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Original Article

Investigation of a Fused in Sarcoma Splicing Mutation in a Chinese Amyotrophic Lateral Sclerosis Patient

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ABSTRACT: ***Objective:*** Genetic mutations of fused in sarcoma (*FUS)* causing amyotrophic lateral sclerosis (ALS) may disrupt mRNA splicing events. For example, the *FUS* c.1394-2delA variant was reported in two western ALS patients, but its molecular mechanism is unclear. In this study, we aim to investigate *FUS* splice site mutations in Chinese ALS patients. ***Methods:*** Sanger sequencing was used to identify *FUS* splicing mutations in Chinese ALS patients. We combined a deep learning tool (SpliceAI), RNA sequencing, and RT-PCR/RT-qPCR to analyze the effect of *FUS* c.1394-2delA mutation on RNA splicing and expression. AlphaFold was used to predict the protein structure of mutant FUS. In transfected cell lines, we used immunofluorescence to assess cytoplasmic mislocalization of mutant FUS protein. ***Results:*** We identified a *de novo FUS* splice acceptor site mutation (c.1394-2delA, p. Gly466Valfs*14) in one Chinese sporadic ALS patient, which is linked to exon 14 skipping, and upregulated total *FUS* mRNA expression. The *FUS* splice site mutation was predicted to be translated into a truncated protein product at C-terminal. *In vitro* studies revealed that the *FUS* mutation increased cytoplasmic mislocalization in both HEK293T and SH-SY5Y cells. ***Conclusions:*** We identified a *de novo FUS* splicing mutation (c.1394-2delA, p. Gly466Valfs*14) in 1 out of 233 Chinese ALS patients. It caused abnormal RNA splicing, upregulated gene expression, truncated FUS translation, and cytosolic mislocalization. Our findings suggested that *FUS* splice site mutation is rare in Chinese ALS patients and extended our knowledge of molecular mechanisms of the *FUS* c.1394-2delA mutation.

RÉSUMÉ : Étude d'une mutation affectant des sites d'épissage dans le cas de protéines de fusion d'un sarcome chez un patient chinois atteint de sclérose latérale amyotrophique. ***Objectif :*** Les mutations génétiques des protéines de fusion d'un sarcome (PFS), lesquelles sont à l'origine de la sclérose latérale amyotrophique (SLA), peuvent perturber les sites d'épissage de l'ARN messager. À titre d'exemple, la variante c.1394-2delA des PFS a été signalée chez deux patients occidentaux atteints de SLA mais son mécanisme moléculaire demeure nébuleux. Dans la présente étude, nous entendons étudier les mutations des sites d'épissage des PFS chez des patients chinois atteints de SLA. ***Méthodes :*** Le séquençage génétique de Sanger a été utilisé pour identifier les mutations des sites d'épissage des PFS chez des patients chinois atteints de SLA. Nous avons aussi combiné un outil d'apprentissage profond (SpliceAI), le séquençage de l'ARN messager et la méthode dite « RT-PCR/RT-qPCR » pour analyser l'effet de la mutation des PFS (variante c.1394-2delA) sur l'épissage et l'expression de l'ARN messager. Le programme AlphaFold a été par ailleurs utilisé pour prédire la structure protéique des PFS mutantes. Dans les lignées cellulaires transfectées, nous avons utilisé l'immunofluorescence pour évaluer la mauvaise localisation cytoplasmique des PFS mutantes. ***Résultats :*** Nous avons identifié une mutation *de novo* du site accepteur d'épissage des PFS (c.1394-2delA, p.Gly466Valfs*14) chez un patient chinois atteint de SLA sporadique, laquelle est liée à un saut de l'exon 14, ce qui a augmenté l'expression totale de l'ARN messager des PFS. La mutation des sites d'épissage des PFS est supposée se traduire par un produit protéique tronqué à l'extrémité C-terminale. Des études *in vitro* ont aussi révélé que les mutations des PFS augmentait la mauvaise localisation cytoplasmique dans les cellules HEK293T et SH-SY5Y. ***Conclusions :*** Nous avons identifié une mutation *de novo* de l'épissage des PFS (c.1394-2delA, p.Gly466Valfs*14) chez un des 233 patients chinois atteints de SLA. Cette mutation a provoqué un épissage anormal de l'ARN messager, une expression génétique régulée, une traduction tronquée des PFS ainsi qu'une mauvaise localisation cytoplasmique. En somme, nos résultats suggèrent que les mutations des sites d'épissage des PFS sont rares chez les patients chinois atteints de SLA. Ils permettent également d'élargir nos connaissances quant aux mécanismes moléculaires de la mutation c.1394-2delA des PFS.

