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Sequence analysis of four vitamin D family genes (*VDR, CYP24A1, CYP27B1* and *CYP2R1*) in Vogt-Koyanagi-Harada (VKH) patients: identification of a potentially pathogenic variant in *CYP2R1*

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Abstract

Background: VKH is a rare autoimmune disease. Decreased level of vitamin D has recently been found to be involved in the pathogenesis of
Vogt-Koyanagi-Harada (VKH) disease. This study was designed to screen the vitamin D pathway genes for pathogenic mutations, if any, in VKH patients.

Methods: Genomic DNA was extracted from blood samples collected from patients with VKH disease and healthy controls. Entire coding region, exon-intron junctions of four genes were sequenced in DNA from 39 Saudi VKH patients and 50 ethnically matched healthy individuals. All patients and controls were unrelated.

Results: Vitamin D levels in VKH patients were found either insufficient (21–29 ng/mL) or deficient (<20 ng/mL). Sequencing analysis of the *VDR, CYP24A1, CYP27B1* and *CYP2R1* detected twelve nucleotide changes in these genes in our cohort of 39 patients; 4 of which were non-coding, 6 were synonymous coding and 2 were non-synonymous coding sequence changes. All synonymous coding variants were benign polymorphisms with no apparent clinical significance. A non-synonymous coding sequence variant (c.2 T > C; p.1Met?) found in *VDR* is an initiation coding change and was detected in control individuals as well, while another variant (c.852G > A; p.284 M > I) found in *CYP2R1* is predicted to be disease causing by mutationtaster software. This potentially pathogenic variant was found in 17 out of 39 VKH patients.

Conclusions: Screening of four Vitamin D pathway genes in 39 VKH patients shows that a potentially pathogenic sequence variant in CYP2R1 may cause VKH in a subset of patients. These findings support the previous observation that low vitamin D levels might play a role in VKH pathogenesis and mutations in genes involved in vitamin D anabolism and catabolism might be of importance in
VKH pathobiology.

Keywords: Vogt-Koyanagi-Harada, Vitamin D, Genes, Mutations

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Background

Vogt-Koyanagi-Harada (VKH) syndrome is a rare multisystem autoimmune disease that affects melanin containing tissues, including the eye, inner ear, meninges and skin. The disease is characterized by bilateral uveitis associated with a varying degree of auditory, neurological and cutaneous manifestations [1–3]. VKH affects more frequently people with darker skin pigmentation. Asians, Native Americans, Middle Easterners and Hispanics are most frequently affected [4]. It predominates in patients aged between 20 and 50 years with a female:male ratio of 2:1.

The classic clinical course is characterized by severe bilateral granulomatous panuveitis, hypoacusis and meningitis in addition to cutaneous involvement with poliosis, vitiligo and alopecia. If not treated appropriately it results in severely decreased vision or even leads to blindness [2, 5, 6]. Although the exact cause of VKH disease remains unclear, studies have shown that an autoimmune response directed against melanocytes plays a major role in the onset of this disease [7, 8]. Although a number of HLA and non-HLA genes have been shown to be associated with VKH [9–12] the genetic basis of VKH still remain illusive. Therefore, further studies on the association of autoimmune modulatory genes may yield informative data for the genetic background of VKH disease.

Vitamin D is produced in the skin or obtained from the diet [13]. Its receptor has been found in the immune cells, and some immune cells are able to produce Vitamin D3 [14–17]. The biologically active metabolite of Vitamin D3, $1,25(OH)_2D_3$, has been shown to have immunomodulatory action alongside its role in the bone and calcium metabolism [18, 19]. Vitamin D receptor (VDR) gene negative mice showed a significantly increased susceptibility to several autoimmune diseases, such as autoimmune encephalomyelitis [20, 21], autoimmune uveitis [22] and allergic asthma [23]. Moreover, multiple studies found decreased levels of serum Vitamin D in several human autoimmune diseases, such as multiple sclerosis [24–26], rheumatoid arthritis [27, 28], Behçet's disease [29], Graves disease [30] and systemic lupus erythematosus [31]. It has been reported that decreased $1,25(OH)_2D_3$ level may play a role in the development of VKH disease [32]. Vitamin D deficiency, compromising the immunoregulatory action leading to the autoimmune diseases like VKH could result from either environmental factors or defect in genes concern with Vitamin D metabolism pathway or both. We, therefore, entertained the hypothesis that genetic variation in the Vitamin D genes could be associated with VKH disease.

