

Identification of novel *FUS* and *TARDBP* gene mutations in Chinese amyotrophic lateral sclerosis patients with HRM analysis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive loss of motor neurons. More than 30 genes have been linked to ALS to date, including *FUS* and *TARDBP*, which exhibit similar roles in RNA metabolism. This study explored the use of high-resolution melting (HRM) analysis to screen for *FUS* and *TARDBP* mutation hotspot regions in 146 Chinese ALS patients, which achieved 100% detection. Two *FUS* mutations were observed in two different familial ALS probands, a missense mutation (p.R521H) and a novel splicing mutation (c.1541+1G>A). Five *TARDBP* mutations were identified in six ALS patients, including a novel 3'UTR mutation (c.*731A>G) and four missense mutations (p.G294V, p.M337V, p.G348V, and p.I383V). We found that *FUS* mutations were present in 1.4% of Chinese ALS patients, whereas *TARDBP* mutations were responsible for 4.1% of Chinese ALS cases. Here, we describe the accuracy of using highly sensitive HRM analysis to identify two novel *FUS* and *TARDBP* mutations in Chinese sporadic and familial ALS cases. Our study contributes to the further understanding of the genetic and phenotypic diversity of ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset progressive neurodegenerative disease and is characterized by selective degeneration of upper and lower motor neurons in the brain and spinal cord [1]. The ALS median incidence rate is approximately two per 100,000 persons [2]. Median age of symptom onset is 55 years, and the average survival time is 3-5 years [3]. Approximately 10% of ALS cases are patients with familial ALS (fALS), whereas the remaining 90% are sporadic ALS (sALS) [4].

To date, more than 30 genes have been linked to ALS. However, only a few, including SOD1, C9ORF72,

FUS, and *TARDBP*, have been identified as causative ALS genes [5, 6]. The pathogenesis of ALS remains unclear; however, one of the possible mechanisms underlying ALS is defective RNA metabolism and homeostasis [7]. Recent studies have shown that *FUS* and *TARDBP* RNA-binding proteins may contribute to ALS pathogenesis [8], and that most of these causative *FUS* and *TARDBP* mutations cluster in the C-terminus of proteins, called mutation hotspot regions [9, 10].

Sanger sequencing and next-generation sequencing for mutation screening in large samples are time consuming and expensive. In contrast, here we screen the *FUS* and *TARDBP* mutation hotspot regions in 146 ALS cases

using the rapid, highly sensitive screening method, high-resolution melting (HRM) analysis. HRM analysis is a recently developed genetic analysis method for fast, high-throughput post-PCR analysis. During high-resolution melting analysis, melting curves are produced using dyes that fluoresce in the presence of double-stranded DNA (dsDNA) and specialized instruments designed to monitor fluorescence during heating; as the temperature increases, the fluorescence decreases, producing a characteristic melting profile. The shape differences in melting curves, obtained as fluorescence difference plots, are used to distinguish between mutations and controls [11, 12].

In this study, we demonstrated that highly sensitive HRM analysis can be used for screening FUS and TARDBP mutations and identified two novel mutations in Chinese sALS and fALS cases.

RESULTS

Sensitivity and HRM analysis

Excluding synonymous mutations and normal single-nucleotide polymorphisms, HRM analysis identified eight pathogenic mutations in 146 patients (Table 1). Similarly, targeted sequencing also detected the eight pathogenic mutations, confirming the mutation detection sensitivity of HRM analysis was 100% in this cohort. These mutations included two different FUS pathogenic mutations in two different ALS patients and five TARDBP pathogenic mutations in six ALS patients, which included two novel mutations. Therefore, FUS mutations were present in 1.4% of Chinese ALS patients, whereas TARDBP mutations were responsible for 4.1% of Chinese ALS cases.

FUS mutations and clinical characteristics

Figure 1A shows the detected novel splicing mutation, c.1541+1G>A (Figure 1A), located at the splice donor site of the FUS intron 14. The guanine of the wild-type donor site is highly conserved across species (Figure 1B). A functional splicing reporter minigene assay confirmed the novel splicing mutation result in a frameshift, leading to truncated FUS protein p.G466VfsX14 (Figure 1C).