Keywords: ALS; FUS; RNA splicing; Cytoplasmic mislocalization

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Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disease, characterized by the loss of upper and lower motor neurons in the brain and spinal cord.[1] More than 50 fused in sarcoma (*FUS*) mutations have been reported, accounting for about 5.8% familial ALS (fALS) and 1.3% sporadic ALS (sALS) cases in China.[2] The majority of *FUS* mutations occur in exons 5–6 and exons 13–15 of different protein domains, suggesting variable pathogenic mechanisms.[3] For example, FUS-Arg244Cys, FUS-Arg514Ser, and FUS-Arg521Cys mutations cause DNA damage by impairing FUS interaction with histone deacetylase 1 (*HDAC1)* and reducing double-strand breaks repair efficiency,[4,5] FUS-Pro525Leu causes abnormal RNA metabolism by inhibiting splicing of minor introns,[6] and C-terminal mutations usually cause cytoplasmic mislocalization of FUS.[7]

Pathogenic genetic mutations in the intron/exon flanking regions (e.g. classical splicing sites) may lead to complete exon skipping, intron retention, or the introduction of a new splice site within an exon or intron.[8] Splice site mutations are DNA sequence changes that alter or abolish correct mRNA splicing during the process of precursor mRNA maturation.[9] It has been estimated that about 15% of human disease-causing mutations may disrupt mRNA splicing.[10] For example, a C/T change at position 6 in exon 7 of survival of motor neuron 2 (*SMN2*) gene causing frequent skipping of exon 7 that produces an inactive and unstable protein lacking the last 16 amino acids, caused spinal muscular atrophy.[11]

So far, few splice site mutations in *FUS* have been reported in the ALSoD database with unclear pathogenicity. The *FUS* splicing site mutations (IVS13-2A > G, p. Gly466Valfs*14; c.1542-2A > C, p. Gly515Serfs*8; c.1542-1G > T, p. Gly515Serfs*8) at C-terminal were reported to cause exon skipping, truncated protein products, and cytosolic mislocalization.[12–14] Previously, two independent studies reported the *FUS* c.1394-2delA variant in western ALS patients. Briefly, the heterozygous *FUS* c.1394-2delA variant was found in 1 out of 301 German ALS patients,[15] and 1 out of 391 US ALS patients.[16] However, the frequency of *FUS* c.1394-2delA variant in Chinese ALS patients is unclear, and its functional mechanisms are largely unknown.

In this study, we aimed to investigate FUS splice site mutations in Chinese ALS patients and reported one Chinese sALS patient carrying the *FUS* splice acceptor site mutation (c.1394-2delA, p. Gly466Valfs*14). We also characterized the molecular mechanisms of this splice site *FUS* mutation at mRNA and protein levels.

Materials and Methods

Participants

The included patients were diagnosed with ALS according to the revised EI Escorial criteria[17] in the ALS clinic at Huashan Hospital (Shanghai, China). The parents of the *FUS* splice mutation carrier were also included. Age of onset was defined as the age of initial appearance of symptoms. The functional impairment of the proband was assessed according to the Revised Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R).[18] This study was approved by the ethical review boards of Huashan Hospital (Shanghai, China).

Genetic Analysis

We collected the peripheral blood using the EDTA-containing vacuum blood collection tube (BD Vacutainer, 367863, USA). Genomic DNA was extracted using the QIAGEN DNA extract kit (QIAGEN, 51206, Germany) according to the standard protocols. We amplified all coding regions of Cu/Zn superoxide dismutase (*SOD1*), exon 6 of TAR DNA binding protein (*TARDBP)* and selected *FUS* coding regions (exon 5, exon 6, exons 13–14, and exon 15) for polymerase chain reaction (PCR), followed by sequencing with the ABI-3730XL genetic analyzer (Applied Biosystems, USA). We used CodonCode Aligner software (V.10.0.2) to analyze the amplicon sequences.