In Saudi Arabia, VKH is a common cause of uveitis [33] but no study has been conducted to explore the role of vitamin D pathway genes in VKH pathogenesis. In the present study, we screened Vitamin D metabolism pathway genes (*VDR, CYP24A1, CYP27B1 and CYP2R1*) to examine the possible involvement of variation in these genes with VKH disease in Saudi patients. We identified a novel missense variant in *CYP2R1* in VKH patients which might be responsible for low vitamin D level in these patients. Overall, our results showed that a variation/polymorphisms in Vitamin D pathway genes tested here are not responsible for VKH in Saudi population. However, we detected a variant in *CYP2R1* gene that may be pathogenic for VKH disease.

Methods

Subjects

All subjects were recruited from Magrabi Hospital Almadinah Almunawarah. We collected 39 VKH patients and 50 control individuals for this study. All affected and control individuals signed informed written consent prior to start of the study. In case of minor, consent was taken from parents. All patients were examined clinically by a senior ophthalmologist and diagnosed as VKH. Revised diagnostic criteria has been used for VKH diagnosis [3]. Systemic observations for vertigo, poliosis and alopecia, vitiligo, hearing impairment and tinnitus were recorded for all VKH patient. Ethical approval for the study was obtained from the IRB of the Center for Genetics and Inherited Diseases (CGID), Taibah University Almadinah Almunawarah. All experimental procedures were conducted in accordance with the tenets of the Declaration of Helsinki.

All patients were diagnosed using slit lamp biomicroscopy while cornea was found clear. Fundus examination was carried out using indirect ophthalmoscope and a 20× diopter aspheric lens.

Blood collection and gDNA extraction

In this study, we screened 39 VKH patients and 50 controls for 4 vitamin D pathway genes. Peripheral blood samples of 6 ml was collected from each of the patients and the controls in EDTA tubes. Extraction of genomic DNA was performed using Qiagen blood mini kit. DNA was quantified using Maestro spectrophotometer.

Vitamin D measurement

Serum levels of 25-hydroxyvitamin D3 (25OHD3) were measured in all 39 VKH patients and 50 controls by radioimmunoassay using the Wallac 1470 Gamma Counter (Wallac Inc, Gaithersburg, MD, USA). 25OHD3 level of >30 ng/mL was considered normal. Vitamin D deficiency was defined as a serum level of 25OHD of ≤20 ng/mL and insufficiency as a serum level between 21–29 ng/mL.

