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De novo p.G696S mutation in *COL4A1* causes intracranial calcification and late-onset cerebral hemorrhage: A case report and review of the literature

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1 Title

- 2 *De novo* p.G696S mutation in *COL4A1* causes intracranial calcification and late-onset cerebral
- 3 hemorrhage: A case report and review of the literature

4

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- 1
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- 4
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- 7TORCH, and Carbazochrome

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1 Abstract

 $\mathbf{2}$ Background: The collagen type IV alpha 1 chain (COL4A1) is an essential component of the 3 basement membrane in small vessels. Pathogenic variants in COL4A1 cause perinatal cerebral hemorrhages in an autosomal-dominant fashion. However, little is known about the long-term 4 $\mathbf{5}$ outcomes of patients with mildly affecting COL4A1 mutations. 6 *Case report:* We report a 17-year-old boy, who presented with recurrent intracranial hemorrhages in $\overline{7}$ the periventricular white matter. He had been followed-up as a child with cerebral palsy bearing 8 intracranial calcifications, developmental delay and epilepsy. Screening tests in infancy provided negative results for intrauterine infections. Severe motor and cognitive deficits persisted after 9 10admission. Carbazochrome was introduced on day 19 of admission, which appeared to prevent extension and reactivation of cerebral hemorrhages for over 6 months after discharge. 11 12Results: Targeted sequencing of NOTCH3 and TREX1 excluded causal mutations in these genes. The whole-exome sequencing revealed that he carried a de novo mutation in COL4A1 13(p.Gly696Ser). An overview of the literature for 345 cases with COL4A1 mutations supported 1415evidence that p.Gly696Ser is associated with the unique phenotype of late-onset hemorrhage among patients with COL4A1-associated cerebral angiopathy. 1617Conclusions: This case first demonstrates that infants with COL4A1-associated 18leukoencephalopathy and calcifications have a risk for developing the rupture of small vessels in the cerebral white matter after 10 years of age. 1920

21 (216 words)

22

1 Introduction

 $\mathbf{2}$ COL4A1-associated vasculopathy represents a group of systemic disorders affecting predominantly the small vessels (Gould et al., 2005; Gould et al., 2006). Accumulating 3 evidence shows that mutations in COL4A1 causes a broad spectrum of vascular phenotypes in 4 the brain, retina and other organs (Magnin et al., 2014; Meuwissen et al., 2015; Rannikmae et $\mathbf{5}$ al., 2015; Rannikmae et al., 2017; Weng et al., 2012; Yoneda et al., 2012; Yoneda et al., 2013; 6 $\overline{7}$ Zagaglia et al., 2018). 8 The COL4A1 gene is located at chromosome 13q34 (Cutting et al., 1988). This gene encodes the collagen type IV alpha protein 1, an essential component of the vascular basement 9 10membrane. The COL4A1 protein harbors a highly conserved motif of 'glycine (Gly)-X-Y repeats' in the triple-helix domain, which provides the indispensable structure for the monomer 11 12protein to form heterotrimer complexes of alpha chains (Solomon et al., 1987). The heterotrimers of alpha chains contribute to the development of supramolecular networks of 13collagen type IV in the basement membrane (Gould et al., 2005; Gould et al., 2006; Poschl et 1415al., 2004). Notably, the majority of patients with COL4A1-associated vasculopathy carry mutations that alter the coding sequences of Gly-X-Y repeats (Zagaglia et al., 2018). Impaired 1617assemblies of collagen IV fibers are thus considered to be the common mechanism underlying the vasculopathy (Yoneda et al., 2012; Yoneda et al., 2013). 18

19 Despite an increasing number of cases manifesting variant phenotypes of

20 COL4A-associated vasculopathy, few reports have demonstrated the long-term outcomes of

affected individuals from infancy to the late-teen age. Here we report a case with a *de novo*

22 mutation in *COL4A1*, who experienced late-onset intracranial hemorrhage. This case suggests

that patients with *COL4A1* mutations have a lifelong risk for the development of intracranial

hemorrhages.

