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CYP26B1 AND ITS IMPLICATIONS IN LYMPHANGIOGENESIS: LITERATURE REVIEW AND STUDY OF RARE VARIANTS IN TWO FAMILIES

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ABSTRACT

CYP26B1 *is a member of the cytochrome P450 family and is responsible for the breakdown of retinoic acid forwhich appropriate levels are important for normal development of the cardiovascular and lymphatic systems. In a cohort of 235 patients with lymphatic malformations, we performed genetic testing for the* CYP26B1 *gene. These probands had previously tested negative for known lymphedema genes. We identified two heterozygous missense CYP26B1 variants in two patients. Our bioinformatic study suggested that alterations caused by these variants have no major effect on the overall stability of* CYP26B1 *protein structure. Balanced levels of retinoic acid maintained by* CYP26B1 *are crucial for the lymphatic system. We identified that* CYP26B1 *could be involved in predisposition for lymphedema. We propose that* CYP26B1 *be further explored as a new candidate gene for genetic testing of lymphedema patients.*

Keywords: *CYP26B1* (Cytochrome P450 Family 26 Subfamily B Member 1), NGS (Next Generation Sequencing), lymphedema, genetic diagnosis, lymphvascular genomics, lymphangiogenesis, retinoic acid

CYP26B1 is a gene that encodes for CYP26B1 (Cytochrome P450 Family 26 Subfamily B Member 1), also known as *P450RAI2.* It is one of three isoforms of *CYP26 (CYP26A1, CYP26B1 and CYP26C1)* identified in humans (1-3). The gene consists of eight exons and covers about 18 kbp. It also includes a 3'UTR of almost 3 kb (4). The *CYP26B1* gene maps to 2p13.2; after transcription and translation, CYP26B1 protein is transported to the

endoplasmic reticulum. CYP26B1 is a monooxygenase responsible for the synthesis of cholesterol, steroids and other lipids. It plays an important role in cell proliferation, differentiation, and apoptosis and in many developmental processes.

The lymphatic vasculature is an essential component of the lymphatic system. It is composed of lymphatic capillaries, pre-collecting lymphatic vessels, and collecting lymphatic vessels (5). Lymphatic vessels drain interstitial fluid, lipids, and other macromolecules and collaborate with the immune system. Fluid collected by the lymphatic system is delivered to the lymph nodes and/or returned to the blood circulatory system (6). Impairment of this balanced homeostasis can cause disorders such as lymphedema and other inflammatory diseases (7).

Lymphedema is a disorder caused by dysfunction of the lymphatic system. It can be caused by impaired function of lymph nodes or malformation of lymphatic vessels (LVs). It leads to a blockage of the flow of lymphatic fluid and fluid build-up in tissues (8). Proper development and function of LVs is therefore important.

Lymphatic capillaries are specialized vessels surrounded by a discontinuous basement membrane and cell junctions that permit entry of fluid and immune cells (9). It is unclear how they are formed. Animal models, such as mice and zebrafish embryos, provided the insights that LVs are formed from lymphatic endothelial cells (LECs), which differentiate at least in large part from the cardinal and embryonic veins (10,11). Lymphatic vessels differ from blood vessels under normal conditions in lacking perivascular cells like pericytes and vascular smooth muscle cells although these features may be acquired in pathologic states. There is nonetheless a clear connection between the development of lymphatic vessels and blood vessels. *CYP26B1* plays a role in both.

Retinoic acid (RA) is an active derivative of vitamin A (retinol, ROH), synthesized by retinaldehyde dehydrogenase (RALDH) (12) and degraded by members of the cytochrome P450 family 26 (CYP26s) *(Fig. 1)* (13,14). Retinol is a crucial regulator of gene expression in biological processes such as cell proliferation and differentiation. Appropriate levels of RA are important for development of the cardiovascular and lymphatic systems and other organs. Retinoic acid levels in the body are regulated by various enzymes, including CYP26B1 (15). CYP26B1 plays a key role in the regulation of retinoid levels in vascularization processes (15).

The importance of RA in lymphatic vascular development was demonstrated by Bowles and colleagues (16), who detected RA in the cardinal vein of a mouse model and showed that lower signaling activity of RA is associated with LEC progenitors (16). This finding suggests that RA levels might need to be low to ensure appropriate development of LECs from their LEC progenitors. Choi et al showed that RA promotes proliferation and migration of LECs and therefore lymphangiogenesis in a mouse model (17). They also demonstrated that 9-cis-RA has positive effects on lymphatic vessel regeneration (17).

