De novo mutations in GFAP cause Alexander disease: clinical features, fMRI and functional analysis

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Title page

De novo mutations in *GFAP* cause Alexander disease: clinical features, fMRI and functional analysis

Running head: De novo mutations in Alexander disease

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Abstract

Background: Alexander disease (AxD) is a progressive and fatal neurological disorder characterized by white matter degeneration and Rosenthal fibers inclusions, which was caused by *GFAP* mutations. Since the first description of AxD, more than 550 cases have been reported. We identified two patients with de novo mutations in *GFAP* gene causing bulbospinal AxD, and further investigated the pathogenicity of identified variants.

Results: Two de novo mutations in *GFAP* gene were identified (c.214G>A, p.E72K and c.1235C>T, p.T412I) by whole exome sequencing, of which c.1235C>T was the first reported. Clinical assessment revealed that both probands similarly presented bulbar symptoms, pyramidal signs and white matter atrophy in periventricular regions. We conducted a novel data-driven method to analyze fMRI characteristics. Increased brain functional connectivity in occipital and posterior parietal cortex was first discovered in AxD. Grey matter atrophy was also observed in one patient. Functional studies showed that the solubility of p.T412I GFAP was lower than wild type, while p.E72K and p.T412I were at a similar level to wild type, suggesting that mutations located in the tail domain could decrease the solubility of GFAP. Abnormal inclusions of mutant GFAP were colocalized with ubiquitin, 20S proteasome, protein 1 light chain 3-II (LC3-II) and lysosome. Western blotting indicated the ubiquitin-proteasome pathway was blocked while autophagy was activated as a mechanism for degrading aggregates.

Conclusions: *GFAP* mutations identified in our study were associated with AxD and accompanied by protein functional alterations. Our findings further expand the clinical and genetic spectrum of AxD.

Keywords: Alexander disease, GFAP, mutation, autophagy, accumulation

Background

Alexander disease (AxD) is a progressive and fatal neurological disorder characterized by white matter degeneration and astrocytic cytoplasmic inclusions, termed Rosenthal fibers(RFs). These inclusions contain glial fibrillary acidic protein (GFAP), small heat shock proteins hsp27 and α B-crystallin. In 2001, Messing and colleagues identified *GFAP* as the candidate gene for AxD and act in a gain-of-function manner [1]. Since then, more than 550 cases have been reported, mostly missense mutations[2]. In general, AxD was determined as infantile, juvenile and adult forms. However, nonspecific symptoms seen in infantile and adult AxD may lead to misdiagnosis. Yoshida et al. proposed a new guideline which based on the neurological and MRI findings, of which AxD has been classified into three types: cerebral form (type 1), bulbospinal form (type 2) and intermediate form (type 3). Type 1 is characterized by delayed psychomotor development, convulsions, macrocephaly and leukoencephalopathy with frontal lobe predominance on brain imaging. Type 2 patients with atrophy of medulla oblongata or cervical cord on MRI generally showed muscle weakness, hyperreflexia and distinct bulbar dysfunction. Type 3 is an intermediate form which contains various symptoms of type 1 and type 2 [3]. Whereas the featured clinical manifestations and brain MRI provide clues to diagnosis, the definitive diagnosis for AxD still requires genetic analysis or brain biopsy.

The pathogenic mechanisms responsible for AxD remain undefined. Mutated GFAP could implicate intracellular vesicle regulation and organelle distribution, leading to abnormal accumulations of eosinophilic cytoplasmic inclusions [4]. Aggregate-prone proteins unsuccessfully corrected by chaperones are likely to be ubiquitylated and subsequently recognized by protein degrading pathways, such as the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP) [5]. However, mutant GFAP can inhibit proteasome activity and lead to increased levels of ubiquitinated

proteins. This inhibition in turn activates intracellular stress response, which up-regulate transcription and activate autophagy to regulate protein levels.

Here, we identified two patients with de novo mutations in *GFAP* gene causing bulbospinal AxD. Based on detailed clinical, neuroimaging and genetic analysis, functional investigations were further conducted to determine whether identified variants are disease-causing.

Results

Clinical findings

Patient P3433 was a 29-year-old woman featured as gait disturbance with a 19-year-duration. She had no issues of developmental retardation or psychomotor abnormalities. Initially, she presented with gait disorders caused by weakness in left lower limb. She was diagnosed with scoliosis (Fig. 1A) at the age of 14. Then she received corrective surgery for scoliosis (Fig. 1B) due to progressive walking difficulties. Likewise, her brain imaging showed mild leukoencephalopathy. With disease progression, she had to rely on a wheelchair because of spasticity and balance impairment. Meanwhile, she began to suffer dysarthria, urinary dysfunction and aspiration pneumonia. There was no consanguineous marriage in her family. Neurological examination revealed strabismus, extraocular muscle movement disorder, several beats of bilaterally horizontal nystagmus and cerebellar ataxia. Muscle tension and strength of lower extremities were significantly decreased. Tendon reflexes were normal in upper limbs and absent in lower limbs. The bilaterally pathologic signs were positive. She showed dysmetria in the finger-to-nose test on both sides. Her sensory system and cognitive functions were normal. Laboratory examinations revealed unremarkable findings. A de novo missense mutation in *GFAP* gene (NM_002055), c.214G>A (p.E72K) (Fig. 1C), which had been reported was identified in our study [6]. This variant was absent in 1000 Genome Project (1000g, <http://browser.1000genomes.org>), Exome

