Variant discovery in patients with Mendelian vascular anomalies by next-generation sequencing and their use in patient clinical management

Raul Mattassi, MD,[a] Elena Manara, PhD,[b] Pier Giuseppe Colombo, MD,[c] Sofia Manara, MD,[c] Antonella Porcella, MD,[d] Giulia Bruno, MD,[d] Alice Bruson, PhD,[e] *and* Matteo Bertelli, MD,[b] *Castellanza, Bolzano, Rozzano, and Rovereto, Italy*

ABSTRACT

Objective: An accurate "molecular" diagnosis and classification of similar but distinct diseases is sometime challenging but often crucial for the definition of the appropriate patient medical management and treatment as well as for genetic counseling and risk assessment in families. The advent of next-generation sequencing (NGS), which analysed all known disease-associated genes in parallel in a cost- and time-effective manner, eased this process of disease definition and also for vascular anomalies that are a heterogeneous group of vascular tumors and congenital circulatory malformations and often characterized by overlapping phenotypes.

Methods: We designed a NGS-based screening of the 25 currently most prevalent genes identified in patients with vascular anomalies with Mendelian inheritance and applied this panel to study the DNA of 150 patients affected with vascular anomalies for autosomal recessive and autosomal dominant variants and to analyse the paired blood and DNA from intralesional biopsy specimens in 17 patients for somatic unbalance. Results were confirmed with Sanger sequencing.

Results: We identified 14 pathogenic variants in 13 of 150 patients. Eight variants were previously reported as a disease-causing variant, and six were new. In 55 additional probands we detected 75 variants with unknown significance. Moreover, a previously reported somatic variant was detected in five of 17 available tissue biopsy specimens.

Conclusions: Our results show that many genes can cause a wide variety of syndromic and nonsyndromic disorders, confirming that genetic testing by NGS is the approach of choice to diagnose heritable vascular anomalies, especially, but not only, when an intralesional biopsy specimen is available. The identification of the causative genes and the possibility of tracing somatic variants in tissues provide important information about etiology, patient clinical management, and treatment, and it could highlight otherwise unsuspected clinical situations. (J Vasc Surg 2018;67:922-32.)

Clinical Relevance: The prompt and correct identification of the causative gene variant in those uncertain phenotype or complex cases of patients affected by vascular anomalies is of inestimable value in order to provide the appropriate clinical management, monitoring, and treatment of patients. Genetic testing by next-generation sequencing of blood DNA or tissue DNA could be fundamental in helping clinicians determine the right disease and take the appropriate therapeutic decision. The identification of variants could provide prognostic or therapeutic information, directing a personalized patient care with development of specific small-molecule therapies, with the aim of increasing efficacy of traditional therapeutic methods.

Vascular anomalies with Mendelian inheritance are a heterogeneous group of circulatory alterations, charac terized by morphologic-structural or functional defects, or both, of varied nature, severity, and extent.[1,2] They comprise vascular tumors (hemangiomas) and vascular malformations. Hemangiomas are benign, highly proliferative lesions involving aberrant localized growth of capillary endothelium that grows during the first year of age and then spontaneously regress over time. Most hemangiomas occur sporadically, but some families with autosomal dominant inheritance have been reported.[3]

Vascular malformations are rare and affect ~0.3% of the population.[4] They are subdivided depending on the type(s) of vessel(s) affected.[5] Most are sporadic (ie, without family history), but familial cases exist transmitted as an autosomal recessive or dominant trait. Sporadic forms usually present with a single lesion, whereas more lesions are observed in familial cases. In addition, evidence for a few genes is also accumulating to support a paradominant model of inheritance in which development of lesions depends on the combination of a germline hereditary variants and a somatic second-hit. Somatic variants have been identified in venous, cerebral cavernous, and glomuvenous malformations.[3]

From the Center for Vascular Malformations, "Stefan Belov", Clinical Institute Humanitas "Mater Domini", Castellanza[a]; the MAGI Euregio, Bolzano[b]; the Department of Pathology, Humanitas University, Clinical Institute Humanitas, Rozzano[c]; the Laboratory of Clinical Analysis, Clinical Institute Humanitas "Mater Domini", Castellanza[d]; and the Magi's Lab, Rovereto.[e]

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Correspondence: Elena Manara, PhD, Magi Euregio, via Maso Della Pieve 60/A, Bolzano/Bozen 39100, Italy (e-mail: elena.manara@assomagi.org).

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For some lesions, the clinical features are often characteristic, but in some cases there may be atypical clinical or radiologic findings, and a definitive diagnosis may be difficult, requiring histologic evaluation of tissue samples.[6] Incorrect nomenclature and misdiagnoses are indeed frequently experienced by patients with vascular anomalies because they may present overlapping phenotypes and can occur in association with other symptoms (ie, in syndromes) or with high variability and extension. In addition, despite being present since birth, the anomalies do not immediately become evident; thus, they are often inappropriately evaluated and managed.

During the last 10 years, major advances have been made in identifying the genetic bases of vascular anomalies, shedding light on the fact that variants in different genes could give rise to the same malformation and that variants in the same gene can originate in different phenotype. A more accurate "molecular" diagnosis, being available thanks to the advances obtained in next-generation sequencing (NGS) techniques, has allowed for a better and more rapid understanding of the deregulated downstream pathways that could be targeted for therapy and to defining better recommendations for patient monitoring to early identify risks of health problems.

In addition, the availability of a genetic test can help in the classification of those complex clinical cases or in the evaluation of a presymptomatic phenotype and can give people more information for making decisions about their health and their family's health. Therefore, in this study we aimed to analyze in parallel all of the genes known to be involved in vascular anomalies with Mendelian inheritance in a large cohort of 150 Italian patients affected by vascular malformation or hemangiomas and enrolled in a single hospital. Genomic DNA was analyzed by a NGS using a Illumina (San Diego, Calif) custom-made oligonucleotide probe library to study a panel of 25 genes. In addition, we sequenced 17 intralesional biopsy specimens to test for the presence of somatic variants, because accumulating evidence supports the notion that the analysis of tissue samples may provide prognostic or therapeutic information, directing a personalized patient care with the development of specific small-molecule therapies.[7,8]

ARTICLE HIGHLIGHTS

- **Type of Research:** Single center prospective cohort study
- **Take Home Message:** DNA of 150 patients with vascular anomalies was sequenced by next generation sequencing identifying 89 variants. DNA from biopsy specimens identified the pathogenic variant in five of 17 patients.
- **Recommendation:** Genetic variants can be determined in a number of patients with congenital vascular lesions, and could provide prognostic or therapeutic information, directing a personalized patient care with the aim of increasing efficacy of traditional treatments.

METHODS

Study patients. This study enrolled 150 patients after clinical assessment of congenital vascular malformations or hemangiomas conducted at the Clinical Institute Humanitas, Castellanza (VA), Italy. Enrollment criteria were three of the following: (1) family history with Mendelian inheritance, (2) neonatal or congenital appearance of lesions, (3) presence of vascular anomalies with exclusion of those induced by teratogen of chemical, physical, or biologic origin (ie, those patients presenting vascular anomalies that could be attributed, as assessed during anamnesis, to treatment with a teratogenic drug were excluded from the study), (4) whenever diagnostic criteria for each disease (as defined by Genereview or Orphanet) were met. The study excluded patients affected by lymphedema.

All patients received genetic counselling to explain the risks and benefits of genetic testing, signed a written informed consent, including the authorization to use anonymized genetic results for research and publication, and were asked to provide a blood sample. For 17 patients, one intralesional biopsy specimen was available in addition to their blood DNA for screening.

Sample processing. Genomic DNA from blood samples was extracted following standard procedures using the MagPurix Blood DNA Extraction Kit (Zinexts Life Science, New Taipei City, Taiwan) and was extracted from biopsy samples using the MagPurix Tissue DNA Extraction Kit.

Custom panel design. A custom-made oligonucleotide probe library was designed to capture all coding exons and flanking exon/intron boundaries (±15 bp) of 114 genes known to be associated with a large group of cardiovascular and lymphatic diseases from the literature or databases (Human Gene Mutation Database Professional [Qiagen, Redwood City, Calif], Online Mendelian Inheritance in Man [OMIM; Johns Hopkins University, Baltimore, Md], Orphanet [Paris, France], National Center for Biotechnology Information GeneReviews, National Center for Biotechnology Information PubMed [Bethesda, Md], and specific databases). The 25 genes known to be involved in vascular malformation (Table I) were included in the analyzed panel. The genomic coordinates of the probes are provided in Supplementary Table I (online only). The DNA probe set, complementary to the target regions (GRCh37/hg19), was designed using the specific online tool, Illumina DesignStudio (Nextera Rapid Capture Custom Assay Technology; http://designstudio.illumina.com/Home/SelectAssay/), and was

Table I. List of vascular anomalies associated genes analysed in this study[a]

