Six SQSTM1 mutations in a Chinese amyotrophic lateral sclerosis cohort

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Abstract
The purpose of this study was to identify SQSTM1 gene mutations, estimate survival based on the progression rate of the revised amyotrophic lateral sclerosis functional rating scale (ALSFRS-R) score (ΔFS), and characterize the relationships between SQSTM1 mutations and clinical phenotypes in Chinese ALS patients. We sequenced the SQSTM1 gene in 35 familial ALS patients, 436 sporadic ALS patients, and 384 healthy controls. SQSTM1 gene mutations were screened with PCR and direct sequencing; the correlations between genotype and phenotype and the progressive ALSFRS-R ratio were analyzed. Results revealed six heterozygous missense mutations in 471 ALS patients: c.241 G>A (p.E81K), c.717 C>A (p.N239K), c.889 G>A (p.G297S), c.1116 G>C (p.E372D), c.1162 C>T (p.P388S) and c.1175 C>T (p.P392 L). The gender ratio was 1:1, and the limb was the site of disease onset in mutation-positive patients. Notably, the ΔFS analysis revealed that the risk of death or tracheostomy was significantly increased in SQSTM1 mutation carriers (p<0.05).

In conclusion, E81K, N239K, G297S, E372D, P388S and P392 L were detected in the PB1, TRAF6, PEST and UBA domains, which are important to p62 function and prone to ALS. The incidence of ALS caused by the SQSTM1 mutation has increased from 30 to 35 worldwide.

Key words: Amyotrophic lateral sclerosis, SQSTM1 gene, mutation, clinical features

Introduction
Amyotrophic lateral sclerosis (ALS) is a syndrome of progressive deterioration involving the corticospinal tract, brainstem and anterior horn cells of the spinal cord, with more than 60% of patients dying within three years of presentation (1). Approximately 90% of ALS cases are clinically sporadic ALS (SALS) with unknown causes, while approximately 5–10% of ALS has genetically heterogeneous causes (familial ALS, FALS). Mutations in the Cu/Zn superoxide dismutase gene (SOD1) are well-known causes of ALS, accounting for approximately 20% of FALS and 1% of SALS cases (2,3). Approximately 8–10% of FALS cases reflect mutations in TARDBP and FUS/TLS genes. Expanded GGGGCC repeats in C9orf72 have been identified in Caucasian ALS cohorts, occurring in 21.7%–57.9% of FALS patients (4–6). However, lower frequencies of C9orf72 repeat expansions have been reported in eastern Asian ALS patients (7–10), and an undetermined percentage of mutations in CPTN, PGRN, UBQLN2, VCP, PFN1 and ARHGEF28 have also been reported (11–20).

The hallmarks of the pathology detected in spinal motor neurons of most ALS patients include ubiquitin, ubiquilin-2 (14), SOD-1 (21,22), TDP-43 (TAR DNA-binding protein 43) (23,24), FUS/TLS (12,13), neurofilament, peripherin and ubiquitin-binding protein p62 (25). p62 plays an important role in protein degradation via proteasomes and autophagy, which is observed in neuronal and glial ubiquitin-positive inclusions in Alzheimer’s disease, Parkinson’s disease, multiple-system atrophy and ALS (25–27). p62-knockout mice develop memory loss after
neurodegeneration caused by the accumulation of hyperphosphorylated tau and neurofibrillary tangles (28). The SQSTM1 gene encodes seques-
tosome 1/p62 (440 amino acids), which comprises several different domains including PB1, ZZ, TRAF6, PEST, and UBA, and which facilitate the scaffolding of this protein for the regulation of ubiquination (29). To maintain intracellular homeostasis, p62 participates in the degradation of protein aggregates and cytoplasmic bodies via selective autophagy through its PB1, LIR, and UBA domains (30). SQSTM1 has been screened as a candidate gene in eight studies of ALS patients (31–38).

The median survival of ALS patients from symptom onset ranges from two to four years, but the survival of individual ALS patients widely varies and is difficult to predict in individual cases. The revised amyotrophic lateral sclerosis functional rating scale (ALSFRS-R) is a sensitive tool for the evaluation of the functional status and disease progression in ALS patients, and it is widely used in clinical trials and clinical settings (39–41). We assessed how well survival time could be predicted by the ALSFRS-R score ratio (ΔFS), which is calculated as the change in the ALSFRS-R score between symptom onset and the date of diagnosis (42). We also considered the change in the ALSFRS-R score over the last three months (ΔFS recent), as this value is sensitive enough to analyze survival and can easily be applied in a busy multidisciplinary clinical setting (43). We sequenced the SQSTM1 gene in a large Chinese cohort with sporadic and familial ALS, recorded clinical information and the ALSFRS-R score ratio for the mutation-positive patients, and confirmed the increased frequency of the SQSTM1 mutation in ALS.

