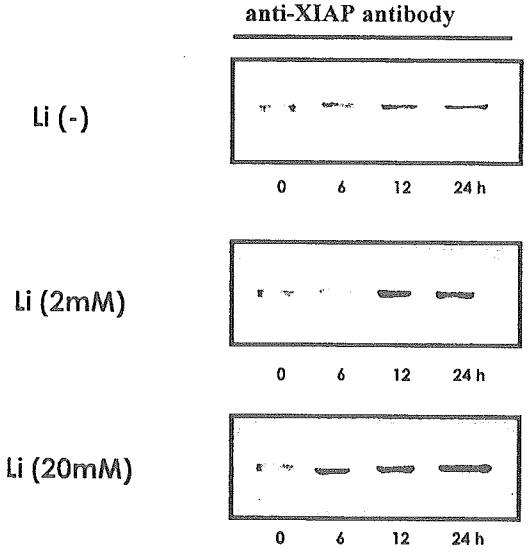

Fig. 1 Increased expression of XIAP in SY5Y cells treated with lithium chloride
Human neuroblastoma SH-SY5Y cells were cultured in the presence and absence of lithium chloride (2 mM and 20 mM). Cells were collected and lysed after 0, 6, 12, and 24 hours. The supernatants were analyzed by Western blot employing anti-XIAP antibody. Increased expression of XIAP was observed in cells treated with lithium chloride by dose-dependent manner.

ることを報告してきた[21)22)24)]．そこで，今年度は，XIAPの発現を逆に促進する因子を検討した．

リチウムは臨床的には主に躁病に用いられる薬剤であるが，タウ蛋白をリン酸化する酵素の1つであるグリコーゲンシンターゼキナーゼ-3（Glycogen Synthase Kinase-3（GSK-3））の阻害機能があることが判明し，タウ蛋白のリン酸化制御の側面からも研究が行われている[10)12)]．また，neuroprotectionの作用があることも知られ，そのメカニズムとしてはProtein Kinase B（PKB）の活性化やBcl-2の発現促進なども報告されている[1)2)]．今回我々はリチウムによるXIAPの発現の影響を検討し，リチウムおよびGSK-3阻害剤はXIAPの発現を亢進させることを見いだした．このことは，リチウムによるneuroprotectionのメカニズムの1つとして，アポトーシス阻害機能を有するXIAPの発現の亢進があることを示唆している．

方　　法

まず，培養細胞に対するXIAPの発現の検討を行うためSY5Y神経芽細胞腫を5％ウシ胎児血清を含むD-MEM/F-12培地にて培養し，2 mMおよび20 mMの塩化リチウムを添加し24時間まで経過を追った後，細胞を集めた．また，昨年度5 μMの$A\beta_{25\text{-}35}$によって，XIAPの発現が低下することを確認しているので，リチウムがそれにどのような影響を与えるかを見るために，SY5Y神経芽細胞腫に5 μM $A\beta_{25\text{-}35}$および5 μM $A\beta_{25\text{-}35}$と2 mM塩化リチウムを添加して24時間後，細胞を集めた．さらに，リチウムの作用機序を検討するために，市販のGSK-3阻害剤（10 μMのGSK-3 Inhibitor-IおよびーII（Calbiochem社））を添加して3時間後に細胞を集めた．集めた細胞はバッファー（100 mM PIPES, pH6.8, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 25 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/ml aprotinine, 5 μg/ml leupeptine, 0.1％ Triton-X100）にて溶解し，そのlysatesを200K × Gにて遠心し，supernatantを得た．このsupernatantの各50 μgをポリアクリルアミドゲルの各レーンにアプライして，抗XIAP抗体（R&D社）を用いたウエスタンブロットを行いXIAPの発現を検討した．

そして，リチウムのneuroprotectionの効果を確認するために，SY5Y神経芽細胞腫に2 mMの塩化リチウムを添加して24時間培養したものとそうでないものを用意し，これにさまざまな細胞死ストレス（10 μMおよび50 μMの過酸化水素，25 μMの$A\beta_{25\text{-}35}$）を添加した．そして24時間後の細胞死のレベルについてLive and Dead Assay（Molecular Probe社）を用いて検討した．

結　　果

SY5Y神経芽細胞腫に2 mMおよび20 mMの塩化リチウムを添加したところ，時間とともにXIAPの発現量は増加した．この効果は濃度依存的であった（**Fig. 1**）．次に，同じ細胞に5 μMの$A\beta_{25\text{-}35}$を添加して24時間後のXIAPの発現は低下していたが，同時に2 mMの塩化リチウムを添加していた細胞では，逆にXIAPの発現は増加していた

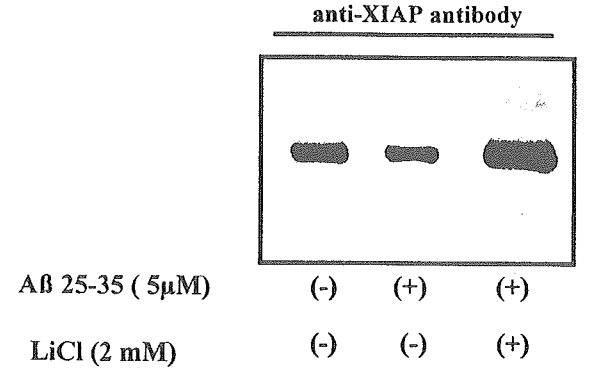

Fig. 2 Decreased expression of XIAP is reversed by lithium
SH-SY5Y cells were cultured in the presence of amyloid β 25-35 or in the presence of combination of amyloid β 25-35 and 2 mM lithium chloride. Cells were collected and lysed after 24 hours. The supernatants were analyzed by Western blot employing anti-XIAP antibody. Decreased expression of XIAP was observed in cells treated with amyloid β 25-35 , however this effect was reversed by lithium chloride.

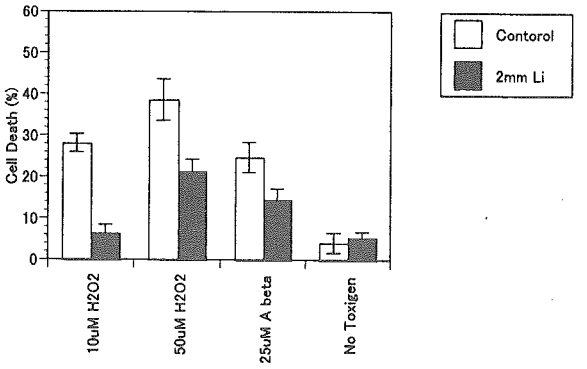

Fig. 4 Decreased cytotoxicity in SY5Y cells treated with lithium
SH-SY5Y cells were cultured in the presence and absence of 2 mM lithium chloride for 24 hours. Then the cells were treated with hydrogen peroxide (10 μM and 50 μM), and 25 μM amyloid β 25-35 peptide for 24 hours. And live and dead cells were counted. Dead cells were observed in cells treated with hydrogen peroxide and amyloid β 25-35 peptide, however decreased cytotoxicities were observed when cells were pretreated with 2 mM lithium chloride.