Gene[b]	RefSeq no.	Gene/locus OMIM no.	Conditions	Mode of inheritance
ABCC6	NM_001171, NM_001079528	603234	Pseudoxanthoma elasticum;	AR
			Pseudoxanthoma elasticum, forme fruste	AD
ACVRL1	NM_000020, NM_001077401	601284	Hereditary hemorrhagic telangiectasia	AD
ANTXR1	NM_018153, NM_032208, NM_053034	606410	Hemangioma of infancy	AD, P
AKT1	NM_005163, NM_001014432, NM_001014431	164730	Proteus syndrome	SM
GNAQ	NM_002072.4	600998	Capillary malformations;	SM
			Sturge-Weber syndrome	SM
CCM2	NM_001167935, NM_031443, NM_001167934, NM_001029835	607929	Cerebral cavernous malformations	AD, P
COL3A1	NM_000090.3	120180	Ehlers-Danlos syndrome	AD
DUSP5	NM_004419	603069	Hemangioma of infancy	SM
ENG	NM_001114753, NM_001278138, NM_000118	131195	Hereditary hemorrhagic telangiectasia	AD
FBN1	NM_000138.4	134797	Marfan syndrome	AD
FLT4	NM_182925, NM_002020	136352	Hemangioma of infancy	SM
GLMN	NM_053274.2	601749	Glomuvenous malformations	AD, P
KDR	NM_002253.2	191306	Hemangioma of infancy	AD, P
KRIT1	NM_194455, NM_194454, NM_004912, NM_194456, NM_001013406	604214	Cerebral cavernous malformations	AD, P
PDCD10	NM_007217, NM_145859, NM_145860	609118	Cerebral cavernous malformations	AD, P
PIK3CA	NM_006218.2	171834	CLOVES, Venous malformations	SM
			Cowden, Cowden-like syndrome	AD
PTEN	NM_000314.5	601728	Proteus-like syndrome	AD, SM
			Cowden syndrome	AD
RASA1	NM_002890.2	139150	Capillary malformations;	AD, P
			Capillary-arteriovenous malformation	AD, P
			Parkes Weber syndrome	AD
SLC2A10	NM_030777.3	606145	Arterial tortuosity syndrome	AR
SMAD3	NM_005902, NM_001145104, NM_001145103, NM_001145102	603109	Loeys-Dietz syndrome	AD
SMAD4	NM_005359.5	600993	Hereditary hemorrhagic telangiectasia/ juvenile polyposis	AD
TEK	NM_000459, NM_001290078, NM_001290077	600221	Multiple cutaneous and mucosal venous malformations	AD, P
TGFB2	NM_003238, NM_001135599	190220	Loeys-Dietz syndrome	AD
TGFBR1	NM_004612, NM_001130916	190181	Loeys-Dietz syndrome	AD
TGFBR2	NM_003242, NM_001024847	190182	Loeys-Dietz syndrome	AD

AD, Germline autosomic dominant; *AR*, germline autosomic recessive; *CLOVES*, Congenital lipomatous overgrowth, vascular malformations, and epidermal nevi; *P*, paradominant inheritance (ie, germinal and somatic second hit); *SM*, sporadic somatic mosaic.
[a]For each gene the relative RefSeq and Online Mendelian Inheritance in Man (OMIM) accession number, associated condition and type of mutation was reported.
[b]Expansions for the genes listed are available at U.S. National Library of Medicine, Genetics Home Reference (https://ghr.nlm.nih.gov/gene).

optimized to improve the coverage of low-performance target regions. The panel was generated with 5106 capture probes over 2010 targets, 586 bp in size.

Library preparation, targeted capture, and sequencing. In-solution target enrichment was performed according to the manufacturer's protocol using the Nextera Rapid Capture Enrichment kit (Illumina). Briefly, 5 ng of genomic DNA was simultaneously fragmented and tagged by Nextera transposon-based shearing technology. Limited-cycle polymerase chain reaction (PCR) was performed to incorporate specific index adaptors to each sample library. A total of 500 ng of each indexed DNA library was combined with the 12-plex library pool and then hybridized with target-specific biotinylated probes. The libraries were subsequently captured using streptavidin magnetic beads and underwent a second round of hybridization, capture,

PCR amplification, and PCR clean-up. The final enriched pooled libraries, with sizes mainly distributed between 500 and 600 bp, were quantified using the Qubit method (Invitrogen, Carlsbad, Calif), and sample quality was verified using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, Calif). A MiSeq personal sequencer (Illumina) was used to perform 150 bp paired-end reads sequencing according to the manufacturer's instructions.

Data analysis. The raw read data in fastq format, generated by the Ilumina MiSeq 2.5 reporter software, was analyzed to generate the final set of sequence variants using an in-house pipeline that includes the following modules: mapping, duplicate read removal, indel realignment, quality calibration, coverage analysis, variant calling, and annotation. A full description of the pipeline is provided in the Supplementary Methods (online only). To verify the presence of somatic variants in the vascular anomalies biopsy specimens, we developed a pipeline to detect somatic single nucleotide variants using a subtractive correction method described previously.[9] For each position, the base read frequency of the constitutive tissue was subtracted from the base read frequency for the affected tissue. Variants were selected for further analysis using the bioinformatics software mentioned above only if the variant read frequency percentage was at least 5% higher or lower in the biopsy vs blood in order to isolate only those variants in unbalance between the germ line and somatic variation.[10] To identify somatic variant with high confidence, we imposed at least a ≥100-times coverage and an allele frequency ≥0.05.

Variant filtering and prioritization. Variants were selected for Sanger validation on the basis of the following criteria: (1) previously reported in Human Gene Mutation Database and HumsVar database; (2) present in Single Nucleotide Polymorphism database, Exome Variant Server, and 1000 Genome Project with allelic frequency ≤0.03. A variant was denoted as pathogenic and most likely to be disease causing if the following criteria were met: (1) the sequence change (or the amino acidic residue involved) has previously been documented to be pathogenic, (2) it results in a shift of the open reading frame of the transcript, (3) it introduces a premature stop codon, (4) it changes the canonical splice-site sequence, and (5) the variation induces a start- or stop-loss.

Moreover new missense variants having an allelic frequency of <0.01 in db single nucleotide polymorphism and with deleterious effects predicted by at least two of three in silico pathogenicity prediction tools (SIFT [Sorting Intolerant From Tolerant; http://sift.jcvi.org/www/SIFT_enst_submit.html], PolyPhen-2 [Polymorphism Phenotyping version 2; http://genetics.bwh.harvard.edu/pph2/index.shtml], and Mutation Taster [http://www.mutationtaster.org/]) were considered potentially pathogenic variants.

Sanger validation and sequencing of poorly covered target regions. Target region coverage of <10 reads was further analyzed by bidirectional Sanger sequencing (CEQ8800 Sequencer; Beckman Coulter, Fullerton, Calif) according to the manufacturer's protocols. Sanger sequencing was performed to confirm each predicted pathogenic variants using genomic DNA from different aliquots of blood samples and to perform family cosegregation analyses in available family member.

To validate the presence of variants in somatic tissue we performed Sanger sequencing directly when the variant was present in at least 15% of reads, and when lower we performed a TOPO TA cloning (Thermo Fisher Scientific, Waltham, Mass) of the PCR amplicon, followed by sequencing of the plasmid according to the manufacturer's instruction.

RESULTS

NGS analysis of blood DNA. Molecular genetic analysis was performed on a large cohort of 150 unrelated Italian patients with clinical findings of vascular anomalies. In this study, we performed NGS analysis in a validated panel of 114 genes encompassing a large group of cardiovascular and lymphatic diseases, including 25 known vascular anomalies-related genes (Table I). An average of 1.3 ± 0.2 M mappable reads per sample were obtained, resulting in mean coverage of targeted bases of 236 ± 22× per sample. A 10- to 25-fold average was achieved for 99% ± 0.0% and 98.4% ± 0.0% of the targeted region, respectively.

The analysis of the entire cohort led to the identification in 68 patients of 89 variants, among those, missense variants were more frequent (68 of 89), followed by 6 nonsense, 5 affecting the vicinity of splicing sites, 4 variants in introns, 4 deletions, and 2 duplications. The analysis of the entire cohort led to the definition of the candidate variant that could explain the observed phenotype in 13 of 150 individuals (9%). For 82 patients (55.5%) we could not rule out any causative variant in the genes analyzed in the panel, whereas for 55 probands we found 75 variants of uncertain significance (Supplementary Table II, online only). Among these variants, 21 have supporting evidence of pathogenicity and could be described as potentially pathogenic by at least two prediction tools, although further analysis including cosegregation, copy number variation and functional studies are needed to determine the actual pathogenicity of these variants (highlighted in gray in Supplementary Table I, online only).

Patients who were considered resolved are included in Table II. The variations identified were new in five patients and known from the literature in eight patients. Regarding the known variants, just in four of eight patients we found correspondence between the clinical suspect and the variants identified, highlighting the importance of using an NGS approach to analyze those

Table II. Germ line variants in disease genes with an established association with vascular anomalies[a]

	Patient ID	Gene[b]	Protein substitution	Nucleotide substitution	Exon/intron	RefSeq	Heredity	Type of mutation
1	R217 (F)	GLMN	p.(Glu347*)	c.1039G>T	Exon 11	NM_053274	AD	N
2	R845 (M)[c,d]	PTEN	p.(Arg130*)	c.388C>T	Exon 5	NM_000314	AD	N
3	R846 (M)	TGFBR1	p.(Arg294Ilefs*38)	c.880_881del	Exon 5	NM_004612	AD	F
4	R847 (F)[d]	GLMN	p.(Lys205*)	c.613A>T	Exon 6	NM_053274	AD	Ne
5	R848 (F)	ABCC6	p.(Arg391Gly)	c.1171A>G	Exon 9	NM_001171	AR	M
		ABCC6	p.(Glu125Lys)	c.373G>A	Exon 4	NM_001171	AR	M
6	R849 (F)	GLMN	p.(Cys36*)	c.108C>A	Exon 3	NM_053274	AD	N
7	R850 (M)	RASA1	p.(Tyr528Cys)	c.1583A>G	Exon 11	NM_002890	AD	M
8	R158 (M)	ACVRL1	p.(Met438Lys)	c.1313T>A	Exon 9	NM_000020	AD	M
9	R473 (M)	RASA1	p.(Asp667Argfs*3)	c.1998dup	Exon 19	NM_002890	AD	F
10	R562 (M)	GLMN	p.(Cys36*)	c.108C>A	Exon 3	NM_053274	AD	N
11	R851 (M)[d]	GLMN	p.(Cys36*)	c.108C>A	Exon 3	NM_053274	AD	N
12	R852 (M)	FBN1	p.(Gly301Val)	c.902G>T	Exon 9	NM_000138	AD	M
13	R126 (F)[d]	GLMN	p.(Thr442Tyrfs*10)	c.1323dup	Exon 15	NM_053274	AD	F

