The Genetic Basis of Vascular Anomalies

3

Ha-Long Nguyen, Laurence M. Boon, and Miikka Vikkula

Introduction

Vascular anomalies are localized lesions that arise from aberrant regulation and establishment of the vasculature. There are two categories of vascular anomalies: vascular tumors and malformations. Vascular tumors are characterized by an overactive endothelium and consist mainly of hemangiomas. The endothelium of vascular malformations is more quiescent, and this category comprises a large number of subtypes. Based on clinical, radiological, and histological evaluations, they are subdivided according to the vessel type affected as venous, arteriovenous, capillary, lymphatic, and combined lesions (e.g., capillary-venous malformation). Additionally, vascular malformations may be a major or minor part of the phenotype in syndromes. For example, in Klippel-Trenaunay syndrome (KTS), extensive capillary-lymphatico-venous malformation (CLVM) is associated with hypertrophy, whereas in PTEN hamartoma tumor syndrome (PHTS), variable defects, from macrocephaly to penile freckles, are accompanied by abnormally vascularized lesions.

The mode of development (familial vs. sporadic) of each vascular anomaly varies widely; however, knowledge of this trait influences the treatment regimen and patient education provided. In some cases, the most frequent form of the disease is familial (e.g., glomuvenous malformation and hereditary hemorrhagic telangiectasia). Therefore, special consideration must be given to evaluation of risk

H.-L. Nguyen
Human Molecular Genetics, de Duve Institute, Brussels, Belgium

L. M. Boon · M. Vikkula (✉)
Human Molecular Genetics, de Duve Institute, Brussels, Belgium

Center for Vascular Anomalies, Cliniques Universitaires Saint-Luc, Catholic University of Louvain, Brussels, Belgium
e-mail: miikka.vikkula@uclouvain.be

C. C. Trenor III, D. M. Adams (eds.), *Vascular Anomalies*,
https://doi.org/10.1007/978-3-030-25624-1_3

to develop the disease in a (not-yet-born) family member and management of follow-up to detect newly developing lesions. In other instances, the disease seems to occur exclusively sporadically (e.g., KTS or CLVM; Sturge-Weber syndrome; lymphatic malformation). Thus, management is largely done on a case-by-case basis. For the majority of vascular anomalies, the lesions predominantly appear sporadically, though a variable fraction (1–20%) may be familial forms (e.g., capillary malformation, venous malformation, cerebral cavernous malformations).

The extent of disease most likely reflects the level of dysregulation caused by the mutations. Weak effects can be present in all cells and from the single cell stage onward (as in germline mutations of inherited forms), whereas mutations with strong effects are likely incompatible with life in the heterozygous stage. This may be due either to embryonic vascular defects critical to early embryogenesis or to more widespread defects if present in all cell types and not only restricted to a limited number/type of cells (e.g., endothelial cells).

The familial or inherited vascular anomalies were crucial in providing the first insights into the pathogenesis of vascular anomalies. The first somatic mutations were identified in sporadically occurring venous malformations. They were point mutations in the TIE2 receptor encoding gene *TEK* [1] (Fig. 3.1). This was based on traditional Sanger sequencing of DNA extracted from resected lesions. As less than 50% of the alleles were mutant (often only around 10%, the minimum detection level of Sanger sequencing), RNA-based screens were done to enrich for the endothelial-specific TIE2 transcripts. This allowed enhanced sensitivity for mutation detection and pointed out the heterogeneity of the causative mutation within affected tissues. It also highlighted the need for extremely sensitive mutation detection in various vascular anomaly tissues to find causative (or associated) changes [2].

With the advancement of massively parallel sequencing, it is now possible to perform high-throughput screens on tissue samples. Targeted panels allow sequencing thousands of times a limited number of genes (high vertical coverage) and result in a sensitive detection rate of 1% for mutant alleles. In tandem, digital droplet PCR (ddPCR) does the same in a performance- and cost-efficient manner for known mutations, on the basis of specific probes synthesized for each variant to be tested. This vastly improved the ability to reveal causative or associated nucleotide variants (mutations) in lesional tissues that account for most of the venous, capillary, lymphatic malformations, and arteriovenous malformations.

