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Review

Biomedicine and diseases: the Klippel-Trenaunay syndrome, vascular anomalies and vascular morphogenesis

A. A. Timur [a, b], D. J. Driscoll [c] and Q. Wang [a, b, d, *]

[a] Center for Molecular Genetics, ND40, Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195 (USA), Fax: +1 216 444 2682, e-mail: wangq2@ccf.org
[b] Center for Cardiovascular Genetics, Department of Cardiovascular Medicine, The Cleveland Clinic Foundation, Cleveland, Ohio 44195 (USA)
[c] Division of Pediatric Cardiology, Mayo Clinic, Rochester, Minnesota 55905 (USA)
[d] Huazhong University of Science and Technology Human Genome Research Center, Wuhan, Hubei 430074 (P. R. China)

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Abstract. Vascular morphogenesis is a vital process for embryonic development, normal physiologic conditions (e.g. wound healing) and pathological processes (e.g. atherosclerosis, cancer). Genetic studies of vascular anomalies have led to identification of critical genes involved in vascular morphogenesis. A susceptibility gene, *VG5Q* (formally named *AGGF1*), was cloned for Klippel-Trenaunay syndrome (KTS). *AGGF1* encodes a potent angiogenic factor, and KTS-associated mutations enhance angiogenic activity of AGGF1, defining 'increased angiogenesis' as one molecular mechanism for the pathogenesis of KTS. Similar studies have identified other genes involved in vascular anomalies as important genes for vascular morphogenesis, including *TIE2*, *VEGFR-3*, *RASA1*, *KRIT1*, *MGC4607*, *PDCD10, glomulin*, *FOXC2*, *NEMO*, *SOX18*, *ENG*, *ACVRLK1*, *MADH4*, *NDP*, *TIMP3*, *Notch3*, *COL3A1* and *PTEN*. Future studies of vascular anomaly genes will provide insights into the molecular mechanisms for vascular morphogenesis, and may lead to the development of therapeutic strategies for treating these and other angiogenesis-related diseases, including coronary artery disease and cancer.

Key words. Blood vessels; vasculogenesis; angiogenesis; vascular anomalies and malformations; Klippel-Trenaunay syndrome (KTS); *VG5Q*; *AGGF1*.

Introduction

Blood vessels are intricate networks of tubes that transport blood throughout the entire body. A closed blood vascular system efficiently carries nutrients, gases, wastes, hormones, metabolites, as well as immune cells, to and from distant actively metabolizing tissues. Blood vessel formation is a vital and dynamic physiological process for normal tissue growth, such as embryonic development, wound healing, placenta formation after fertilization and menstrual cycle. When the formation of blood vessels is unregulated or misregulated, numerous malignant, ischemic, inflammatory, infectious and immune disorders evolve. Diseases associated with pathogenic blood vessel formation can be characterized or caused by excessive or abnormal blood vessel formation, such as vascular malformations, cancer and age-related macular degeneration, or by insufficient new blood vessel formation or vessel regression such as ischemia in heart and brain, hypertension and neurodegeneration [1]. Understanding of blood vessel formation and its regulation at the cellular, molecular and genetic levels will provide information critical for the prognosis and therapy of these diseases. On the other hand, genetic and molecular

* Corresponding author.

studies of diseases associated with abnormal blood vessel formation will provide fundamental understanding of vascular morphogenesis, development and growth. Here, we review advances in molecular genetic studies of a congenital vascular disorder, Klippel-Trenaunay syndrome (KTS), which led to the molecular cloning of a novel angiogenic factor VG5Q. VG5Q stands for the vascular gene on chromosome 5Q, and it is the first angiogenic factor gene identified by a human genetic approach. We also update the molecular genetics of other vascular disorders. Please note that upon recent recommendation by the International Gene Nomenclature Committee, we have changed the official name for *VG5Q* to *AGGF1* (angio genic factor with G patch and FHA domains 1).

Blood and lymphatic vessels

The vascular system consists of blood vessels and lymphatic vessels (lymphatics). The blood vascular system consists of arteries, capillaries and veins. Walls of the circulatory vessels (blood or lymphatic) are composed mainly of endothelial cells (ECs) surrounded by basement membrane and mural cells [pericytes and vascular smooth muscle cells (SMCs)] that are embedded in an extracellular matrix (ECM). They differ in the blood pressure they hold and the thickness of the vascular SMC layer.

Arteries

Arteries are strong, elastic and/or muscular vessels. Large arteries branch progressively into thinner small arteries and arterioles. The wall of a large artery consists of three layers of tunics, the intima, media and adventitia. Tunica intima is composed of ECs resting on a connective tissue membrane which is rich in elastic and collagen fibers. Tunica media has a thick layer of SMCs and elastic connective tissue. Smooth muscle fibers encircle the tube. The outer layer, tunica adventitia, consists of fibroblasts with irregularly arranged elastic and collagenous fibers. This layer attaches the artery to the surrounding tissues, which can be muscle, adipose or other types. Arterioles have also three layers but have a decreased ratio of mural cells. The wall of a pre-capillary arteriole consists of only ECs and SMCs surrounded by a small amount of elastic connective tissue. The SMCs in the walls of arteries and arterioles are innervated by the sympathetic branches of the autonomic nervous system (ANS). Vasomotor impulses cause SMCs to contract by reducing the diameter of the vessels. If these impulses are inhibited, SMCs relax and the diameter of the vessels increases, which is known as vasodilation. Changes in the diameters of arteries and arterioles greatly influence blood flow and pressure.

Capillaries

Capillaries are the most abundant blood vessel in the body. They form connections between the arterioles and the smallest venules. Capillaries consist of a single layer of ECs surrounded by basement membrane and a layer of pericytes embedded within the EC basement membrane. Because of their wall structure, they are the main site of exchange of gases, nutrients and metabolic by-products between blood and the tissue fluid surrounding the body cells. Endothelial cell-cell junctions play a role in the permeability of the capillary walls that varies from tissue to tissue depending upon the permeability requirements of perfused organs [2].

Veins

Venules continue from the capillaries and merge to form veins. The walls of most veins are similar to those of arteries in that they are composed of three distinct layers, the intima, media and adventitia. Because the middle layer is poorly developed, veins have thinner walls that contain fewer SMCs and less elastic tissue than arteries. Many veins, particularly those in the arms and legs, have flap-like valves. Valves are open as long as the blood flow is toward the heart and closed if it is in the opposite direction. Veins also function as blood reservoirs that can be drawn upon in time of need. If a hemorrhage with a drop in blood pressure occurs, the muscular walls of the veins are stimulated by the sympathetic nervous system. The venous constriction augments cardiac preload, helping to raise the blood pressure, and ensures a normal blood flow.

