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ARTICLE

Presence of Rare Variants is Associated with Poorer Survival in Chinese Patients with Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder with phenotypic and genetic heterogeneity. Recent studies have suggested an oligogenic basis of ALS, in which the co-occurrence of two or more genetic variants has additive or synergistic deleterious effects. To assess the contribution of possible oligogenic inheritance, we profiled a panel of 43 relevant genes in 57 sporadic ALS (sALS) patients and eight familial ALS (fALS) patients from five pedigrees in east China. We filtered rare variants using the combination of the Exome Aggregation Consortium, the 1000 Genomes and the HuaBiao Project. We analyzed patients with multiple rare variants in 43 known ALS causative genes and the genotype–phenotype correlation. Overall, we detected 30 rare variants in 16 different genes and found that 16 of the sALS patients and all the fALS patients examined harbored at least one variant in the investigated genes, among which two sALS and four fALS patients harbored two or more variants. Of note, the sALS patients with one or more variants in ALS genes had worse survival than the patients with no variants. Typically, in one fALS pedigree with three variants, the family member with three variants (*Superoxide dismutase 1* (*SOD1*) p.V48A, *Optineurin* (*OPTN*) p.A433V and *TANK binding kinase 1* (*TBK1)* p.R573H) exhibited much more severe disease phenotype than the member carrying one variant (*TBK1* p.R573H). Our findings suggest that rare variants could exert a negative prognostic effect, thereby supporting the oligogenic inheritance of ALS.

Keywords Amyotrophic lateral sclerosis · Oligogenic inheritance · Survival · Superoxide dismutase 1 · TANK-binding kinase 1 · Optineurin

Abbreviations

ACMG	The American College of Medical Genetics and Genomics
ALS	Amyotrophic lateral sclerosis
ALS2	Amyotrophic lateral sclerosis 2
ALSdb	ALS data browser
ALSod	Amyotrophic Lateral Sclerosis online Database
ANG	Angiogenin
ANXA11	Annexin A11
ATXN2	Ataxin 2
C9orf72	Chromosome 9 open reading frame 72
C21orf2	Cilia- and flagella-associated protein 410
CAMTA1	Calmodulin-binding transcription activator 1
CCNF	Cyclin F
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain containing 10
CHMP2B	Charged multivesicular body protein 2B
CYLD	Cylindromatosis
DCTN1	Dynactin subunit 1
DNAJC7	DnaJ heat shock protein family (Hsp40) member C7
ELP3	Elongator acetyltransferase complex subunit 3

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EPHA4	Ephrin type-A receptor 4
ERBB4	Erb-B2 receptor tyrosine kinase 4
ExAC	The Exome Aggregation Consortium
fALS	Familial amyotrophic lateral sclerosis
FTD	Frontotemporal dementia
FIG4	FIG4 phosphoinositide 5-phosphatase
FUS	Fused in sarcoma
GLT8D1	Glycosyltransferase 8 domain containing 1
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1
HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1
KIF5A	Kinesin family member 5A
LGALSL	Lectin galactoside-binding-like protein
MATR3	Matrin 3
NEFH	Neurofilament heavy chain
NEK1	NIMA-related kinase 1
OMIM	Online Mendelian inheritance in man
OPTN	Optineurin
PFN1	Profilin 1
PRPH	Peripherin
sALS	Sporadic amyotrophic lateral sclerosis
SETX	Senataxin
SIGMAR1	Sigma non-opioid intracellular receptor 1
SNP	Single-nucleotide polymorphisms
SOD1	Superoxide dismutase 1
SPG11	Spastic paraplegia 11
SPTLC1	Serine palmitoyltransferase long chain base subunit 1
SQSTM1	Sequestosome 1
TARDBP	TAR DNA-binding protein
TBK1	TANK-binding kinase 1
TIA1	Cytotoxic granule-associated RNA-binding protein
TUBA4A	Tubulin Alpha 4a
UBQLN2	Ubiquilin 2
UNC13A	Unc-13 Homolog A
VAPB	Vesicle-associated membrane protein-associated protein B/C
VCP	Valosin-containing protein
WES	Whole-exome sequencing

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder with a substantial heritable component (Goutman et al. 2022a). Mendelian familial ALS (fALS) accounts for 10–15% of individuals with the disease. Approximately, 90% of cases are sporadic and do not have any family history. The pathogenesis of ALS remains inadequately understood. Recent studies have raised a gene–time–environment hypothesis, which posits that genetic predisposition interacts with environmental exposures over time leading to ALS (Al-Chalabi and Hardiman 2013; Goutman et al. 2022b). Heredity plays an important role in the cause of ALS, even in sporadic ALS (sALS) patients. The heritability of sALS has been estimated to be 12–21% in genome-wide association studies (Fogh et al. 2014; Keller 2014) to as high as 61% in twin studies (Al-Chalabi et al. 2010). The first ALS gene, *Superoxide dismutase 1* (*SOD1*), was discovered through linkage analysis in 1993 (Rosen et al. 1993). Moreover, over the past decade, the number of genes associated with ALS has increased dramatically. Currently, more than 150 genes have been identified to be related to ALS (http://alsod.iop.kcl.ac.uk); among them are approximately 20 genes are with high penetrance, such as *SOD1*, *Chromosome 9 open reading frame 72* (*C9ORF72*), *TAR DNA-binding protein* (*TARDBP*) and *Fused in sarcoma* (*FUS*), whereas the rest are reported to increase the risk of ALS (Shatunov and Al-Chalabi 2021).

Accordingly, lines of evidence have proposed an oligogenic basis of ALS (Al-Chalabi et al. 2017; van Blitterswijk et al. 2012). The hypothesis was firstly developed from the fact that asymptomatic carriers are common even in pedigrees with variants in high-penetrant genes such as *SOD1*. We have reported p.Gly141Ala mutation in the *SOD1* gene associated with incomplete penetrance (Dong et al. 2020). Description of compound heterozygous and recessive *SOD1* mutations suggests that oligogenic inheritance may account for incomplete penetrance (Gentile et al. 2021; Kuuluvainen et al. 2019). Subsequently, a series of studies have reported the coexistence of multiple variants in ALS causal genes in both sALS and fALS patients (Cady et al. 2015; Dols-Icardo et al. 2018; Giannoccaro et al. 2017; McCann et al. 2020; Morgan et al. 2017; Naruse et al. 2019; Pang et al. 2017; Scarlino et al. 2020; Shepheard et al. 2021). Among them, several studies suggested that the additional variant in the ALS causative gene may influence ALS phenotypes such as the onset of age (Cady et al. 2015; Naruse et al. 2019; Shepheard et al. 2021), survival (Pang et al. 2017; Scarlino et al. 2020), and co-occurrence with dementia (Dols-Icardo et al. 2018; Giannoccaro et al. 2017) or parkinsonism (Giannoccaro et al. 2017).

