

Clusterin as a Blood Biomarker for Diagnosis of Mild Cognitive Impairment and Alzheimer's Disease

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ABSTRACT

With increasing global life expectancy, Alzheimer's disease will become an increasingly prevalent health problem. The development of biomarkers that predict risk for both Alzheimer's disease and mild cognitive impairment will be useful for early diagnosis of dementia. To date, no surrogate blood biomarker exists to classify between Alzheimer's disease/mild cognitive impairment and normal controls or Alzheimer's disease and mild cognitive impairment/normal control as a diagnostic parameter. In this study, we analyzed serum levels of amyloid- β 40 ($A\beta_{40}$), amyloid- β 42 ($A\beta_{42}$), clusterin (CLU) and p97 using ELISA kits from 157 subjects with normal cognition, mild cognitive impairment and Alzheimer's disease. We found a significant increase in serum levels of $A\beta_{42}$ ($P < 0.05$) and serum clusterin ($P < 0.001$) between normal and Alzheimer's disease subjects and between normal and mild cognitively impaired subjects. In contrast, serum $A\beta_{40}$ and p97 levels did not differ significantly between all groups. We also used receiver operating characteristic curves to determine the cut-off point of $A\beta_{42}$ and clusterin to differentiate either cognitively normal from cognitively impaired subjects (both Alzheimer's disease and mild cognitive impairment) or cognitively normal and mild cognitively impaired subjects from those with Alzheimer's disease. Only clusterin with 84% sensitivity, 75% specificity and good accuracy of diagnosis showed promise for diagnosing patients with cognitive impairment (Alzheimer's disease and mild cognitive impairment).

Keywords: Alzheimer's disease, Mild cognitive impairment, Biomarker, Clusterin, $A\beta_{42}$

INTRODUCTION

With increasing global life expectancy, Alzheimer's disease (AD) will become an increasingly prevalent health problem. Alzheimer's disease is the most common type of dementia; approximately one in eight people over the age of 65 years are at risk of developing Alzheimer's disease (Kidd, 2008). Currently, accurate diagnosis of Alzheimer's disease is based on medical history, neurological tests and clinical examination criteria (McKhann et al., 1994; APA, 1994). To date, there is no reliable diagnostic test for Alzheimer's disease. In addition, no adequate treatments to cure or prevent disease progression exist. Thus, early detection of preclinical Alzheimer's disease among mild cognitive impaired (MCI) patients may provide greater opportunity for early treatment and monitoring of disease progression (Morris, 2005).

The majority of the studies of accepted Alzheimer's disease biomarkers to date have been carried out using samples of cerebrospinal fluid obtained by lumbar puncture (Blennow and Hampel, 2003). This is an invasive procedure, particularly in elderly people, which is unsuitable for routine laboratory testing. Recently, development of diagnostic tests for Alzheimer's disease and mild cognitive impairment has begun to focus on blood biomarkers (Ray et al., 2007; German et al., 2007). There is evidence that Alzheimer's disease-associated amyloid- β ($A\beta$) proteins, and in particular amyloid- β 42 ($A\beta_{42}$), are the main components of amyloid senile plaques in Alzheimer's disease pathogenesis (Selkoe, 1994). $A\beta_{42}$ is initially deposited in the Alzheimer's patient's brain instead of the more predominant amyloid- β 40 ($A\beta_{40}$) (Younkin, 1995). Mayeux and colleagues found that plasma $A\beta_{42}$ may be elevated many years before the onset of sporadic Alzheimer's disease (Mayeux et al., 1998). Another study suggested that an increased level of $A\beta_{42}$ in the plasma of Alzheimer's patients may be involved in the pathology of Alzheimer's disease in the brain (Abdullah et al., 2009). Besides $A\beta$, other plasma/serum proteins have been reported to be associated with Alzheimer's disease. For example, proteins in the apolipoprotein family, such as apolipoprotein E (ApoE) and apolipoprotein J (ApoJ), or clusterin, are expressed in both brain and plasma, and bind to $A\beta$ with high affinity (Wilson et al, 2008; Bell et al., 2007). Reports have also linked melanotransferin (MTf), or p97, with Alzheimer's disease. Levels of p97 in the serum of Alzheimer's patients are often higher than those in non-demented elderly controls or in patients with other dementias (Kim et al., 2001; Ujiie et al., 2002).

