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Genetic analysis by whole exome sequencing (WES) in a patient with retinal disorder: A Case Study

Ebrahim Rakhshani [1], Mohammad Areesh[2], Farzaneh Salimi[3], Dor-Mohammad Kordi-Tamandani[4*]

[1,3] Department of Biology, Faculty of Science, University of Sistan and Baluchestan, Zahedan, Iran. E-mail: ebrahim.rni99@gmail.com , farzanehsalimi66@gmail.com.

[2] Department of Eye Surgery, Faculty of Ophthalmology, Zahedan University of Medical School , Zahedan, Iran. E-mail: m_areesh@yahoo.com.

[4*] *Corresponding author*, Professor, Department of Biology, Faculty of Science, University of Sistan and Baluchestan, Zahedan, Iran. E-mail: dor_kordi@science.usb.ac.ir.

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A B S T R A C T

Objective: Retinal hemangioblastoma is part of the symptoms of von Hippel-Lindau (VHL) syndrome, which occurs due to mutations in the VHL gene located on chromosome 3. This gene encodes a protein that plays important roles such as regulating the expression of oxygen-related genes, cilia formation, extracellular matrix formation, etc. Mutations in this gene cause hemangioblastomas of the retina, brain, and spinal cord, as well as a wide range of symptoms such as kidney and pancreatic cysts, etc.

Materials and Methods: This is a laboratory and experimental study in which a female patient suddenly developed blindness in her right eye at the age of 23. After ophthalmological examinations by specialists, blood was taken from the patient to find the main cause of the disease and genetic study, and DNA extraction was performed using the saturated salt method. Then, whole exome sequencing (WES) was performed, and finally, to confirm the candidate mutation and the results of WES, Sanger sequencing was performed.

Results: Based on the findings of this study, a mutation occurred in chromosome number 3 and at position Chr3-10191469 A G:NM_000551:exon3: c.464-2A>G of the VHL gene, in which a nucleotide with a guanine base was replaced by an adenine nucleotide. This mutation is pathogenic and its inheritance is autosomal dominant, and the patient's genotype for this gene was heterozygous, which was directly related to his phenotype and led to the development of von Hippel-Lindau syndrome and impaired retinal function in his right eye.

Introduction

The retina is the nerve part of the eye and the third (innermost) layer, which is very thin, about half a millimeter in diameter. This important part of the eye converts light that passes through the cornea and lens into nerve signals using special cells that are sent to the brain and the back of the head by the optic nerve, where it is finally transformed into an image (Boll, 1977). There are two types of photoreceptor cells in the retina. These cells (cones

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and rods) enable our sense of sight by converting light rays into signals that are transmitted to the brain. There is a very high concentration of cone photoreceptor cells in the macula, which are responsible for color vision and our ability to see fine details (Cornish et al., 2021). Cones are responsible for producing color and fine detail, while rods provide peripheral vision, motion, and shades of gray. Rods are located mostly outside the macula, and cones are located inside it (Kaplan, 2007).

Inherited retinal disorders (IRDs) comprise a heterogeneous group of genetic disorders that affect the retina. Caused by mutations in over 280 genes, IRDs result in visual impairment due to dysfunction and destruction of photoreceptors, the retinal pigment epithelium, or the choroid (Hu et al., 2021). The prevalence of these disorders is approximately 1:1380, with an estimated 5.5 million people worldwide affected. Currently, IRDs are largely incurable, but the landscape of treatment options is rapidly changing for these diseases, which, if left untreated, lead to severe visual impairment and blindness (Ben-Yosef, 2022). The most common inherited retinal disorders include:

1. Retinitis pigmentosa: Retinitis pigmentosa is a group of eye disorders caused by mutations in more than 45 genes that affect the retina (Hartong et al,. 2006). The disease causes dysfunction of light-receptor cells and degeneration of the retinal pigment epithelium. The first sign of RP is usually loss of night vision, called night blindness, which then gradually causes blind spots in peripheral vision. (Liu et al,. 2022).

