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Research Article

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Combined Mutations of DMD and CFTR Genes in an Azerbaijani Family

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Abstract: A 9 year old boy with the obvious traits of Duchene muscular dystrophy disease is a resident of Masalli region of Azerbaijan, located in the south-east foothill of Talysh Mountains. Family members such as mother, father and sister of this index patient were examined. To diagnose all members of the family, biochemical analysis was conducted for quantitative analysis of creatine phosphokinase in blood serum and genealogical survey identified inherited disease cystic fibrosis in first cousin of this index patient . Quantitative evaluation of creatine phosphokinase in blood serum was performed for all family members of the index patient ., and identified high values: in index patient (2298 U/L, norm - 38-137 U/L), in his mother (879 U/L, norm - 26-140 U/L) and his sister (852 U/L, norm - 26-140 U/L), whereas his father had normal range values of creatine phosphokinase in blood (53,1 U/L, where norm - 38-137 U/L). Diagnosis of Duchenne muscular dystrophy disease was confirmed in index patient in hemizygous state. His mother and sister were found as heterozygous carriers of the (Duchenne muscular dystrophy) DMD gene. Molecular genetic analysis of the DMD gene (MLPA) identified mutation in the mother and sister of the index patient. Mutation type was nonsense, and classified as pathogenic class. Molecular genetic analysis of the DMD gene showed a gain of mutations, consisting of two copies encompassing exon 03 to 09 in the index patient, mother and his sister. The identified two different mutations of DMD gene in Azerbaijani family: fragment deletion of exon 45 in three sibs from Astara region of Azerbaijan, located in the south-east of the country, and deletion encompassing exons from 8 to 20 in 10-year-old boy in Balakan region, located in the north-west of the Republic Mutation type is a nonsense mutation and classified as pathogenic of class 1. Inherited cystic fibrosis in heterozygous state was additionally identified in the index patient, in father and sister. The screening of identified mutations may serve as a prenatal diagnostic tool to carefully plan the prophylaxis in patients with cystic fibrosis. Furthermore, our studies may serve as a basis for the future investigation of many aberrant molecular mechanisms and regulatory pathways. The study of Duchenne and Becker muscular dystrophy resulted in one of the first successful attempts at reverse genetics, better described as positional cloning, in humans. Discovery and subsequent analysis of the gene mutation that results in the clinical disorder led to the discovery of the encoded protein, dystrophin. This coinage set a precedent for the naming of proteins discovered by positional cloning of human disease genes: for example, huntingtin, emerin, and ataxin.

