Title	Burden of rare variants in ALS genes influences survival in familial and sporadic ALS
Author(s)	Pang, SYY; HSU, SJ; Teo, KC; LI, Y; Kung, MHW; Cheah, KSE; Chan, D; Cheung, KMC; Li, M; Sham, PC; Ho, SL
Citation	Neurobiology of Aging, 2017, v. 58, p. 238.e9-238.e15
Issued Date	2017
URL	http://hdl.handle.net/10722/248305
Rights	This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

Neurobiology of Aging 58 (2017) 238.e9–238.e15

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Burden of rare variants in ALS genes influences survival in familial and sporadic ALS

Shirley Yin-Yu Pang [a,1], Jacob Shujui Hsu [b,c,1], Kay-Cheong Teo [a], Yan Li [b,c], Michelle H.W. Kung [a], Kathryn S.E. Cheah [d], Danny Chan [d], Kenneth M.C. Cheung [e], Miaoxin Li [b,c,f,g,*], Pak-Chung Sham [b,c,**], Shu-Leong Ho [a,***]

[a] Division of Neurology, Department of Medicine, University of Hong Kong, Hong Kong, P.R. China
[b] Department of Psychiatry, University of Hong Kong, Hong Kong, P.R. China
[c] Centre for Genomic Sciences, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong, P.R. China
[d] School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong, P.R. China
[e] Department of Orthopaedics & Traumatology, University of Hong Kong, Hong Kong, P.R. China
[f] Department of Medical Genetics, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, P.R. China
[g] Key Laboratory of Tropical Disease Control (SYSU), Ministry of Education, Guangzhou, P.R. China

ARTICLE INFO

Article history:
Received 10 March 2017
Received in revised form 29 May 2017
Accepted 11 June 2017
Available online 20 June 2017

Keywords:
ALS
Genetics
Next generation sequencing
Survival

ABSTRACT

Genetic variants are implicated in the development of amyotrophic lateral sclerosis (ALS), but it is unclear whether the burden of rare variants in ALS genes has an effect on survival. We performed whole genome sequencing on 8 familial ALS (FALS) patients with *superoxide dismutase 1 (SOD1)* mutation and whole exome sequencing on 46 sporadic ALS (SALS) patients living in Hong Kong and found that 67% had at least 1 rare variant in the exons of 40 ALS genes; 22% had 2 or more. Patients with 2 or more rare variants had lower probability of survival than patients with 0 or 1 variant ($p = 0.001$). After adjusting for other factors, each additional rare variant increased the risk of respiratory failure or death by 60% ($p = 0.0098$). The presence of the rare variant was associated with the risk of ALS (Odds ratio 1.91, 95% confidence interval 1.03–3.61, $p = 0.03$), and ALS patients had higher rare variant burden than controls (MB, $p = 0.004$). Our findings support an oligogenic basis with the burden of rare variants affecting the development and survival of ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by the degeneration of motor neurons, progressive paralysis, and death. The world-wide incidence of ALS is 0.3–7.0 per 100,000 per year (Cronin et al., 2007) and in Hong Kong, the incidence of ALS was estimated to be 0.6 per 100,000 per year (Fong et al., 2005). Five to 10% of cases are familial. There are more than 20 known causative genes, with more showing association with risk of disease (Leblond et al., 2014). Mutations in *superoxide dismutase 1 (SOD1)* and *chromosome 9 open reading frame 72 (C9ORF72)*, the 2 most common causes of familial ALS (FALS) in populations of European ancestry, account for about half of familial cases collectively (Finsterer and Burgunder, 2014). Mutations in ALS genes are also found in 10% of patients with sporadic ALS (SALS) (Renton et al., 2014). While twin studies from European populations showed ALS heritability to be around 61% in sporadic cases (Al-Chalabi et al., 2010), genome-wide association analyses showed that common single nucleotide polymorphisms (SNPs) contribute to 8.5%–12% of SALS's heritability (Fogh et al., 2014; van Rheenen et al., 2016). The difference in these heritability estimates, or "missing heritability," suggests that there is a notable genetic role in ALS that remains to be elucidated.

There is considerable variability in ALS phenotype: age and site of onset, relative degree of upper and lower motor neuron involvement, and rate of progression (Swinnen and Robberecht,

* Corresponding author at: Department of Medical Genetics, Zhongshan School of Medicine, Sun Yat-sen University, Room 903, Medical Science and Technology Building, Guangzhou, P.R. China. Tel.:+86-2087335080.
** Corresponding author at: Centre for Genomic Sciences, Li Ka Shing Faculty of Medicine, University of Hong Kong, 6th Floor, The Hong Kong Jockey Club Building for Interdisciplinary Research, 5 Sassoon Road, Pokfulam, Hong Kong. Tel.: (852) 2831 5425; fax: (852) 2818 5653.
*** Corresponding author at: Division of Neurology, Department of Medicine, Queen Mary Hospital, University of Hong Kong, 102 Pok Fu Lam Road, Pokfulam, Hong Kong. Tel.: (852) 2855 3315; fax: (852) 2974 1171.
E-mail addresses: limiaoxin@mail.sysu.edu.cn (M. Li), pcsham@hku.hk (P.-C. Sham), slho@hku.hk (S.-L. Ho).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.neurobiolaging.2017.06.007

2014). We previously described a Chinese kindred of FALS with *SOD1* mutation with considerable intrafamilial phenotypic variability (Fong et al., 2006; Ng et al., 2008). Identification of patients with mutations in more than 1 gene has led to the hypothesis of an oligogenic model of ALS, with 4.0% of FALS and 1.3% of SALS patients carrying multiple variants in 1 study (Kenna et al., 2013). Patients harboring rare variants in more than 1 gene had disease onset 10 years earlier than subjects with variants in a single gene (Cady et al., 2015). We, therefore, hypothesized that there is an oligogenic influence on survival in familial and SALS. We characterized the presence and burden of rare variants in 40 ALS genes in 8 FALS and 46 SALS patients.

2. Patients and methods

2.1. Subjects

ALS patients diagnosed and followed up at Queen Mary Hospital, Hong Kong since 1997 were enrolled. They all fulfilled the criteria for definite ALS, probable ALS, or probable ALS—laboratory-supported based on the revised El Escorial criteria (Brooks et al., 2000).

2.1.1. Familial ALS

Eight ALS patients from 4 Chinese families in Hong Kong were included. They have been previously confirmed to harbor c.449T>C:p.I150T *SOD1* mutation. Five (F19, F21, F204, F310, and F331) came from the same kindred (Supplementary Fig. 1). FA-III-16, FB-III-9, and FC-I came from 3 different families in Hong Kong.

2.1.2. Sporadic ALS

Forty-six SALS patients were included. Information on sex, age, and site of onset, treatment with riluzole, and survival as defined by time from symptom onset to ventilation (noninvasive or invasive) or time to death (non-ventilated subjects), whichever is earlier, were recorded. We measured survival in terms of time from symptom onset to ventilatory failure or death, whichever is earlier, to avoid the confounding effect of ventilatory support on survival. Genetic relatedness was measured by pi-hat.

