Original Article

Increased Expressions of Plasma Galectin-3 in Patients with Amyotrophic Lateral Sclerosis

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Abstract

Background: High expressions of galectin-3 were identified recently in the end stage of amyotrophic lateral sclerosis (ALS) patients, which suggested that immune reactivity and inflammatory mechanisms might play an important role in the pathogenesis of ALS. The purpose of this study was to investigate plasma galectin-3 levels in different groups and stages of ALS patients and the association with related clinical characteristics.

Methods: A total of 51 patients with ALS and 60 normal controls (NCs) were recruited in this study. Plasma galectin-3 levels were determined using the enzyme-linked immunosorbent assay. Patients with ALS were divided into several groups according to their clinical characteristics: gender, type of disease onset, duration of disease, and clinical conditions of disease. Statistical analyses of the differences of galectin-3 levels between groups and the association with the clinical characteristics of disease were performed.

Results: As compared with the NCs (201.64 [22.35–401.63] ng/ml), plasma galectin-3 levels were significantly elevated in the patients with duration >12 months (341.17 [69.12–859.22] ng/ml, $P < 0.05$), and the patients with limb onset of disease (254.14 [69.12–859.22] ng/ml, $P < 0.05$); however, no difference was found in the patients with duration ≤12 months (250.62 [109.77–334.92] ng/ml, $P > 0.05$), and the patients with bulbar onset of disease (251.79 [109.20–404.76] ng/ml, $P > 0.05$). In addition, galectin-3 levels were significantly increased in the female patients (263.27 [123.32–859.22] ng/ml, $P < 0.05$) while no difference was found in the male patients (220.39 [69.12–748.73] ng/ml, $P > 0.05$). The further statistical analyses showed that plasma galectin-3 levels were positively correlated with the duration of disease ($r = 0.293$, $P = 0.037$).

Conclusions: Plasma galectin-3 levels were significantly increased in ALS patients with limb onset of disease, especially in ALS female patients, and positively correlated with the duration of disease, which suggested that plasma galectin-3 might be an interesting and useful factor associated with ALS.

Key words: Amyotrophic Lateral Sclerosis; Galectin-3; Immune; Inflammatory

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and devastating neurodegenerative disease characterized by selective loss of motor neurons in the motor cortex, brain stem, and spinal cord.[1] The mechanisms underlying the loss of motor neurons are complex and have to be clarified yet. A variety of hypotheses on the pathogenesis of ALS have been proposed so far, such as apoptosis, excitotoxicity, immune reactivity, inflammatory mechanism, mitochondrial dysfunction, protein aggregation, and oxidative stress.[2] And immune reactivity and inflammatory mechanism are considered to be the especially important factors.[3,4]

Some previous studies identified the mutations in the superoxide dismutase 1 (SOD1) gene caused immune and inflammation abnormalities in animal models of ALS.[5,6] Some pathological studies found the immune abnormalities in the central nervous system (CNS),

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blood, and cerebrospinal fluid (CSF) of the patients with ALS.[7] Other studies in the patients with ALS found that the expression of C-reactive protein (CRP), interleukin (IL)-6, IL-8, and macrophage chemotactic protein-1 was increased,[4,8,9] which demonstrated a systemic pro-inflammatory condition and adaptive immune system responses in ALS. Furthermore, the autopsies of the ALS patients showed that galectin-3 levels in the spinal cord and brainstem homogenates were significantly higher than the normal controls (NCs).[10] A study on SOD1 (G93A) mutant mice showed that early disease was associated with astrogliosis while late disease was correlated with microglial activation.[5] The elevated galectin-3 levels by microglia in a mouse model of ALS was related to the progression of disease.[11] It has been identified that galectin-3 plays an important role in plentiful cellular functions including apoptosis, cellular adhesion, cell migration, cell growth or differentiation, innate or adaptive immune response, and inflammation.[12-14] Despite of these studies indicating the involvement of galectin-3 in the pathophysiological processes of ALS, whether galectin-3 plays a protective, anti-inflammatory response in ALS has not been clarified up to now.

Biomarkers of ALS were originally identified in 1965. They have been shown to impact diagnosis, understanding of pathogenic mechanisms, and efficiency of therapeutic clinical trials.[15] Although many biomarkers have been derived from CSF and blood so far, none of the known biomarkers can accurately distinguish ALS from the NCs, including some protein biomarkers in CSF and blood, which showed a bit contradictory results.[16] Group analyses that stratify the patients with ALS by gender, type of disease onset, duration of disease and so on might be helpful in identifying biomarkers of ALS.

