Chapter 4
The Genetic Basis and Molecular Diagnosis of Vascular Tumors and Developmental Malformations

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Vascular Malformations

Vascular malformations, unlike vascular tumors, are present at birth (although not always evident), grow in proportion with the child, and do not regress. Although benign, they often present extremely challenging, lifelong management problems for patient and physician. Vascular malformations are defined by the affected vessel(s) and include capillary, venous, arterial, lymphatic, and combined malformations. Some are deadly, while others merely cosmetically or functionally disabling. Most are sporadic in occurrence, but more rare familial forms have revealed genetic abnormalities that not only suggest eventual therapies but also reveal basic control points in vasculogenesis (Table 4.1) [1–4].

Capillary Malformations (CMs)

Capillary malformations are slow-flow vascular malformations present at birth that grow with an individual, do not regress on their own, and have a normal rate of endothelial cell turnover. These contrast to infantile capillary hemangiomas

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Table 4.1 Molecular diagnostic targets and associations of vascular tumors and developmental malformations [1–4]

Vascular anomaly	Gene/locus	Location	Inheritance
Capillary/venulocapillary malformation			
Sturge-Weber syndrome (leptomeningeal and cutaneous venulocapillary malformation, aka "port-wine stain")	*GNAQ	9q21	Somatic
Non-syndromic port-wine stain	*GNAQ	9q21	Somatic
Arteriovenous malformations			
Capillary malformation-arteriovenous malformation (CM-AVM)	*RASA1	5q14.3	AD
Parkes Weber syndrome	*RASA1 (in subset)	5q14.3	AD
Hereditary hemorrhagic telangiectasia			
HHT1	*ENG	9q34.11	AD
HHT2	*ACVRL1/ALK1	12q13.13	AD
HHT3	Unknown	5q31.3-q32	AD
HHT4	Unknown	7p14	AD
Juvenile polyposis/HHT syndrome (JP/HHT)	*SMAD4	18q21.2	AD
HHT5/atypical HTT	*BMP9/GDF2	10q11.22	Association only
HBT	Unknown	CMC1/5q14	Association only
Angiokeratoma			
Fabry disease	*GLA	Xq22.1	XD
Progressive patchy capillary malformations			
Angioma serpiginosum	Unknown	Xp11.3-Xq12	Association only
Familial cerebral cavernous malformations (CCM)			
CCM1	*KRIT1	7q21.2	AD
CCM2	*Malcavernin/CCM2	7p13	AD
CCM3	*PDCD10	3q26.1	AD
CCM4	Unknown	3q26.3-27.2	

Venous malformations			
Sporadic Venous malformations (VM)	*TEK/TIE2	9p21.2	Somatic
Familial venous malformations cutaneous-mucosal (VMCM)	*TEK/TIE2	9p21.2	AD
Glomuvenous malformation (GVM)	*Glomulin/GLMN	1p22.1	AD
Verrucous venous malformation (VVM)/verrucous hemangioma	MAP3K3	17q23.3	Somatic
Lymphatic malformations, lymphedemas, and complex syndromes			
CLOVES	*PIK3CA	3q26.32	Somatic
Klippel-Trenaunay syndrome (KTS)	*PIK3CA	3q26.32	Somatic
Fibro-adipose vascular anomaly (FAVA)	*PIK3CA	3q26.32	Somatic
Macrocephaly-capillary malformation (M-CM)	*PIK3CA	3q26.32	Somatic
Microcephaly-capillary malformation (MICCAP)	*STAMBP	2p13.1	AR
Nonne-Milroy syndrome	*FLT4/VEGFR3	5q34-35	AD/AR
Primary hereditary lymphedema (Nonne-Milroy-like syndrome)	*VEGFC	4q34	AD
Hypotrichosis lymphedema telangiectasia (HLT)	*SOX18	20q13.33	AD/AR
Primary hereditary lymphedema	*GJC2/Connexin 47	1q41-42, 4q34	AD
Lymphedema distichiasis	*FOXC2	16q24.1	AD
Primary lymphedema with myelodysplasia (Emberger syndrome)	*GATA2	3q21.3	AD
Primary generalized lymphatic anomaly (Hennekam syndrome)	*CCBE1	18q21.32	AR
Microcephaly with or without chorioretinopathy, lymphedema, or mental retardation syndrome	*KIF11	10q23.33	AD
Lymphedema-choanal atresia	*PTPN14	1q41	AR
Proteus syndrome	*AKT1	14q32.33	Somatic

(continued)

Table 4.1 (continued)

Vascular anomaly	Gene/locus	Location	Inheritance
Vascular tumors			
Infantile hemangioma	Unknown	5q31-33	Association only
Epithelioid hemangioendothelioma (EHE)	WWTR1-CAMTA1, YAP1-TFE3	t(1;3)(p36.23;q25.1), t(11;X)(q13;p11)	
Spindle cell hemangioma (Maffucci syndrome)	*IDH1, *IDH2	2q34, 15q26.1	Somatic
PTEN hamartoma tumor syndrome (PHTS)			
Bannayan-Riley-Ruvalcaba syndrome (BRRS)	*PTEN	10q23.3	AD
Cowden syndrome (CS)/Cowden-like syndrome	*PTEN, *SDHB, *SDHD, KLLN	10q23.3	AD
"Proteus-like" syndrome	*PTEN	10q23.3	AD
PTEN-related Proteus syndrome (PS)	*PTEN	10q23.3	AD