2.1.3. Controls

A total of 699 exomes from volunteer-based healthy controls in Hong Kong were used for case-control association tests. Whole exome sequencing (WES) was performed using Illumina TruSep Exome capture kits with 48× coverage overall followed by the same procedures as ALS subjects. Only protein-coding regions were included for downstream analyses. Principal component analysis was performed to check the population stratification between ALS and control (Chang et al., 2015; Yang et al., 2011).

2.2. Patient consents

This study has been approved by the Hong Kong Hospital Authority/Hong Kong West Cluster Institutional Review Board Ethics Committee and all participants gave written informed consent to participate.

2.3. Genotyping and rare variants filtering

Whole genome sequencing (WGS) was performed on 8 FALS patients and WES was performed on 46 SALS patients using Nimblegen SeqCap EZ Human Exome Kit v3.0 capture kits. Illumina Hiseq 2000 system with 100 bp pair-end reads was used on both WGS and WES. The sequencing coverage reached at least 33× and 95×, respectively. The GATK (The Genome Analysis Toolkit) best practices pipeline was adopted for sequencing data processing and variant calling analysis (McKenna et al., 2010). Another independent sample in the same sequencing batch consisting of 28 controls from the same population, using the same capture kits and sequencing machine, was used to filter out any potential batch effect and system bias. Variant annotation and filtering were performed by KGGSeq (Hsu et al., 2016; Li et al., 2012). As ALS is a rare disease, only nonsynonymous variants with minor allele frequency (MAF) of less than 0.1% across all populations in Exome Aggregation Consortium (ExAC) dataset were selected (http://exac.broadinstitute.org). All of the variants had a sequencing quality score (Phred) of at least 50 and a mapping quality score of at least 20. Only those alternative alleles with more than 25% of total reads were considered as heterozygous alleles. Phred-scaled p-value using Fisher's exact test (FS) was less than 60, which excluded strand bias.

2.4. ALS candidate genes

Forty protein-coding genes: 14 causative ALS genes, 24 susceptibility risk genes (Leblond et al., 2014), and 2 recently described genes from a large-scale ALS exome study (Cirulli et al., 2015) were examined for novel and rare variants (Supplementary Table 1).

2.5. Survival analysis

The Kaplan-Meier survival analysis was used to measure the effect of rare variant burden on the time to ventilator or death. We defined the patients started on noninvasive ventilatory support (NIV) as reaching the endpoint of ventilatory failure since in our center, NIV support was only initiated in patients with respiratory failure. All but 1 patient who received NIV had expressed their wish against intubation, and none of them would have survived much longer without NIV support. After adjusting for onset age, censorship, sex, onset region, inheritance (familial or sporadic), and riluzole treatment, Cox regression was performed to estimate the proportional hazard ratio of rare variant burden on survival.

2.6. Rare variant association test

Rare variant association tests between case and control were performed by RVTESTS (Zhan et al., 2016) and KGGSeq-integrated SKAT package (Ionita-Laza et al., 2013) on 3 levels: single variant, gene, and gene set. Collapsing and combining rare variants followed by Fisher's exact test (CMCFisher), upweighing rare variant using inverse frequency from controls (Madsen-Browning), and Sequencing Kernel Association Test (SKAT and SKATO) were selected to evaluate rare variant burden association.

3. Results

3.1. Familial ALS

The mean (SD) age of onset of the 8 FALS patients (3 males, 5 females) was 41.4 (±8.71) years. Only F19 had bulbar onset. Six subjects reached the endpoint of requiring ventilatory support or death after a median survival of 30.5 (interquartile range 11–68) months (Table 1).

Survival time was largely variable in the 8 familial patients among the 4 kindreds we have analyzed. From the same kindred, F19 had the shortest survival of 3 months, followed by F21 and F204 (11 months). F310 and F331 had longer survival of 37 (and still alive) and 74 months, respectively. FA-III-16, FB-III-9, and FC-I, from 3 different families, had longer survival of 76, 50, and 80 months respectively.

In the exons of 40 ALS genes, F19 had 2 additional rare variants: *ALS2* p.L520F and *DAO* p.P103L (Table 2; Supplementary Table 2). F21 had 1 additional rare variant in *DAO* (p.P103L). F204 had 1

Table 1
Demographics of FALS and SALS patients

Total number of ALS	FALS (8)	SALS (46)
Mean age of onset (y) (SD)	41.4 (±8.71)	58.1 (±13.45)
Ethnic Chinese	8 (100%)	44 (95.7%)
Male sex	3 (37.5%)	28 (60.9%)
Bulbar onset	1 (12.5%)	20 (43.5%)
Riluzole use	0	2 (8.7%)
Surviving patients	2 (25%)	6 (13.0%)
Median Time to ventilator/death (mo) (IQR)	30.5 (11–68)	34 (13–42)
Number of healthy controls	699	
Male sex	305 (44%)	
Mean age	55.5 (±8.27)	

Key: ALS, amyotrophic lateral sclerosis; FALS, familial ALS; IQR, interquartile range; SALS, sporadic ALS; SD, standard deviation.

additional rare variant in *C9ORF72* (p.G465R, MAF = 0.00009). We excluded this variant from the downstream survival and case-control association analyses because only 74.9% of the control subjects had adequate coverage for this locus (vs. 100% of cases). FB-III-9 had 3 additional rare variants (*NEK1* p.P287A, *SETX* p.M2324I, and an additional *SOD1* variant p.D93G). In contrast, F331 and FA-III-16 who survived for more than 70 months, and the 2 surviving subjects (F310, FC-I), had no additional rare variant. Overall, FALS patients with 2 or more rare variants appear to have shorter time to ventilator dependence or death.

