Novel mutations in three families confirm a major role of COL4A1 in hereditary porencephaly

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Received 4 June 2005
Revised version received 9 August 2005
Accepted for publication 10 August 2005
Published Online First 17 August 2005

Background: Porencephaly (cystic cavities of the brain) is caused by perinatal vascular accidents from various causes. Several familial cases have been described and autosomal dominant inheritance linked to chromosome 13q has been suggested. COL4A1 is an essential component in basal membrane stability. Mutations in COL4A1 have been described in hereditary porencephaly but have been ascribed to a perinatal parenchymal insult to the brain, and a familial predisposition with dominant inheritance has been widely documented. Occurrence of other complaints such as stroke, vascular aneurysm, and migraine in pedigrees with familial porencephaly has suggested a genetic predisposition to "vascular liability." It has been suggested that thrombophilia is associated with familial porencephaly, but trauma, maternal disease, and infections are also considered risk factors for sporadic porencephaly. There has been discussion as to whether the occurrence of porencephaly only at specific periods of intrauterine life (the end of gestation or in general after the 20th gestational week) might be caused by the mutation of a gene specific for brain development. A locus for familial porencephaly has recently been described on chromosome 13qter. The description of a mouse model where a Col4a1 mutation leads to perinatal cerebral haemorrhage and to porencephaly has shed light on the pathogenesis of this disorder. Collagen IV is important for structural integrity and function of basement membrane. Together with COL4A2, COL4A1 is the most abundant component of type IV collagen in basement membrane. These two proteins assemble to form a heterotrimeric triple helix of the type α1.1α1.2(IV), forming an hexamer with itself or with a triple helix α1α1α1. Myoptic and pituitary defects when thrombophilia is associated with familial porencephaly, but have been caused by the mutation of a gene specific for brain development or in general after the 20th gestational week. There has been discussion as to whether the occurrence of porencephaly only at specific periods of intrauterine life (the end of gestation or in general after the 20th gestational week) might be caused by the mutation of a gene specific for brain development. A locus for familial porencephaly has recently been described on chromosome 13qter. The description of a mouse model where a Col4a1 mutation leads to perinatal cerebral haemorrhage and to porencephaly has shed light on the pathogenesis of this disorder. Collagen IV is important for structural integrity and function of basement membrane. Together with COL4A2, COL4A1 is the most abundant component of type IV collagen in basement membrane. These two proteins assemble to form a heterotrimeric triple helix of the type α1.α1α1.2(IV), forming an hexamer with itself or with a triple helix α1α1α1. Mice bearing a heterozygous Col4a1 mutation leading to in-frame deletion of exon 40, are prone to brain haemorrhage at birth. This mutation leads to the synthesis of an abnormal COL4A1 which cannot be properly secreted outside the cell. In two families, mutations in conserved glycines of the Gly-X-Y repeats in the triple helix domain of COL4A1 have been associated with porencephaly. One of the issues raised in familial dominant porencephaly concerns individuals who appear to be non-manifesting obligate carriers. It has been suggested that magnetic resonance imaging (MRI) fails to identify asymptomatic carriers. In contrast, other observations suggest that subcortical and periventricular white matter lesions resembling gliosis are present in obligate carriers and might aid carrier detection. Another issue concerns the occurrence of strokes at older age in congenital porencephaly families, as previously reported.

We report three novel mutations in COL4A1 in three unrelated Dutch families with an autosomal dominant predisposition to porencephaly. Clinical and MRI findings of two of these families have been described in detail previously.

METHODS
Mutation analysis of COL4A1

Written informed consent was obtained from all subjects. Genomic DNA was isolated from peripheral blood using standard protocols.

The primers were designed to amplify the 52 exons including at least 50 bases of flanking genomic sequences based on the reference sequence of COL4A1 as deposited in GenBank (accession number for the mRNA NM_001845 and for the COL4A1 gene Entrez GeneID 1282).

