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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

特許第3515988号　発明の名称：物忘れ自己診断システムおよびその装置　出願番号：特願2001-281442　出願年月日平成13年9月17日　登録日：平成16年1月30日

2. 実用新案登録

なし。

3.その他

なし。

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平成16年度 分担研究報告書

痴呆のスクリーニング及び早期診断法の確立に関する臨床研究

(分担研究課題名：アルツハイマー病患者の尿および血清における酸化ストレスマーカーの検討)

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研究要旨

アルツハイマー病（Alzheimer Disease, AD）の早期診断を可能にする生物学的診断マーカーの確立のためには、AD の病理学的カスケードの最上流に位置する普遍的な変化を解明することが重要である。われわれは、孤発性・家族性 AD および関連疾患の剖検脳を用いて、酸化ストレス（Oxidative Stress, OS）が AD の変性過程において早期段階の変化であることを明らかにした。続いて、OS マーカーとして尿中 8-hydroxydeoxyguanosine、血清 CoQ10 酸化率（ubiquinol に対する ubiquinone の比）、および serum total antioxidant status（STAS）を測定し、AD 患者では尿や血清を用いて OS の増加を検出できることを明らかにした。今年度はこれらの OS マーカーの早期診断マーカーとしての有用性を検討する目的で、(1) mild cognitive impairment(MCI)～最軽度 AD 群（10 例、平均 71 歳）：clinical dementia rating（CDR）が 0.5 で mini-mental state examination(MMSE)が 24 点以上、(2) 軽度～高度 AD 群（25 例、平均 70 歳）：CDR が 1 以上あるいは MMSE が 23 以下、および(3)健常対照群（39 例、平均 65 歳）の 3 群を対象に検討した。その結果、血清 CoQ10 酸化率および STAS は、MCI～最軽度 AD 群でも軽度～高度 AD 群と同様に対照群に比べて有意に変化していた。以上のことから、これらの血清 OS マーカーが AD の早期診断に有用である可能性があり、今後、疾患特異性についても検討すべきであると考えられた。

キーワード：アルツハイマー病、酸化ストレス、生物学的診断マーカー

A. 研究目的

アルツハイマー病(Alzheimer Disease, AD)の早期診断を可能にする生物学的診断マーカーの確立のためには、AD の病理学的カスケードの最上流に位置する変化を解明することが重要である。従来、AD の生物学的診断マーカーとしては、AD 脳で観察される老人斑や神経原線維変化の主要構成蛋白であるアミロイドβやタウに注目が集まっていた。もし AD 脳において老人斑や神経原線維変化の形成に先行する普遍的な変化を見い出すことができれば、早期診断や発

症前診断法の確立に寄与することが期待される。

近年、AD の病態に酸化ストレス(Oxidative Stress, OS) が関与していることを示唆する報告が相次いでいるが、われわれは、孤発性および家族性 AD や AD 型の脳病理が併存するレビー小体型痴呆の剖検脳を用いて、神経細胞内の酸化的傷害が AD 型の変性過程において早期段階の普遍的な変化であることを明らかにしてきた(平成13年度および平成14年度の成果)。続いてわれわれは、患者の尿や血清中で測定可能な OS マーカーとして、尿中 8-hydroxydeoxyguanosine (8-OHdG)、血清 CoQ10 酸化率(ubiquinol に対する ubiquinone の比)、および serum total antioxidant status (STAS) を測定したところ、AD 患者でこれらが健常対照群に比べて有意に変化していることが明らかになった(平成15年度の成果)。

今年度はこれらの OS マーカーの早期診断マーカーとしての有用性を検討する目的で、AD の前段階と考えられる mild cognitive impairment (MCI) や最軽度の AD における OS マーカーの変化に注目した。

B. 研究方法

臨床的診断により、対象を以下の 3 群、すなわち、(1) MCI～最軽度 AD 群(10 例、平均 71 歳):clinical dementia rating (CDR) が 0.5 で mini-mental state examination (MMSE) が 24 点以上、(2) 軽度～高度 AD 群(25 例、平均 70 歳):CDR が 1 以上あるいは MMSE が 23 以下、および(3) 健常対照群(39 例、平均 65 歳)に分けて検討した。各群の対象から朝食前に尿および静脈血を採取し(解析まで－70℃で保存)、以下の OS マーカーについて検討した。すなわち、OS 強度の指標として尿中 8-OHdG (ELISA 法) および血清 CoQ10 酸化率 (HPLC 法) を測定し、OS に対する防御能力の指標として STAS (比色法) を測定した。

統計解析は、ANOVA (post hoc Fisher's PLSD) を用いて 3 群間の比較を行った。

(倫理面への配慮)

旭川医科大学倫理委員会の承認(平成15年1月17日承認、受付番号104)のもとに研究を行った。各対象例からの採血・採尿にあたっては、文書にて本研究の趣旨を患者および保護者(対照群は本人のみ)に説明し、研究に対する協力の同意を得た。協力の同意の有無によって、患者が臨床上、不利益を被ることがないように配慮した。

C. 研究結果

尿中 8-OHdG は、対照群、MCI～最軽度 AD 群、軽度～高度 AD 群の順に、7.9(4.2)、9.1(5.2)、11.9(7.4) ng/ml, [平均(標準偏差)]であり、対照群に比較して軽度～高度 AD 群で有意に高値であったが ($p < 0.01$)、対照群と MCI～最軽度 AD 群の間には有意差がなかった。

血清 CoQ10 酸化率は、対照群、MCI～最軽度 AD 群、軽度～高度 AD 群の順に、4.8(0.9)、7.6(2.4)、7.9(2.3) [平均(標準偏差)]であり、対照群に比

較してMCI～最軽度AD群（$p < 0.05$）あるいは軽度～高度AD群（$p < 0.02$）で有意に高値であった。

STASは、対照群、MCI～最軽度AD群、軽度～高度AD群の順に、1398(129)、1188(152)、1169(106) μM[平均（標準偏差）]であり、対照群に比較してMCI～最軽度AD群（$p < 0.001$）あるいは軽度～高度AD群（$p < 0.0001$）で有意に低値であった。

