Serum ferritin is a candidate biomarker of disease aggravation in amyotrophic lateral sclerosis

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Abstract. Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease. The mechanism that defines the loss of neurons in ALS is still not clearly understood, and there is no effective therapy to block its progression. Previous studies indicate that a disorder of iron homeostasis exists in ALS and based on this, the change of serum iron and ferritin and the association between iron metabolism and clinical features in Chinese Han patients with ALS was further investigated in the present study, in order to define its pathogenesis. Two cohorts were established: An ALS group consisting of 24 patients and a control group consisting of 38 healthy volunteers. Venous blood samples were collected for serum iron and ferritin analysis. The results indicated that the levels of serum iron were significantly decreased in patients with ALS (P<0.05), while there was no significant difference in serum ferritin between the ALS and control groups. However, the levels of serum ferritin were increased significantly in ALS patients with bulbar-onset (vs. limb-onset in females), dysphagia (vs. without dysphagia), longer disease duration (>12 months vs. ≤12 months in males) and lower ALS Functional Rating Scale-Revised score (<33 vs. ≥33; P<0.05). These results suggested that there was dysregulation of iron metabolism in Chinese Han patients with ALS and that serum ferritin may be a candidate biomarker of aggravation in these patients.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive, lethal disease characterized by degeneration of motor neurons in the cortex, brainstem and spinal cord (1,2). ALS is among the most common types of neurodegenerative disorder, with an incidence of approximately 2-3/100,000 individuals in Europe (3,4). The majority of cases of ALS are sporadic, while 5-10% are familial (5,6). With the current therapy (riluzole, 100 mg/day), the typical survival time for patients is 3-5 years following diagnosis (7).

Previous studies have indicated that the loss of neurons in ALS results from a complex interplay among oxidative injury, excitotoxic stimulation, aggregation and/or dysfunction of critical proteins, and genetic factors (8-12). However, to the best of our knowledge, the mechanism that defines the loss of neurons in ALS is not clearly understood. Further studies have demonstrated the following: A disorder of iron homeostasis exists in ALS (13-17); there is increased iron in the spinal cord of patients with ALS (18,19); the iron level in the cerebrospinal fluid of patients with ALS was higher than in control subjects (20,21); and serum ferritin levels were increased in patients with ALS (22-26). However, it remains unconfirmed whether a high level of ferritin is specific to ALS. A number of studies have reported that patients with elevated serum ferritin have a shorter survival time (25); however, another study concluded that ferritin levels are unrelated to the survival of patients with ALS (26).

In the present study, the levels of serum iron and ferritin in Chinese Han patients with ALS and control subjects were measured, and the association between iron metabolism and clinical features was studied in order to implicate iron metabolism in Chinese Han patients with ALS.

Materials and methods

Subjects. Two cohorts were established as follows: An ALS group consisting of 24 patients, 14 males and 10 females (42-69 years old; mean, 51.96±7.57 years), all diagnosed with ALS according to El Escorial criteria (27); and a control group consisting of 38 healthy volunteers, 28 males and 10 females (50-62 years old; mean, 53.32±3.28 years), recruited from the local community. Participants who had iron deficiency anemia, acute inflammation, a history of chronic kidney disease, neurologic or psychiatric diseases, or neurologic sequelae induced by brain trauma were excluded. All subjects were of Chinese Han ethnicity. All blood samples were collected for analysis

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of serum iron and ferritin, and then serum iron and ferritin was compared between those patients with limb-onset and bulbar-onset, with dysphagia and without dysphagia, disease duration ≤12 months and >12 months, and ALS Functional Rating Scale-Revised (ALSFRS-R) score <33 points and ≥33 points. The ALSFRS-R is among the most frequently used scales in the neurological and functional assessment of patients with ALS, and survival time is reportedly shorter in ALS patients with lower ALSFRS-R scores (28). The range of ALSFRS-R scores was 20-42 with a mean 33.04±6.89 in the present study, and therefore patients with an ALSFRS-R score <33 points were separated from those with a score ≥33 points for analysis, as in previous studies (28,29).

Each subject that agreed to enroll in the study reviewed and signed an informed consent form prior to their participation. The study protocol and consent forms were approved by the Medical Ethics Committee of Linyi People's Hospital, Linyi, China.

