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Characterization of a novel 21-kb deletion, CFTRdele2,3(21 kb), in the *CFTR* gene: a cystic fibrosis mutation of Slavic origin common in Central and East Europe

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Abstract We report a large genomic deletion of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, viz., a deletion that is frequently observed in Central and Eastern Europe. The mutation, termed *CFTR*dele2,3(21 kb), deletes 21,080 bp spanning introns 1–3 of the *CFTR* gene. Transcript analyses have revealed that this deletion results in the loss of exons 2 and 3 in epithelial *CFTR* mRNA, thereby producing a premature termination signal within exon 4. In order to develop a simple polymerase chain reaction assay for this allele, we defined the end-points of the deletion at the DNA sequence level. We next screened for this mutation in a representative set of European and European-derived populations. Some 197 CF patients, including seven homozygotes, bearing this mutation have been identified during the course of our study. Clinical evaluation of *CFTR*dele2,3(21 kb) homozygotes and a comparison of compound heterozygotes for ΔF508/*CFTR*dele2,3(21 kb) with pairwise-matched ΔF508 homozygotes indicate that this deletion represents a severe mutation associated with pancreatic insufficiency and early age at diagnosis. Current data show that the mutation is particularly common in Czech (6.4% of all CF chromosomes), Russian (5.2%), Belorussian (3.3%), Austrian (2.6%), German (1.5%), Polish (1.5%), Slovenian (1.5%), Ukrainian (1.2%), and Slovak patients (1.1%). It has also been found in Lithuania, Latvia, Macedonia and Greece and has sporadically been observed in Canada, USA, France, Spain, Turkey, and UK, but not in CF patients from Bulgaria, Croatia, Romania or Serbia. Haplotype analysis has identified the same extragenic CF-haplotype XV-2c/KM.19 "A" and the same infrequent intragenic microsatellite haplotype 16–33–13 (IVS8CA-IVS17bTA-IVS17bCA) in all examined CFTRdele2,3(21 kb) chromosomes, suggesting a common origin for this deletion. We conclude that the 21-kb deletion is a frequent and severe CF mutation in populations of Eastern- and Western-Slavic descent.

Introduction

Cystic fibrosis (CF) is an autosomal recessive exocrinopathy characterized by the abnormal transport of ions and fluid across epithelial cell membranes (Welsh et al. 1995). Clinical symptoms of the classical form of CF include progressive obstructive sino-pulmonary disease, dysfunction of pancreatic exocrine secretion, increased sweat chloride concentration, and male infertility (Welsh et al. 1995). The estimated incidence of CF is approximately 1 in 2500 newborns in Caucasian populations. Partial forms of CF have been described, such as congenital absence of vas deferens, idiopathic chronic pancreatitis, and chronic obstructive bronchopulmonary disease, all of which reflect the extensive allelic heterogeneity at the CF locus (Dean and Santis 1994; Zielenski and Tsui 1995; Estivill 1996).

Mutations of the cystic fibrosis transmembrane-conductance regulator (*CFTR*) gene are causative for both classic and atypical presentations of the disease. The

CFTR gene was identified 10 years ago (Rommens et al. 1989; Riordan et al. 1989). *CFTR* spans approximately 250 kb at chromosomal region 7q31.3 (Rommens et al. 1989) and consists of 27 exons (Zielenski et al. 1991) that encode a membrane protein of 1480 amino acids. The CFTR protein functions as a cAMP-regulated chloride channel in exocrine epithelia (Riordan et al. 1989; Welsh et al. 1995).

Over 800 mutations have so far been described in the *CFTR* gene (CFGAC at http:www.genet.sickkids.on.ca/cftr/). The most frequent Caucasian CF mutation, termed ΔF508, is a 3-bp deletion in exon 10 resulting in the loss of phenylalanine at position 508 of the CFTR protein (Kerem et al. 1989). Although a group of relatively common CF alleles occurs in certain populations, the majority of mutations are rare (Cystic Fibrosis Genetic Analysis Consortium 1994). However, a considerable portion of CF alleles has eluded detection despite extensive screening by current polymerase chain reaction (PCR)-based mutation detection techniques (Claustres et al. 1993; Dörk et al. 1994; Bonizzato et al. 1995; Verlingue et al. 1995; Angelicheva et al. 1997; Casals et al. 1997; Tzetis et al. 1997; Onay et al. 1998). In the present study, we report a large genomic deletion that accounts for many of the previously unidentified CF chromosomes in Central and Eastern Europe.

Materials and methods

CF patients for whom one or two *CFTR* mutations remained unidentified after mutation screening and/or scanning were ascertained for the study in the participating centers. Following informed consent, genomic DNA was extracted from nucleated peripheral blood cells of the patients and, where possible, of their parents. The ΔF508 deletion and other common CF mutations were examined at each participating center by applying a population-specific mutation panel and utilizing established techniques for their routine molecular genetic diagnosis.

Microsatellite haplotypes of intragenic markers IVS8(CA), IVS17b(TA), and IVS17b(CA) were determined by a multiplex PCR protocol (Morral and Estivill 1993), followed by sizing on an ALF sequencer (Pharmacia), an ABI 377 sequencer (Perkin Elmer), or a direct blotting device (MWG; Mekus et al. 1995).

