

## ANALYSIS OF EXTRA- AND INTRAGENIC MARKER HAPLOTYPES AS PART OF MOLECULAR DIAGNOSIS OF CYSTIC FIBROSIS IN PATIENTS FROM SERBIA

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**Abstract** — Cystic fibrosis (CF) is the most common fatal autosomal recessive disease in Caucasians. Since characterization of F508del, the predominant mutation in different countries, more than 1500 mutations have been discovered in the CFTR gene, including a large number of polymorphisms. After molecular screening of 222 CF patients from Serbia, we detected 21 different CFTR mutations, F508del being the most frequent (69.59% of CF alleles). A total of 21 mutations cover almost 80% of CF alleles in this group. Since the molecular basis of CF is highly heterogeneous in our population, studying the haplotype association with normal and CF chromosomes could be very helpful in all cases where one or both mutations remain unidentified. Haplotype analysis was done using six diallelic sites and one tetranucleotide repeat (XV2C-KM19-MP6D9-J44-IVS6a(GATT)-M470V-T854T) on 99 F508del, 90 non-F508del, and 105 normal chromosomes. Strong linkage disequilibrium was observed for CFTR mutations and one haplotype (1-2-2-1-6(2)-1-1), while normal chromosomes were mostly associated with another (1-1-2-1-6(2)-1-2). The obtained results show that in most cases it would be possible with this group of polymorphisms to separate normal chromosomes from chromosomes which carry the CFTR mutation. As far as prenatal diagnosis of cystic fibrosis is concerned, haplotype analysis can be used as a helpful method of indirect diagnosis in families at risk with one or both CF alleles uncharacterized.

**Key words:** Cystic fibrosis, CFTR gene, mutation, haplotype analysis

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### INTRODUCTION

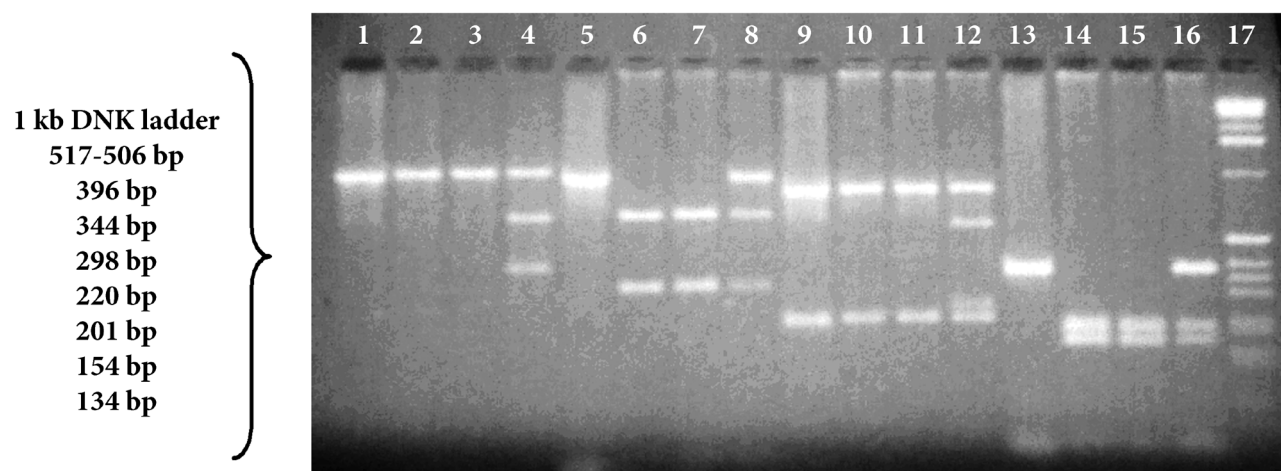
Cystic fibrosis (CF; MIM# 219700) is the most common fatal autosomal recessive genetic disease in Caucasians (1 in 2000-3000 newborn affected). Mutations in the gene encoding the CF transmembrane conductance regulator (CFTR; MIM# 602421) cause both classic and atypical forms of the disease (Bobadilla et al., 2002). Worldwide collaboration has resulted in discovery of almost 1500 mutations and a large number of polymorphisms, F508del being the most common in all populations, with a mean frequency of 66.8%. A group of relatively common CF alleles occurs in certain populations, but the majority of mutations are rare, being either private or limited to a small number of individuals (CFGCA, 2007).