Keywords: Inherited Diseases, Exon, DMD Gene, CFTR Gene, Protein

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1. INTRODUCTION

According to WHO data, there are more than 6000 inherited diseases that have already been studied. The major part of identified genetic diseases is monogenic inherited diseases, which are caused by the mutations in only one gene [1,5]. Inherited Duchenne muscular dystrophy disease is one of the monogenic diseases. The symptoms usually appear before age 6 and may appear as early as infancy. Typically, the first noticeable symptom is delay of motor milestones, including sitting and standing independently. The mean age for walking in boys with Duchenne muscular dystrophy is 18 months. There is a progressive muscle weakness of the legs and pelvic muscles, which is associated with a loss of muscle mass (wasting). This muscle weakness causes a waddling gait and difficulty in climbing stairs. Muscle weakness also occurs in the arms, neck, and other areas, but not as severely or as early as in the lower half of the body [2,3,4,6]. Calf muscles initially enlarge and the enlarged muscle tissue is eventually replaced with fat and connective tissue (pseudohypertrophy). Muscle contractures occur in the legs, making the muscles unusable because the muscle fibers shorten and fibrosis occurs in connective tissue. Occasionally, there can be pain in the calves. Symptoms usually appear in boys aged 1to 6. There is a steady decline in muscle strength between the ages of 6 and 11 years. By age 10, braces may be required for walking, and by age 12, most boys are confined to a wheelchair. Bones develop abnormally, causing skeletal deformities of the spine and other areas. Muscular weakness and skeletal deformities frequently contribute to breathing disorders. Cardio-myopathy (enlarged heart) occurs in almost all cases, beginning in the early teens in some, and in all after the age of 18 years. Intellectual impairment may occur, but it is not inevitable and does not worsen as the disorder progresses. Few individuals with DMD live beyond their 30s. Breathing complications and cardiomyopathy are common causes of death[27]. Pseudohypertrophic muscular dystrophy, a severe X-linked recessive hereditary disorder mainly affecting the male, includes (DMD, OMIM #310200) and Becker muscular dystrophy (BMD, OMIM #300376) with the incidences of 1 in 3600 and 1 in 185,181, respectively, in live baby boys. BMD is featured by mild symptoms and long survival time that is close to the normal human lifespan in most patients, DMD patients usually have severe and fatal symptoms mainly including progressive muscular atrophy and myasthenia complicated with gastrocnemius muscle pseudohypertrophy [35]. DMD onset usually occurs between 3 and 5 years old, followed by loss of standing and walking ability before the age of 12 years and death heart failure or respiratory failure before the age of 20 years. The study of Duchenne and Becker muscular dystrophy resulted in one of the first successful attempts at reverse genetics, better described as positional cloning, in humans. Discovery and subsequent analysis of the gene mutation that results in the clinical disorder led to the discovery of the encoded protein, dystrophin. This coinage set a precedent for the naming of proteins discovered by positional cloning of human disease genes: for example, huntingtin, emerin, and ataxin. DMD severely affects young men's health and heavy mental and economic burdens. At present, there is no effective treatment for DMD, thus.[25] Cystic fibrosis is also monogene disease and inherited in an autosomal recessive manner. Cystic fibrosis (CF) is an inherited disorder that causes severe damage to the lungs, digestive system and other organs in the body. Cystic fibrosis affects the cells that produce mucus, sweat and digestive juices. These secreted fluids are normally thin and slippery. But in people with CF, a defective gene causes the secretions to become sticky and thick. Instead of acting as lubricants, the secretions plug up tubes, ducts and passageways, especially in the lungs and pancreas [12,19]. Many different defects can occur in the gene. The type of gene mutation is associated with the severity of the condition [17]. Children need to inherit one copy of the gene from each parent in order to have the disease. If children inherit only one copy, they won't develop cystic fibrosis. However, they will be carriers and could pass the gene to their own children [18]. (Cystic fibrosis transmembrane conductance regulator) The *CFTR* gene, found at the q31.2 locus of chromosome 7, is 230,000 base pairs long, and creates a protein that is 1,480 amino acids long [22,24]. More specifically, the location is between base pair 117,120,016 and 117,308,718 on the long arm of chromosome 7, region 3, band 1, subband 2, represented as 7q31.2. Structurally, the *CFTR* is a type of gene known as an ABC gene [15,23,36]. The product of this gene (the CFTR protein) is a chloride ion channel important in creating sweat, digestive juices, and mucus. This protein possesses two ATP-hydrolyzing domains, which allows the protein to use energy in the form of ATP [9,16,19]. It also contains two domains comprising six alpha helices apiece, which allow the protein to cross the cell membrane [36]. A regulatory binding site on the protein allows activation by phosphorylation, mainly by cAMP-dependent protein kinase.[21] The carboxyl terminal of the protein is anchored to the cytoskeleton by a PDZ domain interaction. [13,14.20,] The size of the gene is 190 kb and covers 27 exons. CFTR gene plays a role in synthesis of transmembrane protein sized 170 kDa. For now more than 1700 mutations of CFTR gene have been identified [21]. In exon 11, the mutation between nucleotides 1521-1523 causes deletion of phenylalanine at position 508, which is first nucleotide-binding domain of CFTR gene. Deletion of phenylalanine 508 forces the first nucleotide-binding domain into a conformation, which inhibits protein dimerization, reducing channel production and function. Previously we and others showed several types of CFTR gene mutations, such as del121kb, delf508, del1501507, 1677delTA, 2143delT, 2184insA, 394delTT, 3821delT, G542X, W1282X, N1303K, L138ins, R334W and 3849+10kb C→T, which represent 76% of mutations[7,11,18.] However, genetics of and cystic fibrosis for patients in Azerbaijan are understudied and remain elusive. In this study, we addressed a molecular genetic testing of both DMD and CFTR genes in one family, where the index patient has already clinical manifestations of Duchenne muscular dystrophy (DMD) disease.

