Original Article

Three novel mutations in Chinese patients with *CSF1R*-related leukoencephalopathy

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Background: *CSF1R*-related encephalopathy refers to adult-onset leukodystrophy with neuroaxonal spheroids and pigmented glia (ALSP) due to *CSF1R* mutations, which is a rare autosomal dominant white matter disease including two pathological entities, hereditary diffuse leukoencephalopathy with spheroids (HDLS) and pigmentary orthochromatic leukodystrophy (POLD). The aim of this study was to identify additional causative mutations in the *CSF1R* gene and clarify their pathogenic effects.

Methods: Whole-exome sequencing was conducted for nine Chinese patients diagnosed with possible ALSP based on clinical and neuroimaging findings from March 2014 to June 2020 at Xuanwu Hospital (Beijing, China). Variant pathogenicity was assessed according to the American College of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG/AMP) Standards and Guidelines.

Results: Mean ± standard deviation (range) age of disease onset in the nine patients was 39.22±9.63 [25–54] years. Four of the nine patients were male, and four out of nine had a remarkable family history. Seven *CSF1R* mutations were identified in the nine patients; four (p.G17C, p.R579Q, p.I794T and c.2909_2910insATCA) have been previously reported, while three (p.V613L, p.W821R and c.2442+2_2442+3dupT) were novel. Of the latter, two (p.V613L and p.W821R) were likely pathogenic and 1 (c.2442+2_2442+3dupT) was of uncertain significance according to ACMG/AMP criteria.

Conclusions: These findings expand the mutational spectrum of ALSP and provide a basis for future investigations on etiologic factors and potential management strategies for this disease.

Keywords: Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP); pigmented orthochromatic leukodystrophy (POLD); hereditary diffuse leukoencephalopathy with spheroid (HDLS); colony-stimulating factor 1 receptor (*CSF1R*); novel mutation; mutational hotspot

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Introduction

CSF1R-related leukoencephalopathy is adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) in patients positive for *CSF1R* gene mutation. ALSP is an autosomal dominant white matter disease characterized by progressive dementia, personality changes, parkinsonism, or seizures as primary clinical symptoms, and including hereditary diffuse leukoencephalopathy with axonal spheroids (HDLS) and pigmented orthochromatic leukodystrophy (POLD), which have many clinical and

pathological similarities (1-3). To date, 147 patients with *CSF1R*-related leukoencephalopathy have been reported worldwide (4).

The colony-stimulating factor 1 receptor (*CSF1R*) gene on chromosome 5q32, which was identified as a causative gene in HDLS in 2011, encodes a transmembrane tyrosine kinase receptor that is mainly expressed in microglia in the brain and participates in their development and maintenance (3). Mutations in *CSF1R* may lead to microglia dysfunction and pathologic manifestations (5). Most *CSF1R* mutations have been reported in Europe, the United States, and Japan and include 94 missense mutations, 13 splicing mutations, 6 deletion/insertion mutations, 1 code shift mutation, and 1 nonsense mutation (4). Exons 12–22 of the *CSF1R* gene encode the tyrosine kinase domain (TKD) of *CSF1R* and are hotspots for mutations leading to functional impairment of the receptor (3,6). To our knowledge, at least 21 mutations have been reported in China, 18 located on exon 12–22 including 14 missense mutations, 1 nonsense mutation and 3 insertion/deletion mutations; A further three were intronic splicing mutations, located on intron 2 to 3, intron 13 to 14 and intron 17 to 18 (4,7-14). Although novel variants are occasionally reported, neither the functional consequences of all known *CSF1R* mutations nor the full genetic spectrum of mutations causing ALSP have yet been fully elucidated.

In this study, we analyzed the genetic profile of nine Chinese patients diagnosed with possible ALSP in order to identify possible novel causative mutations in the *CSF1R* gene and evaluate their pathogenic effects. This article is presented in accordance with the Materials Design Analysis Reporting (MDAR) reporting checklist (available at http://dx.doi.org/10.21037/atm-21-217).

Methods

Patients

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Approval of the study protocol and for ethical considerations was granted by the Ethics Committee and local Institutional Review Board of Xuanwu Hospital, Capital Medical University (approval number 2014019). Written informed consent was obtained from all participants or their guardians prior to the start of the study. All protocols were carried out in accordance with relevant guidelines and regulations for the use of human subjects in research.

