Electronic letter

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Distribution of *CFTR* gene mutations in cystic fibrosis patients from Estonia

EDITOR—Cystic fibrosis (CF) is the most common recessive genetic disorder affecting about 1 in 2500 white live births with a carrier frequency of 1 in 25.[1] The incidence of this disease has been found to be somewhat lower in Nordic countries,[2] especially Finland.[3] To date more than 800 mutations have been identified in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (http://www.genet.sickkids.on.ca/cftr/). The major mutation, ΔF508, accounts for 72.8% of the CF chromosomes in northern and central European countries,[4] but the frequencies and types of mutations in different populations vary considerably depending on the ethnic and geographical origin of the population tested.

In our previous report, the incidence of CF was estimated to be about 1 in 4500 live births in Estonia.[5] The present study was undertaken to identify the whole spectrum of *CFTR* gene mutations in Estonian patients.

Thirty families with CF patients were studied. We think the patients should account for most of the CF cases in Estonia, as they attend the central hospitals, but the exact number of CF patients is not known. Diagnostic criteria were based on repeated positive sweat chloride tests (>60 mmol/l) and on typical findings of pulmonary/gastrointestinal disease. In addition, 20 more patients suspected of having CF, with either undefined pancreatitis or a broad spectrum of respiratory diseases and borderline (40-60 mmol/l/l) or normal (<40 mmol/l) sweat chloride values, were analysed for mutations in the *CFTR* gene.

First, several known mutations were tested directly by the heteroduplex analysis (HA; ΔF508, 394delTT, polyT variants in IVS8), restriction digestion (RD; G551D, R553X, 1811+1.6kbA→G, L206W, 3849+10kbC→T), and amplification refractory mutation system (ARMS, kits from Cellmark Diagnostics, UK; G542X, 621+1G→T, N1303K). As a result of this screening, only two mutations were found: ΔF508 was found in 31 (51.7%) alleles and 394delTT in eight (13.3%) alleles (table 1).

In parallel to direct testing, scanning of all 27 exons and their flanking sequences was undertaken by single strand conformational polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) analyses. The primers and PCR conditions used for amplification of most of the exons for SSCP analysis were published by Zielenski *et al*.[8] In order to obtain DNA fragments with an optimal length, some PCR products were digested with appropriate restriction enzymes. Different primers were used for exons 9,[9] 14b,[10] 17a,[11] and 20.[12] For DGGE analysis, the primers, PCR, and electrophoresis conditions used have been published elsewhere.[13 14] DNA samples, displaying PCR fragments with altered mobility in SSCP or DGGE analyses, were sequenced using Thermo Sequenase™ DNA polymerase and [α-^{33}P] labelled ddNTP terminators (Amersham).[15] As a result of scanning of the *CFTR* gene exon by exon, eight rare mutations were found (table 1). Each of these mutations was detected once, except for S1235R, which was found in two heterozygotes. All of these mutations have been described previously in other populations. The CF genotypes identified in our patients are shown in table 2. In addition to the disease causing mutations, 18 different polymorphisms were detected.[26] Two of these are novel, 405+42A/G in intron 3 and 1110A/G in exon 7 (Ala326Ala). Both of them were found by DGGE assay. The latter creates a restriction site for *Hha*I.

Table 1 Mutations identified in CF patients from Estonia

Mutation	*Exon*	*No of chromosomes*	*Frequency (%)*	*Method*	*Reference*
ΔF508	10	31	51.7	HA	6
394delTT	3	8	13.3	HA	7
359insT	3	1		SSCP	16
3659delC	19	1		SSCP	17
E217G	6a	1		DGGE	18
H117C	4	1		SSCP	19
I1005R	17a	1		SSCP	19
R1066H	17b	1		DGGE	20
S1196X	19	1		DGGE	21
S1235R	19	2		DGGE	22
	Unidentified	12			
	Total	60			

Table 2 Genotypes of the 30 CF patients from Estonia

No of patients	*Genotype*
8	ΔF508/ΔF508
6	ΔF508/394delTT
1	ΔF508/I1005R
1	ΔF508/359insT
1	ΔF508/3659delC
1	ΔF508/H117C
1	ΔF508/R1066H
1	ΔF508/S1196X
1	394delTT/394delTT
2	S1235R/U*
3	ΔF508/U
1	E217G/U
3	U/U

*Unidentified mutation

We did not find any mutations in the 20 patients with atypical disease and suspected of having CF.