Higher levels of RA have been reported in *Cyp26b1*-null mice. Silencing of *Cyp26b1* abolished the machinery necessary for breakdown of RA, leading to constantly elevated levels of RA. The number of lymphatic endothelial progenitor cells subsequently increased in the cardinal vein. Other lymphatic abnormalities were also observed, including hyperplastic blood-filled lymph sacs and hyperplastic dermal lymphatic vessels (16). Other animal models have also been used to study *CYP26B1*. High RA levels in *Xenopus laevis* embryos are associated with excessive lymphangiogenesis, which may lead to obstruction of lymph flow (18).

Human polymorphisms in *CYP26B1* have been associated with different diseases. Fransen et al reported an association between a *CYP26B1* variant and Crohn's disease. This chronic inflammation of the gut has been linked to vitamin A metabolism. The authors showed that probands homozygous for *CYP26B1* variant rs2241057 are at higher risk of developing Crohn's disease because retinoic

TABLE 1
Human Phenotype Associated with CYP26B1

Gene	Function	OMIM disease	Inheritance	Lymphatic phenotype
CYP26B1	Protein coding	614416, Craniosynostosis with radiohumeral fusions and other skeletal and craniofacial anomalies	Autosomal recessive	Dysregulation of RA, high levels of RA, excessive lymphangiogenesis [16,20]

TABLE 2
Primary Amino Acid Sequence for Which Templates were Searched and for Which Models were Built

MLFEGLDLVSALATLAACLVSVTLLLAVSQQLWQLRWAATRDKSCKLPIPKGSMGFPLIGETGH
WLLQGSGFQSSRREKYGNVFKTHLLGRPLIRVTGAENVRKILMGEHHLVSTEWPRSTRMLLGP
NTVSNSIGDIHRNKRKVFSKIFSHEALESYLPKIQLVIQDTLRAWSSHPEAINVYQEAQKLTFRM
AIRVLLGFSIPEEDLGHLFEVYQQFVDNVFSLPVDLPFSGYRRGIQARQILQKGLEKAIREKLQCT
QGKDYLDALDLLIESSKEHGKEMTMQELKDGTLELIFAAYATTASASTSLIMQLLKHPTVLEKL
RDELRAHGILHSGGCPCEGTLRLDTLSGLRYLDCVIKEVMRLFTPISGGYRTVLQTFELDGFQIP
KGWSVMYSIRDTHDTAPVFKDVNVFDPDRFSQARSEDKDGRFHYLPFGGGVRTCLGKHLAKL
FLKVLAVELASTSRFELATRTFPRITLVPVLHPVDGLSVKFFGLDSNQNEILPETEAMLSATV

acid is not effectively catabolized in these individuals. This defect leads to accumulation of RA and promotion of lymphangiogenesis and inflammation. In contrast, the absence of this allele is linked to protection against inflammation (19).

Another *CYP26B1* polymorphism has been implicated in atherosclerosis or chronic inflammatory disease of the blood vessels. Polymorphism rs2241057 was found to affect *CYP26B1*-regulated levels of retinoids and possibly the development of atherosclerosis (20). Since the development of blood and lymphatic vessels is closely linked, it is reasonable to speculate that polymorphisms in CYP26B1 may also affect lymphatic vessel development and function *(Table 1)*.

MATERIALS AND METHODS

Clinical Evaluations

We analyzed samples from 246 Caucasian patients diagnosed with primary lymphedema in hospitals across Italy. The patients were included retrospectively in our study. No consanguinity was reported in the families. Clinical diagnosis of lymphedema was according to generally approved clinical criteria. DNA was extracted from saliva or blood of probands for genetic testing. All data were collected as part of routine diagnosis.

Genetic Analysis

A custom-made oligonucleotide probe library was designed to capture all coding exons and flanking exon/intron boundaries (±15 bp) of 29 genes known to be associated with lymphedema. Our panel included the candidate gene *CYP26B1*. Variants with likely clinical significance identified in DNA of probands were confirmed by bidirectional Sanger sequencing on a CEQ8800 Sequencer (Beckman Coulter).

