

Association of a structural variant within the *SQSTM1* gene with amyotrophic lateral sclerosis

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Abstract

Objective

As structural variations may underpin susceptibility to complex neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), the objective of this study was to investigate a structural variant (SV) within sequestosome 1 (*SQSTM1*).

Methods

A candidate insertion/deletion variant within intron 5 of the *SQSTM1* gene was identified using a previously established SV evaluation algorithm and chosen according to its subsequent theoretical effect on gene expression. The variant was systematically assessed through PCR, polyacrylamide gel fractionation, Sanger sequencing, and reverse transcriptase PCR.

Results

A reliable and robust assay confirmed the polymorphic nature of this variant and that the variant may influence *SQSTM1* transcript levels. In a North American cohort of patients with familial ALS (fALS) and sporadic ALS (sALS) ($n = 403$) and age-matched healthy controls ($n = 562$), we subsequently showed that the *SQSTM1* variant is associated with fALS ($p = 0.0036$), particularly in familial superoxide dismutase 1 mutation positive patients ($p = 0.0005$), but not with patients with sALS ($p = 0.97$).

Conclusions

This disease association highlights the importance and implications of further investigation into SVs that may provide new targets for cohort stratification and therapeutic development.

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Glossary

ALS = amyotrophic lateral sclerosis; **CI** = confidence interval; **fALS** = familial ALS; **GWAS** = genome-wide association study; **I/D** = insertion/deletion; **mRNA** = messenger RNA; **ONS** = olfactory neurosphere derived; **OR** = odds ratio; **sALS** = sporadic ALS; **SOD1** = superoxide dismutase 1; **SQSTM1** = sequestosome 1; **RT-PCR** = reverse transcriptase PCR; **SV** = structural variant.

A growing body of literature indicates that structural variants (SVs) are important in complex diseases because their role in gene expression and messenger RNA (mRNA) regulation is now emerging.^{1–4} Given the potential implication of SVs in neurodegenerative disorders,^{5–7} an investigation of SVs within associated genes may provide insight toward the pathogenic mechanisms involved and enable the identification of new therapeutic targets.

Mutations in sequestosome 1 (*SQSTM1*), and aggregation of the *SQSTM1*-encoded p62 protein, have been identified in patients with amyotrophic lateral sclerosis (ALS) and other neurodegenerative diseases.^{8–13} P62 is a multifunctional protein that binds ubiquitin and is involved in autophagy, proteasomal degradation of ubiquitinated proteins, mitophagy, and cellular signaling.^{14–16} Variants within *SQSTM1* or surrounding *SQSTM1* may contribute to the diverse presentation observed between the patients with ALS. How mutations in *SQSTM1* influence ALS is not fully understood; however, it is believed that structural changes to the protein may affect adapter function of *SQSTM1* to the LC3 protein in the nascent autophagosome and impair autophagy of proteins that are not recyclable by the proteasome.¹⁷ Other cellular systems may also be affected such as ubiquitin binding and regulation of cellular processes including DNA repair, endocytosis, and signal transduction.^{8,14}

In the current study, a potential influential insertion/deletion (I/D) within intron 5 of *SQSTM1* was identified using an *in silico* short SV evaluation algorithm.¹⁸ Reverse transcriptase PCR (RT-PCR) analysis revealed a link between the I/D and altered transcript levels. An exploratory association study in a sporadic and familial North American ALS cohort established an association with familial ALS (fALS), particularly superoxide dismutase 1 (*SOD1*) mutation-positive patients but not sporadic ALS (sALS). We further examined whether this SV was associated with age of disease onset and duration in *SOD1* mutation-positive patients and found no statistically significant association.

Methods

Standard protocol approvals, registrations, and patient consents

This study was approved by the ethical standards of the relevant institutional review board, the Human Research Ethics Committee of the University of Western Australia (RA/4/20/5308). Participants were enrolled after informed consent was obtained. Clinical data were collected according to the Health

Insurance Portability and Accountability Act Standards of Confidentiality and Disclosure and approved by the Northwestern University institutional review board (STU0012722/CR5_STU00012722) and the Duke University institutional review board (PRO00040665/323682). All patients were diagnosed by board-certified neurologists and met the revised El Escorial World Federation of Neurology criteria for diagnosis with ALS.¹⁹

SV identification

An SV evaluation algorithm was used to locate the variant within the gene, *SQSTM1*.¹⁸ Detailed methods are described in the supplementary material (see e-methods, links.lww.com/NXG/A229).

