Published in final edited form as:

J Craniofac Surg. 2009 March ; 20(Suppl 1): 703–706. doi:10.1097/SCS.0b013e318193d7a0.

FROM BLUE JEANS TO BLUE GENES

Laurence M. Boon, MD, PhD^{1,2} and Miikka Vikkula, MD, PhD²

¹ Center for vascular Anomalies, Division of Plastic Surgery, Cliniques universitaires St Luc Avenue Hippocrate 10, B-1200 Brussels

² Laboratory of Human Molecular Genetics, de Duve Institute, Université catholique de Louvain, Avenue Hippocrate 74, 5th floor, B-1200 Brussels, Belgium

Abstract

Cutaneous venous anomalies are common. They are blue in color and vary in size, number and location, and account for the majority of consultations at specialized interdisciplinary clinics for vascular anomalies. Venous lesions are clinically important as they cause pain, dysfunction, destruction of adjacent tissues and esthetic concern. Only resection and sclerotherapy are helpful, although not always curative. Understanding etiopathogenesis could help design animal models and develop novel therapeutic approaches.

Dr Mulliken envisioned a project to uncover the genetic basis of an inherited form of venous malformation in a large New England family. Recruitment of two young fellows resulted in a collaborative project that unraveled the searched-for-gene and its mutation. This was an opening for a new era in the field of vascular anomalies. Two blue genes' mutations were discovered, which account for the majority, if not all, of the inherited forms of venous anomalies, but other genes as well, for rheologically diverse lesions. Differential diagnosis and management has improved, and animal models are being made. This was achieved thanks to Dr Mulliken, who inspired two young investigators in blue jeans to find two blue genes.

Keywords

vascular anomaly; genetic; angioma; venous malformation; glomangioma

Prologue

In September 1993, when we left from our respective capital cities, Brussels, and Helsinki, to come to Boston, Laurence Boon was to be a fellow with Dr. John B Mulliken at the Children's Hospital to specialize in the care of patients with vascular anomalies. Miikka Vikkula was coming to the laboratory of Dr. Bjorn Olsen in the Department of Cell Biology, Harvard Medical School, to continue his MD- PhD training as a post-doc. His aim was to acquire knowledge in constructing transgenic mice in the field of vascular biology and extracellular proteins. We came to Boston thanks to the recommendations of our mentors, Dr. Romain Vanwijck and Dr. Leena Peltonen-Palotie, both longtime friends and colleagues, of our new chiefs. Little did we know where this would lead.

Discovery of the First Blue-Causing Gene (VMCM)

A catalyzer for the events to follow was Dr. Matthew Warman, an Instructor in the Olsen lab, and close friend of John Mulliken. John knew of a family with multiple venous malformations

(Fig. 1A). Matt suggested a project in the Olsen lab to identify the genetic cause. What a perfect project for the new fellows! Thus, on a bright Saturday morning, the day after our arrival, we were introduced to each other in Dr. Mulliken's office. The project was launched, but it turned out to become a much longer undertaking than initially thought.

Dr. Mulliken had collected blood samples of some family members. More blood had to be collected during numerous trips to different parts of New England. DNAs were extracted and numerous short tandem repeat genetic markers, made detectable by radioactive marking, were analyzed for all the DNAs. This was systematic and tedious, compared to the current power of SNP chips. As Miikka had experience in molecular genetics and linkage mapping, whereas Laurence was new to the field, we spent a lot of time together. There were only negative results after 3 months of running PCRs and acrylamide gels. But a broken gel, which contained the marker D9S171, showed genotypes, which, when analyzed in a certain way, gave positive LOD scores. We got excited and decided to run the marker again immediately, as the left-over reactions were still radioactive. To speed up the analyses, we put the dried gels with films in the freezer, and went out for late dinner. At midnight we came back to develop the film, and see if the marker was linked. Eureka! During the following weeks we analyzed all the available markers in this chromosome 9p locus, and found that the causative gene must lie in a 24 centiMorgan locus¹. This was clear evidence for a gene and a mutation, at least in this rare family with autosomal dominantly inherited mucocutaneous venous malformations.

