A new complex allele of the *CFTR* gene partially explains the variable phenotype of the L997F mutation

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Purpose: To evaluate the role of complex alleles, with two or more mutations in *cis* position, of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene in the definition of the genotype-phenotype relationship in cystic fibrosis (CF), and to evaluate the functional significance of the highly controversial L997F *CFTR* mutation. **Methods:** We evaluated the diagnosis of CF or *CFTR*-related disorders in 12 unrelated subjects with highly variable phenotypes. According to a first *CFTR* mutational analysis, subjects appeared to be compound heterozygotes for a classic mutation and the L997F mutation. A further *CFTR* mutational analysis was conducted by means of a protocol of extended sequencing, particularly suited to the detection of complex alleles. **Results:** We detected a new [R117L; L997F] *CFTR* complex allele in the four subjects with the highest sweat test values and CF. The eight subjects without the complex allele showed the most varied biochemical and clinical outcome and were diagnosed as having mild CF, *CFTR*-related disorders, or even no disease. **Conclusions:** The new complex allele partially explains the variable phenotype in CF subjects with the L997F mutation. *CFTR* complex alleles are likely to have a role in the definition of the genotype-phenotype relationship in CF. Whenever apparently identical *CFTR*-mutated genotypes are found in subjects with divergent phenotypes, an extensive mutational search is mandatory. ***Genet Med* 2010:12(9):548–555.**

Key Words: *CFTR, complex alleles, cystic fibrosis, genotype-phenotype relationship, mutational search*

Cystic fibrosis (CF) is a recessive autosomal disease caused by cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Genbank accession NG_016465) mutations.[1,2] Clinical manifestations of CF vary to such an extent that some mono- or oligosymptomatic phenotypes have to be distinguished from polysymptomatic classic CF, as occurs with *CFTR*-related disorders (*CFTR*-RD).[3] It is generally accepted that the phenotypical severity of CF or *CFTR*-RD is essentially correlated with the residual function of the *CFTR* gene,[4] which is produced by intragenic variability due to the large number of mutations and to the even larger number of combinations of these mutations. However, the influence of modifier genes as a source of extragenic variability on the final phenotype has been observed[5] when a high phenotypical variability occurs in subjects with identical *CFTR*-mutated genotypes. An aspect of the genotype-phenotype relationship that has not yet been fully addressed is the involvement of complex alleles as a further source of *CFTR* genetic variability. An allele is called complex when it carries two (or more) mutations in *cis*. The most widely used protocols for a mutational search within the *CFTR* gene, whether they be protocols based on mutational panels including well-characterized classic *CFTR* mutations or gene scanning protocols, are designed in such a way as to stop when two mutations on different alleles are found. Thus, additional mutations that may be present in *cis* with the already found mutations may escape detection. Because of this common limitation of mutational search protocols, the mutated genotypes of CF-affected subjects with varying clinical presentations may appear identical despite the presence of unrevealed complex alleles that might explain the divergent phenotypes. As regards the consequences of an unrevealed complex allele, a distinction must be made between finding the two-*CFTR* mutations already known to cause disease on both alleles, which may result in an unclear genotype-phenotype relationship, and finding one classic mutation on one allele and one sequence variation with little or conflicting functional data on the other, which may instead result in diagnostic errors or misclassification of the sequence variation. Although the *CFTR* mutation L997F has been known since 1992, it is still highly controversial from the functional point of view, and its pathogenetic role has not yet been fully elucidated. L997F was initially believed to be a polymorphism[6] then it was been reported to cause both CFTR-RD and no disease. The presence of L997F has been linked to pulmonary diseases,[7,8] disseminated bronchiectasis,[9,10] neonatal hypertrypsinaemia with normal sweat test,[11–14] idiopathic pancreatitis,[12,15–17] and congenital absence of vas deferens.[18] It has also been suggested that L997F negatively influences the clinical course of primary sclerosis cholangitis.[19] In subjects with idiopathic pancreatitis, the L997F *CFTR* mutation has also been found to be associated with mutations of the serine protease inhibitor Kazal type 1/pancreatic secretory trypsin inhibitor (*SPINK1*) gene, namely the N34S mutation[20] and a deletion encompassing the entire gene.[21] However, there is also evidence suggesting that homozygosity for L997F does not cause disease.[22,23] The frequency of L997F in the general population has been reported to be ~0.5%,[14,24] which is neither high enough to clearly exclude its pathogenetic role nor so low as to strongly support the pathogenetic hypothesis.