We searched the international databases dbSNP and Human Gene Mutation Database Professional for all nucleotide changes. *In silico* evaluation of the pathogenicity of changes in the sequence of *CYP26B1* was performed using the Variant Effect Predictor tool and MutationTaster. Minor allele frequencies (MAF) were checked in the Genome Aggregation Database (gnomAD). All variants were evaluated according to American College of Medical Genetics and Genomics guidelines (21). Detailed pre-test genetic counseling was provided to all subjects, who were then invited to sign specific informed consent according to Institutional Review Board guidelines to use their anonymized genetic results for research.

TABLE 3
Clinical Features of Female Probands with *CYP26B1* Variants

Age	Clinical features	Age of onset	Family history of lymphedema	Variant nomenclature
55	lower limb lymphedema, nanism	infancy	NO	NM_001277742.1:c.37G>A p.(Ala13Thr)
55	left lower limb edema	48 years	NO	NM_001277742.1:c.1284G>T p.(Glu428Asp)

TABLE 4
Characterization of the Identified *CYP26B1* Variants

Variants	dbSNP	Varsome	Mutation Taster	Polyphen	Frequency
+NM_001277742.1:c.37G>A NP_001264671.1:p.Ala13Thr	/	Likely Benign	disease causing	Potentially damaging	/
NM_001277742.1:c.1284G>T NP_001264671.1:p.Glu428Asp	rs202200784	Likely Benign	disease causing	benign	0.00003

In Silico Analysis

The primary amino acid sequence of CYP26B1 in FASTA format *(Table 2)* was used to search for template libraries in Swiss model template library (SMTL) version 2019-10-24 and Protein Data Bank (PDB) release 2019-10-18 (22) with BLAST (Basic Local Alignment Search Tool) (23) and HHBlits (24) for evolution-related structures matching the given CYP26B1 sequence. Models based on target-template alignment were built using ProMod3 of the SWISS-MODELING server (25). Coordinates conserved between the target and the template were copied from the template to the model. Insertions and deletions were remodeled using a fragment library. Side chains were then rebuilt. Finally, the geometry of the resulting model was regularized using the CHARMM27 force field (26). If loop modeling with ProMod3 failed, an alternative model was built with PROMOD-II (27). Global and per-residue model quality was assessed using the QMEAN scoring function (28). BioVia Discovery Studio Visualizer ver17.2 (29) was used to visualize the modeled protein, to mutate the targeted amino acids and to analyze molecular level interactions.

RESULTS

Clinical and Genetic Assessment

Of the 246 samples, 235 tested negative for known lymphedema genes. Negative samples underwent a second test for possible new candidate genes, including *CYP26B1*. Two patients with two heterozygous variants in the *CYP26B1* gene were identified. Both cases were sporadic and had no family history of lymphedema. *Table 3* shows the clinical features of these patients.

The first female proband was diagnosed with lymphedema in infancy and showed lymphedema of the. lower limbs and restricted growth. The missense variant NM_001277742.1:c.37G>A was identified. It has no dbSNP ID and allele frequency is unknown. PolyPhen describes this variant as possibly damaging *(Table 4)*.

The second female proband showed left lower limb edema that manifested at age 48 years. The missense CYP26B1 variant was identified as NM_001277742.1:c.1284G>T. It is characterized in dbSNP as rs202200784 and has a total frequency of 0.00003 according to GnomAD. As shown in , two out of three predictors (Mutation Taster and Polyphen) report the variants as potentially damaging. Both

TABLE 5
Top Ten Selected Models for CYP26B1

Template	Seq Identity	Oligo-state	Founby	Method	Resolution	Seq similarity	Coverage	Description
2ve3.1.A	35.33	monomer	BLAST	X-ray	2.10Å	0.38	0.85	PUTATIVE CYTOCHROME-P450 120
6a16.1.A	26.98	monomer	HHblits	X-ray	2.00Å	0.33	0.86	Cytochrome P450 90B1
6a15.1.A	27.46	monomer	HHblits	X-ray	1.79Å	0.34	0.85	Cytochrome P450 90B1
2ve4.1.A	34.17	monomer	HHblits	X-ray	2.40Å	0.37	0.86	PUTATIVE CYTOCHROME-P450 120
6a17.1.A	27.40	monomer	HHblits	X-ray	2.30Å	0.34	0.86	Cytochrome P450 90B1
5eah.1.A	23.04	monomer	HHblits	X-ray	2.54Å	0.31	0.87	Lanosterol 14-alpha demethylase
6a18.1.A	27.40	monomer	HHblits	X-ray	2.48Å	0.34	0.86	Cytochrome P450 90B1
5ese.1.A	23.04	monomer	HHblits	X-ray	2.20Å	0.31	0.87	Lanosterol 14-alpha demethylase
4ze2.1.A	23.04	monomer	HHblits	X-ray	2.30Å	0.31	0.87	Lanosterol 14-alpha demethylase
6mcw.1.A	22.58	monomer	HHblits	X-ray	2.40Å	0.32	0.85	Cytochrome P450 51

variants are reported on Varsome as Likely Benign, with BP1 and BP4 supporting criteria.