To scan for several known mutations simultaneously, a multiple allele specific primer extension (MASPE) method was developed.[23] Thirty eight mutations were chosen: 30 of the most common mutations world wide[24] and eight mutations of regional interest (394delTT, 3821delT, 2143delT, 2184insA, 3732delA/K1200E, R117C, and I1005R), previously detected in Scandinavia,[2] Russia,[25] or Germany.[19] Fifteen target exons were amplified to get the templates for primer extension.[26] Owing to the dense location of the mutation sites in seven exons, both DNA strands were used as templates to avoid overlapping of the extension primers. Therefore, the 22 PCR products of 50 µl were divided into four sets: (1) exons 3, 4, 10, 11, 19, and 21; (2) exons 3, 4, 9, 10, 11, 12, and 17a; (3) exons 4, 7, 11, 13b, 14b, 19, and intron 19; and (4) exons 5, 7, 11, 13, 19, and 20. The four sets of PCR products were immobilised on Dynabeads M-280 Streptavidin (Dynal, Oslo) via biotin to get single strand templates. The extension primers were designed to anneal the corresponding templates up to the base located 5' of the base to be assayed. These primers were also divided into four sets, corresponding to the template mixtures. To distinguish between the mutant and wild type sequence, the oligonucleotides were annealed to the templates and the labelled complementary ddNTPs were added by using DNA polymerase. Twelve µl of the corresponding primer mix and 48 µl of 5 × reaction buffer (Sequenase® 2.0, USB) were added to 180 µl of template bound magnetic beads. The mixture was heated to 80°C,

cooled slowly to 30°C, and then divided into four sets of 60 µl. To each set, 6 µl of 0.1 mol/l DDT, 2.4 µl of 100 mmol/l $MnCl_2$, 8 µl of one of the four fluorescein labelled ddNTPs (F-ddNTP, DuPont NEN), 16.6 µl of H_2O, and 3 µl of diluted Sequenase® version 2.0 polymerase (USB) were added. F-ddATP, F-ddCTP, and F-ddGTP were used at 0.5 µmol/l concentration and F-ddUTP at 1 µmol/l concentration. Sequenase® version 2.0 polymerase (USB) was used at 0.1-0.2 U per reaction. The extension reactions continued at room temperature for seven minutes. The magnetic beads were collected and washed twice as recommended by the manufacturer (Dynal). The beads from reaction sets 1 to 4 were pooled into four tubes so that each of them contained the extension products with the same ddNTPs. The products were eluted in formamide and analysed by electrophoresis using an automated DNA sequencer ALF (Pharmacia Biotech). Data were collected using ALFwin Version 1.00 software (Pharmacia Biotech) and analysed with AlleleLinks Version 1.00 analysis software (Pharmacia Biotech).

