A new identified COL4A2 mutation contributes to astrocyte activation by activating JAK/STAT signaling in epilepsy

Chun-Hui Hu[1], Bo Wang[2], and Yi Wang[1]

[1]Children's Hospital of Fudan University
[2]Shenzhen Second People's Hospital

December 9, 2022

Abstract

COL4A2 is the encoding gene of the α2 chains of type IV collagen, and missense mutations of COL4A2 is correlated with multiple diseases. However, the association of COL4A2 mutations with epilepsy remains elusive. Here, we aimed to explore the function of COL4A2 mutations in the development of epilepsy. We performed a full spectrum of family-enhanced whole-exome sequencing on a family lineage and examined the genetic change on COL4A2 gene. The kainic acid (KA)-induced in vivo model and lipopolysaccharide (LPS)-induced in vitro model were established. The production and secretion of inflammation cytokines were measured by qPCR, western blotting, and ELISA assay. Neuron damages and astrocyte activation were checked by Nissle and GFAP immunofluorescence staining. One heterozygous variant was detected in the COL4A2 gene: c.1148C>T(p.Pro383Leu). COL4A2 mutation significant induced the exacerbation of seizures and impaired the learning and memory phenotype of the KA rats. COL4A2 mutation promoted the hippocampal astrocyte activation, enhanced hippocampal neuronal injury in the rats. The levels of iNOS, COX-2, IL-1β, IL-6, and TNF-α elevated in KA-treated rats and LPS-treated astrocytes were further induced by COL4A2 mutation. Mechanically, COL4A2 mutation stimulated JAK/STAT signaling. JAK2 and STAT3 phosphorylation was promoted by COL4A2 mutation and JAK/STAT signaling inhibitor WP1066 could blocked the effect in primary astrocytes and CTX-TNA cells. These data indicated that a new identified COL4A2 mutation contributed to astrocyte activation by activating JAK/STAT signaling in epilepsy. Our findings provided a novel mechanism and treatment target of COL4A2 related epilepsy.

A new identified COL4A2 mutation contributes to astrocyte activation by activating JAK/STAT signaling in epilepsy

Chunhui Hu[1], Bo Wang[2], Yi Wang[1,*]

[1] Department of Neurology, Children's Hospital of Fudan University, National Children's Medical Center, Shanghai, China

[2] Department of Pediatrics, Shenzhen Second People's Hospital, Shenzhen, China

[*]Address correspondence to:

Yi Wang. Department of Neurology, Children's Hospital of Fudan University, National Children's Medical Center, Shanghai, China.

E-mail: yiwang@shmu.edu.cn

Abstract

COL4A2 is the encoding gene of the α2 chains of type IV collagen, and missense mutations of COL4A2 is correlated with multiple diseases. However, the association of COL4A2 mutations with epilepsy remains

elusive. Here, we aimed to explore the function of COL4A2 mutations in the development of epilepsy. We performed a full spectrum of family-enhanced whole-exome sequencing on a family lineage and examined the genetic change on COL4A2 gene. The kainic acid (KA)-induced in vivo model and lipopolysaccharide (LPS)-induced in vitro model were established. The production and secretion of inflammation cytokines were measured by qPCR, western blotting, and ELISA assay. Neuron damages and astrocyte activation were checked by Nissle and GFAP immunofluorescence staining. One heterozygous variant was detected in the COL4A2 gene: c.1148C>T(p.Pro383Leu). COL4A2 mutation significant induced the exacerbation of seizures and impaired the learning and memory phenotype of the KA rats. COL4A2 mutation promoted the hippocampal astrocyte activation, enhanced hippocampal neuronal injury in the rats. The levels of iNOS, COX-2, IL-1β, IL-6, and TNF-α elevated in KA-treated rats and LPS-treated astrocytes were further induced by COL4A2 mutation. Mechanically, COL4A2 mutation stimulated JAK/STAT signaling. JAK2 and STAT3 phosphorylation was promoted by COL4A2 mutation and JAK/STAT signaling inhibitor WP1066 could blocked the effect in primary astrocytes and CTX-TNA cells. These data indicated that a new identified COL4A2 mutation contributed to astrocyte activation by activating JAK/STAT signaling in epilepsy. Our findings provided a novel mechanism and treatment target of COL4A2 related epilepsy.

Keywords: Epilepsy; Astrocyte; COL4A2; Mutation; JAK/STAT signaling.

INTRODUCTION

Epilepsy is one of the most prevalent neurological conditions with an incidence of more than 0.5% worldwide (Steriade et al., 2020). Approximately 70 million people were affected by this disease (Thijs et al., 2019). And elder people and infants tend to have higher risk than other people (Perucca et al., 2018). The epilepsy is commonly due to neurological malfunction-caused synchronized excess firings in the certain region of brain, and characterized by recurrent disturbances of consciousness and muscle spasms and convulsions (Falco-Walter et al., 2020). The etiology of epilepsy is classified as infectious, immune, structural, metabolic, genetic and unknown factors. Recent studies reported that neuroinflammation is the pathological signature of central nervous system diseases including epilepsy (Galan, 2021; Devinsky et al., 2013; Vezzani et al., 2013). Proinflammatory cytokines such as tumor necrosis factor alpha, interleukin 1-beta (IL-1β), and IL-6 were increased in the $Gabrg2^{+/Q390X}$ knockin mice, providing the first link of neuroinflammation between genetic epilepsy associated with an ion channel gene mutation and acquired epilepsy (Shen et al., 2020). Astrocytes are characteristic glial cells responsible for nutrition support, blood-brain barrier formation, extracellular ion equilibrium, and synaptic plasticity (Karve et al., 2016; Araki et al., 2021). Stimulation with sustained inflammation, damage-associated molecular pattern, and reactive oxidative species cause astrocytes activation, which exhibits upregulation of glial fibrillary acidic protein (GFAP) protein, could promote glutamate excitatory toxicity and mitochondrial dysfunction (Terrone et al., 2017; Xian et al., 2019). JAK/STAT signaling has been implicated as important regulator of epilepsy. GFAP-positive astrocytes were increased by JAK/STAT activation (Wang et al., 2020; Hixson et al., 2019; Feng et al., 2019). LncRNA H19 could modulate astrocytes activation for prevention of epilepsy through the JAK/STAT signaling (Han et al., 2018).

