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Decreased level of 5-methyltetrahydrofolate: A potential biomarker for pre-symptomatic amyotrophic lateral sclerosis

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ABSTRACT

Background: Several studies have reported that homocysteine (Hcy) is associated with amyotrophic lateral sclerosis (ALS), a neurodegenerative disease without special biomarkers for early diagnosis. Here, we examined the levels of Hcy, folic acid and its metabolic molecule 5-methyltetrahydrofolate (5-MTHF) in SOD1^{G93A} transgenic mouse model of ALS in an attempt to determine whether the change in those molecules can be used as potential biomarkers for the disease.
Methods: According to the disease progression, SOD1^{G93A} transgenic mice were divided into early stage group (30 d); pre-symptom group (60 d); symptom group (90 d) and terminal stage group (120 d). LC–MS/MS was used to measure the level of Hcy, folic acid and 5-MTHF in the plasma, spinal cord and cortex of the ALS transgenic SOD1^{G93A} mice at different disease stages. Nissl staining was used to detect the motor neurons survival in the anterior horn of the spinal cord of the SOD1^{G93A} mice.
Results: In this study, we demonstrated that the level of 5-MTHF is significantly decreased in the plasma, spinal cord and cortex at the early stages of pre-symptomatic ALS transgenic SOD1^{G93A} mice while folic acid is decreased at the middle to late stages of the disease. Furthermore, we found that the level of Hcy is markedly elevated after the motor symptoms appeared in the ALS mice.
Conclusion: Our study suggests that decreased 5-MTHF level may be a potential biomarker for the early stage of the disease in the ALS mice, which may warrant further validating study of 5-MTHF level in ALS patients.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive, lethal neurodegenerative disorder characterized by massive loss of spinal cord and cortical motor neurons [1,2]. About 10% of the ALS patients are familial (fALS), and mutations in the *Cu/Zn superoxide dismutase* (*SOD1*) gene cause 20% of fALS [3]. Although tremendous effects have been made in recent years to identify the biomarker of the disease so that the diagnosis could be established in the earlier stage of the disease, no marker has been proved to be sensitive, specific and reliable for ALS. Our previous animal study found that the plasma homocysteine (Hcy) level was significantly increased in SOD1^{G93A} ALS model and treatment with Hcy-lowing drug folic acid in the ALS mice can markedly delay the onset of the disease and prolong the lifespan of the ALS mice [4]. Recently, Zoccolella et al. reported that Hcy level was significantly higher while folic acid level was significantly lower in clinical ALS patients [5]. Therefore, we hypothesize that the folic acid and its transmethylation cycle in ALS patients may be altered at the early stage of the disease, leading to Hcy accumulation at the late stage of the disease. To determine whether folic acid and its metabolic molecule 5-methyltetrahydrofolate (5-MTHF) are sensitive biomarkers to the early ALS, we measured the levels of folic acid and 5-MTHF in the plasma, spinal cord and cortex in different stages of the disease in transgenic SOD1^{G93A} mice. We also determined the Hcy plasma level in the ALS mice. In parallel with the biochemical assays, we examined the clinical manifestation and spinal cord motor neurons to correlate the changes in the 5-MTHF, folic acid and Hcy with the disease stages. We document that the level of 5-MTHF is significantly decreased in the plasma, spinal cord and cortex at the early stages of pre-symptomatic ALS transgenic SOD1^{G93A} mice, which may suggest that alteration in 5-MTHF level may be a sensitive biomarker for early diagnosis of ALS.

2. Materials and methods

2.1. Subjects

SOD1^{G93A} transgenic mice were purchased from Jackson's Lab. The initial motor impairment sign of the disease in the hemizygous

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transgenic mice usually starts at the age of 90 d, followed by paralysis at the age of 120 d resulting from massive motor neurons (MNs) loss in the spinal cord [3]. According to the pathological stages, SOD1^{G93A} transgenic mice were divided into four groups: early stage group (30 d) when the animal has minimal loss of MNs; pre-symptom group (60 d) when the animal has moderate decrease in the number of MNs; symptom group (90 d) when the animal has clinical paralysis and significant loss of MNs; and terminal stage group (120 d) when the animal is near death and MNs in the spinal cord are lost above 70%. We used age-matched wild-type littermates as four control groups. Each group consisted of eight mice. Animal care and procedures were performed in accordance with the Laboratory Animal Care Guidelines approved by the Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences.