Aggregation Consortium (ExAC), gnomAD (<http://gnomad-old.broadinstitute.org>) and our controls. p.E72K was predicted damaging by PolyPhen2 (probability score 0.945), damaging by SIFT (score: 0.001) and disease-causing by Mutationtaster (probability score 1.000). According to the ACMG Guidelines, this variant was classified as likely pathogenic.

Patient P4288 was a 33-year-old man suffering slurred speech and progressive gait disturbance for two-year duration. At the beginning, he displayed an abnormal gait and external rotation of the left foot. Imaging examinations revealed thoracic spinal cord thinning and tethered cord syndrome. Surgical decompression of cauda equina nerve was performed and symptoms were slightly improved after operation. About one year later, he presented with swaying while walking caused by progressive spasticity and muscle weakness. He started to rely on a wheelchair and showed dysphagia occasionally. Physical examinations revealed speech disfluency and horizontal nystagmus. Muscle strength in lower limbs were decreased. Muscle tension of upper limbs was increased, with the left-side relatively higher. Pathologic reflexes of both sides were positive. Examinations of coordinate movement were difficult to complete. There was no significant abnormality in cognition and mental states. Laboratory investigations were nearly normal. A novel variant, c.1235C>T (p.T412I) (Fig. 1D), were detected in patient P4288 which was absent in 1000g, ExAC and gnomAD, as well as controls. SIFT (score 0.001), Polyphen2 (score 0.519) and mutationtaster (probability score 1.000) predicted the variants were damaging, possibly damaging and disease-causing, respectively. According to ACMG Guidelines, it was classified as likely pathogenic variant.

Neuroimaging findings

The brain MR imaging of the two probands displayed WM lesions in the bilateral corona radiata, centrum semiovale and the regions surrounding the 4th ventricle (Fig. 1E to F). Volume estimation

based on WM tissue probability map in CAT12 suggested 17.33 ml of WM hyperintensity in patient P3433, while the TIV was 1218.15 ml. Comparatively, minor WM lesions were found in patient P4288.

The ages of control group ranged from 23 to 45 years old, with an average of 30.4 and standard deviation of 7.5. The averaged TIV was 1481.34 ml, with standard deviation of 113.83 ml. After adjusting for the TIVs, patient P3433 had atrophy mainly in the bilateral putamen and thalamus in the GM analysis. Consistent with leukodystrophies in the Alexander disease, atrophic WM was observed in the corona radiata, centrum semiovale, cerebellopontine angle and medulla. Patient P4288 presented similar pattern of atrophy in WM, while no significant GM difference was observed (Fig. 1G to H).

Regarding the neural activity, higher ALFF in cerebellar vermis, cerebellopontine angles, occipital and posterior parietal cortex was observed in patient P3433, overlapping with higher DC and Reho in cerebellum and occipital cortex. Increased DC distributed in both frontal and posterior parietal cortex. In P4288, higher ALFF, DC and Reho was overlapped in similar regions. Increased DC was additionally observed in the bilateral insula (Fig. 1I to J).

Variants functional analysis

The protein levels of GFAP-WT/Mut were detected by transfecting GFAP-GFP plasmids into HEK293T cells. The soluble GFAP of p.T412I group was lower than the WT group; on the contrary, p.T412I group showed a higher level in the insoluble fraction than the WT. There was no significant level difference between WT, p.E72K and positive control (p.R239C) in the soluble and insoluble fractions (Fig. 2A). In immunostaining, the WT exhibited diffuse distribution with a few aggregates of GFAP proteins throughout cytoplasm, while mutant GFAP (p.E72K, p.T412I and positive control) appeared as punctate aggregations in perinuclear areas (Fig. 2B).