2. MATERIALS AND METHODS

2.1 *Patients*

All patients have free and informed written consent to participate in this study, conforming to the ethical standards adhering to the local Institutional Review Boards and the Declaration of Helsinki. The ethics committee of Azerbaijan National Academy of Science, Genetic Resources Institute (ANAS GRI) (approval number 58-8/17) has approved the study. Patient of 9 old boy with the obvious traits of Duchenne muscular dystrophy disease is a resident of Masalli region of Azerbaijan, located in the south-east foothill of Talysh Mountains. Family members: mother, father and sister of the index patient. were examined. To diagnose all members of the family for, biochemical analysis was

conducted for quantitative analysis of creatine phosphokinase in blood serum and genealogical survey identified inherited disease cystic fibrosis in first cousin of index patient. Therefore, all family members of the index patient underwent molecular-genetic research for DMD and CFTR genes.

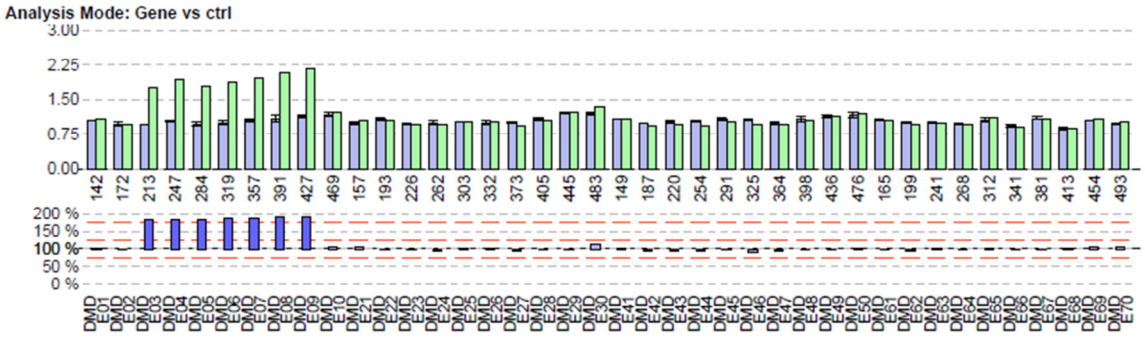
2.2 *Gene analysis*

DMD genes were analyzed by an amplicon-based next-generation sequencing approach. The amplicons cover the entire coding region and the highly conserved exon-intron splice junctions (minimum coverage of >20x for every amplicon). Missing regions or regions of poor quality are completed with classical Sanger sequencing to achieve 100% coverage. Variants of relevance identified by NGS are continuously and individually in-house validated for quality aspects; those variants which meet our internal QC criteria (based on extensive validation processes) are not validated by Sanger. The reference sequence is/ sequences are: DMD: NM_004006.2. MLPA (multiplex ligation-dependent probe amplification) analyses were performed using SALSA MLPA probemix P034-B2/P035-B1 provided by MRC-Holland to test for deletions or duplications within or including DMD genes[25]. stranded DNA capture baits against approximately 36.5 Mb of the human coding exome (targeting >98% of the coding RefSeq and Gencode v28 regions, which was obtained from the human genome build GRCh37/hg19 May 2018) were used to enrich target regions from fragmented genomic DNA with the Twist Human Core Exome Plus kit. The generated library is sequenced on an Illumina platform to obtain at least 20x coverage depth for >98% of the targeted bases. An in-house bioinformatics pipeline, including read alignment to GRCh37/hg19 genome assembly, variant calling and annotation, and comprehensive variant filtering is applied. All disease-causing variants reported in HGMD®, in ClinVar and CentoMD® as well as all variants with minor allele frequency (MAF) below 1% in gnomAD database considered. The investigation for relevant variants is focused on coding exons and flanking +/-20 intronic bases. All potential modes of inheritance patterns are considered. Provided family history and clinical information are used to evaluate identified variants of their pathogenicity and causality, and are categorized into classes 1-5 (see above). All variants related to the phenotype of the patient, except benign or likely benign variants, are reported. ANAS GRI has established stringent quality criteria and validation processes for variants detected by NGS quality single nucleotide variants and all relevant deletion/insertion variants are confirmed by Sanger sequencing. Consequently, we warrant a specificity of >99.9% for all reported variants.