While galectin-3 is a secretory protein, the former studies on CSF suggested that galectin-3 might be a biomarker of ALS, but failed to reveal the change of galectin-3 in different stages of ALS.[10] The CSF samples were obtained using lumbar puncture (commonly called spinal tap); the blood samples were obtained using venipuncture. Lumbar puncture is a little inconvenient and invasive compared with the low cost and easily access of venipuncture. Besides, all of the foregoing studies on galectin-3 in the tissues of ALS patients were from the postmortem (end-stage) of ALS. That encouraged us to set up a suitable source for clinical measurement of galectin-3 in patients with ALS.

In this study, we evaluated plasma galectin-3 levels in different stages of ALS, which were rarely investigated before as far as we know. We expected that plasma galectin-3 levels are correlated with the clinical characteristics of disease. The results validated our expectation very well, suggesting that plasma galectin-3 might be a useful factor which might reveal the underlying pathogenesis of ALS.

Methods

Between July 2010 and December 2014, 51 patients with ALS (ALS group) and 60 NCs (NC group) were enrolled in this study. All cases were recruited from the Department of Neurology and the Department of Geriatric Medicine of Affiliated Nanjing Brain Hospital of Nanjing Medical University. This study was approved by the Ethics Committees of Affiliated Nanjing Brain Hospital of Nanjing Medical University (Clinical Trials Government Identifier: NCT201006). All patients provided a written informed consent.

All cases were examined according to the standardized protocol, which included a general medical and neurological examination by a neurologist. The ALS was diagnosed based on the physical findings and electromyography. The cases were diagnosed according to the El Escorial criteria of ALS.[17] There were 38 patients with "clinically definite ALS" and 13 patients with "clinically probable ALS" according to the criteria. Patients with inflammatory, gastrostomy, or a predicted forced vital capacity <50% were excluded from the study. Patients with evidence of a systemic inflammation on clinical examinations or serum biochemical tests (e.g., increased number of white blood cells, high levels of CRP, or erythrocyte sedimentation rate) were excluded from the study. Patients who used nonsteroidal anti-inflammatory drugs, steroids, or statins during the last 2 months before enrollment were excluded from the study.

The neurological functional assessment of the ALS group was recorded by the revised version of Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R) at the time of diagnoses.[18,19] According to this scale, ALS patients were scored from 0 to 48 points and divided into two groups: the patients with a mild clinical condition (over 24 points according to ALSFRS-R) and the patients with a severe clinical condition (up to 24 points according to ALSFRS-R).[20] Besides, the patients were divided into two groups according to the type of disease onset: the patients with limb onset and the patients with bulbar onset.[20] The duration of ALS was 2–96 months; the average of the duration was 19.8 months. According to the duration of disease, two groups of patients were isolated: the patients with duration ≤12 months and the patients with duration >12 months.[20]

Within 30 min after admission, the plasma samples were collected into the plastic tubes with the ethylenediaminetetraacetic acid as an anticoagulant, centrifuged for 15 min at 1000 × *g*, 2°C–8°C, and then stored at −80°C until the analyses were carried out. The levels of galectin-3 in plasma were determined using the commercially available enzyme-linked immunosorbent assay kit for human galectin-3 (Cusabio Biotech Co., Ltd, Wuhan, Hubei, China) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using the SPSS statistics 23.0 (IBM Corp., Armonk, NY, USA). Whether the data follow the normal distribution or not was performed

with Kolmogorov-Smirnov test. The nonparametric Mann-Whitney *U*-test and Wilcoxon signed-rank test were used to examine the difference between two groups because the data were not normally distributed. Correlation analyses were performed using the Spearman's rank correlation to investigate whether there is an association of plasma galectin-3 levels with the clinical characteristics of disease. Galectin-3 levels are expressed in ng/ml, as median (range). A value of $P < 0.05$ was considered statistically significant.

Results

The clinical characteristics of the ALS group and the NC group are shown in Table 1. All the patients with ALS were divided into groups according to their clinical characteristics: male ($n = 26$) and female ($n = 25$), duration ≤12 months ($n = 26$) and duration >12 months ($n = 25$), bulbar onset ($n = 13$) and limb onset ($n = 38$), and mild clinical condition ($n = 39$) and severe clinical condition ($n = 12$). The age and the male: female ratio between the NC group and each of the ALS group were not statistically different ($P > 0.05$).

