Nine cystic fibrosis patients homozygous for the CFTR nonsense mutation R1162X have mild or moderate lung disease

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Abstract

The clinical course of nine cystic fibrosis patients homozygous for the CF gene nonsense mutation R1162X was investigated. Since this mutation should lead to an interruption in the synthesis of the cystic fibrosis transmembrane regulator (CFTR) protein, a severe clinical course was expected. All patients showed pancreatic insufficiency, while the course of the lung disease was mild to moderate. These results suggest that this form of truncated CFTR protein, still containing the regulatory region, the first ATP binding domain, and both transmembrane domains, could be partially working in the lung tissues.

Among them, the R1162X nonsense mutation¹⁵ is relatively frequent in north-eastern Italy¹⁴; therefore patients carrying this mutation on both their genes are more easily found. The R1162X mutation is characterised by the substitution of the amino acid arginine in position 1162 of CFTR with a UGA termination codon.¹⁵

Here we report our results on the correlation between the R1162X mutation and the clinical manifestations of cystic fibrosis, including meconium ileus, pancreatic function, lung involvement, growth rate, etc, as shown by the nine patients identified so far in our population who are homozygotes for the mutation. The possible pathophysiological role of the R1162X mutation is discussed.

Cystic fibrosis (CF) is an autosomal recessive disease with a prevalence of about 1 in 2000 live births and a carrier frequency of approximately 1 in 23 in Caucasian populations.¹ At the end of 1989, the cystic fibrosis gene was identified. On the basis of the nucleotide sequence, the CF gene was predicted to encode a 1480 amino acid membrane glycoprotein, the 'cystic fibrosis transmembrane conductance regulator' (CFTR).²⁻⁴ The deduced amino acid sequence suggests that CFTR has several functionally important regions, including two ATP binding domains, two transmembrane domains, and a highly charged domain with several potential sites for phosphorylation by protein kinases. Its structure shows homology with the ATP binding cassette transport systems.⁵ The function of CFTR is still unknown, even if recent studies confirm that it may well be involved in cAMP activated chloride ion transport.^{6,7}

The identification of naturally occurring deleterious gene mutations and the characterisation of their relation to disease phenotypes could help us to understand the functional importance of CFTR and of its putative domains. For this purpose, patients homozygous for a specific mutation are needed, in which the phenotypic expression of the disease may be ascribed to that specific gene alteration. Unfortunately, a useful number of homozygous patients can be collected only when the particular mutation being investigated reaches a high frequency in a given population. Therefore, only the clinical correlation of the most common CF mutation, $\Delta F508$,⁸⁻¹⁰ has been described in detail so far.¹¹⁻¹³

Several other mutations have been detected, but none of them seems to be very common.¹⁴

Subjects and methods

DNA TYPING

Data were collected from 187 unrelated Italian patients with cystic fibrosis attending the CF Centre in Verona. Blood samples for DNA genotyping were obtained in each family from the parents and one affected child. The diagnosis of CF was confirmed by at least two positive sweat tests, performed according to the method of Gibson and Cooke.¹⁶ Genomic DNA was prepared from whole blood by standard methods. All patients were analysed for the $\Delta F508$ gene deletion and for the R1162X nonsense mutation. Primers for PCR were selected from the flanking intron sequence of exon 10⁴ and of exon 19¹⁵ for analysis of $\Delta F508$ and R1162X, respectively. PCR was performed according to standard protocols in a thermal cycler (Perkin Elmer Cetus Co, USA). The amplified fragments corresponding to the region of $\Delta F508$ were subjected to heteroduplex analysis.¹⁷ The presence of the R1162X mutation was investigated by restriction digestion with *DdeI*, followed by fragment visualisation in 2% Nusieve agarose minigels.

Clinical assessment

PATIENTS

Out of 187 unrelated CF patients analysed for genetic mutations, nine were found to be homozygous for the R1162X mutation including a couple of brothers. This group consisted of five males and four females. Retrospective data on sweat test values, clinical features, and pancreatic involvement at diagnosis were collected. Current data at the last visit include growth and nutritional status, respiratory function, pulmonary radiological findings, and

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bacterial cultures of deep sputum. A tenth R1162X/R1162X patient was not considered because he was being treated in a different centre.

