H63D CG genotype of \textit{HFE} is associated with increased risk of sporadic amyotrophic lateral sclerosis in a single population

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This paper describes the genetic etiology of sporadic amyotrophic lateral sclerosis in a single population. Polymerase chain reaction-restriction fragment length polymorphism and DNA sample sequencing of 3 common \textit{HFE} gene variants (C282Y and H63D and S65C) were performed on 10 randomly selected samples of H63D gene variant (124 patients with sporadic amyotrophic lateral sclerosis) and 10 wild types of H63D samples (210 controls). The C282Y and S65C gene variant were absent. There were 24 cases (7.18%) with H63D heterozygous variants, including 16 cases (13%) in the sporadic amyotrophic lateral sclerosis group and 8 cases (4%) in the healthy control group. The polymorphism frequency of the H63D gene variant in the sporadic amyotrophic lateral sclerosis group was significantly different than that in the control group ($P < 0.05$), and the difference at allele level, which is still more significant ($P < 0.05$). H63D gene variant could be a risk factor for sporadic amyotrophic lateral sclerosis in a single population. The results showed \textit{HFE} gene variants play a role in the occurrence of sporadic amyotrophic lateral sclerosis, but its effect should be carefully estimated.

Keywords
Neurogenetics; amyotrophic lateral sclerosis; motor neuron disease; \textit{HFE} gene; polymorphism

1. Introduction

Amyotrophic lateral sclerosis (ALS) is an idiopathic and fatal neurodegenerative disease of the human motor system. Like other neurodegenerative diseases, about 10% of ALS is classified as familial diseases, while the remaining 90% are considered sporadic because they appear to occur randomly throughout the community (Rowland and Shneider, 2001). Further study on the genetic etiology of ALS provides basic insights into the cellular mechanism of neuronal degeneration, and also contributes to the design and testing of disease modeling and targeted therapy. Therefore, it is not surprising that a large number of resources have been used to search for pathogenic variants. Sporadic ALS is suspected to be associated with genetic susceptibility to environmental risk factors (Talbott et al., 2016).

\textit{SOD1} is the first gene found to cause ALS (Rosen, 1993). With the rapid development of technology, gene discovery has been dramatically accelerated. Some new ALS genes have also been found, including \textit{TARDBP} (Sreedharan et al., 2008), \textit{ profilin 1} (PFN1) gene (Wu et al., 2012), \textit{C9orf72} (Renton et al., 2011), \textit{valosin-containing protein} (VCP) gene (Johnson et al., 2010), \textit{UBQLN2} (Deng et al., 2011), and other genes.

The existence of the \textit{SOD1} variant leads to the theory of the role of oxidative stress in the pathogenesis of the disease. One of the leading causes of oxidative stress is the disorder of cellular iron homeostasis. Improper cell iron management and oxidative stress are associated with many neurodegenerative diseases. One of the mechanisms by which cells cannot regulate iron status properly is through variants in the \textit{HFE} gene (Pietrangelo, 2004). It was found that H63D variant cells had the phenotype of promoting glutamate toxicity (Mitchell et al., 2011), which was one of the causes of ALS. The p. His 63 Asp polymorphism of the \textit{HFE} gene (c.187C $> G$, rs1799945) was found to accelerate the disease progression of \textit{SOD1} transgenic mouse (Nandar et al., 2014). The H63D variant in the \textit{HFE} gene has been found to increase the risk of ALS in several populations (Eun et al., 2015; He et al., 2011; Sutedja et al., 2007), but the conclusion was inconsistent (Chiò et al., 2015; Li et al., 2014; Praline et al., 2012).

In this paper, we determine the relationship between the C282Y (p.Cys 282 Tyr, A at rs1800562 instead of G), H63D (p. His 63 Asp, G at rs1799945 instead of C), and S65C (p.Ser65Cys, T at i3002468 instead of A) variants in \textit{HFE} gene (chromosome 6p21.3) and the risk of sporadic ALS (SALS) in a single population.
### Table 1. The basic information on genotyping assays of HFE