2. Leber congenital amaurosis: Leber congenital amaurosis (LCA) is a group of monogenic inherited retinal degenerations that usually present with early onset and severe visual impairment, with a prevalence of 1:40,000. People with this disorder usually have severe visual impairment from infancy. Symptoms include photophobia, nystagmus, hyperopia, and strabismus. (Cideciyan et al,. 2019).

3. Cone-rod dystrophy (CRD): This type of IRD affects both cones and rods. As the cones and rods gradually deteriorate, people with this condition experience vision loss over time. The first symptoms usually occur in childhood and may include blurred vision and photophobia. Other symptoms include blind spots in the center of vision, loss of the ability to see colors, and loss of lateral or peripheral vision (Hamel ,2007).

4. Stargardt disease: Stargardt disease (STGD) is one of the most common macular dystrophies, with a prevalence of approximately 1:10,000. Age of onset is an important marker, meaning that the earlier the onset, the more severe the course of the disease. Onset usually occurs in childhood or early adolescence, around 10–15 years of age. The disease causes damage to the macula and impairs clear, direct vision (Tsang et al,. 2018).

5. Choroidermia: One of these IRDs is choroideremia, which is caused by a mutation in the CHM gene that results in the absence of the Rab protein (REP1), which causes the death of retinal pigment epithelium cells and photoreceptor destruction. Choroideremia is a vision-reducing condition that affects mostly men, and over time, a person develops tunnel vision and loses the ability to see details. (Ong ,2019).

6. Von Hippel-Lindau Syndrome (VHL): The first pieces of the puzzle of VHL syndrome began to be identified in the late 19th century. The disease is named after the German ophthalmologist Eugen von Hippel and the Swedish pathologist Arvid Lindau (Hudler et al,. 2022). VHL is an inherited disease with an incidence of 1 in 36,000 live births and a penetrance of more than 90%. The inheritance pattern of the disease is autosomal dominant (Gläsker et al,. 2018). VHL disease is caused by mutations in the VHL tumor suppressor gene, located on the short arm of chromosome 3 (3p25-6), which encodes a protein (pVHL) that plays a role in cellular oxygen sensing. (The primary function of the pVHL protein is to downregulate VEGF) (Wiley et al,. 2019). The highly vascular nature of VHL-associated neoplasms can be understood in light of recent findings that the *VHL* gene product (pVHL) inhibits the accumulation of hypoxia-inducible mRNAs, such as the mRNA encoding vascular endothelial growth factor (VEGF), under normoxic conditions. This property of pVHL appears to be related to its ability to bind to complexes containing elongin B, elongin C, and colin 2 (Cul2). Elongin C and Cul2, based on their homology to Skp1 and Cdc53, are suspected of targeting specific proteins for covalent modification with ubiquitin and thus for degradation (Maher et al,. 1997).

The *VHL* gene was isolated in 1993 using a positional cloning strategy by a group at the National Cancer Institute led by Marston Linehan, Michael Lerman, and Brett Zbar, in collaboration with Eamonn Maher, then at the University of Birmingham, England. Most people with VHL inherit one mutated copy of the VHL gene from one affected parent and one normal copy of the gene from the other parent. In some cases, the mutation is a de novo germline or somatic cell mutation of the *VHL* gene, and is therefore present in the absence of a family history of the disease (Latif et al,. 1993). People with VHL develop benign and malignant tumors, including retinal and central nervous system hemangioblastomas, renal cell clear cell carcinoma (RCC), pheochromocytoma, pancreatic neuroendocrine tumors, and endolymphatic sac tumors (ELSTs) (Louise et al,. 2022). Retinal hemangioblastomas occur very frequently in VHL patients. Between 49 and 62% of VHL patients develop retinal hemangioblastomas. Hemangioblastomas are highly vascular tumors of the CNS that predominate in the posterior fossa (Chittiboina et al,. 2015). Despite their frequent sporadic appearance, hemangioblastomas can be the first manifestation of VHLD. Early diagnosis of VHLD is crucial for adequate screening and follow-up for other manifestations of the disease and for genetic counseling (Bamps et al,. 2013). Based on their 2017 studies, Varshney and colleagues found that retinal hemangioblastomas, although not the most common tumor in VHL, are often the first manifestation. The median age of onset is 21 years, which is the lowest among other clinical presentations. All patients presenting with a retinal hemangioblastoma are likely to have bilateral lesions by the age of 56 years. Complications of retinal hemangioblastoma include blindness or severe visual impairment in 5 to 8 percent of VHL patients. The disease can occur in the periphery or optic nerve, both of which can cause symptoms due to fluid accumulation under the retina and the formation of hard deposits in the macula (Varshney et al,. 2017).