The comparisons of plasma galectin-3 levels in the ALS group and with the NC are listed in Table 2. The preliminary statistical analyses found that galectin-3 levels in all the ALS patients were not different as compared with the NCs (254.14 [69.12–859.22] vs. 201.64 [22.35–401.63] ng/ml, $P = 0.05$). However, the further statistical analyses revealed the differences between the ALS group and the NC group elaborated hereafter. In addition, compared with the NC group, galectin-3 levels in the ALS group with mild clinical condition and the ALS group with severe clinical condition were not different at all (253.62 [69.12–859.22] vs. 201.64 [22.35–401.63] ng/ml, $P > 0.05$; 263.27 [109.99–531.92] vs. 201.64 [22.35–401.63] ng/ml, $P > 0.05$).

As shown in Figure 1a, compared with the NC group, galectin-3 levels in the ALS group with duration >12 months were significantly increased (341.17 [69.12–859.22] vs. 201.64 [22.35–401.63] ng/ml, $P < 0.05$). However, no significant difference was found between the ALS group with duration ≤12 months and the NC group (250.62 [109.77–334.92] vs. 201.64 [22.35–401.63] ng/ml, $P > 0.05$). Furthermore, as shown in Figure 1b, compared with the ALS group with duration ≤12 months, galectin-3 levels in the ALS group with duration >12 months were increased significantly (341.17 [69.12–859.22] vs. 250.62 [109.77–334.92] ng/ml, $P < 0.05$).

As shown in Figure 1a, compared with the NC group, galectin-3 levels in the ALS group with limb onset were significantly increased (254.14 [69.12–859.22] vs. 201.64 [22.35–401.63] ng/ml, $P < 0.05$). The further analyses in the ALS group with limb onset showed that significant difference of galectin-3 levels was found in the ALS group with duration >12 months and the ALS group with duration ≤12 months (481.30 [69.12–859.22] vs. 251.79 [116.03–334.92] ng/ml, $P < 0.05$) [Figure 1b]. However, no significant difference was found between the ALS group with bulbar onset and the NC group (251.79 [109.20–404.76] vs. 201.64 [22.35–401.63] ng/ml, $P > 0.05$). The same result was found between the ALS group with limb onset and the ALS group with bulbar onset (254.14 [69.12–859.22] vs. 251.79 [109.20–404.76] ng/ml, $P > 0.05$).

As shown in Figure 1a, galectin-3 levels were significantly increased in the ALS female group compared with the NC group (263.27 [123.32–859.22] vs. 201.64 [22.35–401.63] ng/ml, $P < 0.05$), while there was no significant difference between the ALS male group and the NC group (220.39 [69.12–748.73] vs. 201.64 [22.35–401.63] ng/ml, $P > 0.05$). Moreover, there was no significant difference between the ALS female group and the ALS male group (263.27 [123.32–859.22] vs. 220.39 [69.12–748.73] ng/ml, $P > 0.05$). The comparisons of plasma galectin-3 levels between the ALS groups and the NCs are listed in Table 3.

The association between galectin-3 levels and the duration of disease is shown in Figure 1c, suggesting a significant positive correlation between them ($r = 0.293$, $P = 0.037$). However, there was no statistically significant correlation between galectin-3 levels and the severity of clinical conditions.

Table 1: Clinical characteristics of the ALS groups and the normal controls

Groups	Number of patients	Age (years)	Male/female, *n*
Controls	60	55.52 ± 8.99	32/28
ALS			
Total	51	54.84 ± 10.04	26/25
Duration ≤12 months	26	55.58 ± 8.56	14/12
Duration >12 months	25	54.08 ± 11.51	12/13
Bulbar onset	13	59.77 ± 12.11	7/6
Limb onset	38	52.76 ± 8.89	19/19
Mild clinical condition	39	53.59 ± 9.48	20/19
Severe clinical condition	12	57.67 ± 12.04	6/6

Values were *n* or mean ± SD. ALS: Amyotrophic lateral sclerosis; SD: Standard deviation.

Discussion

As far as we know, this study indicated that plasma galectin-3 levels were related to the clinical characteristics of disease, associated with limb onset of disease and the female patients with ALS. Moreover, there was a positive correlation between plasma galectin-3 levels and the duration of disease, suggesting that plasma galectin-3 might be a very interesting factor associated with ALS.