Lymphatics

The lymphatic system consists of lymphatic capillaries and lymphatic vessels that carry lymph and participate in the nutritional processes of organs. Interstitial or extracellular fluid is formed by leakage of blood plasma through minute pores of the capillaries. There is a continual interchange of fluids of the blood and tissue spaces with a free interchange of nutrients and other dissolved substances. Most of the tissue fluid returns to the circulatory system by means of capillaries, which feed into larger veins. However, large protein molecules, as well as white blood cells, dead cells, bacterial debris, infected substances, and larger particulate matter, pass through the porous walls of the lymphatic capillaries and, thus, enter the lymphatic circulatory system with the remainder of the tissue fluid. The interstitial fluid entering the capillaries is called lymph. The lymphatic capillary wall consists of a single layer of ECs, lymphothelium. D2-40, podoplanin, prox-1 and LYVE-1 are the new markers specific to the lymphothelium [3, 4]. Lymphatic capillaries merge to form lymphatic vessels or lymphatics. They are similar to veins in structure and also have valves. Lymphatic

vessels merge to form larger lymphatic trunks and finally lymphatic ducts.

Vasculogenesis, angiogenesis, vessel maturation and lymphangiogenesis

During embryogenesis, blood vessels are formed via two processes: vasculogenesis and angiogenesis. Vasculogenesis is defined as the process in which mesoderm cells are induced to differentiate into hemangioblasts and ECs, which then assemble into a primitive tubular network called the primary capillary plexus [5, 6]. Hemangioblasts are the common progenitors of ECs and hematopoietic cells. These two cell lines carry common markers and share similar signaling pathways. Several signaling proteins, including VEGFR-1, VEGFR-2, SCL/tal-1, Cbfa2/Runx1/AML1, GATA-2, CD31 (PECAM) and CD34, are induced during vasculogenesis and hematopoiesis [7]. Then, angioblasts and other endothelial progenitors differentiate into arterial and venous ECs that form a capillary plexus. The Notch pathway was found to promote arterial fate by repressing venous differentiation [8–10]. One of the ephrin family transmembrane ligands, Eph-B2, marks future arterial but not venous endothelium cells, while one of the receptors for ephrin-B2, i.e. Eph-B4, marks venous endothelium, at the earliest stages of capillary plexus formation [11–13].

The primary capillary plexus later undergoes a remodeling and sprouting process called angiogenesis, and is transformed into a complex network. Further nonsprouting development accompanied by recruitment of smooth muscle cells and changes in size and mural structure leads to the formation of arteries, capillaries and veins, each with their own function and characteristics. Sprouting angiogenesis initiates with vasodilation, which is presumably stimulated by hypoxia, and it then involves an increase in the permeability of the ECs, allowing extravasation of plasma proteins that lay down a provisional scaffold for migrating ECs [14, 15]. To migrate, ECs need to loosen the cell-cell junctions [2] and to relieve surrounding cells and matrix support by proteolytic degradations. Proliferating ECs migrate to distant sites and a lumen is formed. Nonsprouting angiogenesis takes place by intussusceptions in which ECs proliferate within a vessel, resulting in splits in the lumen or by fusion and splitting of capillaries [16].

The maturation of nascent vessels involves stabilization of the vessels by recruiting surrounding cells (pericytes and vascular SMCs) and generating an ECM. The vascular network continues to develop and mature by growth, branching, remodeling and pruning of its different segments in response to the demands of specific tissues and organs. Another step of vessel maturation is tissue- and organ-specific specialization of wall and network structure. This procedure involves arterio-venous determination, homotypic and heterotypic junction formation, and EC differentiation to form organ-specific capillary structures [17].

Lymphangiogenesis refers to the formation of lymphatic vessels. Although angiogenesis plays a critical role in the progression of tumors, lymphangiogenesis may be even more important to metastatic spread. Embryonic lymphatic vessels originate from blood vessels. Lymphatic ECs originate from the cardinal vein during embryonic development [17].

Protein factors regulating vasculogenesis, angiogenesis, vessel maturation and lymphangiogenesis

A number of protein factors that play an important role in the regulation of vasculogenesis, angiogenesis and vascular maturation have been identified by biochemical approaches or transgenic/knockout mouse studies.

1) The best known class of angiogenic growth factors is the vascular endothelial growth factors (VEGFs), including VEGF-A, VEGF-B, VEGF-C and VEGF-D. VEGF-A is one of the most important angiogenic factors, and is required for the earliest stages of vasculogenesis. VEGF-A knockout mice fail to develop blood islands, ECs and major vessels [18]. The concentration of VEGF-A in cells is strictly regulated as the deletion of one single copy of the gene causes embryonic lethality in mice.

   VEGF-A carries out its biological functions by binding to its receptors, VEGFR-1 and VEGFR-2. VEGFR-2 is an early embryonic marker for the formation of vasculature. Mouse embryos lacking VEGFR-2 die in utero as a result of an early defect in the development of hematopoietic and endothelial cells [19]. VEGFR-1 plays a role later. Mice lacking VEGFR-1 produce angioblasts, but cannot assemble angioblasts into functional blood vessels [20]. VEGF-B was recently shown to be capable of promoting angiogenesis through its receptor VEGFR-1 and the activation of Akt and eNOS-related pathways [21]. VEGF-C is a lymphatic-specific growth factor, and VEGF-D is involved in both angiogenesis and lymphangiogenesis, discussed in detail below.
2) Receptor tyrosine kinases Tie-1 and Tie-2, and Tie-2 ligands angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are another class of molecules important for blood vessel formation. Ang-1, its receptor Tie2 and Ang-2 (the natural antagonist for Tie2) do not seem to be essential for vasculogenesis but instead important for angiogenesis [22–27]. Disruption of the Tie2 gene, Ang-1 or transgenic overexpression of Ang-2 all lead

to embryonic lethality as a result of defects in angiogenesis. Ang-1 stabilizes vessel walls and makes them less leaky by promoting interactions between ECs and the surrounding cells and ECM [27]. In the absence of VEGF, Ang2 acts as an antagonist of Ang1 and destabilizes vessels, leading to vessel regression. In the presence of VEGF, Ang2 facilitates vascular sprouting [17]. Tie-1 promotes EC survival possibly through activation of phosphatidylinositol-3-kinase (PI3K) and Akt [7]. The ligand for Tie-1 has not yet been identified.