Unfortunately, to date, there is no surrogate blood biomarker for classification between Alzheimer's disease, mild cognitive impairment and controls as a diagnostic parameter. The aim of this study was to compare the serum protein biomarkers, including $A\beta_{40}$, $A\beta_{42}$, clusterin and p97, between subjects with normal cognition, mild cognitive impairment and Alzheimer's disease for diagnosis and monitoring the progression in Alzheimer's disease by using an enzyme-linked immunosorbent assay (ELISA). This may then prove useful as a diagnostic tool for Alzheimer's disease and improve diagnostic accuracy of early Alzheimer's disease. An ideal biomarker should reflect the neuropathology of Alzheimer's disease and can be used in monitoring the progression of the disease and the

efficacy of therapy for Alzheimer’s disease. Moreover, the focus of this interest is not only limited to patients with Alzheimer’s disease, but also extends to those individuals at increased risk for Alzheimer’s disease, mild cognitive impairment.

Table 1. Demographic characteristics of the subject groups.

	Normal Subject	MCI	Mild AD	Moderate AD	Severe AD	*P value
Total sample	32	33	30	30	32	-
Sex (M/F)	9/23	12/21	14/26	10/20	11/21	0.169
Age	74.9±7.20	74.1±7.05	76.3±7.98	78.2±8.17	76.4±(10.01)	0.633
MMSE-Thai Score	28.90±1.04	25.77±2.47	20.67±2.91	14.72±3.16	8.96±4.03	<i>P</i> < 0.001

Note: Values are expressed as mean ± SD, M = male, F = female, **P* < 0.05 was considered statistically significant

MATERIALS AND METHODS

Subjects

The study population consisted of 157 subjects in three groups: 92 with Alzheimer’s disease (mild AD, n= 30; moderate AD, n=30; severe AD, n=32), 33 with mild cognitive impairment and 32 cognitively normal subjects. Clinically diagnosed patients with Alzheimer’s disease and mild cognitive impairment were recruited from Suanprung Psychiatric Hospital, Chiang Mai, Thailand. All patients underwent a standard clinical assessment, including neurological, physical and psychiatric examinations. Criteria for controls were absence of memory complaints or other cognitive symptoms, preservation of general cognitive functioning and no active neurological or psychiatric disease. The Mini-Mental State Examination-Thai Version 2002 score (MMSE-Thai score) was added for investigation and classification of cognitive function in Alzheimer’s disease, mild cognitive impairment and normal subjects. The Ethics Committees at Suanprung Psychiatric Hospital and the Faculty of Associated Medical Sciences, Chiang Mai University approved the study. Demographic characteristics of the subjects are shown in Table 1.

Sample collection

Five milliliters of venous blood samples were collected from all participants. After clotting, blood was centrifuged at 1,800 g for 10 minutes. Serum samples were aliquoted into microcentrifuge tubes and stored at -70°C before analysis.

Serum protein analysis of Aβ₄₀, Aβ₄₂, clusterin and p97 by ELISA

A concentration of Aβ₄₀, Aβ₄₂, clusterin and p97 were measured by ELISA techniques in Alzheimer’s disease, mild cognitive impairment, and normal subject serum samples, as instructed by the manufacturer. Samples were measured in duplicate. All samples had undergone only one freeze-thaw cycle and were analyzed within 12 months of collection.

Table 2. Concentration of A β_{40} , A β_{42} , clusterin (CLU) and p97 levels in subject groups.