ALS is a fatal neurodegenerative disease with few therapeutic options; currently, riluzole is the only drug to treat ALS approved by the Food and Drug Administration of USA. Once patients progress to the late stage, there are no efficacious treatment options. Therefore, in disease

Table 2: Comparisons of plasma galectin-3 levels between the ALS and normal control groups

Groups	Number of patients, *n*	Galectin-3 levels (ng/ml), median (range)	P_n	P_c
Control	60	201.64 (22.35–401.63)	0.004	–
ALS, total	51	254.14 (69.12–859.22)	<0.001	0.050 (ALS, total vs. controls)
ALS, duration ≤12 months	26	250.62 (109.77–334.92)	0.009	0.652 (ALS, duration ≤12 months vs. controls)
ALS, duration >12 months	25	341.17 (69.12–859.22)	0.200	0.006 (ALS, duration >12 months vs. controls)
				0.020 (ALS, duration ≤12 months vs. ALS, duration >12 months)
ALS, bulbar onset	13	251.79 (109.20–404.76)	0.200	0.655 (ALS, bulbar onset vs. controls)
ALS, limb onset	38	254.14 (69.12–859.22)	<0.001	0.037 (ALS, limb onset vs. controls)
				0.400 (ALS, bulbar onset vs. ALS, limb onset)
ALS, limb onset, duration ≤12 months	19	251.79 (116.03–334.92)	0.049	0.642 (ALS, limb onset, duration ≤12 months vs. controls)
ALS, limb onset, duration >12 months	19	481.30 (69.12–859.22)	0.200	0.005 (ALS, limb onset, duration >12 months vs. controls)
				0.043 (ALS, limb onset, duration >12 months vs. ALS, limb onset, duration ≤12 months)
ALS, mild clinical condition	39	253.62 (69.12–859.22)	<0.001	0.080 (ALS, mild clinical condition vs. controls)
ALS, severe clinical condition	12	263.27 (109.99–531.92)	0.103	0.260 (ALS, severe clinical condition vs. controls)
				0.900 (ALS, mild clinical condition vs. ALS, severe clinical condition)
ALS, male	26	220.39 (69.12–748.73)	0.013	0.789 (ALS, male vs. controls)
ALS, female	25	263.27 (123.32–859.22)	<0.001	0.005 (ALS, female vs. controls)
				0.600 (ALS, male vs. ALS, female)

P_n values were estimated by Kolmogorov-Smirnov test; P_n<0.05 means that the data were not following the normal distribution. P_c values were estimated by Mann-Whitney *U*-test; P_c<0.05 means that the difference between two groups was statistically significant. ALS: Amyotrophic lateral sclerosis.

Table 3: Comparisons of plasma galectin-3 levels between the ALS (segmentation) and normal control groups

Groups	Number of patients, *n*	Galectin-3 levels (ng/ml), median (range)	P_n	P_c
Control	60	201.64 (22.35–401.63)	0.004	–
ALS, duration ≤12 months, male	14	187.95 (109.77–334.92)	0.200	0.440
ALS, duration ≤12 months, female	12	251.79 (168.15–293.22)	0.158	0.116
ALS, duration >12 months, male	12	390.16 (69.12–748.73)	0.108	0.202
ALS, duration >12 months, female	13	341.17 (123.32–859.22)	0.200	0.004
ALS, bulbar onset, male	7	152.51 (109.20–341.17)	0.200	0.160
ALS, bulbar onset, female	6	285.78 (223.39–404.76)	0.200	0.024
ALS, limb onset, male	19	254.14 (69.12–748.73)	0.074	0.266
ALS, limb onset, female	19	254.14 (123.32–859.22)	<0.001	0.030
ALS, bulbar onset, female, duration ≤12 months	3	251.79 (223.39–293.23)	<0.001	0.294
ALS, bulbar onset, female, duration >12 months	3	341.17 (278.34–404.76)	<0.001	0.029
ALS, limb onset, female, duration ≤12 months	9	251.79 (168.15–267.17)	0.165	0.314
ALS, limb onset, female, duration >12 months	10	392.00 (123.32–859.22)	0.138	0.026

P_n values were estimated by Kolmogorov-Smirnov test; P_n<0.05 means that the data were not following the normal distribution. P_c values were estimated by Mann-Whitney *U*-test; P_c<0.05 means that the difference between two groups was statistically significant. ALS: Amyotrophic lateral sclerosis.