COL4A1 and COL4A2 are the encoding gene of the α1 and α2 chains of type IV collagen, respectively, which is the structural components of basement membrane (Chung et al., 2021; Meuwissen et al., 2015; Liu et al., 2020). Studies have suggested that missense mutations of COL4A1 and COL4A2 are correlated with multiple diseases (Zagaglia et al., 2018; Boyce et al., 2021). For example, COL4A1 and COL4A2 gene mutation was spotted on patients with ischemic cerebral lesions (Maurice et al., 2021). A novel COL4A2 mutation was recently identified to be correlated with cortical development malformations (Neri et al., 2021). Besides, COL4A1 mutation has been revealed to induce perinatal cerebral hemorrhage and porencephaly (Yaramis et al., 2020). It has been recognized that epilepsy is a clinical feature of porencephaly, however, the correlation between COL4A1 and COL4A2 and epilepsy has not been specified.

In this research, we performed family-enhanced whole-exome sequencing to determine COL4A2 mutation in clinical peripheral blood samples, and established an epilepsy rat model to evaluate the effects of COL4A2

mutation on epilepsy progression. Our findings may provide novel therapeutic strategies for epilepsy.

2 MATERIALS AND METHODS

2.1 Materials

The project ethics were approved by the Ethic Committees of Children's Hospital of Fudan University (No. 503). All experiments involving human subjects were in accordance with the ethical standards of the institutional and/or national research committee and with the Declaration of Helsinki. Kainic acid and lipopolysaccharide (LPS) were brought from Sigma (MO, USA). JAK/STAT inhibitor (WP1066) was brought from Selleck (CA, USA). The Adenovirus (AdV) vectors vectors that contain wild type (COL4A2 WT) and mutated (COL4A2 MUT) COL4A2 were synthesized by Gene Pharma (Shanghai, China). Primary antibodies against GFAP (ab7260; Abcam, USA), IL-1β (ab254360; Abcam, USA), IL-6 (ab233706; Abcam, USA), TNF-α (ab183218; Abcam, USA), c-Myc (ab32072; Abcam, USA), JAK2 (ab108596; Abcam, USA), STAT3 (ab68153; Abcam, USA), p-JAK2 (ab32101; Abcam, USA), p-STAT3 (ab76315; Abcam, USA), iNOS (ab178945; Abcam, USA), and COX-2 (ab179800; Abcam, USA) were provided by Abcam (CA, USA).

2.2 Rat model

Sprague–Dawley (SD) rats that weighted 200 to 220 g were brought from Vital River Laboratoryand housed with a 12/12-hour light/dark cycle. Rats were then randomly divided into experimental groups with 5 in each group. To establish the status epilepticus (SE) rat model, KA (1 μg/μl, 1 μl) was stereotaxically injected into the amygdala section of rats at a speed of 0.2 μl/min using a microsyringe. Rats in sham group were injected with saline as control. For treatment, the AdV vectors that contain COL4A2 WT or COL4A2 MUT (2.0×10^{12} PFU) were injected into the right dorsal hippocampus using a microsyringe 2 weeks before the establishment of SE model. The rats were sacrificed 10 days after the establishment of KA model and the brain tissues were collected for further experiments. All animal experiments were performed under the authorization and followed the guideline of Ethic Committee of Children's Hospital of Fudan University.

2.3 Racine grading

Seizure behavior was observed and graded according to Racine scale (Racine, 1972) (Wu et al., 2020) by two independent observers: stage 1: freezing; stage 2: head nodding; stage 3: forelimb clonus or transient myoclonic jerk; stage 4: sustained forelimb clonus, rearing with lordotic standing posture and mouth foaming; stage 5: rearing and falling over. The duration and times of seizures were observed and recorded with in 3 hours after KA injection. Seizure duration was calculated from rats exhibited seizure stage 4 to the time point when rats moved around the cage freely. Rats that scored stage 4 and stage 5 were included in KA group and used for subsequent experiments.

2.4 Morris water maze test

To check the behaviors of epilepsy rats, we performed a Morris water maze test. A platform was set in the middle of a water pool. Rats were trained to find the hidden platform within 120s every day for 7 consecutive days. 24 hours after the last training, the rats were placed a random site and released to find the platform. The time to reach the platform (escape latency) and the times to cross the platform were recorded.

2.5 Nissl staining

The ipsilateral hippocampus tissues were sectioned and dehydrated in xylene and a serial of ethanol, washed with PBS, stained in 1% toluidine blue (G3668; SolarBio, Beijing, China) for 40 minutes. Then the sections were rehydrated and observed under a microscope (Leica, Germany).

2.6 Immunofluorescence (IF) staining

The ipsilateral hippocampus tissues sections were coated by OCT and dehydrated. For IF staining, the tissues were incubated with anti-GFAP primary antibodies (ab7260; Abcam, USA) at 4 for one night. Next day, the tissues were probed with Alexa Fluor 488 and Alexa Fluor 633 (ab150083; Abcam, USA) at room

temperature for 40 minutes. The nuclei were stained with DAPI. Fluorescence was captured by microscope (Leica, Germany).

2.7 Cell lines and in vitro model

The rat astrocytes CTX-TNA were brought from SAIBAIKANG, and maintained in DMEM medium (Thermo, USA) that contains 10% FBS (Hyclone, USA) and 1% penicillin-streptomycin (SolarBio, China). For isolation of primary astrocytes, new-born SD rats were purchased form Vital River Laboratory and proceeded following a published study (Sun et al., 2015). Cells were stimulated with 100 ng/mL LPS for 24 hours to induce neuroinflammation.

2.8 Cell proliferation and apoptosis

The proliferation of astrocytes was measured by CCK-8 (C0083; Beyotime, China). In brief, cells were seeded in 96-well plate after transfection and indicated treatment and incubated for the indicated time. Then, CCK-8 reagent was added into each well and incubated for 2 hours. The absorbance values were measured by a microplate reader (Thermo, USA). To determine cell apoptosis, cells were collected after indicated treatment for 48 hours, suspended in binding buffer that contains Annexin V and PI reagent (C1062S; Beyotime, China) for 30 minutes in dark. The samples were then analyzed by a flow cytometer (BD Biosciences, USA).

