Genetic investigation of cerebrovascular disorders:

cerebral cavernous malformations

and

intracranial aneurysms

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ABSTRACT

Cerebral Cavernous Malformations (CCM) and Intracranial Aneurysms (IA) are cerebrovascular disorders that can lead to a hemorrhagic stroke and other neurological problems. CCMs are characterized by abnormally enlarged capillary cavities while IAs are saccular outpouchings of intracranial arteries. CCM is found in approximately 0.4% to 0.9% of the population, while IA is more common (3-6%).

This dissertation aimed to add to the body of research for CCM and IA and was divided into two parts. Initial work focused on the characterization and identification of the genes involved in CCM; the second phase focused on the identification of a susceptibility gene for IA.

In the first phase, the *CCM1*, *CCM2* and *CCM3* genes were characterized in families and in sporadic cases of CCM. In both cohorts, a causative mutation was identified in 71% of the cases. Subsequent MLPA analysis of subjects with no CCM mutations revealed that large genomic deletions and duplications are a common cause of CCM. In addition, investigation of *CCM1* point mutations revealed that these were not simple missense mutations but that they rather activated cryptic splice-donor sites and caused aberrant splicing. Furthermore, the genetic predisposition to CCM in sporadic cases with a single lesion was determined to be different from sporadic cases with multiple malformations. Investigation into the loss of heterozygosity demonstrated a plausible mechanism for CCM pathogenesis involving a second somatic hit at the site of the lesion, suggesting that CCM may be caused by a complete loss of CCM protein function.

In the second phase, a genome-wide scan of a large family and subsequent linkage analysis using a monogenic approach identified a susceptibility locus for IA (*ANIB4*).

As a result of this research, we have greatly contributed to the field of CCM, most specifically to its clinical diagnosis. A greater understanding of the genetics involved in CCM will facilitate and permit better management care for patients. Furthermore, the possibility of identification of a gene with a major effect for IA will give us more insight into which pathways are involved in IA formation.

RÉSUMÉ

Les angiomes caverneux (CCM) et les anévrismes cérébraux (AC) sont deux types de maladies cérébrovasculaires qui peuvent entraîner des hémorragies cérébrales ainsi que d'autres déficites neurologiques. Les CCMs sont caractérisés par une dilatation anormale des cavités capillaires tandis que les ACs sont des agrandissements des parois des artères intracrâniennes. 0.4% à 0.9% de la population est atteinte de CCM tandis que l'AC est une maladie plus commune (3% à 6%). La recherche a donc été divisée en deux parties: les CCMs et les ACs. Premièrement, les gènes *CCM1*, *CCM2* and *CCM3* ont été caractérisés dans des familles ainsi que dans des cas sporadiques atteints de CCM. Dans les deux cohortes, une mutation causative a été identifiée dans 71% des cas. Une analyse plus poussée, par MLPA, a démontré que de larges délétions et duplications génomiques sont des causes communes des CCM. En plus, une analyse des mutations ponctuelles retrouvées dans le gène *CCM1* a démontré que ce ne sont pas de simples mutations faux-sens mais des mutations qui activent de nouveaux sites d'épissage et causent ainsi de l'épissage anormal. En outre, nous avons déterminé que la prédisposition génétique aux CCM, pour les cas sporadiques ayant un seul angiome, est différente de celle des cas sporadiques qui ont plusieurs angiomes. Une recherche sur la perte d'hétérozygosité a démontré un mécanisme plausible pour la pathogénie des CCM, impliquant une deuxième mutation somatique dans l'angiome et suggérant que les CCM peuvent être provoqués par une perte functionnelle complète des protéines CCM.

Deuxièmement, le criblage du génome et des analyses de liaison génétique utilisant un modèle monogénique ont mené à l'identification d'un *locus* de susceptibilité pour les AC (*ANIB4*).

Les résultats de cette recherche ont considérablement élargi le champ des connaissances des CCM, plus spécifiquement dans le domaine du diagnostic clinique. Une plus grande compréhension de la génétique impliquée dans les CCM facilitera et permettra l'apport de meilleurs soins aux patients. En plus, la possibilité d'identifier le gène ayant un effet important sur les AC, aidera à acquérir plus de connaissances sur les facteurs qui sont impliqués dans la formation des anévrismes cérébraux.

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Cerebral Cavernous Malformation results from a complete loss of *CCM1* function

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Chapter 11

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CLAIMS FOR ORIGINALITY

1. Seven novel and one previously described *CCM1* mutations were identified in a familial CCM cohort contributing to the spectrum of CCM mutations. The frequency of *CCM1* mutations in our cohort was determined and the study confirmed the role of the newly identified *CCM1* exons in the pathogenesis of the disease. In addition, four novel mutations were identified in a cohort of sporadic cases with multiple malformations.

2. Investigation of the only two *CCM1* missense mutations identified, demonstrated that they were in actuality two point mutations leading to activation of cryptic splice-donor sites causing aberrant splicing.

3. A linkage analysis study at the three CCM loci demonstrated the genetic heterogeneity found within a small cohort of families and linked one large family to the *CCM2* locus.

4. Three novel *CCM2* mutations were identified in a familial CCM cohort, contributing to the spectrum of *CCM2* mutations. One mutation was identified in two different families, indicating a possible common ancestor. In addition, the frequency of *CCM2* mutations in our cohort was determined, which suggested a lower *CCM2* contribution than previously thought. Since no *CCM2* mutations were identified in sporadic cases this

also confirmed the lower proportion of *CCM2* mutations contributing to the overall CCM pathogenesis.

5. Four novel *CCM3* mutations were identified, two in a familial CCM cohort and two in sporadic cases, contributing to the spectrum of *CCM3* mutations. The frequency of *CCM3* mutations in our familial and sporadic cohorts was determined and the studies confirmed the role of the newly identified *CCM3* gene in the pathogenesis of the disease.

6. Four genomic rearrangements were identified in sporadic cases with multiple malformations, including a large duplication within the *CCM1* gene and a novel deletion involving the entire coding region of the *CCM2* gene. Consequently, systematic screening for *CCM* deletions and duplications is recommended.

7. Mutations were identified in all CCM genes in individuals with a sporadic presentation of the disorder with multiple CCMs while none were identified in CCM sporadic cases with only one CCM. This indicates that sporadic cases with multiple CCMs should clinically be considered as CCM familial cases.

8. A loss of heterozygosity study has demonstrated the loss of the normal *CCM1* copy in lesions of individuals with a *CCM1* mutation implicating a two-hit mechanism in the formation of a CCM.

9. A new locus (*ANIB4)* for Intracranial Aneurysm (IA) was identified in a large pedigree. Clinical data demonstrated that even though patients share a common genetic cause for the disorder, the location of the aneurysms for each patient was different. A shared haplotype between two families allowed the refinement of the disease region. Further candidate gene analysis will undoubtedly lead to the identification of the first dominant IA gene.

10. In general, this thesis demonstrates an attempt to understand the genetic basis of CCM and IA, two types of cerebrovascular disorders that can lead to hemorrhagic stroke.

ABBREVIATIONS

AA	Amino acid
AC	astrocytes
ANG1	Angiopoietin-1
AT	Abnormal Tissue
BAC	Bacterial artificial chromosome
BL	basal lamina
CCM	Cerebral Cavernous Malformation
CCM1	KRIT1
CCM2	MGC4607
CCM3	PDCD10
cM	centiMorgan
CNS	Central Nervous System
dHPLC	denaturing high-pressure liquid chromatography
EC	endothelial cell
ELN	Elastin
EST	Expressed Sequence Tag
FC	French Canadian
FERM	4.1, ezrin-radizin-moesin
FGF2	Fibroblast Growth Factor 2
FISH	Fluorescent *in situ* hybridization
GFP	Green fluorescent protein
GWS	Genome-wide Scan
HLOD	Heterogeneity Score
HMZ	Homozygous
HTZ	Heterozygous
IA	Intracranial Aneurysm
ICAP1α	Integrin cytoplasmic domain-associated protein-1
IFCAS	International familial cavernous angioma study
LCM	Laser Capture Microdissection
LD	Linkage Disequilibrium
LOD	Logarithm odds of linkage
LOH	Loss of Heterozygosity
Mb	Megabases
MEKK3	Mitogen-Activated Kinase Kinase Kinase 3
MLPA	Multiplex ligation-dependent probe amplification
MMP	matrix metalloproteinase
MNS	maximum nonparametric logarithm of the odds score
MRA	Magnetic Resonance Angiography

MRI	Magnetic Resonance Imaging
MUSICA	Multiple-single cavernous angioma study
NPL	Non-parametric lod score
NT	Normal Tissue
ORF	Open reading frame
OSM	osmosensing scaffold for Mekk3
PC	Pericyte
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PTB	Phosphotyrosine-binding
RAP1A	ras-related protein 1A
SAH	Subarachnoid Haemorrhage
SNP	Single Nucleotide Polymorphism
SSCP	Single-strand conformational polymorphism
TGF-β	Transforming Growth Factor – β
TLOD	Theta LOD score
TIMP	tissue inhibitor of matrix metalloproteinase
TJ	tight junction
VEGF	Vascular endothelial growth factor
VS.	Versus

1 GENERAL INTRODUCTION

Stroke is the third-leading cause of death in Western countries after heart disease and cancer (Minino et al. 2006). There are different types of stroke depending upon how brain blood flow is affected, but the unfortunate and frequent outcome is a loss of brain function. Ischemic strokes are most common and occur when vessels that supply blood to the brain are completely blocked. Hemorrhagic stroke occurs when an intracranial blood vessel ruptures, causing blood to leak into and apply pressure on the brain. Two causes of hemorrhagic stroke, cerebral cavernous malformations (CCM) and intracranial aneurysms (IA) are the focus of this dissertation.

This general introduction begins with the primary research objectives into CCM and IA. Subsequent sections in this chapter outline key areas of the anatomy, development and differentiation of the cerebrovasculature as well as the genetic tools and procedures that have been applied during this investigation.

1.1 Overview of Objectives

OBJECTIVE #1: To further understand the pathogenesis of CCM:

This objective was researched by:

- characterizing the *CCM1* gene in familial and sporadic cases
- screening *CCM2* and *CCM3* candidate genes suspected of acting/interacting within the cerebrovasculature in CCM families that did not harbour a *CCM1* mutation

- studying a mechanism in which *CCM1* could cause the disorder e.g. two-hit hypothesis

OBJECTIVE #2: To further understand the pathogenesis of IA:

This objective was researched by identifying a gene that causes or predisposes the individual to IA

1.2 Vascular system development

The vascular system is one of the most important organs in the body. It is the first functional system to be formed during embryogenesis and comprises the vessels that carry the blood, nutrients and waste in our bodies. The vascular system is constructed and shaped through the processes of vasculogenesis and angiogenesis (Risau 1997). Vasculogenesis is the process that forms new blood vessels and it takes place in early embryogenesis. It is then followed by angiogenesis, which is involved in the growth of new blood vessels from pre-existing vessels. Angiogenesis primarily occurs during embryonic growth and development but also occurs during wound healing. In addition, it occurs in females during menstruation and pregnancy.

Vasculogenesis occurs in three phases and depends upon the differentiation and proliferation of splanchnic mesoderm cell precursors (see figure 1.1)(Sabin 1920). In the first phase, the precursor cells are stimulated by a series of growth factors to become hemangioblasts, which group to form blood islands (Shalaby et al. 1995). Ultimately, hemangioblasts differentiate to become cells of the vasculature or blood cells depending on their position within the island.

In the second phase of vasculogenesis, cells in the outer ring of the island (now known as angioblasts) multiply and differentiate into endothelial cells that will later line the blood vessel wall. Cells remaining inside the original blood island become hematopoietic stem cells. The final phase of vasculogenesis involves the endothelial cells further organizing together to form the vessel tube and joining with other tubes to form the capillary network (Shalaby et al. 1995).

At least three growth factors may be responsible for initiating vasculogenesis: fibroblast growth factor (FGF2) (Flamme and Risau 1992; Ribatti et al. 1995), vascular endothelial growth factor (VEGF) (Millauer et al. 1993; Fong et al. 1995; Shalaby et al. 1995; Ferrara et al. 1996) and angiopoietin-1 (Ang1) (Davis et al. 1996; Suri et al. 1996; Vikkula et al. 1996).

Angiogenesis impacts the vasculature by fusing capillaries into larger veins and arteries or splitting and multiplying existing capillaries (Hanahan 1997; Risau 1997) (see figure 1.1). Several growth factors are involved in this process and act on the extracellular matrix and endothelial cell bands. VEGF acts first to locally degrade the matrix and loosen the cell bands. Once free, the cells branch out and multiply from these points like a new limb on a tree. The matrix and endothelial cell bands are later restabilized by TGF-β (Transforming Growth Factor – β) and PDGF (Platelet-Derived Growth Factor) respectively (Lindahl et al. 1997).

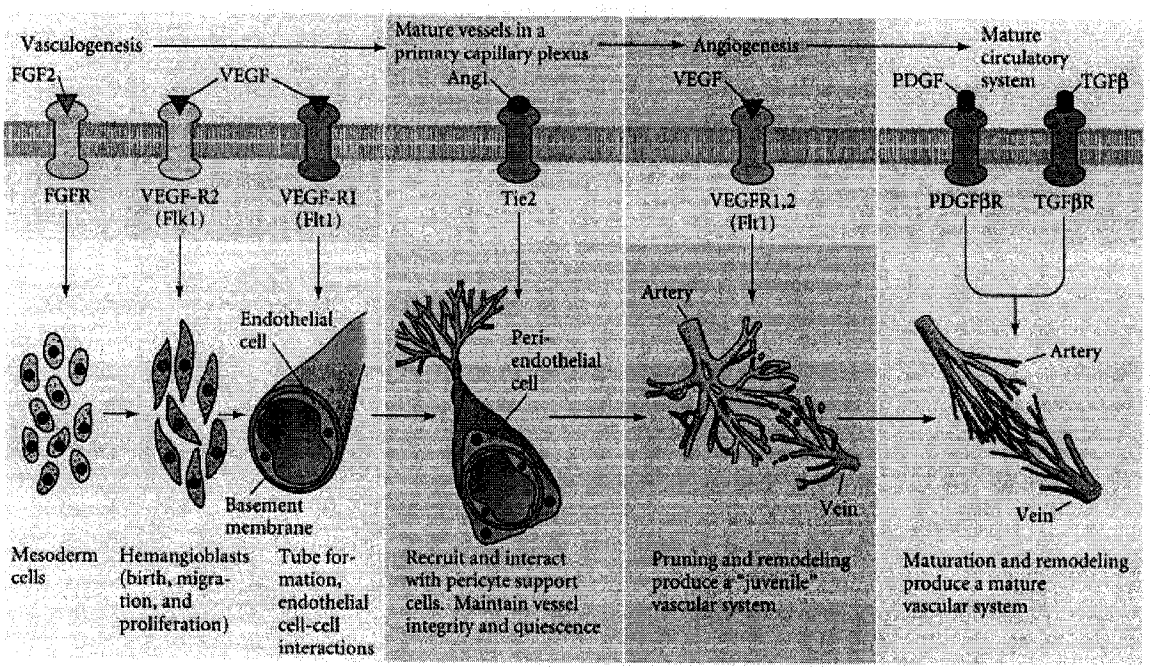


Figure 1.1: Vascular system developmental overview.

Reproduced from Developmental biology, 6th edition (Gilbert 2000).

More than 20 endogenous regulators of angiogenesis have been described, including growth factors (Cao et al. 2003), matrix metalloproteinases (MMP) and their inhibitors (TIMP) (Brooks et al. 1998), cytokines (Park et al. 2002), and integrins (Zhang et al. 2001b).

When the balance between these factors is altered, pathological angiogenesis occurs causing either an increase in the formation of blood-vessels (e.g. during tumour growth) or insufficient angiogenesis (e.g. during coronary artery disease, stroke, and delayed wound healing).

In reality, the development of the vascular system is not fully understood. As a result, vasculogenesis and angiogenesis oversimplify the complexities of blood flow organization. Vessel remodelling requires cell proliferation and regression as

well as programmed branching and migration. In addition, each vessel needs to differentiate into either an artery or vein, which can then be further subdivided into large vessels, venules, arterioles and capillaries. Furthermore, supporting cells, smooth muscle cells and pericytes (undifferentiated mesenchymal-like cells) are needed to ensure the stability of the vessels formed (Folkman and D'Amore 1996).

1.3 Anatomy of the blood-brain barrier

The blood-brain barrier (BBB) is a specialized system that protects the brain from harmful substances in the blood stream while allowing the passage of required nutrients. Unlike peripheral capillaries that allow the relatively free exchange of substances across and between cells, the BBB strictly regulates transport into the brain via both physical (tight junctions) and metabolic (enzyme) barriers. Anatomically, the BBB is composed of the cerebral capillary network (the blood vessel endothelium), astrocytes, pericytes, neurons and the extracellular matrix (Hawkins and Davis 2005).

The lumen (where the blood flows) of the capillary is formed by a single endothelial cell (EC) that wraps around and interconnects itself through a tight junction (TJ) (see figure 1.2). Pericytes (PC) are attached to the outside surface of the EC and both of these cells are surrounded by the basal lamina (BL). The BL is a thick membrane composed of extracellular matrix proteins such as collagen type

IV and fibronectin (Farkas and Luiten 2001). Astrocyte (AC) foot processes fully ensheathe the cerebral blood vessels, BL and PC (Hawkins and Davis 2005).

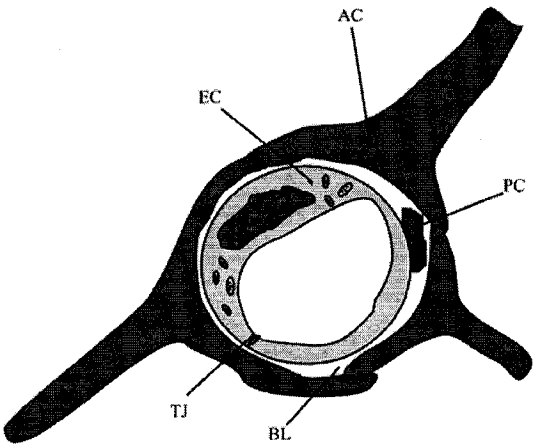


Figure 1.2: Schematic cross-sectional representation of a cerebral capillary.

EC: endothelial cell, PC: pericyte, BL: basal lamina, AC: astrocytes and TJ: tight junction. Reproduced from (Hawkins and Davis 2005).

Astrocytes, possibly through neuronal signalling (Zonta et al. 2003), are thought to be critical in the development and maintenance of the BBB (Willis et al. 2004). As well, interaction between developing vascular tissue and nervous tissue is critical to the development of the BBB (Stewart and Wiley 1981).

Knowledge of the cranial vascular development and composition can give us insight into the possible causes that can trigger the growth of an intracranial aneurysm or of a cerebral cavernous malformation.

1.4 Positional cloning for a Mendelian trait

In the year 1866, the monk Gregor Mendel (1822-1884) elucidated the genetic transmission of single characters through selective breeding of the garden pea plant. Phenotypes with a clear mode of inheritance (recessive, dominant, X or Y-link) are therefore called Mendelian traits, while phenotypes with unclear mode of inheritance are called complex traits. Positional cloning is the process of mapping the location of a human disease gene by linkage analysis in order to determine the underlying causative gene (see figure 1.3). It has led to the identification of many genes, including the genes for cystic fibrosis (Kerem et al. 1989; Riordan et al. 1989), neurofibromatosis type 1 (Wallace et al. 1990), and ataxia telangiectasia (Savitsky et al. 1995).

1.4.1 Mapping requirement

One of the most important requirements for mapping a gene involved in a disorder is the sample size of genetically similar material. Large families are preferred because one can safely assume that all affected family members share the same mutation at a single locus. Therefore, a large family containing many affected individuals also provides the most power to detect the general location of a gene with a major effect on disease. Genetic samples from smaller families are easier to collect, particularly for relatively common disorders, but they carry the risk of genetic heterogeneity (the same disease phenotype may be caused by mutation in multiple genes).

Another important requirement for linkage analysis (see next section) is the identification of a clear segregating phenotype. A clear and unambiguous phenotype is critical since the positional cloning process can be unsuccessful due to a clinical misdiagnosis. Once suitable families are identified, blood is collected and a family history is taken.

Before any genetic linkage studies are performed, several steps must initially be completed. First, gross chromosomal abnormalities are excluded by karyotyping the cells of the affected patient. Second, the possibility of linkage to any known loci linked with the disease needs to be excluded (explained below). Provided the family studied is free of chromosomal abnormalities and there is no linkage to loci related to the disease, the process of positional cloning can then be started.

1.4.2 Linkage analysis

Linkage analysis begins with a genome-wide scan (GWS) of family members' DNA. A GWS genotypes a set of polymorphic satellite markers evenly spaced within the genome. Linkage analysis is then carried out on the results of the GWS. A marker segregating perfectly with the disease phenotype indicates that the marker is physically close to the disease gene and is desirable since it provides a starting location for investigation.

Since the mode of inheritance is known for a Mendelian trait, statistical analysis is performed with a parametric model using two-point and multi-point linkage analyses:

1. Two-point linkage analysis compares the disease locus with one marker at a time and can be performed by the computer programs FASTLINK (Lathrop et al. 1984; Cottingham et al. 1993; Schaffer et al. 1994; Schaffer 1996), and SLINK/FASTSLINK (Ott 1989).

2. Multipoint linkage analysis compares the disease locus with multiple markers and can be performed by using GENEHUNTER (Kruglyak et al. 1996). Although multipoint is preferable to two-point analysis, statistical constraints sometimes make it unfeasible.

These programs predict the likelihood of a marker or a set of markers being linked to the disease (recombination fraction $\theta=0$) or them not being linked (recombination fraction $\theta=0.5$). The ratio of these two likelihoods is converted into a LOD score (logarithm of the odds) and gives the odds of linkage. In the scientific community, a LOD score below -2 indicates that the marker is not linked to the disorder while a LOD score greater than 3 is indicative of linkage. Thus a LOD score of 3 [$\log_{10}(1000) = 3.0$], implies the odds of the marker being linked to the disease is 1000:1 (with a 5% chance of error). Alternatively a LOD score of -2 [$\log_{10}(0.01) = -2$] implies the odds of the marker not being linked is 100:1 (with a 5% chance of error). A LOD score greater than 1.5 merits further analysis by genotyping additional markers in that particular region.

A candidate locus for a Mendelian trait is established when all markers within the locus are segregating with the disease and all other loci have been excluded. Usually, the critical candidate gene interval is delimited by recombinant events that have occurred on the disease chromosome. The candidate region is reduced as

much as possible by genotyping more polymorphic markers around the borders. This is called fine mapping.

In addition, linkage disequilibrium (LD) mapping can be applied to further narrow down the candidate region. Large segments of LD can be seen in young populations with few ancestors (i.e. French Canadian, Hispanic-American), while very small segments of LD are seen in older and larger populations (Lonjou et al. 2003; Morton 2003).

Generally, the mapping of recessive genes in consanguineous families is easier because one can search for marker homozygosity and one can be more certain about recombinant events (Botstein and Risch 2003).

1.4.3 Bioinformatics

Recent advances in the human genome project make physical mapping of a candidate region unnecessary, since almost all of the human sequence is publicly available on-line. Databases such as Ensembl (www.ensembl.org), NCBI (www.ncbi.nlm.nih.gov) and UCSC (http://genome.ucsc.edu) are screened to obtain raw sequence and data on known, predicted genes and ESTs (expressed sequence tags). In some instances, where genes are not fully annotated, other tools, such as open reading frame (ORF) finding programs and protein domain homology comparison, can be used to validate the gene. A list of all the current tools can be found in the Bioinformatic Links Directory (Fox et al. 2006) (http://bioinformatics.ubc.ca/resources/links_directory). Furthermore, access to the mouse, zebrafish and other model organism genomes allows the comparison

to human data for gene structure determination. Sequence homologies are performed using BLAST (Altschul et al. 1997), in order to align available human and other species ESTs to identify intron-exon borders and regulatory sequences. Expression data is also used to determine if the genes are expressed in the right tissues (SAGE database: www.ncbi.nlm.nih.gov/SAGE/). Genes are then prioritized for mutation screening based on known or suggested function. And lastly, the genes are screened by amplifying each exon using PCR (polymerase chain reaction) with intronic primers. Each PCR product is finally sequenced in the hope of finding a causative variant.

1.4.4 Mutations

Coding changes are the most common type of mutation found in human disorders. There are several types of coding changes including missense, nonsense, splicing, small deletions and insertions. Together, they account for more than 90% (58554/64251) of the known mutations listed in The Human Gene Mutation Database of the Institute of Medical Genetics of Cardiff (www.hgmd.cf.ac.uk/ac/index.php). The remaining 10% (5697/64251) of mutations are categorized as gross deletions or insertions, rearrangements and repeat variations. While the absolute number of mutations continues to change, this ratio is unlikely to change greatly. It is for this reason, that most screening efforts are concentrated on the coding sequences of genes.

1.4.5 Validation

When a probable mutation is identified, several steps are performed in order to validate it. First, segregation of the mutation with the disease haplotype is confirmed within the linked family. Second, the prevalence of the variant is assessed in a control population. Third, the gene identified is screened in other families with the same disorder in the hopes of finding new mutations. Lastly, functional and tissue expression studies can be performed for additional support.

1.4.6 Complications

Positional cloning can be confounded by misdiagnosis, loci heterogeneity, complex inheritance, variable expressivity and phenocopies which can all make the disease gene difficult or even impossible to find.

1.4.7 Benefits

Gene identification provides valuable information for both the medical and scientific communities. Scientifically, knowledge of the relevant protein gives us insight into the molecular and physiological processes involved in a disorder and may lead to a better understanding of its pathophysiology. Clinical benefits of gene identification include the possible development of simple and accurate diagnostic tests, improved treatment protocols, and the opportunity for pre-natal or pre-symptomatic diagnosis for individuals at risk of carrying the disease gene.

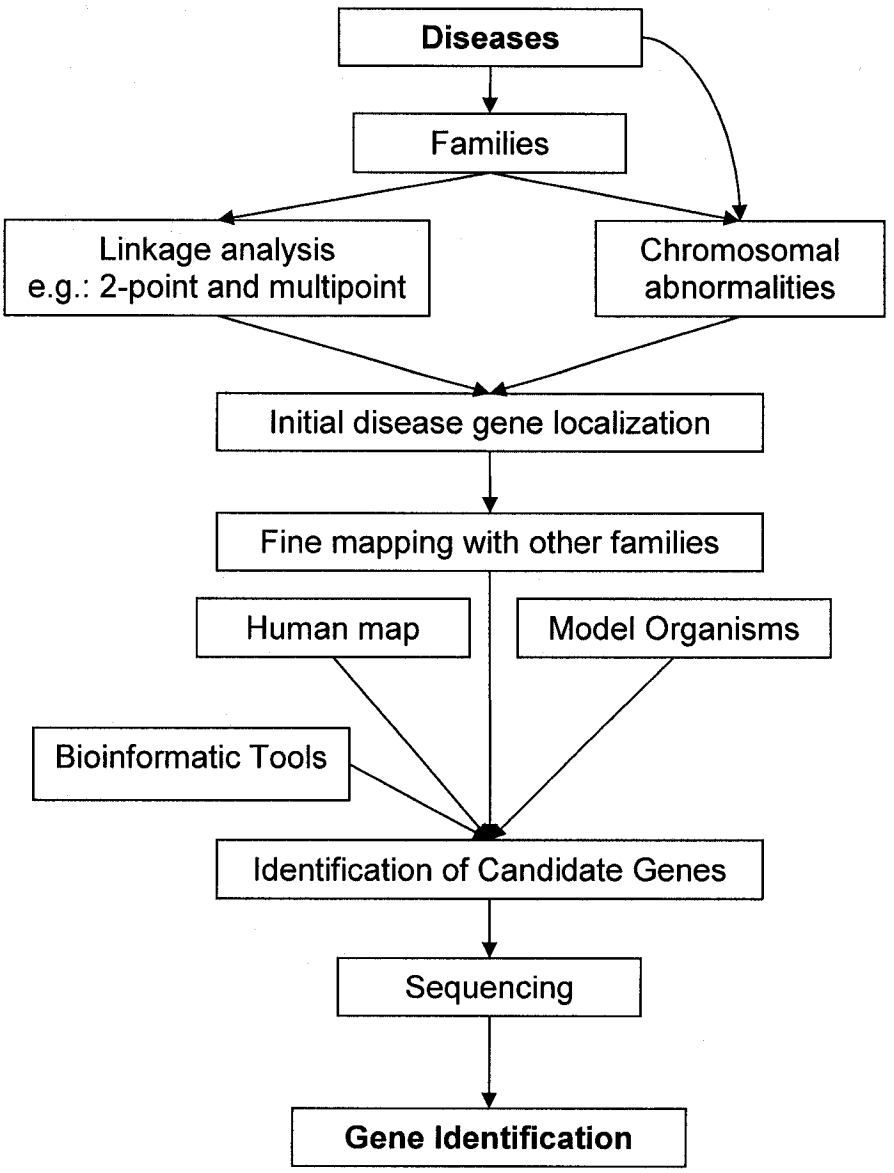


Figure 1.3: Gene identification flow chart for Mendelian disorders.

2 CEREBRAL CAVERNOUS MALFORMATIONS

2.1 Natural history

Cerebral cavernous malformations (CCM [OMIM 116860]) are found in approximately 0.4% to 0.9% (Porter et al. 1999) of the population and represent approximately 9% to 35% of cerebral vascular lesions (Fritschi et al. 1994). Fortunately, symptomatic disease is considerably less common. Symptomatic individuals typically present between the ages of 20 and 40 with seizures but CCMs have also been found in infants (Braga et al. 2006). Patients can also present with brain haemorrhage, focal neurological deficits and/or headaches, attributable to a single or multiple lesions (Porter et al. 1997; Moriarity et al. 1999). Lesions have also been described outside of this age bracket and show no sex predominance (Labauge et al. 1998). There is growing evidence that suggests that pregnancy may increase the risk for CCM haemorrhage (Flemming et al. 2003; Safavi-Abbasi et al. 2006).

2.2 Pathology

Cerebral cavernous malformations are characterized by abnormally enlarged capillary cavities without intervening brain parenchyma and are embedded in a dense collagenous matrix (Russell and Rubenstein 1989). The abnormally thin-walled capillaries contain blood at various stages of organization, are lined by a single layer of endothelium, lack mature vessel wall elements and are prone to haemorrhage (Gunel et al. 1996b). They are also characterized by the absence or

abnormality of blood-brain barrier components (Wong et al. 2000; Clatterbuck et al. 2001; Tu et al. 2005). One of the abnormal components is the poorly formed or absent junctions between endothelial cells. Pericytes are also rarely found at the lesion site. In addition, neither astrocytic foot processes nor normal nervous tissue are present within the lesion (Clatterbuck et al. 2001).

The cerebral cortex is the most common place for lesions to occur but they have also been found in the brainstem, spinal cord, cranial nerves and cerebral ventricles (Giombini and Morello 1978; Chadduck et al. 1985; Del Curling et al. 1991; Aiba et al. 1995). Generally, lesions are found throughout the nervous system in rough relationship to the volume of nervous tissue and consequently, lesions within the cerebral hemisphere are the most common (Dubovsky et al. 1995). Lesions have been nicknamed "popcorn" lesions because they resemble popcorn on Magnetic Resonance Imaging (MRI) (see figure 2.1). MRI reveals a characteristic image of fluctuating signal intensity surrounded by a dark ring attributable to hemosiderin deposition (Gunel et al. 1996b). Hemosiderin-loaded macrophages, gliosis and calcification are also common findings, suggesting recurrent bleeding (Gil-Nagel et al. 1996).

CCMs can also be associated with other cutaneous and visceral malformations (retina (Sarraf et al. 2000; Couteulx et al. 2002), skin (Labauge et al. 1999; Eerola et al. 2000), liver and pancreas (Gil-Nagel et al. 1996)). They can vary in size from a few millimeters to several centimeters and their diameter can grow and change shape over time; on rare occasions they can also undergo spontaneous involution (Siddiqui and Jooma 2001).

In addition, increases in endothelial expression of matrix metalloproteinases 2 and 9 (MMP-2, -9) and tissue inhibitor matrix metalloproteinase 2 (TIMP-2) have been observed in CCMs, suggesting that the matrix stability may be affected, which possibly results in hemorrhage (Fujimura et al. 2006).

