Mutation analysis of the *CFTR* gene in Slovak cystic fibrosis patients by DHPLC and subsequent sequencing: identification of four novel mutations

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Abstract. Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder in Caucasians. Its incidence is approximately 1 : 2500 newborns. CF is caused by mutations in the transmembrane conductance regulator (*CFTR)* gene, which encodes an important chloride ion channel. The disease affects the respiratory, digestive and reproductive systems. To date more than 1550 mutations and polymorphisms have been identified throughout the *CFTR* gene, making the DNA diagnosis more difficult. Rapid accurate identification of *CFTR* gene mutations is important for confirming the clinical diagnosis, for cascade screening in families at risk for CF, for understanding the correlation between genotype and phenotype, and moreover it is also the only means for prenatal diagnosis. Individuals suspect of CF are in Slovakia presently screened for the presence of 30 common mutations, giving mutation detection rate only approximately 48%. To increase the detection rate we applied a gene scanning approach using DHPLC system for analysing specifically all *CFTR* exons. The fragments showing abnormal elution profiles were subsequently sequenced to characterize the DNA change. We have identified a total of 28 different mutations up to present not found in Slovak CF patients, and 17 different polymorphisms. Four mutations (G437D, H954P, H1375N, and 3120+33G>T) are novel, not yet found in any other CF patient all over the word.

Key words: DHPLC — Mutation detection — Cystic fibrosis — *CFTR* gene — Sequencing

Introduction

Cystic fibrosis (CF) is, with an incidence of approximately 1 : 2500, the most common lethal autosomal recessive disorder among Caucasians. It affects the respiratory, digestive and reproductive systems. There is not yet an effective cure for CF and the life expectancy of patients is significantly reduced. Early diagnosis and symptomatic treatment, however, significantly increase their mean age of survival (Sims et al. 2007). CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which encodes a chloride ion channel (Kerem et al. 1989; Riordan et al. 1989; Rommens et al. 1989). To date more than 1550 mutations and polymorphisms have been identified throughout the *CFTR* gene (Cystic Fibrosis Mutation Database, http://www.genet.sickkids.on.ca/cftr). In populations of European origin the most common mutation is the F508del. In Slovak patients it accounts for 59.4% of all *CFTR* mutations found so far (Kádaši et al. 1992, 1997). However, there is great mutational heterogeneity in the not-F508del chromosomes, the vast majority of which being either private or limited to a small number of patients.

Rapid and accurate identification of *CFTR* gene mutations is important for confirming the clinical diagnosis, for cascade screening in families at risk for CF, for understanding the correlation between genotype and phenotype, and moreover it is also the only means of prenatal diagnosis. However, due to the extensive mutation heterogeneity of the *CFTR* gene, to reach the highest mutation detection rate possible,

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search for any mutation within the whole gene is necessary. For this purpose several screening methods have been used (SSCP, DGGE), among which denaturing high performance liquid chromatography (DHPLC), has emerged as one of the most sensitive one (Le Maréchal et al. 2001; D'Apice et al. 2004). In addition it provides a high degree of automation and throughput.

Individuals suspect of CF in Slovakia are presently screened for the presence of 30 common mutations, giving the detection rate of only approximately 48% of all CF chromosomes. For diagnostic purposes it is a very low figure, considering the detection rate of about 95%, recommended by Consensus Conference organized by the European Cystic Fibrosis Society (Castellani et al. 2008).

The aims of this work were:

- to optimize the application of DHPLC for the *CFTR* gene scanning by defining suitable conditions for PCR amplification and DHPLC analysis,
- to use DHPLC for detection of *CFTR* gene mutations in a set of CF-suspect individuals, in which two causative mutations could not been identified by the previously used methods,
- to evaluate the efficiency of DHPLC analysis in detection of *CFTR* gene mutations in the Slovak population.

Materials and Methods

DNA samples

Genomic DNA was isolated from peripheral blood leukocytes using phenol-chloroform extraction followed by ethanol precipitation. The concentration and purity of DNA samples was measured by the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA). DNA samples were then diluted to the stock concentration of 75 ng/μl.

We analysed DNA samples of 85 non-consanguineous Slovak patients that had previously been tested for the presence of the most common CF mutations, due to suspicion of CF. In these patients, 10 different mutations were identified in one copy: F508del (65 patients), G542X (4 p.), R117H (3 p.), 3849+10kbC→T (3 p.), I148T (2 p.), R347P (2 p.), R553X (2 p.), N1303K (2 p.), 711+1G→T (1 p.), and 1717-1G→A (1 p.).

