

# Phenotypic Spectrum of *COL4A1* Mutations: Porencephaly to Schizencephaly

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**Objective:** Recently, *COL4A1* mutations have been reported in porencephaly and other cerebral vascular diseases, often associated with ocular, renal, and muscular features. In this study, we aimed to clarify the phenotypic spectrum and incidence of *COL4A1* mutations.

**Methods:** We screened for *COL4A1* mutations in 61 patients with porencephaly and 10 patients with schizencephaly, which may be similarly caused by disturbed vascular supply leading to cerebral degeneration, but can be distinguished depending on time of insult.

**Results:** *COL4A1* mutations were identified in 15 patients (21%, 10 mutations in porencephaly and 5 mutations in schizencephaly), who showed a variety of associated findings, including intracranial calcification, focal cortical dysplasia, pontocerebellar atrophy, ocular abnormalities, myopathy, elevated serum creatine kinase levels, and hemolytic anemia. Mutations include 10 missense, a nonsense, a frameshift, and 3 splice site mutations. Five mutations were confirmed as de novo events. One mutation was cosegregated with familial porencephaly, and 2

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mutations were inherited from asymptomatic parents. Aberrant splicing was demonstrated by reverse transcriptase polymerase chain reaction analyses in 2 patients with splice site mutations.

**Interpretation:** Our study first confirmed that *COL4A1* mutations are associated with schizencephaly and hemolytic anemia. Based on the finding that *COL4A1* mutations were frequent in patients with porencephaly and schizencephaly, genetic testing for *COL4A1* should be considered for children with these conditions.

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Type IV collagens are basement membrane proteins that are expressed in all tissues, including the vasculature. *COL4A1* ( $\alpha 1$  chain) and *COL4A2* ( $\alpha 2$  chain) are the most abundant type IV collagens, and form heterotrimers with a 2:1 stoichiometry ( $\alpha 1\alpha 1\alpha 2$ ).<sup>1</sup> Mutations in *COL4A1* and *COL4A2* cause sporadic and hereditary porencephaly, a neurological disorder characterized by fluid-filled cysts in the brain that often cause hemiplegia or tetraplegia.<sup>2–4</sup> In addition, a variety of clinical phenotypes, including small vessel disease affecting the brain, eyes, and kidneys, are associated with *COL4A1* abnormality<sup>5,6</sup>; neonatal porencephaly and adult stroke,<sup>7</sup> sporadic extensive bilateral porencephaly resembling hydranencephaly,<sup>8</sup> periventricular leukomalacia with intracranial calcification,<sup>9</sup> HANAC (hereditary angiopathy with nephropathy, aneurysm, and muscle cramps) syndrome,<sup>10,11</sup> Axenfeld–Rieger anomaly with leukoencephalopathy, and adult stroke and intracerebral hemorrhage.<sup>12–14</sup> Notably, *COL4A1* mutations were present in 2 patients with muscle–eye–brain/Walker–Warburg syndrome (MEB/WWS), which is characterized by ocular dysgenesis, neuronal migration defects, and congenital muscular dystrophy, suggesting that *COL4A1* is also involved in normal cortical and muscular development in humans.<sup>15</sup> Consistent with this hypothesis, a mouse model of a heterozygous *COL4A1* mutation (*Col4a1*<sup>+/- $\Delta$ ex40</sup>) showed ocular dysgenesis, cortical neuronal localization defects, and myopathy, along with cerebral hemorrhage and porencephaly.<sup>2,15</sup> The phenotypic spectrum of *COL4A1* mutations is expanding; however, the whole spectrum of systemic phenotypes and the incidence of *COL4A1* mutations associated with porencephaly has not been systemically examined.

In this study, we screened for *COL4A1* mutations in 61 patients with porencephaly and 10 patients with schizencephaly, which may be similarly caused by disturbed vascular supply leading to cerebral degeneration, but can be distinguished depending on time of insult.<sup>2–4,16,17</sup> *COL4A1* mutations were identified in 10 patients with porencephaly and 5 patients with schizencephaly, who showed a variety of associated findings, including intracranial calcification, focal cortical dysplasia (FCD), ocular abnormalities, pontocerebellar atrophy, myopathy, elevated serum creatine kinase levels, and hemolytic anemia. Our study demonstrated the importance of genetic testing for *COL4A1* in children with porencephaly or schizencephaly.