Table 1 Clinical characteristics and genetic variants identified in VKH patients

Patient ID	Age/Sex	Age at onset	Vitamin D level	Clinical description	Variant identified
VKH1	40–45/M	40–45 (A)	14.4 ng/mL	De-pigmented fundus OU, Keratoconus OU, Diffuse vitiligo, Poor improvement of VA on treatment	CYP24A1 (g. 632 T > G; c.234 T > G) VDR (g.63937 T > C; p.1Met?, g.65058 T > C; c.1056 T > C)
VKH2	16–20/M	16–20 (C)	16 ng/mL	De-pigmented fundus OU, Vertigo, Tinnitus, Improved VA on treatment	VDR (g.63937 T > C; p.1Met?) CYP2R1 (c.852G > A; p.284 M > I)
VKH3	10–15/F	6–10 (A)	23 ng/mL	De-pigmented fundus OU, Band keratopathy, Vertigo, Tinnitus, Maintained VA, Vitiligo, Poliosis	CYP27B1 (g.2989C > T) VDR (g.63937 T > C; p.1Met?, g.64978 G > T; -49 int 9G > T)
VKH4	16–20/M	17 (A)	13 ng/mL	De-pigmented fundus OU, CNVM, Vertigo, Tinnitus, Decrease hearing, Improved VA on treatment	CYP27B1 (g.2989C > T) VDR (g.64978 G > T; -49 int 9G > T)
VKH5	30–35/M	30–35 (A)	17 ng/mL	De-pigmented fundus OU, Improved VA on treatment	CYP24A1 (g.512G > T; c.114G > T, g. 632 T > G; c.234 T > G, g.821C > T)
VKH6	46–50/F	36–40 (A)	14 ng/mL	De-pigmented fundus OU, Peripheral Anterior Synechea, Improved VA on treatment, Vitiligo	CYP2R1 (c.852G > A; p.284 M > I) CYP24A1 (g.821C > T)
VKH7	20–25/F	20–25 (A)	22 ng/mL	De-pigmented fundus OU, Cataract, Improved VA on treatment	VDR (g.63937 T > C; p.1Met?)
VKH8	56–60/F	56–60 (C)	26 ng/mL	De-pigmented fundus OU, Improved VA on treatment	CYP2R1 (c.852G > A; p.284 M > I)
VKH9	30–35/F	30–35 (A)	16 ng/mL	De-pigmented fundus OU, Improved VA on treatment, Alopecia, Vitiligo	CYP24A1 (g.512G > T; c.114G > T)
VKH10	50–55/M	50–55 (A)	11 ng/mL	De-pigmented fundus OU, Cataract, Improved VA on treatment	CYP24A1 (g. 632 T > G; c.234 T > G)
VKH11	16–20/F	16–20 (A)	15 ng/mL	De-pigmented fundus OU, Improved VA on treatment	CYP2R1 (c.852G > A; p.284 M > I) VDR (g.63937 T > C; p.1Met?)
VKH12	36–40/M	36–40 (Acute)	24 ng/mL	De-pigmented fundus OU, Improved VA on treatment	CYP27B1 (g.2989C > T)
VKH13	56–60/F	56–60 (A)	22 ng/mL	De-pigmented fundus OU, Improved VA on treatment	CYP24A1 (g.512G > T; c.114G > T, g.2989C > T)
VKH14	40–45/F	30–35 (A)	25 ng/mL	De-pigmented fundus OU, Improved VA on treatment, Alopecia, Vitiligo	VDR (g.65058 T > C; c.1056 T > C)
VKH15	16–20/F	10–15 (A)	13 ng/mL	De-pigmented fundus OU, Improved VA on treatment, Alopecia, Vitiligo, Poliosis	VDR (g.63937 T > C; p.1Met?)
VKH16	30–35/F	10–15 (A)	12 ng/mL	De-pigmented fundus OU, Improved VA on treatment, Vitiligo	CYP24A1 (g.2989C > T)
VKH17	20–25/M	16–20 (A)	17 ng/mL	De-pigmented fundus OU, Peripheral Anterior Synechea, Improved VA on treatment, Vitiligo	VDR (g.64978 G > T;–49 int 9G > T) CYP24A1 (g.2989C > T)
VKH18	30–35/M	30–35 (A)	25 ng/mL	De-pigmented fundus OU, Keratoconus OU, Diffuse vitiligo, Poor improvement of VA on treatment	CYP2R1 (c.852G > A; p.284 M > I) CYP24A1 (g.2989C > T)
VKH19	40–46/M	36–40 (A)	17 ng/mL	De-pigmented fundus OU, Band keratopathy, Vertigo, Tinnitus, Maintained VA, Vitiligo, Poliosis	CYP2R1 (c.852G > A; p.284 M > I) CYP24A1 (g.512G > T; c.114G > T, g.2989C > T)
VKH20	20–25/F	20–25 (A)	13 ng/mL	De-pigmented fundus OU, Cataract, Improved VA on treatment	VDR (g.64978 G > T;–49 int 9G > T)
VKH21	50–55/M	46–50 (C)	26 ng/mL	De-pigmented fundus OU, Peripheral Anterior Synechea	CYP24A1 (g.2989C > T)
VKH22	30–35/F	30–35 (A)	12 ng/mL	De-pigmented fundus OU, Improved VA on treatment, Alopecia, Vitiligo	CYP2R1 (c.852G > A; p.284 M > I) VDR (g.64978 G > T;–49 int 9G > T)
VKH23	16–20/F	16–20 (A)	17 ng/mL	De-pigmented fundus OU, Cataract, Improved VA on treatment	CYP27B1 (g.2989C > T)
VKH24	30–35/F	30–35 (A)	23 ng/mL	De-pigmented fundus OU, Improved VA on treatment, Alopecia, Vitiligo	CYP2R1 (c.852G > A; p.284 M > I)