2.9 Quantitative real time PCR (qPCR) assay

Total RNA was extracted using Trizol lysis buffer. A total of 1μg RNA was transcribed to cDNA using PrimeScript RT reagent Kit (Takara, Japan), following the manufacturer's protocol. The qPCR was performed using SYBR Green PCR Kit (Takara, Japan). Relative RNA expression to β-actin as endogenous reference gene was measured following the $2^{-\Delta\Delta\hat{}\tau}$ method.

2.10 Western blotting

The proteins were extracted from astrocytes and tissues using RIPA buffer (Beyotime, China). The equal amounts of proteins were loaded and divided by SDS-PAGE gel, shifted to NC membranes, blocked by 5% nonfat milk, and hatched with corresponding primary antibodies against iNOS (ab178945; Abcam, USA), COX-2 (ab179800; Abcam, USA), IL-1β (ab254360; Abcam, USA), IL-6 (ab233706; Abcam, USA), TNF-α (ab183218; Abcam, USA), JAK2 (ab108596; Abcam, USA), p-JAK2 (ab32101; Abcam, USA), STAT3 (ab68153; Abcam, USA), p-STAT3 (ab76315; Abcam, USA) and GAPDH (ab8245; Abcam, USA) at 4 overnight. Then, the protein bands were incubated with HRP-conjugated secondary anti-rabbit (ab6721; Abcam, USA) and anti-mouse (ab6728; Abcam, USA) antibodies. After reaction with ECL solution (WBKLS0500; Millipore, USA), the bands were visualized using a gel image system.

2.11 Enzyme-linked immuno sorbent assay (ELISA)

The blood samples were obtained from the rat tails and serum samples were collected after centrifugation. The IL-1β, IL-6 and TNF-α levels in serum and cell culture medium were measured by ELISA assay kits (Thermo, USA) following manufacturer's instruction.

2.12 Statistical analysis

Data were presented as means ± SD of three replicates. SPSS (ver. 19.0) software was used for statistical analysis. Comparisons between two or more groups were analyzed by using Student's t -test and on-way ANOVA analysis. Statistical significance was set as $p < 0.05$.

3. RESULTS

3.1 Identification of COL4A2 mutation

The proband was a 6 months boy first-born from a full term pregnancy and natural birth. The pregnancy history, delivery history and family history of this boy were unremarkable. The boy could not raise his head and sit at present. He started to have epileptic spasms at 3 months old. Nodding episodes and flexion of

both upper limbs occurred with no apparent cause, which occurred 6 to 8 times a day. He had isolated or clusters of epileptic spasms. The seizures are mainly after waking up from sleep. But his parents didn't take it seriously until 6 months. When she was 6 months, she was sent to our hospital. She had no morphological facial and other congenital abnormalities. Gesell developmental scale scores showed severe developmental retardation. Video EEG showed hypsarrhythmia during wake and sleep at 6 months (Fig S1A and B). Routine laboratory tests and brain MRI were normal(Fig S1C). Whole-exome sequencing of a family lineage identified a COL4A2 mutation(Fig S1D). We performed a full spectrum of family-enhanced whole-exome sequencing on the submitted peripheral blood samples, and analyzed clinically relevant point mutations, small insertional deletions and copy number variants. One heterozygous variant was detected in the COL4A2 gene: c.1148C>T(p.Pro383Leu), and this sequence change resulted in the replacement of nucleotide 1148 with T(c.Pro383Leu) in the COL4A2 gene. C was replaced with T (c.1148C>T), resulting in a change in amino acid 383 from proline to leucine (p.Pro383Leu). This mutation is a de novo missense mutation, which has not been reported before. The guidelines of the American College of Medical Genetics and Genomics and various predictions (SIFT:D, Polyphen2_HDI:V:P) suggest that this variant is a likely pathogenic mutation and indicated a high probability of deleterious effects of the mutation on gene/white matter structure or function. Then she was given oral prednison and became seizure-free 4 weeks later. The prednison was gradually stopped after 6 months. At last follow up, he remained seizure-free at 1year and 6 months.

3.2 COL4A2 mutation induces the exacerbation of the seizures and contributes to learning and memory impairments in epilepsy rats

Next, to further evaluate the function of COL4A2 mutation in epilepsy, we established a KA-induced epilepsy rat model and treated rats with AdV vectors that contain COL4A2 WT or COL4A2 MUT. The treatment with mutated COL4A2 significantly induced the times of stage 4 and 5 severe seizures ($p < 0.05$; Fig. 1A) and the duration ($p < 0.01$; Fig. 1B) compared with KA group within 3 h after the injection of KA. Then Morris water maze assay was conducted to evaluate the learning and memory of epilepsy rats. We observed that the overexpression of WT and MUT COL4A2 did not affect the escape latency and swimming speed of rats in sham group ($p > 0.05$; Fig. 1C and D). In the KA rats, MUT COL4A2 notably elevated the escape latency ($p < 0.01$, Fig. C) without change the speed of rats ($p > 0.05$; Fig. 1D), whereas the WT COL4A2 had no such effects. Consistently, the swimming distance of rats in KA group was notably decreased comparing with that in the sham group, and administration of MUT COL4A2 further enhanced this phenotype ($p < 0.01$; Fig. 1E and F). Moreover, epileptic rats that treated with MUT COL4A2, but not the WT COL4A2, took less time in the target area and pass cross the platform fewer times than that of KA rats ($p < 0.01$; Fig. 1G and H). These results demonstrated that mutated COL4A2 exacerbated the impaired seizures, learning ability and memory of epilepsy rats. Meanwhile, the protein and RNA levels of iNOS, COX-2, IL-1β, IL-6, and TNF-α elevated in KA-treated rats were remarkably induced by COL4A2 mutation in the model ($p < 0.05$; Fig. 1I-K).