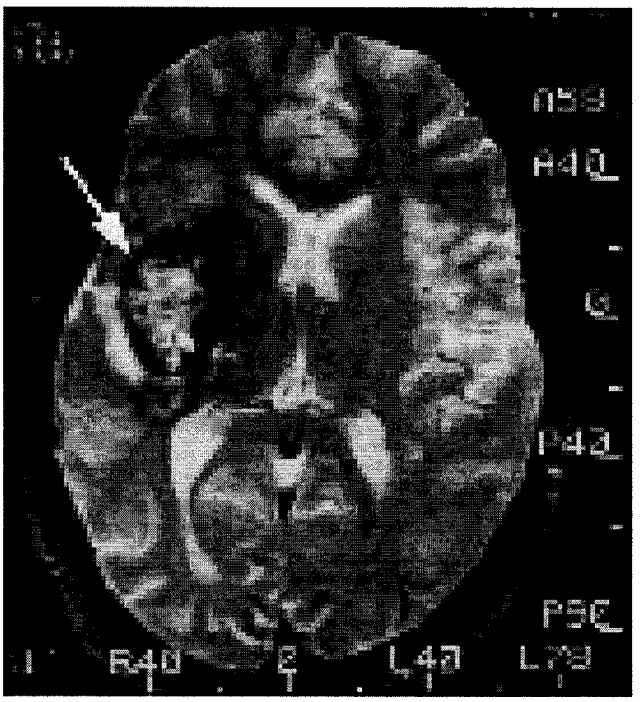


Figure 2.1: Magnetic Resonance Imaging of Cerebral Cavernous Malformation

Modified from the Center for Inherited Neurovascular Diseases website **www.cind.org/details.html**

2.3 Treatment options

Therapy options for CCM can include anti-epileptic drug therapies and/or surgical excision of selected lesions (Gunel et al. 1996b). Surgeries are most beneficial in young patients harbouring deep (e.g. brainstem) lesions, but they are difficult to remove. Although superficial lesions are easily excisable, surgical intervention is only seriously considered if these lesions carry a high risk of haemorrhaging or creating/exacerbating a focal neurological deficit.

2.4 Genetic history

CCM most commonly arises sporadically but can also be dominantly inherited. There is no difference in the pathological features or clinical presentation of the sporadic and familial forms. Most families present with multiple lesions and studies show that 30 to 50% of patients may in fact have the familial form (Labauge et al. 1998). Imaging of asymptomatic members of affected families shows that CNS lesions can be clinically silent, and because all first degree relatives of patients with CCM may not be screened radiologically, the ratio of truly sporadic cases to familial cases may be overestimated.

A familial form of CCM became apparent in the Hispanic-American population (of Mexican descent), where more than 50% of individuals with a lesion had at least another affected family member (Gunel et al. 1996b). Genome scan analyses performed on this population found that almost all Hispanic-American families shared the same haplotype at markers located on chromosome 7q21-q22 (Dubovsky et al. 1995; Gunel et al. 1995; Marchuk et al. 1995; Gunel et al. 1996a) and therefore predicted a founder mutation. By using informative recombinants in these families, the region was reduced and a mutated *CCM1* gene was found to be the cause of the disease (Laberge-le Couteulx et al. 1999; Sahoo et al. 1999) and as hypothesized, the same mutation was found in all of the Hispanic-American families who shared the same haplotype.

Furthermore, a genome scan analysis performed on 20 non-Hispanic-American families found that there were probably three CCM loci (Craig et al. 1998): *CCM1* on chromosome 7q21-q22, *CCM2* on 7p13-p15, and *CCM3* on 3q25.2-q27

(Gunel et al. 1996b; Chen et al. 2002). The data predicted that 40% of the families would link to *CCM1*, 20% to *CCM2* and 40% to *CCM3* and these predictions were accepted by most in the field.

2.4.1 CCM1

The *CCM1* gene encodes the protein Krev Interaction trapped 1 (*KRIT1*) which was identified as a binding partner of KREV1 (*RAP1A*) through a two-hybrid screen (Serebriiskii et al. 1997). Originally, the gene was characterized as having only twelve exons which were identified by alignment of the *CCM1* mRNA with a BAC {HSA000120 (Serebriiskii et al. 1997)} (Laberge-le Couteulx et al. 1999). However, it was later discovered that the gene had not been fully characterized and that its 5' end was incomplete. The full form of the *CCM1* gene now consists of 19 exons spanning 47,132 base pairs of genomic DNA. The start codon is located in exon 4 and when the transcript is translated it produces a protein of 736 amino acids. The protein is expressed in astrocytes, neurons and various epithelial cells in adults as well as in vascular endothelial cells during early angiogenesis. (Denier et al. 2002; Guzeloglu-Kayisli et al. 2004a; Guzeloglu-Kayisli et al. 2004b).

The Krit1 protein contains four ankyrin repeats, a FERM (4.1, ezrin-radizin-moesin) domain and three NPxY motifs. The ankyrin repeats, found in the middle of the protein, are thought to be involved in protein-protein interaction. The FERM homology domain located in exons 14-18 has been found in other proteins that link cytoplasmic proteins to transmembrane proteins. And, although the Krit1

protein carboxyl-terminal domain was originally identified as a binding partner of KREV1, the full length Krit1 does not seem to interact with it and hence may not be biologically relevant (Sahoo et al. 2001). However, there is evidence to suggest that RAP proteins may act as regulators of cell morphogenesis *in vivo* (Asha et al. 1999). The NPxY motif found at the amino terminal has been shown to interact with the PTB domain of the integrin cytoplasmic domain-associated protein-1 (ICAP1α), which is part of the integrin signaling pathway (Zawistowski et al. 2002) (see figure 2.2). In parallel, the PTB domain of ICAP1α has been shown to directly interact with an NPxY motif of β1 integrins, which are involved in adhesion between cells and also to the extracellular matrix (Chang et al. 1997).

Ccm1 knockout mice ($Ccm1^{-/-}$), which die in mid-gestation, suggest an essential role for Krit1 in arterial morphogenesis and identity (Whitehead et al. 2004). Downregulation of artery specific markers, Efnb2, Dll4 and Notch4, was observed. Mice with a heterozygous mutation in the Ccm1 gene ($Ccm1^{+/-}$) (mimicking the human disorder) did not develop any CCM (Plummer et al. 2004). However, 55% of double mutant mice with a heterozygous Ccm1 mutation and homozygous p53 mutation ($Ccm1^{+/-}Trp53^{-/-}$) presented with features similar to CCM. No lesions were observed in the $Trp53^{+/-}$ and $Trp53^{-/-}$ mutant mice, indicating that the p53 tumor suppressor gene is not sufficient to develop CCM. However, this suggests that cell cycle regulators can act as modifiers in the pathogenesis of CCM.

2.4.2 *CCM2*

The *MGC4607* gene, found on 7p13, has been identified as the *CCM2* gene (Liquori et al. 2003; Denier et al. 2004). The gene spans 76,282 base pairs of genomic DNA, contains 10 coding exons and codes for the malcavernin protein. This protein is 444 amino acids in length and contains a phosphotyrosine-binding domain (PTB) located at amino acids 66 to 224, which seem to be conserved in other species. Paralleling Ccm1 expression in embryos and later stages, Ccm2 mRNA expression was shown in neurons (Petit et al. 2006; Plummer et al. 2006) and in astrocytic foot processes terminating on cerebral blood vessels (Seker et al. 2006).

The murine ortholog of malcavernin, OSM (osmosensing scaffold for Mekk3) was shown to modulate the Mekk3 dependent p38Mapk activation by directly interacting with MEKK3, Rac and actin, induced by hyperosmotic shock (Uhlik et al. 2003). Preliminary experiments suggest that OSM may have the capacity to bind phospholipids and peptides, a known function to PTB domain containing proteins (Uhlik et al. 2005).

Contrary to Ccm1, transgenic mice with a heterozygous mutation in the Ccm2 gene ($Ccm2^{+/-}$), develop CCMs in a low penetrance manner (Plummer et al. 2006).

2.4.3 *CCM3*

The Programmed Cell Death 10 (*PDCD10*) gene, found on 3q26.1, has been identified as the *CCM3* gene (Bergametti et al. 2005). This gene spans 50,899

base pairs of genomic DNA and contains seven coding and three non-coding exons. The gene is translated into a 212 amino acid protein and does not seem to contain any obvious known domains. Like Ccm1 and Ccm2, murine Ccm3 mRNA expression was shown in neuronal cells at embryonic and adult stages (Petit et al. 2006). In addition, Ccm3 expression coincided with that of Ccm2 in meningeal and parenchymal cerebral vessels. The *PDCD10* protein has been implicated in the apoptosis pathway, which is an essential process in blood vessel morphogenesis (Busch et al. 2004).

2.5 A possible pathway involved in CCM

In a similar fashion to ICAP1α, Krit1 interacts with the PTB domain of malcavernin through one of its NPxY motifs (see figure 2.2) (Zawistowski et al. 2005). ICAP1α and malcavernin do not competitively bind to the same NPxY motif within Krit1 but may instead influence its subcellular localization. ICAP1α may act to predominantly sequester the *CCM1* protein in the nucleus, while malcavernin sequesters it in the cytoplasm. Interestingly, the only *CCM2* missense (L198R) mutation, found in the PTB domain, disrupts the Krit1 and malcavernin protein interaction (Zawistowski et al. 2005).

In addition, evidence suggests that Krit1, malcavernin and MEKK3 interact to form a tertiary complex critical for p38 mitogen-activated protein kinase activation and/or regulation of integrin-mediated adhesion by ICAP1α (Zawistowski et al. 2005). Knockout mice lacking Mekk3 or p38α Map kinase

have been shown to have significant defects in placental angiogenesis and in brain blood vessel development (Adams et al. 2000; Yang et al. 2000).

While the first two CCM genes have been linked together, their link to the third gene, *CCM3*, remains elusive (see figure 2.2). Research in apoptosis has implicated the CCM3 protein in its pathway (Busch et al. 2004). This provides a possible link to the other CCM proteins since apoptosis in smooth-muscle cells has been shown to be mediated by a β1 integrin signalling cascade (Wernig et al. 2003).

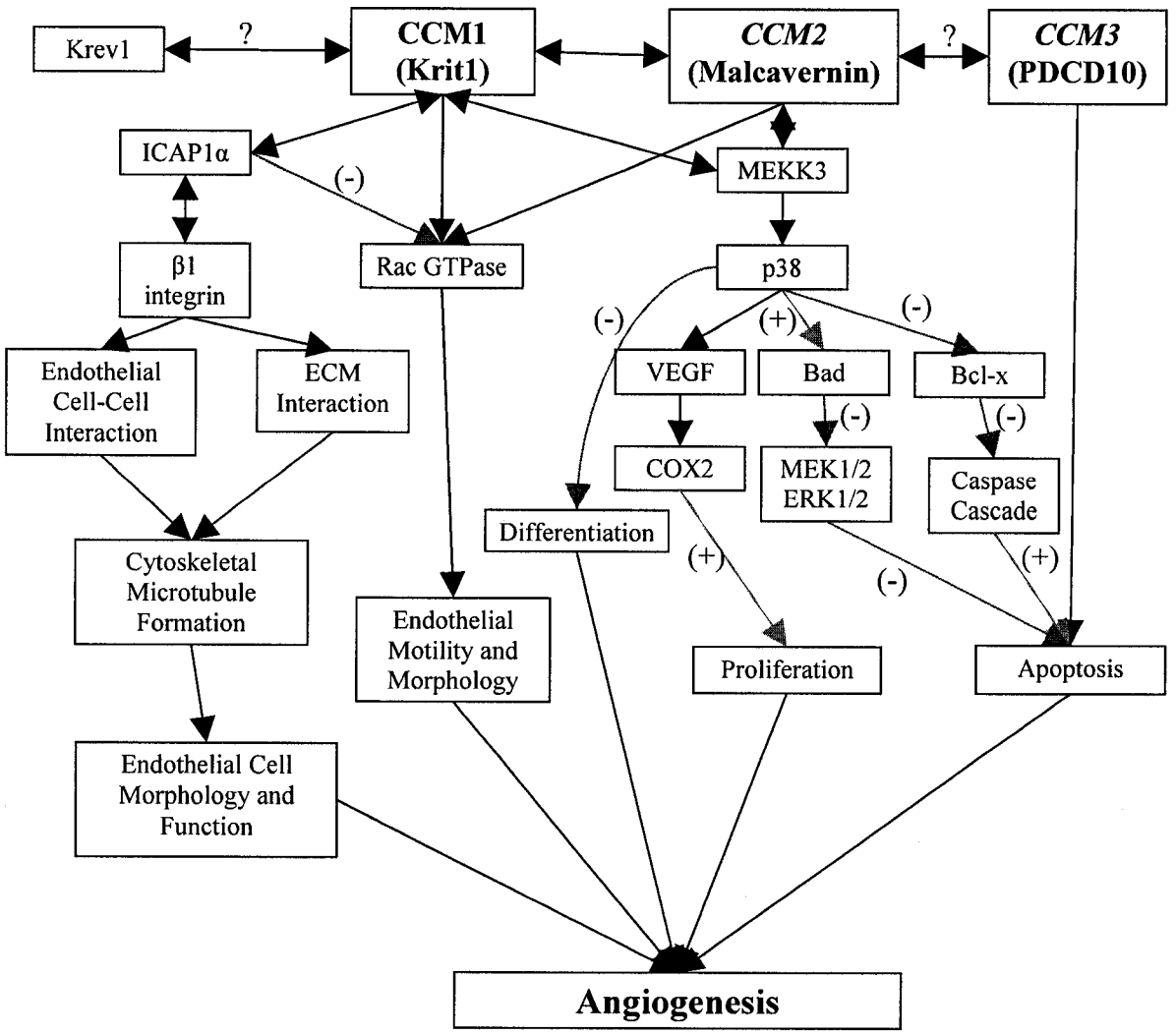


Figure 2.2: Possible pathway diagram for CCM

Red arrow or (-): inhibits, Green arrow or (+): activates, double head arrow: binding

2.6 Models of pathogenesis

Two possible models of pathogenesis have been proposed for CCM formation: the 2-hit hypothesis model or the model of haploinsufficiency.

The two-hit hypothesis is an inactivating (loss-of-function) model, where the remaining functional allele is somatically mutated in a tumour suppressor-like mechanism, akin to Knudson's two-hit hypothesis (see figure 2.3) (Knudson et al. 1975). It is the preferred model because it would explain the reason why only a small number of lesions develop and why their frequency increases in an age-dependent manner in affected members of CCM families.

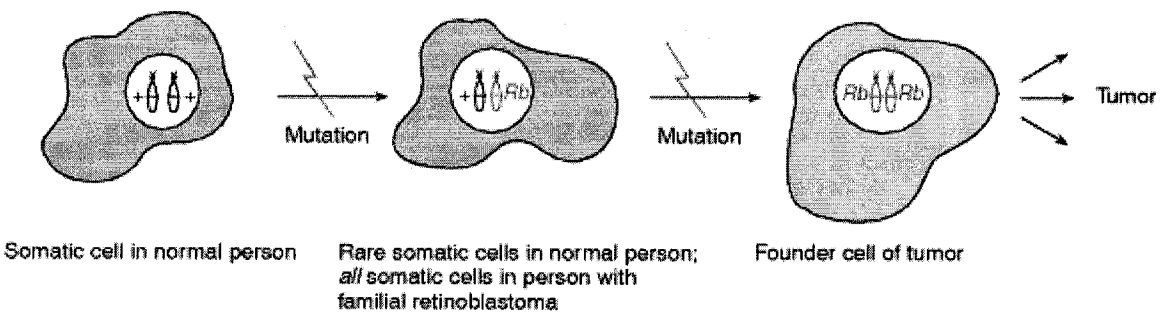


Figure 2.3: Knudson's two-hit hypothesis for Retinoblastoma.

Modified from Human Molecular Genetics 2 (Strachan and Read 1999)

The haploinsufficiency model occurs when a 50% reduction in the level of the gene function causes an abnormal phenotype. Although only relatively few genes show haploinsufficiency [Waardenburg syndrome Type 1 (Tassabehji et al. 1994)], this could occur if the CCM proteins interact with other proteins in a fixed stoichiometry, or if they compete with other proteins to determine a

developmental or metabolic switch. However, haploinsufficiency effects are sensitive to changes in all interacting partners and often show highly variable expression. This is not observed in CCM and this model, although possible, is probably unlikely.

2.7 Cohort of patients

CCM patients were separated into those who had a family history of the disorder and those who did not. All participants read and signed the consent form approved by the Committee for the Protection of Human Subjects at Dartmouth College (Hanover, NH). Most willing patients were located in Europe and a handful was collected from Canada, the United States, Japan and Israel.

Familial form: Criteria for inclusion in the International Familial Cavernous Angioma Study (IFCAS) were either neuroradiologic diagnosis by MRI or histopathologically proven CCM of the CNS in at least two family members. Fifty-three families were collected over the course of the study as part of this cohort, to a total of 339 individuals.

Sporadic form: Criteria for inclusion in the study on sporadic cases included: (i) neuroradiologic diagnosis of either single or multiple CCMs by MRI; (ii) histological verification of at least one neuroradiologically diagnosed CCM; and (iii) no familial history for typical clinical manifestations of CCMs such as seizures, haemorrhage of the CNS, focal neurological deficits, and headache. These criteria yielded twenty-one sporadic cases with one single malformation

and fourteen sporadic cases with multiple malformations over the course of the study.

2.8 Objectives

As initially mentioned in chapter 1, this research aimed to further understand the pathogenesis of CCM by: (i) characterizing the *CCM1* gene in familial and sporadic cases, (ii) screening *CCM2* and *CCM3* candidate genes in CCM families that did not harbour a *CCM1* mutation and finally (iii) studying a mechanism in which *CCM1* could cause the disorder e.g. the two-hit hypothesis.

3 *CCM1* GENE ANALYSIS

Reference:

D.J. Verlaan, W.J. Davenport, H. Stefan, U. Sure, A.M. Siegel, and G.A. Rouleau (2002) Cerebral cavernous malformations: Mutations in Krit1. *Neurology* 58:853–857

and

Reference:

D.J. Verlaan, S.B. Laurent, U. Sure, H. Bertalanffy, E. Andermann, F. Andermann, G.A. Rouleau and A.M. Siegel (2004) *CCM1* mutation screen of sporadic cases with cerebral cavernous malformations. *Neurology* 62: 1213-1215

3.1 Rationale

Originally, the *CCM1* gene was thought to consist of only twelve coding exons (Laberge-le Couteulx et al. 1999; Sahoo et al. 1999), however one year later, four additional coding and three additional non-coding exons were identified upstream in *CCM1* (Zhang et al. 2000a; Sahoo et al. 2001). This new discovery prompted the re-investigation of the *CCM1* gene in both old and newly identified CCM families in an effort to i) find new mutations in the additional exons of the *CCM1* gene and ii) to determine the relative frequency *CCM1* mutations cause CCM.

The first study (section 3.2) consisted of screening 27 families and 11 sporadic cases for mutations in the *CCM1* gene. Next, (section 3.3) 34 patients with no family history of the disease were assessed for *CCM1* mutations. Patients in the latter study were separated into two groups based on their lesion presentation: 21 cases had one single malformation and 14 cases had multiple malformations.

3.2 Cerebral cavernous malformations: mutations in Krit1

3.2.1 Abstract

Objective: To find mutations in the recently identified additional exons of the *Krit1* gene that causes *CCM1*, a disease characterized by the formation of cerebral cavernous malformations (CCM). To determine the relative frequency with which *Krit1* mutations cause CCM as well as recharacterize the mutations reported in the literature.

Methods: Twenty-seven families and 11 apparently sporadic individuals affected with CCM were screened for mutations in the *Krit1* gene. The gene was screened by single stranded conformation polymorphism, and variants were sequenced. Familial segregation of the mutations was determined.

Results: In familial samples, two new mutations in the novel upstream exons and six additional mutations in the previously identified exons were identified. No mutation was found in any of the sporadic individuals.

Conclusions: Results demonstrate that the frequency of mutations found in *Krit1* is 47% in the families studied and the frequency may increase as more mutations are detected. Mutations are evenly distributed in the gene and do not seem to be limited to structural domains present in Krit1. This is in accordance with the model that *Krit1* could be a tumor suppressor gene.

3.2.2 Introduction

Cerebral cavernous malformations (CCM) are found in 0.1% to 0.5% of the population and represent approximately 10% to 20% of cerebral vascular lesions (Rigamonti et al. 1988). These vascular malformations are characterized by abnormally enlarged capillary cavities without intervening brain parenchyma (Russell and Rubenstein 1989). They occur as single or multiple malformations that lead to focal neurological signs, hemorrhagic strokes, or seizures (Rigamonti et al. 1988). The vascular malformations can arise sporadically or may be dominantly inherited. Three CCM loci have been mapped: *CCM1* on 7q21-q22 (Dubovsky et al. 1995; Gunel et al. 1995; Marchuk et al. 1995) accounts for 40% of the cases, *CCM2* on 7p13-p15 for 20%, and *CCM3* at 3q25.2-q27 for 40% (Craig et al. 1998). Individuals usually become symptomatic between 20 and 40 years of age, although lesions have been described in all age groups with no sex predominance (Labauge et al. 1998). Imaging of asymptomatic members of affected families shows that CNS lesions can be clinically silent, and because all first degree relatives of patients with CCM may not be screened radiologically, the ratio of truly sporadic cases to familial cases may be overestimated. So far, only one gene has been identified, *Krit1*, for *CCM1* (Sahoo et al. 1999).

The Krev Interaction Trapped 1 (Krit1) protein contains three ankyrin repeats and a FERM domain. The ankyrin repeats are thought to interact with Krev-1/rap1a, a member of the Ras family of GTPases (Serebriiskii et al. 1997), whereas the FERM domain of the erzin/radixin/moesin protein family is thought to regulate cytoskeletal/plasma membrane interactions. So far, the combination of

ankyrin/FERM domains has not been found in any other protein. Many frameshifts, nonsense mutations, missense mutations, and invariant splice junction mutations have been described in *Krit1* (Table 3.1).

The clinical phenotype of multiple lesions in familial cases and single lesions in sporadic cases, the preponderance of nonsense mutations, and the structure of the protein suggest that CCM may be caused by somatic mutations of the remaining functional gene in familial cases in a tumour suppressor-like mechanism. Explaining the effect of *Krit1* mutations, which are limited to vascular lesions in the CNS, will require further definition of Krit1 function.

More recently, the presence of three additional noncoding and four new coding upstream exons of the *Krit1* gene was confirmed by computational and experimental analyses, bringing the total number of coding exons to 16 (Zhang et al. 2000a; Eerola et al. 2001; Sahoo et al. 2001). These new exons encode 207 additional amino acids for a total protein length of 736 amino acids. To confirm a role for these new upstream exons in the pathogenesis of *CCM1* and to estimate the relative prevalence of *Krit1* mutations in familial CCM, we screened these exons for mutations in our families.

3.2.3 Methods

Patients: DNA from 27 families and 11 apparently sporadic individuals affected with CCM were screened for mutations in *Krit1*. In all cases, DNA was collected with informed consent, and the study was approved by the Committee for the Protection of Human Subjects at Dartmouth College (Hanover, NH). Families

were located in Switzerland, United States, Japan, Israel, Germany, United Kingdom, and Canada, and all participated in the International Familial Cavernous Angioma Study (IFCAS). Criteria for inclusion in our IFCAS study were neuroradiologic diagnosis by MRI (figure 3.1) or histopathologically proven CCM of the CNS in at least two family members.

Gene analysis: DNA was obtained from whole blood or from cultured lymphocytes by phenol and chloroform extraction. Each of the 16 *Krit1* coding exons was amplified by PCR with intronic primers and labelled with 35S-dATP. PCR-amplified products were analyzed by single stranded conformation polymorphism (SSCP) using two separation conditions: 9.5% acrylamide gels containing 5% glycerol and 25% mutation detection enhancement gels (FMC Bioproducts, Rockland, ME). Samples producing a different migration pattern on polyacrylamide gels were reamplified by PCR, separated on 2% agarose gels, and purified using QIAEX II (Qiagen, Ontario, Canada). Direct cycle sequencing was then performed using the Thermo sequenase radiolabeled terminator cycle sequencing kit (USB, Cleveland, OH). Sequence products were run on high resolution 6% denaturing acrylamide gels. Segregation of the mutations with the affected phenotype was confirmed by screening the remaining family members by SSCP or direct sequencing.

3.2.4 Results

Part 1. We had previously screened the DNA of 29 families and 5 sporadic cases affected with CCM for mutations in the *Krit1* gene (Davenport et al. 2001). Ten

new mutations were then identified as well as the previously identified common Hispanic American mutation (Sahoo et al. 1999) in 10 families and 1 sporadic case. In Part 1 of this study, the four new upstream coding exons (4–7) of *Krit1* were screened in the remaining 18 families and 4 sporadic cases where no mutation was detected. One family was not screened because the *CCM1* locus has been excluded by linkage analysis (data not shown). A deletion of 4 base pairs in exon 5, leading to a nonsense codon, was detected in the IFCAS-20 family (151delAAAG) (see table 3.1). No other mutation was found in the rest of these families or in the sporadic individuals.

Part 2. Since our last report, nine additional IFCAS families and seven more sporadic cases were collected. We have screened these cases for *Krit1* mutations in all of the 16 coding exons (4–19). Six novel and one previously described mutation were detected in seven families. One deletion (1813delT) and one insertion (1964insAAGT) cause frameshifts leading to a premature stop codon. One previously described transition (Sahoo et al. 1999) (T1611A) and one transversion (G1890A) cause nonsense mutations. Two invariant splice acceptor site mutations in exon 13 (IVS13-2delATG, IVS13-1G3A) and a missense mutation (A410G) were also detected. This missense mutation changes a conserved aspartic acid to a glycine in exon 7. This A410G was not found in 200 matched control chromosomes and is only the second missense mutation currently reported in Krit1. No mutation was detected in the sporadic patients. Pedigrees of the families in which we found mutations are shown in figure 3.2. Table 3.1 presents a summary of all mutations published, showing nucleotide positions from the first base in the coding portion of exon 4 and amino acid position numbers

from the first codon of the newly published human Krit1 mRNA (AF296765). Figure 3.3 is a representation of the *CCM1* gene showing all reported mutations. The mutations are separated into four categories: frameshifts, nonsense mutations, missense mutations, and invariant splice junction mutations.

3.2.5 Table

Table 3.1: Summary of Krit1 mutations reported here and in the literature

Table Summary of Krit1 *mutations reported here and in the literature*

Mutations	Exon	Predicted effect	Reference
151delAAAG	5	Frameshift	*
152delAAGT	5	Frameshift	10
410A→G	7	D137G	*
601G→C	8	Q201E	13
699delG†	8	Frameshift	17
IVS9-1GG→TT	9	Invariant splice acceptor site altered	13
IVS9-1G→A	9	Invariant splice acceptor site altered	13
813G→A	9	W271X	13
843insC	9	Frameshift	18
IVS9+2T→C	9	Invariant splice donor site altered	17
858G→A	10	W286X	18
902C→G	10	S301X	8
1034delA	11	Frameshift	18
1060C→T	11	Q354X	13
IVS11+1G→A	11	Invariant splice donor site altered	8
1179delAAATATT	12	Frameshift	13
1204delAACAA	12	Frameshift	10
1211G→A	12	W404X	18
1229delT	12	Frameshift	18
IVS13-2A→G	13	Invariant splice acceptor site altered	8
IVS13-2delATG	13	Invariant splice acceptor site altered	*
IVS13-1G→A	13	Invariant splice acceptor site altered	*
1277delGAAT	13	Frameshift	18
1362delTC	13	Frameshift	10,19
1363C→T	13	Q455X	8,10,13,20
1487dupATCTGGATCCT	14	Frameshift	20
1513C→T‡	14	Q504X	13
1595delTT	15	Frameshift	13
1608del26bp	15	Frameshift	18
1611T→A	15	Y537X	8,*
1657insA	15	Frameshift	13
1710delA	15	Frameshift	8
1813delT	16	Frameshift	*
1867insC	17	Frameshift	18
1879C→T	17	Q627X	18
1890G→A	17	W630X	*
1935delT	17	Frameshift	8
1940delA	17	Frameshift	18
1944del84bp	17	28-amino acid deletion	18
1964insAAGT	17	Frameshift	*
IVS18-12A→G	18	Invariant splice acceptor site altered	18
2042delTT	18	Frameshift	13

* This report.
† Hyperkeratotic cutaneous capillary venous malformation.
‡ Skin angiomas.

References for Table 3.1:

8: (Sahoo et al. 1999), 10: (Sahoo et al. 2001), 13 : (Davenport et al. 2001), 17: (Eerola et al. 2000), 18 : (Laberge-le Couteulx et al. 1999), 19 :(Lucas et al. 2001), and 20 : (Zhang et al. 2000b).

3.2.6 Figures

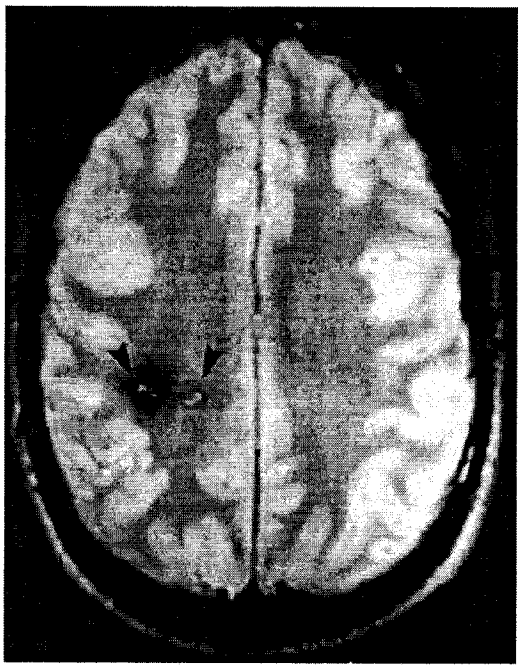


Figure 3.1: Typical MRI of an individual showing two cerebral cavernous malformations.

The left malformation has bled.

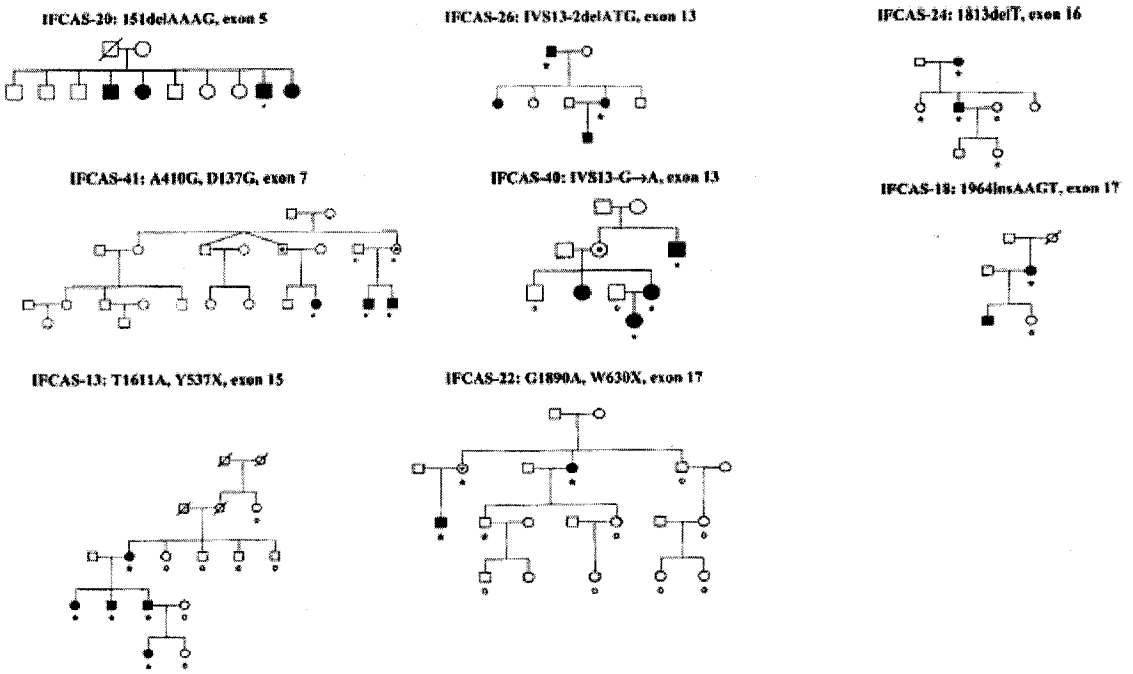


Figure 3.2: Pedigrees of families found to have cerebral cavernous malformation-1 mutations by International Familial Cavernous Angioma Study (IFCAS) numbers.