Table 1 Clinical characteristics and genetic variants identified in VKH patients *(Continued)*

VKH25	46–50/F	36–40 (A)	27 ng/mL	De-pigmented fundus OU	CYP2R1 (c.852G > A; p.284 M > I)
VKH26	40–45/M	36–40 (C)	12 ng/mL	De-pigmented fundus OU, Peripheral Anterior Synechea, Improved VA on treatment, Vitiligo	VDR (g.63937 T > C; p.1Met?) CYP2R1 (c.852G > A; p.284 M > I)
VKH27	56–60/M	50–55 (C)	16 ng/mL	De-pigmented fundus OU	CYP27B1 (g.2989C > T)
VKH28	26–30/F	26–30 (A)	25 ng/mL	De-pigmented fundus OU, Cataract, Improved VA on treatment	CYP24A1 (g.512G > T; c.114G > T)
VKH29	40–45/F	40–45 (C)	14 ng/mL	De-pigmented fundus OU, Improved VA on treatment, Alopecia, Vitiligo	CYP27B1 (g.2989C > T)
VKH30	50–55/M	26–30 (A)	25 ng/mL	De-pigmented fundus OU, Keratoconus OU, Diffuse vitiligo, Poor improvement of VA on treatment	CYP2R1 (c.852G > A; p.284 M > I) VDR (g.63937 T > C; p.1Met?)
VKH31	26–30/F	20–25 (A)	15 ng/mL	De-pigmented fundus OU, Improved VA on treatment	CYP2R1 (c.852G > A; p.284 M > I) VDR (g.63937 T > C; p.1Met?)
VKH32	40–45/F	16–20 (A)	27 ng/mL	De-pigmented fundus OU	CYP2R1 (c.852G > A; p.284 M > I)
VKH33	40–45/M	36–40 (C)	12 ng/mL	De-pigmented fundus OU, Peripheral Anterior Synechea, Improved VA on treatment, Vitiligo	CYP2R1 (c.852G > A; p.284 M > I) VDR (g.63937 T > C; p.1Met?)
VKH34	50–55/M	50–55 (C)	16 ng/mL	De-pigmented fundus OU	CYP27B1 (g.2989C > T)
VKH35	26–30/F	20–25 (A)	25 ng/mL	De-pigmented fundus OU, Cataract, Improved VA on treatment	CYP24A1 (g.512G > T; c.114G > T)
VKH36	40–45/F	40–45 (C)	14 ng/mL	De-pigmented fundus OU, Improved VA on treatment, Alopecia, Vitiligo	CYP27B1 (g.2989C > T)
VKH37	26–30/F	20–25 (A)	15 ng/mL	De-pigmented fundus OU, Improved VA on treatment	CYP2R1 (c.852G > A; p.284 M > I) VDR (g.63937 T > C; p.1Met?)
VKH38	40–45/F	26–30 (A)	27 ng/mL	De-pigmented fundus OU	CYP2R1 (c.852G > A; p.284 M > I)
VKH39	46–50/F	26–30 (C)	26 ng/mL	De-pigmented fundus OU, Improved VA on treatment	CYP2R1 (c.852G > A; p.284 M > I)

