Development of a Novel Urine Alzheimer-Associated Neuronal Thread Protein ELISA Kit and Its Potential Use in the Diagnosis of Alzheimer's Disease

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Background: Alzheimer's disease (AD) is an age-related chronic degenerative disease that damages the nervous system. A non-invasive and simple method for early detection of AD is extremely important for the diagnosis and prognosis of AD. Thus, we aimed to develop an enzyme-linked immunosorbent assay (ELISA) kit to detect urine Alzheimer-associated neuronal thread protein (AD7C-NTP), and to evaluate its clinical value for the diagnosis of AD. **Methods:** Immunogenic AD7C-NTP peptide fragments were synthesized by the solid-phase method and used for immunizing mice or rabbits to generate anti-AD7C-NTP antibodies. The urine AD7C-NTP ELISA kit was then established; the generated mouse anti-AD7C-NTP antibody was used as a capture antibody, the biotin-labeled rabbit anti-AD7C-NTP antibody was used as a detection antibody, and avidin labeled by horseradish peroxidase was used as a sub-

strate. The first morning urine specimens of 121 AD patients and 118 age-matched controls were collected, and the urine AD7C-NTP levels were detected by the above ELISA kit. **Results:** Mouse and rabbit anti-AD7C-NTP antibody ELISA titer was found to be 1:8,000 and 1:32,000, respectively. A single band with a relative molecular mass of 41 kDa was found in human brain specimens by Western blot assay, which was identified as AD7C-NTP antibody. The urine AD7C-NTP concentration of the AD patients was higher than that of the age-matched controls, the sensitivity was 89.3% and the specificity was 84.7%. **Conclusions:** Our study demonstrated that our newly developed urine AD7C-NTP ELISA kit has suggested potential for diagnosing AD in a Chinese population, suggesting it may be a useful diagnostic kit for detecting early AD. J. Clin. Lab. Anal. 30:308–314, 2016. © 2015 Wiley Periodicals, Inc.

Key words: Alzheimer-associated neuronal thread protein; enzyme-linked immunosorbent assay; urine; Alzheimer disease

INTRODUCTION

Alzheimer's disease (AD) is a progressive and neurodegenerative disease that predominantly affects the

cognitive functions in the elderly (1, 2). The number of people with AD will increase dramatically in the next

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40 years (3). The current diagnosis of AD is based on clinical manifestations combined with comprehensive evaluation, such as magnetic resonance imaging (MRI) and a neuropsychological examination scale (4). The latency of AD, however, is long and it usually takes more than 3 years from the onset of symptoms to diagnosis (5). Thus, looking for noninvasive biomarkers with high sensitivity and specificity is of great importance to improve the early diagnosis of AD, provide opportunities for drug intervention, and slow down the progression of the disease.

Alzheimer-associated neuronal thread protein (AD7C-NTP) is expressed by neurons in the brain, and has been found to colocalize with neurofibrillary tangles (NFTs) and malnutritional axons (6). Increased AD7C-NTP expression is an early event in AD, which occurs before the NFTs formation; that is, the expression of AD7C-NTP can be found in neurons in the absence of NFTs (7). The level of AD7C-NTP in the cerebral spinal fluid and urine of AD patients is significantly higher than that in normal people (8). The AD7C-NTP level is positively correlated with the degree of dementia, and its level gradually increases with the extension of AD course (9). Some researchers have therefore suggested that AD7C-NTP could be used as a peripheral biomarker for the early diagnosis of AD (10).

Several human AD7C-NTP ELISA kits have been developed for laboratory use in the world; however, they are too expensive for most researchers in China. In this study, we designed an AD7C-NTP ELISA kit for detection of urine AD7C-NTP in AD patients and healthy controls, and evaluated the value of this method for clinical application. This is the first independent development of an AD7C-NTP ELISA kit for clinical diagnosis of AD in China.

MATERIALS AND METHODS

Experimental Procedure

Experimental animals

Two male New Zealand white rabbits (2,000 g each) and ten male 7-week-old Kunming mice were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. All animals were caged individually under controlled conditions: a light-dark cycle (12:12 h, lights on starting at 06:00 am), temperature (23°C), relative humidity (60–65%), and food and water ad libitum. Human brain specimens were received from the Holland Brain Institute. All animal use protocols were approved by the Institutional Animal Care and Ethics Committee of Xuanwu Hospital of Capital Medical University, China.