AD autosomal dominant, *AR* autosomal recessive, *XD* X-linked dominant

*Clinical testing available in the United States and/or internationally as of June 1, 2015, according to the GTR: Genetic Testing Registry at NCBI and www.GeneTests.org

which are highly proliferative, appear after birth, and have a characteristic rapid growth, slow involution, and marked cellular increase in endothelial cells [5, 6]. Capillary malformations occur in approximately 0.3 % of newborns in the United States and in 0.1–2 % of newborns worldwide [7]. Isolated capillary malformations do not increase mortality; however, they do cause disability secondary to facial disfigurement. One study has shown that psychosocial difficulties not only persist into adulthood, they actually get worse [8]. The most common capillary malformation is called a "port-wine stain" (aka nevus flammeus, OMIM 163000), resulting from a congenital malformation of the superficial vessels of the dermis, which is present at birth. Sturge-Weber syndrome is characterized by a port-wine stain of the face combined with capillary-venous malformations in the eye and brain (leptomeningeal angioma) [9]. Port-wine stains and Sturge-Weber syndrome are caused by a somatic mutation in the guanine nucleotide binding protein (GNAQ) on chromosome 9q21 [10]. A single-nucleotide variant in GNAQ (c.548G>A, p.Arg183Gln) was identified in 88 % of affected tissue samples from individuals with Sturge-Weber syndrome and 92 % from nonsyndromic port-wine stains [10]. Port-wine stains generally grow in proportion to the child and do not tend to involute spontaneously. Histologically, these vessels are lined by flat, endothelial cells, similar to the vessels found in the skin. They stain for endothelial antigens such as von Willebrand factor and collagenous basement membrane proteins. There are a paucity of mitotic cells similar to normal dermal vessels, indicating their turnover is not increased. They are found in the reticular dermis, with a mean depth of 0.46 mm [11]. There are both sporadic and familial forms that occur (Table 4.1).

Arteriovenous Malformations

Mutations in RASA1 Result in CM-AVM, AVF, and Parkes Weber Syndrome

Distinct from the nevus flammeus lesion is the autosomal dominant fast-flow capillary malformation-arteriovenous malformation (CM-AVM) characterized by AVM's and small, multifocal cutaneous vascular marks that may have a surrounding pale halo [12, 13]. Three distinct pathologies have been defined, all resulting from RASA1 mutations: (1) capillary malformation-arteriovenous malformation (CM-AVM, OMIM 608354), (2) arteriovenous fistula (AVF), and (3) Parkes Weber syndrome (PSW, OMIM 608355). Recent studies have found that these lesions result from loss-of-function mutations in the RASA1 gene [14]. Mutations in RASA1 would be present in a number of cell types, resulting in alterations in proliferation, migration, and survival, including endothelial cells that largely make up the malformation. The RASA1 gene encodes a protein that stimulates the GTPase activity of RAS p21, controlling cellular proliferation and differentiation. Mutations causing CM-AVM have a high penetrance, nearly 98 % [13]. Nearly 30 % of the

time, CM-AVM lesions have associated vascular anomalies that are clinically more serious, including fast-flow anomalies like arteriovenous malformation (AVM), arteriovenous fistulas (AVF), or Parkes Weber syndrome (PWS) [13]. Eighty percent of the AVM and AVF are found in the head and neck regions [13]. For these reasons, a brain MRI is performed on patients presenting with CM-AVM found anywhere on the body. The heterogeneity of this disease process may be due to the requirement of additional somatic genetic hits or second mutations acquired [15]. Diagnosis of these three syndromes is made by sequencing the RASA1 gene, which is performed by a number of laboratories (Table 4.1).

Hereditary Hemorrhagic Telangiectasia

Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber syndrome is an autosomal dominant disorder characterized by telangiectases and arteriovenous malformation (AVM) (OMIM 187300 and 600736) [16]. Several genes in the TGF-b/BMP signaling pathway are involved in hereditary hemorrhagic telangiectasia, including the genes endoglin (ENG), activin A receptor type II-like 1 (ACVRL1/ALK1), and SMAD4 [17]. Genetic variations in ENG, ACVRL1/ALK1, and SMAD4 cause HHT1, HHT2, and the combined juvenile polyposis/HHT (JP/HHT) syndrome, respectively (Table 4.1) [18–20]. Two additional loci, 5q31 and 7p14, are associated with HHT3 and HHT4, but the genes are unknown [21, 22]. Most of the disease-causing mutations identified in ENG, ACVRL1/ALK1, and SMAD4 are null alleles, resulting in haploinsufficiency and reduced protein levels in patients suffering from hereditary hemorrhagic telangiectasia [17]. Germline mutations are most common, but de novo mutations and mosaicism in ENG and ACVRL1/ALK1 have been reported [23]. In a few cases of telangiectases with atypical distribution, mutations in the bone morphogenetic 9 (BMP9) gene (GDF2) were identified [24]. In addition, genetic variants in PTPN14 and ADAM17 appear to be genetic modifiers of hereditary hemorrhagic telangiectasia that influence clinical severity [25, 26]. Molecular diagnostic testing is available for detection of mutations, large deletions, and duplications in ENG, ACVRL1/ALK1, SMAD4, and GDF2/BMP9. Endoglin and ACVRL1/ALK1 mutations are present in the majority of cases with an indicated suspicion of HHT, whereas SMAD is detected in less than 2 % of cases [17]. BMP9/GDF2 mutations appear to be rare, in less than 1 % of hereditary hemorrhagic telangiectasia. As such, ENG and ACVRL1/ALK1 are often tested first, simultaneously, with reflex to SMAD testing if results are negative for both genes [17].

Hereditary benign telangiectasias (HBT) are considered the benign form of hereditary hemorrhagic telangiectasia, characterized by asymptomatic lesions that can cause mild cosmetic disability [27]. HBT has been mapped to the CMC1 locus on chromosome 5q14 [27], although subsequent studies suggest that this phenotype may have other genetic causes as well [28].