## Patients and Methods

### Patients

A total of 61 patients with porencephaly including a previous cohort with porencephaly,<sup>4</sup> and 10 patients with schizencephaly including a patient who also had porencephaly were analyzed for *COL4A1* mutations. Schizencephaly is defined as transmantle clefts bordered by polymicrogyria in adjacent cortex.<sup>18</sup> The clefts extended through the entire hemisphere, from the ependymal lining of the lateral ventricles to the pial covering of the cortex.<sup>19</sup> The clefts are further divided into those with closed lips and those with open lips. In the clefts with closed lips, the walls affix each other directly, obliterating the cerebrospinal fluid space within the cleft at that point.<sup>20</sup> *COL4A2* mutations were negative for these patients. Genomic DNA was isolated from blood leukocytes according to standard methods, and amplified using an illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK). The DNA of familial members of patient 6 was isolated from saliva samples using Oragene (DNA Genotek, Kanata, Ontario, Canada). Experimental protocols were approved by the committee for ethical issues at Yokohama City University School of Medicine. All patients were investigated in agreement with the requirements of Japanese regulations.

### Mutation Analysis

Exons 1 to 52, covering the entire *COL4A1* coding region, were examined by high-resolution melting (HRM) curve analysis. Samples showing an aberrant melting curve pattern in the HRM analysis were sequenced. Polymerase chain reaction (PCR) primers and conditions are shown in Supplementary Table S1. All novel mutations were verified using original genomic DNA, and screened in 200 Japanese normal controls by HRM analysis. For the family showing de novo mutations, parentage was confirmed by microsatellite analysis, as previously described.<sup>21</sup> Biological parents were confirmed if >4 informative markers were compatible and other markers showed no discrepancy.

### Reverse Transcriptase-PCR

Reverse transcriptase (RT)-PCR using total RNA extracted from lymphoblastoid cell lines (LCL) was performed essentially as previously described.<sup>22</sup> Briefly, total RNA was extracted using RNeasy Plus MiniKit (Qiagen, Tokyo, Japan) from LCL with or without 30  $\mu$ M cycloheximide (CHX; Sigma, Tokyo, Japan) incubation for 4 hours. Four micrograms total RNA was subjected to reverse transcription, and 2  $\mu$ l cDNA was used for PCR. Primer sequences are ex20-F (5'-CCCCAAAGGTTTCC CAGGACTACCA-3') and ex22-R (5'-GTCCGGGCTGACAT TCCACAATTC-3'; for patient 4); and ex22-F (5'-GAATTC-CAGGGCAGCCAGGATTTAT-3') and ex24-R (5'-CATCTCT GCCAGGCAAACCTCTGT-3'; for patient 7). DNA of each

PCR band was purified by QIAEXII Gel extraction kit (Qiagen; for patient 4) and E.Z.N.A. poly-Gel DNA Extraction kit (Omega Bio-Tek, Norcross, GA; for patient 7), respectively.

## Results

### Mutation and RT-PCR analysis

*COL4A1* abnormalities were identified in 15 patients (Fig 1 and Table). Nine mutations occurred at highly conserved Gly residues in the Gly-X-Y repeat of the collagen triple helical domain. Interestingly, a missense mutation (c.4843G>A [p.Glu1615Lys]) at an evolutionarily conserved amino acid and a nonsense mutation (c.4887C>A [p.Tyr1629X]) were found in the carboxy-terminal noncollagenous (NC1) domain. The other 4 mutations include a frameshift mutation (c.2931dupT [p.Gly978TrpfsX15]) and 3 splice site mutations (c.1121-2dupA, c.1382-1G>C, and c.1990+1G>A). None of these mutations was present in 200 Japanese normal controls, and Web-based prediction tools suggested that these mutations are pathogenic (Supplementary Table S2). The c.2842G>A (patient 1), c.3976G>A (patient 2), c.4887C>A (patient 8), c.2689G>A (patient 13), and c.1990+1G>A (patient 14) mutations occurred de novo. The c.3995G>A mutation (patient 3) was not found in the mother's DNA (the father's DNA was unavailable). The c.1121-2dupA (patient 4) and c.2931dupT (patient 6) mutations were found in the asymptomatic fathers. c.1963G>A (patient 10) was found in familial members affected with porencephaly as well as asymptomatic carriers, suggesting incomplete penetrance of the mutation (Supplementary Fig S1). The remaining patients' parental DNA was unavailable.

To examine the mutational effects of the 2 splice acceptor site mutations (c.1121-2dupA and c.1382-1G>C), RT-PCR and sequencing were performed (see Fig 1). c.1121-2dupA caused the deletion of exon 21 from the wild-type *COL4A1* mRNA, resulting in an in-frame 55-amino acid deletion (p.Gly374\_Asn429delinsAsp). The effect of c.1382-1G>C was more complicated. There were 3 PCR products amplified from LCL treated with CHX, which inhibits nonsense-mediated mRNA decay (NMD). The middle band corresponded to the wild-type allele. The sequence of the lower mutant band showed a 33bp insertion of intron 22 and an 84bp deletion of all of exon 23 from the use of cryptic splice acceptor and donor sites within intron 22. The change of amino acid sequence from this mutant transcript was a deletion of 29 amino acids and an insertion of 12 amino acids (p.Gly461\_Gly489delinsValHisCysGlyAsp-PheTrpSerHisValThrArg). The upper band was only observed in CHX-treated LCL, but was not evident in