To shed light on the general issue of the role of complex alleles in the genotype-phenotype relationship in CF, we studied the functional and clinical significance of the L997F *CFTR* mutation. For this purpose, we studied 12 unrelated compound heterozygotes for a classic *CFTR* mutation on one allele and, according to a first *CFTR* mutational analysis, apparently only L997F on the other allele. These 12 subjects also had somewhat different phenotypes as assessed by biochemical and clinical studies. They underwent complete sequencing of all *CFTR*

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exons, adjacent intronic zones, and 5′-flanking regions to verify whether their phenotypic variability was linked to any additional *CFTR* sequence variations in *cis* with L997F.

MATERIALS AND METHODS

Selected subjects and genetic testing cascade

The study is a retrospective study that does not require ethics committee approval at our institution. Informed consent was obtained from subjects included.

We selected, from a larger group of subjects with a diagnosis or presumptive diagnosis of CF, 12 unrelated subjects who had a classic mutation and the L997F mutation in different alleles of the *CFTR* gene. In the analysis of the larger case series, the genetic testing cascade included a first-level genetic analysis using the CF-oligonucleotide ligation assay (OLA) mutational panel, a second-level genetic analysis using a multistep-extended *CFTR* sequencing protocol (which analyzes successive groups of exons), and an analysis of the parents to verify allelic segregation (see also, "Mutational analysis" section). This mutational search was interrupted when two mutations in different alleles were found. The 12 subjects studied here instead underwent the extended *CFTR* mutational analysis described later even when two segregating mutations had previously been found. This mutational search protocol is more suited to the detection of complex alleles.

Biochemical, microbiologic, and clinical characterization

The subjects enrolled underwent: a sweat test, with a quantitative pilocarpine iontophoresis method,[25] using the Macroduct device (Delcon, Italy) for sweat collection and the PCL M3 chloride analyzer Jenway (VWR International, Italy) for measurement; a pancreatic function evaluation by dosage of fecal elastase 1[26] (using the pancreatic fecal elastase test; Meridian Bioscience, Italy); and a clinical examination. Nutritional status was evaluated by weight and height centiles. Liver disease was assessed by physical inspection, dosage of serum liver enzymes (aspartate aminotransferase, alanine aminotransferase, and γ-glutamyltransferase with common methods), and hepatopancreatic ultrasonography. Pulmonary status was evaluated by forced expiratory volume in the first second (FEV1) assessment (for subjects aged 8-years old, unless differently indicated) and chest radiographs according to the Brasfield scoring system.[27] Microbiologic status was evaluated by bacteriologic analysis of oropharyngeal cultures by deep suctioning, using common and shared laboratory approaches.[28] CF and *CFTR*-RD diagnoses were performed according to current guidelines.[3,29,30] Whenever applicable, semen analysis was performed according to World Health Organization (WHO) guidelines.[31]

According to the recent guidelines,[30] the sweat test in subjects up to 6 months of age was considered negative if <30 mEq/L, pathologic if ≥ 60 mEq/L, and borderline in the 30–59 mEq/L range; for all other subjects, the sweat test was considered negative if <40 mEq/L, pathologic if ≥ 60 mEq/L, and borderline in the 40–59 mEq/L range. Although Table 1 only shows the mean value of repeated sweat test measurements (from two to four) on enrollment and during follow-up, the patients age was taken into account when they were classified as having a negative, borderline, or pathologic sweat test. We classified the severity of CF as follows: mild CF if FEV1 $>70\%$ and Bransfield score >15; moderate CF if FEV1 ranged from 40% to 70% and Bransfield score <15; and severe CF if FEV1 $<40\%$ and Bransfield score <15.

Mutational analysis

DNA was extracted from peripheral blood using the QIAamp DNA blood midi kit (QIAGEN, Hilden, Germany). The CF-OLA assay (Abbott, Wiesbaden, Germany),[32] including the 32 most common mutations of the *CFTR* gene worldwide, was used as a first-level genetic analysis. A 96-well formatted method for the sequencing of the 5′-flanking region, all exons, and the adjacent intronic regions of the *CFTR* gene was used as a second-level genetic analysis.[33] The $(TG)_mT_n$ polymorphic tract in the *CFTR* intron 8 was studied by sequencing, as previously described.[34] The BigDye Terminator version 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) was used for sequencing. The first-level OLA products and the second-level sequences were run by an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems). Electropherograms were analyzed by specific Genotyper (for OLA) and Seqscape (for sequencing) templates. Segregation of the mutations found was always verified by analysis of the parents. The *CFTR* molecular genetic data were interpreted and are reported according to the updated European recommendations.[3]

The state of conservation of both the L997 and R117 aminoacidic residues was studied by comparing the following 10 species using CLUSTALW software: human, mouse, rat, macaque, bovine, sheep, pig, dog, rabbit, and xenopus.