The first step in improving the path to diagnosis, informed treatment, and improved counseling for affected families is whole exome sequencing (WES). WES refers to the sequencing of all protein-coding genes in the genome, which accounts for about 1 to 2 percent of the entire genome. Since about 85% of molecular mutations causing genetic diseases in humans occur in exome regions, performing this test (WES) is of great help in finding these mutations and the genetic clues of the disease, and is one of the most complete genetic tests used to find the genetic cause and variant of most genetic diseases, including hereditary retinal disorders (Jelin et al,. 2018).

Material and Methods

Sampling and DNA extraction

After receiving the code of ethics from the University of Sistan and Baluchestan, in cooperation with the Al-Zahra Ophthalmology Center in Zahedan, blood was taken from a patient with a retinal disorder, with his consent and cooperation, and transferred to the genetic laboratory for testing. Then, using the necessary materials and a specific kit, the DNA extraction process was performed using the saturated salt method. The patient's DNA must be pure for genetic testing and analysis and must be free of any cracks or breaks. To measure the purity of the extracted DNA, the optical absorption of the DNA molecule can be used using the Nanodrop 2000 device. The DNA molecule has maximum absorption at a wavelength of 260 nm, and on the other hand, the protein molecule has absorption at a wavelength of 280 nm. Therefore, the quality and purity of DNA is measured with a ratio of 260 to 280, so that the result of this fraction should be between 1.8-2.0, and anything lower than that indicates the presence of impurities in the extracted DNA molecule.

Whole Exome sequencing

After quality control, the DNA molecule must be fragmented to create a gene library, which is done by cryo- or enzymatic methods. Adapters are then ligated to both ends of the DNA fragments, followed by targeted enrichment (purification of the adapter-ligated fragments) to capture protein-coding exons. This process can be performed using solution-based or array-based methods, both of which rely on hybridization absorption. In the most commonly used solution-absorption approach, biotinylated probes complementary to the exonic regions of interest are added to the sample. The DNA sequences are denatured by heating, then the double-stranded molecules are reformed by cooling the sample. Non-coding sequences and

some exonic sequences are re-annealed to a complementary sample strand, while other protein-coding DNA fragments are ligated to a probe. Subsequently, streptavidin-coated magnetic beads are added to the sample, to which the probes are attached via a biotin molecule at one end. When the sample is placed on a separator or magnetic stand, the beads carrying the foreign regions of interest are attracted. Most of the unwanted and unauthorized DNA sequences can be removed by removing the supernatant, and after a series of washing steps, only the foreign regions of interest remain. Array-based target enrichment methods work similarly to solution-based approaches. The main difference is that the probes are bound to a microarray rather than being added to the sample. When the sample is denatured and added to the microarray, most of the exomic sequences hybridize to the probes, and unbound fragments can be aspirated and discarded. It should be noted that the target enrichment steps for capturing exons are usually performed using a kit from a specialized manufacturer. An example of a solution-based kit is the Twist Human Core Exome Kit (Twist Bioscience). The protein-coding exon sequences obtained at the end of the target enrichment process must be sequenced to identify mutations. The read sequences are aligned with the reference genome by BWA (Wheeler – Burrows 0.5.9v, Alignment) software and mutations are identified by specific software. The last step is data analysis. Although WES sequences only a part of the entire genome, a large amount of data is generated. Its interpretation is done with computational algorithms and bioinformatics methods, for which SAMtools and Annovar software are used.

