Mutational analysis of 206 families with cavernous malformations

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Object. A gene contributing to the autosomal-dominant cerebral cavernous malformation (CCM) phenotype, \textit{KRIT1} \protect\footnote{an acronym for \textit{Krev Interaction Trapped 1}}), has been identified through linkage analysis and mutation screening. The authors collected blood samples from 68 patients with familial CCM and 138 patients with apparently sporadic CCM as well as from their families, in an effort to characterize the prevalence and spectrum of disease-causing sequence variants in the \textit{KRIT1} gene.

Methods. The authors used single-strand conformational polymorphism analysis to identify genomic variants in \textit{KRIT1}, which were sequenced to determine the specific mutation. Among 43 Hispanic-American kindreds who immigrated to the southwestern US from northern Mexico, 31 share an identical founder mutation. This Q455X mutation is found in 18 (86\%) of 21 persons with a positive family history and in 13 (59\%) of 22 persons with apparently sporadic CCM. This mutation was not found among 13 persons with CCM who were recruited from Mexico. These findings establish the key role of a recent founder mutation in Hispanic persons with CCM who live in the US.

Although nearly all Hispanic families in the US in which there are multiple CCM cases linked to the \textit{CCM1} locus, only 13 of 25 non-Hispanic CCM-carrying families have displayed evidence of linkage to the \textit{CCM1} locus. Among these 13 families, the authors identified eight independent mutations in nine kindreds. They identified four additional mutations among 22 familial CCM kindreds with no linkage information, bringing the total number of independent mutations to 12. Inherited \textit{KRIT1} mutations were not detected among 13 persons with CCM who were recruited from Mexico. These findings establish the key role of a recent founder mutation in Hispanic persons with CCM who live in the US.

Conclusions. All mutations were nonsense mutations, frame-shift mutations predicting premature termination, or splice-site mutations located throughout the \textit{KRIT1} gene, suggesting that these are genetic loss-of-function mutations. These genetic findings, in conjunction with the clinical phenotype, are consistent with a two-hit model for the occurrence of CCM.

**KEY WORDS** • cavernous angioma • cerebral cavernous malformation • \textit{KRIT1} gene

Cerebral cavernous malformation represents a brain and spinal cord vascular disorder characterized by abnormally dilated vascular channels lined by a single layer of endothelium. Histological examination of these lesions demonstrates dilated capillary beds without intervening neural structures or smooth muscle, as well as hemosiderin deposits indicating prior hemorrhage.\textsuperscript{24,25} The disease has been recognized as a common clinical entity since the advent of MR imaging. Although these common neurovascular lesions affect almost 0.5\% of the population, only 20 to 30\% of these individuals experience symp-

\begin{footnotesize}
\textsuperscript{Abbreviations used in this paper: CCM = cerebral cavernous malformation; MDE = Mutation Detection Enhancement; PCR = polymerase chain reaction; SSCP = single-strand conformational polymorphism; TBE = Tris-borate electrophoresis.}
\end{footnotesize}
Mutations in the \textit{CCMI} gene

toms.\textsuperscript{19} Symptomatic patients typically present with sei-
zures, stroke, headaches, or, rarely, frank hemorrhage. The
causes of such a diverse presentation among patients suf-
fering from CCMs remains unclear, and little is known re-
garding the molecular pathogenesis of the disease.

Ten to twenty percent of caucasian patients with CCM
have a first-degree relative with the disease; this proportion
increases to 50\% among Hispanic Americans, representing a
20- to 100-fold increase in risk compared with the gener-
al population.\textsuperscript{13} These findings suggest that genetic factors
contribute to the pathogenesis of CCM, and an initial link-
age analysis in Hispanic-American families has localized a
gene causing CCM, \textit{CCM1}, to chromosome 7q21.\textsuperscript{7,10} Sub-
sequent studies have demonstrated a founder effect among
both familial and apparently sporadic cases in this popu-
lation, proving that all these persons have inherited an identi-
cal mutation from a common ancestor,\textsuperscript{12} refining the linked
region to a 4 cM interval at 7q21.\textsuperscript{14} Positional cloning ex-
periments have ultimately identified one of the genes locat-
ed in this region, \textit{KRIT1}, as the \textit{CCM1} gene.\textsuperscript{1,2,4,6,15,17,21,23,24,27}

The function of the \textit{KRIT1} protein is unknown. The
\textit{KRIT1} gene was incidentally cloned using a yeast two-hy-
brid screen with Krev1 (Rap1A) as bait. A small guanosine
triphosphatase with significant homology to Ras, Krev1 is
thought to act as a tumor suppressor gene within the Ras-
signaling cascade.\textsuperscript{22} Thus \textit{KRIT1} is an acronym for the Krev
Interaction Trapped 1 gene.