3.2. Sporadic ALS

The mean (SD) age of onset in SALS was 58.1 (±13.45) years including censored patients, and 60.9% of the patients were male (Table 1). Twenty patients (43.5%) had bulbar onset. Two patients

Table 2
Rare single nucleotide variants detected among 40 ALS genes in ALS patients

Gene	Mutation	Minor allele frequency			Database, ALSoD/alsdb	ALS versus control, OR (95%CI)[b]	*p* value[b]	SampleID	Survival
		ALS (n = 54)	Control (n = 699)	ExAC (n = 60,706)					
Rare mis-sense SNVs in familial ALS patients									
ALS2	p.L520F	0.019	0.004	0.000098	−/−	4.36 (0.08–55.41)	0.258	F19;	3
NEK1	p.P287A	0.056	0.012	0.0001	−/−	5.05 (0.84–21.88)	0.038	FB-III-9	50
SETX	p.M2324I	0.019	0	0.00002	−/−	..	..	FB-III-9	50
DAO	p.P103L	0.037	0.004	0.00036	−/−	8.50 (0.70–75.88)	0.047	F19; F21	3; 11
SOD1	p.D93G	0.019	0	0.00001	+/−	..	..	FB-III-9	50
SOD1[*]	p.I150T	0.148	0	N	−/−	..	..	F19; F21; F204; F310; F331; FA-III-16; FB-III-9; FC-I	3; 11; 11; 37[c];74; 76; 50; 80[c]
C9orf72[a]	p.G465R	0.019	0.0019	0.00009	−/−	9.79(0.12–770.67)	0.178	F204	11
Rare mis-sense SNVs in sporadic ALS patients									
TARDBP	p.G298S	0.037	0	N	+/+	..	..	MND_19; MND_40	18; 10
TARDBP	p.M337V	0.019	0	N	+/+	..	..	MND_4	164
TARDBP	p.S375G	0.019	0	0.00002	−/+	..	..	MND_37	15
KIFAP3	p.F694L	0.019	0.006	0.00007	−/−	2.96 (0.06–30.59)	0.337	MND_64	3
DCTN1	p.D1199N	0.038	0.007	0.00015	−/−	5.42 (0.50–34.11)	0.081	MND_17; MND_72	13; 49[c]
DCTN1	p.G1013A	0.019	0	0.00001	−/−	..	..	MND_60	72[c]
DCTN1	p.Q701R	0.019	0.009	0.00047	−/−	2.17 (0.05–18.42)	0.408	MND_58	11
DCTN1	p.A457T	0.02	0	0.00004	−/−	..	..	MND_34	72
PLCD1	p.M412R	0.019	0	0.00007	−/−	..	..	MND_17	13
NEK1	p.L413P	0.019	0.006	0.00011	−/−	3.27 (0.07–33.81)	0.311	MND_37	15
NEK1	p.P287A	0.056	0.012	0.0001	−/−	5.05 (0.84–21.88)	0.038	MND_51; MND_64	38; 11
ARHGEF28	p.A168T	0.019	0	N	−/−	..	..	MND_58	11
ARHGEF28	p.A717V	0.019	0.001	0.000196	−/−	12.70 (0.16–997.92)	0.142	MND_8	83
ARHGEF28	p.G1029W	0.019	0	N	−/−	..	..	MND_54	48
ARHGEF28	p.M1261T	0.019	0	0.00002	−/−	..	..	MND_14	44
SQSTM1	p.V144I	0.019	0	0.00006	−/−	..	..	MND_47	42
SQSTM1	p.G411S	0.019	0	0.00005	−/+	..	..	MND_49	24
PON2	p.S31F	0.019	0.002	0.00002	−/−	11.60 (0.15–912.09)	0.154	MND_57	34
VCP	p.G157R	0.019	0	N	−/−	..	..	MND_73	40[c]
SETX	p.N1100S	0.019	0	0.00001	−/−	..	..	MND_24	12
SETX	p.M627T	0.019	0.004	0.00012	−/−	4.36 (0.08–55.41)	0.258	MND_59	31
ITPR2	p.M2443T	0.019	0.007	0.000196	−/−	2.53 (0.05–23.25)	0.37	MND_24	12
ITPR2	p.V1952A	0.019	0	0.00001	−/−	..	..	MND_37	15
PRPH	p.L118M	0.043	0.023	0.000397	−/−	1.98 (0.21–9.04)	0.302	MND_18; MND_52	1; 20
TBK1	p.L94S	0.019	0	N	−/−	..	..	MND_51	38
TBK1	p.H336R	0.019	0	N	−/−	..	..	MND_47	42
DAO	p.T269I	0.019	0	N	−/−	..	..	MND_25	12
ATXN2	p.A1023V	0.019	0	0.00007	−/−	..	..	MND_32	41
APEX1	p.G8R	0.019	0.003	0.00012	−/−	6.54 (0.11–127.61)	0.2	MND_74	19[c]
APEX1	p.E110G	0.019	0	0.00001	−/−	..	..	MND_10	37
SPG11	p.L1982S	0.019	0.025	0.000699	−/−	0.76 (0.02–5.02)	1.000	MND_55	24
SPG11	p.P194L	0.019	0.005	0.00007	−/−	3.95 (0.07–50.28)	0.279	MND_39	42
UNC13A	p.P960S	0.019	0	N	−/−	..	..	MND_25	12
NEFH	p.T642M	0.019	0.025	0.00085	−/+	0.78 (0.02–5.19)	1.000	MND_55	24

Key: ALSdb, (http://alsdb.org/index.jsp, Last Update: Oct 2016); ALSoD, (http://alsod.iop.kcl.ac.uk/, Last Update: Sep 2015); DB, the variant has been reported in ALS database; ExAC, Exome Aggregation Consortium V3; N, Novel variant in ExAC; OR (95% CI), Odds ratio with 95% confidence interval; SNV, single nucleotide variant; ··, All control subjects are reference homozygous.

[*] *p* value <0.0001.

[a] This SNV has been excluded from downstream analyses since only 74.8% of control had adequate coverage.

[b] Fisher's exact test.

[c] Alive.