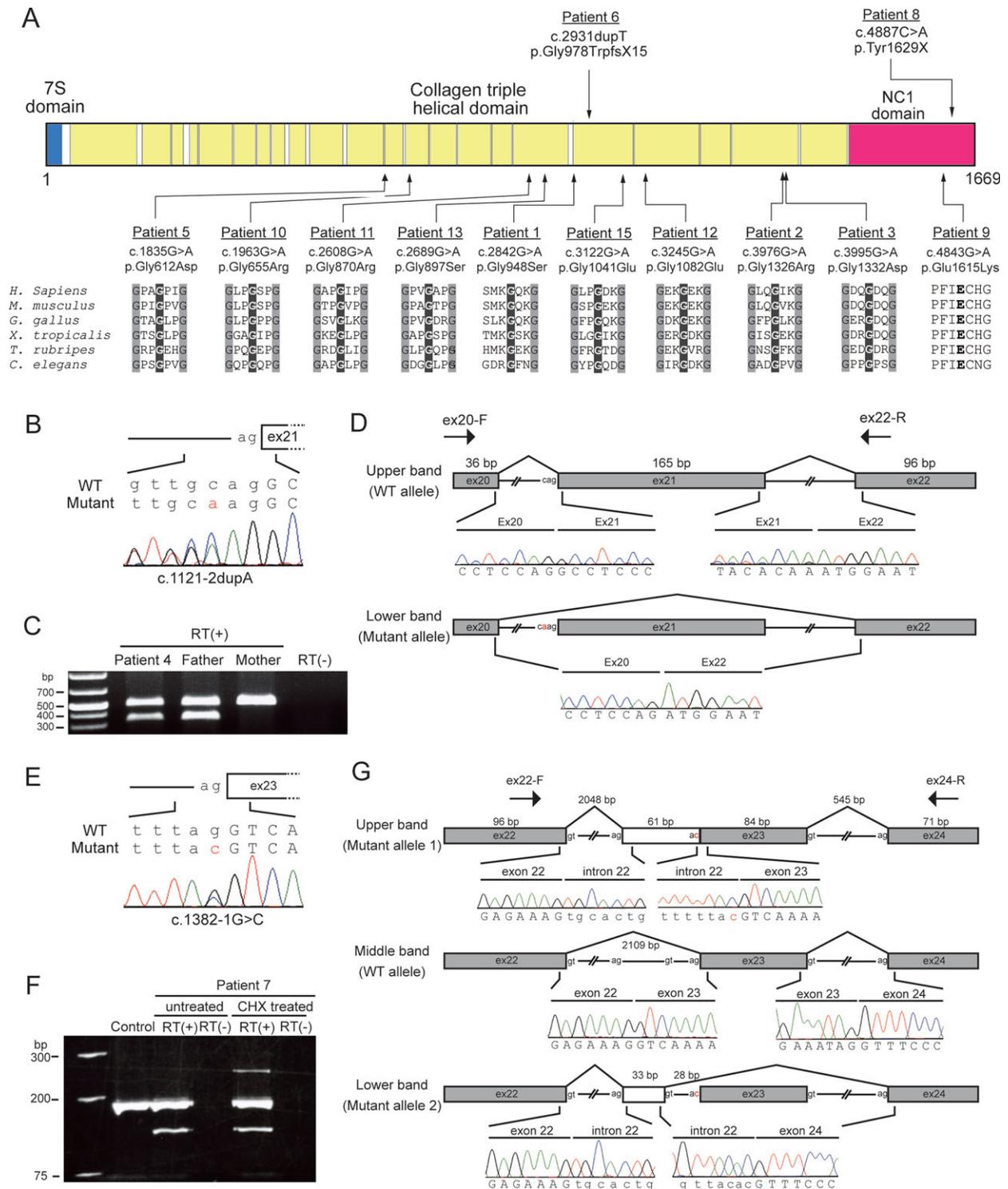
the untreated LCL, suggesting that this mutant transcript may undergo NMD. Sequencing of the upper band showed a 61bp insertion of intron 22 from the use of a cryptic splice acceptor site within intron 22, as mentioned above. The product of this mutant transcript leads to a frameshift, creating a premature stop codon (p.Gly461ValfsX31), which is consistent with degradation of the mutant transcript by NMD.