Tests	Normal Subject	MCI	Mild AD	Moderate AD	Severe AD
A β_{40} (pg/ml)	14.37 \pm 9.37	16.30 \pm 14.22	15.10 \pm 9.94	20.99 \pm 16.09	15.58 \pm 11.15
A β_{42} (pg/ml)	1.26 \pm 1.97	1.91 \pm 2.13*	1.97 \pm 2.02*	1.30 \pm 1.44*	1.59 \pm 1.81*
CLU (ng/ml)	83.25 \pm 24.78	106.61 \pm 57.24**	121.91 \pm 70.94**	110.33 \pm 36.45**	113.73 \pm 62.93**
p97 (pg/ml)	1002 \pm 599.82	995 \pm 698.21	1114 \pm 792.17	956 \pm 720.67	1350 \pm 899.87

Note: Values are expressed as mean \pm SD, * $P < 0.05$, ** $P < 0.00$

A β_{40} & A β_{42}

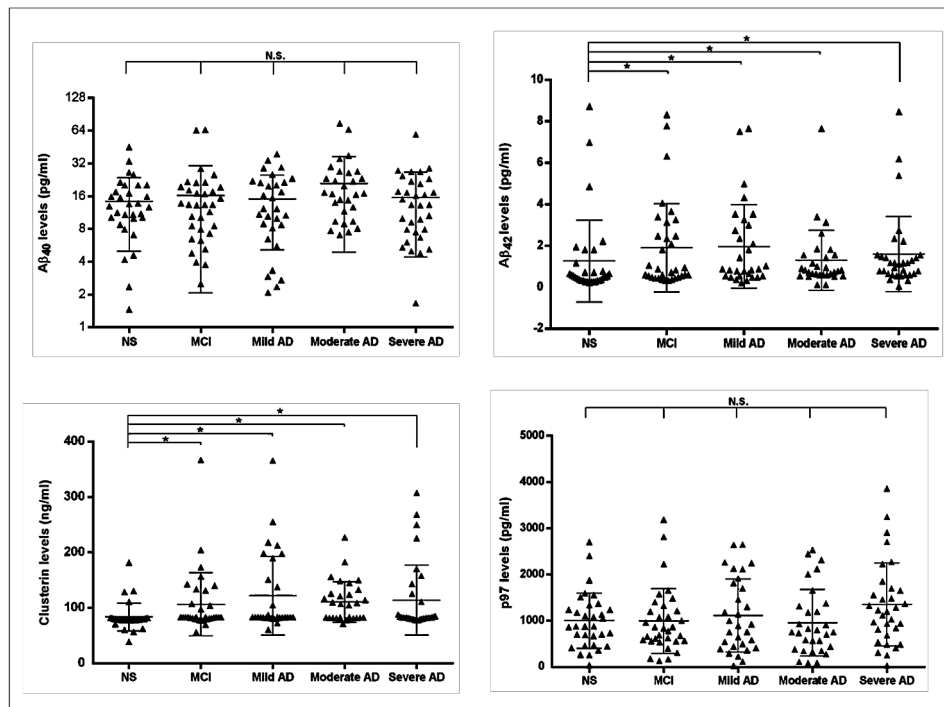
Serum A β_{40} and A β_{42} levels were measured using sandwich ELISA kit (Invitrogen, Camarillo, USA). The ELISA plates were pre-coated with monoclonal antibody specific for the NH₂-terminus of Hu A β_{40} and A β_{42} . Briefly, 50 μ l of A β peptide standards, controls or serum samples were added into the microplate wells. Fifty microliters of Hu A β_{40} or A β_{42} detection antibody were then pipetted into the wells. After incubating for 3 hours at room temperature with shaking, a solution of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was added and incubated for 30 min at room temperature with shaking. Finally, 100 μ l of substrate was added and the absorbance was read at 450 nm on a microplate reader.

Clusterin

Serum clusterin levels were detected using a human clusterin competitive ELISA Kit (Adipogen, Incheon, Korea). The wells of a 96-well ELISA plate were coated with human clusterin recombinant protein. Briefly, 50 μ l of serum samples, human clusterin standard or control sample were added into microplate wells followed by the addition of 50 μ l of clusterin detection antibody. After 1 hour incubation at 37°C, a solution of HRP-conjugated anti-rabbit IgG was added and incubated for 1 hour at 37°C. Finally, 100 μ l of substrate was added and the absorbance was read at 450 nm on a microplate reader.

p97

Serum p97 levels were estimated using sandwich ELISA kit (USCN Life Science Inc., China). The 96-well ELISA plates were pre-coated with anti-MTf (p97) antibodies. Briefly, 100 μ l of standard blank or serum sample were added into microplate wells. Then 100 μ l of p97 biotin-conjugated detection antibody were pipetted into the wells. After 1 hour incubation at 37°C, a solution of avidin conjugated to HRP was added and incubated for 30 min at 37°C. Finally, 90 μ l of substrate was added and the absorbance was read at 450 nm on a microplate reader.