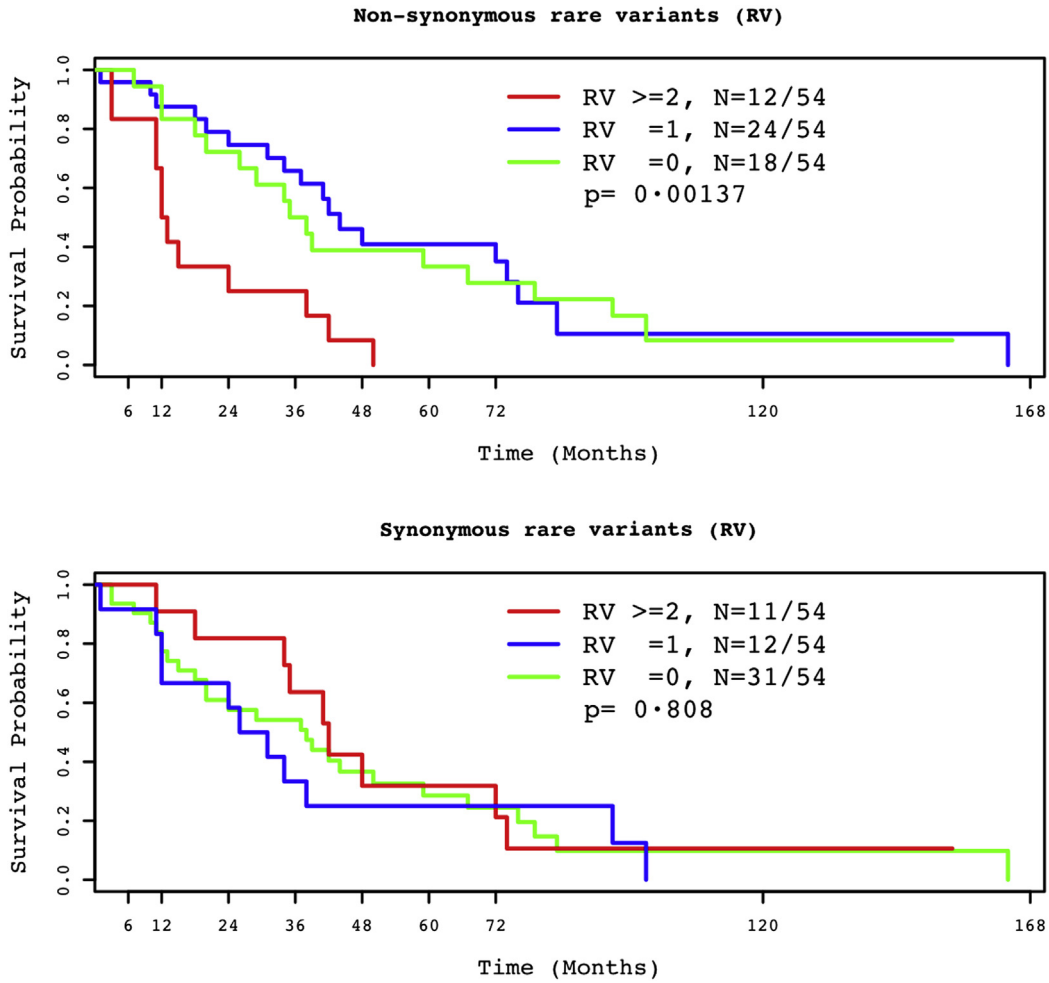

Fig. 1. Kaplan-Meier survival curves for ALS patients stratified by nonsynonymous and synonymous rare variants. The differences were evaluated by the log-rank test with 2 degrees of freedom. Abbreviations: RV, rare variant; N, number of patients.

received riluzole for at least 1 month. Forty patients had reached the endpoint after a median of 34 (interquartile range 13–42) months. Twenty-eight patients (60.8%) had at least 1 rare variant within the 40 ALS genes and 9 (19.5%) had 2 or more rare variants (Supplementary Table 3).

3.3. Novel and rare variant identifications

Of the 40 genes examined, novel or rare variants (MAF $\leq$0.001) were found in 21 genes. Thirty-nine mis-sense variants, occurring in 52 alleles, were identified. Nine novel variants that have not been described in the ExAC (Table 2) occurred in 10 SALS patients. Five patients with a novel variant (*VCP* p.G157R, *TARDBP* p.G298S, *TARDBP* p.M337V or *ARHGEF2* p.G1029W) had no additional variants, suggesting a possible causal effect of these variants in ALS. Excluding the *SOD1* mutation, 5 (13.2%) and 3 (7.9%) of the remaining 38 rare variants have been previously reported in **ALSdb** (http://alsdb.org/) and **ALSoD** (http://alsod.iop.kcl.ac.uk/) (Abel et al., 2012) databases, respectively. No variants were found to be significantly associated with survival probability at the variant or gene level.

The most frequently found variant occurred in *NEK1* (p.P287A) in 1 FALS and 2 SALS patients (1 with short survival and 2 with long survival). A rare variant in *DAO* (p.P103L) was found in 2 FALS patients (F19 and F21), both with a survival <12 months. Two rare variants were found in *ITPR2* in 2 SALS patients (p.M2443T, p.V1952A), both with a survival <18 months. A novel variant in *TARDBP* (p.G298S) was shared by 2 SALS patients (MND19 and MND40) and both survived <18 months. Another patient (MND4) with long survival (164 months) had a different rare variant in *TARDBP* (p.M337V). Patients sharing the same rare variants had pi-hat values ranging from 0 to 0.022, indicating that they are likely to be genetically independent (Supplementary Table 4).

3.4. Effect of variant burden on survival

FALS patients with survival of less than a year all had additional rare variants. The patient (F19) with the shortest survival had the highest number of rare variants. These observations suggested that the burden of rare variants may have an effect on survival, with each additional variant reducing survival probability. Combining FALS and SALS patients, we found that higher variant burden is associated with lower survival probability (log-rank test X^2 (2, $N = 54$) = 13.2, $p = 0.00137$, Fig. 1). Patients with 2 or more rare variants had reduced survival probability compared with patients with 0 or 1 variant (log-rank test X^2 (1, $N = 54$) = 13.1, $p = 0.00029$). At 15 months after symptom onset, only 33% (4/12) patients with 2 or more variants were alive without requiring ventilator support, whereas 86% (36/42) of patients with 0 or 1 rare variant were alive. We also performed survival analysis on SALS patients only, with

Table 3
Rare variant association for ALS and control

Variant type	RV burden	ALS (N = 54)	Control (N = 699)	Odds ratio (95% CI)	*p* value (CMCFisher)	*p* value (MB)
Synonymous	RV = 0	31	425	1.15 (0.63–2.09)	0.666	0.055
	RV ≥1	23	274			
Nonsynonymous	RV = 0	18	356	1.91 (1.03–3.61)	0.033*	0.004**
	RV ≥1	36	343			

Significance Code: $^{*}p < 0.05$, $^{**}p < 0.005$.
Key: ALS, amyotrophic lateral sclerosis; CMCFisher, Collapsing and combine rare variants followed by Fisher's exact test; MB, Upweight rare variant using inverse frequency from controls by Madsen-Browning; OR, odds ratio; RV, rare variant.

similar results (log-rank test X^2 (2, $N = 46$) = 10.8, $p = 0.004$). If we only included patients who were intubated or who had died as reaching the endpoint, the result was also similar (log-rank test $p = 0.00027$).

After adjusting for age of onset, sex, inheritance (sporadic or familial), and treatment with riluzole, rare variant burden remains to be associated with survival, with each additional rare variant increasing the risk of death/ventilator dependence by 60% (Cox regression, $p = 0.0097$). Bulbar onset increases the hazard risk of death/ventilator dependence by 48% compared with spinal onset (Cox regression, $p = 0.033$).

3.5. Effect of rare variants on risk of developing ALS

Next, we examined if the presence of rare variants in our gene set contributes also to the risk of developing ALS. Principal component analysis for common variants (MAF >5%) confirmed that ALS patients and healthy controls had similar genetic background (Supplementary Fig. 2). Of 54 patients, 35 (65%) had at least 1 nonsynonymous rare variant among the 40 selected ALS genes, with an average rare variant burden of 0.96, which is higher than the 0.68 in controls (odds ratio 1.91, 95% CI 1.03–3.61, see Table 3). Rare variant burden was associated with ALS (CMCFisher, *p*-value = 0.033; Madsen-Browning, *p*-value = 0.0041). The sequence kernel association test by SKAT-O, which was designed to improve power for a mixed set of risk and protective variants, was not significant (*p*-value 0.094), implying that the rare variants identified in our study had predominantly deleterious effects in ALS patients.