The minor allele frequency was obtained from the gnomAD database (Version 2.1.1) (http://gnomad-sg.org) ($n = 15{,}708$ Genomes data) and the China Metabolic Analytics Project (ChinaMAP) database (http://www.mbiobank.com) ($n = 10{,}588$ Genomes data). We used the SpliceAI deep learning algorithm (https://github.com/Illumina/SpliceAI)[19] to predict the effect of mutation on splicing. The software sets a cutoff of 0.8 to indicate a high-precision prediction result.

Prediction of Protein Structure

AlphaFold deep learning algorithm was used to predict protein structures of FUS carrying wild type or mutant variant (https://github.com/deepmind/alphafold#running-alphafold).[20] We used the ranked_0.pdb file for further analysis, which contained the prediction result with the highest confidence. The results were visualized by PyMol (Version 2.5.2) software.

RNA Sequencing and RT-PCR

Lymphocytes were isolated from 3 ml EDTA-containing venous blood using Lymphoprep™ (Stem Cell, Germany) and density-gradient centrifugation. Total RNA extraction from lymphocytes was performed using the total RNA extraction kit (Aidlab, China). 1 μg RNA was used for RNA sequencing at BerryGenomics Co., Ltd. In brief, the Illumina Ribo-Zero Plus rRNA Depletion Kit (Illumina, 20037135, USA) was used to remove the ribosomal RNA and TruSeq Stranded mRNA kit (Illumina, RS-122-2001, USA). Strand-specific sequencing libraries were prepared and sequenced by the Illumina Novaseq 6000 platform (Illumina, San Diego, USA). The alternative splicing was analyzed by LeafCutter using the RNA sequencing data with the default setting, which is available on https://github.com/davidaknowles/leafcutter/.[21] In short, the RNA sequencing reads were aligned by STAR (Version 2.7.10a).[22] Reads that span exon–exon junctions and map with a minimum of 6 nt into each exon were extracted from the bam files with filter_cs.py script. Intron clustering was performed using the leafcutter_cluster.py script. We used the leafcutter_ds.R script to calculate the differential excision of *FUS* (wild type *vs* mutant).

To validate abnormal RNA splicing event, we used the PrimeScript™ RT reagent Kit (Takara Bio, Japan) to convert RNA to cDNA. *FUS*-specific primers targeting the exon 14 were used for RT-PCR (Supplementary Figure 1). The PCR products were separated by 2% agarose gel electrophoresis and visualized using iBrightCL1000 Imager (Thermo Fisher Scientific, USA). We then cut the target gel band, extracted the cDNA by the gel extraction kit (TianGen, China) and sequenced it with the ABI-3730XL genetic analyzer (Applied Biosystems, USA).

Preparation of Plasmids and Cell Culture

The human full-length coding sequence of wild-type *FUS* (NM_004960.3) was amplified from pEGFP-N1-FUS/TLS-FLAGC (addgene:60362) and was cloned into pEGFP-C1 vector at the XhoI/BamHI restriction site. The cDNA of the patient

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was used as a template to generate the truncated mutant *FUS* sequences and cloned into the XhoI/BamHI site of the pEGFP-C1 vector. HEK293T cells (purchased from Cell Bank of Chinese Academic of Science) and SH-SY5Y cells (a gift from Dr. Jian Wang in Fudan University) were cultured in DMEM or DF12 supplemented with 10% fetal bovine serum (FBS, Gibco) and Glutamax (Gibco), MEM non-essential amino acids solution (Gibco), and 1% penicillin/streptomycin (Gibco) respectively.