PANCREATIC FUNCTION

Exocrine pancreatic function was studied by duodenal intubation and pancreatic stimulation test (Pancreozymin and secretin bolus 2 U/kg body weight). Output over 30 min/kg body weight of bicarbonate (gasanalytic method), trypsin, chymotrypsin, and lipase (titrimetric method) were determined¹⁸ as previously described.¹¹

Digestive function was studied by a three day fat balance or by 24 hour steatorrhoea determination with analysis of fecal fat according to the method of Van Der Kamer, modified by Jeejeebhoy *et al.*,¹⁹ as previously described.¹¹

CLINICAL STATUS, GROWTH, AND NUTRITION

Clinical status was evaluated by the Shwachman and Kulczycki score.²⁰ On this score the best value is 100. Height and weight centiles and weight expressed as a percentage of ideal weight for height were computed with the use of the tables of Tanner *et al.*²¹

MECONIUM ILEUS AND OTHER COMPLICATIONS

Ileal obstruction at birth requiring medical or surgical therapy was defined as meconium ileus. Other major complications typically associated with CF were investigated, including meconium ileus equivalent, liver disease and cholelithiasis (clinical, biochemical and ultrasonographic investigations), glucose intolerance (OGTT test) and diabetes, haemoptysis, pneumothorax (PNX), lobar atelectasis, nasal polyposis, and allergic bronchopulmonary aspergillosis (ABPA).

PULMONARY DISEASE

Respiratory function was assessed in the patients over 6 years of age by the following index: FVC (forced vital capacity), FEV1 (forced expiratory volume in one second), and FEF 25–75 (forced expiratory flow rate in the middle half of expiration), expressed as a percentage of normal predicted values for height and sex, according to Hibbert *et al.*²²

Pulmonary radiographs were evaluated using the Chrispin-Norman score²³: it ranges from 0 (no damage) to 38 which is the worst score. Onset of *Pseudomonas* colonisation was defined as the first 6 month period in which sputum cultures were repeatedly positive for *Pseudomonas aeruginosa*.

ASSESSMENT OF THE DEGREE OF PULMONARY DISEASE

In order to define the degree of pulmonary disease of the R1162X homozygotes, their last chest radiograph score values were compared to the longitudinal score centiles of a cohort of

CF patients. This cohort consisted of all the CF patients diagnosed in the Veneto and Trentino regions from 1.10.73 to 31.12.81 and all treated at the Verona CF Centre. They were checked by chest radiography nearly every six months. The 25th, 50th, and 75th centiles of the Chrispin-Norman score values were computed for 20 month subgroups from 50 to 210 months of age (individual scores within the subgroups were averaged).

Furthermore the last Chrispin-Norman score values of the nine R1162X homozygotes were compared to the last score values of 37 unselected $\Delta F508$ homozygotes.

Results

Out of 187 unrelated patients analysed for the presence of the $\Delta F508$ and R1162X mutations, nine homozygotes for the latter mutation were found, including a couple of brothers. Their present ages range from 5 to about 24 years.

The clinical features of these patients are shown in the table. Two of them (nos 1 and 3) were detected through neonatal screening performed with blood spot immunotrypsin and meconium lactase assays.²⁴ Two were diagnosed through meconium ileus (nos 4 and 9), four through digestive symptoms, and only one through respiratory as well as digestive ones (no 6). One of them (no 5) had borderline sweat test results.

All the patients showed pancreatic involvement at birth. The exocrine pancreatic function, investigated in six cases by duodenal intubation, was characterised by very decreased output of enzymes and bicarbonate, and a high degree of fat loss was shown in all nine patients: all had been treated with pancreatic enzyme supplementation since diagnosis. Only two adolescent subjects had poor nutritional status (nos 5 and 7) and another had very short stature at 12.7 years.

The onset of respiratory symptoms occurred in all but one of the subjects after the first year of life. One of them developed pneumothorax at 16 years; he is now 18 years old and no important deterioration in respiratory function or symptoms has been observed so far. No other major respiratory complications like haemoptysis or lobar atelectasis were recorded in the whole group. Nasal polyposis was present in two sibs (nos 3 and 7) and allergic bronchopulmonary aspergillosis (ABPA) occurred in two cases (nos 4 and 5).

Lung function at the last test showed a moderately obstructive picture in three patients (nos 5, 6, and 7); there was mild obstruction in the oldest patients (nos 8 and 9) and normal or near normal respiratory function in the youngest ones (nos 2, 3, and 4). The individual chest x ray score at the last visit was compared to that of a CF cohort (fig 1): eight out of nine patients showed values lower than the 50th centile of the reference CF population. $\Delta F508$ frequency in the cohort was 48%. When compared to an unselected group of $\Delta F508$ homozygotes the x ray scores of the R1162X homozygotes were in the mid-low range of the reference group (fig 2).

Main clinical features of the R1162X homozygous CF patients.