AD, Germline autosomic dominant; *AR,* germline autosomic recessive; *dbSNP,* single Nucleotide Polymorphism database; *F,* female (Patient ID); *M,* male (Patient ID); *NA,* not available.
[a]For each mutation there is a description of the nucleotide and amino acid substitution, of the type of mutation (Nonsense [*N*]); Missense (*M*); frameshift (*F*), if it has been previously reported or not (new or known), and an evaluation of pathogenicity by Mutation Taster, Sorting Intolerant From Tolerant (SIFT) and PolyPhen-2 (Polymorphism Phenotyping version 2). Mutation Taster score: polymorphism (*P*), disease-causing (*DC*); SIFT score: tolerated (*T*), deleterious (*De*), damaging (*D*); PolyPhen-2 score: benign (*B*), possibly damaging (*PoD*, less confident prediction), probably damaging (*PrD*, more confident prediction). MAF%, minor allele frequency in percent in European American population from Exome Variant Server.
[b]Expansions for the genes listed are available at U.S. National Library of Medicine, Genetics Home Reference (https://ghr.nlm.nih.gov/gene).
[c]Patient whose pedigree is available.
[d]Patients with additional variation (not reported).

patients affected with vascular anomalies. The newly identified variant introduced an early stop codon in the sequence in all patients except one. These last variants occurred in conserved amino acids that have been previously linked to vascular malformation but with a different amino acid change. These new variants are expected to be pathogenic also in our patients. The variant p.(Met438Lys) modifies a methionine residue of activin A receptor like type 1 (ACVRL1) that, when altered (p.[Met438Arg][11] and p. [Met438Thr]),[12] led to the development of hemorrhagic telangiectasia.

Specifically, the gene that was found majorly mutated in our cohort was the glomulin (*GLMN*) gene (6 of 13 patients). Three patients presented the same sequence alteration (p.[Cys36*]) that has already been described by different groups.[13-15] The other variants in *GLMN* (p.[Glu347*] and p.[Lys205*]) have not been previously described; however, they are supposed to be pathogenic because glomuvenous malformations are caused by a variation that leads to a loss of function of GLMN, and in our patients, the sequence change introduced an early stop codon that led to the loss of almost half and more than half of the protein, respectively.

Variation in the Ras p21 protein activator 1 (*RASA1*) gene are associated to capillary malformation-arteriovenous malformations with autosomic dominant heredity. This gene was found mutated in two of 13 patients. The variant p.(Tyr528Cys) was reported by Revencu et al,[16] but the variant p.(Asp667Argfs*3) is described for the first time here. The pathogenicity of this variant resides in the fact that it led to the formation of a truncated isoform that loses 40% of the wild-type protein.

We also found a patient harboring a variant (p.[Arg294Ilefs*38]) in the gene encoding a serine/threonine kinase receptor for transforming growth factor-β (*TGFBR1*), this variant has not been described previously and presents evidence of pathogenicity because it leads to the formation of an early truncated protein. In addition, we found a patient harboring a variation in the large fibrillin-1 (*FBN1*) gene (p.[Gly301Val]), previously identified as pathogenic by Franken et al.[17] This gene encode for fibrillin, a protein

Table II. Continued.

New/known	Mutation Taster	SIFT	PolyPhen-2	Reference	dbSNP accession number	MAF%	Phenotype correspondence
New	…	…	…	…	…	…	
Known	…	…	…	Cowden disease[1]	rs121909224	NA	No
New	…	…	…	…	…	…	
New	…	…	…	…	…	…	
Known	DC	D	PrD	Pseudoxantoma elastico[2,3]	rs72653762	0.006695	No
Known	DC	D	PrD	Pseudoxantoma elastico[4]	rs28492767	NA	No
Known	-	-	-	Glomuvenous malformations[5]	-	-	Yes
Known	DC	D	PrD	Capillary malformation-arteriovenous malformation[6]	rs145752649	0.000308	Yes
New	DC	D	PrD	…	…	…	
New	…	…	…	…	…	…	
Known	…	…	…	Glomuvenous malformations[5]			Yes
Known	…	…	…	Glomuvenous malformations[5]			Yes
Known	DC	T	benign	Marfan syndrome[7]	rs142888621	0.02	No
New	…	…	…	…	…	…	

of the connective tissue, frequently found mutated in Marfan syndrome.

Only one patient in our cohort was characterized by harboring two missense variants in the recessive gene adenosine triphosphate binding cassette subfamily C member 6 (*ABCC6*). Those variants have been already reported in patients affected by pseudoxanthoma elasticum.[18]

The phosphatase and tensin homolog gene (*PTEN*) was found harboring the variant p.(Arg130*) in one patient. This variant has been described to occur in Cowden disease (OMIM 158350),[19] an autosomal dominant cancer syndrome caused by the very deleterious effect on the tumor suppressor protein PTEN that increases the predisposition to develop benign and malignant neoplasms. This patient harbored also another variant in Krev interaction trapped protein 1 (*KRIT1*) with unknown predicted value. The segregation analysis on the proband's relatives (Fig 1) helped in the definition of the disease-causative variants and to the exclusion of the substitution in *KRIT1* p.(Arg26Gln) as pathogenic.

Among all the described patients, four individuals, whose pedigrees were available (Table II), presented an

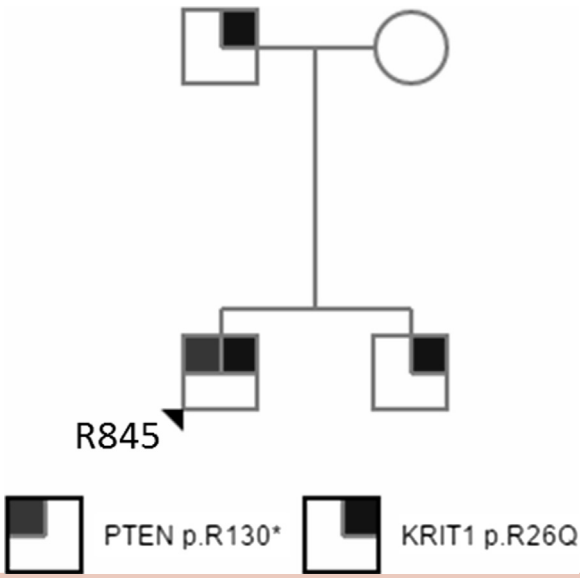

Fig 1. Pedigree and genotype of an Italian family showing the segregation of the known p.(R130*) phosphatase and tensin homolog (*PTEN*) variant with the affected phenotype, and lack of segregation of a new p.(R26Q) Krev interaction trapped 1 (*KRIT1*) variant.

Table III. Variants in disease genes with an established association with the reported phenotype identified in biopsy of patients with vascular anomalies[a]

Patient ID	Clinical suspect	Gene[b]	RefSeq	Nucleotide variant	Protein variant	New/known	Reference
R548	Venous	TEK	NM_000459	c.3319_3320del	p.(Y1108*)	Known	8
R853	Capillary	GNAQ	NM_002072	c.548G>A	p.(Arg183Gln)	Known	9
R851	Glomuvenous	GLMN	NM_053274	c.108C>A	p.(Cys36*)	Known	
R854	Cavernous	TEK	NM_000459	c.2740C>T	p.(Leu914Phe)	Known	10
R221	NA	PIK3CA	NM_006218	c.1633G>A	p.(Glu545Lys)	Known	11

NA, Not available.
[a]For each variant it is detailed the gene involved and the RefSeq, the nucleotide and amino acid substitution, if it has been previously reported or not (new or known), the genomic position, the coverage both for the germline and somatic analysis, the germline and somatic unbalance and the difference.
[b]Expansions for the genes listed are available at U.S. National Library of Medicine, Genetics Home Reference (https://ghr.nlm.nih.gov/gene).

additional sequence variation with uncertain predictive value and that could not recapitulate the disease phenotype. Those variations were affecting genes such as *ABCC6*, TEK receptor tyrosine kinase (*TEK*), and AKT serine/threonine kinase 1 (*AKT1;* data not shown).

NGS analysis of biopsy specimen. A biopsy specimen of the malformation was available in 17 patients and was used for detection of a possible local hit compounding the effects of inherited pathogenic alleles. The average NGS coverage for samples analyzed for somatic hits was 478.3 ± 45 reads. A 10- to 25-fold average was achieved for 99.7% ± 0.0% and 99.7% ± 0.0% of the targeted region, respectively. Moreover 96.8% ± 0.7% of the target had a coverage of 100 times. In five patients, the results obtained by the analysis of the biopsy were interesting (Table III and Fig 2). Patient R851 presented in the germinal DNA a pathogenic variant in GLMN. We analyzed the biopsy specimen because it is described that this gene could have a somatic second hit.[20,21] In this individual, we identified an increase in the unbalance between the germline and the specimen DNA, suggesting the occurrence of a second hit, altering the second intact allele locally.

Patients R548 and R854 presented a somatic variation in the *TEK* gene. In the first patient, the variant p.(Tyr1108*)[22] led to an early termination of the protein and deleted an important phosphorylation site involved in downstream signaling and interaction with docking protein 2 (DOK-R). In the second individual, the variant p.(Leu914Phe) is described as one of the somatic variants more commonly found in vascular malformation.[23,24]

In patient R221 we highlighted the presence of a sequence alteration in phosphatidylinositol-4,5-bispho sphate 3-kinase catalytic subunit alpha (*PIK3CA*) The variant identified in our cohort is within the highly conserved helical domain of phosphatidylinositide 3-kinase-α (PI3K) and has been associated to an increased catalytic activity resulting in enhanced downstream signaling and oncogenic transformation in vitro.[25] Another patient (R853) presented a missense variant p.(Arg183Leu) in guanine nucleotide-binding protein G(q) already described to occur somatically in capillary malformations.[26]

In conclusion, we detected a somatic variant in five of 17 patients, and in four patients the identified variant seemed sufficient to explain lesion formation, whereas it represented a second hit in the last patient. Therefore, with both the blood and the biopsy sequencing used in our approach, we identified in 17 of 150 patients (11%) the variants that most likely are associated with the disease (Tables II and III).