Three interesting genes were known reside in this locus: *Interferon α , β* and the putative tumor suppressor gene *MTS1*. None of them proved to be mutated. Luckily, another patient with multiple cutaneous venous malformations similar to those observed in the family visited Dr. Mulliken's clinic. As family history was positive for vascular malformations (VMs) in family members, another trip outside of Boston was organized. To our surprise, the name of the first family was mentioned during this reunion, and we realized that the two families were distantly related. Thus, it was possible to repeat the genetic marker analyses using DNAs from 35 new family members, and to narrow the linked locus to 8cM².

A new candidate gene encoding the endothelial specific receptor tyrosine kinase (*TIE2* / *TEK*) had been localized to 9p21³. Using two melanoma cell lines, with proven homozygous deletions in the 9p region, we discovered that the *TIE2* gene must be located in between the IFN gene cluster and marker D9S3. Further proof for the *TIE2* gene to be in our linked interval was obtained from PCR analyses of YAC clones from the region (positive for D9S169), the smallest of which was only 560kb in length. *TIE2* became our prime candidate gene. However, the structure of the gene was unknown. This was the pre-human-sequence era. We decided to try to amplify the *TIE2* transcript for Epstein-Barr-virus transformed lymphoblasts, as they had been shown to produce low levels of various transcripts, called "illegitimate transcription". Complementary DNAs were reverse-transcribed using total RNA from the patients' cells, and overlapping fragments of the cDNA were sequenced. A single nucleotide change (2545C→T) was identified. It was a non-synonymous change leading to an arginine-to-tryptophan substitution in the encoded protein. Moreover, the amino acid was conserved among species. The nucleotide change also co-segregated with the disorder, i.e. all affected family members had the mutation, whereas none of the unaffected ones or 138 controls had it. In collaboration with Dr Kermit Carraway in Lewis Cantley's laboratory in Boston, we expressed the mutant and wild-type receptors in insect cells using baculoviruses. The mutant receptor was overphosphorylated when compared to the wild-type receptor. We concluded that because of a gain-of-function, this point mutation in *TIE2/TEK*, causes abnormal venous endothelial cell behaviour in a way that gives rise to the histologically distended mucocutaneous vein-like vessels, which often lack investing mural cells². We had completed the first step of the project, and decided to take "the Big Step" – we became engaged to marry. Fortunately, John had introduced us to each other !

Discovery of the Second Blue-Causing Gene (GVM)

There were other patients with multiple venous anomalies who visited Dr. Mulliken's clinic, and who admitted there were others in the family who had similar blue lesions. More trips in New England were made, and a half-dozen of the families were recruited to the studies. Nevertheless, genetic marker analyses of the 9p region showed that none of them were linked to the *TIE2* gene. Since we also excluded linkage to the *TIE2* homologue (*TIE1*) on chromosome 1p33–34, we started a new random genomic screen. We began by testing the remainder of chromosome 1, and identified positive LOD scores on the short arm.

In fall 1995, it was time for Laurence to return to Brussels to finish her training in plastic surgery. The studies continued for 1.5 years as an over-the-Atlantic project. After inquiries to half-a-dozen Belgian institutes, Miikka was able to establish his own group in February, 1997. He moved to Brussels to join Laurence. The multidisciplinary vascular anomalies center was developed by Laurence, while Miikka's laboratory was established in the de Duve Institute, Université catholique de Louvain, Brussels. These genetic studies of vascular anomalies began to flourish in continued collaboration with Drs. Mulliken and Olsen. We were also married in 1997.

The recruitment of patients by Dr. Mulliken was reinforced by the Laurence's clinic in Brussels. By 1999, five families were analyzed, leading to the delineation of the VMGLOM locus on 1p21–22 to a 4–6 Mbp region⁴. By (immuno)histochemistry, these lesions had abnormal looking, rounded, smooth muscle cell alpha actin – positive mural cells, called glomus cells. We coined the term “glomovenous malformation” (GVM). The 1p21–22 locus was subjected to positional cloning using YAC, BAC and PAC clones, which were used to identify new genetic markers⁵. Further recruitment of families was aided by a number of specialists in the field of vascular anomalies (particularly Dr. Odile Enjolras of Paris, France). The novel markers, enabled identification of linkage disequilibrium in seven of 12 families, narrowing the locus further to only 1.48 Mb⁶. Finally, a piece of an uncharacterized gene was identified in the physical contig, and screens of this gene unraveled premature STOP codon mutations in several families⁷. We named the gene *glomulin* (*GLMN*). There were no functional data on this gene or the encoded protein. We could only hypothesize that due to the phenotypic similarity between Venous Malformation-Capillary Malformation (VMCM) and GVM, glomulin might somehow be linked to the functional pathway of *TIE2*. We now know that glomulin is an intracellular protein in vascular smooth muscle cells, whereas *TIE2* is membranous receptor in endothelial cells⁸. Therefore, direct interaction is excluded, whereas indirect communication is still possible.

