DOI: 10.1515/cipms-2018-0037

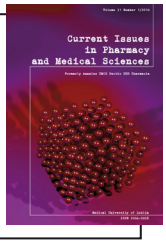
Curr. Issues Pharm. Med. Sci., Vol. 31, No. 4, Pages 200-203

sciendo

Current Issues in Pharmacy and Medical Sciences

Formerly ANNALES UNIVERSITATIS MARIAE CURIE-SKLODOWSKA, SECTIO DDD, PHARMACIA

journal homepage: http://www.curipms.umlub.pl/



Developing a diagnostic test to identify the selected mutation within the CFTR gene that determines the onset of cystic fibrosis

Angelika Szymczak[1]*, Piotr Ksiazek[1], Sylwia Mojsym-Korybska[1], Wojciech Skorupa[2], Albertyna Zbikowska-Machul[3]

[1] Department of Public Health Medical University of Lublin, Chodzki 1, 20-093 Lublin, Poland
[2] Clinic of Tuberculosis and Lung Diseases in Warsaw, Plocka 26, 01-138 Warszawa, Poland
[3] Independent Public Health Care Center in Puławy, Bema 1, 24-100 Pulawy, Poland

ARTICLE INFO

Received 14 March 2018
Accepted 24 May 2018

Keywords:
CF – cystic fibrosis,
CFTR – cystic fibrosis transmembrane conductance regulator,
EnaC – epithlial sodium channel,
ROMK – renal outermedullary potassium channel,
ORCC – outwardly rectifying chloride channel,
CBAVD – congenital bilateral absence of the vas deferent,
WHO – World Health Organization,
ECFTN – European Cystic Fibrosis Society,
PCR – polymerase chain reaction.

ABSTRACT

Cystic fibrosis is one of the most common genetic diseases among Caucasians due to its prevalence. Modern methods of molecular diagnostics and treatment of the disease allow to prolong the life of patients. In order to apply the appropriate treatment, the genetic basis of this disease should, however, first be known. The most common and the most severe mutation present in the CFTR gene (60-70% of cases) takes the form of an allele. This is responsible for the deletion of phenylalanine in position 508 (Δ508) of the CFTR protein. Determination of mutations in the CFTR gene using molecular techniques makes it possible to identify the causes of the disease in people who do not show the characteristic symptoms of cystic fibrosis.

INTRODUCTION

Cystic fibrosis is one of the most common genetic diseases among Caucasians due to its prevalence. There are approximately 1,200 diagnosed CF patients in Poland. However, it is estimated that the number of patients is greater, because many patients with similar symptoms are treated for other diseases. Modern methods of molecular diagnostics and treatment of diseases allow patients to prolong life and give a chance to improve its quality. In order to apply the appropriate treatment, the genetic basis of the disease should first be known.

The development of diagnostic techniques has made it possible to identify many genetic diseases that are conditioned by chromosomal aberrations or epigenetic changes. Depending on the changes occurring in the genome, various molecular techniques are used to identify genetic diseases.

Among the molecular biology techniques most commonly used in clinical diagnosis include: polymerase chain reaction (PCR), gel electrophoresis and its varieties, cloning of DNA fragments, DNA sequencing, Southern blotting, hybridization, Northern blot, in situ hybridization, FISH (fluorescent in situ hybridization) and DNA microarrays.

METHODS AND IDENTIFICATION OF THE SELECTED MUTATION OF THE CFTR GENE

The study was conducted on biological material obtained from 18 patients with cystic fibrosis. The genetic test was carried out with valid consent expressed by the Bioethical Commission. Each patient received an informed consent to participate in the study.

* **Corresponding author**
e-mail: angied@interia.pl

The aim of the work was to develop a diagnostic test for the identification of a selected mutation within the CFTR gene that determines the onset of cystic fibrosis.

