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The Regulatory Region Polymorphisms of the MTHFR Gene Are Not Associated with Alzheimer's Disease

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Key Words
Alzheimer's disease · MTHFR gene · Regulatory region · Genetics · Polymorphisms

Abstract
Recent epidemiological studies have emphasized the impact of elevated blood homocysteine levels on the pathogenesis of Alzheimer's disease (AD). In spite of a significant impact of a MTHFR C677T polymorphism on the blood homocysteine levels, the association between the C677T polymorphism and AD remains controversial. Therefore, other unidentified genetic factor(s) that regulate blood homocysteine levels may exist. Here, we have analyzed the 5′-upstream region of the MTHFR gene and examined AD patients (n = 223) and nondemented individuals (n = 323) for polymorphisms in the 5′-upstream region of the MTHFR gene. We identified two polymorphisms (–713G/A and –393C/A, upstream of the start codon). We found no significant relationship between AD and the 5′-upstream region polymorphisms of the MTHFR gene. Thus, our study does not reinforce the hypothesis of an independent involvement of the MTHFR gene upstream region polymorphisms in AD risk.

Introduction

Methylene tetrahydrofolate reductase (MTHFR) is a pivotal enzyme for DNA synthesis and homocysteine (Hcy) remethylation. A C677T polymorphism has been well characterized with regard to thermolability and reduced activity which lead to an increased concentration of plasma total Hcy [1]. The TT genotype of the C677T mutation has been reported to relate to various diseases and differences in drug metabolism, including neural tube defects, cardiovascular diseases, cancers, migraine, and *L*-dopa treatment [2–4]. Recent epidemiological studies have been discussing that increased levels of plasma Hcy are one of the independent risk factors for the development of Alzheimer's disease (AD) [5–8]. However, it is yet unclear whether elevated plasma Hcy levels finally lead to AD, clinically and pathologically. In spite of the evident effect of the C677T TT genotype (or the T allele) on increased Hcy levels, the impact on the development of AD has been controversially discussed [9–12].

Recent studies have reported that the MTHFR gene has multiple promoters, leading to variable-length transcripts [13–15]. A putative 70-kD MTHFR1 polypeptide is thought to be a major functional transcript; however, a 77-kD MTHFR2 polypeptide has conserved amino acid sequence in human, pig, and mouse and is the predominant form of the pig liver enzyme. Therefore the 77-kDa MTHFR2 polypeptide may have similar properties to

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those of the MTHFR1 polypeptide or may have alternative properties based upon its additional amino terminus polypeptides [13–15].

We postulated that polymorphism(s) in the regulatory region of the MTHFR gene may have a potential impact on the transcriptional regulation of the MTFHR gene and/or the metabolism of MTHFR polypeptides. Thus, we screened the regulatory region of the MTHFR gene and performed genetic case-control studies on the identified polymorphisms to evaluate associations with AD.

Patients and Methods

Patients and Controls

The study refers to a total of 546 Japanese subjects in a western region of Japan, composed of 223 individuals with a clinical diagnosis of AD and 323 cognitively normal controls. The cognitively normal controls included the individuals without any neurological diseases or with some minor neurological diseases (e.g., viral meningitis, headache, etc.), excluding other neurodegenerative diseases (e.g., Parkinson's disease, cerebrospinal degeneration, etc.). The profiles for AD and controls are detailed in table 1. The diagnosis of AD was determined clinically according to DSM-III-R and the NINCDS-ADRDA criteria (provable AD). The age at onset was defined on the basis of the first appearance of clinical symptoms.

DNA Collection and Analysis

After informed consent had been obtained, blood samples of each subject were taken by venous puncture. Leukocyte DNA was isolated with a standard phenol-chloroform method. We generated three primer sets covering a 2.5-kb upstream region of the MTHFR gene according to a genomic sequence in the NCBI database (accession No. NT–021937). The primer sequences (size of the PCR product) and PCR conditions were as follows: PCR1, MTHFR-Pro-F1: 5′-TGAACTTGGGTCTGGCTATTTT-3′, MTHFR-Pro-R1: 5′-ACACATCAAGACACCTGAGTGG-3′ (797 bp); PCR2, MTHFR-Pro-F2: 5′-TGCACCTGTGTGTGTATTTCAG-3′, MTHFR-Pro-R2: 5′-GAAACAGTTCGTGCACAGGAT-3′ (953 bp), and PCR3, MTHFR-Pro-F3: 5′-AGTGTAACTCCCAATGGCTACC-3′, MTHFR-Pro-R3: 5′-CCAGGTGAGGTTCATTTCTTTC-3′ (1,074 bp), all with the same PCR conditions: initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 39 s, and extension at 72 °C for 50 s, and a final extension at 72 °C for 5 min using a hot start PCR kit (TaKaRa, Kyoto, Japan) and a model 9700 thermal cycler (Applied Biosystems Japan, Tokyo, Japan). The PCR products were purified and subjected to sequential analysis using a Big-Dye cycle sequence kit (Amersham Bioscience Japan, Tokyo) and a model ALF automated sequencer (Applied Biosystems Japan).

Sequence analysis of the regulatory region of the MTHFR gene revealed two novel polymorphisms: the –713G/A and –393C/A polymorphisms in the upstream region of the originally reported initiation codon (fig. 1). Importantly, the –393C/A polymorphism results in a putative amino acid substitution from aspartate to glutamate at codon 2 of the MTHFR2 polypeptide (A2G/MTHFR2) (fig. 1). For the genetic case-control study of the identified polymorphisms on the PCR1 reaction, we employed a double digest restriction fragment length polymorphism analysis using *ApaI* and *ApaLI*. The –713G/A mutation abolishes an *ApaI* site, and the –393C/A mutation abolishes an *ApaLI* site. These polymorphisms were visualized and distinguished by 2% agarose gel electrophoresis. Apolipoprotein E (ApoE) genotypes were determined by a standard *HhaI* RLFP method [16, 17].

Statistics

Hardy-Weinberg equilibrium was confirmed for both populations. The allele frequencies were compared using the chi-square test. The number of positive individuals having at least one polymorphism was also compared using the chi-square test. Possible interactions between the allele of each polymorphism and the ApoE ε4 allele were determined using logistic regression analysis with age, sex, and at least one ApoE ε4 allele as covariates. Odds ratios were calculated with exact 95% confidence intervals (CI); p values and significance considerations are two-sided and subject to a significance level of 5%. Analyses were performed by the SPSS statistical package (Japanese version 11; SPSS Japan, Tokyo).

Table 1. Profiles for AD patients (n = 223) and controls (n = 323)

Age years	n	Mean age ± SD years	Females %	ApoE ε4 carriers, %
AD patients				
≤64	34	60.2±3.1	52.9	44.1
≥65	189	77.0±6.8	73.5	49.7
Total	223	74.5±8.8	74.6	48.9
Controls				
≤64	57	59.4±4.1	78.9	28.1
≥65	266	76.0±6.8	73.7	15.0
Total	323	73.1±9.0	70.4	17.3

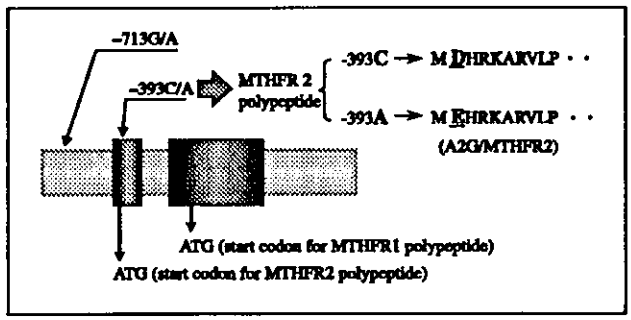

Fig. 1. Schematic representation of the –393C/A and –713G/A polymorphisms. The –393C/A polymorphism results in a putative amino acid substitution at codon 2 of the MTHFR2 polypeptide (A2G/MTHFR2).