Immunofluorescence

HEK293T and SH-SY5Y cells were plated on 20 mm Glass Bottom Cell Culture Dishes (NEST, 801001, China) and transfected with wild type or mutant FUS plasmids using Lipofectamine 2000 (Thermo Fisher Scientific, USA).[23] After 36 h of plasmids transfection, we washed cells with phosphate-buffered saline and fixed cells with 4% cold paraformaldehyde for 15 min at room temperature. We used the Hoechst 33342 (Thermo Fisher Scientific, H3570, USA) to label the nuclei and Nikon's Eclipse Ti2 inverted microscope confocal system (Nikon, Japan) to acquire images at 60× magnification with a Z stack interval of 2 µm. ImageJ software was used to quantify the intensity of the GFP fluorescence (NIH, USA, https://imagej.nih.gov/ij/). Briefly, we selected the cells with the nucleus stained by Hoechst and GFP for quantifying the intensities. The cytoplasmic FUS intensities were calculated by the total GFP intensities minus the GFP intensities of the nucleus.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was reverse transcribed to cDNA using the PrimeScript™ RT reagent Kit (Takara Bio, Japan). RT-qPCR was performed in a 20-µl reaction volume using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) reagent (Takara Bio, Japan) and specific primers (Supplementary Table 1) in Bio-Rad CFX96 Real-Time PCR Detection System (Bio-rad, USA). The expression level of total *FUS* mRNA was estimated based on the $2^{-\Delta\Delta CT}$ method and was normalized to the expression level of *GAPDH* housekeeping gene to obtain a relative expression level.

Statistics

We used the Wilcoxon rank sum nonparametric test (R Version 4.0.4) to analyze the mean cytoplasmic/total FUS ratio between wild-type and mutant groups. P value < 0.05 was accepted as statistically significant.

Results

Identification of the FUS Splicing Mutation in Chinese ALS Patients

In 233 Chinese ALS patients (Table 1), we found that one sporadic case carried a heterozygous *FUS* splicing mutation (NM_004960.4, chr16:31202282, c.1394-2delA, p. Gly466Valfs*14, rs1555509569) (Figure 1(A) and (B)). Sanger sequencing excluded other mutations of *SOD1* or *TARDBP* in the proband. The healthy parents were further analyzed and did not carry this variant, suggesting that the *FUS* c.1394-2delA is a *de novo* variant (Figure 1(B)). It was absent in the gnomAD and ChinMAP databases. The proband has a younger sister [II-2] (39 years old), who had no complaint of any neurological problems and refused any genetic tests.

Table 1: Characteristics of 233 Chinese ALS patients

Clinical features	fALS	sALS
Cases, *n* (%)	40 (17.1)	193 (82.8)
Sex, *n* (%)	22 (55.0)	113 (58.5)
Age at onset, mean ± SD (year)	48.1 ± 12.0	50.91 ± 13.2
Limb onset, *n* (%)	35 (87.5)	175 (90.6)
Bulbar onset, n (%)	5 (12.5)	18 (9.3)

Abbreviations: ALS: amyotrophic lateral sclerosis; fALS: familial ALS; sALS: sporadic ALS; N, number; M: male; SD: standard deviation.

Clinical Presentation

The pedigree of this family is presented in Figure 1(A). The proband (II-1) developed muscle weakness in the right upper limb at the age of 39. The symptoms spread quickly to the left upper limb. Seven months later, he developed dysarthria, dysphagia, and dyspnea, with fasciculation in four limbs and obvious atrophy in both upper limbs. Neurologic examination at 11 months after initial symptoms showed reduced muscle strength of both distal and proximal upper extremities (4/5). Deep tendon reflexes were reduced in upper limbs and brisk in lower limbs. Hoffmann and Babinski's signs were not present. His ALSFRS-R score was 40/48 with normal cognition and significant weight loss. Electromyography showed acute and chronic denervation in the upper and lower limb, thoracic, and sternocleidomastoid muscles. Blood biochemical tests (routine blood test, thyroid function, retinal and liver function, and immune-related antibodies [anti-ANA, anti-ENA, anti-CCP, anti-ACA, and anti-ANCAs]) and related examinations (cerebral magnetic resonance imaging and cerebrospinal fluid basic analyses) were normal. His blood gas analysis showed the high pressure of carbon dioxide (PaCO2: 46.3 mmHg) which indicated decreased respiratory function. The ALSFRS-R score of the patient was 15/48 at 17 months after symptom onset. The patient underwent tracheostomy 19 months after symptom onset due to respiratory failure.