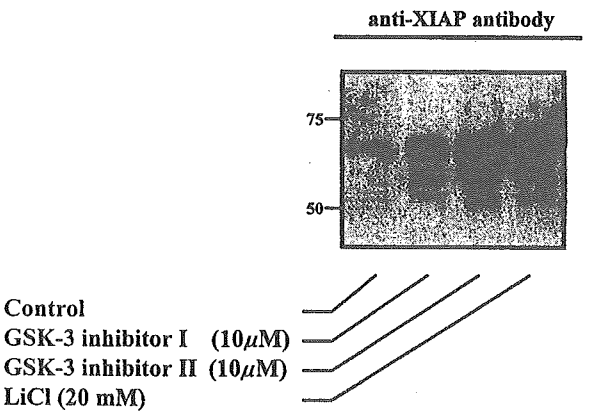

Fig. 3 Increased expression of XIAP by GSK-3 inhibitors
SH-SY5Y cells were cultured in the presence of 20 mM lithium chloride or 10 μM GSK-3 inhibitore I or II. Cells were collected and lysed after 3 hours. The supernatants were analyzed by Western blot employing anti-XIAP antibody. Increased expression of XIAP was observed in cells treated with GSK-3 inhibitore I or II, and also with lithium chloride.

(**Fig. 2**). さらに，GSK-3 阻害剤（10 μM の GSK-3 Inhibitor-I および -II）を添加して 3 時間後の XIAP の発現量を検討したところ，20 mM の塩化リチウムを添加した場合と同様に XIAP の発現は増加していた（**Fig. 3**）．最後に，SY5Y 神経芽細胞腫に 2 mM の塩化リチウムを添加したものとそうでないものを 24 時間培養し，さまざまな細胞死ストレスを与えると，10 μM および 50 μM の過酸化水素の場合も，25 μM の $A\beta_{25\text{-}35}$ の場合も，細胞死レベルは減少していた（**Fig. 4**）．以上のことから，リチウムは XIAP の発現を亢進させること，その機序は GSK-3 の阻害であること，そしてリチウムによる neuroprotection のメカニズムの 1 つとしてアポトーシス阻害機能を有する XIAP の発現の亢進があることが示唆された．

考　　察

我々は今までに,神経細胞死とタウ蛋白異常リン酸化のメカニズムをプロテインフォスファターゼや GSK-3 およびストレス関連 MAP キナーゼとの関わりから詳細に検討してきた[16)17)18)19)20)23)]．その中

で，アポトーシスのメカニズムに興味をいだき，内因性に細胞内に存在しカスパーゼを抑制する蛋白として知られるIAPの1つであるXIAPについて研究を行ってきた[21)22)24)]．そして，このXIAPが過剰発現する細胞ではアポトーシスを誘導するような細胞死ストレスのもとでその細胞死を抑制すると同時にアポトーシスに伴うタウ蛋白の脱リン酸化を抑制すること，N末端のMetの除かれたタウ蛋白と特異的に結合しその機能を抑制する可能性があること，そしてAβによってXIAPの発現が抑制されることを報告してきた．今回の研究は，XIAPの発現を逆に促進する因子としてリチウムを見出し，その検討を行ったものである．

そもそもリチウムは躁病に対する薬剤として約半世紀使用されてきたが，さまざまな生物学的作用を有し，抗躁効果の作用機序は未だ明らかではない．有力な機序としては，フォスファチジルイノシトールモノフォスファターゼ阻害作用，結果として細胞内イノシトール枯渇作用というものが知られている[8)]．しかし，抗躁効果との関連性はないようであるがリチウムの重要な薬理作用としてGlycogen Synthase Kinase-3（GSK-3）の阻害機能があることもよく知られるようになり，タウ蛋白のリン酸化制御や，詳細な機序は不明であるがAβ産生制御の側面からも研究が行われるようになった[10)12)13)]．また，リチウムには近年neuroprotection作用があることが報告されるようになり，この機序としてはProtein Kinase B（PKB）の活性化やBcl-2の発現促進などが報告されている[1)2)]．

今回の我々の研究は，XIAPの発現を促進する因子としてリチウムを見出したものであったが，今回の実験結果からはその作用機序としてはGSK-3の阻害がまず考えられる．発現量の促進に関しては，通常の転写レベルでの亢進と翻訳レベルでの亢進，そして分解速度の低下がその理由として考えられる．XIAPの発現は他の蛋白と異なり，通常の転写レベルでの制御に加えて，その遺伝子の5'-末端にIRES（Internal Ribosome Entry Sites）という配列を持つことから翻訳レベルでの制御が重要であるとされている．このIRESによる制御を介して，培地のアミノ酸除去などのストレス刺激によってmRNAから蛋白への翻訳量が増大することが知られているが，これは細胞死ストレスに対する生体反応として備わっている可能性が高い[3)9)]．今回発見した，発現亢進を誘導するリチウムは細胞死ストレスとは異なるため，神経変性疾患に対して治療的に応用されうる可能性を有している．GSK-3の機能とIRESを介した翻訳制御についてはまだ報告はないが，興味深い可能性があると思われ，さらに検討が必要と考えられる．また，リン酸化酵素が分解に関与する可能性としては，ある蛋白がリン酸化によりユビキチン化が亢進し，そのため分解が促進する例もあり，この点についてもさらに検討が必要と考えられる．

以上より，リチウムによるXIAPの発現の影響を検討し，リチウムおよびGSK-3阻害剤はXIAPの発現を亢進させることが示唆された．このことは，リチウムによるneuroprotectionのメカニズムの1つとして，アポトーシス阻害機能を有するXIAPの発現の亢進があることをも示唆している．以前の検討から，少なくとも，アポトーシス阻害因子という細胞死の過程に逆の働きをしているものも関与している可能性が示唆されていることから，今後提起された疑問点を解明するための検討を行うことにより，さまざまな神経変性疾患におけるアポトーシス阻害因子をターゲットにした治療および診断的方法の検討が必要と考えられる．

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ABSTRACT

Study on diagnosis and therapeutics for Alzheimer disease in relation to modified-tau and inhibitor of apoptosis protein

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Amyloid β (Aβ) and tau protein are abnormally accumulated protein in Alzheimer disease (AD). Previously we reported tau protein could bind with XIAP (X chromosome-linked inhibitor of apoptosis), one of intrinsic anti-apoptotic proteins, and low dose of A β attenuated expression of XIAP. Here we show lithium and GSK-3 inhibitors up-regulate the expression of XIAP. This finding might support the mechanisms of neuroprotective effects of lithium, and XIAP might be one of therapeutic targets in neurodegenerative disease including AD.
(**Ann. Rep. Mitsubishi Pharma Res. Found.** 2005, 37 : 69 ～ 74)

Chapter 23

Cerebrospinal fluid phosphorylated tau protein at serine 199 is a useful diagnostic biomarker in Alzheimer's disease and mild cognitive impairment

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INTRODUCTION

Our recent studies of biological markers in Alzheimer's disease (AD) have focused specifically on analysis of cerebrospinal fluid (CSF) tau protein levels and amyloid β-protein ending at amino acid 42.[1–4] Although CSF total tau (t-tau) level in AD was significantly higher than in controls, there were overlaps between AD and non-AD dementias.[1–3] One possible explanation is that the enzyme-linked immunosorbent assay (ELISA) kit we used detects not only phosphorylated but also normal tau. Therefore, we developed the sandwich ELISA system for phosphorylated tau at serine 199 (p-tau 199) in CSF[5] and examined 236 cases with AD, 206 cases with non-AD demented and non-demented disease controls, and 95 age-matched normal controls.[6]

SUBJECTS AND METHODS

Table 23.1 shows a summary of the patients' demographic data. We surveyed a total of 537 CSF samples. We also examined CSF p-tau 199