2.2. Determination of folic acid, 5-MTHF, and Hcy levels

All mice were deeply anesthetized with 10% chloral hydrate. Blood sample (500 μL) was obtained from the left ventricle of the heart. Plasma was separated and prepared in the anti-oxidative mixture containing 50 μg/mL ascorbic acid and 50 μg/mL 2-mercaptoethanol to prevent oxidation during storage at −80 °C. Half sample was used for folic acid and 5-MTHF analysis; the other half was used for Hcy analysis. Then, all mice were sacrificed by trans-cardiac perfusion with phosphate buffered saline (PBS). After sacrificed, the spinal cords (L_{4-5}) and cortex (frontal lobe and temporal lobe) were rapidly removed and put into 5% physiological saline which contained the anti-oxidative mixture. LC–MS/MS equipped with Shimadzu LC-10AD pump (Kyoto, Japan) and API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) was used to measure the levels of folic acid and 5-MTHF in the samples according to Garbis' procedure [6]. Hcy level was determined by LC–MS/MS as previously described in detail [4].

2.3. Nissl staining

All mice were anesthetized with chloral hydrate and sacrificed by trans-cardiac perfusion with phosphate buffered saline (PBS). The spinal cord (L_{4-5}) was removed, postfixed overnight in 4% paraformaldehyde and subsequently dehydration in 30% sucrose for 48 h.

Lumbar spinal cord (L_{4-5}) was embedded in optimal cutting temperature compound and frozen at −80 °C. Serial transverse sections (10 μm thickness) of the lumbar segment (L_{4-5}) were cut and mounted on gelatin coated slides. Serial sections (200 slices) were stained with cresyl violet for Nissl staining, and then sections were dehydrated in a graded alcohol series, cleared in xylene and covered by glass slide. Sections were photographed under the light microscopy. We counted two sides of the anterior horn on every third section between the L_4 and L_5 levels of spinal cord of all the four group mice [7]. An examiner who was blinded to the experimental design counted the anterior horn cells that met all of the following criteria: (1) neurons located in the anterior horn ventral to the line tangential to the ventral tip of the central canal; (2) neurons with a maximum diameter of 20 μm or more; and (3) neurons with a distinct nucleolus [8,9].

2.4. Statistics

All data are expressed as mean ± S.E.M. Data (the level of Hcy, folic acid and 5-MTHF) were analyzed using a Student's *t*-test with significance reported at the level of $P<0.05$. The number of the motor neuron in different groups was analyzed by ANOVA and *P* value less than 0.05 was considered significant.

3. Results

3.1. The alteration of folic acid, 5-MTHF and Hcy levels in different disease stages of ALS mice

We used LC–MS/MS to measure the levels of folic acid and 5-MTHF in the plasma, spinal cord and cortex in mice of all eight groups. Our results showed that the level of folic acid was significantly reduced in the plasma, spinal cord and cortex in the ALS mice at the terminal stage but not at the early, pre-symptom, and symptom stages as compared with the age-matched control groups (Table 1). Interestingly, we found out that the level of 5-MTHF was significantly decreased to 75.4 ± 9.2% in the plasma ($P<0.01$), 59.2 ± 19.8% in the spinal cord ($P<0.05$), and 66.0 ± 12.9% in the cortex ($P<0.05$) of SOD1^{G93A} mice at the early stage (30 d) as compared with the same age-matched controls (Table 1). The decrease of 5-MTHF level was also significant in ALS mice at the pre-symptom stage (74.0 ± 10.5% in the plasma, $P<0.01$; 57.3 ± 17.8% in the spinal cord, $P<0.05$; and 64.1 ± 10.8% in the cortex, $P<0.05$), at the symptom stage (68.3 ± 10.7% in the plasma, $P<0.01$; 52.5 ± 14.1% in the spinal cord, $P<0.01$; and 59.8 ± 13.7% in the cortex, $P<0.01$), and at the terminal stage (64.1 ± 13.8% in the plasma, $P<0.01$; 51.2 ± 11.2% in the spinal cord, $P<0.01$; and 58.2 ± 11.8% in the cortex, $P<0.01$) as compared with the age-matched WT mice (Table 1).