3) Integrins and their ligands form another class of regulatory proteins which are critical for EC survival and vascular development. They mediate cell-cell interactions and cell-ECM adhesion [7].
4) Other important angiogenic factors include fibroblast growth factor-2 (basic FGF, bFGF, FGF-2), platelet-derived growth factor (PDGF) and transforming growth factor-beta.
5) Angiogenesis is dependent on a delicate balance between activators and inhibitors during vessel formation. In addition to angiogenic growth factors (described above) that strongly promote angiogenesis, there are antiangiogenic factors that block angiogenesis. Examples include thrombospondin-1 (TSP-1), metalloproteinase inhibitors (TIMPs), angiostatin and endostatin [17, 28–33].
6) The VEGF-C/VEGF-D/VEGFR-3 signaling pathway plays a central role in control of lymphangiogenesis [34]. LYVE-1 (lymphatic vessel endothelial hyaluronan receptor) and VEGFR-3 mark lymphatic vessels in the embryo and adult [35]. The homeobox gene *Prox-1* regulates lymphatic sprouting and budding [36]. The Syk-SLP76 pathway triggers separation of embryonic lymphatic and blood vessels [37]. Ang2 is thought to be involved in the maturation and patterning of lymph vessels. It was also shown that neuropilin 2 (NRP2) is required for formation of small lymphatic vessels and capillaries [38]. For an overview of the molecular mechanisms involved in lymphangiogenesis, see [39] and [40].

Vascular anomalies

The processes of vessel formation and vascular morphogenesis are precisely regulated, and disruption of these processes or developmental errors affecting them leads to a heterogeneous group of vascular anomalies, including KTS (discussed later).

Vascular anomalies are broadly classified into vascular tumors and vascular malformations [41-43]. Vascular tumors include hemangiomas of infancy, tufted angiomas, Kaposiform hemangioendotheliomas, infantile hemangioendotheliomas and spindle cell hemangioendotheliomas. Vascular malformations include telangiectasia, capillary, venous, arterial and lymphatic malformations, and combined or mixed vascular malformations, such as Klippel-Trenaunay, Parkes Weber and Servelle Martorell syndromes.

Hemangiomas are benign vascular tumors of infants, and are in fact the most common tumor of infancy, occurring in 1.1–2.6% of newborns. For infants at 1 year of age, the prevalence rate is as high as 10–12%. Hemangiomas of infancy usually are not present at birth, become visible within 1–4 weeks of neonatal life, and grow rapidly (proliferative) up to 18 months of age and then begin to regress (involute). Hemangiomas have endothelial hyperplasia. They can be superficial, deep or both.

Vascular malformations are usually, but not always, obvious at birth, grow proportionally with the patient and rarely, if ever, regress. They have a single layer of endothelium. Except for some lymphatic lesions, they do not respond to steroids. Vascular malformations can be subcategorized according to the channel type and rheology as either slow-flow or fast-flow [44]. They can be single-channel (arterial, venous, capillary or lymphatic) or combined-channel (arteriovenous, capillary-lymphatic, capillary-venous, capillary-lymphatic-venous or lymphatic-venous) malformations. The combined vascular malformations are often associated with bony and/or soft tissue overgrowth (hypertrophy). KTS is an extensive combined malformation comprising capillary, lymphatic and venous malformations associated with overgrowth of the affected extremity.

Clinical features of KTS

KTS (MIM 149000) [45–48] is defined as a congenital vascular disorder comprised of (i) capillary malformations, (ii) venous malformations or extensive distribution and early onset of the varicose veins, and (iii) hypertrophy of the affected tissues. Lymphatic malformations also occur in some KTS patients (11% of cases). The presence of two of the three cardinal features is sufficient to make a KTS diagnosis [47]. Figure 1 shows some features with which the KTS patients typically present. Vascular malformations in KTS are the slow-flow type. Significant arteriovenous fistulae do not occur in KTS [49].

Capillary malformations (CMs, also known as portwine stains) are the most common cutaneous vascular malformation in KTS, and occur in 98% of KTS patients [47]. CMs are red to purplish in color and can be present on any part of the body. They consist of an increased number of abnormal ectatic capillaries in the papillary dermis. The walls of the capillaries are thin, and the ECs are flat [44]. Capillary malformations in KTS can be accompanied by lymphatic and venous malformations. Lymphatic malformations occur in 11% of KTS patients, and 10%

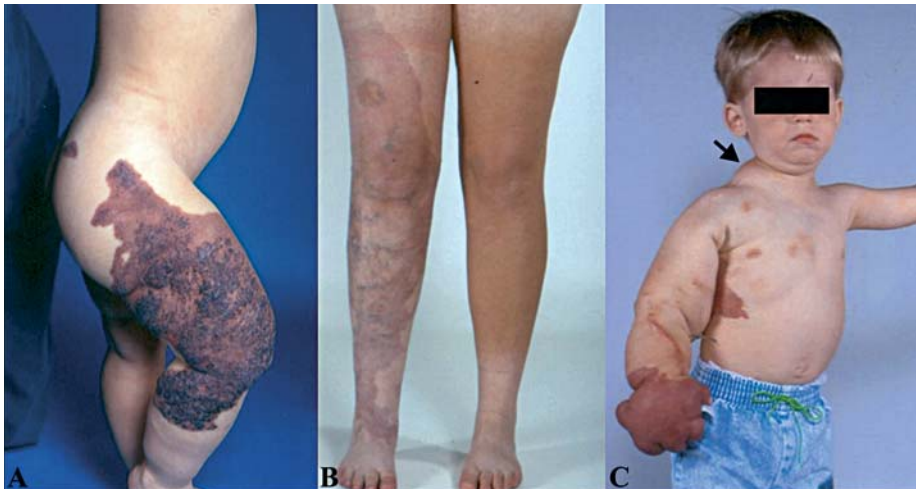

Figure 1. Klippel-Trenaunay syndrome (KTS). *(A)* Extensive combined capillary (port-wine satin)-lymphatico-venousomal formation of the right lower extremity. These may be flat, or elevated as in this case. *(B)* Capillary malformation (portwine stain) and varicose veins. *(C)* Hypertrophic right arm and trunk together with capillary and venous malformations. Subcutaneous hypertrophy also is present (arrow).

may degenerate into lymphedema [47]. Lymphatic malformations are a defect of cutaneous and subcutaneous lymphatic vessels. A lymphatic malformation is composed of dilated lymphatic vessels filled with clear proteinaceous fluid, and not connected to normal lymphatic vessels [44].

