Novel mutations in three families confirm a major role of \textit{COL4A1} in hereditary porencephaly.

G. Breedveld\textsuperscript{1}, I.F. de Coo\textsuperscript{2}, M.H. Lequin\textsuperscript{3}, W.F.M. Arts\textsuperscript{2}, P. Heutink\textsuperscript{4}, D.B. Gould\textsuperscript{5}, Simon W.M. John\textsuperscript{5}, B. Oostra\textsuperscript{1} and G.M.S. Mancini\textsuperscript{1}\textsuperscript{*}

Author Affiliations:
Departments of Clinical Genetics (1), Child Neurology (2) and Radiology (3), Erasmus University Medical Center, PO Box 1738; 3000 Rotterdam, The Netherlands
Department of Human Genetics (4), Section of Medical Genomics, VU University Medical Center, Amsterdam, The Netherlands
The Howard Hughes Medical Institute and The Jackson laboratory (5), Bar Harbor, Maine, 04609, USA

*Corresponding author address:
Grazia M.S. Mancini
Department of Clinical Genetics
ErasmusMC
P.O. Box 1738
3000 DR Rotterdam, The Netherlands
Tel: +31-10-4087266
Fax: +31-10-4367133
g.mancini@erasmusmc.nl

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Abstract

**Introduction:** Porencephaly (cystic cavities of the brain) is caused by perinatal vascular accidents of different cause. Several familial cases have been described and autosomal dominant inheritance, linked to chromosome 13q has been suggested. COL4A1 is an essential component for basal membrane stability.

**Methods:** Mouse mutants bearing an in-frame deletion of exon 40 of Col4a1 show perinatal death with hemorrhage and porencephaly in survivors. Report of inherited mutations in COL4A1 in two families has demonstrated that familial porencephaly may have the same cause in humans.

**Results:** We report three novel COL4A1 mutations in three unrelated Dutch families, two missense mutations of glycine residues predicted to result in abnormal collagen IV assembly and one mutation predicted to abolish the traditional COL4A1 start codon. The last mutation was also present in an asymptomatic obligate carrier with white matter abnormalities on brain MRI.

**Discussion:** Our observation confirms COL4A1 as a major locus for genetic predisposition to perinatal cerebral hemorrhage and porencephaly and suggests variable expression of COL4A1 mutations.
Introduction

The term porencephaly is used to indicate a fluid filled cavity in the brain. Congenital porencephaly often causes hemiplegia, but also tetraplegia, epilepsy, mental retardation, and dystonia, optic and pituitary defects when involving deep midline structures. Encephaloclastic (disruptive) porencephaly has been ascribed to perinatal parenchymal insult to the brain, and familial predisposition with dominant inheritance has been widely documented. Occurrence of other complaints like stroke, vascular aneurism and migraine in pedigrees with familial porencephaly has suggested a genetic predisposition for “vascular liability”. Thrombophilia has been suggested to be associated with familial porencephaly, but together with trauma, maternal disease and infections is considered a risk factor also for sporadic porencephaly. It has been discussed whether the occurrence of porencephaly only in specific periods of intrauterine life (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 been recently described on chromosome 13qter. The description of a mouse model where a Col4a1 mutation leads to perinatal cerebral hemorrhage and to porencephaly has shed light on the pathogenesis of this disorder. Collagen IV is important for structural integrity and function of basement membrane (BM). COL4A1 is together with COL4A2 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.α1.α2(IV), forming an hexamer or with itself or with a triple helix α5.α5.α6(IV). Mice bearing a heterozygote Col4a1 mutation leading to in-frame deletion of exon 40, are prone to brain hemorrhage 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 MRI scans fail 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 reported. Here we report three novel mutations in COL4A1 in three unrelated Dutch families with autosomal dominant predisposition to porencephaly. Clinical and MRI findings of two of these families have previously been described in detail.

Materials and methods

Mutation analysis 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
GeneBank (accession number for the mRNA NM_001845 and for the COL4A1 gene Entrez GeneID 1282).

Amplification reactions (exon 3 – exon 52) were performed in 21 µl containing 1x Invitrogen PCR buffer, 1.5mM 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. Exon 1 and 2 were amplified in 20 µl containing 1x 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: 7’30” 95°C; 10 cycles of 30” denaturation 94°C, annealing 68°C minus 1°C per cycle, 1’ extension 72°C followed by 25 cycles of 30” denaturation 94°C, annealing 58°C, 1’ extension 72°C; final extension 5’ 72°C. The designed primer sequences are available upon request (G.B.).