Filled circle or square: affected; open circle or square: not known to be affected; small filled circle inside circle or square: obligate carrier; asterisk: mutation; small open circle: no mutation; diagonal line: individual deceased.

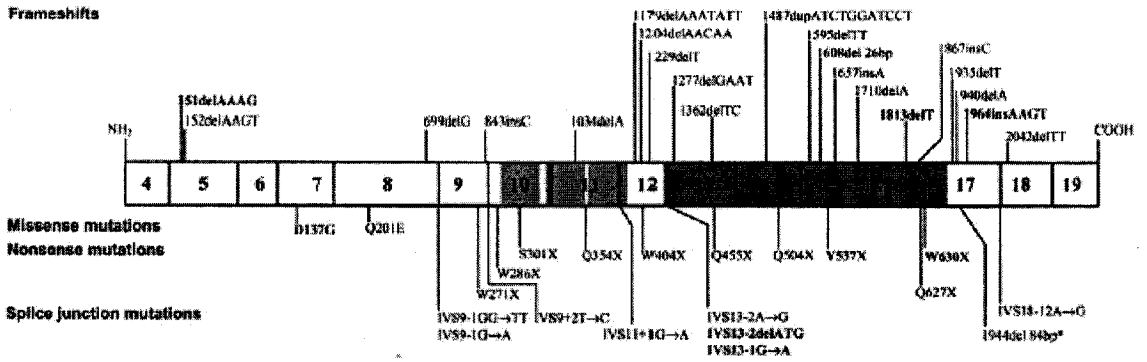


Figure 3.3: Representation of the Krit1 protein containing all of the reported mutations (exons 4 to 19).

Light grey: ankyrin repeats; dark grey: FERM domain. Frameshifts (above figure) are numbered using nucleotide sequence. Other mutations (below figure) are numbered using amino acid sequence. *Deletion of 28 amino acids. The new mutations described in this report are written in boldface.

3.2.7 Discussion

We are reporting seven novel mutations and one previously described mutation in the *Krit1* gene of CCM-affected individuals. In combination with our previous study, approximately 47% (18/38) of our CCM families harbour *Krit1* mutations, which is higher than the 40% predicted by linkage studies (Dubovsky et al. 1995). This number may still be an underestimate since SSCP gels may not detect all mutations either because of inadequate screening sensitivity of the two-gel method or the inability to detect large deletions. This indicates that *Krit1* mutations may be more frequent than previously estimated, which could have important implications for genetic diagnosis and care. In addition, two of the eight mutations found were in the newly identified coding exons of *Krit1*, demonstrating the exons' importance in Krit1 function and pathogenesis of CCM. To date, a total of 42 *Krit1* mutations have been reported (see table 3.1). Frameshifts account for 50% (21/42) of the observed mutations, 24% (10/42) are nonsense mutations, 19% (8/42) are changes in the invariant splice junctions, 5% (2/42) are missense mutations, and the remaining mutation (1/42) consists of an 84-base pair deletion. The mutations seem to be evenly distributed from exon 9 to 18 (see figure 3.3) and do not seem to be limited to the structural ankyrin/FERM domains present in Krit1. However, we suspect that it is likely that more mutations will be found in the upstream exons as other CCM families will be screened for them. Even distribution of mutations can be seen in tumour suppressor genes such as in neurofibromatosis type 2 (NF2) (Rouleau et al. 1993)

and retinoblastoma (RB1) (Murphree and Benedict 1984), which is in accordance with the model that *Krit1* is a tumour suppressor.

Only two missense mutations have been detected so far. They are found only 191 base pairs apart in exons 7 and 8. Interestingly, one of the missense mutations (Q201E) that we had previously reported as a 5' untranslated region variant destroys a predicted *N*-myristoylation site, which is thought to be needed for plasma membrane interaction. No important structural feature has been detected in that region by the SMART program (Simple Modular Architecture Research Tool: http://smart.embl-heidelburg.de/smart), though this region may become important with additional molecular studies. With the discovery of the new exons, the effects of the mutation on the coding sequence can be more accurately determined.

That 40 of 42 mutations lead to a substantial alteration of the gene product support a loss of function mechanism. If *Krit1* acts as a tumour suppressor, a loss of growth control and formation of a malformation could arise. Further investigation such as loss of heterozygosity or "double hit" studies in malformations would give us some insight into the Krit1 mechanism and indicate whether or not it is a tumour suppressor. More research is needed to fully understand the pathogenesis and implications of CCM and the function of Krit1. In addition, identification of the genes responsible for *CCM2* and *CCM3* will help in understanding the mechanism leading to vascular malformation.

3.2.8 Acknowledgment

The authors thank the families for their cooperation and their physicians for referring them for participation in the study.

3.3 *CCM1* mutation screen of sporadic cases with cerebral cavernous malformations

3.3.1 Abstract

Cerebral cavernous malformations (CCM) are CNS vascular anomalies associated with seizures, headaches, and hemorrhagic strokes. The *CCM1* gene was screened in 35 sporadic cases with either single or multiple CCM. It was found that 29% of the individuals with multiple CCM have a *CCM1* mutation, whereas cases with only one malformation have none. Sporadic cases with multiple malformations warrant the same approach as individuals who have a familial history of CCM.

3.3.2 Introduction

Cerebral cavernous malformation (CCM; MIM 116860) is a common disorder characterized by abnormally enlarged capillary cavities in the brain without intervening normal parenchyma (Rigamonti et al. 1988). It is found in 0.1 to 0.5% of the population and represents 10 to 20% of the cerebral vascular lesions (Rigamonti et al. 1988). CCM most often occurs sporadically but may also be inherited dominantly with incomplete penetrance. Patients may have single or multiple malformations that can lead to focal neurologic signs, hemorrhagic strokes, seizures, or sometimes death (Rigamonti et al. 1988). Patients can be

either managed conservatively or treated with surgical resection when lesions cause recurrent haemorrhage or seizures.

Mutations in the gene *CCM1*, located on chromosome 7q21.2, account for nearly all Hispanic and approximately 40% (Craig et al. 1998) of non-Hispanic familial CCM cases. All *CCM1* mutations found to date are predicted to result in a truncated protein (Verlaan et al. 2002a; Verlaan et al. 2002b). Two other loci were found by linkage analysis: *CCM2* on 7p13-p15 and *CCM3* on 3q25.2-q27, calculated to account for 20 and 40%, respectively, of all remaining familial cases (Craig et al. 1998).

We assessed the incidence of mutation in the *CCM1* gene in individuals harbouring single or multiple cavernous malformations and who have no family history of the disease. For this purpose, individuals were screened for mutation in the *CCM1* gene by denaturing high-performance liquid chromatography (dHPLC).

3.3.3 Methods

Patients: DNA was collected from 21 sporadic cases with one single malformation and 14 sporadic cases with multiple malformations. All individuals participated in the International Familial Cavernous Angioma Study and were located in Switzerland and Germany. In all cases, DNA was collected with informed consent, and the study was approved by the Committee for the Protection of Human Subjects at Dartmouth College (Hanover, NH). Criteria for inclusion in the study were 1) neuroradiologic diagnosis of either single or

multiple cavernous malformations by MRI (including T1, T2, and gradient echo sequences); 2) histological verification of at least one neuroradiologically diagnosed cavernous malformation; 3) no familial history for typical clinical manifestations of cavernous malformations such as seizures, haemorrhage of the CNS, focal neurologic deficits, and headache; and 4) no unexplained death of a family member at an early age (as putative indicator for a fatal haemorrhage of a cavernous malformation).

Mutation detection by dHPLC and sequencing: For dHPLC of the gene *CCM1*, patient DNA was extracted from peripheral blood lymphocytes by standard methods. The *CCM1* gene contains 16 coding and 3 noncoding exons (Sahoo et al. 2001). Each of the 16 coding exons was amplified by PCR with intronic primers (Dupre et al. 2003). PCR-amplified products were denatured by heating to 95 °C for 5 minutes, followed by cooling to room temperature over a 45-minute period to enhance heteroduplex formation. The sequences of these fragments were analyzed using Wavemaker software package (Transgenomic, Omaha, NE). dHPLC detects 96% of all single-base substitutions as well as insertions and deletions one to several base pairs in length compared with single-strand conformational polymorphism (SSCP), which has a power of mutation detection of 85% (Xiao and Oefner 2001). Each variant found by the dHPLC was reamplified by PCR and sequenced on an ABI 3700 sequencer, according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

3.3.4 Results

A total of 35 sporadic cases were screened for mutation in the *CCM1* gene. Of the 14 cases with multiple malformations, 4 (29%) have a *CCM1* mutation leading to truncation of the protein (see table 3.2). Subject 3 has a nonsense mutation in exon 9 (Trp271Stop) (Verlaan et al. 2002a), created by a G-to-C transition at nucleotide position 813, predicted to lead to a protein missing most of its domains. Subject 14 has an invariant splice donor site mutation in exon 16 (IVS16+2T→A), and Subject 34 has an invariant splice acceptor site mutation in exon 8 (IVS8-2A→G). Subject 32 has a nonsense mutation in exon 15 (Glu567Stop), created by a G-to-T transversion at nucleotide position 1,699, predicted to lead to a truncated protein with half of its FERM (band 4.1, ezrin, radixin, moesin) domain missing. In contrast, only a missense mutation was found in 1 of the 21 individuals with a single malformation. Subject 7 has a Lys479Thr missense in exon 14 due to an A-to-C transversion at nucleotide position 1,436. The lysine, which is found in the FERM domain, is conserved within the mouse protein. The variation could not be found in 96 control subjects of European descent. Yet, the only missense mutations ever reported in familial cases actually led to splicing errors (Verlaan et al. 2002b). Unfortunately, the effect of this missense on splicing was not tested, as blood was no longer available from this subject. However, this variation does not seem to be in a conserved sequence for splicing. It is likely to be a rare polymorphism, but further molecular testing would be required to be certain.

3.3.5 Table

Table 3.2: Mutations in sporadic cases with cerebral cavernous malformations

Table Mutations in sporadic cases with cerebral cavernous malformations

Subject no.	Mutation	Exon	Predicted effect	Diagnosis, no. of malformations
3*	813G→C	9	Trp271Stop	Multiple
14	IVS16+2T→A	16	Invariant splice donor site altered	Multiple
32	1699G→T	15	Glu567Stop	Multiple
34	IVS8-2A→G	8	Invariant splice acceptor site altered	Multiple
7	1436A→C	14	Lys479Tyr	Single

* This mutation has already been published.[9]

3.3.6 Discussion

The gene *CCM1* encodes for a 736–amino acid protein called the Krev interaction trapped 1 (Krit1) protein. Krit1 contains three ankyrin repeats, a FERM domain, and an NPxY (Asn-Pro-x-Tyr) motif. Molecular studies show that the NPxY motif seems to modulate a strong interaction with the integrin cytoplasmic domain–associated protein 1 (icap1α), a protein involved with β1-dependent angiogenesis (Zawistowski et al. 2002). In addition, Krit1 has been shown to be a microtubule associated protein that may help determine endothelial cell shape and function in response to cell–cell and cell–matrix interactions by guiding cytoskeletal structure (Gunel et al. 2002).

The chance of finding a deleterious mutation in an individual with multiple malformations (4 in 14) compared with an individual with only a single malformation (0 in 21) is significant (p=0.004, Student t-test); 29% (what we found) and 40% (the expected value) are not statistically different (p=0.361, Student t-test). Hence, our results show that sporadic cases with multiple malformations seem to harbour *CCM1* mutations in approximately the same proportion that familial cases harbour *CCM1* mutations. By extension, mutations in the candidate genes *CCM2* and *CCM3* may account for the cases where no *CCM1* mutation was found. The *CCM1* mutations found in these sporadic individuals are either germline mutations or have been inherited from an asymptomatic parent. Clinically, these individuals may be considered as CCM familial cases that have a higher recurrence risk of malformation formation and a 50% chance of offspring transmission. MRI or other CCM screening method of

the patient's family may also be appropriate for early detection diagnosis. In contrast, sporadic cases with only one malformation do not seem to have any *CCM1* mutation, and their malformation may have been caused by a one-time random mutational event in the *CCM1* gene at the site of the malformation or by mutation in other genes. These cases would have a much lower risk of malformation formation recurrence and little chance of offspring transmission.

A similar study (Reich et al. 2003) that included 64 sporadic cases with a single malformation, 6 sporadic cases with multiple malformations, and 2 individuals from a CCM family for a total of 72 patients found that none of the cases had a *CCM1* mutation. Their results for patients with only one malformation seem to be concordant with our data, suggesting that an inherited *CCM1* mutation is not the cause of the disease. However, the fact that they did not find any mutation in their cases with multiple malformations does not contradict our study but could simply suggest a small sample size or missed mutation due to the limitation of their mutation detection technique (SSCP) (Xiao and Oefner 2001).

Although there are many new molecular findings, the pathologic mechanism of *CCM1* still remains unknown. There are at least two possible mechanisms hypothesized for malformation development. The first mechanism is Knudson's second hit hypothesis in which the normal allele is deactivated, and the second one is a haploinsufficiency model. Further studies will need to confirm either one of these theories.

3.3.7 Acknowledgment

The authors thank the patients and the physicians who referred them for their participation in this study.

4 INVESTIGATION OF *CCM1* MISSENSE MUTATIONS

Reference:

D.J. Verlaan, A.M. Siegel, and G.A. Rouleau (2002) *Krit1* Missense Mutations Lead to Splicing Errors in Cerebral Cavernous Malformation. *American Journal of Human Genetics* 70:1564–156

4.1 Rationale

All *CCM1* mutations discovered in the literature, cause truncation of their respective proteins except for two missense mutations that we had previously reported (D137G and Q201E) (Davenport et al. 2001; Verlaan et al. 2002a). Because of the prevalence of deleterious mutations reported within the *CCM1* gene, we hypothesized that these missense mutations were likely point mutations affecting either the splicing mechanism or altering an important protein domain. In this chapter, the effects of *CCM1* point mutations on splicing were investigated.

4.2 *Krit1* missense mutations lead to splicing errors in cerebral cavernous malformation

4.2.1 Abstract

At least 40% of families affected with cerebral cavernous malformation have a mutation in *Krit1*. We previously identified two point mutations in *Krit1* leading to changes in amino acids (D137G and Q201E) in two different families. Further RNA analysis reveals that both point mutations actually activate cryptic splice-donor sites, causing aberrant splicing and leading to a frameshift and protein truncation. To date, no simple missense mutations have been detected in *Krit1*.

4.2.2 Letter

Cerebral cavernous malformation (CCM [MIM 116860]) is a common autosomal disorder, characterized by abnormally enlarged capillary cavities in the brain without intervening normal parenchyma (Russell and Rubenstein 1989). They occur as single or multiple malformations that lead to focal neurologic signs, hemorrhagic strokes, or seizures. CCMs are found in 0.1%–0.5% of the population and represent 10%–20% of cerebral vascular lesions (Rigamonti et al. 1988). Three genetic loci have been defined: *CCM1* on chromosome 7q21-q22 (Dubovsky et al. 1995; Gunel et al. 1995; Marchuk et al. 1995), *CCM2* on 7p13-p15, and *CCM3* on 3q25.2-q27 (Craig et al. 1998). To date, only one gene has been identified: *Krit1,* for *CCM1* (Laberge-le Couteulx et al. 1999; Sahoo et al. 1999), which is responsible for ≥40% of CCM cases. The Krit1 protein has 736

amino acids (Zhang et al. 2000a; Eerola et al. 2001; Sahoo et al. 2001) and contains three ankyrin repeats, one FERM (Band 4.1, ezrin, radixin, moesin) domain, and one NPxY (Asn-Pro-x-Tyr) motif. It has been recently demonstrated that Krit1 shows a strong interaction with the integrin cytoplasmic domain–associated protein 1 (*ICAP1α*), a protein involved with β1-dependent angiogenesis, through its NPxY motif (Zhang et al. 2001b).

All *Krit1* mutations, except two point mutations predicted to lead to an amino-acid change in two different families (D137G in family IFCAS-41, and Q201E in family IFCAS-28) (Davenport et al. 2001; Verlaan et al. 2002a), lead to a truncated and presumably inactive protein. Both families are part of the International Familial Cavernous Angioma Study (IFCAS), which was approved by the Committee for the Protection of Human Subjects at Dartmouth College. Because of the prevalence of deleterious mutations reported in the *Krit1* gene, we further investigated these two missense mutations, for effects on splicing.

Total RNA was extracted from cultured lymphocytes immortalized with the Epstein-Barr virus, for each member of the families, using an RNeasy mini kit (QIAGEN). A cDNA library was synthesized by RT-PCR, using hexanucleotides (pdN_6). The cDNA sequences encompassing the mutations were PCR amplified by use of exonic primers and were electrophoresed on 2% agarose gel.

The affected members of IFCAS-41 (fig. 4.1*A*) are heterozygous for an A→G substitution (fig. 4.1*B*) in exon 7 at the nucleotide position 410 of the coding sequence. The migration pattern of the cDNA (fig. 4.1*C*) shows that affected individuals have two different-sized alleles, whereas the unaffected individual is homozygous for the larger allele. This result suggests that the substitution may

lead to truncation of the transcript. Sequencing of the different cDNA alleles shows that alternative splicing is occurring in the mutated allele (fig. 4.1*D*). The A→G shift creates an alternative splice site that, when used, results in premature splicing of exon 7 and in splicing of exon 8 at the correct position but in an incorrect reading frame. This would result in a frameshift event, leading to a truncated protein of 138 amino acids that has 2 novel amino acids and contains no structural domains of Krit1.

The affected members of IFCAS-28 (fig. 4.2*A*) are heterozygous for a C→G substitution (fig. 4.2*B*) in exon 8 at nucleotide position 601 of the coding sequence. Similar to IFCAS-41, the migration pattern of the cDNA (fig. 4.2*C*) of IFCAS-28 shows that affected individuals have two different-sized alleles, whereas the unaffected individual is homozygous for the larger allele. This result suggests that the substitution may truncate the transcript. Sequencing of the different cDNA alleles shows that alternative splicing is occurring in the mutated allele (fig. 4.2*D*). The C→G shift creates an alternative splice site that, when used, results in premature splicing of exon 8 and in splicing of exon 9 at the correct position but in an incorrect reading frame. This results in a frameshift, which is predicted to lead to a truncated protein of 201 amino acids that has a novel amino acid and contains only the NPXY motif.

The fact that the RT-PCR products from the normal and mutant alleles ("a" and "b," respectively, in figs. 4.1*C* and 4.2*C*) are of similar intensity suggests that most of the mutant allele is alternatively spliced. In the present study, we present two examples of point mutations in the coding sequence that activate a cryptic

splice-donor site motif (fig. 4.3) that is used preferentially over the downstream authentic splice site.

Thus, all *Krit1* mutations associated with CCM that have been published to date are predicted to result in a truncated protein. This observation suggests that Krit1 protein function needs to be severely impaired for pathogenesis and that no single amino acid change results in a loss of function sufficient to cause CCM. In addition, our findings stress the importance of examining all point mutations, including silent ones, to determine whether they activate a cryptic splice-donor site motif.

4.2.3 Figures

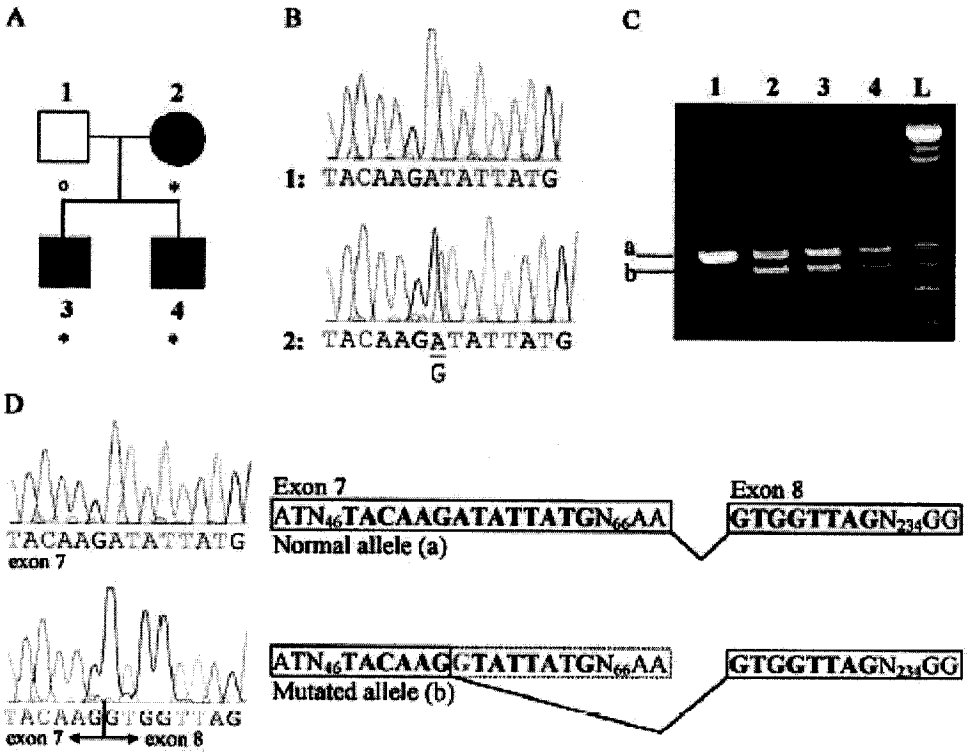


Figure 4.1: DNA and mRNA analysis for IFCAS-41

A, Pedigree of IFCAS-41. The blackened symbols denote affected individuals, and the unblackened square denotes an individual not known to be affected. Asterisks denote mutations, and the small unblackened circle denotes an absence of mutation. *B,* Genomic DNA sequences of unaffected (1) and affected (2) individuals. The affected individual carries an A→G substitution at nucleotide position 410 of the coding sequence. *C,* cDNA migration pattern of the normal (a) and mutated (b) alleles, for each member of the IFCAS family. *D,* cDNA sequences of the normal and mutated alleles. The mutated allele causes cryptic splicing, as is illustrated in the diagram.

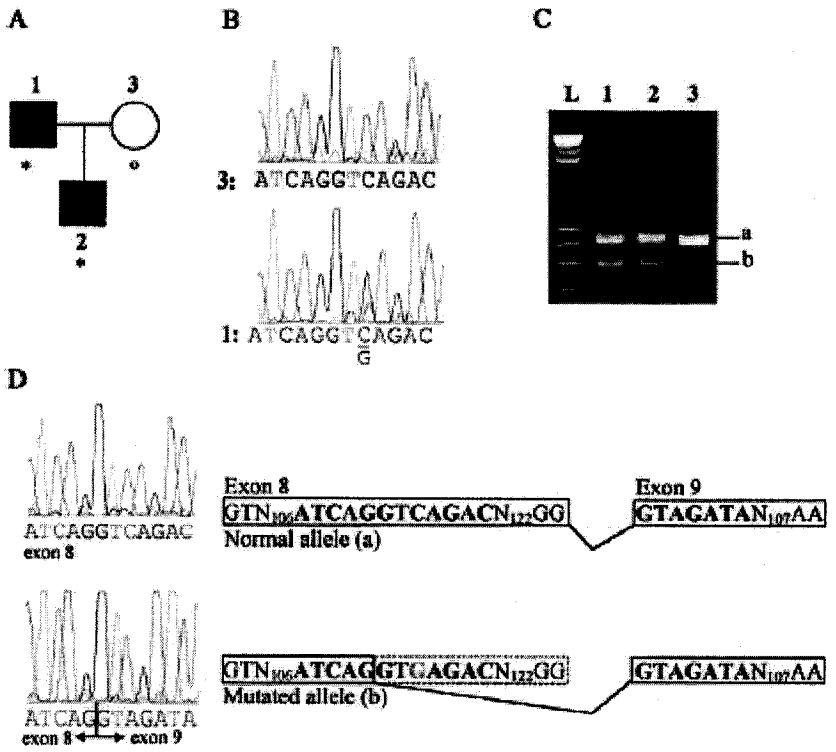


Figure 4.2: DNA and mRNA analysis for IFCAS-28.

A, Pedigree of IFCAS-28. Definitions of symbols are the same as in figure 1. *B,* Genomic DNA sequences of a normal (3) and affected (1) individuals. The affected individual carries a C→G substitution at nucleotide position 601 of the coding sequence. *C,* cDNA migration pattern of the normal (a) and mutated (b) alleles, for each member of the IFCAS family. *D,* cDNA sequences of the normal and mutated alleles. The mutated allele causes cryptic splicing, as is illustrated in the diagram.

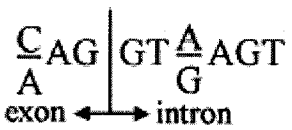


Figure 4.3: Splice-donor site consensus sequence

The A→G substitution in IFCAS-41 changed the first nucleotide of the intron, whereas the C→G substitution in IFCAS-28 changed the third nucleotide of the intron.

4.2.4 Acknowledgments

We would like to thank the families and the physicians who referred the families, for participating in the IFCAS study. We would like to thank Drs. Collette Hand and André Toulouse for their insightful comments and precious aid.

4.2.5 Electronic-database information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CCM [MIM 116860])

5 LINKAGE ANALYSIS

Reference:

N. Dupré, D.J. Verlaan, C. Hand, S.B. Laurent, G. Turecki, J.W. Davenport, N. Acciarri, J. Dichgans, A. Ohkuma, A.M. Siegel, and G.A. Rouleau (2003) Linkage to the *CCM2* locus and genetic heterogeneity in familial CCM. *Canadian Journal of Neurological Sciences* 30:122-128

5.1 Rationale

Following our screening studies of the *CCM1* gene, mutations were undetected in 53% of the families. Of these families, five were large enough to potentially establish linkage to the *CCM2* or *CCM3* loci and merited further research. Genotyping of polymorphic markers found at the different loci was conducted to identify families that were linked to either the *CCM2* or *CCM3* loci. Linkage in any of these families would allow us to move forward in our intent to start a gene identification project for either the *CCM2* or *CCM3* loci.

5.2 Linkage to the *CCM2* locus and genetic heterogeneity in familial cerebral cavernous malformation

5.2.1 Abstract

Background: Cerebral cavernous malformation (CCM) is a form of intracranial vascular disease that may arise sporadically or be dominantly inherited. Linkage studies have revealed genetic heterogeneity among the dominantly inherited forms suggesting the existence of at least three loci called *CCM1*, *CCM2* and *CCM3*.

Methods: In the present study, we screened five families with dominantly inherited CCM for *CCM1* gene mutations with denaturing high performance liquid chromatography (dHPLC). Then, we performed linkage analysis and haplotyping on these five families using highly polymorphic markers at the candidate CCM loci.

Results: None of the five families tested with dHPLC were found to have mutations in the *CCM1* gene. Based on haplotyping, we identified three families segregating alleles for *CCM2*, while two families segregated alleles for *CCM3*. Using linkage analysis, we could confirm that one family (IFCAS-1) had a positive LOD score of 2.03 ($p<0.0001$) at the *CCM2* locus using marker D7S678.

Conclusions: The present study is the first one to replicate linkage at the *CCM2* locus and provides a fifth family identified as such. It also supports the concept of genetic heterogeneity in CCM, identifying four other families that showed no mutations in the *CCM1* gene.

5.2.2 Résumé: Liaison au locus *CCM2* et hétérogénéité dans les CCM familiales.

Contexte: La malformation caverneuse cérébrale (CCM) est une forme de maladie vasculaire intracrânienne qui survient de façon sporadique mais qui peut aussi avoir un mode d'hérédité dominant. Des analyses de liaison ont montré une hétérogénéité génétique parmi les formes dont l'hérédité est dominante, suggérant l'existence d'au moins trois locus, *CCM1*, *CCM2* et *CCM3*.

Méthodes: Dans cette étude, nous avons évalué cinq familles présentant une CCM à hérédité dominante pour déterminer la présence de mutations dans le gène *CCM1* au moyen de la chromatographie en phase liquide à haute performance dénaturante. Nous avons ensuite procédé à une analyse de liaison et à un haplotypage dans ces cinq familles au moyen de marqueurs très polymorphes des gènes candidats CCM.

Résultats: Aucune mutation dans le gène *CCM1* n'a été démontrée dans les cinq familles étudiées au moyen de marqueurs très polymorphes. Nous avons identifié par haplotypage trois familles où il y a ségrégation d'allèles de *CCM2* avec la maladie et deux familles où il y a ségrégation d'allèles de *CCM3* avec la maladie. Nous avons confirmé au moyen de l'analyse de liaison, qu'une famille avait un LOD score positif de 2,03 ($p<0,0001$) au locus *CCM2* en utilisant lemarqueur D7S678.

Conclusions: Cette étude est la première à reproduire une liaison au locus *CCM2* et identifie une cinquième famille dans laquelle la maladie est liée à ce gène. Elle

supporte également le concept d'une hétérogénéité génétique dans la CCM en identifiant quatre autres familles où on n.a pas décelé de mutation dans le gène *CCM1*.

5.2.3 Introduction

Cerebral cavernous malformation (CCM; OMIM 116860) is a dominantly inherited form of intracranial vascular disease with a prevalence of up to 0.5% in the general population (Otten et al. 1989; Del Curling et al. 1991). A proportion of patients affected by these lesions become symptomatic, presenting usually between 20 and 40 years of age with intracranial haemorrhage, focal neurological deficits or seizures. Patients can be managed either conservatively or treated with surgical resection when lesions cause recurrent haemorrhage or seizures (Maraire and Awad 1995; Siegel et al. 2000). Little is known about the pathophysiology of these lesions which have very characteristic features on magnetic resonance imaging (MRI), and are described pathologically as dilated sinusoidal vascular spaces surrounded by a collagenous matrix devoid of mature vessel elements (Perl and Ross 1989; Russell and Rubenstein 1989).

Both autosomal dominantly inherited and sporadic forms of the disease are recognized (Rigamonti et al. 1988). Among Hispanic Americans with CCM, there is evidence of a founder mutation in the *CCM1* gene (chr. 7q21-q22) that accounts for almost all familial cases (Gunel et al. 1996a). In non-Hispanic kindreds, however, while some support linkage to *CCM1*, families have been described for which linkage to this locus is excluded (Gunel et al. 1996b; Craig et al. 1998). Multilocus linkage on non-Hispanic kindreds has revealed linkage to two

additional loci referred to as *CCM2* (chr. 7p15-13) and *CCM3* (chr. 3q25.2-27) (Craig et al. 1998). At the present stage, the gene responsible for *CCM1* (coding for KRIT1) has been identified (Laberge-le Couteulx et al. 1999), while the genes responsible for *CCM2* and *CCM3* remain unknown (Craig et al. 1998). The present study evaluated linkage in five non-Hispanic kindreds that participated in the International Familial Cavernous Angioma Study (IFCAS) (Siegel et al. 1998; Verlaan et al. 2002a) to assess the role of previously identified loci.

5.2.4 Subjects

Twenty-nine families were identified in kindreds collected from the IFCAS (Siegel et al. 1998). These families were from Switzerland, Italy, United States, Japan and Germany. Neurological signs and symptoms included visual deficit, paresis, seizure, paresthesia and headache. Diagnoses were based on MRI or computed tomography (CT) in at least two affected family members and in some cases biopsy, surgical excision or autopsy. Asymptomatic subjects with no history of stroke, seizure disorder or focal neurological deficit were classified as unaffected. Index cases of each family were screened for *CCM1* gene mutations, and 11 were identified (Davenport et al. 2001). Of the remaining families, five were chosen for linkage analysis at the known CCM loci since they were large enough to yield potentially significant LOD scores and were not found to have *CCM1* gene mutations. These families were from Switzerland (IFCAS-1, IFCAS-4, IFCAS-31) and from the United States of Irish descent (IFCAS-35).

5.2.5 Methods

Mutation detection

Families were screened for mutations in the *CCM1* gene using denaturing high performance liquid chromatography (dHPLC). After informed consent, blood samples were taken from each subject and DNA was extracted from peripheral blood by standard methods. Each of the 16 *CCM1* coding exons was amplified by polymerase chain reaction (PCR) with intronic primers (Table 5.1). Polymerase chain reaction-amplified products were denatured by heating to 95°C for five minutes, followed by cooling to room temperature over a 45 minute period to enhance heteroduplex formation. The sequences of these fragments were analyzed using Wavemaker software package (Transgenomics, USA). Each variant found by DHPLC was re-amplified by PCR and directly sequenced on an ABI 3700 automated sequencer using BigDye chemistry, according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA, USA).

