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MOLECULAR-GENETIC RESEARCH OF CFTR (CYSTIC FIBROSIS TRANSMEMBRANE REGULATOR) GENE MUTATIONS AMONG CYSTIC FIBROSIS PATIENTS IN THE AZERBAIJAN PAPULATION

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ABSTRACT

In this study five CFTR gene mutations were identified, that shows high frequency significance. It could be accepted as a specific mutation panel for Azerbaijan population and, hence, could be recommended as a mutation panel for cystic fibrosis disease diagnostics. Cystic fibrosis is the mostly spread monogenous disease with autosome recessive inheritance. There is no special data on incidence and genetics of the said disease among children in Azerbaijan Republic.

Thus, we put up a goal to study CFTR (Cystic Fibrosis Transmembrane regulator gene) gene mutations for population of Azerbaijan Republic, using molecular-genetic method complex. A group of 149 children (78 boys, 71 girls) was studied. Children were in the age range between 18 months and 9 years old. We did family study to find out cases similar to cystic fibrosis. We did sweat test for all of them. To take task on the goal discussed we started the following tests. A part of analysis were tested on ROTOR-GENE apparatus. To do that, a panel of 6 mutations of CFTR gene: delF508, W1282X, N1303K, delT2143, 3849+10kb C→T and del2,3-21kb) was used. The different part of analyses have been carried out by means of fluorimetric methods, liquid chromatography and mass-spectrometry.

For the first time, five mutations for CFTR gene was identified in Azerbaijan 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%). We were first to describe mutation 965, T>C (Leu322Pro) in Azerbaijan, which has no reference sequence results in NCBI. Kids diagnosed as cystic fibrosis patients consisted 5.37% to all children under our study.

The molecular diagnostics methods and choriocentesis method using specific primers to identify CFTR gene the following mutations: 1521-1523 exon 11; 1210-1211T>G intron 9; 328 G>A exon 4; 1000 G>T exon 8 and 965 T>C. 149 children aged from 18 months to 9 years old were selected to undergo specific sweat test, positive results of which speak for cystic fibrosis disease. 8 patients turned to be positive. And during these studies 5 CFTR gene mutations were identified, that shows high frequency significance. It could be accepted as a specific mutation panel for Azerbaijan population and, hence, could be recommended as a mutation panel for cystic fibrosis disease diagnostics. To prophilaxy the cystic fibrosis disease, it is recommended to screen genetically newborns, to consult medical-genetically risky families, and to carry out prenatal diagnostics during pregnancies for those families.

Keywords: inherited diseases, cystic fibrosis, CFTR gene, nucleotide, amino acid

INRODUCTION

As to WHO (World Health Organization) data, there are more than 6000 inherited diseases that have been already studied. The major part of identified genetic diseases consist of monogene natured ones. It is understood from their names that one gene is mutated and causes monogene natured inherited disease. There are around 5000 monogene inherited diseases [1,2].

Cystic fibrosis for the first time was recognized as a separate disease by Dorothy Andersen in 1938. Gene responsible for cystic fibrosis was cloned in 1989. Thanks to that the nature of mutation became able to be cleared up and improved method to indicate carriers. The gene mutation is a disturbance of protein structure and function that got the name of cystic fibrosis transmembrane conductance regulator (CFTR). The sequence of that is clotting of excretory glands secretion, difficulties in secretion evacuation and change of its physical-chemical characteristics, that in its turn makes the disease pattern. Changes in the pancreas gland, respiratory system, gastro-intestinal tract are already registered in prenatal period and progressively are being increased with the age. Exretion of the viscous secretion with exocreane glands leads to difficulties of its evacuation and stagnation with the following widening of the duct glands, atrophy of gland tissue and development of the progressive fibrosis. Activity of the intestinal enzymes and pancreas gland is significantly lowered. Alongside with sclerosis formation in organs, function of fibroblasts disturbance takes place. It is stated that fibroblasts in patients with cystic fibrosis produce ciliary factor or M-factor which possesses anti-ciliary activity – it disturbs epithelial cilia function [3,4].

Cystic fibrosis is the widest spread monogene inherited disease. Cystic fibrosis is autosome recessive inheritance type disease [5,6].

Frequency of the disease (homozyous form) is 1:2500-5000 in newborns. Heterozygous carriers are born as 1:25-30 [7].