Index patients were recruited as a continuous series of nine cases clinically diagnosed with possible ALSP at Xuanwu Hospital from March 2014 to June 2020, according to established criteria (15). A standardized clinical interview was conducted, family history recorded, and a physical examination performed at the time of admission, with 1–6 years of outpatient or telephone follow-up. All patients underwent routine laboratory testing and no abnormalities were detected. Four patients completed neurophysiologic examinations including Mini Mental State Examination and/or Montreal Cognitive Assessment. All patients underwent a magnetic resonance image (MRI) scan, and five underwent a computed tomography (CT) scan.

Whole-exome sequencing

Whole-exome sequencing was conducted using total DNA samples obtained from each patient using the SureSelect Human All Exon V6 Kit (Agilent Technologies, Santa Clara, CA), according to the manufacturer's protocol. After library preparation and capture, pooled libraries were sequenced (Hiseq-2000; Illumina, San Diego, CA, USA). Sequence reads were aligned to the human genome (GRCh37/hg19) and assembled for single nucleotide polymorphism calling and subsequent analysis using Burrows–Wheeler Aligner software (http://bio-bwa.sourceforge.net/). Variants were annotated using Realigner Target Creator in the Genome Analysis Toolkit (16) and ANNOVAR (17). EasyExonPrimer (18) was used to generate primers for PCR validation of exome sequencing derived *CSF1R* gene variants. Sanger sequencing of protein-coding regions was conducted according to standard protocols. PCR products were purified and then sequenced in both directions using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and the resulting products purified and analyzed using an ABI3730xl Genetic Analyzer (Applied Biosystems). DNA sequence variants were identified using Seq-Pilot software (JSI, Kippenheim, Germany) and named based on known sequences (accession numbers NM_005211.3 and NP_005202.2).

Pathogenicity prediction analyses of CSF1R variants

In silico prediction of the functional effects of missense mutations was carried out using Polymorphism Phenotyping v2 (PolyPhen2) (19), Sorting Intolerant From Tolerant (SIFT) (20), Mutationtaster (21) and the likelihood ratio test (LRT) (22). Protein sequence alignment was performed

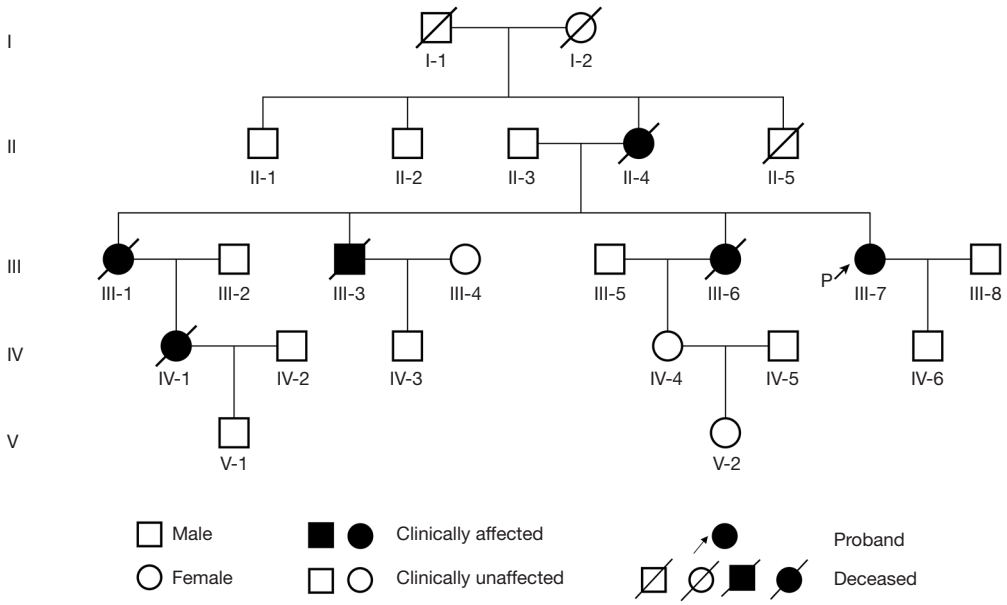

Figure 1 Pedigree of Family 1 with ALSP. The family pedigree included 23 individuals across five generations. Females and males are represented by circles and squares, respectively. Black and white symbols represent clinically affected and unaffected individuals, respectively. Diagonal lines indicate a deceased individual. The proband is indicated by an arrowhead. The proband's mother (II-4), siblings (III-1, III-3, and III-6), and niece (IV-1) were clinically affected but all passed away.