4. Discussion

The clinical diagnosis of ALS relies on features of upper and lower motor neuron degeneration. Patients demonstrate high phenotypic variability, suggesting that ALS is a heterogeneous disorder. About 20 genes have been identified as genetic causes of FALS. Some phenotypic correlations have been made based on the specific genetic mutation, but identification of causal mutation remains difficult as most families have a small number of affected individuals with wide phenotypic variability (Yamashita and Ando, 2015). Furthermore, about 30%–50% of families have no mutation in any of the known ALS genes. Clearly, a notable genetic component contributing to the development and clinical course of ALS, even in the relatively more homogeneous FALS patients, remains unknown.

Our ALS gene list consists of genes that have been reported as causative or susceptibility genes in the literature. There is evidence that the frequencies of genetic mutations in Asian ALS patients are different from those in patients of European ancestry. For example, repeat expansion in *C9ORF72* is the most common genetic mutation in Caucasian ALS patients but is rare in Asians; conversely, *SOD1* mutations are more common in Asians (Kim et al., 2016; Nakamura et al., 2016; Soong et al., 2014). For this study, we have included genes that are commonly mutated in Asian ALS patients.

In our 8 FALS patients sharing the same *SOD1* mutation, there was wide variability in survival. Therefore, we looked for additional genetic variants which may explain this variability. We observed that FALS patients with additional rare variants had shorter survival. Combining FALS and SALS patients, we confirmed that patients with 2 or more variants had shorter survival, suggesting an accumulative effect of rare genetic variants on reducing survival probability. This was confirmed by gene set analysis, which showed that variant burden within these 40 ALS genes was associated with survival probability.

We included FALS patients in the gene set association analyses (Table 3) as there is no evidence that familial forms of ALS are clinically different from sporadic ones. For the survival analysis, apart from F19 and F21 who were siblings, and F204 who was a cousin (Supplementary Fig. 1), all the other FALS patients had low pi-hat values indicating that they are genetically independent. More importantly, if we exclude all FALS patients, the survival analysis is still significant (log-rank sum test $p = 0.004$).

Bulbar onset increased risk of ventilator dependence or death by 47% compared with spinal onset ($p = 0.03$). Notably, rare variant burden had an even larger effect, with each additional rare variant increasing the risk of ventilator dependence/death by 60% ($p = 0.0098$). The Kaplan-Meier survival curve showed that patients with 2 or more variants had reduced survival compared with patients with 0 or 1 variant ($p = 0.0003$). Although no individual gene or variant is associated with survival, rare variant burden has a contribution to shorter survival, supporting an oligogenic basis in the progression of ALS.

We also showed in this study that ALS patients more often had rare variants within the 40 ALS genes than healthy controls. This suggests that deleterious variants across a set of biologically relevant genes may be responsible for the development of ALS. Our findings are in accordance with another study which showed that there was a significant enrichment of deleterious rare or novel alleles in ALS patients compared with controls, and that 60% of SALS patients had rare or novel variants in 169 ALS genes (Couthouis et al., 2014). With fewer candidate genes, we showed that 60.8% of SALS had at least 1% and 19.5% had 2 or more rare variants. This observation confirmed the importance of these 40 ALS risk genes in disease etiology.

In a large-scale WES study, *DAO* was found to correlate with survival time (Cirulli et al., 2015). In our study, rare variants in *DAO* were identified in 2 FALS patients and 1 SALS patient, all with short survival of less than 1 year. Similarly, we found 2 rare variants in *ITPR2* in 2 SALS patients, both with short survival. Our findings suggest that *DAO* and *ITPR2* may be important in modifying disease course and deserve further studies. We also confirmed the importance of *NEK1* and *TBK1*, 2 recently implicated ALS genes, as variants in both genes have been identified in our ALS patients. Although no single gene achieved genome-wide significance, after Bonferroni correction for 40 candidate genes, we found that *TARDBP* was associated with ALS (CMCFisherExact, *p*-value = 0.00002; Madsen-Browning permutations, *p*-value = 0.00004), as were *C9ORF72* (SKAT-O, $p = 0.00032$) and *DAO* (SKAT-O, $p = 0.0011$).

Oligogenic inheritance of ALS has been proposed based on observations of familial and sporadic patients with mutations in 2 ALS

genes (Lattante et al., 2012; van Blitterswijk et al., 2012). An earlier study which examined 17 ALS genes in ALS patients showed that 27.8% of SALS patients had 1 or more rare variants (Cady et al., 2015). Looking at the exons of these 17 genes in our patients, we saw a comparable proportion (30.4%) with 1 or more rare variants. The previous study showed no association between rare variant burden and disease duration. In contrast, using our list of 40 genes, with a smaller sample size, we showed that rare variant burden was associated with reduced survival. This is likely because our expanded gene list conferred more power to detect associations between clinical features and rare variant burden.

Genome-wide association studies have estimated that common genetic variants account for 8.5%–12% of heritability of ALS (Fogh et al., 2014; van Rheenen et al., 2016). Using genome-wide complex trait analysis looking at common variants in ALS patients, another study further increased the heritability estimate to 21% (Keller et al., 2014), while twin studies showed ALS heritability to be around 61% (Al-Chalabi et al., 2010). The "missing heritability" is likely due to rare variants, and our study confirmed that a notable proportion of SALS patients carried rare variants in ALS risk and susceptibility genes.

Just as we have shown that rare variants can influence survival in ALS, common variants may also play a role. A genome-wide association study carried out in patients from Europe and the United States identified common variants in *CAMTA1* to be associated with survival (Fogh et al., 2016). In our 54 patients, 5 carried the rare nonsynonymous variant in *CAMTA1:* F21, F310, and MND64 carried the same rare variant (p.V949L) and had survival of 11, 37, and 3 months, respectively. F331 carried a p.M401I variant and survived for 74 months, whereas MND59 carried a p.A1407G variant and survived for 31 months. All 3 variants are located more than 630kb away from the reported common variant (rs2412208). Further studies may elucidate the role of *CAMTA1* in modifying the disease course of ALS.

There are some limitations to our study. Our sample size is small. However, because all patients were seen in a single center, we have detailed phenotype information enabling analysis for genotype-phenotype correlation. We only examined the exons of 40 genes for nonsynonymous mis-sense mutations. Repeat expansions, variants located in noncoding regions, and copy number variations would not be detected in our study. We did not screen for the hexanucleotide repeat expansion in *C9ORF72*, the most common genetic cause of ALS in the West, but this mutation is exceedingly rare in Chinese, Koreans, and Japanese (He et al., 2015; Kim et al., 2016; Nakamura et al., 2016). Similarly, repeat expansions in *ATXN2* and copy number variations in *SMN1* were not included in our study. Although it is not known if nonsynonymous single nucleotide variants in *C9ORF72* and *ATXN2* cause ALS, we nevertheless included these genes in our analysis to look for the presence of variants in the exons of these genes in ALS patients. In fact, a recent study which included 469 Japanese ALS patients identified an single nucleotide variant in *C9ORF72* in 1 patient, while no repeat expansion was detected (Nakamura et al., 2016). There are various models which provide pathogenicity predictions of variants (Supplementary Table 5), but different models give different predictions and the results are not always consistent. Hence, in our analysis, we treated all rare variants equally and did not take into account whether some variants may be more deleterious than others. Survival analysis suggests that inheritance (familial versus sporadic) did not influence survival. FALS patient with only the *SOD1* mutation had similar survival as SALS patients with 0 or 1 variant. Rather, it is the burden of rare variants that was found to affect survival.