Preparation of AD7C-NTP antigen

The N- and C-terminal antigenic determinant polypeptide fragments of AD7C-NTP were synthesized by the solid-phase method using a 431-A polypeptide automatic synthesizer (ABI, Carlsbad, California), and then these fragments were purified by high performance liquid chromatography (600E; Waters, Milford, Massachusetts) (11).

The above polypeptide fragments were coupled with hemocyanin to produce the immunogen. Rabbits were immunized with AD7C-NTP C-terminal antigenic determinant polypeptide fragments (1 mg/rabbit), while Kunming mice were immunized with AD7C-NTP N-terminal antigenic determinant polypeptide fragments (0.1 mg/mouse) after being fully emulsified with an equal amount of complete Freund's adjuvant as the basic immunity. Strengthened immunity was taken 2 weeks after basic immunity, the AD7C-NTP C-terminal antigenic determinant polypeptide fragments (0.5 mg/rabbit, 0.05 mg/mouse) or AD7C-NTP N-terminal antigenic determinant polypeptide fragments (0.5 mg/rabbit, 0.05 mg/mouse) were fully emulsified with an equal amount of incomplete Freund's adjuvant. The above strengthened immunity total numbered 5 or 6. Antibody titer was detected from ear blood of rabbits and tail blood of mice at 10–14 days after each immunization. Blood serum of rabbits was collected by carotid intubation and peritoneal effusion of mice was collected after the mice were inoculated with hepatoma H22 cells (ATCC) in order to get more antibody, freeze dried (Virtis freeze drier; SP Scientific), and placed in a –20°C refrigerator.

Identification of AD7C-NTP antibody

The antibody titer, location of protein expression, and relative molecular mass of AD7C-NTP were identified by ELISA, immunohistochemical staining, and Western blot assay, respectively.

Preparation of biotinylated antibody

Antibody was diluted by adding 1 ml of 0.1 M sodium bicarbonate. Biotin-N-hydroxysuccinimide ester (BNHS; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing) was dissolved in dimethylformamide (DMF) at a concentration of 20 mg/ml. Then, 1 ml antibody solution was incubated with 20 µl of BNHS. After 2 hr of reaction at room temperature, the mixture was subjected to dialysis against Phosphate buffered saline (PBS) for 24 hr at 4°C. The dialyzed biotinylated antibody was then used after titration of working concentrations.

Establishment of AD7C-NTP ELISA assay

Based on the principle of sandwich ELISA, mouse anti-AD7C-NTP antibody was used as a coating antibody,

biotinylated rabbit anti-AD7C-NTP antibody was used as a detection antibody, and horseradish peroxidase labeled avidin and chromogenic substrate were added afterwards. The brand name of the ELISA plate is Corning, USA. The absorbance value (OD) was measured at 450 nm with an LP400 plate reader (Barster, France). The urine AD7C-NTP concentration was calculated according to the standard curve of human recombinant AD7C-NTP protein.

Patients and Methods

Participants

In order to evaluate the value of clinical application of urine AD7C-NTP ELISA kit, AD patients ($n = 121$) were selected from the Department of Neurology at the Xuanwu Hospital of Capital Medical University and the Department of Geriatrics at the Beijing Chaoyang Hospital, China. All AD patients met National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimers Disease and Related Disorders Association (NINCDS-ADRDA) criteria for probable dementia on clinical grounds (12). Age-matched healthy participants ($n = 118$) were selected from the Department of Health Examination Center at the Xuanwu Hospital of Capital Medical University, China. All participants underwent a standardized clinical assessment, which included a medical history, and a physical and neurological examination together with mini mental state examination (MMSE) tests, Montreal Cognitive Assessment (MoCA) tests, psychometric evaluation, and MRI (13). Inclusion criteria: healthy participants with normal cognitive function. Exclusion criteria: a head MRI examination showing local lesions of the brain; cognitive impairment; metabolic syndrome; hypertension; diabetes; cancer; recent acute infection; severe cardiac, liver, or kidney dysfunction; cerebrovascular disease; severe Parkinson's disease; depression or anxiety; hypothyroidism; intracranial space-occupying; alcohol dependence; cognitive dysfunction after traumatic brain injury; or drug abuse. Informed consent was obtained for all participants. This clinical investigation was approved by the Ethics Committee at the Xuanwu Hospital of Capital Medical University, Beijing, China.