Table 2. -713 G/A and -393 C/A polymorphisms in the regulatory region of the MTHFR gene in controls (n = 323) and AD patients (n = 223) – comparisons of genotype distributions and allele frequencies and concurrence of both polymorphisms

		n	-393 C/A genotype		
			CC	AC	AA
	Controls	323	278 (86.1%)	44 (13.6%)	1 (0.3%)
	AD patients	223	199 (89.2%)	21 (9.4%)	3 (1.3%)
-713 G/A genotype					
AA	Controls	259 (80.2%)	216	42	1
	AD patients	187 (83.9%)	166	18	3
AG	Controls	64 (19.8%)	62	2	-
	AD patients	34 (15.2%)	31	3	-
GG	Controls	0 (0.0%)	0	-	-
	AD patients	2 (0.9%)	2	-	-

Table 3. MTHFR promoter polymorphisms (P0, P1 and P2) in controls and AD patients – genotype distribution and allele frequency

	Genotype distribution						Allele frequency		
	P0/P0	P0/P1	P0/P2	P1/P1	P1/P2	P2/P2	P0	P1	P2
Normal controls (n = 323)									
n	216	61	42	0	2	1	0.83	0.07	0.10
%	67.2	18.9	13.0	0.0	0.6	0.3			
AD patients (n = 223)									
n	166	31	18	2	3	3	0.85	0.05	0.08
%	74.4	13.9	8.1	0.9	1.3	1.3			

P0 = -713G/-393C (wild); P1 = -713*A*/-393C; P2 = -713G/-393*A*.

Results

Genotype distributions and allele frequencies of the -713G/A and -393C/A polymorphisms are shown in table 2. We did not find genotype combinations which would indicate the concurrence of both polymorphisms. Accordingly, the concurrence of both polymorphisms shown in table 2 indicates that the two polymorphisms never occurred on the same allele in our samples and that the regional alleles for this region of the MTHFR gene are divided into three types. Genotype distribution and allele frequencies are shown in table 3.

As expected, the ApoE ε4 allele was associated with a highly significant risk for developing AD, as estimated by logistic regression analysis (odds ratio OR = 4.8, 95% CI 3.2–7.1, age and gender adjusted). Statistical analysis failed to show a significant difference between AD patients and controls in the genotype (P0/P0, P0/P1, P0/P2, P1/P1, P1/P2, and P2/P2) distributions and allele (P0, P1, and P2) frequencies (table 3). The number of positive individuals having at least one polymorphism also showed no statistical difference (data not shown). Logistic regression analysis (adjusted by age, gender, and presence of at least one ApoE ε4 allele) did not confirm significant influences for either polymorphism (P1: OR = 0.735, p = 0.210, 95% CI 0.445–1.189, absence versus presence of P1; P2: OR = 0.697, p = 0.213, 95% CI 0.395–1.230, absence versus presence of P2).

Discussion

This is the first study identifying polymorphisms in the 5'-upstream region of the MTHFR gene and analyzing the possible involvement of the identified polymorphisms in AD. The -393C/A polymorphism corresponded to a putative amino acid substitution at codon 2 of the MTHFR2 polypeptide (A2G/MTHFR2). We failed to confirm a positive association between this polymorphism and AD. However, since the A2G/

MTHFR2 polymorphism potentially impacts on function or processing of the MTHFR2 polypeptide, further characterization of the A2G/MTHFR2 polypeptide will be needed. The A2G/MTHFR2 polymorphism may be related to other diseases that are involved in the metabolism of Hcy.

Similarly, no positive associations between –713G/A polymorphism and AD were observed. The sequences surrounding the –713G/A polymorphism do not correspond to any putative transcription factor binding elements, and the G/A nucleotide substitution does not produce an alternative transcriptional regulator site, as estimated by online TFSEARCH analysis [http://www.cbrc.jp/research/db/TFSEARCH.html]. Therefore, the –713G/A polymorphism may be a nonfunctional single-nucleotide polymorphism. However, the identification of such regulatory consensus sites is imprecise, and ultimately functional studies are needed.

It has been discussed whether or not Hcy has been thought to play an important role in the development of AD [5–8]. Assuming that dysmetabolism of Hcy is a risk factor for the development of AD, MTHFR is predicted to be implicated in the pathomechanism of AD. However, the effects of the common exonic polymorphisms (C677T and A1298C) on the pathogenesis of AD have not been firmly demonstrated.

The mRNA expression levels of the MTHFR gene are regulated by the complicated processing of the 5′-upstream region and the 3′-untranslated region [13–15]. In the 3′-downstream region, the MTHFR gene is reported to have multiple polyadenylation sites, resulting in multiple mRNA species having differently sized 3′-untranslated regions. Since the tissue specificity of mRNA expression has been reported to be determined by these complicated expression regulations, the expression levels or metabolism of MTHFR may differ between the central nervous system and the peripheral organs. Assuming that MTHFR is associated with the pathogenesis of AD through Hcy homeostasis, alternative genetic factors may exist. Recently, we proposed that a haplotype formed by the combination of exonic nonsynonymous polymorphisms (C677T, A1298C, and A1793G) is protective against the development of late-onset AD, whereas a conventional 677TT genotype (or 677T allele) is not associated with late-onset AD [18]. Therefore, further investigations to identify other genetic regulators that affect the MTHFR gene expression combined with exonic polymorphisms (haplotypes) will be required.

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Elevated interleukin-6 levels in cerebrospinal fluid of vascular dementia patients

Wada-Isoe K, Wakutani Y, Urakami K, Nakashima K. Elevated interleukin-6 levels in cerebrospinal fluid of vascular dementia patients. Acta Neurol Scand 2004 DOI: 10.1111/j.1600-0404.2004.00286.x

Objectives – To investigate a possible implication of inflammatory processes in the development of dementia in cerebrovascular disease. *Patients and methods* – We examined the levels of interleukin-6 (IL-6) in the cerebrospinal fluid (CSF) of patients with Alzheimer's disease (AD) ($n = 26$), ischemic cerebrovascular disease without dementia (CVD) ($n = 11$), vascular dementia (VD) ($n = 11$), and other neurological disorders ($n = 21$) using sensitive enzyme-linked immunosorbent assay. *Results* – The CSF concentrations of IL-6 were significantly elevated in patients with VD compared with those of patients with AD or CVD. *Conclusion* – The CSF IL-6 levels are increased in patients with VD, suggesting that inflammatory mechanisms may be involved in the development of cognitive decline in some patients with cerebrovascular disease. CSF IL-6 may be a biological marker for dementia in cerebrovascular disease.

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Key words: vascular dementia; Alzheimer's disease; cerebrovascular disease; interleukin-6; cytokines; tau protein; cerebrospinal fluid; biological marker

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Vascular dementia (VD) is a common cause of dementia in Japan (1). However, the mechanisms of clinical cognitive deterioration in patients with cerebral ischemia are not completely understood. Several recent studies have provided insight into the possible role of inflammatory processes in the development of brain ischemia and multi-infarct cognitive impairment, as demonstrated by the accumulation of inflammatory cells and mediators in the ischemic brain (2–5). Vila et al. (6) reported that interleukin-6 (IL-6) participates in the acute-phase response that follows cerebral ischemia and that an association exists between high levels of IL-6 and early neurological worsening. A case–control genetic study reported a positive association of the -174 G/C IL-6 gene polymorphism and the risk of multi-infarct dementia (7). These data led the hypothesis that inflammatory mechanisms play a crucial role in the pathogenesis of the development of dementia in cerebrovascular disease. Cerebrospinal fluid (CSF) levels of IL-6 are elevated in central nervous system (CNS) infections and non-infectious CNS inflammatory diseases, indicating that levels of IL-6 in the CSF reflect the inflammatory processes (8–10). Little has been reported about the IL-6 levels in the CSF of patients with VD. Previous studies reported that these levels did not differ from those of controls (11–13). However, some patients with VD in the previous study investigated by Yamada et al. (13) showed high CSF concentrations of IL-6. In order to clarify the association of inflammatory mechanisms with VD, we examined the CSF levels of IL-6 in patients with VD as well as in patients with Alzheimer's disease (AD) and ischemic cerebrovascular disease without dementia (CVD).

