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Short Report

Does cystic fibrosis neonatal screening detect atypical CF forms? Extended genetic characterization and 4-year clinical follow-up

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The neonatal screening protocol for cystic fibrosis (CF) is based on a first determination of blood immunoreactive trypsin (IRT1), followed by a first level genetic test that includes the 31 worldwide most common mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (DNA31), and a second determination of blood immunoreactive trypsin (IRT2). This approach identifies, in addition to affected subjects, a high proportion of newborns with hypertrypsinaemia at birth, in whom only one mutation is identified and who have a negative or borderline sweat test and pancreatic sufficiency. Although it has been suggested that hypertrypsinaemia may be caused by a single CFTR mutation, whether such neonates should be merely considered as healthy carriers remains a matter of debate as hypertrypsinaemia at birth may be a biochemical marker of a CFTR malfunction because of a second mild mutation. We analyzed, by means of an extended sequencing protocol, 32 newborns who tested positive at an IRT1/DNA31/IRT2 screening protocol and in whom only one CFTR mutation was found. The results obtained demonstrate that 62.5% of these newborns were also carrying a second mild CFTR mutation. The high proportion of compound heterozygous subjects, combined with the results of a 4-year follow-up in nine of these subjects all of whom displaying initial CF clinical symptoms, suggest that it may be possible to use the IRT1/DNA31/IRT2 protocol of neonatal screening to identify newborns with atypical forms of CF. In view of these findings, an extended genetic search for subjects with compound heterozygosity and a periodic clinical assessment should be considered.

L Narzi[a], G Ferraguti[b], A Stamato[a], F Narzi[a], SB Valentini[a], A Lelli[c], I Delaroche[c], M Lucarelli[b], R Strom[b] and S Quattrucci[a]

[a]Department of Paediatrics, Cystic Fibrosis Centre, and [b]Department of Cellular Biotechnologies and Haematology, University of Rome "La Sapienza", Rome, Italy, and [c]Central Laboratory, Italian Red Cross, Rome, Italy

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Corresponding author: Serena Quattrucci, Centro Fibrosi Cistica Regione Lazio, Clinica Pediatrica, Universitàdi Roma "La Sapienza", Viale Regina Elena 324, 00161 Roma, Italia.
Tel./fax: +39-06-49979295;
e-mail: serena.quattrucci@uniroma1.it

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Early diagnosis of cystic fibrosis (CF) improves access to genetic counselling as well as quality of life and survival by allowing early treatment and preventing malnutrition (1–4).

One of the most powerful tools for early diagnosis of CF appears to be an IRT1/DNA31/IRT2 neonatal screening protocol (5). According to this protocol, all newborns who test positive for a first IRT determination undergo a first-level genetic test (6) as well as a second IRT test. Newborns who test positive for either the genetic test or the IRT2 test are enrolled by the CF centre, where a diagnosis based on the presence of two CF mutations and/or a positive sweat test is made. In some newborns enrolled through the IRT1/DNA31/IRT2 protocol, however, the DNA31 step detected only one, or even no, cystic fibrosis transmembrane conductance regulator (CFTR) mutation, possibly linked to borderline sweat test values, which thus raises a diagnostic dilemma (7): in addition to detecting CF-affected subjects with CFTR mutations on both alleles, neonatal screening protocols also identifies heterozygous subjects, the higher IRT value at birth being linked to

the presence of a single CFTR mutation (8–12). These subjects are usually considered as healthy carriers. Neonatal hypertrypsinaemia may, however, also be a biochemical manifestation of a CFTR malfunction because of a second mild mutation. In this newborn population, several authors have recently detected a varying prevalence of second CFTR mutations that are uncommon and/or functionally poorly characterized (8, 13–17). These findings raise issues concerning genetic selection strategies as well as the genotype–phenotype correlation.

The aim of this work was to evaluate, by means of a highly sensitive sequencing method (18), the possible presence of a second mild CFTR mutation or variant tract in a group of heterozygous newborns who tested positive for the IRT1/DNA31/IRT2 screening protocol but had a negative or borderline sweat test and to establish relative frequencies of double mutants and healthy heterozygous carriers. The problems of correct diagnostic classification and adequate therapeutic approaches are obviously all the more relevant, if we consider the high proportion of double mutants identified.