Total RNA was isolated from nasal polyp samples of deletion carriers by acid guanidinium/phenol-chloroform extraction (Chomczynski and Sacchi 1987). A sample of 1 μg total RNA was reverse-transcribed in a 15-μl volume by using random hexamer primers and the First Strand cDNA Synthesis Kit (Pharmacia/Amersham). One-fifth of the cDNA was subsequently amplified in a 20-μl reaction by using primers T1 (5'-GGTCTTTGGCATTAGGAGCTTG-3', 5'-UTR) and T17 (5'-TTCATCAAATTTGTTCAGGTTGTTG-3', exon 5). The reverse-transcription/PCR (RT-PCR) products were separated by 2% NuSieve agarose gel electrophoresis.

For pulsed-field gel electrophoresis (PFGE) analysis, agarose-embedded DNA samples prepared from fresh blood samples were partially digested with *Sal*I (New England BioLabs) and separated by electrophoresis in a CHEF DR11 apparatus with 0.5×TBE (1×TBE=0.09 M TRIS-borate, 0.002 M EDTA, pH 8.3) at 10°C, 6 V/cm, and a 120° reorientation angle, with the following pulse times: two linear ramps from 5 s to 30 s over 18 h and from 5 s to 80 s over 20 h. The gel was stained with ethidium bromide, denatured, and transferred to Hybond N+ membranes (Amersham). *CFTR* fragments were detected by hybridization with a radiolabeled *CFTR* cDNA probe spanning exons 7–24 (T16–4.5, ATCC).

The deletion breakpoints were narrowed down in the sample of a homozygous patient by using primer pairs designed from the sequence of human BAC clone 068P20 containing *CFTR* introns 1–3 (Ellsworth et al. 2000; Genbank accession no. AC000111). A total of 12 amplicons located in introns 1 and 3 (nucleotide positions 29987–30100, 32255–32507, 35353–35732, 36329–36690, 37486–37912, 38910–39210, 58203–58346, 59154–59498, 60169–60533, 60974–61404, 63497–63923, and 65117–65414) were analyzed by PCR in order to assess the extent of the deletion. Direct sequencing was performed by using the Sequenase 2.0 PCR product sequencing kit (USB/Amersham) or the BigDye Terminator Sequencing Kit (Perkin Elmer).

Following end-point identification, we developed a rapid simple duplex PCR assay for screening of the *CFTR*dele2,3(21 kb) mutation. Primers 2,3F (5'-GAGCTTCTGAAATTAATTGACCAC-3') and 2,3R (5'-GAACCCATCATAGGATACAATG-3'), which flank the deletion breakpoints, amplify a 207-bp product in the presence of the deletion, whereas control primers 3i-5 and 3i-3 generate a 309-bp product containing *CFTR* exon 3 (Zielenski et al. 1991). The duplex PCR ensures an internal amplification control and can distinguish between homozygotes and heterozygotes for the deletion. A standard (32 cycles) PCR protocol was followed with primer annealing set at 57°C. PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium-bromide staining. The specificity of the assay could be confirmed by sequencing the 207-bp junction fragment or by restriction enzyme analysis with *Bsp*HI, which produces fragments of 113 bp and 94 bp. The 309-bp internal control product exhibited weaker intensity than the 207-bp junction fragment when equal concentrations of primer pairs were used. Despite this amplification bias helping to avoid false negative results, it may include the risk of misinterpreting heterozygotes as apparent homozygotes for the deletion, in particular with respect to low-molecular-weight DNA preparations. Although this was not observed in our study, it is recommended that each apparent homozygote should be verified by another separate amplification of exons 2 or 3.

Clinical variables of general outcome, including age at diagnosis, sweat chloride concentrations (in millimoles per liter, measured by pilocarpine iontophoresis), weight, and height, and categorical data on pancreatic status were obtained for most of the patients at their respective centers. The majority of research groups also obtained data on the respiratory status of the patients, assessed by tests of forced vital capacity (FVC), forced expiratory volume in 1 s (FEV1), and the colonization of the airways by *Pseudomonas aeruginosa*. An extended genotype-phenotype comparison of pairwise-matched patients with compound heterozygosity ΔF508/*CFTR*dele2,3(21 kb) versus ΔF508 homozygosity was conducted for 66 Czech and 30 German patients who had been treated at specialized CF centers in Hannover, Rostock, and Prague for long periods. Each of the patients with genotype ΔF508/*CFTR*dele2,3(21 kb) was matched with the ΔF508 homozygote closest in age, within 1 year, and of the same sex. Statistical analysis of continuous variables was performed by using the Mann-Whitney U-rank test (age at diagnosis) or the two-tailed Wilcoxon pair rank test (sweat chloride concentration and lung function parameters). In cases of dichotomous variables, Fisher's exact test was used to compare proportions. *P*-values were two-tailed, and probabilities of less than 0.05 were considered significant.

Results

Several lines of evidence pointed to the existence of a frequent genomic deletion spanning *CFTR* exons 2 and 3. First, these two exons were resistant to amplification with established primer pairs of the CF Genetic Analysis Consortium (Zielenski et al. 1991) in DNA samples from single Turkish, Spanish, and Canadian CF patients. These patients later proved to be homozygous for the deletion.