The novel splicing mutation was identified in a 51-year-old male patient whose daughter previously died from ALS. Initially, he presented with neck weakness and dysphagia. After 5 months, lower right limb weakness emerged and gradually increased without cognitive impairment. The patient died 13 months after onset of symptoms. A 20-year-old female patient, the daughter of the 51-year-old patient, experienced lower right limb

weakness, which quickly developed in all four extremities within 1 year. After 12 months, she died of respiratory failure. Figure 1D shows the same mutation was detected in the sister of the 51-year-old patient's sister and shared a similar clinical phenotype; the disease duration of this patient was only 8 months.

A heterozygous missense substitution, c.1562G>A (p.R521H, Figure 2A), was identified in an index fALS patient who began suffering from progressive weakness in the upper-right limb at 41 years of age. Twelve months later, the weakness developed in other limbs. Then, he presented with dysarthria and dysphagia after 13 months and without cognitive impairment. The patient died 3 years after onset of symptoms. The patient's brother who shared the same mutation (patient III1) also had a similar clinical phenotype and disease duration. Patient III2, as well as several family members of patients III1 and III3, also share the mutation; however, they did not show any clinical characteristics of ALS until now (Figure 2B).

TARDBP mutations and clinical characteristics

Five TARDBP mutations were observed in six unrelated ALS patients (Figure 3A), which included a novel 3'UTR mutation (c.*731A>G) and four known causative mutations (p.G294V, p.G348V, p.M337V, and p.I383V). According to the UCSC Genome Browser, the TARDBP 3'-UTR site c.*731 is highly conserved through the evolutionary spectrum from human to chicken (Figure 3B), supporting its functional importance. MicroRNA (miRNA) binding searches of TARDBP through the TargetScanHuman database showed binding of miR-376a directly to the 3'UTR variant region. The novel c.*731A>G TARDBP mutation was expected to interfere with the normal binding of miR-376, and then disorder the expression of TARDBP mRNA. However, we sequenced this region of the patient's parents and confirmed this was a de novo mutation. In addition, the mutation was not observed in available variation databases, including mutation database and SNP databases such as the National Center for Biotechnology Information (NCBI) Single Nucleotide Polymorphism Database (<http://www.ncbi.nlm.nih.gov/projects/SNP/>), Exome Aggregation Consortium (ExAC) database (<http://exac.broadinstitute.org>), and ALS Online Genetics Database (<http://alsod.iop.kcl.ac.uk/>) [13]. According to American College of Medical Genetics and Genomics guidelines, it meets de novo criteria PS2 (in a patient with the disease and no family history), PM2 (absent from controls or at extremely low frequency if recessive in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium), and PP4 (patient's

Table 1. Clinical data of ALS patients carrying pathogenic mutations in FUS and TARDBP genes.

Gene	Nucleotide change	Amino acid change	Type of mutation	Onset; age of onset;gender	Disease duration	ALS family history	References
FUS	c.1541+1G>A	-	splicing	Bulbar; 51; M	1 y	Yes	This study
FUS	c.1562G>A	p. R521H	missense	Spinal; 41; M	3 y	Yes	[14]
TARDBP	c.881G>T	p. G294V	missense	Bulbar, Spinal; 61; M	0.5 y	No	[15, 16]
				Bulbar; 53; M	1.5+y	No	
TARDBP	c.1009A>G	p. M337V	missense	Spinal; 58; M	1+y	Yes	[17]
TARDBP	c.1043G>T	p. G348V	missense	Spinal; 24; M	10+y	No	[18]
TARDBP	c.1147A>G	p. I383V	missense	Spinal; 45; F	3 y	No	[19]
TARDBP	c. *731A>G	-	3'UTR	-,N/A;M	-	No	This study

The symbol + indicates disease in progress.

Key: ALS, amyotrophic lateral sclerosis; F, female; M, male; N/A, information not available

phenotype or family history is highly specific for a disease with a single genetic etiology) for classifying pathogenic variants. We suggest this mutation is a “likely pathogenic” for the previously stated reason.

The TARDBP p.G294V mutation is located in the sixth exon of the gene and was present in two unrelated male sALS patients. The patient began undergoing dysarthria with weakness in the upper right limb. Five months later, the weakness developed in the upper left limb, and dysphagia occasionally occurred. He died 6 months after onset of symptoms. At age 53, the second patient experienced symptoms primarily characterized by dysarthria. Neither ALS patient carrying the p.G294V mutation showed signs of cognitive dysfunction.