25

26 Case report

Kinoshita K. COL4A1-associated vasculopathy

1	A 17-year-old boy was transferred to our department by ambulance, with symptom of
2	acutely worsening left hemiplegia and emotional explosion. He was the second offspring of healthy,
3	non-consanguineous parents. His parents and older sister did not have neuromuscular or genetic
4	diseases. He was born at 39 weeks of gestation without asphyxia. His birth weight was 3,055 g
5	(-0.21 standard deviation, SD), height 48.8 cm (-0.11 SD), and head circumference 32.4 cm (-0.64
6	SD). He received phototherapy twice for neonatal hyper bilirubinemia. On one day of age,
7	chemistry serum revealed that creatine kinase (CK) was elevated to 3,507 IU/L (normal: 59 -248).
8	Because the CK value was normalized in one month, the abnormal data were considered a transient
9	increase after the fetal distress.
10	At one month of age, he had a convulsive seizure lasting 1 min. A head computed
11	tomogram detected the calcifications in the basal ganglia and periventricular white matters (Figure
12	1A, left). Antibodies against toxoplasmosis, rubella, cytomegalovirus, and herpes simplex were
13	negative. Metabolic disorders were considered unlikely from the blood tests for serum calcium,
14	activated vitamin D, amino acids, and urinary organic acid profiles. Funduscopic examination
15	excluded the aberrant vasculature and pigmentation in the retina. Lactate (8.9 mg/dl) and pyruvate
16	(0.67 mg/dl, cut off: 0.9) levels in the spinal fluids were normal at four months of age.
17	During infancy, he sat unaided from 23 months, and spoke meaningful words from 12
18	months of age. He began to show involuntary movements of the limbs and trunk, and to take
19	frequently the dystonic posture from two years of age. Because of the increased frequency and
20	prolonged duration, epileptic seizures were suspected; however, electroencephalogram (EEG) did
21	not show the evidence for the paroxysmal activity. At three years of age, a follow-up magnetic
22	resonance imaging (MRI) detected the high-intensity signals on T2-weighted image in the right
23	dominant bilateral frontal lobe (Figure 1A, right). Biochemical analysis showed no accumulation of
24	very-long-chain fatty acids in leukocytes, excluding the diagnosis of adrenoleukodystrophy.

which carbamazepine was introduced. Serial EEG recordings revealed the epileptiform discharges 26

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that appeared from the right central to parietal regions at this age. The treatment response to carbamazepine was favorable, but he stopped the medication with his decision at 15 years of age. Recurrence of seizure was not observed thereafter. Although his daily activity was limited on wheelchair, he had attended the special support school before the onset of present illness. He had acquired writing skills, and was able to compose sentences at the equivalent level of second grade in elementary school. Because of the motor disability, his intelligence quotient had not been exactly evaluated before.

Five days before admission, his parents noticed that he was unable to move his 8 9 arms and legs smoothly after playing indoor baseball as the extracurricular activities. He 10had no accident of head trauma during the sport activity. These symptoms did not recover and became more evident over the next 4 days. On the day of admission, he began to show 11 12dysarthria, to be easily excited and to show anger with uncontrollable screaming in a short 13temper. These emotional explosions suddenly and recurrently appeared without certain triggers. He was transported to our hospital by ambulance. On admission, his height was 1415156 cm (-2.5 SD) and body weight was 41 kg (-2.3 SD). Neurological examination showed 16the left spastic hemiplegia and left facial paralysis. Cranial nerves were unaffected. Although his consciousness and vital signs remained unaltered, he kept showing the upset 1718attitude and expressing loud vocalization without sound reasons for the next two weeks. The cranial MRI on the first day of admission revealed acute hemorrhagic lesions in the 1920right basal ganglia, which extended to the white matter of radiate crown (Figure 1B). No 21aneurysms were shown in MR angiography. Although his neurological symptoms did not 22progress, serial studies of MRI on the 12th and 19th days of admission showed that the 23hemorrhagic lesions extended to the white matters adjacent to the right lateral ventricle 24(Figure S1A, B). However, no midline shift or other signs of increased intracranial pressure were suspected in these series of MRIs. Coagulation tests did not show elongation 2526of prothrombin time or activated partial thromboplastin time throughout the period of

1 admission, either.