Linkage analysis and haplotyping

Linkage was performed using highly informative di-, tri-, and tetranucleotide polymorphic markers retrieved either from the Cooperative Human Linkage Center (CHLC) (http://lpg.nci.nih.gov/CHLC/) or from the Centre d'Étude du Polymorphisme Humain (CEPH) (http://www.cephb.fr/). Primer sequences were obtained either from the CHLC or from the GDBTM Human Genome Database (http://www.gdb.org/). Each primer pair was amplified according to specific PCR conditions and labelled with 35S-dATP. Polymerase chain reaction-amplified

products were transferred on 6% denaturing acrylamide gels and visualized on autoradiography film. Marker allele frequencies were obtained from the CEPH database and map distances between markers were determined from the constructed linkage map of Marshfield (http://research.marshfieldclinic.org). For haplotyping of *CCM1*, we used markers D7S2410, D7S646, D7S1789, and D7S1813; for *CCM2*, markers D7S2846, D7S510, D7S521 and D7S678; for *CCM3*, markers D3S3053, D3S3041, D3S1571, and D3S1262. For linkage analysis of *CCM1*, we used markers D7S2410, D7S1813, and D7S646; for *CCM2*, D7S2846, D7S510, D7S667, and D7S678; for *CCM3*, D3S1571, D3S1754, D3S3041, and D3S3053. Linkage analysis was performed with the MLINK program of the FASTLINK v.3.0P analytical package (Cottingham et al. 1993; Schaffer et al. 1994) using an autosomal dominant inheritance model with 90% non age-dependant penetrance and 0.1% phenocopy. This model had been used before in previous similar studies (Dubovsky et al. 1995; Craig et al. 1998) considering that precise epidemiological data is still lacking. Empirical P value was obtained using the SIMULATE program and the replicates were analyzed using MSIM program of the SLINK package (Ott 1989; Weeks and Lathrop 1990). Ten thousand replicates were carried out, using the corresponding genetic model and marker information from linkage computation.

5.2.6 Results

The families large enough to perform linkage analysis were IFCAS-1, IFCAS-4, IFCAS-31, IFCAS-33, and IFCAS-35. They were first screened for mutations in

the *CCM1* gene with the dHPLC method which has more than 90% sensitivity (O'Donovan et al. 1998). None of the five families were found to have mutations in the *CCM1* gene. Then we used highly polymorphic markers to perform haplotyping in each individual family for each known CCM loci (*CCM1*, *CCM2*, and *CCM3*). The segregation pattern of each marker in each family was established (Figure 5.1). For IFCAS-1, IFCAS-31 and IFCAS-33, haplotypes supported segregation of markers with the *CCM2* locus but not with the *CCM1* or *CCM3* loci. For IFCAS-4 and IFCAS-35, haplotypes supported segregation of markers with the *CCM3* locus but not with *CCM1* or *CCM2* loci. Then, we performed two-point linkage analysis in each family for each locus (Table 5.2). A maximum LOD score of 2.03 at $\theta = 0$ ($p < 0.0001$) was obtained for IFCAS-1 with marker D7S678, 1.07 cM telomeric from the point of highest LOD score reported by Craig et al. (Craig et al. 1998) at marker D7S521, therefore supporting linkage of the IFCAS-1 family to the *CCM2* locus. We also performed multipoint linkage analysis in IFCAS-1 at the *CCM2* locus with markers D7S510, D7S678, and D7S667 (Figure 5.2). Linkage was excluded for IFCAS-1 at the *CCM1* locus and for IFCAS-35 at the *CCM2* locus. Otherwise, none of the other families showed significant LOD scores that could allow definite exclusion or confirmation of linkage.

5.2.7 Tables

Table 5.1: Primers for *KRIT1* coding exons

Table 1: Primers for PCR amplification and sequencing of KRIT1 coding exons for CCM1 gene screening (all primers read 5' to 3').

Exons	Forward primers (sense)	Reverse primers (antisense)
4	TTGAGTAGTTGAACAGTAAAGATG	AAAATAATGGGCAGAGACCTAAA
5	TATGCAGCTAGAGTTGAGAAAGAC	CTAGGGAACTACACTTCACATCAA
6	TTTGCATTTATCAGTTTTTATTAG	TGCCTTCCCTCCTCATT
7	CAAGGTCACAGAGCTAGTCATCAC	ACCCAGGCCAGGACAACCTTA
8	CACTTTCGAATGGCTACTTCTACC	ACTGTACCAGGCCTTCATGTTTAT
9	TGACAAAGCTCTTAATGGGT	GACTACAATGCATACAAATTGC
10	AAACAATTTTTACAGTCCTGTTG	AGAACAGTCTTGAAAAGAAGGA
11	CATTTCAGATGATCTTTTTAGG	TGTCATTACTTGTTATTCACTGCT
12	ATTGGATGACATTTTCCCTT	AGCCATCTAATCGTCTTTCC
13	AGCACATGAAGTTGAAGGAA	CCCAAAAAGGAATAATGAGG
14	GAAGTGCAGACAGTTTAATACAAA	CTCAACAGATTTTGTGCATTT
15	GCTTTTTCTTTTCCCATATT	TAGCACAAGACCATGCATAA
16	CGTTACTGAAAGCCATTTGT	CAGGACTATAAATTTAATCTACCTCTG
17	CAATGGTACATTTTCCTTTCA	AGGTTGGTACTGTTGTTTTAACT
18	CTGAACTATTATATTTAGAGCAGACA	CACAATAGTTTATGAAGTCCAAA
19	CCCAATGTCATGAATTTCC	GCTCGGCCAAAAGTAATA

Table 5.2: Two-point LOD scores at CCM loci

Table 2: Two-point Lod scores for ranges of theta obtained for each family at loci CCM1, CCM2 and CCM3.

Theta		0.00	0.01	0.05	0.10	0.20	0.30	0.40
IFCAS-1								
CCM1	D7S2410	-5.18	-2.52	-1.13	-0.58	-0.16	-0.04	-0.01
	D7S1813	-4.19	-1.44	-0.61	-0.24	0.06	0.11	0.05
	D7S646	-3.65	-0.95	-0.25	0.01	0.16	0.13	0.05
CCM2	D7S2846	0.95	0.96	0.94	0.88	0.66	0.37	0.10
	D7S510	1.21	1.21	1.18	1.10	0.83	0.48	0.13
	D7S667	1.13	1.13	1.08	0.99	0.70	0.37	0.09
	D7S678	2.03	2.02	1.94	1.79	1.38	0.85	0.28
CCM3	D3S1571	0.35	0.37	0.40	0.39	0.31	0.18	0.05
	D3S1754	0.55	0.57	0.60	0.59	0.49	0.32	0.12
	D3S3041	0.52	0.54	0.56	0.55	0.45	0.28	0.10
	D3S3053	-1.49	-0.41	0.29	0.53	0.59	0.41	0.15
IFCAS-4								
CCM1	D7S2410	0.87	0.85	0.77	0.66	0.45	0.24	0.07
	D7S1813	0.04	0.05	0.09	0.11	0.10	0.06	0.02
	D7S646	-0.03	-0.03	-0.03	-0.03	-0.02	-0.01	0.00
CCM2	D7S2846	-3.84	-1.85	-1.06	-0.69	-0.31	-0.12	-0.03
	D7S510	-0.40	-0.39	-0.35	-0.28	-0.16	-0.07	-0.02
	D7S667	-0.47	-0.46	-0.40	-0.32	-0.18	-0.08	-0.02
	D7S678	-0.30	-0.29	-0.25	-0.20	-0.11	-0.05	-0.01
CCM3	D3S1571	-0.03	-0.03	-0.02	-0.02	-0.01	-0.01	0.00
	D3S1754	0.82	0.80	0.72	0.61	0.40	0.21	0.06
	D3S3041	0.82	0.80	0.72	0.61	0.40	0.21	0.06
	D3S3053	0.82	0.80	0.72	0.61	0.40	0.21	0.06
IFCAS-33								
CCM1	D7S2410	0.19	0.19	0.16	0.13	0.08	0.04	0.01
	D7S1813	-0.69	-0.60	-0.37	-0.22	-0.08	-0.02	0.00
	D7S646	0.30	0.30	0.27	0.24	0.17	0.09	0.03
CCM2	D7S2846	0.33	0.31	0.26	0.20	0.10	0.04	0.01
	D7S510	0.30	0.29	0.24	0.18	0.09	0.03	0.00
	D7S667	0.48	0.47	0.42	0.36	0.24	0.12	0.03
	D7S678	0.20	0.19	0.15	0.11	0.04	0.01	0.00
CCM3	D3S1571	-0.73	-0.63	-0.39	-0.23	-0.08	-0.02	0.00
	D3S1754	-1.01	-0.85	-0.52	-0.32	-0.13	-0.05	-0.01
	D3S3041	-1.01	-0.85	-0.52	-0.32	-0.13	-0.05	-0.01
	D3S3053	-0.30	-0.28	-0.22	-0.17	-0.09	-0.04	-0.01

Continued over ...

Table 5.2 (continued)

Table 2: Two-point Lod scores for ranges of theta obtained for each family at loci CCM1, CCM2 and CCM3 continued ...

IFCAS-31								
CCM1	D7S2410	-2.91	-0.92	-0.30	-0.08	0.05	0.07	0.05
	D7S1813	-1.37	-1.29	-1.03	-0.78	-0.43	-0.22	-0.09
	D7S646	0.35	0.34	0.31	0.28	0.21	0.14	0.07
CCM2	D7S2846	-0.80	-0.72	-0.46	-0.25	-0.03	0.05	0.05
	D7S510	0.38	0.38	0.36	0.33	0.26	0.18	0.08
	D7S667	-0.32	-0.27	-0.15	-0.06	0.04	0.07	0.05
	D7S678	-0.35	-0.32	-0.21	-0.12	0.00	0.05	0.05
CCM3	D3S1571	-3.42	-1.41	-0.71	-0.42	-0.16	-0.04	0.01
	D3S1754	-2.36	-0.94	-0.32	-0.11	0.03	0.06	0.04
	D3S3041	-0.02	-0.01	0.03	0.06	0.09	0.09	0.06
	D3S3053	0.10	0.10	0.08	0.07	0.04	0.02	0.00
IFCAS-35								
CCM1	D7S2410	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	D7S1813	0.30	0.30	0.28	0.26	0.20	0.15	0.08
	D7S646	0.30	0.30	0.28	0.26	0.20	0.15	0.08
CCM2	D7S2846	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	D7S510	-3.45	-1.69	-1.00	-0.70	-0.40	-0.22	-0.10
	D7S667	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	D7S678	-3.45	-1.69	-1.00	-0.70	-0.40	-0.22	-0.10
CCM3	D3S1571	0.18	0.18	0.16	0.15	0.12	0.08	0.04
	D3S1754	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	D3S3041	-0.18	-0.17	-0.15	-0.13	-0.10	-0.06	-0.03
	D3S3053	0.30	0.30	0.28	0.26	0.20	0.15	0.08

5.2.8 Figures

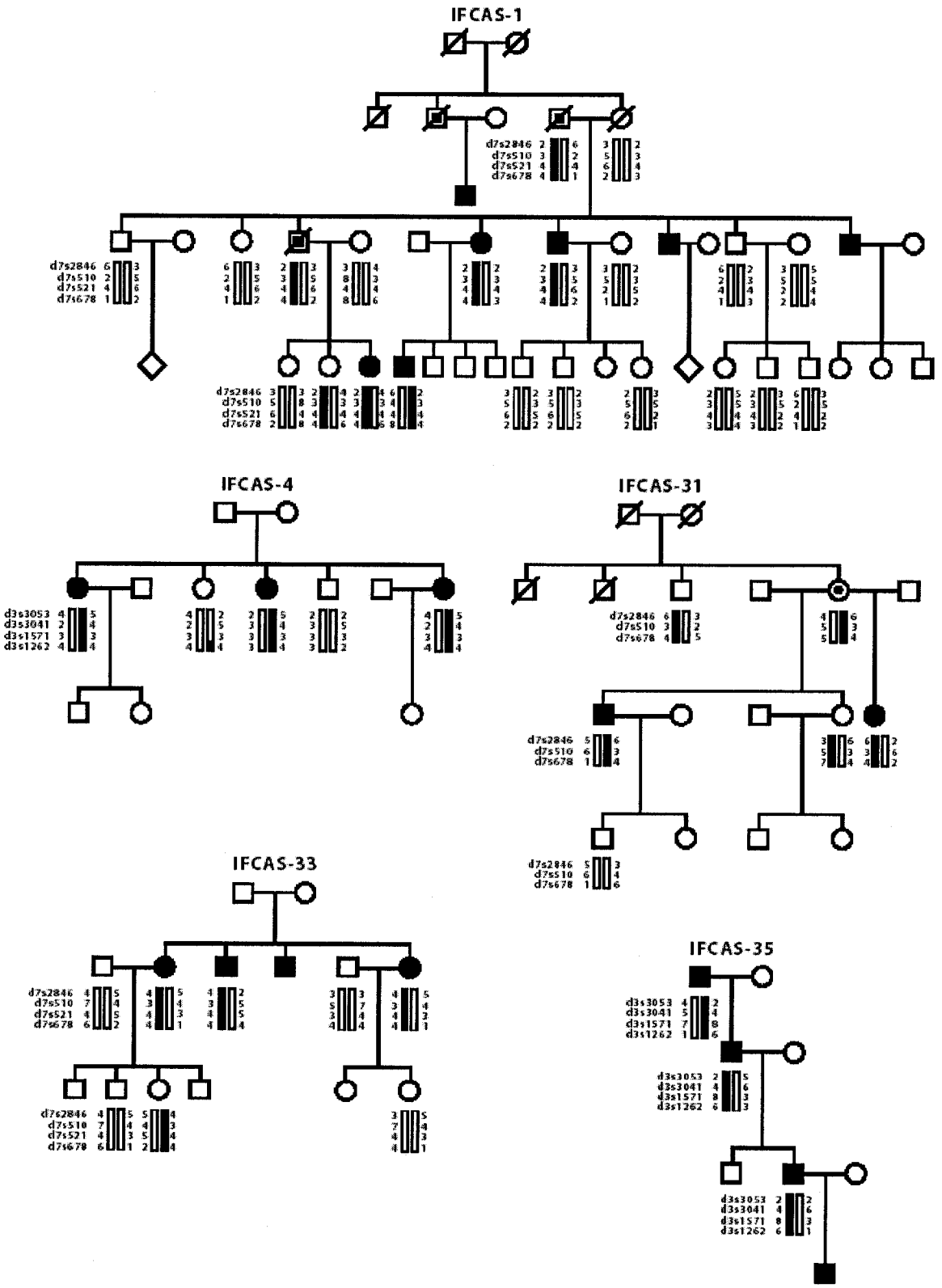


Figure 5.1: Haplotypes of IFCAS-1, IFCAS-31, IFCAS-33 (*CCM2* locus) and IFCAS-4, IFCAS-35 (*CCM3* locus).

Black symbols indicate subjects considered affected based on MRI or pathological diagnosis. Open symbols indicate subjects considered unaffected based on clinical evaluation. Open symbols with black dots indicate obligate carriers. Crossed symbols indicate deceased subjects.

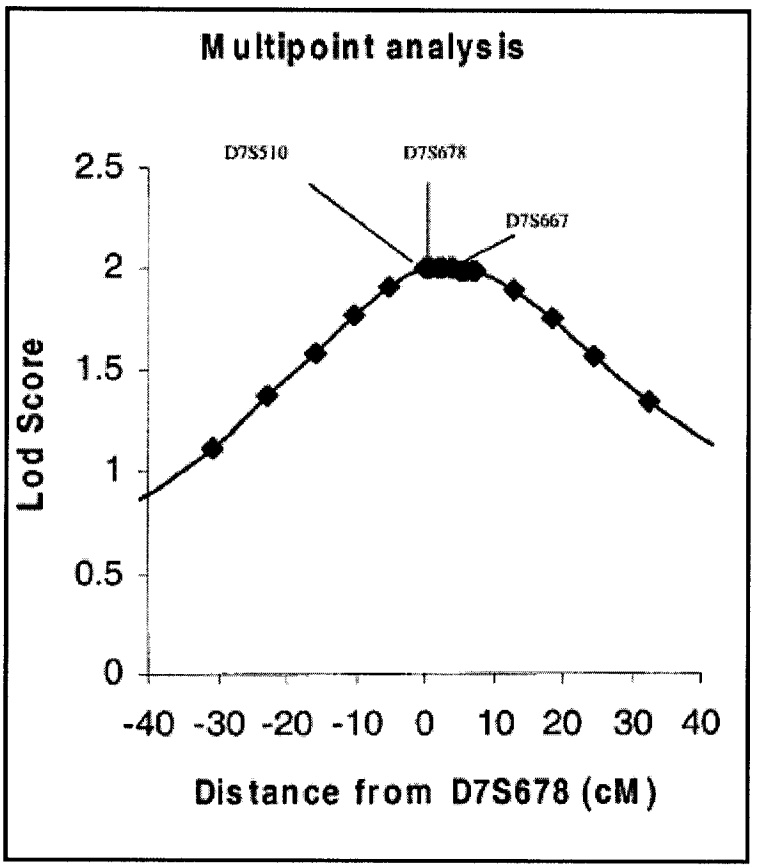


Figure 5.2: Multipoint analysis for IFCAS-1

Multipoint analysis of linkage performed for *CCM2* with IFCAS-1 using markers D7S510, D7S678 and D7S667. The highest LOD score (2.01) is 1.07 cM telomeric from marker D7S521.

5.2.9 Discussion

In the present study, we report linkage of one family (IFCAS-1) to the *CCM2* locus. IFCAS-1 was tested for mutations in the *CCM1* gene, and none was found. Additionally, linkage was excluded at the *CCM1* locus for this family, while it was confirmed at the *CCM2* locus with a positive LOD score and supportive empirical p value. As for the other families, they are unlikely to be related to the *CCM1* locus, since they have been screened for mutations in the *CCM1* gene with dHPLC, which provides more than 90% sensitivity (O'Donovan et al. 1998). Additionally, haplotypes did not suggest linkage to the *CCM1* locus. For IFCAS-31 and IFCAS-33, haplotypes supported linkage to the *CCM2* locus, while for IFCAS-4 and IFCAS-35 haplotypes supported linkage to the *CCM3* locus. Obviously, these cannot be considered as definitive proof of linkage, since the LOD scores were not significant because of the small sample size in each family. Previous studies have shown evidence of genetic heterogeneity in CCM. Laberge et al. (Laberge et al. 1999) showed that in the French population, the proportion of families linked to the *CCM1* locus was of 65%. Gunel et al (Gunel et al. 1996b) reported two non-Hispanic families showing no evidence of linkage to the *CCM1* locus. Craig et al (Craig et al. 1998) reported definite linkage in three families to the *CCM1* locus, in four families to the *CCM2* locus, and in two families to the *CCM3* locus. Our study thus provides evidence supporting genetic heterogeneity in familial CCM, as well as being the first report replicating the existence of the *CCM2* locus and suggesting that other families may also be linked to the *CCM2* and *CCM3* loci. The recent description of the *CCM1* gene (Laberge-le Couteulx et

al. 1999) will assist greatly in the identification of other CCM genes that might have sequence homology to *CCM1* or that might be acting on the same biochemical pathway. We are presently carrying out candidate gene screening for the *CCM2* and *CCM3* loci, which should allow us to identify the gene responsible for these disorders. As other genes are identified, we will need to investigate further the pathogenesis of cavernous angioma formation to understand why, for example, while all blood vessels harbour inherited CCM mutations, only a small number of lesions develop.

5.2.10 Acknowledgements

We thank the families involved in these studies and the physicians who referred them to us. AMS was supported by the Schweizerischer Nationalfonds (Swiss National Research Foundation), the Kommission zur Förderung des akademischen Nachwuchses des Kt.Zürichs and the Julius Klaus-Stiftung. GAR is supported by the Canadian Institute of Health Research.

6 *CCM2* GENE ANALYSIS

Reference:

D.J. Verlaan, S.B. Laurent, D.L. Rochefort, C.L. Liquori, D.A. Marchuk, A.M. Siegel and G.A. Rouleau (2004) *CCM2* mutations account for 13% of cases in a large collection of kindreds with hereditary cavernous malformations. *Annals of Neurology* 55:757-758

and

Reference:

D.J. Verlaan, S.B. Laurent, G.A. Rouleau and A.M. Siegel (2004) No *CCM2* mutations in a cohort of 31 sporadic cases. *Neurology* 63:1979.

6.1 Candidate gene screen for the *CCM2* locus

Since results from our linkage study were not entirely conclusive, and we could not accurately conclude which families and loci were linked, only two candidate genes (*TXBP151* and *CAB66721)* were screened for *CCM2* (see table 6.2). These candidate genes were selected because of their location on the border of a chromosomal translocation event in two different patients with a sporadic presentation of the disease. This method for identifying chromosomal anomalies had proven successful on at least one occasion for Kurotaki et al in sporadic cases of Sotos syndrome (Kurotaki et al. 2002).

Twenty families that did not have a *CCM1* mutation were screened for *TXBP151* and *CAB66721* by denaturing High Performance Liquid Chromatography (dHPLC). Variants were subsequently sequenced (Applied Biosystems, Foster City, CA). Sequence traces were viewed and analyzed by the program Seqman (DNAstar package).

The first of the two genes screened was *TXBP151* (Tax1 [human T-cell leukemia virus type I] binding protein 1) (De Valck et al. 1999). The second and larger gene that was screened was a hypothetical protein called *CAB66721.* It is now known that the product of the *CAB66721* gene (now called *ELMO1*) is involved in cytoskeletal rearrangements required for phagocytosis of apoptotic cells and cell motility (Gumienny et al. 2001).

Table 6.1: Candidate genes screened for *CCM2*

Gene	# of Exons	Protein (aa)	Genomic DNA spanned
TXBP151	18	474	89,072 bp
CAB66721	22	727	594,490 bp

While no significant variants were found within these genes during our experiments, we were approached by another group that was actively screening *CCM2* candidate genes. DNA samples from families who were linked or possibly linked to the *CCM2* region were subsequently sent to them for further testing and our collaborators successfully identified the *CCM2* gene as being the gene *MGC4607* (Liquori et al. 2003).

6.2 Screening *CCM2* for mutations in CCM families

Mutations in the *MGC4607* gene identified by Liquori and colleagues (Liquori et al. 2003) provided the opportunity to locate mutations in the identified *CCM2* gene and to determine the relative frequency of *CCM2* mutations that caused CCM. In the first study (section 6.3) 21 IFCAS families were screened for mutations. In the second study (section 6.4) the *CCM2* gene was screened in 21 sporadic cases containing one single malformation and an additional 10 sporadic cases containing multiple malformations.

6.3 *CCM2* mutations account for 13% of cases in a large collection of kindreds with hereditary cavernous malformations

6.3.1 Letter

Cerebral cavernous malformation (CCM) is a common disorder characterized by abnormally enlarged vascular cavities in the brain without intervening normal parenchyma (Russell and Rubenstein 1989). It is found in 0.1% to 0.5% of the population and represents 10% to 20% of cerebral vascular lesions (Rigamonti et al. 1988). CCM can be inherited dominantly or can occur sporadically and can develop as single or multiple malformations that lead to focal neurological signs, hemorrhagic strokes, seizures, or sometimes death (Siegel et al. 1998). Recently, mutations in the *MGC4607* gene were found in families that showed linkage to the *CCM2* locus (Liquori et al. 2003). Consequently, 21 families that are part of the International Familial Cavernous Angioma Study (IFCAS) were screened for mutations in this new *CCM2* gene. Criteria for inclusion in the study were neuroradiological diagnosis by magnetic resonance imaging or histological verification of at least one neuroradiologically diagnosed cavernous malformation of the central nervous system in at least two family members.

The *CCM2* gene codes for the malcavernin protein (Liquori et al. 2003). It contains 444 amino acids and has a predicted phosphotyrosine-binding (PTB)

domain located at amino acids 66 to 224. All 10 exons were screened for mutation by denaturing high performance liquid chromatography (dHPLC) (Transgenomics, Omaha, NE). Each variant found by the dHPLC then was subsequently sequenced (Applied Biosystems, Foster City, CA). Exon 1 was not screened by dHPLC because of high GC content but was directly sequenced.

We report the identification of three novel mutations segregating in four different families (Figure 6.1). An invariant splice donor site mutation (IVS1+1G→A) was found in exon 1 and an invariant splice acceptor site mutation (IVS3-1G→A) was found in exon 3. In exon 2, a nonsense mutation at amino acid 19 (Arg19Stop) was found to be segregating in two European families, which may indicate a common founder. Degradation of the mRNA may occur through the nonsense mediated decay mechanism unless it is translated leading to a severely shortened protein with no PTB domain. To date, 45 IFCAS families have been screened for mutations in *CCM1* and *CCM2*. Twenty-two (49%) of these families segregate a *CCM1* mutation (Verlaan et al. 2002a), whereas 6 (13%) families contain a *CCM2* mutation (two families previously reported (Liquori et al. 2003)). There remain 17 (38%) families with no *CCM1* or *CCM2* mutations but which will almost certainly have mutations in the non-identified *CCM3* gene, and possibly in some as yet unidentified CCM locus. More work will be needed, such as the identification of the *CCM3* gene, to further understand the pathological mechanisms involved in the formation of CCMs.

We thank the patients and the physicians for their participation in this study.

6.3.2 Figure

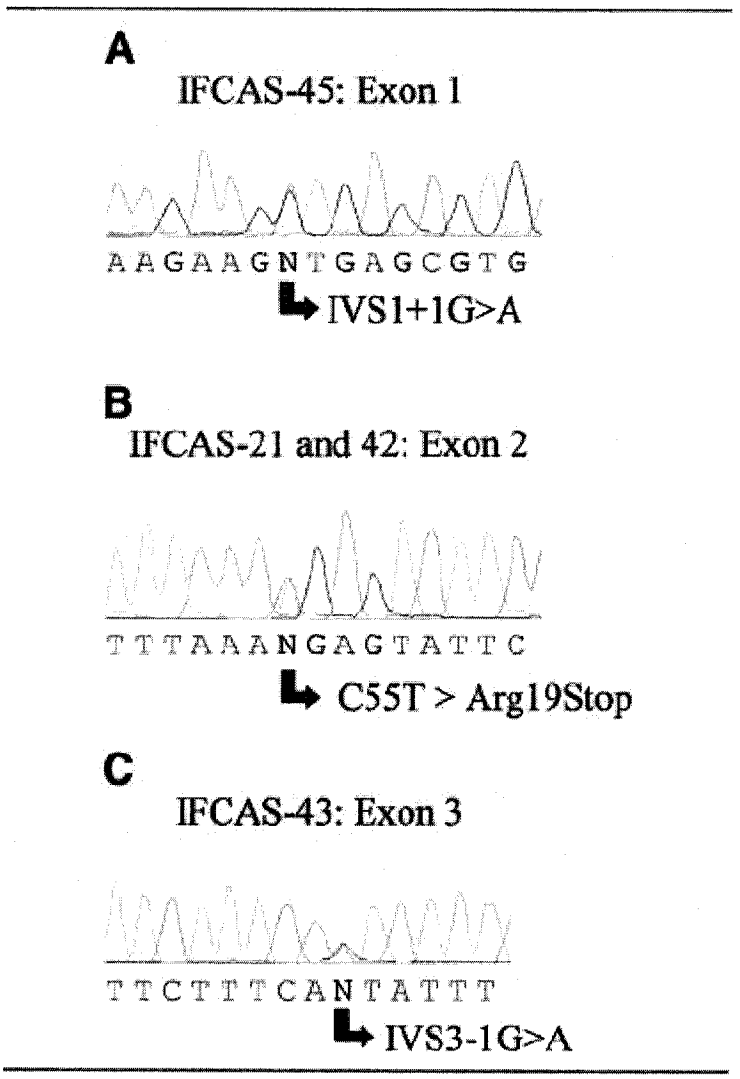

A
IFCAS-45: Exon 1
AAGAAGNTGAGCGTG
IVS1+1G>A
B
IFCAS-21 and 42: Exon 2
TTTAAANGAGTATTC
C55T > Arg19Stop
C
IFCAS-43: Exon 3
TTCTTTCANTATTT
IVS3-1G>A

Figure 6.1: Sequence traces of *CCM2* mutations.

(A) Invariant splice donor site mutation leading to a shortened protein with no phosphotyrosine-binding (PTB) domain. (B) A C to T transversion causing a nonsense mutation leading to degradation of the mRNA or to shortened protein with no PTB domain. (C) Invariant splice acceptor site mutation leading to deletion of exon 3 and part of the PTB domain. IFCAS= International Familial Cavernous Angioma Study.

6.4 No *CCM2* mutations in a cohort of 31 sporadic cases

6.4.1 Introduction

Cerebral cavernous malformation (CCM; MIM 116860) is a common disorder that is found in 0.1 to 0.5% of the population and represents ~10 to 20% of cerebral vascular lesions (Rigamonti et al. 1988). However, symptomatic disease is considerably less common. The lesions, which affect the brain vasculature, are characterized by abnormally enlarged capillary cavities in the brain without intervening normal parenchyma (Russell and Rubenstein 1989). Most often, CCM occurs sporadically, and individuals develop only one malformation. In familial CCM, the disorder is dominantly inherited, and individuals often develop multiple malformations. Symptoms include focal neurologic signs, hemorrhagic strokes, seizures, headaches, or sometimes death (Rigamonti et al. 1988). Patients are treated conservatively or with surgical resection when lesions cause recurrent haemorrhage or seizures.

Three genetic loci have been defined (Craig et al. 1998): *CCM1* on chromosome 7q21-q22 accounting for 40% of all familial cases, *CCM2* on 7p13-p15 accounting for 20%, and *CCM3* on 3q25.2-q27 for 40%. Subsequently, the *CCM1* gene has been identified and has been shown to encode the Krit1 protein (Laberge-le Couteulx et al. 1999). More recently in *CCM2* families, a novel gene, MGC4607, has been found that encodes malcavernin, a protein with a phosphotyrosine-binding domain.

We previously screened the *CCM1* gene in a cohort of 21 sporadic cases with a single malformation and 14 sporadic cases with multiple malformations (Verlaan et al. 2004c). Of the 14 cases with multiple malformations, 4 cases had a mutation leading to a truncated protein, which suggested that sporadic cases with multiple malformations seem to harbour *CCM1* mutations in approximately the same proportion that familial cases harbour *CCM1* mutations. In contrast, none of the 21 sporadic cases with a single malformation had a mutation, suggesting that the malformation may have been caused by a one-time random mutational event in one of the CCM genes.

Mutations in the *MGC4607* gene recently were found in CCM families, which show linkage to the *CCM2* locus (Liquori et al. 2003). Therefore, we decided to screen the *CCM2* gene for mutation in the 21 sporadic cases with one malformation and the remaining 10 sporadic cases with multiple malformations.

The *CCM2* gene found at locus 7p13 contains 10 coding exons and codes for the malcavernin protein (Liquori et al. 2003). Malcavernin is 444 amino acids long with a hypothetical molecular weight of 48.8 kDa and contains a predicted phosphotyrosine-binding domain (PTB) located at amino acids 66 to 224. It is expressed in the brain and is highly expressed in skeletal muscle, heart, and liver with minimal or no expression in colon and lung (Liquori et al. 2003).

6.4.2 Methods

In all cases, DNA was collected with informed consent, and the Committee for the Protection of Human Subjects at Dartmouth College (Hanover, NH) approved the

study. Individuals were located in Switzerland and Germany. Criteria for inclusion in the study were 1) neuroradiologic diagnosis of either single or multiple cavernous malformations by MRI; 2) histological verification of at least one neuroradiologically diagnosed cavernous malformation; and 3) no familial history for typical clinical manifestations of cavernous malformations, such as seizures, haemorrhage of the CNS, focal neurologic deficits, and headache.

Each of the 10 *CCM2* exons was amplified by PCR with intronic primers. Exons 2 to 10 were screened by denaturing high performance liquid chromatography (dHPLC) by denaturing the PCR product by heating to 95° C for 5 minutes, followed by cooling to room temperature during a 45-minute period to enhance heteroduplex formation. Each variant found by the dHPLC was reamplified by PCR and sequenced on an ABI 3700 sequencer (Applied Biosystems, Foster City, CA). Exon 1 was not screened by dHPLC because of the high guanine cytosine content of the fragment but was directly sequenced.