The biological material was collected in the form of a cytological smear from the oral cavity. DNA isolation was performed using isolation kits. The assessment of the presence of the ΔF508 mutation in the CFTR gene was performed using two methods:

1. Polymerase chain reaction (PCR).
2. DNA sequencing.

A cytological swab was collected by using sterile swabs. The swab was obtained from patients in duplicate. DNA isolation was carried out according to the original protocol.

The PCR reaction was carried out using proprietary designed oligonucleotides.

3. Three variants of the starters have been designed:

- 1 pair – oligonucleotides to obtain amplicon in patients with at least one normal allele within the CFTR gene, i.e. lacking the CTT deletion characteristic of the ΔF508 mutation of the primers: MUKO1L-MUKO1R and MUKO1L-MUKO2R,
- 2 pair – oligonucleotides to obtain amplicon in patients with at least one abnormal allele within the CFTR gene, i.e. a CTT deletion characteristic of the ΔF508 mutation of the primer names: MUKO1L-MUKO3R and MUKO1L-MUKO4R,
- 3 pair – oligonucleotides to obtain an amplicon containing the site of the ΔF508 mutation in the CFTR gene to know the DNA sequence of this genomic region›s starter names: MUKO5L-MUKO5R.

4. The primers were designed in the Primer3 Inpvt program (version 0.4.0).

People with allele lacking CTT deletion – 2 alternative MUKO1L starter pairs were designed – MUKO1R and MUKO1L-MUKO2R.

People with CTT deletion (ΔF508 mutation) – 2 alternative MUKO1L starter pairs were designed – MUKO3R and MUKO1L-MUKO4R.

Primers that allow the amplification of the gene section in which the mutation is present – the MUKO5L primer pair-MUKO5R.

Detection of the resulting PCR product was obtained by electrophoresis in a 1.5% agarose gel. The next stage of this study was the sequencing of nucleic acids.

RESULTS AND CONCLUSIONS

In this work, three exemplary fluorograms obtained by DNA sequencing were presented: Figure 1 presents heterozygote with deletion and without CTT deletion (14),

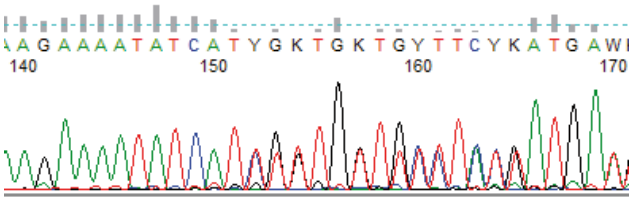


Figure 1. Fragment of the fluorogram obtained by sequencing the sample 14 - heterozygote: CTT deleted allele and allele without CTT deletion

Figure 2 presents a homozygote without CTT deletion (15),

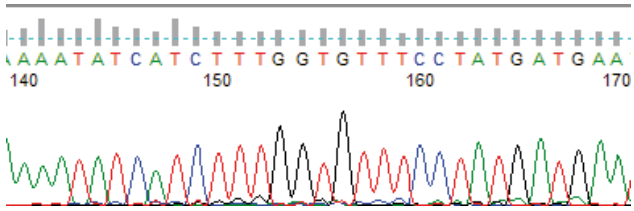


Figure 2. Fragment of a fluorogram obtained by sequencing the sample 15 - homozygote without CTT deletion

Figure 3 presents a CTT deletion homozygote (16).

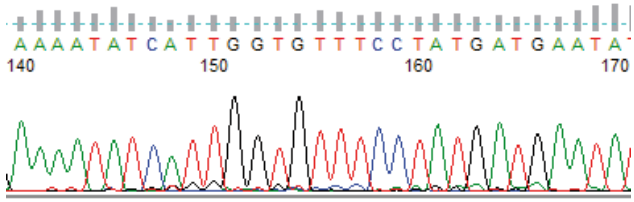


Figure 3. Fragment of fluorogram obtained by sequencing sample 16 - homozygote with CTT deletion