PCR amplification and Sanger sequencing

Detailed methods are described in the supplementary material. Sequences of all primers used for this study are outlined in table e-1 (links.lww.com/NXG/A229).

Olfactory neurosphere-derived cells

Culturing information can be found in the supplementary data.

RT-PCR and densitometry

Detailed methods are described in the supplementary material. Sequences of all primers used for this study are detailed in table e-1 (links.lww.com/NXG/A229).

ALS and healthy control participants

A cohort of 196 fALS, 207 sALS, and 562 healthy control participants were recruited into the Neurologic Diseases Registry, Northwestern University, Chicago, USA, and Duke University, North Carolina, USA. The 196 fALS cases from Northwestern University belong to 74 different families with possible within-family genetic correlations. The mutation data for all fALS patients are detailed in table e-2 (links.lww.com/NXG/A229).

Statistics

Data are reported as mean \pm SD where appropriate. Statistical differences in genotype proportions for independent cases were assessed using Pearson χ^2 test with Yates correction. Proportions and ages of onset involving familial groups were analyzed using the mixed effects regression models to account for possible within-family correlations. Durations were analyzed using Cox proportional hazards models with clustering to account for the correlations. Analyses were carried out in IBM SPSS Statistics version 25.0 (IBM Co., Armonk, NY) and R version 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria).

Data availability

Study data for the primary analyses presented in this report are available on reasonable request from the corresponding author.

Results

Identification of a polymorphic variant within the *SQSTM1* gene

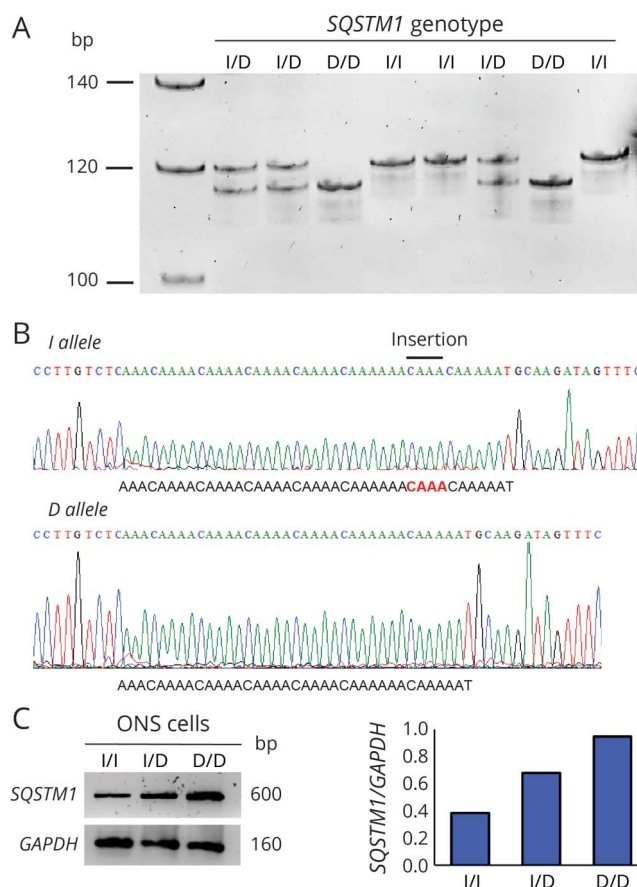
An SV evaluation algorithm was used to identify the genetic variant located within *SQSTM1*. The identified cytosine adenine adenine adenine (CAAA) I/D within intron 5 of *SQSTM1* was evaluated on the NCBI database; although the region had been previously reported (NC_0000005.0), no disease associations were established. The recorded NCBI data demonstrated uncertainty to its polymorphic nature; currently, the region is recorded as a series of insertion/deletions of varying length, with no allelic frequency data. Conventional polyacrylamide gel electrophoresis revealed a biallelic variant, consisting of a heterozygous I/D genotype in lanes 2, 3, and 7, a homozygous D/D genotype in lanes 4 and 8, and a homozygous I/I genotype in lanes 5, 6, and 9 (figure 1A). Sanger sequencing confirmed that the I allele contained a CAAA insertion when compared with the D allele (figure 1B). To determine the effect of the CAAA SV on *SQSTM1* expression, *SQSTM1* was assessed in a panel (n = 3) of olfactory neurosphere-derived (ONS) cells obtained from the healthy controls. Semiquantitative RT-PCR revealed a stepwise increase in the level of *SQSTM1* transcript, with the I/I genotype yielding the lowest levels and the I/D and D/D genotypes producing a 1.77-fold and 2.47-fold relative increase in *SQSTM1* transcript, respectively (figure 1C).