The discovery of the two genes gave a new precise marker for clinical distinction between glomovenous malformations (previously called “glomangioma”) and sporadic venous malformations. In the past, only the histological presence of glomus cells was used; often these cells could not be seen. We compared a large number of lesions from GVM and VM patients from three vascular anomalies centers (Boston, Brussels, and Paris) and determined a set of clinical characteristics that can help to differentiate the two lesions⁹ (Fig. 1). We identified differences in aspect, color and compressibility. Importantly, GVMs are often painful on palpation and more superficial. This contraindicates the use of elastic garments for GVMs, whereas they are often useful for VMs. On the other hand, resection of a GVM is often easier than for a VM because GVMs do not extend to the underlying tissues. Coagulation abnormalities in large, and some small, multifocal VMs, is another parameter that can help differentiate and manage these lesions¹⁰. Follow-up of some GVM patients showed that some lesions resemble capillary malformations in the newborn. They are thin, reddish in color, and only with time do they darken and thicken, to obtain the more characteristic cobblestone

appearance of GVM¹¹. It is possible that laser therapy at this early age could help treat these superficial, plaque-like GVMs.

With our large network of clinical collaborators, we have been able to analyze over 100 families with GVMs¹² (Brouillard et al., unpublished). It seems that GVMs are always caused by a *glomulin* mutation and that they are invariably inherited. As GVMs account for about 5% of VMs, the risk of having an inherited lesion in any given patient needs to be considered. *De novo* mutations occur in a small percentage of patients. Some of the mutations are common between different families, as suggested by linkage disequilibrium identified in linkage analyses and used to delimit the genetic locus. Screening of these mutations by specific tests can currently give a genetic confirmation for the clinical diagnosis in about 70% of patients. The rest of the patients and families have private mutations^{12–14} (Brouillard et al., unpublished).

Why are Venous Anomalies Localized?

Since the identification of linkage between VMCM and 9p, we wondered why the patients in the family, who carry an inheritable, apparently dominant mutation, only have localized, multifocal lesions¹. The mutation should be present in all cells, and one would expect all endothelium to be affected. But, only venous vessels of a certain, small caliber seem to be abnormal. In addition, of those small (sub)cutaneous veins, only few, in certain locations, are affected. The vessel-type specificity of the pathophysiological phenotype could be explained by differences in expression of other (epigenetic) factors, e.g. phosphatases that regulate the activity of the TIE2 receptor. However, there is no evidence for such differences to explain the localized nature of the lesions. We hypothesized that an expressed lesion may have undergone a second mutational event, as in Knudson's double-hit model for retinoblastoma¹. According to Knudson's theory, an inherited germline *RB1* mutation and an acquired, somatic mutation are needed for retinoblastomas to develop¹⁵. Somatic mutations that affect only a limited number of cells could explain why all veins are not affected, and why lesions are localized. As we all carry millions of somatic mutations, it is only a question of risk where such mutations occur. A person carrying an inherited (germline) mutation in all cells could acquire cells in which both alleles of the gene are mutated. These "localized" dysfunctional cells would be able to promote the development of venous malformations in areas of active angiogenesis, especially during embryogenesis and childhood. Blood vessels and their lining endothelium become stable and non-proliferative in the adult. General characteristics of inherited venous malformations fit with these data, in accordance with activity of angiogenesis and frequency of somatic mutations. Most of the lesions are present at birth, although small additional lesions appear with age, and the penetrance is high, with a maximum at about 20 years of age.