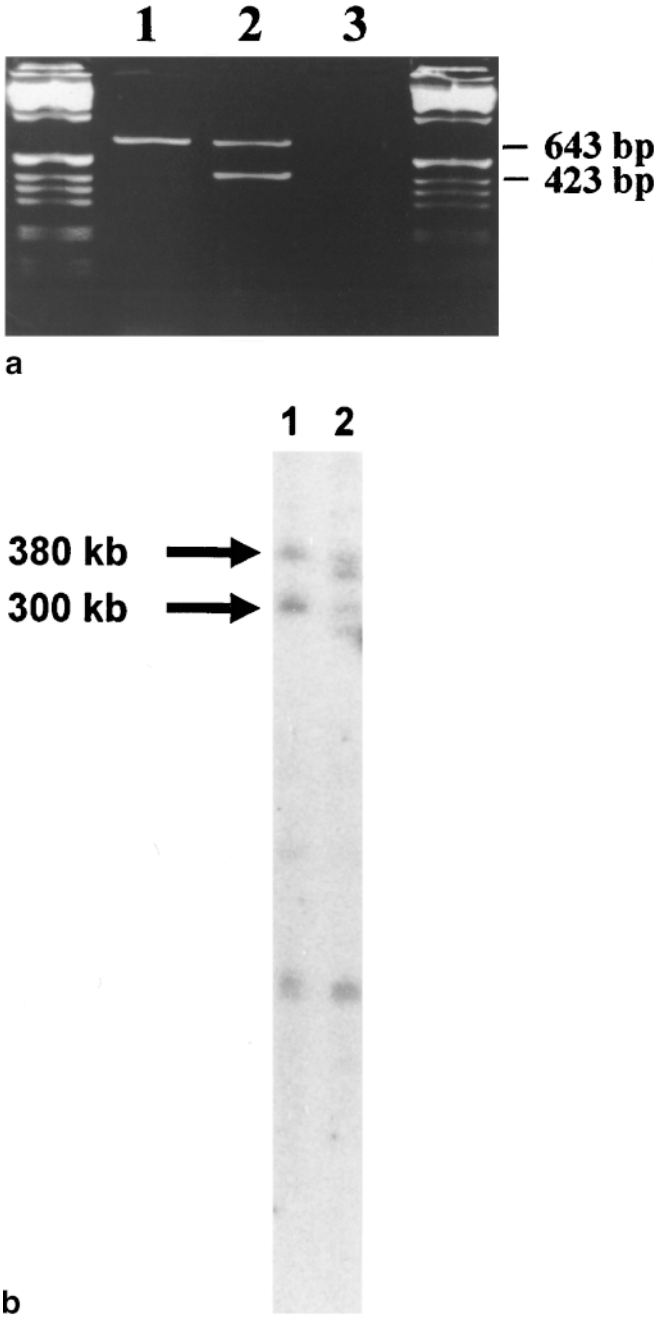

Fig. 1 **a** Detection of mutation *CFTR*dele2,3(21 kb) by RNA analysis. RT-PCR was performed on epithelial *CFTR* mRNA from the nasal polyp of a patient heterozygous for G551D and *CFTR*dele2,3(21 kb). RT-PCR product spanning exons 1–5 was analyzed by 2% agarose electrophoresis. The patient's sample (*lane 2*) shows an additional band of 423 bp lacking exons 2 and 3, whereas the wildtype product is 643 bp (*lane 1*, control). *Lane 3* Water control, *outmost lanes* size marker (kb ladder, Gibco BRL). **b** Detection of mutation *CFTR*dele2,3(21 kb) by pulse field gel electrophoresis and Southern blotting. Genomic DNA was digested with *Sal*I prior to electrophoresis. The sample of the heterozygous patient (*lane 2*) exhibits mobility shifts in two *Sal*I fragments, which both are consistent with the presence of a deletion of 20–25 kb

Second, a combined loss of these exons was found in about half of the nasal epithelial *CFTR* mRNA transcripts from a German CF patient who was compound heterozygous for G551D and the presumed deletion (Fig. 1a). At a later stage of the study, the same pattern was observed in nasal epithelial samples from two further patients who were compound heterozygous for the deletion allele and the R347P or ΔF508 mutations, respectively. Third, Southern blot hybridization of PFGE gels revealed aberrant bands in samples of several German CF patients with unknown CF mutations, these being consistent with a genomic deletion in the range of 20–25 kb (Fig. 1b). Haplotype comparison of Canadian, German, Spanish, and Turkish patients suspected of carrying the "two-exon" deletion revealed that they carried the same extragenic XV2c/KM19 haplotype "A" (Estivill et al. 1987) and shared an identical intragenic microsatellite haplotype "16–33–13" (IVS8CA-IVS17bTA-IVS17bCA). This microsatellite haplotype is rare in the general population and accounts for only 1.7% of CF chromosomes in Caucasians (Morral et al. 1996).

The endpoints of the deletion could be resolved at the genomic level by using sequence information provided by the Chromosome 7 Sequencing Project (Ellsworth et al. 2000; Genbank accession no. AC000111). Primer pairs, specific for selected regions of *CFTR* introns 1 and 3, were used to establish the extent of the deletion in a DNA sample of a homozygous patient and subsequently to amplify and sequence the junction fragment. Positive signals were obtained with 10 primer pairs covering regions in introns 1 and 3 (nucleotide positions 29987–30100, 32255–32507, 35353–35732, 36329–36690, 37486–37912, 59154–59498, 60169–60533, 60974–61404, 63497–63923, and 65117–65414), whereas the PCR product could only be amplified from controls but not from the patient DNA with two primer pairs (38910–39210, 58203–58346). A junction fragment, spanning nucleotides 37486–59498, was amplified, and the resulting 933 bp PCR product was sequenced (Fig. 2a). The deletion spans 21.08 kb and includes about 25% of intron 1, the complete exon 2, intron 2, and exon 3, and approximately 45% of intron 3 (Fig. 2b). The sequences at the deletion breakpoints were not homologous, except that a short sequence of four pyrimidines (5'-CTTT-3') was identical at both breakpoints (Fig. 2a). At the level of the cDNA, the omission of exons 2 and 3 is "out-of-frame" and causes a premature translation termination signal at codon 106 within exon 4.

