Original Article
Plasma long noncoding RNA 51A as a stable biomarker of Alzheimer's disease

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Abstract: Background: Recently, large numbers of long noncoding RNAs (lncRNAs) have been detected in the mammalian nervous system. These circulating lncRNAs have been reported as potential biomarkers for neurodegenerative disorders and processes affecting the central nervous system. This study was conducted to investigate the potential role of plasma lncRNA 51A as a diagnostic biomarker for Alzheimer's disease (AD). Methods: Plasma samples were obtained from 70 AD patients and 90 age- and gender-matched controls. The lncRNA 51A level in plasma was measured by real-time quantitative reverse transcriptase PCR. Moreover, the correlation between the MMSE scores and the level of plasma lncRNA 51A expression was analyzed. Results: We found that lncRNA 51A was upregulated in plasma of AD patients compared with that of controls. In addition, lncRNA 51A was stable in plasma. By receiver operating characteristic curve analysis, the area under the receiver operating characteristic curve was 0.844, sensitivity was 0.839 and specificity was 0.729. Importantly, lncRNA 51A was negatively correlated with the Mini Mental State Examination scores in AD patients. Conclusions: Our results indicate that plasma lncRNA 51A may serve as a useful biomarker for the diagnosis of AD.

Keywords: Alzheimer's disease, lncRNA 51A, plasma, biomarker

Introduction

Alzheimer's disease (AD), a neurodegenerative disorder, is the most common form of dementia, estimated to affect 27 million individuals worldwide [1]. It is a slowly progressive degenerative disease with memory loss and other cognitive dysfunction. Therefore, early detection of AD is of particular significance to reduce the progression of cognitive dysfunction. Recently published data have demonstrated high sensitivity of AD detection by measuring concentrations of protein biomarkers such as total-tau, phospho-tau and Aβ1-42 in the cerebral spinal fluid. However, this examination method is not widely used for diagnosis because of its invasiveness [2]. Therefore, the discovery of stable and more conveniently detected biomarkers is highly desirable.

Long noncoding RNAs (lncRNAs), RNA molecules longer than 200 bp that lack coding capability, can regulate the expression of associated genes at transcriptional, posttranscriptional, and epigenetic levels [3, 4]. Previous studies have shown that lncRNA expression is frequently dysregulated in disease, including AD. For example, lncRNA n341006, which associates with genes involved in the protein ubiquitination pathway, was found to be downregulated in AD [5].

Circulating RNAs, including miRNAs and lncRNAs, are emerging as potential candidates for non-invasive diagnostic applications in plasma or serum [6]. They have already been proposed as potential diagnostic tools for many diseases due to their stability and convenience of detection in plasma or serum. For example, LINC00152 is detected in plasma and has been reported as a novel blood-based biomarker in gastric cancer [7]. In addition, serum miR-125b is downregulated in patients with AD and may serve as a useful noninvasive biomarker for AD [8].

A previous study has shown that lncRNA 51A is upregulated in AD brain samples [9]. In this present study, lncRNA 51A was detected in plasma samples of AD patients as well as

Table 1. Comparison of clinical characteristics and relative expression level of lncRNA 51A in AD group and control group

Index	AD group (n = 70)	control group (n = 90)	P
Sex (male/female)	33/37	48/42	0.437
Age (years)	73.24 ± 4.81	74.28 ± 4.18	0.148
MMSE scores	12.81 ± 4.77	29.27 ± 0.35	< 0.001
lncRNA 51A	4.79*	1.05*	< 0.001

*The data were shown in mean of $2^{-\Delta\Delta Ct}$.

healthy controls. The primary aim of the study was to investigate whether lncRNA 51A in plasma could be used as a novel, noninvasive diagnostic biomarker for AD patients.

Materials and methods

Patients and samples

Our study group comprised 70 sporadic AD patients (age onset > 65 years) and 90 healthy controls (age > 65 years) matched for age and gender. The patients were recruited from the outpatient clinic and the Department of Neurology at the 3rd Xiangya Hospital of Central South University in Hunan Province from July 2012 to 2015. The control group comprised healthy people without dementia, tested in the Health Management Center of the 3rd Xiangya Hospital at the same time. All participants are from the Chinese Han population of the Hunan Changsha region. A clinical diagnosis of probable Alzheimer's disease was established according to the criteria of National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA) [10]. Clinical data and peripheral blood samples were obtained from the members of both groups. Subjects with significant illness, including type 2 diabetes mellitus, coronary heart disease, ischemic stroke, hemorrhagic stroke, infectious disease, cancer, and glaucoma, were excluded from both cases in our study. The study protocol was approved by the local Ethics Committee of the 3rd Xiangya Hospital, and informed consent was acquired from all subjects or from their caregivers.