Statistical analysis

The comparison between the mean sweat test values of the groups with or without the complex allele was performed by means of the Student *t*-test, using the data analysis tool of Excel software (Microsoft, Redmond, WA).

RESULTS

Genetic analysis, biochemical features of the mutations found, and state of conservation of L997F and R117L residues

Five different *CFTR* mutations were found (those included in the CF-OLA panel were confirmed by sequencing) on one allele of the subjects analyzed (Table 1). F508del (c.1520_1522delTCT), the most common CF mutation worldwide, is a trinucleotide deletion resulting in the loss of phenylalanine in position 508 of the CFTR protein in the cytoplasmic first nucleotide-binding domain. W1282X (c.3846G>A) is a nonsense mutation that produces a truncated CFTR form. G85E (c.254G>A), S549R(A>C) (c.1645A>C), and L320V (c.958T>G) are missense mutations located in the first membrane spanning domain-first transmembrane segment, in the first nucleotide-binding domain, and in the first membrane spanning domain-fifth transmembrane segment, respectively. F508del, W1282X, G85E,[35] and S549R(A>C)[36] are considered classic *CFTR* mutations that cause a severe CF form when in homozygosity or compound heterozygosity with another classic mutation. The L320V mutation has previously been found in two subjects with congenital bilateral absence of the vas deferens, with no other symptoms[37]; to our knowledge, no other phenotypical description of this mutation has been published.

On the other allele (allelic segregation was confirmed by analysis of the parents), L997F (c.2991G>C) is a conservative aminoacidic substitution: both amino acids are nonpolar. Because the position of L997F is in the second membrane spanning domain-ninth transmembrane segment, both amino acids fit the hydrophobic transmembrane environment. The R117L (c.350G>T) was found in four subjects on the same allele as L997F (as assessed by parents' analysis); it is a nonconservative

Table 1 C*FTR* genotypes, sweat test values, and clinical assessment of the subjects studied

					Upon enrollment					
								Respiratory manifestations		
Subject	Genotype	Sex	Average sweat test value[a] (mEq/L)	Semen analysis	Age	Cause	Clinical symptoms (without therapy)	By FEV1	By rx	Pulmonary bacterial isolates
1	F508del/[R117L; L997F]	M	90 ± 9	OA	5 yr	Symptoms	Dehydration events, bronchopneumonia, rhinosinusitis	Moderate	Moderate	Absent
2[c]	F508del/L997F	F	56 ± 8	ND	2 mo	Neonatal screening	Absent	Too young	Mild	Absent
3	F508del/L997F	M	42 ± 5	Too young	5 mo	Neonatal screening	Absent	Too young	Absent	Absent
4[c]	F508del/L997F	F	35 ± 4	ND	1 mo	Neonatal screening	Absent	Too young	Mild	Absent
5[c]	F508del/L997F	M	32 ± 1	Too young	2 mo	Neonatal screening	Absent	Too young	Absent	Absent
6	F508del/L997F	M	22 ± 3	Too young	8 yr	Symptoms	Bronchopneumonia, bronchitis, rhinosinusitis	Absent	Mild	Absent
7	G85E/[R117L; L997F]	M	102 ± 10	OA	7 yr	Symptoms	Productive cough	Too young	Mild	*S. aureus*
8	G85E/L997F	M	21 ± 4	Too young	11 mo	Neonatal screening	Productive cough	Too young	Mild	*S. aureus* (sporadic)
9	W1282X/[R117L; L997F]	M	96 ± 4	OA	33 yr	Symptoms	Cholelithiasis, productive cough, bronchopneumonia	Mild	Moderate	*S. aureus, P. aeruginosa*
10	W1282X/[R117L; L997F]	F	80 ± 5	ND	36 yr	Symptoms	Bronchopneumonia	Moderate	Moderate	*P. aeruginosa*
11	L320V/L997F	M	77 ± 5	Too young	3 yr	Symptoms	Rhinosinusitis	Too young	Absent	Absent
12[c]	S549R(A>C)/L997F	M	39 ± 6	Too young	2 mo	Neonatal screening	Absent	Too young	Absent	Absent

No subject had either pancreatitis or liver disease. Classification of pulmonary symptoms by FEV1 is as follows: absent, >90%; mild, from 70% to 90%; moderate, from 40% to 70%; severe, <40%. Classification of pulmonary symptoms by chest x-ray is as follows: absent, no radiological signs; mild, limited air trapping or peribronchial infiltration; moderate, dense areas or bronchiectasis restricted to one lobe; severe, dense areas or bronchiectasis in both hemithoraxes. The severity of cystic fibrosis was classified as reported in "Materials and Methods—Biochemical, microbiologic, and clinical characterization" section.