| Polymorphism | Primer forward | Restriction enzyme | PCR products | Base change | Digestion product | Mutations |
|--------------|----------------|-------------------|--------------|-------------|-------------------|-----------|
| C282Y        | 5'-TGGCAAGGGTAAACAGATCC-3' (forward) | RsaI             | 387bp        | G→A        | 247bp             | Wild      |
|              | 5'-CTCAGGCACTCTCTCAACC-3' (reverse) |                  | 140bp        |            | 111bp             | Homozygous|
| S65C         | 5'-ACATGTAAAGGCCTGTTCG-3' (forward) | HinfI             | 208bp        | A→T        | 147bp             | Homozygous|
|              | 5'-GCCACATCTGGCTTGAAATT-3' (reverse) |                  | 61bp         |            | 147bp             | Heterozygous|
| H63D         | 5'-ACATGTAAAGGCCTGTTCG-3' (forward) | MboI              | 208bp        | C→G        | 138bp             | Heterozygous|
|              | 5'-GCCACATCTGGCTTGAAATT-3' (reverse) |                  | 70bp         |            | 138bp             |           |

### Table 2. Demographic characteristic of the study populations

| Variable                  | Patients n = 124 | Controls n = 210 | P-Value |
|---------------------------|------------------|------------------|---------|
| Male, n                   | 69               | 133              | 0.165a  |
| Female, n                 | 55               | 77               |         |
| Mean age (SD), y          | 50.00 (10.09)    | 45.02 (10.64)    | 0.66b   |
| Spinal onset, n           | 103              |                  |         |
| Bulbar onset, n           | 21               |                  |         |
| Symptom duration 36m      | 98               |                  |         |
| Symptom duration > 36m    | 26               |                  |         |
| Age at disease onset ≤ 45y| 48               |                  |         |
| Age at disease onset > 45y| 76               |                  |         |

Note: a: P-value for Chi-square test; b: P-value for t-test.

### 2. Materials and methods

#### 2.1 Patients population

One hundred twenty-four patients (69 males and 55 females) with SALS were recruited from the Second Hospital of Hebei Medical University, the Peking University Third Hospital, the West China Hospital, Sichuan University, and the Third Hospital of Hebei Medical University. Patients met the El Escorial criteria for definite, probable, or possible ALS (de et al., 2008). Two hundred ten healthy people matched by age, gender, and nationality were included as the control group. All patients and controls were of Han ethnicity. The ethics committee approved the research plan of the second hospital of Hebei Medical University. All individuals gave written informed consent.

#### 2.2 Genotyping

Venous blood (5 ml) was collected from patients and healthy subjects, anticoagulated with sodium citrate, and stored in a refrigerator at 4 °C. DNA was extracted according to the instructions of the blood genomic DNA extraction kit within 1 week after blood collection. The DNA of the final product was stored in a refrigerator at -80 °C before use. The genotypes of C282Y, S65C, and H63D, were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and the necessary information of HFE polymorphism genotyping was shown in Table 1. 10 samples of H63D variant and 10 wild type H63D samples were randomly selected for PCR amplification and DNA sequencing.

### 2.3 Statistical analysis

SPSS 21 version software was used for statistical processing. \( \chi^2 \) test was used to analyze the frequency distribution of genotypes between the healthy control group and the SALS group. Hardy-Weinberg equilibrium analysis was performed. \( T \)-test was used for the age difference between the SALS group and the healthy control group, and the \( \chi^2 \) test was used for gender difference. The odds ratio (OR) and the 95% confidence interval (CI) of the relative risk were calculated by the unconditional logistic regression method.

### 3. Results

The baseline characteristic was shown in Table 2. There was no statistical difference in age and gender between the control group and the patient group \(( P > 0.05)\), so a further comparison can be made. The frequency of the H63D allele was in Hardy-Weinberg equilibrium in the patient and control group, respectively \(( P > 0.05)\) (Table 3). There were 24 cases (7.18%) with H63D heterozygous variants, including 16 cases (13%) in the SALS group and 8 cases (4%) in the healthy control group.

The polymorphism frequency of the H63D variant in the SALS group was significantly different from that in the control group \(( P < 0.05)\). The frequency of CG genotype \(( c.187 C > G)\) in the SALS group was significantly higher than that in the control group \(( P < 0.05)\), and the difference at allele level, which was still more significant \(( P < 0.05)\) (Table 4). Non-conditioned logistic regression test showed that the heterozygous variant (CG genotype) of H63D was independently associated with an increased risk of developing SALS \(( \text{odds ratio} 3.74, \text{95\% CI} 1.55-9.02, P = 0.003)\).