Primer Design

Primer sequences are required as oligonucleotide sequences to perform PCR and bastion sequencing. Software such as Primer3Plus and NCBI Primer BLAST can be used to create primer sequences. Then, BLAST software was used to examine the specificity of the primer to ensure that the designed primer did not bind to sites other than the intended site and had high specificity.

Table 1-Primers used to confirm the candidate mutation.

Gene	Primer	Sequence (5' - 3')	Junction temperature (annealing)	Length of amplified strand
VHL	Forward	CAAGCCCAGCCCATTTCAAG	60°	501 bp
	Reverse	TGAAACTAAGGAAGGAACCAGTC		

PCR

The polymerase chain reaction consists of three main stepsThe polymerase chain reaction consists of three main steps, which were performed using a vortex (Thermo Fisher Scientific, MA) and thermocycler (DLAB, Germany). The first step, during which the two DNA strands are unwound from each other, is called primary annealing. The second step has three parts: secondary annealing at the beginning of each cycle, primer binding, and primary amplification by the Taq DNA polymerase enzyme. The third step represents the final amplification of the polymerase. The temperature and time of each step are shown in Table 2.

Table 2- PCR reaction steps.

Number of cycles	Steps		Temperature	Time
1	Stage One	Initial denaturation	95°	5 m
35	Stage Two	Denaturation	94°	30 s
		Annealing	60°	30 s
		Extension	72°	40 s
1	Stage Three	Final Extention	72°	5 m

Electrophoresis

One of the important methods in the process of genetic screening is electrophoresis, which can separate nucleic acid fragments based on their electrical charge, molecular weight, shape, and size by passing through an electric field. Due to their negative charge, nucleic acid fragments move from the cathode to the anode after being placed in an electric field that has two electrodes, positive (cathode) and negative (anode). The smaller the size and lower the molecular weight of the fragments, the faster they can pass through the pores and cracks of the agarose gel and reach the positive electrode. For this purpose, the Gel Doc device (Bio-Rad, CA) was used. Ultimately, the fragments with a lower molecular weight are closer

to the cathode than the others, and the others are placed next to them according to size.

Sanger Sequencing

After the PCR reaction and electrophoresis are completed, for Sanger sequencing, the product must first be washed and cleaned of any buffer, enzyme, nucleotide, etc. contamination. The PCR product was sent to Niagen Noor Company in Tehran along with 10 microliters of Forward 10 micromolar primer, and after two weeks, two files in ABI format containing the chromatogram and a FASTA file containing the read nucleotides were received.

For Sanger sequencing, primer sequence, template DNA, DNA polymerase, dNTPS (containing regular nucleotides) and ddNTPS (containing die-deoxy nucleotides) are required. The steps of the Sanger are as follows: first, the two strands of the template DNA are unwound, then primers are attached to the two DNA strands, and DNA polymerase extends the strands until it randomly inserts a fluorescently labeled dideoxynucleotide, which causes the newly made DNA strand to terminate (complementary). In trench sequencing, chemically modified bases called dideoxynucleotides are introduced along with the regular nucleotides, which results in the DNA being randomly terminated by incorporating a dideoxy base, which results in the production of all possible fragments of the target sequence. The fragments are sorted by their molecular weight, which is done using capillary electrophoresis. Each die-deoxy base is labeled with a fluorescent dye, for example, adenine is labeled in blue, thymine in red, guanine in yellow, and cytosine in green. This allows the last base to be determined by the device and creates a sequential readout of the nucleotides in the template DNA sequence.

