POSSIBILITIES OF MOLECULAR BIOPSY IN DIFFERENTIAL DIAGNOSIS OF PULMONARY ARTERIAL HYPERTENSION: CASE DESCRIPTION AND LITERATURE REVIEW

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Precision studies of pulmonary arterial hypertension (PAH) have led to identification of the subgroup of pulmonary venoocclusive disease (PVOD). The article describes the clinical case of the hereditary form of PAH in a 53-year-old woman. Using next-generation sequencing we found biallelic mutations in the EIF2AK4 gene – c. C2965T variant in the heterozygous state resulting in the non-synonymous substitution in the position 989 of the protein (p.Arg989Trp) and the non-described variant c.859+1G>A in the exon 7 in the heterozygous state. Taking into consideration the genetic data, the diagnosis in this case was changed from idiopathic PAH to PVOD. So, implementation of molecular genetic methods in routine clinical examination of patients with PAH can greatly improve and speed up the diagnostic process.

Keywords: pulmonary arterial hypertension, pulmonary venoocclusive disease, EIF2AK4 gene mutations

Precision studies of pulmonary arterial hypertension (PAH) have led to identification of the subgroup of pulmonary venoocclusive disease (PVOD) [1]. This subgroup also contains idiopathic and hereditary types of PVOD. The main difference of this form of PAH from other forms is its recessive heritability. The frequency of PVOD in the population is believed to be one to two cases per 1 million of the population. However, the actual prevalence of PVOD could be significantly higher because identification of this form is clinically difficult. PVOD was described in 1934 for the first time, but until 1966, its diagnosis was established only by postmortem histology [2]. PVOD is characterized by progressive fibrosis and proliferation of neointimal septal pulmonary veins and preseptal venules, and this is often accompanied by dilatation and proliferation of pulmonary capillaries. Multiple intravascular thrombi are usually found, which can be recanalized. Hypertrophy of the arteriolar wall in PVOD is similar to that of idiopathic PAH, but plexiform lesions are absent. PVOD is characterized by enlargement of the lymph nodes and dilation of lymphatic vessels. Interestingly, connective tissue disorders are sometimes inexplicably complicated with development of the venoocclusive phenotype of PVOD.

The most precise method for diagnosis of PVOD is lung biopsy, but it is contraindicated because of the high risk of the procedure. Therefore, this disease is most often detected with pulmonary transplantation or in postmortem histology. In vivo, the diagnosis of PVOD is extremely complicated.

In this report, we present the possibilities of using molecular genetic testing as a type of molecular biopsy that allows this diagnostic problem to be solved in least some patients in a relatively simple and rapid manner.

Case. A 53-year-old woman complained of exercise-induced dyspnea for at least 5 years. Her tolerance to exercise gradually decreased to functional class III. The diagnosis of PAH was established after 4 years. This time is much longer than that in other countries in which the diagnostic period is currently 2 years [3]. Sildenafil treatment (60 mg) for 7 months did not improve the patient’s condition. The six-minute walk test was 291 m. Dyspnea by the Borg scale at the beginning of the test was 0.5 and after the test it was 4. Pulse oximeter saturation decreased from 91 % to 73 % and her blood pressure reaction was normal. An echocardiography study showed considerable enlargement of the right heart chambers. The right ventricle/left ventricle basal diameter ratio was 1.3 and the left ventricular eccentricity index was 0.5. Right ventricular hypertrophy (thickness of the free wall of 7 mm) and a reduction in RL contractile function (FAC: 31 %, TAPSE: 14 mm) was also found. The tricuspid valve regurgitation rate was 4.0 m/s. Data of invasive evaluation of pulmonary hemodynamics were as follows: pulmonary artery pressure (systolic/diastolic/mean), 46/33/39 mm Hg; right atrial pressure, 2/0/1 mm Hg; and right ventricular pressure, 30/11/18 mm Hg. Furthermore, pulmonary capillary wedge pressure was 8.7 Wood Units, and total vascular resistance was 25 Wood Units. Mixed venous oxygen saturation in the left atrium was 66 % and oxygen saturation in arterial blood was 92 %. Acute pulmonary vasodilator testing with inhaled iloprost 10 μg was negative. The family history of the patient was as follows. Her brother suffered from a heart condition with severe shortness of breath and died at 28 years old (he might have also had PAH), and her sisters (58 and 46 years old) are healthy. Her father is 85 years and mother is 83 years old, and both are still alive and do not have PAH. Her first cousin on her father’s side suddenly died at 35 years old, and the cause is unknown. Therefore, we can assume a family history of PAH in this patient (Figure), with a recessive type of inheritance of this disease.