3.3 COL4A2 mutation induces hippocampal astrocyte activation and neuronal injury in epilepsy rats

We next examined tissues damage by Nissl staining of hippocampus. As shown in Figure 2A, the pyramidal cells in sham group were darked-stained and the cytoplasm was abundant of Nissl bodies. In the hippocampus from KA rats, we observed disordered neurons and reduced cytoplasmic Nissl body, and the treatment with MUT COL4A2 exacerbated this effect (Fig. 2A). Meanwhile, we observed the elevated expression of GFAP, indicating the hippocampal astrocyte activation in KA-treated rats, and COL4A2 mutation enhanced this activation (Fig. 2B and C). Noteworthy, we found that the expression and phosphorylation of JAK2 and STAT3 were notably elevated in KA rats compared with the sham group, and COL4A2 mutation further induced the activation of JAK2/STAT3 (Fig. 2D). These data indicated that COL4A2 mutation induced inflammation and neuronal injury in epilepsy rats possibly via modulation the JAK2/STAT3 signaling.

3.4 COL4A2 mutation promotes inflammation and astrocyte activation in vitro

Next, we further assessed the function of COL4A2 mutation in LPS-treated primary astrocytes and CTX-

TNA cells. Interestingly, we failed to observe the effect of COL4A2 and COL4A2 mutation on the viability and induced apoptosis in primary astrocytes (Fig. 3A and B) and CTX-TNA cells (Fig S2A). COL4A2 mutation enhanced astrocyte activation in primary astrocytes (Fig. 3C) and CTX-TNA cells (Fig S2B). In addition, expression of COX-2 and iNOS was induced by COL4A2 mutation in primary astrocytes (Fig. 3D and E) and CTX-TNA cells (Fig S2C and D). Consistently, the intracellular levels and secretion of IL-1β, IL-6, and TNF-α were induced by COL4A2 mutation (Fig. 3F-L and Fig. S2E-K). The activation of JAK2 and STAT3 were also observed in primary astrocytes (Fig. 3M) and CTX-TNA cells (Fig S2L).

3.5 COL4A2 mutation promotes hippocampus injury and inflammation by stimulating JAK/STAT signaling *in vivo*

To elucidate the role of JAK2/STAT3 signaling in mutated COL4A2-stimulated epilepsy, we used JAK/STAT signaling inhibitor WP1066 for further study. We observed that administration of WP1066 suppressed the times ($p < 0.01$; Fig. 4A) and duration ($p < 0.05$; Fig. 4B) of seizures compared with the COL4A2 mutation group. WP1066 decreased the COL4A2 mutation-induced escape latency ($p < 0.01$; Fig. 4C) and increased swimming speed ($p < 0.01$; Fig. 4D) of KA rats. The swimming distance ($p < 0.001$; Fig. 4E and F), number of times pass through platform ($p < 0.01$; Fig. 4G), duration of stay at the target ($p < 0.001$; Fig. 4H) were all significantly increased upon WP1066 treatment, comparing with the COL4A2 mutation group. Besides, the inhibition of JAK/STAT signaling suppressed the COL4A2 mutation-induced production of inflammatory cytokines (Fig. 4I and J). The results from Nissl staining demonstrated that WP1066 treatment alleviated the COL4A2 mutation-induced neuron injury in KA rats (Fig. 5A). Results from immunofluorescence staining and western blotting showed suppressed GFAP level in COL4A2 mutation group after inhibition of JAK/STAT signaling (Fig. 5B and C). The suppressed activation of JAK2 and STAT3 under WP1066 treatment was validated (Fig. 5D).

3.6 COL4A2 mutation promotes inflammation and astrocyte activation by stimulating JAK/STAT signaling *in vitro*

Then, we observed that the astrocyte activation was induced by COL4A2 mutation but WP1066 treatment reversed the effect in primary astrocytes (Fig. 6A) and CTX-TNA cells (Fig. S3A). The COL4A2 mutation increased iNOS, COX2, IL-1β, IL-6, and TNF-α levels in primary astrocytes (Fig. 6B-G) and CTX-TNA cells (Fig. S3B-G), in which WP1066 was able to attenuate the effect in the model. The inhibited JAK2 and STAT3 by WP1066 were also observed in primary astrocytes (Fig. 6H) and CTX-TNA cells (Fig. S3H).

4. DISCUSSION

Epilepsy is a prevalent neurological disorder worldwide and COL4A2 is the encoding gene of the α2 chains of type IV collagen, and the missense mutations of COL4A2 is correlated with a wide spectrum of phenotypes. It has been reported that COL4A2 mutations regulates secretion of COL4A1 and COL4A2 in hemorrhagic stroke (Jeanne et al., 2012). A Novel COL4A2 mutation cause familial malformations of cortical development (Neri et al., 2021). COL4A2 mutation is correlated with familial porencephaly and small-vessel disease (Verbeek et al., 2012). A mutation COL4A2 leads to autosomal dominant porencephaly with cataracts (Ha et al., 2016). COL4A2 mutation affects adult onset recurrent intracerebral hemorrhage and leukoencephalopathy (Gunda et al., 2014). At present, COL4A2 mutation related phenotypes include prenatal and neonatal intracranial hemorrhage, cerebral perforating malformation, cerebral perforating malformation with cataract, focal cortical dysplasia, cerebral fissure malformation, childhood and youth stroke, sporadic delayed hemorrhagic stroke (Meuwissen et al., 2015; Ha et al., 2016). COL4A1 and COL4A2 mutations share common and variable phenotypes varying from mild to severe (Hausman-Kedem et al., 2021). COL4A2 mutations are mainly missense mutations with incomplete penetrance, in which epilepsy with brain perforating malformation is an important clinical feature (Chung et al., 2021; Itai et al., 2021). Nevertheless, the effect of COL4A2 mutations on epilepsy is still obscure. In this study, we performed a full spectrum of family-enhanced whole-exome sequencing on a family lineage, and one denovo heterozygous variant was detected in the COL4A2 gene. We discovered the novel function of a COL4A2 mutation in promoting epilepsy. This patient was diagnosed with west syndrome. However, his brain MRI was normal. So we believe west syndrome without

structural brain malformation is the new phenotype of COL4A2.