 $\mathbf{2}$ Based on an assumption that capillary hemorrhages led to the development of neurological symptoms and the progressive neuroimaging features, we introduced the dripping infusion of 3 carbazochrome from 19 days of admission (Figure S1A). His emotional condition became 4 $\mathbf{5}$ stationary from the third week of admission, while MRI on the 26th day of admission no more 6 revealed expansion of hemorrhagic lesions to the surrounding parenchyma. He was discharged on $\overline{7}$ the 33rd day of admission. Because the left hemiplegia and dysarthria were not ameliorated by the 8 day of discharge, he started daily rehabilitation. He has continued the medication of oral carbazochrome after discharge. Follow-up MRIs were taken at three and six months after discharge, 9 10which did not show the extension of the hyperintense signals of the white matter lesions or the 11 recurrence of hemorrhage.

12

13 **Results**

The characteristic white matter lesions in this patient led us to suspect the diagnosis of 1415cerebral autosomal dominant arteriopathy with subcortical infarct and leukoencephalopathy (CADASIL)(Matsubara et al., 2015; Pippucci et al., 2015). We therefore tested whether he carried 16mutations in NOTCH3. However, the targeted sequencing did not show the presence of pathogenic 1718variants in this gene. We have also excluded the possibility that he had mutations in the coding regions of TREX1, the gene associated with Aicardi-Goutieres syndrome. We therefore took the 1920strategy of whole-exome sequencing (WES). 21Overall, the whole-exome sequencing analysis (WES) achieved 93.8% of 20× coverage 22with mean read depths of 86.7 per exon. The proband WES identified 8,340 variants of minor allele

frequency at < 0.05 according to the Human Genetic Variation Database

24 (http://www.hgvd.genome.med.kyoto-u.ac.jp/). Of 725 indels, nonsense or missense mutations, four

variant calls were annotated to be pathogenic or likely pathogenic (Table S1). Among them, a

26 missense mutation was located at exon 28 in the COL4A1 gene

(NM_001845.5:c.2086G>A:p.Gly696Ser). We have reported this variation data to ClinVar 1 $\mathbf{2}$ (https://www.ncbi.nlm.nih.gov/clinvar/) with submission ID: SUB6404908. Either of two 3 variants (OPTN and ITGB3) was unlikely to contribute to his phenotype. Another variant (CREBBP) was not validated by Sanger sequencing, indicating an error in the 4 whole-exome sequencing (Table S1). We validated the presence of the mutation in DNA of $\mathbf{5}$ 6 this boy, but not in his parents, using the Sanger method (Figure 2A). We thus confirmed $\overline{7}$ that the mutation occurred *de novo*, and that he manifested the neurological symptoms with 8 imaging features of COL4A1-related disorder. KOC 9

Literature review 10

To gain further insight into the pathogenic impact of the p.Gly696Ser mutation in this case 11 12(Figure 2B), we collected the literature information for the mutation spectra of *COL4A1* mutations. According to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), a total of 345 patients 13with COL4A1 mutations have been documented thus far (Figure S2). Among them, 70 patients 1415(21%) harbored missense or truncating mutations at the coding sequences for glycine, while 80 16mutations (23%) were located at the X or Y positions in the Gly-X-Y motif (Figure 2B). Of 82 non-coding mutations, 30 (9%) were found in the promoter, enhancer or 5'-untranslated regions and 171852 (15%) in introns or splicing junctions. Copy number variations were identified in 86 patients (25%). 19