Measurement of serum iron. Venous blood samples (5 ml) were collected at Linyi People's Hospital (Linyi, China) between 2014 and 2016, and placed upright for 2 h for clotting at room temperature. Then, the samples were centrifuged at 3,000 x g for 15 min, and the supernatant serum was collected for analysis of serum iron and ferritin. Subjects did not have a fever or other signs of inflammation when the blood samples were taken. Iron reagent kit (catalogue no. 467910) was purchased from Beckman Coulter, Inc. and the iron assays were performed on a UniCel DxC 800 Synchron Clinical System (both from Beckman Coulter, Inc., Brea, CA, USA). Iron concentration was measured by an end-point method. In brief, iron is released from transferrin by decomposition of acetic acid and then reduced to ferrous ion by hydroxylamine and sulfhydryl acetate, which combines with zinc-iron by complexation. The proper quantities of samples and reagents were automatically distributed in proportion to a reaction cup by the Synchron System. The proportion of samples to reagents was 1:8. The absorbance at 560 nm was then measured; the change of this absorption is directly proportional to the iron concentration of the samples. Subsequently, the system calculated the iron concentration. All tests were conducted according to the manufacturer's instructions and the standard operating procedures at the Biochemistry Department of Linyi People's Hospital. The reference range of serum iron was 8.1-32.6 μmol/l.

Measurement of serum ferritin. Ferritin detection kit (catalogue no.: 04491785) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Ferritin was measured on a Roche Cobas E601 Immunology analyzer (Roche Diagnostics GmbH). Serum samples (10 μl) were reacted with the biotin monoclonal specific antibody of ferritin, and the specific monoclonal antibody of ferritin was tagged with the ruthenium complex to form sandwich complexes. Following the addition of particles coated with streptavidin, the complexes combined with them to form a solid phase through interaction between biotin and streptavidin. The reaction mixture was placed in a measuring cell, and particles were adsorbed onto the surface of the electrode by magnetism. Procell/Procell M (catalogue

no. 04880340190; Roche Diagnostics GmbH) was used to remove the uncombined substances. Chemiluminescence was produced by applying voltage to the electrode, which was then measured by a photomultiplier. Results were calculated using a calibration curve, which was produced by a two-point calibration and master curve (provided by the reagent barcode). All tests were conducted according to the manufacturer's instructions and standard operating procedures at the Immunology Department of Linyi People's Hospital. The reference ranges of serum ferritin were as follows: 30-400 ng/ml for males and 13-150 ng/ml for females.

Statistical analysis. Results are expressed as the mean ± standard deviation. Statistical analyses were performed using the unpaired t-test and Wilcoxon rank-sum test, depending on the normality of the data. The rank-sum test was used when comparing serum ferritin between male control and ALS patients, and the unpaired Student's t-test was used to analyze all other datasets. Difference was considered to be significant at P<0.05. Statistical analysis was performed using the SAS System for Windows v8 (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics. The patients with ALS in the current study included 10 definite ALS and 14 probable ALS diagnoses. A total of 16 patients exhibited signs of limb-onset ALS and 8 patients signs of bulbar-onset ALS. Dysphagia symptoms were apparent in 17 patients (71%) when collecting blood samples. All patients had sporadic ALS. Table I presents the clinical characteristics of the included patients. No significant difference in age was identified between the ALS patients and control subjects (P=0.4129).

Serum iron and ferritin in patients with ALS and the control group. As illustrated in Table II, the serum iron of the ALS group was significantly lower than that of the control group (P=0.0074), while there was no significant difference in serum ferritin between the groups (male, P=0.3504; female, P=0.1068; Table III). However, the mean levels of serum ferritin in the ALS group were higher than those in the control group for both male and female patients; therefore, it was indicated that serum ferritin was increased in patients with ALS.

Serum iron and ferritin in patients with limb-onset and bulbar-onset ALS. When comparing the serum iron and ferritin levels between patients with limb-onset and bulbar-onset ALS, no significant difference in serum iron was identified (P=0.0586; Table IV). There was also no significant difference in the levels of serum ferritin between male patients with limb-onset and bulbar-onset ALS (P=0.2257; Table V); however, the serum ferritin levels of female patients with bulbar-onset ALS were significantly higher than those of female patients with limb-onset ALS (P=0.008; Table VI).

Serum iron and ferritin in ALS patients with and without dysphagia. On comparing the serum iron and ferritin between ALS patients with and without dysphagia, no significant difference in serum iron was observed (P=0.7659; Table IV).

Table I. Clinical characteristics of included ALS patients.