with UniProt (https://www.uniprot.org) to determine whether sequences were evolutionarily conserved across different species including *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Danio rerio* (zebrafish), *Canis lupus familiaris* (dog), *Bos taurus* (bovine), and *Macaca mulatta* (rhesus macaque). Variant frequency was evaluated using Genome Aggregation Database (gnomAD), 1000 Genomes Project, and Exome Aggregation Consortium databases. All novel variants were classified as pathogenic/likely pathogenic, benign/likely benign, or uncertain significance, according to the 2015 American College of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG/AMP) Standards and Guidelines (23). All analyses were performed on the Seqmax (https://www.seqmax.com/; accessed March 2020) and Pubvar (https://www.pubvar.com; accessed March 2020) platforms.

Statistical analyses

Continuous data were represented as means ± standard deviation, dichotomous data were as values. Mean age of onset between male and female were compared via *t*-test. All statistical analyses were performed using IBM SPSS Statistics® v22.0.0.0 (SPSS Inc., Chicago, IL, 2013). P values less than 0.05 were regarded as statistically significant.

Results

Demographic and clinical characteristics of patients

A series of nine ALSP patients with *CSF1R* mutations from independent families were included in our study. Four of the nine (44.4%) patients were male. Mean ± standard deviation (range) age of disease onset in the nine patients was 39.22±9.63 [25–54] years. No significant difference was found in the mean age at onset between male and female patients (male *vs.* female, 33.50±8.06 *vs.* 43.80±8.82 years; P=0.114). Four patients had a remarkable family history that suggested an autosomal dominant pattern of inheritance. Pedigree analysis of the family of Patient 1 including 23 individuals from five generations was conducted (*Figure 1*). The patient's mother (II-4), siblings (III-1, III-3, and III-6), and niece (IV-1) were clinically affected but had all died, while her 32-year-old son (IV-6) did not show any symptoms. The mother of Patient 2 had had symptoms similar to the proband but was also deceased. The family history of two other patients (Patients 8 and 9) have been previously reported (11) and are

not described in detail here.

The clinical characteristics of the patients are summarized in *Table 1*. Six of the nine patients presented with cognitive decline, one had personality change, one had parkinsonism, and one had walking difficulties as initial symptoms. Seven of the nine patients presented with cognitive decline, seven with pyramidal signs, and four with parkinsonism; two had personality changes, and one had epilepsy as clinical symptoms.

Brain MRI scans revealed that all patients had white matter lesions and brain atrophy; six had thinning of the corpus callosum, three had enlarged ventricles, and two had persistent limited diffusion on diffusion-weighted imaging (DWI). The brain MRI scans of Patients 1, 2, and 4 are shown in *Figure 2*. Four of the five patients who underwent CT scanning and had subcortical calcifications (see Figures S1,S2).

All patients were followed up for 1–6 years; six are still alive and three had died at the last follow-up. For the three deceased patients, mean ± standard deviation disease duration from onset until death was 4.33±2.01 years (2, 5, and 6 years), and all experienced progressive deterioration of brain function and became mute and bedridden, with incontinence and dysphagia, and required nasogastric intubation at the terminal stage of illness.

Genetic analysis

Seven *CSF1R* mutations were detected in our nine ALSP patients, including three novel variants (p.V613L, p.W821R and c.2442+2_2442+3dupT) and four known mutations (p.G17C, p.R579Q, p.I794T and c.2909_2910insATCA). Notably, p.I794T was present in three patients (Patients 7, 8, and 9). Genetic screening results and protein sequence alignments with other vertebrate species are shown in *Figure 3*.

The three novel variants and prediction of their pathogenicity are summarized in *Table 2*. Two missense mutations (p.V613L and p.W821R) affected the functional TKD of CSF1R (PM1) and were absent in healthy population (PM2). These mutations are in residues highly conserved across species and computational predictions support a deleterious effect (PP3). As the phenotypes and family histories of the patients indicated a disease with single-gene etiology (PP4), the variants were interpreted as likely pathogenic (PM1+PM2+PP3+PP4). The intronic splice-site mutation c.2442+2_2442+3dupT was determined to be of uncertain significance (PM2). Thus, according to ACMG/AMP guidelines for sequence variant interpretation, the p.V613L and p.W821R mutations were predicted to be likely pathogenic, whereas c.2442+2_2442+3dupT was interpreted as of uncertain significance.