Subjects and methods

The subjects were 26 patients with AD (mean age ± SD 66.8 ± 8.2 years), 11 patients with CVD (70.0 ± 6.2 years), 11 patients with VD (74.5 ± 4.5 years), and 21 patients with other neurological disease (OND) (68.4 ± 5.8 years). Assessments of these patients included a carefully examined medical history, physiological examination, drug inventory, neurological examination, comprehensive cognitive evaluation with the use of the Functional Assessment Staging of Alzheimer's disease (FAST staging), the Mini-Mental State Examination (MMSE), neuroimaging assessments of CT scan or MRI and single photon emission computed tomography of the head, and routine laboratory tests, such as blood analysis and biological

examination. Patients who satisfied the Diagnostic and Statistical Manual of Mental Disorders, third edition-revised (DSM-III-R) (14) and the diagnostic criteria of the National Institute of Neurological and Communicative Disorders Association (NINCDS-ADRDA) (15) and those scoring 4 points or less on Hachinski's ischemic score (16) were diagnosed as having AD. Patients who satisfied the DSM-III-R and the ADDTC criteria for ischemic VD (17) and those scoring 7 points or more on Hachinski's ischemic score were diagnosed as having VD. All the patients with VD showed stepwise deterioration of cognitive function and one or more infarcts outside the cerebellum detected by neuroimaging. The CVD group was defined as patients who had a history of stroke episode and with CT scan or MRI findings of infarcts without dementia. OND patients consisted of seven patients with Parkinson's disease (PD), four patients with amyotrophic lateral sclerosis (ALS), four patients with spinocerebellar degeneration, two patients with peripheral neuropathy, two patients with tension-type headache, one patient with myopathy and one patient with essential tremor. OND patients did not show any cognitive impairment. After informed consent from patients or their families, CSF was collected by lumbar puncture. CSF samples were stored at −80°C until assay. Collections of CSF from the patients with CVD or VD were performed during the chronic phase of the diseases when the progression of neurological deterioration was no longer observed. CSF IL-6 levels were determined in duplicate, using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine; R&D Systems, Inc, Minneapolis, MN, USA). CSF total tau protein levels were measured using ELISA kit (Innogenetics, Gent, Belgium). Statistical significance was analyzed by one-way ANOVA, followed by *post hoc* tests. Correlation was analyzed by Spearman rank correlation test.

Results

As shown in Fig. 1, the concentrations of IL-6 in the CSF of VD patients were 5.67 ± 1.7 pg/ml (mean ± SE); those of patients with AD were 2.53 ± 0.87 pg/ml; those of patients with CVD were 2.15 ± 0.38 pg/ml; and those of patients with OND were 3.15 ± 0.67 pg/ml. Significantly elevated levels of IL-6 were found in the CSF of patients with VD compared with those in the CSF of patients with AD, CVD, and OND. There was no significant difference in CSF IL-6 levels between AD patients and CVD and OND patients. There were not a correlation between CSF IL-6 levels and

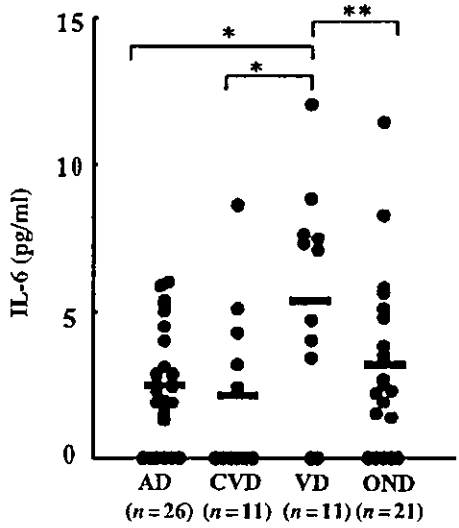

Figure 1. Interleukin-6 levels in the cerebrospinal fluid of the patients with Alzheimer's disease, ischemic cerebrovascular disease without dementia, vascular dementia, and other neurological disease. The horizontal bar indicates the mean level. Statistical differences were calculated using one-way ANOVA followed by a *post hoc* test; $*P < 0.01$, $**P < 0.05$.

Table 1 Total tau levels in cerebrospinal fluid

Disease	Mean ± SE (pg/ml)
AD	236.0 ± 17.2
CVD	115.0 ± 39.3
VD	116.8 ± 28.1
OND	126.4 ± 16.4

AD, Alzheimer's disease; CVD, cerebrovascular disease without dementia; VD, vascular dementia; OND, other neurological disorders.

MMSE scores in VD patients (data not shown). The levels of total tau protein in CSF were shown in Table 1. Significantly elevated levels of tau protein were found in the CSF of patients with AD compared with those in the CSF of patients with VD ($P < 0.01$). There were not significant differences between CSF tau levels in VD and those in CVD or OND.

Discussion

Interleukin-6 was described initially by Hirano (18) as a B cell differentiation factor usually derived from T cells and it can also be produced by astrocytes and microglia in the CNS (9, 19, 20). CSF levels of IL-6 were examined in patients with infectious or non-infectious inflammatory diseases of the CNS. CSF levels of IL-6 were also examined in patients with neurodegenerative disorders. In particular, controversial results ranging from no changed (21, 22), to increased (23) or decreased

(13) levels of IL-6 in the CSF have been reported in AD patients. Differences in sample size, selection of patients with AD and control subjects, or experimental procedures may account for these varying results. Our results show that there are no significant differences in the CSF levels of IL-6 between patients with AD and patients with CVD or OND. The OND group included patients with PD and ALS, in which higher levels of CSF IL-6 have been reported (23, 24). Indeed, the patients with the two highest levels in OND group in our study were a PD patient and an ALS patient, but CSF IL-6 levels were not altered in other patients with ALS or PD. We conclude that the levels of IL-6 are not altered in patients with AD, and that CSF IL-6 may not be a biological marker for the diagnosis of AD. On the contrary, we obtained significantly higher CSF levels of IL-6 in patients with VD, but not in patients with CVD who did not have dementia. However, previous reports indicated that CSF levels of IL-6 in VD patients were not significantly elevated (11, 12). It has been reported that patients with VD are heterogeneous and diagnosis criteria for VD are not interchangeable (25). Selection of patients may account for these differences. Not all the patients with VD showed higher levels of IL-6 in CSF in this study, but we used the DSM-III-R and ADTTC criteria for diagnosis of VD and employed probable cases in this study and these cases also showed significant lower levels of total tau protein in CSF compared with AD patients, suggesting that clinical diagnosis of VD patients was sufficient to segregate AD patients from VD cases. Recent reports indicated inflammatory process might be involved in cerebrovascular disease. Higher baseline levels of CSF IL-6 were shown to be related to early neurologic worsening in ischemic stroke, independent of the initial size topography or mechanism of ischemic infarction (6, 26). A genetic association of IL-6 polymorphism with multi-infarct dementia (7) and activation of the micoglia in Binswanger's disease, a form of VD has been shown (3). The increased intrathecal production of granulocyte-macrophage colony stimulating factor (GM-CSF), a cytokine that stimulates microglial cell growth and has inflammatory properties, has been found in patients with VD (4). Taken together with our result, the inflammatory activations in the CNS might be associated with some part of VD patients and measurement of CSF IL-6 might provide a clue to differential diagnosis of dementia. Our study is a cross-sectional design, further studies using a longitudinal design with large samples are necessary to support these results.

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A haplotype of the methylenetetrahydrofolate reductase gene is protective against late-onset Alzheimer's disease

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Abstract

Epidemiological studies have shown that elevated plasma homocysteine (Hcy) levels play an important role in the pathogenesis of Alzheimer's disease (AD). In spite of the evidence that a C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene elevates plasma Hcy levels, the impact of the C677T polymorphism on the development of AD is controversial. Here, we performed a genetic case-control study in a Japanese population to investigate whether three polymorphisms of the MTHFR gene, C677T (Ala222Val), A1298C (Glu429Ala), and A1793G (Arg594Gln), are associated with the development of late-onset AD (LOAD). In our study, the MTHFR gene had four major regional haplotypes: Haplotype A (677C-1298A-1793G), Haplotype B (677T-1298A-1793G), Haplotype C (677C-1298C-1793G), and Haplotype D (677C-1298C-1793A). The frequency of Haplotype C in LOAD was significantly lower than that in control group. Furthermore, the benefit conferred by the presence of at least one Haplotype C was stronger in LOAD patients who lacked the ApoE ε4 allele (OR = 0.293; 95% CI = 0.115–0.744; $P = 0.010$). The results indicate that Haplotype C of the MTHFR gene is protective against the development of LOAD.