Although oligogenic inheritance is reported in several studies, further studies in different ethnic populations are crucial. In this study, to assess the contribution of possible oligogenic inheritance, by profiling a panel of 43 relevant genes in 57 sALS patients and eight familial fALS patients from five pedigrees in east China, we have demonstrated that ALS patients can carry more than one variant in ALS causative genes, and that the presence of rare variants is associated with poorer survival in both sALS patients and an ALS pedigree. Our findings suggested that rare variants

could exert a negative prognostic effect, supporting the oligogenic inheritance of ALS.

Materials and Methods

Subjects

Between 2011 and 2020, ALS patients who were diagnosed and followed up at Huashan Hospital, Shanghai were systematically enrolled. All subjects were evaluated by neuromuscular specialists and fulfilled the criteria for definite ALS, probable ALS, or probable ALS-laboratory-supported based on the revised El Escorial criteria (Brooks et al. 2000). Data on sex, age, site of onset, and treatment with riluzole were recorded and those on survival, defined as the time from symptom onset to permanent assisted ventilation ($\geq$ 23 h per day noninvasive ventilation), tracheostomy, or death, whichever is earlier, were recorded. Written informed consent was obtained from all the participating subjects.

Controls

An exon database named "HuaBiao" (https://www.biosino.org/wepd), which contains deep sequencing ($>100\times$) of 5000 healthy samples collected mainly from three representative Han Chinese populations at Zhengzhou, Taizhou and Nanning, was used for case–control association tests (Hao et al. 2021). High-quality reads (mean Phred score > 30) and high sequencing depth of samples (mean depth > 100×) were processed by the same procedures as ALS subjects. Only protein-coding regions were included for downstream analyses.

Genotyping and Rare Variant Filtering

Genomic DNA was extracted from the whole blood of individual subjects according to standard protocols. Next-generation sequencing (NGS) library preparation and whole-exome sequencing (WES) was performed using commercial assay kits (NGS Library Prep kits and Nimblegen SeqCap EZ Human Exome Kit v3.0 kits, Roche). NGS was performed by Illumina HiSeq system with 140 bp of paired-end reads. The mean sequencing depth of the targeted exome region reached at least 60× in both cases and controls. The WES data were processed through the Genome Analysis Toolkit best practice pipeline before variant calling, and HaplotypeCaller was used (www.biostars.org/) to call the variants in parallel. Variant annotation and filtering were performed by ANNOVAR with sorting intolerant from torelant (SIFT, http://sift.jcvi.org/) and PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), and by SnpEff with GENCODE (http://snpeff.sourceforge.net/) as an annotation database. Population frequencies for each variant were determined in dbSNP, the 1000 Genomes Project, and the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org). Under the "rare disease, rare mutation" criteria, only non-synonymous variants with minor allele frequency (MAF) of less than 0.01% across all populations in ExAC and 1000 Genomes Project datasets were selected. All of the variants had a sequencing quality score (Phred) of at least 50 and a mapping quality score of at least 20.

ALS Candidate Genes

Genes for the ALS panel were selected from the Amyotrophic Lateral Sclerosis online Database (ALSoD, http://alsod.iop.kcl.ac.uk/) and the Online Mendelian Inheritance in Man (OMIM, http://www.omim.org/) databases and two latest studies (Goutman et al. 2022b; Mohassel et al. 2021). Forty-one causative ALS genes (*Amyotrophic lateral sclerosis 2* (*ALS2*), *Angiogenin (ANG)*, *Annexin A11* (*ANXA11*), *Cilia and flagella associated protein 410 (C21orf2*), *Calmodulin binding transcription activator 1 (CAMTA1)*, *Cyclin F (CCNF)*, *Coiled-coil-helix-coiled-coil-helix domain containing 10 (CHCHD10)*, *Charged multivesicular body protein 2B (CHMP2B)*, *Cylindromatosis (CYLD)*, *Dynactin subunit 1 (DCTN1)*, *DnaJ heat shock protein family member C7 (DNAJC7)*, *Elongator acetyltransferase complex subunit 3* (*ELP3*), *Ephrin type-A receptor 4 (EPHA4)*, *Erb-B2 receptor tyrosine kinase 4 (ERBB4)*, *Fig4 phosphoinositide 5-phosphatase (FIG4)*, *Fused in sarcoma (FUS)*, *Glycosyltransferase 8 domain containing 1 (GLT8D1)*, *Heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1)*, *Heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1)*, *Kinesin family member 5A (KIF5A)*, *Lectin galactoside binding like protein (LGALSL)*, *Matrin 3 (MATR3)*, *Neurofilament heavy chain (NEFH)*, *NIMA related kinase 1 (NEK1)*, *Optineurin (OPTN)*, *Profilin 1 (PFN1)*, *Peripherin (PRPH)*, *Senataxin (SETX)*, *Sigma non-opioid intracellular receptor 1 (SIGMAR1)*, *Superoxide dismutase 1 (SOD1)*, *Spastic paraplegia 11 (SPG11)*, *Serine palmitoyltransferase long chain base subunit 1 (SPTLC1)*, *Sequestosome 1 (SQSTM1)*, TAR DNA binding protein (*TARDBP)*, *TANK binding kinase 1 (TBK1)*, Cytotoxic granule associated RNA binding protein (*TIA1)*, *Tubulin alpha 4A (TUBA4A)*, *Ubiquilin 2 (UBQLN2)*, *Unc-13 homolog A (UNC13A)*, *Vesicle-associated membrane protein associated protein B/C (VAPB),* and *Valosin containg protein (VCP)*) were examined for rare variants. The *Chromosome 9 open reading frame 72 (C9orf72)* repeat expansion and *Ataxin 2 (ATXN2)* repeat expansion were analyzed in all the patients using repeat-primed polymerase chain reactions.

Statistical Analysis

Statistical analyses were performed in R v4.0.3 to identify any association between clinical variables including sex, age at disease onset, site of disease onset (bulbar or spinal), and disease duration. A χ^2 analysis was performed between sex and site of onset, whereas Welch's *t*-tests were performed between the age of onset and both sex and site of onset. The Kaplan–Meier survival analyses were performed between disease duration and both sex and site of onset. Survival was defined as the time from symptom onset to permanent assisted ventilation (≥ 23 h per day noninvasive ventilation), tracheostomy, or death. In addition, a linear regression model was fitted between age of onset and duration.

Whether the number of ALS-implicated variants carried by individuals influenced their clinical presentation was also assessed. To identify rare and novel single-nucleotide polymorphisms (SNPs) that might be overrepresented in sALS subjects, Fisher's exact tests were used to compare each candidate SNV's allele frequency in sALS versus controls. One-way analysis of variance was used to assess the association between the number of rare non-synonymous variants (Non-syn RV) and age at onset. The Kaplan–Meier survival analyses were performed between disease duration and the presence of both non-synonymous rare variants (Non-syn RV) and synonymous rare variants (Syn RV). After adjustment for onset age, sex and site of onset, the hazard ratio between the number of rare variants and survival status was estimated by Cox regression. All the hazard ratios in this paper were estimated by penalized regression model using glmnet v4.1 to correct left-truncation bias that might arise from death before being able to undergo the genetic tests (McGough et al. 2021).

