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THE ROLE OF MOLECULAR-GENETIC RESEARCH IN THE DIAGNOSIS OF HEREDITARY POLYPOSIS SYNDROME

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To determine the molecular genetic cause of the disease in a patient with colon oligopolyposis, whole exome sequencing was performed. The c.333+5G/C variant was detected in the BMPR1A gene. The functional significance of the found variant was elucidated, which demonstrated exon elongation at the mRNA level. This made it possible to confirm the diagnosis of juvenile polyposis in the patient.

Keywords: oligopolyposis, NGS, BMPR1A gene, mRNA, cDNA, reverse transcription.

Introduction. About 3-5% of all cases of colorectal cancer are caused by hereditary oncological syndromes, including Lynch syndrome (OMIM:120435) [12], familial adenomatous polyposis (OMIM:175100) [14], MutYH-associated polyposis (OMIM:608456) [16], Peutz-Jeghers syndrome (OMIM:175200) [15] and juvenile polyposis (OMIM:174900) [3,13]. These syndromes have different clinical manifestations, the course of the disease, as well as in molecular genetic characteristics [11]. In this regard, the clinical picture often helps in the initial choice of a target for molecular genetic investigation – a gene with possible pathogenic variant causing the disease. However, in some cases, the clinical manifestations of the disease are unclear, so they may correspond to several hereditary syndromes simultaneously. At the same time, if molecular diagnostics of genes in which pathogenic variants are most often found is ineffective, next-generation sequencing becomes the option of choice, allowing for the study of the whole exome or genome [4]. A feature of whole exome or whole genome sequencing is the identification of a wide variety of variants, while difficulties arise in interpreting previously undescribed variants and it is not always possible to verify them as pathogenic (in such cases they are called variants of unknown significance) [6]. Only use of additional advanced molecular genetic analysis allows finding out the functional significance of such variants.

Patients and Methods. A male 28-years old patient with periodic blood mixture in stools was checked up by the proctologist at the local hospital. During colonoscopy, numerous polyps were revealed in the bowel. The patient was directed to the federal hospital with diagnosis “familial adenomatous polyposis” and there he received genetic counseling. According to the family history, mother of the patient at the age of 45 was diagnosed with the infiltrative ulcerative stomach cancer, which caused her death; besides there were some cases of oncological diseases in maternal relatives, but without accurate data on tumor site and the time of their occurrence, and the degree of kinship. Colonoscopy revealed 19 polyps throughout the bowel with different sizes and shapes (some on wide bases, and some on a long thin stalks) from 0.3 to 3.5 cm in diameter, while only 6 of them were more than 1 cm in diameter. The endoscopic picture corresponded to adenomatous polyps; the biopsy from the largest polyps revealed fragments of tubular and villous adenomatous structures with low-grade epithelial dysplasia. The patient underwent endoscopic removal of the largest polyps (>1 cm in diameter); the histological examination of the removed polyps revealed tubular adenomas with low-grade epithelial dysplasia, villous adenoma with low-grade epithelial dysplasia, as well as 2 polyps with juvenile structure. Thus, the clinical picture of the patient's disease could correspond to both adenomatous polyposis syndrome (an attenuated form of familial adenomatous polyposis or *MutYH*-associated polyposis) and “mixed” polyposis, in which both adenomatous and juvenile polyps could be detected in the bowel [19]. To verify the diagnosis, a molecular genetic study was conducted, with venous blood as a material. The patient was under medical supervision in accordance with the observation protocols and gave written informed consent for the study. This study corresponded to the ethical principles of the Helsinki Declaration and was approved by the local Ethics Committee of the NMRC of Coloproctology of the Health Ministry of Russia, Moscow, the Russian Federation.

DNA extraction. The samples of patient's DNA were extracted from the venous blood using an automatic MagNaPure Compact station (Roche, Switzerland), using the MagNa Pure Compact Nucleic Acid Isolation Kit I (Roche, Switzerland), according to the manufacturer's protocol.