Templates for the Direct Sequencing reactions were cleaned from dNTP’s and primers using 2 µl ExoSAP-IT (USB) during 15 minutes at 37°C followed by a 15 minutes inactivation step at 80°C. Direct sequencing of both strands was performed using Big Dye Terminator chemistry ver.3.1 (Applied Biosystems) as recommended by the manufacturer. Fragments were loaded on an ABI3100 automated sequencer and analysed with DNA Sequencing Analysis (ver.3.7) and SeqScape (ver.2.1) software (Applied Biosystems).

Templates for the SNaPshot reactions (3389G>A; exon 39 and 4267G>C; exon 48) were cleaned from dNTP’s and primers using 2 µl ExoSAP-IT (USB) during 15 minutes at 37°C followed by a 15 minutes inactivation step at 80°C. About 20 ng of pooled product was used in a primer extension reaction including the following primers

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Exon</th>
<th>Extension primer</th>
<th>strand</th>
<th>Sz bp</th>
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<tr>
<td>3389G&gt;A</td>
<td>39</td>
<td>caaaggctcctccaggattggcatccctg</td>
<td>sense</td>
<td>32</td>
</tr>
<tr>
<td>4267G&gt;C</td>
<td>48</td>
<td>tctctatctctaggctcaagaggttccca</td>
<td>sense</td>
<td>32</td>
</tr>
</tbody>
</table>

Reactions were done 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 TrisHCl; 5 mM MgCl₂ pH 9. Additional thermal cycling was performed 40 cycles 10” 95°C; 5” 50°C and 30” 60°C. Removal of the 5’ phosphoryl groups was performed using 1 unit of Shrimp Alkaline Phosphatase (SAP) (Roche) during 30’ 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 5’ 95°C, placed on ice and loaded on an ABI3100 Genetic Analyzer (Applied Biosystems). Fragments were analysed using GeneMapper V3.0 software (Applied Biosystems).

Digestion of exon 1 PCR product was performed by adding 1x NEBuffer 4 (BioLabs) and 10 units of NcoI, incubation 2 hours 37 °C. The 1A>T mutation generates a NcoI restriction size cutting the 539 bp sized exon 1 in two fragments of 317 and 222 bases respectively. Fragments were analyzed on a 2% agarose gel.
Case reports
Pedigrees are shown in Figure 1. Family A and B have previously been described as respectively family A and B by Mancini et al (2004)\textsuperscript{13}.

**Family A:** patient A-IV-1 showed at the age of 1.5 years a left-sided hemiparesis and an OFC at –1SD. A brain MRI at that age showed a 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 at proton density imaging, suggesting gliosis (see figure 3 in ref. 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, at the age of 5 months neglect for the right arm was noted. 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. A 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 ref. 13).

A brain MRI in Patient A-IV-2 was performed at the age of 4 years because of recurrent attacks of vertigo and 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. Her brain MRIs showed in the periventricular white matter bilaterally scattered areas of high signal intensity at T2 weighted images but no porencephaly (see figure 5 in ref. 13).

Both patients A-II-3 and A-II-4 are known with congenital hemiplegia. Patient A-II-3 suffered from a stroke at the age 49 yr. From medical records a cystic dilatation of the ventricle was present on CT (scan not revised by the authors). Patient A-II-4 suffers from migraine attacks. A brain CT-scan of patient A-II-4 at the age of 40 years showed a diffuse dilatation of the right lateral ventricle, mostly abnormal in the parietal and occipital areas (see figure 6 in ref 13).

**Family B:** patient B-III-1 was examined at the age of 6 yr because of unexplained mild left-sided hemiparesis and a moderate psychomotor retardation. The pregnancy and birth were uneventful. A brain MRI at the age of 8 yr showed a thin corpus callosum, a 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 ref. 13).