NGS genetic test and clinical management. We identified in two patients an otherwise unsuspected clinical situation that led to a personalized clinical management of the patients and to a more accurate planning of follow-up (Table IV).

Patient R846 presented a truncating variation in *TGFBR1* that led to the loss of more than one-third of the sequence. This variant suggested the presence of a Loeys-Dietz syndrome type I.[27] The initial clinical suspect for this patient was a congenital venous malformation of the lower limbs and pelvic floor. The definition of a pathogenic variant in *TGFBR1* led the clinician to closely monitor the patient by a constant echocardiographic examination because the patient's life was at risk for aortic dissection, which may be fatal.

Patient R845 came into the clinic with arteriovenous malformations since the age of 8 that affected the right thigh, the gluteus, and the back. Genetic testing revealed two variations, both potentially pathogenic: the *KRIT1* gene, which is characteristic of cerebral cavernous malformation, and the *PTEN* gene, which is mainly found mutated in Proteus, Proteus-like syndrome, or Cowden syndrome.[28] Analysis of segregation in the family confirmed that the pathogenic variant was the one affecting PTEN (Fig 1), therefore inferring that the patient was affected by Cowden syndrome. Clinical management, in this specific case, envisages prevention through periodic monitoring for early identification of tumors.

Table III. Continued.

Germline coverage	Germline Phred score	Somatic coverage	Somatic Phred score	Genomic position	Germinal unbalance	Somatic unbalance	Difference
291	999	356	999	chr9:27229176-27229177	CT: 100%	CT: 77%; del: 23%	23%
253	999	353	30	chr9:77797577-77797577	G: 100%	G: 93%; A: 7%	7%
38	222	44	222	Chr1:92297461-92297461	G: 47.4%; T: 52.6%	G: 31.8%; T: 68.2%	15.6%
206	30	246	6	chr9:27212760-27212760	C: 100%	C: 84%; T:16%	17%
206	999	245	30	chr3:179218303-179218303	G: 100%	G: 94%; A: 6%	6%

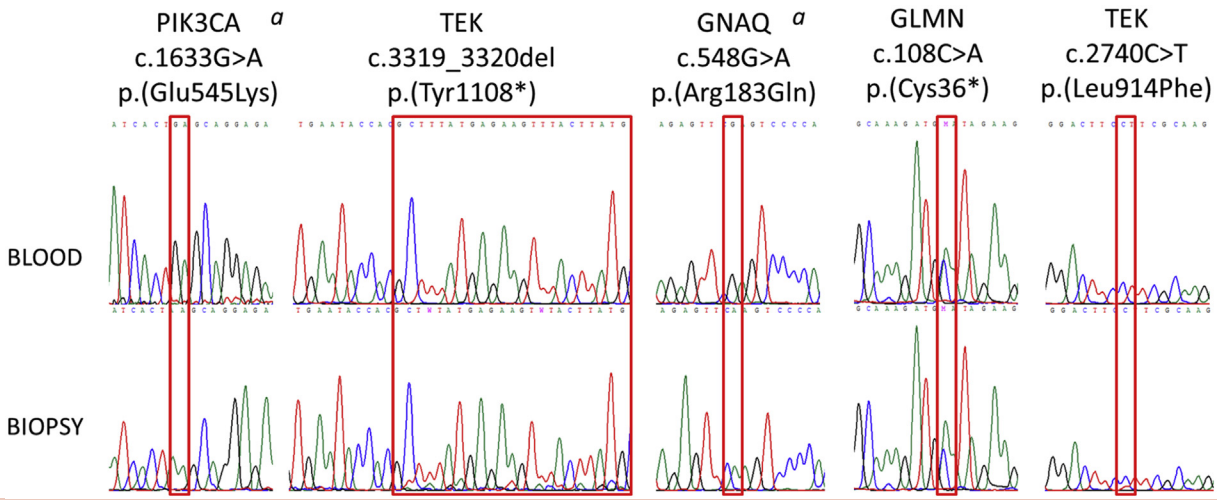

Fig 2. Sanger validation of variants identified in the biopsy sample. For each reported variant, the sequence has been performed for the DNA extracted from the blood and from the biopsy sample. [a]Sequencing after cloning. *GLMN,* Glomulin; *GNAQ,* G protein subunit alpha Q; *PIK3CA,* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *TEK,* TEK receptor tyrosine kinase.

Table IV. Patients whose genetic diagnosis was beneficial for their therapeutic intervention

Patient	Clinical suspect	Age of onset	Anatomical district	Variation (RefSeq; nucleotide variant; protein variant)	Gene[a]	Genetic finding	Genetic final diagnosis
R846 (M)	Venous malformations	Congenital	Lower limbs, pelvic floor	NM_004612.2: c.880_881del: p.(Arg294Ilefs*38)	TGFBR1	Loeys-Dietz syndrome type 1	x
R845 (M)	Arteriovenous malformations	8 years	Right thigh, gluteus, back	NM_000314: c.388C>T: p.(Arg130*) NM_194456: c.77G>A: p.(Arg26Gln)	PTEN KRIT1	Cowden syndrome Cerebral cavernous malformations	x

M, Male.
[a]Expansions for the genes listed are available at U.S. National Library of Medicine, Genetics Home Reference (https://ghr.nlm.nih.gov/gene).

DISCUSSION

For diseases such as heritable vascular anomalies in which the symptoms are extremely variable and with various extension, overlapping phenotypes are recurrent and could mislead to an incorrect management of patients, the frequency of sporadic cases is elevated, and a plethora of different genes, when mutated, are responsible of their onset, there is a great need to opt for NGS genetic testing over classical sequencing to analyze all known vascular malformation-associated genes in parallel, increasing substantially the detection rate with the most time and cost effective choice. NGS genetic testing

should be the method of choice, especially when early clinical symptoms can be misleading or when the prompt identification of the altered pathway is auspicated to begin the right clinical management.

In this study, we screened a large cohort of 150 Italian patients affected by vascular anomalies with Mendelian inheritance enrolled in a single hospital, analyzing 25 vascular malformation and vascular tumor-associated genes. To our knowledge we are the first in Italy to apply the NGS technology to a large cohort of patients affected by vascular malformation and hemangiomas, excluding lymphedema. The overall detection rate found in our samples is 9% if considering just the analysis of the germline variants, but the percentage reaches 11% if analysing also, when available, an intralesional biopsy specimen. In our cohort we identified variations prevalently in the *GLMN* gene in 4% (6 of 150). This percentage could seem low, but in the literature all together, 381 index cases affected by glomuvenous malformation are reported.[29] In our cohort, 55 patients (37.0%) presented variants that, to our current knowledge, cannot completely explain the patient's phenotype or are of unknown significance. In addition, tests in 82 patients (55%) were negative for all known variants, indicating that in these disorders more effort is needed to define those genes responsible for the vascular disease.

In addition, the NGS technology is not void of limitation: it fails to identify large deletions or insertions, suggesting multiplex ligation-dependent probe amplification assay as a complementary technique that assess copy number variations especially in recessively inherited genes or in those with paradominant inheritance. We can also not exclude that patients negative to genetic testing carry a pathogenic variant in a gene not included in the panel currently used. Considering the assumption that many vascular lesions seem to have an etiopathogenesis similar to that of tumors, as previously suggested by Knudson,[30] tissue analysis is surely the second fundamental step required for an accurate diagnosis of patients with vascular malformations and hemangiomas.

The sporadic vascular anomalies forms, indeed, have been described to be sometimes caused by somatic mosaic variations in those genes or pathways, or both, also implicated in their rare, inherited counterparts or to occur as a consequence of a second hit locally acquired that leads to the biallelic loss of function of the mutated gene.[22-24,31] We detected variants in five of 17 analyzed tissues. In four of the five patients, the somatic variant identified seemed sufficient to explain the lesion formation, without the need of a second hit,[24,31,32] whereas for the remaining patient, we detected an increase in the percentage of reads with the variation between blood and biopsy from 50% to 68%. This could mean that there has been a deletion in the glomulin gene or the acquisition of the same variant in the opposite allele.

Interestingly, one of the five variants somatically identified was located in the *PIK3CA* gene. Somatic activating variations in PIK3CA, the catalytic subunit of PI3K, are frequently observed in several common human tumor types[33] and have also been reported in vascular malformation.[31] Variants in this gene are often activating, leading to abnormal cell growth, angiogenesis, and survival.[34] To note, PI3K inhibitors as cancer potential therapeutic agents have been developed, and some of them have reached clinical trials[35]; therefore, patients harboring variations in *PIK3CA* might benefit from repurposing of those inhibitors.

In addition, it is important to state that the number of patients with identified pathogenic variants could be slightly underestimated as a result of the inherent weakness of our approach, which scrutinizes a heterogeneous tissue with a perfectible sensitivity of the method: resection of the affected tissue areas or the use of a deep sequencing approach could help in the identification of pathogenic variants present at lower allelic frequencies.