なお、尿中8-OHdG、血清CoQ10酸化率、およびSTASのいずれにおいても、MCI～最軽度AD群と軽度～高度AD群との間には有意差は認められなかった。

D. 考察

AD および関連疾患の剖検脳における検討から、酸化的傷害はAD型の変性過程において早期段階の普遍的な変化であることが示唆されている。したがって、OS マーカーは、AD 早期診断マーカーとして重要な候補の1つであると考えられる。昨年度までの検討によって、AD患者の尿および血清においてOS強度の増加やOSに対する防御能力の低下が認められ、AD患者では尿や血清を用いてOSの増加が検出されることが明らかになっている。

今回、ADの前段階と考えられるMCIあるいは最軽度のADにおいて、OS強度の指標である血清CoQ10酸化率の増加やOSに対する防御能力の指標であるSTASの低下が認められたことから、これらの血清OSマーカーがADの早期診断に有用である可能性が示唆された。

今後、尿および血清中OSマーカーの疾患特異性、すなわち、AD以外の痴呆性疾患や神経疾患とADの鑑別診断上の有用性について検討を加えることにより、尿および血清中OSマーカーのAD生物学的診断マーカーとしての重要性が証明されることが期待される。

E. 結論

血清中のOSマーカーであるCoQ10酸化率やSTASは、MCI～最軽度のADにおいて軽度～高度のADと同様に変化しており、ADの早期診断上、これらのOSマーカーが有用である可能性が示唆された。

F. 健康危険情報

なし。

G. 研究発表

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H. 知的財産権の出願・登録状況

なし。

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Amyloid-β down-regulates XIAP expression in human SH-SY5Y neuroblastoma cells

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Recent observations suggest that amyloid-β (Aβ), a major constituent of senile plaques, induces apoptosis in cultured neuronal cells. However, the concentration of Aβ that leads to neuronal cell death is much higher (10–25 μM) than that in the cerebrospinal fluid of normal controls or AD patients (nM order). As reported here, we found that subtoxic concentrations (100–500 nM) of Aβ(1-42) can down-regulate the expression of the X-linked inhibitor of apoptosis (XIAP) in human SH-SY5Y neuroblastoma cells, and that vulnerability to oxidative stress caused by Aβ(1-42) is attenuated by over-expression of XIAP. These results suggest that down-regulation of XIAP expression in response to subtoxic, more physiological concentrations (100–500 nM) of Aβ(1-42) increases vulnerability to oxidative stress. *NeuroReport* 15:851–854 © 2004 Lippincott Williams & Wilkins.

Key words: Alzheimer's disease (AD); Amyloid β (Aβ); Apoptosis; Oxidative stress; X-linked inhibitor of apoptosis (XIAP)

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, characterized by extracellular senile plaques that are composed of the deposition of amyloid-β (Aβ), the formation of neurofibrillary tangles, with abnormally phosphorylated tau as their major component, and a reduction in neuronal cells. Aβ is a 39–43 amino acid peptide derived from processing of the amyloid precursor protein (APP). The importance of Aβ in the pathogenesis of AD has been indicated by several findings. Most important, mutations in presenilin-1, -2 and APP, which are involved in familial forms of AD, lead to an increase in the amyloidogenic form of Aβ [1]. Although it is still controversial whether this increase in Aβ formation is sufficient to cause nerve cell degeneration in AD, the neurotoxic effects of Aβ have been demonstrated both *in vitro* and *in vivo* [2–5]. Recent studies have also shown that in cultures of neurons exposed to Aβ, dying cells display the characteristics of apoptosis [6]. It has been suggested that Aβ-induced apoptosis involves oxidative stress and perturbation of intracellular calcium homeostasis [3,7]. However, the Aβ concentration that leads to apoptosis is much higher (10–25 μM) than the physiological concentration (nM order).

Apoptosis is regulated by several gene products, including the members of the caspase family, Apaf-1 and related proteins, the Bcl-2 family, and the inhibitor of apoptosis (IAP) family [8–11]. Members of the IAP family are intrinsic cellular suppressors of apoptosis and are represented by highly conserved members found in a wide range of locations, from insect viruses to mammals. The most potent human IAP is the X-linked inhibitor of apoptosis (XIAP), whose mechanism of action involves direct inhibition of caspase 3, 7 and 9, which are key proteases of the apoptotic cascade. Furthermore, XIAP is ubiquitously expressed, whereas expression of HIAP1 or HIAP2 is lowest in the CNS [11]. Given the potency of XIAP as inhibitor of apoptosis and previous findings that subtoxic, high physiological concentrations of Aβ increases vulnerability to oxidative stress, at least in part through an increase in the expression ratio of Bax/Bcl-2 [13], we decided to examine the effects of subtoxic concentrations of Aβ on the expression of XIAP.

MATERIALS AND METHODS

Preparation of gene construct: The XIAP gene, a gift from Dr J.D. Ashwell (National Institute of Health, Bethesda, USA), was cut with *Bam*HI and *Not*I restriction enzymes and inserted with a linker into the pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) vector cut with *Nhe*I and *Not*I.

Cell line, culture conditions, and transfections: SH-SY5Y human neuroblastoma cells were kindly donated by Dr J.L. Biedler (Sloan Kettering Institute, New York, NY, USA) and were cultured in DMEM/F12 containing 5% fetal calf serum (FCS). Cultures were transfected with the XIAP gene or a mock vector gene employing optimum-minimum essential medium with Lipofectamine2000 (Gibco, Rockville, MD, USA). Stably transfected cells were selected in 600 μg/ml G418 (Invitrogen, Carlsbad, CA, USA) for 3 weeks. Pools of ~100 colonies were used to avoid bias of single cell colonies.

Treatment of cell cultures with Aβ: Fibrillar Aβ1-42 (Sigma-Aldrich, Tokyo, Japan) and control reverse peptide Aβ42-1 (BACHEM, King of Prussia, PA, USA) were prepared by incubation at 200 μM in sterile water at 37°C for 3 days.

Cells were plated for 3 days before treatment with Aβ. Confluent cultures (about 60% confluence) were used and were placed in fresh media 2 h before treatment. Aβ peptides were added to the cultures and incubated for 12, 24 and 48 h.

Oxidative stress on cell cultures pre-exposed to Aβ: Cells were treated for 48 h with fibrillar Aβ1-42 (500 nM) as described above. H_2O_2 (0.5 μM) or 5 nM 4-hydroxynonenal (HNE) was then added to the cultures and incubated for 3 h.