management, early diagnosis and treatment of ALS are important. Nevertheless, early biomarkers, which would ideally be useful in disease monitoring, are very limited up to now. Immune reactivity and inflammatory mechanisms play important roles in the pathogenesis of ALS. Ibudilast is a phosphodiesterase inhibitor, which affects the function of lymphocytes, glial cells, and inhibits the release of tumor necrosis factor-α (TNF-α) by inflammatory cells.[21] In 2015, a report from the American Academy of Neurology showed that the coadministration of ibudilast and riluzole was safe and tolerable in patients with ALS,[22] which could improve ALS function and delay progression. There is a clear evidence of immune responses in ALS patients. A study using SOD1 (G93A) rats (an animal model of ALS) showed that immunomagnetically isolated microglia were in different CNS regions at different points in the progression of disease. The authors also found that at the end stage of disease, microglias were characterized by high expressions of galectin-3, and the concomitant downregulated expressions of inflammatory factors such as TNF-α and IL-6. Moreover, galectin-3 or osteopontin-positive microglia were restricted only to the ventral horns of spinal cord and the regions with

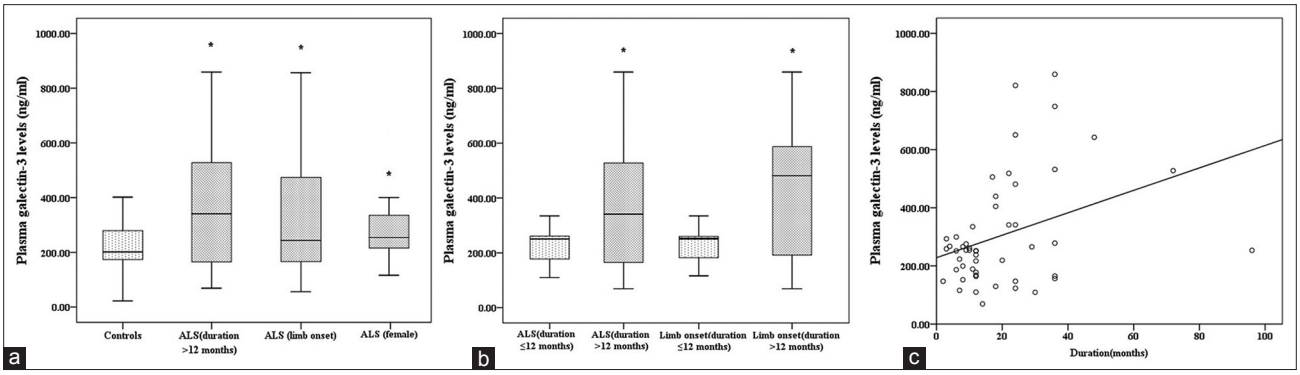

Figure 1: Comparisons of plasma galectin 3 levels between the ALS and normal control groups. (a) Comparison between the ALS group with duration >12 months and the normal controls (341.17 [69.12–859.22] vs. 201.64 [22.35–401.63] ng/ml, $P_c = 0.006$, $P_c < 0.05$); Comparison between the ALS group with limb onset and the normal controls (254.14 [69.12–859.22] vs. 201.64 [22.35–401.63] ng/ml, $P_c = 0.037$, $P_c < 0.05$); Comparison between the female ALS group and the normal controls (263.27 [9123.32–859.22] vs. 201.64 [22.35–401.63] ng/ml, $P_c = 0.005$, $P_c < 0.05$). (b) Comparison between the ALS group with duration ≤12 months and the ALS group with duration >12 months (250.62 [109.77–334.92] vs. 341.17 [69.12–859.22] ng/ml, $P_c = 0.02$, $P_c < 0.05$); Comparison between the ALS group of limb onset with duration ≤12 months and the ALS group of limb onset with duration >12 months (251.79 [116.03–334.92] vs. 481.30 [69.12–859.22] ng/ml, $P_c = 0.04$, $P_c < 0.05$). (c) Correlation between the plasma levels of galectin 3 and the duration of ALS ($n = 51$), $r = 0.293$, $P_a = 0.037$, $P_a < 0.05$. P_c values were estimated by Mann-Whitney *U*-test; $P_c < 0.05$ means that the difference between two groups was statistically significant. P_a values were estimated by Spearman's rank correlation; $P_a < 0.05$ means that the association between two factors was statistically significant. Galectin 3 levels were expressed as median (range). ALS: Amyotrophic lateral sclerosis.

severe motor neurons degeneration.[23] Other researchers used SOD1 (G93A) or galectin-3 transgenic mice to evaluate the role of galectin-3 in a mouse model of ALS; the results suggested that although the deletions of galectin-3 did not change the onset of disease, it resulted in a greater rapid progression, and more severely impaired neurological symptoms at all stages of disease.[11] Zhou *et al*.[10] found increased levels of galectin-3 in the spinal cord tissues from the patients with ALS in comparison to the NCs. These data suggested that the elevation in galectin-3 as the disease progresses might play a protective, anti-inflammatory role in innate immune responses to ALS.