Note: All data are shown. Horizontal bars represent the mean \pm SD values.

Figure 1. Scatter plot comparison of serum $A\beta_{40}$, $A\beta_{42}$, clusterin and p97 levels in Alzheimer's disease (AD), mild cognitive impairment (MCI) and cognitive normal subject (NS) groups as measured by ELISA. (A) No significant difference of serum $A\beta_{40}$ was observed among all five groups. (B) Significant difference in serum $A\beta_{42}$ in Alzheimer's and mild cognitive impairment patients was demonstrated compared to normal cognitive subjects ($P < 0.05$). (C) Significant difference in serum clusterin in Alzheimer's and mild cognitive impairment patients was demonstrated compared to normal cognitive subjects ($P < 0.001$). (D) No significant differences of serum p97 was observed among all five groups.

Statistical analysis

Statistical comparison of age, sex and MMSE-Thai score were evaluated by one-way analysis of variance (ANOVA). The significance of differences between Alzheimer's disease groups, mild cognitive impairment group and cognitive normal subject group were evaluated using non-parametric Kruskal-Wallis and Mann-Whitney U tests. Results of the analyses were considered significant at P-value less than 0.05. The relationship between sensitivity and specificity for the different groups of patients (Alzheimer's disease and mild cognitive impairment) versus cognitive normal subject or Alzheimer's disease versus mild cognitive impairment and cognitive normal subject was described using receiver operating

characteristic curve analysis (Greiner, 1996). The receiver operating characteristic curve was calculated with 95% confidence intervals.

Table 3. Receiver operating characteristic curve analysis of A β ₄₂ and clusterin in serum for discriminating patients with cognitive impairment (Alzheimer's disease and mild cognitive impairment) and cognitive normal subjects.

Parameter	Area under the curve	Best cutoff point	Sensitivity %	Specificity %
A β ₄₂	0.685	> 0.49 (pg/ml)	84%	50%
Clusterin	0.814	> 80.23 (ng/ml)	84%	75%

Table 4. Receiver operating characteristic curve analysis of A β ₄₂ and clusterin in serum for discriminating patients with Alzheimer's disease compare to mild cognitive impairment and cognitive normal subjects.

Parameter	Area under the curve	Best cutoff point	Sensitivity %	Specificity %
A β ₄₂	0.603	> 0.49 (pg/ml)	88%	38.5%
Clusterin	0.687	> 80.23 (ng/ml)	87%	49.2%

RESULTS

Sample characteristics

A summary of the demographic data is given in Table 1. According to their clinical assessment, individuals were grouped as either Alzheimer's disease (mild, moderate and severe), mild cognitive impairment or cognitively normal. All groups were age- and sex- matched. Interestingly, a significant decrease in MMSE-Thai score was found in Alzheimer's disease and mild cognitive impairment groups compared with cognitive normal subject group ($P < 0.001$).

Concentrations of serum A β ₄₀, A β ₄₂, clusterin and p97 in Alzheimer's disease, mild cognitive impairment and cognitive normal subject

Mean \pm SD values for serum A β ₄₀, A β ₄₂, clusterin and p97 levels in Alzheimer's disease, mild cognitive impairment and cognitive normal subject groups are summarized in Table 2. The Alzheimer's disease groups were divided into three subgroups, including mild Alzheimer's disease, moderate Alzheimer's disease and severe Alzheimer's disease. There were no significant differences among all five groups in serum A β ₄₀ and p97 levels. On the other hand, there was a significant increase in serum levels of A β ₄₂ ($P < 0.05$) and clusterin ($P < 0.001$) between Alzheimer's disease groups and cognitive normal subjects and between mild cognitive impairment and cognitive normal subjects (Table 2 and Figure 1).