Materials and methods

For all mutational analyses, DNA was extracted from peripheral blood using the QIAamp DNA blood midi kit (QIAGEN, Hilden, Germany). The polymerase chain reaction/oligonucleotide ligation assay/sequence-coded separation technique (6) was applied to search for the 31 most common CF mutations (DNA31, first-level analysis, see supplementary materials and methods on the web). $(TG)_m$ and T_n tracts (for definition, see Discussion) were studied by a sequencing method that allows the simultaneous resolution of the $(TG)_mT_n$ haplotypes of both alleles (19). The extended mutational search (second level analysis) was performed using a 96-well formatted method for the sequencing of the 5′-flanking region, all the exons and the adjacent intronic regions of the CFTR gene (18) (see supplementary materials and methods on the web). Segregation of the mutations in double-mutant newborns was verified by parents' analysis.

For 7 years (1998–2004), an IRT1/DNA31/IRT2 screening protocol for CF (5, 20, 21) was applied to 167,000 newborns in the Lazio region (see supplementary materials and methods on the web); 182 subjects among those positive to the screening were enrolled by the regional CF centre and submitted to (i) a sweat test, using a quantitative pilocarpine iontophoresis method (22); (ii) a pancreatic function evaluation, using the stool chymotrypsin test (Chymo, Roche, Mannheim, Germany) (23) and (iii) a clinical examination. For the purposes of this study, we enrolled, from the aforementioned 182 subjects (with transient or persistent hypertrypsinaemia at birth) and referred to the CF centre for diagnosis, a random group of 32 neonates who had no other CF symptoms, who had a negative ($[Cl^-] < 30$ mmol/l) or borderline ($[Cl^-]$ 30 − 59 mmol/l) sweat test and in whom only one CFTR mutation had been detected in the preliminary genetic assay. Neonates with persistent hypertrypsinaemia but no mutations to the basic DNA31 panel and negative sweat test were not enrolled.

All 20 families of double-mutant neonates were recalled for a 4-year follow up. Among these, only nine accepted and underwent a clinical assessment, including evaluation of nutritional status by weight and height centiles, laboratory assessment including serum liver enzyme levels (aspartate aminotransferase, alanine aminotransferase and γ-glutamyltransferase), evaluation of liver disease by hepato-pancreatic ultrasonography, if clinical hepatomegaly or abnormal liver enzyme levels are present (24), evaluation of microbiological status by oropharyngeal cultures through deep suctioning (see supplementary materials and methods on the web) and evaluation of chest radiographs according to Chrispin and Norman's protocol (25).

A group of 94 subjects selected from the general population, mainly comprising healthy blood donors and the non-carrier partner of intermediate risk couples, were analyzed to assess the frequency of uncommon mutations found by the extended search.

Results

A total of 277 newborns tested positive for the IRT1/DNA31/IRT2 protocol (all positive at the IRT1 and some at the IRT2 as well) (5). Of these, 182 were evaluated by the CF centre. A total of 44 (24.2%) were diagnosed as being affected by typical CF, 28 (15.4%) were considered as non-affected ($[Cl^-] < 30$ mmol/l and no mutation found by the DNA31 panel) and 110 (60.4%) were found to be heterozygous for a common CF mutation. Thirty-two newborns without clinical symptoms of CF, randomly selected from the 110 heterozygous subjects, were examined by means of our extended protocol of mutational analysis (18) to investigate the possible presence of a CFTR genotype with mutations on both alleles. Of these 32 subjects (Table 1), 13 (40.6%) also tested positive for the IRT2 and were therefore considered

Table 1. CFTR genotypes, IRT2 and sweat test values of the 32 newborns analyzed

Newborn	CFTR genotype	IRT2	Sweat test (mmol/l [Cl^-]) at enrolment
True heterozygous subjects			
1	N1303K/+	Negative	18
2	2183AAtoG/+	Negative	11
3	G85E/+	Positive	19
4	F508del/+	Negative	21
5	F508del/+	Negative	20
6	R117H/+	Negative	6
7	1717-1GtoA/+	Positive	7
8	W1282X/+	Negative	14
9	2789+5GtoA/+	Negative	23
10	N1303K/+	Negative	19
11	F508del/+	Negative	14
12	G542X/+	Negative	39
		% of positivity = 16.7%	Average ± SD = 18 ± 9
Compound heterozygous subjects			
13	F508del/D806G	Positive	24
14	F508del/D836Y	Negative	12
15	R347P/R1162L	Negative	18
16	F508del/P5L $(TG)_{11}T_5$	Negative	16
17	F508del/L997F	Positive	32
18	R347P/D1152H	Positive	42
19	F508del/P5L	Negative	42
20	2789+5GtoA/711+3AtoG	Positive	33
21	F508del/P5L	Positive	39
22	F508del $(TG)_{12}T_7/(TG)_{12}T_5$	Negative	23
23	N1303K/S1235R $(TG)_{12}T_7$	Negative	30
24	F508del/L997F	Positive	34
25	F508del/$(TG)_{12}T_5$	Negative	34
26	R117H/$(TG)_{12}T_7$	Positive	22
27	F508del/P1013L	Positive	8
28	F508del/L997F	Negative	28
29	N1303K/$(TG)_{12}T_5$	Positive	13
30	F508del/L997F	Positive	50
31	R1162X/P5L	Negative	31
32	L997F/S549R(AtoC)	Positive	38
		% of positivity = 55.0%	Average ± SD = 29 ± 12