Table 23.1 Summary of patients' demographic data

	No. of patients	*Age* (years)	*Gender* (M/F)
Alzheimer's disease (AD)	235*	71 ± 9	66/172
Normal control	95	57 ± 16	51/44
Neurological disease control	122	59 ± 13	70/52
Frontotemporal dementia (FTD)	16*	63 ± 12	9/7
Progressive supranuclear palsy	21	63 ± 7	10/11
Corticobasal degeneration	15	64 ± 4	8/7
Dementia with Lewy body (DLB)	13*	63 ± 10	8/5
Vascular dementia	23	71 ± 6	16/7
Meningoencephalitis	18	51 ± 21	7/11
Creutzfeldt–Jakob disease (CJD)	11*	71 ± 6	6/5

* Two patients with AD, one patient with FTD, one patient with DLB and four patients with CJD were confirmed by autopsy

levels in a population with mild cognitive impairment (MCI). The MCI group was later subdivided into two different categories. One category was that which eventually later progressed to AD (progressive MCI). The other category was that which later did not progress to AD (non-progressive MCI). Memory complainers were patients who complained about memory disturbance, but were not demented. These constituted the control group. CSF samples were taken into polypropylene tubes by lumbar puncture after informed consent was obtained from each patient and/or family members. Bloody or traumatic CSF samples were excluded from this study. After centrifugation at 1500 rpm for 10 min, the aliquots were stored at –80°C until analysis. CSF levels of p-tau 199 were measured by a sensitive sandwich ELISA.[5,6] CSF level of t-tau protein was measured using the sandwich ELISA assay provided by the Innogenetics Company, Belgium.[7]

RESULTS

CSF p-tau 199 levels in the AD group were significantly elevated ($p < 0.001$) compared to those in all the other non-AD groups, including patients with acute neurological conditions such as meningoencephalitis and Creutzfeldt–Jakob disease (CJD) (Figure 23.1). On the other hand, CSF t-tau levels were occasionally very high in the meningoencephalitis and CJD groups,

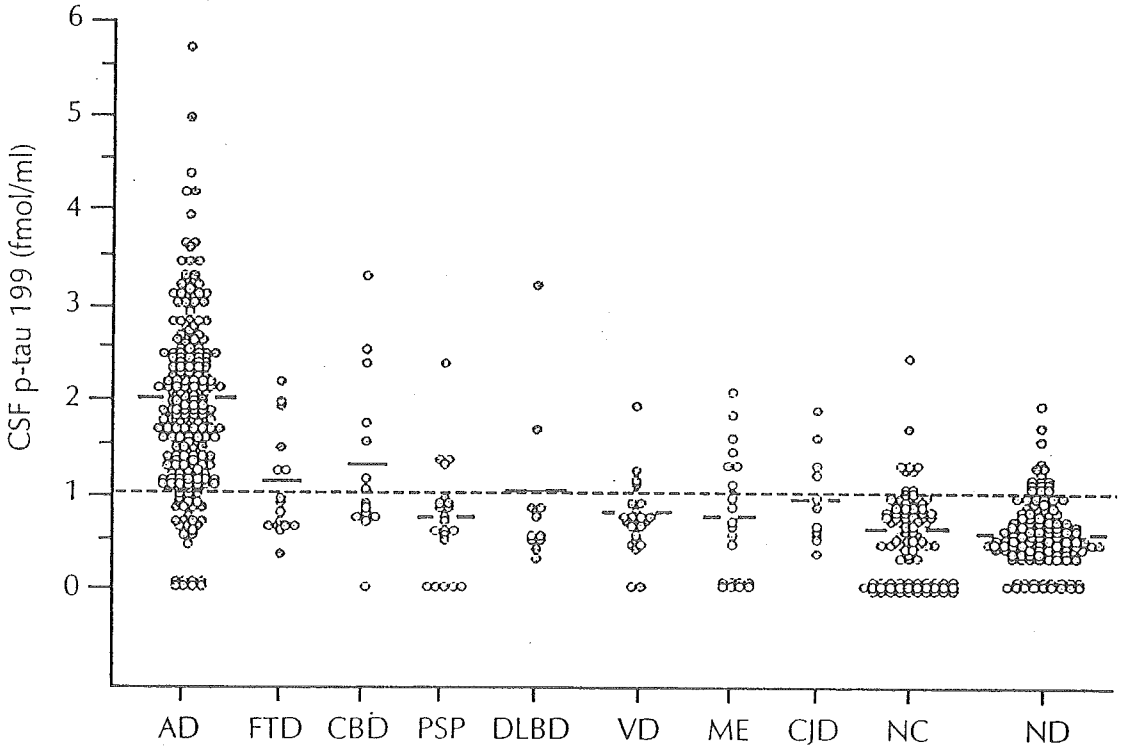

Figure 23.1 The results of cerebrospinal fluid (CSF) phosphorylated tau at serine 199 (p-tau 199) levels, among groups with Alzheimer's disease (AD), frontotemporal dementia (FTD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), dementia with Lewy body disease (DLBD), vascular dementia (VD), meningoencephalitis (ME), Creutzfeldt–Jakob disease (CJD), normal controls (NC) and neurological disease controls (ND)

although most CSF t-tau levels were significantly increased in the AD group compared to normal control groups (Figure 23.2).

A receiver operating characteristics (ROC) curve analysis demonstrated that CSF p-tau 199 was more amenable than CSF t-tau to differentiating between AD and non-AD subjects (Table 23.2).

The results of CSF p-tau 199 levels in the progressive MCI group were significantly