Treated with proteasome inhibitor (MG132), GFAP proteins trended to decline especially in WT,

p.E72K and positive control groups in the soluble fraction, while the 20S proteasome complexes remained unchanged among all groups (Fig. 2C). Meanwhile, GFAP in mutant and positive control groups were completely colocalized with ubiquitin and 20S, while in WT group these proteins distributed uniformly in cytoplasm with no colocalization (Fig. 2D to E). We also tested the autophagy flux by lysosome inhibitor (BafAI). After treatment, the increased level of LC3-II was observed in soluble mutant groups and positive control, while the overexpression of GFAP-WT could partly contribute to autophagy (Fig. 2F). Furthermore, the soluble GFAP level of mutant groups showed a upregulated tendency after BafAI treated, especially in the p.T412I and positive control group (Fig. 2G). By immunofluorescence, mutant GFAP nearly co-expressed with LC3 and lysosome (labeled by LAMP1) (Fig. 2H to I).

Discussion

Before the present study, a total of 135 mutations in *GFAP* gene (NM_002055) have been reported, including 122 missense mutations, 1 splicing, 2 regulatory, 3 small deletion, 5 small insertion and 3 small indel (Fig. 3), according to the Human Gene Mutation Database. The GFAP protein comprises an N-terminal head domain, a central rod domain and a C-terminal tail domain, which are important for assembling cellular intermediate filament (diameter, 10nm) [7]. The rod domain manifesting a lower conservation in intermediate filaments contains four α -helical segments (1A, 1B, 2A and 2B) [8]. Pathogenic mutations were scattered all over the domains of GFAP protein. However, the genotype-phenotype correlations appear to be complicated. The variants affecting the hot spot amino acids (R79, R88, R239, R416) accounted for more than half of identified patients [6]. The R79, R88 and R239 mutations are probably associated with early-onset patients [3], while R239 mutations could induce more severe manifestations [6]. Moreover, there is a great deal of variability in clinical

severity of AxD, even among individuals carrying the same mutation. Patient P3433 with p.E72K (c.214G>A) mutation which is located close to R79, showed symptoms relatively earlier and more in line with the adult-onset form, such as bulbar symptoms, autonomic dysfunction and gait disturbance. Patient P4288 with p.T412I (c.1235C>T) variant, located close to R416, presented a rapidly progressive clinical course. Analysis of the patients with an R416 change showed no significant genotype-phenotype associations, supporting that mutations in tail domain of GFAP exhibit varied clinical courses and severities [8]. What accounts for these variabilities remains obscure. There might be genetic modifiers or environmental factors affect clinical phenotypes [9].

A novel data-driven method was used in our study to explore the atrophic pattern and spontaneous brain functional network activity in AxD patients. Patient P3433 and P4288 showed a similar pattern of atrophy in WM mainly involving periventricular regions. It has been proposed that the subventricular region was most vulnerable to the pathogenesis of AxD [10]. About one-third of the patients displayed abnormal signals in the periventricular rim [11] which may be associated with the abnormal aggregation of Rosenthal fibers in subependymal regions [12]. Moreover, patient P3433 with early age onset also presented GM atrophy mainly in the bilateral putamen and thalamus, which has not been reported in AxD. GM volume loss may be related to long-term disability [13] and interprets physical disabilities better than WM lesions [14]. Several mechanisms may underlie the GM damage including iron deposition, mitochondrial failure, WM lesions induced retrograde degeneration and meningeal inflammation [15, 16]. Patient P3433 suffered more severe WM atrophy, thus the overload and collapse of brain network could also explain the atrophy of GM [17]. For the first time, we evaluated alterations in spontaneous brain functional network activities in our patients by three different types of data-driven analyses, ReHo, DC and ALFF. The results showed the ReHo, DC and ALFF values seem to be

consistently increased in occipital and posterior parietal cortex, indicating a higher amount of communication between these regions and the rest of the brain network. Increased functional connectivity has been interpreted as a compensatory mechanism or reorganization of the brain work [18].

It is notable that variant p.T412I induced abnormal aggregations and significantly decreased solubility of GFAP, similar to the consequences caused by variant p.S393I (c.1178G>T) [19] and p.R416W (c.1246C>T) [20]. Both p.T412I, p.S393I and p.R416W are located in tail domain which is highly conserved and important for stabilizing filament-filament interactions [21]. Filament disorganizations may enhance the stability of the assembled protein, which could result in resistance to salt extraction [22]. HEK 293T cells contain endogenous intermediate filaments such as vimentin and keratin which may have effect on the assembly of filaments and GFAP network formation [22], but more research was needed. We likewise observed 20S complexes colocalized with ubiquitylated GFAP accumulations, indicating mutation did not disrupt GFAP binding to 20S complexes, but the degradation of GFAP through the UPS seemed to be blocked. Previous studies demonstrated that overexpression of GFAP impairs proteasome activities and results in decreased protein turnover [23], especially mutant oligomer GFAP proteins produce a stronger inhibition [24]. The loss of proteasome activity may also arise from the shift of 20S complexes from soluble fraction to insoluble fraction [24]. Proteasome dysfunction may establish a positive feedback loop that would further increase GFAP protein levels and accumulations [23]. However, some studies showed proteasome inhibitors could induce the reduction of GFAP gene transcription and other intermediate filaments expression [25], as we observed a decrease in GFAP levels in HEK 293T cells. This effect may only occur during the early stage (not after 7 days) of GFAP expression which is more susceptible to proteasome inhibition [24],