CFTR, cystic fibrosis transmembrane conductance regulator.

to have a persistent hypertrypsinaemia since birth. None of these newborns had a [Cl^-] > 50 mmol/l, while 13 (40.6%) had a [Cl^-] ranging from 28 to 50 mmol/l. All 32 newborns displayed pancreatic sufficiency, with stool chymotrypsin values >8.4 U/g (wet weight).

Five of the 31 mutations included in the basic genetic panel were found to occur in more than 1 of the 64 alleles examined (Table 1 and Fig. 1): F508del in 15 alleles (23.4%), N1303K in four alleles (6.3%), 2789 + 5G->A in two alleles (3.1%), R117H in two alleles (3.1%), and R347P in two alleles (3.1%).

No additional mutations besides those already found in the first level were detected by the extended genetic analysis in 12 (37.5%) heterozygous newborns (Table 1). Twenty alleles carrying CFTR mutations or variant tracts were instead found in the 20 (62.5%) remaining newborns (Table 1). The L997F and the P5L mutations were found, respectively, in five (7.8%) and four (6.3%) alleles, which is a significantly higher frequency than that found in the general population (see Discussion) ($\chi^2 = 10.6$, d.f. = 1, $p < 0.01$ and $\chi^2 = 11.7$, d.f. = 1, $p < 0.01$, respectively). All the other mutations were found on single alleles (Table 1

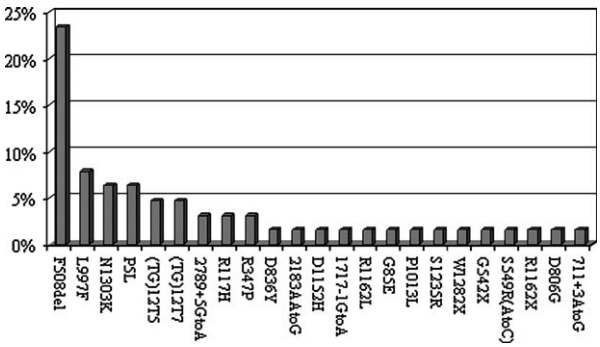

Fig. 1. Allelic frequencies of the cystic fibrosis transmembrane conductance regulator mutations found.

and Fig. 1). The $(TG)_{12}T_5$ and $(TG)_{12}T_7$ variant tracts were each found in three alleles (4.7%); two of the three $(TG)_{12}T_7$ tracts occurred, however, on complex alleles that also carried an exonic CFTR mutation (Table 1). On the basis of these genetic results, it may be possible to divide the hypertrypsinaemic subjects into two subgroups (Table 1): true heterozygous subjects (with only one affected allele) and those with a compound heterozygosity (with two distinct CFTR mutations or variant tracts located on different alleles, as assessed by parents' segregation analysis).

The pathogenic role of the majority of the mutations found is well established, whereas that of some of the uncommon mutations has yet to be fully elucidated (see Discussion). A preliminary analysis of the pathogenic role of a mutation may be made by comparing its frequency between target and general populations. This type of analysis has already been performed for some of the uncommon mutations found in this work (R1162L, S1235R and L997F, see Discussion), while we performed frequency studies for D836Y, P1013L, P5L and D806G. None of these last four mutations were found in 188 alleles of a general population. We also assessed the frequency of L997F in our general population, finding one allele with this mutation, which is similar in frequency (0.5%) to that found in a previous study (see Discussion). In addition, an extended genetic analysis did not detect any other mutations in the alleles carrying the seven mutations listed above (with the exception of S1235R, which was found in a complex allele with the $(TG)_{12}T_7$ variant tract).