Urine sample collection

Patients with renal disease and urine abnormalities were excluded from the study. First morning urine samples of participants were taken before breakfast. Ten milliliters of urine was collected from each participant and immediately placed in a 4°C refrigerator.

Determination of urine AD7C-NTP concentration

According to the above AD7C-NTP ELISA assay, 100 μ l of standard or urine specimen was added to the pre-coated plate and incubated at 37°C for 1 hr. Following five consecutive wash steps with PBS, 100 μ l biotinylated rabbit anti-AD7C-NTP antibody was added and incubated at 37°C for another 1 hr. One hundred milliliters of horseradish peroxidase labeled avidin was added after five times of washing with PBS and incubated at 37°C for 30 min. Finally, 50 μ l of chromogenic reagent A and B was added in turn after five times of washing with PBS, and then incubated at 37°C for 15 min. The reaction was stopped by adding 50 μ l of sulfuric acid as stop buffer. The OD value of each sample was measured at 450 nm with an LP400 plate meter.

Statistical Analysis

An unpaired *t*-test was conducted with SPSS 11.5 software for Windows (Chicago, IL). Data are expressed as mean $\pm$ SEM. A rank-sum test was used for nonnormal distribution data. A *P*-value < 0.05 was considered statistically significant.

RESULTS

AD7C-NTP Antibody Identification

The ELISA titer of mouse and rabbit anti-AD7C-NTP antibody was determined to be 1:8,000 and 1:32,000, respectively. Figure 1 shows that AD7C-NTP immunopositive cells existed in the mouse frontal lobe and hippocampus as evidenced by immunohistochemical staining with the above two antibodies. Western blot detection of the hippocampus and temporal lobe of human brain by both mouse and rabbit anti-AD7C-NTP antibody showed a distinct single band with the relative molecular mass of 41 kDa, which is in accordance with the relative molecular weight of AD7C-NTP (Fig. 2).

Stability Test of the Coated ELISA Plate

The results showed that there was no significant reduction in the anti-AD7C-NTP antibody activity of coated plate following 37°C for 4 days or 4°C for 8 months (reduction $7.18\% \pm 1.2\%$ vs. $6.80\% \pm 0.9\%$, all $< 12\%$, $P > 0.05$; Fig. 3).

Methodology of AD7C-NTP ELISA Detection

Optimization of ELISA conditions includes the following aspects.

Fig. 1. Immunohistochemical staining of AD7C-NTP in the mouse frontal lobe and hippocampus. The expression of AD7C-NTP in the mouse frontal lobe (A) and hippocampus (B) was detected by immunohistochemistry using mouse and rabbit anti-AD7C-NTP antibodies, respectively. The AD7C-NTP immunopositive cells are stained in brown color. Scale bar = 50 µm.

Fig. 2. AD7C-NTP is expressed in the temporal lobe and hippocampus of AD patients. The protein expression of AD7C-NTP in the temporal lobe (A) and hippocampus (B) of AD patients was measured by Western blot assay using specific mouse anti-AD7C-NTP antibody. 1:6,000, 1:6,000 dilution of anti-AD7C-NTP antibody; 1:3,000, 1:3,000 dilution of anti-AD7C-NTP antibody; M, prestained protein standard.

Specificity

There was no cross-reaction between AD7C-NTP and the phosphorylation of Tau protein or A β 42 (both < 0.4 ng/ml).

Precision

Two samples of AD7C-NTP at concentrations of 2 and 8 ng/ml had an intrabatch Coefficient of variance (CV) of 3.8% and 4.5%, and interbatch CV of 7.6% and 6.8%.

Standard curve and sensitivity

As shown in Figure 4A, there was a very good linear relationship between AD7C-NTP concentration (0–10 ng/ml) and OD value ($R^2 > 0.99$, $P < 0.001$), which indicates that the credibility is very high.