A noteworthy result of our study was that we identified in some patients an otherwise unsuspected clinical situation that led to a personalized clinical management of the patients and to a more accurate planning of follow-up. Loeys-Dietz syndrome is a very rare disorder, and its prevalence is unknown. It has autosomal dominant inheritance and results from variations on the *TGFBR1*, *TGFBR2*, mothers against decapentaplegic homolog 3 (*SMAD3*), and *TGFB2* genes.[35] In our cohort, we managed the diagnosis of a patient harboring a *TGFBR1* variation, a relevant result considering the total number of reported individuals in OMIM that present variants in this gene. For this patient we suggested further clinical and diagnostic study, because patients affected by Loeys-Dietz syndrome are characterized by rapidly progressive aortic and arterial tortuosity and aneurysmal disease and therefore require constant monitoring.

Cowden syndrome has an estimated prevalence of 1/200,000 in the population and is associated in 25% with germinal variants in the *PTEN* oncosuppressor gene. The function of PTEN is to inhibit processes of the PI3K/Akt/mechanistic target of rapamycin pathway that lead to cell proliferation by dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate to phosphatidylinositol 4,5-bisphosphate. Genetic analysis of subject R845 revealed two variations, both potentially pathogenic, despite the clear difference in the final effect: the *KRIT1* gene, is characteristic of cerebral cavernous malformation, whereas the *PTEN* gene, is mainly found mutated in Proteus, Proteus-like syndrome, or Cowden syndrome.[28] Analysis of segregation in the family confirmed that the patient was affected with Cowden syndrome (Fig 1). Clinical management of these patients envisages prevention through periodic monitoring for early identification of tumors.

CONCLUSIONS

Our results show that genetic testing by NGS is the approach of choice to diagnose heritable vascular anomalies, especially, but not only, when an intralesional biopsy specimen is available, given the high frequency of somatic variants detected in our cohort (5 of 17 [29%]). The identification of the causative gene in those with an uncertain phenotype or in complex cases is of inestimable value to provide the appropriate clinical management, monitoring, and treatment; in addition, it provides important information to help people make decisions about having children or to identify genetic disorders early in life so treatment can be started as early as possible.

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AUTHOR CONTRIBUTIONS

Conception and design: RM, EM, MB
Analysis and interpretation: EM, AB, MB
Data collection: RM, EM, PC, SM, AP, GB, AB, MB
Writing the article: EM
Critical revision of the article: RM, EM, PC, SM, AP, GB, AB, MB
Final approval of the article: RM, EM, PC, SM, AP, GB, AB, MB
Statistical analysis: Not applicable
Obtained funding: Not applicable
Overall responsibility: RM

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APPENDIX (online only).

Supplementary Methods

Data analysis. The raw read data in fastq format, generated by the Illumina MiSeq 2.5 reporter software (San Diego, Calif), was analyzed to generate the final set of sequence variants using an in-house pipeline that includes following modules: mapping, duplicate read removal, indel realignment, quality calibration, coverage analysis, variant calling, and annotation. In brief, the sequencing reads were mapped to the genome build hg19 using the Burrows-Wheeler Aligner (BWA version 0.7.5a-r405; http://bio-bwa.sourceforge.net/)[1] with default settings. Next, duplicate fragments were marked and eliminated with the MarkDuplicates Genome Analysis Took Kit (GATK) tool (version v2.5-2-gf57256b; https://software.broadinstitute.org/gatk/).[2,3]

The BAM alignment files generated were refined by local realignment and base quality score recalibration using the RealignerTargetCreator and IndelRealigner GATK tools. Statistical and coverage analysis of final BAM files was performed using SAMTools and BEDTools.[4] Reads aligned to the designed target regions (coding exons and 15 bp flanking of gene-disease subpanel) were collected for variant calling and subsequent analysis. The following data per sample were generated by coverage analysis: average read depth, low coverage target regions ($<10\times$); % of target bases with coverage $\geq 10\times$.

Sequence variant calling was performed using three single nucleotide polymorphism and genotype calling tools: GATK UnifiedGenotyper, Varscan (version 2.3)[5] and Bcftools of SAMTools (version 0.1.19-44428cd). The output data from the three variant callers was joined and converted to a standard vcf file using a custom script. Called variants were annotated using Annovar software[6] with the aid of information from publicly available databases (database for allele frequency data: 1000 Genomes Project [http://www.1000genomes.org/], Single Nucleotide Polymorphism database [http://www.ncbi.nlm.nih.gov/projects/SNP/], and Exome Variant Server [evs.gs.washington.edu/EVS] databases; variant-disease association databases: Human Gene Mutation Database [HGMD], HumsVar [http://omictools.com/humsavar-tool], and LOVD [Leiden Open Variation Database]). The potential deleterious effect of missense variants was determined by using various in silico prediction algorithms (SIFT [Sorting Intolerant From Tolerant, http://sift.jcvi.org/www/SIFT_enst_submit.html], PolyPhen-2 [Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/index.shtml] and Mutation Taster [http://www.mutationtaster.org/]).

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Supplementary Table I (online only). Target regions of the 25 genes related to vascular anomalies[a]

Chromosome	Start	End	Gene[b]
chr5	180030176	180030405	FLT4
chr5	180035265	180035299	FLT4
chr5	180035952	180036068	FLT4
chr5	180036889	180037040	FLT4
chr5	180038315	180038494	FLT4
chr5	180039490	180039626	FLT4
chr5	180039995	180040125	FLT4
chr5	180041052	180041194	FLT4
chr5	180043351	180043504	FLT4
chr5	180043884	180044009	FLT4
chr5	180045754	180045935	FLT4
chr5	180046005	180046124	FLT4
chr5	180046237	180046381	FLT4
chr5	180046649	180046784	FLT4
chr5	180047157	180047323	FLT4
chr5	180047593	180047730	FLT4
chr5	180047860	180048022	FLT4
chr5	180048090	180048267	FLT4
chr5	180048526	180048919	FLT4
chr5	180049715	180049854	FLT4
chr5	180050919	180051076	FLT4
chr5	180052853	180053046	FLT4
chr5	180053095	180053280	FLT4
chr5	180055866	180056014	FLT4
chr5	180056243	180056442	FLT4
chr5	180056680	180056850	FLT4
chr5	180056927	180057120	FLT4
chr5	180057209	180057352	FLT4
chr5	180057539	180057814	FLT4
chr5	180058666	180058793	FLT4
chr5	180076472	180076560	FLT4
chr3	30648360	30648484	TGFBR2
chr3	30664675	30664780	TGFBR2
chr3	30686223	30686422	TGFBR2
chr3	30691746	30691967	TGFBR2
chr3	30713114	30713944	TGFBR2
chr3	30715581	30715753	TGFBR2
chr3	30729860	30730018	TGFBR2
chr3	30732896	30733106	TGFBR2
chr9	80336223	80336444	GNAQ
chr9	80343414	80343598	GNAQ
chr9	80409363	80409523	GNAQ
chr9	80412420	80412579	GNAQ
chr9	80430516	80430701	GNAQ
chr9	80537061	80537276	GNAQ
chr9	80646000	80646166	GNAQ
chr9	27109573	27109655	TEK
chr9	27157813	27158155	TEK
chr9	27168477	27168618	TEK
chr9	27169459	27169642	TEK
chr9	27172598	27172760	TEK
chr9	27173204	27173375	TEK
chr9	27180222	27180381	TEK
chr9	27183441	27183623	TEK
chr9	27185467	27185642	TEK
chr9	27190511	27190703	TEK
chr9	27192471	27192636	TEK
chr9	27197297	27197612	TEK
chr9	27202802	27203132	TEK
chr9	27204893	27205078	TEK
chr9	27206564	27206805	TEK
chr9	27209103	27209244	TEK
chr9	27212689	27212910	TEK
chr9	27213466	27213610	TEK
chr9	27217670	27217771	TEK
chr9	27218759	27218830	TEK
chr9	27220031	27220158	TEK
chr9	27228188	27228318	TEK
chr9	27229140	27229245	TEK
chr7	91830034	91830133	KRIT1
chr7	91830605	91830752	KRIT1
chr7	91842493	91842730	KRIT1
chr7	91843190	91843308	KRIT1
chr7	91843909	91844106	KRIT1
chr7	91851200	91851382	KRIT1
chr7	91852120	91852307	KRIT1
chr7	91855018	91855156	KRIT1
chr7	91855824	91856011	KRIT1
chr7	91863747	91863921	KRIT1
chr7	91864106	91864252	KRIT1
chr7	91864701	91864975	KRIT1
chr7	91865711	91865871	KRIT1
chr7	91866965	91867088	KRIT1
chr7	91870291	91870481	KRIT1
chr7	91871332	91871464	KRIT1
chr1	218520028	218520404	TGFB2
chr1	218536660	218536774	TGFB2
chr1	218578495	218578689	TGFB2
chr1	218607408	218607571	TGFB2
chr1	218607664	218607805	TGFB2
chr1	218609296	218609504	TGFB2
chr1	218610669	218610853	TGFB2
chr1	218614530	218614719	TGFB2
chr4	55946092	55946345	KDR
chr4	55948107	55948223	KDR

(Continued)

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Supplementary Table I (online only). Continued.