Keywords: MTHFR gene; Haplotype; C677T; A1298C; A1793G; Association; Protective; Alzheimer

1. Introduction

Alzheimer's disease (AD) is one of the major neurodegenerative diseases in elderly population. Recent epidemiological studies have demonstrated that elevated levels of plasma homocysteine (Hcy) may play an important role in the pathogenesis of AD [3,19]. However, the detailed pathomechanism by which elevated plasma Hcy levels finally lead to AD is still uncertain.

Methylenetetrahydrofolate reductase (MTHFR; MIM *607093, EC 1.5.1.20) is one of the central enzymes for DNA synthesis and Hcy metabolism. In spite of the evidence that the C677T TT genotype (or the T allele) increases Hcy levels (particular in folate deficiency state) [8], its impact on the development of AD has been controversial [2,13,15]. No association studies of a second common polymorphism, A1298C (Glu429Ala) with AD were conducted.

Recent full genome scan studies have demonstrated the multiple candidate loci for late-onset AD (LOAD) including chromosome 1 [9,12,14]. The MTHFR gene locates chromosome 1p36.3 [5] and is predicted to be susceptible to LOAD. Our study was designed to evaluate whether the polymorphisms or the combined haplotypes of the MTHFR gene have an impact on the development of LOAD. We examined three MTHFR gene polymorphisms, C677T (Ala222Val), A1298C (Glu429Ala), and A1793G (Arg594Gln) (NCBI dbSNP cluster ID: rs2066462, rs1801131, and rs2274976, respectively), and the regional haplotypes derived from the three polymorphisms in a LOAD group and a control group.

2. Materials and methods

The study enrolled subjects from a western region of Japan. The diagnosis of AD was determined clinically according to the DSM-III-R and NINCDS-ADRDA criteria. Age at onset was defined by the appearance of the first clinical symptoms.

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Table 1
Primer sequence, annealing temperature, PCR product size, restriction endonuclease, and digestion pattern for the MTHFR C677T, A1298C, and A1793G polymorphisms

Position	Exon	Sequence 5′–3′	Annealing temperature (°C)	Size (bp)	Nuclease	Digestion pattern (bp)
C677T	Ex4-F	AGTCCCTGTGGTCTCTTCATC	58	387	Gain of *Hin*fI site	152/235
	Ex4-R	GGAGATCTGGGAAGAACTCAG				
A1298C	Ex7-MF	AGATGTGGGGGGAGGAGCTGACCAGTGC*AG	62	175	Gain of *Fnu*4HI site	28/147
	Ex7-MR	GCCCCA**CAGCCTGGCCTA**CAGCT				
A1793G	Ex11-F	TTGGAGAGCCCTGTTAATCTTG	58	390	Loss of *Bsr*BI site	125/264
	Ex11-R	AGAGACACGAAGGAGAGTGGAG				

* Mismatch position (A–C) for creation of an artificial *Fnu*4HI site.
** Mismatch positions (both of C–A) for abolishment of *Fnu*4HI sites.

After informed consent was given, blood leukocyte DNA was isolated using the standard phenol–chloroform method. We generated the three primer sets according to a genomic sequence in the NCBI database (GenBank accession No: AF257484). Table 1 depicts each of the primer sequences, the annealing temperature, the PCR product length, the restriction enzymes for PCR-restriction fragment length polymorphism (RFLP) analysis, and the digestion pattern. "Hot start" PCR reactions for each primer set were performed using a supplied kit (TaKaRa, Japan) in standard PCR reaction conditions. The amplified PCR products for each primer set were subjected to RFLP analysis by agarose electrophoresis. ApoE genotypes were determined by a standard *Hha*I RFLP analysis [6,7].

The Hardy–Weinberg equilibrium was confirmed for both populations. The allele frequencies and the number of positive individuals having at least one polymorphism or haplotype were compared using the chi-square test. The possible effects of each haplotype on LOAD were determined using logistic regression analysis with age, sex, and the presence of at least one ApoE ε4 allele as covariates. We also stratified the data sets into two types of subjects: subjects possessing at least one ApoE ε4 allele and subjects without an ApoE ε4 allele. Odds ratios (ORs) were calculated with exact 95% confidence intervals (CIs). *P* values and significance considerations are two-sided and subject to a significance level of 5%. Analyses were performed with the SPSS statistical package (Japanese version 11).

3. Results

A total of 307 Japanese subjects from a western region of Japan were enrolled, including 129 individuals with a clinical diagnosis of LOAD and 178 cognitively normal controls (CTLs). The mean age at onset of patients with LOAD was 74.4 years (65–85, S.D. = 5.4), and 76.0% were women. The corresponding values of the CTL group were 74.4 (65–85, S.D. = 4.5), and 73.0% were women.

Concurrences of the genotype distributions of C677T with A1298C indicated complete linkage disequilibrium between the polymorphisms at nucleotide 677 and nucleotide 1298, as previously reported [10,22]. Correspondingly, we did not detect the combinations of 677CT and 1793AA, 677TT and 1793AA, or 677TT and 1793AG, indicating complete linkage disequilibrium between all of these polymorphisms. Nor did we detect combinations of 1298AA and 1793AG, 1298AA and 1793AA, or 1298AC and 1793AA, indicating that the 1793A polymorphism is a concomitant allele to the 1298A allele. Thus, the 1298A and 1793A allele are always in the *cis* configuration. From these results, we divided the regional haplotypes of the MTHFR gene into four haplotypes, which we named Haplotype A (wild type 677C-1298A-1793G), Haplotype B (677T-1298A-1793G), Haplotype C (677C-1298C-1793G), and Haplotype D (677C-1298C-1793A) (Fig. 1); this results in eight diplotypes (genotypes). Further data analysis was performed using these haplotypes. The diplotype distributions between AD and CTL did not statistically differ because of a small number of cases for each diplotype (Table 2). Logistic regression analysis adjusted by age, gender, and the presence of at least one ApoE ε4 allele demonstrated a significant protective effect of Haplotype C (OR = 0.426; 95% CI = 0.220–0.827; $P = 0.012$, presence of at least one Haplotype C versus absence of Haplotype C), whereas the presence

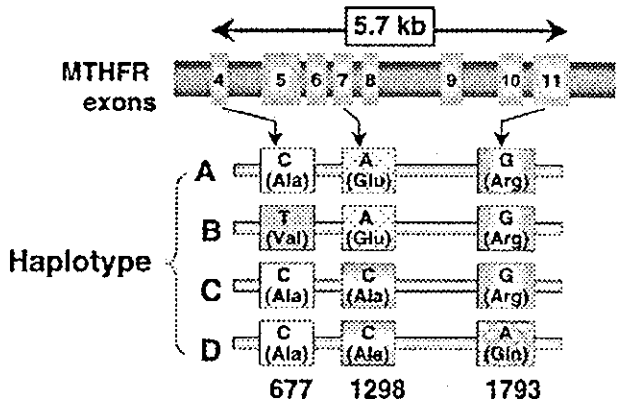

Fig. 1. A schematic representation of the estimated regional haplotypes of the MTHFR gene.

Table 2
Diplotype distribution and haplotype frequency for exon 4 to exon 11 of the MTHFR gene

	Diplotype distribution (%)									
	AA	AB	AC	AD	BB	BC	BD	CC	CD	DD
AD	25 (19.4)	49 (38.0)	5 (3.9)	9 (7.0)	17 (13.2)	6 (4.7)	10 (7.8)	3 (2.3)	3 (2.3)	2 (1.6)
CTL	31 (17.4)	49 (27.5)	17 (9.6)	10 (5.6)	25 (14.0)	18 (10.1)	18 (10.1)	3 (1.7)	6 (3.4)	1 (0.6)

Table 3
Logistic regression analysis of at least one of each haplotype adjusted by age, gender, and at last one ApoE ε4 allele

	d.f.	Odds ratio	95.0% CI	*P* value
Haplotype B	1	0.921	0.553–1.532	0.750
Haplotype C	1	0.426	0.220–0.827	0.012*
Haplotype D	1	1.023	0.549–1.906	0.943

* Statistically significant.