Linear relationship

There was a good linear relationship between AD7C-NTP OD value and multiple dilution ratios ($R^2 = 0.996$).

Fig. 3. Stability test of the coated ELISA plate. The reduction of anti-AD7C-NTP antibody activity of coated plate following between the two groups, 37°C for 4 days and 4°C for 8 months, were compared.

Fig. 4. The concentration of urine AD7C-NTP. (A) The standard curve of urine AD7C-NTP ELISA detection. (B) The area under the curve of urine AD7C-NTP ELISA detection. When the best critical point of the ROC curve of urine AD7C-NTP is 1.5 ng/ml, the sum of sensitivity and specificity reaches the maximal value; that is, at a sensitivity of 89.3% and specificity of 84.7%, the area under the curve is 0.926 and the diagnostic value is the highest.

Measurement of Human Urine AD7C-NTP Level by ELISA

There was no difference in age between the AD group and the healthy group ($t = 1.535$, $P = 0.126$). The concentration of urine AD7C-NTP of the AD group was higher than that of the control group (2.25 ± 0.83 ng/ml vs. 0.82 ± 0.21 ng/ml, $P < 0.0001$). The area under the curve of urine AD7C-NTP ELISA detection is shown in Figure 4B. When the best critical point of Receiver operating characteristic curve (ROC) curve of urine AD7C-NTP is 1.5 ng/ml, the sum of sensitivity and specificity reaches the maximal value; that is, at a sensitivity of 89.3% and specificity of 84.7%, the area under the curve is 0.926 and the diagnostic value is the highest.

DISCUSSION

This is the first independent research and development of a human AD7C-NTP kit for clinical diagnosis of AD in China. Our research group has been devoted to the development of a urine AD7C-NTP diagnostic kit in recent

years (14–16). We aimed to develop an AD diagnostic kit with independent intellectual property rights, simple and convenient detection, low price, and acceptance by a vast number of patients. In this study, we synthesized various peptides fragments with a self-designed antigenic determinant and used them to immunize animals, and subsequently prepared a specific anti-AD7C-NTP antibody for the ELISA kit. The urine AD7C-NTP ELISA kit has a sensitivity of 89.3% and a specificity of 84.7%. The ELISA kit can distinguish AD patients from normal controls effectively, which is expected to be used in the clinical diagnosis of AD.

The main clinical manifestations of AD are cognitive dysfunction, memory loss, and personality disorders. Pathological features of this disease include diffuse cortical atrophy, senile plaques deposition, NFTs formation, and neuronal loss (17). If early diagnosis of AD can be achieved, preventative measures can be taken to improve patient prognosis. However, biomarkers that can help early diagnosis of AD are scarce. In 1997, De la Monte isolated a membrane-associated phosphoprotein, AD7C-NTP, which is a member of the NTPs (18). Afterwards, elevated levels of the AD7c-NTP were found in cortical

neurons, brain tissue extracts, cerebrospinal fluid (CSF), and urine early in the course of AD neurodegeneration (10, 19).

A previous study using a competitive ELISA method to detect the AD7C-NTP level in human urine specimens showed that the mean assay measurement in the AD group was significantly higher than that in the non-AD dementia control group and in the nondementia control group (20). Also, the mean assay measurement in early AD cases was significantly lower than that in other AD cases (20). However, the experimental process is complex and is not convenient for clinical use. Thus, in this study, we designed a human urine AD7C-NTP ELISA kit. The kit's application in clinical work will make the diagnosis of AD more convenient due to its advantages of simplicity of operation, acceptable price, and stable reliability. Our study shows the specificity of our kit is 84.7%, which is lower than other kits available (with a specificity of 90%; (21)). The reason for the lowered specificity may be due to the different preparation methods of antigen and antibody, which in turn make the production of our ELISA kit more simple and convenient compared to other kits. Further study is needed to verify the application value of our urinary AD7C-NTP ELISA kit in the early diagnosis of AD.

CONCLUSION

Our study demonstrated that our newly developed urine AD7C-NTP ELISA kit has high specificity and sensitivity in the diagnosis of AD in a Chinese population, suggesting it may be useful for detecting early AD.

