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VARIANT OF RARE HERMANSKY — PUDLAK SYNDROME ASSOCIATED WITH GRANULOMATOUS COLITIS: DIAGNOSTICS, CLINICAL COURSE AND TREATMENT

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***Aim*: To study the relationship between the genotype and the phenotype in the patients with Hermansky — Pudlak syndrome (HPS) associated with granulomatous colitis; to monitor clinical course of the disease for adequate treatment, cancer surveillance and genetic counseling. *Materials and Methods:* The diagnosis of HPS is established by physical examination, chest X-ray, computed tomography, endoscopic examination with biopsy, and laboratory tests, including histology, baseline laboratory blood, urine and feces tests, determination of ASCA-C and ANCA antibodies using an ELISA. Molecular genetic testing for *HPS* gene mutations, R702W, G908R, L1007fs and P268S mutations in *NOD2* gene, and TaqI variant of the *VDR* gene were carried out. *Results:* We report 2 cases of HPS from unrelated families. Both were complicated by inflammatory bowel disease with pathologic features of Crohn's disease refractory to antibiotics and corticosteroids. One patient (family 1) with Ashkenazi Jewish ancestry had pathogenic variant of the *HPS-4* gene in exon 8, mutation P268S of *NOD2* genes and "Tt" genotype of TaqI variant of the *VDR* gene. Another patient (family 2) carried two mutations P268S and G908R of *NOD2* gene, and had a large paraovarian cyst diagnosed. No consistent success with the standard medical therapy, used for treating granulomatous colitis, associated with HPS, in presented cases was achieved. Patients needed surgical interventions at a young age and a long-term surveillance of the probable development of tumors and other complications. Azathioprine at 2 mg/kg/day and mesalazine 3 g/day were used with some positive effect for prevention of Crohn's disease postoperative recurrence. *Conclusion:* The occurrence of perianal lesions, the histopathological findings and the results of the molecular genetic analysis confirmed the mutations P268S and G908R of *NOD2* gene in these cases suggest that HPS was truly associated with Crohn's disease variant with early onset and severe course. The search for the molecular causes of the disease in some individuals may help in the development of new therapeutic and surgical approaches, as well in the improvement of understanding of premalignant inflammatory conditions in a large bowel.**

***Key Words*: Hermansky — Pudlak syndrome, Crohn's disease, *HPS, NOD2* and *VDR* genes mutations, therapeutic treatment, surgical interventions, cancer surveillance.**

Inflammatory bowel disease (IBD) is among the top three high-risk conditions for colorectal cancer, similar to familial adenomatous polyposis and hereditary non-polyposis colorectal cancer [1]. In case of early and severe onset of IBD in the childhood groups, the disease may be caused by mutations in genes responsible for severe monogenic disorders such as Hermansky — Pudlak syndrome (HPS) [2]. HPS is a rare autosomal recessive disorder consisting of a triad of manifestations of albinism, increased bleeding tendency secondary to platelet dysfunction, and systemic complications associated with accumulation of ceroid lipofusion [3, 4]. The prevalence of the variant of HPS — HPS-1 in northwestern Puerto Rico is 1/1,800 population [5, 6], but in non-Puerto Rican populations the prevalence of syndrome is estimated at 1–9/1,000,000 (www.orpha.net), but in Ukraine it is not known.

Originally described in 1959 by Drs. Hermansky and Pudlak, HPS is now known to be a disease of lysosome-related organelles [7]. The disorder usually presents in early childhood, but may present at older ages, with tyrosinase-positive oculocutaneous albinism (varying degrees of hypopigmentation), reduced visual acuity (often at/below the level of legal blindness), horizontal nystagmus, easy bruising of soft tissues, epistaxis, prolonged bleeding after dental extraction, surgery or childbirth. Women may present with medically significant menstrual bleeding [8, 9]. Differential diagnoses include other forms/causes of oculocutaneous albinism, *i.e.*, X-linked ocular albinism, Chediak — Higashi syndrome, Griscelli syndrome, Cross syndrome, pulmonary fibrosis and hemophagocytic lymphohistiocytosis [10].