Varicose veins occur in 72% of patients with KTS [47]. Persistence of the embryonic lateral vein is very common in KTS (56%) [47]. Deep vein anomalies include aneurysmal dilation, duplication, hypoplasia, aplasia, and external compression of veins by fibrous bands or anomalous vessels [50–54]. Visceral vascular malformations can involve the liver, kidney, bladder, rectum and lower gastrointestinal (GI) tract, retroperitoneum, pericardium, spine and lung. They can cause severe bleeding.

Limb hypertrophy occurs in 67% of the KTS patients, of whom 88% involve the lower extremities [47]. The hypertrophy can involve girth and/or length. Hypertrophy also can occur in the thorax, pelvis, abdomen, head and neck. Macrodactyly can occur.

Genetics of KTS

Most KTS cases are sporadic. However, capillary malformations and varicose veins have been reported among family members of the KTS patients [49, 55–57]. In addition, there have been three reported chromosomal abnormalities in three different KTS patients, two balanced translocations t(5;11)(q13.3;p15.1) and t(8;14)(q22.3;q13), and an extra supernumerary ring chromosome 18 [58–60]. Translocation t(8;14)(q22.3;q13) and the ring chromosome 18 were shown to arise de novo. These findings suggest that genetic factors contribute to the pathogenesis of KTS. The finding of three different cytogenetic defects associated with KTS may suggest that KTS is genetically heterogeneous, and several different genes may be involved in different cases of KTS.

Due to the sporadic nature of KTS, it is technically challenging to identify or clone a gene for KTS using the large family-based positional cloning approach. Thus, we focused on translocation t(5;11)(q13.3;p15.1) (fig. 2A) for identifying a gene for KTS [58]. Our hypothesis was that translocation t(5;11) alters the structure or expression of a gene at one of the two chromosomal breakpoints during the formation of the translocation, which then leads to the development of KTS. No genes were identified in a 100-kb area of the chromosome 11p15.1 breakpoint; however, the chromosome 5q13.3 breakpoint was found to be located in the promoter region of a novel gene, which we named as *VG5Q* (vascular or vasculogenesis gene on 5q; now renamed AGFF1, angiogenic factor with G-patch and FHA domains 1) [61]. The 5q13.3 breakpoint is 1644 bp upstream from the start codon (fig. 2B) and was shown to increase the expression of *AGGF1* by threefold [61].

A case-control association study was then carried out with 130 KTS patients and 200 matched controls. A mutation in *AGGF1*, E133K, was identified in 5 of 130 KTS patients, but not in 200 normal controls [61]. The clinical features of the five gene carriers are shown in table 1. A statistically significant association between mutation E133K and the risk to the development of KTS was established ($P = 0.009$). Mutation E133K was later shown to increase the angiogenic activity of AGGF1 [61]. These results demonstrate that mutation E133K of *AGGF1* is a functional mutation that acts by a gain-of-function mechanism (increased angiogenesis). These results establish

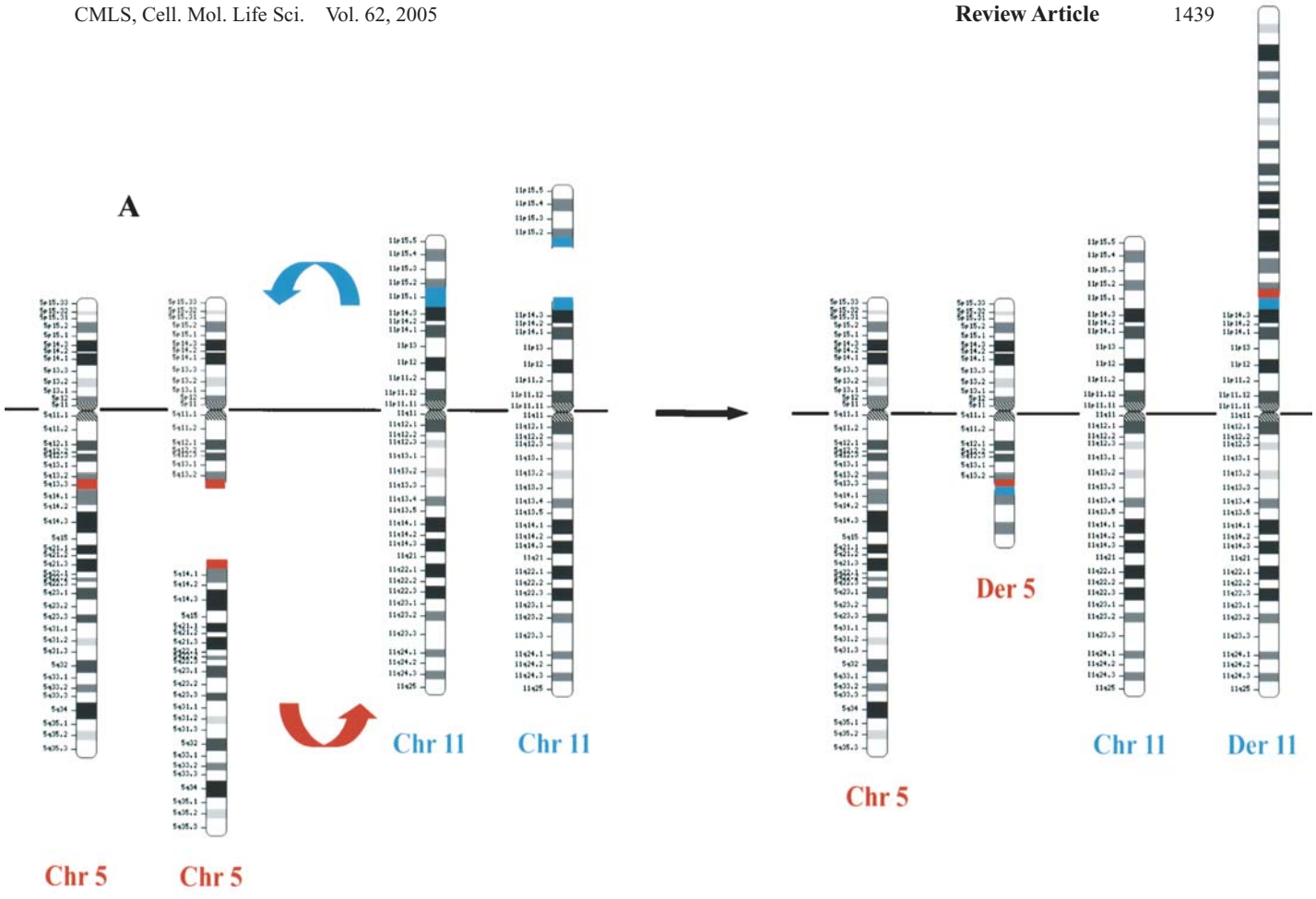

Figure 2. *(A)* Translocation, t(5;11)(q13.3;p15.1), associated with KTS. Breakages occur on one of the chromosomes 5 and 11 at the bands q13.3 (red) and p15.1 (blue), respectively. Broken pieces are exchanged between chromosomes involved, resulting in the formation of two abnormal chromosomes, derivative chromosomes 5 and 11 (Chr, chromosome; Der, derivative). *(B) AGGF1* at the 5q13.3 breakpoint. The 5q13.3 breakpoint is 1644 bp upstream from the start codon (ATG) of *AGGF1*. *AGGF1* contains 14 exons. *(C)* AGGF1 protein structure. There are four putative functional domains; coiled-coil, OCRE (octamer repeat), FHA (Forkhead-associated) and G-patch domains.