In 80's of the last century, CFTR gene structure of cystic fibrosis disease was identified and studied by means of molecular-genetic methods. Gene CFTR locates on the long arm of the seventh chromosome (7q31). The size of the gene is 190 kb and covers 27 exons. CFTR gene takes part in synthesis of transmembrane protein sized 170 kDa [8].

For now more than 1700 mutations of CFTR gene have been identified, and most of them are rarely encounted. Cystic fibrosis has autosome-recessive inheritance type [9].

Pathogenic variants in the CFTR gene are causative for cystic fibrosis. 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 cystic fibrosis. Meconium ileus occurs at birth in 15%-20% of newborns with cystic fibrosis. More than 95% of males with cystic fibrosis are infertile (OMIM®: 219700; GeneReviews - PMID: 20301428) [10,11].

70% of all identified mutations of CFTR gene present one and the same mutation. In exon 11 in place of protein biosynthesis the mutation between nucleotides 1521-1523 causes deletion of Phenialanine in position 508. Since the synthesized protein is not structurally normal, it cannot come out from endomplazmatic reticulum and, changes are observed in its tolerance and activity [12].

All types of mutations of CFTR gene are identified as point mutations, manor and major deletions, transversions, inversions, etc. Around half of all identified mutations are missense mutations [13].

The following mutations consist 76% of CFTR gene mutations: del121kb, delf508, del1501507, 1677delTA, 2143delT, 2184insA, 394delTT, 3821delT, G542X, W1282X, N1303K, L138ins, R334W and 3849+10kb C→T [14].

Altogether, protein synthesized by CFTR gene regulates chloride channels' activities which are located in apical membranes of epithelial cells. Disease damages function of lungs and pancreas. Diagnostic sign is increase of clorides and sodium values in kid's sweat. Only 2% of patients have got normal values of chlorides in sweat with tipical features of the disease. In these cases, molecular-genetic methods are used to identify mutation in CFTR gene [15,16].

CFTR gene mutations causing cystic fibrosis inherited disease have not been identified for Azerbaijan Republic population.

Thus, we put up a goal to study CFTR gene mutations for population of Azerbaijan Republic, using molecular-genetic method complex for further preventive prenatal diagnostics of the affected families.

MATERIAL AND METHODS

For every patient 1 ml venouse blood has been sampled into an Eppendorf tube with EDTA anticoagulant solution. Later on it was absorbed to special DBS (dried blood spots) cards and dried up for an hour at room temperature, only then has been sent to the laboratory for further analysis.

Sampling has been carried out both in children patients and their parents at the same time. So, parents gave their signed consent for that.

The genome DNA was separated from DBS cards from venous blood. For this purpose, venous blood samples were taken from 8 patients after showing positive results for sweat test. Patients were between 18 months-9 years old out of 149 pediatric patients (Figure 1).

The concentration and intactness of the separated genome DNA was tested in 0.7% agarose gel. The genome DNA was PCR separately for protein-encoding exons of the CFTR gene. Positive PCR samples, that have been got by electrophoresis in the agarose gel, were purified by enzymatic method (Figure 2).

Polymerase chain reaction was carried out in a fol-lowing conditions: denaturation at 96 °C for 30 seconds; annealing at 55 °C for 30 seconds; extension at 75 °C for 1 min. This cycle was repeated 25 times, 72 °C for 10 min. and 4 °C pause. The PCR was carried out on a Professional Thermocycler Biometra system (Biometra Biomedizinische Analytik GmbH, Göttingen, Germany). A pair of for-ward and reverse primers was used for each genomic fragment. For the purification of DNA fragments after the first stage of PCR, a set of magnets was used: Agencourt AMPure XP PCR purification and SPRIPlate 96 Super Magnet Plate (Beckman Coulter Inc., Beverly, CA, USA). The second am-plification of the purified DNA fragments was carried out in the following condition: denaturation at 95 °C for 30 seconds; annealing at 55 °C for 30 seconds; extension at 77 for 2 min. This cycle was repeated 25 times, and 72 °C for 10 min. and 4 °C pause.

The nucleotide sequence of purified fragments was studied in GENOME Lab GeXP™ Sequencing (SCIEX, Brea, CA, USA).

Positive Cycle Sequencing PCR samples, got by agarose gel electrophoresis, are purified by BIGDye XT dye remover. The purified gene samples were read by the Automatic DNA sequencing AB13130xI Analysis System. The obtained nucleotide sequences were read out with Seqscape V.2.7. programme (Applied Biosystems, Foster City, CA, USA; http:/tools.thermofisher. com/content/sfs/manuals/4401740.pdf) compared to normal CFTR nucleotide sequence by Blast Ce NCBI, and then polymorphisms and relative mutations were identified (Figure 3).