Results

Pedigree of the patient

The Figure below (Fig 1) is related to the pedigree of the patient, who is female and suddenly became blind in the right eye at the age of 23. As is clear from the figure below, all family members, including parents, brothers, sisters and children, were healthy and had no family history of this disorder, and only the patient suffered from blindness in the right eye due to a retinal disorder. Given the available evidence, it is highly likely that the mutation is de novo, which may have occurred after the formation of the egg cell.

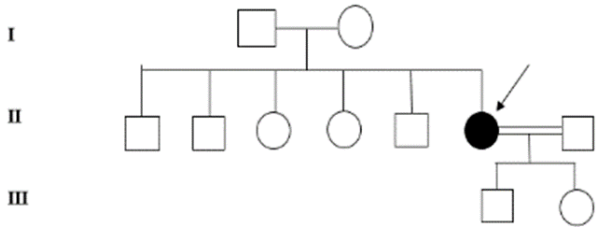


Fig 1-Pedigree of the patient.

Whole exome sequencing

To perform whole exome sequencing (WES), since unfortunately this test is not performed in most genetic centers in our country, the patient's blood was sent to Macrogen, South Korea, for evaluation, and the patient's DNA was analyzed using the WES method. After the sequencing steps, the file was sent to us in BAM format. Then, variants were identified using SAM tools and GATK software, and the results were interpreted as a VCF file by ANNOVAR.

According to the studies performed, the c.464-2A>G variant in the *VHL* gene associated with the patient's phenotype was identified as heterozygous, the details of which can be seen in the table below (Table 3).

Table 3- Results from WES.

Genes	Position/Variant	Zigosity	Inheritance	Disease	Classification ACMG/clinVar
VHL	Chr3-10191469 A G: NM_000551:exon3: c.464-2A>G	Het (Hetrozigosity)	AD (Autosomal dominant)	Von Hippel-lindau syndrome	Pathogenic

After performing WES test and examining the variants found and comparing them, it was finally determined that the variant related to the patient's phenotype was related to chromosome number 3 and occurred at position Chr3-10191469 A G:NM_000551:exon3: c.464-2A>G of the *VHL* gene, in which a nucleotide with a guanine base was replaced by an adenine nucleotide. The inheritance pattern of the *VHL* gene is autosomal dominant, which is expressed as a heterozygous (impure) in the patient's genotype and leads to the disease phenotype.



Fig 2- Details and evaluation of the location of the variant found by the Franklin by genoox database, indicating the pathogenicity of the mutation.

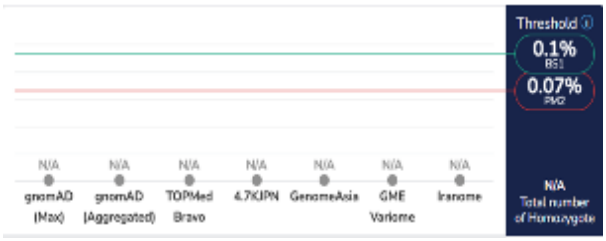


Fig 3- Population Frequencies (PM2 threshold: Allele frequency is extremly rare in control databases compared with disorder prevalence assosciated with gene. Variants above this threshold are possibly pathogenic. BS1 threshold: Allele frequency in control databases is greater than expected for disorder associated with gene. Variant above this threshold are more likely of being benign.)

Review of primer design and Primer BLAST results

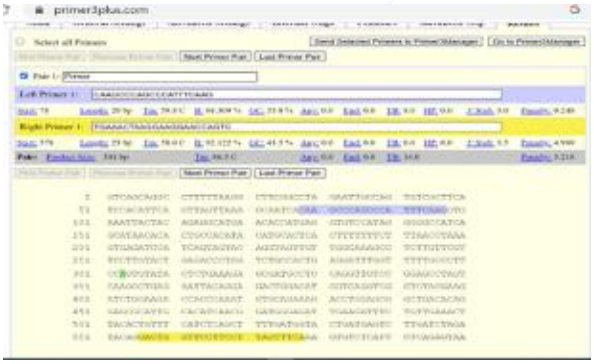


Fig 4- To begin the PCR polymerase chain reaction and Sanger sequencing, it is necessary to design an oligonucleotide sequence called a primer, which is done using Primer3 software.