Results

Subject Phenotypic Characteristics

Demographic characteristics for all 57 sequenced sALS subjects and eight fALS subjects from five pedigrees are shown in Table 1. The mean (SD) age of onset was 55.0 ± 10.7 years in sALS patients and 43.1 ± 7.7 in eight fALS patients. Of sALS and fALS patients, 68.4% and 60% were male, respectively. Bulbar onset was present in 14 sALS patients (24.6%) but not in fALS patients. Riluzole was administered in 46 sALS patients and all fALS patients for at least one month. After a median of 29 (interquartile range 19–45) months, 53 sALS patients had reached the end point. The follow-up time of eight fALS patients (i.e., Pedigree A: III7 and IV4, Pedigree B:III1, Pedigree C: II7, Pedigree D: III18, three members of Pedigree E not shown) was short, thus, only two patients reached the end point, the survival time of which was 24 months (Pedigree A, III7) and 47 months (Pedigree E, III2) (Table 1, Fig. 1).

Table 1 Demographics of ALS patients

	sALS patient	fALS patients
Self-reported Han Chinese	57 (100%)	8 (100%)
Male sex, num (%)	39 (68.4%)	3 (37.5%)
Bulbar onset, num (%)	15 (26.3%)	0 (0%)
Age at onset, year mean ± SD	55.0 ± 10.7	43.1 ± 7.7
Riluzole use	46 (80.7%)	8 (100%)
Survival, month median (IQR)	29 (19–45)	35.5

ALS, amyotrophic lateral sclerosis; sALS, sporadic ALS; fALS, familial ALS; IQR, interquartile range

Statistical analyses were performed to identify any association between clinical variables, namely sex, age at disease onset, site of disease onset and disease duration (Fig. 2a–f). There was no significant association between gender and any of the other three indicators (Fig. 2a–c); however, there was a high propensity for women to develop bulbar onset and for men to present with spinal onset (Fig. 2c). The cases with bulbar onset were more likely to have later age of onset ($p = 0.0159$, Fig. 2d) and reduced life expectancy ($p = 0.031$, Fig. 2e). There was also a tendency that patients with later onset present shorter disease duration (Fig. 2f).

Rare Variant Identifications

Of the 43 causative genes examined, 43 missense variants with MAF < 0.01% in ExAC and 1000 Genomes were filtered, as shown in Table 2. The pathogenicity of each variant was evaluated according to the American College of Medical Genetics and Genomics (ACMG) standards (Richards et al. 2015). Considering the heterogeneity of common SNPs in different ethnic populations, the frequency of the loci found in the HuaBiao database was also explored, which consists of 5000 healthy Han Chinese. Fisher's exact tests were performed to calculate whether there was a significant difference in the frequency of these SNPs between the ALS cohort and the Chinese healthy population. We identified 13 of the variants with MAF < 0.01 in the ExAC and 1000 Genomes, which are relatively common ($p > 0.05$) in the Chinese population (Table 2, common variants in the Chinese population), whereas the other 30 variants in 16 different genes are rare in both Chinese and Caucasian populations (Table 2, rare variants in the Chinese population). Some synonymous variants of 30 genes were also found (Supplementary Table 1).

Patients with Multiple Rare Variants

After the exclusion of the 10 relatively common variants in the Chinese population, all eight fALS patients from

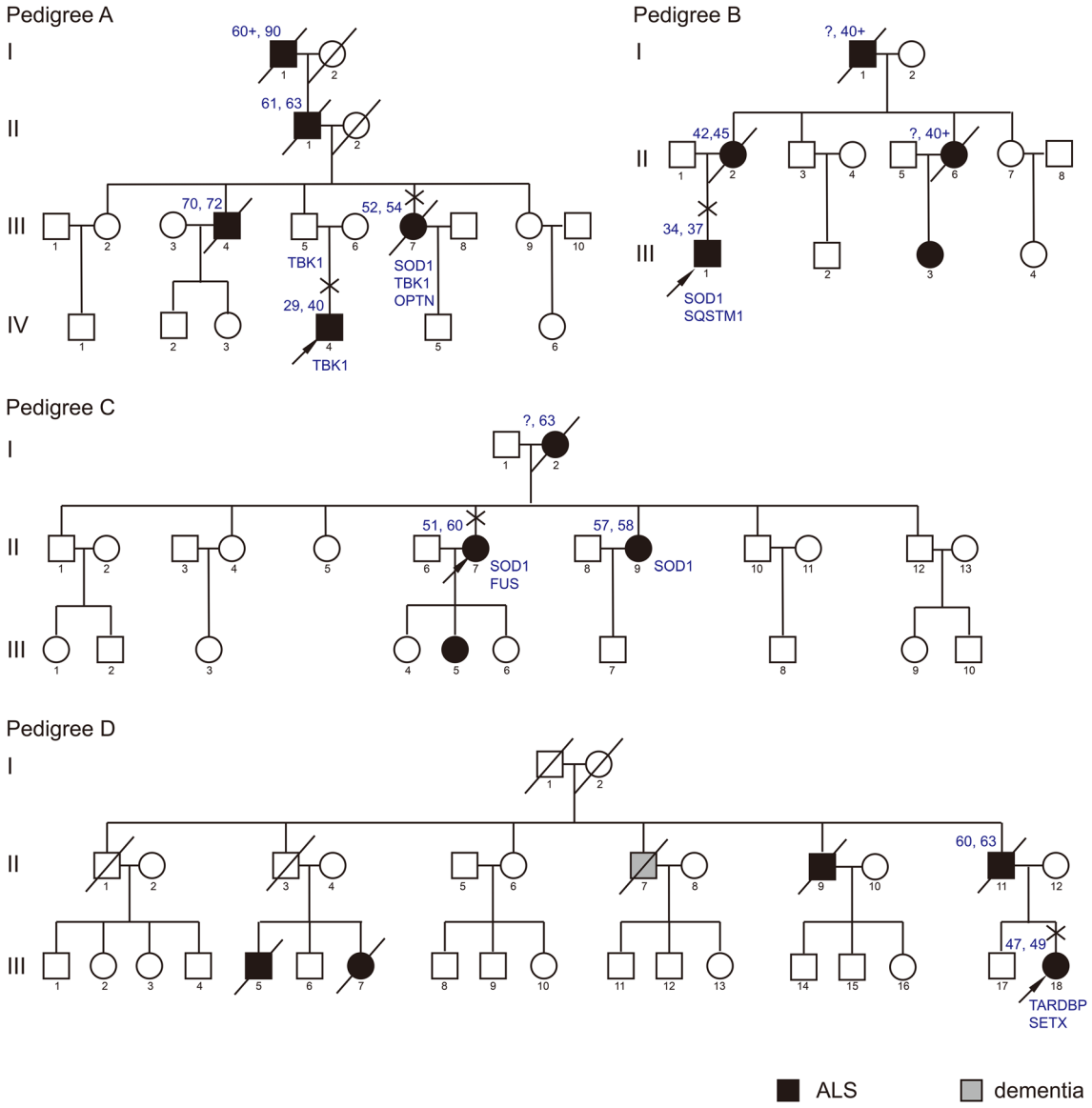

Fig. 1 ALS pedigrees with multiple variants of interest. Examined subjects are indicated by the crosses above the pedigree symbol. The arrows indicate the probands. The age at onset and the age at death or current age are indicated at the top of the symbols. The variants harbored are indicated at the bottom of the symbols. *ALS* amyotrophic lateral sclerosis

