microsatellite haplotype for IVS8CA, IVS17bTA, and IVS17bCA showed three different associations with mutation R1066C: 17-7-17 (6 chromosomes), 16-33-13 (3 chromosomes), and 16-31-13 (1 chromosome).

DISCUSSION

The information on the clinical features of patients with missense mutations is important for the clinical prognosis of the patients and for genetic counselling in the families. Since most of the missense mutations are rare, the information about their severity depends on the study of a large number of cases and/or the analysis of patients that are homozygous for these mutations. The current clinical data for missense mutations derived from a relatively large number of cases are limited to a few mutations: N1303K (Osborne et al., 1992; CF genotype-phenotype Consortium 1993), R117H (CF genotype-phenotype Consortium 1993), P205S (Chillón et al., 1993), A455E (Gan et al., 1995), L206W (Desgeorges et al., 1995), R334W (Estivill et al., 1995), and G85E (Vázquez et al., 1996). Data on homozygous patients for missense mutations have been obtained for G551S

TABLE 1. **Comparison of Clinical Features Between Cystic Fibrosis Patients With R1066C/Any Mutation and ΔF508/ΔF508**

Parameter	Genotype R1066C/any[a]		Genotype ΔF508/Δ508
No. of patients	21		82
Sex: female/male	10/11		36/46
	Mean ± SD (no. studied)		Mean ± SD (no. studied)
Current age-year	11.05 ± 6.93 (21)	$P < 0.01$	7.72 ± 5.26 (82)
Age at diagnosis-year	2.57 ± 4.63 (21)		2.22 ± 2.91 (82)
Sweat Cl-mEq/l	112.44 ± 28.80 (18)		104.40 ± 15.70 (80)
%ile-height	32.19 ± 35.03 (18)		30.70 ± 26.04 (80)
%ile-weight	33.68 ± 35.95 (19)		27.99 ± 24.19 (80)
Chrispin-Norman	12.50 ± 8.07 (12)	$P < 0.005$	6.60 ± 6.26 (63)
Shwachman-Kulcycki	69.46 ± 17.88 (15)	$P < 0.0004$	83.11 ± 11.79 (72)
FEV1-% predicted	64.06 ± 22.81 (15)		74.80 ± 23.06 (41)
FVC-% predicted	72.20 ± 20.26 (15)		82.31 ± 20.03 (41)
	No. positive/no. studied (%)		No. positive/no. studied (%)
Lung colonization with bacterial pathogens	14/20 (70.0)		46/80 (57.5)
Meconium ileus	1/21 (4.7)		9/81 (11.1)
Dehydration	2/19 (10.5)		10/68 (14.7)
Pancreatic insufficiency	21/21 (100.0)		79/81 (97.5)
Other clinical features	13/21 (61.9)	$P < 0.02$	29/82 (35.4)

[a]Mutations were: ΔF508 (14 cases), R1066C (2), R334W (1), G542X (1), 712-1G>T (1), 711+1G>T (1), and 3905insT (1); FEV1, forced expiratory volume in 1 sec; FVC, forced vital capacity.

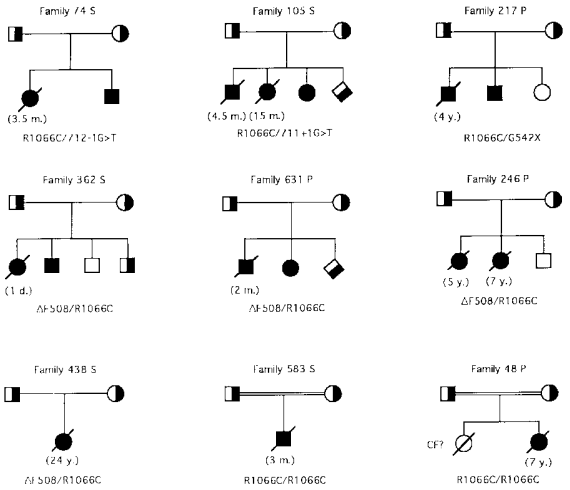

FIGURE 1. **Genealogies of nine families with CFTR mutation R1066C and deceased CF patients. A total of 11 CF patients bearing the R1066C mutation were deceased, including two homozygous patients. The age of death of the patients is indicated in parentheses (d, day; m, month; y, year).**

(Strong et al., 1991), E92K (Nunes et al., 1993), S549N (Curtis et al., 1993), I175V (Romey et al., 1994), A559T (McDowell et al., 1995), and G85E (Vázquez et al., 1996). One example of the importance of the analysis of a large number of patients is mutation R334W, for which the study of a large number of patients has clearly defined R334W as a late onset PI mutation with inter- and intrafamilial variability (Estivill et al., 1995), whereas initial data (including functional studies) suggested that it was a PS mutation (Welsh and Smith, 1993).