After primer design, to verify and ensure the specificity and correct binding of the primer to the desired part of the nucleotide sequence, primer blast was performed using the NCBI Primer BLAST software, the results of which can be seen in Fig 5.



Fig 5- Primer Blast result and details.

Analysis of PCR results using gel electrophoresis

After performing the PCR reaction, the resulting products were transferred to an agarose gel for electrophoresis to find the desired fragment with a specific size that was predetermined by the software. Then, with the help of Ladder, the desired fragment, which is 501 bp in this study, was identified (Fig 6). Identification of the desired fragment indicates that the primer was designed correctly and the length of the amplified fragment was equal to the length of the fragment that was determined by the software. Finally, for trench sequencing, the desired fragment was sent to Niagene Company in Tehran along with the primer.

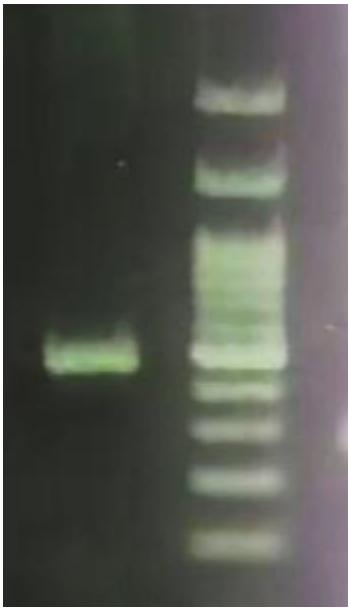


Fig 6- Image of the electrophoresis gel and observation of the 501 bp band.

Results of Sanger sequencing and confirmation of whole-exome sequencing results

After the results of whole-exome sequencing and the mutation on the *VHL* gene are determined, Sanger sequencing of the *VHL* gene is required to ensure the obtained result and confirm it. For trench sequencing and PCR reaction, first the reference sequence of the *VHL* gene was determined by the NCBI scientific database and a pair of primers was designed based on it to amplify this region of the patient's genome. Figure 6 shows the location of the mutation and the primers on the *VHL* gene.

TGTGTATTTTGTAGAGACGGGGTTTCGCCATGTTGCCAGGCTGGTCTCGA
ACTCCTGAGCTCAGGCGATCTACTGACGTTGGCCTCCCAAAGTGTTGGGA
TCACAGGCATGAACCACCATGCCTGGCCAGGGCCTGTTCCTCTTTATGTG
GTCTCTCTAGCAGGGTAGCTCAGGGCTTTCAAAAGTATAAAAGCAGAAGT
CAGCAGGCCTTTTTAAGGCTTCGGCCTAGAATTGCCAGTGTCGCTTCATC
CACATTCAGTTAGTTAAAGCAATCACAAGCCCAGCCCATTTCAAGGTGAA
ATTACTACAGAGGCATGAACACCATGAGGTGTCCATAGGGGGCCATCAGC
ATAACACACTGCCACATACATGCACTCACTTTTTTTCTTTAACCTAAAGT
GAGATCCATCAGTAGTACAGGTAGTTGTTGGCAAAGCCTCTTGTTCGTTC
CTTGTACTGAGACCCTAGTCTGCCACTGAGGATTTGGTTTTTGCCCTTCC
AGTGTATACTCTGAAAGAGCGATGCCTCCAGGTTGTCCGGAGCCTAGTCA
AGCCTGAGAATTACAGGAGACTGGACATCGTCAGGTCGCTCTACGAAGAT
CTGGAAGACCACCCAAATGTGCAGAAAGACCTGGAGCGGCTGACACAGGA
GCGCATTGCACATCAACGGATGGGAGATTGAAGATTTCTGTTGAAACTTA
CACTGTTTCATCTCAGCTTTTGATGGTACTGATGAGTCTTGATCTAGATA
CAGGACTGGTTCCTTCCTTAGTTTCAAAGTGTCTCATTCTCAGAGTAAAA
TAGGCACCATTGCTTAAAAGAAAGTTAACTGACTTCACTAGGCATTGTGA
TGTTTAGGGGCAAACATCACAAAATGTAATTTAATGCCTGCCCATTAGAG
AAGTATTTATCAGGAGAAGGTGGTGGCATTTTTGCTTCCTAGTAAGTCAG
GACAGCTTGTATGTAAGGAGGTTTGTATAAGTAATTCAGTGGGAATTGCA