while the relationship between proteasome inhibition and GFAP still needs explorations. As we had seen, the degradation of GFAP mutants could be restored by lysosome inhibitor. Autophagy acts as a complementary route to degrading aggregated proteins in this disease [26]. The GFP-tags and overexpression of GFAP plasmid may increase the formation of GFAP accumulations and then increase the pathology [23], which increased the difficulty to detect the differences between WT and mutants. It remains to be undefined whether other compensatory mechanisms exist for regulating the damaged proteins.

Conclusions

To summarize, we identified two cases of bulbospinal AxD patients presenting with bulbar symptoms and pyramidal tract signs. Two de novo variants (c.214G>A, p.E72K and c.1235C>T, p.T412I) in *GFAP* gene were identified, and one novel variant (c.1235C>T) was first described. Increased brain functional connectivity in occipital and posterior parietal cortex was discovered in two probands, while grey matter atrophy mainly in the bilateral putamen and thalamus was observed in one patient, broadening the spectrum of clinical phenotypes of AxD. *GFAP* mutations lead to abnormal accumulations and proteasome dysfunction, as well as autophagy. The autophagy-lysosome pathway plays a role in degrading toxic GFAP accumulations to maintain intracellular homeostasis.

Materials and Methods

Participants

Two probands (P3433 and P4288) identified with AxD were enrolled. This study had received ethics approval from the Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China. All participants had signed informed consents.

Genetical analysis

Genomic DNA was extracted by phenol-chloroform method. 200 healthy subjects were enrolled as normal controls. Whole exome sequencing was performed for 2 probands. The human genome reference sequence (GRCh37/hg19) were used to map the sequence reads. On the basis of public databases of normal human variation (1000g, ExAC and gnomAD), all the variants of which Minor Allele Frequency (MAF) is higher than 1% were filtered. Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2>), SIFT (<http://provean.jcvi.org>) and Mutationtaster (<http://www.mutationtaster.org>) were used to predict the pathogenicity of mutation. American College of Medical Genetics and Genomics (ACMG) Guidelines were used to interpret and classify variants. Sanger sequencing was carried out to confirm the putatively pathogenic variants.

MRI acquisition, preprocessing and statistical analysis

Two probands were scanned on a magnetic resonance system (Ingenia, Philips MR system, Dutch) with an 8-channel based-array head coil, with 15 normal individuals (5 male, 10 female participants) as controls. The protocol included a 3-dimension high-resolution turbo field echo T1-weighted sequence for neuroanatomy (sagittal slice orientation; matrix = 256×256; repetition time = 7.2 ms; echo time = 3.3 ms; flip angle = 7°; slice thickness=1 mm; slice number = 192). Resting-state blood oxygen level dependent (BOLD) MRI used T2*-weighted echo planar imaging sequence (240 functional images, sagittal slice orientation; 39 slices; slice thickness = 3.5 mm; matrix = 64×64; repetition time = 2000 ms; echo time = 30 ms; flip angle = 90°). The two patients also had T2-weighted fluid attenuated inversion recovery (FLAIR) sequence to recognize white matter lesions better.

The T1-weighted anatomical image was firstly segmented into grey matter (GM), white matter (WM) and cerebrospinal fluid using computational anatomy toolbox (CAT) 12 (<http://www.neuro.uni-jena.de>) within SPM 12 (v6685; <http://fil.ion.ucl.ac.uk/spm>) in MATLAB 2014b

environment (<https://www.mathworks.com>), with reference to tissue probabilistic maps in MNI (Montreal Neurological Institute) space. WM hyperintensity was also estimated by GM-WM tissue probability map in CAT12. Voxel based morphology analysis was performed between each patient and normal controls by t test for GM and WM respectively, with total intracranial volume (TIV) as covariate. For multiple comparison correction, we used false discovery rate (FDR) correction at $p < 0.05$.

Regarding the fMRI, the first 40 fMRI images of each subject were discarded. The remaining 200 images were realigned to adjust head motion, co-registered to the anatomical image and normalized into MNI space using a modified MATLAB toolbox “Data Processing & Analysis of Brain Imaging (DPABI, version 3.0)” [27]. We computed mean amplitude of low frequency fluctuation (ALFF) to represent individuals’ regional neural activities [28], regional homogeneity (Reho) and degree centrality (DC) representing the quantity of functional connections of a region [29, 30]. Individual participants’ weighted DC values were from for all voxels in standard space with DPABI. Mean ALFF and voxel-wise centrality values were also compared between one patient and controls with t test adopting FDR of $p < 0.05$, with voxel-based morphometry of GM as covariate volume.