[a]Each sweat test value is the mean of repeated sweat test measurements (from 2 to 4) on enrollment and during follow-up.

[b]The Bransfield score ranges from 25, no disease, to 0, highly severe disease (for reference see "Materials and Methods" section).[27]

[c]These four subjects have already been partially described[14] and are included here merely for comparison purposes.

[d]All the subjects had pancreatic sufficiency with the exception of Subject 1 who had initial PS, which gradually evolved into pancreatic insufficiency from 12 years of age.

OA, obstructive azoospermia; ND, not determined; PI, pancreatic insufficiency; PS, pancreatic sufficiency.

Table 1 *Continued*

Subject	At follow-up: Age	Clinical symptoms (with therapy whenever necessary)	Respiratory manifestations: By FEV1	Respiratory manifestations: By rx	Pulmonary bacterial isolates	Chest radiograph evaluation (Bransfield score)[b]	Diagnosis
1	31 yr	Asthmatic crisis, relapsing hemoptysis, bronchopneumonia, rhinosinusitis	Severe	Severe	*S. aureus, P. aeruginosa*	13	CF severe with late PI[d]
2[c]	6 yr	Pharyngitis, abdominal pain	Too young	Mild	Absent	21	*CFTR*-RD
3	7 yr	Productive cough	Too young	Moderate	Absent	19	*CFTR*-RD
4[c]	7 yr	Rhinosinusitis, pharyngitis	Absent	Mild	*S. aureus* (sporadic)	20	*CFTR*-RD
5[c]	4 yr	Absent	Too young	Absent	Absent	23	No disease
6	10 yr	Rhinosinusitis	Absent	Mild	*S. aureus* (sporadic)	17	CF mild with PS
7	26 yr	Nasal polyposis, rhinosinusitis	Mild	Mild	*S. aureus*	21	CF mild with PS
8	5 yr	Productive cough	Too young	Mild	*S. aureus* (sporadic)	17	CF mild with PS
9	42 yr	Broncopneumonia	Moderate	Moderate	*S. aureus*, *P. aeruginosa*	14	CF moderate with PS and late-onset
10	47 yr	Acute respiratory failure, oxygen therapy under stress, bronchopneumonia	Severe	Severe	*P. aeruginosa*	8	CF severe with PS and late-onset
11	11 yr	Rhinosinusitis	Absent	Mild	Absent	20	*CFTR*-RD
12[c]	4 yr	Productive cough, rhinitis, bronchitis	Too young	Mild	Absent	21	*CFTR*-RD

No subject had either pancreatitis or liver disease. Classification of pulmonary symptoms by FEV1 is as follows: absent, >90%; mild, from 70% to 90%; moderate, from 40% to 70%; severe, <40%. Classification of pulmonary symptoms by chest x-ray is as follows: absent, no radiological signs; mild, limited air trapping or peribronchial infiltration; moderate, dense areas or bronchiectasis restricted to one lobe; severe, dense areas or bronchiectasis in both hemithoraxes. The severity of cystic fibrosis was classified as reported in "Materials and Methods—Biochemical, microbiologic, and clinical characterization" section.

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OA, obstructive azoospermia; ND, not determined; PI, pancreatic insufficiency; PS, pancreatic sufficiency.

substitution: a positively charged amino acid is substituted by a nonpolar one. Because the position of R117L is in the first extracellular loop exposed to the aqueous extracellular environment, the substituting amino acid will have a lower fit than the original one. Both L997 and R117 aminoacidic residues are completely conserved in the 10 species analyzed.

Among the subjects analyzed, we found the following $(TG)_mT_n$ polymorphic genotypes: 5 $(TG)_{10}T_9/(TG)_{10}T_9$, 4 $(TG)_{10}T_9/(TG)_{11}T_7$, 2 $(TG)_{10}T_9/(TG)_{10}T_7$, and 1 $(TG)_{11}T_7/(TG)_{11}T_7$. No $(TG)_{12}$ or T_5 variant tracts were found. Both the isolated L997F and the [R117L; L997F] complex allele were associated with the 470V allele in all subjects.