Angiokeratoma

Angiokeratomas are capillary vascular malformations that are characterized as papules or plaques. These benign cutaneous lesions appear as small raised nodules that are red to blue and can be quite dark. A systemic form of angiokeratomas, known as angiokeratoma corporis diffusum, has been linked to metabolic disorders such as Fabry disease and fucosidosis and is transmitted in an autosomal dominant pattern (OMIM ID 60041). In the description of the systemic disease, skin lesions could be as limited to a few localized angiokeratomas on the scrotum, to more than 100 lesions over the limbs and trunk [29]. Single solitary lesions have also been reported on the tongue and oral cavity [30, 31]. Fabry disease is caused by genetic variations in alpha-galactosidase (GLA). Over 300 mutations have been identified in GLA, as well as several GLA gene rearrangements and mRNA processing defects [3]. Genetic testing is available to confirm diagnosis of a metabolic storage disorder, such as Fabry disease, in an individual with clinical and biochemical phenotype.

Progressive Patchy Capillary Malformations and Focal Dermal Hypoplasia

Progressive patchy capillary malformations (aka angioma serpiginosum) are a rare X-linked dominant congenital disorder [32]. It is characterized by increased numbers of dilated, thickened capillaries located sub-epidermally [32]. These findings are associated with mild hair and nail dystrophy and esophageal papillomas [32]. Affected individuals have a 112 kilobase deletion at Xp11.23 loci, which contains up to eight genes [33] (summarized in Table 4.1). However, genetic testing for progressive patch capillary malformation/angioma serpiginosum specifically is not currently available since the genetic basis has not been confirmed. A previously unrelated disorder, called focal dermal hypoplasia (FDH), is caused only by sporadic or inherited mutations and deletions in the PORCN gene, one of the genes located in the Xp11.23 loci missing in progressive patchy capillary malformations. FDH manifests itself as dermal atrophy, cutaneous and esophageal papillomas, as well as hand, eye, and skeletal anomalies [33–35]. While genetic testing for progressive patch capillary malformations does not exist, genetic testing for candidate genes, specifically PORCN, FTSJ1, and EBP, is available. However, not enough is currently understood about the genetics causing this disease to currently warrant use of these tests.

Familial Cerebral Cavernous Malformations (CCMs)

Cerebral cavernous malformations are familial vascular malformations [36] characterized by clustered capillary caverns within a single endothelium layer without normal brain parenchyma or mature vessel wall elements interrupting. The diameter

of these vessels can vary from just a couple of millimeters to several cm. The cavern (channel) size and number progressively increase over time. The lesions themselves may develop de novo as demonstrated by serial MRIs and brain biopsies, demonstrating the evolutionary nature of CCMs [37].

CCMs occur in 0.4–0.5 % of the general population and are responsible for 5–15 % of all vascular malformation found in the cerebral cortex [36, 38, 39]. While CCM is found in children, the majority of patients do not have symptoms until they are adults. Patients generally present at a mean age of 30–40, with women presenting more commonly with hemorrhage and neurologic deficits [38, 39]. Up to 25 % remain symptom-free their entire life [40, 41], although other investigators have suggested this is an underestimation. Otten et al. [42], for example, reported an absence of symptoms in 90 % of CCMs identified at autopsy. The remaining 50–75 % present with a variety of symptoms, including cerebral hemorrhage, headaches, focal neurologic defects, and seizures [39]. CCM can be considered familial if these defects are seen in at least two family members or there are disease-causing mutations that are identified in genes associated with CCM. In cases without a family history, sporadic mutations are believed to account for the disease.

Clinical Diagnosis

The diagnosis of CCM is made by identifying blood flow through cavernous malformations. Not all malformations may be seen by angiography, making other modalities, such as MRI, more important in diagnosis [36]. T1 and T2 weighted images will show a characteristic popcorn pattern of variable intensities. On T2 or gradient echo, a dark hemosiderin ring at the periphery of the lesion may be seen, suggestive of a previous hemorrhage. Histopathologic examination and molecular testing are also utilized in making the diagnosis of CCM. Histologically, CCMs are identified by vascular malformations consisting of clustered, enlarged channels with a single layer of endothelium without intervening mature vessel wall elements or brain parenchyma [43–45].

Molecular Diagnosis of CCM

Familial CCM is associated with mutations in KRIT1, CCM2, and PDCD10 (Table 4.1), which account for 60–80 % of patients with CCM [46–49]. A putative locus for CCM has been proposed by Liquori et al. to be in the 3q26.3-27.2 region, although the specific gene involved has not been identified [48]. A single mutation in the KRIT1 gene, C1363T, is a common mutation found in 70 % of affected families of Hispanic heritage [50–52]. No other common mutation has been identified in other subgroups.

Molecular Pathogenesis of CCM

CCM1/KRIT1

CCM1 is caused by mutations in the gene KRIT1, located on 7q11.2-q21. The gene is comprised of 16 exons. Seventy four mutations have been described: 50 % are frameshifts, 24 % involve invariant splice junction changes, 24 % nonsense mutations, and 1 involves a deletion [53–56]. These mutations are distributed from exons 9 to 18 and are hypothesized to have a loss-of-function phenotype, since most result in a premature termination codon [55, 56]. The KRIT1 protein has tumor suppressor function and plays a critical role in early angiogenesis and the formation of vessels [57]. Mice lacking CCM1 are embryonic lethal, apparently due to its effects on the vasculature, more than its support for neurons [58].

CCM2/Malcavernin

The CCM2 gene product is the protein malcavernin, composed of 10 coding exons that encode a scaffold protein that functions in the p38 MAPK signaling cascade. Malcavernin interacts with the SMAD specific E3 ubiquitin ligase (SMURF1) through binding via a phosphotyrosine-binding domain to promote RhoA degradation [59]. Malcavernin is integral to normal cytoskeletal structure, cell-cell interactions, and formation of the lumen by endothelial cells [60].