PCR and Sanger Sequencing. Polymerase chain reaction and sequencing by the Sanger method of *APC* and *MutYH* genes were carried out according to the method presented in previously published papers [2,18].

MLPA Analysis. The detection of extended rearrangements was carried out by the multiplex test-dependent ligase reaction with subsequent amplification (MLPA) using reagent kits: 1. SALSA MLPA P043-APC v.D1; 2. SALSA MLPA Probemix P158 JPS; SALSA MLPA Probemix P378 MUTYH (MRC-Holland, Netherlands) according to the manufacturer's protocol.

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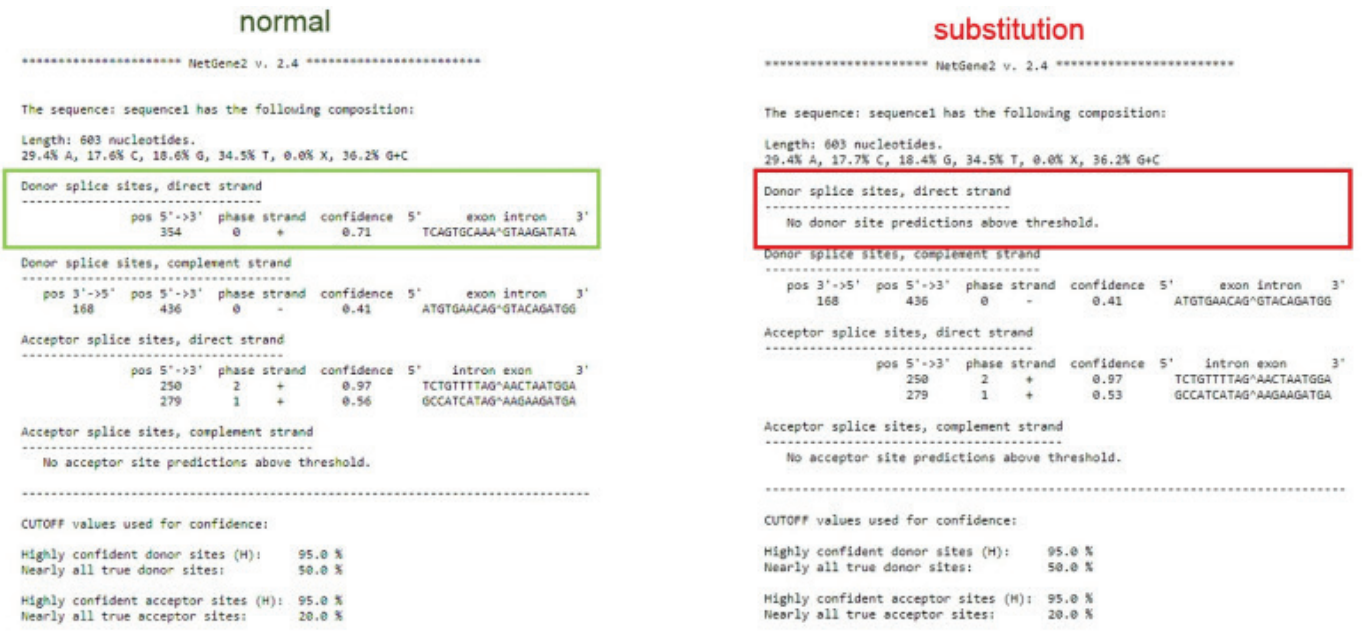

Fig. 1. The result of the analysis of possible outcomes in the case of variant c.333+5G>C using the NetGene2 splicing site prediction program: prediction of variant c.333+5G is a green frame; prediction of variant c.333+5C is a red frame