The three known mutations are not described in detail here. The p.I794T mutation (accession no. 29813) is recorded in clinVar (https://www.ncbi.nlm.nih.gov/clinvar/) as a pathogenic variant, while p.G17C, p.R579Q and c.2909_2910insATCA are likely pathogenic, as previously reported (11,13).

Discussion

In this study, we identified three novel mutations in the *CSF1R* gene in Chinese patients with ALSP, including two missense mutations [p.V613L (exon 13) and p.W821R (exon 19) and one splicing site mutation (c.2442+2_2442+3dupT (intron 17–18)]. Among them, two were likely pathogenic variants [p.V613L (exon 13) and p.W821R (exon 19)] and one was a variant of uncertain significance [c.2442+2_2442+3dupT (intron 17–18)].

The mean disease duration of our patients was 4.3 years, which was shorter than the average disease duration (6.8 years) previously reported (24). There are some possible explanations for this discrepancy. First, the mean age of onset was 39.2 years in our patients, compared with 43 years in previously reported patients, and it is established that neurodegenerative genetic diseases may progress rapidly if onset occurs at an early age. Second, mean disease duration was calculated from a very small number of patients (only three) who died during our study. Finally, the previously reported sample comprised 26 Japanese patients with ALSP and 96 symptomatic carriers, based on reports published worldwide, whereas we focused on only Chinese patients in our institution; hence it is possible that regional, medical care condition, and/or ethnic variations contributed to the observed difference. Sex-dependent structural and functional differences in microglia have been convincingly demonstrated in some experimental studies, and female patients with CSF1R-related leukoencephalopathy were reported to develop clinical symptoms significantly earlier than males in some studies (24-26); however, no significant difference was found according to sex in our study, possibly because of the small sample size.

The two identified missense mutations [p.V613L (exon 13) and p.W821R (exon 19)] are located in the *CSF1R* gene mutation hotspot at exons 12–22, which encodes the TKD. Thus, these amino acid substitutions could perturb

Table 1 Clinical characteristics and neuroimaging features of the nine ALSP patients in this study

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9
Sex	F	F	F	F	M	M	F	M	M
Age of onset, years	51	32	54	41	25	29	41	37	43
Age at death, years	–	–	–	–	31	–	46	39	–
Family history	(+)[a]	(+)[a]	(–)	(–)	(–)	(–)	(+)[a]	(+)[a]	(–)
Initial symptoms									
Cognitive decline	(+)	(+)	(+)	(–)	(+)	(–)	(+)	(+)	(–)
Personality changes	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(–)	(–)
Parkinsonism	(–)	(–)	(+)	(–)	(–)	(–)	(–)	(–)	(–)
Epilepsy	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
Walking difficulties	(–)	(–)	(–)	(+)	(–)	(–)	(–)	(–)	(+)
Clinical symptoms									
Cognitive decline	(+)	(+)	(+)	(+)	(+)	(–)	(+)	(+)	(–)
Personality changes	(–)	(+)	(–)	(–)	(–)	(+)	(–)	(–)	(–)
Parkinsonism	(–)	(+)	(+)	(+)	(–)	(–)	(+)	(–)	(–)
Epilepsy	(–)	(–)	(–)	(+)	(–)	(–)	(–)	(–)	(–)
Walking difficulties	(–)	(–)	(–)	(+)	(–)	(–)	(–)	(–)	(+)
Pyramidal signs	(+)	(+)	(+)	(+)	(–)	(+)	(–)	(+)	(+)
MMSE	20	NA	NA	11	28	NA	NA	NA	27
MoCA	12	NA	NA	NA	NA	NA	NA	NA	23
MRI findings									
White matter lesions	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Atrophy	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Thinning of corpus callosum	(+)	(+)	(+)	(+)	(+)	(–)	(–)	(–)	(+)
Enlargement ventricles	(+)	(+)	(–)	(+)	(–)	(–)	(–)	(–)	(–)
Persistent limited diffusion on DWI	(–)	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(+)
Calcification on CT	(+)	NA	NA	NA	(+)	(+)	NA	(–)	(+)
CSF1R mutation	c.1837G>T[b] p.V613L	c.2461T>C[b] p.W821R	c.1736G>A[b] p.R579Q	c.2442+2_2442+3dupT[b]	c.49G>T[c] p.G17C	c.2909_2910insAT[C]Ac	c.2381T>C[c] p.I794T	c.2381T>C[c] p.I794T	c.2381T>C[c] p.I794T