CCM3/PDCD10

The PDCD10 (programmed cell death protein 10) gene has 10 exons, with the coding region starting in exon 4. This gene is an evolutionarily conserved protein that interacts with the serine/threonine protein kinase MST4 to regulate the ERK pathway [61]. At least seven mutations have been described in eight families, including a deletion of the entire gene and abnormal splicing of exon 5, three nonsense mutations, and two splice-site mutations [46]. Recent studies have implicated PDCD10 in cardiovascular development, in part by stabilizing the VEGF receptor [62–64] and by its role in apoptosis [65].

Molecular Testing for CCM

Clinical testing of KRIT1, CCM2, and PDCD10 is available (Table 4.1). In non-Hispanic individuals with a positive family history or multiple CCMs, KRIT1 mutations could be found in 43 % of probands with at least one affected relative or multiple CCM lesions detected by MRI [53]. Mutations in KRIT1 were identified in 30 % of

individuals with multiple CCMs with no family history [66]. Thirteen to 20 % of patients with familial CCM have mutations in the CCM2 gene [54, 55, 67, 68]. In individuals with no family history of CCM, no Malcavernin/CCM2 mutations were found in one report [66]. While initial studies suggested 40 % of patients with familial CCM may be linked to PDCD10 [55, 67], more recent studies suggest this might be an overestimate. In patients with no mutation in KRIT1 or Malcavernin/CCM2 by sequence analysis, PDCD10 sequence analysis found between 7 % and 40 % [46–49]. Since 20–40 % of individuals from families with CCM do not have mutations in KRIT1, Malcavernin/CCM2, or PDCD10, linkage analysis to the CCM4 locus may be warranted [48]. However, this is done on a research testing basis only.

Genetic Counseling

Since familial CCM is autosomal dominant, identifying CCM mutations necessitates investigation of both parents and siblings. Many patients diagnosed with CCM have an asymptomatic parent. Alternatively, an affected proband may have CCM resulting from a de novo gene mutation. How often this occurs has not been studied thoroughly, as only one de novo germline mutation has been reported [69]. Caution should be taken when there is a negative family history because of the failure to recognize the disorder (asymptomatic), a reduced penetrance in the parent with a disease-causing mutation, and the possibility of an early death of an affected family member. Screening of parents may be performed by MRI [70].

The risk to the siblings of a proband depends on the presence of mutations in the proband's parents. If a proband's parent has a mutation, there is a 50 % risk to the siblings of inheriting the mutation. Offspring of a proband also has a 50 % risk of inheriting the mutations. If a parent is found to have a disease-causing mutation, it is recommended that family members be tested. If a mutation is not detected in the parents, the risk to the sibling is low as germline mosaicism has not been reported. In this case, it is likely that the proband has a de novo mutation, although alternate possibilities such as an undisclosed parent may be possible as well. Prenatal testing and preimplantation diagnosis are available for pregnancies at increased risk or for family planning (Table 4.1).

Venous Malformations

Venous anomalies are slow-flow lesions characterized by abnormal vascular channels lined by flat but continuous endothelial cells and smooth muscle cells that can be immature and deficient in number [71]. Venous anomalies are categorized into sporadic venous malformation (VM), familial venous malformations-multiple cutaneous or mucosal (VMCM), and glomuvenous malformation (GVM). VM, VMCM, and GVM are clinically distinguishable with regard to

physical appearance, histology, genetic analysis, and management [71–74]. VM and VMCM are associated with gain-of-function mutations in receptor *TIE2*, whereas GVM is caused by loss-of-function mutations in the *glomulin* gene. These molecular signatures have allowed physicians to more accurately diagnose vascular anomalies and offer better managed treatment and care.

Sporadic Venous Malformation (VM) and Familial Cutaneous and Mucosal Venous Malformation (VMCM)

Most venous malformations (94 %) are sporadic vascular malformations (VMs) with unifocal (93 %) or multifocal (1 %) presentation. VMs are light to dark blue lesions that are compressible on palpation [72, 74]. They grow proportionally with the child and can be more invasive to the muscles, tissues, and organs [74].

Inherited VMCMs (MIM# 600195; Orpha.net #2451) are hereditary vascular malformations often characterized by small, multifocal lesions that have a bluish purple color [72]. VMCMs account for approximately 1 % of all vascular anomalies. VMCMs are also known as venous malformations-multiple cutaneous and mucosal, cutaneomucosal, and mucocutaneous venous malformations. VMCMs demonstrate autosomal dominant inheritance with incomplete penetrance. Therefore, each child of an affected individual will have a 50 % risk of inheriting the disease-causing mutation.

TIE2 Mutations in VM and VMCM

To date, receptor tyrosine kinase *TIE2* (also known as TEK) is the only gene known to be associated with VM and VMCM. *TIE2* is a receptor tyrosine kinase almost exclusively expressed in vascular endothelial cells (OMIM 600221). The *TIE2* gene maps to human locus 9p21 and consists of 23 exons that span over 121 kb of the genome. *TIE2* has a well-documented role in embryonic development, primarily through the binding of the growth factor ligand angiopoietin-1 (ANG1), which triggers downstream signaling pathways required for angiogenesis and vasculogenesis.

Mutations in *TIE2* have been identified in familial and sporadic VMCM cases. The most common mutation is the arginine to tryptophan substitution at position 849 (R849W) in the kinase domain of *TIE2*, with an incidence of 60 % [72]. The R849W *TIE2* mutation was identified by segregation in two different families with dominantly inherited vascular malformation [75]. Subsequent studies confirmed the R849W mutation and identified seven additional *TIE2* germline mutations in different families (Y897S, Y897C, R915H, R918C, V919L, A925S, K1100N) [76–78].