A mutational analysis of \textit{KRIT1} in affected Hispanic-
American kindreds has demonstrated the founder mutation to be 2105C→T, which inserts a stop codon in exon 14.\textsuperscript{14,21} Although this founder mutation in \textit{KRIT1} is responsible for virtually all CCM cases among Hispanic Americans of Mexican descent, mutations in \textit{KRIT1} only account for approximately 30\% of CCMs among non-Hispanic popula-
tions.\textsuperscript{12} Subsequent genome-wide linkage studies in this latter pop-
ulation identified two additional loci, \textit{CCM2} on 7p and
\textit{CCM3} on 3q.\textsuperscript{3} The \textit{CCM2} and \textit{CCM3} genes are not yet
known and the phenotype of patients of all three loci is clin-
ically indistinguishable.

In this study we screened for mutations in \textit{KRIT1} in 21
Hispanic and 47 non-Hispanic kindreds with a family his-
tory of CCM, as well as in 35 Hispanic and 103 non-His-
panic persons whose CCM is apparently sporadic.

\textbf{Clinical Material and Methods}

\textit{Reverse Transcription–PCR}

Whole-cell RNA was extracted from lymphocyte cell
lines by using Trizol reagent according to the manufac-
turer’s protocol (Gibco BRL, Gaithersburg, MD). Complen-
tary DNA was generated by adding 1 \(\mu\)l of Oligo-dT
primer to 1 \(\mu\)g of whole messenger RNA with the aid of
M-MuLV reverse transcriptase (Roche Applied Scienc-
es, Indianapolis, IN). Polymerase chain reaction was per-
formed on 2 \(\mu\)l of reverse transcription reaction agent
with primers designed to amplify a segment of the transcript cov-
ering both the newly identified coding sequence and the
published \textit{KRIT1} sequence. The resulting ampiclon was se-
quenced using dideoxyribonucleotide chain termination on
a DNA sequencing system (ABI 373 instrument; Applied
Biosystems, Framingham, MA).

\textbf{Family Collection}

This study was approved by the Human Investigation
Committee at Yale University (Protocol No. 7680). At the
present time we have recruited a total of 961 individuals
from 206 families (Figs. 1 and 2). Index cases were col-
lected and screened rigorously for a family history based
not only on CCM lesions, but also on history of stroke or
seizure, and/or positive findings on a magnetic resonance
image. Fourteen of 21 Hispanic kindreds with a positive
family history, 10 of 34 Hispanic persons with apparently
sporadic CCM, and 20 of 47 non-Hispanic familial cases
were reported on previously.\textsuperscript{5,11}

\textbf{Preparation of Genomic DNA}

Ten milliliters of venous blood was collected in tubes
containing either acid citrate–dextrose or ethylenediamine
tetraacetic acid and was shipped at ambient temperature by
overnight courier to the laboratory. The DNA was prepared
from whole blood by lysis of nuclei, which was accom-
plished using sodium dodecyl sulfate and proteinase K, fol-
lowed by phenol–chloroform extraction and ethanol precip-
tation. The DNA was resuspended in water containing 10
mM Tris (pH 8) and 0.1 mM ethylenediamine tetraacetic
acid. Samples were aliquotted and diluted to 200 \(\mu\)g/ml, af-
fter which they were frozen at \(-80^\circ\)C.