Table 1. Clinical findings of KTS patients with AGGF1 mutation E133K.

Patient ID	CM	VM	LM	Hypertrophy	Others
QW576	+	+	N/A	left leg; girth	hemorrhage in right knee
QW731	+	+	+	right foot; soft tissue hypertrophy	–
QW1251	+	+	+	right leg; girth and length	café au lait spots
QW1441	+	+	N/A	right leg; length	–
QW1592	+	+	N/A	right leg; girth and length	–

CM, capillary malformations; VM, vascular malformations; LM, lymphatic malformations; N/A, data not available.

AGGF1 as the first susceptibility gene that confers a risk for development of KTS.

Translocation t(8;14)(q22.3;q13) arose de novo [59], which suggests that another pathogenic gene for KTS may be located on chromosome 8q22.3 or 14q13. The breakpoint on chromosome 8q22.3 has been defined to a <5-cM interval flanked by markers *AFMA082TG9* and *GATA25E10*, and the 14q13 breakpoint within a 1-cM region between sequence-tagged sites (STSs) *WI-6583* and *D14S989* [59]. The specific vascular gene at either 8q22.3 or 14q13 is expected to be identified in the near future.

The KTS-associated de novo mosaic supernumerary ring chromosome 18 implicates a potential vascular and/or overgrowth gene located on the chromosome 18 [60]. The ring chromosome 18 r(18) was mostly derived from the short arm of chromosome 18, and its size is estimated to be approximately 10 cM. Further analyses of the genes on the r(18) may lead to the identification of a new KTS gene.

AGGF1 is a novel angiogenic factor

The full-length *AGGF1* complementary DNA (cDNA) encodes a novel protein with 714 amino acids with a high level of expression in ECs, vascular smooth muscle cells (VSMCs) and osteoblasts (MG-63) [61]. Strong AGGF1 protein expression was detected in blood vessels embedded in various tissues, including the heart, kidney, tail and limb. AGGF1 protein contains a coiled-coil motif (amino acids 19–85), a forkhead-associated domain (FHA, amino acids 435–508) and a G-patch domain (amino acids 619–663) (fig. 2C). The coiled-coil motif may be involved in protein-protein interactions. The roles of the FHA domain and G-patch domain in AGGF1 are not clear. The FHA domain may be involved in phospho-dependent protein-protein interactions [62], whereas G-patch domains have been implicated as RNA-interacting modules [63]. Recently, an OCRE (OCtamer REpeat) motif (amino acids 197–256) (fig. 2C) was identified in AGGF1, and the authors suggested that this motif may be involved in RNA metabolism and/or in signaling pathways activated by the tumor necrosis factor (TNF) superfamily of cytokines [64].

At the cellular level, AGGF1 is mainly localized in the cytoplasm and around the nucleus, though signal was also detected inside the nucleus [61]. Multiple assays showed that AGGF1 secreted outside the endothelial cell when angiogenesis starts (when endothelial cells are grown on matrigel-coated plates), although weak secretion signal was also detected with endothelial cells cultured on plastic dishes [61]. Our recent results suggest that AGGF1 can secrete outside bacterial cells containing overexpressed recombinant AGGF1 [X. Tian and Q. Wang, unpublished data]. The molecular mechanisms for trafficking and secretion of AGGF1 remain to be established.

With the chick chorioallantoic membrane (CAM) angiogenesis assay, we found that the purified AGGF1 protein (75 ng/μl) promoted strong angiogenesis (fig. 3A) [61]. Angiogenesis was also observed around the discs which were spotted with VEGF-A (100 ng/μl) as a positive control (fig. 3A) [61]. These results suggest that, similar to VEGF-A, AGGF1 strongly promotes angiogenesis, indicating that AGGF1 is a potent angiogenic factor [61]. This conclusion is supported by our recent finding that *AGGF1* delivered as a transgene by an adenovirus vector promoted strong angiogenesis in a matrigel angiogenesis assay and in a mouse skeletal muscle angiogenesis assay in vivo [S. You and Q. Wang, unpublished data]. The molecular mechanism for AGGF1-mediated angiogenesis is not clear. On the cellular level, AGGF1 can promote weak endothelial cell proliferation and can bind to endothelial cells (cell adhesion); on the molecular level, AGGF1 can bind to another angiogenic factor, TWEAK, and there may be a cell surface receptor for AGGF1 [61]. These hypotheses warrant future studies.

Molecular mechanism for the pathogenesis of KTS

Histological studies showed an increase in both the number and diameter of the venules in the dermis and subdermal fat and widespread hypertrophy of the smooth muscle in the walls of subcutaneous veins [65]. The blood flow in the affected limb is greater than the unaffected one, and the increased blood flow is related to the presence of a nevus on the affected limb [65]. MRI angiography data also revealed the distorted architecture of the vascular system, indicating a defect in the process of vascular growth and remodeling [55, 66]. Our results showing that translocation t(5;11) increases expression of *AGGF1*, and that KTS mutation E133K in *AGGF1* promotes stronger angiogenesis than wild-type *AGGF1*, suggest that the molecular mechanism for the pathogenesis of KTS is ‘increased’ angiogenesis (fig. 3B). The increased angiogenesis theory is supported by the histological features of KTS and magnetic resonance imaging (MRI) findings described above.

The sporadic occurrence of KTS and the mosaic pattern of KTS features may be explained genetically by the concept of paradominant inheritance proposed by Happle [67–69]. Based on this hypothesis, KTS would be caused by a defect in a lethal gene. Homozygotes for the mutation cannot survive, and they die during early embryogenesis. Heterozygous individuals are phenotypically normal. Therefore, the gene can be transmitted unperceived through many generations until a somatic mutation or ‘second hit’ occurs in the developing embryo, causing loss of heterozygosity and leading to the formation of a cell population being homozygous or heterozygous for the mutation.