Table 1. List of results of PCR amplification of a CFTR gene fragment using primers MUKO1L-MUKO1R, MUKO1L-MUKO2R, MUKO1L-MUKO3R, MUKO1L-MUKO4R with data obtained on the basis of DNA sequence analysis

Determination of samples	Amplification of the stripped allele CTT deletion		Amplification of the allele with CTT deletion		Data obtained based on sequencing
	Starters MUKO1L/ MUKO1R	Starters MUKO1L/ MUKO2R	Starters MUKO1L/ MUKO3R	Starters MUKO1L/ MUKO4R	
1	-	-	-	-	Heterozygote: allele 1: CTT deletion; allele 2: no CTT deletion
2	-	-	-	-	Heterozygote: allele 1: CTT deletion; allele 2: no CTT deletion
3	-	-	-	+	-
4	-	-	-	-	Homozygote: both alleles with CTT deletion
5	-	-	-	-	Homozygote: both alleles with CTT deletion
6	-	+	-	-	-
7	-	+	-	-	Heterozygote: allele 1: CTT deletion; allele 2: no CTT deletion
8	-	+	-	+	Heterozygote: allele 1: CTT deletion; allele 2: no CTT deletion
9	-	-	-	-	-
10	-	+	-	+	-
12	+	+	+	+	Heterozygote: allele 1: CTT deletion; allele 2: no CTT deletion
13	-	-	-	-	-
14	-	+	-	+	Heterozygote: allele 1: CTT deletion; allele 2: no CTT deletion
15	-	+	-	+	Homozygote: both alleles do not contain CTT deletions
16	-	-	-	+	Homozygote: both alleles with CTT deletion
17	-	+/-	-	+	Heterozygote: allele 1: CTT deletion; allele 2: no CTT deletion
18	-	+	+	+	Homozygote: both alleles with CTT deletion
20	+	+	+/-	+	Heterozygote: allele 1: CTT deletion; allele 2: no CTT deletion

Signs:
(+) DNA amplicon was obtained
(-) no DNA amplicon was obtained

1. Among the designed methods, DNA sequencing with a pair of MUKO5L and MUKO5R primers made it possible to determine the genotype within exon 10 of the CFTR gene in the largest number of patients.
2. Using the PCR method, we managed to determine the genotype in less than half of the analyzed samples.
3. Among the two primer pairs analyzed, which were designed to amplify the ΔF508 DNA fragment within the CFTR gene, better results were obtained with the MUKO1L and MUKO2R oligonucleotides.
4. Among the two primer pairs analyzed, which were designed to amplify the DNA fragment lacking the ΔF508 mutation within the CFTR gene, better results were obtained using the MUKO1L and MUKO4R oligonucleotides.
5. The results obtained indicate the benefits of using two methods of analysis of the ΔF508 mutation in the CFTR gene simultaneously. However, the DNA sequencing method should be considered as a reference.

DISCUSSION

The incidence of CFTR gene mutation in Caucasians is 1/25. The most frequent finding is the F508del mutation in which a deletion of three nucleotides in exon 10 results in the loss of phenylalanine at position 508 of the CFTR protein. Most cases of this disease are caused by the F508del mutation in both gene alleles. The presence of the F508del mutation and other mutations (complex heterozygote) is usually the cause of the formation of disorders of varying severity of symptoms. It is dependent on the effect of particular mutations on the structure and function of the chloride channel and the sum of the effects caused by mutations. In the Polish population, in addition to the F508del mutation, the following mutations are found more often than 1% of patients with cystic fibrosis: 3949 + 10kbC > T (2.6%), G542X (2.5%), N1303K (1.7%), 1717 – IG > A (1.7%), R553X (1%), CFTR dele2, 3 (21kb) (2%) [1,2].