VKH Vogt-Koyanagi-Harada, *M* Male, *F* Female, *A* Acute, *C* Chronic

Sequencing of vitamin D pathway genes

gDNA was diluted to 20 ng/ul concentration and PCR amplification of coding regions of all four genes was performed using primers flanking exons following a protocol used earlier [34]. Primer sequences are available on request. Bidirectional sequencing of all fragments was carried out using BigDye (Applied Biosystems, Foster city, CA) chain termination chemistry. Fragments were then separated on AB 3500 genetic analyzer (Life Technologies). All sequenced fragments were analyzed using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and compared to the reference sequences of corresponding genes from UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway).

In house controls were used and it was ensured that controls are healthy individuals without having any ocular disease(s) or previous ophthalmic surgeries.

Results

Clinical description of subjects

In this study, 39 unrelated VKH patients (Table 1) and 50 unrelated controls were screened for four genes by Sanger sequencing. Of the 39 VKH patients there were 27 females and 12 males with a mean age of 32.58 years. Of the 50 controls there were 29 females and 21 males with a mean age of 34.75.

History of ocular trauma and ocular surgery before the onset of the disease was ruled out in all patients. No sign of optic atrophy was found in all VKH patients. Distribution of eye involvement is bilateral affecting the whole middle layer of the eye (Pan-uveitis OU). All patients had depigmented fundus OU from mild to severe.

Best Characterized Visual Acuity (BCVA) in all VKH patients was observed in the range of 20/20–20/200 (BCVA). Patients with BCVA of 20/200 had severe depigmentation of the retina and significant retinal pigmentation of epithelium (RPE) changes in the macular area. Some patients developed complications including cataract, glaucoma, subretinal neovascular membranes and two patients developed subretinal fibrosis as well. Recurrence was observed in four cases.

All patients and controls were screened for serum vitamin D levels. Most of the patients were found Vitamin D deficient (Table 1). Among 50 controls, 12 were found Vitamin D deficient, 23 were Vitamin D insufficient and 15 showed Vitamin D levels of more than 30 ng/mL.

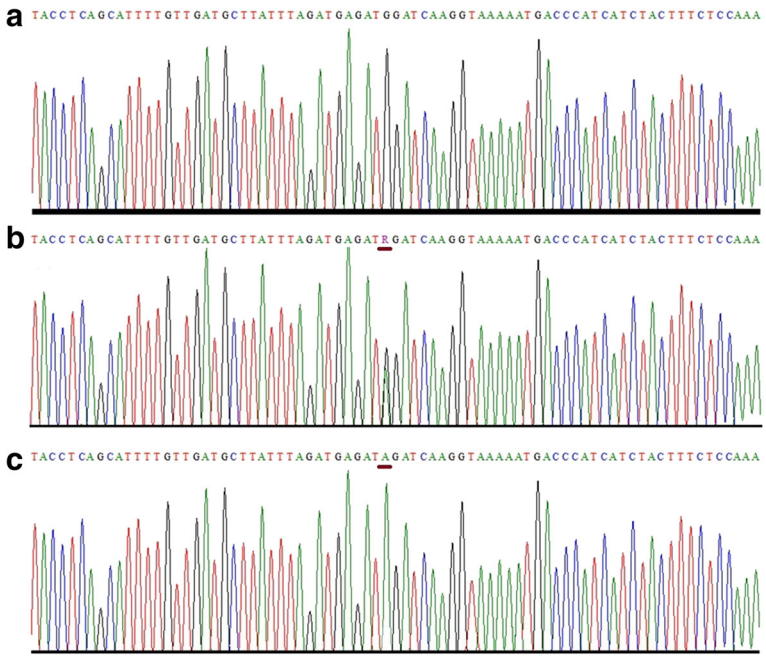

Fig. 1 Sequence analysis of potentially pathogenic variant (c.852G > A) in *CYP2R1* gene. Partial DNA sequence of *CYP2R1* gene from (**a**) control individual, showing wild type sequence (**b**) a heterozygous carrier and (**c**) a homozygous (VKH patients) showing a transition (G > A). Mutated position is underlined