### Clinical Features

The clinical information for individuals with *COL4A1* mutations is summarized in the Table, and their representative brain images are shown in Figure 2 and Supplementary Fig S2. *COL4A1* mutations were identified in 10 of 61 patients with porencephaly (16.4%). Of note, *COL4A1* mutations were identified in 5 of 10 patients with schizencephaly (50.0%), revealing a novel association between *COL4A1* mutations and schizencephaly. Thirteen patients were born at term, and 2 patients (patients 1 and 12) were born at preterm. Their body weight was normal at birth except for 5 patients (patients 3, 4, 9, 12, and 15) who were below  $-2.0$  standard deviations. The occipitofrontal circumference was available in 12 patients, and 6 patients (patients 2, 3, 6, 13, 14, and 15) were below  $-2.0$  standard deviations. Two patients (patients 11 and 12) were confirmed to have an antenatal hemorrhage as previously reported.<sup>23,24</sup> Among associated findings with *COL4A1* mutations, a patient showed FCD that was histologically demonstrated (Fig 3A–F). In addition, hemolytic anemia was found in 5 of 15 patients, suggesting that hemolytic anemia may be a novel feature associated with *COL4A1* mutation. Pontocerebellar atrophy along with severe bilateral porencephaly was observed in 2 patients, and a patient showed cerebellar hypoplasia. Previously reported magnetic resonance imaging and systemic findings associated with *COL4A1* mutations were also observed, including intracranial calcification (7 of 15), myopathy (1 of 15; see Fig 3G, H), ocular abnormalities (4 of 15), and elevated serum creatine kinase levels (6 of 15), confirming that these features are useful signs for *COL4A1* testing. Case reports are available in the Supplementary Data.

### Discussion

We found a total of 15 novel mutations in this study. Nine mutations occurred at highly conserved Gly residues in the Gly-X-Y repeat of the collagen triple helical domain, suggesting that these mutations may alter the collagen IV  $\alpha 1\alpha 1\alpha 2$  heterotrimers.<sup>1,25</sup> We reported for the first time 2 mutations (a nonsense and a missense change) in the NC1 domain. The nonsense mutation



**FIGURE 1: COL4A1 mutations in patients with porencephaly or schizencephaly. (A)** Functional domains of COL4A1 protein. The locations of 12 mutations, including 10 missense mutations (bottom), a nonsense mutation, and a frameshift mutation (top) are indicated by arrows. The 7S domain is highlighted with blue and the NC1 domain with red. Gly-X-Y repeats within the collagen triple helical domain are highlighted with yellow. All of the missense mutations occurred at evolutionary conserved amino acids. The positions of the conserved Gly residues in the Gly-X-Y repeats are highlighted in gray. Homologous sequences were aligned using CLUSTALW (<http://www.genome.jp/tools/clustalw/>). **(B)** The c.1121-2dupA mutation in intron 20 is colored red. Sequences of exons and introns are presented in upper and lower cases, respectively. **(C)** Reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis of patient 4 and his parents. **(D)** Schematic presentation of the wild-type (WT; upper) and mutant (lower) transcripts and primers used for analysis. A single band (500bp), corresponding to the WT allele, was amplified using the mother's cDNA template. Conversely, a lower band was detected from the cDNA from the patient and his father. In the mutant transcript, the 165bp exon 21 was deleted. Sequences of exons and introns are presented in upper and lower cases, respectively. **(E)** The c.1382-1G>C mutation in intron 22 is colored red. **(F)** RT-PCR analysis of patient 7 and a control. **(G)** Schematic representation of the WT and mutant transcripts, and primers used for analysis. A single band (183bp), corresponding to the WT allele, was amplified using a control cDNA template. Conversely, upper and lower bands were detected from the patient's cDNA. The upper band (244bp), which was observed only in cycloheximide (CHX)-treated cells, had a 61bp insertion of intron 22 sequences, leading to a frameshift. Absence of the upper band in untreated lymphoblastoid cell lines strongly suggests that the mutant transcript may undergo nonsense-mediated mRNA decay. The lower band had a 33bp insertion of intron 22 and 84bp deletion of the whole of exon 23, leading to an in-frame 51bp deletion.