We designed a PCR-based assay to screen for this allele in genomic DNA samples from European and European-derived CF patients by utilizing the established duplex-PCR format (see above and Fig. 2c). CF research groups from Albania (patients were tested in Rome), Austria (South-Eastern, Graz), Belarus (Minsk, patients were tested in Prague), Belgium (Leuven), Bulgaria (Sofia), Canada I (Central, Toronto), Canada II (East, Halifax), Canada III (West, Vancouver), Croatia (Zagreb), Czech Republic (Bohemia and Moravia, Prague), Estonia (Tartu), France I (Brittany, Brest), France II (South Cen-

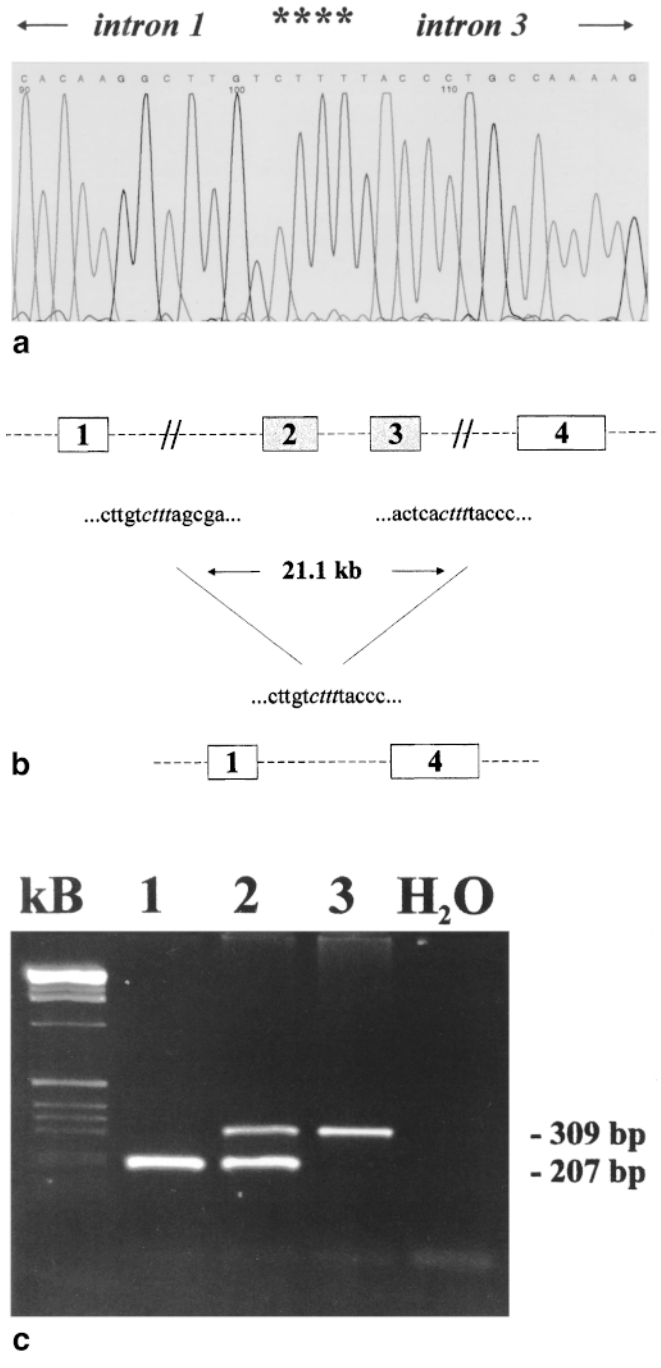

Fig.2 **a** Direct sequencing of the junction fragment obtained from genomic DNA of a patient homozygous for the *CFTR*dele2,3(21 kb) deletion. The four-base-sequence shared by both deletion endpoints in intron 1 and 3, respectively, is marked by *asterisks*. **b** Schematic illustration of the 21.1-kb deletion resulting in the loss of exons 2 and 3 (not drawn to scale). Exons are depicted as *boxes*. The four-base sequence shared by both deletion endpoints in intron 1 and 3, respectively, is shown in *italics*. **c** Duplex PCR-based assay to identify carriers of the *CFTR*dele2,3(21 kb) deletion. The presence of mutation *CFTR*dele2,3(21 kb) yields a 207-bp product, whereas the presence of exon 3 yields a 309-bp product. *Lane 1* Patient homozygous for *CFTR*dele2,3(21 kb), *lane 2* patient heterozygous for *CFTR*dele2,3(21 kb), *lane 3* control individual without mutation *CFTR*dele2,3(21 kb), *outmost lanes* size marker (kb ladder, Gibco BRL, *left*), water control (*right*)

Table 1 Allele frequencies of the *CFTR* dele2,3(21 kb) mutation in various populations. All participating groups screened at least those CF samples for the CFTRdele2,3(21 kb) mutation in which one or both mutations had previously remained undetected by their respective routine diagnostic assays. Allele frequencies are given as percentages of total CF chromosomes in the respective population cohort (*n.s.* not significant)