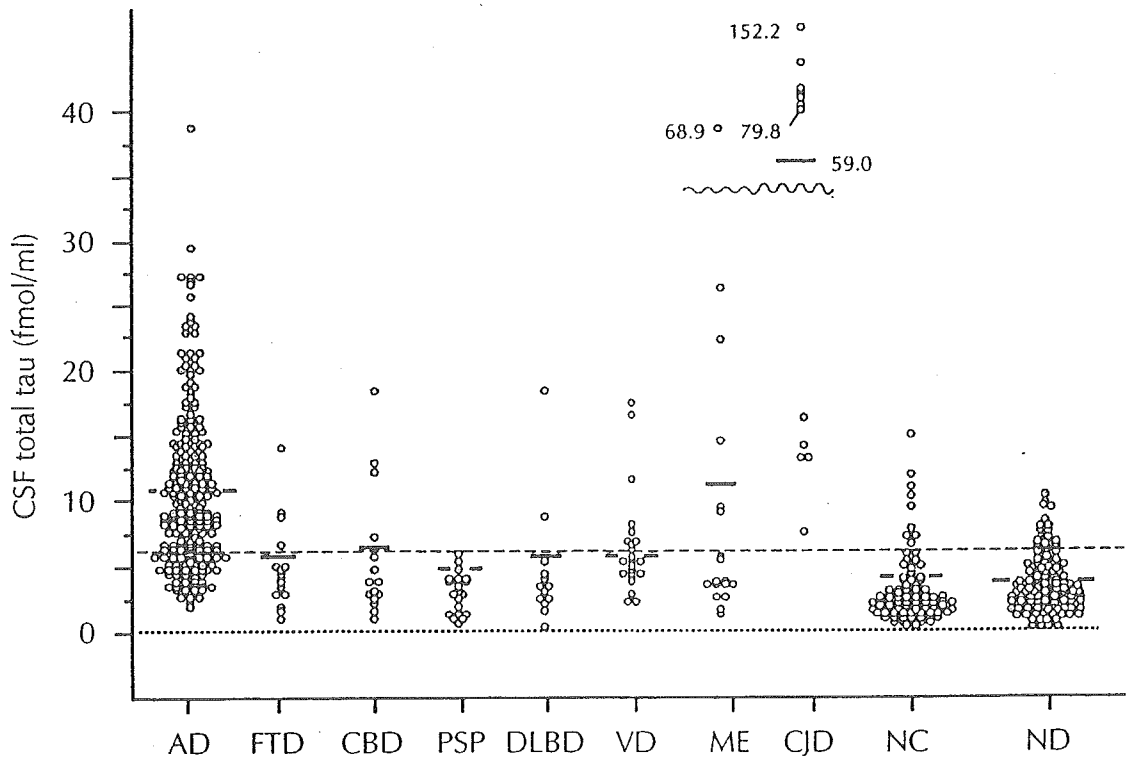

Figure 23.2 The results of cerebrospinal fluid (CSF) total tau levels, among groups with Alzheimer's disease (AD), frontotemporal dementia (FTD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), dementia with Lewy body disease (DLBD), vascular dementia (VD), meningoencephalitis (ME), Creutzfeldt–Jakob disease (CJD), normal controls (NC) and neurological disease controls (ND)

Table 23.2 Receiver operating curve analysis

	Cut-off level (fmol/ml)	*Sensitivity* (%)	*Specificity* (%)
Alzheimer's disease vs. neurological disease controls and normal controls			
Total tau	4.8	82.7	82.0
p-tau 199	0.96	87.3	87.4
Alzheimer's disease vs. others			
Total tau	6.0	77.1	77.6
p-tau 199	1.05	85.2	85.0

p-tau 199, phosphorylated tau at serine 199

elevated ($p < 0.001$) compared to those in the non-progressive MCI and the control groups (Figure 23.3). We thus propose that CSF p-tau 199 may also be useful for the diagnosis of MCI as it is for AD.

DISCUSSION

In the present study, we examined CSF p-tau 199 levels in a total of 570 living ($n = 562$) or autopsy-confirmed ($n = 8$) subjects with AD and other dementing disorders that resemble AD, as well as normal and neurological diseased controls. A combination of HT-7 (phosphorylation-independent monoclonal antibody; Innogenetics) and the anti-p-tau 199 antibody anti-PS199 allowed us to detect and quantitate CSF levels of the p-tau 199 by a newly constructed sandwich ELISA.[5,6] We reported p-tau 199 to be elevated in AD using different diagnostic antibodies that uniquely recognize specific phosphorylation epitopes of tau. We also monitored the CSF t-tau levels side by side in the same patients to assess and compare the sensitivity and specificity by ROC. Here, it should be noted that CSF p-tau 199 is not only the first biomarker that exceeds (over 85%) both sensitivity and specificity as a sole biomarker of AD, but also meets many other recommended criteria as an ideal biomarker.[8] The improvement of the diagnostic accuracy using CSF p-tau 199 seems to be accomplished not only by enhancing the lowest detection limit but also by eliminating a subset of non-AD patients with high CSF t-tau levels. Indeed, it is noteworthy that a subset of CJD patients with extremely high CSF t-tau levels showed only a mild elevation or an elevation under the cut-off level of CSF p-tau 199.

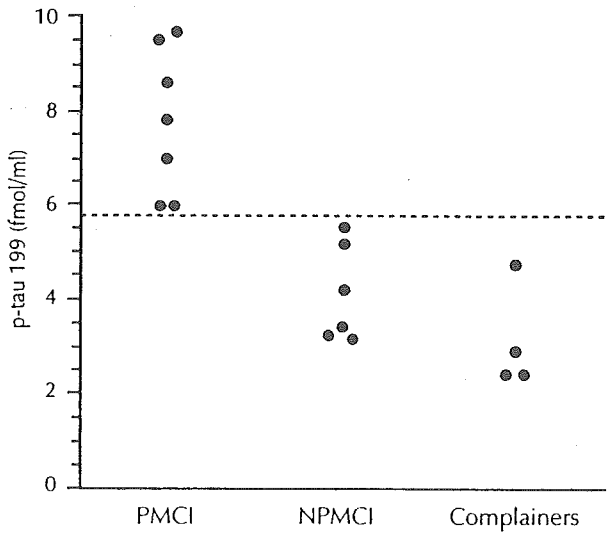

Figure 23.3 The results of cerebrospinal fluid (CSF) phosphorylated tau at serine 199 (p-tau 199) in mild cognitive impairment (MCI). PMCI, progressive MCI (Alzheimer's disease; AD); NPMCI, non-progressive MCI (non-AD). With a cut-off of 5.8 fmol/ml, sensitivity for PMCI 100% (7/7), and specificity for NPMCI 100% (6/6)

Nonetheless, our study suggests that there might be a limitation even in the use of the p-tau assay for a clear-cut distinction to be made between AD and certain tauopathies. In fact, the CSF p-tau 199 levels were over the cut-off value in approximately 30% (16/52) of the non-AD tauopathy group (Figure 23.2). Additional studies reported that pathological tau isoforms purified from tauopathy brains were occasionally hyperphosphorylayed at serine 199.[9,10] Therefore, the CSF p-tau 199 testing may be less accurate in distinguishing AD from other types of degenerative dementia or tauopathies. New and/or modified biomarkers would be necessary to differentiate AD from tauopathies in the future.[11,12]

A substantial proportion of subjects with MCI later developed clinical AD.[13] At autopsy, subjects with MCI showed a broad spectrum of morphological brain changes including typical AD pathological characteristics. Therefore, MCI

partly represents a predementia stage of AD. To maximize the benefit of therapeutic strategies, it is important to identify AD at the stage of MCI. Biochemical markers will be required to establish the diagnosis of MCI.[14] This study showed that CSF p-tau 199 levels in the progressive MCI group were significantly elevated compared to those in the non-progressive MCI and the control groups. We found that CSF p-tau 199 increased in an early stage of AD, the so-called MCI state, and confirmed that CSF p-tau 199 may be useful for the diagnosis of MCI as well as AD.

CONCLUSION

Our results suggest that CSF p-tau 199 is useful for an early diagnosis of AD.

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REVIEW ARTICLE

Studies on diagnostic markers for Alzheimer's disease

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Key words: *acetylcholine receptor α7, cerebrospinal fluid, genetic polymorphism, phosphorylated tau protein, touch panel computer.*

Abstract

In recent years, Alzheimer's disease (AD) has increased in incidence in Japan and elsewhere, and the marketing of donepezil hydrochloride (Aricept®) has allowed for the treatment of AD. These circumstances have encouraged the development of and research in markers for the early diagnosis of AD. Currently, the measurement of phosphorylated tau protein in the cerebrospinal fluid is considered to provide the most reliable and useful diagnostic marker for AD. For this purpose, a screening test using a touch panel computer can be recommended. The results of our study also suggest that the analysis of acetylcholine receptor α7 genetic polymorphism may be useful as a marker in the treatment with acetylcholine esterase inhibitors.

INTRODUCTION

In recent years, Alzheimer's disease (AD) has increased in incidence in Japan and elsewhere, and it accounts for about half the dementing diseases.[1,2] The recent marketing of donepezil hydrochloride (Aricept®) has allowed AD to be treated, and researchers have reported its usefulness.[3,4] Thus, the key to the treatment of AD is whether it can be diagnosed early and reliably. Unfortunately, AD is currently diagnosed solely by exclusion, and the development of diagnostic markers that are more easily accessible to everyone is highly desired. Researchers have tried many approaches in the development of diagnostic markers, of which tau protein-related ones have yielded the best results. This paper reports on the studies of tau protein-related markers and other markers.