Study has been carried out at ANAS Institute of Genetic Resources, “Laboratory of human genetics” and AFGENE laboratory in Baku, Azerbaijan.

Molecular genetic analysis have been carried out in AFGENE laboratory in Baku, Azerbaijan. That was Whole Exome Sequencing (CentoXome GOLD®). Method is as follows: Double 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 on 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 in CentoMD® as well as all variants with minor allele frequency (MAF) below 1% in gnomAD

database are considered. The investigation for relevant variants is focused on coding exons and flanking +/-20 intronic bases. All potential modes of inheritance patterns are considered. In addition, provided family history and clinical information are used to evaluate identified variants with respect to their pathogenicity and causality, and are categorized into classes 1 - 5. All variants related to the phenotype of the patient, except benign or likely benign variants, are reported.Low 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 [17].

A part of analysis were tested on ROTOR-GENE apparatus (QIAGEN, USA) in "Laboratory science" Chair at the Azerbaijan State Doctors' Advanced Training Institute after A.Aliyev. To do that, a panel of 6 mutations of CFTR gene: delF508, W1282X, N1303K, delT2143, 3849+10kb C→T and del2,3-21kb was used.

RESULTS

Examination has been provided for 149 pediatric patients who applied to Republic Children's Hospital at Scientific Research Pediatry Institute under Ministry of Health, and outpatient departments in the different areas of the republic. At the same time material was collected during the field work in the regions of Azerbaijan and cities of Baku, Lankaran, Masalli, Astara and Sheki. The age of patients varied from 18 months up to 9 years of age. Among those 149 patients: 78 are boys and 71-girls. All of them had suspicion for cystic fibrosis disease (Figure 1).

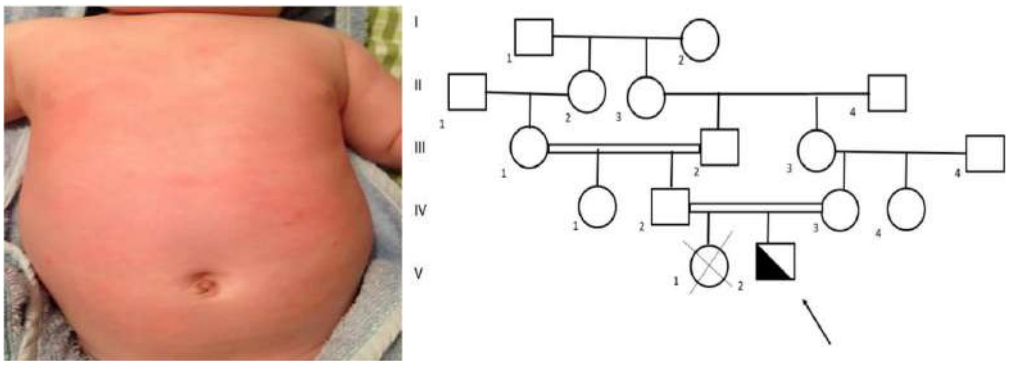


Figure 1. A newborn with cystic fibrosis (heterozygous mutation L322P (965T>C) in exon 8 of the CFTR gene)