6.4.3 Results and discussion

Our results show that of the 21 cases with a single malformation and of the 10 cases with multiple malformations, none had a *CCM2* mutation. It is most possible that mutations in the candidate gene *CCM3* or unidentified CCM locus may account for the cases in which no *CCM2* mutation was found. Because our sample size is small, our study does not imply that *CCM2* mutations are not a cause of CCM in sporadic individuals but may rather indicate that the *CCM2* gene may account for only a small proportion of CCM cases. We also observed this in

another study in which we screened the *CCM2* gene in a large collection of kindred affected with CCM; of the 45 families, 49% had *CCM1* mutations, 13% had *CCM2* mutations, and 38% had neither (Verlaan et al. 2004a). To further understand the pathologic mechanisms involved in the formation of CCMs, more research—such as the identification of the *CCM3* gene and functions of malcavernin and Krit1—will be needed.

6.4.4 Acknowledgments

The authors thank the patients for their participation in this study.

7 *CCM3* GENE ANALYSIS

Reference:

D.J. Verlaan, J. Roussel, C.E. Elger, A.M. Siegel and G.A. Rouleau (2005) *CCM3* mutations are uncommon in cerebral cavernous malformations. *Neurology* 65:1982-1983.

7.1 Candidate gene screen for the *CCM3* locus

7.1.1 Rationale

Over the course of our previous experiments, it was determined that 17 CCM families possessed no identifiable mutation within the *CCM1* or *CCM2* genes. Unfortunately, due to the small family size of this remaining group, determining absolute linkage to the *CCM3* locus was not likely.

The *CCM3* locus, found at 3q25.2-q27, is located between the genetic markers D3S3053 and D3S1262 (see figure 7.1).

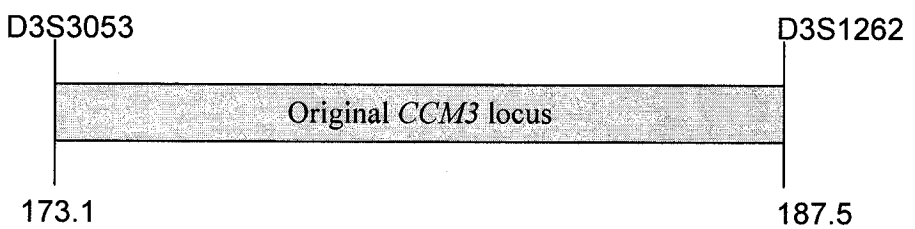


Figure 7.1: Map of the original *CCM3* linkage interval.

Numbers indicate the location on chromosome 3 in Mb.

This region contains more than 70 known genes, so a *restrictive approach* was needed in order to identify the *CCM3* gene. Our approach was based on the observation that certain mutations in *CCM1* and *CCM2* were independently identified in multiple families suggesting a shared haplotype and/or origins. Two

examples of this occurrence are the frameshift mutation 1362delTC found in the *CCM1* gene (Lucas et al. 2001; Sahoo et al. 2001) and the nonsense *CCM2* mutation Arg19X identified in two different families of European descent (Verlaan et al. 2004a). This pattern represented a potential opportunity to investigate *CCM3* with the remaining 17 families based on genetic homogeneity.

7.1.2 Hypothesis

Since certain mutations in the *CCM1* and *CCM2* genes have been identified in more than one family, we hypothesized that it may therefore be possible that some of the remaining 17 families shared a common mutation in the *CCM3* gene. By studying the ancestry of the families, we determined that this might be feasible in three families of German ancestry and six families of Italian ancestry. Twenty-two polymorphic markers were genotyped within the *CCM3* locus in the nine families using methods previously described in Chapter 5.

7.1.3 Results

Haplotyping revealed that two sets of haplotypes were shared between D3S3037 and D3S2312 (see table 7.1). One haplotype set (¥) was shared by two Italian families (IFCAS 4 and 5) and the other set (φ) was shared by two separate Italian families (IFCAS 16 and 38). A third and smaller shared haplotype (я) was also observed in two Italian families (IFCAS 5 and 16) between markers D3S1232 and D3S1262. No obvious shared haplotype was observed within the German

families. We also observed that two families (IFCAS 5 and 9) seemed to reduce the *CCM3* interval by excluding the region above the D3S3037 marker and one family (IFCAS-33) excluded the region below marker D3S1571.

Table 7.1: Disease haplotype of European families genotyped at the *CCM3* locus

Markers	Location (Mb)	German families			Italian families					
		33	8	9	15	4	5	48	38	16
D3S3053	173.1	3	3		3	4		3	3	3
D3S2421	176.4	6	6		8	6		2	3	7
D3S3041	177.8	6	5		6	4		5	6	4
D3S2412	178.4	1	6		3	1		6	6	1
D3S3715	178.7	4	3		6	3		3	4	4
D3S3037	178.8	7	7		7	8		10	11	8
D3S3730	179.9	5	5	9	4	**9¥**	**9¥**	8	**8φ**	**8φ**
BAF53A	180.6	C	T	T	C/T	**C**	**C/T**	T	**T**	**T**
D3S3699	180.6	2	5	5	2	**2**	**2**	5	**5**	**5**
D3S3565	180.8	3	3	3/2	2	**3**	**3**	3	**2**	**2**
PEX5R	180.8	G	G	G	G/A	**G**	**G/A**	A	**G**	**G**
D3S3662	181.7	1	2	1	2	**2**	**2**	2	**2**	**2**
FXR	182.0	G/A	A	G/A	G	G	A	A	**A**	**A**
D3S2312	182.7	4	1	5	4	6	4	4	9	8
D3S1232	182.7	5	4	3	4	4	4	7	7	5
D3S2314	183.5	4	4	3	4	1	**4¤**	3	3	**4¤**
D3S1521	184.5	4	2	2	1	1	**2**	2	1	**2**
D3S1618	184.7	2	6	2	2	4	**4**	3	2	**4**
D3S1571	184.9		3	3/8	8	3	**1**	3	3	**1**
D3S1262	187.5		6	6	8	5	6	5	4	8

Since the two possible shared haplotypes (¥ and φ) were situated between D3S3037 and D3S2312, our gene screening effort focused on genes within this interval. Based on the UCSC freeze of May 2004 (http://genome.ucsc.edu), seventeen genes were located within that interval (Table 7.2). Genomic sequences from the *CCM3* locus were obtained from the databases to determine the structure of the candidate genes and design PCR primers for screening. Each gene segment

from the four affected individuals (in their respective families) was amplified using PCR, and the products were sent for sequencing at the Genome Québec Innovation Centre. Sequence traces were viewed and analyzed using the program Seqman (DNAstar package).

Table 7.2: Genes found between markers D3S3037 and D3S2312

	Gene Name	# Exons	Location	Description
D3S3037			178762515	
1	*KCNMB2*	5	179597400	large conductance calcium activated potassium channel β2 subunit
2	*WIG1*	6	180063634	P53 target zinc finger protein
3	*PIK3CA*	20	180237517	Phosphatidylinasitol-4,5-bisphosphate 3 kinase catalytic subunit, involved in ovarian cancer
4	*KCNMB3*	6	180281468	Potassium large conductance calcium activated channel β3 subunit
5	*ANC_2H01*	6	180362463	Kruppel-like zinc finger protein
6	*MFN1*	18	180415762	Mitofusin 1, mitochondrial fusion, essential for embryonic devel.
7	*GNB4*	10	180439767	Guanine nucleotide binding protein beta subunit 4
8	*BAF53A*	14	180601644	?
9	*MRPL47*	17	180601657	Mitochondrial 39S ribosomal protein L47
10	*NDUFB5*	6	180643496	NADH-Ubiquinone oxidoreductase SGDH subunit, mitochondrial precursor
11	*AK055159*	1	180664278	weakly similar to TLM protein
12	*USP13*	21	180691853	Ubiquitin carboxyl-terminal hydrolase 13
13	*PEX5R*	15	180839755	PXR2b
14	*FLJ00166*	12	181640917	Micronuclear linker histone polyprotein with TPR domain
15	*AL122120*	25	181652708	Similar to myosin, exp in brain
16	*FXR1*	17	182001634	Fragile X mental retardation syndrome related protein 1
17	*LOC131118*	6	182023250	Similar to RIKEN cDNA 1810055d05
D3S2312			182712074	

While the most promising genes were screened first (such as *AL122120* and *USP13*), all genes were eventually screened. Unfortunately, no causative variants were found during this screening. Without positive results from the ¥ and φ shared haplotype sets, the focus shifted to the third shared haplotype set (я) located

between markers D3S1232 and D3S1262 (see figure 7.2). This interval subsequently revealed thirteen additional genes (Table 7.3).

Table 7.3: Genes found between markers D3S1232 and D3S1571

	Gene Name	# Exons	Location	Description
D3S1232			182740679	
1	*SOX2*	1	182750988	SRY (sex determining region Y)-box 2
2	*ATP11B*	9	183936001	Phospholipid-transporting ATPase
3	*DCUN1D1*	9	183981472	Squamous cell carcinoma-related oncogene
4	*MCCC1*	19	184053920	methylcrotonoyl-Coenzyme A carboxylase 1
5	*LAMP3*	6	184160915	Lysosomal-associated membrane protein 3.
6	*B3GNT5*	2	184308370	candidate for lactotriaosylceramide synthase
7	*MCF2L2*	30	184216743	Rho family guanine-nucleotide exchange factor
8	*AK097976*	2	184529710	?
9	*KLHL6*	8	184526270	Kelch-like 6
10	*KLHL24*	8	184836105	Kelch-like, DRE1 protein
11	*AK090720*	1	184842684	?
12	*YEATS2*	31	184898300	Yeats domain-containing protein 2
13	*MAP6D1*	3	184854582	?
D3S1571			184860826	

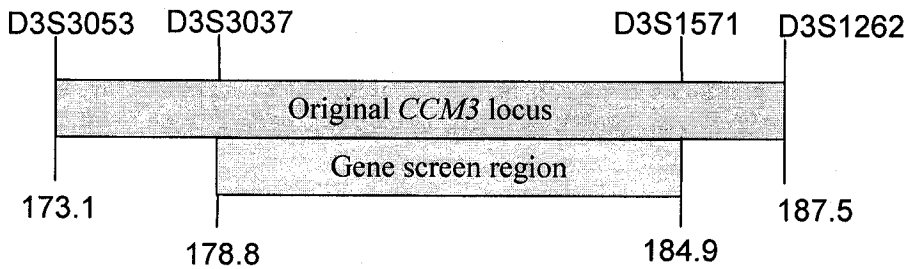


Figure 7.2: Candidate gene screen region compared to the original *CCM3* linkage interval.

Numbers indicate the location on chromosome 3 in Mb.

7.1.4 Discussion

In total, 30 genes were screened in four families. Most of them were completely screened and some were partially screened. However during this time, Bergametti and colleagues identified the *CCM3* gene to be the *PDCD10* gene (Bergametti et al. 2005). Interestingly, this gene is not in the interval reported by (Craig et al. 1998) The *PDCD10* gene is actually located 4.35 Mb upstream of the D3S3053 which was the uppermost limit of the locus. It appears that one of the families which pointed to the *CCM3* locus in the original linkage (Craig et al. 1998) was actually linked to the *CCM1* locus and hence shifted the locus' left border to the right (see figure 7.3).

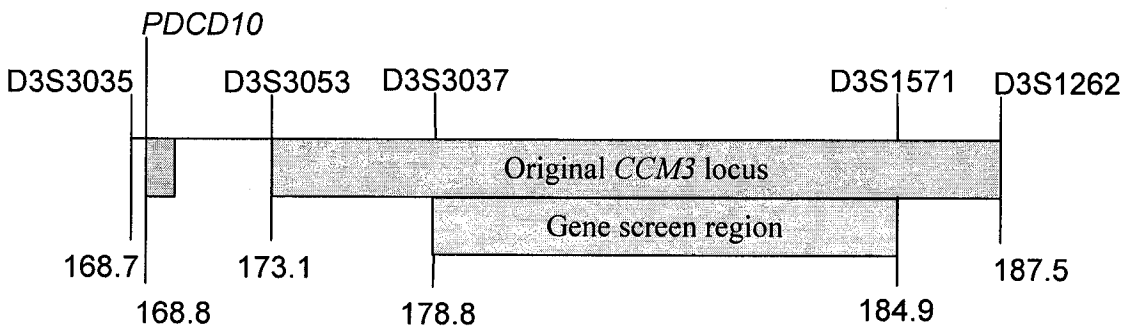


Figure 7.3: Location of the *PDCD10* gene to the original *CCM3* linkage interval. Numbers indicates the location on chromosome 3 in Mb.

7.2 Screening *CCM3* for mutations in CCM families

Since Bergametti et al. discovered mutations in the *PDCD10* gene in families that showed linkage to the *CCM3* locus (Bergametti et al. 2005), the focus of research shifted to i) find mutations in the identified *CCM3* gene and ii) to determine the relative frequency with which *CCM3* mutations caused CCM.

In section 7.3, the *CCM3* gene was screened for mutations in 15 IFCAS families. In section 7.4, the *CCM3* gene was screened for mutations in 21 sporadic cases containing one single malformation and 10 sporadic cases containing multiple malformations.

7.3 *CCM3* mutations are uncommon in cerebral cavernous malformations

7.3.1 Abstract

Cerebral cavernous malformations (CCMs) are characterized by abnormally enlarged capillary cavities without intervening brain parenchyma. Mutations in the gene *PDCD10* have been found in CCM families linked to the *CCM3* locus. The authors screened this gene in 15 families that did not have a *CCM1* or *CCM2* mutation. Only two novel mutations were found, suggesting that mutations in this gene may only account for a small percentage of CCM familial cases.

7.3.2 Introduction

Cerebral cavernous malformations (CCMs [OMIM 116860]) are found in approximately 0.5% of the population and represent 10% to 20% of cerebral vascular lesions (Rigamonti et al. 1988). Symptomatic disease is considerably less common. These vascular malformations are characterized by abnormally enlarged vascular cavities in the brain without intervening normal parenchyma (Russell and Rubenstein 1989). Single or multiple malformations may develop, which can lead to focal neurologic deficits, hemorrhagic strokes, seizures, or death (Rigamonti et al. 1988). These patients can be treated either conservatively or with surgical resection when lesions cause recurrent hemorrhage or seizures. Individuals often

become symptomatic between 20 and 40 years of age, although lesions have been described in all age groups, with no sex predominance. MRI of asymptomatic members of affected families often shows that CNS lesions can be clinically silent, and because all first-degree relatives of patients with CCM may not be screened radiologically, the ratio of truly sporadic cases to familial cases may be overestimated.

Three genetic loci have been previously identified (Craig et al. 1998): *CCM1* on 7q21-q22, *CCM2* on 7p13-p15, and *CCM3* on 3q25.2-q27. The *CCM1* gene (Laberge-le Couteulx et al. 1999) codes for the Krit1 protein (KREV interaction trapped 1), whereas the *CCM2* gene (Liquori et al. 2003), also known as *MGC4607*, codes for the Malcavernin protein. Recently, mutations in the *PDCD10* gene were found in families that showed linkage to the *CCM3* locus (Bergametti et al. 2005). Consequently, we sought to determine whether mutations in this gene might be the cause of disease in 15 CCM families that do not have a *CCM1* or a *CCM2* mutation.

7.3.3 Methods

DNA was collected from families participating in the International Familial Cavernous Angioma Study (IFCAS), which was approved by the Committee for the Protection of Human Subjects at Dartmouth College (Hanover, NH). In all cases, blood samples were collected with informed consent. Inclusion criteria were diagnosis by MRI or histologic verification of at least one neuroradiologically diagnosed cavernous malformation of the CNS in at least two

family members. DNA was extracted from peripheral blood lymphocytes by standard methods. In some cases, total RNA was extracted from cultured lymphocytes immortalized with the Epstein–Barr virus, using an RNeasy mini kit (QIAGEN). A complementary DNA library was synthesized by reverse-transcriptase PCR, using hexanucleotides (pdN_6).

In all of the probands, each of the ten *CCM3* exons was amplified by PCR with intronic primers and directly sequenced on an ABI 3700 sequencer, according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA). In seven of the probands, the coding portion of the *PDCD10* messenger RNA (mRNA) transcript was sequenced as well.

7.3.4 Results

Fifteen familial probands affected with CCM were screened for mutations in the ten exons of the *PDCD10* gene. Two novel mutations were detected in two families (figure 7.4). The first mutation, which is found in exon 7, is a transversion of a C to a T at nucleotide 283, which produces a nonsense mutation at amino acid 95 (C283T, Arg95Stop) (figure 7.5A). The second mutation is an invariant splice acceptor site mutation where an A is mutated to a G (IVS8-2A>G) located in exon 8 (figure 7.5B). Both mutations are predicted to lead to a truncated protein.

7.3.5 Figures

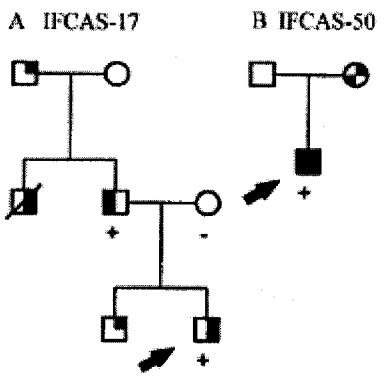


Figure 7.4: Familial segregation of the *CCM3* mutations in (a) IFCAS-17 and (b) IFCAS-50

Asymptomatic hemorrhage seizure liver angioma, Blackened symbols are affected individuals while non-blackened symbols are individuals not known to be affected. Black arrows indicate probands. The + indicates the presence of a mutation and the – indicates that there is no mutation. Figure 2: Sequence traces of *CCM3* mutations (a) A C to T transversion at nucleotide 283 causes a nonsense mutation at amino acid 95 (b) A change from an A to a G causes an invariant splice acceptor site mutation.

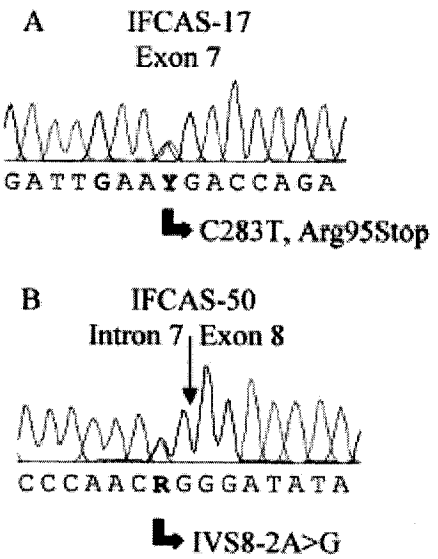


Figure 7.5: Sequence traces of *CCM3* mutations in familial CCM.

(A) A C to T transversion at nucleotide 283 causes a nonsense mutation at amino acid 95. (B) A change from an A to a G causes an invariant splice acceptor site mutation.

7.3.6 Discussion

The *CCM3* gene (*PDCD10*), found at the locus 3q25.2-q27, was recently discovered to code for the programmed cell death 10 protein and contains ten exons (Bergametti et al. 2005). The resulting protein contains 212 amino acids but does not seem to contain any known domain. Probands of 15 families affected with CCM, but that did not have a *CCM1* or a *CCM2* mutation, were screened for mutations in this *PDCD10* gene.

We identified two novel mutations found in two different families (see figure 7.4). The first mutation is a nonsense mutation (Arg95Stop), whereas the second is an invariant splice acceptor site mutation (IVS8-2A>G) (see figure 7.5). These types of mutations are identical to those found in *CCM1* (Verlaan et al. 2002a) and in *CCM2* (Liquori et al. 2003; Verlaan et al. 2004a), they are deleterious mutations predicting to lead to truncation of the protein with possible inactivation of the protein. Similar to *CCM1* and *CCM2*, a genotype–phenotype pattern may prove difficult to establish because all mutations reported seem to result in a loss of normal protein.

In addition, because all mutations reported are deleterious and an individual with a CCM mutation may have multiple lesions in different areas of the brain, a loss of heterozygosity mechanism may be more probable than one of haploinsufficiency.

Initially, an estimated proportion for CCM was 40% for *CCM1*, 20% for *CCM2*, and 40% for *CCM3* (Craig et al. 1998), and hence a higher proportion of *PDCD10* mutation might be expected in the presented cohort. It is possible that some

mutations have been missed because sequencing of the mRNA transcript was not done in all cases and large genomic deletion would have been undetected by the methods used. However, our study is comparable to the original *PDCD10* study (Bergametti et al. 2005) where, out of 20 families, 12 did not have a *CCM1*, *CCM2*, or *CCM3* mutation. This may suggest a high level of undetected large genomic deletions within that region or that there is another unidentified CCM gene.

Unfortunately, because of the small size of the families, it was not possible to perform linkage analysis to establish whether the families are really linked to the known loci (where mutations have been undetected) or whether these families are linked to another gene.

To date, 46 IFCAS families have been screened for mutations in the three CCM genes. Twenty-five (54%) of these families segregate a *CCM1* mutation (Verlaan et al. 2002a) (not all data published), 6 families (13%) contain a *CCM2* mutation (Liquori et al. 2003; Verlaan et al. 2004a), and only 2 families (4%) segregate a *CCM3* mutation. There remain 13 families (29%) that have no detectable mutations.

Molecular studies have shown that the *CCM1* gene protein, Krit1, binds to a phosphotyrosine binding (PTB) domain containing protein called icap1α (integrin cytoplasmic domain-associated protein), which also binds to the β1-integrin cytoplasmic domain (Zhang et al. 2001b). By extension, an interaction between the PTB domain of Malcavernin, the *CCM2* gene product, and with Krit1 or with the β1-integrin cytoplasmic tail may be possible (Liquori et al. 2003). Because the programmed cell death protein does not seem to have any known domains, no

similar link is yet apparent. To date, one study has implicated the programmed cell death protein in the mechanism of apoptosis (Busch et al. 2004); however, the protein has not been studied in detail. More research is needed to further understand the pathologic mechanisms involved in the formation of cerebral cavernous malformations.

7.3.7 Acknowledgments

The authors thank the patients and the physicians who referred them for their participation.

7.4 Two *CCM3* mutations in a cohort of 31 sporadic cases

7.4.1 Rationale

After the *CCM3* gene was identified, our research focus shifted from families affected by CCM to sporadic cases. Two cohorts of sporadic cases were subsequently screened for mutations; 21 cases with one malformation and 10 sporadic cases with multiple malformations. The methods utilized were the same as the ones found in Section 7.3.3.

7.4.2 Results

Ten sporadic cases affected with multiple CCMs were screened for mutations in the ten exons of the *PDCD10* gene. One novel and one previously identified (Verlaan et al. 2005) mutations were detected in two probands (see figure 7.6). The first mutation, is an invariant splice acceptor site mutation where an A is mutated to a G (IVS8-2A>G) located in exon 8 (see figure 7.6a). The second mutation, found in exon 9, is a deletion of a T at nucleotide 501 (501delT), which leads to frameshifting (see figure 7.6b). Both mutations are predicted to lead to a truncated protein. No *CCM3* mutations were identified in the single malformation cohort.

7.4.3 Figure

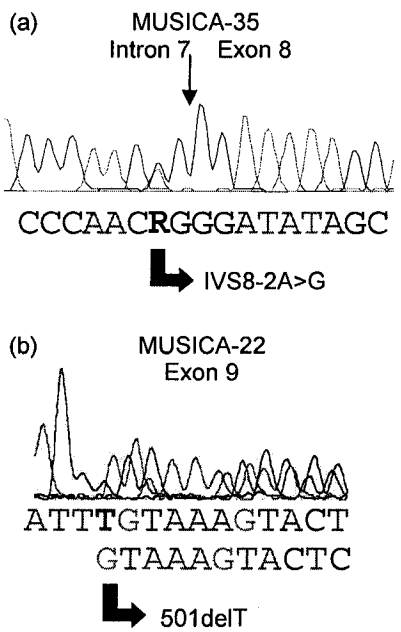


Figure 7.6: Sequence traces of *CCM3* mutations in sporadic cases.

(a) A change from an A to a G causes an invariant splice acceptor site mutation.

(b) Deletion of a T at nucleotide 501 causes a frameshift.

7.4.4 Discussion

The *CCM3* gene (*PDCD10*), found at the locus 3q25.2-q27, was recently discovered to code for the programmed cell death 10 protein and contains ten exons (Bergametti et al. 2005). The resulting protein contains 212 amino acids but does not seem to contain any known domain. Ten sporadic cases with multiple CCMs and 21 sporadic cases with a single CCM, but with no *CCM1* or a *CCM2* mutation, were screened for mutations in this *PDCD10* gene.

One novel and one previously identified mutations were found in two different cases with multiple CCMs. The first mutation is an invariant splice acceptor site mutation (IVS8-2A>G) whereas the second is a deletion mutation (501delT) (see figure 7.6). Both mutations are predicted to lead to a truncated protein. Not surprisingly, no *CCM3* mutations were identified in the single malformation cohort.

Interestingly, the splice mutation was also identified in another family (Verlaan et al. 2005). Haplotyping markers found around the *CCM3* gene will help us determined if the mutation as arisen twice independently or if the sporadic case and the family share a common ancestor.

The fact that we found two mutations in our multiple malformation cohort was unexpected since no *CCM2* mutation had previously been found in this patient population. Nevertheless, this study confirms the involvement of the *PDCD10* gene in CCM and demonstrates that having a small cohort of patients may yield different results. In the end, four of fourteen (29%) individuals with multiple CCMs had a *CCM1* mutation (Verlaan et al. 2004c), none had a *CCM2* mutation

(Verlaan et al. 2004b) and two had a *CCM3* mutation (14%). It remains to be seen if the eight remaining cases with no detectable mutation have a mutation in another CCM causative gene or whether they have a mutation that was not detectable by the methods utilized.

8 MLPA ANALYSIS OF CCM SAMPLES

Reference:

U. Felbor, S. Gaetzner, D.J. Verlaan, R. Vijzelaar, G.A. Rouleau and A.M. Siegel (2007) Large germline deletions and duplication in isolated cerebral cavernous malformation patients *Neurogenetics* 8:149-153

8.1 Rationale

At this stage of our research, many cases screened for CCM mutations had no detectable mutation. Due to the number of unresolved cases, an alternative method of detection was employed for a new degree of sensitivity. Multiplex ligation-dependent probe amplification (MLPA) technique was used for the detection of large genomic rearrangements within genes.

In this study, the *CCM1*, *CCM2* and *CCM3* genes of the remaining eight sporadic cases with multiple CCM were analysed using MLPA to more accurately determine the extent of involvement of the CCM genes in our cohort.

8.2 Large germline deletions and duplication in isolated cerebral cavernous malformation patients

8.2.1 Abstract

Cerebral cavernous malformations (CCM) are vascular lesions that predispose to headaches, seizures, and hemorrhagic stroke. Hereditary CCMs are usually associated with the occurrence of multiple CCMs and occur with a frequency of 1:2000 to 1:10000. In this study, eight isolated cases with multiple CCMs but no *CCM1-3* point mutation were analyzed using the multiplex ligation-dependent probe amplification assay. Four genomic rearrangements were identified including a previously unreported large duplication within the *CCM1* gene and a novel deletion involving the entire coding region of the *CCM2* gene. Consequently, systematic screening for *CCM* deletions/duplications is recommended.

8.2.2 Introduction

Cerebral cavernous malformations (CCM) consist of abnormally dilated, immature blood vessels with a lobulated appearance. Due to their fragile cytoarchitecture, they tend to rupture and to cause intracranial haemorrhages. Clinical symptoms include recurrent headaches, seizures, and hemorrhagic stroke and may occur in early childhood. However, CCMs generally manifest during the second to fourth decade of life. Neurosurgical resection is usually indicated in the

following situations: rapid cavernoma growth, repetitive haemorrhages or a major haemorrhage, occurrence of intractable epilepsy or neurological deficits (Raychaudhuri et al. 2005).

Patients with familial CCM often present with multiple lesions whose number and size increase with age. The prevalence of familial CCM has been estimated to be 1:2000 – 1:10000 (Denier et al. 2006). Familial CCM is caused by autosomal dominant, inactivating mutations in three genes, *CCM1*, *CCM2*, and *CCM3*. A mutation detection rate of 94% is achieved for familial CCM if RNA-based screening techniques are used in addition to exon-by-exon sequencing. At least 57% of isolated cases with multiple lesions also harbour detectable mutations in *CCM1*, *CCM2* or *CCM3* (Denier et al. 2006). One explanation for the lack of detectable mutations in the remaining patients is the existence of large genomic rearrangements which escape detection by direct sequencing and RT-PCR-analyses of coding regions. Only a few genomic deletions have thus far been identified (Table 8.1) (Denier et al. 2004; Bergametti et al. 2005; Gaetzner et al. 2006). These have been associated with incomplete penetrance and the same intrafamilial phenotypic variability ranging from complete lack of symptoms to fatal haemorrhage (Gaetzner et al. 2006) has been observed for patients with small mutations.

We have applied the multiplex ligation-dependent probe amplification (MLPA) technique which permits the detection of large genomic rearrangements (Schouten et al. 2002) to a previously analyzed Swiss cohort of 14 isolated patients affected with multiple cerebral cavernous malformations (Verlaan et al. 2004c). In this cohort, only four *CCM1* and two *CCM3* mutations were previously identified

((Verlaan et al. 2004c) and unpublished data). Using an exon scanning mutation detection strategy based on denaturing high-pressure liquid chromatography (dHPLC) as well as direct sequencing, none of these individuals revealed a mutation in the *CCM2* gene (Verlaan et al. 2004b). We here demonstrate the identification of two previously overlooked genomic *CCM2* deletions as well as one large *CCM1* deletion and one multi-exon duplication within the *CCM1* gene applying the MLPA method.

8.2.3 Methods

DNA was collected with informed consent and approval of the Committee for the Protection of Human Subjects at Dartmouth College (Hanover, NH). Criteria for inclusion in the study were 1) neuroradiologic diagnosis of multiple CCMs by MRI, 2) histologic verification of at least one neuroradiologically diagnosed CCM, and 3) negative family history for typical clinical manifestations of CCMs. This cohort is referred to as Swiss cohort, since the majority of individuals analyzed were Swiss patients attending an epilepsy outpatient clinic.

MLPA analyses were performed according to the manufacturer's instructions using two MLPA kits (SALSA MLPA Kits P130 & P131 CCM, MRC Holland). *CCM1-3* MLPA analyses of three control individuals in each test and the eight patient samples were carried out according to the manufacturer's instructions using an ABI Prism 310 genetic analyzer and GeneMapper 3.7 data collection software. In addition to visual examination of the peaks, MLPA signals were exported to Microsoft Excel. The individual peak area of each PCR product was

divided by the mean peak area of all internal controls. Then, the quotient of each patient sample was divided by the mean of five corresponding quotients from healthy controls.

Primers for long-range PCR were *CCM1*ex17forward (5'-ATCGTACCTG TTACCAAACTG-3') and *CCM1*ex7reverse (5'-ATAAATAATG ATGCTTCTCTGC-3'). The following *CCM2* SNPs were analyzed: rs 2107732, 2304689, 11552376, 11552377, 2289366, 2289367, 2289368, 2304691, 3214691.

8.2.4 Results

Heterozygous changes in exon copy number were observed in four individuals. One patient suffered from partial epilepsy with complex partial seizures due to multiple CCMs since the age of 24 years (Fig. 8.1). In this patient, an increase in copy number of *CCM1* exons 7-17 was found suggesting a large, multi-exon duplication of *CCM1* exons 7 through 17. Notably, this duplication was observed independently in contiguous MLPA probes in both MLPA kits (Fig. 8.2E, F) and not in controls (Fig. 8.2A, C, D) or further patients (Fig. 8.2B, G-M). To confirm this first large duplication within a *CCM* gene, long-range PCR was performed with primers localized in exons 17 and 7 of the *CCM1* gene. An 1853 bp PCR product could be amplified from the patient's DNA but not from control DNA (Fig. 8.3). Sequencing of the entire product revealed breakpoints at positions c.1818+455 and c.263-1259 in introns 17 and 6, respectively (data not shown). This novel multi-exon duplication event likely leads to a *CCM1* frameshift mutation (p.N607EfsX6).

In a further patient with multiple CCMs manifesting with partial epilepsy at the age of 16 years, a heterozygous deletion of all *CCM1* exons was found while *CCM2* and *CCM3* peaks and ratios did not differ between patient and controls (Fig. 8.2G, H). A similar deletion of the *CCM1* gene was previously detected in a German CCM family and confirmed with three informative single nucleotide polymorphisms (Gaetzner et al. 2006). It could be demonstrated that the two *CCM1* gene deletions had occurred twice independently since the Swiss patient did not share the German disease haplotype 2-3-3-2 based on the order of microsatellite markers D7S2410-D7S1813-D7S2189-D7S646 linked to the disease locus (data not shown).