Population	No. of mutant chromosomes detected/no. of total CF chromosomes	Percentage	Center
Albania	0/15	0	Rome
Austria	12/470	2.6	Graz
Belarus	3/90	3.3	Minsk/Prague
Belgium	0/230	0	Leuven
Bulgaria	0/392	0	Sofia
Canada (I)	3/1268	0.2	Toronto
Canada (II)	1/400	0.3	Halifax
Canada (III)	2/676	0.3	Vancouver
Croatia	0/276	0	Zagreb
Czechia	40/628	6.4	Prague
Estonia	0/60	0	Tartu
France (I)	1/2800[a]	<0.1	Brest
France (II)	1/980[a]	0.1	Montpellier
France (III)	1/1500[a]	<0.1	Lyon
Germany (I)	21/1400	1.5	Hanover
Germany (II)	6/242	2.5	Rostock
Germany (III)	0/212	0	Leipzig
Germany (IV)	5/290	1.7	Freiburg
Germany (V)	3/200	1.5	Münster
Germany (VI)	1/124	0.9	Magdeburg
Germany (VII)	1/200	0.5	Erlangen
Greece	2/718	0.3	Athens
Italy	0/225	0	Verona
Latvia	1/36	2.8	Riga
Lithuania	2/94[a]	2.1	Vilnius
Macedonia	2/167	1.2	Skopje
Poland (I)	14/764	1.8	Poznan
Poland (II)	6/586	1.0	Warsaw
Romania	0/50	0	Manchester
Russia (I)	38/668	5.8	Moscow
Russia (II)	12/294	4.1	St. Petersburg
Slovakia	3/282	1.1	Bratislava
Slovenia	2/132	1.5	Ljubljana
Spain	2/1280	0.2	Barcelona
Sweden	0/120	0	Goteborg
Turkey (I)	1/174	0.6	Istanbul
Turkey (II)	2/30	n.s.	Hanover
Ukraine	5/419	1.2	Kiev
UK	2/1720[a]	0.1	Manchester
USA	2/1372	0.2	Chapel Hill
Uruguay	0/104	0	Montevideo
Yugoslavia	0/398	0	Belgrade

[a]The ethnic descent of the identified carrier was followed more closely. One patient identified in Britanny was of Tunisian descent and had inherited the mutation from his Russian mother; the single patient from Southern France had inherited the deletion from the patient's German mother; the single patient identified in Lyon had a Polish father; out of two cases detected in Lithuania, one was a Polish patient and one a Lithuanian who originated from the territory of Poland; both patients detected in the UK were of European non-British descent, one having an Austrian mother, the other having a paternal grandfather from Poland and great-grandfather from Germany

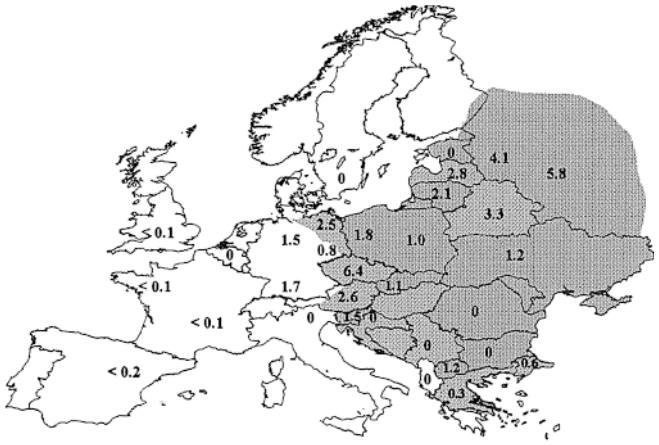

Fig. 3 Maximum spread of Slavic populations during the first millenium AD and current distribution of the *CFTR*dele2,3(21 kb) mutation. The geographic distribution of the *CFTR*dele2,3(21 kb) mutation throughout Europe is given with its allele frequencies on CF chromosomes in the countries of the study participants (see Table 1). Geographic regions inhabited by Slavic populations during the first millenium are shown as *shaded areas*

tral, Montpellier), France III (Lyon), Germany I (Northern and Central, Hannover), Germany II (North-Eastern, Rostock), Germany III (Eastern, Leipzig), Germany IV (Southern, Freiburg), Germany V (Western, Münster), Germany VI (Eastern, Magdeburg), Germany VII (Southern, Erlangen), Greece (Athens), Italy (Northeastern, Verona), Latvia (Riga), Lithuania (Vilnius), Macedonia (Skopje), Poland I (Western, Poznan), Poland II (Central, Warsaw), Romania (Eastern, patients were tested in Manchester), Russia (Central, Moscow), Slovakia (Bratislava), Slovenia (Ljubljana), Spain (Catalunya, Barcelona), Sweden (Central, Göteborg, patients were tested in Toronto), Turkey (Western, Istanbul), Ukraine (Kiev), UK (Manchester), USA (South-Eastern, Chapel Hill), Uruguay (Montevideo, patients were tested in Toronto) and Federal Republic of Yugoslavia (Belgrade) participated in the study. Only patients with verified forms of classical CF were screened at these centers to determine the allele frequencies listed in Table 1.