DNA amplification

All 27 coding exons along with intron-exon boundaries of the *CFTR* gene were amplified in 27 PCR reactions using primers previously described by Le Marechal et al. (2001). Novel primer design was chosen for exons 6b and 9 (primers CFTR-ex6bF, CFTR-ex9F) to include the whole exons along with intron-exon boundaries and thus improving the sensitivity. These primers were designed by using Primer3 software (http://frodo.wi.mit.edu). Further, three other primers were considered unnecessarily long and were shortened (CFTR-ex15F, CFTR-ex17aR, CFTR-ex23F). The primers, summarized in Table 1, were purchased from Sigma-Genosys (Germany).

The PCR for each of the 27 *CFTR* exons was performed in a 30 μl reaction volume containing 1× concentrated S1.5 buffer (final concentration of $MgCl_2$ was 1.5 μmol/l), 0.25 μmol/l of both primers, 67 μmol/l of each dNTP, 0.75 U Taq DNA polymerase, and approximately 75 ng DNA. All 27 exons were amplified using one of two touchdown PCR protocols previously described by Le Marechal et al. (2001). Cycling conditions were as follows: initial denaturation at 94°C for 3 min, 14 touchdown cycles with annealing temperature decreasing from 57/63°C by 0.5°C per cycle (denaturation at 94°C for 20 s, annealing for 40 s, primer extension at 72°C for 45 s), and 25 cycles at the final touchdown annealing temperature (50/56°C – Table 1) and a final elongation step at 72°C for 7 min. All reactions were carried out using the GeneAmp PCR system 2700 (Applied Biosystems, USA) and XP Cycler (Bioer Technology, China).

DHPLC analysis

Prior to DHPLC analysis, to enable the formation of heteroduplexes, PCR products were denatured at 94°C for 5 min. and then gradually reannealed by decreasing the temperature by 0.5°C per 0.5 min to 54°C (in 80 steps).

DHPLC analysis was performed using the Transgenomic WAVE 3500 System equipped with a DNASep column (Transgenomic, Inc., UK). The temperature of the oven for the optimal separation of heteroduplex molecules in each amplicon was deduced from the melting profile of the amplicon sequence computed by the Navigator software (Transgenomic). All exons were analyzed at temperatures that corresponded to approximately 80–90% of α-helical fraction; if different melting domains were present in the exon, it was analyzed at 2 or 3 different temperatures (Table 1). Aliquots of 5 μl crude PCR samples were loaded onto a preheated DNASep column. DNA was eluted from the column by a linear acetonitrile gradient in 0.1 mmol/l triethylamonium acetate (TEAA) buffer, pH 7, at a constant flow rate of 0.9 ml/min. The gradient was formed by mixing buffer A (0.1 mmol/l TEAA) and buffer B (0.1 mmol/l TEAA and 25% acetonitrile). For each fragment, the gradient of buffer B was automatically computed by the Navigator software. Elution of DNA was detected by 260 nm UV absorbance. DHPLC chromatograms were analyzed using the Navigator software. Samples with extra peaks or with a difference in peak appearance were scored as positive.

Table 1. PCR (primer sequences, final touchdown annealing temperatures) and DHPLC (oven temperature) analysis conditions for 27 exons of the *CFTR* gene