TABLE 2. **Clinical Features of Two Cystic Fibrosis Patients Homozygous for CFTR Mutation R1066C**

Parameter	Patient	
	583 S	48P
Genotype	R1066C/R1066C	R1066C/R1066C
Sex	male	female
Current age-year	0.25[a]	7.00[a]
Age at diagnosis-year	0.16	2.00
Sweat Cl-mEq/l	100	75
%ile-height	–	–
%ile-weight	3.9 (<3)	–
Chrispin-Norman	–	–
Shwachman-Kulcycki	–	–
FEV1[b]-% predicted	–	–
FVC[c]-% predicted	–	–
Lung colonization	no	yes
Meconium ileus	no	no
Dehydration	no	no
Pancreatic insufficiency	yes	yes
Other clinical features	–	–

[a]Patients deceased.
[b]Forced expiratory volume in 1 sec.
[c]Forced vital capacity.

The analysis of the 19 patients heterozygous for mutation R1066C and the two homozygous patients provides useful clinical information on the severity of this mutation. No significant differences were detected concerning the age at diagnosis, sweat Cl^- values, and the percentiles of height and weight between the R1066C/any mutation group and the ΔF508/ΔF508 group. The current age was significantly higher in the R1066C/any mutation group ($P < 0.01$), as compared with the ΔF508/ΔF508 group. Worse values for lung radiological involvement (Chrispin-Norman) and general status (Shwachman-Kulcycki) were observed in the R1066C/any mutation group ($P < 0.005$ and $P < 0.0004$). A slightly worse, but not significantly, lung function was found in the R1066C/any mutation group when compared with the ΔF508/ΔF508 patients. No significant differences were observed concerning sex, meconium ileus (4.7% vs. 11.1%), or dehydration (10.5% vs. 14.7%). All the patients with the R1066C mutation were PI. The proportion of patients with lung colonization by bacterial pathogens was slightly higher, but not significantly, in the R1066C/any mutation group (70.0%) as compared with the ΔF508/ΔF508 group (57.5%). Other clinical complications were significantly higher in the R1066C/any mutation patients ($P < 0.02$) than in the ΔF508/ΔF508 group. Among these features we found bronchiectasia (23.8%), liver disease (23.8%), and nasal polyps (9.5%). Of special interest and deserving further investigation is liver disease, since only 2–5% of CF patients by large develop this aspect of the phenotype (Dean and Santis, 1994).

In all the patients heterozygous for mutation R1066C, the second CF mutation can be considered as severe (ΔF508, G542X, 712-1G→T, 711+1G→T, 3905insT, and R334W). Thus if the patients had a mild phenotype, this effect could be attributed to the R1066C mutation. However, all the patients had a severe CF phenotype. Also, the two patients homozygous for mutation R1066C died at the ages of 3 months and 7 years old, which further confirms the involvement of this mutation in determining a severe phenotype. Finally, of the 28 patients identified with the R1066C, 11 were deceased between a few hours after birth and 24 years, with a mean age at death of 4.5 years, further supporting the severity of mutation R1066C.

Mutation R1066C is an arginine to cysteine change due to a C→T transition at nucleotide position 3328 in exon 17b (MSD2) of the *CFTR* gene (Fanen et al., 1992). The three microsatellite haplotypes associated with mutation R1066C make mutation analysis based on microsatellites difficult as

compared with other CF mutations (Morral et al., 1994), and it also might suggest that mutation R1066C has originated independently in different genetic backgrounds. Two other changes have been described in the same codon: R1066H, G→A at nucleotide 3329 (Férec et al., 1992); and R1066L, G→T at nucleotide 3329 (Mercier et al., 1993). Thus this CpG dinucleotide can be considered a hot spot for mutation (Morral et al., 1994).

The three CF mutations described in codon 1066 of *CFTR* are associated with different phenotypes. Férec et al. (1992) described a R1066H/1078delT patient with PI. Mercier et al. (1993) identified a patient with the R1066L mutation and PI. Brancolini et al. (1995) found three heterozygous patients: R1066H/1717-1G→A, R1066H/G542X and R1066C/ΔF508 all PS. Finally, Mercier et al. (1995) reported the R1066H mutation associated with male infertility. Thus the data obtained for single patients are often inconclusive for defining the clinical implications of a given CFTR mutation. In the case of mutations at codon 1066, it is now clear that mutation R1066C is a severe CF mutation, but further patients with mutations R1066H and R1066L should be analyzed before conclusions could be made about the severity of mutations at codon 1066.

Although it is clear that R1066C in the second MSD of CFTR is a severe mutation, the worse clinical phenotype of patients with this mutation could be due to a slightly longer survival of the patients. This could favour the development of complications and the deterioration of lung function (higher morbility), which does not occur in those patients with a more severe phenotype and a rapid evolution of the disease (higher mortality).

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