A novel heterozygous deletion of all contiguous *CCM2* exons was reproducibly identified in another case while *CCM1* and *CCM3* exons showed no copy number changes (Fig. 8.2I, K). In support of these results, none of the nine intragenic SNPs analyzed revealed heterozygosity. This patient with multiple supratentorial CCMs experienced an ataxia due to an additional infratentorial CCM at age 50. Finally, two adjacent MLPA probes designed for *CCM2* exon 1 and the 5'-upstream region were reduced in the fourth patient (Fig. 8.2L, M) who presented with partial epilepsy with simple partial seizures due to multiple CCMs at the age of 19 years. Two large deletions including the first exon of the *CCM2* gene have previously been published (Denier et al. 2004). It remains to be determined whether a common founder exists for the Swiss patient and one of the two French families.

8.2.5 Figures

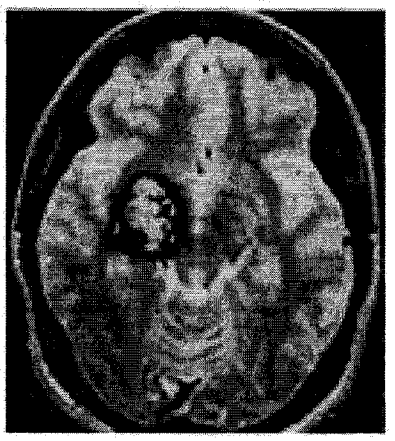


Figure 8.1: MRI of a CCM patient

T1-weighted magnetic resonance imaging shows the typical appearance of a cavernous malformation (hypo/hyperintense) with perilesional hemosiderin deposits (hypointense rim) in the right temporal lobe.

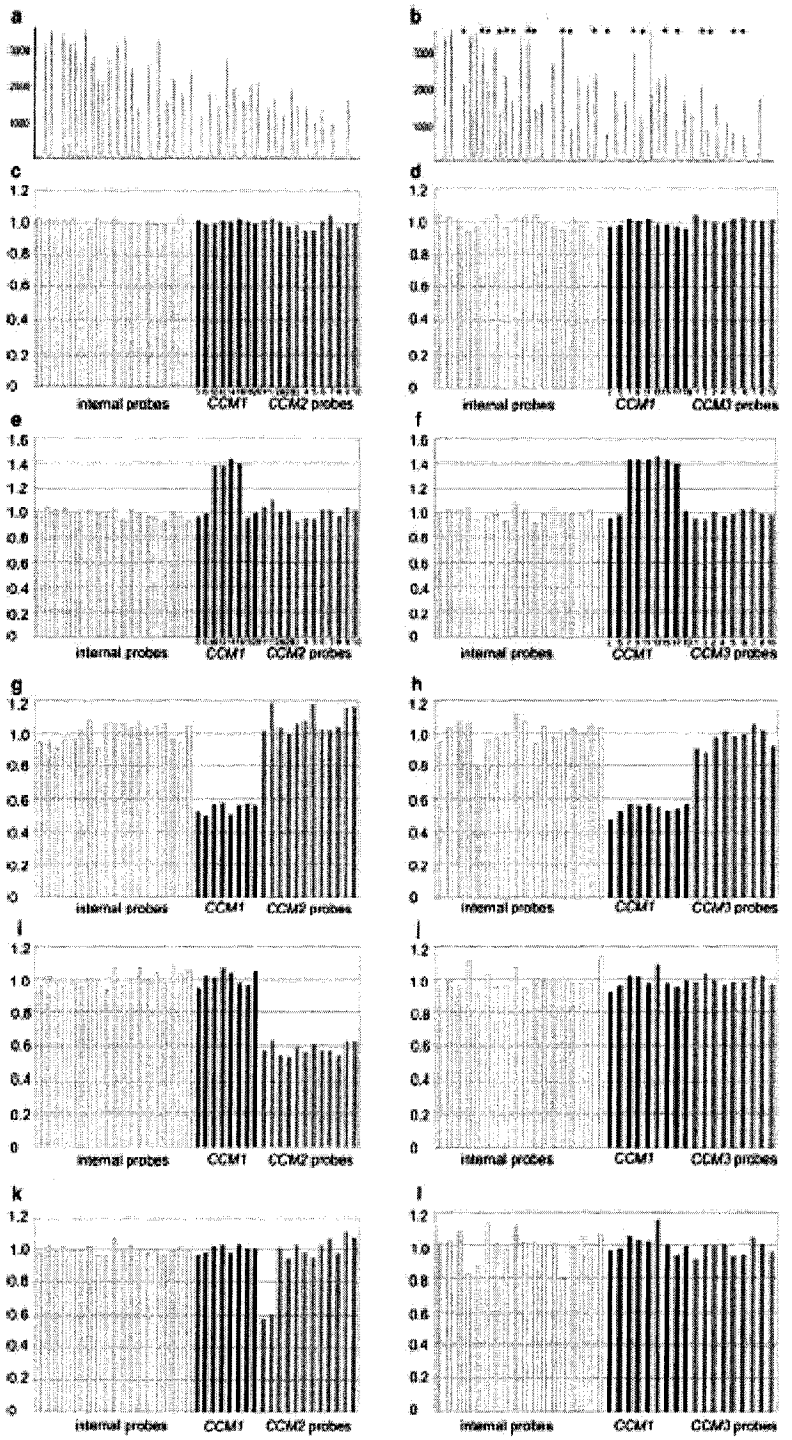

a
b
c
d
e
f
g
h
i
j
k
l
internal probes
CCM1
CCM2 probes
CCM3 probes

Figure 8.2: MLPA data

MLPA data demonstrating heterozygous copy number changes in four sporadic cases. Example of (A) normal and (B) pathological MLPA raw data (SALSA MLPA Kit P130). The electropherograms show reduced peaks for all *CCM2* exons in the proband (dots) while *CCM1* peaks (asterisks) are comparable between control and patient. Peaks from internal controls are not highlighted. (C-M) Quantitative analyses demonstrate that the relative peak areas are either increased by 50% (E, F) or decreased to approximately 50% (G-M) in affected codons when compared to internal (white) and external controls (C, D). All MLPA analyses were performed in duplicate. Numbers below the columns in (C-F) indicate the *CCM1-3* exons analyzed. (E, F) Duplication of *CCM1* exons 7-17, (G-M) deletions of the *CCM1* (G, H) and *CCM2* (I, K) genes as well as *CCM2* exon 1 and the 5'-untranslated region (L, M).

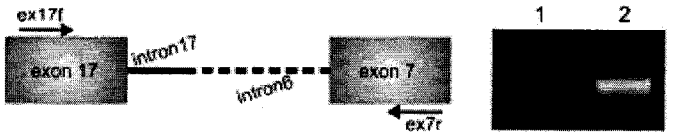


Figure 8.3: Confirmation of the *CCM1* duplication.

PCR amplification of almost the entire intron 17 fused to the 3' 1258 bp of intron 6 using primers *CCM1*ex17forward (ex17f) and *CCM1*ex7reverse (ex7r) yielding a 1853 bp product in patient (2) but not in control DNA (1) upon separation on an agarose gel.

8.2.6 Table

Table 8.1: Genomic CCM deletions/duplications

Table Genomic CCM deletions/duplications

No.	Gene	Exons involved	Type of mutation	Reference
1	*CCM1*	2-20[a)]	deletion	(5)
2	*CCM1*	2-20	deletion	this paper
3	*CCM1*	7-17	duplication	this paper
4	*CCM1*	18[b)]	deletion	(12)
5	*CCM2*	1-10 + 5'-UTR	deletion	this paper
6	*CCM2*	1 + 5'-UTR	deletion	(3)
7	*CCM2*	1 + 5'-UTR	deletion	(3)
8	*CCM2*	1 + 5'-UTR	deletion	this paper
9	*CCM3*	1-10	deletion	(4)
10	*CCM3*	5[c)]	deletion	(4)

[a)] exon numbering according to NM_194456 for *CCM1*, (3) for *CCM2*, and (4) for *CCM3*.

[b,c)] in-frame 84 and 174-bp cDNA deletions

UTR = untranslated region

References for Table:

3: (Denier et al. 2004), 4: (Bergametti et al. 2005), 5: (Gaetzner et al. 2006), 12: (Laberge-le Couteulx et al. 1999)

8.2.7 Discussion

Using MLPA, two novel and two recurrent genomic alterations were found in a total of 14 isolated patients with multiple lesions. In this group, four *CCM1* and two *CCM3* intragenic mutations had been previously identified ((Verlaan et al. 2004c) and unpublished data). Thus, our mutation detection rates for the three genes (*CCM1*: 6/14 = 43%; *CCM2*: 2/14 = 14%; *CCM3*: 2/14 = 14%) are in agreement with the results obtained from the large French cohort published most recently: *CCM1*, *CCM2*, and *CCM3* mutations were found in 53%, 15%, and 10% of combined multiple and isolated CCM (Denier et al. 2006). Compatible with the French results of 57% for isolated patients with multiple CCMs after direct sequencing, polymorphic marker and transcript analyses, the combination of sequencing and MLPA analyses enabled us a mutation detection rate of 71%. Our results therefore underline that the majority of patients with multiple CCMs harbour germline mutations which can be transmitted to their offspring.

Neuroimaging has demonstrated that 77% (17/22) of isolated patients with multiple CCMs had an asymptomatic parent with small CCMs (Labauge et al. 1998). The identification of *CCM* mutations permits us to determine if the mutation has been inherited from an asymptomatic parent. Predictive genetic testing can then be offered to further at risk family members prior to onset of symptoms. However, the clinical course of the disease remains unpredictable. We did not notice phenotypic differences between subjects with small inactivating mutations and large genomic rearrangements. Furthermore, wide intrafamilial

clinical variability has been described for small and large *CCM* mutations (Gaetzner et al. 2006).

Only a few *de novo* mutations have been published more recently (Lucas et al. 2001; Bergametti et al. 2005; Liquori et al. 2006). Somatic mosaicism due to postzygotic *de novo* mutations may in part explain the obvious discrepancy of mutation detection rates between familial and isolated CCM cases. The undiagnosed 6% of familial cases (Denier et al. 2006) may be attributable to the occurrence of mutations within regulatory regions of *CCM1-3*, the existence of a fourth *CCM* gene or limitations of the screening methods applied.

Five genomic *CCM* deletions had previously been identified by one French laboratory that applied RNA-based mutation analysis complemented with analysis of polymorphic markers (Laberge-le Couteulx et al. 1999; Denier et al. 2004; Bergametti et al. 2005) (Table 8.1). With the use of MLPA in a small German cohort (Gaetzner et al. 2006) and the Swiss cohort presented here, the total percentage of known genomic *CCM* rearrangements increased from 4% (5 out of 140 published *CCM* mutations (Felbor et al. 2006)) to 7% (10/145; table 8.1). It can be anticipated that a systematic screen for genomic alterations in other cohorts will result in a higher proportion of this type of mutations which can be caused by homologous recombination and non-homologous DNA end-joining (Lieber et al. 2003; Shaw and Lupski 2005). Future studies will have to reveal whether it might be more cost-effective to perform MLPA prior to full sequence analyses of *CCM1-3*.

8.2.8 Acknowledgements

The authors thank the patients for their cooperation. Ute Felbor receives an Emmy Noether-grant from the Deutsche Forschungsgemeinschaft (Fe 432/6-5) and is supported by a grant from the Bavarian Genome Network. The experiments comply with the current German laws.

9 INVESTIGATION OF THE "2-HIT" HYPOTHESIS IN CCM SAMPLES

Submitted to *Annals of Neurology* (January 2007)

Reference:

Dominique J. Verlaan, Marie-Christine Guiot, Daniel L. Rochefort, Adrian M. Siegel and Guy A. Rouleau

Title: Cerebral Cavernous Malformation results from a complete loss of *CCM1* function

9.1 Rationale

Given that affected members of *CCM* families all inherit one mutated CCM allele, it is unclear why only a small number of lesions develop and why their frequency increases in an age-dependent manner. There are several scientific hypotheses that try to explain the underlying mechanism for CCM pathogenesis. One proposed mechanism is a biallelic loss of function of the gene due to a germline mutation and a somatic "second hit" of the remaining functional allele in a tumour suppressor-like mechanism. Research thus focused on this loss of heterozygosity (LOH) according to this "second hit" hypothesis in four cerebral cavernous malformations of two individuals who were found to have a *CCM1* mutation.

9.2 Cerebral cavernous malformation results from a complete loss of *CCM1* function

9.2.1 Abstract

Cerebral cavernous malformation (CCM [MIM 116860]) is a common disorder found in approximately 0.5% of the population and represents approximately 10% to 20% of all cerebral vascular lesions (Rigamonti et al. 1988). While most cases are sporadic, more than 20% (Porter et al. 1997; Moriarity et al. 1999) are inherited in an autosomal dominant fashion. An intriguing feature of the inherited form of CCM is the focal and stochastic formation of the vascular lesions. A possible model of pathogenesis for this disease is an inactivating (loss-of-function) model, akin to Knudson's two hit hypothesis (Knudson et al. 1975). To test this hypothesis, loss of heterozygosity (LOH) was investigated in the *CCM1* region in four cerebral cavernous malformations of two individuals who where found to both have a frameshifting *CCM1* mutation. Loss of heterozygosity was found in both cases and in Case 2, loss of heterozygosity always occurred on the same allele suggesting that the deletion occurs non-randomly. These results support a loss-of-function model for *CCM1*, with a germline mutation inactivating one copy of *CCM1* and somatic inactivation of the remaining wild-type copy in familial cases.

9.2.2 Introduction

Cerebral cavernous malformation (CCM [MIM 116860]) is a common disorder characterized by abnormally enlarged capillary cavities in the brain without intervening normal parenchyma (Russell and Rubenstein 1989). They are well circumscribed, often encapsulated and can be associated with other cutaneous and visceral malformations {retina (Sarraf et al. 2000; Couteulx et al. 2002), the skin (Labauge et al. 1999; Eerola et al. 2000), the liver and pancreas (Gil-Nagel et al. 1995)}. They can vary in size from a few millimetres to several centimetres in diameter. MRI reveals a characteristic image of heterogeneous signal intensity surrounded by a dark ring attributable to hemosiderin deposition (Gunel et al. 1996b). Symptomatic individuals typically present between the ages of 20 and 40 with seizures, brain haemorrhage, focal neurological deficits and/or headaches, attributable to a single or multiple lesions (Porter et al. 1997; Moriarity et al. 1999). However, lesions have been described in all age groups with no sex predominance. These vascular malformations most commonly arise sporadically or may be dominantly inherited and most families present with multiple lesions (Labauge et al. 1998). Patients can be managed either conservatively or treated with surgical resection when lesions cause recurrent haemorrhage or seizures.

There have been three genetic loci defined for Cerebral Cavernous Malformation: *CCM1* on chromosome 7q21-q22 (Dubovsky et al. 1995; Gunel et al. 1995; Marchuk et al. 1995), which encodes for the Krev Interaction Trapped 1 (Krit1) protein, *CCM2* on 7p13-p15, which encodes for malcavernin (Liquori et al. 2003) and *CCM3* on 3q25.2-q27, which encodes for the programmed cell death protein

10 (Bergametti et al. 2005). It is thought that all of the mutations found to date in the CCM genes result in the truncation of their respective protein.

Given that all blood vessels harbour one inherited *CCM1* mutated allele in affected members of *CCM1* families, it is unclear why only a small number of lesions develop and why their frequency increases in an age-dependent manner. An underlying mechanism for CCM pathogenesis could be a biallelic loss of function of the gene due to a germline mutation and a somatic "second hit" of the remaining functional allele in a tumour suppressor-like mechanism. For this reason, loss of heterozygosity (LOH) was investigated in four cerebral cavernous malformations of two individuals who where found to have a *CCM1* mutation.

9.2.3 Materials and methods

Study population: The first case presented with a familial history and multiple lesions. The second case presented with no familial history but with multiple lesions. These individuals are part of the IFCAS study (Siegel et al. 1998) approved by the Committee for the Protection of Human Subjects at Dartmouth College (Hanover, NH).

Laser capture microdissection: Five microns sections from formalin fixed paraffin embedded tissues were stained with Hematoxylin and Eosin and were thoroughly dehydrated. Abnormal vessel walls and normal brain parenchyma were isolated using laser microdissection (Pix Cell II system, Arcturus, Mountain View California). Enzymatic digestion were carried in 10mM M Tris HCl pH:8,

in 1 mM EDTA, 1% Tween 20 at 37°C for 18 hours to collect the DNA. (Figure 9.1)

DNA pre-amplification: To increase the amount of DNA for genotyping, the material obtained from the cerebral cavernous malformation cells, was pre-amplified. The reaction included 1-2 ul DNA, 6ul 10X buffer (QIAGEN), 4mM DTT, 2.5 mM MgCl2, 10mM of each dNTPs, 5U of Taq polymerase (QIAGEN), 400uM of each random primer (15-mer oligonucleotides in which any one of the four possible bases could be present at each position), and distilled water to 60ul. PCR amplification was performed as follows: 5 min at 94°C; 50 cycles: 1 min at 94°C, 2 min at 37°C, and 4 min at 55°C (Torra et al. 1999).

***CCM1* gene screen:** The pre-amplified DNA extracted from the abnormal tissue of the malformations and the surrounding normal tissue was used to amplify *CCM1*, which contains 3 non-coding and 16 coding exons. Each of the coding exons was amplify by PCR using intronic primers. Products were sequenced on an ABI 3700 automated sequencer using the BigDye chemistry, according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, Ca, USA).

Marker amplification: Genotyping was performed using tri or tetra microsatellite markers found around the *CCM1* gene (D7S627, D7S1813, D7S1789 and D7S623). Each marker was amplified with nested primers

according to specific PCR conditions and labelled with ^{35}S-dATP. PCR-amplified products were transferred on 6% denaturing acrylamide gels and visualized on autoradiography film.

9.2.4 Results

DNA from normal and abnormal tissue of paraffin-embedded cerebral cavernous malformations was collected by laser-capture microdissection (Figure 9.1) and pre-amplified to increase the amount of genetic material. All samples were screened for mutation in each of the 16 coding exons of the *CCM1* gene (Zhang et al. 2000a; Eerola et al. 2001; Sahoo et al. 2001). Case 1 has a germline mutation of an 1179delAAATATT mutation in exon 15 and Case 2 has a 1717delCAAGGTTTCinsGT$_6$A mutation in exon 12. Both mutations lead to frameshifting and to a presumably inactive protein.

A heterozygote pattern of the normal and mutated alleles is seen, shown in fig. 9.2b and 9.3b, when DNA from the normal brain parenchyma surrounding the malformations is sequenced. Control sequences can be seen in fig. 9.2a and 9.3a. In the abnormal tissue of the malformation in Case 1, shown in fig. 9.2c, there is a homozygote pattern of the mutated allele demonstrating a loss of heterozygosity. In the abnormal tissue of the malformation of Case 2, shown in fig. 9.3c, the pattern is not entirely homozygote yet there is an excess of the mutated allele showing a partial loss of heterozygosity. In all probability the presence of the normal allele in the abnormal tissue has been caused by contamination with

normal tissue, as it is difficult to separate the normal surrounding tissue and the abnormal tissue of a cavernous malformation.

Subsequently, each sample was genotyped around the *CCM1* gene using the microsatellite markers D7S627, D7S1813, D7S1789 and D7S623. Loss of heterozygosity was found at the *CCM1* locus for both case 1 and 2, both of which harbour multiple lesions (fig. 9.4 and table 9.1). In the multiple lesions of case 2, loss of heterozygosity always occurred on the same allele suggesting that the deletions occurred on the normal allele in a non-random fashion.

9.2.5 Table

Table 9.1: Loss of heterozygosity results

Table: Loss of heterozygosity results										
Markers	D7S627		D7S1813		D7S1789		*CCM1* mutation		D7S623	
Location	92Mb		93.3Mb		93.4Mb		93.5Mb		94.5Mb	
Cases	NT	AT	NT	AT	NT	AT	NT	AT	NT	AT
1	Htz	Hmz	Htz	Hmz	Htz	Hmz	Htz	Hmz	Htz	Hmz
2[a]	Htz	Hmz	Hmz	Hmz	Htz	Hmz	Htz	Hmz[b]	Htz	Hmz

NT: Normal Tissue, AT: Abnormal Tissue, Htz: heterozygote, Hmz: homozygote

[a]In case 2, three different malformations were studied. LOH always occurred on the same allele.

[b]not a 100% homozygote, see fig 3c.

9.2.6 Figures

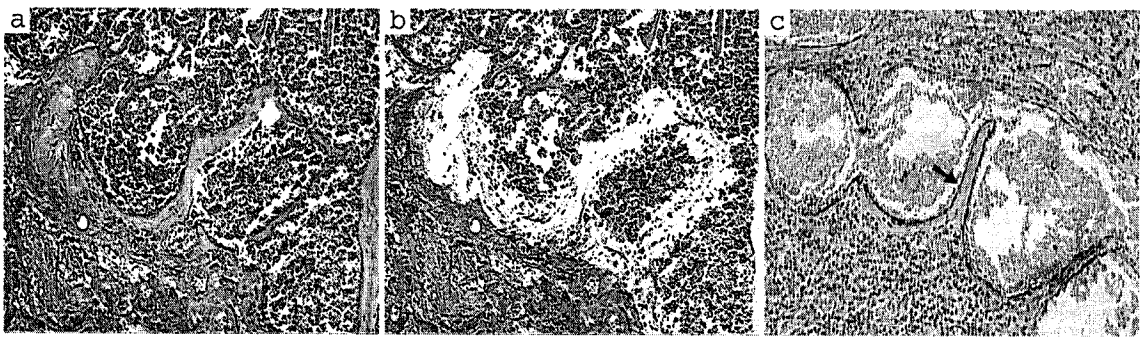


Figure 9.1: Microscopic field before and after LCM

a: Hematoxylin and Eosin stain showing dilated vascular channels with fibrotic walls lined with endothelium. No brain parenchyma was present within the lesion. b: Same microscopic field after LCM. The fibrotic walls lined with endothelium have been removed. c: Immunohistochemistry for Factor VIII confirms the presence of endothelial cells (arrow).

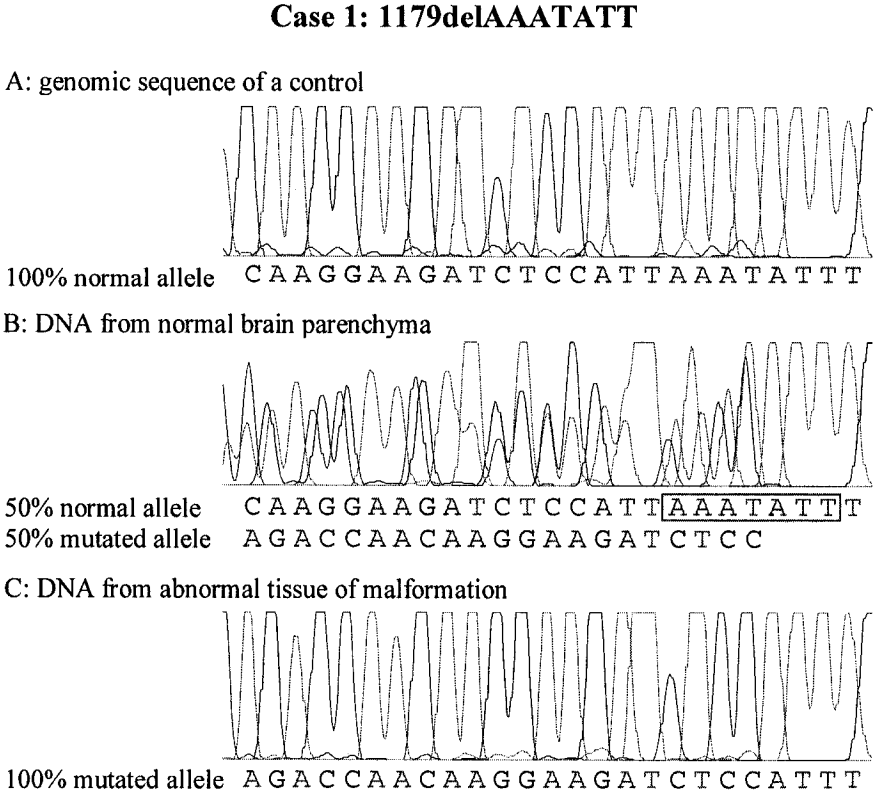


Figure 9.2: *CCM1* mutation analysis in case 1

A. genomic sequence of the normal allele in a control. B. Normal brain parenchyma: DNA sequence from a lesion of Case 1 showing a heterozygote pattern of the normal allele and the mutated allele carrying the frameshifting 1179delAAATATT mutation. C. Abnormal Tissue: Homozygote pattern of the DNA sequence of the mutated allele found in the malformation.

Case 2: 1717delCAAGGTTTCinsGT$_6$A

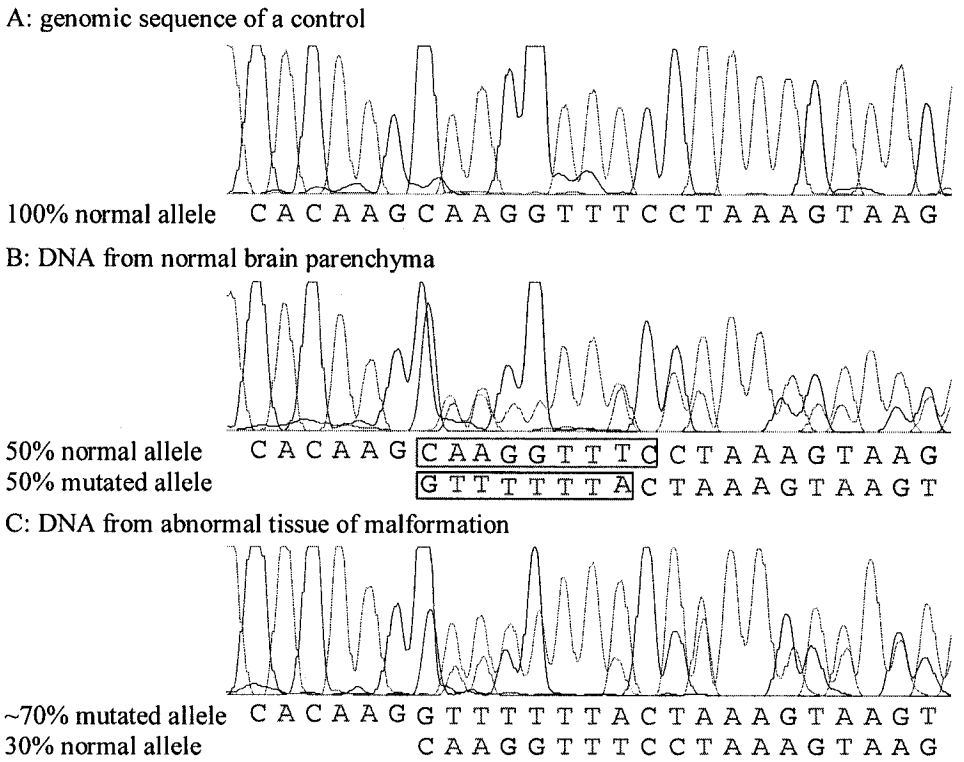


Figure 9.3: CCM1 mutation analysis in case 2

A. genomic sequence of the normal allele 1 in a control. B. Normal brain parenchyma: DNA sequence from a lesion of Case 2 showing an heterozygote pattern of the normal allele and the mutated allele carrying the frameshifting 1717delCAAGGTTTCinsGT$_6$A mutation. C. Abnormal Tissue: Pattern shows an excess of the mutated allele in the malformation.

Figure 9.4: Example of loss of heterozygosity found at marker D7S623 in case 2. The abnormal tissue (AT) of the malformation has lost the upper allele and is now homozygote for the smaller allele. The normal brain parenchyma (NT) contains both alleles in equal quantity.

9.2.7 Discussion

These results suggest that a somatic *CCM1* mutation in a *CCM1* family would result in a malformation since a germline *CCM1* mutation already exists in the same cell. A result of this model is that two independent somatic *CCM1* mutations within single cells, which is a rare event, may lead to the sporadic malformations that we find in the general population hence the occurrence of solitary lesions. Accordingly, a germline mutation is necessary but insufficient for the formation of a malformation, while the somatic mutation is the rate-limiting step for lesion formation.

Support for loss of heterozygosity has been demonstrated in two previous research papers. In the first study, the authors studied mice ($Ccm1^{+/-}Trp53^{-/-}$) that were heterozygous for a Ccm1 mutation and homozygous for loss of the tumour suppressor Trp53 (p53) (Plummer et al. 2004). Deactivation of the p53 tumour suppressor has been shown to increase the rate of somatic mutation (Jacks et al. 1994) and hence would increase the chance of a second somatic hit. Cerebral vascular lesions were observed in 55% of these mutant animals but none were observed in the controls of differing genotypes, suggesting that the normal copy of the Ccm1 gene was inactivated.

In the second study, a CCM lesion with an inherited *CCM1* nonsense mutation was studied (Gault et al. 2005). The researchers demonstrated the occurrence of a new somatic deletion found within the lesion and established that the *CCM1* mutations were biallelic. Hence, consistent with a two-hit hypothesis, they have

demonstrated that formation of a CCM may be due to complete loss of the *CCM1* protein function in cells critical for vascular integrity maintenance.

Evidently, the extent that this model may operate to account for all of the malformations in CCM disease will need to be assessed in future studies.

9.2.8 Acknowledgments

The authors thank the patients and the physicians who refer them for their participation in this study. GAR is supported by the CIHR. This work was supported by the FRSQ-FCAR.

10 INTRACRANIAL ANEURYSMS

10.1 Natural history

Intracranial aneurysms (IA) are saccular outpouchings of intracranial arteries that occur most commonly at arterial bifurcations (OMIM: 105800). Based on a series of autopsies, unruptured IAs are present in approximately 3-6% of the population older than 30 (Schievink 1997). The probability of an IA increases with age but does not usually manifest until well into adulthood. Aneurysms are only very rarely symptomatic unless they rupture, which typically results in a subarachnoid haemorrhage (SAH). About 90% of SAH cases are caused by a ruptured IA (Ronkainen et al. 1997). The lifetime risk of having a ruptured aneurysm is between 0.5 and 1%, representing about 10% of all strokes. IA is a serious condition because the first manifestation is almost always a rupture, leading to death in 50% of cases and serious morbidity in an additional 30% of cases (Molyneux et al. 2002; van der Voet et al. 2004).

Familial predisposition is the strongest risk factor for the development of IA (Rinkel 2005). Cigarette smoking has also been identified as a risk factor for SAH (Bonita 1986; Koskinen and Blomstedt 2006). A number of studies show a clear dose-response curve between increasing cigarette consumption and increased risk of SAH. Some studies have also implicated hypertension with increased risk of SAH, while others have failed to confirm this association (Bonita 1986; Longstreth et al. 1992).

10.2 Pathology

Pathologically, IAs are characterised by a very thin or absent tunica media (middle layer of the artery that is made up of smooth muscle and elastic tissue) and internal elastic lamina (surrounds the tunica media) (Schievink 1997). IAs are thought to occur at the bifurcations of large intracranial arteries because they are exposed to greater blood flow pressures and undergo a subsequent and premature weakening of the blood vessel wall. Beyond this basic explanation, pathophysiology of the formation of IA is still not completely understood.

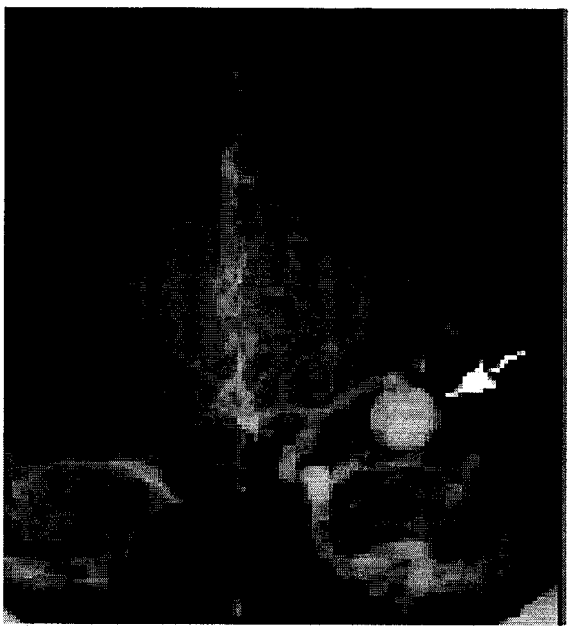


Figure 10.1: Angiogram of an Intracranial Aneurysm

Modified from the Center for Inherited Neurovascular Diseases website **www.cind.org/details.html**

10.3 Treatment options

There are two main options for the treatment of aneurysms. The first method is microvascular surgical clipping, which consists of placing a metal clip across the neck of the aneurysm in order to prevent it from rupturing. The second method is endovascular coiling, which consists of passing a catheter through an artery and depositing detachable platinum coils of various length and diameter within the aneurysm to exclude it from circulation. Both methods are routinely performed and the choice of treatment depends mostly on the size and location of the aneurysm in addition to the area of expertise of the neurosurgeon. In cases where the risk of rupture is low, periodic monitoring may be preferred to surgical treatment.