To determine if the level of Hcy was altered before the disease onset, we examined the level of plasma Hcy by LC–MS/MS in eight different groups of mice. We found that Hcy level in ALS mice was significantly increased by 130.0 ± 17.7% (6.84 ± 0.4 vs 4.04 ± 0.27 μmol/L; $P<0.05$) and 169.6 ± 24% (5.11 ± 0.33 vs 3.93 ± 0.45 μmol/L; $P<0.01$) at symptom and terminal stages as compared with the age-matched controls, respectively (Table 2). However, the level of Hcy in ALS mice was only moderately elevated without statistical significance by 110.5 ± 12.1%

Table 1
The levels of folic acid and 5-MTHF in the plasma, spinal cord and cortex are significantly decreased in ALS mice.

Group	30 d		60 d		90 d		120 d	
	WT	ALS	WT	ALS	WT	ALS	WT	ALS
Folic acid level (ng/mL)								
Plasma	3.73 ± 0.23	3.5 ± 0.21	3.61 ± 0.34	3.38 ± 0.19	3.42 ± 0.22	3.22 ± 0.18	3.48 ± 0.54	2.61 ± 0.25*
Spinal cord	0.71 ± 0.13	0.66 ± 0.07	0.71 ± 0.08	0.65 ± 0.12	0.69 ± 0.15	0.69 ± 0.08	0.68 ± 0.07	0.53 ± 0.03*
Cortex	0.66 ± 0.11	0.67 ± 0.12	0.68 ± 0.1	0.66 ± 0.06	0.7 ± 0.05	0.62 ± 0.12	0.69 ± 0.09	0.56 ± 0.12*
5-MTHF level (ng/mL)								
Plasma	38.7 ± 2.68	29.2 ± 2.56**	38.2 ± 3.43	28.3 ± 3.57**	37.3 ± 4.43	24.7 ± 5.34**	36.9 ± 5.01	22.4 ± 4.63**
Spinal cord	12.6 ± 2.25	8.94 ± 2.1*	12 ± 1.78	8.23 ± 1.5*	11.7 ± 1.73	7.41 ± 1.04**	10.9 ± 0.76	7.24 ± 0.91**
Cortex	14.8 ± 0.76	10.4 ± 1.4*	14.2 ± 0.89	9.88 ± 1.03*	13.2 ± 2.13	8.1 ± 1.62**	12.8 ± 2.07	7.92 ± 1.92**

Values represent the mean ± S.E.M. $N=8$ in each group.
*$P<0.05$ when compared with age-matched WT group.
**$P<0.01$ when compared with age-matched WT group.

Table 2
Hcy plasma level (μmol/L) is significantly increased in ALS mice.

Group	30 d	60 d	90 d	12 d
WT	3.8 ± 0.24	3.86 ± 0.58	3.93 ± 0.45	4.04 ± 0.27
ALS	4.23 ± 0.32	4.23 ± 0.38	5.11 ± 0.33*	6.84 ± 0.42**

Values represent the mean ± S.E.M. $N = 8$ in each group.
*$P<0.05$ when compared with age-matched WT group.
**$P<0.01$ when compared with age-matched WT group.

(4.20 ± 0.38 μmol/L vs 3.86 ± 0.58 μmol/L; $P>0.05$) at early stage and by 111.0 ± 11.5% (4.23 ± 0.32 μmol/L vs 3.80 ± 0.24 μmol/L; $P>0.05$) at pre-symptom early stage as compared with the age-matched WT groups (Table 2).

3.2. The relationship of the levels of 5-MTHF, folic acid, Hcy and MNs degeneration

We analyzed the levels of 5-MTHF, folic acid, Hcy in the plasma, spinal cord and cortex in different disease stages. Our results demonstrated that the decrease of 5-MTHF level started at the early stage of disease (30 d) and became more significant with the disease progression (Fig. 1 C), while the level of Hcy was increased at the symptom stage and became extremely higher at the terminal stage (Fig. 1 D). Meanwhile, the level of folic acid was reduced after the symptom onset and decreased significantly at the terminal stage of the disease (120 d) (Fig. 1 E). Furthermore, Nissl staining revealed a drastic decrease of MNs in the anterior horn of the spinal cord at the symptomatic and terminal stages of ALS mice compared with the age-matched controls (654 ± 31 vs 792 ± 34, $P<0.01$; 216 ± 23 vs 812 ± 42, $P<0.01$ Fig. 1 A, B). Thus, our results demonstrated that the decrease of 5-MTHF started before the degeneration of the MNs, which suggested that the level of 5-MTHF might be the potential biomarker for the pre-symptomatic ALS in the transgenic $SOD1^{G93A}$ mice. Furthermore, our study may show a possible correlation between decreased 5-MTHF, reduced folic acid, increased Hcy and lost MNs.