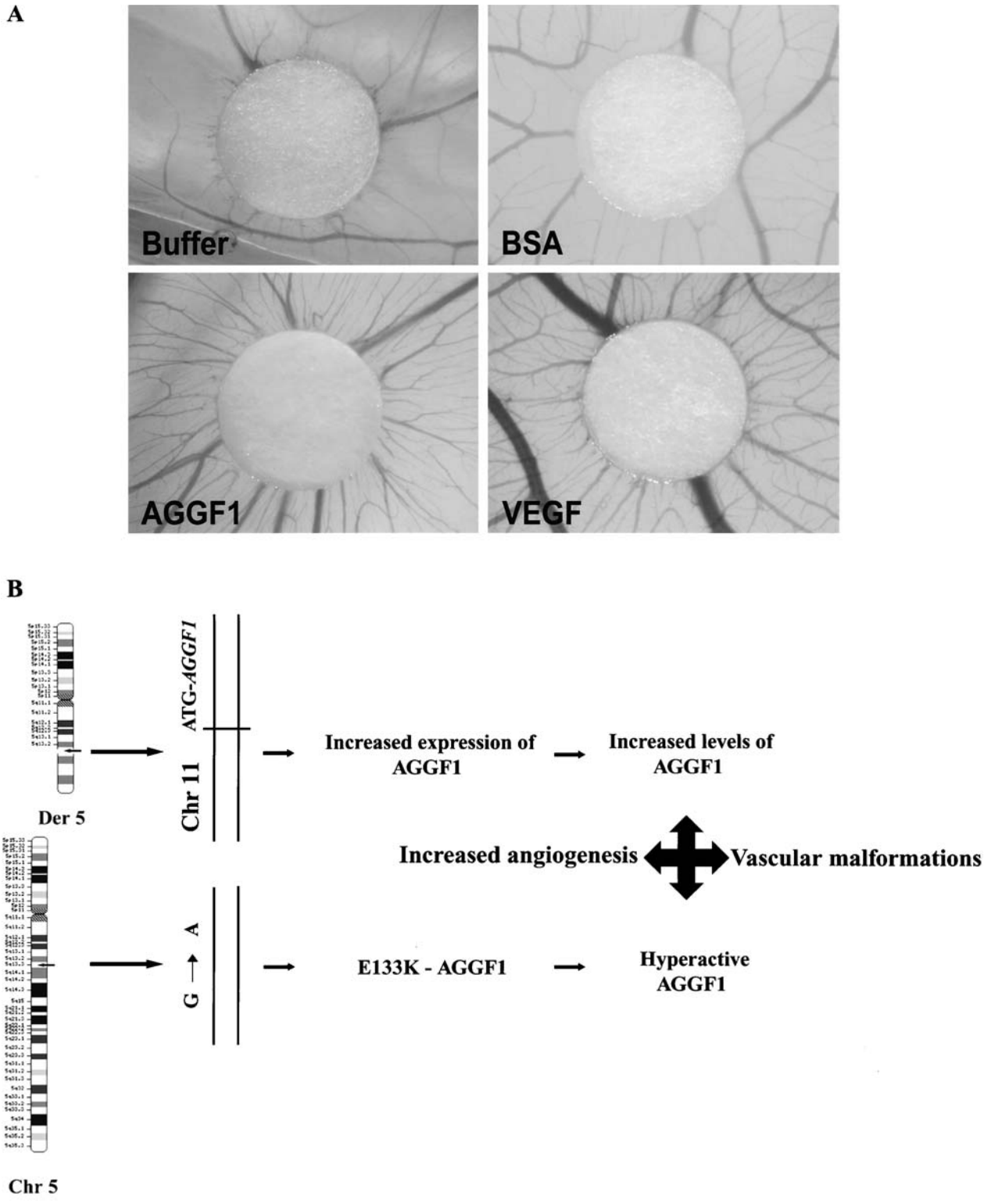

Figure 3. *(A)* AGGF1 is an angiogenic factor in chick chorioallantoic membrane (CAM) angiogenesis assays. Buffer (the same elution fraction as purified AGGF1, but from bacteria with the empty expression vector) and BSA were negative controls, and VEGF-A (100 ng/µl) was used as a positive control. AGGF1 protein (75 ng/µl) promoted strong angiogenesis. (Adapted from Tian et al. [61] with the permission of Nature Publishing Group). *(B)* A molecular mechanism for the pathogenesis of KTS. Small arrows on the chromosomes show the location of *AGGF1*. Translocation of the chromosome 11 sequences upstream of *AGGF1* causes a threefold increase in expression of *AGGF1,* resulting in increased levels of the protein. Mutation E133K increases the angiogenic activity of AGGF1. Both effects are expected to result in increased angiogenesis, which leads to the development of vascular malformations in KTS patients (Chr, chromosome; Der, derivative).

Genetics of other vascular and vascular/overgrowth anomalies

Molecular genetics studies have identified several disease-causing genes for other vascular anomalies. Table 2 lists some important genes identified for several vascular and vascular/overgrowth anomalies. Two of these genes have been linked to vascular morphogenesis before the human genetics studies, and they are the *TIE2* gene on chromosome 9p21 and the *VEGFR3* gene on 5q35.3. Gain-of-function mutations in *TIE2* cause multiple cutaneous and mucosal venous malformation (VMCM) (MIM 600195) [70]. Inactivating mutations in *VEGFR3* cause lymphedema type I [71]. The link of genes in table 2 to vascular morphogenesis was all initially identified by the human genetics approach.

CMs (MIM 163000) are the most common cutaneous vascular malformation, present in 0.3% of the newborns [72]. CMs also occur in several combined vascular anomalies associated with hypertrophy, as in the case of KTS. Heterozygous inactivating *RASA1* mutations were detected in families with CMs with either arteriovenous malformation, arteriovenous fistula or Parkes Weber syndrome (CM-arteriovenous malformation, CMAVM) (MIM 608354) [73]. *RASA1* codes for p120-RasGTPase-activating protein (p-120-RasGAP) which catalyzes intrinsic GTPase activity of Ras. It is a downregulator of the Ras/mitogen-activated protein kinase (MAPK)-signaling pathway, which mediates cellular growth, differentiation and proliferation from various receptor tyrosine kinases on cell surfaces [74, 75]. The p120-RasGTPase also binds to p190-RhoGAP which directs signaling to the cytoskeleton [76], and to Rap1a, which is involved in integrin signaling-mediated cellular adhesion [77, 78].