Cell viability assay: Surviving and dead cells were visualized and counted in three fields (600–700 cells) per well in a blinded manner with the aid of the Live/Dead Eukolight Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA). Data show dead cells as a percentage of total cells.

Assessment of caspase activity: Analysis of caspase activity was performed with the Caspase Colorimetric Protease Assay Kit according to the manufacturer's instructions (MBL, Nagoya, Japan). After exposure to Aβ and oxidative stress, the culture medium was aspirated, and the cells washed with phosphate buffered saline, lysed in lysis buffer, and centrifuged at 10 000 × *g* for 10 min. Aliquots of 100 μg supernatant were added to the reaction buffer and incubated at 37°C for 2 h. The reaction buffer was supplemented with 200 μM DEVD-pNA, a colorimetric substrate for caspase-3 and -7. Production of pNA was monitored with a microplate reader at an absorbance of 405 nm. Colorimetric blanks containing no cellular extracts were subtracted from the values obtained.

Antibodies: Anti-XIAP polyclonal antibody was purchased from R&D Systems (Minneapolis, MN, USA) and Anti-Actin polyclonal antibody from Sigma-Aldrich (Tokyo, Japan).

Western blots: Cells were lysed in 100 mM PIPES pH 6.8, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinine, 5 μg/ml leupeptine, 25 mM NaF, 1 mM Na_3VO_4, 0.1% Triton-X100 on ice, and centrifuged at 200 000 × *g* for 30 min. The supernatants were used for Western blot analysis. Aliquots of 50 μg supernatant were separated by SDS-PAGE and transferred to nitrocellulose membranes. Peroxidase-labeled anti-rabbit IgG was used as the secondary antibody, and membranes were developed by means of ECL (Amersham Biosciences, Buckinghamshire, UK).

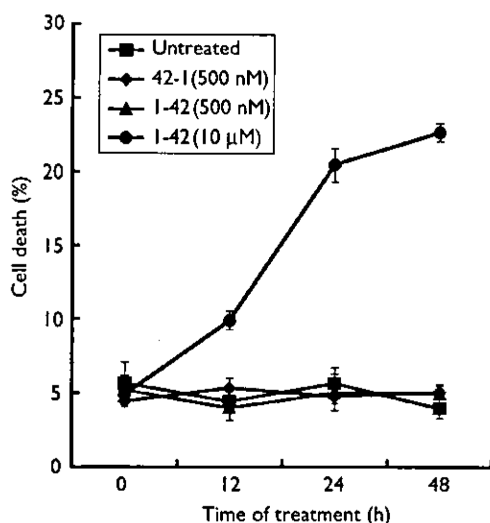

Fig. 1. Cell death in SH-SY5Y cells treated with Aβ. Fibrillar Aβ1-42 (500 nM) did not cause high levels of cell death in SH-SY5Y cells. SH-SY5Y cells were exposed to fibrillar Aβ1-42 (500 nM and 10 μM), and Aβ42-1 (500 nM) for 12 h, 24 h, and 48 h. Surviving and dead cells were visualized and counted with the Live/Dead Eukolight Viability/Cytotoxicity Kit. Data show dead cells as a percentage of total cells ($n=3$, mean ± s.e.m.).

RESULTS

Cell death in SH-SY5Y cells treated with Aβ: SH-SY5Y cell death after exposure to Aβ was quantified by calsein

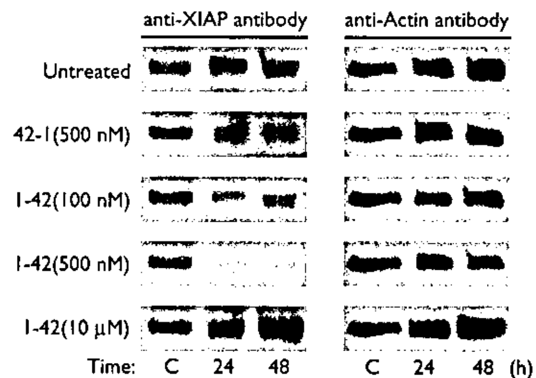

Fig. 2. Western blot analysis showing expression of XIAP and Actin after treatment with Aβ. Concentration-dependent down-regulation of XIAP expression by fibrillar Aβ1-42 at subtoxic concentrations. SH-SY5Y cells were treated with fibrillar Aβ1-42 (100 nM, 500 nM and 10 μM) and Aβ42-1 (500 nM) for the indicated number of hours.

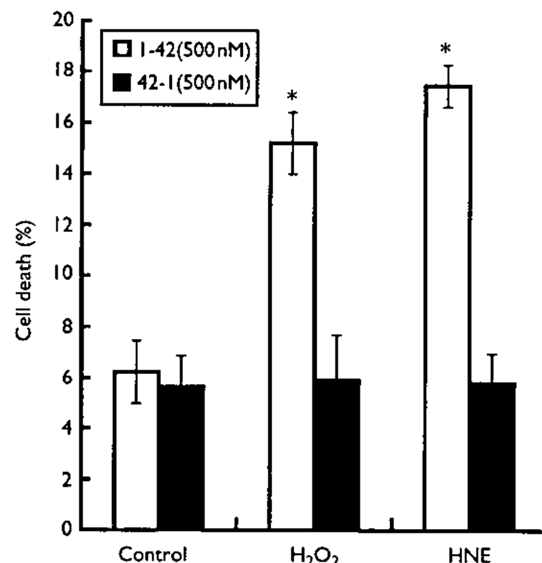

Fig. 3. Susceptibility to oxidative stress in SH-SY5Y cells pretreated with fibrillar Aβ1-42 but not in those pretreated with Aβ42-1. EthD-1 staining indicates increased cell death in cells pretreated with fibrillar Aβ1-42 (500 nM) for 48 h and then submitted to low oxidative stress levels, by exposure to 0.5 μM H_2O_2 or 5 nM HNE. In contrast, the cells treated with the control peptide Aβ42-1 (500 nM) did not show an increase in sensitivity to low levels of oxidative stress. Data show dead cells as a percentage of total cells ($n=3$, mean ± s.e.m.). $*p < 0.05$: significantly different from respective controls (two sample t-test).

acetoxymethylester/EthD-1 double staining. Uptake of EthD-1 indicates membrane leakage, a terminal stage of neuronal degeneration, which also occurs after neuronal apoptosis. There was no significant difference between fibrillar Aβ1-42 (500 nM), Aβ42-1 (500 nM), and untreated cells. Fibrillar Aβ1-42 (500 nM) did not cause high levels of cell death in SH-SY5Y cells, although in cells treated with 10 μM fibrillar Aβ1-42, an increase in cell death with time was observed (Fig. 1).