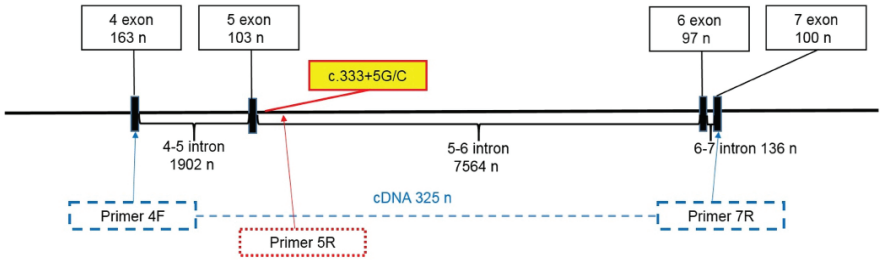

Fig. 2. Exon-intron region of the site from 4 to 7 exons of the BMPR1A gene. A yellow rectangle with a red frame is the name and location of the variant c.333+5G>C. The blue dotted frames indicate the location of the primers on the cDNA. The red dot frame shows the location of the primer in the intron after replacing c.333+5G>C.

Whole exome sequencing. We used 100 ng of total genomic DNA. The stages of sample preparation were carried out, followed by enrichment of exome regions using the Illumina Truseq Exome protocol and sequencing on the NextSeq550 platform with a reading length of 2*75 nctd (Illumina, USA).

mRNA extraction. mRNA was extracted from the patient's blood using the MagNa Pure Compact RNA Nucleic Acid Isolation Kit (Roche, Switzerland), using an automatic MagNaPure Compact station (Roche, Switzerland).

Reverse Transcription. The reverse transcription reaction was performed using the Thermo Scientific RevertAid Reverse Transcriptase kit (Thermo Scientific, Latvia).

PCR and Sanger Sequencing. DNA fragments were amplified by polymerase chain reaction (PCR). Primers of the *BMPR1A* gene were selected using the Primer3Plus program (https://www.primer3plus.com /): 4F – AGCACCAGAGGATACCTTGC; 7R – AATGAGCAAAACCAGCCATC; 5F – aacatgctagctacaattattgtga 5R – ggtgtacacatcgctgtatgttc (large caps – exon, small caps – intron).

Results. A sequential molecular genetic study of the *APC* and *MutYH* genes, including the search for extended deletions/insertions, did not reveal the presence of pathogenic variants. As a further diagnostic search, we decided to conduct a whole-exome sequencing. As a result, a variant c.333+5G>C, located in the intron (between exon 5 and 6) of the *BMPR1A* NM_004329.3 gene, was identified and then confirmed by Sanger sequencing.

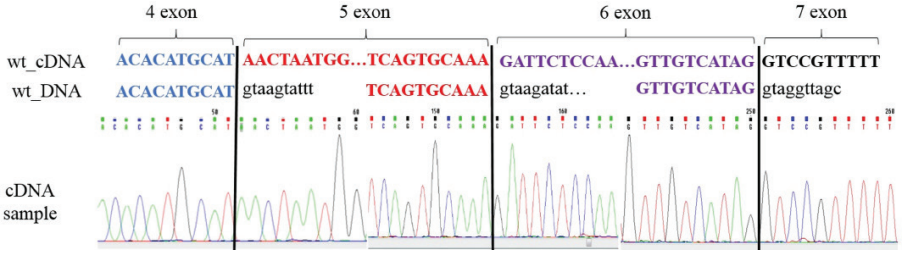

Рис. 3. Электрофореграмма участка кДНК пациента с вариантом c.333+5G/C в гене *BMPR1A*. В рис.3-4: wt_кДНК – референсный фрагмент кДНК гена *BMPR1A*; wt_ДНК – референсный фрагмент ДНК гена *BMPR1A* (ensembl.org)

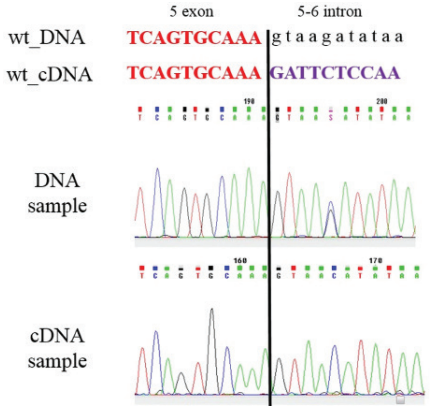

Рис. 4. Электрофореграммы участков ДНК и кДНК пациента с вариантом c.333+5G/C в гене *BMPR1A*

To find out the functional significance, an *in silico* analysis was performed using the NetGene2 splicing site prediction program (http://www.cbs.dtu.dk/services/NetGene2). It was found that variant c.333+5G>C leads to the loss of the donor splicing site and, accordingly, the variant may be functionally significant (Figure 1).