Unfortunately, resected tissues from VMCM families have not been available for study, as these families are rare. Dr. Mulliken resected a VMCM of one of his patients in the original, large VMCM family². Screening of the DNA extracted from this tissue not only unraveled the inherited *TIE2* mutation, but also another mutation, a small deletion in the part of the gene that codes for the extracellular domain of the receptor (Limaye et al., submitted). This mutation is not present in the blood-extracted DNA of this patient, suggesting that it is a somatic second hit. Moreover, a glomuvenous malformation operated by Laurence was analogously screened for the *glomulin* gene. Again, the inherited mutation was present in combination with a second mutation, which was not present in lymphocytes⁷. Similar somatic mutations have been identified in additional glomuvenous malformations, as these lesions are more amenable to study due to their higher prevalence (Aerts et al., in preparation). It is becoming clear that the Knudson's double-hit theory is applicable to inherited VMs, and inhibition of occurrence of somatic mutations should limit the development of lesions. It also suggests that any partial intervention in an inherited venous malformation (VMCM or GVM), which would increase

local angiogenesis, could lead to re-emergence of the lesion, and only complete removal of the double-mutant cells would be curative. These ideas are consistent with surgical therapy of these lesions.

Afterthoughts

Dr. Mulliken's curiosity and natural ability to recognize phenotypes initiated the genetic studies on the pathophysiology of vascular anomalies. From the initial project focused on a single New England family with inherited blue spots has evolved a program involving families and patients affected by various kinds of vascular anomalies all around the world. We have identified genes for cutaneous vascular anomalies associated with cerebral cavernous malformation¹⁶, congenital primary lymphedema¹⁷, and primary lymphedema associated with hypotrichosis and telangiectasias¹⁸. Moreover, we have identified inherited capillary malformations to be linked to 5q and recognized an association between atypical capillary malformations and arteriovenous malformations (CM-AVM)^{19–21}. Many of these patients have fast-flow lesions, intracerebral arteriovenous malformations (AVMs) or arteriovenous fistulas (AVFs), vein of Galen aneurismal malformations, Parkes Weber syndrome or AVM, making identification of these patients and families important²².

The field of vascular anomalies was revealed by Mulliken's seminal biological classification of vascular anomalies²³. These discoveries have had enormous impact on the diagnosis and management of these patients. Animal models are being made with the hope that they can be used in future studies to develop new therapeutic protocols. Moreover, data are emerging that the genetic findings may also be applicable to the often more frequent, non-inherited forms of vascular anomalies, at least to VMs (Limaye et al., submitted). There is new promise for all the patients who visit the interdisciplinary clinics for vascular anomalies. Dr. Mulliken's work continues into our next generation. He is the godfather of our daughter.

Acknowledgments

We are grateful to all the family members for their invaluable contributions. These studies were partially supported by the Interuniversity Attraction Poles initiated by the Belgian Federal Science Policy, network 5/25 and 6/05; Concerted Research Actions (A.R.C.) – Convention No 02/07/276 and No 07/12-005 of the Belgian French Community Ministry; the National Institute of Health, Program Project P01 AR48564; EU FW6 Integrated project LYMPHANGIOGENOMICS, LSHG-CT-2004-503573; the F.N.R.S. (Fonds national de la recherche scientifique) (to M.V., a “Maître de recherches du F.N.R.S.”). We thank Ms. Liliana Niculescu for secretarial help.

References

1. Boon LM, Mulliken JB, Vikkula M, et al. Assignment of a locus for dominantly inherited venous malformations to chromosome 9p. *Hum Mol Genet* 1994;3:1583–1587. [PubMed: 7833915]
2. Vikkula M, Boon LM, Carraway KL, et al. Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. *Cell* 1996;87:1181–1190. [PubMed: 8980225]
3. Dumont DJ, Anderson L, Breitman ML, et al. Assignment of the endothelial-specific protein receptor tyrosine kinase gene (TEK) to human chromosome 9p21. *Genomics* 1994;23:512–513. [PubMed: 7835909]
4. Boon LM, Brouillard P, Irrthum A, et al. A gene for inherited cutaneous venous anomalies (“glomangiomas”) localizes to chromosome 1p21–22. *Am J Hum Genet* 1999;65:125–133. [PubMed: 10364524]
5. Brouillard P, Olsen BR, Vikkula M. High-resolution physical and transcript map of the locus for venous malformations with glomus cells (VMGLOM) on chromosome 1p21–p22. *Genomics* 2000;67:96–101. [PubMed: 10945476]
6. Irrthum A, Brouillard P, Enjolras O, et al. Linkage disequilibrium narrows locus for venous malformation with glomus cells (VMGLOM) to a single 1.48 Mbp YAC. *Eur J Hum Genet* 2001;9:34–38. [PubMed: 11175297]