The different types of vascular lesions are now known to be largely due to disrupted (endothelial) receptor intracellular signaling pathways. Mutations have been identified in proteins such as receptor tyrosine kinases, G-protein-coupled receptor adaptor molecules, and various proteins involved in the PI3kinase(K)–AKT, MAPK, and SMAD signaling pathways (Fig. 3.1). This has opened a new era of potential for the management of vascular anomalies that could only be dreamed of 25 years ago, when all these studies were initiated. We can now start to hypothesize, develop, and test targeted molecular therapies, and the first reports show promising efficacy in ameliorating patients' quality of life [3, 4].

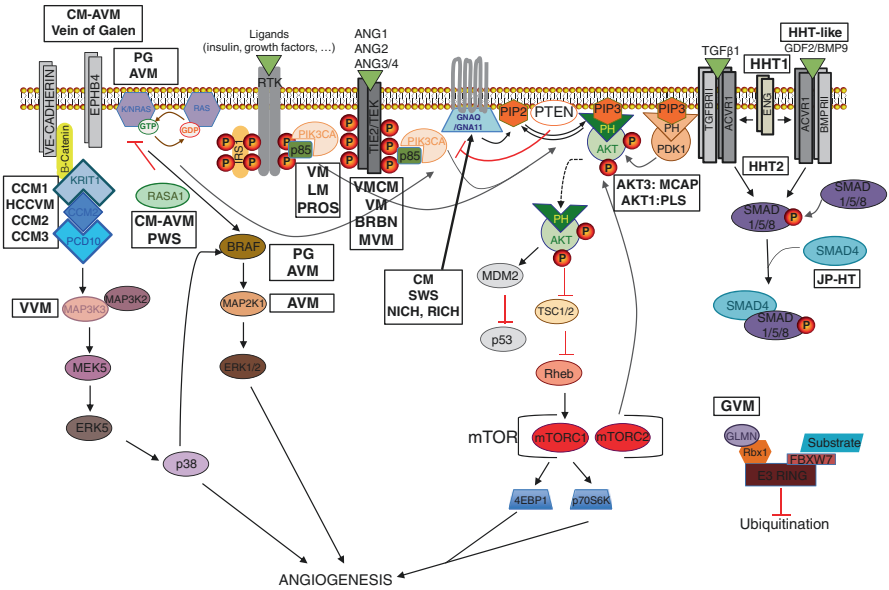

Fig. 3.1 Summary of vascular anomalies and associated syndromes that are linked to molecules of essential signaling pathways within the vasculature. Mutations within genes that code for proteins that are involved in the MAPK pathways have been found for capillary malformation (CM), arteriovenous malformation, AVM, CM-AVM, Vein of Galen, cerebral cavernous malformation (CCM)1–3, hyperkeratotic cutaneous capillary-venous malformation (HCCVM), verrucous venous malformation (VVM), pyogenic granuloma (PG), Parkes-Weber syndrome (PWS), Sturge-Weber syndrome, rapidly involuting congenital hemangiomas (RICH), and noninvoluting congenital hemangiomas (NICH). Meanwhile, for venous malformation (VM), lymphatic malformation (LM), PIK3CA-related overgrowth spectrum (PROS), mucocutaneous VM (VMCM), multifocal VM (MVM), blue rubber bleb nevus syndrome (BRBN), megalencephaly-CM (MCAP), and Proteus syndrome (PLS), the PI3K/AKT signaling is affected. Hereditary hemorrhagic telangiectasia (HHT) results from dysregulation of TGF-β superfamily signaling, more specifically via the BMP ligands. Glomuvenous malformations (GVM) are linked to glomulin, a protein that may have a more generalized role as a part of the machinery involved in FBW7-mediated protein ubiquitination and degradation