4. Discussion

Biomarkers are very important indicators of normal and abnormal biological processes. It is believed that biomarkers have great potential in predicting chances for diseases, aiding in early diagnosis, setting standards for the development of new remedies to treat diseases and indicating the responses to potential treatments such as antisense RNA or SiRNA against SOD1 for ALS. Up to now, no proposed biomarkers have been demonstrated to meet the desired criteria in the diagnosis of ALS. Using the $SOD1^{G93A}$ transgenic mouse model, we found that the levels of folic acid and its transmethylation metabolite 5-MTHF were significantly lower in the ALS mouse model. Most interestingly, the decreased level of 5-MTHF was detected in plasma, spinal cord and cortex of the ALS mice at the early non-symptomatic stage prior to the folic acid reduction, indicating that the change in 5-MTHF level may be a potential biomarker for early diagnosis of pre-symptomatic ALS.

It remains unclear how 5-MTHF and folic acid are significantly altered in the blood and CNS tissues, and why the alteration in 5-MTHF occurs much earlier than that of folic acid in the ALS mouse model. 5-MTHF is an important intermediate metabolite in the folic acid and Hcy metabolic pathway [10,11]. In the remethylation of Hcy, folic acid converts to 5-MTHF, which acts as a methyl donor to Hcy [11]. This conversion requires tetrahydrofolate (THF) transformation to 5,10-methylenetetrahydrofolate that is then formed into 5-MTHF by methylenetetrahydrofolate reductase (MTHFR) [11]. We suspect that MTHFR enzyme activity may be directly affected by the SOD1 mutations or other pathogenetic molecules related to ALS. When MTHFR is down-regulated, it may immediately influence the conversion of 5-MTHF while folic acid can easily obtain from food to compensate the reduction at the early stage of the disease in the ALS mice. Future measurement of MTHFR activity is needed to test the hypothesis.

Folic acid, a water-soluble vitamin of the B complex group, is required for optimal health and plays key roles in a variety of bio-physiological processes, including the maintenance and repair of the genome functions, regulation of gene expression, amino-acid metabolism, neurotransmitter synthesis, and myelin formation [10]. It has been known for decades that folic acid deficiency can cause nervous