Aβ down-regulates XIAP expression: To determine whether XIAP expression is involved in the Aβ-induced neurotoxic mechanism, the expression level of XIAP protein in SH-SY5Y cells exposed to Aβ was investigated by western blotting. Treatment with fibrillar Aβ1-42 at subtoxic concentrations (100–500 nM) reduced XIAP protein levels in a concentration-dependent manner compared to cells treated with Aβ42-1 control and untreated cells. Fibrillar Aβ1-42 at a toxic dose (10 μM) did not down-regulate but could have induced XIAP expression (Fig. 2).

XIAP attenuates vulnerability to oxidative stress: To determine whether cells exposed to Aβ were more vulnerable to an age-dependent secondary insult such as oxidative stress, we first treated the SH-SY5Y cells with subtoxic (500 nM) Aβ1-42 for 48 h and then used 0.5 μM. H_2O_2 or 5 nM HNE to expose them to low oxidative stress levels. The Aβ-treated cells proved to be more vulnerable to oxidative stress than untreated cells (Fig. 3, Fig. 4b).

To determine whether XIAP expression is involved in the mechanism of this Aβ-induced increase in vulnerability to

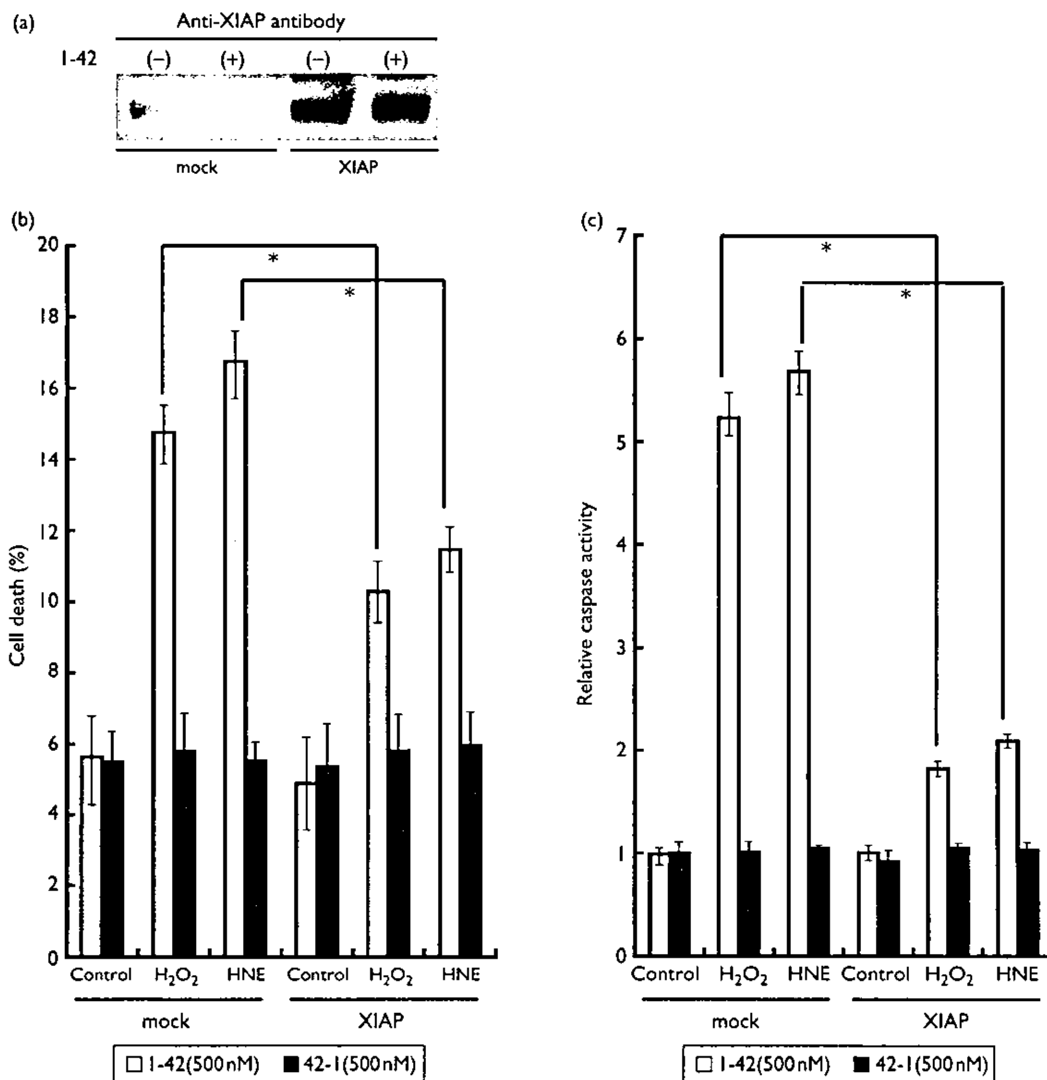

Fig. 4. XIAP attenuates vulnerability to oxidative stress. (a) The levels of XIAP expression in transfected cell lines were determined by Western blotting. In cells showing over-expression of XIAP, no change was observed after treatment with fibrillar Aβ1-42 (500 nM) for 48 h. (b) Susceptibility to oxidative stress in SH-SY5Y cells pretreated with fibrillar Aβ1-42 was attenuated by over-expression of XIAP. SH-SY5Y cells were stably transfected with the XIAP gene or a mock vector gene and exposed to 0.5 μM H_2O_2 or 5 nM HNE after treatment with fibrillar Aβ1-42 (500 nM) or control peptide Aβ42-1 (500 nM) for 48 h. Surviving and dead cells were visualized and counted with the Live/Dead Eukolight Viability/ Cytotoxicity Kit. Data show dead cells as a percentage of total cells ($n=3$, mean $\pm$ s.e.m.). *$p < 0.05$: significantly different from mock (two sample t-test). (c) Activation of caspase activity induced by oxidative stress after treatment with Aβ was inhibited by over-expression of XIAP. Caspase activity was measured by observing the cleavage of DEVD-pNA, the colorimetric substrate for caspase-3 and -7. Data show caspase activity in cells relative to that in Aβ42-1 treated cells transfected with the mock vector gene ($n=3$, mean $\pm$ s.e.m.). *$p < 0.05$: significantly different from mock (two sample t-test).