10.4 Genetic history

Evidence suggests that part of the basis for developing IA is genetic (Leblanc et al. 1994). Among first degree relatives of patients with SAH, the risk of a ruptured IA is three to seven times higher than in the general population (Bromberg et al. 1995; Schievink et al. 1995; Raaymakers et al. 1998). In a study of 412 ruptured aneurysms in the Saguenay-Lac-Saint-Jean region of Quebec, 30% occurred in families (Mathieu et al. 1996). Multiple aneurysms occur in 20-30% of the patient population, and often in families with a history of the disease, suggesting genetic predisposition to the development of IA in certain cases. Familial aneurysms tend to rupture at an earlier age than sporadic

aneurysms and may be smaller in size when they rupture (Schievink 1997). In addition, familial aneurysms are often concordant for site in the cerebral vasculature and are often followed by the formation of a new aneurysm (Lozano and Leblanc 1987; Ronkainen et al. 1995). In most families with IA, only 2 or 3 members are known to be affected and the inheritance pattern is unclear. While segregation analysis of published pedigrees suggests autosomal dominant transmission with reduced penetrance (Schievink 1997), some studies report that in aneurysm families, siblings are at greatest risk, suggesting possible recessive inheritance. Clearly, the susceptibility to IA should be considered a complex trait. Nonetheless, some large families have been described, suggesting the existence of highly penetrant alleles in one or more predisposing genes.

10.5 Genetic linkage studies

To date, four genetic linkage studies performed on different populations have been published. At least one IA susceptibility locus was identified in each experiment.

10.5.1 *ANIB1* locus

A genome-wide linkage study of IA was performed in 104 Japanese affected sib-pairs in which positive evidence of linkage on chromosomes 5q22-31 (maximum LOD score [MLS] 2.24), 7q11 (MLS 3.22) and 14q22 (MLS 2.31) was found

(Onda et al. 2001). The most significant linkage was detected at D7S2472, in the vicinity of the elastin gene (*ELN*), a candidate gene for IA. They found that the haplotype between the intron-20/intron-23 polymorphism of *ELN* was strongly associated with IA ($P=3.81x10^{-6}$), and homozygous patients are at high risk (P=0.002), with an odds ratio of 4.39. Suggestive confirmation of this locus was found when using 13 extended families from Utah (Farnham et al. 2004) and utilizing a recessive affected-only model (multipoint TLOD=2.34, at D7S2421, corrected P=0.001). However, the linkage was not replicated (LOD: -8.04, NPL: -0.643) in a study of 14 Japanese families with 64 members (Yamada et al. 2003).

10.5.2 *ANIB2* locus

A 15cM genome scan was conducted on 48 Finnish affected sib-pairs and found LOD scores of 2.63 on 19q13 and 2.08 on the X chromosome. An additional four loci yielded LOD scores above 1 (Olson et al. 2002). This same group replicated their findings for the chromosome 19 locus using an additional 91 sib pairs and 83 other affected relative pairs (van der Voet et al. 2004). Suggestive linkage was observed in both independent sample sets, and linkage was significant in the combined set at 70cM (LOD score 3.50; P=0.00006) and at 80cM (LOD score 3.93; P=0.00002). They predict that the most likely location for a gene predisposing to IAs in the Finnish population is in a region with 95% confidence interval of 11.6 cM (P=0.00007) centered 2.0 cM proximal to D19S246. This region contains about 135 genes. Another group independently replicated the

19q13 linkage in another 29 Japanese families (MNS 2.05) (Yamada et al. 2004), making this the most replicated IA locus.

10.5.3 *ANIB3* locus

A GWS linkage analysis of ~10 000 SNPs (Single Nucleotide Polymorphisms) was performed on an American family of 10 affected individuals (6 were available for the analysis, 4 were deceased) (Nahed et al. 2005). Using a dominant model with a high penetrance of 99%, a LOD score of 4.2 was calculated for locus 1p34.3-p36.13 which is defined by markers D1S199 and D1S496. The locus spans about 15Mb and contains approximately 240 genes including the following potential candidate genes: polycystic kidney disease-like 1, brain-specific angiogenesis inhibitor 2, fibronectin type III domain-containing gene and collagen type XVI α1. Two other potential loci, 1q31-1q41 and 2p11-2p14 were also identified.

10.5.4 *ANIB5* locus

A genome-wide linkage analysis was performed in a large Dutch consanguineous pedigree where 7 out of 20 siblings had an intracranial aneurysm (Roos et al. 2004). Genome-wide multipoint linkage analysis showed a significant LOD score of 3.55 when using an autosomal recessive model with a penetrance of 70%. Their locus is found on chromosome 2p13 defined by the markers D2S2206 and D2S2977. The locus spans 7cM and contains about 150 genes. Testing of four

other unrelated Dutch families revealed that one other family might be linked to this locus. No evidence of linkage was found in the other 3 families suggesting further genetic heterogeneity.

10.6 Candidate gene approach

Studies searching for the genetic basis of aneurysm formation have largely focused on candidate genes encoding for enzymes and proteins that are either key components of the arterial wall or involved in mechanisms for blood vessel remodelling. As previously mentioned, the structural integrity of the arterial wall depends on the interrelated extracellular matrix proteins, such as collagen, elastin and matrix metalloproteinases (Gaetani et al. 1999; Peters et al. 1999; Yoon et al. 1999). Protease inhibitors regulate the degradation of these proteins by proteolytic enzymes, and an imbalance between them may predispose an individual to aneurysm formation (Schievink 1998). Unfortunately, at the conclusion of gene screening, only a few genes have shown a moderate positive association (see following Table). Frequent conflicting results have prevented a single gene from being consistently identified as a candidate gene.

Table 10.1: Summary of candidate gene association and functional studies

Gene	Location	Association	# of cases vs. controls	Study
Interleukin-1β	2q14	Yes	231/231 Polish	(Slowik et al. 2006)
Collagen type 3 A2	2q31	No	55 cases Mix	(Kuivaniemi et al. 1993)
		Yes	19/15 N/A	(Brega et al. 1996)
		No	41/41 Dutch	(van den Berg et al. 1999)
		Reduced or absent in skin and intracranial and temporal arteries		(Neil-Dwyer et al. 1983) (Ostergaard and Oxlund 1987) (van den Berg et al. 1997)
		Reduced/irregular/shorter reticular fibres in intracranial and extracranial arteries		(Hegedus 1984) (Ostergaard et al. 1987) (Chyatte et al. 1990)
		Overexpression		(Peters et al. 2001)
		Reduced expression		(Kassam et al. 2004)
Versican	5q14.3	Yes	617/639 Dutch	(Ruigrok et al. 2006)
Lysyl oxidase	5q23.2	No	25 FIA European White	(Hofer et al. 2004)
		No	172/192 Japanese	(Yoneyama et al. 2003)
FGF1	5q31	No	172/192 Japanese	(Yoneyama et al. 2003)
Fibrillin 2	5q23-31	No	172/192 Japanese	(Yoneyama et al. 2003)
Lipoprotein(a)	6q25-26	Yes	50FIA/50 Irish Caucasian	(Roberts et al. 2001)
Elastin	7q11.23	Yes	78FIA+925IA/192 Japanese sib-pairs	(Onda et al. 2001)
		No	301FIA+175SIA/235 European white	(Hofer et al. 2003)
		Yes	167/167 N/A	(Ruigrok et al. 2004)
		No	120/172 German white	(Krex et al. 2004a)
		Yes	14FIA Utah white	(Berthelemy-Okazaki et al. 2005)
		Defective internal elastic lamina		(Cajander and Hassler 1976)
		Disruption of elastin fibres in skin biopsies		(Grond-Ginsbach et al. 2002)
		Overexpression		(Peters et al. 2001)
Collagen type 1 A2	7q22.1	Yes	115/293 Japanese	(Yoneyama et al. 2004b)
		Overexpression		(Peters et al. 2001)

Gene	Location	Association	# of cases vs. controls	Study
eNOS	7q36	Size	52/90 Caucasian	(Khurana et al. 2003)
		Vasospasm	51/90 Caucasian	(Khurana et al. 2004)
		Rupture	58rup/49unrup Caucasian	(Khurana et al. 2005)
		No	336/224(Japanese) 191/191 (Korean)	(Akagawa et al. 2005)
		No Rupture	297rup/108rup/176 Japanese	(Krischek et al. 2006)
Endoglin	9q34.11	Yes	82/114 Japanese	(Takenaka et al. 1999b)
		No	121/124 German white	(Krex et al. 2001)
		No	172/192 Japanese	(Onda et al. 2003)
		No	98/191 European White	(Peters et al. 2005)
		No	119/119 Polish	(Pera et al. 2005)
MMP1, 3, 12	11q22-q23	No	57/174 Finnish	(Yoon et al. 1999)
		No	92/158 European white	(Zhang et al. 2001a)
SERPINA3	14q32.1	Yes	180/263 Polish	(Slowik et al. 2005)
α1 antitrypsin	14q32.1	No	72 cases England/USA	(St Jean et al. 1996)
		No	195/195 (Japanese) 189/94 (Korean	(Yoneyama et al. 2004a)
		Ratio elastase vs. antitrypsin elevated		(Baker et al. 1995)
MMP2	16q12.2	No	125/234 Caucasian	(Pannu et al. 2006)
		Expressed in medial smooth muscle cells		(Caird et al. 2006)
NADPH Oxidase	16q24	No	113/53 German white	(Krex et al. 2003b)
Angiotensin-Converting Enzyme	17q23	Yes	83/104 Japanese	(Takenaka et al. 1998)
		Yes	258/299 White East Anglican	(Keramatipour et al. 2000)
		Yes	90/128 Polish	(Slowik et al. 2004)
		No	162/143 Caucasian USA	(Pannu et al. 2005)
MMP9	20q13.2	Yes	98/191 European White	(Peters et al. 1999)
		No	92/158 European White	(Zhang et al. 2001a)
		No	40/44 German White	(Krex et al. 2004b)
		Yes	125SIA/234 Caucasian	(Pannu et al. 2006)
		Increased expression and activity		(Kim et al. 1997)
Phospholipase C	20q12	No	72 cases Japanese	(Takenaka et al. 1999a)
Heme-oxygenase 1	22q13	Yes	69/230 Caucasian	(Morgan et al. 2005)

Gene	Location	Association	# of cases vs. controls	Study
TIMP 1,2,3	Xp11.23 17q25 22q12.3	No	44/44 German White	(Krex et al. 2003a)
		Overexpression of TIMP3		(Peters et al. 2001)

10.7 Cohort of patients

Subjects were considered affected if an IA or an infundibulum was documented either by standard angiography or surgical ablation. Subjects were excluded if they had a personal diagnosis or family history of polycystic kidney disease, Ehlers-Danlos syndrome type IV, neurofibromatosis type 1 or Marfan syndrome (Schievink 1997). Patients with these disorders have an increase risk for aneurysm formation. Patients with a fusiform aneurysm of a major intracranial artery or of other intracranial vascular malformations were also excluded. In all instances, a complete family and medical history was obtained, (including consumption of cigarettes and history of hypertension). In instances where two or more individuals within family had documented IA, all first-degree relatives were screened by magnetic resonance angiography (MRA) or CT angiography. Suspected IAs detected using MRA or CT-angio were confirmed by conventional angiography. Over one hundred families were collected over the course of the study.

10.8 Rationale

Previously, the occurrence of intracranial aneurysms (IAs) was believed to be either non-genetic in nature or simply a complex trait. Although the majority of IAs fit this description, segregation of IA in families has been described in the literature.

IA and CCM can appear very similar when looking at familial cases (see table 10.2). For example, both occur in cerebral blood vessels and their lesions share the ability to grow in size from a few millimetres to a few centimetres, contract and occasionally undergo spontaneous involution (Siddiqui and Jooma 2001). In addition, many families present with multiple lesions while sporadic individuals usually present with one (Labauge et al. 1998). Finally, CCM is inherited dominantly and there is also some evidence that some form of IA is as well (Schievink 1997).

Table 10.2: Comparison of IA and CCM characteristics presenting with a family history

Characteristics	**Cerebral Cavernous Malformations**	**Intracranial Aneurysms**
Damage	Vessel wall malformation	Vessel wall rupture
Location	Venous flow	Arterial flow
Family history	High probability of multiple lesions	High probability of multiple lesions
Risk of recurrence	High	High
Age of onset	20-40 yrs old, earlier than in sporadic CCM cases	Earlier than in sporadic cases of IA
Penetrance	Reduced (70%)	Low

Based on the similarities between IA and CCM, a hypothesis was formulated that IA may be dominantly inherited in some families and could be considered as a Mendelian Trait based on several characteristics shared with CCM.

10.9 Objectives

As initially mentioned in chapter 1, based on the hypothesis that a portion of the reported familial aggregation for IA is due to genetic factor(s), a collection of affected families was used to identify a gene that causes or predisposes to IA.

11 IDENTIFICATION OF A NEW IA LOCUS

Reference:

Verlaan DJ, Dubé MP, St-Onge J, Noreau A, Roussel J, Satge N, Wallace MC, Rouleau GA. (2006) A new locus for autosomal dominant intracranial aneurysm, *ANIB4*, maps to chromosome 5p15.2-14.3. *Journal of Medical Genetics* Jun;43(6):e31.

11.1 A new locus for autosomal dominant intracranial aneurysm, *ANIB4*, maps to chromosome 5p15.2-14.3

11.1.1 Abstract

Background: Intracranial aneurysms (IA) are dilatations of intracranial arteries that occur most commonly at arterial bifurcations. Unruptured IAs are present in approximately 1–2% of the population aged over 30 years of age. Aneurysms are only rarely symptomatic unless they rupture, which typically results in a subarachnoid haemorrhage associated with high morbidity and mortality.

Methods: A large French Canadian (FC) family (Aneu60) was identified which contained 12 affected individuals with intracranial aneurysms. Nine of the affected patients and three unaffected individuals were sent for an 8 cM genomewide scan. Multipoint and two-point methods were used to analyse the scan data by using a dominant parametric model.

Results: We identified an IA susceptibility locus (*ANIB4*) located on chromosome 5p15.2-14.3. The locus was found by genome-wide linkage analysis and follow up analyses provided a maximum multipoint LOD score of 3.57 over the region. An identical haplotype segment of 7.2 Mb was found in a second FC pedigree and contributes to the refinement of the candidate gene interval.

Conclusions: Our results indicate that there is a major gene locus on chromosome 5p.

11.1.2 Introduction

Intracranial aneurysms (IA) are dilatations of cerebral arteries that occur most commonly at arterial bifurcations (MIM 105800). The lifetime incidence of IA is in the order of 1–2% (Rinkel et al. 1998), while the lifetime risk of rupture is between 0.5% and 1%, representing about 10% of all strokes (Schievink 1997). Rupture of an IA causing a subarachnoid haemorrhage (SAH) occurs with a frequency of between 6 and 8 per 100 000 in most Western populations (Linn et al. 1996); typically the incidence of SAH is higher among women than among men (Lozano and Leblanc 1987). IA usually occurs in adults where the incidence increases with increasing age. It is a serious condition because the first manifestation is almost always rupture, leading to death in 50% of cases and severe morbidity in an additional 30% of cases (Heiskanen 1986; King et al. 1994). Pathologically, IA are characterised by a very thin or absent tunica media and internal elastic lamina of the vascular wall. Because IA usually occur at the bifurcations of large intracranial arteries, which are exposed to the greatest pressures, premature weakening of the blood vessel is thought to predispose to IA (Stehbens 1983). Environmental factors, such as cigarette smoking and alcohol consumption, have been identified as risk factors for SAH (Longstreth et al. 1992).

Evidence suggests that genetics contribute to the development of IA (Leblanc et al. 1994). Among first degree relatives of patients with SAH, the risk of a ruptured IA is three to seven times higher than in the general population (Bromberg et al. 1995; Schievink et al. 1995; Raaymakers et al. 1998). In a study

of 412 ruptured aneurysms in the Saguenay-Lac-Saint-Jean region of Quebec, Canada, 30% aggregated within families (Mathieu et al. 1996). In addition, multiple aneurysms occur in 20–30% of patients (often those with a family history), suggesting that these individuals are particularly predisposed to the development of IA (Ellison and Love 1998). Familial aneurysms tend to be smaller in size, may rupture at an earlier age, and are more often followed by the formation of a new aneurysm compared to sporadic aneurysms (Lozano and Leblanc 1987; Schievink et al. 1994; Ronkainen et al. 1995).

Several patterns, including both recessive and dominant models of inheritance, have been described, suggesting a level of genetic heterogeneity (Schievink et al. 1994; Wills et al. 2003). In addition, several groups have studied familial IA by performing genome scan studies on sibships (Onda et al. 2001; van der Voet et al. 2004) and on large families (Roos et al. 2004; Yamada et al. 2004; Nahed et al. 2005). Different possible susceptibility loci for familial IA have been described, but no gene has yet been identified. In this study, we performed a genome-wide linkage analysis in a large French Canadian (FC) pedigree affected with IA in order to identify a susceptibility locus for this disorder.

11.1.3 Methods

Study inclusion

Twenty nine FC families, with at least two affected individuals, were enrolled in this study, which was approved by the Montreal General Hospital Research Ethics Committee. After informed consent, blood samples were taken from 231 subjects

and DNA was extracted from peripheral blood by standard methods. The total number of affected individuals was 112, of whom 54 were enrolled in the study and 19 were genetically reconstructable. Subjects were considered affected if an IA was documented either by standard angiography or surgical ablation or if they had an infundibulum (Martins et al. 2002; Cowan et al. 2004). Subjects were excluded if they had a personal diagnosis or family history of polycystic kidney disease (MIM 173900), Ehlers-Danlos syndrome type IV (MIM 130050), neurofibromatosis type 1 (MIM 162200), Marfan syndrome (MIM 154700) (Schievink 1997), a fusiform aneurysm of a major intracranial artery, or other intracranial vascular malformations. In all instances, a complete family and medical history was obtained, including cigarette consumption and history of hypertension. During the enrolment of these families, a large family (Aneu60) was identified, which contained 12 affected individuals, nine of whom were available for analysis (three are deceased).

Linkage analysis

Samples from nine affected and three unaffected individuals from the Aneu60 family were sent for an 8 cM genome-wide scan performed by the company deCODE (Reykjavik, Iceland). A panel of 531 highly polymorphic evenly spaced microsatellite markers was used and the genotyping success rate was more than 95%. The disease segregation within the family was compatible with Mendelian inheritance (fig 11.1), and a parametric LOD score approach was used to test for linkage. To analyse the genome scan markers, a test using an affecteds-only approach was conducted with an autosomal dominant model, a phenocopy

frequency of 0.01, a penetrance of 0.8, and a disease allele frequency of 0.001. Multipoint linkage of the autosomes was performed using GENEHUNTER version 2.1_r5 beta (Kruglyak et al. 1996) and two point linkage for the X chromosome was calculated using MLINK from the FASTLINK 3.0P package (Cottingham et al. 1993). Analyses were performed using deCODE allele frequencies for the genome-wide analysis and were set to CEPH frequencies (Murray et al. 1994) for fine mapping linkage.

Fine mapping

Additional genotyping was performed using polymorphic markers obtained from the Marshfield genetic map (http://research.marshfieldclinic.org/genetics) (Ghebranious et al. 2003). Each primer pair was amplified according to specific polymerase chain reaction (PCR) conditions and the product was labelled with nucleotide 35S-dATP. The PCR products were separated on 6% denaturing polyacrylamide gels and detected by exposure to autoradiographic film. The alleles were assigned on the basis of their size in accordance with CEPH data and with comparison to an M13mp18 sequence ladder. Marker location was obtained from the UCSC physical map (May 2004 assembly: http://genome.ucsc.edu).

Additional analysis of other FC families

Ten additional FC families (family trees are available from a supplemental figure at http://www.jmedgenet.com/supplemental) were tested for linkage on 5p15.2-14.3. All families were genotyped using markers spanning the critical region defined by the markers D5S2095 and D5S2031 using the markers D5S2095,

D5S667, D5S1991, D5S1954, D5S1963, D5S1997, D5S268, D5S2096, and D5S2031. Haplotypes and heterogeneity scores (HLOD) were calculated using GENEHUNTER version 2.1_r5 beta (Kruglyak et al. 1996).

11.1.4 Results

Aneu60 is an FC family that contains 12 individuals affected with IA that seems to segregate according to a monogenic inheritance pattern (fig 11.1). Clinical information for the Aneu60 kindred indicated that aneurysm location was not specific to a particular area, although four of the nine documented aneurysms were located at the middle cerebral artery (table 11.1). In addition, two of the patients had aneurysms in two different locations. The age at diagnosis varied between 34 and 56 and most of the affected individuals were smokers. Following the deaths of individuals II:2 and III:11, many aneurysms were discovered when descendents of II:2 underwent preventive screening by magnetic resonance angiography (MRA). However, as only a few descendents of II:7 have undergone MRA screening, other aneurysms have probably remained undiagnosed. This may explain why there seem to be only two affected individuals in that branch.

The genome-wide scan analysis revealed that the strongest evidence for linkage was found around microsatellite markers D5S1486 and D5S2081 on chromosome 5p with a multipoint LOD score of 2.69. Two other regions revealed LOD scores higher than 2, D12S2081 (LOD=2.12) and D16S418 (LOD=2.24). These three regions were further investigated by genotyping every individual from the family included unaffected members. For chromosome 5, segregation revealed a disease haplotype for every affected individual in the Aneu60 family (fig 11.1) and

maximum multipoint LOD score analysis gave 3.57 at marker D5S1954 (fig 11.2). For chromosomes 12 and 16, a disease haplotype did not segregate with every affected individual and multipoint LOD scores were reduced to 0.35 and 0.97, respectively.

We investigated the chromosome 5 region in 10 other FC families in the hope that they would be linked to this same region. Calculations for all families together, including the Aneu60 family, resulted in a maximum multipoint HLOD score of 3.42 and an α value of 0.40. Haplotype analysis was performed to determine if a common haplotype was being observed in the potentially linked families. The analysis revealed that one of the 10 families (Aneu57) shares a 7.2 Mb haplotype segment identical to the Aneu60 haplotype between D5S2095 and D5S1963 (table 2).

11.1.5 Figures

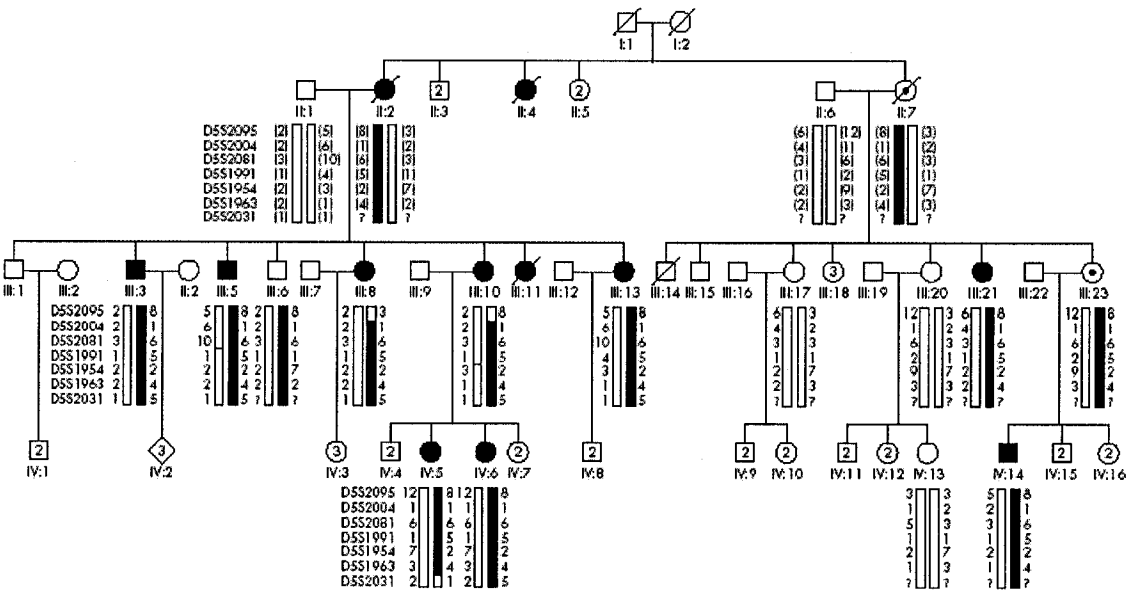


Figure 11.1: Pedigree of the French Canadian Aneu60 kindred segregating intracranial aneurysm.

Filled symbols indicate affected individuals, while empty symbols indicated individuals not known to be affected. A small dot within a symbol denotes an obligate carrier. Parentheses signify an inferred genotype. The disease haplotype is shown by a black bar. The upper recombinant can be found in individual III:8 and the lower recombinant in individual IV:5.

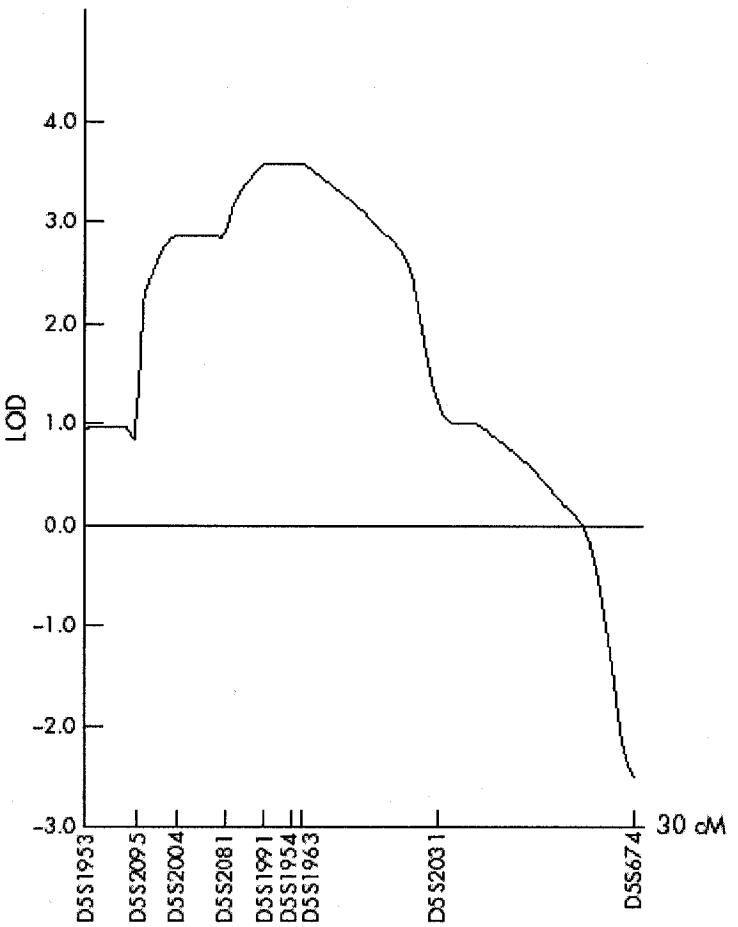


Figure 11.2: Multipoint LOD score of Aneu60 on 5p15.2-14.3.

11.1.6 Supplemental figure

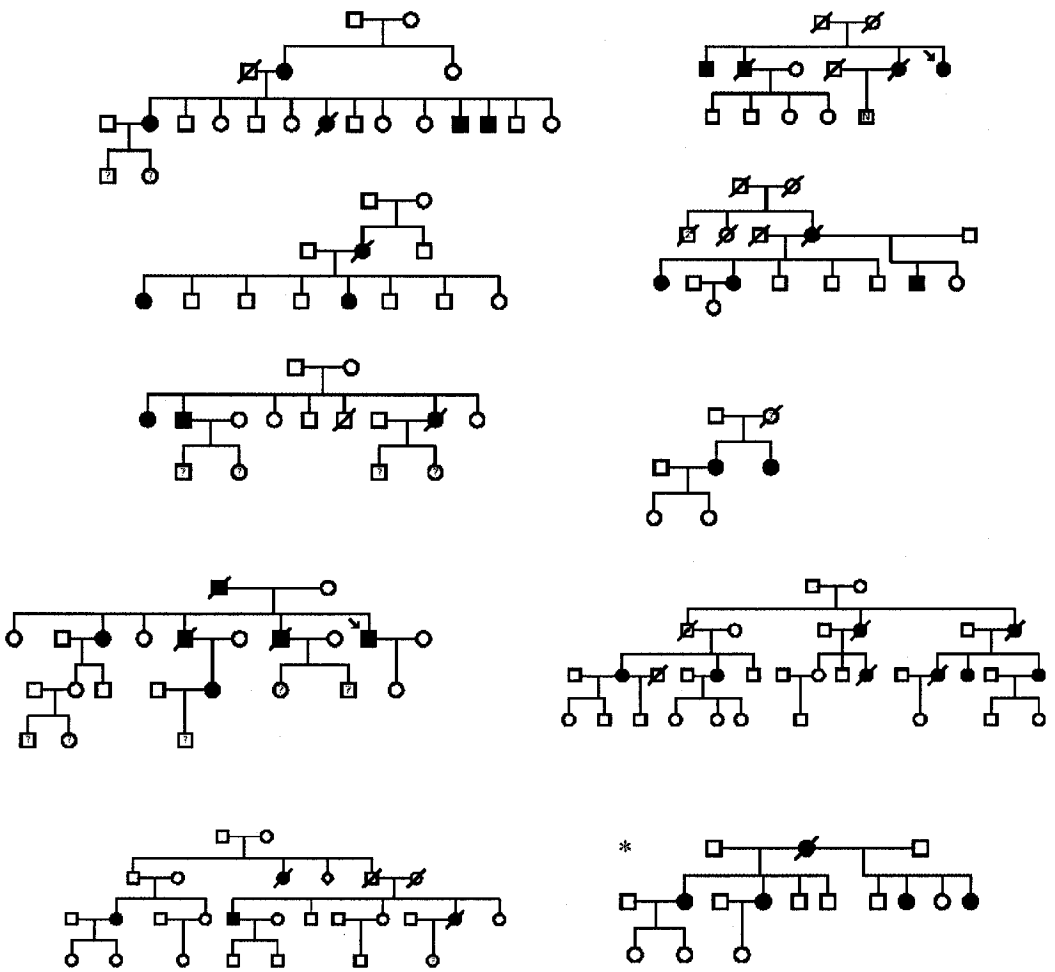


Figure 11.3: Supplemental figure

Family trees of the 10 additional French Canadian families used to test the *ANIB4* locus.

Blackened symbols are affected individuals while non-blackened symbols are individuals not known to be affected. * : Aneu57

11.1.7 Tables

Table 11.1: Clinical features of affected members of the Aneu60 family

Table 1 Clinical features of affected members of the Aneu60 family

ID	Aneurysm location	Age at diagnosis (years)	Smoker	Ruptured	Clipped
II:2	NA (SAH)	55	Y	Y	N
II:4	NA (SAH)	42	S	Y	N
III:3	L infundibulum on posterior communicating artery	49	Y	N	N
III:5	R MCA	42	Y	N	Y
III:8	L superior cerebellar artery	48	Y	N	Y
	L posterior communicating artery			N	Y
III:10	R MCA	55	S	N	Y
III:11	NA (SAH)	56	NA	Y	N
III:13	L MCA bifurcation	41	Y	N	Y
	R paraophthalmic intercavernous			N	N
III:21	L intracavernous carotid artery	53	Y	N	N
IV:5	R infundibulum on posterior communicating artery	40	Y	N	N
IV:6	R ophthalmic artery	34	Y	N	Y
IV:14	L MCA	45	Y	N	N

L, left; MCA, middle cerebral artery; N, no; NA, not available; R, right; S, smokes on social occasions; SAH, died of subarachnoid haemorrhage; Y, yes.

Table 11.2: Haplotype analysis of families Aneu60 and Aneu57

Table 2 Haplotype analysis of families Aneu60 and Aneu57

Markers	Mb	cM	Aneu60	Aneu57	Allele length (bp)	CEPH allele frequency
D5S2095	9.44	19.67	**8***			
D5S2004	10.55	21.81	*1*	*1*	211	29%
D5S2081	13.53	24.48	*6*	*6*	197	50%
D5S1991	14.93	26.73	*5*	*5*	233	3.6%
D5S1954	15.87	28.30	*2*	*2*	225	52%
D5S1963	16.68	28.76	4	1		
D5S2031	21.15	36.25	**5***			

*Alleles in bold indicate that a recombinant occurred within the family. Italics indicate haplotype that is shared by Aneu60 and Aneu57. Allele lengths were down-coded according to CEPH.