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CONFLICT OF INTEREST

There are no ethical/legal conflicts involved in the article.

ABBREVIATIONS

AD = Alzheimer's disease
AD7c-NTP = Alzheimer-associated neuronal thread protein

CSF = cerebrospinal fluid
ELISA = enzyme-linked immunosorbent assay
NTPs = neuronal thread proteins

REFERENCES

1. Marilyn SA, Steven TD, Dennis D, et al. The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 2011;7:270-279.
2. Sun F, Mao X, Xie L, David AG, Kunlin J. Neuroglobin protein is upregulated in Alzheimer's disease. *J Alzheimers Dis* 2013;36: 659-663.
3. Hebert LE, Weuve J, Scherr PA, Evans DA. Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. *Neurology* 2013;80:1778-1783.
4. Huisain MM, Garrett RK. Clinical diagnosis and management of Alzheimer's disease. *Neuroimaging Clin N Am* 2005;15:767-777.
5. Barnes DE, Yaffe K, Byers AL, McCormick M, Schaefer C, Whitmer RA. Midlife vs late-life depressive symptoms and risk of dementia: differential effects for Alzheimer disease and vascular dementia. *Arch Gen Psychiatry* 2012;69:493-498.
6. de la Monte SM, Carlson RI, Brown NV, Wands JR. Profiles of neuronal thread protein expression in Alzheimer's disease. *J Neuropathol Exp Neurol* 1996;55:1038-1050.
7. de la Monte SM, Wands JR. Neurodegeneration changes in primary central nervous system neurons transfected with the Alzheimer-associated neuronal thread protein gene. *Cell Mol Life Sci* 2001;58:844-849.
8. Kahle PJ, Jakowee M, Teipel SJ, et al. Combined assessment of tau and neuronal thread protein in Alzheimer's disease CSF. *Neurology* 2000;54:1498-1504.
9. de la Monte SM, Ghanbari H, Ghanbari K, et al. AD7C-NTP biomarker for Alzheimer's disease. *Alzheimers Rep* 1999;2:327-332.
10. de la Monte SM, Wands JR. The AD7C-NTP neuronal thread protein biomarker for detecting Alzheimer's disease. *J Alzheimers Dis* 2001;3:345-353.
11. Marcarelle LA, Bertozzi CR. *Chem Eur J* 1999;5:1384-1390.
12. Jack CR Jr, Albert MS, Knopman DS, et al. Introduction to the recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 2011;7:257-262.
13. Sarah TP, Jose M, Linda B, et al. MoCA, ACE-R, and MMSE versus the National Institute of Neurological Disorders and Stroke—Canadian Stroke Network Vascular Cognitive Impairment Harmonization Standards Neuropsychological Battery after TIA and stroke. *Stroke* 2012;43:464-469.
14. Wang HJ, Wang R. AD7c-NTP-A new biomarker for Alzheimer's disease. *Chin J Geront* 2007;27:699-701.
15. Wang HJ, Zhao ZW, Ji ZJ, et al. Coexistence of AD7C-NTP and insulin receptor in CA1 area of hippocampus in mouse. *J Capit Univ Med Sci* 2007;24:324-327.
16. Zhang ZP, Ji ZJ, Zhao ZW, et al. Existence and significance of AD7C-NTP in the brain of patients with Alzheimers disease and in different species of animals. *J Capit Univ Med Sci* 2008;29:159-161.
17. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 2002;297:353-356.

18. Monte SM, Ghanbari K, Frey WH, et al. Characterization of the AD7C-NTP cDNA expression in Alzheimer's disease and measurement of a 41-kD protein in cerebrospinal fluid. *J Clin Invest* 1997;100:3093–3104.
19. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 2002;297:353–356.
20. Munzar M, Levy S, Rush R, Averbach P. Clinical study of a urinary competitive ELISA for neural thread protein in Alzheimer disease. *Neurol Clin Neurophysiol* 2002;2002:2–8.
21. Jian Z, Chun-Hua Z, Rong-Jie L, et al. Accuracy of urinary AD7c-NTP for diagnosing Alzheimer's disease: a systematic review and meta-analysis. *J Alzheimers Dis* 2014;40:153–159.