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Abbreviations used: ANCA-C – anti-neurotrophic cytoplasmic antibodies; ASCA – anti-*Saccharomyces cerevisiae* antibodies; AP-3 – adaptor complex-3; ATG16L1 – autophagy related 16-like 1 gene; BLOCs – biogenesis of lysosome related organelle complexes; EO-IBD – early onset of inflammatory bowel disease; HPS – Hermansky – Pudlak syndrome; IBD – inflammatory bowel disease; NOD2 – nucleotide oligomerization domain containing 2; PCR – polymerase chain reaction; VEO-IBD – very early onset of inflammatory bowel disease; VDR – vitamin D receptor.

HPS is characterized by a storage pool deficiency of platelets and can be caused by mutations in one of several genes: *HPS1* (10q23.1), *AP3B1* (5q14.1; causing HPS-2), *HPS3* (3q24), *HPS4* (22q11.2–q12.2), *HPS5* (11p15-p13), *HPS6* (10q24.32), *DTNBP1* (6p22.3; HPS-7), and *BLOC1S3* (19q13; HPS-8). The product of AP3B1 codes for the beta 3A subunit of adaptor complex-3 (AP-3), involved in vesicle formation and protein sorting. The function of the other HPS gene products is unknown, but they interact with each other in biogenesis of lysosome-related organelle complexes (BLOCs); BLOC-2: HPS3, HPS5 and HPS6; BLOC-1: HPS7 and HPS8. Targeted analysis for the *HPS3* splice site variant c.1163+1G>A can be performed first in individuals of Ashkenazi Jewish ancestry. HPS1 and HPS4 form a protein complex called BLOC-3. Affected lysosome-related organelles include melanosomes in melanocytes, delta granules in platelets, lamellar bodies in pulmonary type II cells, and secretory granules in T-cells. Of the eight genotypic subtypes (HPS-1 to HPS-8), the first subtype (HPS-1) is the most common [11]. HPS types 1 and 4 are associated with gastrointestinal complications related to granulomatous colitis, enterocolitis, ileitis, intestinal fistulization or granulomatous perianal disease that is pathologically and phenotypically indistinguishable from Crohn's disease [12–14]. These observations suggest that the colitis of HPS is due to the development of classical Crohn's disease. Therefore, it is possible that treatments known to be effective for Crohn's disease would be effective for HPS-associated enterocolitis [15]. Complications may include in HPS-1 or HPS-4, pulmonary fibrosis also. Pulmonary fibrosis is the most serious complication and usually presents in the fourth or fifth decade. HPS is associated with solar keratoses (premalignant lesions), lung and skin cancer (basal cell carcinoma and squamous cell carcinoma) [16, 17].

Crohn's disease is characterized by changes in intestinal microbiota, focal translocation of bacteria across the mucosal barrier, altered mucosal response to bacterial invasion, development of chronic granulomatous inflammation. Genetic factors leading to a leaky epithelial barrier and impaired mechanisms of phagocytosis and autophagy. Whatever is the particular combination of factors in each patient, the common result is a granulomatous inflammation and activation of T cell immunity [18]. The innate immune receptor nucleotide oligomerization domain containing 2 (*NOD2*) was the first gene associated with Crohn's disease [19, 20]. Three mutations (R702W, G908R and L1007fs) in the *NOD2* coding region were demonstrated to be associated with Crohn's disease in affected patients that carried at least one variant [21]. Genetic susceptibility to Crohn's disease shows significant ethnic differences. The mechanisms linking *NOD2* variants to the risk of Crohn's disease are not fully clear. Another strong association with Crohn's disease regards the autophagy related 16-like 1 (*ATG16L1*) gene [22].

HPS to be very rare, but it is expected that in the severe form of early onset of IBD (EO-IBD) including Crohn's disease in the childhood groups, genetic factors play a significant role in pathogenesis. In EO-IBD the disease tend to be much more severe and much more difficult to control with conventional therapies, compared with adult-onset IBD. Increasing evidence suggest a stronger genetic contribution to these forms compared with adults. Some patients with EO-IBD or very early onset (VEO-IBD) may have developed intestinal inflammation as a part of a monogenic disease, usually a primary immunodeficiency disease [2]. In fact, these cases may account, at least in part, for the phenomenon of missing heritability in IBD, which is the inability to explain all the genetic contribution to IBD based solely on the additive effect of common risk gene variants [23]. Distinguishing monogenic forms among VEO-IBD is a crucial importance to allow the best treatment. The pathogenesis of inflammation in chronic granulomatous disease could also be attributed to a deficiency of autophagy, leading to autoinflammatory response dominated by IL-1 release [24].