No significant differences between the two groups of hypertrypsinaemic subjects (true heterozygous and compound heterozygous) emerged from the average values of either IRT1 or IRT2 determinations, or from the stool chymotrypsin test (data not shown, all Student's *t*-test NS). By contrast, the frequency of positive IRT2 tests was significantly higher ($\chi^2 = 4.6$, d.f. $= 1$, $p < 0.05$) in the compound heterozygous group (55.0%) than in the true heterozygous group (16.7%). The sweat test values at birth were also significantly higher (Student's *t*-test $= 2.9$, d.f. $= 30$, $p < 0.01$) in the compound heterozygous group (average $[Cl^-] = 29$ mmol/l) than in the true heterozygous group (average $[Cl^-] = 18$ mmol/l) (Table 1).

If neonates were partitioned accordingly by sweat test values, greater than or equal to 30 mmol/l of $[Cl^-]$ ($n = 12$) or below this value ($n = 20$), significant differences in the frequency of double mutants (91.7% *vs* 45.0%, respectively, $\chi^2 = 7.0$, d.f. $= 1$, $p < 0.01$), as well as expected in sweat test value (37 ± 6 SD *vs* 17 ± 6 SD, respectively, Student's *t*-test $= 9.3$, d.f. $= 30$, $p < 0.01$) was evident. The frequency of IRT2 positivity resulted not significantly different.

A 4-year follow-up was performed on nine compound heterozygous newborns (clinical findings in Table 2), randomly selected from the 20 available. The sweat test values increased in eight of these subjects over the 4-year interval, the mean overall value of the all nine subjects rising significantly from 30 mmol/l of chloride at birth to 44 mmol/l (paired Student's *t*-test $= 2.5$, d.f. $= 8$, $p < 0.05$). Seven of nine newborns exhibited clinical symptoms because of involvement of the upper airways, nasal turbinate hypertrophy or development of sinus disease. Moreover, seven showed mild respiratory signs because of involvement of the lower airways, with bronchial thickening and air trapping. One of the two newborns with a normal clinical examination exhibited radiological signs (patient 18). Chrispin's score, used to assess the chest X-ray, revealed mild lung abnormalities (mean value 5 ± 3 SD). The patient's growth was normal in all cases except two subjects, who were at 18th and 20th centiles of weight and were found to be intolerant to cow's milk protein(s). Five of the nine subjects, those with clinical hepatomegaly (see Materials and methods), underwent a hepato-pancreatic ultrasonography, which revealed some degree of liver involvement, i.e. hepatomegaly (2 subjects), initial focal biliary cirrhosis (2 subjects), and thickening of portal spaces (1 subject), although serum liver enzyme levels were normal. None of the children were chronically colonized with *Pseudomonas aeruginosa* in the airways. In conclusion, all nine subjects developed at least one initial symptom of CF during the follow-up period.

Discussion

The majority of the mutations found (F508del, R347P, D1152H, 2789 + 5G->A, 711 + 3A->G, N1303K, R117H, R1162X, S549R(A->C), 2183AA->G, G85E, 1717-1G->A, G542X, and W1282X) have an established pathogenic role (26–44). The $(TG)_{12}T_5$ and $(TG)_{12}T_7$ variant tracts may act as pathogenic by altering splicing patterns, at least in atypical mono/oligosymptomatic forms of CF (45–47). By contrast, the pathogenic role of some of the uncommon mutations found (P5L, D836Y, P1013L, D806G, L997F, S1235R, and R1162L) is still a matter of debate (15, 17, 28, 32, 48–58). The P5L, D836Y, P1013L, and D806G mutations were found to be absent from the general population in the search performed in this work. L997F, S1235R and