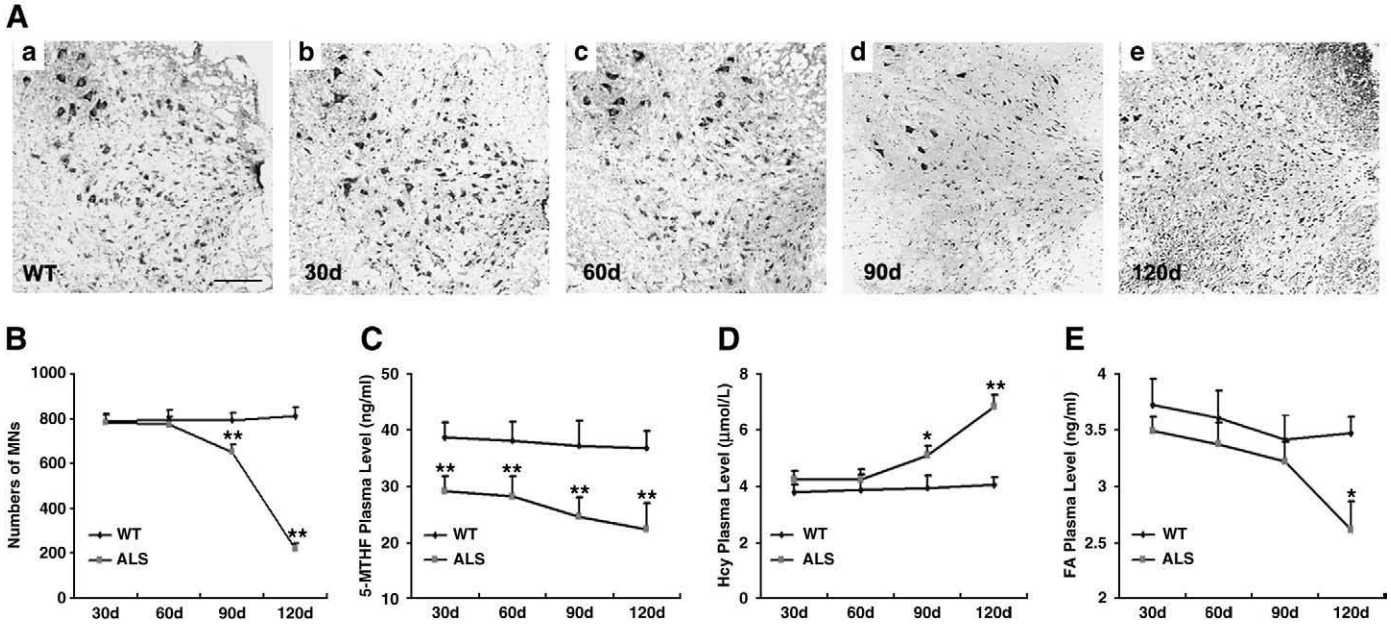

Fig. 1. Nissl staining in the anterior horn of the spinal cord of ALS mice and WT mice. (A): Nissl staining of MNs in the anterior horn of WT (a), 30 d ALS (b), 60 d ALS (c), 90 d ALS (d) and 120 d ALS mice (e); (B) quantitative analysis of MNs in the spinal cord anterior horn of the ALS mice and age-matched WT mice ($n=5$, each group; **, $P<0.01$); quantitative analysis of 5-MTHF level (C), Hcy level (D) and folic acid level (E) in different disease stages of ALS mice and age-matched WT mice ($n=8$, each group; *, $P<0.05$; **, $P<0.01$).

system defects and numerous neurological disorders [10,11]. In addition, folic acid deficiency can elevate plasma Hcy levels and sensitize CNS to neurotoxin-induced neuronal injury [10–12]. High Hcy level itself may contribute to the SOD1 mutation induced mitochondrial impairment and MNs degeneration [13]. The present study in ALS mice and Zoccolella's report in clinical ALS patients [4,5] support a correlation between alteration in folic acid–5-MTHF cycle and ALS. Folic acid supplement has been shown to protect the MNs degeneration and attenuate the increased level of Hcy in the $SOD1^{G93A}$ transgenic ALS mice [4], further implying a role of folic acid in the pathogenesis of ALS.

To further determine whether the change of Hcy level is associated with folic acid–5-MTHF cycle, we examined the level of Hcy in different stages of ALS mice. Our data showed that the level of Hcy was altered only after motor symptom onset. These findings indicate that the change in Hcy level may be a consequence of folic acid–5-MTHF deficiency.

Although $SOD1^{G93A}$ transgenic mice have been commonly used animal model of ALS, it remains uncertain whether it can represent the sporadic ALS which accounts for nearly 90% of clinical ALS patients [3]. However, the fact that elevated Hcy has been reported in human ALS argues that our finding may be generally relevant [5].

Alteration in Hcy level has been reported in association with aging [14], cardiovascular diseases, Alzheimer's disease and Parkinson's disease [15]. These reports suggest that monitoring Hcy level alone does not help diagnose ALS, track the disease progression or monitor the effectiveness of treatments. Based on the results from our study, we propose that a combination of such changes in 5-MTHF, folic acid and Hcy levels might be a meaningful indicator for the early sign of the disease. Furthermore, our study may provide useful information which lead to further clinical study to determine whether ALS patients have folic acid deficiency [5].

In summary, we report the alteration of 5-MTHF level in plasma and CNS tissues might be an early biochemical sign of disease in ALS animal model, which may warrant future investigation to determine whether such finding can be validated in clinical ALS patients and can be used to help early diagnosis and respond to the specific treatment to the disease. Furthermore, application of folic acid and 5-MTHF might be considered as an alternative therapy for this devastating disease.

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