Exon	Primer name	Sequence 5'→3'	Amplicon length (bp)	Final annealing temperature (°C)	Oven temperature (°C)
1	CFTR-ex1F CFTR-ex1R	TTgAgCggCAggCACC gCACgTgTCTTTCCgAAgCT	182	56	61.9 63.4
2	CFTR-ex2F CFTR-ex2R	CAAATCTgTATggAgACC CAACTAAACAATgTACATgAAC	194	50	55.3 57.5
3	CFTR-ex3F CFTR-ex3R	gAAATAggACAACTAAAATA ATTCACCAgATTTCgTAGTC	259	50	54.0 56.2
4	CFTR-ex4F CFTR-ex4R	CACATATggTATgACCCTCT TTgTACCAgCTCACTACCTA	437	56	56.0 57.7
5	CFTR-ex5F CFTR-ex5R	gTTgAAATTATCTAACTTTC AACTCCgCCTTTCCAgTTgT	192	50	53.5 55.1
6a	CFTR-ex6Af CFTR-ex6aR	TCCTTTTACTTgCTTTCTTTCA TATgCATAgAgCAgTCCTggTT	344	50	57.2 59.5
6b	CFTR-ex6bF CFTR-ex6bR	TgACTTAAAACCTTgAgCAgTTCTT gAggTggAAgTCTACCATgA	300	56	54.2 56.1
7	CFTR-ex7F CFTR-ex7R	TgCTCAgATCTTCCATTCCAAg AACTgATCTATTgACTgAT	390	50	54.3 58.0 61.0
8	CFTR-ex8F CFTR-ex8R	AATgCATTAATgCTATTCTgATTC AgTTAggTgTTTAgAgCAAACAA	190	56	53.7 55.0
9	CFTR-ex9F CFTR-ex9R	ggCCATgTgCTTTTCAAACTA CTTCCAgCACTACAAACTAgAAA	377	56	55.4
10	CFTR-ex10F CFTR-ex10R	TgATAATgACCTAATAATgAT CATTCACAgTAgCTTACCCA	363	50	50.0 55.5
11	CFTR-ex11F CFTR-ex11R	TgCCTTTCAAATTCAgATTgAgC ACAgCAAATgCTTgCTAgACC	197	56	56.4 57.8
12	CFTR-ex12F CFTR-ex12R	gAATCgATgTggTgACCATATTgT CCAgTAgggCAgATCAgATTTgA	366	50	54.5
13	CFTR-ex13F CFTR-ex13R	TgCTAAAATACgAgACATATTgC TACACCTTATCCTAATCCTAT	906	50	53.8 57.0 59.1
14a	CFTR-ex14aF CFTR-ex14aR	CACAATggTggCATgAAACT gTATACATCCCCAAACTATCT	256	56	55.5 56.5
14b	CFTR-ex14bF CFTR-ex14bR	gggAggAATAggTgAAgAT TACATACAAACATagTggATT	175	50	58.6 60.6
15	CFTR-ex15F CFTR-ex15R	CggAAATTCAgTAAgTAACTTTgg AgCCAgCACTgCCATTAgAAA	401	56	56.2 57.7
16	CFTR-ex16F CFTR-ex16R	CTgAATgCgTCTACTgTgATCCA TgTgggATTgCCTCAggTTT	401	56	53.3
17a	CFTR-ex17aF CFTR-ex17aR	AATCACTgACACACTTTgTCCACTT ggCTCTTATAgCTTTTTTACAAgATg	281	56	56.1 57.5
17b	CFTR-ex17bF CFTR-ex17bR	AATgACATTTgTgATATgAT CTTAAATgCTTAgCTAAAgT	379	50	54.7 58.0
18	CFTR-ex18F CFTR-ex18R	AgTCgTTCACAgAAgAgAgA AATgACAgATACACAgTgACCCTCA	312	50	56.2
19	CFTR-ex19F CFTR-ex19R	gTgAAATTgTCTgCCATTCT ACTCCATATAATAAAACATgTgTg	449	50	57.0
20	CFTR-ex20F CFTR-ex20R	ATCTTCCACTggTgACAggA AAAgACAgCAATgCATAACAA	400	50	57.3
21	CFTR-ex21F CFTR-ex21R	AATgTTCACAAgggACTCCA CAAAAgTACCTgTTgCTCCA	477	56	52.2 55.1
22	CFTR-ex22F CFTR-ex22R	ATCAATTCAAATggTggCAggT AATgATTCTgTTCCCACTgTgCT	370	56	58.5
23	CFTR-ex23F CFTR-ex23R	CggCAAggTAAATACAgATCAT gCAggAACTATCACATgTgA	250	56	54.3 57.2
24	CFTR-ex24F CFTR-ex24R	TCCCTgCTCTggTCTgACCTgC CATgAggTgACTgTCCCACgAg	329	56	59.3 61.2