In various immune-related diseases low serum vitamin D levels have been reported pointing to an immunoregulatory role. Vitamin D and its receptor (VDR) are known to interact with different players of the immune homeostasis by controlling cell proliferation, antigen receptor signaling, and intestinal barrier function. In European Caucasian patients, a significantly higher frequency of the TaqI polymorphism (genotype "tt") was reported in Crohn's disease compared to healthy control individuals [25]. This finding was replicated in German IBD patients where the "tt" genotype was significantly more frequent in fistulizing and stenosing Crohn's disease. *VDR* polymorphisms have been identified in various diseases, such as cancer or cancer risk [26].

The aim of this work is to study the relationship between the genotype and the phenotype in the patients with HPS associated with granulomatous colitis; to monitor clinical course of the disease for adequate treatment, cancer surveillance and genetic counseling.

MATERIALS AND METHODS

We report the one patient with HPS from family 1 (case 1) and one patient with this syndrome from family 2 (case 2) which were complicated by IBD. The diagnosis of HPS is established by physical examination, chest x-ray, high-resolution computed tomography, endoscopic examination with biopsy samples from different part of the large bowel, baseline laboratory blood, urine and feces tests and molecular genetic analysis. Laboratory tests were performed for proper treatment: general and biochemical blood tests, calprotectin determination, anti-*Saccharomyces cerevisiae* antibodies (ASCA), anti-neurotrophic cytoplasmic antibodies (ANCA-C). Immunoglobulin G (IgG) ASCA and ANCA-C were measured by using an ELISA in serum samples from patient (case 1). For the possibility

of tuberculosis of the intestine a tuberculin skin test was performed, which was negative.

Molecular genetic testing

This study was approved by the hospital local ethics committee and all the patients provided written informed consent. Blood samples were taken and DNA was extracted from the leukocytes of the peripheral blood cells with the phenol purification method [27].

Targeted analysis for the *HPS-4* variant can be performed in patient of Ashkenazi Jewish ancestry. Molecular genetic testing approaches included serial single-gene testing, using a multigene panel. Identification of biallelic pathogenic variant in *HPS-4* confirms the diagnosis if clinical features are inconclusive. Targeted sequence analysis of *HPS-4* for the patient was carried out based on the severity of clinical findings, such as oculocutaneous albinism and granulomatous colitis. The identification of *HPS* gene mutation (case 1) was carried out in the Medical Genetic Scientific Center (Moscow, Russia).

Molecular genetic testing for four mutations (R702W, G908R, L1007fs and P268S) in the *NOD2* gene was carried out in the Institute of Human Genetics of the Polish Academy of Sciences (Poznań, Poland).

A polymerase chain reaction (PCR)-restriction fragment length polymorphism technique was used to identify the mutations, which was confirmed by sequencing.

The variants of *NOD2* gene associated with Crohn's disease are c.2104 C>T (p.R702W), c.2722G>C (p.G908R), and 3020insC (p.L1007fs) localized in exons 4, 8 and 11, corresponding to leucin rich repeat protein domain or adjacent region [28, 29]. The exons 4, 8, 11 of *NOD2* gene were amplified using the following primers, designed by Primer3 software:

Exon 4F 5'AGTGCACAGCTTGTGAATGG3',
Exon 4R 5'GCTCCCACACTTAGCCTTGA3',
Exon 8F 5'CCACTCTGGGATTGAGTGGT3',
Exon 8R 5'TCCATTGCCTAACATTGTGG3',
Exon 11F 5'GGACAGGTGGGCTTCAGTAG3',
Exon 11R 5'CCTCAAAATTCTGCCATTCC3'.

Protocol was performed as previously described [30, 31]. For the amplification reaction was used a touchdown PCR protocol, consisting in 1 cycle of 3 min of denaturation at 94 °C, after which the DNA was amplified during 39 cycles, of which 14 cycles consisted of 20 s of denaturation at 94 °C, 40 s of annealing at 62 °C, decreasing 0.5 °C each cycle, and 45 s of extension at 72 °C; then 25 cycles of denaturation at 94 °C for 20 s, 40 s of annealing at 55 °C and 45 s of extension at 72 °C. After amplification, the reaction mixture was subjected to a final cycle of 7 min of extension at 72 °C. PCR products were subjected to sequence analysis performed on both strands with an automated procedure using the 3100 Genetic Analyzer (Applied Biosystems). PCR fragments were sequenced using the same primers used for PCR amplification.