Clinical data and laboratory results		Patients								
		1	2	3	4	5	6	7	8	9
Sweat test (mEq/kg)	Na Cl	118 109	80 100	101 103	92 105	51 68	104 105	91 93	80 94	133 114
Age at diagnosis (y)		0.1	1.2	0.1	0.1	0.3	0.3	0.4	6	0.1
Current age (y)		5	7.1	8.2	12.7	15.5	16.9	18.1	18.2	23.9
Diagnosis by										
Neonatal screening		+		+						
Meconium ileus					+	+		+	+	+
Only digestive symptoms			+							
Respiratory and digestive symptoms							+			
Pancreatic involvement at diagnosis										
Pancreatic secretion (output/30'/kg)										
Bicarbonate*		—	—	5.3	—	2.4	—	0.0	—	2.7
Lipase†		—	—	17.9	—	2.1	3.4	0	0	0
Trypsin‡		—	—	2.7	—	0	9	0	8	0
Fat absorption coefficient (%)*§		—	74	57	32	76	—	56	—	36.5
Steatorrhea (g/24h)		14.6	—	—	18.2	—	9.9	—	22	—
Current weight (kg)		18.2	21.6	28.4	32.8	46.8	50.8	43.2	54.2	46.4
(Centile)		(50)	(25–50)	(75–90)	(3–10)	(3–10)	(10–25)	(<3)	(10–25)	(3–10)
Height (cm)		112	123	138	138	169	163	161	171	156
(Centile)		(75)	(75)	(>97)	(3)	(25–50)	(50–75)	(25–50)	(25–50)	(10)
Weight/height (% predicted)		94	92	89	103	83	90	79	90	98
Current respiratory function										
CVF (% predicted)		—	86	97	95	63	79	83	85	92
FEV1 (% predicted)		—	94	94	81	45	58	44	76	75
FEF 25/75 (% predicted)		—	114	93	56	20	29	13	58	33
Current Chrispin-Norman chest x ray score¶		5	7	4	14	12	9	14	16	16
Age at <i>Pseudomonas</i> colonisation (y)		None	6	None	5	4	13	4	None	12
Current Shwachman-Kulczycki clinical score**		90	83	97	76	56	69	68	73	75
Complications										
						Nasal polypoidosis	Diabetes, ABPA	ABPA	Nasal polypoidosis	PNX

* Output mEq/30'/kg (normal values >23). † Output U/1/30'/kg (normal values >35). ‡ Output mU/30'/kg (normal values >20). § Normal values >92%. || Normal values <4 g/24 h. ¶ 38 is the worst score. **100 is the best score. ABPA=allergic bronchopulmonary aspergillosis. PNX=pneumothorax.

Pseudomonas colonisation occurred in six out of nine patients and its onset was recorded from 4 to 13 years of age (table). Another complication, besides two cases of meconium ileus, was insulin requiring diabetes starting at 10 years in a young CF patient (no 4).

The clinical score allowed us to classify the patients at the last check up, according to the original Shwachman-Kulczycki evaluation criteria, as follows: the three youngest patients (under 9 years) excellent or good and the remaining subjects (over 12 years) mild or fairly good.

Figure 1 Chest x ray score centiles in a cohort of 121 CF patients diagnosed in Veneto and Trentino regions from 1.10.73 to 31.12.81. Chest x ray score according to Chrispin-Norman (38 is the worst score). The 25th, 50th, and 75th centiles were computed for 20 month subgroups from 50 to 210 months of age. Individual scores within the subgroups were averaged.

Discussion

In this paper we describe nine patients with the R1162X nonsense mutation in each of their CFTR genes: this condition has not been described to date. This mutation involves the second half of the CFTR protein, located in exon 19 of the CF gene. Usually, nonsense mutations in human genes are associated with decreased levels of mutant messenger RNA.^{25,26} Furthermore, the truncated protein produced by a nonsense mutation can be difficult to isolate, probably because of instability and rapid degradation.²⁷ In the case of CF the difficulty of analysing the mutant gene product is increased by the fact that the CFTR gene is better expressed in selected tissues that are not easy to investigate, such as sweat gland, pancreatic, and tracheobronchial epithelial cells.

In other genetic diseases, such as haemophilia,²⁸ the presence of nonsense mutations is frequently associated with a more severe clinical picture when compared to that associated with missense mutations. However, all the patients described here so far seem to show a mild or moderate clinical course. All but one of

Figure 2 Last chest x ray scores in nine R1162X homozygotes compared with 37 unselected Δ F508 homozygous CF patients. The least square regression curve for Δ F508 homozygotes is shown ($r=0.15$).

them had pulmonary symptoms after the first year of life and after diagnosis the severity of the lung disease does not seem to have increased dramatically, even though all the subjects had pancreatic insufficiency from birth.

A general problem in the evaluation of pulmonary involvement in CF is the difficulty of defining the profile of the lung disease when dealing with single or very few cases, since the disease progresses with age and the influence of treatment on genetic factors remains unclear.