Cell culture and transfection

The cDNAs for identified variants in *GFAP* (NM_002055) were inserted into the pcDNA3.1-GFP plasmids to express GFP-tagged fusion proteins. HEK 293T cells were grown in Dulbecco’s modified Eagle’s medium supplement with 10 % Fetal bovine serum (FBS) (Gibco) and 1% penicillin streptomycin (P/S) (Invitrogen). 24h after plating, cells were transfected with GFAP-GFP wild-type (GFAP-WT) or mutant (p.E72K and p.T412I) plasmid DNA using Lipofectamine 3000 transfection reagent (Invitrogen). Moreover, a hot spot mutation (c.715C>T, p.R239C) [8] identified as

disease-causing was set as positive control. 24h after transfection, cells were collected to extract proteins for western blot. As for pharmacological treatment, MG132 (proteasome inhibitor) (10 uM) [24] (Cell Signaling Technology) and Bafilomycin AI (BafAI, lysosomal inhibitor) (5nM) [31] (Sigma) were added to post-transfected cells respectively and incubated for 12h to extract proteins, with DMSO as the vehicle control.

Western blotting and immunofluorescence

Cells were collected and lysed in radioactive immunoprecipitation assay (RIPA) buffer (Beyotime) with protease inhibitors for protein extraction. After centrifuged at 13000 g for 20min under 4 °C, cell lysates were separated into two parts, the supernatant as the soluble fraction, and the sedimentation as the “insoluble” fraction. The insoluble fraction was dissolved by denaturing protein solubilization reagent (Invent). Equal amounts of protein were diluted in 6X SDS-PAGE Sample Loading Buffer (Beyotime) and were then separated by 12% SDS-PAGE. The protein levels of EGFP-GFAP were detected by anti-GFP antibody (1:2500, AVES). Anti-proteasome 20S (1:2000, Enzo Life Science) and anti-ubiquitin antibodies (1:1000, Cell Signaling Technology) were used for the analysis of the UPS. Anti-LC3B (1:1000, Cell Signaling Technology) and anti-LAMP1 antibodies (1:1000, Cell Signaling Technology) were used to detect the ALP. We chose 20S and LC3 to undergo western blot, respectively. Using anti-GAPDH antibody (1:1000, Cell Signaling Technology) to normalize sample loading and transfer. Then the blots were incubated with secondary HRP-conjugated antibodies (1:5000, Beyotime) and subjected to chemiluminescence detection.

For immunofluorescence, 48h after transfection, cells were fixed with 4% PFA, blocked with 10% serum and 0.3% Triton X-100 in PBS for 60min, and incubated with primary antibodies to detect the UPS and the ALP mentioned above in blocking solution at 4°C overnight. Then cells were stained with

the Alexa Fluor 594 secondary antibodies (1:1000, Life), and nucleic acid was stained with DAPI (1:10000, Life). Cells were visualized under a Zeiss 710 confocal microscope.

Statistical analysis

Results were analyzed statistically (mean $\pm$ S) using GraphPad Prism version 8.0.1 for Windows. One-way ANOVA was used to compare data of multiple experimental groups. Two-way ANOVA was used to analyze relative protein levels after two different treatments. For all tests, * represents as $p < 0.05$, ** as $p < 0.01$, and *** as $p < 0.001$.

Abbreviations

AxD: Alexander disease; GFAP: glial fibrillary acidic protein; UPS: ubiquitin-proteasome system; ALP: autophagy-lysosomal pathway; ACMG: American College of Medical Genetics and Genomics; GM: grey matter; WM: white matter; CAT: computational anatomy toolbox; TIV: total intracranial volume; DPABI: Data Processing & Analysis of Brain Imaging; MNI: Montreal Neurological Institute; ALFF: amplitude of low frequency fluctuation; Reho: regional homogeneity; DC: degree centrality; FDR: false discovery rate; WT: wild-type; Mut: mutant; BafAI: Bafilomycin A1

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Authors' contributions

XXS and JWJ were responsible for data acquisition, analysis and interpretation of data, study design, statistical analysis, drafting the manuscript. WTT, FXZ, ZYZ, XJH and XLL contributed to data acquisition, analysis and interpretation of data. BYL, HDT and LC designed this study and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and material

All the data used and analyzed for this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

Consent for publication

All patients and authors agreed on the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Reference

1. Brenner M, Johnson AB, Boespflug-Tanguy O, Rodriguez D, Goldman JE, Messing A. Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease. *Nature genetics*. 2001;27(1):117-20.
2. Srivastava S, Naidu S. Alexander Disease. 2002 Nov 15 [Updated 2015 Jan 8]. In: Adam MP, Ardinger HH, Pagon RA, et al., editors. *GeneReviews* ® [Internet]. Seattle (WA): University of

Washington, Seattle; 1993-2020.

