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Negative sweat test in hypertrypsinaemic infants with cystic fibrosis carrying rare *CFTR* mutations

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Abstract Persistent hypertrypsinaemia in newborn screening for cystic fibrosis (CF) recognises subjects at high risk to be affected. Diagnosis is confirmed by a positive sweat test and/or by the presence of two mutations in the cystic fibrosis transmembrane regulator gene. The aim of the present study was to evaluate the occurrence of a negative sweat test (chloride < 60 mmol/l) during the first months of life, in hypertrypsinaemic infants, which would lead to a delayed diagnosis. We reviewed clinical charts of CF patients born between January 1993 and September 1998, when the neonatal screening programme consisted of an immunoreactive trypsinogen (IRT)/DNA (F508del) + IRT strategy. Laboratory and clinical data were collected for patients diagnosed after 12 months of life. Out of 446,492 newborns, 104 CF patients were diagnosed giving an overall incidence of 1:4293. Of these, six had a blood IRT level above the cut off value (99th percentile) and a negative sweat test in the first trimester of life. At a mean age of 3.5years, the patients were again referred to our CF Centre for re-evaluation in order to confirm or exclude the disorder. Molecular analysis identified the following genotypes: F508del/A309D, F508del/3849 + 10kbC→T, F508del/R117H (in two patients), R117H/L997F, and F508del/R117L. *Conclusion:* infants with cystic fibrosis bearing a spectrum of mild cystic fibrosis transmembrane regulator gene mutations may present as hypertrypsinaemic newborns with a sweat chloride within the normal range. Reference values for normal sweat test during the first months of life should be revised. A wide molecular genetic analysis is recommended for newborns presenting persistent hypertrypsinaemia and a sweat test result > 30 mmol/l in order to diagnose atypical forms of the disease.

Keywords *CFTR* gene · Cystic fibrosis · Delayed diagnosis · Sweat test

Abbreviations *b-IRT* blood-immunoreactive trypsinogen · *CF* cystic fibrosis · *CFTR* cystic fibrosis transmembrane regulator · *DGGE* denaturing gradient gel electrophoresis · *OLA* oligonucleotide ligation assay

Introduction

Newborn screening for cystic fibrosis (CF) recognises subjects with persistent hypertrypsinaemia in the first months of life [5]. Diagnosis is confirmed by a positive sweat test (chloride > 60 mmol/l) and/or by the presence of two changes in both the cystic fibrosis transmembrane regulator (CFTR) gene [19].

Diagnostic delay has been reported for patients bearing specific *CFTR* gene mutations such as R117H, D1152H, 3849 + 10kbC→T, A455E and 2789 + 5G→A, which were associated with a non-pathological sweat chloride level [3, 6, 9,13]. The aim of our study was to evaluate whether some of the infants diagnosed by means of neonatal screening at our centre, although correctly selected by persistent neonatal hypertrypsinaemia, had received a delayed diagnosis because of a sweat chloride concentration < 60 mmol/l which is presently considered the upper limit of the reference range. We also investigated whether normal sweat chloride values could be related to peculiar *CFTR* mutations.

Patients and methods

The study was carried out in CF patients born between January 1993 and September 1998 in the Lombardy Region (Northwestern

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Italy) and regularly followed at the Milan CF Centre. Data of all these patients are collected in a national database, the Italian CF National Registry [17]. Patients with a CF diagnosis beyond 1 year of age were selected and their neonatal screening results were reviewed. There were six true false-negative patients (5.7%) with blood immunoreactive trypsinogen (b-IRT) levels in the normal range in the first sample or at recall who are subject of a separate report [16].

Six patients were persistently hypertrypsinaemic and had a sweat chloride < 60 mmol/l during the first trimester of life: their clinical features (symptoms at diagnosis) and laboratory data, with particular regard to neonatal screening results and sweat test values at different ages were reviewed. The neonatal screening strategy in the considered period was a two-step, two-tiered programme consisting in detection of b-IRT followed by genetic analysis for identification of the F508del *CFTR* gene mutation, by means of polyacrylamide gel electrophoresis on Guthrie cards, in all hypertrypsinaemic infants (IRT/DNA + IRT). The sweat test was performed using quantitative pilocarpine iontophoresis [11].