The FUS Splicing Mutation is Linked to Altered RNA Splicing and Upregulated Gene Expression

Functional mechanisms of the *FUS* splice site mutation are largely unclear. SpliceAI analysis suggested that the *FUS* mutation (c.1394-2delA) may alter the normal splicing at chr16:31202284 (hg19) (SpliceAI = T|FUS|0.15|1.00|0.00|0.00|37|3|5|29), suggesting that the variant may cause a loss of splice acceptor. Sequencing of lymphocyte RNA revealed aberrant splicing leading to the skipping of *FUS* exon 14 (Figure 1(C)). The agarose gel electrophoresis of *FUS* cDNA products from the proband (II-1) revealed two PCR bands corresponding to a 393 bp (containing exon 14) and a 245 bp band (Figure 1(D)). Sanger sequencing chromatogram of the mutant products showed the skipping of *FUS* exon 14 (148bp) (Figure 1(E)). In addition, the mutation carrier showed elevated total *FUS* mRNA expression level (Supplementary Figure 2) when compared to the *FUS* expression of lymphocytes of the parents and another independent neurologically healthy individuals carrying the wild-type allele. RNA-seq further confirmed the upregulation of FUS expression in the mutation carrier (Supplementary Figure 3).

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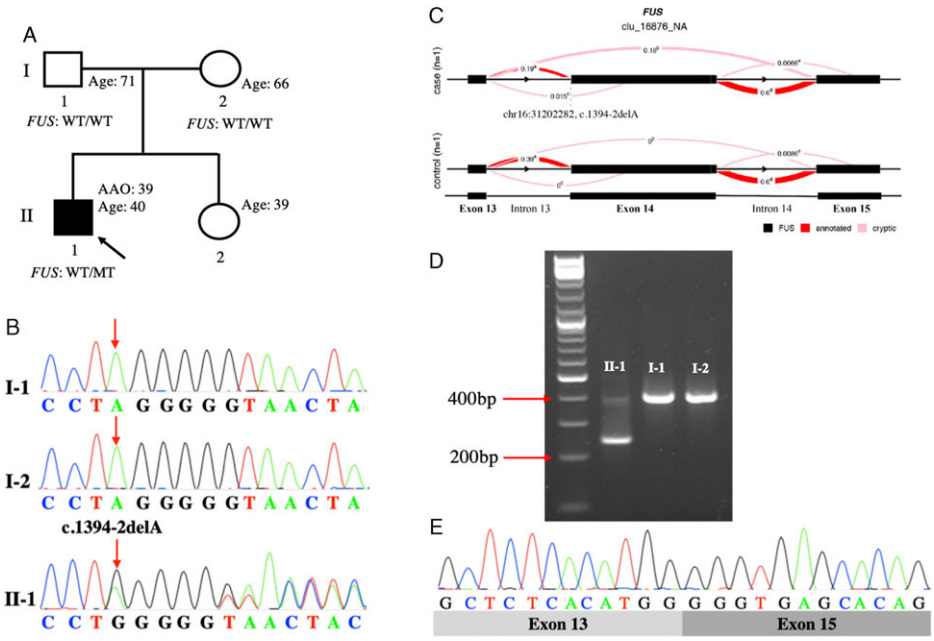

Figure 1: A novel *FUS* mutation affected RNA splicing in a sporadic ALS patient. (A) The pedigree of the investigated family. Proband was indicated by the arrowhead, and the age of family members and age at onset (AAO) were indicated. FUS genotype status was indicated as WT (wild type), MT (mutant); (B) Chromatography of Sanger sequencing. It showed that the proband carried FUS c.1394-2delA variant; (C) Visualization of altered RNA splicing of FUS exon13-exon15. The RNA sequencing data of the proband and one healthy control sample was processed by the LeafCutter tool (https://github.com/davidaknowles/leafcutter/). Location of the mutation site was indicated; (D) Agarose gel electrophoresis of FUS cDNA product. The patient II-1 (lane 1) and the health parents (I-1 and I-2, lane 2–3) had different FUS mRNA transcripts; and the patient had two dominant cDNA bands, corresponding to the normal transcript (393 bp) and the abnormal transcript (~245 bp); (E) The Sanger sequencing chromatogram of the smaller cDNA product (~245 bp on the gel). It validated the skipping of FUS exon 14 in the proband.