In patients with refractory epilepsy, single-cell transcriptomics and surface epitope detection in human brain epileptic lesions identified pro-inflammatory mechanisms and revealed a pro-inflammatory microenvironment, including extensive activation of microglia and infiltration of other pro-inflammatory immune cells. At the same time, immunotranscriptome found the expression of some differential genes, including cytokine related genes CCL2, CCL3, CCL4 and CCL7, chemokine related genes CXCR2, CXCR3, CXCR4 and CXCR6, and collagen genes COL1A2, COL3A1, COL4A1, COL4A2, COL4A5, COL5A1, COL5A3 and COL9A2. Among them, there is differential expression of COL4A2 involved in the formation of basement membrane in neurovascular units (Meuwissen et al., 2015). Previous findings have shown that activated astrocytes played an important role in driving epileptogenesis independently of neurons (Boison et al., 2018). In our study, for the treatment, this patient received sound effect with prednisone, so we reasonably hypothesized that astrocyte activation and neuroinflammation may be involved in the progression of COL4A2 related epilepsy.

To identify the function of COL4A2 mutation in epilepsy, experiments in vivo model indicated that COL4A2 mutation significant impaired the seizures, learning and memory phenotype of the rats. We showed that COL4A2 mutation affects epilepsy and cognitive function. Further experiments with in vitro and in vivo model both indicated elevated the secretion of inflammatory cytokines, including the iNOS, COX2, IL-1β, IL-6, and TNF-α. We also showed the presence of pro-inflammatory microenvironments in the rats' hippocampus associated with COL4A2 epilepsy. Because there was no increase of pro-inflammatory cytokines in our patient's peripheral blood, so we speculate that the increased pro-inflammatory cytokines are limited to the patients' brain region. As is well known, JAK2/STAT3 signaling is highly conservative in evolution and considered to be the most important one in JAK/STAT signaling pathway (Jaskiewicz et al., 2020), so we examined this signaling pathway. As expected, we found COL4A2 mutation remarkably induced astrocyte activation and activation of JAK2/STAT3 signaling. We identified that astrocyte activation and neuroinflammation are important pathological features in COL4A2 related epilepsy, in addition to neuronal damage. Interestingly, we failed to observe the impact of COL4A2 or COL4A2 mutation on the viability and apoptosis of primary astrocytes and CTX-TNA cells. It implies that COL4A2 mutation may mainly affect neuroinflammation signaling.

It is well-recognized that GFAP elevation is the first step of astrocyte hypertrophy in epilepsy (Savaş et al., 2021; Li et al., 2021). And the activation of JAK2/STAT3 signaling has been indicated to induce the formation of GFAP - positive glia (Lee et al., 2010). Moreover, the JAK and STAT3 signaling has been indicated to mediate the LPS-induced inflammatory responses (Yu et al., 2014; Yu et al., 2009). Following the phosphorylation of JAK2 and STAT3, the homo- or heterodimers of phosphorylated STATs translocate into the nucleus and modulate the transcription of multiple pro-inflammatory cytokines, such as iNOS, COX2, and IL6 (Zegeye et al., 2018; Yeung et al., 2018). Moreover, it has been reported that lncRNA H19 promotes activation of hippocampal glial cells by JAK/STAT signaling in a rat model of temporal lobe epilepsy (Han et al., 2018). MicroRNA-183 regulates hippocampal neuron injury in epilepsy rats by targeting JAK/STAT signaling (Feng et al., 2019). LncRNA-UCA1 represses the activation of astrocyte by JAK/STAT signaling in the temporal lobe epilepsy (Wang et al., 2020). In the present study, we identified COL4A2 mutation stimulated JAK/STAT signaling. JAK2 and STAT3 phosphorylation was promoted by COL4A2 mutation and JAK/STAT signaling inhibitor WP1066 could blocked the effect in epilepsy rats, primary astrocytes and CTX-TNA cells. The astrocyte activation was induced by COL4A2 mutation but WP1066 treatment reversed the effect. The COL4A2 mutation increased IL-1β, IL-6, and TNF-α levels in epilepsy rats, primary astrocytes and CTX-TNA cells, in which WP1066 was able to attenuate the effect in the model. These data suggested that the COL4A2 mutation may affect epilepsy by targeting JAK/STAT signaling, which also indicated neuroinflammation as the new mechanism in the pathology of genetic epilepsy.

5. CONCLUSION

In conclusion, we discovered that a new identified COL4A2 mutation contributed to astrocyte activation by activating JAK/STAT signaling in epilepsy. These findings help to better understand how astrocyte activation contributes to the pathology of COL4A2 related epilepsy. In the future, astrocytes may be the

alternative targets for more efficient anti-seizure medications.

AUTHOR CONTRIBUTION STATEMENT

Chunhui Hu and Bo Wang conceived the study. Chunhui Hu designed the study and wrote the manuscript. Yi Wang supervised and contributed to the revisions and approval of the manuscript.

ETHICS APPROVAL STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. The project ethics were approved by the Ethic Committees of Children's Hospital of Fudan University (No. 503). All experiments involving human subjects were in accordance with the ethical standards of the institutional research committee and with the Declaration of Helsinki. All animal experiments were performed under the authorization and followed the guideline of Ethic Committee of Children's Hospital of Fudan University.

ACKNOWLEDGEMENTS

We appreciate the patient participating in this study. Moreover, I express my heartfelt gratitude for the hard work of all team members.

FUNDING

This work was funded by Shanghai Municipal Science and Technology Major Project,International Human Phenome Program (Phase I) - Study of the Whole Phenome of Refractory Epilepsy (Grant No. 2017SHZDZX01)and Shanghai Municipal Science and Technology Major Project (No.2018SHZDZX03) and ZJLab.

CONFLICTS OF INTEREST

There are no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw data will be provided, as and when required.