oxidative stress, we studied the effect of overexpression of XIAP on oxidative stress resistance. SH-SY5Y cells were stably transfected with a mock vector gene or the XIAP gene (Fig. 4a) and exposed to 0.5 μM H_2O_2 or 5 nM HNE after treatment with Aβ1-42 for 48 h. Vulnerability to oxidative stress caused by Aβ at a subtoxic concentration (500 nM) was attenuated by over-expression of XIAP (Fig. 4b). To further investigate the relationship between the attenuation of vulnerability and inhibition of caspase activity by XIAP, caspase activity was measured by observing the cleavage of DEVD-pNA, the colorimetric substrate for caspase-3 and -7. Activation of caspase activity induced by oxidative stress after treatment with Aβ was found to be inhibited by over-expression of XIAP (Fig. 4c). This led to the hypothesis that over-expression of XIAP attenuates vulnerability to oxidative stress, in part through inhibition of caspases.

DISCUSSION

Aβ is a major constituent of senile plaques, which are a hallmark of AD, and the production of Aβ might be a primary cause of neuronal cell death that leads to cognitive dysfunction in AD [1]. Recent observations suggest that Aβ induces apoptosis in cultured neuronal cells [6], and that the concentration of Aβ that leads to neuronal cell death is much higher than that observed in the cerebrospinal fluid of normal controls or AD patients [3,4,6,7]. Bax is a pro-apoptotic and Bcl-2 an anti-apoptotic factor involved in the process of apoptosis [9]. It has been reported that subtoxic concentrations of Aβ increase vulnerability to oxidative stress, at least in part through an increase in the expression ratio of Bax/Bcl-2 [13].

In this study we examined the possibility that Aβ at a subtoxic, more physiological concentration increases vulnerability to age-dependent secondary insults such as oxidative stress through changing the expression of XIAP. Aβ at subtoxic concentrations was found to down-regulate XIAP expression (Fig. 1, Fig. 2). Furthermore, over-expression of XIAP attenuated vulnerability to oxidative stress caused by subtoxic concentrations of Aβ (Fig. 3, Fig. 4). An increase in the XIAP protein level was observed in cells treated with Aβ at a toxic dose. Expression of the XIAP protein is translationally regulated via internal ribosome entry site (IRES) elements located in the 5′ untranslated region (5′UTR) [14] and IRES-regulated proteins are synthesized under a variety of stress conditions, including hypoxia, serum deprivation, amino acid deficiency, and apoptosis [12,15,16]. The increase in the XIAP protein level observed in cells treated with toxic Aβ is therefore thought to be caused by Aβ-induced apoptosis. It remains unclear how Aβ down-regulates XIAP expression. Recently, it was reported that degradation of XIAP is mediated by proteasome or calpain [17,18]. It is possible that in cells treated with Aβ, XIAP has readily been degraded by activation of proteasome or calpain. It is also possible that the IRES-regulated synthesis of XIAP is interfered with in cells treated with Aβ. Further study is needed to determine how Aβ down-regulates XIAP expression.

CONCLUSION

Our findings suggest that down-regulation of XIAP expression in response to Aβ at subtoxic, more physiological concentrations increases vulnerability to oxidative stress. When combined with the observation of an increase in the expression ratio of Bax/Bcl-2, these findings may offer an explanation for the neurodegenerative mechanisms of AD caused by low concentrations of Aβ.

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ORIGINAL ARTICLE

C677T polymorphism of methylenetetrahydrofolate reductase gene affects plasma homocysteine level and is a genetic factor of late-onset Alzheimer's disease

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Abstract

Background: Elevated plasma homocysteine levels are known as a risk for atherosclerotic vascular disease and venous thrombosis and have been shown as a risk for late-onset Alzheimer's disease (LOAD).

Method: To examine the effect of genetic factors predisposing to elevated plasma homocysteine levels on the occurrence of LOAD, we determined the genotype of a C677T polymorphism of methylenetetrahydrofolate reductase (MTHFR) gene and a variable number tandem repeat (VNTR) spanning exon 13–intron 13 boundary of cystathionine β-synthase (CBS) gene in patients with LOAD and community-based control subjects.

Results: Logistic regression indicated that the MTHFR-T allele was a risk for LOAD ($P < 0.05$), independently from apolipoprotein E-ε4 (APOE-ε4) allele. Kaplan–Meier tests showed that in APOE-ε4 non-carriers, individuals with the MTHFR-TT genotype have occurences of LOAD earlier than those with the MTHFR-CC genotype ($P < 0.05$). Multiple regression analysis indicates that MTHFR-T allele increases plasma homocysteine levels ($P = 0.0002$), while the number of X chromosomes decreases ($P = 0.01$). Plasma homocysteine level was not correlated with age, plasma albumin reflecting nutritional condition, and the dose of APOE-ε4 allele. The CBS-20 VNTR allele showed the same trend to increase plasma homocysteine level as the MTHFR-T allele, but a risk effect for LOAD was not evident.

Conclusion: A genetic propensity for elevated plasma homocysteine levels, explained by the MTHFR-T allele encoding defective enzymatic function, is involved in the development of LOAD, particularly in APOE-ε4 non-carriers, and that homocysteine metabolism could be a preventive target to LOAD in the elderly.