TABLE: Clinical features of patients with COL4A1 mutations

| Cases | Age | Sex | Mutation                         | Inheritance         | Brain MRI/<br>CT findings   | CP | Epi | Ocular<br>features                | Family<br>history | ID | Hyper-CK | Other    |
|-------|-----|-----|----------------------------------|---------------------|---|----|-----|-----------------------------------|-------------------|----|----------|----------|
| 1     | 14y | M   | c.2842G>A<br>(p.Gly948Ser)       | de novo             | Bilateral POCE,<br>calcification,<br>hemosiderin<br>deposition  | Q  | +   | -                                 | -                 | +  | -        |          |
| 2     | 18m | M   | c.3976G>A<br>(p.Gly1326Arg)      | de novo             | Bilateral SCZ,<br>calcification,<br>hemosiderin<br>deposition   | Q  | +   | -                                 | -                 | +  | -        |          |
| 3     | 15m | M   | c.3995G>A<br>(p.Gly1332Asp)      | Absent in<br>mother | Unilateral SCZ,<br>calcification,<br>hemosiderin<br>deposition  | H  | +   | -                                 | -                 | +  | -        |          |
| 4     | 6y  | M   | c.1121-2dupA <sup>1)</sup>       | Paternal            | Unilateral POCE   | H  | +   | -                                 | -                 | +  | -        | FCD      |
| 5     | 2m  | F   | c.1835G>A<br>(p.Gly612Asp)       | ND                  | Bilateral SCZ,<br>calcification,<br>thin CC, thin<br>brain stem,<br>cerebellar atrophy,<br>absence of SP,<br>hemosiderin<br>deposition,<br>multicystic<br>encephalomalacia, | Q  | +   | Optic<br>nerve<br>hypoplasia      | -                 | +  | +        | HA       |
| 6     | 7y  | M   | c.2931dupT<br>(p.Gly978TrpfsX15) | Paternal            | Unilateral POCE   | H  | +   | -                                 | -                 | +  | +        |          |
| 7     | 12y | F   | c.1382-1G>C <sup>2)</sup>        | ND                  | Unilateral POCE   | H  | +   | -                                 | -                 | +  | +        | Myopathy |
| 8     | 10y | M   | c.4887C>A<br>(p.Tyr1629X)        | de novo             | Unilateral POCE   | H  | +   | -                                 | Hematuria         | +  | -        |          |
| 9     | 3m  | F   | c.4843G>A<br>(p.Glu1615Lys)      | ND                  | Bilateral POCE,<br>calcification,<br>hypoplastic CC,<br>hemosiderin<br>deposition, thin   | Q  | +   | Microphthalmia<br>Corneal opacity | -                 | +  | -        | VSD, HA  |

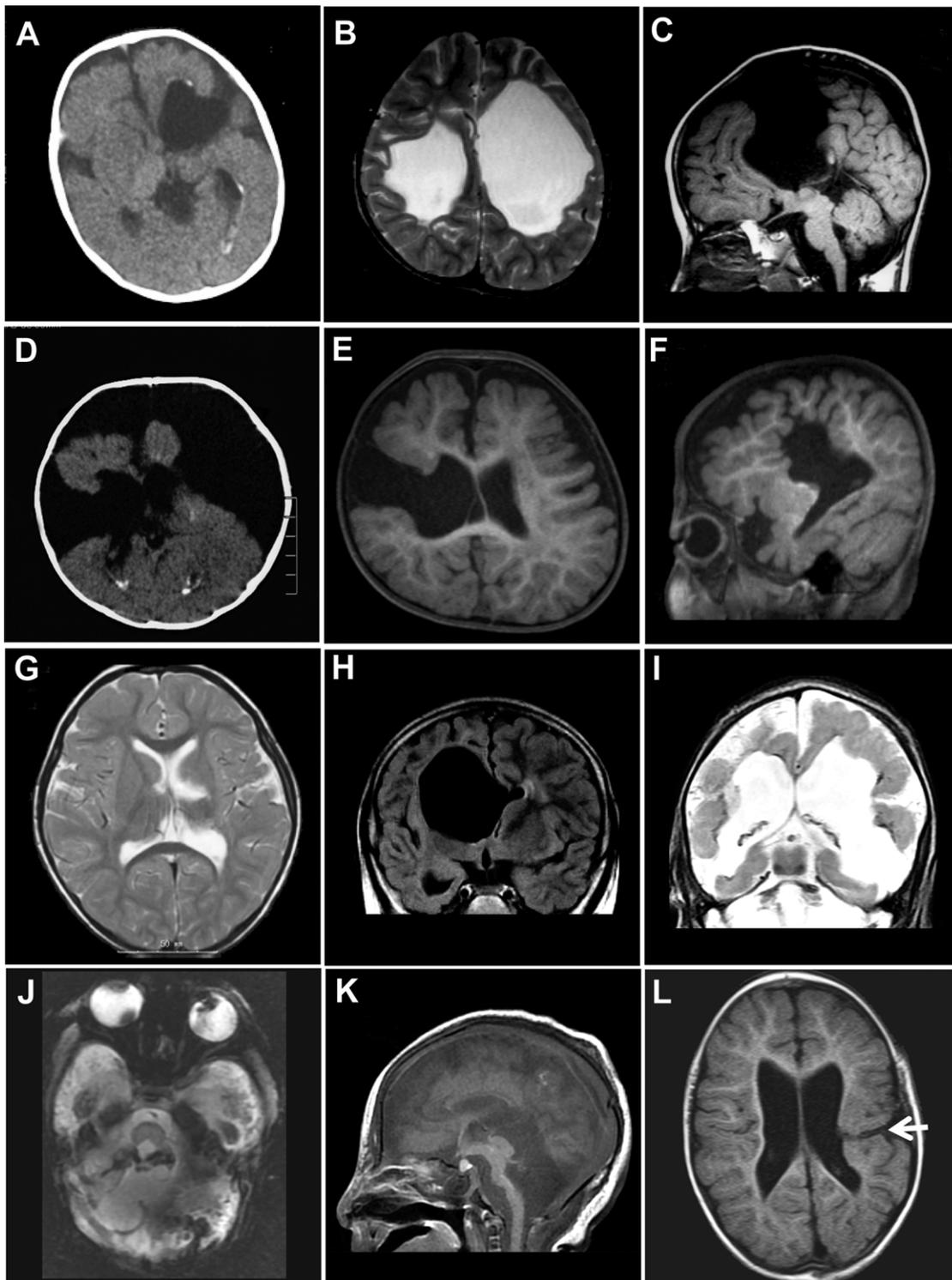
TABLE (Continued)