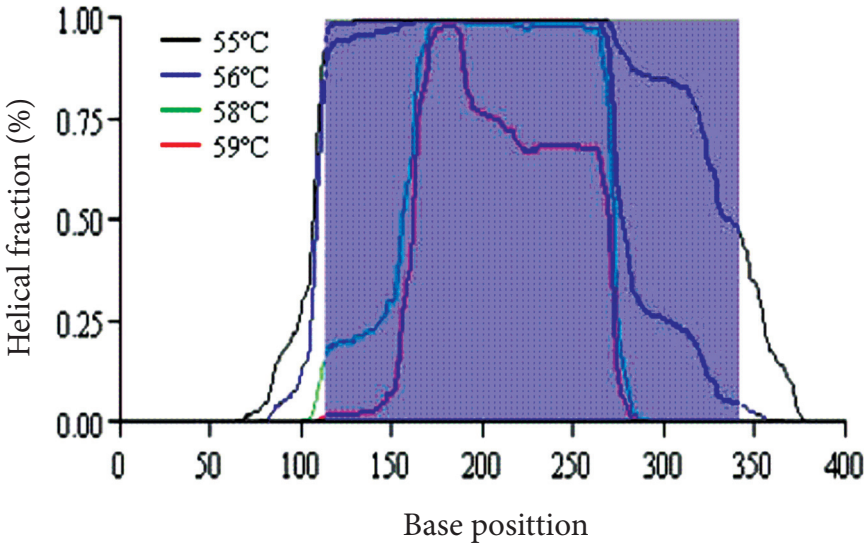

Figure 1. Melting profile of exon 17b of the *CFTR* gene. The coding sequence, represented by the blue rectangle contains two melting domains. The area of 5'and 3'ends start to melt at 55°C, whereas the central area starts to melt at 58°C (the amplicon was analyzed at 54.7°C and 58°C).

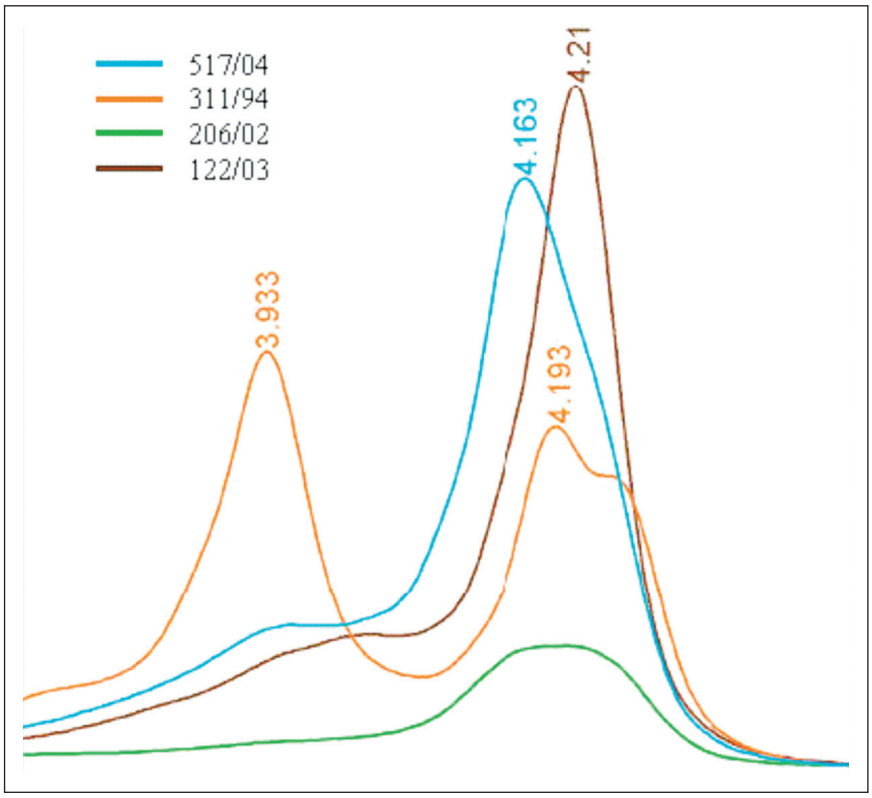

Figure 2. Elution profiles of exon 17b corresponding to three different *CFTR* mutations in heterozygous state (sample 517/04 – polymorphism 3272-93T/C; sample 311/94 – mutation 3272-26A→G; sample 206/02 – mutation R1066L) compared to a normal wild-type sample (122/03). DHPLC analysis was performed at 54.7°C.

Sequencing

Samples showing abnormal elution profiles were reamplified from genomic DNA. PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (protocol available at www.promega.com/tbs/tb308/tb308.pdf).