Fig. Pedigree of patient C. All cases of the disease are concentrated in one generation.
We performed targeted sequencing. DNA extraction was performed using sets for isolating genomic DNA from blood on QiaAMP spin columns (Qiagen, USA). Synthesis of DNA libraries from DNA samples for Illumina MiSeq was performed using the KAPA HyPerPlus kit (KAPA, USA). The following stages for each of the samples were performed: enzymatic DNA fragmentation, polishing of the ends, ligation of specific sequencing adapters with unique barcodes for each of the samples, and polymerase chain reaction (PCR) from flanking primers. Enrichment with the desired gene fragments (coding regions) using a selective DNA capture technique with synthesized probes was performed with a synthetic enrichment panel of NimbleGen (Roche, USA). This process included the following steps: equimolar mixing of DNA libraries for subsequent enrichment, hybridization with synthetic probes, sorption of hybridized DNA libraries on streptavidin magnetic particles, and PCR from flanking primers. Quality control of the amplified DNA library was performed by capillary gel electrophoresis using a BioAnalyzer 2100 on HighSensitivity sets (Agilent, USA). The concentration of the amplified DNA libraries was obtained by the fluorimetric method using a Qubit DNA fluorimeter (Invitrogen, USA). The concentration of the amplified enriched DNA libraries for subsequent enrichment, hybridization with specific sequencing adapters with unique barcodes for each of the samples were performed: enzymatic DNA fragmentation, «polishing of the ends», ligation of each of the samples were performed: enzymatic DNA fragmentation, «polishing of the ends», ligation of synthetic probes, sorption of hybridized DNA libraries on streptavidin magnetic particles, and PCR from flanking primers. Units of concentration were recalculated on the basis of capillary electrophoresis data. The enriched DNA library was then sequenced on a platform of Illumina MiSeq by pairwise reads of 2-160 in accordance with the manufacturer’s recommendations using a set of reagents for 300 cycles [4, 5]. A panel of genes that are involved in development of cardiovascular disease (involvement in the pathogenesis of PAH: BMP2, BMPR1B, BMPR1B, CAV1, EIF2AK4, ENG, FOXF1, GDF2, KCNA5, KCNK3, NOTCH3, RASA1, SMAD1, SMAD4, SMAD9, TOPBP1) was used for target next-generation sequencing. We found a variant of a nucleotide sequence, which was previously described, in patients with PVOD, in exon 21 of the EIF2AK4 c.2965T gene in the heterozygous state. This result in the appearance of a non-synonymous substitution in the 989 position of the protein (p.Arg989Trp) [6]. The frequency of the detected variant in the control sample ExAC was not higher than 0.01%. Additionally, we found a variant of the nucleotide sequence c.859+1G>A in exon 7 of the EIF2AK4 gene (eukaryotic translation initiation factor 2 alpha-4 kinase 4, a eukaryotic translational factor 2 alpha-4 kinase factor) in the heterozygous state. This leads to a violation of the splice site and has not been previously described. The frequency of the detected variant of the nucleotide sequence in the control sample of ExAC is unknown. Therefore, our patient had two mutations in the EIF2AK4 gene (biallelic mutations), which is a common finding in patients with PVOD, and confirms the recessive nature of inheritance of this disease.