[a]Family history. Patient 1: mother, siblings, and niece were clinically affected but all died; Patient 2: mother was clinically affected; Patient 8: father and brother were clinically affected; Patient 9: father was a heterozygous mutation carrier and was clinically affected. [b]Novel mutations identified in this study; [c]Known mutations. (+), abnormal; (–), normal; CSF1R, colony-stimulating factor 1 receptor (NM_ 005211.3); CT, computed tomography; F, female; HDLS, hereditary diffuse leukoencephalopathy with spheroids; M, male; MMSE, Mini Mental State Examination; MoCA, Montreal Cognitive Assessment; MRI, magnetic resonance imaging; DWI, diffusion-weighted imaging; NA, not available.

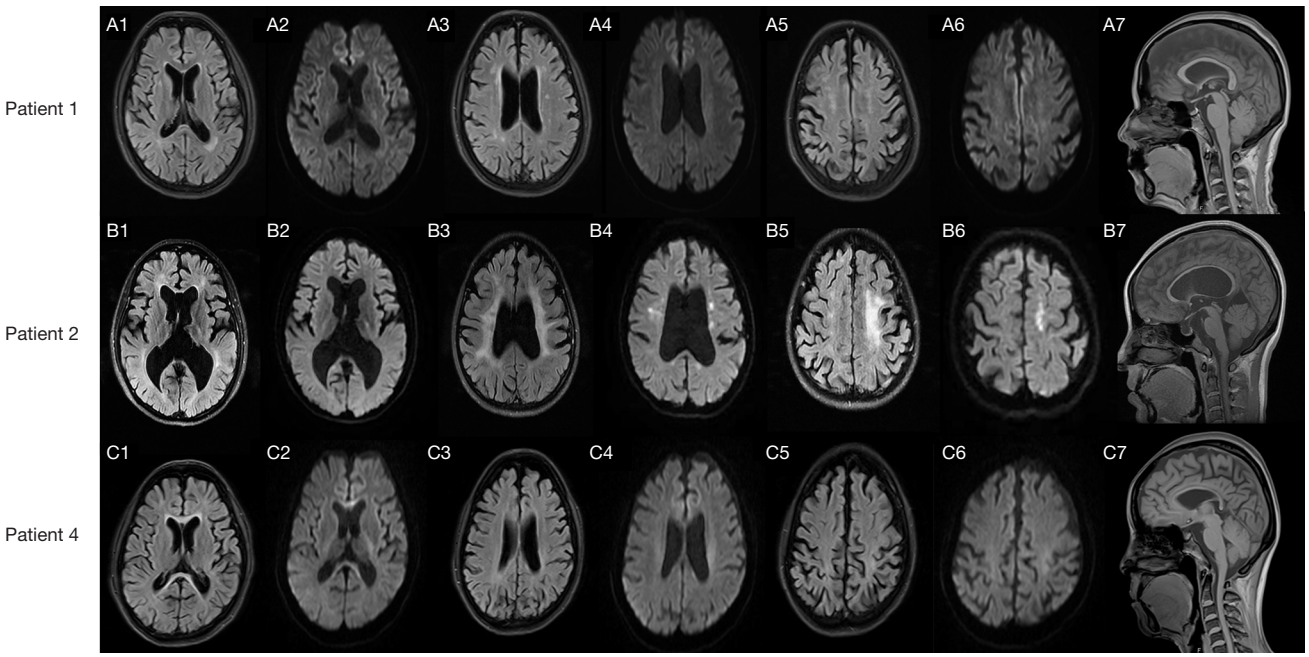

Figure 2 Brain MRI findings of three patients with ALSP. The first and second columns show the T2 fluid-attenuated inversion recovery (FLAIR) and corresponding diffusion-weighted imaging (DWI) images at the level of the third and lateral ventricles. The third and fourth columns show T2 FLAIR and DWI images at the level of the lateral ventricles. The fifth and sixth columns show T2 FLAIR and DWI images at the level of the centrum semiovale. The last column show T1-weighted images in the sagittal view. Images A1–A7 (Patient 1, 2 years after disease onset) revealed bilateral periventricular leukodystrophy and global brain atrophy, especially in the frontal and temporal lobes. Images B1–B7 (Patient 2, 7 months after disease onset) show asymmetric confluent T2 hyperintensities in periventricular regions and centrum semiovale, with some areas of restricted diffusion; apparent generalized cerebral atrophy of central and cortical gray matter and enlargement of the lateral ventricles; and thinning of the corpus callosum. Images C1–C7 (Patient 4, 6 months after disease onset) show prominent hyperintensities in the corpus callosum and brain atrophy.