3. Yoshida T, Sasaki M, Yoshida M, Namekawa M, Okamoto Y, Tsujino S, et al. Nationwide survey of Alexander disease in Japan and proposed new guidelines for diagnosis. *Journal of neurology*. 2011;258(11):1998-2008.
4. Jones JR, Kong L, Hanna MGt, Hoffman B, Krencik R, Bradley R, et al. Mutations in GFAP Disrupt the Distribution and Function of Organelles in Human Astrocytes. *Cell Rep*. 2018;25(4):947-58 e4.
5. Kwon YT, Ciechanover A. The Ubiquitin Code in the Ubiquitin-Proteasome System and Autophagy. *Trends Biochem Sci*. 2017;42(11):873-86.
6. Prust M, Wang J, Morizono H, Messing A, Brenner M, Gordon E, et al. GFAP mutations, age at onset, and clinical subtypes in Alexander disease. *Neurology*. 2011;77(13):1287-94.
7. Hol EM, Capetanaki Y. Type III Intermediate Filaments Desmin, Glial Fibrillary Acidic Protein (GFAP), Vimentin, and Peripherin. *Cold Spring Harbor Perspectives in Biology*. 2017;9(12):a021642.
8. Li R, Johnson AB, Salomons G, Goldman JE, Naidu S, Quinlan R, et al. Glial fibrillary acidic protein mutations in infantile, juvenile, and adult forms of Alexander disease. *Ann Neurol*. 2005;57(3):310-26.
9. Messing A. Alexander disease. *Handb Clin Neurol*. 2018;148:693-700.
10. Sawaishi Y. Review of Alexander disease: beyond the classical concept of leukodystrophy. *Brain Dev*. 2009;31(7):493-8.
11. Balbi P, Salvini S, Fundaro C, Frazzitta G, Maestri R, Mosah D, et al. The clinical spectrum of late-onset Alexander disease: a systematic literature review. *Journal of neurology*. 2010;257(12):1955-62.

- 353 12. van der Knaap MS, Ramesh V, Schiffmann R, Blaser S, Kyllerman M, Gholkar A, et al. Alexander
354 disease: ventricular garlands and abnormalities of the medulla and spinal cord. *Neurology*.
355 2006;66(4):494-8.
- 356 13. Eshaghi A, Prados F, Brownlee WJ, Altmann DR, Tur C, Cardoso MJ, et al. Deep gray matter
357 volume loss drives disability worsening in multiple sclerosis. *Ann Neurol*. 2018;83(2):210-22.
- 358 14. Roosendaal SD, Bendfeldt K, Vrenken H, Polman CH, Borgwardt S, Radue EW, et al. Grey
359 matter volume in a large cohort of MS patients: relation to MRI parameters and disability. *Mult Scler*.
360 2011;17(9):1098-106.
- 361 15. Eshaghi A, Marinescu RV, Young AL, Firth NC, Prados F, Jorge Cardoso M, et al. Progression of
362 regional grey matter atrophy in multiple sclerosis. *Brain*. 2018;141(6):1665-77.
- 363 16. Calabrese M, Magliozzi R, Ciccarelli O, Geurts JJ, Reynolds R, Martin R. Exploring the origins
364 of grey matter damage in multiple sclerosis. *Nat Rev Neurosci*. 2015;16(3):147-58.
- 365 17. Minagar A, Barnett MH, Benedict RH, Pelletier D, Pirko I, Sahraian MA, et al. The thalamus and
366 multiple sclerosis: modern views on pathologic, imaging, and clinical aspects. *Neurology*.
367 2013;80(2):210-9.
- 368 18. Widjaja E, Zamyadi M, Raybaud C, Snead OC, Smith ML. Abnormal functional network
369 connectivity among resting-state networks in children with frontal lobe epilepsy. *AJNR Am J*
370 *Neuroradiol*. 2013;34(12):2386-92.
- 371 19. Chen YS, Lim SC, Chen MH, Quinlan RA, Perng MD. Alexander disease causing mutations in
372 the C-terminal domain of GFAP are deleterious both to assembly and network formation with the
373 potential to both activate caspase 3 and decrease cell viability. *Exp Cell Res*. 2011;317(16):2252-66.
- 374 20. Perng MD, Su M, Wen SF, Li R, Gibbon T, Prescott AR, et al. The Alexander Disease–Causing

375 Glial Fibrillary Acidic Protein Mutant, R416W, Accumulates into Rosenthal Fibers by a Pathway That
 376 Involves Filament Aggregation and the Association of α B-Crystallin and HSP27. 2006;79(2):197-213.

377 21. Yoshida T, Sasayama H, Nakagawa M. The process of inducing GFAP aggregates in
 378 astrocytoma-derived cells is different between R239C and R416W mutant GFAP. A time-lapse
 379 recording study. *Neurosci Lett*. 2009;458(1):11-4.