Peripheral blood (7 ml) was collected from patients and controls in sodium heparin tubes and immediately subjected to the three-spin protocol (1500 rpm for 30 min, 3000 rpm for 5 min, and 4500 rpm for 5 min) to prevent contamination by cellular nucleic acids. We had access to

information that could identify individual participants during or after data collection.
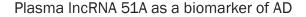
Real-time PCR

The plasma was separated from venous blood within 12 hours. The plasma sample was further resolved by a 15 min centrifugation at 12000 rpm at 4°C to completely remove cell debris. Following the manufacturer's protocol, RNA was extracted using Trizol Reagent (Takara, Japan). The reverse transcription reaction was carried out using the PrimeScript II cDNA synthesis kit (Takara, Japan) in 10 μl reaction containing 3 μl of RNA extract. For cDNA synthesis, the reaction mixtures were incubated at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min and then held at 4°C. The quantitative detection of lncRNA was performed using SYBR Green as implemented in the ABI StepOne Real-Time PCR System (Applied Biosystems), and the reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. The sequences of 51A forward and reverse primers were 5'-TGGGAGAGTCAGCATCTTGAAG-3' and 5'-TG-TACAGTCAGACAAGAGGTGTGTGTAT-3', respectively. As an endogenous control, expression of the human glyceraldehyde 3 phosphate dehydrogenase (GAPDH) gene was examined. The sequences for human GAPDH primers were 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAG-ATGGTGATGGGATTTC-3'. All samples were measured in duplicate and the mean values were calculated for further statistical analysis.

Statistical analysis

Statistically significant differences between groups were determined by the two-tailed Student's t-test. All statistical analyses were carried out using SPSS version 19.0 (IBM, Armonk, NJ, USA) and expressed as mean ± SD. A value of $P < 0.05$ was considered significant. A receiver operating characteristics (ROC) curve was plotted to determine how well the expression level of lncRNA 51A discriminated between AD samples and healthy control samples. MMSE score was compared between AD patients and controls using the Mann-Whitney test. The correlations between the variables were assessed with the Spearman's correlation coefficient.
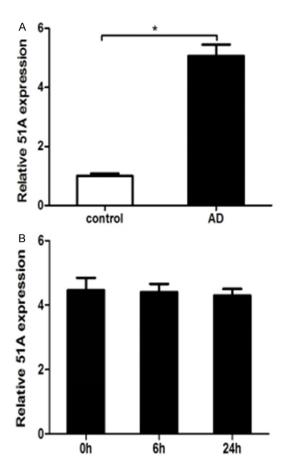
Figure 1. Validation of AD-related lncRNA 51A expression as circulating biomarkers. A. The expression of lncRNA 51A in 20 pairs of AD and healthy controls plasma. Differential expression of lncRNA 51A in AD plasma was compared with those of normal plasma. B. LncRNA 51A expression with prolonged room temperature incubation time. *P < 0.05 compared with the control group.
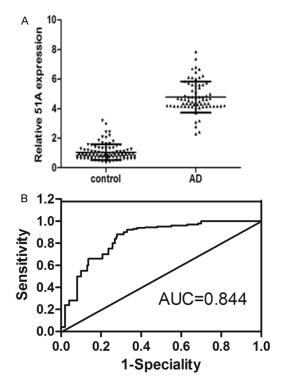

Figure 2. Evaluation of 51A levels in plasma samples of patients with AD. A. Plasma 51A levels in 70 consecutive AD patients and 90 healthy controls. Using a real-time RT-PCR assay, difference in plasma 51A expression between AD patients and normal healthy controls was determined (P < 0.0001). B. The ROC curve analysis for discriminative ability between AD cases and normal controls (AUC = 0.844, sensitivity: 0.839, specificity: 0.729).

Results

Clinical data comparison between AD and control groups

Clinical characteristics of the study population are shown in **Table 1**. No significant differences were found between the AD and control groups in terms of sex and mean age. However, lncRNA 51A expression was significantly upregulated in AD plasma compared to healthy plasma. In addition, MMSE scores in AD patients were lower than in healthy patients.

LncRNA 51A was upregulated in AD patients and was stable in plasma

To determine the expression level of lncRNA 51A in plasma, we investigated the lncRNA ex-

pression in the plasma of 20 AD patients and 20 healthy controls. As a result, we found that plasma lncRNA 51A was significantly upregulated in AD plasma compared with healthy controls (**Figure 1A**, *P* < 0.05). We then determined the stability of the lncRNA in the plasma. Plasma samples were incubated at room temperature for 0, 6, and 24 h to determine whether lncRNA 51A could be degraded under these conditions. Results indicated that incubation time (**Figure 1B**, *P* > 0.05) had a negligible effect on plasma level of lncRNA 51A, which suggests that lncRNA 51A is stable in plasma.