**Discussion.** In our case, genetic testing clarified the exact cause of PAH, which was the hereditary form of PVOD. An important feature of this form of PAH is the independence of sex for penetrance. In our case, PVOD was also found in the family representatives of both sexes. In Japan, PVOD is diagnosed in men more often than in women [7].

PAH associated with PVOD is characterized by rapid progression. A test with vasodilators has no predictive value for the effectiveness of calcium antagonists and can cause pulmonary edema (similar to other drugs in patients with PAH). This reaction is considered a pathognomonic sign of this form of PAH, but it is not observed in all patients. Moreover, two clinically morphological phenotypes of PVOD have recently been described [3]. One of these phenotypes, which has less histological changes, is characterized by a positive response to vasodilators and a better prognosis. Prolonged (>15 years) survival of patients after manifestation of symptoms of PVOD has been observed, which suggests that a different rate of progression is possible [8].

There are no specific signs to help differentiate this form from other types of PAH. Nevertheless, certain symptoms of PVOD in some cases allow an intravital diagnosis. These symptoms include a marked decrease in the diffusing capacity of the lungs for carbon monoxide, the presence of hypoxemia at rest, and severe desaturation during physical exertion. Alveolar hemorrhage that develops because of postcapillary block leads to an increase in hemosiderin concentrations in fluid obtained from bronchoalveolar lavage [9]. Alveolar hemorrhage can also lead to an increase in hemosiderin concentrations in alveolar macrophages according to the Golde scale [10]. A chest computed tomography scan can show a combination of an increase in mediastinal lymph nodes, the symptom of «frosted glass,» and smooth thickening of interlobular septa. At the same time, specificity of this sign is low, because it is found in other types of PAH [11].

There are few data on the effectiveness of different groups of specific drugs for PVOD [12, 13]. There is also a concern that such treatment can cause pulmonary edema. In our case, sildenafil treatment for 7 months did not result in clinical improvement, while echocardiography indicators indicated continued remodeling of the right heart. Specific signs for Pulmonary Hypertension in France. The diagnosis of PVOD was confirmed by biopsy data from lung transplantation in eight families and the diagnosis was based on clinical data in five families. In all of the families described, at least two affected siblings were detected from healthy parents, which confirmed the autosomal recessive type of inheritance. The age of diagnosis of PVOD ranged from 11-50 years, which is consistent with our case (symptoms appeared in 48 years). PVOD rapidly progressed in almost all cases. In all families, as well as in 20% of sporadic cases of PVOD, either homozygous carriage of recessive mutations or heterozygous carriage of combined mutations, which cause a significant decrease in protein function, was detected. The EIF2AK4 gene is present in all eukaryotes and participates in initiation of gene expression. The molecular mechanism for developing PVOD remains unknown. However, the serine/threonine protein kinase GCN2 that is encoded by this gene and involved in phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 is involved in regulating translation processes in response to stressor stimuli, providing cell resistance to inflammation, and oxidative stress. However, how the loss of function of this kinase leads to such specific changes in the microcirculation remains unclear [15].

Pathological variants in the EIF2AK4 gene are found in the majority of patients with family forms of PVOD and in 25% of sporadic cases of this disease. In patients with
PVOD, the presence of mutations in EIF2AK4 suggests a more severe course of this disease and mortality at an earlier age [14]. Because of difficulties of intravital diagnosis and certain selection in referral centers of pulmonary hypertension, as well as a small number of observations, more favorable cases of PVOD might not be fixed.