Molecular genetic testing for variant TaqI of the *VDR*-3 gene was carried out in the Institute of Hereditary Pathology of the National Medical Academy of Sciences (Lviv, Ukraine). For the amplification reaction the PCR program mode (Saiki et al., 1983) was used on the termocyclers "AMPLY-4" ("Biokom", Moscow, Russia). Oligonucleotide primers had been synthesized in the Institute of Bioorganic Chemistry of the Russian Academy of Sciences (Moscow, Russia). Analysis of TaqI variant of the *VDR-3* gene using 35 cycles of PCR with the following parameters were performed: 94 °C 1 min, 60 °C 1 min, 72 °C 1 min. As a nucleators the following primers were used: 5'-CAGAGCATGGACAGGGAGCAA-3' and 5'-GCAACTCCTCATGGCTGAGGTCTC-3'. The restrictases and the thermostable Taq-polymerase produced by the company "Fermentas" (Vilnius, Lithuania). The specificity of PCR products and the analysis of restrict fragments by means of electrophoresis in 2 to 3% agarose gel were performed.

RESULTS AND DISCUSSION

Case 1. A 15-year-old girl of Ashkenazi Jewish ancestry with hallmark findings of HPS was admitted to the proctology department of the University Hospital complaining of 1-year history of recurrent episodes of abdominal pain, general weakness, frequent defecations (6–7 times/day) with intermittent blood and pain of the perineum area. Physical examination revealed an albino girl with whitish hair, pale and unpigmented skin and strabismus. Ocular examination showed horizontal nystagmus with reduced vision and no pigmentation of the iris. Cardiopulmonary examinations were normal. Chest x-ray and a high-resolution computed tomography didn't show any signs of pulmonary fibrosis. No signs of hemorrhagic diathesis were observed at presentation. An 18-year-old brother of the proband also has HPS. The pedigree of proband is shown in Fig. 1.

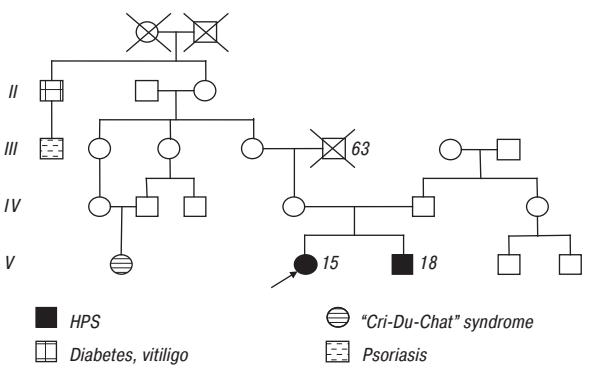

Fig. 1. The pedigree of proband of Ashkenazi Jewish ancestry with HPS

Laboratory blood tests showed hypochromic anemia, leukopenia, and thrombocytopenia. An increased level of calprotectin (283 ml/g) was observed, as well as higher ASCA IgG (14.286 units/ml) titers, and normal ANCA-C (5.38 units/ml) titers. The combination of a positive ASCA test with a negative ANCA-C test has a positive predictive value of 96% and a specificity of 97% for Crohn's disease [32]. ASCA+ patients have higher frequency of mutant *NOD2* alleles. Higher ASCA titers were associated with higher probabilities stricturing/penetrating of Crohn's disease behavior. This quantitative marker may prove useful in risk-stratifying patients to more aggressive anti-inflammatory therapies [33].

Colonoscopy revealed both severe segmental colitis with numerous deep ulcers with sparing of the remainder of the colon, hemorrhages in the submucosal layer, and stricture in sigmoid colon. This endoscopic appearance is highly reminiscent of Crohn's disease. Upper endoscopy showed erosive gastritis — the catarrhal bulbitis. Contrast enhanced computed tomography of the abdomen showed thick-walled loops of sigmoid colon, without evidence of perforation. Histologic findings of biopsy samples from the large bowel showed focal chronic inflammation, irregular villous architecture, and granuloma formation with no obvious ceroid deposition. All the mucosal biopsies from the different colonic segments, included those from ulcer bases, were negative for *Mycobacterium tuberculosis*.