Genetic analysis was initially performed by PCR and oligonucleotide ligation assay (OLA) [10], then an extended analysis of the *CFTR* gene was performed using denaturing gradient gel electrophoresis (DGGE) analysis and sequencing as previously reported [1]. An allele-specific PCR assay was used to distinguish the 5T, 7T and 9T alleles. Amplification products were visualised on ethidium bromide stained 4% agarose gels.

Results

During the study period, a total of 446,492 newborns were screened of whom 8,126 presented a b-IRT level above the cut-off value (99th percentile) and underwent a recall test (recall index: 1.82%). Of the hypertrypsinaemic newborns, 77 CF infants were identified having a pathological sweat chloride above 60 mmol/l, 15 patients presented with meconium ileus, six subjects born in the same period were true false-negatives in the neonatal screening programme (neonatal b-IRT in the normal range), and six infants discharged as false-positives in the screening programme were diagnosed beyond I year of life, giving an overall CF incidence of 1:4293 (104 cases).

The six infants (two males) were considered as false-positives because they presented persistent hypertrypsinaemia, but repeated sweat test were within the normal (< 40 mmol/l) or borderline (40–60 mmol/l) ranges in the first trimester of life. Clinical assessment at the time of the first sweat test did not revealed any CF-related symptoms in any patients. F508del was identified in five chromosomes within the neonatal screening programme and the patients were diagnosed as carriers; the parents were offered genetic counselling and genetic analysis.

At a median age of 3.5 years (range 18 months–6.5 years) the patients were again referred to our CF Centre. They were re-evaluated for CF because of respiratory symptoms (patient 1), recurrent nasal polyps requiring surgery (patient 2), positive familial history (patient 3), or because their families asked for a wide genetic analysis in view of further pregnancies (patients 4, 5, 6) (Table1). Genetic analysis performed with PCR/OLA assay identified two other mutations (R117H in three patients and 3849 + 10kbC→T in one). A subsequent expanded analysis of the *CFTR* gene, by means of DGGE analysis and sequencing, was performed on the remaining three chromosomes and identified the following *CFTR* alterations: R117L, L997F, and A309D. The 5T allele was identified only in patient 4.

According to the current criteria [19], a diagnosis of CF was confirmed in all subjects. However, on a repeat sweat test, only two patients showed abnormal chloride values (patients 1 and 2), although the sweat chloride concentration found in patient 3 (R117H-7T/F508del) was higher than that found in previously performed sweat tests. All patients underwent clinical assessment: all presented pancreatic sufficiency and growth was in the normal range in all but one (patient 4). Patients 1 and 2 presented lung disease and isolated severe nasal polyps respectively, whereas patients 3 and 5 (R117H-7T/F508del) suffered from recurrent upper respiratory infections, showing only bronchial thickening in the lower lobes on a chest X-ray film. Following adequate treatment (inhaled anti-inflammatory and bronchodilator therapy plus chest physiotherapy, and antibiotics, if needed), all patients showed improvement in their respiratory symptoms.

Table 1 Diagnostic features of patients

Patient number	Sex	First IRT (ng/ml) (cut-off)	Second IRT (ng/ml) (cut-off)	Sweat test chloride (mmol/l)	Age at sweat test	Age at re-evaluation	Symptoms	Repeat sweat test chloride (mmol/l)	Genotype
1	M	47 (40)	39 (30)	43	4 months	3 years and 3 months	Chronic respiratory	64	ΔF508/A309D
2	M	174 (55)	112 (40)	< 60	4 months	6 years and 6 months	Severe nasal polyposis	68	ΔF508/3849 + 10kbC→T
3	F	56 (55)	64 (40)	34	4 months	5 years and 4 months	Recurrent upper airways infections	55	ΔF508/R117H-7T
4	F	84 (80)	102 (40)	55	4 months	4 years	No symptoms	Not determined	R117H-5T/L997F
5	F	142 (80)	81 (40)	37	3 months	20 months	Recurrent upper airways infections	47	ΔF508/R117H-7T
6	F	90 (80)	55 (40)	36	2 months	18 months	No symptoms	49	ΔF508/R117L

Discussion

Our retrospective evaluation of patients diagnosed beyond 1 year of age at our centre over a ca. 6-year period shows that hypertrypsinaemic newborns carrying at least one "mild" *CFTR* mutation may have a chloride sweat test below 60 mmol/l and a delayed CF diagnosis. Rare mutations in the *CFTR* gene were identified in six patients showing increased b-IRT on newborn screening and a normal sweat test: R117H (three cases), R117L, A309D, L997F and the intronic alteration 3849 + 10kbC→T. In the whole CF population followed at the Milan CF Centre (580 patients), R117L, A309D and L997F have never been identified before, whereas R117H and 3849 + 10kbC→T account for only 0.51% and 0.68% of alleles, respectively.