As a single entity, Parkes Weber syndrome (PKWS, MIM 608355) [79] is very similar to KTS. It is characterized by cutaneous CMAVMs in association with hypertrophy of the affected limbs [80]. Several differences distinguish these two syndromes [81]. In contrast to KTS, the vascular malformations in PKWS are fast flow and involve arterial malformations. Lymphatic malformations are rare in PKWS. The hypertrophy involved in PKWS occurs mostly in the length of the extremities.

Type 1 cerebral CMs (CCM1) (MIM 116860) are caused by loss-of-function mutations in the *KRIT1* gene [82, 83]. *KRIT1* or *CCM1* gene codes for Krev interaction trapped-1 protein, which was identified as a binding partner of Rap1a [84], an antagonist of Ras transformation [85]. KRIT1 also binds to ICAP (integrin cytoplasmic domain-associated proten-1), implying a process of integrin signaling-mediated cellular adhesion in the pathogenesis of CCM [86]. It is worth noting that some CCM family members with *KRIT1* mutations also have hyperkeratotic cutaneous capillary-venous malformations [73, 87]. The second CCM gene was identified as a novel gene, *MGC4607* on 7p15-p13, which encodes a protein, mal-

Table 2. Genetics of several vascular and vascular/overgrowth anomalies.

Disease	Chromosome	Gene
Venous malformations, multiple cutaneous and mucosal (VMCM)	9p21	*TIE2* [70]
Lymphedema type I (Nonne-Milroy lymphedema)	5q35.3	*VEGFR3* [71]
Capillary malformation-arteriovenous malformation (CMAVM)	5q13.3	*RASA 1* [73]
Cerebral capillary malformation (CCM)		
CCM1	7q11.2-q21	*KRIT1* [82, 83]
CCM2	7p15-p13	*MGC4607* [88, 89]
CCM3	3q25.2-27	*PDCD10* [90]
Glomuvenous malformation (GVM)	1p22-p21	*glomulin* [91]
Lymphedema-distichiasis (LD) syndrome	16q24.3	*FOXC2* [92]
OL-EDA-ID	Xq28	*NEMO* [93]
Hypotrichosis-lymphedema-telangiectasia syndrome (HLTS)	20q13.33	*SOX18* [94]
Hereditary hemorrhagic telangiectasia (HHT) (Osler-Rendu-Weber disease)		
type 1 (HHT1)	9q34.1	*endoglin (ENG)* [95]
type 2 (HHT2)	12q11-q14	*ACVRLK1* [96]
Juvenile polyposis/hereditary hemorrhagic telangiectasia	18q21.1	*MADH4* [97]
Coats' disease (retinal telangiectasis)	Xp11.4	*NDP* [98]
Age-related macular degeneration: Sorsby's fundus dystrophy (SFD)	22q12.1-q13.2	*TIMP3* [102]
Cerebral arteriopathy (CADASIL)	19p13.2-p13.1	*Notch3* [104]
Ehlers-Danlos syndrome, vascular type (type IV)	2q31	*COL3A1* [105]
Proteus and Proteus-like syndromes	10q23.31	*PTEN* [107–110]
Macrocephaly, multiple lipomas, hemangiomata	10q23.31	*PTEN* [112]
Bannayan-Zonana syndrome (BZS)		
Bannayan-Riley-Ruvalcaba syndrome (BRSS)		
Riley-Smith syndrome (RSS)		
Ruvalcaba-Myhre-Smith syndrome (RMSS)		
Klippel-Trenaunay syndrome (KTS)	5q13.3	*AGGF1* [61]

cavernin, with a phosphotyrosine binding domain, a domain found in ICAP1α (a binding partner of KRIT1). Malcavernin and KRIT1 may be members of a protein complex involved in integrin signaling [88, 89]. The third CCM gene, *CCM3*, has been identified as *PDCD10* (programmed cell death 10, TFAR15) on 3q26-27 [90]. The role of the PDCD10 gene in vascular morphogenesis remains to be investigated.

Glomuvenous malformations or glomangiomas (venous malformations with smooth muscle-like glomus cells) (MIM 138000) are caused by loss-of function mutations in the *glomulin (FAP48)* gene coding for an FK506-binding protein (FKBP)-associated protein of 48 kD [91].

One form of hereditary lympedema is the lymphedema-distichiasis syndrome (LD) (MIM 153400). It involves lymphedema together with the presence of double rows of eyelashes. Other complications may include cardiac and skeletal abnormalities. LD is caused by inactivating mutations in the *FOXC2 (MFH-1)* gene, which codes for a forkhead transcription factor [92]. On the other hand, lymphedema with osteoporosis, ectodermal dysplasia (anhidrotic) and immunodeficiency, called OL-EDA-ID (MIM 300301), was found to be caused by a mutation in the stop codon of the nuclear factor-kappaB (NF-κB) essential modulator gene (*NEMO*) [93]. The mutation results in expression of a protein that is 27 amino acids longer than the wild-type protein. It was shown that reduced NF-κB signaling is a possible mechanism for OL-EDA-ID [93]. Another interesting combination of lymphedema with hypotrichosis (sparse hair) and telangiectasia (abnormal dilation of capillaries and arterioles) (MIM 607823) was found to be caused by inactivating mutations in the *SOX18* gene, which belongs to the *SOX* (Sry-type high-mobility group box) gene family. This gene family codes for transcription factors required for diverse developmental processes such as cardiac development, sex and neural determination [94].

Hereditary hemorrhagic telangiectasia (HHT) or Osler-Rendu-Weber disease is a vascular dysplasia leading to telangiectases and AVMs of skin, mucosa and viscera (lung, liver and brain). The mucosal complications involve epistaxis and GI bleeding. There are two types of the disease, HHT1 (MIM 187300) and HHT2 (MIM 600376). HHT1 patients have an earlier onset of epixtaxis and telengiectasis than those with HHT2, and only HHT1 patients have a high frequency of pulmonary AVMs. The inactivating mutations in the *endoglin (ENG)* gene, which encodes an accessory TGFβ receptor, were found to cause HTT1 [95]. HHT2 was caused by loss-of function mutations in the *ACVRLK1 (ALK1)* gene, which codes for activin receptor-like kinase type 1 [96]. Both proteins are involved in the TGFβ signaling pathway. A combined syndrome of juvenile polyposis and HHT (MIM 175050) was found to be associated with mutations in the *MADH4* gene, which encodes SMAD4 [97]. This protein is an integral downstream effector of TGFβ signal transduction.