\textit{Linkage Analysis}

Index cases were initially collected and screened using
mutational analysis for \textit{KRIT1} mutations. Linkage informa-
tion, therefore, was not collected for 22 non-Hispanic index
cases with a positive family history. For the remaining fam-
ilies, in whom no \textit{KRIT1} mutation was identified, high-
ly polymorphic di-, tri-, and tetranucleotide repeat marker
loci were genotyped using PCR. Primers for each locus
were designed from available published sequences and
synthesized by personnel at the Keck Biotechnology Re-
source Laboratory at Yale University. One primer of each
pair was 5’ tagged with either 6-FAM, HEX, or TET phos-
phoramidite dyes. The markers used for the three CCM loci
were previously described:\textsuperscript{5} for \textit{CCM1} at 7q22, D7S1813,
D7S1789, and D7S646 spanning 5 cM; for \textit{CCM2}, D7S2846, D7S510, D7S521, D7S667, and D7S478 span-
ning 22 cM; and for \textit{CCM3}, D3S3053, GATA18H05, GATA14G12, GGAA3H06, and D3S1262 spanning 22
cM. Genotypes were determined with the aid of an ABI 377
instrument. Pairwise and multipoint linkage analysis was
performed, as previously described,\textsuperscript{7} using appropriate soft-
ware (FASTLINK [version 3.0P; available from Rice Uni-
versity at FTP://softlib.cs.rice.edu] and LINKAGE [version
5.11\textsuperscript{6}] run on a Sun Sparcstation 20 (Sun Microsystems,
Palo Alto, CA).

\textit{Single-Strand Conformational Polymorphism Analysis and DNA Sequencing}

We used SSCP to screen for molecular variants in can-
didate genes in patients with CCM. In brief, specific primers
were used to amplify a segment of genomic DNA contain-
ing a coding sequence. The products were labeled by in-
cision of 1 \(\mu\)Ci of \(^{32}\)P deoxyctydosine triphosphate in the
PCR reaction. Products were denatured and then fractionat-
ed under denaturing conditions via electrophoresis on three
different gels under three empirically derived conditions: 1) 0.5 × MDE gel, 0.6 × TBE buffer, electrophoresis at 50 W constant power at room temperature; 2) 0.5 × MDE gel, 0.6 × TBE buffer, electrophoresis at 50 W at 4°C; and 3) 5% acrylamide (37.5:1 acrylamide/bis acrylamide) gel, 0.5 × TBE buffer at 15 W at 4°C. Amplicons were detected by exposure of the gel to x-ray film (Eastman Kodak Co., Rochester, NY). Variant amplicons were excised from the gel and eluted in water. These products were used for a second round of PCR amplification with the same primers, after which the product was subjected to DNA sequence analysis by dideoxyribonucleotide chain termination with the aid of an ABI 373 instrument.

Results

Mutation Analysis

We screened 206 index cases and other affected members of the kindreds for mutations in the KRIT1 gene, including previously unrecognized upstream coding exons identified by blasting the gene sequence against human and mouse Expressed Sequence Tag databases.8,20,26 In 56 of the index cases, the patients were of Hispanic descent and in 150 the patients were of non-Hispanic descent (Fig. 1). Forty-three of the 56 Hispanic-American index cases of Mexican descent were collected from kindreds living in the US with 21
Mutations in the CCM1 gene

We identified the Q455X founder mutation in 18 (86%) of these 21 families. No mutation was identified in the remaining three kindreds. Twenty-two remaining cases were apparently sporadic with no known family history. We identified the founder mutation in 13 (59%) of these cases; no mutations were identified in the remaining nine. Every case that had the conserved Hispanic haplotype11—with or without family history—had the Q455X founder mutation.

To determine if the founder mutation could be traced back to Mexico, the original source of migration to the southwestern US, 13 Mexican families with CCM from this region were collected and screened. These families did not have the conserved haplotype or the founder mutation.

During our initial screen of the 150 non-Hispanic kindreds, 47 (31%) reported a positive family history. Linkage analysis was performed in 25 of these families in which at least three affected members were available for study; 13 showed evidence of linkage to the CCM1 locus. Eleven of these families had a posterior probability greater than 0.9 for linkage to the KRIT1 locus and we identified mutations in nine of these 11. We found the identical mutation in two presumably independent kindreds; however, further analysis of these two kindreds revealed that they share a common ancestor, suggesting that this mutation was inherited by descent. In the remaining two kindreds among the original 13 with evidence for linkage to the CCM1 locus, we did not identify KRIT1 mutations by SSCP analysis.