The sequencing reaction was performed with the BigDye Terminator v1.1 cycle sequencing kit in a 10 μl volume containing 25–30 ng DNA template, 2 pmol selected primer, 0.5 μl RR mix and 1 × BD buffer. Cycle sequencing was performed on the Veriti Thermal Cycler (Applied Biosystems, USA) using the recently described STeP cycle sequencing protocol (Platt et al. 2007). To remove excess dye terminator, extension products were purified by ethanol/sodium acetate precipitation. An aliquot of 12.5 μl Hi-Di formamide was added to the purified sequencing products, after which the products were denatured at 94°C for 5 min and analyzed by capillary electrophoresis on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequencing data were processed by the Sequencing Analysis Software v5.2 (Applied Biosystems) and were analyzed using the Chromas software (Technelysium, Australia).

Results

DHPLC analysis

The melting profiles were studied by using the Navigator software; the majority of the amplicons (18) showed two different melting domains and were therefore analyzed at two different temperatures (e.g. exon 17b; Figure 1), seven exons were analyzed at a single temperature, and we have chosen three temperatures for exons 7 and 13 (Table 1).

When the DHPLC chromatographs were analyzed by the Navigator software, abnormal elution profiles (differing from the wild-type homozygous samples) were observed in 23 of 27 exons (for exons 8, 14b, 17a, and 23 no abnormal profiles were found). Examples of three different atypical (heterozygous) profiles and a homozygous profile observed during analysis of exon 17b are illustrated by Figure 2. The presence of profiles different from wild type was rare for the majority of exons, however, for exons 6b, 9, 10, 14a and 24 the heterozygous profiles were frequent due to the presence of common polymorphisms. Since in all of the 85 tested samples one mutation was found prior to our work, these mutations were used as positive controls of our DHPLC analysis. Indeed, all the mutations situated in the amplified sequence led to the formation of atypical elution profiles of the particular amplicons. Mutation 3849+10kbC→T could not be identified because it lies outside the chosen amplicons.

Sequencing

For the five highly polymorphic amplicons (6b, 9, 10, 14a, 24) we sequenced all the samples showing less common heterozygous profile, and 4 samples for each common heterozygous profile. For the remaining 22 exons, we sequenced all samples showing an atypical profile and 4 samples with a homozygous profile as negative controls. Sequencing confirmed the presence of a mutation or a polymorphism in all amplicons that had showed abnormal elution profiles; in the homozygous controls no mutations were found. We identified the second mutation in 27 samples (32%), one sample contained 3 mutations (ΔF508, E831X, R851X). Overall we identified 28 different mutations, from which 18 were found for the first time in Slovak CF patients (Table 2), 4 of them (G437D, H954P, 3120+33G→T, H1375N) have not been previously described elsewhere in the world. In addition we identified

Table 2. Mutations identified first time in Slovak CF patients

Mutation	Location	Number
R31C	exon 2	1
306insA	exon 3	1
712-1G→T	intron 5	2
G437D	exon 9	1
2184insA	exon 13	4
R764X	exon 13	1
2143delT	exon 13	1
E831X	exon 14a	1
R851X	exon 14a	1
H954P	exon 15	1
3120+33G→T	intron 16	1
3272-26A→G	intron 17a	4
R1066L	exon 17b	1
3600+2insT	intron 18	1
S1159F	exon 19	1
L1227S	exon 19	1
4108delT	exon 22	1
H1375N	exon 22	1

Table 3. Identified less common polymorphisms

Polymorphism	Location	Number
125G/C	exon 1	5
R75Q	exon 3	4
875+40A/G	intron 6a	6
IVS8-5T	intron 8	9
L467F	exon 10	2
1716G/A	exon 10	1
I807M	exon 13	1
3041-71G/C	intron 15	3
3272-93T/C	intron 17a	5
R1162L	exon 19	2
P1290P	exon 20	6

17 different DNA polymorphisms, the less common ones are summarized in Table 3. The very frequent polymorphisms included TTGA repeats (intron 6a), 1001+11C/T (in 6b), 1342-12(GT)n (in 8), poly-T tract variations (in 8), M470V (exon 10), 2694T/G (ex 14a), and 4521G/A (ex 24).

Discussion

We have identified 4 novel *CFTR* mutations, previously not reported in any other population, which can extend the database of known mutations.