While increasing number of genes have been implicated in ALS, individual gene variants are so rare that even studies with large sample sizes often cannot definitively confirm causal effect or association with clinical features. The findings from our study suggest that development and progression of ALS may involve more than 1 genetic mutation. A previous study showed that there was a linear relationship between log incidence and log age in ALS in 5 populations, consistent with a multistep model (Al-Chalabi et al., 2014). This would be in agreement with our finding of an oligogenic influence on the prognosis of ALS, with each additional rare variant increasing the risk of mortality, suggesting that development and progression of ALS is indeed a multi-hit process. Our findings highlight the importance of looking into the possible interaction among known ALS genes which could collectively affect disease progression. Cellular pathways and potential pathogenic mechanisms, such as disruption of protein degradation and RNA metabolism defects, are already emerging from the plethora of genetic factors so far identified (Taylor et al., 2016), with implications on possible therapeutic targets. Further studies in elucidating cellular pathways shared by our 40 ALS genes may identify therapeutic targets to prolong survival. Furthermore, a potential clinical application of our results is to predict and stratify survival in ALS patients using their genetic profile, which may be useful in future interventional trials to more accurately measure the efficacy of treatment in prolonging survival.

Disclosure statement

The authors report no conflict of interest.

Acknowledgements

The authors would like to thank Timothy Shin Heng Mak, PhD, Centre for Genomic Sciences, University of Hong Kong and Prof. Hung Fat Tse, MD, PhD, Department of Medicine, University of Hong Kong who provided sequencing data from controls.

This work was supported by Liu Po Shan/Dr Vincent Liu Endowment Fund for Motor Neurone Disease, the Henry G. Leong Professorship in Neurology, the Donation Fund for Neurology Research, Hong Kong Research Grants Council grants AoE/M-04/04&T12-708/12-N, European Community Seventh Framework Programme Grant on European Network of National Schizophrenia Networks Studying Gene-Environment Interactions (EU-GEI), the HKU Seed Funding Programme for Basic Research 201411159172, Health and the Medical Research Fund - Full Grant 01121436.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2017.06.007.

References

Abel, O., Powell, J.F., Andersen, P.M., Al-Chalabi, A., 2012. ALSoD: a user-friendly online bioinformatics tool for amyotrophic lateral sclerosis genetics. Hum. Mutat. 33, 1345–1351.

Al-Chalabi, A., Calvo, A., Chio, A., Colville, S., Ellis, C.M., Hardiman, O., Heverin, M., Howard, R.S., Huisman, M.H., Keren, N., Leigh, P.N., Mazzini, L., Mora, G., Orrell, R.W., Rooney, J., Scott, K.M., Scotton, W.J., Seelen, M., Shaw, C.E., Sidle, K.S., Swingler, R., Tsuda, M., Veldink, J.H., Visser, A.E., van den Berg, L.H., Pearce, N., 2014. Analysis of amyotrophic lateral sclerosis as a multistep process: a population-based modelling study. Lancet Neurol. 13, 1108–1113.

Al-Chalabi, A., Fang, F., Hanby, M.F., Leigh, P.N., Shaw, C.E., Ye, W., Rijsdijk, F., 2010. An estimate of amyotrophic lateral sclerosis heritability using twin data. J. Neurol. Neurosurg. Psychiatry 81, 1324–1326.

Brooks, B.R., Miller, R.G., Swash, M., Munsat, T.L. World Federation of Neurology Research Group on Motor Neuron Diseases, 2000. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. Amyotroph. Lateral Scler. Other Mot. Neuron Disord 1, 293–299.

Cady, J., Allred, P., Bali, T., Pestronk, A., Goate, A., Miller, T.M., Mitra, R.D., Ravits, J., Harms, M.B., Baloh, R.H., 2015. ALS onset is influenced by the burden of rare variants in known ALS genes. Ann. Neurol. 77, 100–113.

Chang, C.C., Chow, C.C., Tellier, L.C.A.M., Vattikuti, S., Purcell, S.M., Lee, J.J., 2015. Second-generation PLINK: rising to the challenge of larger and richer datasets. GigaScience 4, 7.

Cirulli, E.T., Lasseigne, B.N., Petrovski, S., Sapp, P.C., Dion, P.A., Leblond, C.S., Couthouis, J., Lu, Y.F., Wang, Q., Krueger, B.J., Ren, Z., Keebler, J., Han, Y., Levy, S.E., Boone, B.E., Wimbish, J.R., Waite, L.L., Jones, A.L., Carulli, J.P., Day-Williams, A.G., Staropoli, J.F., Xin, W.W., Chesi, A., Raphael, A.R., McKenna-Yasek, D., Cady, J., Vianney de Jong, J.M., Kenna, K.P., Smith, B.N., Topp, S., Miller, J., Gkazi, A., FALS Sequencing Consortium, Al-Chalabi, A., van den Berg, L.H., Veldink, J., Silani, V., Ticozzi, N., Shaw, C.E., Baloh, R.H., Appel, S., Simpson, E., Lagier-Tourenne, C., Pulst, S.M., Gibson, S., Trojanowski, J.Q., Elman, L., McCluskey, L., Grossman, M., Shneider, N.A., Chung, W.K., Ravits, J.M., Glass, J.D., Sims, K.B., Van Deerlin, V.M., Maniatis, T., Hayes, S.D., Ordureau, A., Swarup, S., Landers, J., Baas, F., Allen, A.S., Bedlack, R.S., Harper, J.W., Gitler, A.D., Rouleau, G.A., Brown, R., Harms, M.B., Cooper, G.M., Harris, T., Myers, R.M., Goldstein, D.B., 2015. Exome sequencing in amyotrophic lateral sclerosis identifies risk genes and pathways. Science 347, 1436–1441.

Couthouis, J., Raphael, A.R., Daneshjou, R., Gitler, A.D., 2014. Targeted exon capture and sequencing in sporadic amyotrophic lateral sclerosis. PLoS Genet. 10, e1004704.

Cronin, S., Hardiman, O., Traynor, B.J., 2007. Ethnic variation in the incidence of ALS: a systematic review. Neurology 68, 1002–1007.