the structure of the CSF1R protein, resulting in disease (PM1). Further, these mutations were absent from the gnomAD, 1000 Genomes Project and Exome Aggregation Consortium databases (PM2). Additionally, amino acids in this region are highly conserved across species, and the identified variants were predicted to have disease-damaging effects (probably damaging, damaging, disease-causing and deleterious) using four in silico analysis tools (PolyPhen-2, SIFT, Mutationtaster, and LRT) (PP3). Patients 1 and 2, harboring p.V613L and p.W821R, respectively, had the classic manifestations of ALSP, including memory deficit, speech dysfunction, and white matter lesions, according to neuroimaging, as well as remarkable family history (PP4); hence, the variants identified thus met the criteria for likely pathogenic (two pieces of moderate and two pieces of supporting evidence).

The splice-site variant, c.2442+2_2442+3dupT, was absent from healthy populations, according to searches of gnomAD, 1000 Genomes Project, and Exome Aggregation Consortium databases (PM2); however, it did not meet any other ACMG/AMP criteria for pathogenicity and was therefore considered as a variant of uncertain significance (one piece of moderate evidence). Nonetheless, as reported previously, mutations on intron 17 to 18 have frequently been associated with functional impacts, harboring half (5/10) of the known splice-site variants associated with ALSP (5,27-31). We also identified c.2442+2_2442+3dupT, a +2 splice-site mutation comprising a duplicated thymidine adjacent to the guanine thymidine splice donor of intron 17 to18; whether or not this mutation causes a deleterious effect on the wide-type donor site splicing signal remains unknown. Previous functional studies provide some evidence supporting its potential influence on splicing of the *CSF1R* gene transcript or causing exon 18 skipping and functional impairment of CSF1R, as previously reported for c.2442+2T>C (29,30); Moreover, *CSF1R* expression levels