Among 259 patients harboring coding or non-coding single nucleotide variations 2021(SNVs), 141 (54%) showed porencephaly or other types of inborn malformations, such as 22hydranencephaly and schizencephaly (Figure 2C). As previously described, the syndromic 23phenotype of hereditary angiopathy, nephropathy, aneurysms and cramps (HANAC) were 24associated with, but not restricted to, the integrin-binding regions (Figure 2D, Table S2) 25(Alamowitch et al., 2009). Among those with intracranial hemorrhages and systemic 26vasculopathy, only three patients were identified to show the late-onset intracranial

hemorrhages, as observed in our case ("Late-onset ICH", Table S2). There was no information
about the inheritance of these mutations in all three patients. Notably, one of these patients had an
identical mutation to that of our case, and the other two patients carried SNVs encoding Pro352 and
Arg538 (Meuwissen et al., 2015; Weng et al., 2012). The present case confirmed that the amino acid
substitution of Gly696 with Ser was associated with an atypical phenotype of vasculopathy among
the SNVs that occurred at Gly-coding sequences in *COL4A1*.

7

8 Discussion

In this report, we described a case with *COL4A1*-associated vasculopathy, who showed 9 10recurrent intracerebral hemorrhages after 17 years of follow-up period. This case gives an alert that a small group of patients with specific *COL4A1* mutations show only an intracranial calcification in 11 12infancy without apparent cerebral hemorrhage or systemic involvement. This report also demonstrated that they had a persistent risk for the rupture of small vessels in the cerebral white 13matter after long intervals. We interpreted that a new series of hemorrhagic event occurred at 17 1415years of age because the acute onset of hemiplegia and his neurological symptoms were correlated 16with the MRI findings after admission. However, we cannot exclude the possibility that we missed the critical time points of subclinical hemorrhage in the basal ganglia that might have been already 1718present before 17 years of age. In this regard, the present case implicated that children with COL4A1-associated vasculopathy need continuous monitoring of MRI for minimizing the risk for 1920the rupture of small vessels.

Mutations in *COL4A1* have been reported to determine the clinical phenotype of small vessel disease. Typically, the nephropathy, tortuosity of retinal arteries and muscular cramps are the diagnostic hallmarks for the HANAC syndrome, the generalized form of *COL4A1*-associated vasculopathy (<u>Alamowitch et al., 2009; Takenouchi et al., 2015</u>). Our case showed only corneal opacities, but not aberrant retinal vasculature or optic nerve hypotrophy, on ophthalmologic tests. Nephropathy was absent, either. Muscular cramps were not evident until the present admission.

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Nonetheless, the transient elevation of CK in early infancy was retrospectively considered
 to be an informative clinical parameter for diagnosing this disease as formerly described
 (Plaisier et al., 2007).

Recent studies have shown that periventricular leukomalacia and calcifications 4 $\mathbf{5}$ were the neuroimaging clues for the diagnosis of COL4A1-associated vasculopathy. With 6 this regard, we have initially suspected the diagnosis of pseudo-TORCH, including $\overline{7}$ Aicardi-Goutieres syndrome (AGS) (Crow et al., 2006; Rice et al., 2007; Tonduti et al., 8 2018). Although the white matter lesions of the two disorders might be hard to distinguish from each other at their early stages, patients with AGS have been reported to show the 9 10 unique findings of MRI, such as temporal lobe swelling and progressive cortical atrophy (Livingston and Crow, 2016; Livingston et al., 2013). Because the patient lacked these 11 12findings, we valued the utility of follow-up MRI studies until the genetic tests confirmed the diagnosis. 13Through the literature search, we found 151 (58%) of 259 patients with SNVs in 1415COL4A1 had amino acid substitutions in triple-helix (Gly-X-Y) repeats (Abe et al., 2017; Borlot et al., 2017; Khalid et al., 2018; Malik et al., 2018; Miyatake et al., 2018; 16Rannikmae et al., 2015; Rannikmae et al., 2017; Saskin et al., 2018; Zagaglia et al., 2018). 1718Among them, the majority of patients show inborn malformations, such as porencephaly or schizencephaly (Takenouchi et al., 2015; Yoneda et al., 2013). Thus, a single-amino acid 1920substitution in Gly-X-Y repeats can affect the whole protein structure and/or the 21triple-helix-forming functions of COL4A1. Given that the three previous cases showed the 22late-onset hemorrhages (Takenouchi et al., 2015; Yoneda et al., 2013), the COL4A1 protein 23in the basement membrane plays a critical role not only in the embryonic development of 24vascularization, but also in the postnatal maintenance of vessel walls (Plaisier et al., 2007). 25Our case was considered to develop the hemorrhage at 17 years of age with an inborn 26vulnerability and the postnatal susceptibility to the rupture of the cerebral small vessels.