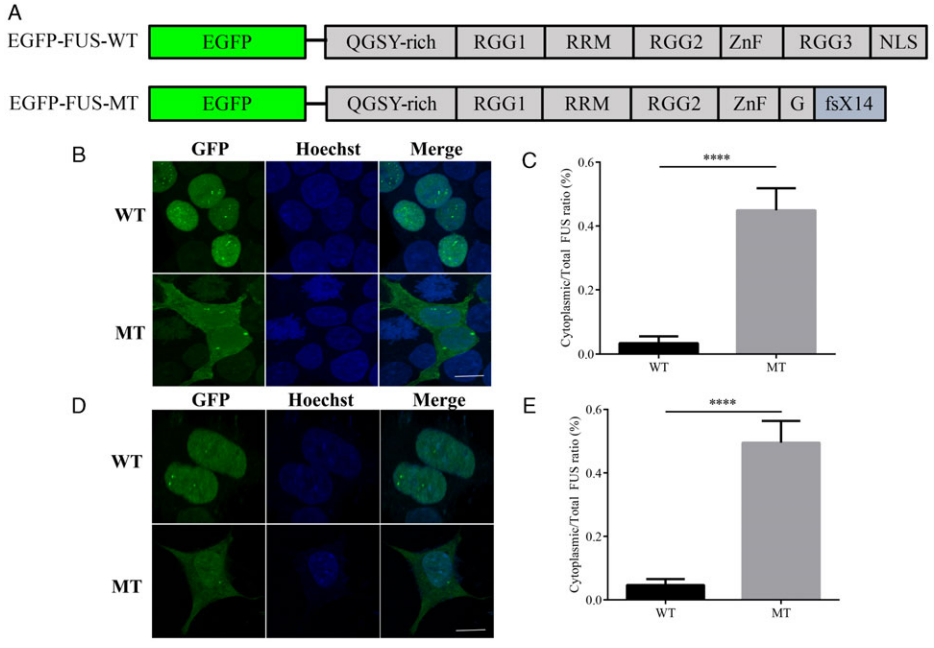

Figure 2: Mislocalization of FUS in cell lines. (A) Schematic diagram of the EGFP-FUS fusion protein of different FUS plasmids. The protein domains of FUS are indicated by different boxes. The G in the mutant plasmid represents the truncated last RGG domain; (B) In HEK293T cells, mutant FUS caused mislocalization of FUS into the cytoplasm. GFP: EGFP-FUS fusion protein, Hoechst: nuclear staining, Merge: colocalization of GFP signal with the nuclear Hoechst fluorescence; (C) The histogram of the mean ratio of cytoplasmic/total FUS intensities in HEK293T cells (mean ratio: 0.45 vs 0.03 in MT and WT groups); (D) In SH-SY5Y cells, mutant FUS caused mislocalization of FUS into the cytoplasm. GFP: EGFP-FUS fusion protein, Hoechst: nuclear staining, Merge: colocalization of GFP signal with the nuclear Hoechst fluorescence; (E) The histogram of the mean ratio of cytoplasmic/total FUS in SH-SY5Y cells (mean ratio: 0.50 vs 0.05 in MT and WT groups); Scale bar: 10 μm; $p < 10^{-4}$.

The FUS Splicing Mutation Induces Prominent Cytosolic Mislocalization

The *FUS* c.1394-2delA mutation was predicted to cause the frameshift and premature termination in exon 15, leading to a truncated FUS (p. Gly466Valfs*14) (Supplementary Figure 4), which might cause the loss of the nuclear localization signal (NLS) domain (Figure 2(A)).

Since the C-terminus of FUS is a non-classical Proline/Tyrosine-nuclear localization signal (PY-NLS) domain and essential for FUS nuclear import, we tested whether the novel *FUS* c.1394-2delA mutation may affect the FUS cytosolic/nucleus mislocalization. In both HEK293T kidney and SH-SY5Y neuronal cell lines, we found that cells with the mutant *FUS* (c.1394-2delA, p. Gly466Valfs*14) showed the mislocalization of nucleus FUS into the cytoplasm ($p < 0.0001$; mean ratio:0.45 *vs* 0.03 in HEK293T cells, 0.50 *vs* 0.05 in SH-SY5Y cells) (Figure 2).