Patient	Sex	Age, years	Diagnosis	Onset site	Disease duration, months	ALSFRS-R score
1	Male	52	Definite	Bulbar-onset	36	25
2	Male	47	Probable	Limb-onset	12	37
3	Male	69	Probable	Limb-onset	30	23
4	Male	45	Definite	Limb-onset	24	31
5	Male	50	Probable	Bulbar-onset	16	39
6	Male	42	Probable	Limb-onset	24	22
7	Male	47	Probable	Limb-onset	24	35
8	Male	62	Definite	Limb-onset	12	41
9	Male	48	Definite	Limb-onset	18	40
10	Male	55	Probable	Limb-onset	12	36
11	Male	48	Probable	Limb-onset	24	36
12	Male	43	Probable	Limb-onset	7	42
13	Male	52	Probable	Bulbar-onset	6	37
14	Male	68	Definite	Bulbar-onset	12	39
15	Female	48	Probable	Limb-onset	30	31
16	Female	64	Definite	Limb-onset	10	20
17	Female	46	Definite	Limb-onset	12	25
18	Female	49	Probable	Bulbar-onset	30	22
19	Female	46	Definite	Bulbar-onset	24	30
20	Female	53	Definite	Bulbar-onset	24	31
21	Female	56	Probable	Limb-onset	28	35
22	Female	45	Probable	Limb-onset	12	36
23	Female	54	Probable	Bulbar-onset	12	39
24	Female	58	Definite	Limb-onset	17	41

ALS, amyotrophic lateral sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised.

Table II. Serum iron of ALS patients and controls.

Group	Number of subjects	Serum iron, μmol/l	P-value
Control	38	19.44±6.47	0.0074
ALS patients	24	14.95±5.77	

ALS, amyotrophic lateral sclerosis.

Table III. Serum ferritin of ALS patients and controls.

Group	Number of subjects	Serum ferritin, ng/ml	P-value
Male control	28	171.35±73.64	0.3504
Male ALS patients	14	221.95±138.20	
Female control	10	93.67±65.88	0.1068
Female ALS patients	10	157.86±99.76	

ALS, amyotrophic lateral sclerosis.

However, the serum ferritin of ALS patients with dysphagia was significantly higher than in those without dysphagia (male, P=0.0195; female, P=0.0357; Tables V and VI).

Serum iron and ferritin in patients with ALS of different disease duration. The serum iron and ferritin in patients with ALS of different disease durations (≤12 months and >12 months) were subsequently compared. No significant difference in serum iron in ALS cases of different disease duration was determined (P=0.3852; Table IV). Serum ferritin was increased significantly in male ALS cases with longer disease duration (P=0.0422; Table V); however, there was no significant difference in serum ferritin in female cases of ALS of different disease duration (P=0.2739; Table VI).

Serum iron and ferritin in ALS patients with different ALSFRS-R scores. By comparing the serum iron and ferritin of ALS patients with different ALSFRS-R scores, it was identified that the levels of serum iron in ALS patients with ALSFRS-R scores <33 points were lower compared with in those patients with ALSFRS-R scores ≥33 points (P=0.0155; Table IV). Additionally, the levels of serum ferritin were increased significantly in ALS patients with ALSFRS-R scores <33 points compared with in those patients with ALSFRS-R scores ≥33 points (male, P=0.0287; female, P=0.0474, Tables V and VI).

Table IV. Serum iron of ALS patients.

Disease characteristic	Number of subjects	Serum iron, μmol/l	P-value
Limb-onset	16	13.39±4.91	0.0586
Bulbar-onset	8	18.08±6.40	
Without dysphagia	7	14.39±4.30	0.7659
With dysphagia	17	15.18±6.38	
Disease duration ≤12 months	10	16.40±8.34	0.3852
Disease duration >12 months	14	13.91±2.81	
ALSFRS-R score <33	10	11.69±4.11	0.0155
ALSFRS-R score ≥33	14	17.28±5.76	

ALS, amyotrophic lateral sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised.

Table V. Serum ferritin of male ALS patients.

Disease characteristic	Number of subjects	Serum ferritin, ng/ml	P-value
Limb-onset	10	251.08±148.71	0.2257
Bulbar-onset	4	149.12±80.75	
Without dysphagia	4	125.68±33.96	0.0195
With dysphagia	10	260.46±146.41	
Disease duration ≤12 months	6	142.46±58.43	0.0422
Disease duration >12 months	8	281.57±153.47	
ALSFRS-R score <33	4	344.70±152.14	0.0287
ALSFRS-R score ≥33	10	172.85±102.45	

ALS, amyotrophic lateral sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised.

Table VI. Serum ferritin of female ALS patients.

Disease characteristic	Number of subjects	Serum ferritin, ng/ml	P-value
Limb-onset	6	97.71±59.42	0.0080
Bulbar-onset	4	248.08±76.69	
Without dysphagia	3	61.63±43.89	0.0357
With dysphagia	7	199.10±87.59	
Disease duration ≤12 months	4	113.40±73.93	0.2739
Disease duration >12 months	6	187.50±109.54	
ALSFRS-R score <33	6	207.11±93.09	0.0474
ALSFRS-R score ≥33	4	83.98±57.28	

ALS, amyotrophic lateral sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised.