ORCID

Chunhui hu ID*https://orcid.org/0000-0003-4096-8035*

References

Araki, T., Ikegaya, Y., & Koyama, R. (2021). The effects of microglia- and astrocyte-derived factors on neurogenesis in health and disease. The European journal of neuroscience, 54: 5880-901. https://doi.10.1111/ejn.14969

Boison, D., & Steinhauser, C. (2018). Epilepsy and astrocyte energy metabolism. Glia., 66: 1235-43. https://doi.10.1002/glia.23247

Boyce, D., McGee, S., Shank, L., Pathak, S., & Gould, D.. (2021). Epilepsy and related challenges in children with COL4A1 and COL4A2 mutations: A Gould syndrome patient registry. Epilepsy behav, 125: 108365. https:// doi.10.1016/j.yebeh.2021.108365

Chung, J., Hamilton, G., Kim, M., Marini, S., Montgomery, B., Henry, J., Cho, A. E., Brown, D. L., Worrall, B. B., & Meschia, J. F. (2021). Rare Missense Functional Variants at COL4A1 and COL4A2 in Sporadic Intracerebral Hemorrhage. Neurology, 97: e236-47.*https://doi.10.1212/WNL.0000000000012227*

Devinsky, O., Vezzani, A., Najjar, S., De Lanerolle, N. C., & Rogawski, M. A. (2013). Glia and epilepsy: excitability and inflammation. Trends Neurosci, 36: 174-84.*https://doi.10.1016/j.tins.2012.11.008*

Falco-Walter, J. (2020). Epilepsy-Definition, Classification, Pathophysiology, and Epidemiology. Seminars in neurology, 40: 617-23.*https://doi.10.1055/s-0040-1718719*

Feng, X., Xiong, W., Yuan, M., Zhan, J., Zhu, X., Wei, Z., Chen, X., & Cheng, X. (2019). Down-regulated microRNA-183 mediates the Jak/Stat signaling pathway to attenuate hippocampal neuron injury in epilepsy rats by targeting Foxp1. Cell Cycle, 18: 3206-22. https://doi.10.1080/15384101.2019.1671717

Galan, F. N., Beier, A. D., & Sheth, R. D. (2021). Advances in Epilepsy Surgery. Pediatric neurology, 122: 89-97.*https://doi.10.1016/j.pediatrneurol.2021.06.004*

Gunda, B., Mine, M., Kovacs, T., Hornyak, C., Bereczki, D., Varallyay, G., Rudas, G., Audrezet, M. P., & Tournier-Lasserve, E. (2014). COL4A2 mutation causing adult onset recurrent intracerebral hemorrhage and leukoencephalopathy. J Neurol, 261: 500-3. https://doi.10.1007/s00415-013-7224-4

Han, C. L, Ge, M., Liu, Y. P., Zhao, X. M., Wang, K. L., Chen, N., Meng, W. J., Hu, W., Zhang, J. G., & Li, L. (2018). LncRNA H19 contributes to hippocampal glial cell activation via JAK/STAT signaling in a rat model of temporal lobe epilepsy. J Neuroinflammation, 15: 103.*https://doi.10.1186/s12974-018-1139-z*

Hausman-Kedem, M., Ben-Sira, L., Kidron, D., Ben-Shachar, S., Straussberg, R., Marom, D., Ponger, P., Bar-Shira, A., Malinger, G., & Fattal-Valevski, A. (2021). Deletion in COL4A2 is associated with a three-generation variable phenotype: from fetal to adult manifestations. Eur J Hum Genet, 29: 1654-62. https://doi.10.1038/s41431-021-00880-3

Ha, T. T., Sadleir, L. G., Mandelstam, S. A., Paterson, S. J., Scheffer, I. E., Gecz, J., & Corbett, M. A. (2016). A mutation in COL4A2 causes autosomal dominant porencephaly with cataracts. Am J Med Genet A, 170A: 1059-63.*https://doi.10.1002/ajmg.a.37527*

Hixson, K. M., Cogswell, M., Brooks-Kayal, A. R., & Russek, S. J. (2019). Evidence for a non-canonical JAK/STAT signaling pathway in the synthesis of the brain's major ion channels and neurotransmitter receptors. BMC genomics, 20: 677. https://doi.10.1186/s12864-019-6033-2

Itai, T., Miyatake, S., Taguri, M., Nozaki, F., Ohta, M., Osaka, H., Morimoto, M., Tandou, T., Nohara, F., & Takami, Y. (2021). Prenatal clinical manifestations in individuals with COL4A1/2 variants. J Med Genet, 58: 505-13.*https://doi.10.1136/jmedgenet-2020-106896*

Jaskiewicz, A., Domoradzki, T., & Pajak, B. (2020). Targeting the JAK2/STAT3 Pathway-Can We Compare It to the Two Faces of the God Janus?. Int J Mol Sci, 21 (21):8261. https://doi.10.3390/ijms21218261

Jeanne, M., Labelle-Dumais, C., Jorgensen, J., Kauffman, W. B., Mancini, G.M., Favor, J., Valant, V., Greenberg, S. M., Rosand, J., & Gould, D. B. (2012). COL4A2 mutations impair COL4A1 and COL4A2 secretion and cause hemorrhagic stroke. Am J Hum Genet, 90: 91-101.*https://doi.10.1016/j.ajhg.2011.11.022*

Karve, I. P., Taylor, J. M., & Crack, P. J. (2016). The contribution of astrocytes and microglia to traumatic brain injury. Br J Pharmacol, 173: 692-702. https://doi.10.1111/bph.13125

Lee, H. S., Han, J., Lee, S. H., Park, J. A., & Kim, K. W. (2010). Meteorin promotes the formation of GFAP-positive glia via activation of the Jak-STAT3 pathway. J Cell Sci, 123: 1959-68. https://doi.10.1242/jcs.063784

Li, X., Yang, C., Shi, Y., Guan, L., Li, H., Li, S., Li, Y., Zhang, Y., & Lin, J. (2021). Abnormal neuronal damage and inflammation in the hippocampus of kainic acid-induced epilepsy mice. Cell Biochem Funct, 39: 791-801. https://doi.10.1002/cbf.3651

Liu, Y., Zhang, J., Chen, Y., Sohel, H., Ke, X., Chen, J., & Li, Y. X. (2020). The correlation and role analysis of COL4A1 and COL4A2 in hepatocarcinogenesis. Aging (Albany NY). 2020, 204-23.*https://doi.10.18632/aging.102610*

Maurice, P., Guilbaud, L., Garel, J., Mine, M., Dugas, A., Friszer, S., Maisonneuve, E., Moutard, M. L., Coste, T., & Héron, D. (2021). Prevalence of COL4A1 and COL4A2 mutations in severe fetal multifocal hemorrhagic and/or ischemic cerebral lesions. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology, 57: 783-9. https:// doi.10.1002/uog.22106