Some 197 CF patients bearing the *CFTR*dele2,3(21 kb) deletion were identified during the course of this work. Our results indicate that this mutation is particularly common in Czech, Russian, Belorussian, Austrian, German, Polish, Ukrainian, Slovenian, and Slovak CF patients (Table 1, Fig. 3). It is the second most common CF mutation, after ΔF508, to be identified in Central and Eastern European CF patients (except for the Baltic population in this region). By contrast, this deletion was only sporadically detected in Western Europe and was absent in Bulgarian, Croatian, Romanian, and Serbian CF patients. It was not found in diverse other populations of non-Slavic origin (Table 1). The geographic distribution of the mutation is similar to the spread of Slavic populations during the first millenium AD (Fig. 3). Low incidence of this mutation was observed in countries such as Canada with high immigration from various ethnic populations including Central and Eastern Europeans.

Seven homozygotes for the *CFTR*dele2,3(21 kb) mutation have been identified in different populations during the course of this study. All of these patients presented with classic symptoms of CF, including pancreatic insufficiency, early age at diagnosis, elevated sweat chloride concentrations, and moderate to severe lung disease (Table 2). In addition, a clinical evaluation of compound heterozygotes showed that the 21-kb-deletion represents a "severe" allele, since all patients with the ΔF508/*CFTR*dele2,3(21 kb) genotype were pancreatic insufficient. A statistical evaluation of clinical parameters of age- and sex-matched Czech and German patients was performed to compare 48 compound heterozygotes for ΔF508/*CFTR*dele2,3(21 kb) with 48 ΔF508 homozygotes (Table 3). Age at diagnosis was significantly lower in the group of patients who were compound heterozygous for ΔF508/*CFTR*dele2,3(21 kb), i.e., median 0.3 years, inner quartiles 0.1–0.7, than for ΔF508 homozygotes, i.e., median 0.6 years, inner quartiles 0.3–3.0 (u=3.368, $P<0.001$). The groups were indistinguishable with respect

Table 2 Clinical presentation of patients homozygous for the *CFTR*dele2,3 (21 kb) mutation (*PI* pancreatic insufficient, *GTT* glucose tolerance test, *S.au. Staphylococcus aureus*, *P.a. Pseudomonas aeruginosa*, *FEV1* forced expiratory volume in 1 s, *VC* vital capacity, *n.d.* not documented)

Ethnic origin	1 Polish	2 Polish-Canadian	3 Spanish	4 Turkish	5 German	6 German	7 Czech
Sex	Male	Female	Female	Female	Female	Male	Female
Present age (years)	15.3	19	11	21	7	11	7
Age at diagnosis (months)	30	11	2	6	At birth	9	4
Sweat chloride (mmoles/l)	70–120	100–135	82	95–103	85	126	79
Height (cm)	155	169	143	153	133	135	121
Weight (kg)	38	61	39	42	26	35	20
Pancreatic status	PI	PI	PI	PI	PI	PI	PI
Meconium ileus	No	No	No	Yes	Yes	No	No
FEV1 (% pred.)	84	n.d.	79	67	n.d.	n.d.	66
VC (% pred.)	82	77	80	61	n.d.	n.d.	72
Lung colonization	*S. au.*	*P.ae.*	None	*P.ae.*	None	None	None
Complications	Nasal polyps				Cholestasis	Nasal polyps	Impaired GTT

Table 3 Matched-pair comparison of clinical phenotype of patients with genotypes ΔF508/ΔF508 and ΔF508/*CFTR*dele2,3(21 kb). Patients with genotypes ΔF508/ΔF508 and ΔF508/*CFTR*dele2,3(21 kb) were pairwise matched by sex and age (*DIOS* distal intestinal obstruction syndrome, *n.s.* not significant)

	ΔF508/ΔF508	ΔF508/CFTRdele2,3(21 kb)	
No. of patients	48	48	
Male/female	24/24	24/24	
Median age at diagnosis (year)	0.6 (range: 0–13.2)	0.3 (range: 0–10.0)	$P<0.001$[a]
Median sweat chloride (mmoles/l)	95 (range: 63–138)	91 (range: 61–130)	n.s.
Pancreatic insufficiency	47/47	45/45	n.s.
Meconium ileus	6/45	12/47	$P=0.133$[b]
DIOS	2/45	2/47	n.s.
P. aeruginosa colonisation	32/46	23/47	$P=0.035$[b]
Hepatobiliary disease	8/43	7/45	n.s.
Diabetes mellitus	4/42	0/42	$P=0.058$[b]

[a]Differences in the evaluated clinical parameters were tested for significance by the Mann-Whitney U-rank test
[b]Differences in the evaluated clinical parameters were tested for significance by Fisher's exact test

to the distribution of sweat chloride concentrations, hepatobiliary disease, and DIOS (distal intestinal obstruction syndrome; Table 3) and did not differ in the distribution of lung function parameters FVC (in % predicted, u=0.14, α=0.88) and FEV1 (in % predicted, u=0.51, α=0.60). Fisher's exact test revealed that the compound heterozygotes were characterized by a lower prevalence of *Pseudomonas aeruginosa* infections (P=0.035) and by a trend toward a lower incidence of secondary diabetes mellitus (P=0.058) and toward a higher incidence of meconium ileus (MI, P=0.133). The higher incidence of MI was significant in the 33 Czech patients (P=0.014), suggesting that population-specific differences may exist in the gastrointestinal expression of CF disease in deletion carriers.