The autosomal recessive nature of inheritance implies that, in the pedigrees of affected probands, parents can remain healthy. This type of inheritance was also observed in our patient. PVOD can develop if two recessive alleles are detected in the genotype of the pathogenic variant of the gene. This was found in a survey of 136 patients with PAH in Spain [16]. Five Roma families with PAH were identified, and several patients had a rapidly progressive form of this disease. In all of the families, a homozygous carrier of the T allele of the variant 3344C>T (p.P1115L) in the EIF2AK4 gene was found. Further analysis of the Spanish register of pulmonary hypertension (REHAP) showed that of 76 patients with PVOD, 19 had signs of the hereditary form [3]. Among these, 18 patients were members of eight gypsy families. Among the 76 patients, 20 homozygous carriers of the pathogenic variant of the EIF2AK4 gene were identified. At the time of the study, penetrance was 90% (PVOD manifested in 18 of 20 patients) [3]. Therefore, ethnic groups with traditions of endogamy can be unique providers of recessive diseases, including PVOD.

However, the family form of PVOD is usually due to carriage of biallelic mutations. At report described two brothers who were diagnosed with PAH and had biallelic mutations in the EIF2AK4 gene in the heterozygous state [17]. The PAH was diagnosed at the age of 20 years in one of the brothers and at 33 years in the other brother.

The use of genetic data for diagnosing PVOD without additional morphological confirmation has been described by Chinese authors [19]. These authors reported a sporadic case of PAH associated with carriage of the mutation c.1392delT (p.Arg465fs) of the EIF2AK4 gene. Their patient also had two mutations in the heterozygous state, which can be considered as a cause of development of pulmonary hypertension. Taking into consideration the genetic data, the diagnosis in this case was changed from idiopathic PAH to PVOD.

In conclusion, implementation of molecular genetic methods in routine clinical examination of patients with PAH can greatly improve and speed up the diagnostic process.

Disclosures: The authors declare no conflict of interest.

Acknowledgment. We thank Ellen Knapp, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

References
4. Chen R., Im H., Snyder M. Whole-exome enrichment sequencing, which was performing in 10 patients with sporadic cases of PVOD, showed pathological variants of the EIF2AK4 gene in two of them. A man whose disease was diagnosed at the age of 15 years was a homozygous carrier of the 1392delT allele. Of particular interest is the case of a woman with a sporadic case of PVOD. She was diagnosed at the age of 22 years, and 10 years later, she had a left atrial pressure of 77 mm Hg. This patient was a homozygous carrier of the allele (860-1) A and mutation of 3438T (Arg1150X). The former allele leads to a violation of the splice site and is similar to the mutation found in our patient. Therefore, these data may support the pathological nature of the genetic variant found.

A family with an autosomal dominant type of inheritance of PAH associated with carriage of the pathological variant of the BMPR2 gene and simultaneous carriage of a mutation in exon 38 of the EIF2AK4 gene has also been described [18]. Carrying the pathological variant of the EIF2AK4 gene proved to be a factor that contributed to penetration of PVOD caused by a mutation in the BMPR2 gene.

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© Group of authors, 2018
UDC 616.711.718:616.831.45-08
DOI – https://doi.org/10.14300/mnnc.2018.13101
ISSN – 2073-8137

CLINICAL CASE OF TREATMENT OF THE LEGG – CALVE – PERTHES DISEASE BY EXTENDED PARASYMPATHETIC BLOCKADE

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КЛИНИЧЕСКИЙ СЛУЧАЙ ЛЕЧЕНИЯ БОЛЕЗНИ ЛЕГГА – КАЛЬВЕ – ПЕРТЕСА МЕТОДОМ ПРОДЛЕННОЙ ПАРАСИМПАТИЧЕСКОЙ БЛОКАДЫ

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This article presents a clinical case of an effective conservative treatment of the Legg – Calve – Perthes disease in girl eight years old. The follow-up period was 7 years. The selected method of treatment was an extended parasym pathetic blockade in the spine lumbar part using a Ropivacaine 0.2 % solution administered daily for 12 hours. The treatment duration was 8 days.

Keywords: Legg – Calve – Perthes disease, non-operative treatment, prolonged epidural analgesia