| Cases | Age  | Sex | Mutation                    | Inheritance            | Brain MRI/<br>CT findings  | CP | Epi | Ocular<br>features  | Family<br>history | ID | Hyper-CK | Other         |
|-------|------|-----|-----------------------------|------------------------|--|----|-----|---------------------|-------------------|----|----------|---------------|
|       |      |     |                             |                        | brain stem, cerebellar atrophy, multicystic encephalomalacia             |    |     |                     |                   |    |          |               |
| 10    | 2y7m | F   | c.1963G>A<br>(p.Gly655Arg)  | Paternal <sup>3)</sup> | Bilateral POCE   | Q  | +   | -                   | POCE, Epi         | +  | -        | -             |
| 11    | 1y   | F   | c.2608G>A<br>(p.Gly870Arg)  | ND                     | Unilateral POCE, calcification   | Q  | +   | Congenital cataract | -                 | +  | -        | -             |
| 12    | 1y5m | M   | c.3245G>A<br>(p.Gly1082Glu) | ND                     | Unilateral SCZ with bilateral POCE, calcification, cerebellar hypoplasia | T  | +   | Congenital cataract | -                 | +  | -        | HA, Hematuria |
| 13    | 3y7m | M   | c.2689G>A<br>(p.Gly897Ser)  | de novo                | Unilateral POCE  | Q  | +   | -                   | -                 | +  | +        | -             |
| 14    | 9m   | F   | c.1990+1G>A                 | de novo                | Unilateral POCE, hemosiderin deposition                                  | Q  | +   | -                   | -                 | +  | +        | HA, Hematuria |
| 15    | 2y   | F   | c.3122G>A<br>(p.Gly1041Glu) | ND                     | Unilateral SCZ, hemosiderin deposition                                   | Q  | +   | -                   | -                 | +  | +        | HA            |

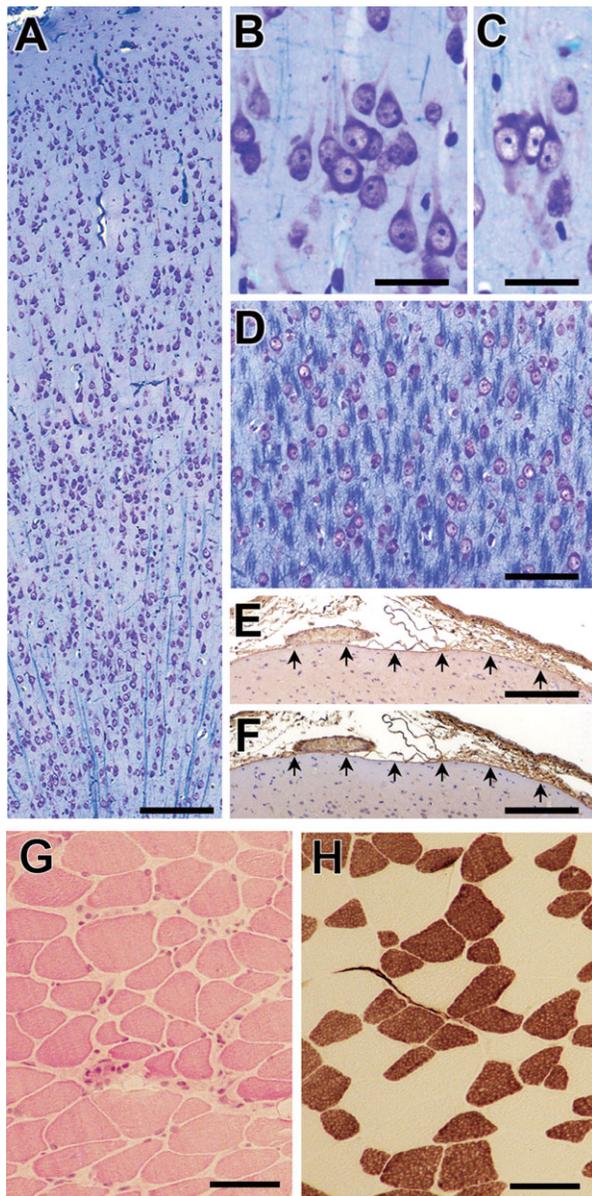
1) p.Gly374\_Asn429delinsAsp change was predicted by mRNA analysis