Methods

Participants

This study included 35 FALS cases (21 males, 14 females; male/female ratio, 1.5:1; mean age of onset ± standard deviation (SD) = 46 ± 11 years) and 436 SALS cases (277 males, 159 female; male/female ratio, 1.74:1; mean age of onset ± SD = 51 ± 12 years). Fourteen probands from 35 FALS patients with SOD1 mutations were observed, and the remaining patients had no mutations in SOD1, TAR-DDB, FUS/TLS or C9orf72. All patients were of Han ethnicity. The patients were registered with the Neurological Department of Peking University Third Hospital from 2011 to 2013. All the patients were diagnosed by board-certified neurologists and met the revised El Escorial criteria for the diagnosis of clinically definite, probable, or laboratory-supported probable ALS (44). Familial ALS was considered for probands with at least more than one first- or second-degree relative with ALS (45). We also studied 384 control subjects (214 males and 170 females; male/female ratio, 1.26:1; mean age of onset ± SD = 47 ± 17 years), and these participants were healthy with no previous personal or family history of neurodegenerative disease. All individuals lived in China and were of Han ethnicity. We followed all ALS patients every three months from the first visit to our hospital.

Approval and registration of standard protocols and patient consent

The study was approved by the institutional review board of Peking University Third Hospital. All individuals enrolled in this study gave written informed consent.

Sequencing analysis of SQSTM1

Genomic DNA was extracted from whole blood using standard protocols (QIAGEN, Valencia, California). The SQSTM1 gene has eight exons, and we designed six intronic primers, covering all eight exons, the intron-exon boundaries, and the promoter region (2 kb upstream from the coding sequence) of the SQSTM1 gene (Table I) (46).

PCR was performed in a final volume of 30 μl, containing 20 ng of genomic DNA, 10 pmol of each primer, and 2×Taq PCR Master Mix (QIAGEN). PCR conditions were as follows: initial denaturation

| Exon    | Primer Sequence                                      |
|---------|------------------------------------------------------|
| Exon 1  | p62e1F: 5’-TCTGCAGGGCGCTCTCGCGCCGCA-3’               |
|         | p62e1R: 5’-CAGGGCCGCTCCCATGCGCGAAGCTG-3’              |
| Exon 2  | p62e2F: 5’-GGGCTGTGAGGTGTCCTTTACAT-3’                 |
|         | p62e2R: 5’-CACCTGGGCTATGTCTCTGTTAA-3’                 |
| Exon 3–4| p62e3F: 5’-CTCACCTAATTGCGCTGAAATTTGTTTG-3’           |
|         | p62e3R: 5’-GGTGGGGGTTATCTGTGAAATTCTCTTT-3’            |
| Exon 5  | p62e5F: 5’-GACAGAGTGTCCGGGTAAAAGGTCGTA-3’             |
|         | p62e5R: 5’-AGGCCAACAAAACCTCCGAGCTGTTCA-3’             |
| Exon 6–7| p62e6F: 5’-ATCCACACGCGTGAGTCGGC-3’                    |
|         | p62e7R: 5’-CTGCAATTACGACAGTCTGTTACCCGCTG-3’           |
| Exon 8  | p62e8F: 5’-AGCTCTGGGCAAGCTCGGACACTG-3’                |
|         | p62e8R: 5’-AGGCGTCAGGAGCGGCGAAGCTG-3’                 |
at 95°C for 5 min, followed by 35 cycles at 95°C for 45 s, 55°C for 40 s, and 72°C for 1 min, and a final elongation at 72°C for 5 min. The PCR products were purified and sequenced at the Tsingke Biotechnology Co., Ltd. (Beijing, China). The forward primer was used for mutation screening, and all mutations were confirmed by reverse sequencing. Sequences were analyzed by DNASTAR Lasergene v7.1. The identified variants were examined using the Short Genetic Variations Database (dbSNP), the Exome Variant Server, and the 1000 Genomes Project. Moreover, the SOD1, TARDBP, FUS/TLS and C9orf72 gene sequences from all ALS patients were examined.

**Software analysis**

A multiple protein alignment was constructed using the HomoloGene site (http://www.ncbi.nlm.nih.gov/homologene/). The PolyPhen 2 (http://genetics.bwh.harvard.edu/pph2/index.shtml), SIFT and PROVEAN (http://sift.bi.a-star.edu.sg/) programs were used to predict the effects on protein structure or function.