Key words: *Alzheimer's disease, apolipoprotein, cystathionine β-synthase, genetic, homocysteine, methylenetetrahydrofolate reductase, risk.*

INTRODUCTION

The major cause of dementia in the elderly is late-onset Alzheimer's disease (LOAD) both among Caucasians and, after 1990s, the Japanese elderly.[1] This trend could be caused by preventive intervention and advanced treatment of cerebrovascular stroke (such as antihypertensive treatment), dietary salt restriction and protein supplement, improved indoor air-conditioning,[2] and global warming. A cohort study in Caucasians showed that elevated plasma homocysteine level is a risk factor for cognitive decline in the elderly, notably for LOAD.[3,4] However, elevated plasma homocysteine level is a risk factor not only for atherosclerotic vascular disease and venous thrombosis but also cerebrovascular disease.[5–9] Plasma homocysteine level is modified by dietary environment; for example, loading of methionine induced elevated plasma homocysteine level under low vitamin B12 supplement.[10] Although, it was noted that both fasting and postmethionine-load

hyperhomocysteinemia is inherited in many of the instances.[11] C677T polymorphism of methylenetetrahydrofolate reductase (MTHFR) gene is one of genetic factors affecting plasma homocysteine levels.[12] Demented patients with multiple infarcts had a higher frequency of the MTHFR-TT genotype than those without.[8] However, it remains undetermined whether the MTHFR-T allele modifies the risk for either vascular dementia or LOAD.[13–16] Cystathionine β-synthase (CBS) gene is another genetic factor to determine plasma homocysteine levels, since a 31-bp variable number tandem repeat (VNTR) spanning the exon 13–intron 13 boundary of the CBS gene is related to plasma homocysteine levels.[17] Subjects with Down syndrome show the appearance of senile plaque in the brain in their thirties, a pathological hallmark of Alzheimer's disease (AD). CBS gene is localized in the critical region of Down syndrome, at 21q22.3,[18] but the genetic association between LOAD and the CBS gene, to our knowledge, has not been reported. To elucidate how genetic factors related to plasma homocysteine level modify the occurrence of LOAD, we performed a case–control study to examine the relation of plasma homocysteine level and genetic polymorphisms modifying plasma homocysteine in patients with LOAD, and evaluated how these genetic factors contribute to the occurrence of LOAD.

SUBJECTS AND METHODS

Subjects

Patients with AD were diagnosed as having probable AD according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA).[19] Elderly subjects, living healthily and independently at home, were recruited in Suita City, Osaka, Japan. No cognitive impairment in these subjects was found by the questionnaire including date, orientation, past history and medical records. After written informed consent to participate in the present study was obtained, peripheral blood was drawn from the patients and these population-based control subjects. The Genome Ethical Committee of Osaka University Graduate School approved this procedure. The age of the patients ($n = 196$) at blood withdrawal was 79.2 years + 7.0 (mean + SD), range 65–98 years, and that of age-matched control subjects ($n = 385$) was 75.4 years + 5.0, range 65–92 years. Age at onset of AD was 74.7 years + 6.9, range 65–94 years. Peripheral blood was drawn into ethylenediaminetetraacetic acid dinatrium (EDTA2Na), and plasma and cell fractions were separated within 6 h. DNA was extracted from the cell fraction using a QIAamp DNA blood kit (Qiagen, Hilden, Germany) and stored at 4°C. Plasma was stored at 80°C until use.

Genotyping

The genotype of the APOE gene was determined using polymase chain reaction–restriction fragment length polymorphism (PCR–RFLP) according to the procedure by Wenham *et al.*[20] The C677T polymorphism of the MTHFR gene was genotyped by a PCR–RFLP method using the *Hinf*I restriction enzyme according to the procedure described previously.[21]

The 31 bp VNTR region in the CBS gene was amplified using primers CBS3: 5′-GGAATGGT GACGCTTGGGAACAT-3′ and CBS4: 5′-ACTTGTAAA GTGGGTGCTTCTCAGC-3′, and PCR product were electrophoresed in 2.5% agarose gel containing ethidium bromide and visualized on an ultraviolet transilluminator. The 31 bp tandem-repeat polymorphic alleles were determined by the comparison with the most frequently observed 796 bp alleles harboring 18 repeats.[17,22]

Plasma homocysteines and albumin

For the patients with LOAD ($n = 77$) we examined the relationship between the allelic doses of the MTHFR-T allele and CBS-repeat doses with plasma total homocysteine level. The concentration of plasma homocysteine was measured by the high-pressure liquid chromatography (HPLC), that included derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (ABD-F) and postcolumn fluorescence detection.[23] To test the effect of nutrition, plasma albumin level was also measured by the BCG method described previously.[24]

Statistics

The allele and genotype frequencies was compared by χ^2-tests, and P-value <0.05 was considered significant after correction by the number of comparisons. Multiple logistic regression of the occurrence of LOAD with the dose of the APOE-ε4, MTHFR-T, and CBS-20 VNTR alleles were examined. Kaplan–Meier survival curves free from LOAD were examined by the Mantel-Cox logrank test. The correlation of plasma

homocysteine level with age, dose of X chromosome, plasma albumin level, dose of the APOE-ε4, MTHFR-T, and CBS-20 VNTR alleles was examined by multiple regression. *P*-values <0.05 was considered significant.

RESULTS

Allele and genotype frequencies of methylenetetrahydrofolate reductase-C766T and cystathionine β-synthase-variable number tandem repeat polymorphisms

We examined the genotype and allele frequencies of the MTHFR-C766T and CBS-VNTR polymorphisms in patients with LOAD and population-based non-demented controls (Tables 1 and 2). Genotype distributions of both of the polymorphisms were in Hardy–Weinberg equilibrium. The genotype frequency of the MTHFR-C766T polymorphism was not significantly different between the groups, but the allele frequency of MTHFR-T allele in the patient group was relatively higher than that in the control group (Table 1). When the subjects were divided into APOE-ε4 carrier and non-carrier, the MTHFR-T allele in the patient group was significantly more frequent than that in the control group in APOE-ε4 non-carriers ($P < 0.02$). The same trend was also found in APOE-ε4 carrier, but not significantly. However, we did not find any significant difference in the CBS-VNTR allele and genotype frequencies between the groups (Table 2).