COL4A1-associated vasculopathy

The information for the therapeutic utility of carbazochrome remains limited. The 1 $\mathbf{2}$ treatment outcome of the present case might simply reflect the spontaneous regression of oozing 3 during the time course, rather than the therapeutic effects. Increasing the number of experiences will provide insight into the efficacy of this treatment option. 4 $\mathbf{5}$ This report has only provided the clinical feature of the second case carrying a rare variant 6 (p.G696S) in COL4A1. The clinical findings may be of use, however, when future genetic studies $\overline{7}$ are directed to analyze the long-term outcomes of infants with mildly affecting variations. 8 Acknowledgments 9 10We thank Ms Tamami Tanaka PhD and Ms Ayumi Tahara for technical assistance, and all the lab members in our department for helpful discussion. This work was supported by JSPS 11 12Kakenhi grant number 17K16271 (YI) and 19K08281 (YS), a Health and Labour Sciences Research Grant on Evidence-based Early Diagnosis and Treatment Strategies for Neuroimmunological 13Diseases from the Ministry of Health, Labour and Welfare of Japan, and Kawano Masanori 1415Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics (YS). 16Consent 1718Written informed consent was obtained from the parents for the genetic tests and reporting the clinical information of the patient. 192021**Conflicts of interest** 22None to disclose.