Venous Malformations

Venous anomalies exemplify the benefits of revealing causative mutations. There are at least five distinguishable entities: the autosomal dominantly inherited glomuvenous malformation (GVM), mucocutaneous venous malformation (VMCM; MIM 600195), sporadically occurring venous malformation (VM; MIM 600221), multifocal venous malformation (MVM), and blue rubber bleb nevus syndrome (BRBN; MIM 112200). GVMs account for about 5% of venous anomaly patients, and there is a clear family history. GVMs are classically present as multifocal small dark-blue cutaneous lesions, which are usually hyperkeratotic [5]. Rarer VMCM is

also characterized by multifocal lesions that are lighter in color, more often subcutaneous and mucosal, and easily compressible on palpation. D-dimers are also elevated. From the three sporadically occurring phenotypes, the rare MVM mimics VMCM, yet lacks a family history. The other two sporadically occurring phenotypes, BRBN and VM are distinct entities. BRBN includes rubbery elevated lesions that are often hyperkeratotic and develop frequently on the hands and soles of the feet. Affected patients are usually born with a *dominant* lesion and grow multiple, up to hundreds, of tiny cutaneous BRBN lesions over time [6]. Gastrointestinal lesions that are prone to hemorrhaging can also form and increase in number with time; thus anemia from chronic blood loss is also prevalent. VM is the most common entity, accounting for roughly 95% of patients with venous malformations. The lesions are unifocal and vary from tiny punctate lesions to large infiltrating ones that can cover an entire extremity. Over 40% of VMs have elevated D-dimers [7].

Identification of genetic mutations has helped better define the signs and symptoms associated with each of these clinical entities. Genetic testing can help classify these patients. VMCM, VM, MVM, and BRBN can all be caused by activating mutations in *TEK*/*TIE2* (MIM 600221), a vascular endothelial-specific receptor essential in angiogenesis and vascular maturation (Fig. 3.1). All mutations that have been discovered are within the intracellular part of the receptor (the kinase and kinase insert domains or the carboxy-terminal tail) and lead to increased ligand-independent receptor autophosphorylation. The predominant mutation in VMs (of which 60% are due to a TIE2 mutation) is a somatic leucine 914-to-phenylalanine (L914F) change [1, 8]. In contrast, the most common inherited mutation in VMCM is an arginine 849-to-tryptophan substitution (R849W) [1, 6]. This weakly activating germline mutation needs somatic second hits in TIE2 to induce lesion formation [1, 6]. MVM patients have an underlying *de novo* mosaic mutation detectable in their blood, and superposed somatic mutations, often occurring on the same allele (in cis) [6]. Double cis somatic changes in TEK are also detected in BRBN. As they have equal frequencies and identities in separate lesions from distant sites within the same patient, activated dissemination of lesions seems to occur in these patients.

PI3K/AKT signaling is the canonical TIE2 signaling pathway, and it is also affected downstream in response to the overactive TIE2 (Fig. 3.1). Human umbilical vein endothelial cells (HUVEC) transfected with the mutations resulted in dysregulation of angiogenic factors linked to this pathway and abnormal EC morphology. This opened the possibility to use sirolimus, which inhibits mTOR downstream of PI3K/AKT signaling, to counteract the effects with very promising preclinical and Phase II clinical data [4, 9]. The importance of the deregulation of this pathway in VM pathogenesis was underscored by the recent discovery that another 20% of VMs are due to activating PIK3CA mutations [10] (Fig. 3.1). These mutations cause inappropriate PI3K activation, and *in vitro* treatment of PIK3CA- and TIE2-mutant cells with a PI3K inhibitor, BYL719, normalized the cells [10]. Thus, this signaling pathway has become a favorable target (in particular PI3K, AKT, and mTOR) for therapeutic intervention. Consequently, Phase III clinical trials have begun using sirolimus. Sirolimus effectively ameliorates patients' quality of life as symptoms become less pronounced, and early data demonstrate reduction in lesional

volume in some patients (Boon et al., unpublished). Similar effects were seen in a compassionate use BYL719 study in PIK3CA-related overgrowth syndromes associated with a vascular anomaly [11].