The *SQSTM1* variant is associated with fALS

Following the identification of the CAAA I/D variant, we proceeded to determine the variant frequency in a cohort of 196 fALS patients, 207 sALS patients, and 562 healthy age-matched controls. The characteristics of the study participants are summarized in table 1, including sex, age, disease duration, and family history of each cohort. Of the ALS cohort, patients with familial mutations (48.6%) were further grouped by affected gene, specifically *C9orf72* (2.5% of patients with ALS), *SOD1* (41.4% of patients with ALS), or *TDP-43* (4.7% of patients with ALS).

There was no difference in the frequency of the *SQSTM1* variant observed between healthy controls and sALS cases ($\chi^2 = 0.032$, df = 2, $p = 0.984$, odds ratio [OR] = 0.974, 95% confidence interval [CI]: 0.660, 1.436). However, there was a difference in *SQSTM1* variant frequency observed between healthy controls and fALS patients ($\chi^2 = 12.791$, df = 2, $p = 0.002$, OR = 1.79, 95% CI: 1.250, 2.562 assuming independence of cases), with the homozygous (I/I) genotype over-represented in the fALS cohort (33.2%) when compared with the controls (21.7%). When analyzed using mixed effects regression models to account for possible within-family correlations, the (I/I) association remained both genotypical and allelic ($p = 0.013$ and $p = 0.0036$, respectively, table 2). This

Figure 1 Characterization of the *SQSTM1* variant



(A) PCR and native polyacrylamide gel electrophoresis across a random selection of control DNA samples and compared against a 100bp ladder. (B) Sanger sequencing of the I allele and D allele. (C) *SQSTM1* transcript levels of *SQSTM1* exon 4-7 analyzed using RT-PCR on RNA from a panel of control ONS cells. Relative densitometry was calculated with *SQSTM1* transcript signal standardized to each respective GAPDH signal. GAPDH = glyceraldehyde 3-phosphate dehydrogenase; I/D = insertion/deletion; ONS = olfactory neurosphere derived cells; RT-PCR = Reverse transcriptase PCR; *SQSTM1* = sequestosome 1.

SQSTM1 association was stronger when examining the subset of fALS patients carrying mutations in *SOD1* ($\chi^2 = 16.754$, df = 2, $p = 0.0002$, OR = 1.869, 95% CI: 1.281, 2.726 assuming independent cases). When accounting for family structures, the *SQSTM1* variant was strongly associated with patients carrying a *SOD1* mutation both genotypically and allelically ($p = 0.001$ and $p = 0.0005$, respectively) (table 2). When analyzed with *SOD1*-ASV mutation-positive patients excluded, the strong *SOD1* association remains ($\chi^2 = 6.801$, $p < 0.009$, OR = 1.830, 95% CI: 1.157, 2.896).

The *SQSTM1* variant is not associated with age at onset of disease or survival in patients with *SOD1*.