As the second stage, mRNA (with concentration of 15 ng/μL) was extracted from the patient's blood, then the reverse transcription reaction was performed, and the amplification of the cDNA fragment from exons 4 to 7 of the *BMPR1A* gene was carried out (Figure 2).

According to our first hypothesis, in the case of the functional significance of the found variant, the loss of an entire exon could be possible and this change should have been registered during sequencing of this site. According to the results of the analysis of the obtained sequencing fragment, there were no changes in the cDNA, and the fragment corresponded to the cDNA reference site (Figure 3).

Another hypothesis suggested that the replacement of c.333+5G>C could lead to the embedding of the intron site located between the 5th and 6th exons, which led to the amplification of the cDNA fragment only from the wild–type allele, since the intron had a large size – 7564 nucleotides. For confirmation of the fact of intron embedding, we amplified a cDNA fragment from the 4th exon to the 5th intron, while the reverse intron primer located further than the replacement of c.333+5G>C site by 82 nucleotides, and the length of the amplified fragment would consist of 348 nucleotides.

It should be noted that in the case of justified our hypothesis the fragment should look like a section of cDNA with an attached intron and a monoallelic variant of c.333+5C. Finally, the study confirmed this hypothesis (Figure 4).

Thus, even despite the lack of data on the number of embedded nucleotides from the intron, it can be stated with full confidence that variant c.333+5G>C of the *BMPR1A* gene leads to the formation of a premature stop-codon, being a pathogenic mutation of the splicing site.

Discussion. Oligopolyposis is a patient's condition with 10 to 99 polyps in the bowel [8]. Depending on the number of the predominant type of polyps, oligopolyposis can be classified into: adenomatous, hamartomic, serrated, and others. In some cases, it is difficult to understand which type of polyps dominate and, accordingly, it is difficult to make a correct diagnosis with further treatment tactics.

We have produced an advanced molecular genetic investigation in a patient who had mainly adenomatous polyps and only 2 juvenile ones. Formally, the patient does not fit the Russian clinical guidelines “Adenomatous polyposis syndrome” developed by Shelygin, Yu. A. et al. [1]. In addition, we could not suspect juvenile polyposis since our patient did not fit the clinical selection criteria described in the clinical guidelines authored by Richard Boland et al. [9]. According to these criteria, a genetic study for the determination of juvenile polyposis should be carried out for:

- *Patients with 5 or more juvenile-polyps in the colon and rectum;*
- *Patients with 2 or more juvenile polyps located in other parts of the gastrointestinal tract, except the large intestine;*
- *Patients with any number of juvenile polyps having 1 or more first-line relatives with juvenile polyposis.*

In addition, single juvenile polyps can occur in 2-3% of children and adolescents [7].

Despite the fact that the clinical manifestation of our patient did not match the Russian criteria for the attenuated form of familial adenomatous polyposis (the presence of 20 to 100 polyps in the bowel, predominantly in the proximal parts) and *MutYH*-associated polyposis (the presence of 20 to 100 polyps), we decided to investigate the *APC* and *MutYH* genes, as the patient's mother had malignant tumor of the stomach at the age of 45, but no information was about the presence or absence of polyps in her bowel. An extended study of these genes, including the MLPA method, revealed no mutations. It is worth to note that the set for the study of large rearrangements of the *APC* gene includes diagnostics of the duplication of the *GREM1* gene (15q13.3) which is associated with mixed polyposis syndrome [10]. For the first time, mixed polyposis syndrome was described in an Ashkenazi Jewish family whose members had polyps of more than one histological type: adenomas, hyperplastic and juvenile [10].