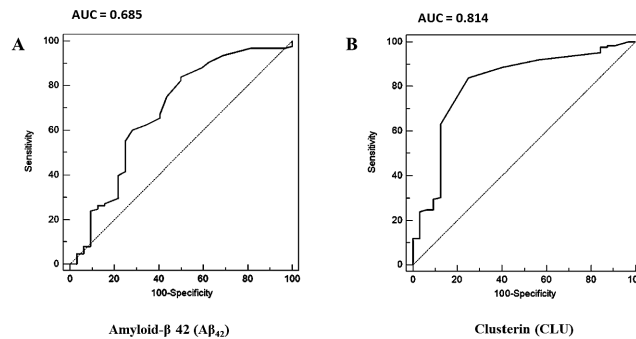


Figure 2. Receiver Operating Characteristic (ROC) curves analysis of $A\beta_{42}$ and clusterin for distinguishing between cognitive impairment subjects (Alzheimer’s disease and mild cognitive impairment) and cognitive normal subjects. **(A)** ROC curve analysis of serum $A\beta_{42}$. Cut-off point and area under ROC curve (AUC) of serum $A\beta_{42}$ was > 0.49 pg/ml and 0.685, respectively. **(B)** ROC curve analysis of serum clusterin. Cut-off point and AUC of serum clusterin was > 80.23 ng/ml and 0.814, respectively.

The receiver operating characteristic curve analysis for determination of serum $A\beta_{42}$ and serum clusterin cut-off value

To identify the optimal serum $A\beta_{42}$ and serum clusterin concentration for discriminating patients with cognitive impairment and cognitive normal subjects, a cut-off value was determined. Using receiver operating characteristic curve analysis, the area under the curve of serum $A\beta_{42}$ is 0.685 and serum clusterin is 0.814 (Figure 2). The optimal cut-off value of serum $A\beta_{42}$ for discriminating Alzheimer’s disease and mild cognitive impairment patients with cognitive normal subjects was 0.49 pg/ml (84% sensitivity and 50% specificity), while that of serum clusterin was 80.23 ng/ml (84% sensitivity and 75% specificity), as summarized in Table 3. In addition, we determined a cut-off point of 0.49 pg/ml for serum $A\beta_{42}$ to differentiate Alzheimer’s disease from mild cognitive impairment and cognitive normal subjects with 88% sensitivity and 38.5% specificity, and 80.23 ng/ml for serum clusterin with 87% sensitivity and 49.2% specificity (Table 4). The area under the curve of serum $A\beta_{42}$ is 0.603 and serum clusterin is 0.687, as summarized in Figure 3. The diagnosis accuracy of serum $A\beta_{42}$ and serum clusterin was sufficient and very good, respectively. Although mild cognitive impairment and Alzheimer’s disease subjects showed good sensitivity of serum $A\beta_{42}$ and serum clusterin more than 80%, at this point the specificity is relatively low.

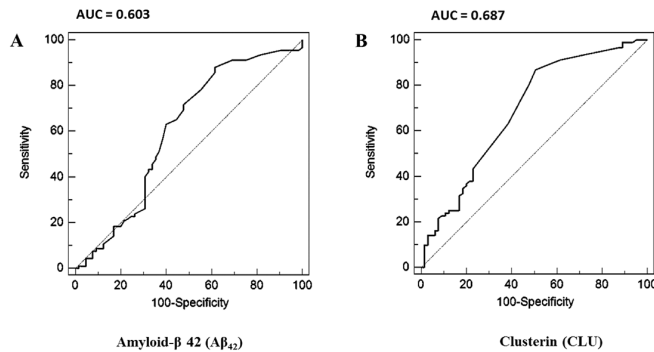


Figure 3. Receiver Operating Characteristic (ROC) curves analysis of $A\beta_{42}$ and clusterin for distinguishing Alzheimer's patients from mild cognitive impairment and cognitive normal subjects. (A) ROC curve analysis of serum $A\beta_{42}$. Cut-off point and area under ROC curve (AUC) of serum $A\beta_{42}$ was > 0.49 pg/ml and 0.603, respectively. (B) ROC curve analysis of serum clusterin. Cut-off point and AUC of serum clusterin > 80.23 ng/ml and 0.687, respectively.