The results of MASPE analysis in normal and mutant samples are shown in fig 1. The peaks, corresponding to specific nucleotide sites, are clearly separated from each other. The pattern of peaks can be easily interpreted, as the oligonucleotide length identifies the mutation site and the incorporated ddNTP identifies the nucleotide at this site. Mutation is indicated by a shift of the peak from the lane, corresponding to extension product on the wild type template, to another lane, thus specifying the change of the nucleotide. For example, the peak in lane A, which corresponds to the 26 bp primer, indicates a normal sequence and the peak with the same size in lane G signals the presence of the 394delTT mutation. The patterns of peaks corresponding to the 394delTT heterozygote and ΔF508 homozygote are shown in figs 1B and C, respectively. The essential point of this method is to use the extension primers, which can be resolved electrophoretically. Our oligonucleotides differed in length from each other by 1 bp, ranging from 16 to 53 bp.[26] Pastinen *et al*[27] used primers that differed in size by three bases to get clear electrophoretic resolution in typing the HLA-DQA1 and HLA-DRB1 genes. We have shown that primers with only 1 bp difference in length can be used successfully. To get optimal resolution, some of the primers were redesigned by changing the length of the oligonucleotides or using an opposite DNA strand, as the base composition of the primer can affect its mobility. We did not find any significant correlation between the length of the extension primers and the efficiency of the reaction. The reaction efficiency depended mainly on the extension primer sequence, its concentration, and the sequence context of the specific test site. The concentration of the extension primers was the main parameter to be adjusted in the optimisation process to achieve equal incorporation of the ddNTPs.[26] The uniformity of peaks was improved by adding Mn^{2+} to the extension reactions. As a result of optimising the method, all mutant samples were unambiguously identified. In principle, the number of mutations to be analysed by this method is determined by the number of the oligonucleotides with unique migration rates in a gel assay. This method can be easily modified to meet the specific *CFTR* mutation spectrum in any population.

To determine the haplotypes associated with the CF and normal chromosomes, two extragenic polymorphic sites, XV2c/*Taq*I[28] and KM.19/*Pst*I,[29] and three intragenic microsatellites, IVS8CA, IVS17BTA, and IVS17BCA,[30] were studied. Haplotype phases were established for 53 normal and 49 mutant chromosomes[26] and 75.5% of mutant chromosomes were found to be associated with diallelic haplotype B. The alleles carrying the ΔF508 and 394delTT mutations were exclusively on the B haplotype. Only 11.3% of the normal chromosomes were on the B haplotype. Nine different microsatellite haplotypes were determined on ΔF508 chromosomes. Among these, 23-31-13 and 17-31-13 together account for 69% of the ΔF508 alleles in Estonia. This is consistent with the published data, in which three haplotypes, 23-31-13, 17-31-13, and 17-32-13, account for 85% of the ΔF508 alleles in Europe.[31] Interestingly, the 394delTT mutation was associated in our population with three different microsatellite haplotypes: 23-36-13, 23-37-13, and 25-36-13. In Finland, 394delTT is associated exclusively with haplotype 23-36-13.[3] The 23-37-13 haplotype was first found on three out of four 394delTT chromosomes in the Flemish population.[32] Haplotype 25-36-13 has been associated with this mutation only in the Estonian population so far.

Other mutations appeared with haplotypes different from those found for ΔF508 and 394delTT mutants at both microsatellite and diallelic markers. 3659delC was associated with C-16-35-13, the same haplotype associated with this mutation in all populations studied, supporting its recent emergence. S1196X was associated with haplotype D-16-7-17, the same in our population and in Russia.[21] Haplotype A-17-32-13 was determined for mutation I1005R. It differs from the one found in Germany by one repeat in the IVS8 locus and could have been derived from it by slippage mechanism.[31] The mutations R117C and R1066H are obviously recurrent and therefore neither diallelic markers nor microsatellites matched to the corresponding haplotypes from the other populations.

As the population of Estonia stems from an admixture of several tribes, Mesolithic, Finno-Ugric, Baltic, German, and Slavic, a heterogeneous distribution of mutations was expected. In this study, two *CFTR* gene mutations were found to be common in Estonia. The major mutation, ΔF508, accounts for 51.7% of the CF chromosomes, which is less than the mean value of 72.8% for northern and central European countries.[4] However, the frequency is relatively similar to the corresponding estimates from neighbouring Russia (61.8-46.3%),[4 33] southern parts of Sweden (50.8% in Stockholm),[2] and southern parts of Norway (59.5% in Oslo).[2] The frequency of ΔF508 is relatively low in other studied Finno-Ugric populations also: 45% in Finland[3] and 50% in Hungary.[34] The deletion 394delTT is remarkably frequent in Estonia, accounting for 13.3% of all the CF chromosomes and ≈28% of the non-ΔF508 chromosomes. This is the second most frequent mutation in several Nordic populations, with a relative frequency of 1.9% in Denmark,[2] 3.2% in Flanders,[32] 6.5% in Sweden,[2] 2.2-5.5% in Norway,[2] and 30% in Finland.[3] It was also found on 1.5% of the CF chromosomes in Russia.[32]