Patient B-III-2 was examined at the age of 2 yr. because of left-sided hemiplegia. At the age of 4 yr she developed focal epilepsy. A brain CT-scan at that age showed a cystic dilatation of the frontal horn and the medial part of the right lateral ventricle (data not shown). She also presents a mild mental handicap.
Neurological examination of patient B-II-4 at the age of 38 yr revealed a mild pyramidal syndrome and Babinski sign at the right lower limb. His brain MRI at the same age revealed a cystic cavity adjacent to the frontal horn of the left lateral ventricle, communicating with the ventricle and contralateral white matter hyperintensity in frontal areas, apparently localized to the frontal watershed area (see figure 8 in ref. 13). His father and his half-sister (patient B-II-1) were known to limp.

Patient B-II-1: her neurological examination at the age of 50 yr revealed left-sided pyramidal signs, while the brain MRI showed bilateral patchy areas of abnormal signal intensity of the cerebral white matter on T2 weighted images, but no porencephalic cyst (see ref. 13, figure 9). Patient B-II-2 has severe 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** consists of a 2 years old boy (C-III-1) and his mother both with congenital right hemiplegia and normal cognition. The pregnancy was uneventful but the delivery was characterized by a protracted duration of expulsion and normal Apgars. His mother (C-II-2) also presented with right-sided hemiplegia and right hand dystonia and atrophy of the right leg from infancy. Mother’s mother is known to have a “thin leg” but has not been investigated. Brain MRI of the boy shows dilatation of the left ventricle in the frontoparietal area without cortex or basal ganglia abnormality. MRI of his mother shows left ventricle dilatation and atrophy of the left thalamus (Figure 2). Coagulation tests, DNA analysis of factor II and factor V Leiden are normal in both mother and son.

**Results**

Clinical and molecular data are summarized in Table 1. Genomic sequencing of COL4A1 revealed the base changes detailed in Table 2. 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. 1 A). Person A-II-3 agreed to share his medical records but refused DNA test. Also person A-II-2, considered an obligate carrier, 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 COL4A1 (Figure 3 A) resulting in no protein or to a translation initiation site moving up- 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 (Figure 3 B), leading to a p. G1130D, which co-segregates with the disease. This mutation is in one of the Gly-X-Y repeats of COL4A1 and was not present in more than 300 control chromosomes and is conserved in 7 different species from primates through nematodes (Figure 4 A). Compared to glycine, the replacing aspartate is a larger amino acid introducing a negatively charged side-chain which probably interferes with triple helix assembly.

In family C we observed a missense c.4267G>C in exon 48 leading to a p. G1423R substitution in both 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. The mutation also disrupts a highly conserved glycine in a Gly-X-Y repeat (Figure 4 B) introducing a large positively charged amino acid. Previously a Gly>Ser and a Gly>Arg changes in Gly-X-Y repeats have been associated to familial porencephaly16. Therefore we conclude that these mutations at Gly-X-Y sites are likely pathogenic.

**Discussion**

Here we report three new mutations in 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 hemorrhage and porencephaly shows an in frame deletion of exon 40 of Col4a1. Mutations in conserved Gly-X-Y repeats of COL4A1 were subsequently found in two other families with autosomal dominant porencephaly16. Immunohistochemistry and electronmicroscopy of Col4a1 mutants indicate impaired secretion of both COL4A1 and COL4A2. Similarly, a mutation of the C.elegans ortholog of Col4a1 (let-2) results in impaired secretion and intracellular accumulation of both let-2 and the Col4a2 ortholog emb-9 24. Mice homozygous for Col4a1Δex40 16 or a null allele20 are not viable and heterozygous null mice have no apparent phenotype20. However 50% of heterozygous Col4a1+/Δex40 mice die following parturition and 18% of the survivors show obvious porencephalic lesions. This suggests that synthesis of a mutant protein in Col4a1+/Δex40 mice has a negative effect on survival. Experimentally, a negative effect of Col4a1+/Δex40 mutation was demonstrated on collagen IV triple helix assembly and secretion 16. Mutations in highly conserved Gly-X-Y domains have been demonstrated in several collagen proteins, leading to a dominant negative effect25,26. Based on these observations, synthesis of an abnormal protein can be predicted in our family 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 -3 position, which is highly conserved with respect to translation initiation sites and suggests that this start site might be used 27. Initiation of translation at this second site would result in synthesis of a protein with 64 amino acid N-terminal truncation but with an intact NC1 domain and collagenous domain. We predict that the NC1 domain of the mutant trimer is able to initiate assembly of heterotrimers but that the heterotrimers would be structurally or functionally abnormal because of the N-terminal truncation. More insight in the effect of this mutation will be found by 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 from our group of the mutant protein also aim at confirming this assumption.