To determine if the *SQSTM1* variant is associated with *SOD1* mutation-positive ALS patient outcomes, age at onset and survival were analyzed. There was no association observed between the age at onset in patients with the I/I (Mean Rank = 87.25), I/D (Mean Rank = 76.40), or D/D (Mean Rank =

Table 1 Characteristics of patients and healthy control participants

Variable	Mean (SD) or n	
	Patients (n = 403)	Controls (n = 562)
Sex		
Male	208	296
Female	195	266
Age (y)	52.38 (12.99)	49.37 (12.56)
Disease duration (mo)	43.21 (46.98)	—
Family history		—
Sporadic	207	
Familial	196	—

84.43) genotypes ($p = 0.2$, mixed model regression, figure 2A). Survival curves were generated to compare durations of each genotypic group (figure 2B). No association was observed between patients carrying each genotype and their survival after taking into account of familial correlations via a clustered Cox regression ($p = 0.55$).

Discussion

A growing body of literature has highlighted SVs, their abundance throughout the human genome, and their potential role in the pathogenesis of ALS and other neurodegenerative diseases.^{20–24} SVs are responsible for greater diversity at the nucleotide level between 2 human genomes than any other form of genetic variations and are three-fold more likely to associate with genome-wide association studies (GWASs) signals than single nucleotide polymorphisms (SNPs).²⁰ SVs that remain cryptic to current sequencing algorithms are likely to account for disease-causing variation in unsolved Mendelian disorders and missing heritability in complex disorders.^{20,23} SVs may affect gene expression and therefore may play an important but understudied role in disease susceptibility.^{20,25} Recent discoveries of SVs as informative disease risk markers for rare genetic disorders provide compelling evidence for ongoing investigation into the association between SVs and rare genetic diseases.^{26–30} The novel bioinformatics SV evaluation algorithm tool prioritizes potential functional/causal SVs within candidate regions identified using GWAS.¹⁸ In ALS and all rare genetic diseases, these highly polymorphic markers are often overlooked, largely because of the limitations in the current gene sequencing platforms such as next-generation sequencing and GWAS, which are primarily designed to detect SNPs.

Two direct lines of evidence link *SQSTM1* to ALS: the first through etiology on account of mutations associated with ALS and second through pathology, where p62 immune-reactive

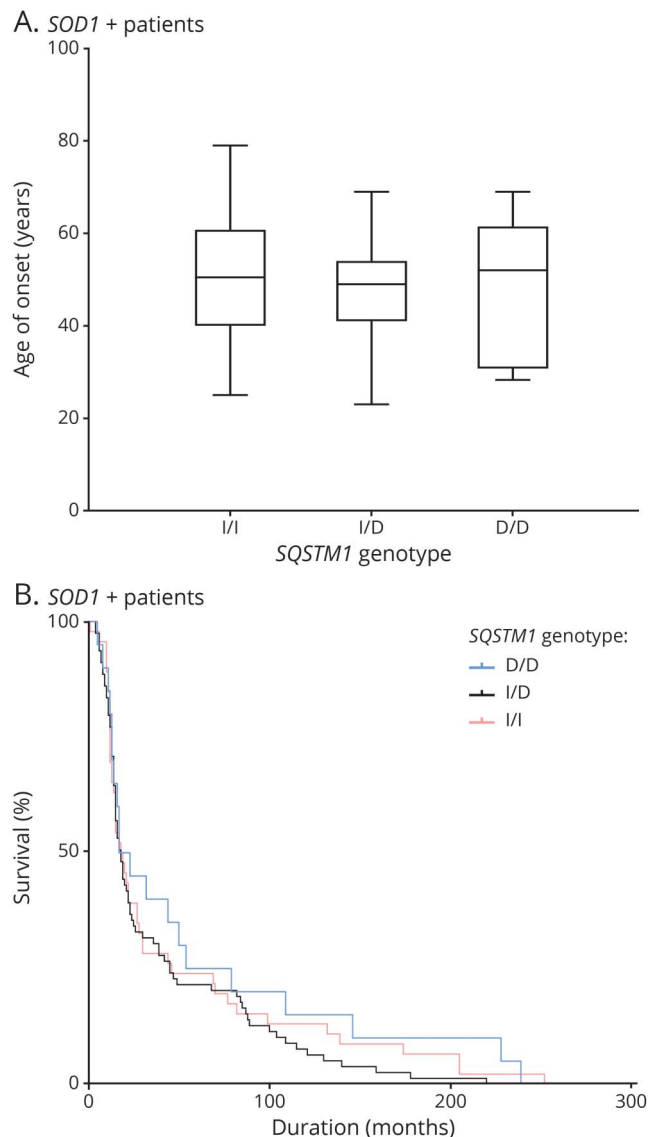
Table 2 Association of *SQSTM1* variant and ALS disease status