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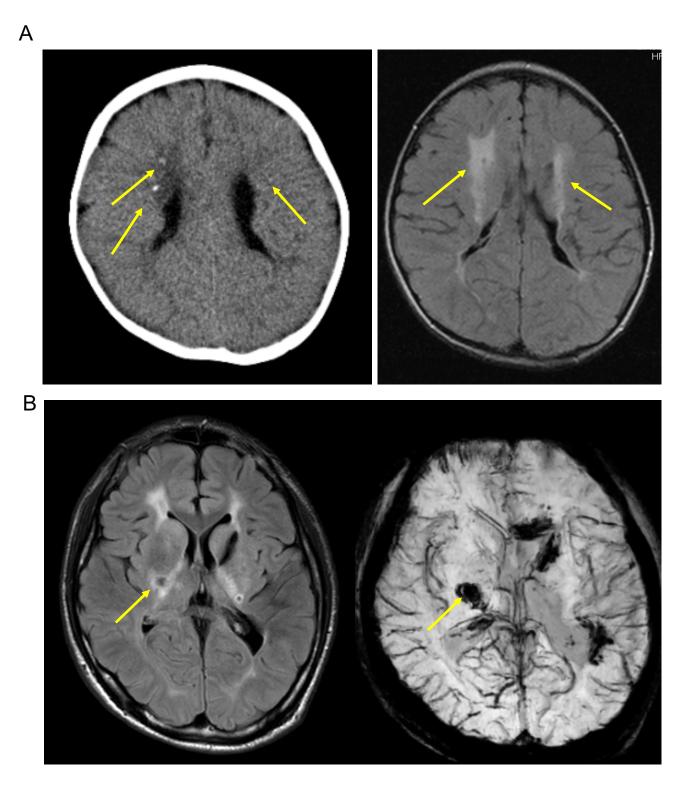
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1	Fig	gure legends
2	Fig	ure 1. Clinical course and the neuroimaging studies for the present case
3	A.	Left: A computed tomography (CT) scan at one months of age. Right: Magnetic resonance
4		imaging (MRI) with fluid-attenuated inversion recovery (FLAIR) at three years of age. Arrows
5		indicate the calcifications on CT and T2-hyperintense lesions on FLAIR image.
6	B.	MRI with the FLAIR (left) and susceptibility-weighted imaging (SWI, right) modalities.
7		Arrows indicate the new hemorrhage in the right posterior limb of internal capsule and basal
8		ganglia.
9		
10	Fig	ure 2. The <i>de novo</i> p.Gly696Ser mutation identified in the present case and the mutation
11		spectrum of patients with COL4A1-associated vasculopathy
12	A.	Sequence chromatograms for father, mother and the present patients. The de novo mutation
13		(G>A, G696S) was annotated on top.
14	В.	Breakdown of mutations identified in patients with COL4A1 vasculopathy. Among a total of
15		259 patients, "Gly" ($n = 70$) and "X or Y" ($n = 81$) represent the mutations located at the
16		positions encoding the amino acid sequences of Gly-X-Y triple-helix repeats; "Coding,
17		non-GXY" denotes those in the other coding regions $(n = 26)$; Those at the promoter, enhancer
18		and 5'-untranslated regions are designated as "Non-coding regions" ($n = 82$). SNVs,
19		single-nucleotide variants.
20	C.	Histograms showing the number of patients with the indicating phenotypes. The type of SNVs
21		are shown over the bar plots. Arrow indicates the three patients who developed late-onset
22		hemorrhages.
23		
24	Suj	pplementary Materials
25	•	Supplementary methods

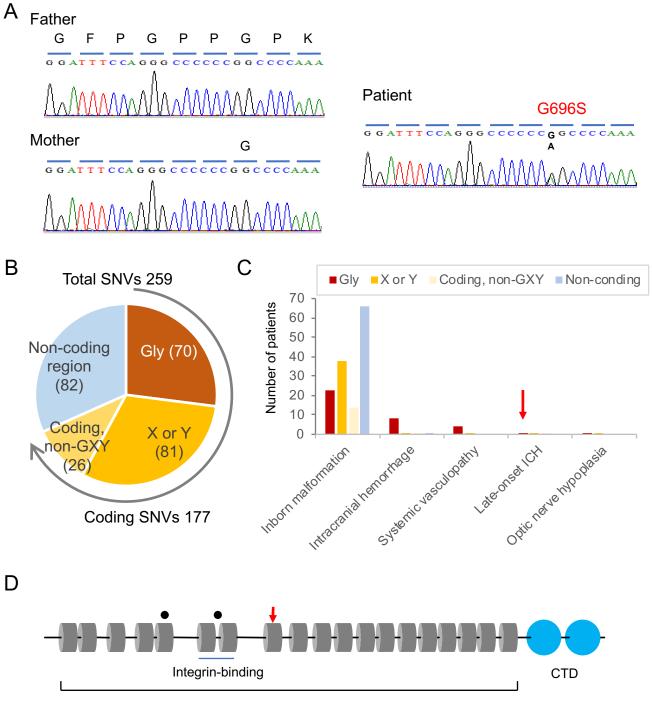
• Figure S1. Clinical course of the present case

- 1 Figure S2. A graphic summary of reported mutations on the amino acid sequence of COL4A1
- 2 Table S1. Four detected variants in the whole-exome sequencing
- 3 Table S2. Summary of reported cases with copy number and sequence variations in COL4A1

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Kinoshita Figure 1



Triple-helix repeats (Gly-X-Y)