Eight somatic "second hit" alterations have also been found in lesions from individuals with multiple sporadic VM [79]. Six of these mutations are novel (Y897H, Y897F, L914F, R915C, R915L, S917I), while two were previously

identified in the germline of patients with VMCM (Y897S and Y897C) [76, 78]. The most common somatic mutation, L914F, is found alone and accounts for 85 % of lesions [72]. The other lesions contain pairs of double mutations in *cis* [72]. The identification of a second somatic mutation in the lesion suggests a paradominant mode of inheritance. Similar to R849W, all *TIE2* mutations are located in the tyrosine kinase domains of the receptor and result in ligand-dependent hyperphosphorylation [72, 78]. In vivo and in vitro studies demonstrate increased *TIE2* activity and ligand-dependent hyperphosphorylation in several mutations, including R849W [75, 76, 78, 79]. Thus, *TIE2* mutations associated with VMCM render a gain-of-function phenotype.

Molecular Genetic Testing

Clinical testing of the *TIE2* gene is available [80] (Table 4.1). Methods include mutation scanning and sequence analysis of variants. In certain cases, prenatal diagnosis may be available for affected families in which a disease-causing mutation has been identified [80]. Germline mutations in VMCM are identified by analysis of genomic DNA isolated from whole blood specimens of individuals with VMCM. To identify the presence of a second somatic mutation specific to the VMCM disorder, genetic analysis of the lesion is important for accurate diagnosis.

Glomuvenous Malformation (GVM)

GVMs are hereditary vascular malformations characterized by small, multifocal lesions with a cobblestone-like appearance that have a pink to purplish dark blue color [71, 72, 81] (MIM #138000; Orpha.net #83454). A venous malformation containing round mural "glomus cells" is diagnosed as a GVM. GVMs account for approximately 5 % of all vascular anomalies [72, 74]. GVMs are also known as venous malformations with glomus cells (VMGLOM), glomangiomatosis, familial or hereditary glomangiomas, and glomus tumors of the skin and soft tissue.

Glomulin Mutations in GVM

G*lomulin* (also known as *GLMN* and *FAP68*) is the only gene known to date that is associated with GVM. In two separate studies, 7 of 12 families with inherited GVM showed linkage to a region of 1.48 Mbp in the VMGLOM locus on chromosome 1p22-p21 [82, 83]. Positional cloning then identified *glomulin* as the gene within the VMGLOM locus that was responsible for GVMs [84, 85]. The *glomulin* gene consists of 19 exons spanning over 55 kb of the genome and encodes a 594-amino

acid protein (also known as FAP68). Previous studies had cloned the incomplete *glomulin* cDNA sequence encoding a predicted 417-amino acid protein of 48 kDa, which is also known as FKBP-associated protein (FAP48) [86].

GVMs demonstrate an autosomal dominant mode of inheritance with incomplete penetrance. Accordingly, the child of an affected individual has a 50 % chance of inheriting the germline mutation. Current evidence supports the hypothesis that all GVMs are caused by loss-of-function mutations in the *glomulin* gene [73, 81]. More than 30 different *glomulin* mutations have been identified in >100 GVM families [81, 87–90]. Eight glomulin mutations are common (i.e., 108C>A, 157delAAGAA, 554delA+556delCCT, and 1179delCAA) and found in >75 % of patients with GVM [88]. There also appears to be a strong founder effect [72, 81]. In a small percentage of patients, a second somatic mutation in *glomulin* (*in trans*) is also seen in combination with the inherited (germline) *glomulin* mutation, suggesting a paradominant mode of inheritance. The remaining mutations have been identified in only one GVM family to date [81]. It appears that all *glomulin* mutations result in truncated glomulin protein, except for one (1179delCCA), which results in a deletion of an arginine at position 394 [81].

Neither in vitro nor in vivo studies have been performed to demonstrate the specific type of functional loss for *glomulin* or definitively determine specific genotype-phenotype associations. This is because the function of *glomulin* remains poorly understood. Furthermore, clinical correlations between specific *glomulin* mutations and the location, extent, and number of vascular lesions have not been made [91]. It is known that glomulin is predominantly expressed in vascular smooth muscle cells (VSMCs) [92]. Glomus cells of GVMs are histologically characterized as abnormally differentiated VSMCs [93], suggesting *glomulin* is involved in VSMC differentiation and its functional loss results in the immature glomus cell phenotype seen in GVM [84].

Molecular Genetic Testing

Clinical testing for GVMs via mutation screening of the GLVM gene is available (Table 4.1). Platforms include analysis of the entire coding region by sequencing and analysis of the entire coding region by mutation scanning. Germline mutations in *glomulin* are identified by analysis of genomic DNA isolated from whole blood specimens. Analysis of genomic DNA from resected tissue is important to identify the presence of a second somatic mutation specific to the GVM lesion. At least one laboratory offers prenatal diagnosis for a known *glomulin* mutation.

Lymphatic Malformations and Lymphedemas

Lymphatic malformations (LM) are focal or extensive lesions of dilated lymphatic channels with abnormal connection to the lymphatic system [88]. They are a major component feature in patients with congenital lipomatous overgrowth with vascular,

epidermal, and skeletal anomaly (CLOVES) syndrome and Klippel-Trenaunay syndrome (KTS). Lymphatic malformations occur sporadically, suggesting the presence of somatic mutations. Recently, five somatic mutations in phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha (PIK3CA) have been identified in patients with lymphatic malformations, CLOVES, KTS, and fibroadipose vascular anomaly (FAVA) [94, 95].