In populations with high heterogeneity, mutation analysis can be facilitated by association between haplotypes for diallelic and microsatellite polymorphisms and CF mutations (Claustres et al., 1996; Kanavakis et al., 2003).

### MATERIALS AND METHODS

Patients included in this study attend the Mother and Child Health Institute of Serbia, Belgrade, Serbia, which is one of the biggest children's hospitals in the country. The CF diagnosis was based on typical clinical manifestations of pulmonary or/and gastrointestinal disease and high levels of sweat chloride concentration (higher than 60 mmol/l).

Genomic DNA was extracted from 222 CF patients and 446 family members by standard pro-



**Fig. 1.** PCR products after restriction digestion (2% agarose gel, 85V, 1.5 h). XV-2c: line 1-undigested sample; lines 2 and 3-samples homozygous for allele 1 (1/1); line 4-heterozygous sample (1/2); KM.19: line 5-undigested sample; lines 6 and 7-samples homozygous for allele 2 (2/2); line 8-heterozygous sample (1/2); J44: line 9-undigested sample; lines 10 and 11-samples homozygous for allele 1 (1/1); line 12-heterozygous sample (1/2); MP6-D9: line 13-undigested sample; lines 14 and 15-samples homozygous for allele 2 (2/2); line 16-heterozygous sample (1/2); line 17- 1 kb DNA ladder.

cedure (Miller et al., 1988).

DNA samples were screened for the presence of CFTR mutations by heteroduplex analysis on PAGE for the presence of F508del (Kerem et al., 1989; Rommens et al., 1990), simultaneous *in vitro* qualitative detection of 29 CFTR mutations frequent in Europe (Elucigene™CF 29 kit, Orchid), DGGE analysis of PCR amplified exons 1-24 (Fanen et al., 1992; Ghaneim et al., 1992; Costes et al., 1993), and sequencing (Sanger et al., 1977).

Haplotype analysis was done for six diallelic sites and one tetranucleotide repeat [XV2C - KM19 - MP6D9 - J44 - IVS6a (GATT) - M470V -T854T] on 189 CF and 105 normal chromosomes.

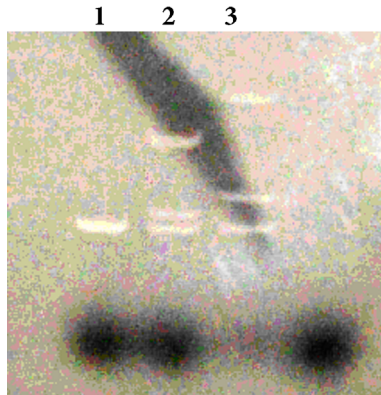
The IVS6aGATT polymorphism was detected by difference in mobility of the PCR product on 8% PAGE gel. Allele 1 showed seven repeats, allele 2 six, and allele 3 eight repeats (Dork et al., 1992).

Other haplotypes were determined after restric-

**Table 1.** Table 1. Allele frequencies of polymorphisms at the CF locus in a group of Serbian normal (N) and CF chromosomes.

Polymorphism	N			F508del CF			"non"-F508del CF		
	No. of chrom.	allele		No. of chrom.	allele		No. of chrom.	allele	
		1 (%)	2 (%)		1 (%)	2 (%)		1 (%)	2 (%)
XV-2C	54	0,55	0,45	42	0,80	0,20	65	0,66	0,34
KM.19	86	0,51	0,49	76	0,11	0,89	80	0,55	0,45
MP6-D9	76	0,46	0,54	57	0,00	1,00	75	0,44	0,56
J44	77	0,69	0,31	76	0,92	0,08	78	0,72	0,28
GATT	85	0,76	0,24*	75	0,04	0,96	79	0,53	0,47
M470V	44	0,80	0,20	43	0,93	0,07	34	0,68	0,32
T854T	73	0,51	0,49	50	1,00	0,00	74	0,62	0,38

\*Allele 3 (8 GATT repeats) was not included in the table, since it was found in only two normal chromosomes.