Glomuvenous malformations (GVM) are caused by loss-of-function mutations in the *glomulin* gene (GLMN; MIM 601749) [12] (Fig. 3.1). The most common change is found in 45% of patients, yet most of the mutations are specific to a single family [13]. Forty different mutations have so far been reported in 162 GVM families. Akin to VMCM, the multifocality of GVM is explained by the principle of paradominant inheritance. The most common "second hit" is an acquired uniparental isodisomy [14]. The exact function of GLMN, particularly in the vasculature, is unknown. It appears to play a major role in regulating differentiation of vascular smooth muscle cells (vSMC), based on the specificity of the GVM phenotype. In vitro studies suggest that it may interact with the transforming growth factor-β (TGF-β) and hepatocyte growth factor (HGF) signaling pathways [15–17]. GLMN may also have a more generalized role in FBW7-mediated protein ubiquitination and degradation [18].

Arteriovenous Malformations

Arteriovenous malformations (AVM) have fast arterial flow, making them more progressive and destructive than other vascular anomalies. They have a strong angiogenic potential, as partial resection often leads to severe deterioration of the lesion with time. The majority of AVMs arises sporadically and may affect any organ. Yet, AVMs are also a prominent feature in patients affected by two inherited disorders: hereditary hemorrhagic telangiectasia (HHT; MIM 187300, 600376, and 175050) and capillary malformation-arteriovenous malformation (CM-AVM; MIM 608354 and 618196).

HHT and CM-AVM have incidences estimated at 1/5000, and 1/10,000, respectively. Based on the identified genes, two major signaling pathways are involved in pathogenesis: TGF-β/SMAD (HHT) and MAPK/ERK (CM-AVM) pathways. However, despite the AVMs having overlapping clinical characteristics, mechanistic cross talk between the two pathogenic pathways regulating angiogenesis has remained elusive.

In HHT, a diagnosis is made by two methods: (1) clinically, if a patient presents with at least three of the four Curaçao criteria, or (2) by using genetic testing, as long as the patient carries a mutation in one of the known 3 HHT genes [19]. There are two other types of HHT, mapped to chromosomes 5 (HHT3; MIM 601101) and 7 (HHT4; MIM 610655) [20, 21], but the causative genes have yet to be identified. The known genes encode proteins of the TGF-β signaling superfamily: the endothelial co-receptor endoglin (ENG; MIM 131195) (HHT1; MIM 187300), the type I receptor activin kinase-like-1 (MIM 601284) (ACVRL1/HHT2; MIM 600376), and the intracellular co-mediator SMAD4 (MIM 600993) [22, 23] (Fig. 3.1). About 80% of patients have a mutation in the first two genes. Over 500 variants for each HHT1 and HHT2 have been reported, with pathological ones (~67 and 50%,

respectively) leading to loss of function [24]. Patients that possess SMAD4 mutations are affected with a combined syndrome of HHT and another autosomal dominant disorder, juvenile polyposis (JP-HT; MIM 175050) (Fig. 3.1). These patients have polyps in the gastrointestinal tract [25].

The underlying pathogenic mechanism for HHT remains controversial. It appears that the bone morphogenetic pathway (BMP) is likely perturbed. It is evidenced that the ligands that bind ACVRL1 are BMP9 and BMP10 [26]. This is supported by the finding that three patients exhibiting HHT-like symptoms harbor mutations in the gene coding for BMP9/GDF2 (MIM 605120) (HHT5; MIM 615506) [27]. Several drugs currently used to treat other diseases are able to alleviate the life-impeding symptoms of HHT, for example, thalidomide, tranexamic acid, and bevacizumab [28–32]. However, how these drugs act in HHT has not been fully explored.