A few compound heterozygous patients carrying mild CF mutations (e.g., I336 K, R347P, 3849+10kbC→T) *in trans* exhibited less severe pulmonary symptoms and were pancreatic sufficient as predicted from the well-documented dominance of milder CF alleles (Kristidis et al. 1993). We also noted that, in addition to those patients with classic CF listed in Table 1, two German adults were carriers of the genotypes R117H/*CFTR*dele2,3(21 kb) and IVS8–5T/*CFTR*dele2,3(21 kb); these patients had initially been diagnosed by urologists as having isolated congenital bilateral absence of vas deferens (Dörk et al. 1997). Similarly, a Czech patient with non-obstructive oligospermia was diagnosed with a *CFTR*dele2,3(21 kb)/unknown genotype. These observations suggest that screening for the new 21-kb deletion could contribute to the genetic diagnosis of male infertility.

Discussion

We have characterized a large genomic deletion that lies within the *CFTR* gene and that accounts for a considerable portion of CF chromosomes in most Central and Eastern European populations. Although it has long been suggested that gross deletions may be found in populations with an incomplete detection rate of CF mutations, only five deletions have been published to date (Morral et al. 1993; Magnani et al. 1996; Chevalier-Porst et al. 1998; Mickle et al. 1998; Lerer et al. 1999). These appear to be rare or restricted to a particular population, and only in two cases have the deletion breakpoints been defined at the *CFTR* nucleotide level (Magnani et al. 1996; Lerer et al. 1999). It has been suggested that the generation of one of these mutations, the 703-bp deletion spanning exon 17b, involves a flanking microsatellite sequence, a direct repeat, and an internal stem-loop structure (Magnani et al. 1996). Similarly, the generation of the other such mutation, the 8.6-kb deletion involving exons 17a–18, seems to be facilitated by an identical 4-bp sequence at the deletion breakpoints (Lerer et al. 1999). In other genetic diseases, the generation of large-scale deletions is often mediated by long direct repeats (Purandare and Patel 1997) or can be facilitated by long palindromic sequences (Akgün et al. 1997). The 21-kb deletion in our study does not appear to meet these latter criteria, but we cannot exclude the possibility that interspersed repetitive elements have acted at a distance, and the associated chromatin structure could also have contributed to the establishment of the deletion breakpoints. The presence of a 4-bp direct repeat (5'-CTTT-3') at the breakpoints of the 21-kb deletion is similar to that reported for the *CFTR* 8.6-kb deletion (Lerer et al. 1999) and may be consistent with a slipped-mispairing mechanism (Esfradiatis et al. 1980). This sequence also matches a topoisomerase I cleavage site (5'-PyTT-3'). Many cross-over points are associated with topoisomerase I cleavage sites, indicating a role of non-homologous recombination in the generation of such large deletions (Bullock et al. 1985; Magnani et al. 1996).

The clinical presentation of carriers of the *CFTR*dele2,3(21 kb) mutation shows that the deletion represents a severe CF allele. Symptoms common to all homozygotes in our study include pancreatic insufficiency, early age at diagnosis, and pulmonary disease. Thus, the *CFTR*dele2,3(21 kb) mutation has features similar to other severe CF alleles, such as ΔF508, N1303 K,

and several nonsense and frameshift mutations (Kristidis et al. 1992; Osborne et al. 1992; Cystic Fibrosis Genotype-Phenotype Analysis Consortium 1993). Furthermore, a pairwise-matched comparison of patients compound heterozygous for the ΔF508/*CFTR*dele2,3(21 kb) genotype with ΔF508 homozygotes demonstrates a significant earlier age at diagnosis in the heterozygotes. The earlier onset of CF has been seen in both German and Czech patients carrying the *CFTR*dele2,3(21 kb) mutation and can only partly be explained by a more frequent occurrence of MI in deletion carriers, as the prevalence of the latter in the Czech patients may depend on population-specific modifying factors (Zielenski et al. 1999). An earlier CF diagnosis does not appear to result in better lung function parameters in the compopund heterozygote group, although the prevalence of *P. aeruginosa* infections is slightly lower. It has previously been noted that pulmonary impairment is highly variable, even among CF patients with the same *CFTR* mutation genotype (Santis et al. 1990; Cystic Fibrosis Genotype-Phenotype Analysis Consortium 1993)

Our observation that *CFTR* mRNA is fully expressed from the deletion allele in nasal epithelial samples of three heterozygous patients indicates that deleterious consequences of the 21-kb deletion act at the post-transcriptional level. Because of a frameshift caused by the combined loss of exons 2 and 3, mRNA translation is predicted to lead to the synthesis of a severely truncated polypeptide lacking all functional CFTR domains. Reinitiation of translation at internal "AUG" codons (e.g., Met150, Met152, and Met156 in exon 4) is an alternative possibility, and amino-terminally shortened CFTR protein has been shown to retain some function in a *Xenopus* oocyte expression system (Piazza Carroll et al. 1995). However, the observed strong association of *CFTR*dele2,3(21 kb) with a pancreatic-insufficient form of cystic fibrosis in our study suggests that such a "rescue" mechanism of reinitiation, if it occurs in vivo, is not sufficient to prevent or mitigate the classic CF phenotype. In COS-7 cells, amino-terminally truncated CFTR has been shown to be incompletely processed and does not reach the plasma membrane, indicating that the amino-terminal portion of CFTR is essential for its proper folding (Prince et al. 1999; Chang et al. 1999). In addition, the amino-terminal cytoplasmic tail of CFTR seems to act as a positive regulator that controls ion-channel gating through an intramolecular interaction with the R-domain; this regulatory activity has been mapped to a cluster of acidic residues (at positions 47, 51, 54, and 58), a region that is deleted in patients with the CFTRdele2,3(21 kb) mutation (Naren et al. 1999).