3. RESULTS AND DISCUSSION

Genetic studies of the DMD and CFTR genes have been successfully studied in the population of most countries Groman et al. (2002) assessed whether alteration in CFTR function is responsible for the entire spectrum of nonclassic CF phenotypes. Extensive genetic analysis of the CFTR gene was performed in 74 patients with nonclassic CF. Furthermore, they evaluated 2 families that each included a proband without identified CFTR mutations and a sib with nonclassic CF to determine whether there was linkage to the CFTR locus and to measure the extent of CFTR function in the sweat gland and nasal epithelium. Of the 74 patients studied, Groman et al. (2002) found that 29 had 2 mutations in the CFTR gene (i.e., were either homozygous or compound heterozygous at the CFTR locus), 15 had 1 mutation, and 30 had no mutations. A genotype of 2 mutations was more common among patients who had been referred after screening for a panel of common CF-causing mutations that had identified 1 mutation than among those who had been referred after screening had identified no such mutations. Comparison of clinical features and sweat chloride concentrations revealed no significant differences among patients with 2, 1, or no CFTR mutations. Haplotype analysis in the 2 families in which 2 sibs had nonclassic CF showed no evidence of linkage to CFTR. Although each of the affected sibs had elevated sweat chloride concentrations, measurements of cAMP-mediated ion and fluid transport in the sweat gland and nasal epithelium demonstrated the presence of functional CFTR. Groman et al. 2002) concluded that factors other than mutations in the CFTR gene can produce phenotypes clinically indistinguishable from nonclassic CF caused by CFTR dysfunction [17]. Castellani et al. (2001) studied 47 neonates with hypertrypsinemia and normal sweat chloride. Thirty-two of the newborns had 1 identified CFTR mutation. Further analysis by DGGE identified additional mutations in 14 of the 32 babies in whom a mutation had previously been found. In 1 case, 2 more CFTR gene mutations were identified. Mutations were identified in 8 of the 15 babies in whom a mutation had previously not been identified.[8] Scotet et al. (2002) evaluated the prenatal detection of CF by ultrasound in more than 346,000 pregnancies in Brittany, France, where the incidence of CF is very high. The authors found that the incidence of CF in fetuses with echogenic bowel was 9.9%, significantly higher than in the general population. Only severe mutations were identified in these fetuses. The ultrasound examination enabled diagnosis of 11% of affected fetuses. Scotet et al. (2002) concluded that CF screening based on ultrasound examination is effective, particularly in populations where the disease is frequent [28,29,30]. De Becdelievre et al. (2011) reported on an 18-year experience of documenting comprehensive CFTR genotypes and correlations with ultrasound patterns in a series of 694 cases of fetal bowel anomalies. A total of 30 CF fetuses and 8 cases compatible with CFTR-related disorders were identified. CFTR rearrangements were found in 5 of the 30 CF fetuses. A second rare mutation indicative of CF was found in 21.2% of fetuses carrying a frequent mutation. The frequency of CF among fetuses with no frequent mutation was 0.43%. Correlation with ultrasound patterns revealed a significant frequency of multiple bowel anomalies in CF fetuses. The association of at least 2 signs of bowel anomaly on ultrasound, including hyperechogenic bowel, loop dilatation, and/or nonvisualization of gallbladder, was observed in 14 of 30 CF fetuses (46.7%) as compared with 61 of 422 (14.5%) non-CF fetuses (P less than 10(-3)). The rare triad of hyperechogenic bowel, loop dilatation, and nonvisualization of the gallbladder was of the highest diagnostic value, with a likelihood ratio of 31.40. Fetuses demonstrating this triad of bowel anomalies should have extensive CFTR sequencing and a search for rearrangements, even if no common mutation is detected.[10] Restrepo et al. (2000) used a reverse dot-blot detection kit to examine the frequency of 16 CFTR mutations among 192 cystic fibrosis alleles in Mexico, Colombia, and Venezuela. The detection efficacy of the panel used was 47.9% in this population. The most prevalent CF allele was delF508 (39.6%). The most common alleles among the others were G542X, N1303K and 3849+10kbC-T (602421.0062). The authors compared their results to