DISCUSSION

Blood-based biomarkers for Alzheimer's disease diagnosis have been widely investigated. However, blood-based biomarkers for Alzheimer's disease diagnosis presented contrary results and it is unclear whether changes in the peripheral blood reflect pathology within the brain (Zetterberg et al, 2010; Mehta, 2007; Irizarry, 2004). In this study, the concentrations of $A\beta_{40}$, $A\beta_{42}$, clusterin and p97 in serum were investigated in Alzheimer's disease, mild cognitive impairment, and cognitive normal subjects who were grouped base on neurological, physical and MMSE-Thai score.

Concordant with previous reports, $A\beta_{42}$ and clusterin in serum of Alzheimer's disease and mild cognitive impairment was significantly elevated compared to normal subjects (Mehta et al., 2000; Assini et al., 2004; Schrijvers et al., 2011; Xing et al., 2012). Several studies claimed that the increase of $A\beta_{42}$ and clusterin in Alzheimer's disease could be useful in improving the progression of Alzheimer's patients (Chiu et al., 2012; Thambisetty et al, 2010; 2012). It has been reported that subjects with normal cognition having a high-plasma $A\beta_{42}$ level were twice as likely to develop Alzheimer's disease as subjects having a low-plasma $A\beta_{42}$ level; and plasma $A\beta_{42}$ levels were elevated in Alzheimer's disease patients compared to controls (Schupf et al., 2007). It has been reported that an increase in the plasma $A\beta_{42}$ level predicted the conversion from normal cognition to mild cognitive impairment, but did not predict conversion from normal cognition to Alzheimer's disease (Blasko et al., 2008). In addition, an increased $A\beta_{42}$ level was detected in women, but not men, with mild cognitive impairment (Assini et al., 2004).

Peripheral concentration of clusterin is associated with changes in patients with Alzheimer's disease and mild cognitive impairment. Our current observations provided evidence linked to high clusterin levels in Alzheimer's disease and mild cognitive impairment which is related to previous study. Thambisetty et al. (2010) showed that high concentrations of plasma clusterin were correlated with the development of entorhinal cortex atrophy, and severity and progression of Alzheimer's disease. Recently, they found that high plasma clusterin levels in mild cognitive impairment were associated with slow rates of brain atrophy (Thambisetty et al, 2012). In addition, association of plasma clusterin with Alzheimer's disease has been recently reported (Schrijvers et al., 2011). Among patients with Alzheimer's disease, higher clusterin levels were associated with more severe disease. Plasma clusterin levels were not related to the risk of incident Alzheimer's disease during total follow-up (Schrijvers et al., 2011). Thus, $A\beta_{42}$ and clusterin in serum were considerably higher in Alzheimer's disease and mild cognitive impairment patients than in cognitive normal subjects. This may be an indication of increased risk for developing Alzheimer's disease.

In contrast, serum $A\beta_{40}$ levels in Alzheimer's disease and mild cognitive impairment were not significantly different compared to cognitive normal subject. Although previous studies have reported elevated plasma $A\beta_{40}$ concentrations in patients with Alzheimer's disease (Mehta et al., 2000; van Oijen et al., 2006), some reports suggested that $A\beta_{40}$ levels were not altered in Alzheimer's disease and mild cognitive impairment patients, which is similar to our study. In a longitudinal study, it appeared that Alzheimer's disease patients at baseline and those who developed Alzheimer's disease during follow-up had significant higher plasma $A\beta_{42}$, but not $A\beta_{40}$ levels, and plasma $A\beta_{42}$, but not $A\beta_{40}$ levels, declined over time in newly acquired Alzheimer's patients (Mayeux et al., 2003). In addition, no difference in plasma $A\beta_{40}$ levels between patients with mild cognitive impairment who later developed Alzheimer's disease and patients with stable mild cognitive impairment or healthy subjects has been reported. Therefore, plasma $A\beta_{40}$ level was not an optimal biomarker in Alzheimer's disease (Hansson et al., 2010)

Kennard et al. (1996) have demonstrated that p97 concentrations are consistently elevated in the serum of Alzheimer's patients, compared with controls. There was no overlap between the groups, and the correlation between age and p97 serum concentration was not significant. Kim DK et al. (2001) showed that serum p97 concentrations were elevated three to four fold in Alzheimer's disease as compared to non-Alzheimer's disease dementia and normal controls. These results support the significance of high serum p97 levels in Alzheimer's disease and its potential utility as a biological marker in Alzheimer's disease. However, we found that p97 levels in serum of Alzheimer's disease and mild cognitive impairment patients were not different from cognitive normal subjects. Two of the limitations of our study were its small sample size and many participants in Alzheimer's disease and mild cognitive impairment groups were not newly diagnosed cases. Thus, the effect of dementia treatment may affect the level of some serum proteins in subjects, including p97.