In Silico Analysis, Template Selection and Model Building

Template search with BLAST and HH-Blits was performed against the SWISS-MODEL template library (SMTL, last update: 2019-10-24, last included PDB release: 2019-10-18). The target sequence was searched with BLAST against the primary amino acid sequence in SMTL. A total of 767 templates matched the target sequence with varied sequence identity and quality percentages. Details of the top 10 templates are shown in *Table 5*.

The 2ve3.1.A template was selected on the basis of sequence identity and similarity to align and query sequences for model building. The resulting model is shown in *Fig. 1*. The Discovery studio visualizer was used to generate Glu428Asp and Ala13Thr mutated structural versions. Molecular level interaction analysis was conducted between native/mutated residues with residue interactions. Snapshots are shown in *Fig. 2a,b.* Details of the residues involved in interactions, along with the type of bonds they formed and bond lengths in angstrom units, are shown in *Tables 6 and 7*, respectively. While there is no difference on length of the bond with the interacting residue for Ala13 and Thr13 *(Table 6)*, the length of the bond between Lys444 and the variants is greater *(Table 7)*.

DISCUSSION AND CONCLUSION

Lymphedema is a chronic disease involving brawny edema, inflammation, and fibrosis due to accumulation of high protein interstitial fluid (8). The incidence of primary lymphedema is approximately at least 1 in 100,000 individuals (30). Despite recent advances, the genetics of lymphedema is still not fully understood. The discovery of new genes with a role in lymphatic system development and/or associated with lymphedema or its predisposi-

TABLE 6
Details of Molecular Interactions of ALA13 and THR13 of the Modeled CYP26B1 Protein with Adjacent Residues

Mutation	Amino acid	Molecular interactions observed	Bond length in angstroms	Bond type
Ala13Thr	Ala13	ALA17:N - ALA13:O	2.93	H-bond
	Thr13	ALA17:N - THR13:O	2.93	H-bond

TABLE 7
Details of Molecular Interactions of a) GLU428 and b) ASP428 of the Modeled CYP26B1 Protein with Adjacent Residues

Mutation	Amino acid	Molecular interactions observed	Bond length in angstroms	Bond type
Glu428Asp	Glu428	LYS444:N - GLU428:O	5.14	H-bond
		SER422:C - GLU428:O	3.22	H-bond
	Asp 428	LYS444:N - ASP428:O	5.59	H-bond
		SER422:C - ASP428:O	3.22	H-bond

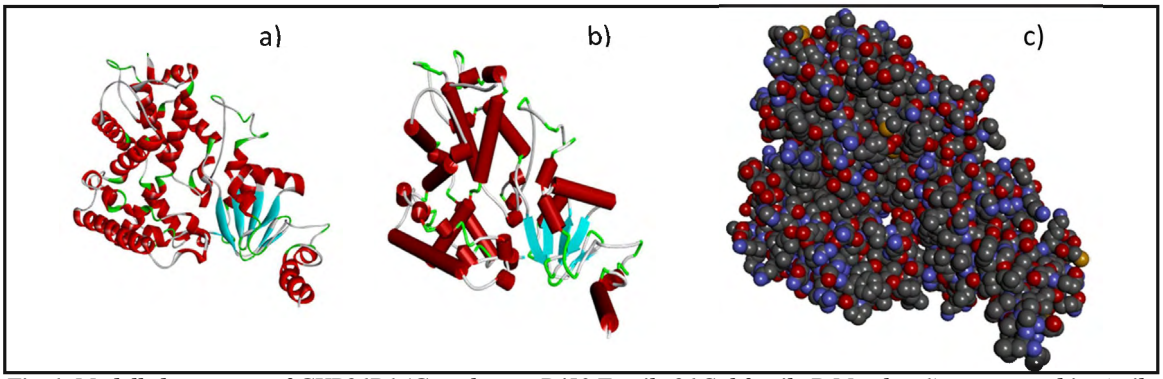

Fig. 1. Modelled structure of CYP26B1 (Cytochrome P450 Family 26 Subfamily B Member 1) represented in a) ribbon b) schematic and c) CPK view. Cyan colored regions represent beta sheets, white regions represent loops and red regions represent alpha helices. Carbon is represented with colored grey, Oxygen is red, Nitrogen is blue, Hydrogen is white, Chlorine is green, Sulfur is yellow, Phosphorus is orange, and Iron is brown.

tion is therefore important.