Large-scale analysis of validation of plasma lncRNA 51A level by comparing AD patients with healthy controls

Next, plasma lncRNA 51A level was examined on a large scale for our validation study using plasma from 70 AD patients and 90 healthy controls by qRT-PCR assays. Results showed
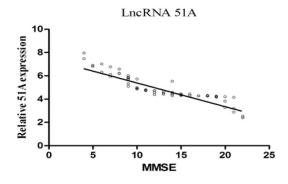
Figure 3. Correlations between plasma lncRNA 51A and MMSE in 70 AD patients. The scatter graph shows a significant negative correlation between the relative level of lncRNA 51A and MMSE score (r = -0.204, P < 0.0001).

that 51A expression was significantly higher in AD patients than in controls (**Figure 2A**, *P* < 0.0001). Representation of the data using an ROC plot showed strong separation between the two groups, with an AUC of 0.844. The sensitivity and specificity were 0.839 and 0.729 respectively (**Figure 2B**).

Correlation between cognitive function and the level of lncRNA 51A

We analyzed the potential correlation between MMSE scores and the level of plasma lncRNA 51A expression. Spearman correlation analysis showed a negative correlation between the relative expression of lncRNA 51A and MMSE scores (**Figure 3**, r = -0.204, *P* < 0.001).

Discussion

A current focus in AD research is the discovery of non-invasive biomarkers for diagnosis of AD. Recent studies have found that specific nucleic acids are detectable in the plasma of many patients with neurodegenerative disorders and therefore may be utilized as a tool for the diagnosis of neurodegenerative disorders including AD [8, 11, 12]. Numerous studies have focused on miRNAs as potential markers for AD diagnosis and prognosis prediction. For example, miR-125b was downregulated in AD and could be a potential marker for AD [8]. Xie *et al.* reported that circulating miR-206 and miR-132 are novel miRNAs upregulated in patients with mild cognitive impairment, and were therefore potential biomarkers for its diagnosis [12]. However, little is known about the diagnostic utility of lncRNAs in the plasma for AD.

Long noncoding RNAs are RNA molecules, longer than 200 nucleotides that lack coding capability. LncRNAs have been shown to regulate the expression of associated genes at transcriptional, posttranscriptional, and epigenetic levels [3, 4]. LncRNA expression levels are strongly associated with development and progression in AD [5]. Thus, differential expression of lncRNAs could be used to diagnose, discover potential treatment targets for, and improve prognosis of patients with AD. Faghihi reported a conserved noncoding antisense transcript for beta-secretase-1 (BACE1) that drives feed-forward regulation of beta-secretase, and affects, and is directly implicated in, the increased abundance of Aβ1-42 in AD [13]. In addition, a long intergenic RNA (lincRNA), primate-specific BC200 RNA (BCYRN1), was found to be expressed in dendritic domains of neurons and downregulated during aging [14].

Sortilin-related receptor 1 (*SORL1*), a risk gene for late-onset AD, is expressed in neurons of the central and peripheral nervous system [15, 16]. Our previous studies showed that a *SORL1* gene polymorphism is closely associated with late-onset Alzheimer's disease [17]. Research indicates that the expression of *SORL1* is downregulated in the brain of AD patients, suggesting a potential role in AD pathogenesis [18]. Ciarlo and colleagues indicated that lncRNA 51A was upregulated in AD brain samples [9], and their experiments further found that the 51A expression drives a splicing shift of *SORL1,* from the synthesis of the canonical long protein variant A to an alternatively spliced protein form. This process, leading to decreased synthesis of SORL1 variant A, leads to impaired processing of amyloid precursor protein (APP), resulting in increased Aβ formation.

Previously, lncRNA 51A was shown to be upregulated in AD brain samples [9]. However, whether lncRNA 51A in plasma could be used as a novel, noninvasive diagnostic biomarker of AD patients was still unknown. To this end, the expression levels of lncRNA 51A were measured in plasma from AD patients and healthy subjects. The results described here demonstrated that the level of lncRNA 51A was significantly higher in plasma from AD patients compared with normal controls. Moreover, lncRNA 51A alone may serve as a novel, noninvasive biomarker for AD with high sensitivity/specificity (83.9%/72.9%). Taken together, these results

provide strong evidence that AD-related lncRNA 51A could be released into the circulation and that its expression profile in plasma could be used as a diagnostic marker for AD. To find a correlation between cognitive function and the level of lncRNA 51A expression, we also found that lncRNA 51A was significantly negatively correlated with the MMSE scores of AD patients.

In conclusion, we have shown that lncRNA 51A is upregulated in the plasma of AD patients compared with those of healthy controls. Studies suggested that there is a vital link between the expression of 51A and *SORL1* splicing; the 51A-dependent alternative splicing of *SORL1* leads to an imbalance in Aβ secretion that could induce diseases like AD. Our findings indicate for the first time that the expression of lncRNA 51A in plasma could be used as a novel and rapid diagnostic and/or prognostic biomarker for AD.

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Disclosure of conflict of interest

None.

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