Chromosome	Start	End	Gene[b]
chr4	55948687	55948817	KDR
chr4	55953758	55953940	KDR
chr4	55955019	55955155	KDR
chr4	55955525	55955655	KDR
chr4	55955842	55955984	KDR
chr4	55956107	55956260	KDR
chr4	55958768	55958896	KDR
chr4	55960953	55961137	KDR
chr4	55961728	55961847	KDR
chr4	55962380	55962524	KDR
chr4	55963813	55963948	KDR
chr4	55964288	55964454	KDR
chr4	55964848	55964985	KDR
chr4	55968048	55968210	KDR
chr4	55968513	55968690	KDR
chr4	55970794	55971166	KDR
chr4	55971983	55972122	KDR
chr4	55972838	55972992	KDR
chr4	55973888	55974075	KDR
chr4	55976554	55976748	KDR
chr4	55976805	55976950	KDR
chr4	55979455	55979663	KDR
chr4	55980277	55980447	KDR
chr4	55981025	55981224	KDR
chr4	55981432	55981593	KDR
chr4	55984755	55984982	KDR
chr4	55987248	55987372	KDR
chr4	55991378	55991475	KDR
chr3	178916598	178916980	PIK3CA
chr3	178917462	178917702	PIK3CA
chr3	178919062	178919343	PIK3CA
chr3	178921316	178921592	PIK3CA
chr3	178922275	178922391	PIK3CA
chr3	178927367	178927503	PIK3CA
chr3	178927958	178928141	PIK3CA
chr3	178928203	178928368	PIK3CA
chr3	178935982	178936137	PIK3CA
chr3	178936968	178937080	PIK3CA
chr3	178937343	178937538	PIK3CA
chr3	178937721	178937855	PIK3CA
chr3	178938758	178938960	PIK3CA
chr3	178941853	178941990	PIK3CA
chr3	178942472	178942624	PIK3CA
chr3	178943734	178943843	PIK3CA
chr3	178947044	178947245	PIK3CA
chr3	178947776	178947924	PIK3CA
chr3	178947997	178948179	PIK3CA
chr3	178951866	178952167	PIK3CA

(Continued)

Supplementary Table I (online only). Continued.

Chromosome	Start	End	Gene[b]
chr12	52306243	52306334	ACVRL1
chr12	52306867	52307149	ACVRL1
chr12	52307327	52307569	ACVRL1
chr12	52307742	52307872	ACVRL1
chr12	52308207	52308384	ACVRL1
chr12	52308993	52309299	ACVRL1
chr12	52309804	52310032	ACVRL1
chr12	52312753	52312914	ACVRL1
chr12	52314527	52314692	ACVRL1
chr15	48703171	48703591	FBN1
chr15	48704750	48704955	FBN1
chr15	48707717	48707979	FBN1
chr15	48712868	48713018	FBN1
chr15	48713739	48713898	FBN1
chr15	48714133	48714280	FBN1
chr15	48717550	48717703	FBN1
chr15	48717920	48718076	FBN1
chr15	48719748	48719985	FBN1
chr15	48720527	48720683	FBN1
chr15	48722852	48723014	FBN1
chr15	48725047	48725200	FBN1
chr15	48726775	48726925	FBN1
chr15	48729142	48729289	FBN1
chr15	48729503	48729599	FBN1
chr15	48729949	48730129	FBN1
chr15	48733902	48734058	FBN1
chr15	48736722	48736872	FBN1
chr15	48737557	48737716	FBN1
chr15	48738887	48739034	FBN1
chr15	48740949	48741105	FBN1
chr15	48744743	48744896	FBN1
chr15	48748818	48748974	FBN1
chr15	48752427	48752529	FBN1
chr15	48755263	48755452	FBN1
chr15	48756080	48756233	FBN1
chr15	48757749	48757905	FBN1
chr15	48757971	48758070	FBN1
chr15	48760119	48760314	FBN1
chr15	48760593	48760746	FBN1
chr15	48762815	48762968	FBN1
chr15	48764732	48764888	FBN1
chr15	48766436	48766589	FBN1
chr15	48766709	48766862	FBN1
chr15	48773836	48773992	FBN1
chr15	48775999	48776155	FBN1
chr15	48777555	48777708	FBN1
chr15	48779256	48779412	FBN1
chr15	48779493	48779649	FBN1

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Supplementary Table I (online only). Continued.

Chromosome	Start	End	Gene[b]
chr15	48780294	48780453	FBN1
chr15	48780549	48780705	FBN1
chr15	48782032	48782290	FBN1
chr15	48784642	48784798	FBN1
chr15	48786385	48786466	FBN1
chr15	48787304	48787472	FBN1
chr15	48787650	48787800	FBN1
chr15	48788281	48788437	FBN1
chr15	48789447	48789603	FBN1
chr15	48791166	48791250	FBN1
chr15	48795968	48796151	FBN1
chr15	48797206	48797359	FBN1
chr15	48800763	48800916	FBN1
chr15	48802225	48802381	FBN1
chr15	48805730	48805880	FBN1
chr15	48807568	48807739	FBN1
chr15	48808364	48808574	FBN1
chr15	48812840	48813029	FBN1
chr15	48818311	48818467	FBN1
chr15	48826261	48826417	FBN1
chr15	48829792	48830020	FBN1
chr15	48888464	48888590	FBN1
chr15	48892320	48892446	FBN1
chr15	48902909	48903038	FBN1
chr15	48905191	48905304	FBN1
chr15	48936787	48936981	FBN1
chr14	105236662	105236772	AKT1
chr14	105237066	105237199	AKT1
chr14	105238686	105238804	AKT1
chr14	105239199	105239444	AKT1
chr14	105239572	105239731	AKT1
chr14	105239776	105239932	AKT1
chr14	105240233	105240332	AKT1
chr14	105241259	105241355	AKT1
chr14	105241397	105241559	AKT1
chr14	105241973	105242151	AKT1
chr14	105242980	105243122	AKT1
chr14	105246409	105246568	AKT1
chr14	105258919	105258995	AKT1
chr10	89624211	89624320	PTEN
chr10	89653766	89653881	PTEN
chr10	89685254	89685329	PTEN
chr10	89690787	89690861	PTEN
chr10	89692754	89693023	PTEN
chr10	89711859	89712031	PTEN
chr10	89717594	89717791	PTEN
chr10	89720635	89720890	PTEN
chr10	89725028	89725244	PTEN

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Supplementary Table I (online only). Continued.

Chromosome	Start	End	Gene[b]
chr10	112257864	112258273	DUSP5
chr10	112262463	112262642	DUSP5
chr10	112266677	112266927	DUSP5
chr10	112269762	112270199	DUSP5
chr20	45338360	45338394	SLC2A10
chr20	45353664	45354978	SLC2A10
chr20	45355487	45355640	SLC2A10
chr20	45357976	45358142	SLC2A10
chr20	45362379	45362488	SLC2A10
chr7	45039917	45039977	CCM2
chr7	45067288	45067411	CCM2
chr7	45077836	45078040	CCM2
chr7	45103501	45103615	CCM2
chr7	45104046	45104260	CCM2
chr7	45108026	45108193	CCM2
chr7	45109409	45109575	CCM2
chr7	45112309	45112397	CCM2
chr7	45113043	45113185	CCM2
chr7	45113853	45114022	CCM2
chr7	45115360	45115671	CCM2
chr2	69240616	69240798	ANTXR1
chr2	69267160	69267262	ANTXR1
chr2	69271858	69271960	ANTXR1
chr2	69297763	69297875	ANTXR1
chr2	69298870	69298934	ANTXR1
chr2	69300138	69300248	ANTXR1
chr2	69302706	69302805	ANTXR1
chr2	69304524	69304635	ANTXR1
chr2	69317975	69318066	ANTXR1
chr2	69329958	69330087	ANTXR1
chr2	69350133	69350233	ANTXR1
chr2	69351681	69351790	ANTXR1
chr2	69372442	69372523	ANTXR1
chr2	69379285	69379411	ANTXR1
chr2	69397364	69397436	ANTXR1
chr2	69399483	69399531	ANTXR1
chr2	69408902	69409028	ANTXR1
chr2	69409609	69409807	ANTXR1
chr2	69420451	69420562	ANTXR1
chr2	69472341	69472632	ANTXR1
chr3	167402080	167402192	PDCD10
chr3	167405006	167405119	PDCD10
chr3	167405387	167405496	PDCD10
chr3	167413368	167413525	PDCD10
chr3	167414781	167414929	PDCD10
chr3	167422614	167422698	PDCD10
chr3	167437834	167437960	PDCD10
chr1	92712071	92712218	GLMN

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Supplementary Table I (online only). Continued.

Chromosome	Start	End	Gene[b]
chr1	92712603	92712716	GLMN
chr1	92713419	92713561	GLMN
chr1	92728404	92728498	GLMN
chr1	92729164	92729304	GLMN
chr1	92730095	92730210	GLMN
chr1	92731960	92732064	GLMN
chr1	92732241	92732313	GLMN
chr1	92733454	92733574	GLMN
chr1	92733646	92733707	GLMN
chr1	92735264	92735348	GLMN
chr1	92737006	92737224	GLMN
chr1	92752031	92752164	GLMN
chr1	92754455	92754723	GLMN
chr1	92755739	92755878	GLMN
chr1	92756959	92757109	GLMN
chr1	92762945	92763101	GLMN
chr1	92763502	92763571	GLMN
chr5	86564253	86564822	RASA1
chr5	86564849	86564887	RASA1
chr5	86627149	86627332	RASA1
chr5	86628308	86628474	RASA1
chr5	86629068	86629169	RASA1
chr5	86633775	86633923	RASA1
chr5	86637091	86637153	RASA1
chr5	86642473	86642556	RASA1
chr5	86645015	86645196	RASA1
chr5	86648958	86649067	RASA1
chr5	86658352	86658503	RASA1
chr5	86659149	86659336	RASA1
chr5	86665614	86665732	RASA1
chr5	86667919	86668027	RASA1
chr5	86669964	86670152	RASA1
chr5	86670641	86670748	RASA1
chr5	86672194	86672397	RASA1
chr5	86672682	86672872	RASA1
chr5	86674197	86674370	RASA1
chr5	86675536	86675682	RASA1
chr5	86676310	86676427	RASA1
chr5	86679514	86679612	RASA1
chr5	86681102	86681221	RASA1
chr5	86682627	86682735	RASA1
chr5	86685194	86685359	RASA1
chr5	86686601	86686715	RASA1
chr9	101867472	101867599	TGFBR1
chr9	101891121	101891397	TGFBR1
chr9	101894775	101895036	TGFBR1
chr9	101900125	101900386	TGFBR1
chr9	101904802	101905000	TGFBR1

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Supplementary Table I (online only). Continued.