five different pedigrees examined had at least one rare non-synonymous variant within the 30 causative ALS genes. Three fALS patients (37.5%) from different families harbored two variants (*SOD1* p.G94R + *SQSTM1* p.G262R; *SOD1* p.F22G + *FUS* p.P151S; *TARDBP* p.G298V + *SETX* p.I1304W) and one patient (12.5%) harbored three variants (*SOD1* p.V48A + *OPTN* p.A433V + *TBK1* p.R573H). The four pedigrees with multiple rare variants are shown in Fig. 1.

Only one out of five pedigrees exhibited monogenic nature, albeit with incomplete penetrance, which we have reported before (Dong et al. 2020). Of the sALS patients, 18 (31.6%) had at least one rare non-synonymous variant within the 30 causative ALS genes; one (3.5%) harbored two variants (*SETX* p.I1520T + *C21orf2* p.R172W), and one harbored three variants (*ALS2* p.E697K + *SPG11* p.C1734F + *NEFH* p.P933S).

Effect of Rare Variants on Survival

Within 43 causative ALS genes, we found that presence of non-synonymous variants is associated with lower survival probability in sALS patients (log-rank test, $p = 0.007$, Fig. 3b), whereas the presence of synonymous variants is not associated with lower survival probability (log-rank test, $p = 0.59$, Fig. 3c). After adjustment for age of onset,

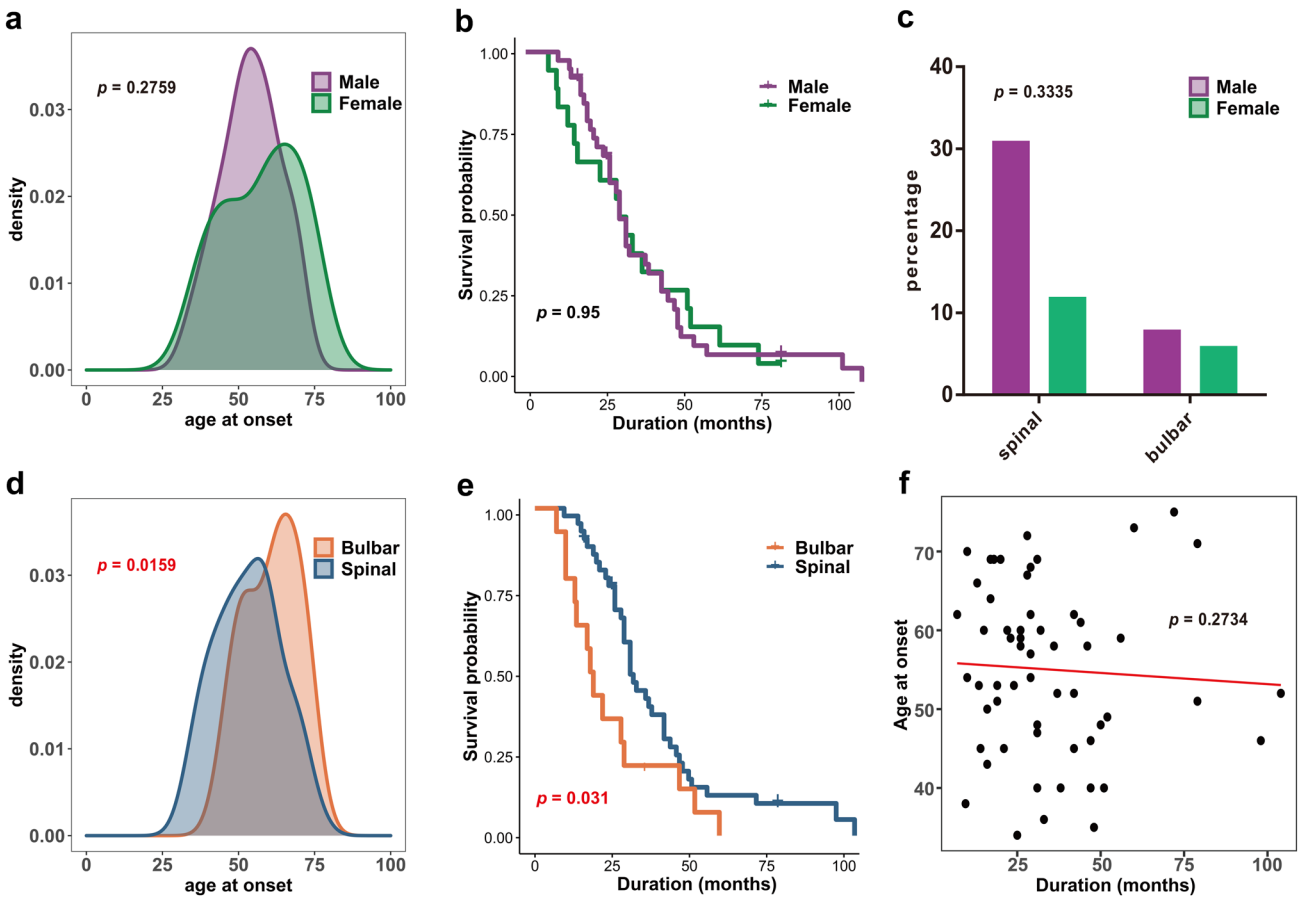

Fig. 2 Statistical analysis of clinical variables for 57 sALS cases. **a** Association between sex and age at onset. **b** Kaplan–Meier survival curves for ALS patients stratified by gender. **c** Association between sex and site of onset. **d** Association between the site of onset and age at onset. **e** Kaplan–Meier survival curves for ALS patients stratified by site of onset. **f** Association between survival and age at onset. ALS, amyotrophic lateral sclerosis; sALS, sporadic ALS

sex and bulbar onset, it was found that the presence of rare non-synonymous still remained associated with survival, increasing the risk of death/ventilator dependence (hazard ratio = 3.93, 95% CI = 1.53–79, $p < 0.05$). Patients with two or more variants did not exhibit reduced survival probability when compared with patients with one variant, which may be explained by the too-small sample size, as only two sALS patients harbored more than one rare variant, the duration of which was 24 and 31 months. Bulbar onset also increased the hazard risk of death/ventilator dependence when compared with spinal onset (hazard ratio = 2.43, 95% CI = 1.23–4.82, $p < 0.05$). However, there is no significance between the number of non-synonymous variants and age at onset (Fig. 3a).