The p.G348V mutation was identified in a 24-year-old juvenile-onset male patient who reported no family history of ALS. During the initial onset of symptoms, the patient showed slight fasciculation in the upper right limb. During the next 3 years, the fasciculation was progressive and developed in the upper left limb. The MRI images show that the anisotropy of cortical spinal tract scores in the left midbrain was indicative of white matter degeneration.

To date, fasciculation and muscle atrophy have been observed in all extremities.

A 45-year-old female patient carrying the p.I383V mutation presented with progressive weakness in the

upper limb, and electromyography results suggested spinal anterior of the lower cervical was injured. After 3 years, the patient died of respiratory failure.

DISCUSSION

Following rapid development of molecular genetics, many genes have been identified with ALS pathogenesis. Although the exact mechanism for ALS is still unknown, several hypotheses include defective RNA metabolism, glutamate excitotoxicity, disruption of membrane trafficking, endoplasmic reticulum stress, mitochondrial dysfunction, and protein misfolding and aggregation [20]. Some mutations in FUS and TARDBP genes, which both play an important role in mRNA transport, axonal maintenance, and motor neuron development, have been reported as causative ALS mutations for disturbing RNA homeostasis [4, 9, 21]. Interestingly, because the vast majority of these causative mutations in FUS and TARDBP cluster in the C-terminus of the proteins, also known as mutation hotspot regions, [9, 10] this suggested we could focus on mutation detection at the mutational hotspot regions, instead of the whole gene.

Although Sanger sequencing and next-generation sequencing are two of most common mutation detection methods for large-scale genomics sequencing samples, they can be cost prohibitive and slow. In this study, we first identified the FUS and TARDBP mutations in

Chinese ALS patients by the fast and highly sensitive screening method, HRM analysis.

Using HRM analysis, we successfully identified five known possible causative mutations, and two novel mutations. The mutation frequency was 1.4% for FUS, and 4.1% for TARDBP in Chinese ALS patients. However, the frequency of our study is inconsistent with previously reported results, [22] which may be due to small sample size and population difference.

We identified two FUS pathogenic mutations in this study. Previous reports identified mutation p.R521H as the highest FUS mutation frequency, accounting for 30%, and experimental results show that the FUS R521H missense mutation could lead to aberrant trafficking and retention of the mutant FUS protein in the cytoplasm [14, 23, 24]. Interestingly, several

relatives and a sibling of the proband also have the p.R521H mutation; however, they did not present with clinically relevant ALS symptoms, which may be related to age or the presence of a recessive inheritance pattern [14, 25].

A novel splicing mutation, c.1541+1G>A, the third splicing mutation linked to ALS in FUS gene [26, 27], produced a truncated FUS protein, p.G466VfsX14, eliminating the nuclear localization sequence (NLS), where most previously published mutations are located [9], and then impair its nuclear import. Previous results show that overexpression of cytoplasmic FUS with deleted NLS results in recapitulation of ALS-like motor neuron abnormalities in mice, [28] indicating this mutation is significant. Furthermore, mutation of a truncated protein lacking the NLS was reported to cause