The gliotic lesions in our families indicate that the time of onset of porencephaly is related to hypoxic-ischemic events occurring in a late stage of pregnancy (after the 20th
week) \(^{13} \). This is compatible with the specific function of COL4A1 in the formation of \(\alpha_1\alpha_1\alpha_2.(IV)\) protomer, which is expressed in early embryonic development (from 32-64 cell stage of mouse embryo) but is not essential for BM deposition. Its essential function is played instead in the maintenance of structural integrity of BMs at later stages, i.e. later fetal life\(^{20}\). Its total ablation leads to embryonic lethality only at E10.5-11.5 because of impaired BM stability \(^{20}\). In this respect, localization of the white matter lesions in areas draining from the vena terminalis in our family A and C and in the watershed area between anterior and medial cerebral artery in patient B-II-4\(^{13}\) (table 1) are compatible with abnormalities of the vascular BM as a consequence of \(COL4A1\) mutations.

Our findings also confirm that in asymptomatic carriers white matter lesions at the MRI can be considered expression (and risk factors?) of \(COL4A1\) mutation. A history of strokes at older age in the patients A-II-3 and B-II-2 could not be related to \(COL4A1\) abnormalities since these patients refused DNA tests. However, recurrent strokes in \(COL4A1\)-related porencephaly have also been observed by Gould et al\(^{16}\) and additional patients need to be tested to prove a relationship. Further evidence is needed to understand whether other neurological complaints in our families like the carotid artery aneurysm of patient A-II-2 \(^{13}\), the recurrent migraine in family A and mental retardation in family B are also related to \(COL4A1\) mutations.

Porencephaly in our families occurs in areas where also traumatic perinatal arterial bleeding (water-shed areas) \(^{14,15}\) or venous thrombotic events occur \(^{13}\) and white matter lesions are seen in asymptomatic carriers of \(COL4A1\) mutations. This suggests that trauma\(^{28,29}\) and thrombophilia\(^{14,30}\) could represent factors influencing the occurrence of cerebral bleeding in \(COL4A1\) mutants. In this case genetic test for \(COL4A1\) mutations in families at risk could add to counseling or additional perinatal care to avoid traumatic delivery.

**Acknowledgement**

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

**Competing interest statement**: the authors declare no competing interests.
References


**Legends to figures**

**Figure 1:**
Pedigrees of families A, B and C with respective mutations in *COL4A1*. Dark symbols indicate individuals with neurological impairment and radiological evidence of porencephaly on MRI or CT-scans. Individual A-II-2 is indicated with a dot as she is obligate carrier and shows white matter lesions at the MRI (see also ref.13, figure 5 for MRI pictures). Similarly, person B-II-1 has pyramidal signs and white matter changes but no porencephaly at MRI. She denied DNA tests. For further explanation see case reports.

**Figure 2:**
Brain MRI of patient C-II-2 and C-III-1.

a) T2 weighted TSE axial image of patient C-II-2 at the age of 25 years, showing a porencephalic enlargement of the left ventricle, ipsilateral atrophy of nucleus caudatus and thalamus and subtle bilateral occipital and right subcortical frontal white matter hyperintensity. b) T2 weighted TSE axial image of patient C-III-1 at the age of 1 year, showing porencephalic enlargement of the left ventricle in the frontal area. Bilateral occipital white matter signal intensity is interpreted as the normal “end-zone” of myelination, but patches of white matter hyperintensity are also visible arount the right frontal horn.

**Figure 3:**
Sequence electropherograms of mutations found in *COL4A1*. Unaffected and affected DNA and amino acid sequences are shown. From top to bottom: Family A, the heterozygous A to T transversion (W) at position 1 (1 A>T) resulting in no protein or to a translation initiation site moving up- or downstream; the absence of translation into an aminoacid is indicated by an asterisk ; Family B, the heterozygous G to A base change (R) at position 3389 (3389 G>A) resulting in a substitution of a glycine by aspartic acid arginine at codon 1130 (G1130D); Family C, the heterozygous G to C base change (S) leading to a substitution of a glycine by arginine at codon 1423 (G1423R).