sALS	Control n (%)	sALS n (%)	p Value
<i>SQSTM1</i> CAAA variant			
I/I	122 (21.7)	44 (21.3)	0.98
I/D	292 (52.0)	109 (52.7)	
D/D	148 (26.3)	54 (26.1)	
I allele	536	197	0.97
D allele	588	217	
Familial ALS	Control n (%)	fALS n (%)	p Value ^a
<i>SQSTM1</i> CAAA variant			
I/I	122 (21.7)	65 (33.2)	0.0013
I/D	292 (52.0)	97 (49.5)	0.50
D/D	148 (26.3)	34 (17.3)	0.08
I allele	536	227	0.0036
D allele	588	165	
<i>SOD1</i> mutation positive	Control n (%)	<i>SOD1</i> n (%)	p Value ^a
<i>SQSTM1</i> CAAA variant			
I/I	122 (21.7)	57 (34.1)	0.001
I/D	292 (52.0)	87 (52.1)	0.99
D/D	147 (26.3)	23 (13.8)	0.014
I allele	536	201	0.0005
D allele	588	133	

Abbreviations: ALS = amyotrophic lateral sclerosis; fALS = familial ALS; I/D = insertion/deletion; sALS = sporadic ALS; *SQSTM1* = sequestosome 1.
^a Each row compared using random effects to account for familial correlation.

inclusions are found in virtually all forms of ALS and ALS-frontotemporal dementia.⁸ We now provide a third link of *SQSTM1* variants to the fALS. *SQSTM1*-encoded p62 protein is a key scaffold involved in cellular signaling and protein degradation through the autophagosome-lysosome system.¹⁷ *SQSTM1*/p62 mutations may confer a toxic gain of function through protein interactions, leading to dysregulation of cell signaling pathways, protein misfolding, and aggregation.⁸ Evidence for this in the literature suggests parallels between p62 and other proteins associated with neurodegeneration, including *SOD1*-linked ALS, *C9orf72*, ubiquitin 2, TDP-43, FUS, optineurin, beta-amyloid, α -synuclein, and tau.^{8,31} Owing to its role in protein degradation, overexpression of p62 has been shown to be protective in some neurodegenerative animal models, but overexpression in a *SOD1* ALS model was found to accelerate disease onset.^{32–34} Taken together, these studies suggest that a fine balance in p62 levels is required for optimal signaling and protein clearance. Consequently, small changes in

Figure 2 Association of the *SQSTM1* variant with age at onset of disease and survival



(A) The median and distribution of age at onset (years) of *SOD1* mutation-positive patients with ALS ($n = 167$) grouped by each *SQSTM1* variant genotype. (B) Kaplan-Meier survival curves of *SOD1* mutation-positive patients, comparing the *SQSTM1* genotypes assuming independent measurements. A robust log-rank test accounting for familial correlation was performed to assess any association between the groups. Survival was measured in months from ALS diagnosis until death. ALS = amyotrophic lateral sclerosis; *SOD1* = superoxide dismutase 1; *SQSTM1* = sequestosome 1.

the level of p62 expression, which may be produced by variants such as the *SQSTM1* intron 5 I/D, could tip the balance of p62 expression, contributing to the disease. Previously identified mutations within *SQSTM1* have been associated with ALS.^{35,36} As such, it has been suggested that these mutations may have a direct role in ALS pathogenesis, presenting as an important target for future therapy.⁸