As in the VMs, the severity of symptoms unpredictably varies among HHT patients, even for those in the same family and mutation. Though a somatic second hit has not been confirmed in patients, mouse models have suggested the combination of three hits to contribute to lesion progression. Conditional knockout of *Acvr1* and *Eng* in adult mice reliably formed AVMs when given a pro-angiogenic stressor, such as wounding or treatment with LPS or VEGF [33–38]. The mouse models suggest that in addition to the predisposing germline mutation and complete localized loss of the gene function, a pro-angiogenic environmental element strongly contributes to lesion formation.

Germline mutations also occur in CM-AVM, either in the RASA1 or the EPHB4 gene, and result in loss of function. The affected gene for CM-AVM1, RASA1 (MIM 139150), encodes the GTPase-activating protein p120-RasGAP [39–41] (Fig. 3.1). The loss of RASA1 causes hyperactivation of the RAS/MAPK signaling pathway, resulting in altered cell proliferation, differentiation, and growth. Mice homozygous for p120rasGap loss die during embryogenesis, whereas embryos mosaic for wild-type and p120RasGap-null cells generate abnormal cutaneous vessels [42]. Additionally, a somatic second hit has been identified in lesional tissue from three CM-AVM1 patients, (1) in a Parkes-Weber syndrome, (2) in a capillary malformation, and (3) in an arteriovenous malformation, underscoring the necessity of complete localized loss of function of p120rasGap for lesions to develop [40, 43, 44].

CM-AVM2 is caused by alterations in the EPH receptor B4 (EPHB4) (MIM 600011), which, along with its ligand Ephrin B2 (EFNB2), plays a major role in arteriovenous differentiation [45, 46]. EPHB4 mutations were also found in sporadic Vein of Galen aneurysmal malformations, which are a subtype of cerebral AVMs [47, 48]. In zebrafish, RASA1 functions downstream of EPHB4, and knocking down either led to enlarged vessels and arrested blood flow [49].

In sporadically occurring AVMs, activating mutations in the KRAS (MIM 190070), NRAS (MIM 164790), BRAF (MIM 164757), and MAP2K1 (MIM 176872) have been found [50–52], further implicating the MAPK signaling in the development of AVMs. The majority of the discovered hotspot mutations are the same as those commonly seen in various types of cancers (e.g., KRAS

glycine-12-to-aspartic acid (G12D)). Thus, it raises the possibility to repurpose currently used therapeutic cancer drugs for treatment of AVMs. A preclinical model showed promise as blood flow was normalized in zebrafish AVM models treated with a BRAF inhibitor, vemurafenib [51].

Capillary Malformations

Capillary malformations (CM; MIM 16300) are the most common vascular malformations, with a reported incidence of 0.3% [53]. They appear sporadically, except in the CM-AVM described above. CM can be an isolated cutaneous lesion or associated with leptomeningeal vascular anomalies (Sturge-Weber syndrome). In both cases, somatic changes in the guanine nucleotide-binding protein G (GNAQ; MIM 600998) are attributed to the disorder, with the most frequent variant being an arginine183-to-glycine (R183Q) substitution [54–56] (Fig. 3.1). This mutation may cause an overstimulation of the MAPK/ERK pathway, as HEK293 cells transfected with mutant GNAQ expressed an increase in ERK activation, compared to control cells [54]. Although it is unknown whether other signaling pathways are involved, ERK inhibition may thus block development of these lesions.

Cerebral Cavernous Malformations, Hyperkeratotic Cutaneous Capillary-Venous Malformations, and Verrucous Venous Malformations

Cerebral cavernous malformations (CCM) are lesions located within the CNS parenchyma. They can be inherited (commonly multifocal) or occur *de novo*. Familial cases are passed on in an autosomal dominant manner, and CCMs also follow paradominant inheritance [57, 58]. Three causative genes are known: Krev interaction trapped-1 (MIM 604214) (KRIT1/CCM1; MIM 116860), malcavernin (MIM 607929) (CCM2; MIM 607929), and programmed cell death 10 (MIM 609118) (PDCD10/CCM3; MIM 603285) (Fig. 3.1). A fourth locus on chromosome 3 has been suggested, but the gene is unknown.