To identify the optimal serum $A\beta_{42}$ and serum clusterin concentration for discriminating patients with cognitive impairment and cognitive normal subjects, receiver operating characteristic curve analysis was used in this study. Our results found that a serum $A\beta_{42}$ level at a cut-off point of 0.49 pg/ml in cognitive impairment patients (Alzheimer's patient and mild cognitive impairment) against cognitive normal subjects had good sensitivity (84%) with low specificity (50%). However, the accuracy of diagnosis was poor (the area under the curve of 0.685). For clusterin, we defined serum clusterin 80.23 ng/ml as the best cut-off point, which gives the same sensitivity (84%) as $A\beta_{42}$ and higher specificity (75%) than $A\beta_{42}$. Interestingly, the accuracy of diagnosis was good (the area under the curve of 0.814). Additionally, we attempted to differentiate patients with Alzheimer's disease from mild cognitive impairment and cognitive normal subjects. The same cut-off points of 0.49pg/ml for serum $A\beta_{42}$ in Alzheimer's disease patients against mild cognitive impairment and cognitive normal subject also had good sensitivity (88%), but low specificity (38.5%). The accuracy of diagnosis was poor (the area under the curve of 0.603). Likewise, the same cut-off points of 80.23 ng/ml for serum clusterin showed 87% sensitivity and 49.2% specificity. The accuracy of diagnosis was poor (the area under the curve of 0.687). In comparison with other studies, Chiu et al. (2012) identified a cut-off point of 16.1 pg/ml for $A\beta_{42}$ to differentiate control subjects from patients (both Alzheimer's disease and mild cognitive impairment) with 85.3% sensitivity and 88.5% specificity and found it a useful biomarker for Alzheimer's disease. Differences between study results may be related to sample size, sampling or timing of the sample collection in relation to the clinical period or stage of disease progression. In accordance with our study, reports have shown significantly increased serum clusterin levels in subjects with mild cognitive impairment and Alzheimer's disease (Schrijvers et al., 2011; Thambisetty et al, 2010; 2012). However, these studies have not addressed the optimal cut-off value, sensitivity and specificity of serum clusterin for distinguishing between cognitive impairment subjects and cognitive normal subjects, nor did they differentiate patients with Alzheimer's disease from mild cognitive impairment and cognitive normal subjects. An ideal biomarker should have a sensitivity of greater than 80% in detecting Alzheimer's disease and a specificity of greater than 80% for distinguishing from other dementias (Ronald and Nancy Reagan Research Institute, 1998). It is difficult to determine the exact normal range and cut-off points of blood biomarker levels that provide both high sensitivity and specificity. Many variable factors can change the numerical data, such as sample size, disease stage, race, therapeutic effects of disease and the analytical process.

CONCLUSIONS

In summary, significantly elevated $A\beta_{42}$ and clusterin levels were observed in Alzheimer's disease and mild cognitive impairment subjects compared to cognitive normal subjects. There were no significant differences in $A\beta_{40}$ and p97 levels between Alzheimer's disease, mild cognitive impairment and cognitive

normal subjects. Receiver operating characteristic curve analyses revealed that only clusterin may be used for diagnosing patients with cognitive impairment with 84% sensitivity, 75% specificity and good accuracy of diagnosis. Although these markers cannot be currently used as diagnostic tests for Alzheimer's disease, our results demonstrated an association between serum $A\beta_{42}$ and serum clusterin concentrations in patients with Alzheimer's disease. Sample size, sampling or timing of the sample collection in relation to the clinical period or stage of disease progression should be considered in future studies to improve sensitivity, specificity and accuracy of clinical diagnosis of Alzheimer's disease among individuals and may also help identify individuals with mild cognitive impairment.

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