7. Brouillard P, Boon LM, Mulliken JB, et al. Mutations in a novel factor, glomulin, are responsible for glomuvenous malformations ("glomangiomas"). *Am J Hum Genet* 2002;70:866–874. [PubMed: 11845407]
8. McIntyre BA, Brouillard P, Aerts V, et al. Glomulin is predominantly expressed in vascular smooth muscle cells in the embryonic and adult mouse. *Gene Expr Patterns* 2004;4:351–358. [PubMed: 15053987]
9. Boon LM, Mulliken JB, Enjolras O, et al. Glomuvenous malformation (glomangioma) and venous malformation: distinct clinicopathologic and genetic entities. *Arch Dermatol* 2004;140:971–976. [PubMed: 15313813]
10. Domp Martin A, Acher A, Thibon P, et al. Association of localized intravascular coagulopathy with venous malformations. *Arch Dermatol* 2008;144:873–877. [PubMed: 18645138]
11. Mallory SB, Enjolras O, Boon LM, et al. Congenital plaque-type glomuvenous malformations presenting in childhood. *Arch Dermatol* 2006;142:892–896. [PubMed: 16847206]
12. Brouillard P, Ghassibe M, Penington A, et al. Four common glomulin mutations cause two thirds of glomuvenous malformations ("familial glomangiomas"): evidence for a founder effect. *J Med Genet* 2005;42(2):e13. [PubMed: 15689436]
13. Brouillard P, Vikkula M. Genetic causes of vascular malformations. *Hum Mol Genet* 2007;16(Spec No 2):R140–149. [PubMed: 17670762]
14. Brouillard, P.; Enjolras, O.; Boon, LM., et al. Glomulin and glomuvenous malformation. In: Epstein, CJ.; Erickson, RP.; Wynshaw-Boris, A., editors. *Inborn Errors of Development*. New York: Oxford University Press; 2008. p. 1561–1565.
15. Knudson AG Jr. Mutation and cancer: Statistical study of retinoblastoma. *Proc Natl Acad Sci USA* 1971;68:820–823. [PubMed: 5279523]
16. Eerola I, Plate KH, Spiegel R, et al. KRIT1 is mutated in hyperkeratotic cutaneous capillary-venous malformation associated with cerebral capillary malformation. *Hum Mol Genet* 2000;9:1351–1355. [PubMed: 10814716]
17. Irrthum A, Karkkainen MJ, Devriendt K, et al. Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. *Am J Hum Genet* 2000;67:295–301. [PubMed: 10856194]
18. Irrthum A, Devriendt K, Chitayat D, et al. Mutations in the transcription factor gene SOX18 underlie recessive and dominant forms of hypotrichosis-lymphedema-telangiectasia. *Am J Hum Genet* 2003;72:1470–1478. [PubMed: 12740761]
19. Eerola I, Boon LM, Watanabe S, et al. Locus for susceptibility for familial capillary malformation ('port-wine stain') maps to 5q. *Eur J Hum Genet* 2002;10:375–380. [PubMed: 12080389]
20. Eerola I, Boon LM, Mulliken JB, et al. Capillary malformation-arteriovenous malformation, a new clinical and genetic disorder caused by RASA1 mutations. *Am J Hum Genet* 2003;73:1240–1249. [PubMed: 14639529]
21. Boon LM, Mulliken JB, Vikkula M. RASA1: Variable phenotype with capillary and arteriovenous malformations. *Curr Opin Genet Dev* 2005;15:265–269. [PubMed: 15917201]
22. Revencu N, Boon LM, Mulliken JB, et al. Parkes Weber syndrome, vein of Galen aneurysmal malformation, and other fast-flow vascular anomalies are caused by RASA1 mutations. *Hum Mutat* 2008;29:959–965. [PubMed: 18446851]
23. Mulliken JB, Glowacki J. Hemangiomas and vascular malformations in infants and children: A classification based on endothelial characteristics. *Plast Reconstr Surg* 1982;69:412–422. [PubMed: 7063565]

Figure 1.

A. Mucocutaneous venous malformation (VMCM) of the tongue. B. Sporadic venous malformation (VM) of the lip. C. Glomuvenous malformation (GVM) of the cheek.

Figure 2.
In the office of John B. Mulliken, MD in the summer of 2008.