**Results**

**Genetic analysis**

We analyzed the SQSTM1 gene in a total of 855 subjects. All subjects were from mainland China. A total of six missense mutations in SQSTM1 coding regions (isoform 1 of NM_003900.4) from six sporadic ALS patients were identified (Table II, Figure 2 a–f), but no mutations in the promoter region were detected. The rate of mutations in SALS was 1.38% (6/436) and no mutations were detected in the FALS and control cohorts. The six mutations were E81K, N239K, G297S, E372D, P388S, and P392L, which have all been defined as rare variants with frequencies of less than 1.0% (47). Four novel missense mutations (N239K, G297S, E372D, P388S) were identified in ALS patients. The missense mutations N239K, G297S, E372D and P388S were not in the dbSNP Short Genetic Variations or expression sequence tag (EST) database of the National Center for Biotechnology Information (NCBI). As shown in Figure 1, p62 is highly conserved in mammals. E81K, N239K, E372D, P388S and P392L were located in conserved regions of p62. G297S was located in a semiconserved region of p62. According to software analysis, the six mutations were potentially pathogenic.

**Patient clinical information**

Six sporadic ALS patients carrying SQSTM1 missense mutations (three female and three male) experienced ALS onset in middle age (39–65 years of age), with a limb as the site of symptom onset, and the disease duration was 11–45 months at the time of the investigation. All patients denied Paget’s disease of bone (PDB) and dementia (Table III). The clinical
features, neurologic examinations and electromyography results of these six ALS SQSTM1 mutation carriers can be found in the online supplementary clinical data to be found online at http://informahealthcare.com/doi/abs/10.3109/21678421.2015.1009466.

Survival analysis

We predicted ALS progression by $\Delta$FS. Four hundred and seventy-one ALS patients were divided into two groups: six SQSTM1 mutation carriers and 465 non-SQSTM1 mutation carriers. We analyzed $\Delta$FS

Table III. Clinical characteristics of patients with the SQSTM1 mutation.

| Mutation | Gender | Age at onset (years) | Site of symptom onset | Duration of disease (months) | Delay to diagnosis (months) | ALSFRS-R First visit | ALSFRS-R Current | $\Delta$FS | $\Delta$FS* | PDB | Dementia |
|----------|--------|----------------------|-----------------------|-----------------------------|---------------------------|---------------------|-----------------|----------|----------|-----|---------|
| E81K     | Female | 58                   | Limb                 | 24                          | 7                         | 42                  | 1.14            | 21                   | 1         | No     | No   |
| N239K    | Female | 39                   | Limb                 | 45                          | 22                        | 37                  | 0.5             | 23                   | 0.67      | No     | No   |
| G297S    | Male   | 59                   | Limb                 | 11                          | 6                         | 43                  | 0.83            | 40                   | 0.67      | No     | No   |
| E372D    | Female | 38                   | Limb                 | 26                          | 11                        | 32                  | 1.45            | Death                | —         | No     | No   |
| P388S    | Male   | 65                   | Limb                 | 32                          | 15                        | 44                  | 0.27            | 28                   | 1         | No     | No   |
| P392L    | Male   | 38                   | Limb                 | 36                          | 8                         | 44                  | 0.5             | 15                   | 1         | No     | No   |

PDB: Paget’s disease of bone; ALSFRS-R: revised amyotrophic lateral sclerosis functional rating scale; $\Delta$FS (progression rate of ALSFRS-R): 48-ALSFRS-R score at first visit/time between first symptom and first examination in months; $\Delta$FS* (the ALSFRS-R score ratio in recent three months) = ratio of ALSFRS-R score between two visits three months apart.
in these two groups with the SPSS 20.0 software and used the Mann-Whitney U-test. The median (25th, 75th percentiles) ΔFS in SQSTM1 mutation carriers was 0.66 (0.27–1.45), and the median ΔFS in non-SQSTM1 mutation carriers was 0.40 (0.00–1.10). The results showed a statistically significant difference between the SQSTM1 mutation carriers group and non-SQSTM1 mutation carriers group (z = –2.024, p = 0.043).

Discussion

Our SQSTM1 genetic analysis identified mutations in Chinese ALS patients. The six mutations corresponded to a frequency of 1.38% (6/436) in SALS, which is much lower than the frequencies previously reported in Caucasian and Japanese SALS patients, but higher than that reported in previous studies on Chinese Han SALS (31–38) patients. We did not observe SQSTM1 mutations in the 35 FALS patients, but 1.76% and 1.11% mutation frequencies have been reported for North American and French FALS patients, respectively, potentially reflecting the different genetic backgrounds and small sample sizes (31,33). The SALS patient sample size in our study was significantly larger than that in other studies, and the Chinese Han ethnicity may have contributed to the lower SQSTM1 mutation frequency.