Table 4
Logistic regression analysis classified by ApoE ε4 status of at least of one each haplotype adjusted by age and gender

	d.f.	Odds ratio	95.0% CI	*P* value
Haplotype B				
ε4 (−)	1	1.180	0.638–2.183	0.598
ε4 (+)	1	0.502	0.176–1.427	0.196
Haplotype C				
ε4 (−)	1	0.293	0.115–0.744	0.010*
ε4 (+)	1	0.592	0.198–1.771	0.349
Haplotype D				
ε4 (−)	1	0.885	0.416–1.884	0.752
ε4 (+)	1	0.977	0.299–3.190	0.969

* Statistically significant.

Haplotype B and Haplotype D conferred no significant advantage (OR = 0.921; 95% CI = 0.553–1.532; $P = 0.750$, presence of at least one Haplotype B versus absence of Haplotype B, and OR = 1.023; 95% CI = 0.549–1.906; $P = 0.943$, presence of at least one Haplotype D versus absence of Haplotype D) (Table 3). Expectedly, the estimated risk of AD in the presence of ApoE ε4 was highly significant (OR = 5.318; 95% CI = 3.153–8.972; $P < 0.001$, presence of at least one ApoE ε4 allele versus absence of ApoE ε4 allele). Subsequent analysis stratified according to the ApoE ε4 status revealed that the protective effect of Haplotype C of the MTHFR gene against LOAD was more prominent in the group lacking the ApoE ε4 allele (OR = 0.293; 95% CI = 0.115–0.744; $P = 0.010$, presence of at least one Haplotype C versus absence of Haplotype C) (Table 4).

4. Discussion

In the present study, we used regional haplotypes to assess the association between polymorphisms (C677T, A1298C, and A1793G) in the gene encoding the MTHFR enzyme and susceptibility to LOAD. We evaluated complete linkage disequilibrium between the 677C and 1298A alleles in our samples as previous reported results [10,22]. Importantly, we found that the 1793G allele always appeared in *trans* with 677T and in *cis* with 1298C. Therefore, we allocated the regional haplotypes of the MTHFR gene into four haplotypes (Fig. 1).

We found that presence of at least one Haplotype C was significantly protective against LOAD (Table 3). Furthermore, the protective effect of Haplotype C was more predominant in ApoE ε4-negative individuals as compared to ApoE ε4-positive individuals (Table 4).

The marked impact of the MTHFR 677T allele in reducing enzyme activity and thermolability and increasing plasma Hcy levels has been well characterized. Although the influence of the 1298C allele (equivalent to Haplotype C) on MTHFR enzyme thermolability has been shown to be negligible, the effects on reducing the enzyme activity in vitro are controversial in independent studies [23,24]. The effects of the 1298C-1793A haplotype (Haplotype D) on the metabolism or activity of MTHFR enzyme have also yet to be discovered.

Biological studies have demonstrated the allele-specific antioxidant potential of ApoE (ε2 > ε3 > ε4) [4,11]. In addition, recent studies using ApoE-deficient transgenic mice have proposed that folate, a major regulatory factor for MTHFR activity and levels of the non-protein Hcy, quenches oxidative damage [20,21]. Therefore, MTHFR dismetabolism and/or inappropriate folate intake may impair the capacity against oxidative stress. We found the enhanced protective effect of Haplotype C of the MTHFR gene in ApoE ε4 lacking individuals, indicating that Haplotype C may have synergic beneficial effects with the negativity of ApoE ε4 against oxidative stress.

In conclusion, we propose that the extended genotypes and haplotypes of the MTHFR gene have important implications for the pathogenesis of LOAD. A negative correlation between the 1298C allele and plasma Hcy levels and an inverse association between Vitamin B-12 status and plasma Hcy have been reported for the 1298C allele [1,10]. In addition, it has been reported that allele or haplotype construction of the MTHFR gene differs according to ethnicity [18] and the polymorphisms of the MTHFR gene have implications for human fertility and dietary folate consumption [16,17]. However, because Haplotype C of the MTHFR gene is a genetic factor that provides protection

against the development of LOAD in the Japanese population, we suggest further analysis of samples from different ethnicities or communities to avoid type 1 (false positive) error. Studies to clarify the effects of the estimated haplotypes on MTHFR metabolism will also be required.

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Neuronal RNA oxidation is a prominent feature of familial Alzheimer's disease

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An in situ approach was used to identify the oxidized RNA nucleoside 8-hydroxyguanosine (8OHG) in the frontal cortex of familial Alzheimer's disease (FAD) with a mutation in presenilin-1 (PS-1) or amyloid β protein precursor (AβPP) gene ($n = 13$, age 47–81 years). Neurons with marked 8OHG immunoreaction in the cytoplasm were widely distributed in the superior/middle frontal gyrus of FAD. Relative intensity measurements of neuronal 8OHG immunoreactivity showed that there was a significant increase in FAD compared with controls ($n = 15$, age 59–81 years), while there was no difference in relative 8OHG between the PS-1 and the AβPP FAD. Interestingly, a presymptomatic case carrying a PS-1 mutation showed a considerable level of relative 8OHG, and the increased levels of neuronal 8OHG in FAD were more prominent in cases with a lower percentage area of Aβ42 burden. These results suggest that oxidative stress is an early event involved in the pathological cascade of FAD.

Keywords: Amyloid β protein precursor; Familial Alzheimer's disease; 8-Hydroxyguanosine; Oxidative stress; Presenilin; RNA

Introduction

Numerous studies have now established the association of neuronal oxidative stress with major neurodegenerative disorders such as Alzheimer's disease (AD) (reviewed in Markesbery and Carney, 1999; Perry et al., 1998; Smith et al., 2000) and Parkinson disease (reviewed in Jenner, 1998; Zhang et al., 2000). We have reported RNA oxidation in vulnerable neurons of sporadic type of AD (Nunomura et al., 1999, 2001), Parkinson disease (Zhang et al., 1999), dementia with Lewy bodies (Nunomura et al., 2002), as well as Down syndrome (Nunomura et al., 2000), which suggests links between oxidative stress and not only age-associated degenerative diseases but also neurodegeneration due to genetic factors.

In this study, we used an in situ approach to identify the oxidized RNA nucleoside 8-hydroxyguanosine (8OHG) in the cerebral cortex of familial AD (FAD) with a mutation in presenilin-1 (PS-1) or amyloid β protein precursor (AβPP) gene. Neurons with marked 8OHG immunoreaction in the cytoplasm were widely distributed in the cerebral cortex of FAD. Importantly, a presymptomatic case carrying a PS-1 mutation (Lippa et al., 1998) showed a considerable level of neuronal 8OHG. Moreover, semiquantitative analysis showed that increased levels of neuronal 8OHG in FAD were more prominent in cases with a lower amount of amyloid β (Aβ), which was immunolabeled with an end-specific antibody for the Aβ1–42 (Aβ42), as we showed in individuals with Down syndrome (Nunomura et al., 2000). These results suggest that oxidative stress is involved in the pathological cascade of FAD especially as an early stage event of the cascade.