Chromosome	Start	End	Gene[b]
chr9	101906998	101907185	TGFBR1
chr9	101908751	101908906	TGFBR1
chr9	101909920	101910081	TGFBR1
chr9	101911446	101911602	TGFBR1
chr15	67358477	67358713	SMAD3
chr15	67430349	67430453	SMAD3
chr15	67457217	67457441	SMAD3
chr15	67457575	67457737	SMAD3
chr15	67459101	67459206	SMAD3
chr15	67462876	67462957	SMAD3
chr15	67473563	67473806	SMAD3
chr15	67477049	67477217	SMAD3
chr15	67479687	67479862	SMAD3
chr15	67482735	67482889	SMAD3
chr16	16243974	16244113	ABCC6
chr16	16244419	16244644	ABCC6
chr16	16248469	16248666	ABCC6
chr16	16248714	16248903	ABCC6
chr16	16251504	16251681	ABCC6
chr16	16253323	16253455	ABCC6
chr16	16255279	16255436	ABCC6
chr16	16256834	16257064	ABCC6
chr16	16259464	16259805	ABCC6
chr16	16263487	16263725	ABCC6
chr16	16267125	16267276	ABCC6
chr16	16269752	16269858	ABCC6
chr16	16271293	16271498	ABCC6
chr16	16272639	16272837	ABCC6
chr16	16276253	16276460	ABCC6
chr16	16276645	16276802	ABCC6
chr16	16278800	16278906	ABCC6
chr16	16280965	16281083	ABCC6
chr16	16282672	16282846	ABCC6
chr16	16284005	16284239	ABCC6
chr16	16286671	16286794	ABCC6
chr16	16291862	16292054	ABCC6
chr16	16295842	16296050	ABCC6
chr16	16297251	16297485	ABCC6
chr16	16302569	16302731	ABCC6
chr16	16306026	16306118	ABCC6
chr16	16308165	16308321	ABCC6
chr16	16313395	16313554	ABCC6
chr16	16313663	16313819	ABCC6
chr16	16315409	16315703	ABCC6
chr16	16317240	16317306	ABCC6
chr2	189839200	189839309	COL3A1
chr2	189849470	189849703	COL3A1
chr2	189849907	189849988	COL3A1

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Supplementary Table I (online only). Continued.

Chromosome	Start	End	Gene[b]
chr2	189850375	189850519	COL3A1
chr2	189851769	189851880	COL3A1
chr2	189852791	189852875	COL3A1
chr2	189853300	189853384	COL3A1
chr2	189854106	189854190	COL3A1
chr2	189854806	189854890	COL3A1
chr2	189855017	189855101	COL3A1
chr2	189855714	189855798	COL3A1
chr2	189856197	189856272	COL3A1
chr2	189856379	189856463	COL3A1
chr2	189856894	189856969	COL3A1
chr2	189857597	189857681	COL3A1
chr2	189858071	189858200	COL3A1
chr2	189858748	189858823	COL3A1
chr2	189858944	189859073	COL3A1
chr2	189859251	189859335	COL3A1
chr2	189859434	189859572	COL3A1
chr2	189859756	189859840	COL3A1
chr2	189860402	189860531	COL3A1
chr2	189860835	189860919	COL3A1
chr2	189861108	189861237	COL3A1
chr2	189861875	189861959	COL3A1
chr2	189862046	189862130	COL3A1
chr2	189862410	189862494	COL3A1
chr2	189862976	189863060	COL3A1
chr2	189863384	189863459	COL3A1
chr2	189863995	189864124	COL3A1
chr2	189864180	189864318	COL3A1
chr2	189864552	189864636	COL3A1
chr2	189866107	189866191	COL3A1
chr2	189866246	189866330	COL3A1
chr2	189867008	189867092	COL3A1
chr2	189867665	189867803	COL3A1
chr2	189868121	189868205	COL3A1
chr2	189868444	189868528	COL3A1
chr2	189868692	189868884	COL3A1
chr2	189868967	189869105	COL3A1
chr2	189870060	189870198	COL3A1
chr2	189870916	189871000	COL3A1
chr2	189871055	189871193	COL3A1
chr2	189871647	189871731	COL3A1
chr2	189872210	189872348	COL3A1
chr2	189872595	189872679	COL3A1
chr2	189872745	189872883	COL3A1
chr2	189873634	189873962	COL3A1
chr2	189874888	189875106	COL3A1
chr2	189875358	189875631	COL3A1
chr2	189876338	189876515	COL3A1
chr9	130577945	130578100	ENG
chr9	130578180	130578347	ENG
chr9	130579412	130579497	ENG
chr9	130580383	130580671	ENG
chr9	130580979	130581126	ENG
chr9	130581885	130581954	ENG
chr9	130582163	130582331	ENG
chr9	130586567	130586740	ENG
chr9	130587063	130587268	ENG
chr9	130587494	130587651	ENG
chr9	130587958	130588154	ENG
chr9	130588773	130588966	ENG
chr9	130591950	130592121	ENG
chr9	130605357	130605539	ENG
chr9	130616552	130616649	ENG
chr18	48573401	48573680	SMAD4
chr18	48575040	48575245	SMAD4
chr18	48575649	48575709	SMAD4
chr18	48581135	48581378	SMAD4
chr18	48584479	48584629	SMAD4
chr18	48584694	48584841	SMAD4
chr18	48586220	48586301	SMAD4
chr18	48591777	48591991	SMAD4
chr18	48593373	48593572	SMAD4
chr18	48602992	48603161	SMAD4
chr18	48604610	48604852	SMAD4

(Continued)

Supplementary Table I (online only). Continued.

chr, Chromosome.
[a]RefSeq University of California, Santa Cruz (UCSC), hg19.
[b]Expansions for the genes listed are available at U.S. National Library of Medicine, Genetics Home Reference (https://ghr.nlm.nih.gov/gene).

Supplementary Table II (online only). Variants in disease genes with a likely association with vascular anomalies[a]

Patient ID	Sex	Gene[c]	RefSeq	Nucleotide substitution	Exon/intron	Protein substitution	Het/Homo
R210	F	ABCC6	NM_001171	c.1540G>A	Exon12	p.(Val514Ile)	Het
R237	F	ENG	NM_000118	c.1538A>G	Exon12	p.(Lys513Arg)	Het
R211[b]	M	FBN1	NM_000138	c.8071G>A	Exon65	p.(Gly2691Ser)	Het
R446[b]	F	GLMN	NM_053274	c.144T>G	Exon3	p.(Ile48Met)	Het
R028[b]	F	TGFB2	NM_003238	c.272G>A	Exon1	p.(Arg91His)	Het
R214	M	PDCD10	NM_145860	c.-126_-125delCT	Intron1-2		Het
R026	F	GLMN	NM_053274	c.436G>A	Exon6	p.(Ala146Thr)	Het
R026[b]	F	PDCD10	NM_145859	c.371G>A	Exon6	p.(Arg124Lys)	Het
R238	F	GLMN	NM_053274	c.271G>A	Exon4	p.(Asp91Asn)	Het
R130[b]	F	CCM2	NM_031443	c.926A>G	Exon9	p.(Lys309Arg)	Het
R130	F	COL3A1	NM_000090	c.2035G>A	Exon30	p.(Ala679Thr)	Het
R142[b]	F	FBN1	NM_000138	c.4270C>G	Exon35	p.(Pro1424Ala)	Het
R186[b]	F	FBN1	NM_000138	c.4270C>G	Exon35	p.(Pro1424Ala)	Het
R822	F	KDR	NM_002253	c.1675A>T	Exon13	p.(Met559Leu)	Het
R027	F	CCM2	NM_031443	c.866G>A	Exon8	p.(Ser289Asn)	Het
R823[b]	F	FBN1	NM_000138	c.4900C>T	Exon40	p.(Pro1634Ser)	Het
R222[b]	F	TGFB2	NM_003238	c.619G>C	Exon3	p.(Val207Leu)	Het
R813	F	RASA1	NM_002890	c.285_305del	Exon1	p.(Ala100_Ala106del)	Het
R813	F	COL3A1	NM_000090	c.1976C>T	Exon28	p.(Pro659Leu)	Het
R813[b]	F	F7	NM_000131	c.1074G>A	Exon9	p.(Met358Ile)	Het
R113	M	KDR	NM_002253	c.1848G>A	Exon13	p.(Met616Ile)	Het
R814[b]	M	GLMN	NM_053274	c.144T>G	Exon3	p.(Ile48Met)	Het
R814	M	ENG	NM_001114753	c.392C>T	Exon4	p.(Pro131Leu)	Het
R134	M	TGFBR2	NM_003242	c.1657T>A	Exon7	p.(Ser553Thr)	Het
R815	F	KDR	NM_002253	c.1325C>T	Exon10	p.(Thr442Met)	Het
R816	M	SMAD4	NM_005359	c.463A>G	Exon5	p.(Ser155Gly)	Het
R129	F	TGFBR2	NM_003242	c.118G>A	Exon2	p.(Asp40Asn)	Het
R442[b]	F	KRIT1	NM_194456	c.167C>T	Exon6	p.(Thr56Met)	Het
R442	F	SLC2A10	NM_030777	c.1309G>A	Exon3	p.(Glu437Lys)	Het
R440	M	GLMN	NM_053274	c.1057T>C	Exon11	p.(Tyr353His)	Het
R440	M	TGFBR1	NM_004612	c.-26_-22del	5′UTR		Het
R440	M	KDR	NM_002253	c.1325C>T	Exon10	p.(Thr442Met)	Het
R817	M	ABCC6	NM_001171	c.3736-1G>A	Exon28		Het
R817	M	ABCC6	NM_001171	c.346-6G>A	Exon4		Het
R818	F	TEK	NM_000459	c.1900C>T	Exon12	p.(Leu634Phe)	Het
R208	F	FLT4	NM_182925	c.376G>A	Exon3	p.(Ala126Thr)	Het
R819[b]	F	RASA1	NM_002890	c.259A>C	Exon1	p.(Thr87Pro)	Het
R216	F	ENG	NM_000118	c.388C>T	Exon4	p.(Pro130Ser)	Het
R824	F	ACVRL1	NM_000020	c.52G>A	Exon2	p.(Val18Met)	Het

NA, Not available; *UTR*, untranslated region.