Interestingly, although the fALS patients were not included in the survival analysis for insufficient sample size, we still observed significant heterogeneity of survival in Pedigree A (Fig. 1). Three family members, that is II1, III4 and III7 in Pedigree A, presented with a typical rapid ALS progression, whereas two other members, I1 and IV4, manifested a prolonged duration of disease. Whole-exome sequencing demonstrated a novel *SOD1* p.V48A (c.T143 > C) variant in addition to the *OPTN* p.A433V (c.C1298 > T) and *TBK1* p.R573H (c.G1718 > A) variants in III7, whereas only the *TBK1* variant was detected in IV4. This finding suggests that the burden of rare variants is associated with survival, consistent with what we found in sALS patients.

Discussion

In this study, we profiled a panel of 43 relevant genes in 57 sALS patients and five ALS pedigrees from east China. Overall, we detected 30 rare variants in 16 different genes and found that the presence of rare variants is associated with poorer survival in both sALS patients and an ALS pedigree with three variants.

Considering the heterogeneity of common SNPs in different ethnic populations, we adopted the HuaBiao database,

Table 2 Rare non-synonymous variants identified in 30 ALS causal genes of 65 followed ALS patients

Gene	Amino acid change	dbSNP150	Minor allele frequency				ALS vs HuaBiao OR (95% CI)	*p*	SIFT	Polyphen2	ALSoD/ ALSdb/ project mine	ACMG	Patient ID	References
			ALS ($n = 65$)	ExAC ($n = 60{,}706$)	1000 Genomes	HuaBiao ($n = 5000$)								
Rare variants in Chinese population														
TARDBP	p.G298V	.	0.00769	0	0	0	.	< 0.0001	D	B	−/−/−	Likely pathogenic	D−III18	Tunca et al. (2020)
DCTN1	p.R651Q	.	0.00769	0	0	0	.	< 0.0001	T	P	−/−/−	VUS	A_87	.
DCTN1	p.Y224F	.	0.00769	0	0	0	.	< 0.0001	T	B	−/−/−	VUS	A_80	.
ALS2	p.L385S	.	0.00769	0	0	0	.	< 0.0001	D	P	−/−/−	VUS	A_2	.
ALS2	p.E697K	rs772918314	0.00769	0	0	0	.	< 0.0001	D	D	−/+/−	VUS	A_94	.
CHMP2B	p.T156A	rs761691146	0.00769	0.0000475	0	0.0003	26.03 (2.670−253.7)	0.0462	T	B	−/−/−	VUS	A_182	.
SQSTM1	p.G262R	rs763179729	0.00769	0.00004946	0	0	.	< 0.0001	T	P	+/−/+	VUS	B−III1	Narain et al. (2018)
SETX	p.M1912T	rs202124856	0.00769	0.00000942	0.0002	0	.	< 0.0001	T	B	−/−/−	VUS	A_54	Kim et al. (2018)
SETX	p.I1520T	rs767954870	0.00769	0	0	0	.	< 0.0001	D	P	−/−/−	VUS	A_86	.
SETX	p.L1304W	.	0.00769	0	0	0	.	< 0.0001	D	D	−/−/−	VUS	D−III18	.
OPTN	p.A433V		0.00769	0	0	0	.	< 0.0001	D	B	−/−/−	VUS	A−III7	.
OPTN	p.D527fs	rs1446908199	0.00769	0	0	0	.	< 0.0001	NA	NA	−/+/−	VUS	A_8	.
KIF5A	p.P1024S	rs1441630174	0.00769	0	0	0	.	< 0.0001	D	B	−/−/−	VUS	A_216	.
TBK1	p.S398P	rs781434264	0.00769	0	0	0	.	< 0.0001	T	P	−/−/−	VUS	A_83	.
TBK1	p.R573H	rs186475789	0.01538	0.00008463	0.0002	0	.	< 0.0001	D	D	−/+/+	VUS	A−III7, A−IV4	Cirulli et al. (2015)
SPG11	p.C1734F	rs780601824	0.00769	0.0003	0	0	.	< 0.0001	D	D	−/−/−	VUS	A_94	.
SPG11	p.Q809P	.	0.00769	0	0	0	.	< 0.0001	T	B	−/−/−	VUS	A_136	.
SPG11	p.H235fs	rs312262719	0.00769	0	0	0	.	< 0.0001	NA	NA	+/−/−	Pathogenic	A_29	Stevanin et al. (2008)
SPG11	p.H86D	rs773123413	0.00769	0.0001	0	0.0003	26.03 (2.670−253.7)	0.0462	T	B	−/−/−	VUS	A_213	.
FUS	p.P151S	rs771163321	0.00769	0.00000824	0	0	.	< 0.0001	D	B	−/−/−	VUS	C-II7	Giau et al. (2019)
FUS	p.R521C	rs121909668	0.00769	0	0	0	.	< 0.0001	D	B	+/+/−	Likely pathogenic	A_173	Kwiatkowski et al. (2009)

Table 2 (continued)