Screening for the *CFTR*dele2,3(21 kb) deletion in a representative group of European and European-derived populations has revealed its unusually high prevalence in Central and Eastern European CF patients. The geographic distribution of the deletion together with the observation of a common CF haplotype for the *CFTR*dele2,3(21 kb)-carrying chromosomes from different populations suggests that this deletion could have originated in a common Slavic ancestral population. According to various historical sources (Bouzek et al. 1982; Vána 1983; Kinder and Hilgermann 1997), the original "Slavic" tribes inhabited the vast Eastern European region, roughly between the Rivers Wisla (Poland) and Dnieper (Ukraine), far into the north of current Belarus and Russia. They also extended into the region adjacent to the Baltic sea, where the unrelated Baltic (Latvia, Lithuania) and Ugrofinnic (Estonia) tribes resided. During the 4th–6th centuries AD, these Slavic tribes began to migrate into Central Europe, while the resident German tribes (e.g., the Markomans and Langobards) started moving to the South, concurrent with the decline of the Roman empire. The historic migration of Eastern Slavs did not go beyond the River Elbe but, in the South, they spread over the entire Balkans, with the exception of Albania, and briefly even invaded the northern parts of Greece (Fig. 3). Therefore, it is not surprising to find the *CFTR*dele2,3(21 kb) mutation in eastern Germany, in the Austrian Steiermark and Carinthia, and in Slovenia, Macedonia, and northern Greece (Bouzek et al. 1982; Vána 1983; Kinder and Hilgermann 1997). Subsequently, in the region of the current Czech Republic and northern Austria, the Slavs assimilated with the remaining Celtic population, as objectively documented by an increased prevalence of the "Celtic" CF mutation, G551D, in Czech and Austrian CF populations (Macek et al. 1991).

Although screening data are not available from all Eastern European countries, it is apparent that the prevalence of this mutation closely follows the historically established presence of Eastern and Western Slavic populations (Fig. 3). The Lausitzer Sorben minority of east Germany and the Steiermark and Carinthia Slavs of Austria were later mostly assimilated by the incoming German populace (Bouzek et al. 1982; Kinder and Hilgermann 1997). The presence of the deletion in Lithuania and Latvia may be attributable to their sizeable Slavic minority or to the close historic Baltic-Slavic associations during the early stage of Slavic ethnogenesis (Vána 1983). However, in this respect, caution must be exerted, because of the small number of investigated CF families in the Baltic region.

Intriguingly, the 21-kb-deletion is not the only CF mutation that exhibits such a frequency gradient. Two frameshift mutations in exon 13 of the *CFTR* gene, viz., 2143delT and 2184insA, originally identified in German CF patients, have subsequently also been found to be common in Russian, Czech, and Polish patients suggesting their common Slavic origin (Dörk et al. 1992, 1994; Verlingue et al. 1995; Estivill et al. 1997). These observations demonstrate the existence of a common subset of CFTR mutations among Central and Eastern European populations of Slavic descent. The observed "Slavic" population distribution pattern is also present in phenylketonuria (PKU), where the major mutation R408 W follows a similar East-to-West cline (Eisensmith et al. 1995).

The absence of the *CFTR*dele2,3(21 kb) mutation in other countries with current "Southern" Slavic populations (e.g., Bulgaria, Croatia, and Serbia) and in ethnically

unrelated Albania and Romania may be attributable to continous extensive population migrations involving other European ethnic groups (e.g., Albanian, Ugrofinnic, and Turkish) or even historic Central-Asian populations (e.g., Avars, Bulgarians, and Huns; Kinder and Hilgermann 1997). Finally, parts of Europe that were not affected by historic Slavic migrations (Bouzek et al. 1982; Kinder and Hilgermann 1997), such as France, UK, and Scandinavia, merely have a sporadic occurrence of this mutation. The presence of the deletion in Canada, USA, and Macedonia can be attributed to the more recent Eastern European immigration or admixture, whereas its absence in Uruguay reflects the predominant Southern European but marginal Eastern European immigration to this region.

In summary, this study has demonstrated the limitations of a PCR-based genomic approach to comprehensive mutation detection as the sole screening strategy. Multi-exon deletions in compound heterozygotes are difficult to detect by genomic PCR-based screening protocols, because of amplification of a non-deleted allele that "masks" the lack of a PCR product corresponding to the deletion site. Therefore, the use of alternative screening approaches, such as implemented RNA transcript analysis, may facilitate identification of otherwise undetectable mutations. Such deletions may constitute a significant portion of the entire mutation spectrum in a particular ethnic group (e.g., *CFTR*dele2,3(21 kb) in Eastern and Western Slavs, or *CFTR*dele17a-18 in Arabs). For instance, inclusion of this deletion in the screening panel in Czech CF families increased the mutation detection rate to over 95% (M. Macek Jr et al., in preparation). Our study has also documented the usefulness of the combination of historic/archeaological data with modern population genetic approaches in the study of population origins, migration, and similarities. Moreover, a multi-center approach to genotype-phenotype studies yields more accurate objective clinical correlations of a given CF allele. Finally, from a practical point of view, screening for the *CFTR*dele2,3(21 kb) deletion should significantly improve the molecular genetic diagnosis of CF in families of Slavic descent.

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