The SQSTM1 mutations in our study had low frequencies compared with other genes involved in ALS, such as SOD1, TARDBP, FUS/TLS and C9orf72. We offer several lines of evidence suggesting that the variants found in our ALS cohort may be pathogenic. First, these variants were not present in the control cohort (768 chromosomes), the dbSNP Short Genetic Variations database, or the 1000 Genomes Project database. Secondly, the variants were detected in the PB1, TRAF6, PEST and UBA domains, which have a high level of evolutionary conservation. Thirdly, we evaluated the risk of death or tracheostomy based on ΔFS. Finally, the clinical syndrome obviously worsened during follow-up.

The range of age at clinical syndrome onset in ALS patients with SQSTM1 mutations widely varied, from 38 to 65 years. The gender ratio was 1:1. Further analysis revealed that ALS syndrome typically occurred in the limbs, with weakness and atrophy. At the first visit, ΔFS ranged from 0.27 to 1.45, and when the disease involved bulbar symptoms ΔFS increased, with a high risk of death or tracheostomy. The survival analysis demonstrated that ALS patients with the SQSTM1 mutation have increased risk of death or tracheostomy. Because the sample size of the SQSTM1 mutation group was very small, more data will be needed in the future.

SQSTM1 mutations have been associated with PDB. Currently, six mutations (A381V, P387L, A390X, P392L, G411S, G425R) are known to be common to ALS and PDB (48). In the present study we observed no family or personal history of PDB in the ALS and control cohorts.

SQSTM1 mutations might confer both loss of function and gain of function through novel protein interactions and the subsequent deregulation of cell signaling pathways. The PB1 domain is a crucial evolutionarily conserved dimerization/oligomerization domain that organizes homodimers and heterodimers. This domain interacts with liposomes and proteasomal Rpt-1 protein, and it triggers the degradation of ubiquitinated p62 cargo proteins via the 26S proteasome complex in neurons (49).

Patient 1 was a SQSTM1 E81K carrier, and the p. E81K substitution occurs in the PB1 domain. Clinically, patient 1 had upper motor neuron (UMN) and lower motor neuron (LMN) dysfunction in the bulbar, cervical, thoracic and lumbar regions. The PolyPhen 2, SIFT, and PROVEAN software programs demonstrate that the mutation is deleterious on the structure and function of p62. Thus, SQSTM1 E81K might be causative for ALS. The p.N239K mutation is in the binding site of TRAF6 domain, which mediates p62 binding to TRAF6 by E3 ubiquitin ligase, and E3 ubiquitin ligase triggers protein polyubiquitination via Keap-1 (50,51). The software programs demonstrated that p.N239K is deleterious to p62, and patient 2 displayed UMN and LMN damage in the cervical, thoracic and lumbar regions. Thus, we predicted that p.N239K might decrease the interaction between p62 and Keap-1, thereby inhibiting early autophagosome formation and the subsequent autophagic protein clearance in neurons. The SQSTM1 G297S carrier was patient 3, who displayed UMN and LMN damage in cervical and lumbar regions, and this residue is close to PEST1. Because of disease deterioration, we predicted that p.G297S might influence p62 function in neurons, but its mechanism is unknown. Patient 4 was a SQSTM1 E372D carrier, which was much higher compared with other patients with SQSTM1 mutations. The syndrome rapidly progressed, and the duration of disease was 26 months. Patient 4 had UMN and LMN damage in the bulbar, cervical and lumbar regions. The p.E372D mutation occurred in the PEST domain, which functions as a proteolytic signal for rapid degradation and reduces the intracellular half-life of proteins (52). p.E372D might not lead to proteolytic signals for degradation and cannot regulate the target of caspases in neurons. The common syndrome of patients 5 and 6 is UMN and LMN dysfunction in the bulbar, cervical, thoracic and lumbar regions. p.P388S and p.P392L are located in the UBA domain, which binds and transports polyubiquitinated proteins for proteasomal degradation. The p.P388S mutation might affect an apparently unstructured region at the N-terminus of the UBA domain (Figure 3) (53). UBA domain mutations may affect the binding of monoubiquitin or polyubiquitin chains, resulting in the dysregulation of ubiquitin-mediated processes.
and the accumulation of ubiquitin-positive protein aggregates in neuronal cells (54).

In summary, we found new mutations in the SQSTM1 gene in ALS patients of Chinese origin and analyzed mutation-survival risks using clinical characteristics. Our findings provide an overview of the occurrence of SQSTM1 mutations in China. Interestingly, the frequencies of these mutations in Chinese patients in the present study were different from other studies. Thus, further screening and functional studies of these mutations are needed to confirm their involvement in ALS.

Acknowledgements

This study was supported by a grant from the National Natural Sciences Foundation of China.

Declaration of interest: The authors report no conflicts of interest.

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Supplementary clinical data.