Genotype-phenotype relationship

The measurement of the Cl^- concentration in the patients sweat is considered as a functional test that measures the in vivo residual ability of CFTR to Cl^- transport (the higher the sweat test, the lower the CFTR functionality). The 12 unrelated subjects examined yielded varying sweat test values (Table 1), ranging from 21 to 102 mEq/L, and varying clinical manifestations. In six subjects, the diagnosis of CF was clear since their enrollment and was fully confirmed by follow-up: one had a severe form (Subject 1), another two had a moderate or severe form (Subjects 9 and 10, respectively), while the remaining three had a mild form (Subjects 6, 7, and 8). Subjects 9 and 10 already displayed, on enrollment, a near-complete form of CF, which worsened in both cases during follow-up; because of the late age of symptom onset, they were classified as having a moderate or severe form with late-onset, respectively. A further five subjects (Subjects 2, 3, 4, 11, and 12) were classified as having *CFTR*-RD, generally with symptoms that were mild on enrollment but worsened during follow-up, without ever reaching a full diagnosis of even mild CF. One subject (Subject 5), who did not display any clinical symptoms either on enrollment or during follow-up, was classified as not having disease.

If one considers only the first two mutations found, the six subjects with apparently the same F508del/L997F genotype (Table 1, Subjects 1–6) showed highly varying sweat test values, ranging from 22 to 90 mEq/L, as did the two subjects (Subjects 7 and 8) with apparently the same G85E/L997F genotype, who had sweat test values of 102 mEq/L and 21 mEq/L, respectively. By contrast, the sweat test values in the two subjects with apparently the same W1282X/L997F genotype (Subjects 9 and 10) were more similar (96 mEq/L and 80 mEq/L, respectively). However, the extended genetic analysis detected the R117L mutation on the same allele as the L997F mutation in Subjects 1, 7, 9, and 10, thereby revealing a new complex allele of the *CFTR* gene (an example of the sequencing analysis is shown in Fig. 1). When the presence of this complex allele of the *CFTR* gene was taken into account, an excellent agreement between genotype and the sweat test values was found. The four subjects with the [R117L; L997F] complex