Gene	Amino acid change	dbSNP150	Minor allele frequency				ALS vs HuaBiao OR (95% CI)	*p*	SIFT	Polyphen2	ALSoD/ ALSdb/ project mine	ACMG	Patient ID	References
			ALS ($n = 65$)	ExAC ($n = 60{,}706$)	1000 Genomes	HuaBiao ($n = 5000$)								
UNC13A	p.P431L	rs554093295	0.00769	0.0000335	0.0002	0.0001	79.35 (4.905–1284)	0.0027	T	P	−/−/−	VUS	A_34	.
SOD1	p.E22G	.	0.00769	0	0	0	.	< 0.0001	D	P	+/−/+	Pathogenic	C−II7	Siddique and Deng (1996)
SOD1	p.V48A	.	0.00769	0	0	0	.	< 0.0001	D	D	+/−/−	Pathogenic	A−III7	Fujisawa et al. (2012)
SOD1	p.G94R	rs121912437	0.00769	0	0	0	.	< 0.0001	D	B	+/−/−	Pathogenic	B−III1	Elshafey et al. (1994)
SOD1	p.E134X	.	0.00769	0	0	0	.	< 0.0001	NA	NA	−/−/−	Likely patho-genic	A_57, A_192	.
SOD1	p.G142A	.	0.02308	0	0	0	.	< 0.0001	D	D	+/−/−	Pathogenic	E−III2, E−III7, E−III11	Dong et al. (2020)
C21orf2	p.R172W	rs139038672	0.00769	0.0000816	0	0.0001	79.35 (4.905–1284)	0.0027	D	D	−/−/+	VUS	A_86	.
NEFH	p.T642M	rs117258406	0.00769	0.0009	0.0036	0	.	< 0.0001	T	P	−/−/−	VUS	A_104	Zhang et al. (2018a)
NEFH	p.P933S	rs777317391	0.00769	0.0007	0	0	.	< 0.0001	D	B	−/−/−	VUS	A_94	.
Common variants in Chinese population														
ERBB4	p.I658F	rs190654033	0.01538	0.0003	0.000599	0.00575	2.716 (0.6489–11.37)	0.3967	D	D	−/−/−	VUS	A_178, A_124	Naruse et al. (2019)
NEK1	p.P287A	rs35222922	0.01538	0	0.0002	0.00535	2.908 (0.6935–12.19)	0.3509	T	B	+/−/−	Likely benign	A_140, A_36	Pang et al. (2017)
SETX	p.P1331L	rs11243731	0.00769	0.0005	0.00299	0.00665	1.150 (0.1572–8.419)	0.686	D	D	+/−/+	Likely benign	A_99	Kim et al. (2018)
SETX	p.E756V	rs202036078	0.00769	0.0000189	0.0002	0.0008	9.750 (1.201–79.14)	0.2544	D	D	+/−/−	VUS	A_50	Zhang et al. (2018b)
SETX	p.E813D	rs190841601	0.00769	0.0000677	0.000998	0.00131	6.089 (0.7842–47.28)	0.4389	T	P	−/−/−	VUS	A_83	Kim et al. (2018)
ANXA11	p.R386G	rs146222704	0.00769	0	0	0.00293	5.372 (0.7489–36.48)	0.4845	D	D	−/−/−	VUS	A_207	.
ANXA11	p.T321N	rs76806315	0.00769	0.0006	0.000399	0.00807	1.902 (0.2670–13.55)	0.9674	T	B	−/−/+	Likely Benign	A_50, A_69	Nahm et al (2020)

Table 2 (continued)

Gene	Amino acid change	dbSNP150	Minor allele frequency				ALS vs HuaBiao OR (95% CI)	*p*	SIFT	Polyphen2	ALSoD/ ALSdb/ project mine	ACMG	Patient ID	References
			ALS (n = 65)	ExAC (n = 60,706)	1000 Genomes	HuaBiao (n = 5000)								
ANXA11	p.G89S	rs201713183	0.00769	0.0002	0.000599	0.00414	3.792 (0.5293–26.27)	0.6588	T	D	–/–/–	VUS	A_218	.
SPG11	p.S2065P	rs568406743	0.00769	0.0003	0.000399	0.00262	3.037 (0.4056–22.74)	0.7839	T	B	–/–/–	VUS	A_104	.
SPG11	p.L1982S	rs185665930	0.00769	0.0007	0.0018	0.00736	1.067 (0.1460–7.799)	0.6461	D	D	–/–/–	VUS	A_116	Kim et al. (2016)
SPG11	p.Y396C	rs3759875	0.02307	0.0003	0.0014	0.00585	4.052 (1.236–13.28)	0.053	T	B	–/–/–	Likely benign	A_34, A_36, A_204	.
CCNF	p.L223R	rs372723774	0.00769	0.0003	0.000998	0.00373	2.096 (0.2831–15.52)	0.9858	D	D	–/–/–	VUS	A_105, A_136	.
NEFH	p.A380T	rs201416955	0.00769	0.0005	0.0014	0.00716	1.069 (0.1463–7.819)	0.6473	T	D	–/–/–	Benign	A_124	Zhang et al. (2018a)

VUS, Variant of uncertain significance

which consists of 5000 healthy Han Chinese as controls. Cases and control WES were performed by utilizing the same library preparation kits. However, we were unable to perform variant calling jointly in the case and control datasets. We identified 13 variants that were rare in the ExAC and 1000 Genomes but were relatively common ($p > 0.05$ by Fisher exact test) in the Chinese population. Interestingly, eight of the 13 variants have been reported before in other Amyorophic lateral slerosis and Frontotemporal dementia (ALS-FTD) studies (Kim et al. 2016, 2018; Nahm et al. 2020; Naruse et al. 2019; Pang et al. 2017; Zhang et al. 2018a, b), namely *ERBB4* p.I658F, *NEK1* p.287A, *SETX* p.1331L, *SETX* p.E756V, *SETX* p.E813D, *ANXA11* p.T321N, *SPG11* p.L1982S and *NEFH* p.A380T (Table 2, common variants in the Chinese population). Our study suggests that these variants may not be directly associated with the disease, whereas the other 30 variants that are rare in the ExAC, 1000 Genomes and HuaBiao database may be causally associated with the disease. Among them, 12 variants (*TARDBP* p.G298S, *SQSTM1* p.G262R, *SETX* p.M1912T, *TBK1* p.R573H, *SPG11* p.H235fs, *FUS* p.P151S, *FUS* p.R520C, *SOD1* p.E22G, *SOD1* p.V48A, *SOD1* p.G94R, *SOD1* p.G142A, and *NEFH* p.T642M) have been reported in ALS-FTD studies (Cirulli et al. 2015; Dong et al. 2020; Elshafey et al. 1994; Fujisawa et al. 2012; Giau et al. 2019; Kim et al. 2018; Kwiatkowski et al. 2009; Narain et al. 2018; Siddique and Deng 1996; Stevanin et al. 2008; Tunca et al. 2020; Zhang et al. 2018a), and three variants (*ALS2* p.E697K, *OPTN* p.D527fs, and *C21orf2* p.R172W) were not reported, but recorded in ALS databases (ALSoD, ALSdb or Project MinE). Fifteen variants (*DCTN1* p.R651Q, *DCTN1* p.Y224F, *ALS2* p.L385S, *CHMP2B* p.T156A, *SETX* p.I1520T, *SETX* p.L1304W, *OPTN* p.A433V, *KIF5A* p.P1024S, *TBK1* p.S398P, *SPG11* p.C1734F, *SPG11* p.Q809P, *SPG11* p.H86D, *UNC13A* p.P431L, *SOD1* p.E134X, and *NEFH* p.P933S) were novel, which have not been reported in ALS-FTD studies or recorded in ALS databases. Our study provided support for the association of these variants with disease. However, because it is difficult to conclusively demonstrate pathogenicity without pedigree information, most of the rare variants we identified were classified as variants of uncertain significance according to the ACMG. Eight rare variants (*TARDBP* p.G298V, *SPG11* p.H235fs, *FUS* p.R521C, *SOD1* p.E22G, *SOD1* p.V48A, *SOD1* p.G94R, *SOD1* p.E134X and *SOD1* p.142A) are classified as pathogenic or likely pathogenic (Table 2). Apart from *SOD1* p.E134X, all other variants have been reported. Of note, *SPG11* p.H235fs was heterozygous, whereas *SPG11* mutations are usually autosomal recessive. Resultantly, the clinical significance of *SPG11 p.H235fs* is uncertain.