2) Two alternative protein changes were predicted by mRNA analysis: p.Gly461\_Gly489delinsValHisCysGlyAspPheTrpSerHisValThrArg and p.Gly461ValfsX31. y, years; m, months; M, male; F, female; ND, Not determined; POCE, porencephaly; SCZ, schizencephaly; CC, corpus callosum; SP, septum pellucidum; CP, cerebral palsy; H, hemiplegia; T, Triplegia; Q, quadriplegia; Epi, epilepsy; ID, intellectual disability; CK, creatine kinase; FCD, Focal cortical dysplasia; HA, Hemolytic anemia; VSD, ventricular septal defect

3) Co-segregation of the p.Gly655Arg mutation with porencephaly was confirmed.



**FIGURE 2:** Computed tomography (CT) scan (A, D) and magnetic resonance imaging (MRI; B, C, E–L) of patients with *COL4A1* mutations. (A–C) Images of patient 1. (A) The CT scan shows calcification along with the dilated lateral ventricular wall. (B) T2-weighted and (C) T1-weighted images (WIs) at 5 years of age showing bilateral porencephaly. (D) The CT image of patient 2 with schizencephaly shows calcification of the lateral ventricular wall and brain parenchyma. (E, F) T1-WIs of patient 3 show unilateral schizencephaly at 15 months of age. (G) T2-WI of patient 4 at 3 years of age shows parenchymal defect of the left thalamus and basal ganglia due to subependymal hemorrhage. (H) Fluid-attenuated inversion recovery image of patient 7 at 6 years of age showing unilateral porencephaly. (I) T2-WI, (J) T2\*-weighted gradient-echo image (WGRE), and (K) T1-WI of patient 9. (I) The MRI at 2 months of age shows bilateral porencephaly with low-intensity lesions along with a deformed ventricular wall, which has hemosiderin deposition and calcification. (J) T2\*-WGRE showing hemosiderin deposition in the atrophic cerebellum. The atrophic pontocerebellar structures are also shown in (K). (L) T1-WI of patient 15 showed schizencephaly in the left hemisphere at 2 years of age.



**FIGURE 3:** Histopathological features of the resected frontal tissue of patient 4 (A–F) and biopsied rectus abdominis muscle of patient 7 (G, H). (A) Low-magnification view of the cortex showing architectural abnormalities. (B, C) Two examples of neuronal clustering. (D) Many neurons scattered within the subcortical white matter. (E, F) Two serial sections demonstrating the superficial layer of the cortex. Note that the basal lamina of the pia mater (arrows in each panel) is continuously labeled with antibodies against collagen type IV (E) and laminin (F). (A–D) Klüver–Barrera stain. (E, F) Immunostained and then counterstained with hematoxylin. (G) Hematoxylin and eosin staining showing variation in fiber size, slightly increased endomysial connective tissue, and internal nuclei. (H) Adenosine triphosphatase (pH 4.5) staining showing type 2B fiber deficiency. There was no increase in number of type 2C fibers. Scale bars indicate 175 $\mu$ m (A, E, F), 30 $\mu$ m (B, C), 80 $\mu$ m (D), and 30 $\mu$ m (G, H).

would cause a truncation of the NC1 domain rather than mRNA degradation by NMD as the mutation was located within 50bp of the exon–intron boundary of the

second to last exon (exon 51).<sup>26</sup> The NC1 domains are the sites for molecular recognition through which the stoichiometry of chains in the assembly of triple-helical formation is directed<sup>1</sup>; therefore, these 2 mutations may alter the assembly of the collagen IV  $\alpha1\alpha1\alpha2$  heterotrimers. In addition, the effect of 2 splice site mutations was examined using LCL, suggesting that in-frame deletion/insertion mutant protein should be produced. Thus, it is highly likely that impairment of the collagen IV  $\alpha1\alpha1\alpha2$  heterotrimer assembly caused by mutant  $\alpha1$  chain is a common pathological mechanism of *COL4A1* mutations. The c.2931dupT mutation found in patient 6 and his father might cause severe truncation of *COL4A1* protein. It is possible that the truncation of *COL4A1* protein can also impair  $\alpha1\alpha1\alpha2$  heterotrimer assembly similar to substitutions of conserved Gly residues in the Gly-X-Y repeat. Alternatively, the mutant transcript might undergo NMD, and haploinsufficiency of *COL4A1* might cause a weakness of basement membrane. Biological analysis using patients' cells will clarify these possibilities.