Another telangiectasia-related disease, Coats' disease or retinal telangiectasis, was shown to be caused by a somatic mutation in the *NDP* gene, which encodes Norrie disease protein (Norrin) [98]. The disease is characterized by a defect of retinal vascular development, leading to vessel leakage, subretinal exudation and retinal detachment. The consequent retinal detachment often results in progressive visual loss. Norrie disease (MIM 310600), on the other hand, is an X-linked recessive disorder in which affected males are blind at birth or in early infancy. The ocular findings involve bilateral retinal folds, retinal detachment, vitreous hemorrhage and bilateral retrolental masses consisting of hemorrhagic vascular and glial tissue (vitreoretinal dysplasia). Patients also develop progressive sensorineural deafness and varying degrees of developmental delay. More than 100 different mutations of the *NDP* gene have been identified in Norrie disease, suggesting an angiogenic role for *NDP* [99]. It has been proposed that retinal telangiectasis is secondary to somatic mutation in the NDP gene [98]. Molecular modeling of NDP revealed a protein structure similar to that of TGFβ and other cysteine-knot growth factors [100].

The hereditary macular dystrophies are progressive degenerations of the central retina and a cause of irreversible visual loss. Among these disorders, Sorsby's fundus dystrophy (SFD) (MIM 136900) is very similar to age-related macular degeneration (AMD), a major cause of blindness in the elderly population of Western countries. The bilateral central visual loss occurs during the fourth or fifth decade of life. Sorsby et al. described five families with a fundus dystrophy [101]. The dystrophy became manifest at about the age of 40 years; the earliest manifestations were color vision deficits and abnormal yellow-white deposits, followed by a central macular lesion with edema, hemorrhage and exudates. In subsequent years, atrophy with pigmentation of the central area and extension occurred peripherally. The choroidal vessels became exposed and appeared somewhat sclerotic. Within about 35 years after onset, the entire fundus was involved. The choroidal vessels disappeared by this stage, and the terminal picture was one of extensive choroidal atrophy. The disease is caused by mutations, likely inactivating, in the *TIMP3* gene [102]. The gene codes for a protein called metalloproteinase-3, which is a matrix-bound inhibitor of matrix metalloproteinases (MMPs). MMPs play a major role in angiogenesis by degrading the ECM and activating growth factors through their degradative activity, thus facilitating EC migration. TIMP3 inhibits angiogenesis by blocking the binding of VEGF to its receptor VEGFR-2 [103].

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (MIM 125310) is thought to be the most common form of hered-

itary stroke disorder. It is characterized by recurrent transient ischemic attacks, strokes and vascular dementia. Other symptoms involve migraine, mostly with aura, psychiatric disturbance, cognitive decline and epilepsy. Arteriopathy is characterized by progressive degeneration of vascular SMCs of small arteries and the accumulation of granular osmiophilic material (GOM) and Notch3 protein within the cell surface of these cells. Notch3 is a member of Notch transmembrane receptors. Its expression is highly restricted to vascular SMCs. Mutations in the *Notch3* gene were found to cause CADASIL [104]. However, the molecular mechanisms by which the mutant Notch3 proteins lead to the disease are still not clear.

The vascular form of Ehlers-Danlos syndrome (type IV) (MIM 130050) is characterized by joint and dermal manifestations and proneness to spontaneous rupture of bowel, intestine and large arteries (e.g. splenic, pulmonary, renal). Patients have also a striking facial appearance, easy bruising and translucent skin with visible veins. Some patients have cerebral vascular complications, including intracranial aneurysms, spontaneous carotid cavernous sinus fistulas and dissection of cervical arteries. The disease is caused by mutations in the *COL3A1* gene, which encodes type III procollagen [105]. Collagen III is an important component of the arterial walls that provides tensile strength to the tissues.

Proteus syndrome (PS) is a complex hamartomatous disorder characterized by asymmetric and disproportionate overgrowth of body parts (macrocephaly, gigantism of hands and feet), connective tissue nevi, epidermal nevi, dysregulated adipose tissue and vascular malformations. Vascular malformations include vascular hamartomas and capillary-venous malformations [106]. Interestingly, germline mutations in the tumor suppressor gene *PTEN* (phosphatase, tensin homologue, deleted on chromosome TEN) were identified in up to 20% of PS cases (MIM 176920) and around 50% of Proteus-like syndrome (PSL) patients [107–110]. *PTEN* encodes a lipid phosphatase that mediates cell cycle arrest and apoptosis [107]. It was fist described in Cowden syndrome (CS) (MIM 158350), which is characterized by multiple hamartomas and a risk of breast, thyroid and endometrial carcinomas [111]. *PTEN* mutations are also associated with other syndromes (MIM 153480) that clinically overlap and are characterized by macrocephaly, lipomatosis, hemangiomata, arteriovenous malformations and developmental delay [112] (table 2).

Summary

Significant progress has been made in the molecular genetics studies of a number of vascular anomalies. Disease-causing or susceptibility genes have been identified for multiple cutaneous and mucosal venous malformations, lymphedema type I, capillary malformations associated with arteriovenous malformations, cerebral capillary malformations, glomuvenous malformations, hereditary hemorrhagic telangiectasia, some forms of lymphedema and telangiectasia, SFD, cerebral arteriopathy, vascular-type Ehlers-Danlos syndrome, Proteus and Proteus-like syndromes and some clinically overlapping syndromes associated with macrocephaly, lipomas and hemangiomata, and, finally, KTS (table 2). These findings make genetic testing possible for some patients and/or families with these diseases. Identification of the disease genes for vascular anomalies also offers interesting targets for investigating the molecular mechanisms involved in vascular morphogenesis, growth and development. The functional roles of *TIE2*, *VEGFR-3* and *AGGF1* in vasculogenesis and angiogenesis have been identified, but the clear functions of other vascular anomaly genes listed in table 2 remain to be identified. The functional studies of vascular anomaly genes may lead to the development of therapeutic options for treating these vascular malformations as well as more common diseases, including cancer, diabetic blindness, age-related macular degeneration, rheumatoid arthritis, psoriasis and other conditions with excessive angiogenesis, or coronary artery disease, stroke and delayed wound healing with insufficient angiogenesis or abnormal vessel regression. For example, our finding that AGGF1 is an angiogenic factor may facilitate the development of therapeutic angiogenesis, in which AGGF1 is delivered as a recombinant protein or as a transgene by adenovirus vectors to promote collateral growth of blood vessels for patients with ischemic heart disease or peripheral vascular diseases. Our finding that knockdown of *AGGF1* expression blocks vessel tube formation may lead to the development of antiangiogenic therapies designed to block angiogenesis and to treat cancer and macular degeneration with excessive angiogenesis.

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