Lymphedemas are characterized by a defect in lymphatic drainage, which results in accumulation of lymphatic fluid in the interstitial space and severe swelling of the lower extremities [72, 88]. Lymphedemas can be sporadic or inherited as an incompletely penetrant autosomal dominant trait or recessive form (see Table 4.1). Studies in families with inherited forms of lymphedema have identified several genes causing the disorders. Nonne-Milroy syndrome is an early onset, primary congenital form of hereditary type I lymphedema that is autosomal dominantly inherited. Loss-of-function mutations in *FLT4/VEGFR3* at chromosome location 5q34-35 are associated with autosomal dominant Nonne-Milroy syndrome, as well as recessively inherited lymphedemas [96, 97]. A mutation in VEGFC, a ligand for VEGFR3, has also been identified in a family with clinical signs resembling Nonne-Milroy syndrome [4]. Lymphedema distichiasis is a syndromic form of primary lymphedema, which is an autosomal dominant disease caused by loss-of-function mutations in *FOXC2* located on chromosome 16p24 [98]. Hypotrichosis lymphedema telangiectasia (HLT) syndrome is inherited as a recessive or dominant trait. Nonsense and homozygous mutations in *SOX18* on chromosome 20q13.33 are responsible for the HLT disorder [99]. More recently, GJC2 (encoding connexin 47) missense mutations were also observed to cause primary lymphedema [100]. The genetic cause and/or locus has been identified for other more rare forms of familial lymphedema. These include *CCBE1* on chromosome 18q21.32, *NEMO* on chromosome Xq28, genetic locus 6q16.2-q22.1, and chromosome 15q, which are associated with Hennekam syndrome, OLEDAID, primary congenital resolving lymphedema, and hereditary lymphedema cholestasis (HLC), respectively (Aagenaes syndrome) (Table 4.1) [101–104]. PTPN14, GATA2, and KIF11 germline mutations are also associated with lymphedemas in choanal atresia, Emberger syndrome, and microcephaly chorioretinopathy, respectively [2].

Vascular Tumors

Vascular tumors are cellular, often proliferative vascular anomalies including infantile hemangiomas (IHs), congenital hemangiomas (RICH and NICH), kaposiform hemangioendotheliomas, tufted angioma, spindle cell hemangioendotheliomas, as well as other rare hemangioendotheliomas and dermatologic acquired vascular tumors [105]. Infantile hemangiomas are the most common tumors of infancy, affecting approximately 4 % of all children and are far more common than vascular malformations. There are also several clinical subtypes and syndromes associated with IHs. An example is PHACE(S) syndrome (posterior fossa malformations, hemangiomas, arterial anomalies, coarctation of the aorta and

cardiac defects, eye abnormalities and sterna defects), which involves facial IHs in association with other anomalies [106, 107]. Hemangiomatosis, PELVIS syndrome, and SACRAL syndrome are other examples [105].

Infantile hemangiomas (OMIM #602089) are comprised of rapidly proliferating endothelial cells that typically appear shortly after birth, grow rapidly during the first year, and then slowly involute over a period of several years. Although all infantile hemangiomas spontaneously regress to a significant degree, most leave clinically significant sequelae, and some are life-threatening. Clinically useful therapeutic options include systemic or local corticosteroids, surgical excision, laser therapy, vincristine, immunomodulatory therapy, and most recently, treatment with beta-blockers such as propranolol. Mechanisms of most medical therapies are largely unknown, and no therapies are universally successful. Recent advances in understanding basic pathogenetic mechanisms yield hope for more effective, specifically targeted therapies.

Diagnosis of Vascular Tumors

Most vascular tumors are differentially diagnosed by their presentation, growth pattern, histological profile, and use of MRI to determine the density and high-flow patterns [105]. Infantile hemangiomas are characterized by strong endothelial GLUT-1 protein expression, which is not seen in other vascular tumors or in normal skin vasculature [108].

The genetics of infantile hemangiomas is poorly understood, although linkage analysis and microarray and cellular studies have identified several important pathogenetic features and candidate genes that may contribute to predilection for the disease. Genome-wide linkage analysis of three unrelated cases of infantile hemangioma led to the identification of a disease locus on chromosome 5q31-33 containing candidate genes FGFGR4, PDGFR-β, and VEGFR3 (Flt-4) [109]. In addition, somatic mutations in the genes VEGFR2 (P1147S), VEGFR3/FLT4 (P954S), TEM8, and DUSP5 genes have been identified in hemangioma tumor tissue [109]. The link between these mutations and disease is not established. It has been hypothesized that germline mutations in TEM8 and KDR represent risk factor mutations for IH and that the combination of these mutations with a secondary somatic hit may trigger the expansion of hemangioma-derived endothelial cells within the lesions [105].

More recent discoveries have been made for other vascular anomalies. For example, a somatic missense mutation (c.1323C>G, p. Iso441Met) in mitogen-activated protein kinase kinase kinase 3 (MAP3K3) has been identified in verrucous venous malformations (verrucous hemangioma) [110]. In addition, the presence of YAP1-TFE3 and WWTR1-CAMTA1 gene fusions has been reported in subsets of epithelioid hemangioendothelioma (EHE) [111, 112], which is a rare malignant soft tissue tumor of variable grade [113].

Somatic mutations in IDH1 or IDH2 have been identified in patients with Maffucci syndrome [114–118]. Maffucci syndrome is a rare congenital disorder characterized by multiple central cartilaginous tumors (enchondromas), similar to

Ollier disease, but with multiple cutaneous spindle cell hemangiomas (SCH) [115]. Common heterozygous point mutations include R132C and R132H in IDH1, as well as the R172S mutation in IDH2. Additional studies found that frequent somatic alterations in 2p22.3, 2q24.3, and 14q11.2 may play a role in causing enchondroma and SCH in patients with Maffucci syndrome [115]. Clinical testing is available for IDH1 and IDH2.

Pharmacogenetics and Treatment of Vascular Anomalies

Managed treatment of vascular anomalies often involves drug therapy, which can cause mild to severe adverse side effects. For example, propranolol is a nonselective beta-adrenergic blocker commonly used to treat cardiac disorders that has gained recent attention in the vascular anomaly community for its off-label use in the treatment of infantile hemangiomas [119–124]. An initial improvement in the size and color of the hemangiomas is observed and the effect is proposed to be secondary to the factors that affect angiogenesis. However, there are known side effects in some patients treated with propranolol, including hypoglycemia, hypothermia, and bradycardia [125].