Materials and methods

Tissue

Brain tissue was obtained at autopsy from 13 clinically and pathologically confirmed cases of FAD according to the CERAD criteria (2 males and 11 females; ages 47–81 years, average 59). Eleven females of the FAD group (ages 47–81 years, average 59) were members of families possessing a PS-1 gene mutation and the other two males of the FAD group (ages 57 and 58 years) were members of families possessing an AβPP gene mutation. Mutations in PS-1 gene in these subjects were found on M146L ($n = 1$), A246E ($n = 4$), L286V ($n = 1$), and C410Y ($n = 5$), and mutations in AβPP gene were found on KM670, 671NL (Swedish mutation; $n = 1$), and A692G (Flemish mutation; $n = 1$). All these FAD subjects died from pneumonia except for a case whose information about cause of death was not available. Another 51-year-old male

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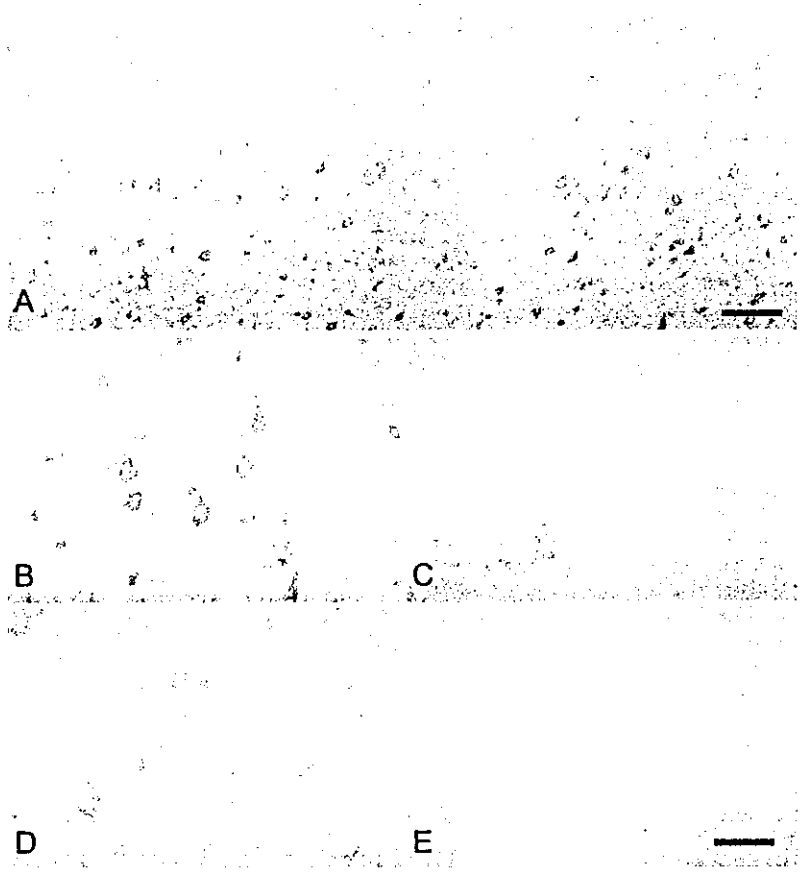

Fig. 1. Oxidized nucleoside 8OHG is abundant in vulnerable neurons in FAD. Neuronal 8OHG immunoreactivity showing cytoplasmic predominance is prominent in the frontal cortex from a case of FAD with a PS-1 gene mutation (C410Y, 55 years old) (A and B). In a case of FAD with an AβPP gene mutation (A692G, 57 years old) (C) and in a presymptomatic case with a PS-1 gene mutation (A264E, 51 years old) (D), moderately positive 8OHG immunoreactivity is observed in neurons of the frontal cortex. Whereas, in a control case (64 years old), the neuronal 8OHG immunoreactivity is faint in the frontal cortex (E). Scale bars, A = 100 μm, B–E = 50 μm.

was a member of a family with a PS-1 gene mutation (A246E) who died from myocardial infarction and had shown no clinical symptoms of dementia immediately before death (Lippa et al., 1998). As for controls, we investigated a consecutive series of 15 subjects without dementia (seven males and eight females; ages 59–81 years, average 66). Causes of death of these controls were internal malignancy (n = 9), leukemia (n = 2), cardiac failure (n = 3), and unknown (n = 1). Postmortem intervals before fixation were 2–19 h (average 8) in the FAD group, 14 h in the presymptomatic case with PS-1 gene mutation, and 3–20 h (average 9) in controls. Duration of dementia was known from clinical records in 12 cases of FAD as 5–25 years (average 11). Slices of the frontal cortex (the superior/middle frontal gyrus) or the temporal cortex (the inferior temporal/occipitotemporal gyrus) from all the subjects were fixed in neutral formalin, dehydrated through graded ethanol followed by xylene, and embedded in paraffin. Six-micron-thick sections were cut and mounted on Silane® (Sigma, St. Louis, MO)-coated glass slides.

Immunocytochemistry and antibodies

Following deparaffinization with xylene, sections were hydrated through graded ethanol. Endogenous peroxidase activity in the tissue was eliminated by a 30-min incubation with 3% H_2O_2 in methanol and nonspecific binding sites were blocked in a 30-min incubation with 10% normal goat serum in Tris-buffered saline (150 mM Tris–HCl, 150 mM NaCl, pH 7.6). To detect oxidized nucleosides, we used a mouse monoclonal antibody against 8OHG, 1F7 (Yin et al., 1995) (1:30; Trevigen, Gaithersburg, MD), after treatment with 10 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) in phosphate-buffered saline (pH7.4) for 40 min at 37°C. Immunostaining was developed by

the peroxidase–antiperoxidase procedure (Sternberger, 1986) by using 0.75 mg/ml 3,3′-diaminobenzidine co-substrate in 0.015% H_2O_2, 50 mM Tris–HCl, pH 7.6 for exactly 10 min. The specificity of 1F7 to 8OHG was confirmed by primary antibody omission or by absorption with purified 8OHG (Cayman Chemical, Ann Arbor, MI) (Nunomura et al., 1999). Although 1F7 recognizes RNA-derived 8OHG as well as DNA-derived 8-hydroxydeoxyguanosine with similar binding affinities (Yin et al., 1995), we have confirmed that 1F7 immunolabeling in neurons in sporadic AD is predominantly in RNA the by pretreatment with DNase or RNase (Nunomura et al., 1999) as well as by immunoelectronmicroscopy, which showed most 8OHG is present in the endoplasmic reticulum (Nunomura et al., 2001). For FAD cases, additional sections were pretreated with RNase-free DNase I (10 U/μl for 2 h at 37°C; Roche, Mannheim, Germany) or DNase-free RNase (0.5 μg/μl for 2 h at 37°C; Boehringer Mannheim) after the proteinase-K treatment. For the detection of Aβ deposition in FAD cases, we used either of mouse monoclonal antibody, BC05 (1:1000; gift of Fukumoto, H., Takeda Chemical Industries, Osaka, Japan) specific for the carboxyl terminus of Aβ1–42 (Aβ42), or BA27 (1:3500; gift of Fukumoto, H.) specific for the carboxyl terminus of Aβ1–40 (Aβ40), with a 5-min pretreatment of 70% formic acid.

Relative scale of 8OHG and Aβ deposition

All measurements were performed in layer III of the cerebral cortex (the superior/middle frontal gyrus or the inferior temporal/occipitotemporal gyrus) using a Q500IW-EX Image Processing and Analysis System (Leica) linked to a SONY CCD Camera (XC-75CE) mounted on a Nikon MICROPHOT-FX microscope. The intensity of immunoreaction with 1F7 was evaluated by measuring the average optical density in an area comprising the cytoplasm and nucleus, as we described previously (Nunomura et al., 1999). Three adjacent fields (each field = 460 × 428 μm) were selected, and in each field of the video camera, five pyramidal neurons sectioned near their equator, based on a section plane that included the nucleolus, were selected and outlined manually so that of the area of the nucleus to cytoplasm was rather constant. The nucleus was included because damage to RNA was nuclear as well as cytoplasmic. The average optical density measurement was obtained for each of the three fields and averaged. Finally, the optical density value was corrected for background by subtracting the optical density of the white matter on the same section. The superior/middle frontal gyrus was available in all 13 FAD cases, while the inferior temporal/occipitotemporal gyrus was available in all 19 controls. Both brain regions were available in two controls and an additional centenarian without dementia, in which levels of the relative 8OHG were similar in both brain regions. In these cases, the ratio of relative 8OHG in the frontal cortex to that in the temporal cortex was 0.78, 0.84, and 1.13 (average 0.92), which meant that regional differences in relative neuronal 8OHG between the frontal and temporal cortices were virtually negligible in controls. Therefore, we used data from the superior/middle frontal gyrus of FAD cases and the inferior temporal/occipitotemporal gyrus of controls for comparison.