Discussion

Our study investigated *FUS* splice site mutations in Chinese ALS patients and identified one Chinese sALS patient carrying the rare *FUS* splice site mutation (c.1394-2delA, p. Gly466Valfs*14), which was previously reported in two western ALS patients,[15,16] with unclear functional mechanisms. In lymphocytes of the patient, we found that the splice site mutation caused the splice acceptor

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loss at intron 13, leading to exon 14 skipping and upregulation of *FUS* gene expression. The mutant allele was predicted to encode a truncated protein. In both kidney and neuronal cell lines, we demonstrated that the *FUS* splice site mutation significantly induced cytosolic mislocalization, which is a key feature of FUS NLS domain mutations.[7,24] Our findings support the pathogenicity of the *FUS* c.1394-2delA mutation in ALS.

A previous study reported a different mutation (g.10747A > G; IVS13-2A > G, p. Gly466Valfs*14) at the same *FUS* genomic locus in an American female sALS patient.[12] It also caused abnormal RNA splicing, truncated FUS product, and cytoplasmic mislocalization of FUS in a mouse neuroblastoma cell line (N2A cells).[12] In addition, both *FUS* c.1394-2delA mutation and the previously reported mutation were absent in the large public datasets (the ChinaMAP and the gnomAD databases), supporting that it might be pathogenic.

FUS is a DNA/RNA-binding protein involved in RNA metabolism processes, including gene transcription regulation, RNA splicing, and transport.[25] Wild-type FUS predominantly resides in the nucleus and shuttles between the nucleus and cytoplasm.[26] Our *in vitro* experiments support that the *FUS* splicing mutation may cause truncated FUS protein lacking the PY-NLS domain, disrupt the interaction between FUS and karyopherin β2 (Kapβ2), and cytoplasmic mislocalization (Figure 2(B)–(E)). The results are in line with the previous report that FUS C-terminal NLS domain is important for FUS nuclear transportation by binding with Kapβ2 in a Ran-sensitive manner,[27] which would form the large TDP-43 negative inclusions and incorporate into the stress granules.[28] Splicing site mutations may cause complete skipping of the exon, retention of the intron, or the introduction of a new splice site within an exon or intron, which could affect the balance of isoforms produced by alternatively spliced exons and cause disease.[10] ALS has been reported to be caused by other splicing mutations, such as annexin A11 (*ANXA11)* c.1086+1G>A by promoting cytoplasmic aggregation of the mutant protein, *SOD1* c.240-7T>G by reducing its stability and enzymic activity, and TANK-binding kinase 1 (*TBK1)* c.1522-3T>G, and c.2066+4A>G by reducing gene expression.[29–31] Our findings support that abnormal splicing is an important molecular mechanism of ALS.

Genetic variants at C-terminal may escape nonsense-mediated RNA decay (NMD),[32] but it is unusual to increase the gene expression level. To maintain its gene expression level *in vivo*, FUS has a unique homeostasis mechanism, in which FUS protein bind to exon 7 and flanking introns of its pre-mRNAs promoting intron retention[33,34] that is followed by the NMD-mediated RNA degradation.[33,34] The *FUS* splicing mutation may disrupt this homeostasis mechanism, because FUS mislocalization reduced the nucleus FUS protein level and may disrupt the intron retention and NMD related degradation, which might be related to upregulated gene expression level observed in this study.

The current study was limited to a moderate sample set of Chinese ALS patients. Future studies should investigate *FUS* splice mutations in a larger sample set with different ethnical backgrounds to understand the mechanisms of splicing mutations in ALS etiology.

In conclusion, we reported a *de novo FUS* splice site mutation (c.1394-2delA, p. Gly466Valfs*14) in 1 out of 233 (0.4%) Chinese ALS patients. It caused abnormal RNA splicing, upregulated gene expression, truncated FUS translation, and cytosolic mislocalization. Our findings suggested the pathogenic mechanism of the *FUS* c.1394-2delA mutation in ALS.

Supplementary Material. To view supplementary material for this article, please visit https://doi.org/10.1017/cjn.2022.336.

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Conflict of Interest. There is no conflict of interest to disclose.

Statement of Authorship. Conceptualization: MZ and YC; Methodology: WLY, YZ, and XLT; Clinical data collection: XC, YMS, YD, and HY; Writing–original draft preparation: WLY, MZ, and XC; Writing—review and editing: MZ and YC.

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