Table 2. Clinical features of the nine newborns followed up for 4 years

Newborn	Genotype	First sweat test (at birth)	Average sweat test (1–4 years)	Symptoms at screening	Symptoms during 4-year follow-up	Chest X-ray	Chrispin score	Weight (centiles)	Height (centiles)	Abdominal ultrasonography	Bacterial isolates
28	F508del/L997F	28	43	Negative	Chronic rhinosinusitis and pharyngitis; sinus disease	Positive	6	100	94	Positive (initial focal biliary cirrhosis)	Negative culture
24	F508del/L997F	34	69	Negative	Hospitalization for severe bronchiolitis; recurrent rhino-pharyngitis, sometimes productive cough	Positive	6	32	43	Positive (hepatomegaly)	Negative culture
30	F508del/L997F	50	62	Negative	Pharyngitis, recurrent abdominal pain	Positive	4	20	79	Positive (thickening of portal spaces)	Negative culture
27	F508del/P1013L	8	20	Negative	Negative	Negative	2	99	76	Positive (hepatomegaly)	Negative culture
31	R1162X/P5L	31	71	Negative	Productive cough, widespread osteoporosis, nasal obstruction	Positive (bronchiectasis)	7	18	98	Positive (initial focal biliary cirrhosis)	*Staphylococcus aureus*
23	N1303K/ S1235R $(TG)_{12}T_7$	30	18	Negative	Sometimes rhinitis	Negative	2	64	96	ND	Negative culture
29	N1303K/$(TG)_{12}T_5$	13	28	Negative	Bronchiolitis, chronic rhinitis, sporadic episodes of cough	Positive	4	59	21	ND	Negative culture
32	L997F/S549R(A->C)	38	41	Negative	Episodically productive cough, chronic rhinitis, bronchitis	Positive	7	56	67	ND	Negative culture
18	R347P/D1152H	42	44	Negative	Negative	Positive	10	44	56	ND	*S. aureus*
	Average ± SD =	30 ± 13	44 ± 20				5 ± 3	55 ± 30	70 ± 26		

ND, not determined.

R1162L have also been shown, in previous reports (49, 54), to be less frequent in the general population (0.5%, a percentage confirmed in this work, 0.3% and 0.3%, respectively). The absence or lower frequency of L997F, S1235R and R1162L in the general population than in our target group is compatible with a possible pathogenic role of these mutations. The L997F and P5L mutations were significantly more frequent in the newborns we studied than in the general population (7.8% and 6.3%, respectively). In all our subjects, each of these seven mutations was found as a unique mutation of the allele studied, with the exception of S1235R, for which a cis-acting effect by the $(TG)_{12}T_7$ variant tract found on the same allele may be hypothesized. The data in the literature on the pathogenic role of these seven mutations are both scarce and problematic, often based on indirect evidence. However, a possible atypical CF causing role of each of them, although with variable phenotypic manifestations, appears at least conceivable (15, 17, 32, 48, 50–52, 54–59) (see supplementary material on the web).

Taken together, these results indicate a probable pathogenic role for all these mutations. Should this conclusion prove to be correct, there would be a high proportion (62.5%) of newborns with hypertrypsinaemia and one CFTR mutation at birth who were subsequently found to be compound heterozygotes, and are consequently more likely to develop CF as adults. This high proportion of compound heterozygotes may be related to our screening cut-off values (20, 21). It should be taken into account that a lower cut-off could select newborns less likely to have a second pathogenetic mutation, resulting in a lower proportion of potentially affected subjects. Indeed, all 9 (out of 20) compound heterozygotes subjects who underwent a prolonged clinical follow-up proved to be pathologically affected to some degree. Although respiratory impairment was moderate, early hepato-pancreatic disease was observed in all four subjects who underwent abdominal ultrasonography, while serum liver enzyme levels were normal.

The sweat test values resulted significantly higher in compound heterozygotes. Although a clear sweat test cut-off value could not be defined, it appeared justified to perform the extended genetic analysis when the $[Cl^-] > 30$ mmol/l. However, because of the considerable overlap the best way to identify double-mutants newborns is extended genetic analysis (see also the supplementary materials on the web on the M470V polymorphism). The high frequency of both L997F and P5L mutation suggests that these should be included in mutational panels that are specific for neonatal screening, at least in Italy.

Three main conclusions can be drawn from these results: firstly, a large proportion of newborns who are positive at the neonatal screening, and are initially characterized as carriers, prove to be compound heterozygotes; secondly, although there are significant differences in some biochemical parameters between true carriers and compound heterozygous subjects, the overlap is too great for these biochemical markers to be used in selection procedures; thirdly, the types of mutations represented in compound heterozygous subjects, as well as the 4-year follow-up findings, indicate that atypical mild forms of CF can be identified by means of a neonatal screening programme; a more extensive assessment of the clinical relevance of these atypical forms combined with a thorough economic analysis of the costs involved may shed light on the priority of this goal.

Supplementary material

Supplementary Materials And Methods
Phenotype Of Uncommon Mutations Found
The M470V Polymorphism

Supplementary materials are available as part of the online article at http://www.blackwell-synergy.com

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