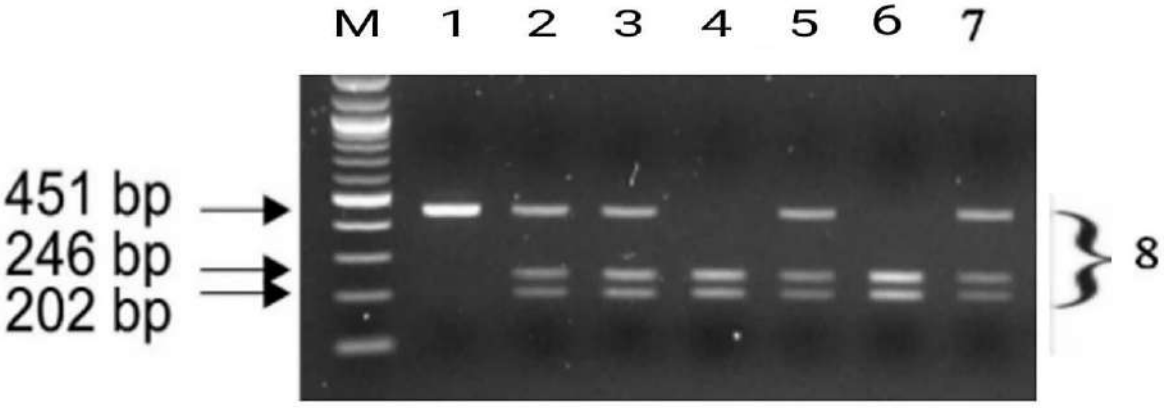


Figure 2. Gel electrophoresis of delF508 mutation detected in CFTR gene: M-DNA marker; 1-normal; 2-heterozygous delF508 mutation (451, 246, 202 b.p. fragments); 3- heterozygous delF508 mutation (451, 246, 202 b.p. fragments); 4- homozygous delF508 mutation (246, 202 b.p. fragments); 5- heterozygous delF508 mutation (451, 246, 202 b.p. fragments); 6- homozygous delF508 mutation in (246, 202 b.p. fragments); 7- heterozygous delF508 mutation (451, 246, 202 b.p. fragments); 8-mutant region (covers the area where fragments 202-451 b.p. are located).

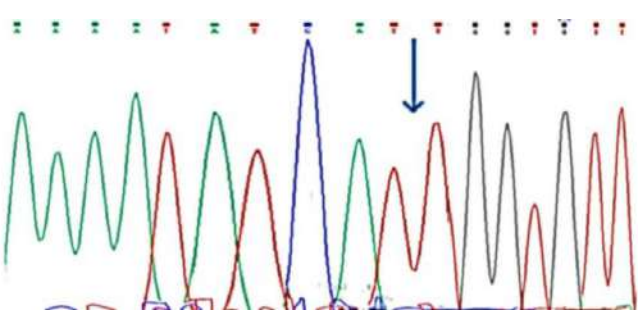
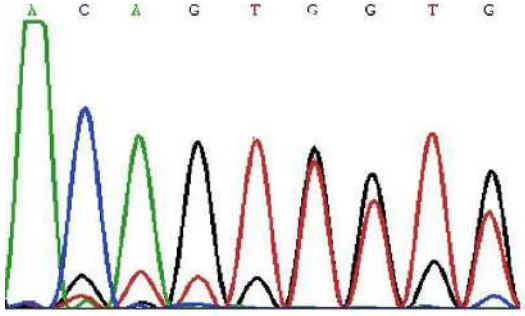
1.  2. 

Figure 3. Electropherogram of changes in the nucleotide sequence of the CFTR gene: the region consisting of the nucleotide sequence AAAATATCAT---TGGTGTTTCC observed with the deletion of the CTT triplet in the 1-delF508 (1521-1523del) mutation (control-AAAATATCATCTTTGGTGTTTCC>carrier of the mutant gene-AAAATATCAT---TGGTGTTTCC); 2- compound heterozygous delf508/5T mutation (control-ACAGTGGTG> mutant gene carrier-ACAGTTTTT);

The Table 1 presents results of 8 cystic fibrosis patients identified during 149 children patients were tested for CFTR gene mutations.

Index patient 1410539– a boy suspicious of Cystic fibrosis is identified during field work in Ganja city. He was hospitalized in children clinic hospital. Patient 1410539 was born in the consanguineous marriage where parents were first cousins. Their fathers were brothers. Patient is the second child and has two sisters and one brother. Prior to that patient has undergone procedure sweat probe that was positive. Molecular genetic DNA analysis identified the following results.

Patient 1410539 is double heterozygote according to two different mutations (compound form). The first nucleotide substitution has taken place in the exon 4 of CFTR gene in position 328. Guanine is changed with Cytosine. As a result of this mutation, protein synthesized changes in position 110 Asparagine aminoacid with Histedine aminoacid (328 G>C, 110 Asp>His).

The second change in CFTR gene happened in the exon 8. In the position 1000of the exon Cytosine nucleotide was substituted with Thymine nucleotide, and in the result protein biosynthesized got change of Arginine amnoacid with Triptophan aminoacid in position 334 (1000 C>T, 334 Arg>Trp). In father of the patient 1410539: 328 G>C (110 Asp>His) CFTR gene mutation was found in heterozyous state, in his mother - 1000 C>T (334 Arg>Trp) mutation also in heterozygous state [18].