In the remaining 22 non-Hispanic kindreds with a positive family history of CCM, we performed KRIT1 mutational analysis on index cases. We identified four novel KRIT1 mutations among these 22 index cases, bringing the total number of independent KRIT1 mutations to 12 (Table 1 and Fig. 3). Screening of all 103 non-Hispanic persons with CCM and no family history did not identify any new germline mutations in KRIT1 in any of these apparently sporadic cases.

The 12 mutations identified here exist in coding and splice-site sequences throughout the KRIT1 gene (Fig. 4). All newly identified mutations in the coding sequence, including mutations in apparently sporadic cases, are nonsense, frame-shift, or splice-site variants that prematurely truncate the encoded protein (Table 1).

Discussion

The discovery that mutations in KRIT1 lead to CCMs is the first of many steps in understanding the pathogenesis of this disease. Here we report the spectrum of mutations leading to the formation of these lesions in Hispanic and non-Hispanic families within our study population. Based on our data, 30% of non-Hispanic families with two or more cases of CCM linked to the CCM1 locus5 and mutations in KRIT1 have been identified in the majority of these cases in our study population (11 of 13 kindreds). If there are three or more affected members within a CCM kindred, the chance of having a KRIT1 mutation increases to 50%. Among non-Hispanic patients in whom a family history has been rigorously excluded, KRIT1 mutations account for none or very few cases of CCM.

TABLE 1
Twelve distinct mutations identified within 150 non-Hispanic kindreds

<table>
<thead>
<tr>
<th>Kindred No.</th>
<th>No. of Persons</th>
<th>Exon</th>
<th>Mutation</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>K2179</td>
<td>2</td>
<td>9</td>
<td>IVS9-1G→T</td>
<td>splice-site mutant</td>
</tr>
<tr>
<td>K2025</td>
<td>3</td>
<td>9</td>
<td>1441delGT</td>
<td>frame-shift</td>
</tr>
<tr>
<td>K2185</td>
<td>3</td>
<td>11</td>
<td>1689delA</td>
<td>frame-shift</td>
</tr>
<tr>
<td>K2172</td>
<td>7</td>
<td>12</td>
<td>1765delAGA</td>
<td>frame-shift</td>
</tr>
<tr>
<td>K2181</td>
<td>5</td>
<td>14</td>
<td>IVS14-1G→T</td>
<td>splice-site mutant</td>
</tr>
<tr>
<td>K2144</td>
<td>5</td>
<td>14</td>
<td>2102delTG</td>
<td>frame-shift</td>
</tr>
<tr>
<td>K2157</td>
<td>2</td>
<td>14</td>
<td>2088insG</td>
<td>frame-shift</td>
</tr>
<tr>
<td>K2154</td>
<td>2</td>
<td>16</td>
<td>2433delGA</td>
<td>frame-shift</td>
</tr>
<tr>
<td>K2158</td>
<td>1</td>
<td>16</td>
<td>2499delCT</td>
<td>frame-shift</td>
</tr>
<tr>
<td>K2195</td>
<td>4</td>
<td>17</td>
<td>2549delC</td>
<td>frame-shift</td>
</tr>
<tr>
<td>K2167</td>
<td>3</td>
<td>18</td>
<td>2632G→A</td>
<td>W630X</td>
</tr>
<tr>
<td>K2138/K2043</td>
<td>16</td>
<td>18</td>
<td>2591G→T</td>
<td>Q617X</td>
</tr>
</tbody>
</table>
Fig. 4. Novel mutations in the KRIT1 coding sequence. Coding exons are represented graphically with new mutations listed in bold above the specific exon. The exon number is identified within each exon, with the size shown by the number of base pairs listed just below.

We did not identify KRIT1 mutations in two of 13 non-Hispanic families, despite evidence for linkage to the CCM1 locus. Both of these families were small and the evidence for linkage was inconclusive, with posterior probabilities of linkage lower than 0.9 for linkage to the CCM1 locus. The CCM in these families is most likely attributable to mutations at another locus such as CCM2 or CCM3. Alternatively, these families could have mutations outside the coding sequence of KRIT1 that escaped detection.