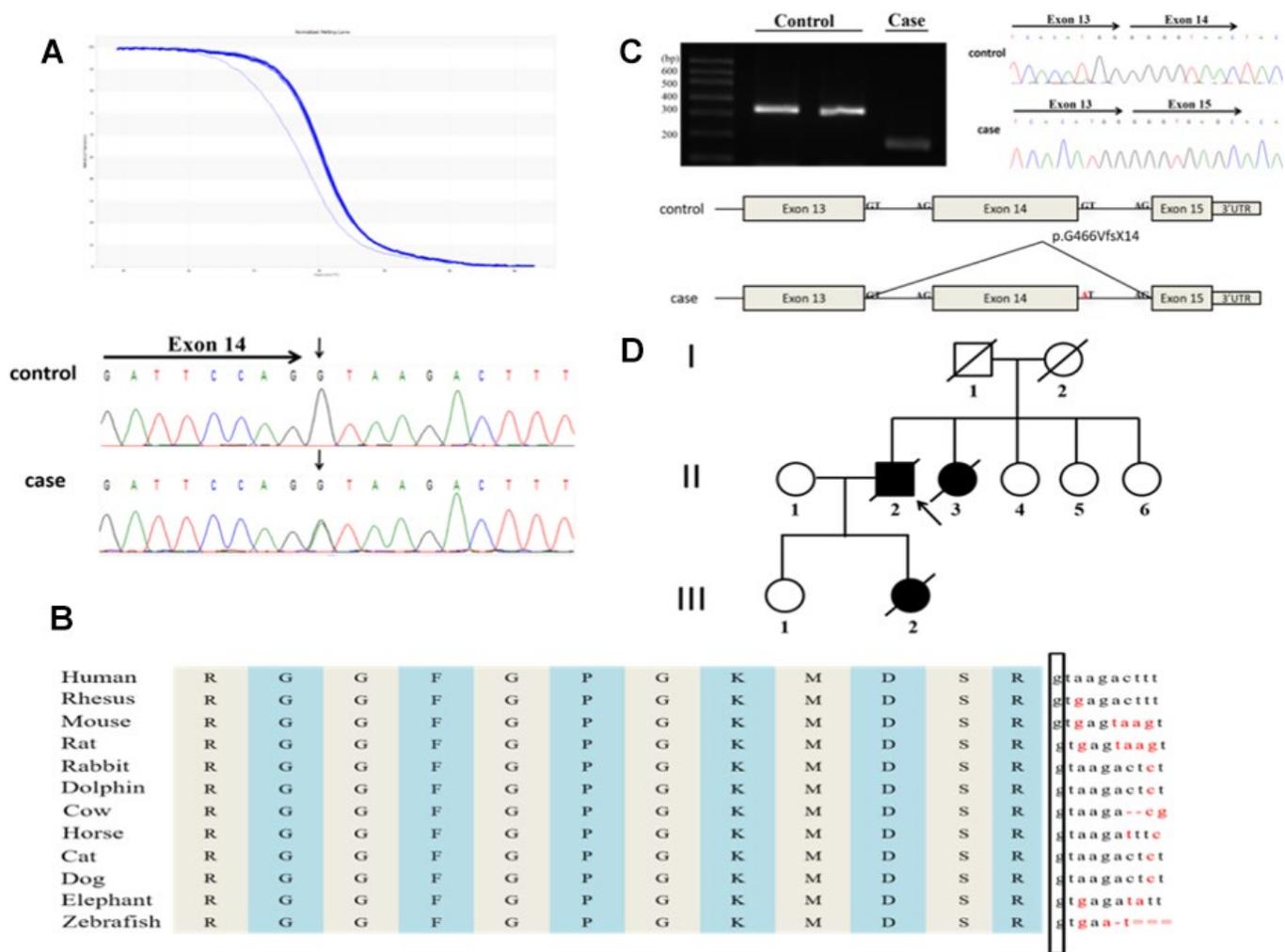


Figure 1. A novel splicing mutation, c.1541+1G>A in the FUS gene identified in a Chinese ALS patient. (A) The novel splicing mutation was identified by HRM analysis and direct sequencing. **(B)** The evolutionary conservation of the splicing mutation c.1541+1G>A are shown. **(C)** Minigene splicing analysis of the novel splice mutation. **(D)** Pedigree of the family. An arrowhead indicates the proband.