Table 1. Results of molecular genetic studies of CFTR gene

Patient No	Gene Mutation	Protein Mutation	Genotype	Method
1431215	1521-1523 exon 11 1210-1211T>G intron 9	Phe508del	Compound	Direct sequencing
1410530	1521-1523del exon 11	Phe508del	Homozigote	Direct sequencing
1410539	328 G>A exon 4 1000 G>T exon 8	Asp>110His Arg>334Trp	Compound	Direct sequencing
1444561	965 T>C	Leu322Pro	Homozigote	Direct sequencing
1431210	1521-1523del exon	Phe508del	Homozig	Genetic panel

	11		ote	
1446891	1521-1523del exon 11	Phe508del	Homozig ote	Genetic panel
1446899	1521-1523del exon 11	Phe508del	Homozig ote	Genetic panel
1446971	1521-1523del exon 11	Phe508del	Homozig ote	Genetic panel

Five patients - 1410530, 1431210, 1446899, 1446891 and 1446971 had got the same deletion in exon 11 of CFTR gene between 1521-1523 nucleotides in hetero- and homozygous form. As a result of the mutation, biosynthesis of protein shows deletion of Phenylalanine aminoacid (Phe508del).

Index patient 1431215, a girl with suspicion to Cystic fibrosis was hospitalized to clinic department of the Pediatric Scientific-Research Institute. Prior to that hospitalization she had a positive sweat test. Patient 1431215 was also born in consanguineous marriage couple who were first cousins. Both parents' mothers are sisters. Patient 1431215 is the last, the third child in the family. She has two elder sisters. Molecular genetic DNA analysis of patient 1431215 has shown the following (Table 1).

DISCUSSION

The CFTR variant c.1210-1211T>G is located in intron 9. It is also known as 5T variant. This variant has been confirmed by Sanger sequencing. It is the most common "mild" CFTR allele. The presence of the splicing variant reduces the level of full-length transcripts especially in Wollfian tissue. According to HGMD Professional 2019.1, this variant in men has previously been as being associated with CBAVD (Congenital Bilateral Absence of Vas Deferentia) (PMID: 8556303), and many other authors. It is classified as risk factor according to the recommendations of ACMG [19,20].

The CFTR variant c.1000 C>T p.(Arg334Trp) causes an amino acid change from Arg to Trp at position 334. According to HGMD Professional 2019.1, this variant has previously been described as disease causing for Cystic fibrosis by Gasparini et al., 1992 (PMID: 2045102), (PMID: 7680769), Welsh et al., 1999 (PMID: 7686820). ClinVar lists this variant as pathogenic (clinical testing/research, Variation ID: 7139) [21].

Index patient 1444561 is a boy suspicious to Cystic fibrosis was identified during field work in Sheki city. Patient was hospitalized to children clinic hospital in Sheki city. He was born in the consanguineous marriage where parents were first cousins. Parents of parents, i.e. one grandma and one grandpa were brother and sister. Patient 1444561 is the first child in the family and has one younger brother. Prior to hospitalization he had a sweat probe, which turned to be positive. Molecular genetic DNA analysis of the patient 1444561showed the following results.

Patient 1444561 has got homozygous form of CFTR gene mutation as a result of change of Thymine nucleotide with Cytosine nucleotide in position 965 (965, T>C). During protein biosynthesis, Leucine aminoacid was substituted with Proline aminoacid in position 322. Prior to us mutation 965, T>C has not been described in Azerbaijan.

Patient 1410530 – a 7-year-old boy – identified a homozygous mutation 1521-1523del in exon 11 by means of direct sequencing. Three nucleotide deletion in protein leads to deletion of Phenylalanine amino acid in position 508 in protein. The same mutation was identified in patients: 1431210, 1446899, 1446891 and 1446971. Some of mutations Phe508del identifications have been done with ROTOR-GENE apparatus. Other part of mutations has been identified through directly sequencing.

Only one (Phe508del) of identified mutations is the mostly spread type. The rest four mutations: 328(G>A), 965(T>C), 1210-1211(T>G) and 1000(G>T) are not the often encountered and treated as rare mutations of CFTR gene.

Identified mutations have been found in intron 9 and exons 4, 8, 9 and 11.

Eight patients have been examined, and out of 16 CFTR genes: 11 identified Phe508del mutated gene (68.75%), two manifested 965,T>C mutated gene (12.5%), the rest each one gene revealed – 1210-1211,T>G (6.25%), 1000, G>T (6.25%) and 328,G>C (6.25%) mutations.

Among tested patients frequency of index patients with cystic fibrosis was 5.37%.