11.1.8 Discussion

The identification of a large FC family (Aneu60) has helped us to identify a susceptibility locus using a monogenic approach for this disorder. The genome-wide scan revealed a susceptibility locus on chromosome 5p for this particular family with a multipoint LOD score of 3.57 (fig 11.2). Further fine mapping of the region allowed the identification of an upper (III:8) and lower (IV:5) recombinant, defining the locus as being from marker D5S2095 to D5S2031 and spanning 11.7 Mb at 5p15.2-14.3 (fig 11.1). There are at least two non-penetrant individuals in Aneu60 (II:7 and III:23) and it is interesting to note that they are both non-smokers. Individual III:6, a 52 year old non-smoking man, carries the upper part of the disease haplotype and although his last MRA was normal, reducing the linked region using this information would not be advisable as IA is not a fully penetrant disease.

The existence of a founder effect in the FC population has been confirmed by many studies (Davignon and Roy 1993; Simard et al. 1994; Heyer and Tremblay 1995; Kibar et al. 1996; Labuda et al. 1996; Brais et al. 1998; Xiong et al. 1999; Engert et al. 2000). This population is particularly well suited for genetic studies due to the relatively small number of ancestors (Desjardins 1990). Many of these founders were related to each other directly or were from a limited number of regions in France further reducing the number of ancestral chromosomes in the FC compared to other outbreed populations. In addition, for socio-economic, religious, and linguistic reasons, the descendants of these founders did not mix with other immigrants for over three centuries. There was also significant

sustained demographic growth of the population, which doubled every 25–30 years. Therefore, a large number of individuals now living in Quebec have inherited chromosomes almost exclusively from a relatively small pool of founders, thus facilitating the use of haplotypes for fine mapping. Haplotyping of 10 other FC families revealed that one family (Aneu57) shares a portion of the Aneu60 disease haplotype. Considering only the shared haplotype, the critical region is reduced from 11.7 to 7.2 Mb (table 11.2). Some smaller families, which could potentially be linked, do not share the Aneu60 haplotype at the marker resolution tested. These other haplotypes could represent other disease haplotypes or the families may not be linked.

There is no evident familial link between Aneu60 and Aneu57. Further genealogical studies will be needed to determine how and if they are related.

The markers D5S2095 and D5S1963 now define the critical region for the 5p locus. This large region of 7.2 Mb seems gene poor as it encompasses only about 25 known genes. Two good candidate genes found within this region, CTNND2 and TRIO, seem to be involved in cell modeling. CTNND2, catenin delta-2 (delta-catenin), may be involved in neuronal cell adhesion and tissue morphogenesis and integrity by regulating adhesion molecules (Medina et al. 2000). In vitro studies have shown that it can induce cell motility and cell scattering in response to hepatocyte growth factor treatment. CTNND2 may also bind to E-cadherin at a juxtamembrane site within the cytoplasmic domain and/or bind to presenilin-1. Its subcellular location seems to be at the adherens junction and it is predominantly expressed in the brain. On the other hand, TRIO is a triple functional domain protein (PTPRF interacting protein) that promotes the exchange of GDP by GTP

(Debant et al. 1996). Together with leucocyte antigen related (LAR) protein, it could play a role in coordinating cell-matrix and cytoskeletal rearrangements necessary for cell migration and cell growth. It is highly expressed in heart, skeletal muscle, brain, pancreas, placenta, liver, kidney, and lung. Screening of these genes is currently underway.

In summary, our results indicate that there is a major gene locus on chromosome 5p, which was named *ANIB4* by the HUGO Gene Nomenclature Committee (Wain et al. 2002). Two families were found to share a common ancestral haplotype, which allowed us to refine our candidate gene region to 7.2 Mb. Nonetheless, our data indicate that there must be other loci involved in the genetic determination of IA in the FC population.

11.1.9 Acknowledgements

We would like to thank the families for participating in this study, the physicians who referred them, and Inge Meijer for careful review of this manuscript.

11.1.10 Electronic-database information

The family trees are given in a supplemental figure at http://www.jmedgenet.com/supplemental. The Center for Medical Genetics web site is at http://research.marshfieldclinic.org/genetics and the UCSC Genome Bioinformatics web site is at http://genome.ucsc.edu.

12 GENE SCREENING FOR *ANIB4*

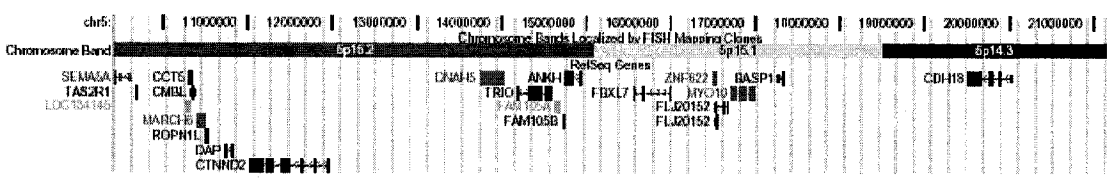
12.1 Rationale

The aim of this study was to screen candidate genes located within the 5p locus in order to find a causative gene for IA. The 11.7 Mb region defined by the Aneu60 family is bounded by the markers D5S2095 and D5S2031 and contains only 21 genes. In figure 12.1, we can see that the region is quite gene-poor, containing three distinct regions with no genes. Alignment with non-human mRNA seems to suggest that there are not many more un-annotated genes. The shared haplotype (table 12.1) between families Aneu60 and Aneu57 is defined by markers D5S2095 and D5S1963 and delimits a critical candidate gene interval of 7.2 Mb with 15 known genes (table 12.3).

Table 12.1: Shared haplotype between families Aneu60 and Aneu57.

Markers	Mb	60	57
D5S2095	9.435	8	
D5S457	10.404	1	1
D5S2004	10.553	1	1
D5S432	10.746	1	1
D5S478	11.034	3	3
D5S667	11.338	3	3
D5S1987	11.435	5	5
D5S2081	13.530	6	6
D5S1991	14.929	5	5
D5S1989	15.679	1	1
D5S1954	15.871	2	2
D5S1992	16.093	1	1
D5S1963	16.684	4	1
D5S2114	16.948	2	2
D5S1997	17.035	8	1
D5S268	17.473	5	5
D5S2096	17.500	1	1
D5S2846	19.921	2	2
D5S2031	21.149	5	

Figure 12.1: Genes located within the *ANIB4* locus from the UCSC genome browser



12.2 Methods

Genomic sequences from the *ANIB4* locus were obtained from the UCSC and NCBI databases to determine the structure of the candidate genes and design intronic PCR primers for screening. All genes were amplified by PCR and sent for sequencing at the Genome Québec Innovation Centre using their 3730XL DNA Analyzer system. Sequence traces were viewed and analyzed by the program Seqman (DNAstar package). Three affected individuals were screened: one each from Aneu60 and Aneu57 (who shared the haplotype) and one individual from family Aneu62, which was considered to be potentially linked but did not share a haplotype.

The genes were screened in priority, based on their involvement in the arterial wall, blood vessel remodelling or expression in appropriate tissues. Although genes were ranked in order of priority for screening, no gene was eliminated from consideration based only on predicted function. In addition, screening suitable candidate genes for mutations was performed in parallel with striving to reduce the size of the disease gene interval.

12.3 Results

We focused our candidate gene identification efforts on the shared haplotype situated between D5S2095 and D5S1963. Based on the UCSC freeze of May 2004 (http://genome.ucsc.edu), fifteen genes were located within that interval (see table 12.2).

Table 12.2: Genes within the *ANIB4* locus defined by the shared haplotype of Aneu60 and Aneu57

Marker	#	Name	Start	Exon	Description
D5S2095			9435000		
	1	SEMA5A	9091858	24	Semaphorin 5A precursor, may act as positive axonal guidance cues.
	2	TAS2R1	9682244	1	taste receptors
	3	LOC134145	10279444	5	Unknown
	4	CCT5	10303370	11	Molecular chaperone
	5	CMBL	10332388	6	Carboxymethylenebutenolidase-like
	6	MARCH-VI	10406827	26	membrane-associated RING-CH protein VI, mRNA turnover, integral nuclear membrane protein
	7	ROPN1L	10495014	5	AKAP-associated sperm protein
	8	DAP	10732342	4	death-associated protein
	9	CTNND2	11024951	22	Neuronal cell adhesion and tissue morphogenesis and integrity by regulating adhesion molecules.
	10	DNAH5*	13745038	79	Dynein, axonemal Primary ciliary dyskinesia disease
	11	TRIO	14196945	57	Promotes the exchange of GDP by GTP. Role in coordinating cell-matrix and cytoskeletal rearrangements necessary for cell migration and cell growth.
	12	FAM105A	14634931	8	Unknown
	13	FAM105B	14717856	7	Unknown
	14	ANKH*	14762018	13	Mutation at the mouse 'progressive ankylosis' (ank) locus causes a generalized, progressive form of arthritis accompanied by mineral deposition, formation of bony outgrowths, and joint destruction.
	15	FBXL7	15553304	4	Binds to phosphorylated proteins and promotes their ubiquitination and degradation.
D5S1963			16684000		

Genes with an * have not been screened because they are associated with other disorders.

The *CTNND2, TRIO, DAP* and *MARCH-VI* genes were screened first as they were deemed the most promising. Unfortunately no causative variants were found. The rest of the genes were screened, except for the two genes involved in known disorders (*DNAH5* and *ANKH*). With no positive results, we decided to screen the genes within the unshared interval located between markers D5S1962 and D5S2031, in case our shared haplotype was accidental. This region contained 6 additional genes, three of which seemed good candidate genes: *MYO10*, *BASP1* and *CDH18* (see table 12.3).

In the end, a total of 19 genes were screened and no causative variants were identified.

Table 12.3: Genes found in the *ANIB4* locus but not within the shared haplotype of Aneu60 and Aneu57

Markers	#	Name	Start	Exon	Description
D5S2095			9435000		
					See table 12.2 for genes
D5S1963			16684000		
	1	ZNF622	16504628	6	Zinc finger-like protein 9
	2	FLJ20152	16527794	9	Unknown
	3	MYO10	16718412	41	IQ calmodulin-binding motif, with Myosin head
	4	FLJ34047	17183136	3	Unknown
	5	BASP1	17270890	2	brain abundant, membrane attached signal protein 1
	6	CDH18	19508913	12	Cadherin: cell adhesion protein
D5S2031			21149000		

12.4 Discussion

There are many possible explanations as to why the causative mutation has not yet been found. The most likely explanation is that our mutation screening was incomplete. For example, the mutation could have been missed when analyzing the sequence traces or the PCR method could have preferentially amplified the normal allele. Having a second reviewer analyse the sequence traces or using another program such as Mutation Surveyor could remedy the former, while the latter one poses more of a problem. Creation of a cell hybrid containing only the disease chromosome could help because the mutation would be in a homozygous state and the normal allele would not be present (Papadopoulos et al. 1995). Furthermore, large deletions spanning whole exons and genes would be detected using this method since it would result in no DNA amplification.

Another limitation to this study was that our candidate gene analysis mostly focused on the coding region with less emphasis on the 5' and 3' UTR (untranslated region). Mutations in the UTR have been found to cause some disorders. As an example, a homozygous point mutation in the 3' UTR of p14 (also known as *MAPBPIP*), causes a human primary immunodeficiency syndrome by decreasing the gene's expression (Bohn et al. 2006). In addition, we did not screen the promoters of any of the candidate genes.

Although we attempted to characterize all genes within the candidate region, it is possible that some were missed. Continued revision and further bioinformatics of the candidate locus will ensure that the region is appropriately annotated and screened.

Another critical explanation is that there could be an underlying problem with our linkage analysis. As mentioned previously, a misdiagnosis or a phenocopy could have confounded our linkage results, making the identification of a disease gene difficult if the initial locus was wrongly defined.

In conclusion, the search for the *ANIB4* mutation continues. Once this gene has been identified, functional studies will be performed. These experiments will be determined using all available information on the gene and its protein.

13 DISCUSSION

13.1 General discussion of the chapters

13.1.1 Mutation analysis of the CCM genes

In total, 46 IFCAS families were screened for mutations in all CCM genes. We successfully identified the underlying genetic cause in 71% of the cases (33/46). Twenty-five of these families (54%) segregated a *CCM1* mutation, 6 (13%) contained a *CCM2* mutation, and only 2 (4%) segregated a *CCM3* mutation. Thirteen (29%) families yielded no detectable mutation after our attempts to locate the underlying genetic cause; however assessment of genomic deletion/insertion using the MLPA technique still remains to be done. Undoubtedly, our mutation identification rate of 71% will go up as deletions/insertions are identified within this last group.

In comparison, the combined techniques of sequencing and MLPA analyses successfully identified the underlying genetic cause in 10 out of 14 (71%) sporadic cases with multiple CCMs. Of this group, six cases (43%) contain a *CCM1* mutation, 2 (14%) have a *CCM2* mutation and 2 (14%) have a *CCM3* mutation. Interestingly, the ratio of mutations uncovered during screening is consistent with a large scale genetic study that found a mutation rate of 78% in 163 French CCM families, when combining sequencing and MLPA analysis (*CCM1*:53%, *CCM2*:15% and *CCM3*:10%) (Denier et al. 2006).

Clearly, it has become evident that gene/exon deletions and insertions are common findings in CCM. Consequently, future screening studies will need to combine both sequencing and MLPA analysis of the three CCM genes.

To this date, it remains unclear why more than 20% of familial CCM patients have no identifiable mutation. Possible explanations include unidentified mutations within the regulatory sequences of CCM genes (i.e. promoters), the involvement of other CCM causative genes or the involvement of modifier genes. In addition, insensitivity of the techniques used may be another cause of a missed coding mutation.

It seems unlikely that another gene can be identified in a large family by traditional positional cloning as few remain that are not already linked to the *CCM1-3* genes. However, a GWS of many small, unlinked families may reveal a common locus if there is collaboration between the different groups who study CCM. Alternatively, screening genes located within the CCM pathway (figure 2.1) may be a possibility. It remains to be seen where CCM genetic research will be headed since it appears that the three major CCM genes have been identified. Possible research directions could include either trying to identify the genetic cause of the remaining cases or possible characterization of the proteins involved in CCM.

13.1.2 Mutation analysis in sporadic cases

As previously mentioned, the genetic cause was identified in 71% (10) of 14 sporadic cases with multiple malformations, demonstrating that these sporadic

cases harbour *CCM1-3* mutations in approximately the same proportion as familial cases. This suggests that mutations found in sporadic individuals with multiple malformations are either germline mutations or have been inherited from an asymptomatic parent. Clinically, these individuals may be considered as CCM familial cases that have a higher recurrence risk of having a malformation and a 50% chance of offspring transmission. MRI or other CCM screening method of the patient's family may also be appropriate for early detection and/or diagnosis.

In contrast to cases with multiple malformations, our screening efforts have been fruitless when trying to identify the genetic cause in 21 sporadic cases with a single lesion. Although this latter cohort was greater in size than the one with multiple malformations, not one single mutation was identified. These data suggest that CCM formation in these individuals may have been caused by a one-time random mutational event in any of the *CCM* genes at the site of the malformation or by mutations in other genes. Clinically, they have a much lower risk of malformation recurrence and little chance of offspring transmission.

13.1.3 Missense mutation analysis

Almost all published mutations associated with CCM lead to a truncated protein. The identification of two missense mutations left us puzzled as to the mechanism by which they would cause the disorder. Further analysis of the two *CCM1* point mutations (D137G and Q201E) demonstrated that both point mutations actually activated cryptic splice-donor sites, causing aberrant splicing and leading to a frameshift and protein truncation. Our analysis of these missense mutations

stresses the importance of a more thorough search when a mutation does not fit the overall mutation pattern for a disorder. In addition, our findings underline the importance of examining all point mutations, including silent ones, to determine whether they activate a cryptic splice-donor site motif.

The need for deeper investigation was also confirmed by another study examining a *CCM2* missense mutation. The authors demonstrated that the missense had a deleterious effect by altering the PTB domain and abolishing *CCM1* and *CCM2* interaction (Zawistowski et al. 2005). These observations suggest that the CCM protein is refractory to small changes.

13.1.4 *CCM2* candidate gene screen

Two genes were screened in our quest to identify the *CCM2* causative gene. These two candidate genes were identified in CCM patients with a sporadic presentation of the disorder but with multiple CCMs. In both cases, a chromosomal translocation event occurred within the *CCM2* locus and the genes identified on the border of the translocation were the ones that were screened. Although, this approach was not successful in our attempt to identify the *CCM2* gene, it has been successful in other disorders such as Sotos syndrome (Kurotaki et al. 2002).

Interestingly, genes *ELMO1* and *TXBP151* are 10 Mb and 20 Mb, away from the causative *CCM2* gene respectively. This may suggest that *CCM2* gene expression may be controlled by distant elements. Studies of chromosome breakpoints in

human diseases show evidence that gene expression may be altered or controlled by distant elements over large chromosomal domains (Kleinjan and van Heyningen 1998). For example, causative chromosomal breaks in patients affected with aniridia (MIM 106210) and campomelic dysplasia (MIM 211970) may occur hundreds of kilobases away from the disease gene (Fantes et al. 1995; Velagaleti et al. 2005). The chromosomal breaks do not physically disrupt the genes, but mainly affect their expression. In addition, transgene expression studies show that correct tissue-specific gene expression can depend on sequences located hundreds of kilobases away from the coding sequence of a gene (Fomin et al. 2004).

While translocation breakpoints may have a reduced value when trying to identify a causative gene, they may be of greater value when trying to identify elements with a long-range effect on gene expression. Clearly, the chromosomal translocation breaks identified in the two CCM patients will have to be further characterized before any hypothesis on its effect on *CCM2* can be formed.

13.1.5 *CCM3* candidate gene screen

A shared haplotype approach was used when trying to identify the *CCM3* causative gene. In chapter 7, genotyping of polymorphic markers found within the original *CCM3* locus, defined by Craig and colleagues (Craig et al. 1998), was performed in 9 small families of European ancestry with no *CCM1* and *CCM2* mutation. Exclusion of the lower and upper regions of the *CCM3* candidate region

was observed in three families and defined the locus to be within markers D3S3037 and D3S1571. Two sets of overlapping haplotypes were observed between markers D3S3037 and D3S2312, while one set was observed between markers D3S1232 and D3S1262 (table 7.1). Thirty genes were almost completely sequenced in four CCM familial cases before the *CCM3* gene was identified by another group (Bergametti et al. 2005). The *CCM3* gene identified as the *PDCD10* gene is located 4.35 Mb upstream of the original *CCM3* locus (figure 7.1).

Unfortunately, the inclusion of small families in linkage analysis can yield erroneous and misleading results. In the original linkage (Craig et al. 1998), one of the families had a greater prior probability of linkage to the *CCM3* locus than to the *CCM1* locus and hence was included in the group of families linked to the *CCM3* locus. A recombinant within this family defined the upper limit of the *CCM3* locus. Unfortunately, this family was later found to have a *CCM1* mutation (personal communication). Hence, small families may lead to errors in linkage analysis.

In addition to reviewing linkage, re-analysis of previously screened genes should also be performed. This is crucial since no *CCM3* mutation was found in any of the families analyzed for haplotype sharing. A more thorough gene analysis, including MLPA, of all the CCM genes in these families needs to be performed. Unfortunately, in this case the shared haplotypes were not indicative of the causative gene's position but probably of the families' ancestry.

In retrospect, it would be interesting to know if the recurrent mutations identified in *CCM1* and *CCM2* arose independently (identical by state) or if they are

actually identical by descent as we had hypothesized. Genotyping markers around the mutations will let us determine that.

13.1.6 The two-hit hypothesis

In chapter 9, we presented evidence that in some cases, Knudson's two-hit hypothesis may be the cause of CCM formation. Knudson first coined the term "two-hit hypothesis" when trying to explain the cellular pathogenesis of retinoblastoma (Knudson et al. 1975). Consequently, the normal alleles of a gene need to be both "hit" by a mutation and become "inactivated" in order for the disorder to occur. In these instances, a dominantly inherited disorder would occur recessively at the cellular level. Usually, the first mutation can either be inherited (in familial cases) or occur somatically (in sporadic cases) while the second mutation is always somatic. For example, a somatic *CCM1* mutation (i.e. deletion, point mutation) in a *CCM1* family would result in the formation of a CCM since a germline *CCM1* mutation already exists in the same cell (figure 2.3). Evidence for this is seen in our study, where a loss of the normal *CCM1* allele was identified in four CCMs from two individuals with a *CCM1* mutation.

Support for the two-hit hypothesis has also been demonstrated in a previous research paper (Gault et al. 2005). In this study, the researchers demonstrated the occurrence of a new *CCM1* somatic deletion found within a lesion and established that it was biallelic to the inherited *CCM1* mutation.

In addition, evidence for a somatic loss of heterozygosity at the Ccm1 locus was demonstrated in a mouse model (Plummer et al. 2004). The authors studied mice

($Ccm1^{+/-}Trp53^{-/-}$) that were heterozygous for a Ccm1 mutation and homozygous for loss of the tumour suppressor Trp53 (p53). Deactivation of the p53 tumour suppressor has been shown to increase the rate of somatic mutations (Jacks et al. 1994) and hence would increase the chance of a second somatic hit. Cerebral vascular lesions were observed in 55% of the double-mutant animals, while none were observed in the controls of differing genotypes, suggesting that the inactivation of the normal copy at the Ccm1 locus has occurred.

Although there is growing evidence to support the two-hit hypothesis and presentation of a small number of lesions developing over time in an age-dependent manner can therefore be explained, it may not be the only mechanism involved in CCM. This model and the model of haploinsufficiency need to be further assessed in future studies.

Interestingly, both models are not mutually exclusive. For example, haploinsufficiency of a CCM protein during development could somehow change the structural integrity of blood vessels making them more susceptible to CCM formation, which could then occur through the two-hit hypothesis process.

13.1.7 Mapping of the *ANIB4* locus

In chapter 11, a novel locus for a dominant form of IA, *ANIB4*, was mapped to chromosome 5p. The identification of a large family (Aneu60) helped us identify this susceptibility locus using a monogenic approach for this disorder. Fine mapping of the region defined the locus between markers D5S2095 to D5S2031, and spanning 11.7 Mb at 5p15.2-14.3. Haplotyping of 10 other FC families

revealed that one family (Aneu57) shares a portion of the Aneu60 disease haplotype. Considering only the shared haplotype, the critical region was reduced from 11.7 to 7.2 Mb. This gene-poor region defined by markers D5S2095 and D5S1963 only contains ~19 genes. At this moment, the disease locus cannot be further reduced unless new affected members (new recombinants) or other linked families are identified. Additional genotyping of other families has revealed that this locus is not a common cause of IA in our cohort.

This is the second locus identified, after *ANIB3*, for a dominant form of IA. Interestingly, three different loci were initially identified in the *ANIB3* linkage analysis. The authors used the unaffected individuals found in the family to narrow it down to one region (Nahed et al. 2005). Since, it is known that the penetrance of this disorder is not 100% it would be cautious not to disregard the other two loci identified.

13.1.8 Gene finding at the *ANIB4* locus

An autosomal dominant form of IA was mapped to chromosome 5p in two French Canadian families. Despite extensive fine-mapping and thorough candidate gene analysis, the causative gene at the *ANIB4* locus remains elusive. There are many possible explanations as to why the causative mutation has not yet been found. The most likely explanation is that our mutation screening was incomplete. Firstly, candidate gene analysis focused mainly on the coding sequences with little emphasis on the UTRs. Although a causative mutation in a UTR is unlikely, all non-coding regions should nonetheless be screened, including promoters and

regions conserved between different species, which are suggestive of regulatory elements. Unfortunately, a large deletion or duplication would not have been identified with the methods utilized. The creation of haploid alleles derived from cell hybrids could have helped identify deletions, while duplications could have been assessed using the Comparative Genomic Hybridization (CGH) technique. In addition, it is possible that the region is much more gene rich than previously thought, or that there is a large genomic aberration or even that the genes have incomplete structural annotations. Unfortunately, LD can also be a source of problems when trying to identify a causative gene in families that are of common ancestry. The causative mutation is much harder to find because only one mutation will be shared between the families.

Another critical explanation is that there could be an underlying problem with our linkage analysis. A misdiagnosis or a phenocopy could have confounded our linkage results, making the identification of a disease gene difficult if the initial locus was wrongly defined. This is still possible, even though the same disease haplotype was discovered in another IA family because the shared haplotype may have occurred by chance, due to the fact that both families are from French Canadian ancestry. However, the identification of the same disease haplotype in other French Canadian IA families will greatly diminish this possibility. Therefore, haplotype analysis of other FC families at this locus is an ongoing process as new families are identified. In addition, replication of this locus in other populations by other researchers will also validate this region as an IA locus.

The search for the *ANIB4* mutation will be continued by reviewing all generated data and by closely monitoring the region for new or unannotated genes. Again, the identification of the *ANIB4* gene and the characterization of its function should help us understand the molecular and physiological processes involved in the formation of an intracranial aneurysm.

13.2 Limitations of the studies

A critical limitation to CCM and IA studies stems from the fact that there are not many large families available for sample collection. Not surprisingly, this is caused by the limited number of available affected families and a low disease penetrance. Death occurs in 50% of IA cases who suffer a ruptured aneurysm, significantly decreasing the number of affected patients that can be collected. In addition, not all family members are willing to participate for fear of retribution from insurance companies or for other personal reasons. The result is often a partially collected family yielding a lower statistical power in linkage analysis.

Usually, DNA is extracted from blood samples and lymphoblastoid cell lines are established for each affected participant. Although lymphoblasts are a continuous and reliable source of RNA and protein, gene expression in those cells may not necessarily be representative of expression in the cerebrovascular system. Unfortunately, the nature of these cerebrovascular disorders only allows a limited access to tissue from patients. The lack of quantity and quality of affected tissue materials (CCM or IA) complicate and limit the characterization of pathogenesis mechanisms in these disorders. For example, if aneurismal tissue from a family

member of Aneu60 were available, expression of the genes located within the *ANIB4* locus could be studied by in-situ hybridization.

The focus of our studies has been primarily on generating genetic data from human families. Alternatively, more functional approaches to these disorders could have been applied. For example, interactions of the CCM proteins with other proteins could have been characterized in a mouse or zebrafish model by developing antibodies against the proteins. Studies of the CCM genes in model organisms have been successful in elucidating the genes' expression and the pathways in which they are involved (Plummer et al. 2004; Seker et al. 2006)

13.3 Efficiency of linkage mapping and positional cloning

The use of linkage mapping as a tool to identify a disease locus has been successful in this study, with the identification of a novel susceptibility locus for IA. In this instance, a monogenic approach was used even though IA is usually considered a complex trait. The identification of a susceptibility locus using a "Mendelianized" form of a complex trait has also been successful in other disorders. This was the method used in the identification of the first breast cancer gene (*BRCA1*: OMIM 113705). In this instance, positional cloning was successful because linkage was based on families with a history of breast cancer and a young age of onset (Hall et al. 1990). Surely the characterization of more familial phenotypes or endophenotypes segregating in a Mendelian manner in complex traits should lead to the identification of more predisposing genes.

One of the major limitations of linkage mapping in humans is the rarity of critical recombinations. In the case of IA, many affected individuals die before blood samples can be collected and asymptomatic patients may be left uncollected. The identification or creation of animals (mice, dog) with similar human disorders or phenotypes offers many advantages to linkage mapping and positional cloning (Starkey et al. 2005). Shorter generation time and higher fecundity rates increase the number of affected cases and by extension the number of potential recombinants available for analysis.

13.4 Potential research avenues

13.4.1 Fluorescent *in situ* hybridization in CCM samples

Another technique that could be employed for detecting a loss of heterozygosity in CCM lesions is fluorescent *in situ* hybridization (FISH). FISH is a cytogenetic technique that detects the presence or absence of specific DNA sequences on chromosomes (Pinkel et al. 1986). Normally, visualization of double spots in a cell, by fluorescence microscopy, corresponds to the probe being hybridized to both copies of the gene. Changes in the number of spots are indicative of chromosomal abnormalities. One spot suggests a deletion while three spots suggest that a duplication has occurred. Fluorescent probes specific to the CCM genes could be tested in lesions and would reveal if large deletions are a cause of CCM.

13.4.2 Zebrafish model for understanding the CCM pathway

The tropical zebrafish (*Danio rerio*) is an excellent model organism, especially for studying vertebrate development and gene function (http://zfin.org/). Its many advantages include a short generation time, a high fecundity rate, an external transparent embryo and uncostly and easy care (Westerfield 2000). Its significant morphological and physiological similarities to mammals make it a great tool to identify disease genes.

Construction of zebrafish transgenic lines that express green fluorescent protein (GFP) is a great tool which facilitates the characterization of embryos by immuno-histochemistry. For example, the line TG(fli1:EGFP), allows continuous *in vivo* observation of the vertebrate embryonic vasculature by driving the expression of enhanced GFP in all blood vessels throughout embryogenesis (Lawson and Weinstein 2002). This transgenic line would be well suited for the study of CCM genes.

One of the techniques employed to study gene effects is the *morpholino knock-down* technique which can decrease a gene's protein expression by 90%. Morpholinos are degradation-resistant modified oligonucleotides that act on a gene's mRNA by either blocking its translation or disrupting its splicing (Gene Tools, LLC http://www.gene-tools.com/]).

This morpholino system has been successfully employed in a wide range of disorders including cardiovascular disease and neurological disorders (Penberthy et al. 2002). For example, a type of vascular disorder, hereditary hemorrhagic telangiectasia type 2 (HHT2), caused by mutations in the *ALK1* gene, was

recently studied (Gu et al. 2006). In this instance, the authors characterized 11 *ALK1* mutations and identified the mechanism of pathogenesis for each mutation.

13.4.3 Microarray expression study for IA

A microarray expression study is another method that could be used to search for potential IA genes. Gene microarray chips are used to measure expression levels for thousands of genes simultaneously (Schena et al. 1995). By comparing the expression profile of RNA extracted from normal cerebral arteries (known to have aneurysms) and normal arteries of similar size from other regions of the body, genes expressed in the cerebral arterial tissues as well as genes that are only expressed in cerebral arteries could be identified. Differentially expressed genes mapping to linked regions (*ANIB4* and other linkages) would be prime candidates for sequencing in a cohort of IA cases.

Furthermore, a microarray chip could also be used to compare the expression profile between normal cerebral arteries and cerebral aneurismal tissue which could lead to the identification of genes directly involved in IA. This method has been successfully employed in the study of abdominal aortic aneurysms (Lenk et al. 2006). Unfortunately, the location and nature of an intracranial aneurysm make tissue recovery difficult or unlikely.

14 CONCLUSION

Over the course of this PhD project, we have tried to understand the genetic causes and mechanisms involved in CCM and IA, responsible for two types of hemorrhagic stroke.

First, we characterized the *CCM1*, *CCM2* and *CCM3* genes in families and in sporadic cases affected with CCM. In both cohorts, a causative mutation was found 71% of the time. A more in depth analysis of cases with no CCM mutations, by MLPA analysis, revealed that large genomic deletions and duplications are a common cause of CCM. In addition, investigation of two simple point mutations revealed the activation of cryptic splice sites. Furthermore, the genetic predisposition to CCM in sporadic cases with a single lesion was determined to be different from sporadic cases with multiple malformations. Second, a loss of heterozygosity study demonstrated that a plausible mechanism for CCM pathogenesis involved a second somatic hit at the site of the lesion, suggesting that CCM may be caused by a complete loss of CCM protein function. Lastly, a susceptibility locus for IA, *ANIB4*, was identified using a monogenic approach to this complex disorder. Gene screening efforts for *ANIB4* and the search for new IA loci using other large families will be continued in our laboratory.

In conclusion, this PhD project has greatly contributed to the field of CCM, most specifically for its clinical diagnosis. A greater understanding of the genetics involved in CCM will facilitate and permit better management care for patients by providing the possibility of better improved treatment and diagnostic options. In

addition, the discovery of a susceptibility locus for IA should lead us closer to the identification of a gene, shedding light on an unknown but hypothesized IA pathway.

Overall, the characterization of the genes involved in the pathophysiology of cerebrovascular disorders will help us understand the pathways involved in the maintenance and regulation of these blood vessels.

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