We did not find any of the mutations more common in European populations, such as G542X (2.6%), N1303K (1.6%), G551D (1.5%), or W1282X (1.0%),[4] which is not surprising, as the relative frequency of these mutations is less than 1% in Nordic countries also. The eight rare mutations found in our patients have all been described previously with frequencies of less than 0.1% in the whole European population. Only 3659delC is more common; mainly found in northern Europe, it comprises 3% of the CF chromosomes in Sweden.[4] Considering the Swedish influence on Estonia in the past, we expect to find this mutation on more than one chromosome in further testing.

In conclusion, the results of an extensive analysis of *CFTR* gene mutations in 60 CF chromosomes from Estonia are presented. About 80% of all mutations were identified. From the 12 unidentified alleles, haplotypes were identified in three, which turned out to be all different, so more mutations can still be found in our population. The

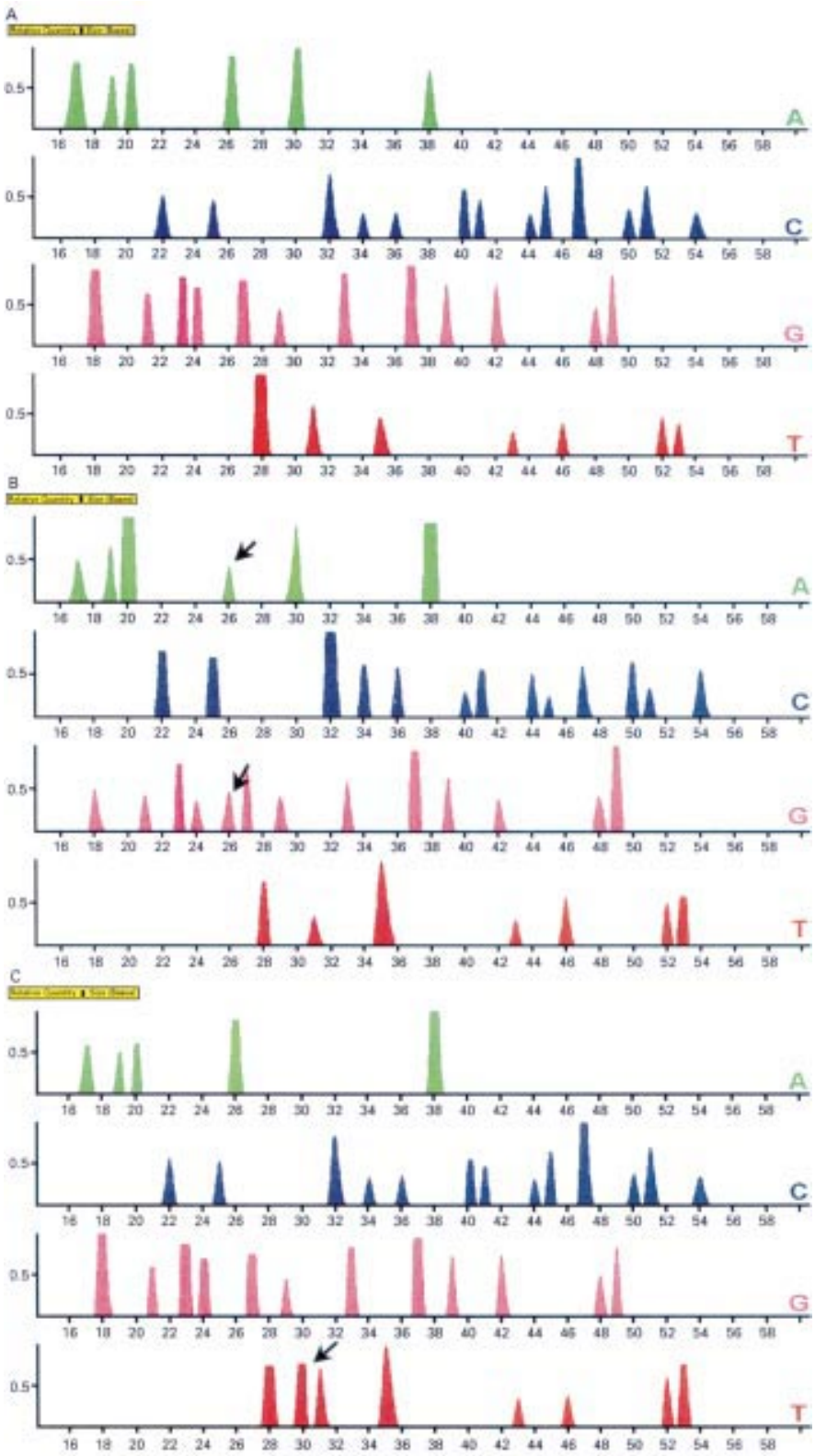

Figure 1 Electrophoretograms of the products of multiple allele specific primer extension analysis for 38 CFTR gene mutations. The measured fluorescence intensity is shown on the vertical axis and the size of extension reaction product (in base pairs) on the horizontal axis. The peaks correspond to the extended mutation specific primers. The mutation sites were checked in the following order from left to right: S549R(17-mer), S549N(18-mer), 2143delT(19-mer), K1200E(20-mer), 3849+10kb C→T(21-mer), R117C(22-mer), N1303K(23-mer), 621+1G→ T(24-mer), G551D(25-mer), 394delTT(26-mer), G542X(27-mer), I1005R(28-mer), 3905insT(29-mer), ΔF508(30-mer), 3821delT(31-mer), W1282(32-mer), R553X(33-mer), R117H(34-mer), 3732delA(35-mer), 2184insA(36-mer), R1162X(37-mer), ΔI507(38-mer), R334W(39-mer), V520F(40-mer), 1717-1G→A(41-mer), 