G437D is a missense mutation produced by a nucleotide exchange of G to A at position 1442 in exon 9, leading to a substitution of glycine to aspartic acid at codon 427. It is a substitution of an uncharged to a negatively charged amino acid located in the cytoplasmic section joining the MSD1 and NBD1 domains. The CF patient carrying this mutation, carries 3849+10kbC→T on the other allele, is 21 years old, was diagnosed at 11.3 year, is pancretic sufficient, and has a milder lung disease. Since 3849+10kbC→T is known to be associated with a milder phenotype (Augarten et al. 1993), the severity of the G437D mutation from this case cannot be presumed.

H954P is a missense mutation involving a nucleotide exchange of A to C at position 2993 in exon 15, leading to a substitution of histidine by proline at codon 954. It is a substitution of a positively charged by an uncharged amino acid located in the MSD2 of the protein. The carrier of this mutation si suspect of CF, carries F508del on the other allele, is 13 years old, the clinical phenotype was not specified.

3120+33G→T is a mutation defined by a nucleotide change of G to T at position 3120+33 in the 5' part of intron 16. The mutation may impair mRNA splicing by affecting a splicing regulatory element (e.g. by disrupting a splicing enhancer or activating a splicing silencer) or it may be a sequence variation with minimal impact on the phenotype. The carrier of this mutation is suspect of CF, carries F508del on the other allele, is 5 years old, the clinical phenotype was not specified.

H1375N is a missense mutation involving a nucleotide exchange of C to A at position 4255 in exon 22, leading to a substitution of histidine to asparagine at codon 1375. It is a substitution of a positively charged to an uncharged amino acid located in the NBD2 of the protein. The patient carried F508del on the other allele, is 51 years old, is pancreatic insufficient with chronic pancreatitis, and has no lung manifestation of the disease. Hence H1375N probably leads to a milder phenotype (because F508del represents a severe mutation).

Screening for the 30 most common mutations of the *CFTR* gene in 335 Slovak patients (that is 670 chromosomes) registered with clinical diagnosis of CF confirmed, up to present, 319 mutation carrying chromosomes, which represents only about 48% of all CF chromosomes. It is obvious that for ensuring effective mutation based diagnostics significantly higher detection rate should be achieved. According to recommendations of the Consensus Conference organized by the European Cystic Fibrosis Society (Castellani et al. 2008) this figure should be at least 95%. DHPLC analysis and subsequent sequencing of the respective amplicons increased the detection rate in our cohort to 66%. Even this figure, however, does not reach the recommended detection rate, nor the rate achieved in other populations using the same approach. For example D'Apice et al. (2004) were able to identify *CFTR* mutations in 88% of CF chromosomes, and even Le Maréchal et al. (2001) report on 98% figure. Several factors can stand in the background. One of them is undoubtedly the sensitivity of DHPLC. Oven temperature is the most important parameter that affects the sensitivity of DHPLC in detecting mutations. In this study, the analysis temperatures for each amplicon were deduced from the melting profiles computed by the Navigator software. Due to the lack of different mutations found in Slovak population prior to our study, the accuracy of oven temperatures proposed by us for the analysis of particular exons could not be empirically verified for all exons (there were only 8 different mutations located in 6 exons identified in our set of DNA samples by previously used methods, however, all of these mutations led to the formation of different elution profiles compared to wild type). Therefore it can not be excluded that some mutations in some amplicons escaped DHPLC detection. On the other hand, we can not exclude a different mutation scale in Slovak CF patients compared to that in populations mentioned above. Namely, disease causing mutations can even be localized in introns, not included into amplicons. Further, according to Férec et al. (2006) large deletions of entire exons or multiple exons would also be missed, because in patients heterozygous for such mutations no heteroduplexes would be formed. Indeed, such deletions have been found to account for about 20% of unidentified CF chromosomes. Analysis of large rearrangements in the *CFTR* gene we plan to accomplish in the near future.

In conclusion, however, it can be stated, that DHPLC approach significantly increases the mutation detection rate in the *CFTR* gene, and considering that it is far less demanding in terms of both financing and laboratory time (processing time) in comparison with mutation detection by sequencing the entire gene. Thus it is beneficial to involve DHPLC approach into cascade screening of *CFTR* mutations in CF patients for whom the screening for the most common mutations in the given population did not reveal both disease causing mutations.

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