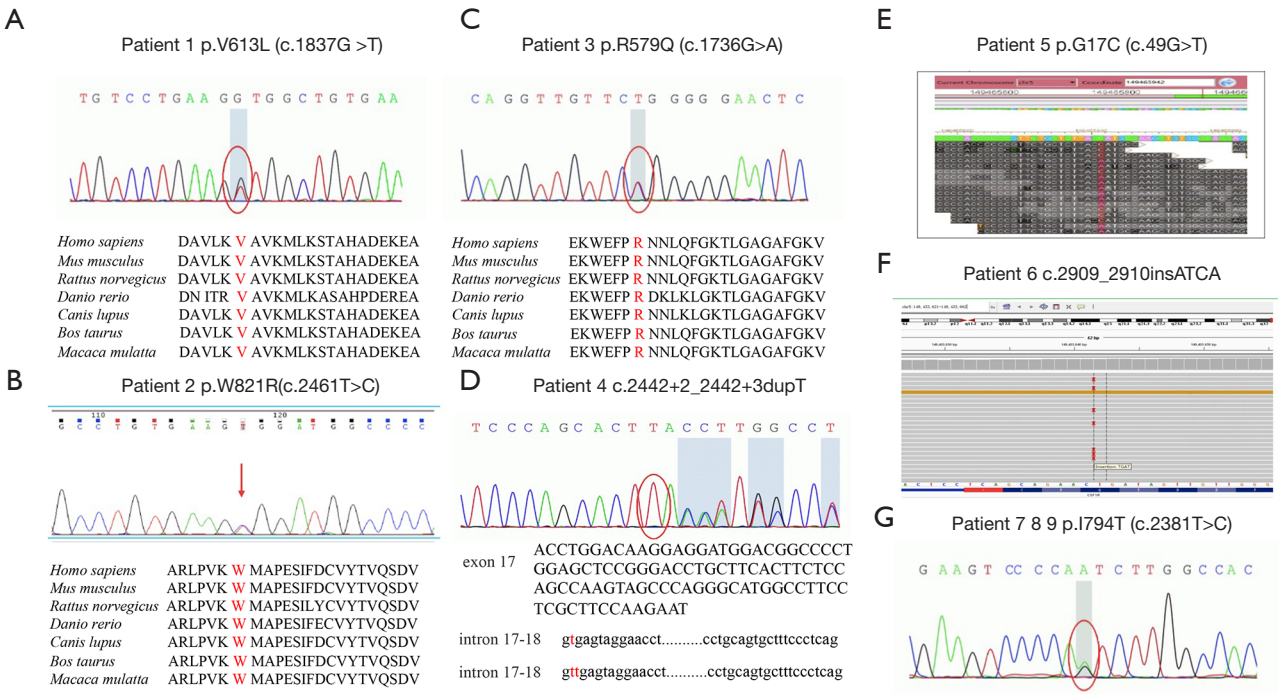

Figure 3 *CSF1R* gene mutation analysis. (A,B,C) Sequencing chromatograms showing three novel missense mutations of *CSF1R* (p.V613L, p.R579Q, and p.W821R). Protein sequence alignment was performed for parts of the TKD to evaluate conservation across species including *Mus musculus* (mouse), *Rattus norvegicus* (rat), *Danio rerio* (zebrafish), *Canis lupus familiaris* (dog), *Bos taurus* (bovine), and *Macaca mulatta* (rhesus macaque). (D) Sequencing chromatogram showing a novel splice site mutation of *CSF1R* (c.2442+2_2442+3dupT). At the beginning of intron 17 to 18, a duplicated thymidine potentially alters the donor site splice signal, likely affecting splicing of the *CSF1R* gene transcript. (E,F,G) Results of sequence analysis showing three known heterozygous mutations of *CSF1R* (p.G17C, c.2909_2910insATCA, and p.I794T). NM_005211.3 and NP_005202.2 were used as reference cDNA and protein sequences, respectively.

were markedly reduced in the brain of patient with the c.2442+1G>T mutation (28), which is located in the same intron as c.2442+2_2442+3dupT. Therefore, we speculate that c.2442+2_2442+3dupT likely influence splicing, thereby causing pathogenic changes; however, as it is not a canonical splice site mutation, additional functional analyses will be required to address this hypothesis in the future.

Our study had some limitations. Firstly, the sample size was small because ALSP is a rare disease in China. Secondly, familial cosegregation (linkage) analyses were not carried out because all affected family members, other than the probands, were deceased and the surviving relatives refused genetic testing. Finally, *in vitro* functional verification of the intronic mutation was not performed; however, this analysis will be conducted in a future study.

In conclusion, we identified three novel mutations in the *CSF1R* gene, two of which are likely pathogenic, and one uncertain significance. Our findings expand the mutational profile of ALSP and provide a basis for future research on the etiology and clinical management of this disease.

Table 2 Pathogenicity prediction analyses of the three novel *CSF1R* variants identified in this study

	Patient 1	Patient 2	Patient 4
Genome location			
Position	149441075	149435682	150056217
CSF1R exon/intron	Exon 13	Exon 19	Intron 17–18
Variant effect			
Base change	c.1837G>T	c.2461T>C	c.2442+2_2442+3dupT
Amino acid change	p.V613L	p.W821R	NA
Domain	Tyrosine kinase domain	Tyrosine kinase domain	NA
Population allele frequency			
Genome Aggregation Database	NR	NR	NR
1000 Genomes Project	NR	NR	NR
Exome Aggregation Consortium	NR	NR	NR
Functional prediction			
Polyphen2	Probably damaging	Probably damaging	NA
SIFT	Damaging	Damaging	NA
Mutation Taster	Disease-causing	Disease-causing	NA
LRT	Deleterious	Deleterious	NA
General prediction			
CADD Phred score	31	28.1	NA
ACMG/AMP criteria	Likely pathogenic	Likely pathogenic	Uncertain significance

ACMG/AMP, American College of Medical Genetics and Genomics and Association for Molecular Pathology; CADD, combined annotation-dependent depletion; CSF1R, colony-stimulating factor 1 receptor (NM_ 005211.3); LRT, likelihood ratio test; NA, not available; NR, not reported; Polyphen2, Polymorphism Phenotyping v2; SIFT, sorting intolerant from tolerant.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-21-217). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee and local Institutional Review Board of

Xuanwu Hospital, Capital Medical University (approval number 2014019) and informed consent was taken from all individual participants.

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