Meuwissen, M. E., Halley, D. J., Smit, L. S., Lequin, M. H., Cobben, J. M., de Coo, R., van Harssel, J., Sallevelt. S., Woldringh, G., & van der Knaap, M. S. (2015). The expanding phenotype of COL4A1 and COL4A2 mutations: clinical data on 13 newly identified families and a review of the literature. Genet Med, 17: 843-53. https://doi.10.1038/gim.2014.210

Neri, S., Ferlazzo, E., Africa, E., Versace, P., Ascoli, M., Mastroianni, G., Cianci, V., Aguglia, U., & Gasparini, S. (2021). Novel COL4A2 mutation causing familial malformations of cortical development. Eur Rev Med Pharmacol Sci, 25: 898-905.*https://doi.10.26355/eurrev_202101_24658*

Perucca, P., Scheffer, I. E., & Kiley, M. (2018). The management of epilepsy in children and adults. The Medical journal of Australia, 208: 226-33. https://doi.org/10.5694/mja17.00951

Savaş, M., Tzartos, J., Küçükali, C., Dursun, E., Karagiorgou, K., Gezen-Ak, D., Türkdoğan, D., Papaconstantinou, A., Başoğlu, S., & Hacıhafızoğlu, N. (2021). Glial fibrillary acidic protein (GFAP)-antibody in children with focal seizures of undetermined cause. Acta Neurol Belg, 121: 1275-80.*https://doi.10.1007/s13760-020-01361-y*

Shen, W., Poliquin, S., Macdonald, R. L., Dong, M., & Kang, J. Q. (2020). Endoplasmic reticulum stress increases inflammatory cytokines in an epilepsy mouse model Gabrg2(+/Q390X) knockin: A link between genetic and acquired epilepsy?. Epilepsia, 61: 2301-12. https://doi.10.1111/epi.16670

Sun, L. H., Yan, M. L., Hu, X. L., Peng, L. W., Che, H., Bao, Y. N., Guo, F., Liu, T., Chen, X., & Zhang, R. (2015). MicroRNA-9 induces defective trafficking of Nav1.1 and Nav1.2 by targeting Navβ2 protein coding region in rat with chronic brain hypoperfusion. Mol Neurodegener, 10: 36. https://doi.10.1186/s13024-015-0032-9

Steriade, C., French, J., & Devinsky, O. (2020). Epilepsy: key experimental therapeutics in early clinical development. Expert Opin Investig Drugs, 29, 373-83.*https://doi.org/10.1080/13543784.2020.1743678*

Terrone, G., Salamone, A., & Vezzani, A. (2017). Inflammation and Epilepsy: Preclinical Findings and Potential Clinical Translation. Curr Pharm Des, 23: 5569-76. https://doi.10.2174/1381612823666170926113754

Thijs, R. D., Surges, R., O'Brien, T. J., & Sander, J. W. (2019). Epilepsy in adults. Lancet, 393: 689-701. https://doi.org/10.1016/S0140-6736(18)32596-0

Verbeek, E., Meuwissen, M. E., Verheijen, F. W., Govaert, P. P., Licht, D. J., Kuo, D. S., Poulton, C. J., Schot, R., Lequin, M. H., & Dudink, J. (2012). COL4A2 mutation associated with familial porencephaly and small-vessel disease. Eur J Hum Genet, 20: 844-51. https://doi.10.1038/ejhg.2012.20

Vezzani, A., Aronica, E., Mazarati, A., & Pittman, Q. J. (2013). Epilepsy and brain inflammation. Exp Neurol, 244: 11-21. https://doi.10.1016/j.expneurol.2011.09.033

Xian, P., Hei, Y., Wang, R., Wang, T., Yang, J., Li, J., Di, Z., Liu, Z., Baskys, A., & Liu, W. (2019). Mesenchymal stem cell-derived exosomes as a nanotherapeutic agent for amelioration of inflammation-induced astrocyte alterations in mice. Theranostics, 9: 5956-75. https://doi.10.7150/thno.33872

Wang, H., Yao, G., Li, L., Ma, Z., Chen, J., & Chen, W. (2020). LncRNA-UCA1 inhibits the astrocyte activation in the temporal lobe epilepsy via regulating the JAK/STAT signaling pathway. J Cell Biochem, 121: 4261-70. https://doi.10.1002/jcb.29634

Wu, Y. J., Chien, M. E., Huang, C. H., Chiang, C. C., Lin, C. C., Huang, C. W., Durand, D. M., & Hsu, K. S. (2020). Transcranial direct current stimulation alleviates seizure severity in kainic acid-induced status epilepticus rats. Exp Neurol, 328: 113264.

https://doi.10.1016/j.expneurol.2020.113264

Yaramis, A., Lochmüller, H., Töpf, A., Sonmezler, E., Yilmaz, E., Hiz, S., Yis, U., Gungor, S., Ipek, P. A., & Edem, P. (2020). COL4A1-related autosomal recessive encephalopathy in 2 Turkish children. Neurology Genetics, 6: e392. https://doi.10.1212/NXG.0000000000000392

Yeung, Y. T., Aziz, F., Guerrero-Castilla, A., Arguelles, S. (2018). Signaling Pathways in Inflammation and Anti-inflammatory Therapies. Curr Pharm Des, 1449-1484.*https://doi.10.2174/1381612824666180327165604*

Yu, H., Lee, H., Herrmann, A., Buettner, R., & Jove, R. (2014). Revisiting STAT3 signalling in cancer: new and unexpected biological functions. Nat Rev Cancer, 14: 736-46.*https://doi.10.1038/nrc3818*

Yu, H., Pardoll, D., & Jove, R. (2009). STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer, 9: 798-809. https://doi.10.1038/nrc2734

Zagaglia, S., Selch, C., Nisevic, J. R., Mei, D., Michalak, Z., Hernandez-Hernandez, L., Krithika, S., Vezyroglou, K., Varadkar, S. M., & Pepler, A. (2018). Neurologic phenotypes associated with COL4A1/2 mutations: Expanding the spectrum of disease. Neurology, 91: e2078-e88. https://doi.10.1212/WNL.0000000000006567