The three CCM proteins interact with each other among several signaling pathways. KRIT1 is involved in the Delta-Notch signaling pathway and regulates endothelial cell (EC)-cell junctions [59], while the delta-like ligand 4 (DLL4) may be targeted by PDCD10. PDCD10 has also been reported to play a role in apoptosis and in the vascular endothelial growth factor (VEGF) signaling pathway [60]. CCM2 is a scaffolding protein for MEKK3/MAP3K3 [61]. Studies in endothelial directed *Krit1* and *CCM2* knockout mice, and evaluation of CCM lesions, suggests the mitogen-activated kinase (MAPK) signaling pathway is affected as the loss of these genes lead to inappropriately active MEKK3 and Kruppel-like factor(KLF)-2 and KLF-4 [62].

Loss of function of CCM1 (KRIT1) is also associated with cutaneous vascular lesions known as hyperkeratotic cutaneous capillary-venous malformations

(HCCVMs) [63, 64] (Fig. 3.1). These lesions are similar to verrucous venous malformations (VVM), which develop sporadically within the skin without associated CCMs. VVMs are caused by activating mutations in the *MAP3K3* gene (MIM 602539) [65] (Fig. 3.1). Thus, MAP3K3 inhibition may be a way to control the development of CCMs, HCCVMs, and VVMs.

Lymphatic Malformations

Lymphatic malformations (LM) are congenital, isolated macro- or micro-cystic lesions that appear sporadically. Exome sequencing was key in identifying activating somatic mutations in the catalytic domain of PIK3CA in lesional tissue [66–68] (Fig. 3.1). As with the 20% of VMs with a PIK3CA mutation, the change causes overstimulation of the PI3K/AKT signaling pathway. Consequently, mutant lymphatic EC isolated from LMs exhibited increased proliferation and sprouting in collagen. Similar to VMs, sirolimus proved effective in improving the quality of life of LM patients [3]. Thus, a new era of treatment now combines conventional therapies with molecular approaches.

Complex and Combined Syndromes

Occasionally, a vascular malformation is present in conjunction with other defects and symptoms, typically connective tissue or bony overgrowth. There are a variety of clinical presentations of such syndromes. Next generation sequencing has been paramount in discovering somatic mutations in the heterogeneous lesions. Many of the identified affected genes are part of the PI3K/AKT signaling pathway. Interestingly, activating alterations in PIK3CA have been implicated in several syndromes. These include congenital lipomatous overgrowth vascular malformations, epidermal nevi, scoliosis/skeletal and spinal (CLOVES; MIM 612918) syndrome, megalencephaly-CM (MCAP; MIM 602501), fibroadipose overgrowth (FAO), and capillary-lymphatico-venous malformation with overgrowth/Klippel-Trenaunay syndrome (KTS; 149000) [69, 70]. Consequently, these are categorized within the PIK3CA-related overgrowth spectrum (PROS) [69], and they have shared molecular pathophysiology despite phenotypic heterogeneity (Fig. 3.1).

Genes encoding proteins of other members of the PI3K/AKT signaling are responsible for additional syndromes. Missense mutations in the serine threonine protein kinase AKT3 (MIM 611223) in some MCAP patients have been found [71]. Activating somatic mutations in AKT1 (MIM 164730) are seen in Proteus syndrome (PLS; MIM 176920) [72] (Fig. 3.1). The role of AKT1 in skin hyperplasia was confirmed in a mouse model, as hyperactivation of AKT in murine skin led to overgrowth [73]. These syndromes and the PROS spectrum may be amenable to management with PI3K-AKT pathway inhibitors, such as sirolimus and BYL719.