To incorporate genetic interactions in risk effects, we performed a logistic regression of the APOE-ε4, MTHFR-T and CBS-20 VNTR alleles for the occurrence of LOAD (Table 3). We detected a significant risk effect of the APOE-ε4 (odds ratio = 5.2, 95% CI = 3.51–7.59, $P < 0.0001$) and also a marginally significant effect of the MTHFR-T allele (odds ratio = 1.4, 95% CI = 1.02–1.85, $P = 0.04$), but the CBS-20 VNTR allele did not show any significant effect. The risk effect of the MTHFR-T allele was more prominent

Table 1 Genotype and allele frequencies of methylenetetrahydrofolate reductase C766T polymorphism in patients with late-onset Alzheimer's disease (LOAD) and controls

	Genotype (frequency)			Allele (frequency)	
Group	CC	CT	TT	C	T
All subjects					
Patients (n = 194)	64 (0.330)	98 (0.505)	32 (0.164)	226 (0.582)	162 (0.418)
Controls (n = 379)	144 (0.380)	193 (0.509)	42 (0.111)	481 (0.635)	277 (0.365)
APOE-ε4(+)					
Patients (n = 102)	38 (0.373)	52 (.510)	12 (0.118)	128 (0.627)	76 (0.373)
Controls (n = 60)	25 (0.417)	30 (0.500)	5 (0.083)	80 (0.667)	40 (0.333)
APOE-ε4(–)					
Patients (n = 92)	26 (0.282)	46 (0.500)	20 (0.217)	98 (0.533)	86 (0.467)*
Controls (n = 319)	119 (0.373)	163 (0.511)	37 (0.116)	401 (0.629)	237 (0.37)

The genotypes of the methylenetetrahydrofolate reductase (MTHFR) polymorphism of two patients and six controls were not determined because of the insufficient polymase chain reaction amplification. Allele frequency of the MTHFR polymorphism in APOE-ε4 (–) group was significantly different between the patients and controls ($P = 0.02$).

Table 2 Genotype frequencies of the cystathionine β-synthase (CBS) polymorphism in patients with late-onset Alzheimer's disease (LOAD) and controls

	Genotype (frequency)				Allele (frequency)		
Group	18/18	18/20	17/18	20/20	17	18	20
All subjects							
Patients (n = 190)	144 (0.758)	45 (0.237)	0 (0.000)	1 (0.005)	0 (0.000)	333 (0.876)	47 (0.124)
Controls (n = 384)	285 (0.742)	91 (0.237)	2 (0.005)	6 (0.016)	2 (0.003)	663 (0.863)	103 (0.134)
APOE-ε4(+)							
Patients (n = 98)	70 (0.714)	27 (0.276)	0 (0.000)	1 (0.010)	0 (0.000)	167 (0.852)	29 (0.148)
Controls (n = 60)	49 (0.817)	9 (0.150)	0 (0.000)	2 (0.033)	0 (0.000)	107 (0.892)	13 (0.108)
APOE-ε4(–)							
Patients (n = 92)	74 (0.804)	18 (0.196)	0 (0.000)	0 (0.000)	0 (0.000)	166 (0.902)	18 (0.098)
Controls (n = 324)	236 (0.728)	82 (0.253)	2 (0.006)	4 (0.012)	2 (0.003)	556 (0.858)	90 (0.139)

The genotypes of the CBS polymorphism of six patients and one controls were not determined because of the insufficient PCR amplification.

among APOE-ε4 non-carriers (odds ratio = 1.5, 95% CI = 1.04–2.14, $P = 0.03$).

Methylenetetrahydrofolate reductase-TT genotype and onset of late-onset Alzheimer's disease

To examine the genetic effect of the defective MTHFR genotype, the common MTHFR-CC genotype and the defective MTHFR-TT genotype was compared for the survival free from LOAD. In all subjects, the subjects with MTHFR-TT genotype showed a trend to develop LOAD faster than those with MTHFR-CC genotype (Fig. 1a). This trend was observed in both APOE-ε4 non-carriers (Fig. 1b) and APOE-ε4 carriers (Fig. 1c). However, the significant difference was only supported in APOE-ε4 non-carriers ($P < 0.05$) (Fig. 1b). We did not find any difference of the survival rate free from LOAD between subjects with the CBS-20/20 VNTR genotype and those with the CBS-18/18 VNTR genotype (data not shown).

Table 3 Logistic regression of doses of the APOE-ε4, methylenetetrahydrofolate reductase (MTHFR)-T and cystathionine β-synthase (CSB)-20 variable number tandem repeat (VNTR) alleles for the occurrence of late-onset Alzheimer's disease (LOAD)

Allele	Odds ratio (*P*-value)	95% Confidence interval
All subjects		
APOE-ε4	5.2*	3.51–7.59
MTHFR-T	1.4**	1.02–1.85
CBS-20 VNTR	0.8	0.55–1.27
APOE-ε4(+) subjects		
APOE-ε4	3.8	0.81–17.9
MTHFR-T	1.1	0.68–1.93
CBS-20 VNTR	1.2	0.61–2.51
APOE-ε4(–) subjects		
MTHFR-T	1.5***	1.04–2.14
CBS-20 VNTR	0.7	0.37–1.16

*$P < 0.0001$, **$P = 0.04$, ***$P = 0.03$.

Correlation of plasma homocysteine level with the methylenetetrahydrofolate reductase-T allele

To examine the effect of age, sex, nutrition, and risk alleles for the occurrence of LOAD on plasma homocysteine level, we performed a multiple regression of these parameters in patients with LOAD (Table 4). Among those, significant correlations with plasma homocysteine level were found in the dose of X chromosome (standerdized coefficient = – 0.266, $P = 0.01$) and that of the MTHFR-T allele (standaerdized coefficient = 0.404, $P = 0.0002$). The dose of the CBS-20 VNTR allele showed a trend to increase the plasma homocysteine level, but marginally not significant (standerdized coefficient = 0.185, $P = 0.08$). No significant effects were found with age, plasma albumin level, and the APOE-ε4 allele.