In this study, we hypothesized that structural variations in *SQSTM1* may uncover novel susceptibility factors that underpin this disease. *SQSTM1* mutations, and p62

aggregation, have been reported not only in ALS but also in other complex neurodegenerative diseases, including Parkinson disease, Paget disease of bone, Alzheimer disease and frontotemporal dementia.^{8–13} A semiquantitative assessment of the mRNA expression revealed a difference in *SQSTM1* transcript levels in the ONS cells carrying each genotype. Neural tissue derived from olfactory mucosa, such as primary ONS cells, provide informative cellular models for neurodegenerative diseases. Primary ONS cells are neural progenitor cells and more accurately reflect motor neuron cellular function than fibroblasts and PBMCs, and unlike postmortem brain tissue, they can be isolated from living patients.^{37,38} As such, the altered *SQSTM1* transcript levels observed in the ONS cells with the I/I genotype may be reflective of altered *SQSTM1* expression in motor neurons. Although this result requires validation in a larger sample size, it does suggest some level of regulation by the SV on *SQSTM1* transcript expression. This may be due to altered transcription efficiency, splicing, or transcript stability, potentially translating to altered protein expression that may contribute to disease pathologies.

An evaluation of the *SQSTM1* variant within a cohort of 403 patients with ALS and 562 healthy age-matched controls revealed an association with fALS disease risk, but not age of disease onset or disease severity. Replications in additional fALS cohorts are necessary to truly elucidate the nature of this variant. Causality has been reported between ALS and *SQSTM1* variants rs796051870, rs776749939, rs796052214, and as such, the ongoing investigation between ALS and *SQSTM1* gene variants is necessary.^{8,39,40} P62 has previously been reported as overexpressed and accumulated in inclusions of sporadic inclusion body myositis, reinforcing its importance in neurodegenerative diseases.¹² We anticipate that as additional SVs are identified, these will further stratify other relevant disease phenotypes such as age at onset, duration, and disease progression.

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Disclosure

Disclosures available: [Neurology.org/NG](https://www.neurology.org/NG).

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Julia Pytte, BSc (Hons)	The Perron Institute for Neurological and Translational Science, Nedlands, Australia	Acquisition of the data, analysis and interpretation, statistical analysis, and critical revision of the manuscript for important intellectual content
Ryan S. Anderton, PhD	The Perron Institute for Neurological and Translational Science, Nedlands, Australia	Study concept and design, statistical analysis, study supervision, and critical revision of the manuscript for important intellectual content
Loren L. Flynn, PhD	Murdoch University, Murdoch, Australia	Study concept and design, analysis and interpretation, study supervision, and critical revision of the manuscript for important intellectual content
Frances Theunissen, MBIomedSc	The Perron Institute for Neurological and Translational Science, Nedlands, Australia	Analysis and interpretation and critical revision of the manuscript for important intellectual content
Leanne Jiang, BSc (Hons)	The Perron Institute for Neurological and Translational Science, Nedlands, Australia	Analysis and interpretation and critical revision of the manuscript for important intellectual content
Ianthe Pitout, PhD	The Perron Institute for Neurological and Translational Science, Nedlands, Australia	Study concept and design and acquisition of the data
Ian James, PhD	Murdoch University, Murdoch, Australia	Statistical analysis and critical revision of the manuscript for important intellectual content
Frank Mastaglia, MD	The Perron Institute for Neurological and Translational Science, Nedlands, Australia	Critical revision of the manuscript for important intellectual content
Ann M. Saunders, PhD	Duke University, Durham, NC	Study concept and design and critical revision of the manuscript for important intellectual content

Appendix (continued)

Name	Location	Contribution
Richard Bedlack, MD, PhD	Duke University, Durham, NC	Study concept and design, acquisition of the data, and critical revision of the manuscript for important intellectual content
Teepu Siddique, MD, DSc (hc), FAAN	Northwestern University, Evanston, IL	Study concept and design, acquisition of the data, and critical revision of the manuscript for important intellectual content
Nailah Siddique, RN, Msn	Northwestern University, Evanston, IL	Study concept and design and critical revision of the manuscript for important intellectual content
P. Anthony Akkari, PhD	The Perron Institute for Neurological and Translational Science, Crawley, Australia	Study concept and design, acquisition of data, analysis and interpretation, and critical revision of the manuscript for important intellectual content and study supervision

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