Vascular Tumors: Congenital Hemangiomas

Vascular tumors, which are largely accounted for by hemangiomas, are defects with hyperactive EC proliferation. Infantile hemangiomas (IH; MIM 602089) are the most common benign pediatric tumors (of vascular endothelial cells), found in 5–10% of children. IHs express the cell surface marker glucose transporter-1 (GLUT1). IH has a propensity toward race and sex, as it is found predominantly in the Caucasian population and three times more often in females. However, a genetic cause is unknown, although familial aggregation is sometimes seen [74–76].

Congenital hemangiomas are much less frequent than IH and differ in that the tumors are fully formed at birth and the ECs are negative for GLUT-1. There are three types of congenital hemangiomas. Rapidly involuting congenital hemangiomas (RICH) regress early in life, sometimes completely involuting within 12–14 months. Noninvoluting congenital hemangiomas (NICH) do not regress. Partially involuting congenital hemangiomas (PICH) are phenotypically between RICH and NICH.

Somatic missense mutations that disrupt the glutamine at amino acid position 209 (Glu20) in both GNAQ and GNA11, which have 90% sequence similarity, were discovered in RICH and NICH (Fig. 3.1). The finding that the same change in GNAQ and GNA11 (c.626A > T) can be observed in RICH and in NICH suggests that additional factors (be it environmental, genetic, developmental context, or others) strongly influence lesional phenotype [77]. Furthermore, the association of specific "hot spot" GNAQ mutations with corresponding vascular anomalies with very different characteristics (RICH/NICH vs. SWS and CM) exemplifies how it is crucial to understand the effects the changes have on underlying molecular signaling pathways. Although both alterations lead to moderate hyperactivation of GNAQ, various factors, such as the downstream signaling pathway, frequency of the mutant allele, and cell type affected apparently contribute to the lesion type that arises.

Pyogenic Granuloma

Pyogenic granuloma (PG), also referred to as lobular capillary hemangioma, is a benign vascular tumor. PGs often appear as singular red or blue papular growths that are susceptible to bleeding, though multifocal cases can occur. They are typically found on the surface of the skin or mucosa along the head and neck [78]. PGs have a propensity toward affecting women and children. Proposed causes for PG development include trauma, infection, or other events stimulating angiogenesis [78, 79].

An uncommon form of PG is associated with CM. Recent findings suggest overactivation of RAS as a culprit [80]. In particular, a c.1799T>A change in BRAF within ECs was implied as a major genetic hit in the progression of secondary PG on CM with a GNAQ R183Q change (Fig. 3.1). A mutation in KRAS further suggests the involvement of the MAPK signaling pathway [81].

Concluding Remarks

The knowledge gained from the oftentimes rare, inherited forms of vascular malformations was essential in opening the doors toward understanding the etiopathogenic causes of vascular anomalies. Since it was demonstrated that the reduced penetrance and variable expressivity for inherited vascular anomalies was due to somatic second hits, the focus was directed toward studies on the sporadically occurring forms focusing on involved tissues. However, such work was previously difficult because somatic changes are present at low allelic frequencies, below the detection threshold of traditional genetic tools, such as Sanger sequencing (which is ~10%). This issue is compounded by the fact that tissues are heterogenous cell populations, and the mutant allele is likely restricted to a certain number/population of cells, e.g., vascular EC. With the development of highly-sensitive, massively parallel sequencing and digital PCR (ddPCR), the ability to detect somatic genetic changes has significantly improved.

For the first time in history, we have insight into the pathophysiological bases of a large number of vascular anomalies, which often pinpoint to the disruption of intracellular signaling pathways in ECs (Fig. 3.1). As the same pathways are implicated in various cancers, and several inhibitors have been developed and used in their treatment, preclinical and clinical trials have been initiated for vascular anomalies. These trials need to be rigorously conducted and documented, in order to objectively evaluate the benefits and side effects, which could differ from cancer patients. Improved quality of life for affected patients is an important goal, thus an essential outcome measure in any such study. It is currently unknown how much a developmental vascular anomaly with continued angiogenic potential can be reduced in size with molecular approaches.

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