For the measurement of the extent of Aβ42 or Aβ40 deposition in FAD cases, three adjacent fields (each field = 624 × 580 μm) were selected to include the same area used to measure 1F7 immunoreactivity in an adjacent serial section. The area of Aβ42 or Aβ40 deposits was determined with gray scale thresholding according to the methods described previously (Hyman et al., 1993). The sum of the areas of Aβ42 or Aβ40 deposits was divided by the total area to give the percentage Aβ42 or percentage Aβ40 burden.

All measurements were done under the same optical and light conditions as well as using an electronic shading correction to compensate for any unevenness that might be present in the illumination. Statistical analysis was performed with Mann–Whitney *U* test and linear regression analysis using StatView 5.0 program (Abacus Concepts, Berkeley, CA).

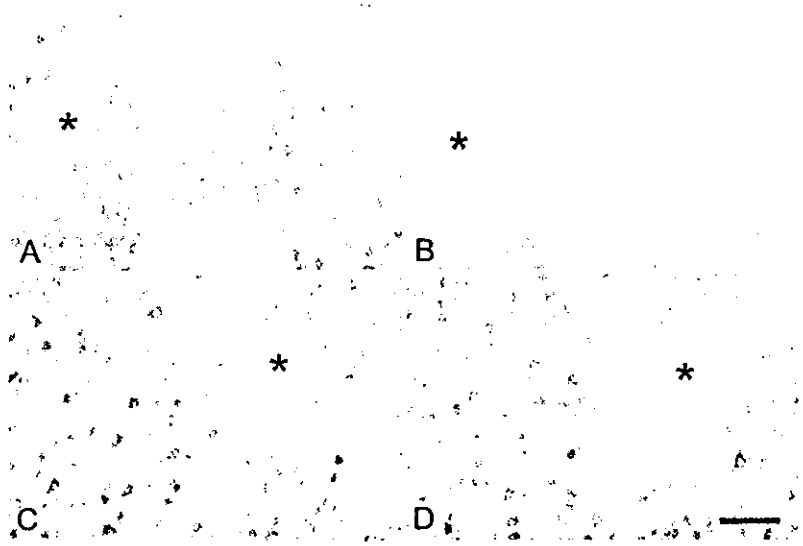

Fig. 2. RNA is a major site of nucleic acid oxidation in FAD. Immunoreaction with 1F7 antibody in FAD (A and C) is greatly diminished by treatment with DNase-free RNase (B), but only slightly by the treatment with RNase-free DNase (D). (A and B) are adjacent serial sections, so are (C and D). *indicates landmark blood vessel. The frontal cortex from a case of FAD with a PS-1 gene mutation (C410Y, 47 years old). Scale bar, 100 μm.

Results

In cases of FAD, 8OHG immunoreactivity was prominent in the neuronal cytoplasm in the superior/middle frontal gyrus (Figs. 1A–C). Neuronal 8OHG immunoreaction was widely distributed throughout the cortical layers (Fig. 1A), while in controls, staining was very low (Fig. 1E). Pyramidal neurons of larger size in the superior/middle frontal gyrus tended to show higher immunointensity of 8OHG in each case of FAD, although individual variation of immunointensity among FAD cases was observed. Moderately positive immunoreaction of neuronal 8OHG was observed in a presymptomatic case carrying a PS-1 gene mutation (Fig. 1D).

To investigate whether the immunoreaction with the 1F7 antibody was derived from oxidized RNA or oxidized DNA or both, we performed nuclease treatment before immunostaining with 1F7. The immunoreaction in the sections of FAD was diminished greatly by DNase-free RNase pretreatment (Figs. 2A and B), but only slightly by the RNase-free DNase pretreatment (Figs. 2C and D), as we demonstrated in sections of sporadic AD and DLB (Nunomura et al., 1999, 2002). Therefore, not only in sporadic AD and DLB but also in FAD is RNA a major site of nucleic acid oxidation.

Relative scale measurements of 8OHG immunoreactivity using a computer-assisted image analysis system demonstrated that the increase was significant in FAD when compared with a control group (Fig. 3A). Because 8OHG immunoreactivities tend to show an age-dependent increase in non-demented individuals (Nunomura et al., 1999), the significant increase in 8OHG immunoreactivity in FAD cases (mean age, 59 years) compared with controls (mean age, 66 years) cannot be explained by the difference in age of subjects between the groups. Neither, these results cannot be explained by neuronal shrinkage, because the average cell profile area remained unchanged between FAD cases (161 μm^2) and controls (148 μm^2). Similar levels of relative 8OHG were demonstrated between the PS-1 and the AβPP FAD (the average of the relative 8OHG = 10.2 (arbitrary units) and 9.6, respectively). Interestingly, a presymptomatic case carrying a PS-1 gene mutation showed a considerable level of relative 8OHG (Fig. 3A). Levels of the relative 8OHG immunoreactivity were not related to postmortem intervals among FAD cases ($P > 0.9$ by linear regression analysis) as well as among controls ($P > 0.9$). Furthermore, an agonal state before death also failed to alter the relative 8OHG immunoreactivity. We found similar average values for the relative 8OHG immunoreactivity in controls who died from internal malignancy ($n = 9$, relative 8OHG = 4.7), leukemia ($n = 2$, relative 8OHG = 7.9), heart failure ($n = 3$, relative 8OHG = 5.9), and unknown ($n = 1$, relative 8OHG = 4.9), as we showed in other series of controls (Nunomura et al., 1999).

When we investigated relationship between percentage area of Aβ 42 or Aβ 40 burden and relative 8OHG levels in FAD, we found a significant inverse correlation between percentage area of Aβ42 burden and relative 8OHG levels, but no significant correlation between percentage area of Aβ40 burden and relative 8OHG levels (Figs. 3B and C), as we observed in Down syndrome (Nunomura et al., 2000). In controls, only seven cases showed Aβ42 burden and only three cases showed Aβ40 burden. No apparent relationship between percentage area of Aβ42 or Aβ40 and the levels of neuronal 8OHG was detected in control subjects (data not shown).

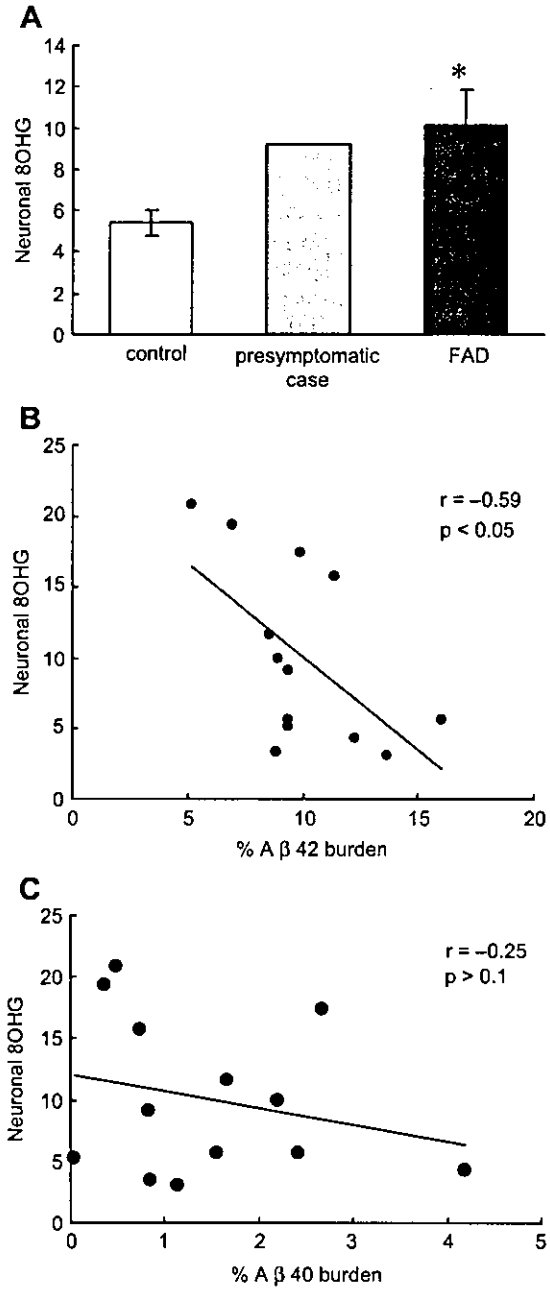

Fig. 3. Increased levels of neuronal 8OHG immunointensity and an inverse relationship between percentage area of Aβ42 burden and neuronal 8OHG immunointensity in the neocortex of FAD. Relative scale measurements reveal that the levels of 8OHG immunoreactivity are significantly increased in FAD cases ($n = 13$, average age 59 years) compared with controls ($n = 15$, average age 66 years) (*$P < 0.05$ by Mann–Whitney U test). A presymptomatic case with a PS-1 gene mutation (51 years old) shows a similar level of neuronal 8OHG immunointensity to the average of the FAD group. Values shown are the averages with SE (A). In FAD cases, there is a significant inverse correlation of percentage area of Aβ42 burden (B), but not percentage area of Aβ40 burden (C), with the levels of 8OHG immunoreactivity by linear regression analysis.