Fig 7- Reference sequence of the *VHL* gene with the mutation site and designed primers marked in red and yellow, respectively.

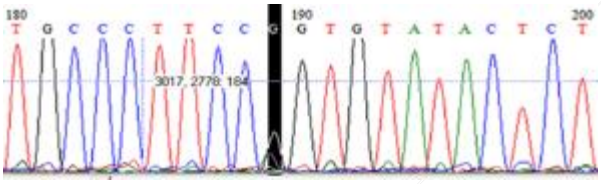


Fig 8- Sequencing analysis by the Sanger method and mutation confirmation.

Based on the results of the Sanger sequencing (Fig 8), the results of the exome sequencing were confirmed and it was determined that a mutation had occurred at the position c.464-2A>G of the *VHL* gene, resulting in a guanine base instead of an adenine base in the *VHL* gene structure, which led to eye disease and retinal disorders in the patient.

Discussion

By performing an exome sequencing test and examining the results, it was determined that the patient had a mutation in chromosome number 3 and at position c.464-2A>G of the VHL gene, and the guanine nucleotide was replaced by an adenine nucleotide. Then, to confirm the candidate mutation and the results of WES, a Sanger sequencing was performed, which confirmed the existence of the above mutation. After analyzing and examining the mutation in the VHL gene, it can be concluded that this mutation is pathogenic and its inheritance is autosomal dominant, and the patient's genotype for this gene is heterozygous, which has a direct relationship with his phenotype and has led to the development of von Hippel-Lindau syndrome and impaired retinal function in his right eye.

Clinical features of von Hippel-Lindau syndrome (VHL) are characterized by hemangioblastomas of the brain, spinal cord, and retina. Renal cysts and clear cell renal cell carcinomas, pheochromocytomas and paragangliomas, pancreatic cysts, neuroendocrine tumors, lymph node tumors, epididymal cystadenomas, and broad ligament tumors are clinical features of von Hippel-Lindau syndrome.

More than 80 forms of syndromic IRDs have been described to date, and more than 200 causative genes have been identified. Given the high degree of phenotypic diversity and phenotypic overlap found in syndromic IRDs, correct diagnosis based on phenotypic features alone is not sufficient, and genetic testing has become the standard for the diagnosis and management of patients with these conditions. Currently, WES is the most popular approach for genetic analysis in patients with monogenic diseases, including IRD.

Based on studies, external beam radiotherapy (EBRT) is a useful option for the treatment of retinal hemangioma secondary to VHL disease that has progressed despite standard therapy. EBRT results in improved visual acuity, reduction of tumor volume, and stabilization of retinal detachment in the majority of patients treated.

Table 4- Evaluation and recommendations after initial diagnosis of von Hippel-Lindau (VHL) syndrome.

Limitations and Challenges

After sampling, due to limitations and infrastructure issues in performing whole exome sequencing within the country, we were forced to send the sample abroad (Macrogene Company, South Korea), which took 4 months and Also, due to the limited budget allocated for

this research, conducting various genetic tests was expensive.

On the other hand, due to lack of access to the patient's parents, we were unable to determine the genotype associated with the VHL gene in the parents.

Ethical consideration

This study was conducted after obtaining full and written consent from the patient, in compliance with all health and ethical standards, and after approval by the Research Ethics Committee of the University of Sistan and Baluchestan with the ID IR.USB.REC.1403.041.

Conflict of interest

The authors declare no conflict of interest.

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