Stężowska-Kubiak [3] reports that the ΔF508 mutation occurs on 56.3% of alleles in people with CF symptoms [3]. Among these patients, the majority were F508del/F508del homozygotes, and the genotype including ΔF508del and known or unknown mutation occurred in 48.7% of all patients [4-6]. However, data on the presence of the ΔF508 mutation obtained in this study show the presence of the F508del/F508del genotype in only 27.7% of the population studied. In the conducted genetic study based on the sequencing technique, it was found that 8 patients with cystic fibrosis are heterozygotes with at least 1 allele with CTT deletion and 2 allele lacking CTT deletion.

The frequency of occurrence of the tested ΔF508 mutation in this study differs from the frequency of its occurrence in the Polish population. This discrepancy is probably due to the smaller number of patients tested for the detection of the ΔF508 mutation in the CFTR gene. Bal *et al*. [4] determined the incidence of the ΔF508 mutation in 84 Polish patients as 47.2%. In the paper by Witta *et al*. [6] the incidence of the ΔF508 mutation in the group of 617 patients was higher than in the studies of Bala *et al*., here – 53.9%. In Pogorzelski's [5] study, the incidence of the ΔF508 mutation was comparable to that of Witt [6] and amounted to 56.4%.

Bobadilla *et al*. [7] situate the population of Poland in a population group characterized by a high frequency of the ΔF508 mutation. This group also includes countries such as Germany (71.8%), Belarus (61.2%), the Czech Republic (70%) and Belgium (75.1%). In the French registry, 43.1% of all patients turned out to be ΔF508 homozygotes, and 36% had the F508del genotype or other known mutation [8]. In the United Kingdom, from 5701 patients – 54.3% were ΔF508 homozygotes, and in total, this mutation occurred in 92% of all respondents [9]. According to Irish data, ΔF508 homozygotes represent 64% of the studied population, and 94% of all patients have at least one allele with this mutation [10]. Similarly in Australia – 94% of the population have at least one allele with the ΔF508 mutation, and 51.4% are homozygous for the molecular defect [11]. Among 3084 patients from Canada – 49.2% have the F508del/F508del genotype, and 39.1% of all patients have at least one allele with this mutation [12]. The German report from 2009 verifies the percentage of the ΔF508 mutation presented in the work of Bobadilla *et al*. [7] – it occurred in a total of 65.7% of all patients with at least one allele with this mutation, and 46.9% of all patients who turned out to be homozygotes [13].

In this work, during the study, a combination of five primer pairs (MUKO1L/MUKO1R, MUKO1L/MUKO2R, MUKO1L/MUKO3R, MUKO1L/MUKO4R, MUKO5L/MUKO5R) was used. The MUKO1L/MUKO1R and MUKO1L/MUKO2R primers were designed to obtain the amplicon in the presence of at least one allele lacking CTT deletion in the CFTR gene. In turn, the MUKO1L/MUKO3R and MUKO1L/MUKO4R primers were designed to obtain the amplicon in the presence of at least one allele with the CTT deletion in the CFTR gene. Finally, the MUKO5L/MUKO5R primers aimed at amplifying the gene section in which the CTT deletion is present.

Among the latest research, the use of preimplantation diagnosis of cystic fibrosis should be noted. Preimplantation diagnostics is a method that allows genetic analysis of oocytes before or after fertilization or embryos before introducing them into the uterus of the future mother [14]. This diagnosis allows the analysis of the more than 30 mutations in the CFTR gene most frequently occurring in Europe [15]. Due to the high heterogeneity of mutations in the CFTR gene, in recent years, the identification of individual mutations has been supplemented with the analysis of highly polymorphic markers surrounding the CFTR gene [16] or intra gene [17].

Moutou *et al*. [17] proposed the use of two polymorphic intra-gene markers (IVS8CA and IVS17bCA) in combination with the identification of the F508del mutation. Goossens *et al*. additionally used an intra-gene marker IVS17bTA and four polymorphic markers that surround the gene (D7S490, D7S486, D7S480 and D7S523) [18]. This approach allows to propose a preimplantation diagnosis of cystic fibrosis in people with unknown or very rare mutations.