Discussion

Iron is established to potentiate the toxic effects of reactive oxygen species by catalyzing the formation of highly reactive hydroxyl radicals from hydrogen peroxide through Fenton chemistry (30,31). Thus, while iron is essential for normal physiology (32), it is also implicated in many pathological processes, including neuron degenerative disorders (33,34).

Iron accelerates β-amyloid aggregation and promotes oxidative damage in Alzheimer's disease (35-37). A number of studies have indicated that iron is increased in both models of ALS and humans with ALS (38-41). Even though it is unclear whether oxidative stress is a primary or secondary cause of neurodegeneration in ALS, previous study has suggested that it is a major contributory factor that leads to chronic motor neuron death (42). Increased iron levels are possibly involved

in oxidative damage through the Fenton reaction in ALS, and iron chelation therapy may have potential in the prevention and treatment of ALS (43,44).

Symptoms of dysphagia, difficulty with self-feeding and fatigue with meals often afflict patients with ALS, making them susceptible to a high risk of malnutrition (45). Dysphagia affects up to 80% of patients with ALS (46), and thus enteral nutrition (EN) and parenteral nutrition (PN) are important for patients with ALS (45). The recommended daily intake of iron for healthy individuals is 10 mg/day, but the iron content of polymeric enteral formulas that are usually employed to meet the nutritional and metabolic needs of patients with ALS ranges from 13 to 24 mg/l (47). Many patients with ALS receive EN, which may exacerbate iron overload. Although previous studies do not report many patients receiving EN, iron overload exacerbated by EN may serve a role in the reported results (26).

In the present study, no significant difference between the ALS and control subjects was identified regarding serum ferritin, which is in contrast to the results of previous studies (22-26); however, the mean levels of serum ferritin in the ALS group were higher than those in the control group. The levels of serum iron in patients with ALS were significantly lower than those in the control group. The reason for this may be attributed to a number of external factors. Environmental factors, including diet, can affect iron homeostasis in the body. The majority of patients with ALS that were enrolled in the present study lived in their own home and could not get help from health workers, or EN or PN treatment, which could result in malnutrition that further influenced their levels of serum iron. By contrast, the Caucasian patients assessed previously lived in developed countries (26), and likely had greater access to treatments and adequate nutrition compared with patients in the present study, which may explain the significant increase in serum ferritin reported (26). Our previous study indicated that iron levels are increased in the motor cortex of patients with ALS by using susceptibility weighted imaging (48). The majority of the patients with ALS in the present study also participated in our aforementioned previous study. Therefore, it may be concluded that the decrease in serum iron levels does not affect the iron levels of the motor cortex in Chinese Han patients with ALS.

Further investigations were conducted in the present study to clarify the effect of onset site, dysphagia, disease duration and ALSFRS-R scores. The results showed that onset site, dysphagia and disease duration did not affect serum iron in patients with ALS. The levels of serum ferritin were increased significantly in male ALS patients with dysphagia, longer disease duration (>12 months) and lower ALSFRS-R scores (<33 points). Furthermore, the levels of serum ferritin were increased significantly in female ALS patients with bulbar-onset, dysphagia and lower ALSFRS-R scores. Intracellular ferritin may be upregulated in response to oxidative stress, and serum ferritin may be increased in patients with ALS due to oxidative stress (25). The results of the present study indicate that serum ferritin is a marker of aggravation in ALS. However, a limitation of this study was the relatively small sample group, and thus data from more patients should be collected for further studies; a further aim is to assess other molecules related to ALS in future study.

In conclusion, serum iron levels of patients with ALS were decreased significantly in the present study, while serum ferritin exhibited an increasing tendency. Furthermore, the levels of serum ferritin were increased significantly in ALS patients with bulbar-onset, dysphagia, longer disease duration and lower ALSFRS-R scores. Collectively the results indicate that dysregulation of iron metabolism exists in Chinese Han patients with ALS, and that serum ferritin may be a candidate biomarker of aggravation in ALS.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JY contributed to study design, to analysis and interpretation of the data, and to writing and revision of the manuscript. NW, YL and HZ performed the tests of serum iron and ferritin. FQ, XW and QZ prepared the blood samples and aided with the testing of serum iron and ferritin. FC and WL analyzed and interpreted the data, and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Medical Ethics Committee of Linyi People's Hospital (Linyi, China). Each subject that agreed to enroll in the study reviewed and signed an informed consent form prior to their participation.

Consent for publication

Written informed consent obtained from all subjects permitted the publication of their data for research purposes following anonymization.

Competing interests

The authors declare that they have no competing interests.

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