Journal of the Neurological Sciences 293 (2010) 102–105

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

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

X. Zhang [a,1], S. Chen [b,1], L. Li [a], Q. Wang [a], W. Le [a,b,c,*]

[a] Insitute of Neurology, Ruijin Hospital, Jiaotong University School of Medicine, Shanghai 200025, China
[b] Department of Neurology, Ruijin Hospital, Jiao Tong University School of Medicine, Shanghai 200025, China
[c] Department of Neurology, Baylor College of Medicine, Houston, TX 77030, USA

ARTICLE INFO

Article history:
Received 16 June 2009
Received in revised form 20 February 2010
Accepted 25 February 2010
Available online 23 March 2010

Keywords:
Amyotrophic lateral sclerosis
Biomarker
Homocysteine
5-Methyltetrahydrofolate

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

* Corresponding author. Postal address: Building 11-1201; Ruijin 2nd Road 197#; Shanghai 200025, China. Tel./fax: +86 21 54669084.
E-mail address: wdle@sibs.ac.cn (W. Le).
[1] These authors contributed equally to this work.

0022-510X/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jns.2010.02.024

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.