Amplification reactions (exon 3 to exon 52) were carried out in 21 μl containing 1× polymerase chain reaction (PCR) buffer (Invitrogen, San Diego, California, USA), 1.5 mM MgCl₂, 0.01% W-1, 250 μM of each dNTP, 1 μM forward primer, 1 μM reverse primer, 0.75 units of platinum Taq DNA polymerase (Invitrogen), and 25 ng genomic DNA. Exons 1 and 2 were amplified in 20 μl containing 1×GCII TaKaRa, 400 μM of each dNTP, 1 μM forward primer, 1 μM reverse primer, 1 unit of LA Taq DNA polymerase (TaKaRa), and 25 ng genomic DNA.
Cycle conditions were: seven minutes 30 seconds at 95°C; 10 cycles of 30 seconds denaturation at 94°C, annealing at 68°C minus 1°C per cycle, one minute extension at 72°C, followed by 25 cycles of 30 seconds denaturation at 94°C, annealing at 58°C, one minute extension at 72°C; and final extension of five minutes at 72°C. The designed primer sequences are available upon request (from GB).

Templates for the direct sequencing reactions were cleaned from dNTPs and primers using 2 μl ExoSAP-IT (US Biochemical, Cleveland, Ohio, USA) during 15 minutes at 37°C, followed by a 15 minutes inactivation step at 80°C. Direct sequencing of both strands was undertaken using Big Dye Terminator chemistry version 3.1 (Applied Biosystems, Foster City, California, USA) as recommended by the manufacturer. Fragments were loaded on an ABI3100 automated sequencer and analysed with DNA Sequencing Analysis (version 3.7) and SeqScape (version 2.1) software (Applied Biosystems).

Templates for the SNaPshot reactions (3389G→A; exon 39, and 4267G→C; exon 48) were cleaned from dNTPs and primers using 2 μl ExoSAP-IT (US Biochemical) during 15 minutes at 37°C followed by a 15 minute inactivation step at 80°C. About 20 ng of pooled product were used in a primer extension reaction including the primers shown in table 1.

Reactions were carried out in 10 μl containing 1 μl SNaPshot multiplex ready reaction mix (Applied Biosystems), 2.5 μM extension primer, and 1 μl ½ term buffer (200 mM Tris HCl; 5 mM MgCl₂ pH 9). Additional thermal cycling was carried out (40 cycles of 10 seconds at 95°C; five seconds at 50°C, and 30 seconds at 60°C). Removal of the 5’ phosphoryl groups was achieved using one unit of shrimp alkaline phosphatase (SAP) (Roche Products, Indianapolis, Indiana, USA) for 30 minutes at 37°C. After the addition of 10 μl Hi-Di formamide (Applied Biosystems) containing GeneScan-120 LIZ size standard (Applied Biosystems) to 1 μl SNaPshot products, samples were heated for five minutes at 95°C, placed on ice, and loaded on an ABI3100 genetic analyser (Applied Biosystems). Fragments were analysed using GeneMapper V3.0 software (Applied Biosystems).

Digestion of exon 1 PCR product was undertaken by adding 1X NEBuffer 4 (New England Biolabs, Beverly, Massachusetts, USA) and 10 units of NcoI, incubating for two hours at 37°C. The 1A→T mutation generates a NcoI restriction site with the 539 base pair (bp) sized exon 1 in two fragments of 317 and 222 bases, respectively. Fragments were analysed on a 2% agarose gel.

Case reports

Pedigrees are shown in fig 1. Families A and B have been described previously, under the same title, by Mancini et al.13

Family A

Patient A-IV-1 showed a left sided hemiparesis and an occipito-frontal head circumference –1 SD from the normal mean at the age of 1.5 years. Brain MRI at that age showed cystic dilatation of the right lateral ventricle in the frontal and parietal area, reaching the cortex and surrounded by a thin layer of white matter. The whole periventricular white matter (down to the occipital area) showed bilateral patches of high signal intensity on T2 weighted images, low signal intensity on T1 weighted images, and hyperintensity on proton density imaging, suggesting gliosis (see figure 3 in reference 13). The right internal capsule was thinned and difficult to identify. There was a clear asymmetry of the cerebral peduncles, with signs of wallerian degeneration of the right peduncle. The left ventricle was not enlarged. The corpus callosum was thin. Coagulation status was normal.