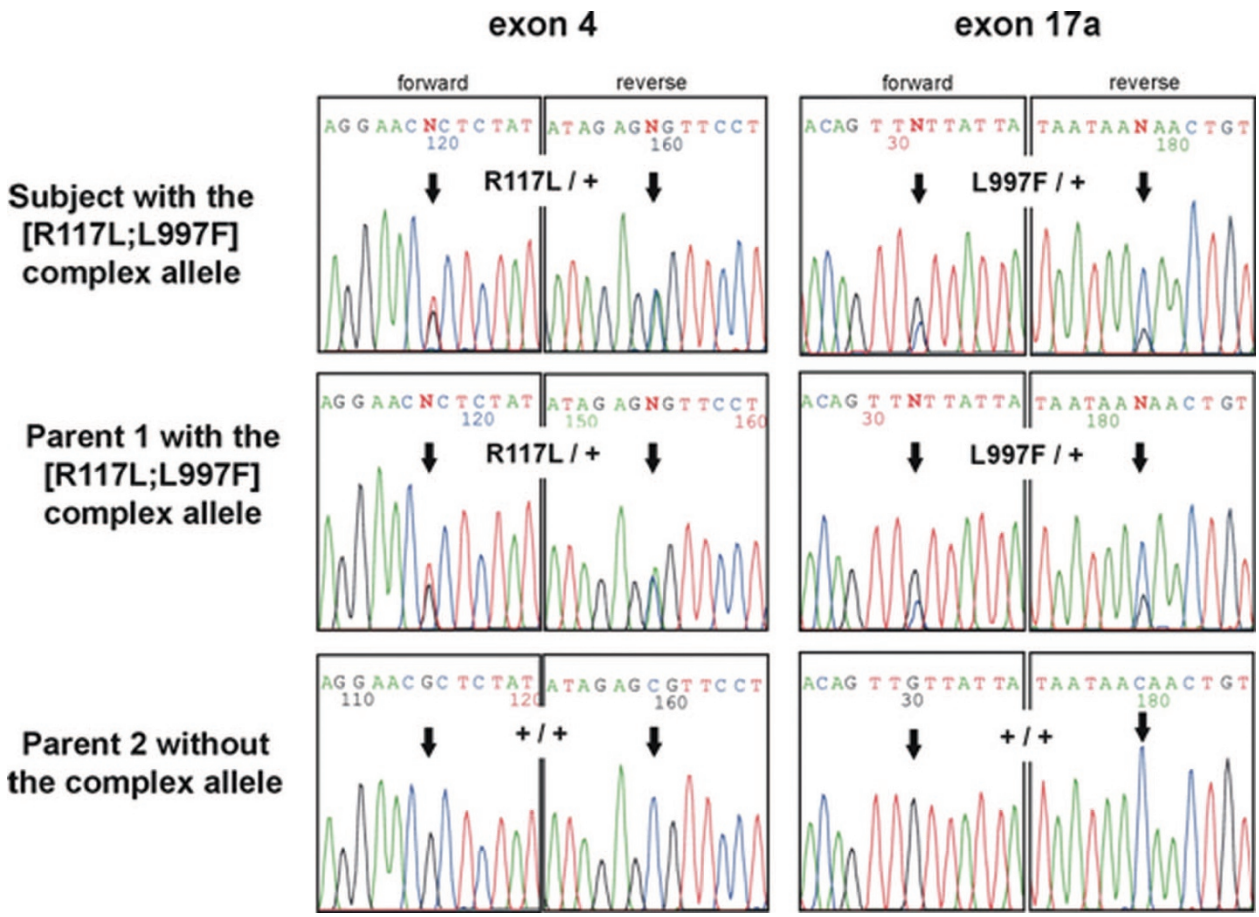

Fig. 1. An example of the complex allele analysis in a family. Some typical sequence electropherograms are shown; the arrows indicate the specific nucleotides.

allele showed the highest sweat test values among the subjects analyzed, even when otherwise identical genotypes were compared (Subject 1 versus 2–6; Subject 7 versus 8). The mean sweat test value in the four subjects with the complex allele (92 mEq/L ± 9 mEq/L) was significantly higher (Student *t*-test $P <$ 0.001) than in the eight subjects without the complex allele (41 mEq/L ± 19 mEq/L). A generally good agreement between the presence of the complex allele and clinical outcome was also found. All four subjects with the complex allele were diagnosed as having from mild to severe forms of CF; none of these four subjects was classified as having either *CFTR*-RD or no disease, as was observed in six of the eight subjects without the complex allele.

The correlation among the complex allele, sweat test values, and clinical outcome is particularly strong if we compare the six subjects with the same F508del mutation on one allele and with or without the complex allele on the other (Table 1). Subject 1, with a F508del/[R117L; L997F] genotype and the highest sweat test value, displayed clinically severe CF with late pancreatic insufficiency, whereas the five subjects with the F508del/L997F genotype and lower sweat test values displayed either a milder form of CF (Subject 6) or *CFTR*-RD (Subjects 2, 3, and 4) or no disease at all (Subject 5). By contrast, the agreement between the presence of the complex allele and clinical outcome was not as good when we compared the two subjects with the same G85E mutation on one allele. Indeed, although the subject with the complex allele (Subject 7) had the highest sweat test value and the subject without the complex allele (Subject 8) had the lowest value in the case series, the clinical outcome in both cases was mild CF. Because the other allele in the two subjects with the W1282X mutation (Subjects 9 and 10) was characterized by the presence of the complex allele, no evaluation of the effect of simple compound heterozygosity was possible. A good correlation was found in these two subjects among the genotype, sweat test, and clinical outcome, as both displayed high sweat test values and clinically moderate or severe CF.

The most varied biochemical and clinical outcome and least clear genotype-phenotype relationship emerged in the eight subjects without the complex allele. Within this group of subjects, Subject 11 was the only one with a pathologic sweat test value; this finding is somewhat surprising as the genotype was expected to be the least severe because it is the only one with fully conservative substitutions on both alleles. Moreover, despite the relatively high sweat test value, the final diagnosis in this subject was of *CFTR*-RD. The five subjects with the F508del/L997F genotype, which were expected to be more severe than the L320V/L997F genotype, represent a paradigm for variability. Indeed, no pathologic sweat test values were found in these subjects; the two subjects with the borderline sweat test values were diagnosed as having *CFTR*-RD (Subjects 2 and 3); of the three subjects with the negative sweat test, one was diagnosed as having mild CF (Subject 6), whereas the other two had a *CFTR*-RD (Subject 4) and no disease (Subject 5). Two other subjects were diagnosed as having either mild CF (Subject 8) or *CFTR*-RD (Subject 12) despite the negative sweat test, the subject with mild CF displaying a lower sweat test value than the subject with the *CFTR*-RD.

None of the subjects had either pancreatitis (excluding the involvement of the *SPINK1* gene[20]) or liver disease. All the subjects had pancreatic sufficiency with the exception of Subject 1; pancreatic sufficiency in this subject gradually evolved into pancreatic insufficiency from the age of 12 years.