DIAGNOSTIC MARKERS FOR AD

The Reagan Institute established to eradicate AD specifies that diagnostic biomarkers for AD must have the following characteristics:[5] They must reflect the disease status, be minimally invasive to the patient and have a high diagnostic accuracy in the differentiation between AD and other dementing diseases; that is, have a detection rate (sensitivity) of more than 80% for AD patients and a non-detection rate (specificity) of more than 80% for non-AD patients. We have previously reported that cerebrospinal fluid total tau protein satisfies most of the above requirements fairly well, but it does not have a sensitivity or specificity of over 80%. However, in combination with amyloid β-protein, it achieves a sensitivity and specificity of over 80% (called the AD index or AD unit).[6,7] The use of total tau protein as a biomarker for AD poses particular problems in that meningoencephalitis and Creutzfeldt–Jakob disease are associated with extremely high levels of total tau protein in the cerebrospinal fluid (Fig. 1).[8] To develop a single marker that meets the above requirements, we analyzed phosphorylated tau protein in the cerebrospinal fluid. Since tau protein in the degenerated neurofibrils in the brains of AD patients is hyperphosphorylated, we postulated that the selective measurement of phosphorylated tau protein would yield better results than the measurement of total tau protein. Focusing on phosphorylation at serine 199, our research group developed a sandwich enzyme-linked immunosor-

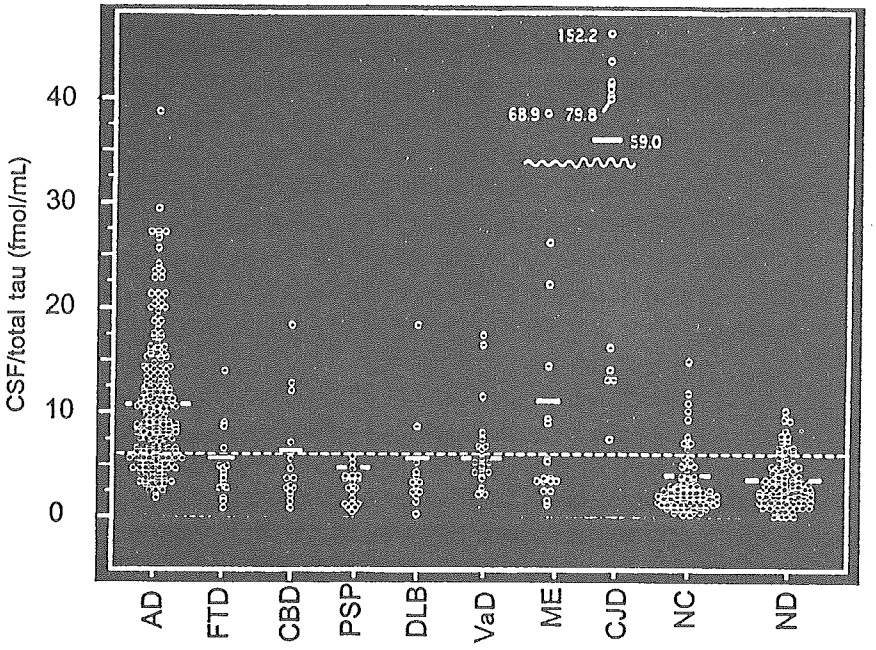

Figure 1 Quantification of total tau in cerebrospinal fluid (CSF). Total tau in CSF was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA) employing anti-tau antibodies. AD, Alzheimer's disease; CBD, corticobasal degeneration; CJD, Creutzfeldt–Jakob disease; DLB, dementia with Lewy body; FTD, frontotemporal dementia; ME, meningoencephalitis; NC, normal controls; ND, non-dementia; PSP, progressive supranuclear palsy; VaD, vascular dementia.

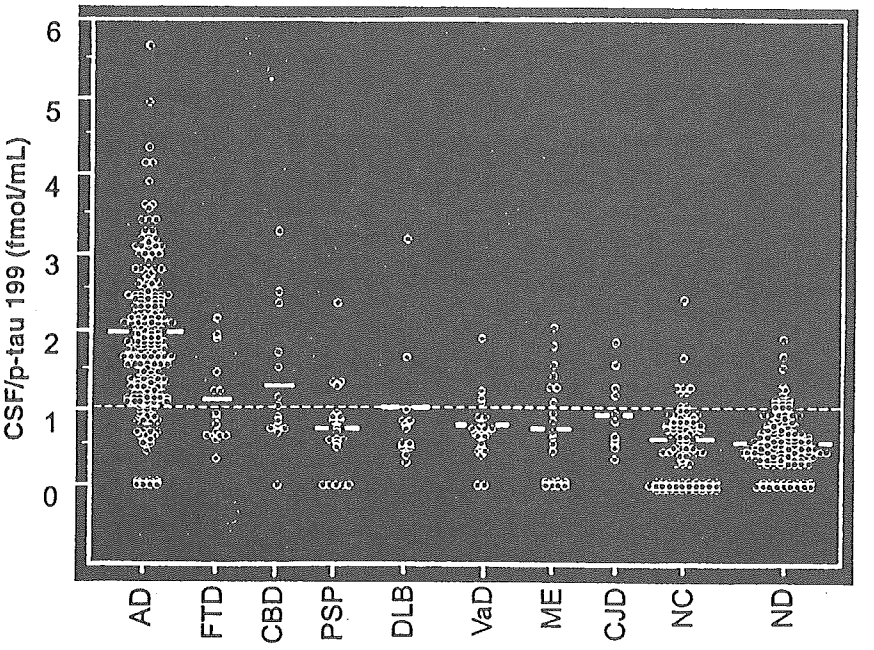

Figure 2 Quantification of phosphorylated tau at Ser 199 in cerebrospinal fluid (CSF). Phosphorylated tau in CSF was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA) employing antiphosphorylated tau antibodies. AD, Alzheimer's disease; CBD, corticobasal degeneration; CJD, Creutzfeldt–Jakob disease; DLB, dementia with Lewy body; FTD, frontotemporal dementia; ME, meningoencephalitis; NC, normal controls; ND, non-dementia; PSP, progressive supranuclear palsy; VaD, vascular dementia.

bent assay (ELISA) to quantify N-terminal fragments of phosphorylated tau protein,[9] which gave better results than total tau protein measurement. In particular, phosphorylated tau protein levels are low in patients with meningoencephalitis and Creutzfeldt–Jakob disease, which are associated with high total tau protein levels (Fig. 2). Thus, the receiver operating characteristic (ROC) analysis also showed improved results, with a sensitivity and specificity of over 80% (Table 1).[10] In addition to our method of quantifying tau protein phosphorylated at serine 199, methods of quantifying tau protein phosphorylated at threonine 181 and threonine 231 have been reported. All these methods have yielded good results, making them the

most reliable diagnostic markers. To further increase the diagnostic accuracy of biomarkers, attempts have been made to increase the ability to differentiate AD from tauopathies typified by corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP),[11,12] such as the method of separately determining the levels of tau isoforms in the cerebrospinal fluid. As tau protein occurs mainly as the 4-repeat isoform in AD, and as the 3-repeat isoform in CBD and PSP, its differentiation is theoretically possible.