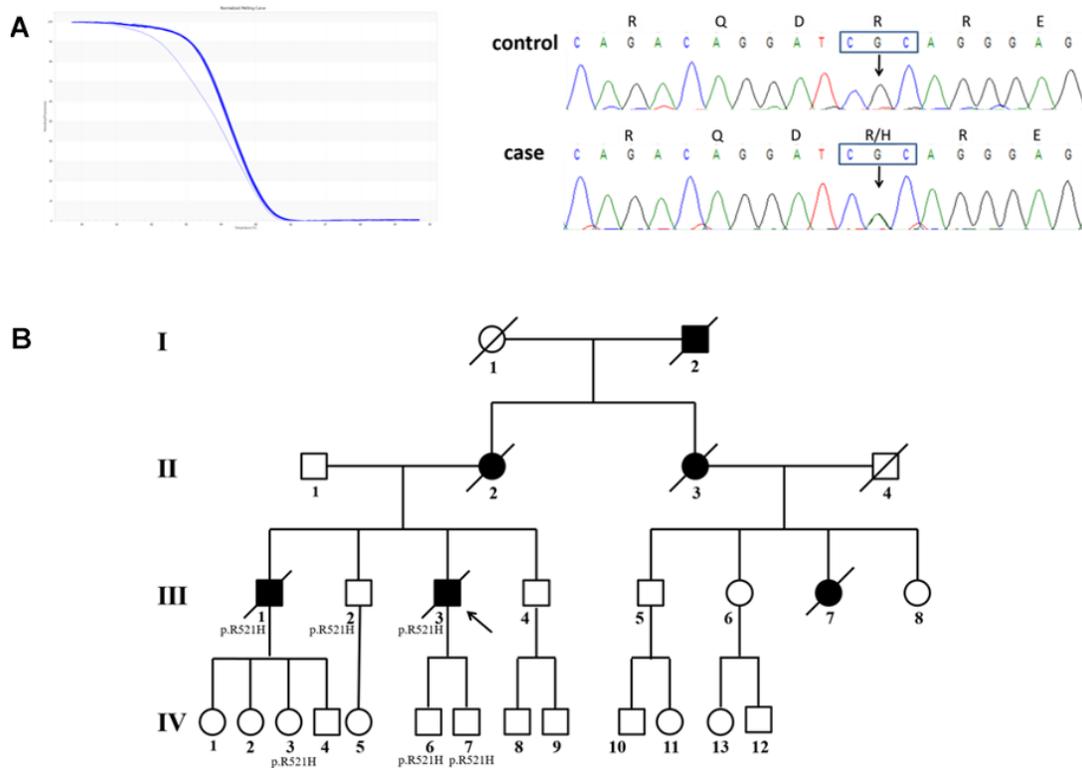


Figure 2. The pedigree with FUS mutation p. R521H. (A) Results of HRM analysis and direct sequencing of patients. (B) Pedigree of the family. An arrowhead indicates the proband. Patients III2, IV3, IV6, and IV7 shared the same mutation, but they did not appear as ALS phenotypes until now.

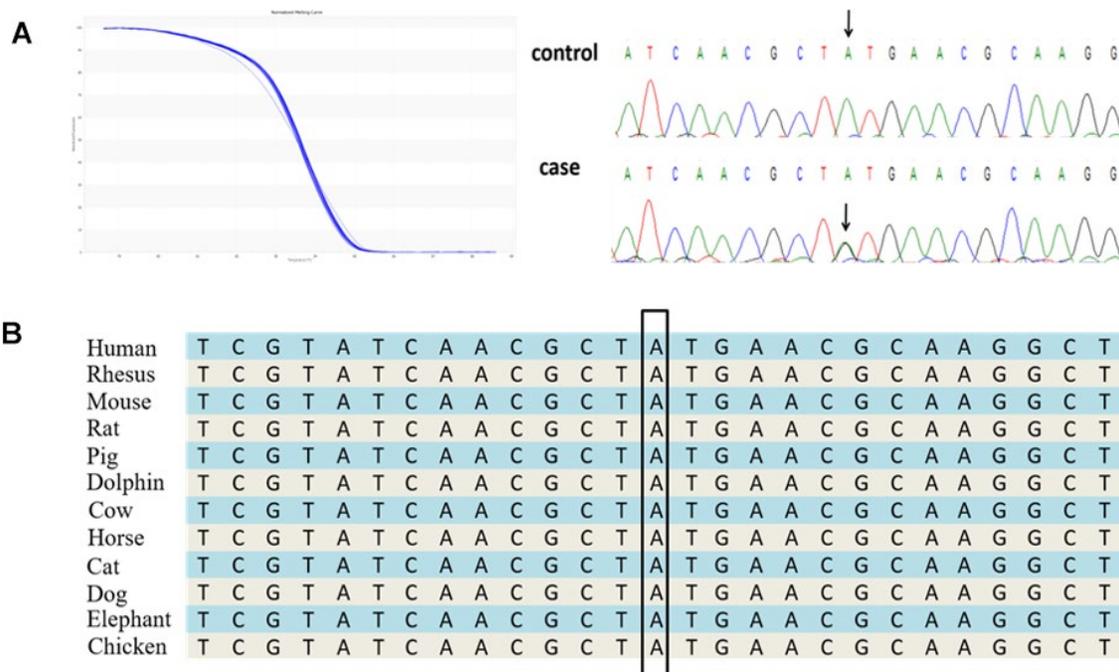


Figure 3. A novel 3'UTR mutation, c. *731A>G in the TARDBP gene identified in a Chinese ALS patient. (A) The novel 3'UTR mutation was identified by HRM analysis and direct sequencing. (B) The evolutionary conservation of the TARDBP 3'UTR mutation c. *731A>G.

juvenile ALS with rapid disease progression [29, 30], which was consistent with the clinical symptoms in our study. Subsequent research is still needed to study the function of the mutation. The novel splicing mutation we found confirms the C-terminal NLS of the FUS gene plays a key role in ALS pathology.