Whole exome sequencing revealed an intronic variant of c.333+5G>C in the *BMPR1A* gene. This variant was not found in the gnomAD population databases (0/250910 alleles). However, it was previously encountered in the study of the Invitae group (rs1554888331), but it was characterized as a variant of unknown significance. It is worth noting that we founded the pathogenic variant IVS3+5G→C in the HGMD (The Human Gene Mutation Database) database [17]. It could be assumed as a similar option. However, a specific name (according to the HGVS nomenclature) is not given in the specified database, therefore, it is not possible to confirm the identity of this variant and c.333+5G>C. In this situation, we performed a study for proving the pathogenicity of a previously unknown variant according to the algorithm of our early investigation [5]. In present work, we also proved the pathogenicity of the unknown variant, because its presence in the *BMPR1A* gene leads to the loss of the splicing site, elongation of the exon and the formation of a premature stop-codon (TAA).

Conclusion. As a result of a complex molecular genetic analysis, it was proved that the variant c.333+5G>C of the *BMPR1A* gene was pathogenic. This gave us ability to confirm a genetically verified clinical diagnosis of juvenile polyposis in a patient with 17 adenomatous and 2 juvenile polyps in the bowel.

The work was carried out within the framework of the state assignment: “Diagnostic of hereditary forms of colorectal cancer by whole exome sequencing and MLPA (multiplex probe-dependent ligase reaction with subsequent amplification) methods.

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HEALTHY LIFESTYLE. PREVENTION

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THE ATTITUDE OF YOUNG PEOPLE OF REPRODUCTIVE AGE TO ETHICALLY CONTROVERSIAL ISSUES OF THE PRENATAL DIAGNOSIS OF HEREDITARY DISEASES

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The article discusses the results of a survey conducted among young people in the City of Yakutsk regarding ethically difficult issues of prenatal DNA-testing for hereditary diseases. Most respondents (74%) consider prenatal diagnosis a necessary procedure. Analysis of attitude of young people to morally ambiguous issues of prenatal diagnosis shows that the decision to terminate pregnancy after prenatal diagnosis is affected by the severity of damage to fetus. Compared to similar survey results in other countries, young reproductive age people in Yakutsk show lower values on the issue of pregnancy termination when confronted with Down's syndrome (49%) and an ethically ambiguous issue of pregnancy termination in case of a deaf child (19%). There is no connection between the opinions of respondents on prenatal diagnosis being a necessary or an unnecessary procedure, and their own desires to terminate pregnancy in case of a disorder.

Keywords: prenatal diagnosis, bioethics, survey, young people, hereditary diseases.

Introduction. Prenatal diagnosis (PND) is a modern means of diagnosing the state of a fetus and detecting possible disorders during pregnancy at different stages of gestation. Various diagnostic methods and their combinations are employed, such as ultrasound, biochemical, cytogenetic, molecular-genetic testing, including invasive and non-invasive methods of fetal examination [2].

According to European guidelines, the objective of PND is providing prenatal diagnostic testing services (for genetic conditions) that enable families to make informed choices consistent with their individual needs and values and which support them in dealing with the outcome of such testing [20].

When conducting a PND for hereditary monogenic disorders, fetal samples obtained via chorionic villus sampling at early stages of pregnancy are processed to extract DNA from cells, after which a molecular-genetic analysis is performed to detect damage (mutations) to genes. There are many different methods of genetic testing available today, from direct PCR diagnosis to detect mutations, to analysis of full genome sequencing of an individual's DNA. Modern genetic testing technologies can detect mutated gene variants and variations of genetic markers, which are connected to disorders based mostly on calculations of probability of disease manifestation. PND is a complicated and expensive procedure which often comes with moral and ethical dilemmas, both for geneticists and families that undergo PND and make a difficult decision to be tested [22]. Main bioethical issues include informed consent for PND, individual autonomy, right to reproductive choice [4]. In case of a risk of severe fetal disorder that makes it non-viable, or high probability of congenital genetic disease, families make emotionally difficult decisions to terminate pregnancy, and it is known that 80 to 90% of families decide to terminate [6,10,18]. The remaining families decide to continue pregnancy with an affected fetus due to their moral values or religious beliefs [13].

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