Pharmacogenetics uses an individual's genotype to help predict drug response, efficacy, and potential adverse drug events. As such, pharmacogenetics could be applied to the IH population during drug therapy. Propranolol is one of the drugs from the top 200 list eliminated by cytochrome P450 (CYP450) enzymes, which contain single-nucleotide polymorphisms (SNPs) that alter enzymatic function [126, 127]. For example, propranolol is metabolized by CYP2D6 and CYP1A2 and enzymes contain SNPs associated with decreased enzymatic activity [128, 129]. Genetic polymorphisms in the β1 and β2-adrenergic receptors, targets of propranolol, have also been identified that affect gene expression, protein function, and response to beta-agonists [130, 131]. Therefore, individuals with SNPs in metabolizing enzymes and/or receptor targets could have an altered drug response to therapy. Pharmacogenetic testing has the potential of offering a direct application to the vascular anomalies population for optimizing treatment and reducing side effects. However, while pharmacogenetic testing is available in several reference and hospital-based laboratories, application to the vascular anomaly population is new and requires additional studies.

PTEN Hamartoma Tumor Syndrome

We have included the PTEN hamartoma tumor syndrome (PHTS) because of the emerging understanding that vascular anomalies are a distinct and common finding in the spectrum of PHTS disease [132]. Vascular anomalies in patients with PTEN

mutations typically manifest as multifocal intramuscular combinations of ectopic fat and fast-flow channels with cerebral developmental venous anomalies particularly common [132]. Arteriovenous malformations have been described in Cowden syndrome [133], which may be due to PTEN's regulation of vascular development and angiogenesis by directly regulating VEGF and other angiogenic factors [134]. So even though small abnormal tumors on the skin and in critical organs dominate the clinical diagnosis of PHTS spectrum diseases, vascular anomalies are a critical feature of this group of diseases.

PTHS is a group of disorders with mutation in the PTEN gene and includes (1) Cowden syndrome (CS), (2) Bannayan-Riley-Ruvalcaba syndrome (BRRS), (3) *PTEN*-related Proteus syndrome (PS), and (4) Proteus-like syndrome. All four of these diseases have in common the presence of hamartomas or abnormal formation of benign tissue "tumors." These "tumors" cause problems primarily due to their location, particularly when they are located on the face and neck, where they can cause significant disfigurement. While these abnormal growths can obstruct any organ in the body, they tend to cause the most health problems when they are located in the hypothalamus, spleen, or kidneys. PTHS must not be confused with Proteus syndrome, which is a rare complex disease characterized by disproportionate bony and soft tissue overgrowth caused by mutations in AKT1. AKT1 activity is regulated by PTEN, so both proteins are involved in intracellular PI3K/Akt signaling [3].

The diagnosis of PTEN hamartoma tumor syndrome (PHTS) is based on specific clinical findings. However, the diagnosis of PHTS is made only when a disease-causing mutation in PTEN is identified. Diagnostic criteria have been developed for Cowden syndrome [135, 136]. Diagnostic criteria for BRRS have not been developed, but are based largely on the cardinal feature of microcephaly, hamartomatous intestinal polyposis, lipomas, and glans penis pigmented macules [137]. *PTEN-related* Proteus syndrome (PS) is widely variable and generally has a mosaic distribution. The manifestations present generally at birth and include cystadenoma of the ovary, various types of testicular tumors, CNS tumor, and parotid monomorphic adenomas. Only 120 people with PS have been identified [138] and diagnostic criteria have been developed by Biesecker et al. [139]. While diagnostic criteria have not been developed for Proteus-like syndrome, it generally includes patients with features of Proteus syndrome, who do not meet the criteria [140].

The phosphatase and tensin homolog (PTEN) protein is a tumor suppressor protein encoded by the *PTEN* gene. PTEN is part of a subclass of phosphatases that have dual activities which remove phosphates from tyrosine as well as serine and threonine. PTEN is critical to the de-phosphorylation of phosphoinositide-3,4,5-triphosphate, resulting in a down-regulation of the downstream Akt signaling pathway. PTEN traffics in and out of the nucleus [141–143] and when in the nucleus inhibits MAPK signaling resulting in an arrest of the cell cycle [144, 145]. In the cytoplasm, PTEN's activity primarily stimulates Akt activity to induce apoptosis [146]. Germline mutations have been identified in exons 1–8 (of 9 total) and include missense, nonsense, splice-site mutations, insertions, and large deletions [147–149]. Approximately 40 % of mutations have

been reported in exon 5 [136]; however, most mutations are unique, but recurrent mutations have been found [147–149].

Clinical molecular testing for disease-causing mutations in PTEN is most commonly performed by analysis of the entire coding region by sequencing (Table 4.1). Many laboratories also perform molecular analyses to identify deletions and duplications, which can be missed by sequencing. Currently, at least one laboratory focuses sequencing on select regions of the PTEN gene in addition to performing FISH-metaphase analysis of mutations (Table 4.1).

Sequence Analysis of PTEN

Nearly all of the missense mutations identified in PTEN are deleterious [136]. Nearly 85 % of patients who meet criteria for Cowden's syndrome and 65 % with a diagnosis of BRRS have a PTEN mutation [149–151]. PTEN mutations are found in 20 % of patients with Proteus syndrome and 50 % of those with a Proteus-like syndrome [136, 148, 152–154]. Another study found that no PTEN mutations could be identified in patients diagnosed with Proteus-like syndrome [155]. These studies suggest that either other genes are involved or else mutations in introns/splice sites or promoters were present and not detected using the methodologies applied. Additionally, deletion and duplications could have been present and not detected. On a research basis, direct sequencing of the promoter regions of PTEN can be performed, which alters the function of the PTEN gene product. Approximately 10 % of CS patients have promoter mutations and no other mutations identified in the coding region [151].