It has been recently suggested that all patients with sweat chloride values within the borderline range (30–60 mmol/l) should be re-evaluated and that the present upper limit of normal sweat chloride concentration in infants be lowered to 40 mmol/l [8,21]. In order to accurately diagnose CF, each laboratory performing sweat tests in infants selected by a positive newborn screening procedure, should establish its own reference ranges, based on a high number of subjects (healthy subjects, heterozygotes, CF patients) [4].

In order to improve the screening programme efficacy and to avoid a diagnostic delay in infants bearing rare mutations, since October 1998 we have modified our screening strategy by adding a PCR/OLA assay for the identification of up to 31 *CFTR* mutations in all hypertrypsinaemic neonates. If we had applied this strategy to the population considered in the present study, we could have recognised 9/12 *CFTR* mutations in the first blood sample. PolyT testing has been recommended to establish whether the R117H mutation is associated with CF, however in our patients the R117H-7T allele was associated with persistent neonatal hypertrypsinaemia, a sweat chloride in the upper borderline range, and recurrent respiratory symptoms in the first years of life. The diagnosis of CF was made in patient 3 after the birth of a sister with persistent neonatal hypertrypsinaemia, a sweat test above 30 mmol/l chloride and the same genotype (F508del/R117H-7T). It is well known that in patients with atypical CF (such as CBAVD, pancreatitis, nasal polyps, ABPA and disseminated bronchiectasis) some of the phenotypic variability can result from an alternative splicing of exon 9 in the *CFTR* gene mediated by tissue specific and/or developmentally controlled changes in the concentration of splicing factors. In monosymptomatic forms of CF, the partial penetrance of the T5 allele can be modulated not only by the TG repeats upstream, but also by variability in the individual tissue concentration of splicing factors [18].

The diagnostic dilemma due to a negative or borderline sweat test in a hypertrypsinaemic newborn could be overcome by new technologies such as assessment of chloride conductance in respiratory and intestinal tissue by determination of nasal potential difference and intestinal current measurements [15]. These measures provide information about the residual function of mutant *CFTR* genes and may be of help in patients in whom a diagnosis of CF is suspected but the sweat test remains inconclusive [7]. Since nasal epithelial dysfunction is present in infants with CF early after birth, nasal potential difference may be determined even in neonates and represent a useful diagnostic adjunct to the sweat test in the early diagnosis of CF [12,20].

In only two of our patients (patients 1 and #2) did a delay in diagnosis have significant clinical consequences (chronic respiratory symptoms without adequate treatment in patient 1 and repeated surgery for nasal polyps in patient 2). However, a longer follow-up is needed to establish the clinical long-term outcome of non-classical CF patients. In addition, studies on genotype-phenotype correlations should help to define the characteristics of the disease in hypertrypsinaemic infants with mild or atypical CF, who may later in life develop minimal or moderate chronic lung disease [14] or chronic idiopathic pancreatitis, and if males, congenital bilateral absence of the vas deferens. Bush and Wallis [2] have proposed that infants detected by neonatal screening but without clinical symptoms in the first months or years of life be considered as "pre-CF" [2], but the issue of classification of subclinical disease remains to be defined. A working group was specifically established by the WHO, ICF(M)A and European CF Society in June 2000 to consider these problems and a diagnostic classification of CF and all conditions associated with *CFTR* mutations has been recently proposed [22].

Further improvement in newborn screening strategies for CF could be reached by adding nasal potential difference or intestinal current measurements for early recognition of non-classical forms of CF, together with a wide molecular genetic study. The early diagnosis of CF patients bearing mild *CFTR* mutations followed by referral to a specialised CF centre may prevent chronic lung disease in adulthood. In the near future, the discussion about the scope and strategies of neonatal screening for CF should also take this point into consideration.

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