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Clinical Study of a Urinary Competitve ELISA for Neural Thread Protein in Alzheimer Disease

■ AD7C-NTP (neural thread protein) is a ≈41-kD brain protein that is selectively elevated in Alzheimer disease (AD). AD7C-NTP is associated with the pathologic changes of AD, and overexpression of the AD7C-NTP gene is associated with cell death similar to that found in the AD brain. A newly developed competitive ELISA (enzyme-linked immunosorbent assay) was tested in urine samples from patients with AD, patients with non-AD dementia, and healthy normal individuals. Mean assay measurement in the AD group (30.1 ± 10.8) was significantly higher than in the non-AD dementia control group (13.4 ± 3.4) and in the nondementia control group (14.8 ± 5.2) ($P < .001$). Mean assay measurement in early-AD cases (25.3 ± 7.6) was significantly lower than in other AD cases (33.9 ± 11.4). Levels of more than 18 units were found in 89% of overall AD cases and in 10% of overall controls. The results further validate urinary AD7C-NTP as a biochemical marker for AD and indicate that the competitive ELISA-format AD7C-NTP test in urine is an accurate method for determining AD7C-NTP levels in AD. ■

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Alzheimer disease (AD) is the most common cause of dementia in the Western hemisphere. The disease is characterized pathologically by prominent atrophy of corticolimbic structures with neuronal loss, neurofibrillary tangle (NFT) formation, aberrant proliferation of neurites, senile plaques, and amyloid deposition in the brain. More than 90% of AD is sporadic. The reported accuracy of antemortem clinical diagnosis of AD is quite variable, and a reliable biochemical marker is needed to help in the accurate and early diagnosis of AD (Molsa et al, 1985; Rocca et al, 1986; Burns et al, 1990; Risse et al, 1990; Gilleard et al, 1992; Mendez et al, 1992; Corey-Bloom et al, 1995; Fleming et al, 1995; Bowler et al, 1998; Lannfelt, 1998; Sandson and Price, 1999; Galton et al, 2000; Diaz, 2001). There is an average delay of nearly 3 years from when initial symptoms appear to when AD is diagnosed (Jorst and Grossberg, 1995). Apolipoprotein E ε4 allele is a genetic risk factor that is not found in 50% of cases of AD (Myers et al, 1996), and τ- and β-amyloid protein measurements in cerebrospinal fluid (CSF) and serum Aβ have significant overlap between AD and non-AD levels (<60% sensitivity, <80% specificity), limiting their usefulness (Pirtilla et al, 1994; Arai et al, 1995; Jensen et al, 1995; Motter et al, 1995; Munroe et al, 1995; Nitsch et al, 1995; Tata et al, 1995; van Gool et al, 1995; Vigo-Pelfrey et al, 1995; Pirtilla et al, 1996; Tamaoka et al, 1996; Iwatsubo, 1998). More recently, other potential AD biomarkers have been described, such as 8-hydroxy-2-deoxyguanosine (Lovell and Markesbery, 2001) and hemeoxygenase-1 (Schipper et al, 2000), but these have yet to be clinically validated. Suggested criteria for potential AD biomarkers have been described (Growdon et al, 1998).

In AD, dementia is caused by cell loss and impaired synaptic function. Cell loss is mediated by factors such as increased apoptosis, predisposition to apoptosis, and impaired mitochondrial function. Neural thread proteins (NTPs) are a novel family of recently characterized brain proteins (de la Monte et al, 1999). AD7C-NTP is a ≈41-kD-membrane–associated phosphoprotein with functions related to neuritic sprouting and cell death (de la Monte et al, 1997). The AD7C-NTP gene is overexpressed in AD beginning early in the course of the disease, and AD7C-NTP accumulation in neurons colocalizes with phospho-τ immunoreactivity (de la Monte, Carlson, et al, 1996; de la Monte et al, 1999). Induction of AD7C-NTP gene expression results in increased cell death mediated by apoptosis, impaired mitochondrial function, and increased cellular levels of the p53 and CD95 pro-apoptosis gene products as occur in AD, and overexpression of AD7C-NTP is associated with increased levels of phospho-τ (de la Monte and Wands, 2001). AD7C-NTP overexpression

probably has a direct role in mediating some of the important cell-death cascades associated with AD neurodegeneration.