We identified several patients who carry more than one variant, accounting for 3.51% of sALS and 50% of fALS patients who we examined, suggesting a possible oligogenic

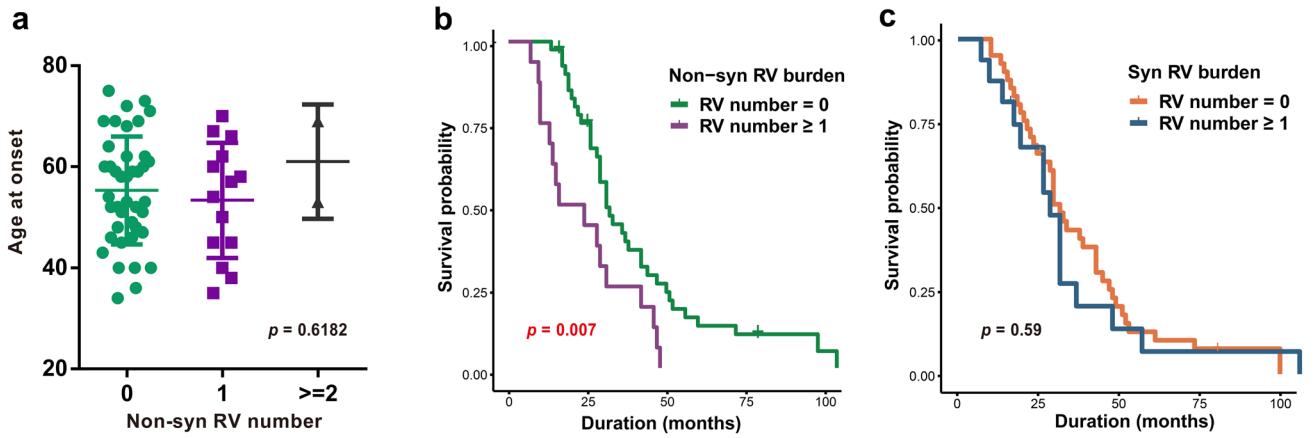

Fig. 3 Effect of rare variants on disease onset and survival. **a** Association between the number of rare non-synonymous variants (Non-syn RV) and age at onset. **b** Kaplan–Meier survival curves for ALS patients stratified by the presence of non-synonymous rare variants (Non-syn RV) in 43 ALS causative genes. **c** Kaplan–Meier survival curves for ALS patients stratified by the presence of synonymous rare variants (Syn RV) in 43 ALS causative genes. ALS, amyotrophic lateral sclerosis

basis. Furthermore, we used a chord diagram to show patients with multiple rare variants (Fig. 4). ALS genes were divided into four categories based on previous literature (Nguyen et al. 2018), including "impaired autophagy/proteostasis", "cytoskeletal defect", "mitochondrial dysfunction and impaired DNA repair", and "disturbed RNA

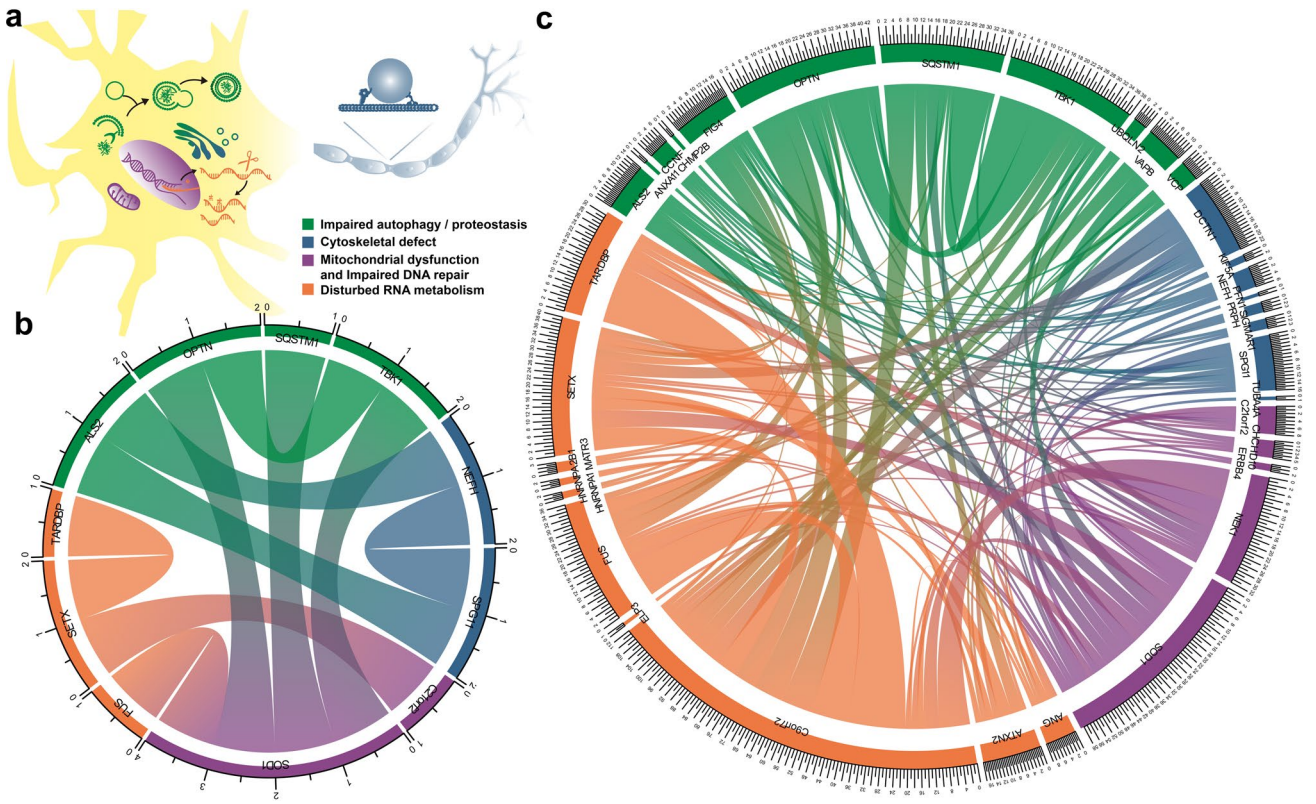

Fig. 4 Patients with multiple rare variants. **a** Biological process affected by mutations in ALS genes: different colors represent different biological processes, and illustrations are also applicable in panels **b** and **c**. **b** Chord diagram illustrating patients with multiple rare variants in our study. The *x*-axis represents the number of times one gene variant was detected alongside another; the width of the chord connecting the two genes represents the number of times they occur together. Different colors represent different biological processes. **c** Chord diagram illustrating reported multiple gene rare variant carriers with ALS or ALS-FTD. ALS, amyotrophic lateral sclerosis