Identification of Deletions and Duplications in PTEN

Individuals with CS who have large deletions have been reported [156]. Additionally, up to 10 % of BRRS patients do not have a mutation that can be detected in the PTEN exons due to deletions within PTEN [151]. Real-time PCR, Southern blotting, Multiplex ligation-dependent probe amplification (MLPA), and other methods that can detect gene copy can be used to determine PTEN deletions or other rearrangements, which PCR-based sequencing is unable to detect.

Non-PTEN Gene Mutations Affecting Susceptibility to Disease

In addition to PTEN mutations, recent early studies have identified mutations in succinate dehydrogenase complex subunit B (SDHB) and succinate dehydrogenase complex subunit D (SDHD), the genes encoding the mitochondrial complex II

protein succinate dehydrogenase, as well as the KLLN (killin) gene in individuals with CS and CS-like disorder [157, 158].

Testing Strategies

Ideally, sequence analysis of PTEN exons 1–9, including the flanking introns, is performed to confirm the diagnosis in a proband (see Table 4.1). Analysis of the PTEN promoter region can be performed next (on a research basis), followed by deletion and duplication analysis (see Table 4.1). Lastly, mutations in the mitochondrial complex II protein succinate dehydrogenase (SDH) have been described in PTEN mutation-negative individuals with CS-like cancers [157]. So sequence analysis of SDH may be warranted, which is available on a research basis for other diseases (testing for SHD B performed for hereditary paraganglioma-pheochromocytoma syndromes).

Differentiating Genetically Related Disorders

One of the challenges of genetically diagnosing Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome (BRRS), *PTEN*-related Proteus syndrome (PS), and Proteus-like syndrome is that mutations in PTEN underlie other diseases with diametrically opposed phenotypes. No other phenotypes other than CS, BRRS, PS, and Proteus-like syndrome are consistently caused by PTEN mutations. Adult-onset Lhermitte-Duclos disease (LDD) can be attributed sometimes to PTEN mutations and characterized by dysplastic gangliocytoma of the cerebellum due to the overgrowth of hamartomas, which can be seen in CS/BRRS. However, PTEN mutations are rarely seen in the LDD form of disease, which presents in childhood [159]. Similarly, PTEN mutations have been reported in ~20 % of people with autism/pervasive developmental disorder and macrocephaly [160, 161]. Several groups have now confirmed this PTEN mutation in 10–20 % of people with autism/pervasive developmental disorder and macrocephaly [161–164].

Genetic Counseling

Patients with PTEN hamartoma tumor syndrome (PHTS) should be advised that the disease is autosomal dominant, so that testing of family members should be recommended. However, since disease is both sporadic and familial, some individuals with CS have no family history, with a rough estimate of 10–50 % having an affected parent [165]. If a proband has an identifiable PTEN mutation, their parents should undergo a clinical evaluation and be tested to identify if PHTS is present.

Testing of proband siblings depends on the genetic status of the parents. If a parent has a PTEN mutation, siblings should be tested since this is an autosomal dominant disease. If neither parent has a PTEN mutation that is identifiable, the proband siblings do not need to be tested. The risk of disease in proband siblings is minimal since mosaicism in the germline has not been reported in PHTS [140]. If the proband does not have an identifiable PTEN mutation, PHTS can be excluded by clinical evaluation of the sibling. Each offspring of a proband has a 50 % chance of inheriting the mutation and developing the PHTS. Therefore, genetic testing should be performed on all offspring of a proband. Prenatal testing is available from many laboratories (see Table 4.1).

Challenges and Future of Molecular Testing

Molecular Diagnostic Challenges

While the genetic cause for several inherited forms of vascular malformations is known, significant challenges facing the field include elucidating the etiology of sporadic forms, identifying somatic mutations that cause rare disorders, and developing therapies [88]. For example, TIE2 mutations account for 40–50 % of VMs, but additional mutations or predisposing genes responsible for the remaining cases are unknown [72]. Another problem is that the genotype-phenotype correlations are not well understood for many disorders, often because the function of the predisposing gene is not known (i.e., *glomulin* mutations associated with GVM). This hinders an understanding of the mechanism by which specific mutations cause disease and the discovery of therapeutic treatments. Finally, a major difficulty within the field is that molecular diagnostic testing for many vascular anomalies is not yet commercially available. Molecular tests that do currently exist are, in most cases, on a research basis only and many of these labs are located in European countries. Furthermore, insurance companies are not often willing to reimburse for genetic testing and turnaround times can be lengthy. This makes testing difficult, cumbersome, and costly for many families. The promising news for the field is that clinical laboratories can provide laboratory developed tests (LDTs) using standard, cost-effective technologies, such as sequencing, allele-specific PCR, and restriction enzymatic digestions [166]. Furthermore, high-density SNP arrays and next-generation sequencing provide the opportunity for high-throughput genotyping, which revolutionizes the way novel and known mutations associated with disease are identified and diagnosed. Advances in sequencing technology have led to a lower error rate, longer read length, and more robust performance, which far exceeds that of standard sequencing using Sanger methodology [167]. The application of next-generation sequencing to vascular disease has the potential to rapidly screen every gene in an affected individual and identify the mutation(s) associated with that particular vascular lesion. The laboratory is also able to identify novel mutations and provide more accurate diagnosis

using technology that reduces tissue heterogeneity. Advances in automated extraction technology have allowed the lab to rapidly extract high-quality DNA from various pathology tissue specimens, both fresh/frozen and formalin-fixed paraffin-embedded (FFPE). Laser-capture microdissection also offers the capability of obtaining lesion-derived material and isolating a specific cell population for subsequent genotype analysis. Such analysis would require punch biopsy or surgical resection of the affected tissue. A limitation lies in the ability to isolate a sufficient number of cells for DNA, mRNA, or protein analysis. Cell culture expansion of isolated cell types is an alternative solution if an inadequate amount of tissue is not available for analysis. However, proper diagnosis based on mRNA on protein analysis will depend on the cells maintaining their phenotype once cultured.

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