Mutation detection

The full coding region, exon-intron junctions and the 5′ and 3′UTRs of *VDR, CYP24A1, CYP27B1* and *CYP2R1* were sequenced in all 39 patients. Controls were screened only for the variants detected in patients. We detected twelve nucleotide changes in both patients and controls (Table 1). Of all these, 4 were non-coding (g.64978G > T in *VDR*, g.2989C > T in *CYP27B1*, g.821C > T and g.15916 T > C in *CYP24A1*), 6 were synonymous coding (c.1056 T > C; 352I > I in VDR, c.114G > T; p.37P > P, c.234 T > G; p.77G > G, c.469C > A; p. 156R > R, c.552C > T; p. 183A > A, c.1125G > A; p. 374P > P in CYP24A1) and 2 were non-synonymous coding sequence changes (c.2 T > C; p.1Met? in *VDR* and c.852G > A; p.284 M > I in *CYP2R1*). All non-coding and synonymous coding variants were benign polymorphisms with no apparent clinical significance. Non-synonymous coding sequence variant (c.2 T > C; p.1Met?) in *VDR* is an initiation coding change and was found in control individuals as well. Homozygous variant (c.852G > A; p.284 M > I) in *CYP2R1* was identified in 17 out of 29 patients and no control individual was found carrying the same variant. This variant (c.852G > A; p.284 M > I) in *CYP2R1* is predicted to be a disease causing by MutationTaster software (Fig. 1). Multiple sequence alignment shows that the amino acid methionine at position p.284 M is evolutionarily conserved (Fig. 2).

Discussion

It is known that vitamin D plays an important role in melanin production and its deficiency has been shown to be associated with skin depigmentation [35]. In VKH patients, melanocytes tends to disappear from the outer

species	match	gene	aa alignment
Human			284 P Q H F V D A Y L D E M D Q G K N D P S S T F
mutated	all conserved		284 P Q H F V D A Y L D E I D Q G K N D P S S T F
Ptroglodytes	all identical	ENSPTRG00000003388	284 P Q H F V D A Y L D E I D Q G K N D P A S T F
Mmulatta	all identical	ENSMMUG00000000761	284 P Q H F V D A Y F D E I D Q G K N D P S S T F
Fcatus	all identical	ENSFCAG00000011383	277 P Q H F V D A Y L D E I D Q G K N D P S S T F
Mmusculus	all identical	ENSMUSG00000030670	284 P H H F V D A Y L D E I D Q G Q N D P L S T F
Ggallus	all identical	ENSGALG00000006050	296 P R H F I D A Y L D E I D C N K N D P E S T Y
Drerio	all identical	ENSDARG00000056587	288 P Q H Y I D A Y L D E I E Q S T P D K A T S F
Xtropicalis	all identical	ENSXETG00000019664	287 P R H F I D A Y M D E I E R N E A D P D S T Y

Fig. 2 Comparison of partial amino acid sequence of human *CYP2R1* with other primates. The shaded Methionine (M) indicates the conserved residue across different species. Isoleucine (I) indicated by an arrow represent the mutated amino acid observed in the VKH patients in this study

layer of the choroid leading to depigmented Dellen-Fuchs scars. Moreover, inflammation of melanocytes of retinal pigment epithelium cause serous retinal detachment.