Thus, as a result of screening done in Baku city of Azerbaijan Republic children hospitals, eight pediatric patients diagnozed with cystic fibrosis were identified for 5 mutations of CFTR gene. One mutation of these, i.e. 965 (T>C) has not been found in NCBI and described prior to us.

Parents of all patients with Cystic fibrosis diagnosis had consanguinity of first cousin type. Grandparents turned to be either brothers, or sisters, or brother and sister. Hence, their marriages were either parallel or cross types of consanguineous marraiges. As to our calculations 8 patients out of 149 suspicious children have got their diagnosis Cystic fibrosis. Phenotypical frequency was 5.37% of all 149 studied kids. Taking into account high frequency of consanguineous marriages in Azerbaijan Republic population (18.9%), as well as significantly high frequency of Cystic fibrosis among children (5.37%) it is required to conduct genetic screening, prospective and retrospective genetic consulting of the disease, and at the same time to carry out prenatal diagnosis by means of choriocentesis method.

To prophylaxy cystic fibrosis in the Republic, we recommend consultations of genetically risky families and their prenatal diagnostics of fetuses during the next pregnancies. They should be carried out by means of molecular diagnostics methods and choriocentesis method using specific primers to identify CFTR gene the following mutations: 1521-1523 exon 11; 1210-1211T>G intron 9; 328 G>A exon 4; 1000 G>T exon 8 and 965 T>C.

CONCLUSION

Pathogenic variants in the CFTR gene are causative for cystic fibrosis (CF), an autosomal recessive disorder. CF 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 (OMIM®: 219700; GeneReviews-PMID: 20301428).The CFTR variant c.328 G>C p.(Asp110His) causes an amino acid change from Asp to His at position 110. According to HGMD Professional 2019.1, this variant has previously been described as disease causing for Cystic fibrosis by Dean et al., 1990 (PMID: 2344617), Weller et al., 2000 (PMID: 10719683), (PMID: 18456578) . ClinVar lists this variant as pathogenic (research, Variation ID: 7108). It is classified as pathogenic (class 1) according to the recommendations of ACMG [22,23].

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 disease causing for Cystic fibrosis by Riordan et al., 1999 (PMID: 2475911) followed by several other authors. ClinVar lists this variant as Pathogenic (clinical testing/research, Variation ID: 7105) and Likely pathogenic (clinical testing, Variation ID: 7105). It is classified as pathogenic (class 1) according to the recommendations of ACMG.

Pathogenic variants in the CFTR gene [CFTR variant c.1210-1211T>G] are causative for cystic fibrosis (CF), an autosomal recessive disorder. CF 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 [24-26].

The CFTR variant c.1000 C>T p.(Arg334Trp) is classified as pathogenic (class 1) according to the recommendations of CENTOGENE and ACMG.

For the first time in Azerbaijan five mutations of CFTR gene: Phe508del, 965,(T>C), 1000 (G>T), 1210-1211 (T>G) and 328 (G>C) have been identified. Gene frequencies for mutations are the following: Phe508del (68.75%), in two 965,T>C (12.5%) and for each one – 1000 G>T (6.25%), 1210-1211,T>G (6.25%) and 328,G>C (6.25%). Mutation 965, T>C (Leu322Pro) has not been described prior to us.

Thus, taking into account high frequency of consanguineous marriages among parents of patients with Cystic fibrosis in Azerbaijan Republic, we recommend to conduct genetic screening of newborns, prospective and retrospective genetic consulting of the disease in genetically risky families and prenatal diagnostics of fetus by means of molecular diagnostics methods and choriocentesis method using specific primers for CFTR gene mutations identification [27-29]. They should be carried out by means of molecular diagnostics methods and choriocentesis method using specific primers to identify CFTR gene the following mutations: 1521-1523 exon 11; 1210-1211T>G intron 9; 328 G>A exon 4; 1000 G>T exon 8 and 965 T>C. 149 children aged from 18 months to 9 years old were selected to undergo specific sweat test, positive results of which speak for cystic fibrosis disease. 8 patients turned to be positive. And during these studies 5 CFTR gene mutations were identified, that shows high frequency significance. It could be accepted as a specific mutation panel for Azerbaijan population and, hence, could be recommended as a mutation panel for cystic fibrosis disease diagnostics.

COMPETING INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Lala Huseynova: study director, conducting experiments, collecting and analyzing data

Raya Hagverdiyeva: collecting and analyzing data

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