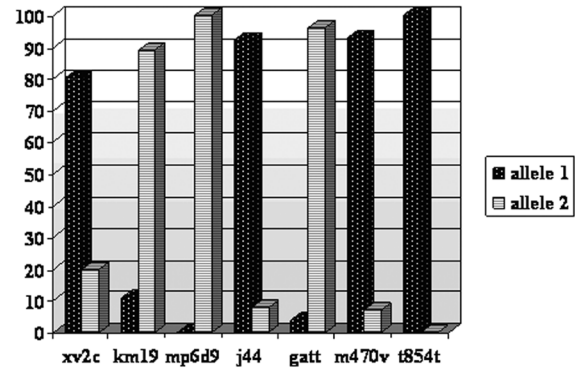
**Fig. 2.** PAGE analysis of PCR products of GATT repeats. Line 1-sample homozygous for allele 2/2 (6/6 repeats); line 2-sample heterozygous for alleles 1/2 (7/6 repeats); line 3-sample heterozygous for alleles 2/3 (6/8 repeats).

tion digests of the RFLP-PCR products were separated by agarose gel electrophoresis. Allele 1 showed the absence of restriction sites, while allele 2 showed their presence (Dork et al., 1992).

## RESULTS

After screening of 222 CF patients from Serbia, we detected 21 different CFTR mutations, F508del being the most frequent (69.59% of CF alleles). A total of 21 mutations cover 80% of CF alleles in this group. Mutation analysis showed the presence of 27 different genotypes; 22 patients (9.90%) had both CF alleles remaining uncharacterized.

Further haplotype analysis was done for 147 members of 44 families at risk. Four diallelic extragenic RFLPs were analyzed: J44 (located less than 10 kb away from the 5' end of the CFTR gene); and



**Fig. 3.** Allele frequencies of polymorphisms associated with the F508del mutation

XV-2c, KM.19, and MP6-D9 (located 175 kb, 125 kb, and 40 kb, respectively, ahead of exon 1 of the gene (Fig. 1)). DNA samples were also analyzed for the presence of two intragenic diallelic single nucleotide polymorphisms (SNPs): M470V (located in exon 10) and T854T (located in exon 14a). Finally, the last polymorphism analyzed was an intragenic tetranucleotide repeat, microsatellite (STR) IVS6aGATT, located at the junction of intron IVS6a and exon 6b (Fig. 2). The allele frequencies are shown in Table 1. All polymorphisms exhibit strong linkage disequilibrium with the F508del mutation, which was the most frequent one in the analyzed group of patients (Fig. 3).

Haplotypes were assigned where the phase could be established by typing both parents and affected child in each CF pedigree. The results showed the presence of 14 different haplotypes, six of them being the most common (Table 2). One (1-2-2-

**Table 1.** Frequencies of the most common marker haplotypes in a group of analyzed N (normal) and CF chromosomes from Serbia.

XV2C-KM19-MP6D9-J44-GATT-M470V-T854T	Haplotype frequency (%)		
	N chrom.	F508del CF chrom.	"non"-F508del CF chrom.
1 - 2 - 2 - 1 - 6 (2) - 1 - 1	7,6	91	15
2 - 1 - 1 - 2 - 7 (1) - 1 - 2	4	-	11
1 - 2 - 2 - 1 - 7 (1) - 1 - 2	9,5	2,58	21
2 - 2 - 2 - 1 - 7 (1) - 2 - 1	12	-	25
2 - 1 - 1 - 1 - 6 (2) - 1 - 2	-	4,7	-
1 - 1 - 2 - 1 - 6 (2) - 1 - 2	36	-	21

1-6(2)-1-1) was found mostly associated with the F508del mutation (91%) because of linkage disequilibrium. The most frequent (36%) haplotype in N chromosomes was (1-1-2-1-6(2)-1-2), while all six of the most common haplotypes were associated with non-F508del CF chromosomes (with frequencies in the range of 11-25%).

### DISCUSSION

Polymorphism analysis is often used as a valid procedure to assess the genetic heterogeneity of a population or to evaluate the origin of a mutation (Morral et al., 1994; Claustres et al., 1996; Morral et al., 1996; Angelicheva et al., 1997; Petreska et al., 1998; Kanavakis et al., 2003). Since the main goal of diagnostic analysis is to allow families at risk to do prenatal diagnosis, studying of haplotype association with normal and CF chromosomes could be very helpful in all cases where one or both CF alleles remain uncharacterized.