We tested 246 primary lymphedema patients for known genes associated with the disease (31). We obtained negative results for 235 samples. These were subsequently tested for possible candidate genes, including *CYP26B1*. We found variants in the *CYP26B1* gene in two of the samples. Both variants were heterozygous missense mutations. One of the variants is listed in dbSNP (rs202200784) with a reported frequency of 0.00003. Both female probands were sporadic cases. No other family members were tested.

In silico analysis of the effect of the variants on the overall structure of CYP26B1 protein demonstrated that gene structure coded with Ala13 has no major difference in stability with respect to mutated Thr13 in terms of direct hydrogen bonding with nearby Ala17. Both bonds have the same bond length of 2.93 Angstroms *(Table 6)*. The same was observed for Glu428Asp, where two direct hydrogen bonds with Lys444 and Ser422 were observed. However, Glu428 formed a slightly stronger

hydrogen bond with Lys144 (5.14 Å) than did Asp428 (5.59 Å) *(Table 7)*. These results suggest that the overall conformation of the protein might be somehow altered by these different interactions with nearby residues, leading to functional defects in the protein *(Fig. 3)*.

To our knowledge, no association between *CYP26B1* and lymphedema has ever been reported. The *CYP26B1* gene is mostly studied in the context of bone development and skeletal

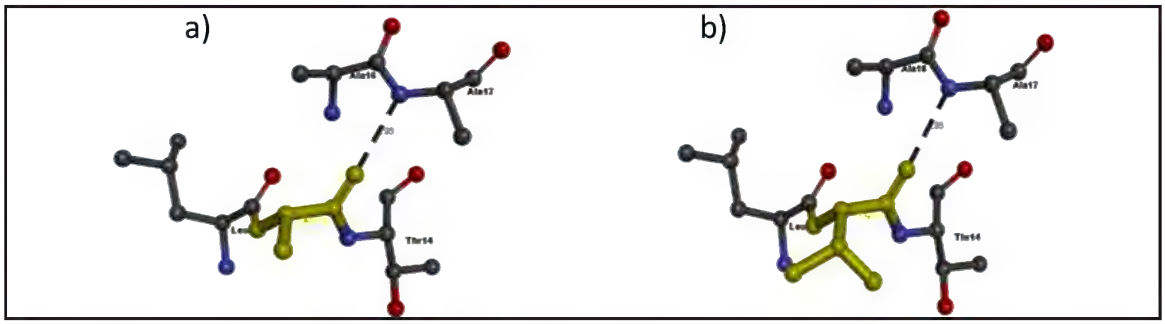

Fig. 2. Molecular interactions of a) ALA13 and b) THR13 (highlighted in yellow) of the modeled CYP26B1 protein with adjacent residues.

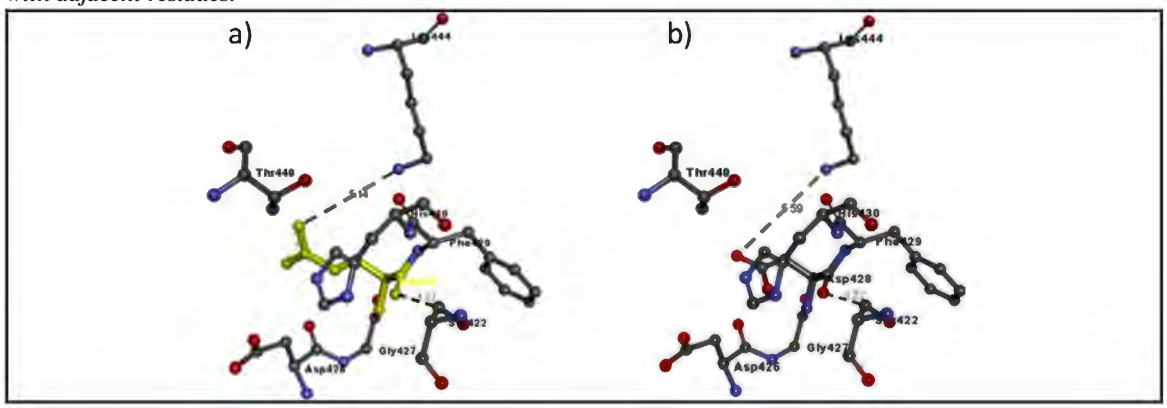

Fig. 3. Molecular interactions of a) GLU428 and b) ASP428 (highlighted in yellow) of the modeled CYP26B1 protein with adjacent residues