In this situation the best course is to match the individual patient with a cohort of CF patients with mixed genotypes but homogeneous for age, treatment, and environment. Alternatively, the patient should be matched with a group of subjects of a well known genotype, homogeneous for the same characters.

Pulmonary features of Δ F508 homozygotes are well known^{12 13 29 30}: a relatively more severe lung involvement has been described by many authors, although there is individual variability. Our data (fig 2) confirm this variability and show the precariousness of a single case match. We investigated both the approaches mentioned above, but for want of genotype characterised cohorts, we think that the comparison of the R1162X homozygotes with a 'mixed genotype' cohort (fig 1), in which chest x ray evaluation was performed longitudinally

over the whole life span, is the best we can do at the moment. Both these approaches suggested mild or moderate lung involvement in R1162X homozygotes, though more information could be obtained from a larger R1162X group followed over a longer period.

It was easier to establish that all the R1162X homozygotes in our series had severe pancreatic insufficiency, as indicated by the low level of pancreatic function and by the high degree of steatorrhoea. The overall better lung picture could not therefore be ascribed to better nutritional status, as might be the case in pancreatic sufficient patients.

It is noteworthy that among 16 R1162X compound heterozygote CF patients we studied, pancreatic sufficiency was found in two patients, one of whom was a compound heterozygote for R347P, and the other had a still unknown mutation. Pancreatic insufficiency was present in the remaining 14 patients, seven of whom were compound heterozygotes for Δ F508. Therefore, it seems that, with regard to the pancreatic involvement, the R1162X mutation, like Δ F508,⁴ acts as a severe lack of function allele, recessive to mild, partially functional ones. R1162X, therefore, when combined with a mild mutation like R347P,³¹ leads to pancreatic sufficiency. When combined with a severe mutation such as Δ F508, pancreatic insufficiency develops.

Some hypotheses could be proposed to explain the minor pulmonary involvement in relation to the severe pancreatic damage. First, the possible presence of alternative splicing could minimise the effect of the R1162X gene mutation. Alternative splicing of the CF gene transcript involving other exons was observed in some CF tissues, but its significance, if any, in the establishment and progression of the disease has not been determined. An alternative splicing involving exon 19 of the CFTR has not been reported. A recurrent tissue specific alternative splicing,³² involving only the tracheobronchial epithelial cells, should be present in the cases described here.

The second possible explanation of the clinical phenotype stems from the observation that the truncated protein created by the R1162X stop signal would still represent a large portion of the CFTR, containing both transmembrane domains, the regulatory region, and the first ATP binding domain, while missing the second nucleotide binding fold, which starts in exon 19. It is possible that this truncated protein is not degraded and is integrated into the cell membrane.³³ In this case, the truncated protein could be partially functional in certain tissues, such as the lung epithelial cells. There is no evidence for such a hypothesis.

The third hypothesis is that the nonsense mutation present in R1162X could be suppressed so that a reduced quantity of CFTR could be produced, as recently suggested from studies on nasal epithelial cells of two patients homozygous for the G542X nonsense mutation.³⁴ In both cases an opal termination codon is involved. In the pulmonary epithelial

cells of the patients described here, such suppression should be more efficient than in pancreatic epithelia.

The fourth hypothesis is that CFTR could be absent, and that other pulmonary epithelial functions of chloride ion transport could partially substitute for the missing function. No CFTR mRNA was detected in bronchial and nasal epithelial cells from a CF patient, a compound heterozygote for mutations R553X and W1316X,³⁵ with severe pancreatic insufficiency and mild pulmonary disease as reported below. In order to determine the pathophysiology of the disease in the patients described here future studies will analyse the expression of the CF gene in different tissues.

Three recent papers are relevant to our findings. Cutting *et al.*³⁶ reported two compound heterozygotes for stop mutations S1255X/G542X and W1316X/R553X (see above), respectively. These mutations are located in exons 20, 11, 21, and 11, respectively. The patients had a mild pulmonary phenotype, meconium ileus, and severe pancreatic disease. Beaudet *et al.*³⁷ described a patient homozygous for mutation G542X with pancreatic insufficiency and pulmonary disease milder than in the average CF patient of comparable age. A patient homozygous for nonsense mutation R553X with lung function 'only moderately affected' at 13.5 years of age was reported by Bal *et al.*³⁸ These reports indicate that mild lung symptoms of the disease are not necessarily restricted to stop mutations occurring only in the terminal half of the CFTR. These reports are in agreement with the results described here, even though it is difficult to evaluate the severity of lung involvement in single cases studied over only a part of their life span.

In conclusion, the results reported here concerning nine Italian patients indicate that a termination mutation in each CF gene may be associated with a mild to moderate course of the disease, even if pancreatic and digestive functions are severely impaired.

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