AD7C-NTP mRNA is upregulated in AD brains compared with control brains, AD7C-NTP levels are higher in brains and CSF of patients with AD than in brains and CSF of controls, and AD7C-NTP immunoreactivity is found in senile plaques, NFTs, degenerating neurons, neuropil threads, and dystrophic neuritic sprouts in brains of patients with AD and Down syndrome (Ozturk et al, 1989; de la Monte et al, 1990; de la Monte and Wands, 1992; de la Monte, Carlson, et al, 1996; de la Monte, Xu, Huchins, and Wands, 1996; de la Monte, Xu, and Wands, 1996; de la Monte et al, 1997). AD7C-NTP accumulation in neurons occurs early in AD neurodegeneration, before NFT formation. AD7C-NTP has been shown to be consistently elevated in the CSF and urine of patients with AD compared with controls (Chong et al, 1992; de la Monte et al, 1992; de la Monte et al, 1997; Fitzpatrick et al, 2000; Kahle et al, 2000). Specificity of the AD7C-NTP elevation in AD was shown in comparison with non-AD neurologic-disease controls, and AD7C-NTP elevation was positively correlated with degree of dementia (de la Monte et al, 1997; de la Monte et al, 1999; Kahle et al, 2000).

We report here data from a clinical study of a new quantitative competitive ELISA (enzyme-linked immunosorbent assay) for AD7C-NTP. Urinary competitive ELISA measurements, performed on first-of-morning urine samples from AD patients, non-AD dementia patients, and nondemented controls, demonstrate the potential utility of this assay.

Methods

Clinical Samples

Urine samples for the possible/probable AD group came from 102 individual physicians throughout the United States. One hundred twenty-two cases were retrospectively studied on a consecutive as-available unselected basis. They were obtained from patients who were being investigated for possible AD by physicians who were independently evaluating the AD7C-NTP competitive ELISA test. The AD7C-NTP test is certified by the Clinical Laboratory Improvements Act (CLIA) of New Jersey (#18755), Florida (#L800013272), and Maryland (#811) and by the US federal CLIA (#21D0917550). The clinical diagnoses for these cases were obtained from the patients' physicians by survey within 4 weeks of urine testing. Diagnostic criteria for the cases used in this study were not controlled, and they may be different from physician to physician, but the study reflects a realistic situation in the field. All the subjects are still living; therefore, postmortem confirmation of the diagnosis has not been possible. Patients with known renal disease or urinary abnormalities were excluded from the study. All results are reported as means and standard deviations. The control groups consisted of 13 patients (Table 1) with non-AD dementia (3 men, mean age 58.7 ± 30.4 y; 10 women, mean age 72.5 ± 13.4 y) and 27 nondemented individuals (12 men, mean age 65.5 ± 11.4 y; 15 women, mean age 63.5 ± 10.5 y). An additional group of 69 consecutive as-available non–age-matched normal control patients were also studied for potential drug interference effects in the assay. The dementia group included a wide variety of cases commonly presenting to physicians. The non-AD dementia group included patients with corticobasal degeneration, delirium, depression, epilepsy, hyperparathyroidism, Lewy body disease, metabolic encephalopathy, multi-infarct dementia, multiple sclerosis, pseudodementia, and psychosis. All clinical data used in the studies were compiled without knowledge of the assay results. Patient information and case diagnoses were unknown to the technologists who received and analyzed the samples.

Materials

Microtiter plates coated with NTP-specific receptor-antibody (Nymox, Maywood, NJ) were used. Alkaline phosphatase conjugated rabbit anti-mouse IgG was obtained from Serex. Saline (TBS) tablets and sodium azide were obtained from Sigma. Other reagents include tris-buffered Chemistrip (Roche), Centricon® YM-10 and YM-100 (Amicon 4205), TBS wash buffer P1379 (Sigma), alkaline phosphatase conjugate stock buffer (Sigma BSA and TBS), sodium

Table 1 Clinical Diagnosis, Age, Sex, and AD7C-NTP Values of Patients with Non–Alzheimer-Disease Dementia