Finsterer, J., Burgunder, J.M., 2014. Recent progress in the genetics of motor neuron disease. Eur. J. Med. Genet. 57, 103–112.

Fogh, I., Lin, K., Tiloca, C., Rooney, J., Gellera, C., Diekstra, F.P., Ratti, A., Shatunov, A., van Es, M.A., Proitsi, P., Jones, A., Sproviero, W., Chiò, A., McLaughlin, R.L., Sorarù, G., Corrado, L., Stahl, D., Del Bo, R., Cereda, C., Castellotti, B., Glass, J.D., Newhouse, S., Dobson, R., Smith, B.N., Topp, S., van Rheenen, W., Meininger, V., Melki, J., Morrison, K.E., Shaw, P.J., Leigh, P.N., Andersen, P.M., Comi, G.P., Ticozzi, N., Mazzini, L., D'Alfonso, S., Traynor, B.J., Van Damme, P., Robberecht, W., Brown, R.H., Landers, J.E., Hardiman, O., Lewis, C.M., van den Berg, L.H., Shaw, C.E., Veldink, J.H., Silani, V., Al-Chalabi, A., Powell, J., 2016. Association of a locus in the *CAMTA1* gene with survival in patients with sporadic amyotrophic lateral sclerosis. JAMA Neurol. 73, 812–820.

Fogh, I., Ratti, A., Gellera, C., Lin, K., Tiloca, C., Moskvina, V., Corrado, L., Sorarù, G., Cereda, C., Corti, S., Gentilini, D., Calini, D., Castellotti, B., Mazzini, L., Querin, G., Gagliardi, S., Del Bo, R., Conforti, F.L., Siciliano, G., Inghilleri, M., Saccà, F., Bongioanni, P., Penco, S., Corbo, M., Sorbi, S., Filosto, M., Ferlini, A., Di Blasio, A.M., Signorini, S., Shatunov, A., Jones, A., Shaw, P.J., Morrison, K.E., Farmer, A.E., Van Damme, P., Robberecht, W., Chiò, A., Traynor, B.J., Sendtner, M., Melki, J., Meininger, V., Hardiman, O., Andersen, P.M., Leigh, N.P., Glass, J.D., Overste, D., Diekstra, F.P., Veldink, J.H., van Es, M.A., Shaw, C.E., Weale, M.E., Lewis, C.M., Williams, J., Brown, R.H., Landers, J.E., Ticozzi, N., Ceroni, M., Pegoraro, E., Comi, G.P., D'Alfonso, S., van den Berg, L.H., Taroni, F., Al-Chalabi, A., Powell, J., Silani, V. SLAGEN Consortium and Collaborators, 2014. A genome-wide association meta-analysis identifies a novel locus at 17q11.2 associated with sporadic amyotrophic lateral sclerosis. Hum. Mol. Genet. 23, 2220–2231.

Fong, G.C., Cheng, T.S., Lam, K., Cheng, W.K., Mok, K.Y., Cheung, C.M., Chim, C.S., Mak, W., Chan, K.H., Tsang, K.L., Kwan, M.C., Tsoi, T.H., Cheung, R.T., Ho, S.L., 2005. An epidemiological study of motor neuron disease in Hong Kong. Amyotroph. Lateral Scler. Other Mot. Neuron Disord 6, 164–168.

Fong, G.C.Y., Kwok, K.H.H., Song, Y.Q., Cheng, T.S., Ho, P.W., Chu, A.C., Kung, M.H., Chan, K.H., Mak, W., Cheung, R.T., Ramsden, D.B., Ho, S.L., 2006. Clinical phenotypes of a large Chinese multigenerational kindred with autosomal dominant familial ALS due to Ile149Thr SOD1 gene mutation. Amyotroph. Lateral Scler. 7, 142–149.

He, J., Tang, L., Benyamin, B., Shah, S., Hemani, G., Liu, R., Ye, S., Liu, X., Ma, Y., Zhang, H., Cremin, K., Leo, P., Wray, N.R., Visscher, P.M., Xu, H., Brown, M.A., Bartlett, P.F., Mangelsdorf, M., Fan, D., 2015. *C9orf72* hexanucleotide repeat expansions in Chinese sporadic amyotrophic lateral sclerosis. Neurobiol. Aging 36, 2660.e1–2660.e8.

Hsu, J.S., Kwan, J.S., Pan, Z., Garcia-Barcelo, M.M., Sham, P.C., Li, M., 2016. Inheritance-mode specific pathogenicity prioritization (ISPP) for human protein coding genes. Bioinformatics 32, 3065–3071.

Ionita-Laza, I., Lee, S., Makarov, V., Buxbaum, J.D., Lin, X., 2013. Sequence kernel association tests for the combined effect of rare and common variants. Am. J. Hum. Genet. 92, 841–853.

Keller, M.F., Ferrucci, L., Singleton, A.B., Tienari, P.J., Laaksovirta, H., Restagno, G., Chio, A., Traynor, B.J., Nalls, M.A., 2014. Genome-wide analysis of the heritability of amyotrophic lateral sclerosis. JAMA Neurol. 71, 1123–1134.

Kenna, K.P., McLaughlin, R.L., Burne, S., Elamin, M., Heverin, M., Kenny, E.M., Cormican, P., Morris, D.W., Donaghy, C.G., Bradley, D.G., Hardiman, O., 2013. Delineating the genetic heterogeneity of ALS using targeted high-throughput sequencing. J. Med. Genet. 50, 776–783.

Kim, H.J., Oh, K.W., Kwon, M.J., Oh, S.I., Park, J.S., Kim, Y.E., Choi, B.O., Lee, S., Ki, C.S., Kim, S.H., 2016. Identification of mutations in Korean patients with amyotrophic lateral sclerosis using multigene panel testing. Neurobiol. Aging 37, 209.e9–209.e16.

Lattante, S., Conte, A., Zollino, M., Luigetti, M., Del Grande, A., Marangi, G., Romano, A., Marcaccio, A., Meleo, E., Bisogni, G., Rossini, P.M., Sabatelli, M., 2012. Contribution of major amyotrophic lateral sclerosis genes to the etiology of sporadic disease. Neurology 79, 66–72.

Leblond, C., Kaneb, H., Dion, P.A., Rouleau, G.A., 2014. Dissection of genetic factors associated with amyotrophic lateral sclerosis. Exp. Neurol. 262 (Pt B), 91–101.

Li, M.X., Gui, H.S., Kwan, J.S., Bao, S.Y., Sham, P.C., 2012. A comprehensive framework for prioritizing variants in exome sequencing studies of Mendelian diseases. Nucleic Acids Res. 40, e53.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., DePristo, M.A., 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303.