[a]List of germ line variants identified in patients with vascular anomalies that however cannot completely explain their phenotype. For each mutation there is a description of the nucleotide and amino acid substitution, an evaluation of pathogenicity by Mutation Taster, SIFT (Sorting Intolerant From Tolerant) and PolyPhen-2 (Polymorphism Phenotyping version 2). Sex: *F*, female; *M*, male; Type of mutation: Missense (*M*), sporadic (*Sp*); Mutation Taster score: polymorphism (*P*), disease-causing (*DC*); SIFT score: tolerated (*T*), deleterious (*De*), damaging (*D*); PolyPhen-2 score: benign (*B*), possibly damaging (*PoD*, less confident prediction), probably damaging (*PrD*, more confident prediction). *MAF%*, minor allele frequency in percent in European American population from Exome Variant Server.

[b]Those are mutations that could be described as potentially pathogenic by at least two prediction tools.

[c]Expansions for the genes listed are available at U.S. National Library of Medicine, Genetics Home Reference (https://ghr.nlm.nih.gov/gene).

Supplementary Table II (online only). Continued.

Type of mutation	New/known	Mutation taster	SIFT	PolyPhen-2	Reference	RS number	MAF%
M	Known	DC	T	B		rs59157279	0.04
M	New	P	T	PrD		NA	NA
M	Known	DC	T	PoD	Lerner-Ellis et al, 2014	rs145105768	NA
M	Known	DC	De	PoD		rs142032681	NA
M	Known	DC	De	PoD		rs10482721	0.08
Del	New	…	…	…			
M	Known	DC	T	B		rs61754623	0.2
M	New	DC	De	PoD		rs769890116	NA
M	Known	DC	T	B		rs144577963	0.4
M	Known	DC	T	PrD		rs535112809	0.02
M	Known	DC	T	B		rs41263773	0.18
M	Known	DC	De	B		rs201273753	NA
M	Known	DC	De	B		rs201273753	NA
M	New	P	T	B		NA	NA
M	Known	P	De	B		rs2289366	1.8
M	New	DC	De	B		NA	NA
M	Known	DC	De	B		rs10482810	0.06
Del	New	…	…	…			
M	New	DC	T	B		NA	NA
M	Known	DC	De	PrD		rs149283257	NA
M	Known	P	T	B		rs753716162	NA
M	New	DC	De	PoD		rs142032681	NA
M	Known	P	T	B	Olivieri et al, 2007	rs139398993	0.98
M	Known	DC	T	B		rs112215250	NA
M	Known	P	T	B		rs766459956	NA
M	New	DC	T	B		NA	NA
M	Known	P	T	B		rs397516837	
M	Known	DC	T	B		rs753631870	NA
M	Known	DC	De	PrD		rs763220502	NA
M	Known	P	T	B		rs149792649	0.1
Del	New	-	-	-			
M	Known	P	T	B		rs766459956	NA
Sp	Known	…	…	…	Ringpfeil et al, 2000; Nitschke et al, 2012		
Sp	Known	…	…	…	Miksch et al, 2005; Schulz et al, 2006		
M	Known	DC	T	B		rs35378598	0.06
M	Known	DC	T	B		rs760182219	NA
M	Known	P	De	PoD		rs147942393	NA
M	Known	P	T	B		rs199840979	NA
M	New	P	T	B		NA	NA

Supplementary Table II (online only). Continued.

Patient ID	Sex	Gene[c]	RefSeq	Nucleotide substitution	Exon/intron	Protein substitution	Het/ Homo
R824[b]	F	FLT4	NM_182925	c.1921C>T	Exon13	p.(Pro641Ser)	Het
R824	F	FLT4	NM_182925	c.3908G>C	Exon30	p.(Gly1303Ala)	Het
R820[b]	M	TEK	NM_000459	c.896A>T	Exon6	p.(Asn299Ile)	Het
R133[b]	M	TGFB2	NM_003238	c.272G>A	Exon1	p.(Arg91His)	Het
R133	M	ABCC6	NM_001171	c.2530A>C	Exon19	p.(Lys844Gln)	Het
R223	F	FBN1	NM_000138	c.7330+10T>G	Intron59-60		Het
R223	F	TGFB2	NM_003238	c.347-14C>G	Intron1-2		Het
R825[b]	F	KDR	NM_002253	c.2245G>A	Exon15	p.(Glu749Lys)	Het
R213[b]	F	KDR	NM_002253	c.406G>A	Exon4	p.(Val136Met)	Het
R826[b]	M	FBN1	NM_000138	c.3368T>A	Exon28	p.(Leu1123Gln)	Het
R826	M	ENG	NM_000118	c.1312-12G>A	Intron		Het
R827[b]	F	ANTXR1	NM_032208	c.1540C>T	Exon18	p.(Pro514Ser)	Het
R828	F	RASA1	NM_002890	c.2487+13T>A	Intron18-19		Het
R829	M	FBN1	NM_000138	c.1177A>G	Exon11	p.(Met393Val)	Het
R830	M	AKT1	NM_005163	c.176-5C>A	Intron3-4		Het
R831	F	RASA1	NM_002890	c.2487+13T>A	Intron18		Het
R832	F	KDR	NM_002253	c.1039C>T	Exon8	p.(Arg347Cys)	Het
R833[b]	M	FBN1	NM_000138	c.8176C>T	Exon65	p.(Arg2726Trp)	Het
R834	F	GLMN	NM_053274	c.1057T>C	Exon11	p.(Tyr353His)	Het
R835	M	TGFBR1	NM_004612	c.457G>A	Exon3	p.(Val153Ile)	Het
R836	F	FBN1	NM_000138	c.6577G>A	Exon54	p.(Glu2193Lys)	Het
R837	M	COL3A1	NM_000090	c.1129G>A	Exon16	p.(Ala377Thr)	Het
R837	M	TEK	NM_000459	c.3281G>C	Exon22	p.(Arg1094Thr)	Het
R838	F	ABCC6	NM_001171	c.2836C>A	Exon22	p.(Leu946Ile)	Het
R838	F	ABCC6	NM_001171	c.3507-3C>T	Intron24-25		Het
R839	M	ABCC6	NM_001171	c.473C>T	Exon4	p.(Ala158Val)	Homo
R840	F	FLT4	NM_182925	c.1921C>T	Exon13	p.(Pro641Ser)	Het
R841	F	ENG	NM_000118	c.392C>T	Exon4	p.(Pro131Leu)	Het
R841	F	ANTXR1	NM_032208	c.1517C>T	Exon18	p.(Pro506Leu)	Het
R842	F	KRIT1	NM_194456	c.707C>T	Exon9	p.(Ser236Leu)	Het
R842	F	FLT4	NM_182925	c.2860C>T	Exon21	p.(Pro954Ser)	Het
R843	M	FLT4	NM_182925	c.76T>G	Exon2	p.(Ser26Ala)	Het
R843	M	COL3A1	NM_000090	c.2035G>A	Exon30	p.(Ala679Thr)	Het
R844	F	PTEN	NM_000314	c.886T>C	Exon8	p.(Cys296Arg)	Het
R844	F	ABCC6	NM_001171	c.3892G>A	Exon28	p.(Val1298Ile)	Het
R821	F	GLMN	NM_053274	c.271G>A	Exon4	p.(Asp91Asn)	Het

Supplementary Table II (online only). Continued.

Type of mutation	New/ known	Mutation taster	SIFT	PolyPhen-2	Reference	RS number	MAF%
M	Known	DC	T	PrD		rs55667289	0.12
M	Known	P	T	B		rs146806202	0.34
M	New	DC	De	B		NA	NA
M	Known	DC	De	PoD		rs10482721	0.08
M	Known	P	T	B		rs201884545	NA
Sp	New	…	…	…			
Sp	New	…	…	…			
M	Known	DC	T	PoD		rs760248367	NA
M	Known	DC	De	PoD		rs35636987	0.24
M	Known	DC	T	PrD		rs201336778	0.02
Int	Known					rs201684408	NA
M	New	DC	T	PoD		NA	NA
Int	Known					rs372369767	0.02
M	New	P	T	B		NA	NA
Int	Known					rs377076374	0.01
Int	Known					rs372369767	0.07
M	Known	P	T	B		rs750983015	NA
M	Known	DC	De	PoD		rs61746008	0.1
M	Known	P	T	B		rs149792649	0.1
M	Known	DC	T	B		rs56014374	0.04
M	Known	DC	T	PoD		rs201361628	NA
M	New	DC	T	PoD		NA	NA
M	New	DC	D	PrD		NA	NA
M	Known	P	T	B	Schulz, Hum Mutat, 2006; Vanakker, Hum Mutat, 2008	rs61340537	1.08
Sp	Known	…	…	…			
M	Known	P	T	B		rs2606921	NA
M	Known	DC	T	PrD		rs55667289	0.12
M	Known	P	T	B		rs139398993	0.15
M	Known	P	T	B		rs200543195	0.0077
M	New	DC	D	PrD		NA	NA
M	Known	DC	T	B		rs34255532	0.34
M	New	DC	T	PoD		rs113995355	0.6
M	Known	DC	T	B		rs41263773	0.18
M	New	DC	T	B			
M	Known	DC	D	PrD		rs63751325	0.04
M	New	DC	T	PrD		rs144577963	0.001384

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