DISCUSSION

Few *CFTR* complex alleles have been found to date, probably owing above all to the lack of a systematic experimental search. In vivo findings and, in some cases, in vitro functional characterizations have been reported for [F508C; S1251N],[38] [R347H; D979A],[39,40] [R74W; D1270N],[41] [G628R; S1235R],[42,43] [M470V; S1235R],[42] [S912L; G1244V],[44] [R117H; $(TG)_mT_n$],[45–47] [R117C; $(TG)_mT_n$],[46] [S1235R; $(TG)_mT_5$],[48] [G576A; R668C],[10,49] [V562I; A1006E],[49] [R352W; P750L],[49] [1198_1203del TGGGCT; 1204G>A],[49] [V754M; *CFTR*dele3_10,14b_16],[50] and [F508del; I1027T].[51] These complex alleles have been found in patients with either CF or *CFTR*-RD, although more often in the former. Isolated inheritance results in *CFTR*-RD, but CF is manifest if both are present in *cis*. Both in vivo and in vitro studies have also highlighted cases in which there is one main mutation with the phenotypical effect that is worsened by a second mutation, which may even be a neutral variant when isolated, as occurs for F508C,[38] R74W,[41] S912L,[44] and M470V.[42] However, different effects have also been described, as in the case of the two M470 and R1235 variants, which give rise to a hyperactive CFTR when present on different alleles but have a suppressive effect when combined on the same allele.[42] In addition, the finding of complex alleles in *CFTR*-RD seems to suggest that a second *CFTR* mutation may even lead to a partial reversion of the phenotype.[43] Indeed, in a reduced number of complex alleles, the effect of the second mutation may partially correct the functional defect, thereby lessening the phenotypical effect, as has been demonstrated for the R553Q mutation in the [F508del; R553Q] complex allele by in vivo[52] and in vitro[53] studies and for the R553M mutation in the [F508del; R553M] complex allele by an in vitro study.[53] A milder phenotypical effect has also been demonstrated for the [R334W; R1158X][54] and [-102T; S549R(T>G)][55] complex alleles if compared with alleles carrying, respectively, isolated R1158X or S549R(T>G).

Both L997 and R117 aminoacidic residues are highly conserved in the 10 species analyzed, which seems to suggest some evolutionary pressure. L997F is the only known mutation of the 997 CFTR amino acid.[37] As reported earlier, when isolated, L997F can cause either *CFTR*-RD (although not CF) or no disease at all.[6–19,22,23] The conservative nature of the L997F substitution (both residues are hydrophobic) in the CFTR second membrane spanning domain-ninth transmembrane segment may constitute the molecular basis for the limited effect of such an isolated L997F. Five different *CFTR* mutations of the 117 CFTR amino acid are known: R117C, R117G, R117H, R117L, and R117P.[37] All these mutations have previously been reported to be more likely to cause *CFTR*-RD than CF.[13,37,46,56] However, R117H and R117C have been shown to yield high sweat test values and CF, even severe, if *cis*-acting with the T_5 variant tract in *CFTR* intron 8.[45,46] If we bear in mind that the pH range of airway surface fluid is pH 6.7–7.0,[57,58] these mutations of the R117 CFTR residue represent both conservative and nonconservative substitutions.

In particular, the R117L is a nonconservative substitution that changes the basic residue to a hydrophobic residue. However, in the absence of other mutations or variants on the same allele, evaluation of the hydrophobic character of the R117 substitution fails to correlate with phenotypical alterations. A possible molecular mechanism for the reduced effect of the isolated R117L mutation may be that only 11 of the 15 amino acids that constitute the first extracellular loop domain in which the R117 residue is located have a charged or a polar side chain, whereas the other four are hydrophobic.

L997F was found in compound heterozygosity with another *CFTR* mutation in six subjects (four of whom had the complex allele) with CF (mild or severe), in five subjects with *CFTR*-RD, and in one asymptomatic subject. A good correlation between genotype, sweat test values, and clinical phenotype was found in the four subjects with the complex allele. In these four cases, the mild effects of the isolated L997F and R117L mutations

cumulate in the complex allele with a *cis*-acting effect, thereby inducing a well-defined, strong effect on both the Cl^- transport (producing the highest sweat test values in the entire case series) and clinical outcome, resulting in CF (from mild to severe). The other eight subjects without the complex allele did not reveal any clear correlation between genotype and phenotype. The high phenotypical variability in these subjects may be explained on at least two levels. The first level is the weak correlation between genotype and CFTR functionality, which clearly emerges in Subjects 2–6, who have the same genotype but very different sweat test values. The second level is the weak correlation between CFTR functionality and clinical outcome, which emerges through several comparisons. One such comparison may be made between the subjects without the complex allele in whom the clinical outcome does not reflect the sweat test values, as in the cases with mild CF and a negative sweat test (Subjects 6 and 8), and in the case with *CFTR*-RD and a positive sweat test (Subject 11). Other ways to highlight this effect are to compare subjects either with similar sweat test values but different disease severity (Subjects 4 and 12 versus 5) or with a similar clinical outcome but different sweat test values (Subjects 2, 3, 4, 11, and 12). There may be several sources of variability on both levels. Although the main CFTR function is Cl^- transport, it has been demonstrated that it is also involved, either directly or indirectly through interaction with other proteins, in other functions, i.e., the transport of other ions, the bacterial clearance or the immune/inflammatory response[2]; a possibly altered balance between the different *CFTR* functions, depending on the *CFTR* mutations, should be taken into account. In the absence of complex alleles, both levels of variability may in general depend on the limited adverse effect of the L997F mutation, which can easily be modified (worsened or improved) by environmental factors and/or modifier genes.[5] A particular class of modifier genes that specifically may influence Cl^- levels are the alternative Cl^- channels.