DISCUSSION

We confirmed that plasma homocysteine level in patients with LOAD is genetically affected by the C766T polymorphism of the MTHFR gene, and that the VNTR length of the CBS gene showed a similar trend to the MTHFR-T allele. The MTHFR-T allele results in an Ala222Val substitution, leading to reduced enzyme activity and increased thermolability, and relates to increased plasma homocysteine level.[21] The increase in the number of a 31-bp VNTR unit of the CBS gene shows a reduced activity of CBS caused by alternative splicing of exon 13, and relates with elevated plasma homocysteine level after

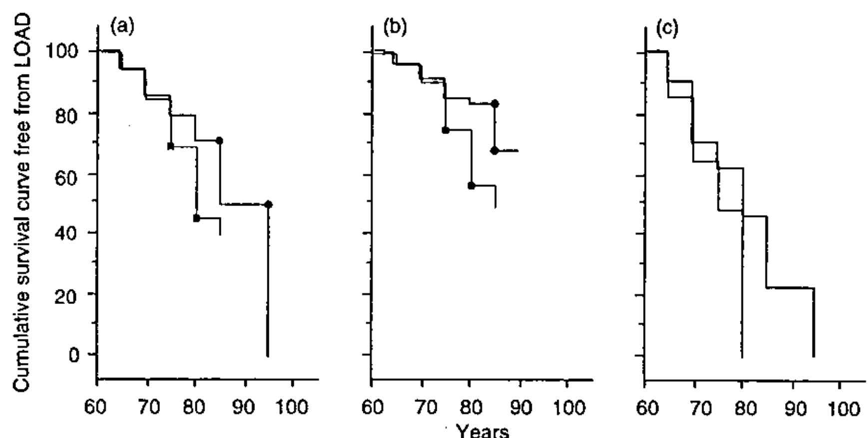

Figure 1 Kaplan–Meier survival curves free from late-onset Alzheimer's disease (LOAD). (●) subjects with the methylenetetrahydrofolate reductase (MTHFR)-CC genotype and (■) those with the MTHFR-TT genotype. Age of subjects is represented by seven groups: 65 years (ranged 65–67 years), 70 (ranged 68–72), 75 (ranged 73–77), 80 (ranged 78–82), 85 (ranged 83–87), 90 (ranged 88–92), and 95 (ranged 93–97). The curves were drawn for (a) all subjects; (b) APOE-ε4 carriers; and (c) APOE-ε4 non-carriers. Logrank test indicated a significant difference of the survival rate free from LOAD between the subjects in APOE-ε4 non-carriers ($P < 0.05$).

Table 4 Multiple regression of plasma homocysteine level with doses of the methylenetetrahydrofolate reductase (MTHFR)-T and cystathionine β-synthase (CBS)-20 variable number tandem repeat (VNTR) alleles

Factor	Coefficient (Standardized)	*t*-value
Age	0.095 (0.089)	0.785
X chromosome	−4.082 (−0.266)	−2.560*
Albumin	0.701 (0.038)	0.352
APOE-ε4	−0.136 (−0.012)	−0.108
MTHFR-T	3.816 (0.404)	3.925**
CBS-20 VNTR	2.574 (0.185)	1.754

*P = 0.01, **P = 0.0002.

methionine loading.[17] A gene–gene interaction between the MTHFR and CBS gene has been shown in plasma homocysteine level.[25]

We found a risk effect and a promotive effect on the occurrence of LOAD by the MTHFR-T allele in APOE-ε4 non-carriers. The same trend was also found in APOE-ε4 carriers, though not significantly. Several reports examined the risk effect of the MTHFR-T allele on the occurrence of LOAD, none of which found any significant effects.[13–16] We showed that the VNTR of the CBS gene did not associate with the occurrence of LOAD. Thus, elevated plasma homocysteine level in LOAD in itself merely reflects one of a vascular factor of LOAD, as shown in cerebrovascular dementia with multiple infarcts.[3,9]

It was well demonstrated that any positive association in LOAD other than APOE-ε4 allele are difficult to replicate, which likely caused by its weak effect. However, selection bias of patients as well as controls often affects the association studies. The frequency of the MTHFR-T allele in Japanese controls was similar to that reported by Wakutani *et al.*[16] We did not include either patients with cerebrovascular changes screened by neuroimaging studies. Therefore, it is unlikely that the patient groups in our study contained vascular dementia that was shown to associate with the MTHFR-T allele.[9] On the other hand, the risk effect of the MTHFR-T allele for coronary heart disease is well demonstrated,[26] and the link between LOAD and coronary heart disease has been noted in relation to cerebral amyloid deposition.[27] Thus, the risk effect for the other diseases might affect the notification of patients with LOAD. Although we found the relation between LOAD and the MTHFR-T allele, the risk effect of the MTHFR-T allele should be carefully confirmed by not only meta-analysis of a large number of case-control studies but prospective cohort studies for aging-related diseases. These studies will elucidate environmental differences to explain the different results of the association studies. Several studies indicated that plasma homocysteine level is related with cognitive function in the elderly,[28,29] though this relation remains controversial.[30,31] Neuroimaging studies evidenced that elevated plasma homocysteine level is related with cortical and hippocampal atrophy in non-demented subjects,[32,33] and with white matter changes in AD cases.[34] It was shown that hyperhomocysteinemia is related to the progression and increasing severity of LOAD.[35] On the other hand, homocysteine enhances the toxicity of beta amyloid in vascular smooth muscle cells, showing the relation of homocysteine with cerebral amyloid angiopathy.[36] One of the unifying mechanisms of this evidence is that elevated plasma homocysteine level could underlie the alteration of microcirculation in the brain, resulting in critically attained threshold of cerebral hypoperfusion.[37]

The CBS gene is localized at 21q22.3, inside the critical region of Down syndrome.[38,39] The brains with Down syndrome show the appearance of senile plaques, a pathological hallmark of LOAD, in their thirties,[40] and the activity of CBS is increased in Down syndrome caused by gene-dose effect, which is opposite to the reduction of the CBS activity in LOAD.[18] However, the content of hydrogen sulfides, endogenously produced from cysteine by CBS, was decreased in the brains with AD, and this decrease was associated with the reduction of S-adenosylmethionine (SAM), a CBS activator, catalyzed from methionine and ATP by methionine adenosyltransferase.[41] It was also reported that AA genotype of A2756G polymorphism of 5-methyltetrahydrofolate-homocysteine S-methyltransferase gene (MTR), also called as methionine synthase and encoded at 1q43, that catalyzes homocysteine to methionine, is an APOE-ε4 allele-independent risk factor for AD.[42] Since we also found that the MTHFR-T allele is an APOE-ε4 allele-independent risk factor for LOAD, folate cycle in homocysteine metabolism is likely to be involved in the development of LOAD. However, it remains undetermined how this metabolic regulation modifies the risk for LOAD.

In conclusion, we showed that the MTHFR-C766T polymorphism, a genetic factor correlated to elevated plasma homocysteine level, is related to the occur-

rence of LOAD. It was supported that control of plasma homocysteine could be beneficial to prevent the elderly from LOAD.

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