population studies from Spain and concluded that an important Spanish contribution is present in CFTR mutations in these 3 countries, but that important regional differences in allele prevalence exist [27]. Wang et al. (2000) found that 7 of 29 Hispanic patients with CF were heterozygous for a single-basepair deletion at nucleotide 3876 resulting in a frameshift and termination at residue 1258 of the CFTR gene (602421.0127). This mutation therefore accounted for 10.3% of mutant alleles in this group. The patients with this mutation had a severe phenotype as determined by age of diagnosis, high sweat chloride,presence of allergic bronchopulmonary aspergillosis, pancreatic insufficiency, liver disease, cor pulmonale, and early death. Wang et al. (2000) also noted that this mutation had not been reported in any other ethnic group [37,38]. Tuffery-Giraud et al. (2009) described a French database of mutations in the DMD gene that includes 2,411 entries consisting of 2,084 independent mutation events identified in 2,046 male patients and 38 expressing females. This corresponds to an estimated frequency of 39 per million with a genetic diagnosis of a 'dystrophinopathy' in France. Mutations in the database include 1,404 large deletions, 215 large duplications, and 465 small rearrangements, of which 39.8% are nonsense mutations. About 24% of the mutations are de novo events. The true frequency of BMD in France was found to be almost half (43%) that of DMD [32,33,34]. Among 624 index cases evaluated for DMD mutations, Oshima et al. (2009) reported that a genomic rearrangement was detected in 238 (38.1%) samples. Deletions were detected in 188 (79.0%), and included 31 cases with single-exon deletions and 157 cases with multi-exonic deletions. Most of the deletions fell between exons 45 and 52 and between exons 8 and 13 of the gene. Duplications were detected in 44 (18.5%) cases, of which 12 involved single exons and 32 multiple exons. Complex rearrangements were detected in 6 (2.5%) cases. The remaining 386 cases showed normal results [26]. Takeshima et al. (2010) reported the results of genomic, cDNA, and chromosome analysis of patients included in a Japanese DMD/BMD database. A mutation in the DMD gene was found in all 442 patients, including deletions and duplications in 270 (61%) and 38 (9%) cases, respectively; nonsense or splice site mutations in 69 (16%) and 24 (5%) cases, respectively; and small deletion/insertion mutations in 34 (8%) cases. X-chromosome abnormalities were identified in 2 cases, and unusual changes were found in 6 cases. The reading frame rule was upheld for 93% of deletion and 66% of duplication mutation cases. Induction of exon skipping was deemed the first priority for treatment of the dystrophinopathy [31]. Quantitative evaluation of creatine phosphokinase in blood serum was performed for all family members of the index patient S.A., and identified high values: in index patient (2298 U/L, norm - 38-137 U/L), in his mother (879 U/L, norm - 26-140 U/L) and his sister (852 U/L, norm - 26-140 U/L), whereas his father had normal range values of creatine phosphokinase in blood (53,1 U/L, where norm - 38-137 U/L). Diagnosis of Duchenne muscular dystrophy disease was confirmed in index patients in hemizygous state. His mother and sister were found as heterozygous carriers of the DMD gene. Molecular genetic analysis of the DMD gene (MLPA) identified mutation in the mother and sister of the index patient. Mutation type was nonsense, and classified as pathogenic class. A large duplication/insertion/gain of copy encompassing these exons has previously been described as causing Duchenne/Becker muscular dystrophy. It is classified as pathogenic (class 1) according to the recommendations of ANAS GRI. Pathogenic variants in the DMD gene are associated with X-linked (DMD – OMIM®: 310200) or (BMD – OMIM®: 300376). Pathogenic variants are associated with muscular dystrophy, an X-linked disorder, ranging from the severe (DMD, OMIM: 310200) to the milder (BMD; OMIM: 300376). DMD is featured by a progressive proximal muscular dystrophy with characteristic pseudo hypertrophy of the calves. The muscular dystrophy that carries the Becker eponym is similar to dystrophy in the distribution of muscle wasting and weakness, which is mainly proximal, but the course is more benign, with age of onset around 12 years; some patients have no symptoms until much later in life. Loss of ambulation also varies from adolescence onward, with death usually in the fourth or fifth decade. In some cases, as in, a degree of mental impairment is present (OMID: 11879882). The onset of DMD usually occurs before age 3 years, and the patient is chair ridden by age 12. The onset of BMD is often in the 20s and 30s and survival to a relatively advanced age is frequent The identified two different mutations of DMD gene in Azerbaijani family: fragment deletion of exon 45 in three sibs from Astara region of Azerbaijan, located in the south-east of the country, and deletion encompassing exons from 8 to 20 in 10-year-old boy in Balakan region, located in the north-west of the Republic. Clinical genealogical analysis of relatives of index patients suggested that cousins of this index patient had already clinical manifestations of cystic fibrosis. Accordingly, diagnosis was confirmed. Taking into account all these data, we attempted to conduct molecular genetic diagnosis of all family members of the index patient. Mutation Phe508del was identified in his father and sister in heterozygous state. The CFTR variant c.1521_1523del p. (Phe508del) is an in-frame deletion of 3 bps in exon 11, which causes the loss of residue Phe at position 508. According to HGMD Professional 2019.1, this variant has previously been described as causing Cystic fibrosis.[21,23] ClinVar lists this variant as pathogenic (clinical testing/ research, Variation ID: 7105) and classified as pathogenic class 1, according to the recommendations of ANAS GRI. Pathogenic variants in the CFTR gene are causative for cystic fibrosis (CF), an autosomal recessive disorder. Cystic fibrosis is a multisystem disease affecting epithelia of the respiratory tract, exocrine pancreas, intestine, hepatobiliary system, and exocrine sweat glands. Morbidities include progressive obstructive lung disease with bronchiectasis, frequent hospitalizations for pulmonary disease, pancreatic insufficiency and malnutrition, recurrent sinusitis and bronchitis, and male infertility. Pulmonary disease is the major cause of morbidity and mortality in CF. Meconium ileus occurs at birth in 15%-20% of newborns with CF. More than 95% of males with CF are infertile.[24,25] Previously, we reported for the first-time, five mutations for the CFTR gene in Azerbaijani population. They are as follows: Phe508del, 965, (T>C), 1000 (G>T), 1210-1211 (T>G) and 328 (G>C). Gene frequencies were equal to: Phe508del (68, 75%), in two 965, T>C (12,5%) and in each of – 1000 G>T (6,25%), 1210-1211, T>G (6,25%) and 328,G>C (6,25%). Only one of 5 identified mutations (Phe508del) is the most widespread type (16-19). The index patient manifested a hemizygous state of covering DMD gene region of exons 3 to 09 corresponding to heterozygous state of Phe508del. Mutation was revealed in the index patient's sister. His mother had heterozygous mutation in DMD and his father carrPhe508del. Fig1.