REFERENCES

1. Dequeker E, Cuppens H, Dodge J, Estivill X, Goossens M, Pignatti PF, et al. Recommendations for quality improvement in genetic testing for cystic fibrosis European Concerted Action on Cystic Fibrosis. *Eur J Hum Genet*. 2000;8(2):S2-24.
2. Dork T, Macek M, Mekus F, Tümmler B, Tzountzouris J, Casals T, et al. Characterization of a novel 21 - kb delection CFTR dele 2, 3 (21kb) in the CFTR gene: a cystic fibrosis mutation on Slavic orgin in common in Central and East Europe. *Hum Genet*. 2000;106:259-68.
3. Stężowska-Kubiak S. *Epidemiological and clinical characteristics of the Polish population of patients suffering from cystic fibrosis.* 2011.
4. Bal J, Maciejko D, Mazurczak T, Potocka A, Krawczak M, Reiss J. Frequency of the cystic fibrosis mutation delta F508 in Poland. *Hum Genet*. 1991;86:329.
5. Pogorzelski A, Witt M, Bal J. CFTR gene mutations in patients with cystic fibrosis in Poland. *Acta Pneumono Allergol Ped*. 1998;2:5-8.
6. Witt M, Bal J, Maciejko D, Mazurczak T. Incidence of CFTR gene mutations in patients with cystic fibrosis in Poland. *Pediatr Pol*. 1997;72:665-8.
7. Bobadilla J, Macek M Jr., Fine J, Farel P. Cystic Fibrosis: a worldwide analysis of CFTR mutations – correlation with incidence data and application to screening. Hum Genet. Mut. 2002;19:575-606.
8. *French CF Registry – Annual Data Report 2007.* Paris: Vaincre la Mucoviscidose and INED; 2010.
9. *UK CF Registry Annual Data Report 2008*. Cystic Fibrosis Trust; 2009.
10. *Annual Report 2007. The Cystic Fibrosis Registry of Ireland*; 2009.
11. *Cystic Fibrosis in Australia 2008. 11th annual report from the Australian Cystic Fibrosis Data Registry.* Australia: Cystic Fibrosis; 2010.
12. www.cysticfibrosis.ca
13. Sens B, Stern M. *Qualitätssicherung Mukoviszidose. Überlich über den Gesundheitszustand der Patient in Deutschland 2009.* Hippocampus Verlag KG;2010.
14. Shamseer L, Adams D, Brown N, Johnson JA, Vohra S. Antioxidant micronutrients for lung disease in cystic fibrosis. *Cochrane Database Syst Rev.* 2010;12:CD007020. DOI: 10.1002/14651858.CD007020.pub2.
15. Sanchez-Garcia J, Benet J, Gutierrez-Mateo C, Luís Séculi J, Monrós E, Navarro J. Multiple mutation analysis of the cystic fibrosis gene a single cells. *Mol Hum Reprod*. 2005;11:463-8.
16. Dreesen J, Jacobs L, Bras M, Herbergs J, Dumolin JCM, Geraedts JPM et al. Multiplex PCR of polymorphic markers flanking the CFTR gene; a general approach for pre-implantation genetic diagnosis of cystic fibrosis. *Mol Hum Reprod*. 2000;6:391-6.
17. Moutou C, Gardes N, Viville S. Multiplex PCR combining deltaF508 mutation and intragenic microsatellites of the CFTR gene for pre-implantation genetic diagnosis (PGD) of cystic fibrosis. *Eur J Hum Genet*. 2002;10:231-8.
18. Goosens V, Sermon K, Lissens W, De Rycke M, Saerens B, De Vos A, et al. Improving clinical preimplantation genetic diagnosis for cystic fibrosis by duplex PCR using two polymorphic markers or one polymorphic marker in combination with the detection of the DF508 mutation. *Mol Hum Reprod*. 2003;9:559-67.