Analysis of six diallelic sites and one STR showed that all of them were in linkage disequilibrium with the most frequent CFTR mutation worldwide, F508del. Almost exclusively, allele 1 of XV-2c, J44, M470V, and T854T, and allele 2 of KM.19, MP6-D9, and IVS6aGATT repeats were found in association with this mutation. This specific diallelic haplotype (1-2-2-1-6(2)-1-1) is commonly associated with F508del in other populations too, supporting the theory of an ancient origin of this mutation as one that arose after one mutational event, and further spreading of it from the south through other parts of the Europe (Dork et al., 1992; Morral et al., 1994; Kanavakis et al., 2003). The same haplotypes were also found in normal and non-F508del CF chromosomes, but in much lower frequencies (7.6% of N and 15% of non-F508del mutations).

The most common (36%) and probably the oldest haplotype associated with normal chromosomes was 1-1-2-1-6(2)-1-2, also found in 21% of CF chromosomes which did not carry the F508del mutation. However, it can be expected that normal chromosomes can be separated from the CF chromosome, mostly due to high frequency of allele 1 of the IVS6aGATT repeat (76% of normal

chromosomes carried allele 1, compared to only 4% of F508del chromosomes and half of non-F508del chromosomes). All six common haplotypes were found in non-F508del chromosomes (11/25%), suggesting heterogeneity and different origin of those mutations. The high mutational heterogeneity and haplotype variability shown in the Serbian population is similar to those found in other populations in our region. This is also consistent with an earlier appearance of CFTR mutations in Southeast Europe, which would allow time for haplotype recombination (Dork et al., 1992; Kanavakis et al., 2003).

Knowledge of the population genetics and chromosomal haplotype of various CFTR mutations would significantly facilitate mutation diagnosis, especially in populations with high mutational heterogeneity, like ours. With respect to prenatal diagnosis, especially in cases where one or even both CF alleles were not identified using direct methods for mutation screening, a combination of methods for detection of CFTR mutations and haplotype analysis could provide rapid, accurate, and reliable prenatal diagnosis for almost all couples at risk of having a child affected with cystic fibrosis in our country.

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## АНАЛИЗА УНУТАР- И ВАНГЕНСКИХ ДНК МАРКЕРА КАО САСТАВНИ ДЕО МОЛЕКУЛАРНЕ ДИЈАГНОСТИКЕ КОД ОБОЛЕЛИХ ОД ЦИСТИЧНЕ ФИБРОЗЕ У СРБИЈИ

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Цистична фиброза (ЦФ) је најчешће аутозомно-рецесивно наследно обољење у популацији Кавказијанаца, са просечном учесталашћу од 1:2000-3000 живорођене деце. Више од 1500 мутација у гену који кодира синтезу ЦФ регулаторног протеина за трансмембранску проводљивост (ЦФТР) основни су узрок ове болести. Применом различитих техника молекуларне генетике, анализирани су ДНК 222 оболелих од ЦФ у Србији. Резултати скрининга ЦФТР гена показали су присуство 21 различите мутације, од којих је Ф508дел била најчешћа (69,59%). Иденти-

фиковане мутације покривале су 80% свих ЦФ алела у нашој популацији. У циљу одређивања хаплотипа, урађена је анализа 6 ди-алелних полиморфизама и једних тетрануклеотидних поновака (ХВ2ц-КМ.19-МП6-Д9-Ј44-ИВС6а(ГАТТ)-М470В-Т584Т) на укупно 189 ЦФ и 105 нормалних хромозома. Хаплотип 1-2-2-1-6(2)-1-1 је био најчешћи код носиоца Ф508дел мутације, што указује на постојање неравнотеже везаности гена. Код хромозома који нису носили мутацију у ЦФТР гену, најчешћи хаплотип је био 1-1-2-1-6(2)-1-2.

На основу добијених учесталости полиморфних алела, анализа хаплотипа у популацији где је хетерогеност ЦФТР мутација велика, као што је случај са нашом, добија практичан значај. Оправ-

дано је применити поменућу анализу у оквиру индиректне дијагностике код високо ризичних породицама у свим случајевима где су један или чак оба ЦФ алела остала неидентификована.