We identified five TARDBP mutations in our ALS cohort, four of which have previously been reported as causative mutations. The p.G294V mutation was previously identified in a sALS patient from Italy and in a fALS patient from Australia, and was predicted to disrupt the glycine-rich domain in the C-terminus, which plays a role in RNA binding and is required for the exon-skipping activity of TARDBP [15, 31]. Previous reports showed the percentages of cells with positive nuclear immunostaining were significantly lower in patients with TARDBP p.G294V mutations than in controls, indicating its importance [16]. In this study, we identified p.G294V mutations in two unrelated sALS patients, for whom the onset of symptoms is characterized by dysarthria. To our knowledge, this is the first Asian population study for this mutation. The p.G348V mutation, which increases the amount of TARDBP protein at the cellular level, was detected in ALS patients from various populations [18, 32–34]. However, there are some differences for the ALS clinical symptoms between the western and Chinese populations. The age of onset for Chinese ALS cases were much younger than previously published western cases. Additionally, the mutation carriers in the Chinese population experienced slower disease progression and longer survival time [34]. Our study confirmed these differences. The third mutation we identified, p.I383V, has been detected in fALS patients from the United States, Turkey, and Taiwan. However, the onset and progression of the disease for the carrier p.I383V are different [19, 35–37]. In our study, the mutation p.I383V was identified from a sALS woman who suffered progressive weakness in the upper limb at the age of 45, and died 3 years later.

The final mutation we identified, the novel 3'UTR mutation c. *731A>G, was a de novo and was not observed in the parents of patients. Additionally, it has not been reported in any mutation databases, and the site c. *731 is highly conserved. According to the software, the mutation may affect the combination of mir-376a for TARDBP and then lead to increasing mRNA levels, which may accelerate disease progression by inducing the formation of cytoplasmic inclusions and/or the dysregulation of RNA metabolism [38]. In addition, a previous study reported a similar 3'UTR mutation linked to ALS [39]. According to the guideline of ACMG, we suggested c. *731A>G as a likely pathogenic mutation for ALS. Unfortunately, we did not

have access to detailed medical records and fresh blood samples.

In conclusion, to our knowledge, this is the first time that HRM analysis was used to identify FUS and TARDBP mutations in Chinese patients with ALS, demonstrating its high sensitivity and accuracy. Our data suggests that HRM analysis is a fast and easy method for mutation screening in large samples. Most important, however, we identified two novel pathogenic ALS mutations, which further expands the ALS-related gene mutation spectrum, and provides data for subsequent studies. We also investigated the frequency of FUS and TARDBP mutations in the Chinese population.

MATERIALS AND METHODS

Subjects

The study included 146 patients with sALS and nine fALS index cases (male: 62.3%; age at onset: 47.9 ± 11.7 years). All patients were recruited from the Department of Neurology, Southwest Hospital, Third Military Medical University, Chongqing between 2012 and 2017. All patients were Han Chinese, and the patients met the El Escorial criteria for ALS diagnosis [40]. Family history was considered positive if the patient had at least one affected relative within three generations [41]. All participants provided informed consent before blood donation. Protocols were in accordance with the ethical standards of the responsible committee on human experimentation and the Helsinki Declaration of 1964 and approved by the institutional ethics committee of the Hospital (equivalent to an Institutional Review Board). This study was carried out in accordance with the approved guidelines. Written informed consent was obtained from all participants. All patients were tested for the C9orf72 GGGGCC expanding mutation with repeat-primed PCR, together with proper positive control and negative control. No C9orf72 GGGGCC expanding mutation was detected in this cohort.

Screening TARDBP and FUS gene mutations by PCR-HRM

We designed PCR primers for HRM analysis to screen all of the mutation hotspot regions containing OMIM Allelic Variants mutations related to ALS (from the UCSC Genome Browser) of FUS (NM_004960.3) and TARDBP (NM_007375.3). Table 2 shows the amplified primer sequence and the length of each expected fragment.