Recently polymorphisms in Vitamin D receptor (*VDR*) and 7-dehydrocholesterol reductase (*DHCR7*) genes have been associated with Behçet's disease [36–38].

Moreover, studies examining *VDR* polymorphisms reported significant associations with diabetes, arthritis, autoimmune diseases and hypertension [39–42]. Significant association between polymorphisms in the *VDR* gene with asthma have also been reported in several genetic association studies [43, 44] but has not been consistently replicated [45]. Feng and colleagues showed significant association of autoimmune thyroid diseases (AITD) with *VDR* gene polymorphisms *TaqI* (rs731236) and *BsmI* (rs1544410) [46].

VDR encodes vitamin D receptor which shows high binding affinity for vitamin D3. Vitamin D3 binding activates VDR and ligand-activated VDR performs its function of gene expression by chromatin modification and the transcription regulation. DHCR7 coverts 7-dehydrocholesterol to cholesterol and, thus, reduces the substrate for vitamin D3 synthesis [47]. DHCR7 mutations have been shown to control vitamin D levels in serum [48, 49]. Similarly, low vitamin D serum level has been associated with VKH disease. Both Behçet's disease and VKH manifest intraocular inflammation (Uveitis) which strengthens the hypothesis that variations in vitamin D pathway genes may cause VKH as well. The current study is based on this hypothesis and hence, we screened four vitamin D family genes (*VDR, CYP24A1, CYP27B1 and CYP2R1*) in 39 VKH patients to detect possible mutations underlying VKH in Saudi population. We identified various population polymorphisms in all these genes (Table 1). However, we identified a novel homozygous missense mutation (c.852G > A; p.284 M > I) in *CYP2R1* gene in 17 VKH patients (Fig. 1). This variant in not present in the homozygous state in 50 control individuals. This mutation changes a conserved amino acid methionine to Isoleucine. In silico analysis predicted that this mutation is probably pathogenic. I-Mutant software (used for prediction of protein stability upon single point mutation) predicted the mutant protein as less stable or with decreased stability [50]. Moreover, we used DUET (predicting effects of mutations on protein stability via an integrated computational approach), SDM (predicting effects of mutations on protein stability and malfunction) and mCSM (predicting the effect of mutation in protein using graph based signatures) for prediction of effect of mutation of the protein and found that this mutation is indeed destabilizing [51–53].

Failure to detect pathogenic variants in other vitamin D genes such as *CYP24A1*, *CYP27B1* and *VDR* does not rule out the possibility that other relevant vitamin D gene mutations could cause VKH in Saudi patients. Deep sequencing in a large number of samples would be required to find if any other Vitamin D pathway gene mutations are associated with VKH disease. Also, the non-genetic factors causing Vitamin D deficiency in these patients should be explored.

Conclusions

These findings support the previous observation that low vitamin D levels might play a role in VKH pathogenesis and mutations in genes involved in vitamin D anabolism and catabolism might be of importance in VKH pathobiology. In conclusion, our study for the first time reports a potentially causative role of *CYP2R1* mutation in VKH disease. Studies on larger cohort of patients are needed to confirm this observation.

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Availability of data and materials

DNA samples and Sanger reads are available on request.

Authors' contributions

MAA, MAS, AB, and SA collected samples and carried out clinical evaluation. AMA, MIS, and SB performed DNA extraction, PCR, genes sequencing and data analysis. SB drafted the manuscript. ML performed vitamin D testing and RNA extraction. All authors have seen and agreed to the content of the manuscript.

Competing interests

All authors declare that they have no competing interests.

Consent to publish

All affected and control individuals signed informed written consent for reporting incidental findings, identifying information and a mutation data underlying the disease.

Ethical approval and consent to participate

Ethical approval for the study was obtained from the IRB of the Center for Genetics and Inherited Diseases (CGID), Taibah University Almadinah Almunawarah. All affected and control individuals signed informed consent for genetic analysis prior to start of the study.

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