380 22. Hsiao VC, Tian R, Long H, Der Perng M, Brenner M, Quinlan RA, et al. Alexander-disease
 381 mutation of GFAP causes filament disorganization and decreased solubility of GFAP. *J Cell Sci*.
 382 2005;118(Pt 9):2057-65.

383 23. Tang G, Xu Z, Goldman JE. Synergistic effects of the SAPK/JNK and the proteasome pathway on
 384 glial fibrillary acidic protein (GFAP) accumulation in Alexander disease. *J Biol Chem*.
 385 2006;281(50):38634-43.

386 24. Tang G, Perng MD, Wilk S, Quinlan R, Goldman JE. Oligomers of mutant glial fibrillary acidic
 387 protein (GFAP) Inhibit the proteasome system in alexander disease astrocytes, and the small heat shock
 388 protein α B-crystallin reverses the inhibition. *J Biol Chem*. 2010;285(14):10527-37.

389 25. Middeldorp J, Kamphuis W, Sluijs JA, Achoui D, Leenaars CH, Feenstra MG, et al. Intermediate
 390 filament transcription in astrocytes is repressed by proteasome inhibition. *FASEB J*.
 391 2009;23(8):2710-26.

392 26. Tang G, Yue Z, Talloczy Z, Hagemann T, Cho W, Messing A, et al. Autophagy induced by
 393 Alexander disease-mutant GFAP accumulation is regulated by p38/MAPK and mTOR signaling
 394 pathways. *Hum Mol Genet*. 2008;17(11):1540-55.

395 27. Yan CG, Wang XD, Zuo XN, Zang YF. DPABI: Data Processing & Analysis for (Resting-State)
 396 Brain Imaging. *Neuroinformatics*. 2016;14(3):339-51.

28. Yang H, Long XY, Yang Y, Yan H, Zhu CZ, Zhou XP, et al. Amplitude of low frequency fluctuation within visual areas revealed by resting-state functional MRI. *Neuroimage*. 2007;36(1):144-52.
29. Eijlers AJ, Meijer KA, Wassenaar TM, Steenwijk MD, Uitdehaag BM, Barkhof F, et al. Increased default-mode network centrality in cognitively impaired multiple sclerosis patients. *Neurology*. 2017;88(10):952-60.
30. Zang Y, Jiang T, Lu Y, He Y, Tian L. Regional homogeneity approach to fMRI data analysis. *Neuroimage*. 2004;22(1):394-400.
31. Bresciani A, Spiezia MC, Boggio R, Cariulo C, Nordheim A, Altobelli R, et al. Quantifying autophagy using novel LC3B and p62 TR-FRET assays. *PLoS One*. 2018;13(3):e0194423.

Figure legend

Fig.1 Genetic and radiologic characteristics of patients who carry de novo variant in *GFAP*. (A-B) Chest radiography of patient P3433. Scoliosis was corrected after the operation. (C-D) Sequence chromatograms of *GFAP* gene of two probands, patient P4288 and patient P3433. (E) Patient P3433 demonstrated hyperintensity in the bilateral corona radiata, centrum semiovale and regions surrounding the 4th ventricle in T2-weighted FLAIR images. (F) In the proband P4288, minor hyperintensity was observed while the distribution of these lesions was similar to patient P3433. (G) Patient P3433 presented atrophy in subcortical grey matter: bilateral putamen, thalamus and cerebellum, as well as atrophic white matter in corona radiata, centrum semiovale, cerebellopontine angle and medulla. (H) Patient P4288 presented insignificant atrophy in grey matter, while the distribution of atrophic white matter was similar to patient P3433. Red/orange: the significantly larger volume of grey matter in

normal controls than the patient. Yellow: the significantly larger volume of white matter in normal controls than the patient. (I) Patient P3433 presented higher ALFF in cerebellar vermis, cerebellopontine angles, occipital and posterior parietal cortex. Increased DC was observed in frontal and posterior cortex, overlapping with Reho mainly in cerebellum and posterior cortex. (J) Patient P4288 presented higher ALFF, DC and Reho in similar regions with patient P3433. Increased DC was additionally observed in bilateral insula. Red/orange: voxels with significantly higher centrality; Blue: voxels with significantly higher amplitude of low frequency fluctuation. Pink: voxels with significantly higher amplitude of regional homogeneity.