SCREENING TEST FOR AD

As described above, the measurement of phosphorylated tau in the cerebrospinal fluid is currently the most reliable marker, but it is not easy to perform a cerebrospinal fluid examination. Therefore, a simple screening test is necessary. One approach is to develop a test that can be performed on blood or urine. Regrettably, no useful markers have been developed so far. Although our group has been measuring blood tau and Aβ levels, we have not achieved satisfactory results. Thus, as another approach, we developed a simple screening method for AD using a touch panel computer.[13] We used questions assessing temporal orientation, delayed recognition and space perception (choosing cubes and triangular prisms), which are sensitive test items. The computer program was developed using Microsoft Visual Basic 6.0, and was made to operate on a PC running a Windows operating system. Hardware with an audio output was used to provide audio information as well as visual information. As elderly individuals are not accustomed to using a mouse, a touch panel was adopted. Incorrect answers were given a mark of 0, and results were graded on a scale of 0 to 15. Tests on 49 AD patients and 30 control subjects showed that almost all the subjects in the control group obtained full marks (with one or two incorrect answers, if any), whereas subjects in the AD group failed to give the correct answer to three or more questions (Fig. 3). Thus, at a cut-off value of 12, the ROC analysis indicated that the screening test has a very high accuracy with a sensitivity of 96% and a specificity of 97%. Because this test can be easily performed anywhere, and is non-invasive, highly sensitive, and highly specific, it is extremely useful.

Table 1 Receiver operating characteristic (ROC) analysis of total tau and phosphorylated tau assays

	Cut-off level	Sensitivity	Specificity
AD vs NC + ND			
Total tau	4.8 fmol/mL	82.7%	82.0%
p-tau 199	0.96	87.3	87.4
AD vs others			
Total tau	6.0 fmol/mL	77.1%	77.6%
p-tau 199	1.05	85.2	85.0

ROC analysis also showed an improved sensitivity and specificity of over 80% in the case of phosphorylated tau in the cerebrospinal fluid (CSF) assay. AD, Alzheimer's disease; NC, normal controls; ND, non-demented controls.

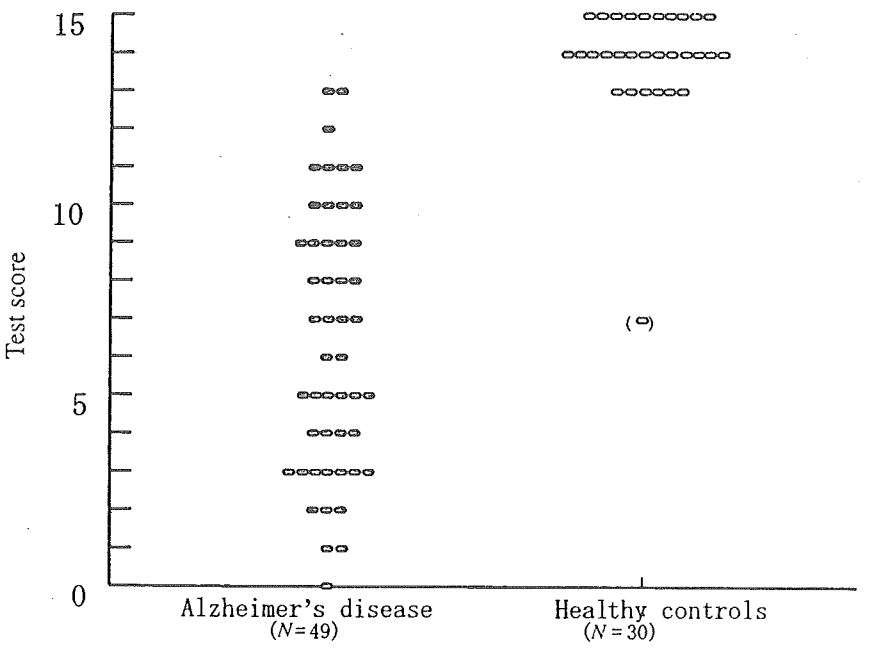

Figure 3 Results of the simple screening test for dementia using a touch panel computer. A simple screening method for Alzheimer's disease (AD) using a touch panel computer was developed. Tests on 49 AD patients and 30 control subjects showed that almost all subjects in the control group obtained full marks.

Table 2 Efficacy of donepezil hydrochloride and acetylcholine receptor (AchR) α7 polymorphism

		Genotype			
	No.	W/W	W/M	M/M	χ^2-test
Responder (improved)	21	10	11	0	$P < 0.05$
Non-responder (not improved)	22	17	5	0	

Donepezil hydrochloride responders were more frequently heterozygous for a 2-base deletion in the AchR α7 gene ($P < 0.05$).

MARKERS FOR THE PREDICTION OF DRUG EFFICACY

With the marketing of donepezil hydrochloride, there is now a treatment for AD. However, responder and non-responder groups exist. Methods to differentiate these groups have also been investigated. As this drug is an acetylcholine esterase inhibitor, we focused our attention on acetylcholine receptor α7 genetic polymorphism, and found that donepezil hydrochloride responders were more frequently heterozygous for a 2-base deletion in the acetylcholine receptor α7 gene ($P < 0.05$, Table 2).[3] This polymorphism requires further analysis not only as a marker for the early detection of AD, but also as a diagnostic marker for predicting the drug efficacy.

CONCLUSION

We consider that the quantification of phosphorylated tau in the cerebrospinal fluid is currently the most reliable and useful biomarker for AD. The method involving the use of a touch panel computer can be recommended as a preliminary screening test. Acetylcholine receptor α7 genetic polymorphism may be a useful marker in the treatment with acetylcholine esterase inhibitors.

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ORIGINAL ARTICLE

Evaluation of a computerized test system to screen for mild cognitive impairment

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Key words: *Alzheimer's disease, computer, dementia, mild cognitive impairment, screening.*

Abstract

Background: Mild cognitive impairment (MCI) refers to the clinical condition between normal aging and Alzheimer's disease (AD) and has a high probability of developing into AD. Early detection of MCI is important because early detection and appropriate follow-up treatment can prevent the disease from progressing. Therefore, MCI is an important candidate for screening and possible intervention.

Methods: We have developed a computerized screening test system to identify cognitive decline. This system consists of six tests (age and year-of-birth validity test, three-word memory test, time orientation test, first modified delayed-recall test, visual working memory test and second modified delayed-recall test). The scores obtained from three groups (MCI patients, AD patients and healthy control subjects) were analyzed to evaluate the sensitivity and specificity required for the screening of MCI.

Results: The system was well accepted by the patients. All of the test procedures were completed within 5 min. Significant group differences in all test results were found. The system has sensitivity and specificity values of 82% and 87%, respectively, when used as a screen for MCI.

Conclusion: The system is useful for the screening of cognitive disorders.

INTRODUCTION

The population of aged persons is increasing along with the extension of the average span of human life. The number of aged persons over 65 years of age is now over 24 million, which accounted for 19% of all Japanese people in 2003. The prevalence rate of dementia was reported to be 7.5% of all people over 65 years of age.[1] This means that the number of persons with dementia is estimated to have reached more than two million in Japan. Therefore, preventing an increase in the number of persons who suffer from cognitive disorders is regarded as an urgent task. Nowadays, a lot of attention is focused on mild cognitive impairment (MCI).[2] MCI refers to the clinical condition between normal aging and Alzheimer's disease (AD) and has a high probability of developing into AD.[3] Early detection of MCI is important because early detection and appropriate follow-up treatment can prevent the disease from progressing.[4] Therefore, MCI is an important candidate for screening and possible intervention.[5,6] In the case of mass screening, the necessary requirements which a test method must fulfill are speed, objectiveness and unbiased results even if the examiner changes. Using a computerized cognitive test system yields some useful advantages; for example, it can provide quick, objective and precise results based on the same standard.[7] We have developed a computerized screening test system to identify cognitive decline. In this paper, we present a description of the system and the results obtained in our study.