**Figure 4:**
Sequence alignment of *COL4A1* orthologs in 7 different species showing conservation of the Gly residues in position 1130 of exon 39 (mutated in family B to an aspartate residue) and in position 1423 of exon 42 (mutated into an arginine residue in family C).
Table 1

Clinical and neuroimaging findings in the porencephaly families (modified from ref. 13)

<table>
<thead>
<tr>
<th>Patients</th>
<th>Laterality of cyst</th>
<th>Localisation</th>
<th>White-matter T2 hyperintensity</th>
<th>Signs and symptoms</th>
<th>Vascular area</th>
<th>COL4A1 mutation</th>
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<tbody>
<tr>
<td>A-IV-1</td>
<td>Unilateral</td>
<td>Right parieto-occipital</td>
<td>Bilateral diffuse, gliosis</td>
<td>Left hemiparesis</td>
<td>Vena terminalis</td>
<td>+</td>
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<tr>
<td>A-III-2</td>
<td>Unilateral</td>
<td>Left frontotemporal</td>
<td>Bilateral diffuse</td>
<td>Right hemiparesis</td>
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<tr>
<td>A-II-4</td>
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<td>Right frontoparietal and occipital</td>
<td>not visible on CT</td>
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<td>Vena terminalis</td>
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<tr>
<td>A-II-2</td>
<td>No cyst</td>
<td>-</td>
<td>Bilateral diffuse, frontal to occipital</td>
<td>None (migraine)</td>
<td></td>
<td></td>
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<tr>
<td>B-III-1</td>
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<td>Right frontotemporal</td>
<td>Left frontotemporal</td>
<td>Left hemiparesis, mental retardation</td>
<td>Vena terminalis</td>
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<td>B-II-4</td>
<td>Unilateral</td>
<td>Left frontal</td>
<td>Right frontal, Centrum semiovale (multiple foci)</td>
<td>Right-side pyramidal signs</td>
<td>Right frontal watershed area</td>
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<tr>
<td>B-III-2</td>
<td>Unilateral</td>
<td>Right frontotemporal</td>
<td>not visible on CT</td>
<td>Left hemiparesis, Focal epilepsy</td>
<td></td>
<td>+</td>
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<tr>
<td>B-II-1</td>
<td>No cyst</td>
<td>-</td>
<td>Bilateral diffuse</td>
<td>Limp; left hyperreflexia</td>
<td></td>
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<tr>
<td>C-II-2</td>
<td>Unilateral</td>
<td>Left frontal</td>
<td>Bilateral occipital, right frontal subcortical</td>
<td>Right hemiplegia and hand dystonia</td>
<td>Vena terminalis and branches of the vena cerebri interna</td>
<td>+</td>
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**Table 2: COL4A1 mutations found in this study**

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<thead>
<tr>
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<td>1</td>
<td>1A&gt;T</td>
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<td>B</td>
<td>39</td>
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<tr>
<td>C</td>
<td>48</td>
<td>4267G&gt;C</td>
<td>GGT-&gt;CGT</td>
<td>G1423R</td>
</tr>
</tbody>
</table>
Figure 4: Alignment of COL4A1 protein homologues at position of missense mutations found in this study

G1130

Homo_sapiens  KGLPGLDGIPvGKGEAGLPG
Pan_troglodytes  KGLPGLDGIPvGKGEAGLPG
Mus_musculus  KGLPGLDGIPvGKGEAGLPG
Canis_familiaris  KGLPGLDGIPvGKGEAGLPG
Gallus_gallus  KGLPGLDGIPvGKGEAGLPG
Tetraodon_nigroviridis  DGIEGPAGPGVQPKGLPG
Caenorhabditis_elegans  KGLPGLDGIPvGKGEAGLPG

G1423

Homo_sapiens  GPTGPRGFPPPGPDGLPG
Pan_troglodytes  GPTGPRGFPPPGPDGLPG
Mus_musculus  GPPGPRGFPPPGPDGLPG
Canis_familiaris  GPPGPRGFPPPGPDGLPG
Gallus_gallus  GPPGPRGFPPPGPDGLPG
Tetraodon_nigroviridis  GPPGPRGFPPPGPDGLPG
Caenorhabditis_elegans  GPPGPRGFPPPGPDGLPG
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