In patient A-III-1, neglect for the right arm was noted at the age of five months. At the age of 15 months a right sided
hemiplegia was diagnosed. She attended regular high school and has remained healthy except for attacks of common migraine. Brain MRI at the age of 27 years showed irregularity of the edge of the middle part of the left lateral ventricle and a porencephalic cystic dilatation and bilateral disseminated white matter lesions, with a secondary wallerian degeneration of the left cerebral peduncle, which was thinned (see figure 4 in reference 13).

In patient A-IV-2, brain MRI was undertaken at the age of four years because of recurrent attacks of vertigo. It was normal.

Patient A-II-2 has migraine and presents with a small right infratentorial meningioma. Angiography of the cerebral arteries also revealed an aneurysm on the top of the right carotid artery. Brain MRI studies showed bilateral scattered areas of high signal intensity on T2 weighted images in the periventricular white matter but no porencephaly (see figure 5 in reference 13).

Patients A-II-3 and A-II-4 are both known to have congenital hemiplegia. Patient A-II-3 had a stroke at the age of 49 years. From the medical records, cystic dilatation of the ventricles was present on CT (scan not reviewed by us). Patient A-II-4 suffers from migraine attacks. Brain CT of patient A-II-4 at the age of 40 years showed diffuse dilatation of the right lateral ventricle, most abnormal in the parietal and occipital areas (see figure 6 in reference 13).

Family B
Patient B-III-1 was examined at the age of six years because of unexplained mild left sided hemiparesis and moderate psychomotor retardation. The pregnancy and birth had been uneventful. Brain MRI at the age of eight years showed a thin corpus callosum, smooth dilatation of part of the frontal horn of the right lateral ventricle, surrounded by a thin wall, and asymmetry of the gyral pattern in the cortex covering the cyst, without overt signs of cortical dysplasia or abnormal signal intensity (see figure 7 in reference 13).

Patient B-III-2 was examined at the age of two years because of left sided hemiplegia.

At the age of four years she developed focal epilepsy. Brain CT at that age showed cystic dilatation of the frontal horn and the medial part of the right lateral ventricle (data not shown). She also has mild mental handicap.

Neurological examination of patient B-II-4 at the age of 38 years showed a mild pyramidal syndrome and positive Babinski sign in the right lower limb. Neurological examination of patient B-II-1 at the age of 50 years revealed left sided pyramidal signs, while brain MRI showed bilateral patchy areas of abnormal signal intensity of the cerebral white matter on T2 weighted images, but no porencephalic cyst (see figure 9 in reference 13). Patient B-II-2 has severe mental retardation, epilepsy, and a normal neurological examination and MRI. Patient B-II-3 experienced a stroke at the age of 55 years but refused access to medical records. Individuals B-II-1, B-II-2, and B-II-3 refused DNA tests.

Family C
Family C consists of a two year old boy (C-III-1) and his mother, both with congenital right hemiplegia and normal cognition. The pregnancy was uneventful, though the Apgar scores were normal. His mother (C-II-2) also had right sided hemiplegia and right hand dystonia with atrophy of the right leg from infancy. The maternal grandmother is known to have a “thin leg” but has not been investigated. Brain MRI of the boy showed dilatation of the left ventricle in the frontoparietal area without cortical or basal ganglia abnormality. MRI of his mother showed left ventricular dilatation and atrophy of the left thalamus (fig 2). Coagulation tests and DNA analysis of factor II and factor V Leiden are normal in both mother and son.