Diagnosis	*Age (y)*	*Sex*	*AD7C-NTP Value (RU/mL)*
Corticobasal degeneration	81	M	6.6
Delirium	71	M	12.4
Depression	81	F	14.3
Depression	84	F	12.5
Epilepsy	24	M	18
Hyperparathyroidism	77	F	15.2
Lewy body disease	70	F	15.9
Metabolic encephalopathy	65	F	15.6
Multi-infarct dementia	81	F	17.2
Multiple sclerosis	44	F	9.3
Pseudodementia	61	F	16.2
Psychosis	90	F	8.7
Psychosis	72	F	13.6

RU indicates recombinant AD7C-NTP units.

azide (Sigma), paranitrophenyl phosphate (Moss), and 0.22 µm syringe filters (Gelman 4192).

Sample Collection and Processing

Using a sterile plastic container, first-of-morning void urine samples were collected. A tablet of Stabilur (Globe Scientific) was added to the urine sample. The urine was immediately refrigerated at 4°C for up to 24 hours before processing. Before sample preparation for the study, a complete urinalysis was carried out by standard methods. Samples were centrifuged at 3000*g* for 15 minutes in a Sorval RC2-B centrifuge to remove cellular debris. The urine was then filtered using a Gelman syringe with a 0.22 µm cellulose acetate (Millipore) filter, and the filtrate was brought to 0.05% sodium azide by adding 0.5% sodium azide 1:10 with filtered supernatant. This solution was then placed in the top of an Amicon Microcon YM-10 and centrifuged at 5000 rpm for 1 hour and then reconstituted to the original volume with TBS. The Amicon YM-10 centrifugation-and-reconstitution step was then repeated twice (30 min/spin). The final retentate was reconstituted to 0.5 mL with TBS. This solution was then placed in the top of an Amicon Microcon YM-100 and was centrifuged at 10,000 rpm for 5 minutes in a Fisher Scientific Marathon 13K/M centrifuge. The volume in the Microcon was diluted 1:4 in TBS.

Assay Procedure

Samples, standards (recombinant AD7C-NTP; de la Monte et al, 1997), and controls (high, medium, low) were each tested in triplicate. Fifty microliters of sample and 50 µL of alkaline phosphatase conjugated rabbit anti-mouse antibody were pipetted into each well, incubated for 60 minutes at room temperature, and then washed 3 times with TBS/Tween 20 (0.05%) buffer. One hundred fifty microliters of PNPP were then added, and the color reaction was read on a Bio Rad 550 microplate reader at 405 nm (Bio Rad, Hercules, CA). Readings were derived from a standard curve constructed with Microplate Manager III using data from wells with recombinant AD7C-NTP. Mean values were compared using the Student *t* test, and receiver operating characteristic (ROC) curves were plotted to maximize the sum of sensitivity and specificity (ROC curves plot sensitivity versus 1-specificity at different cutoff points; Hanley and McNeil, 1983).

Results

Patient data are illustrated in Figure 1 and summarized in Table 2. The mean value in the AD group (30.1 ± 10.8) was significantly higher than in the non-AD dementia control group (13.4 ± 3.4) and in the nondementia control group (14.8 ± 5.2) ($P <$ .001). There was no significant difference between the non-AD dementia control group and the nondemented control group. There were no significant age or sex differences either in the AD group or in the control groups. Data from the 69 random control cases showed no false-positives attributable to a drug effect. Early-AD cases (25.3 ± 7.6) were significantly lower than other AD cases (33.9 ± 11.4) ($P <$ .001). Values of more than 18 recombinant AD7C-NTP units were found in 89% of overall AD cases and in 10% of age-matched overall controls. Values of more than 19 units were found in 86.6% of overall AD cases and in 7.5% of overall controls. Sensitivity and specificity at various index cutoffs are plotted in the ROC curve where the non-AD dementia controls and the nondemented controls have been pooled together as the control group (Fig. 2). At the 18-unit cutoff, sensitivity was 89%, specificity was 90%, positive predictive value was 94.8%, and negative predictive value was 80%. Sensitivity was slightly higher for AD cases (91.3%) than for early-AD cases (86.1%).