Genomic DNA was extracted from peripheral blood leukocytes using standard methods.

Table 2. PCR primer sequences of FUS and TARDBP gene and size of amplified fragment.

Gene	Primer	Primer sequence	Fragment size
FUS	1	5'-GAGGGTAACACTGGGTACAGGAC-3' 5'-GGCTTGGAGAGGCTGGTAAC-3'	160
	2	5'-GGTTATGGCAATCAAGACCAGA-3' 5'-CTGGGTTTCATAGCCACCACT-3'	149
	3	5'- GCTATGATCGAGGCGGCTA -3' 5'-TGGCCTCTGTTCAACTGCTC -3'	158
TARDBP	1	5'-CGGAATATGAAACACAAGTGAAAG-3' 5'-GAATTAGGAAGTTTGCAGTCACAC-3'	82
	2	5'-CCGAACCTAAGCACAATAGCA-3' 5'-TCATCCCACCACCCATATTACT-3'	156
	3	5'-AATATGGGTGGTGGGATGAAC -3' 5'-TGTTGCCTTGGTTTTGGTTATT-3'	160
	4	5'-AATAACCAAAACCAAGGCAACAT-3' 5'-TTGAGCCAAAGCCTCCATTA- 3'	157
	5	5'-GGGTTGTGGTTGGTTGGTATAG-3' 5'-CTGCTGAATATACTCCACACTGAAC-3'	130
	6	5'-GCCATAGGAATACTGTCTACATGCT-3' 5'-CCATATCACAGCCTTGCGTT-3'	152

PCR-HRM was performed with Eco Real-Time PCR System (Illumina) in 10- μ L reaction mixtures comprising 20 ng of DNA, 1 \times PCR buffer, 0.2 mM dNTP, Eva Green (Biotium), 1U of Taq polymerase, and 0.25 μ M each forward and reverse primers. Initial denaturation was performed at 95°C for 5 min, followed by 50 cycles of 95°C for 20 sec, 60°C for 20 sec, and 72°C for 20 sec. The melting curves were obtained under the following conditions: 95°C for 15 sec, 55°C for 15 sec, and with a ramping rate of +0.2°C/sec to 95°C.

Direct DNA sequencing and bioinformatics

To confirmed the genotype of mutations and the specificity of HRM analysis, All PCR products showing an HRM aberrant pattern were analyzed with direct DNA sequencing on a PRISM 377 full-automatic sequencing analyzer (ABI).

When the DNA sequencing result was determined, we first confirmed whether there was a mutation aimed to detect the specificity of HRM analysis. Then, we ensured it was a novel mutation using the HGMD database (<http://www.hgmd.cf.ac.uk/ac>) and the dbSNP database (<http://www.ncbi.nlm.nih.gov/snp>). Finally, to evaluate the potential functional effect of the novel splicing mutation and 3'UTR site mutation, we used the UCSC Genome Browser (<http://genome.ucsc.edu/>),

Target (http://www.targetscan.org/vert_71), Human Splicing Finder (<http://www.umd.be/HSF3/>). To identify known variations, we used ALS Online Genetics Database (<http://alsod.iop.kcl.ac.uk/>) (13), the NCBI Database (<http://www.ncbi.nlm.nih.gov>), and Exome Aggregation Consortium (ExAC) database (<http://exac.broadinstitute.org>).

Targeted sequencing

To verify the sensitivity of HRM analysis, we sequenced all exons of the FUS and TARDBP gene in 146 patients by Illumina Hiseq 3000 system. The target coverage is 98% with an average depth of 100X. All nonsynonymous variants detected were filtered.

Functional splicing reporter minigene assay

Because of the lack of simple RNA, we used splicing reporter minigene assay to confirm the functional effect of the novel splicing mutation. The minigene was constructed by PCR amplification of mutant genomic DNA sequences as previously described [42]. The amplified sequences included exons 13 through 3'UTR of the FUS gene. FUS minigene products were digested with BamH I and Xho I (Thermo) and cloned into the pCDNA3.1(+) reporter vector. The resulting constructs were transfected into

U87 cells. Total cellular RNA was extracted after 24 hours, followed by RT-PCR analysis.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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