Several genes have been proposed as modifier genes[5] that influence the second level of variability (sweat test → clinical outcome). It is these genes that most probably represent, together with complex alleles, the greatest source of variability in CF. However, even at this level, the role of CFTR may be invoked when the sweat test is negative, but the disease is present. Although the L997F mutation seems to exert a limited influence on Cl^- transport (possibly owing to the limited impact of this substitution on the pore structure), it may exert greater influence on other CFTR functions, giving rise to the disease despite having a reduced or even no effect on Cl^- transport. Moreover, when sweat test values are not correlated with clinical outcome, potential tissue-specific differences in the amount of Cl^- transport, and general CFTR malfunction, must be taken into account. In some cases, the level of Cl^- in the sweat may not be proportional to the CFTR functionality in the lung (and other organs), although only in the presence of specific *CFTR* mutations. In such cases, there is little correlation between the clinical outcome and the sweat test.

As no mutational scan technique that detects all the mutations in the *CFTR* gene exists, the presence of some other undetected complex allele could be hypothesized in the subjects analyzed. However, this seems somewhat unlikely as the methodology of mutational search we used, which scans all the exons, adjacent intronic zones, and 5′-flanking of the *CFTR* gene, fails to identify only a small proportion of the *CFTR* mutations (<3%—unpublished results). Any mutations that escape this detection are large deletions and completely intronic mutations that may reveal cryptic exons, both of which are heavy molecular lesions that are most likely to simultaneously produce high sweat test values and a severe clinical outcome.

Another source of variability is age. The six subjects enrolled on the basis of symptoms, including all four subjects with the complex allele, were older than the subjects enrolled by neonatal screening, at both the enrollment and, consequently, follow-up. The fact that the clinical outcome was most severe in these six subjects is probably because they were enrolled before the neonatal screening program had started, their inclusion being exclusively symptom based. Consequently, a worsening in the clinical conditions of those subjects enrolled by means of a neonatal screening protocol cannot be ruled out. This consideration is supported by the fact that a late diagnosis of CF was made in two of the subjects with the complex allele (Subjects 9 and 10), the clinical outcome being classified in one of them as moderate (and not severe) exclusively on account of its late presentation. The late presentation of severe clinical symptoms in subjects with genotypes involving L997F mutation thus seems to be possible.

Although the clinical range in both the group with and the one without the complex allele varies, it also overlaps, from CF mild to CF severe in the former group and from asymptomatic to CF mild in the latter group. These findings shed light not only on the phenotypical variability in CF subjects with the L997F mutation but also, more generally, on the issue of the genotype-phenotype relationship in this disease. The vast majority of CF subjects worldwide undergo a mutational protocol that is interrupted after two mutations have been found on different alleles. Our results highlight the need to search for complex alleles whenever apparently identical *CFTR*-mutated genotypes are found in subjects with a discordant sweat test and clinical outcome who have not undergone a scanning protocol with a high detection rate. In addition, the following practical conclusions may be drawn from these findings. Whenever a L997F mutation is found, the search for the R117L mutation must be undertaken (and vice versa); if the complex allele is found, the onset of CF (in a mild or severe form) with high sweat test value is likely. If the L997F mutation is the only mutation of the allele, the phenotypical results vary to a greater extent: neither mild CF nor *CFTR*-RD can be ruled out, although the disease may even be absent, irrespective of the sweat test value. Because the late-onset of CF is possible even in the presence of the complex allele, particularly when L997F is found alone, a prolonged follow-up is recommended.

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The six subjects enrolled by means of neonatal screening were selected by the two screening centers in the Lazio region (Italy): the central laboratory of the Italian Red Cross of Rome (four subjects) and the screening laboratory of the Department of Experimental Medicine, Sapienza University of Rome (two subjects). The initial genetic characterization of the subject with the L320V/L997F genotype was performed by the Regional Reference Center for Rare Diseases, Department of Pediatrics, University Hospital of Padova (Italy).

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