SUBJECTS AND METHODS

Subjects

Fifty-one outpatients in the memory disorder and dementia clinics at Tottori University Hospital were enrolled for the study. They received neuropsychological tests as well as a neuroimaging examination and other medical checks. The diagnosis of dementia was made according to the *Diagnostic and Statistical Manual of Mental Disorders*, revised third edition (DSM-III-R)[8] criteria, and the diagnosis of AD was based on the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA)[9] criteria. The diagnosis of MCI was made according to the five criteria for amnestic mild cognitive impairment, which are: (i) subjective memory complaints; (ii) impaired memory function for age and education level; (iii) preserved general cognitive function; (vi) intact activities of daily living; and (v) absence of dementia, as previously reported.[3] Finally, 29 patients (eight men and 21 women; mean age of 78.1 ± 5.2 years) were diagnosed with senile dementia of the Alzheimer type (SDAT) and 22 patients (eight men and 14 women; mean age of 72.0 ± 9.6 years) were diagnosed with MCI. We recruited healthy control subjects from among the people who attended the Health Promotion event and from the patients' spouses who visited the hospital. Fifty-five persons (11 men and 44 women; mean age of 72.6 ± 7.3 years) accepted our invitation to participate in the study.

Computer system description

The computer program was developed with Microsoft Visual Basic 6.0 and runs under the Windows operating system on any IBM PC-compatible computer. The computer must have an audio output device because audio instructions are provided along with written instructions. The system has been designed for users who are mainly aged people. Most aged people can hardly operate a computer by using a keyboard and mouse. Therefore, we adopted a touch screen display as an input device so that aged people can operate the system easily. All interactions can be performed by touching the icon shown on the display without using a keyboard and mouse. Throughout the whole process, users are guided not only by text prompts, but also by voice instructions. In this study, we used a Hitachi PC1NH5 laptop computer (Hitachi, Tokyo, Japan) and a Totoku CV511PJ touch screen display (Totoku, Tokyo, Japan).

Details of test procedure

The system presents six tests as follows:

1. Age and year-of-birth validity test

The purpose of this test is to examine the subject's ability to recall his or her age and year of birth. The system will ask the subject for his or her age and year of birth, and the subject is then required to respond by touching the corresponding icons on the screen display. The system awards one point for the correct response.

2. Three-word memory test

The purpose of this test is to assess the immediate memory of the subject. The system will voice three words (for example, apple, swallow and car) and will immediately ask the subject what the words were. Then the system displays nine choice icons and requires the subject to select the three correct icons. The system awards a maximum of three points, one for each correct icon chosen. Before this test ends, the system informs the subject that the following modified delayed-recall test will query the subject for the three words again.

3. Time orientation test

For this test, the system presents four screens in turn, and asks the subject what year, month, date and day it is, respectively. The system awards a maximum of four points, one for each correct response.

4. First modified delayed-recall test

The purpose of this test is to assess the short-term memory of the subject. The system asks the subject to recall the same words voiced in Test 2. Nine choice icons are displayed as in Test 2, but the arrangement is different. The system awards a maximum of three points, one point for each correct answer.

5. Visual working memory test

The purpose of this test is to examine the visual working memory of the subject. Two types of figures are

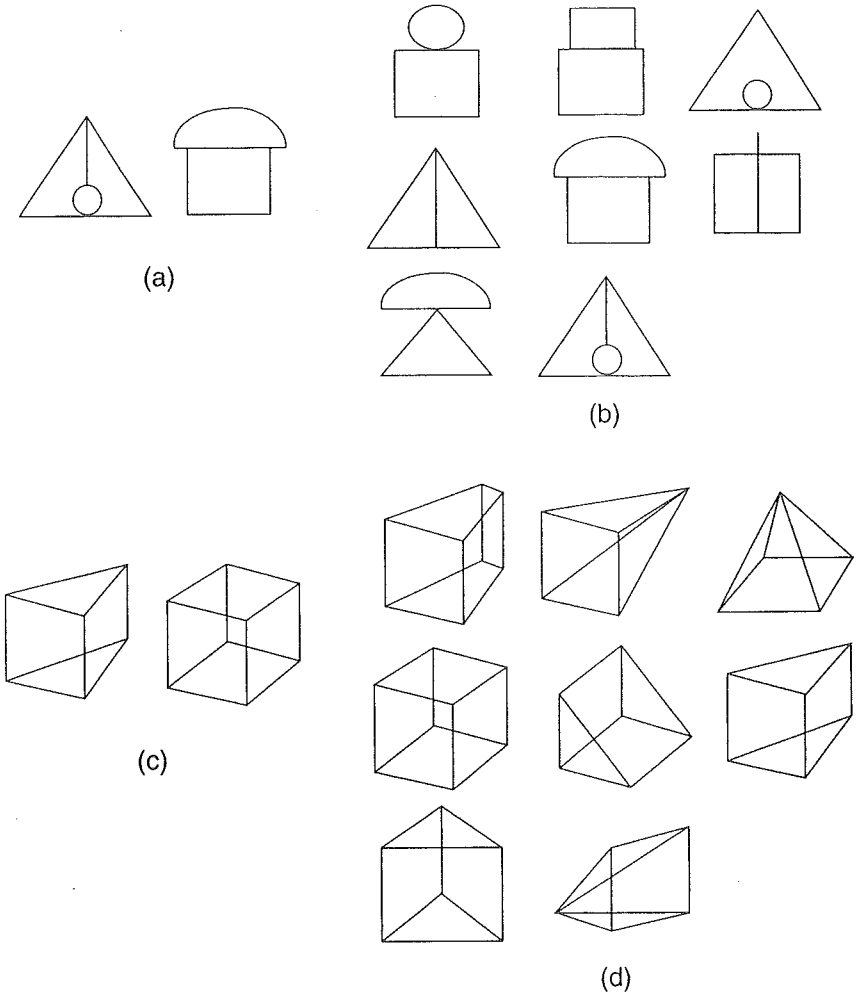

Figure 1 Figures used in the visual working memory test. (a) 2-D target stimuli; (b) 2-D figure choices; (c) 3-D target stimuli; and (d) 3-D figure choices.

used for this test: two-dimensional (2-D) and three-dimensional (3-D). Figure 1 shows the target figures and choices for this test. First, two 2-D target figures are presented for 10 s, after which the system displays eight possible choices of 2-D figures. The subject is then required to select the two previously displayed figures from among the eight choices. One point is awarded for each correct answer, for a maximum of two points. This process is then repeated with 3-D figures.

6. Second modified delayed-recall test

This test also assesses the short-term memory of the subject and is similar to the first delayed-recall test (Test 4, explained above). A maximum of three points are awarded, one for each correct response. For this test, the arrangement of the choice icons is different from that in Test 2 and Test 4.

All tests are usually completed within 5 min and the maximum number of points that can be awarded is 18 points.