The obtained laboratory results and histological examination corresponded to Crohn's disease of large bowel, associated with HPS, and characterized by early onset and severe chronic course. The patient was diagnosed with the chronic paraproctitis with rectovestibular and rectovaginal fistula. The patient underwent laparotomy and imposed a loop transverse colostomy of the left side in the right side of the abdomen. Laparotomic wound closed primarily. After applying a transverse colostomy the patient underwent an operation of perineum fistula incision by Gabriel method on the rectum. Despite the transverse colostomy, the seams on the wound were parted and the wound of the perineum had been healing with a secondary tension. Before and after the operation the patient received such medications: etamsylate, ascorbinic acid with rutin, diosmectite, potassium and magnesium aspartate, and infusion therapy. Her condition has improved. However, after 2 months, despite the treatment, rectovestibular fistulas relapsed. The patient received further antibacterial therapy (ciprofloxacin and metronidazole) for 3 weeks. According literature data fistulizing pattern in Crohn's disease is an independent risk factor for cancer [34]. The patient underwent a left-sided hemicolectomy half a year later at the Center of Coloproctology (Moscow, Russia), because of the chronic perianal complications (Fig. 2).

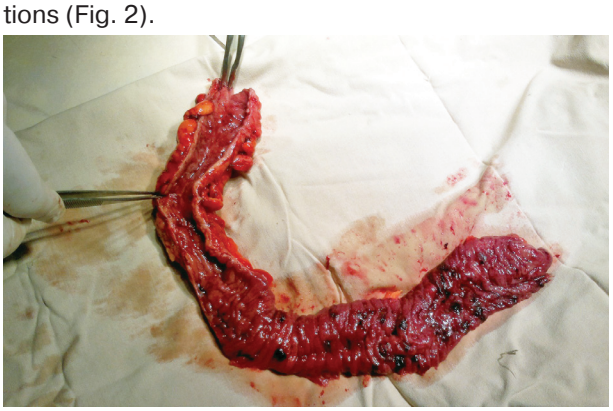

Fig. 2. The preparation of removal large bowel with stricture, deep ulcers and hemorrhage in the submucosal layer

During the last two years, the patient several surgical interventions were performed regarding the persistently unhealed perianal complications. Subsequently, a reconstructive operation of transverse-rectoanastomosis, ileostomy and drainage latex ligation through the fistulas on the perineum was performed. Postoperative wound healed badly. The patient received specific therapy to prevent local complications in the perineum: azathioprine — 50 mg o.d., metronidazole-gel, 5-amynosalicillic acid suppositories. The patient is recommended to: 1) continue azathioprine in the previous dose; 2) start antibacterial therapy parenterally — ciprofloxacin and metronidazole for stopping purulent discharges; 3) use 5-amynosalicillic acid suppositories with metronidazole gel; 4) perform washing of fistulas with antiseptic solutions (dioxisol and betadine); 5) anti-TNF-therapy with natalizumab or adalimumab.

The combination of the patient's specific phenotype with intestinal manifestation similar to Crohn's disease are likely to clinically assume a rare monogenic HPS, probably subtypes 1 or 4, associated with chronic granulomatous colitis. Regarding the features of HPS-4, the collection of genealogical information was performed and molecular genetic studies were conducted. The parents of the proband are healthy, but her brother has expressed signs of the HPS, which confirms the autosomal recessive type of inheritance of the disease.

Molecular genetic analysis shows a marker mutation, a pathogenic variant of the HPS-4 gene in exon 8. This mutation leads to a formation of the premature termination site of translation (chr22: 26864537G>A) in (c.649C>T) 217 codon (p.Arg217Ter), confirming the subtype HPS-4 (OMIM 614073), which is accompanied by chronic granulomatous colitis, the most likely Crohn's disease. The attributed of *HPS-4* pathogenic variant of gene among the in Non-Puerto Rican is ~11.5%. Data based on approximately 278 individuals with HPS of non-Puerto Rican ancestry reported as of July 2017 [16]. No data on detection rate of gene-targeted deletion/duplication analysis are available.

For proper treatment, the patient had been referred to additional molecular genetic studies: the identification of the *NOD2* gene mutations and the Taq1 variant of the *VDR* gene, because patient had very low ionized calcium level at 1.06 mMol/l. The P268S variant of the *NOD2* gene was confirmed to be heterozygous. According to the literature data, this mutation is associated with Crohn's disease in some mostly small populations, such as Ashkenazi Jews [35], representatives of some autonomous groups of China, as well as Iranians and residents of Italy [36, 37]. The three *NOD2* variants are associated with early onset and the presence of one variant allele increases the risk for developing Crohn's disease from 1.5 to 4.3 folds, while two allele variants increase susceptibility to develop the disease from 20 to 40 folds compared with the general population [38]. In patient was confirmed "Tt" genotype of Taq1 variant of the *VDR* gene that also pointed to the disturbance of calcium metabolism. The standard serum chemistry examination shows lowered ionized calcium levels. Literature data highlight the