2789+5G→A(42-mer), 2184delA(43-mer), 1898+1G→A(44-mer), R560T(45-mer), 3849+4A→G(46), A455E(47-mer), G85E(48-mer), 3659delC(49-mer), R347P(50-mer), Q493X(51-mer), 1078delT(52-mer), Y122X(53-mer), 711+1G→T(54-mer). Colour code: green ddATP, blue ddCTP, purple ddGTP, red-ddTTP. (A) Normal sample. (B) Heterozygous sample with mutation 394delTT. The arrows point to two peaks, corresponding to 26 bp extended primer; the one in A lane identifies a normal allele and the other in lane G identifies the mutation. (C) Homozygous sample with mutation ΔF508. The arrow points to the 30 bp peak in lane T, corresponding to the mutant allele.

mutations could be outside the investigated regions, for example, intron mutations, promoter mutations, or large deletions. Although knowledge about the precise distribution of *CFTR* gene mutations in Estonian patients is not complete, it is still valuable for molecular diagnosis and genetic counselling.

The principle of the MASPE method was first presented by A Metspalu, J M Shumaker, and C T Caskey at the 26th Annual Meeting of the European Society of Human Genetics in Paris, 1–5 June 1994. Fluorescent multiplex primer extension method for mutation analysis (Book of Abstracts, abstract No 57). We are grateful to all the patients and their families and we thank all the clinicians and technicians for their cooperation. We especially thank Dr Marianne Schwartz from the Rigshospitalet, Copenhagen and Dr Mireille Claustres from the Institute de Biologie, Montpellier for providing opportunities to work in their laboratories and to learn the methods of SSCP and DGGE. This work was supported, in part, by Open Estonian Foundation grant 592, Estonian Science Foundation grant 2967, Ministry of Education of Estonia grant 0180518s98, and by PECO grant ERBCIPDCT940220 from EC.

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