Zegeye, M. M., Lindkvist, M., Fälker, K., Kumawat, A. K., Paramel, G., Grenegård, M., Sirsjö, A., & Ljungberg, L. U. (2018). Activation of the JAK/STAT3 and PI3K/AKT pathways are crucial for IL-6 trans-signaling-mediated pro-inflammatory response in human vascular endothelial cells. Cell Commun Signal, 16(1): 55. https://doi.10.1186/s12964-018-0268-4

Figure Legends

FIGURE 1 COL4A2 mutation induces the exacerbation of the seizures and contributes to learning and memory impairments in epilepsy rats. Rats were stimulated with KA and treated with wild type (WT) or mutated (MUT) COL4A2. The behaviors were evaluated by Morris water maze test (n = 5 in each group). The (A) times and (B) duration of seizures of rats. (C) The escape time of rats at the 5th day of training. (D) Swimming speed of rats in the space exploration test. (E and F) The (E) swimming path and (F) swimming distance of the rats in the target quadrant of the platform. (G) The residence time of rats in the platform. (H) Calculation of times the rats pass crossed the platform. (I and J) The (I) protein and (J) RNA levels of iNOS, COX-2, IL-1β, IL-6, and TNF-α in hippocampus tissues. (K) ELISA analysis of IL-1β, IL-6, and TNF-α. WT: wild type COL4A2; MUT: mutated COL4A2. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs sham; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ vs KA.

FIGURE 2 COL4A2 mutation induces hippocampal astrocyte activation and neuronal injury in epilepsy rats. (A) Nissl staining analysis. (B) Immunofluorescence analysis of GFAP in hippocampus; Green: GPAF; Blue: DAPI. (C) Western blotting analysis of GFAP in hippocampus. (D) Western blot analysis of JAK2, p-JAK2, STAT3, p-STAT3 in hippocampus of the rats.

FIGURE 3 COL4A2 mutation promotes inflammation and astrocyte activation in primary astrocytes. The primary astrocytes were stimulated with LPS and treated with wild type (WT) or mutated (MUT) COL4A2. (A) Viability of primary astrocytes was measured by CCK-8. (B) Apoptosis of primary astrocytes was measured by flow cytometry. (C) Western blotting analysis of GFAP in primary astrocytes. (D-H) The RNA levels of COX-2, iNOS, IL-1β, IL-6, and TNF-α were measured by qPCR. (I-K) ELISA analysis of secretion of IL-1β, IL-6, and TNF-α. (L and M) Western blot analysis of inflammatory cytokines and JAK2, p-JAK2, STAT3, p-STAT3. *$P < 0.05$; ** $P < 0.01$; *** $P< 0.001$ vs control; # $P < 0.05$; ##$P < 0.01$ vs LPS.

FIGURE 4 COL4A2 mutation stimulates JAK/STAT signaling *in vivo*. Rats were stimulated with KA and treated with wild type (WT) or mutated (MUT) COL4A2 or WP1066. The (A) times and (B) duration of seizures of rats. The behaviors were evaluated by Morris water maze test (n = 5 in each group). (C) The escape time of rats at the 5th day of training. (D) Swimming speed of rats in the space exploration test. (E and F) The (E) swimming path and (F) swimming distance of the rats to find the target quadrant of the platform. (G) The residence time of rats in the platform. (H) Calculation of times the rats pass crossed the platform. (I and J) The (I) protein and (J) RNA levels of iNOS, COX-2, IL-1β, IL-6, and TNF-α in hippocampus tissues. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

FIGURE 5 COL4A2 mutation stimulates JAK/STAT signaling in neurons *in vivo*. (A) Nissl staining analysis. (B) Immunofluorescence analysis of GFAP in hippocampus; Green: GPAF; Blue: DAPI. (C) Western blotting analysis of GFAP in hippocampus. (D) Western blot analysis of JAK2, p-JAK2, STAT3, p-STAT3 in hippocampus of the rats.

FIGURE 6 COL4A2 mutation promotes inflammation and astrocyte activation by stimulating JAK/STAT signaling in primary astrocytes. The primary astrocytes were stimulated with LPS and treated with wild type (WT), mutated (MUT) COL4A2 or WP1066. (A) Western blotting analysis of GFAP in primary astrocytes. (B-F) The RNA levels of COX-2, iNOS, IL-1β, IL-6, and TNF-α were measured by qPCR. (I-K) ELISA analysis of secretion of IL-1β, IL-6, and TNF-α. (G and H) Western blot analysis of inflammatory cytokines and JAK2, p-JAK2, STAT3, p-STAT3. * $P< 0.05$; ** $P < 0.01$; *** $P < 0.001$.

FIGURE S1 Identification of COL4A2 mutation. (A-B) Video EEG showed hypsarrhythmia during sleep and clusters of epileptic spasms at 6 month. (C) T1 Brain MRI was normal at 6 months. (D) One de novo heterozygous missense mutation was detected in the COL4A2 gene: c.1148C>T(p.Pro383Leu).

FIGURE S2 COL4A2 mutation promotes inflammation and astrocyte activation in CTX-TNA cells. The CTX-TNA cells were stimulated with LPS and treated with wild type (WT) or mutated (MUT) COL4A2. (A) Viability of CTX-TNA cells were measured by CCK-8. (B) Western blotting analysis of GFAP in CTX-TNA cells. (C-G) The RNA levels of COX-2, iNOS, IL-1β, IL-6, and TNF-α were measured by qPCR. (H-J) ELISA analysis of secretion of IL-1β, IL-6, and TNF-α. (K and L) Western blot analysis of inflammatory cytokines and JAK2, p-JAK2, STAT3, p-STAT3. * $P< 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs control; # $P < 0.05$; ## $P< 0.01$ vs LPS.

FIGURE S3 COL4A2 mutation promotes inflammation and astrocyte activation by stimulating JAK/STAT signaling in CTX-TNA cells. The CTX-TNA cells were stimulated with LPS and treated with wild type (WT), mutated (MUT) COL4A2 or WP1066. (A) Western blotting analysis of GFAP in CTX-TNA cells. (B-F) The RNA levels of COX-2, iNOS, IL-1β, IL-6, and TNF-α were measured by qPCR. (G and H) Western blot analysis of inflammatory cytokines and JAK2, p-JAK2, STAT3, p-STAT3. * $P< 0.05$; *** $P < 0.001$.