The frequencies of C282Y (GG GA AA) and S65C (AA AT TT) sites of HFE gene in the ALS group and healthy control group were 100%, 0%, 0% and 100%, 0%, 0%, respectively, indicating that C282Y and S65C variants were absent in our study. The RFLP results of negative samples showed that there was no C→G variant. The results of positive samples showed that there was a C→G variant, which was heterozygous.
The finding of increased oxidative stress in the brain disrupts iron homeostasis in the brain. It is associated with the H67D variant of the HFE gene (homologous to H63D in humans) in animal experiments. In animal models, the presence of the HFE gene and ALS is still controversial. There is strong evidence that oxidative stress and plays a vital role in the homeostasis disorder and the pathogenesis of ALS. Although the relationship between homeostasis disorder and oxidative stress and plays a vital role in the development of SALS. The underlying mechanism is that disruption of cholesterol metabolism (Ali-Rahmani et al., 2014). Changes in iron homeostasis and increased oxidative stress were also observed at the cellular level (Lee et al., 2007), and increase glutamate release (Mitchell et al., 2011) and endoplasmic reticulum pressure (Liu et al., 2011). Each of the above mechanisms is considered to be the promoter of ALS pathogenesis (Rothstein, 2009). Therefore, the data suggests that H63D-HFE is a genetic modifier of ALS risk.

Nandar et al. (2014) established a double transgenic mouse line carrying the H67D variant of the HFE gene (homologous with human H63D) and SOD1 (G93A) variant. They found that the H67D variant of the HFE gene can double the survival time and course of disease of transgenic mice. The underlying mechanism is that the H63D variant of the HFE gene changes the pathophysiology of ALS through oxidative stress, gliosis, and cell dysfunction (Rothstein, 2009). Our results suggest that the H63D variant plays a role in the development of SALS. Still, we need to be cautious in extrapolating different geographical regions, as further independent HFE variant association studies are needed.

4. Discussion

We found that the H63D variant was associated with SALS, which is consistent with previous studies (Wang et al., 2004). Our results are consistent with findings in different populations (Eum et al., 2015; He et al., 2011; Sutedja et al., 2007). Compared with the finding of He et al. (2011), we used different amplification primers and different fragment lengths. We used the course of the disease as the stratification factor for statistical analysis. The patient group in this study had different data sources, and the conclusion was complementary to He et al. (2011), which further expands the population data. The results of Li et al. (2014) and Van Rheenen et al. (2013) showed the opposite conclusions and confirmed that the H63D variant was not associated with ALS risk. The difference in the genetic background in ethnicity of the population could explain the difference of experimental results.

SALS was reported to be associated with the H63D variant in Dutch (Eum et al., 2015), and the H63D variant was associated with a higher age of onset. The mean age of onset in Chinese ALS patients was 49.8 to 54.3 years old in hospital-based studies (Chen et al., 2015; Liu et al., 2014, 2019, 2018; Rosenbohm et al., 2018; Wei et al., 2015), indicating that the onset age of ALS patients in China is earlier than that in other countries. The average age of the experimental subjects was 50.00 ± 10.09 years, which was consistent with the other studies. It has been suggested that the age of onset was younger in less developed regions with shorter life expectancy. Older and more severe patients were less likely to be referred to as regional or national referral centers, which partially explained the younger age of onset recorded. Another potential contributing factor was the higher level of environmental pollution in less developed areas (Liu et al., 2018).

The H63D variant in the HFE gene is associated with iron homeostasis disorder and oxidative stress and plays a vital role in the pathogenesis of ALS. Although the relationship between the HFE gene and ALS is still controversial, there is strong evidence in animal experiments. In animal models, the presence of H67D variant of the HFE gene (homologous to H63D in humans) disrupts iron homeostasis in the brain. It is associated with increased oxidative stress in the brain (Nandar et al., 2013) and severe changes in iron homeostasis and increased oxidative stress were observed at the cellular level (Lee et al., 2007), and increase glutamate release (Mitchell et al., 2011) and endoplasmic reticulum pressure (Liu et al., 2011). Each of the above mechanisms is considered to be the promoter of ALS pathogenesis (Rothstein, 2009). Therefore, the data suggests that H63D-HFE is a genetic modifier of ALS risk.

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5. Conclusions

The results suggest that the H63D variant plays a role in the development of SALS. Still, we need to be cautious in extrapolating different geographical regions, as further independent HFE variant association studies are needed.

Ethics approval and consent to participate

The ethics committee approved the research plan of the second hospital of Hebei Medical University. All individuals gave written informed consent.
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Conflict of Interest

The authors promise that there is no conflict of interest in this study.

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