RESULTS
Clinical and molecular data are summarised in table 2.

Genomic sequencing of COL4A1 revealed the base changes detailed in table 3. In family A, an heterozygote mutation in the start codon of the gene1A→T in exon 1 was found cosegregating with the disease in the index patients A-IV-1, A-III-2, and A-II-4 (fig 1A). Subject A-II-3 agreed to share his medical records but refused a DNA test. Subject A-II-2, considered an obligate carrier, also has the same mutation. The c.1A→T change was not found in more than 350 ethnically matched control chromosomes tested and is...
predicted to eliminate the ATG start codon of \textit{COL4A1} (fig 3A), resulting in no protein or in a translation initiation site moving upstream or downstream. The next in-frame ATG can be found 193 nucleotides downstream (base 277 in ref seq NM_01845) and, if used, it may lead to the synthesis of a protein lacking 64 N-terminal amino acids. Although we cannot confirm the synthesis of an abnormal protein in affected family members, disease cosegregation and absence of the mutation in controls suggest pathogenicity.

Sequencing in family B showed a missense c.3389G→A change in exon 39 (fig 3B), leading to a p.G1130D, which cosegregates with the disease. This mutation is in one of the Gly-X-Y repeats of \textit{COL4A1} and was not present in more than 300 control chromosomes and is conserved in seven different species from primates through nematodes (fig 4A). Compared with glycine, the replacing aspartate is a larger amino acid introducing a negatively charged side chain probably interfering with triple helix assembly.

In family C we observed a missense c.4267G→C in exon 48 leading to a p.G1423R substitution in patients C-III-1 and C-II-2. This mutation was not present in the unaffected father (C-II-1) or in 370 ethnically matched control chromosomes.

Table 2 Clinical and neuroimaging findings in the porencephaly families (modified from reference 13)

<table>
<thead>
<tr>
<th>Family</th>
<th>Exon</th>
<th>DNA</th>
<th>Codon effect</th>
<th>Protein effect</th>
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<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>1A→T</td>
<td>ATG→TG</td>
<td>Unknown</td>
</tr>
<tr>
<td>B</td>
<td>39</td>
<td>3389G→A</td>
<td>GGT→GAT</td>
<td>G1130D</td>
</tr>
<tr>
<td>C</td>
<td>48</td>
<td>4267G→C</td>
<td>GGT→GCT</td>
<td>G1423R</td>
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Table 3 \textit{COL4A1} mutations found in this study

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DISCUSSION

We report three new mutations in \textit{COL4A1} in three unrelated Dutch families with dominant porencephaly. Two of the mutations affect highly conserved glycines of Gly-X-Y repeats within the collagenous domain of the protein which is known to interact with COL4A2 to form a collagen IV triple helix.

A mouse model for perinatal cerebral haemorrhage and porencephaly shows an in-frame deletion of exon 40 of \textit{Col4a1}. Mutations in conserved Gly-X-Y repeats of \textit{COL4A1}

were subsequently found in two other families with autosomal dominant porencephaly.24 Immunohistochemistry and electronmicroscopy of \textit{Col4a1} mutants indicate impaired secretion of both \textit{COL4A1} and \textit{COL4A2}. Similarly, a mutation of the \textit{C. elegans} orthologue of \textit{Col4a1} (let-2) results in impaired secretion and intracellular accumulation of both \textit{let-2} and the \textit{Col4a1} orthologue \textit{emb-9}.24 Mice homozygous for \textit{Col4a1}\textsuperscript{ex40} or a null allele20 are not viable, and heterozygous null mice have no apparent phenotype.24 However, 50% of heterozygous \textit{Col4a1}\textsuperscript{ex40} mice die following parturition and 18% of the survivors show obvious porencephalic lesions. This suggests that synthesis of a mutant protein in \textit{Col4a1}\textsuperscript{ex40} mice has a negative effect on survival. Experimentally, a negative effect of \textit{Col4a1}\textsuperscript{ex40} mutation was demonstrated on collagen IV triple helix assembly and secretion.25 Mutations in highly conserved Gly-X-Y domains have been shown in several collagen proteins, leading to a dominant negative effect.22,23 Based on these observations, synthesis of an abnormal protein can be predicted in our families B and C.