Discussion

Recently, an increasing number of in vitro and in vivo studies have suggested that oxidative stress is involved in the pathogenesis of AD and has an involvement in FAD with AβPP, PS-1, or PS-2 gene mutation. Indeed, increased oxidative stress, elevated vulnerability to oxidative stress-induced cell death, and reduced antioxidant defenses have been demonstrated in (i) cell lines expressing mutant human AβPP, PS-1, or PS-2 (Eckert et al., 2001; Guo et al., 1997; Hashimoto et al., 2002; Marques et al., 2003), (ii) transgenic mice expressing mutant human AβPP or PS-1 as well as knock in mice expressing mutant human PS-1 (Guo et al., 1999; LaFontaine et al., 2002; Leutner et al., 2000; Matsuoka et al., 2001; Praticò et al., 2001; Smith et al., 1998; Takahashi et al., 2000), (iii) fibroblasts and lymphoblasts from FAD patients with AβPP or PS-1 gene mutation (Cecchi et al., 2002), and (iv) cerebral cortex of autopsied brain samples from patients with AβPP gene mutation (Bogdanovic et al., 2001). The findings presented here represent the first evidence of increased oxidative damage to RNA in the cerebral cortex neurons of FAD, a finding previously made for the cerebral cortex neurons in sporadic AD and DLB (Nunomura et al., 1999, 2001, 2002) as well as for the substantia nigra neurons of Parkinson's disease (Zhang et al., 1999). Therefore, RNA oxidation is a common phenomenon in vulnerable neurons of sporadic and familial types of AD as well as some disorders classified in the category of synucleinopathy. A recent biochemical study has revealed that some mRNA species are selectively oxidized in the cerebral cortex of AD, and as a biological consequence, abnormal processing of proteins occurred to the oxidized mRNAs when they are expressed in cell lines (Shan et al., 2003). These findings suggest that RNA oxidation itself is directly associated with neuronal deterioration instead of harmless epiphenomenon during the process of neurodegeneration.

Interestingly, a presymptomatic case carrying a PS-1 mutation, whose autopsied cerebral cortex exhibited a substantial amount of Aβ42 deposition but no Aβ40 deposition (Lippa et al., 1998), showed a considerable level of neuronal RNA oxidation. This observation clearly suggests an early involvement of oxidative stress in the pathological cascade of FAD, which corresponds with our previous finding in Down syndrome cases where neuronal RNA oxidation precedes Aβ deposition (Nunomura et al., 2000). The early involvement of oxidative stress in FAD is also supported by experiments examining transgenic mice expressing human AβPP with FAD mutation and showing increased lipid peroxidation before Aβ plaque formation (Praticò et al., 2001).

Furthermore, we found a significant inverse correlation of Aβ42 burden, but not Aβ40 burden, with neuronal RNA oxidation. Again, this observation is completely coincident with the results of our previous study on Down syndrome (Nunomura et al., 2000). Because Aβ 42 deposition is an upstream event in the pathological cascade of FAD (Iwatsubo et al., 1994; Kalaria et al., 1996; Lippa et al., 1998), early involvement of oxidative stress is suggested by the association of Aβ42 burden with the levels of neuronal RNA oxidation. We may be able to explain the inverse correlation between Aβ42 burden and neuronal RNA oxidation when we consider roles of transition metals such as copper and iron, efficient catalysts of oxidation, and zinc, a redox-inert antioxidant. These transition metals are significantly elevated in the neocortex and especially enriched in Aβ plaques of individuals with AD (Lovell et al., 1998). Indeed, Aβ1–42 possesses high affinity for these transition metals and the binding promotes assembly of Aβ (Atwood et al., 1999). The inverse correlation may reflect a possible antioxidant property of the Aβ peptide that chelates copper and iron to keep these transition metals in a redox-inactive form (Kontush, 2001; Zou et al., 2002). Another possible explanation is that the inverse correlation may reflect zinc elevation as a homeostatic antioxidant response to oxidative stress with subsequent abundant Aβ plaques formation (Cuajungco et al., 2000). Because recent studies have suggested that prefibrillar Aβ, but not the Aβ fibril, shows toxicity (Lambert et al., 1998; Walsh et al., 1999), Aβ plaques themselves may represent a fraction of total Aβ in the brain that has been condensed and neutralized and no longer contributes to neurotoxicity. Further investigations on the relationship between intraneuronally accumulated Aβ (Gouras et al., 2000) and oxidative stress markers are necessary to elucidate whether intraneuronal Aβ peptide has pro- or antioxidant property.

Conclusion

We observed prominent nucleic acid oxidation marked by 8OHG immunoreactivity in FAD patients with PS-1 or AβPP gene mutation. 8OHG was mainly restricted to cytoplasmic RNA of vulnerable neurons in FAD as we observed in sporadic AD. Early involvement of RNA oxidation in the pathological cascade of FAD was suggested by a presymptomatic case who carried a PS-1 mutation and showed a considerable level of neuronal RNA oxidation. An inverse correlation of Aβ42 burden with neuronal RNA oxidation in FAD, which was also demonstrated in Down syndrome, might suggest a link between the process of Aβ plaque formation and an effective tissue protective response to oxidative stress.

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タウ蛋白誘導性アポトーシスによる神経変性に対する抑制剤開発研究

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抄録：アルツハイマー病（AD）脳の異常蓄積成分としてタウ蛋白とアミロイドβ蛋白（Aβ）がある．我々はタウ蛋白がアポトーシス阻害蛋白であるX chromosome-linked inhibitor of apoptosis（XIAP）と結合し，その機能を抑制することを報告してきた．一方，Aβは，培養神経細胞に添加されるとアポトーシスを引き起こすが，そのメカニズムは未だ完全には明らかではない．しかし，これまでに低濃度のAβによってアポトーシス促進蛋白のBaxの発現が誘導され，同時にアポトーシス阻害蛋白のBcl-2の発現が抑制されることが報告されている．今回我々は，低濃度のAβによってアポトーシス阻害蛋白であるXIAPの発現が抑制され，更に，XIAPの強制発現により，低濃度のAβによって引き起こされている酸化ストレスへの脆弱性が回復することを見出した．以上のことより，XIAPがAD脳における変性過程に密接に関連する可能性が示唆された．

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Key words : Alzheimer disease, amyloid β, tau protein, XIAP (X-Chromosome-linked Inhibitor of Apoptosis), apoptosis

はじめに

アルツハイマー病（AD）の神経病理学的特徴としては神経原線維変化と老人斑の存在が知られており，前者の構成成分は異常リン酸化タウ蛋白であり，後者の構成成分はアミロイドβ蛋白（Aβ）であることが知られている[3][4][5][18]．これら異常な蓄積物の構成分子が，ADにおける神経変性メカニズムにどのように関与するかについては広く研究がおこなわれているが，完全には解明されていない．細胞死のメカニズムとしてアポトーシスが非常に深く研究されており，このアポトーシスがADに関与するという報告は少なくないが，これに反する報告も少なからず存在する[12][13][24][26]．我々はこのような事情を説明するための仮説として，アポトーシスを誘導するような細胞死ストレスと，それに拮抗する何らかの因子が同時に存在している可能性を想定して研究をおこなってきた．内因性に

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