

Report

***Krit1* Missense Mutations Lead to Splicing Errors in Cerebral Cavernous Malformation**

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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 Q210E) 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*.

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; Günel 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 $\geq 40\%$ of CCM cases. The *Krit1* protein has 736 amino acids (Zhang et al. 2000; 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 (icap1 α), a protein involved with $\beta 1$ -dependent angiogenesis, through its NPXY motif (Zhang et al. 2001).

All *Krit1* mutations, except two point mutations pre-

dicted 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. 2002), 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 a RNeasy mini kit (QIAGEN). A cDNA library was synthesized by RT-PCR, using hexanucleotides (pdN₆). 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. 1A) are heterozygous for an A→G substitution (fig. 1B) in exon 7 at the nucleotide position 410 of the coding sequence. The migration pattern of the cDNA (fig. 1C) 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. 1D). 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

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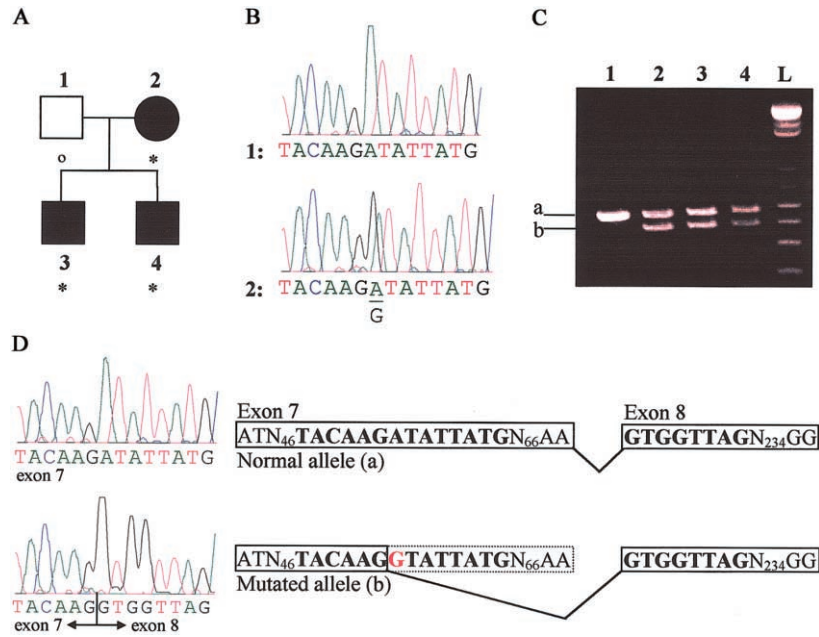


Figure 1 A, Pedigree of IFCAS-41. The blackened symbols denote affected individuals, and the unblackened square denotes an individual not known to be affected. Asterisks denote mutations, and the small unblackened circle denotes an absence of mutation. B, Genomic DNA sequences of unaffected (1) and affected (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.

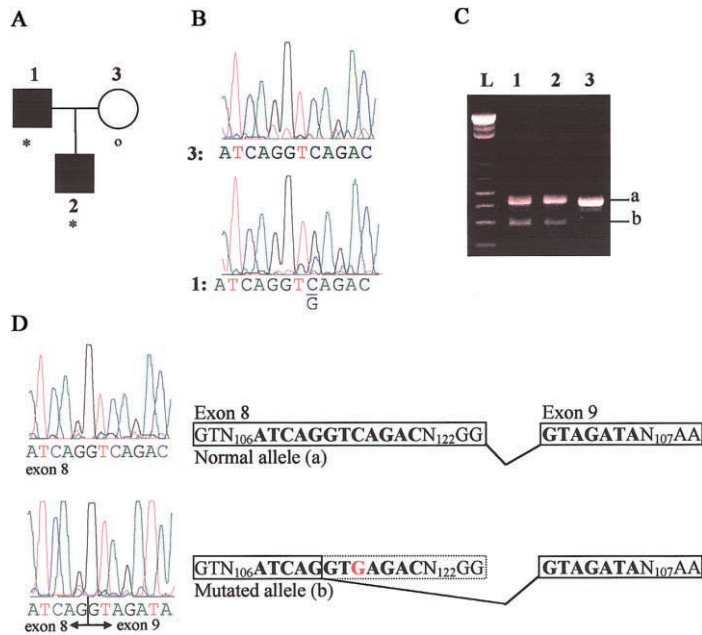


Figure 2 A, Pedigree of IFCAS-28. Definitions of symbols are the same as in figure 1. B, Genomic DNA sequences of a normal (3) and affected (1) individuals. The affected individual carries a C→G substitution at nucleotide position 601 of the coding sequence. C, cDNA migration pattern of the normal (a) 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 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.

has 2 novel amino acids and contains no structural domains of Krit1.

The affected members of IFCAS-28 (fig. 2A) are heterozygous for a C→G substitution (fig. 2B) in exon 8 at nucleotide position 601 of the coding sequence. Similar to IFCAS-41, the migration pattern of the cDNA (fig. 2C) 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. 2D). 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. 1C and 2C) 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. 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.

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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])

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