metabolism" (Fig. 4a). Patients harbored multiple rare variants discovered in our study are presented in Fig. 4b. *SOD1* was the most detected gene, which was detected simultaneously with four other genes, three of which were related to autophagy. We also reviewed reported multiple gene rare variant carriers with ALS or ALS-FTD (Farhan et al. 2018; Keogh et al. 2018; Lamp et al. 2018; Lattante et al. 2021; Liu et al. 2021a, b; McCann et al. 2020; Müller et al. 2018; Narain et al. 2019; Naruse et al. 2019; Nguyen et al. 2018; Pang et al. 2017; Ross et al. 2020; Scarlino et al. 2020; Sghaier et al. 2022; Tripolszki et al. 2019; Vazquez-Costa et al. 2019; Yang et al. 2021; Yilmaz et al. 2022; Zhang et al. 2018a) (Supplementary Table 2, Fig. 3c). *C9orf72* was the most detected gene, accounting for 112 times, which may be because *C9orf72* mutation is the most common known cause of ALS. However, C9orf72 G4C2 expansion is extremely rare in the Chinese population (Zou et al. 2013) and was not detected in our cohort. Similarly, *SOD1* accounted for a large proportion, which was 59 times. It is worth noting that several autophagy genes, including *TBK1*, *OPTN* and *SQSTM1*, the mutations of which were not common in ALS-FTD patients, account for a considerable proportion in multiple gene rare variant carriers, accounting for 38, 42 and 36 times, respectively. The co-occurrence of *TBK1* and *OPTN*, *TBK1* and *SQSTM1* was frequently detected. In addition, these three genes frequently coexist with *SOD1* and genes relating to RNA metabolism, such as *C9orf72* and *FUS*.

Although a series of papers have reported the co-occurrence of variants in ALS genes (Cady et al. 2015; Dols-Icardo et al. 2018; McCann et al. 2020; Morgan et al. 2017; Naruse et al. 2019; Pang et al. 2017; Scarlino et al. 2020; Shepheard et al. 2021), the presence of the oligogenic nature of ALS remains debatable. Notably, the reported percentage of patients who harbored more than one variant varied much, from as low as 1% (Morgan et al. 2017) to as high as 19.5% (Pang et al. 2017). This difference is caused by the inconsistency in the selection of sequenced genes and criteria for filtering potentially pathogenic variants. Some studies adopted very rigorous filtering criteria that only include pathogenic or likely pathogenic variants in high-penetrant genes, whereas other studies, including ours, also had some variants of uncertain significance according to the ACMG standards (Richards et al. 2015). The ACMG standards are very practical for clinical use, but for non-strictly Mendelian diseases such as ALS, only including variants that are classified as pathogenic or likely pathogenic may lead to underestimation of the significance of some variants in low-penetrant or risk genes. Some other studies did not exclude SNPs that were relatively common in the control population, resulting in a falsely high proportion of patients with multiple rare variants. In our study and some other studies (Shepheard et al. 2021), population-matched WES data were adopted to avoid such flaws. In addition, Keogh et al. (2018) suggested that individuals with ALS are more likely to harbor a known genetic risk factor, and it is the burden of these variants in combination with rare benign alleles that is likely to be responsible for some oligogenic associations. Therefore, further mechanistic functional analyses or segregation studies are warranted to scrutinize the pathogenicity of each of the variants that we identified and the synergy of these variants.

Next, we explored whether the presence of rare variants was associated with the survival of ALS patients, and we found that sALS patients with one or more variants in ALS causative genes had worse survival than patients with no variants. In addition, in an ALS pedigree with three variants, the family member III7 with three variants (*SOD1* p.V48A, *OPTN* p.A433V and *TBK1* p.R573H) exhibited much more severe disease phenotype than IV4 who carries one variant (*TBK1* p.R573H). This observation provides strong support for our conclusions in sALS patients. However, Pang and Scarlino reported that patients with two variants exhibited reduced survival probability when compared with patients with one variant (Pang et al. 2017; Scarlino et al. 2020). This phenomenon was not observed in our cohort, possibly because of our insufficient sample size, as only two sALS patients harbored more than one rare variant.

Few studies have examined genotype–phenotypic relationships within patients from the same family, where the genetic background is so uniform that the effects of oligogenic can be seen. Although here we have reported a possible genotype–phenotype correlation within Pedigree A, the pathogenicity of each variant needs to be investigated. The *SOD1* p.V48A variant was recorded in the ALSoD and was reported in a Chinese cohort study (Tang et al. 2019). It was classified as pathogenic according to the ACMG standards. In contrast, the absence of the *SOD1* variant in the proband who presented slow disease progression indicates that *SOD1* is not the only pathogenic gene in this pedigree. We considered the *TBK1* variant to contribute to the disease, as it was recorded in the ALSdb database, and was identified by a genome-wide association study (Cirulli et al. 2015). Patients carrying mutations in *TBK1* and other ALS genes simultaneously have been frequently reported (Liu et al. 2021a), especially in the genes related to the autophagy pathway (*OPTN* and *SQSTM1*) (Black et al. 2017; Dols-Icardo et al. 2018; Lattante et al. 2019; Liu et al. 2021a; Pottier et al. 2015) or RNA homeostasis and trafficking (*C9ORF72*, *FUS*, and *TARDBP*) (Black et al. 2017; de Majo et al. 2018; Muller et al. 2018; van der Zee et al. 2017). These studies as well as ours have suggested that *TBK1* belongs to the genes that might cause an increased risk of developing the disease or an earlier onset of the disease, supporting the oligogenic hypothesis.

Conclusions

In this study, we demonstrated that ALS patients can carry more than one variant in ALS causative genes, and that the presence of rare variants is associated with survival in both sALS patients and an ALS pedigree. Our findings suggested that rare variants could exert a negative prognostic effect, thereby supporting the oligogenic inheritance of ALS.

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Authors' Contributions Included conception and study design (XC and JW), data collection or acquisition (SD, XY, KW, WY, JL, YZ and XL), statistical analysis (SD, XY, KW and YW), interpretation of results (SD, XY, KW, JW and XC), drafting the manuscript work (SD, XY, JW and XC) and approval of final version to be published and agreement to be accountable for the integrity and accuracy of all aspects of the work (all the authors).

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Data Availability The datasets generated from the statistical analyses during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest No potential conflict of interest was reported by the author(s).

Ethical Approval and Consent to Participate The studies involving human participants were reviewed and approved by the Ethics Committee of Huashan Hospital, Fudan University. The patients/participants provided their written informed consent to participate in this study.

Consent for Publication Not applicable.

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