Statistical analysis

Variations between the groups were first analyzed for significance by the Kruskal–Wallis test and then pairs of groups were directly compared using the Mann–Whitney test. Moreover, the differences between the scores obtained from three tests (three-word memory test; first and second modified delayed-recall tests) for each group were examined by the Friedman non-parametric test. These three statistical procedures were performed using statistical software (SPSS version 11 for Windows; SPSS Inc., Chicago, IL, USA). Receiver operating characteristic (ROC) analysis was performed to determine optimal cut-off points for the total score correspond-

Table 1 Demographic characteristics of samples and the results of individual tests for all three groups

	Control	MCI	SDAT
Sample size	55	22	29
Age (years)	72.6 ± 7.3	72 ± 9.6	78.1 ± 5.2
Sex (M/F)	11/44	8/14	8/21
Age and year-of-birth validity test	1 ± 0.00	0.89 ± 0.32	0.66 ± 0.48
Three-word memory test	2.96 ± 0.20	2.83 ± 0.38	2.66 ± 0.61
Time orientation test	3.86 ± 0.35	3.83 ± 0.38	1.69 ± 1.31
First delayed-recall test	2.94 ± 0.24	2.67 ± 0.49	1.76 ± 1.15
Visual working memory test for 2-D figures	1.88 ± 0.33	1.83 ± 0.38	0.97 ± 0.78
Visual working memory test for 3-D figures	1.80 ± 0.40	1.44 ± 0.51	1.31 ± 0.66
Second delayed-recall test	2.86 ± 0.35	2.44 ± 0.51	1.38 ± 0.98
Total score	17.31 ± 0.93	15.94 ± 1.26	10.41 ± 4.06

Results are expressed as: mean ± standard deviation. MCI, mild cognitive impairment; SDAT, senile dementia of the Alzheimer type.

Table 2 Results of the Kruskal–Wallis test showing significant group differences for all tests

	χ^2-value
Age and year-of-birth validity test	20.2**
Three-word memory test	9.39*
Time orientation test	59.3**
First delayed-recall test	35.4**
Visual working memory test for 2-D figures	37.3**
Visual working memory test for 3-D figures	15.7**
Second delayed-recall test	49.3**
Total score	65.0**

*$P < 0.01$, **$P < 0.001$.

ing to the screening between MCI and control subjects.

RESULTS

All subjects could understand how to interact with the computer and could complete the tests by themselves. Table 1 presents the demographic characteristics of samples and the results of individual tests from the three groups. At the group level, the SDAT group performed worse than the other two groups in each of the six tests, and the control group performed best in each of the six tasks. The results of the Kruskal–Wallis test are shown in Table 2 and significant group differences can be found for all tests. The results of the Mann–Whitney test in which the differences for all pairs of groups were compared are shown in Table 3. Significant differences between the control group and the SDAT group were found for all tests. Significant differences between the control group and the MCI group were found for the age and year-of-birth validity test, the visual working memory test for 3-D figures, the second delayed-recall test and the total score. Significant differences between the MCI group and SDAT group were found for the time orientation test, the visual working memory test for 2-D figures, the second delayed-recall test and the total score. The results of the Friedman nonparametric test are shown in Table 4. Significant differences were found in the SDAT and MCI groups at 99.9% level ($P < 0.001$) and 95% level ($P < 0.05$), respectively, and no significant differences were found in the control group. ROC analysis to identify the control and MCI yielded the best sensitivity and specificity values of 82% and 87%, respectively, with a cut-off point of 16.

DISCUSSION

The mini-mental state examination (MMSE)[10] is a widely recognized tool used for the detection of cognitive impairment, and in addition appears to be quite useful for examining patients with an increased risk of dementia (e.g. MCI patients).[11] When we developed the screening system, we referred to the tests used in the MMSE. However, these were originally designed for a method based on face-to-face interviews, so it was difficult to adopt all the tests for the computerized procedure. We paid attention to the tests of memory and the tests of visual working memory which are sensitive neuropsychological measures to detect early cognitive decline. Finally, we assembled the six tests explained above to form the computerized test system.

The mean value of the total score for all tests decreases in this order: control group, MCI group and SDAT group. In the validity test for age and year of

Table 3 Results of the Mann–Whitney test comparing the differences in all pairs of groups

	Control and MCI	Control and SDAT	MCI and SDAT
Age and year-of-birth validity test	*	**	NS
Three-word memory test	NS	**	NS
Time orientation test	NS	**	**
First delayed-recall test	NS	**	NS
Visual working memory test for 2-D figures	NS	**	**
Visual working memory test for 3-D figures	**	**	NS
Second delayed-recall test	**	**	**
Total score	**	**	**

*$P < 0.01$, **$P < 0.001$. MCI, mild cognitive impairment; NS, not significant; SDAT, senile dementia of the Alzheimer type.

Table 4 Results of the Friedman non-parametric test analyzing the differences in the scores from three tests for each group

	χ^2-value
Control	7
MCI	8.22*
SDAT	32.1**

*$P < 0.05$, **$P < 0.001$. MCI, mild cognitive impairment; SDAT, senile dementia of the Alzheimer type.

birth, all control subjects gave the correct answer. However, some MCI patients and one-third of the SDAT patients gave the wrong answer. A person who is unable to give his/her correct age and year of birth can be suspected to have MCI or SDAT. A combination of the three-word memory test and the subsequent delayed-recall test has been proposed as a useful test to screen for dementia.[12] In this study, there were no significant differences between the three-word memory test and the two subsequent modified delayed-recall tests in the control group. However, it is statistically significant that the score for the latter test was worse than that of the earlier test in the MCI and SDAT groups. This result is especially obvious in the SDAT group. It also suggests that these tests are useful for identifying cognitive decline. Visual working memory is also an important factor in the diagnosis of dementia.[13] In this study, we used two types of figures as target stimuli for the visual working memory test: 2-D and 3-D figures. In the case of 2-D figures, there were no statistical differences between the control and MCI groups. However, in the case of 3-D figures, statistical differences were found between the control group and MCI group. As for the MCI patients, they performed as well as the healthy control subjects in the visual working memory test for 2-D figures, but their performance was significantly impaired in the 3-D visual working memory test compared to the control subjects. In contrast, the visual working memory of the SDAT group for 2-D and 3-D figures was significantly impaired compared to the control group. Therefore, the visual working memory test also seems to be a useful indicator in identifying cognitive decline.

Conventional neuropsychological tests, such as the MMSE, involve dialogs between the interviewer and subject. However, it is difficult for a computer to understand what a subject is saying. Therefore, the system can only be designed to recognize a response when the user touches the choice button shown on the computer screen. Showing the answer choices is like giving a small hint and it does not require users to generate answers freely. In the delayed-recall test, presenting cues during the elicit retrieval phase improves the specificity of distinguishing individuals with dementia from unimpaired elderly people.[14] People who are able to answer the question with the help of a hint seem to be able to retain their cognitive ability and they usually get a high score. However, people who obtain a low test score even when hints are given are strongly suspected to be in cognitive decline. The selection of one answer from a range of choices seems to be adequate for the assessment of memory impairment and the method is easily adopted as a computerized procedure. However, computers are unsuitable for the assessment of dialogic ability, such as fluency, and this is a disadvantage of using computers.

We were able to identify MCI patients with an accuracy of 82% in this study, which is similar to the results presented by Darby *et al.*[15] Some other studies using biochemical examinations[16] and medical imaging equipment[17] have distinguished normal aged persons from those with dementia with high sensitivity