Association of a structural variant within the \textit{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 (\textit{SQSTM1}).

Methods
A candidate insertion/deletion variant within intron 5 of the \textit{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 \textit{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 \textit{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.
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.

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 ± SD where appropriate. Statistical differences in genotype proportions for independent cases were assessed using Pearson χ2 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).
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 (CAA) 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 genotypically and allelically ($p = 0.013$ and $p = 0.0036$, respectively, table 2). This SQSTM1 association was stronger when examining the subset of fALS patients carrying mutations in SOD1 ($\chi^2 = 16.754$, df = 2, $p = 0.002$, 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 = 76.40) genotypes vs. the I/I genotype. However, patients with the I allele had a significant shorter survival compared to those with the D allele ($\chi^2 = 76.40$, df = 2, $p < 0.0005$, OR = 1.830, 95% CI: 1.157, 2.896). Furthermore, patients with the I allele had a significantly lower expression of GAPDH compared to the D allele (Mean Rank = 76.40 vs. Mean Rank = 87.25).

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.
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. 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). 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. SVs may affect gene expression and therefore may play an important but understudied role in disease susceptibility. 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. The novel bioinformatics SV evaluation algorithm tool prioritizes potential functional/causal SVs within candidate regions identified using GWAS. 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 inclusions are found in virtually all forms of ALS and ALS-frontotemporal dementia. 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. SQSTM1/p62 mutations may confer a toxic gain of function through protein interactions, leading to dysregulation of cell signaling pathways, protein misfolding, and aggregation. Evidence for this in the literature suggests parallels between p62 and other proteins associated with neurodegeneration, including SOD1-linked ALS, C9orf72, ubiquilin 2, TDP-43, FUS, optineurin, beta-amyloid, a-synuclein, and tau. 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. Taken together, these studies suggest that a fine balance in p62 levels is required for optimal signaling and protein clearance. Consequently, small changes in

### Table 1 Characteristics of patients and healthy control participants

| Variable          | 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               |                   |

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

|        | sALS Control n (%) | sALS n (%) | p Value |
|--------|--------------------|------------|---------|
| **SQSTM1 CAAA variant** |                   |            |         |
| I/I    | 122 (21.7)         | 44 (21.3)  |         |
| I/D    | 292 (52.0)         | 109 (52.7) | 0.98    |
| D/D    | 148 (26.3)         | 54 (26.1)  |         |
| I allele | 536               | 197        |         |
| D allele | 588               | 217        | 0.97    |

|        | sALS Control n (%) | sALS n (%) | p Value |
|--------|--------------------|------------|---------|
| **Familial ALS** |                   |            |         |
| 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        |         |
| D allele | 588               | 165        | 0.0036  |

|        | sALS Control n (%) | SOD1 n (%) | p Value |
|--------|--------------------|------------|---------|
| **SQSTM1 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        |         |
| D allele | 588               | 133        | 0.0005  |

Abbreviations: ALS = amyotrophic lateral sclerosis; fALS = familial ALS; I/D = insertion/deletion; sALS = sporadic ALS; SQSTM1 = sequestosome 1.

* Each row compared using random effects to account for familial correlation.
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.8,13 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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Appendix
Authors

| Name                  | Location                                                                 | Contribution                                                                                       |
|-----------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|
| 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 |
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| 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 |
| Naijah Siddique, RN, Msn | Northwestern University, Evanston, IL                                      | Study concept and design, acquisition of the data, and critical revision of the manuscript for important intellectual content |
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