CSF communicates directly with brain parenchyma and medulla spinals, and thus is an important source of biomarkers that can indicate the presence and extents of ALS. A previous study showed that the patients with ALS had approximately two-fold galectin-3 in CSF as the NCs and other neurological diseases including stroke and dementia.[10] Given the fact that lumbar puncture is painful, invasive, and dangerous, detection methods that are more convenient may be better. Peripheral blood is easy to access and handle and allows multiple tests harmlessly at a low cost. In addition, it is relatively accepted that the integrity of blood-brain barrier and blood-spinal cord barrier is perturbed in ALS.[24,25] So that, the levels of galectin-3 in plasma were determined in this study.

This study indicated that plasma galectin-3 levels were not increased in the whole group of patients with ALS significantly compared to the NC group. However, further analyses showed that galectin-3 levels were increased significantly in the ALS patients with duration >12 months. Moreover, the study showed that there was a positive correlation between galectin-3 and the duration of disease. In SOD1 (G93A) mutant mice, late disease was associated with microglial activation.[5] Besides, the majority of microglia or macrophages expressed galectin-3.[10] In addition, the former studies from the postmortem of ALS showed increased galectin-3 levels compared to the NCs.[10] All the studies indicated that galectin-3 levels in ALS might be elevated in the late stage of disease, which was consisted with our results very well. Furthermore, our data showed that in the ALS patients with limb onset, there were higher galectin-3 levels in the patients with durations >12 months. To the best of our knowledge, this is a rare study that analyzed the relationship between galectin-3 and the duration of disease in ALS so far. Moreover, no former research analyzed the association between galectin-3 and the severity of clinical conditions of ALS. Our study showed the enhanced expressions of galectin-3 potentially correlated with the stage of disease, suggesting that as the disease progresses the immune inflammation is aggravated gradually.

Our data showed that there was a gender difference in plasma galectin-3 levels remarkably, which were only increased in the ALS female patients but not in the ALS male patients as compared with the NCs. Moreover, even in the ALS female patients, galectin-3 levels were only increased when the duration of disease > 12 months, both in ALS patients with bulbar onset and in ALS patients with limb onset. This study demonstrated that there was a significant positive correlation between galectin-3 levels and the duration of disease and further the gender of patients influenced the clinical features of disease. The possible reasons for gender disparity include different immune responsiveness, hormone levels, and biological responses to exogenous toxins.[26-28] However, there was no difference between female ALS patients and male ALS patients in our data, which have to be clarified in the future.

Furthermore, in the study, increased galectin-3 levels were only observed in the ALS patients with limb onset but not

with bulbar onset compared with the NCs. It is well known that the ALS patients with bulbar onset have much more severe clinical conditions than the patients with limb onset. A recent study found that in a mouse model of ALS, after the onset of disease, the frequency of galectin-3 microglia in the spinal cord was significantly elevated compared to the brainstem, which indicated that galectin-3 might play a protective role in ALS.[23] Admittedly, ALS is a disease with very complicated mechanisms, most of which are not clarified yet. Interestingly, our study indicated that the association between plasma galectin-3 and the severity of clinical conditions of ALS was not statistically significant. This might be due to that the ALSFRS-R does not conform with the requirements of measurement.[29] The assessment of stages on the severity of disease is very helpful in prognosis, therapeutic options, and resource planning; recently, a new staging system was proposed.[30]

The main limitation of this work was that it was just a cross-section study. Future studies should be longitudinal so as to evaluate the change of plasma galectin-3 greatly in more stages of ALS. Another limitation was that the number of cases enrolled was small. For ALS, disease heterogeneity is an important factor when performing a biomarker investigation, requiring larger cohorts, and proper patient groups. Therefore, we could not find the difference between gender and the onset of disease in this study, which might be due to the small number of cases enrolled.

In conclusion, this study showed that plasma galectin-3 levels were elevated in ALS patients with limb onset, especially in female patients with ALS, and positively correlated with the duration of disease. Plasma galectin-3 levels were increased with the progression of ALS, which indicated that it potentially plays a protective role. Plasma galectin-3 is a very interesting factor associated with ALS that might be useful in evaluating the progress of ALS and even a potential therapeutic target in the future.

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Conflicts of interest

There are no conflicts of interest.

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