Nakamura, R., Sone, J., Atsuta, N., Tohnai, G., Watanabe, H., Yokoi, D., Nakatochi, M., Watanabe, H., Ito, M., Senda, J., Katsuno, M., Tanaka, F., Li, Y., Izumi, Y., Morita, M., Taniguchi, A., Kano, O., Oda, M., Kuwabara, S., Abe, K., Aiba, I., Okamoto, K., Mizoguchi, K., Hasegawa, K., Aoki, M., Hattori, N., Tsuji, S., Nakashima, K., Kaji, R., Sobue, G. Japanese Consortium for Amyotrophic Lateral Sclerosis Research (JaCALS), 2016. Next generation sequencing of 28 ALS-related genes in a Japanese ALS cohort. Neurobiol. Aging 39, 219.e1–219.e8.

Ng, M.C., Ho, J.T., Ho, S.L., Lee, R., Li, G., Cheng, T.S., Song, Y.Q., Ho, P.W., Fong, G.C., Mak, W., Chan, K.H., Li, L.S., Luk, K.D., Hu, Y., Ramsden, D.B., Leong, L.L., 2008. Abnormal diffusion tensor in non-symptomatic familial amyotrophic lateral sclerosis with a causative superoxide dismutase 1 mutation. J. Magn. Reson. Imaging 27, 8–13.

Renton, A.E., Chio, A., Traynor, B.J., 2014. State of play in amyotrophic lateral sclerosis genetics. Nat. Neurosci. 17, 17–23.

Soong, B.W., Lin, K.P., Guo, Y.C., Lin, C.C., Tsai, P.C., Liao, Y.C., Lu, Y.C., Wang, S.J., Tsai, C.P., Lee, Y.C., 2014. Extensive molecular genetic survey of Taiwanese patients with amyotrophic lateral sclerosis. Neurobiol. Aging 35, 2423.e1–2423.e16.

Swinnen, B., Robberecht, W., 2014. The phenotypic variability of amyotrophic lateral sclerosis. Nat. Rev. Neurol. 10, 661–670.

Taylor, J.P., Brown Jr., R.H., Cleveland, D.W., 2016. Decoding ALS: from genes to mechanism. Nature 539, 197–206.

van Blitterswijk, M., van Es, M.A., Hennekam, E.A.M., Dooijes, D., van Rheenen, W., Medic, J., Bourque, P.R., Schelhaas, H.J., van der Kooi, A.J., de Visser, M., de Bakker, P.I., Veldink, J.H., van den Berg, L.H., 2012. Evidence for an oligogenic basis of amyotrophic lateral sclerosis. Hum. Mol. Genet. 21, 3776–3784.

van Rheenen, W., Shatunov, A., Dekker, A.M., McLaughlin, R.L., Diekstra, F.P., Pulit, S.L., van der Spek, R.A., Võsa, U., de Jong, S., Robinson, M.R., Yang, J., Fogh, I., van Doormaal, P.T., Tazelaar, G.H., Koppers, M., Blokhuis, A.M., Sproviero, W., Jones, A.R., Kenna, K.P., van Eijk, K.R., Harschnitz, O., Schellevis, R.D., Brands, W.J., Medic, J., Menelaou, A., Vajda, A., Ticozzi, N., Lin, K., Rogelj, B., Vrabec, K., Ravnik-Glavač, M., Koritnik, B., Zidar, J., Leonardis, L., Grošelj, L.D., Millecamps, S., Salachas, F., Meininger, V., de Carvalho, M., Pinto, S., Mora, J.S., Rojas-García, R., Polak, M., Chandran, S., Colville, S., Swingler, R., Morrison, K.E., Shaw, P.J., Hardy, J., Orrell, R.W., Pittman, A., Sidle, K., Fratta, P., Malaspina, A., Topp, S., Petri, S., Abdulla, S., Drepper, C., Sendtner, M., Meyer, T., Ophoff, R.A., Staats, K.A., Wiedau-Pazos, M., Lomen-Hoerth, C., Van Deerlin, V.M., Trojanowski, J.Q., Elman, L., McCluskey, L., Basak, A.N., Tunca, C., Hamzeiy, H., Parman, Y., Meitinger, T., Lichtner, P., Radivojkov-Blagojevic, M., Andres, C.R., Maurel, C., Bensimon, G., Landwehrmeyer, B., Brice, A., Payan, C.A., Saker-Delye, S., Dürr, A., Wood, N.W., Tittmann, L., Lieb, W., Franke, A., Rietschel, M., Cichon, S., Nöthen, M.M., Amouyel, P., Tzourio, C., Dartigues, J.F., Uitterlinden, A.G., Rivadeneira, F., Estrada, K., Hofman, A., Curtis, C., Blauw, H.M., van der Kooi, A.J., de Visser, M., Goris, A., Weber, M., Shaw, C.E., Smith, B.N., Pansarasa, O., Cereda, C., Del Bo, R., Comi, G.P., D'Alfonso, S., Bertolin, C., Sorarù, G., Mazzini, L., Pensato, V., Gellera, C., Tiloca, C., Ratti, A., Calvo, A., Moglia, C., Brunetti, M., Arcuti, S., Capozzo, R., Zecca, C., Lunetta, C., Penco, S., Riva, N., Padovani, A., Filosto, M., Muller, B., Stuit, R.J., PARALS Registry SLALOM Group SLAP Registry FALS Sequencing Consortium SLAGEN Consortium NNIPPS Study Group, Blair, I., Zhang, K., McCann, E.P., Fifita, J.A., Nicholson, G.A., Rowe, D.B., Pamphlett, R., Kiernan, M.C., Grosskreutz, J., Witte, O.W., Ringer, T., Prell, T., Stubendorff, B., Kurth, I., Hübner, C.A., Leigh, P.N., Casale, F., Chio, A., Beghi, E., Pupillo, E., Tortelli, R., Logroscino, G., Powell, J., Ludolph, A.C., Weishaupt, J.H., Robberecht, W., Van Damme, P., Franke, L., Pers, T.H., Brown, R.H., Glass, J.D., Landers, J.E., Hardiman, O., Andersen, P.M., Corcia, P., Vourc'h, P., Silani, V., Wray, N.R., Visscher, P.M., de Bakker, P.I., van Es, M.A., Pasterkamp, R.J., Lewis, C.M., Breen, G., Al-Chalabi, A., van den Berg, L.H., Veldink, J.H., 2016. Genome-wide association analyses identify new risk variants and the genetic architecture of amyotrophic lateral sclerosis. Nat. Genet. 48, 1043–1048.

Yamashita, S., Ando, Y., 2015. Genotype-phenotype relationship in hereditary amyotrophic lateral sclerosis. Transl. Neurodegener. 4, 13.

Yang, J., Lee, S.H., Goddard, M.E., Visscher, P.M., 2011. GCTA: a tool for genome-wide complex trait analysis. Am. J. Hum. Genet. 88, 76–82.

Zhan, X., Hu, Y., Li, B., Abecasis, G.R., Liu, D.J., 2016. RVTESTS: an efficient and comprehensive tool for rare variant association analysis using sequence data. Bioinformatics 32, 1423–1426.