*COL4A1* mutations in schizencephaly were first demonstrated in this study. Schizencephaly was used by Yakovlev and Wadsworth in 1946 to describe true clefts formed in the brain as a result of failure of development of the cortical mantle in the zones of cleavage of the primary cerebral fissures.<sup>19</sup> Schizencephaly is differentiated from clefts in the central mantle that arise as the result of a destruction of the cerebral tissues, which they called encephaloclastic porencephalies, now known simply as porencephaly.<sup>19</sup> Schizencephaly has been understood as a neuronal migration disorder, because the clefts are lined by abnormal gray matter, described as polymicrogyria. Conversely, porencephaly is understood to be a postmigration accident resulting in lesions, without gray matter lining the clefts or an associated malformation of cortical development. It has been suggested that both schizencephaly and porencephaly are caused by encephaloclastic regions, and can be distinguished depending on time of insult.<sup>16,17</sup> The present study clearly demonstrated that *COL4A1* mutations caused both porencephaly and schizencephaly, supporting the same pathological mechanism for these 2 conditions.

The genes responsible for FCD have been elusive, despite extensive investigation. The pathological features of the cortical tubers of tuberous sclerosis (TSC) may be indistinguishable from those of FCD. Apart from FCD due to TSC, there is only 1 gene that may explain the genetic basis of FCD, where a homozygous mutation in *CNTNAP2* has been identified in Amish children with FCD, macrocephaly, and intractable seizures.<sup>27</sup> Surprisingly, the present study discovered a patient with FCD

and porencephaly, in whom aberrant splicing was demonstrated and FCD1A was pathologically confirmed using resected brain tissues. A recent report revealed *COL4A1* mutations in 2 patients with MEB/WWS showing cobblestone lissencephaly,<sup>15</sup> and abnormal cortical development has been observed in mouse models of *COL4A1* mutations.<sup>15,28</sup> Thus, it is possible that *COL4A1* mutations are involved in cerebral cortical malformations, including FCD. Identification of a greater number of cases is required to confirm the association between *COL4A1* mutations and cortical malformations in humans.

In a few children, the sequelae were much more severe than would be expected on the basis of their imaging findings. This is of importance when counseling parents with regard to prediction of neurodevelopmental outcome.

Two patients with *COL4A1* mutations showed intracranial calcification, pontocerebellar atrophy, ocular abnormalities, and hemolytic anemia associated with severe bilateral porencephaly (patient 9) or schizencephaly (patient 5). Severe hemorrhagic destructive lesions in the cerebrum were observed in these patients, and T2\* images also showed hemorrhage in the cerebellum, which may have resulted in a thin brainstem and severe cerebellar atrophy. Thus, these 2 patients could be considered as the most severe manifestations affecting the developing brain and eyes. A common feature of the 2 patients is hemolytic anemia of an unknown cause, which required frequent blood transfusions. Five of 15 patients with *COL4A1* mutations showed hemolytic anemia. Interestingly, 2 reports have demonstrated that mouse *Col4a1* mutants showed a significant reduction in red blood cell (RBC) number and hematocrit.<sup>28,29</sup> Given that *Col4a1* mutations lead to hemorrhage, chronic hemorrhage is possibly involved in RBC loss. Alternatively, the *Col4a1* mutation may directly affect blood progenitor cells, as they transmigrate across basement membranes before entering the peripheral blood.<sup>30</sup> Hemolytic anemia in patients with *COL4A1* mutations would imply the latter explanation. Further studies are required to clarify how *COL4A1/Col4a1* mutations are involved in anemia.

In summary, we found 15 mutations in *COL4A1* among 71 patients with porencephaly or schizencephaly, showing an unexpectedly high percentage of mutations (about 21%) in these patients. Fourteen patients with *COL4A1* mutations had no family history of cerebral palsy. The 15 patients with *COL4A1* mutations showed a variety of phenotypes, further expanding the possible clinical spectrum of *COL4A1* mutations to include schizencephaly, FCD, pontocerebellar atrophy, and hemolytic anemia. Genetic testing for *COL4A1* should be

recommended for children with porencephaly and schizencephaly.

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## Authorship

Y.Y. and K.H. contributed equally to this work.

## Potential Conflicts of Interest

Nothing to report.

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