Discussion

The data confirm previous studies that have indicated that AD7C-NTP is a highly sensitive and specific marker for AD (Chong et al, 1992; de la Monte and Wands, 1992; de la Monte et al, 1997; de la Monte et al, 1999; Fitzpatrick et al, 2000; Kahle et al, 2000). The competitive ELISA for AD7C-NTP in urine in this trial has an optimized sensitivity (percentage of AD cases testing positive) of 89% and a specificity (percentage of control cases testing negative) of 90%. These results are similar to those of previous studies—89% sensitivity and 89% specificity in de la Monte et al (1997); 70% sensitivity and 87% specificity in Kahle et al (2000); and 91% sensitivity and 97% specificity in Fitzpatrick et al (2000). It will be useful in future studies to assess the new urinary assay in larger numbers of other neurologic and psychiatric conditions, such as mild cognitive impairment, and other types of dementia, such as Pick disease or Creutzfeldt–Jakob disease.

Earlier studies have shown that elevation of NTP correlates with severity of AD cognitive impairment measured by the Blessed Dementia Scale (de la Monte et al, 1997) and by the Mini-Mental State Examination (Kahle et al, 2000). It is therefore interesting to note in the present study that mean AD7C-NTP urinary measurements were significantly lower in early-AD cases than in other AD cases. This result supports the previous findings that AD7C-NTP is higher with progression of AD.

Compared with the earlier AD7C-NTP research assays (eg, sandwich ELISA), the new competitive ELISA involves far fewer steps (overall technician time of a few hours compared with 2 d) with much