Fig.2 Functional analysis of *GFAP* variants identified in this study. (A) The relative protein levels of GFAP-WT/Mut in soluble fraction and insoluble fraction were detected by western blot (One-way ANOVA; n = 3 separate experiments; ns = non-significance, * $0.01 \leq P < 0.05$, *** $P < 0.001$). (B) Immunofluorescence showed distributions of GFAP-WT were diffuse with few aggregates, while mutant groups (p.E72K and p.T412I) and positive control (p.R239C) were presented as punctate inclusions in perinuclear areas. Scale bar, 10 μ m. (C) After MG132 treatment, the soluble protein levels of 20S remained constant levels among groups while GFAP levels showed a decreased trend especially in WT, p.E72K and positive control groups (Two-way ANOVA; n = 3 separate experiments; * $P < 0.05$, ** $P < 0.01$). (D-E) Immunofluorescence indicating that 20S complex (red, D) and ubiquitin (red, E) in mutant groups and positive control exhibited as inclusions surrounding the nucleus, while diffuse distribution in cytoplasm was shown in WT group. Ubiquitin and 20S complexes were completely colocalized with mutant GFAP (green), respectively. Scale bar, 10 μ m. (F) The soluble levels of GFAP and LC3-II in BafAI-treated group were increased, especially in mutant groups and positive control. (G) After BafAI treatment, the statistical analysis of GFAP levels was shown (Two-way ANOVA; n = 3

441 separate experiments; ns = non-significance, * $P < 0.05$). (H-I) Immunostaining displayed that

442 GFAP-Mut proteins (green) exhibited aggregates and nearly co-expressed LC3 (red) and LAMP1 (red).

443 Scale bar, 10 μm .

444 **Fig.3** Schematic diagram of GFAP structure with identified variants. Mutations identified in our

445 study are in red font.

Figures

Figure 1

Genetic and radiologic characteristics of patients who carry de novo variant in GFAP. (A-B) Chest radiography of patient P3433. Scoliosis was corrected after the operation. (C-D) Sequence chromatograms of GFAP gene of two probands, patient P4288 and patient P3433. (E) Patient P3433

demonstrated hyperintensity in the bilateral corona radiata, centrum semiovale and regions surrounding the 4th ventricle in T2-weighted FLAIR images. (F) In the proband P4288, minor hyperintensity was observed while the distribution of these lesions was similar to patient P3433. (G) Patient P3433 presented atrophy in subcortical grey matter: bilateral putamen, thalamus and cerebellum, as well as atrophic white matter in corona radiata, centrum semiovale, cerebellopontine angle and medulla. (H) Patient P4288 presented insignificant atrophy in grey matter, while the distribution of atrophic white matter was similar to patient P3433. Red/orange: the significantly larger volume of grey matter in normal controls than the patient. Yellow: the significantly larger volume of white matter in normal controls than the patient. (I) Patient P3433 presented higher ALFF in cerebellar vermis, cerebellopontine angles, occipital and posterior parietal cortex. Increased DC was observed in frontal and posterior cortex, overlapping with Reho mainly in cerebellum and posterior cortex. (J) Patient P4288 presented higher ALFF, DC and Reho in similar regions with patient P3433. Increased DC was additionally observed in bilateral insula. Red/orange: voxels with significantly higher centrality; Blue: voxels with significantly higher amplitude of low frequency fluctuation. Pink: voxels with significantly higher amplitude of regional homogeneity.

Figure 2

Functional analysis of GFAP variants identified in this study. (A) The relative protein levels of GFAP-WT/Mut in soluble fraction and insoluble fraction were detected by western blot (One-way ANOVA; $n = 3$ separate experiments; ns = non-significance, $0.01 \leq P < 0.05$, $*** P < 0.001$). (B) Immunofluorescence showed distributions of GFAP-WT were diffuse with few aggregates, while mutant groups (p.E72K and p.T412I) and positive control (p.R239C) were presented as punctate inclusions in perinuclear areas. Scale

bar, 10 μ m. (C) After MG132 treatment, the soluble protein levels of 20S remained constant levels among groups while GFAP levels showed a decreased trend especially in WT, p.E72K and positive control groups (Two-way ANOVA; n = 3 separate experiments; * P < 0.05, ** P < 0.01). (D-E) Immunofluorescence indicating that 20S complex (red, D) and ubiquitin (red, E) in mutant groups and positive control exhibited as inclusions surrounding the nucleus, while diffuse distribution in cytoplasm was shown in WT group. Ubiquitin and 20S complexes were completely colocalized with mutant GFAP (green), respectively. Scale bar, 10 μ m. (F) The soluble levels of GFAP and LC3-II in BafA1-treated group were increased, especially in mutant groups and positive control. (G) After BafA1 treatment, the statistical analysis of GFAP levels was shown (Two-way ANOVA; n = 3 separate experiments; ns = non-significance, * P < 0.05). (H-I) Immunostaining 441 displayed that GFAP-Mut proteins (green) exhibited aggregates and nearly co-expressed LC3 (red) and LAMP1 (red). Scale bar, 10 μ m.

Figure 3

Schematic diagram of GFAP structure with identified variants. Mutations identified in our study are in red font.