The consequence of the mutation in family A is more difficult to predict. One possibility is that this is an effective null allele and a transcript is only produced from the normal allele. However, evidence from model organisms shows no obvious phenotype in heterozygotes for null alleles and suggests that dominant interfering proteins are necessary for pathogenesis. Thus a null mutation is not expected to be pathogenic and it is unlikely that the mutation in family A is a null allele. Following the conserved initiation codon, the next in-frame start codon contains a pyrimidine at the -3 position, which is highly conserved with respect to translation initiation sites and suggests that this start site might be used.27

27 Initiation of translation at this second site would result in synthesis of a protein with a 64 amino acid N-terminal truncation but with an intact NC1 domain and collagenous domain. We predict that the NC1 domain of the mutant trimers is able to initiate assembly of heterotrimers but that the heterotrimers would be structurally or functionally abnormal because of the N-terminal truncation. Further insights into the effect of this mutation will result from the
introduction of this start codon mutation in mice, or functional studies on the COL4A1/2 protein in patients from family A. Future in vitro expression studies of the mutant protein by our group are also aimed at confirming this assumption.

The gliotic lesions in our families indicate that the time of onset of porencephaly is related to hypoxic-ischaemic events occurring in a late stage of pregnancy (after the 20th week). This is compatible with the specific function of COL4A1 in the formation of a \( \alpha_2(IV) \) protomer, which is expressed in early embryonic development (from the 32 to 64 cell stage of the mouse embryo) but is not essential for basement membrane deposition. Instead, its essential function involves the maintenance of the structural integrity of the basement membranes at later stages—that is, later fetal life. Its total ablation leads to embryonic lethality only at E10.5–11.5 because of impaired basement membrane stability. In this respect, localisation of the white matter lesions in areas draining from the vena terminalis in our families A and C and in the watershed area between the anterior and medial...
cerebral arteries in patient B-II-4\(^{11}\) (table 2) are compatible with abnormalities of the vascular basement membrane as a result of \(\text{COL4A1}\) mutations.

Our findings also confirm that in asymptomatic carriers white matter lesions on MRI can be considered an expression of (and perhaps a risk factor for) \(\text{COL4A1}\) mutation. A history of strokes in middle age in patients A-II-3 and B-II-2 could not be correlated with \(\text{COL4A1}\) abnormalities as these patients refused DNA tests. However, recurrent strokes in \(\text{COL4A1}\) related porencephaly have also been observed by Gould et al.,\(^{11}\) and additional patients need to be tested to prove this relation. Further evidence is needed to determine other neurological complaints in our families, such as the carotid artery aneurysm in patient A-II-2,\(^{11}\) recurrent migraine in family A, and mental retardation in family B, are also related to \(\text{COL4A1}\) mutations.

Porencephaly in our families occurs in areas where traumatic perinatal arterial bleeding (watershed areas)\(^{14} 15\) or venous thrombotic events also occur,\(^{11}\) and white matter lesions are seen in asymptomatic carriers of \(\text{COL4A1}\) mutations. This suggests that trauma\(^{19} 20\) and thrombophilia\(^{21} 22\) could be factors influencing the occurrence of cerebral bleeding in \(\text{COL4A1}\) mutants. In this case genetic testing for \(\text{COL4A1}\) mutations in families at risk could aid counselling or suggest the need for additional perinatal care to avoid a traumatic delivery.

ACKNOWLEDGEMENTS

We thank the families participating in this study and referring physicians Dr P W J van Morsvedel and Dr N J Langendoen. We also thank Dr P Govaert for useful discussions.

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Conflicts of interest: none declared

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