importance of vitamin D in different aspects of immune regulation, for example in chronic immune-mediated diseases and cancer. This suggests to considering the metabolite not simply as a vitamin involved in bone and calcium homeostasis, but also an autocrine mediator with an active role in numerous physiological processes, particularly in the innate immune system. Since most studies concerning the calcium status in IBD yielded contradictory data, the discussion has focused in the most recent literature on the possible role of vitamin D as a risk factor for the onset and evolution of gut inflammation [39].

Taking into account the stricture in the anal canal, functioning unhealed fistulas, ineffectiveness of conservative therapy, the risk of malignancy, the patient an operation was proposed — extirpation of rectum, but the patient refused to undergo the intervention and continued conservative therapy.

Case 2. A 26-year-old female patient from the family 2, has HPS phenotype: whitish hair, pale and unpigmented skin, horizontal nystagmus, reduced visual acuity, granulomatous colitis, increased bleeding tendency. Congenital achromatitis and psoriasis were also diagnosed. The first intestinal symptoms appeared at the age of 25 years. All the relatives of the proband are healthy. The detection of mutations in the *HPS* gene was not carried out due to the high cost of the analysis for the patient. The diagnosis of HPS was made clinically. At the age of 26 years, the patient had undergone surgical intervention — the total colproctectomy, because of severe total colitis. The ileoanal pouch was formed, and the ileostoma was made. After half a year the ileostoma was closed. Postoperative period was complicated by bleeding as a result of HPS-associated platelet disorder. The patient had extracolonic concomitant abdominal pathology: chronic pancreatitis and chronic nephritis. Currently, the patient is taking azathioprine at 2 mg/kg/day and mesalazine 3 g/day for prevention of postoperative recurrence of Crohn's disease. Despite the administration of adalimumab after intestinal resection surgery was greatly effective in preventing endoscopic and clinical recurrence of Crohn's disease [40], but using this medication is less frequent in our patients due to the high cost on it.

At the age of 28 years the patient was diagnosed with a non-malignant neoplasm — a paraovarian cyst (15 cm) near the right ovary, caused compression on the intestinal pouch and on the right ureter, partial obstruction and first degree hydronephrosis. This urged a tubo-ovariectomy.

The patient was confirmed to carry two variants of the *NOD2* gene: G908R and P268S in a heterozygous state. The "TT" genotype (wild type) of the Taq1 variant of the *VDR* gene was detected in the patient.

No consistent success with the standard medical therapy was revealed in presented cases, except positive effect of azathioprine in one patient for treatment of Crohn's disease, associated with HPS. These patients need surgical intervention in young age. Insufficient information on pathogenesis, peculiarities of the course and treatment of chronic granulomatous colitis associated with HPS, makes each clinical case an important element in the accumulation of experience for surgeons, gastroenterologists, geneticists and hematologists. EO- and VEO-IBD are often resistant to routine treatment. Therefore, the search for the molecular cause of the disease in some individuals may help in the development of new therapeutic and surgical approaches to treatment. Accurate diagnosis of the HPS subtype has important prognostic and treatment implications.

In cases of HPS-4, associated with chronic severe perianal complications, cramping, increased mucus in the stool and rectal bleeding, patients should be provided with information about preventing basal and squamous cell carcinoma. High-risk patients have a need for ongoing regular follow-up, at least annual, and skin self-surveillance. For the patients at high risk for neoplasia regular ultrasound surveillance is necessary also. Patients with HPS need annual ophthalmologic examination, at least annual examination of the skin for solar keratoses (premalignant lesions) [41]. In cases of HPS-4, associated with pulmonary fibrosis, annual pulmonary function testing is necessary in those older than age 20 years.

Patients with HPS inherited in an autosomal recessive manner need genetic counseling, considering both the risks of severe clinical course of Crohn's disease, it's possible risk of transformation to cancer, and malignancy risks connected with albinism. Each siblings of proband has a 25% chance of being affected, a 50% chance of being an asymptomatic carrier, and a 25% chance of being unaffected and not a carrier. The prenatal diagnosis for pregnancies is possible for those families in which the pathogenic variants have been identified.

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