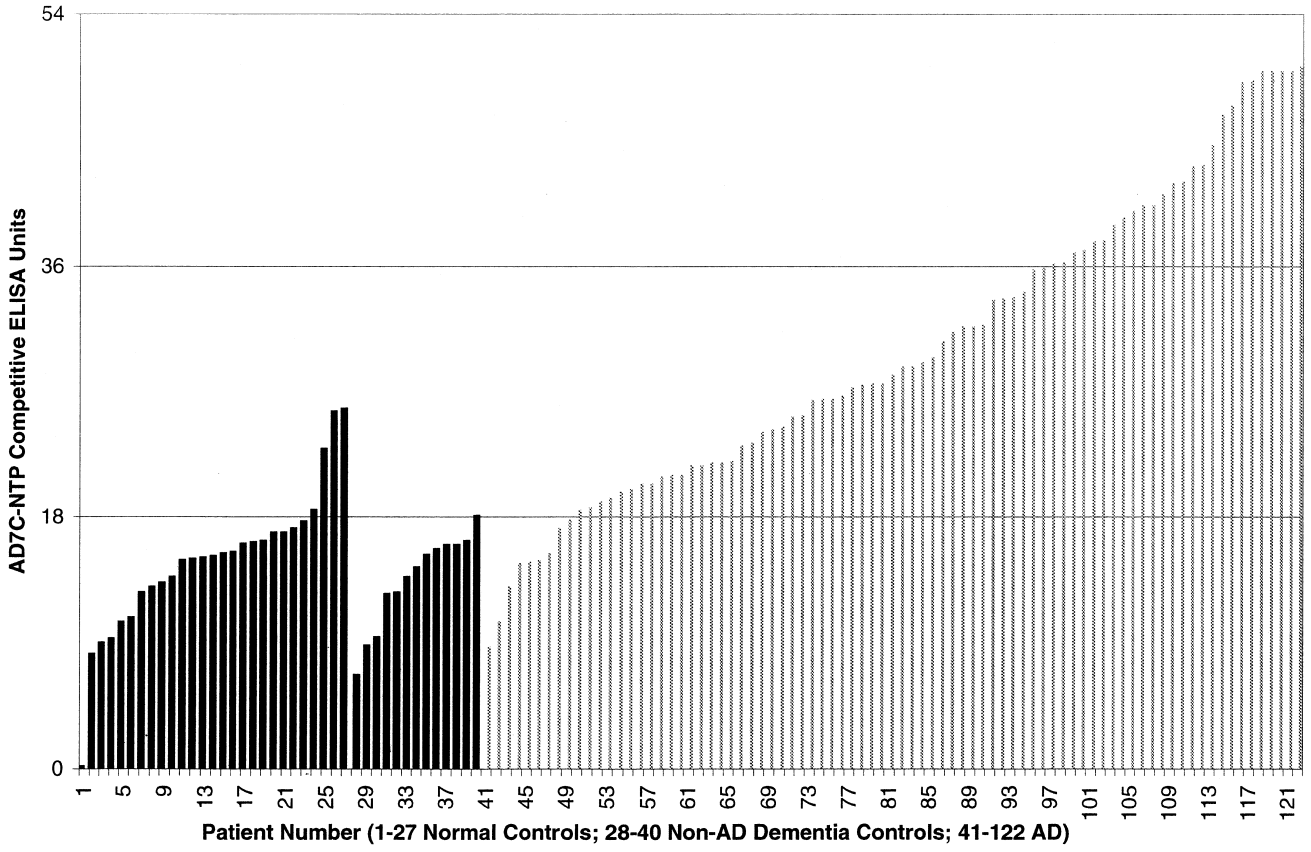

Figure 1 Distribution of competitive ELISA (enzyme-linked immunosorbent assay) AD7C-NTP values in patients with Alzheimer disease and in healthy controls.

Table 2 Population Profiles and Urinary Competitive ELISA Values for AD7C-NTP

Group	*No. Cases*	*Mean Age (y)*	*AD7C-NTP Value (RU/mL)*
AD			
Total	82	69.5 ± 10.8	30.1 ± 10.8*
Early cases	36	72.1 ± 9.1	25.3 ± 7.6*
Excluding early cases	46	67.4 ± 11.6	33.9 ± 11.4*
Controls			
Non-AD dementia	13	69.3 ± 18.0	13.4 ± 3.4
Nondemented	27	64.7 ± 10.8	14.8 ± 5.2

ELISA indicates enzyme-linked immunosorbent assay; RU, recombinant AD7C-NTP units; AD, Alzheimer disease.
*Statistically significant difference ($P < .001$) compared with controls.

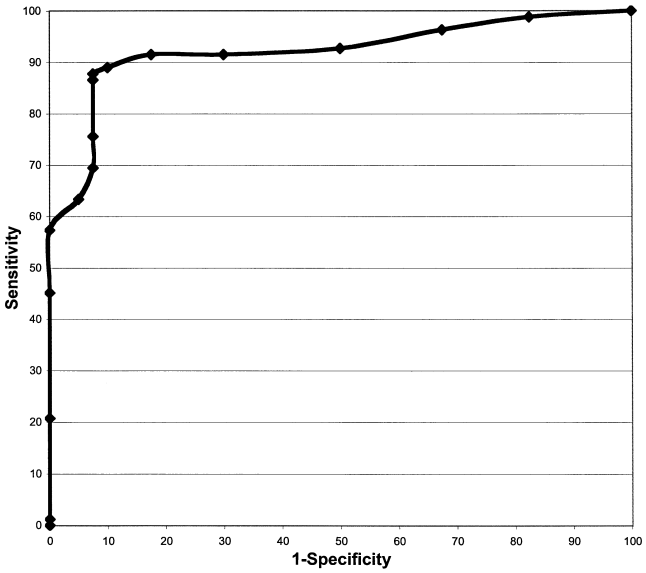

Figure 2 Receiver operating characteristic curve shows sensitivity versus 1-specificity for urinary competitive ELISA (enzyme-linked immunosorbent assay) for AD7C-NTP in patients with Alzheimer disease and in healthy controls.

shorter incubation time (1 h compared with overnight) and has much less potential for technician error. The new urinary competitive ELISA for NTP has significant potential to aid in the evaluation of therapeutics for AD. With an accurate noninvasive peripheral marker that seems to be quantitatively higher

with progression of AD, therapeutic trials can be monitored more frequently and objectively. A technically simple and accurate noninvasive peripheral test such as the new ELISA for NTP will have significant practical use in the routine clinical evaluation of elderly patients at risk for AD.

Conclusion

AD7C-NTP in first-of-morning urine as measured by competitive ELISA is significantly higher in AD patients than in age-matched non-AD demented controls and in nondemented controls, with 89% sensitivity and 90% specificity in this study. AD7C-NTP is further validated as a biochemical marker for AD. Urinary competitive ELISA is an accurate method for determining AD7C-NTP levels in AD.

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