Mix Length	Rf Length	Chr	Chr B	Gene	Loc.	Result	Peak Area	RPA P.	RPA C.	st. Dev	ratio RPA
142	139.55			DMD	E01		15530	1.09	1.05	0.00	103.35
172	172.29			DMD	E02		15617	0.96	0.97	0.04	98.54
213	213.59			DMD	E03	multiple copies	32421	1.76	0.96	0.00	182.94
247	249.36			DMD	E04	multiple copies	37914	1.93	1.04	0.01	185.41
284	284.14			DMD	E05	multiple copies	36654	1.80	0.97	0.05	184.86
319	317.75			DMD	E06	multiple copies	39063	1.89	1.00	0.05	188.80
357	357.22			DMD	E07	multiple copies	40124	1.98	1.06	0.02	187.07
391	388.85			DMD	E08	multiple copies	40449	2.08	1.09	0.07	190.85
427	427.36			DMD	E09	multiple copies	39107	2.17	1.14	0.02	190.15
469	465.51			DMD	E10		19592	1.25	1.19	0.03	104.92
157	156.89			DMD	E21		16023	1.05	1.00	0.02	104.88
193	192.81			DMD	E22		18307	1.05	1.08	0.02	97.19

Fig 1. Index patient's DMD gene MLPA analysis results

4. CONCLUSION

In conclusion, our study identifies for the first-time the existence of combined hemizygous mutations in the region of exons from 03 to 09 of DMD gene and heterozygous Phe508del mutation in CFTR gene in an Azerbaijani family. The screening of identified mutations may serve as a prenatal diagnostic tool to carefully plan the prophylaxis in patients with cystic fibrosis. Furthermore, our studies may serve as a basis for the future investigation of many aberrant molecular mechanisms and regulatory pathways.

5. ACKNOWLEDGMENTS

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6. AUTHORS CONTRIBUTIONS STATEMENT

Dr. Saltanat Aghayeva and Dr. Lala Huseynova conceived and designed the study. Dr. Saltanat Ahghayeva and Dr. Lala Huseynova conceptualized and analyzed the data and necessary inputs with regard to this work and wrote the manuscript. Assistant Raya Hagverdiyeva has played a role in the design of the article. All authors have discussed the methodology and results, contributed to the final manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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