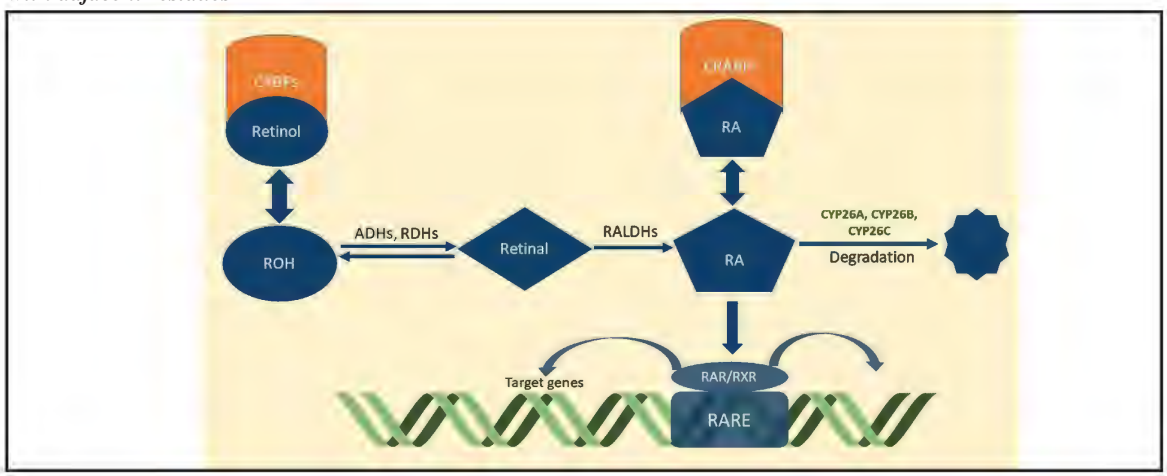

Fig. 4. The metabolism of retinoic acid: Vitamin A (retinol, ROH) is hydrolyzed by alcohol dehydrogenase ADH and retinol dehydrogenase RDH to retinal. Retinal is then oxidized by retinaldehyde dehydrogenase RALDH, creating retinoic acid, the active form of vitamin A. RA is degraded by enzymes of CYP26 family, mainly CYP26B1 (35).

malformations. Laue et al reported a homozygous loss-of-function mutation in *CYP26B1* that causes multiple skeletal defects, long-bone fusions and craniosynostosis in humans (32).

A GWAS led by Kichaev and colleagues (GWAS catalog GCST007841) investigated an association between CYP26B1 variants and body height (33). One of our probands displayed restricted growth, which might be related to the missense variant we identified.

However, the literature also reports a regulatory role of CYP26B1 in the metabolism of retinoic acid *(Fig. 4)*, levels of which are linked directly to lymphangiogenesis (13,15,16). Excessive lymphangiogenesis causes overgrowth of lymphatic vessels, which can lead to lymphedema by impeding the flow of lymph and causing it to accumulate in the tissues (5). CYP26B1 has potential not only as a candidate gene for genetic testing but perhaps also as a therapeutic target. Increasing the degradation of RA in patients with high levels of RA might potentially resolve inordinate lymphangiogenesis and prevent excessive formation of lymphatic vessels, establishing normal lymphatic vessel development.

In conclusion, in this article we review the scientific literature on the relationship between regulation of retinoic acid metabolism by CYP26B1 and development of lymphatic vessels from their LEC progenitors. We suggest that *CYP26B1* might play a significant role in the development of malformations of the lymphatic vessels and in predisposition for lymphedema. Targeted NGS is indeed the best approach to determine genotype-phenotype correlations, segregation and recurrence risk in families, and improvement of diagnostic techniques is needed to offer patients more targeted drugs to treat their lymphatic disorders (34). On the basis of variants described in this paper, we suggest inclusion of *CYP26B1* in the NGS gene panels for testing patients suffering from lymphatic system malformations and lymphedema.

CONFLICT OF INTEREST AND DISCLOSURE

The authors declare no competing financial interests exist.

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