Among Hispanic Americans of Mexican descent, a founder mutation in KRIT1 (Q455X), inherited from a common ancestor, is responsible for CCM1 lesions in the majority of cases. A positive family history predicts nearly a 90% chance of having this founder mutation result in CCM. In apparently sporadic cases, 60% are shown to have this founder mutation.

Including the 12 mutations reported in this series, all but one exist in familial cases. In every instance except one, sequencing data have shown nonsense, point, splice-site, or frame-shift mutations leading to premature stop codons, distributed throughout the KRIT1 gene (Fig. 4).

Interestingly, despite a relatively even distribution throughout the originally identified 12 exons of the KRIT1 gene, only a few of the reported mutations exist in the newly identified 5' exons. These new exons are part of a continuous open reading frame with the original 12 KRIT1 exons, but they might represent alternatively spliced species. Only one mutation at 1342C→G predicts a glycine-to-glutamate missense mutation, although it is unclear if this represents a nonfunctional polymorphism or a functional mutation. The other mutations include two frame-shift mutations located at 893delAAAG and 894delAAGT in exon 6, both predicting premature stop codons, and three more mutations (two frame-shift and one substitution mutations) reported by Cave-Riant, et al.1

Mutations in the KRIT1 gene can lead to a loss of function either through a dominant-negative effect, haploinsufficiency, or a two-hit model. All the mutations identified to date encode a truncated KRIT1 protein. The process of nonsense-mediated messenger RNA decay is expected to lead to degradation of these aberrant transcripts such that the truncated gene products are never produced;8,18 our preliminary results support this conclusion and make dominant-negative models unlikely. We favor the two-hit model over the haploinsufficiency mechanism for several reasons. In the two-hit model, patients with the inherited form of CCM1 are born with one mutant KRIT1 allele, and one wild-type allele, and lose the second allele through a somatic mutation, ultimately leading to complete loss of KRIT1 expression; only the progeny of these cells develop into CCM1 lesions. In contrast, patients with the sporadic form of CCM are born with two wild-type copies of the KRIT1 gene and have to acquire two independent somatic mutations before CCM lesions develop. One result is that these sporadic cases tend to occur in patients who are older and less likely to have multiple CCM lesions than their familial CCM counterparts. Magnetic resonance imaging of patients with familial and sporadic CCM supports this hypothesis, because it has demonstrated a correlation between the number of lesions and family history, as well as the age of the patient. Patients with the sporadic form of CCM almost always have a single lesion, as opposed to patients with the familial form of the disease, in whom multiple lesions are common and clinical awareness of the disease occurs at earlier ages. Lack of correlation between the location of mutations within the KRIT1 gene and clinical symptomatology is also consistent with the notion that loss of function of both alleles of KRIT1 is necessary for the disease phenotype.

These findings have clinical implications. For example, among Hispanic Americans of Mexican descent, nearly all familial and most sporadic cases will have CCM due to a single mutation inherited from a remote founder. As a result, at-risk relatives can be readily identified prospectively, providing the opportunity for premorbid intervention. Further correlation of the spectrum of mutations in CCM with patient phenotype may permit identification of risk factors at the genomic level. Should molecular markers exist that can identify patients at risk for hemorrhage, neurosurgeons will be able to select candidates more appropriately for treatment with early surgical intervention. Similarly, mutations causing a clinically less severe phenotype may allow for expected management. These genotype–phenotype correlation studies for different CCM loci are currently being investigated.

Acknowledgments

We express our sincere gratitude to all patients who participated in this study. We are also deeply grateful to Dr. Rodolfo Ondarza of the Instituto Nacional de Neurologica y Neurocirugia, Mexico City, Mexico, for his contributions to this study.

References

3. Clatterbuck RE, Eberhart CG, Crain BJ, et al: Ultrastructural and immunocytochemical evidence that an incompetent blood-brain...
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...barrier is related to the pathophysiology of cavernous malformations. \textit{J Neurol Neurosurg Psychiatry} 71:188–192, 2001


