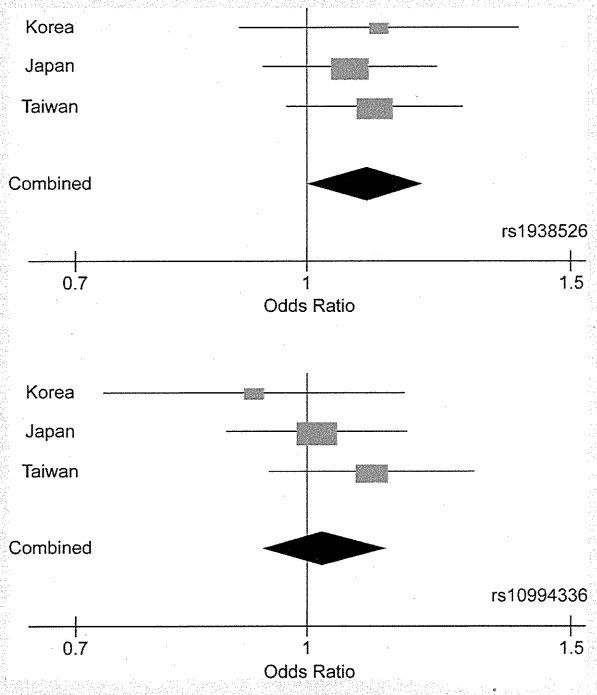

FIG. 1. Forest plots of odds ratios and their 95% confidence intervals for the individual case–control analyses and the meta-analysis.

individually analyzed using each of the three populations, we could not detect any significant difference of allele frequency. However, in the meta-analysis combining the three data sets, rs1938526 showed nominally significant association ($P = 0.048$, OR = 1.09) (Fig. 1). Except for rs1938526 in Korean control population ($P = 0.045$), genotype frequencies of both SNPs in each diagnostic group and each recruited country were within Hardy–Weinberg equilibrium ($P > 0.05$). Although the minor allele frequencies (MAF) were different among three populations, the I^2 statistics were 0% in both SNPs, indicating there was no variation in OR attributable to heterogeneity. Linkage disequilibrium between the two SNPs assessed by D′ was 0.76 in Korean, 0.71 in Japanese and 0.84 in Han-Chinese in Taiwan. The statistical power for detection obtained from overall sample size of our populations were estimated as follows: rs1938526, 98.8% for OR 1.2 and 59.5% for OR 1.1; rs10994336, 97.6% for OR 1.2 and 53.2% for OR1.1.

DISCUSSION

In this study, we detected a nominal association between rs1938526 in *ANK3* and BD, as a result of the meta-analysis using data sets from Korea, Japan, and Taiwan. The G allele of rs1938526 was more frequent in BD cases, in both of our study, using East Asian samples, and Ferreira et al., using Caucasian samples. The statistical power obtained from the overall sample size in this study was substantial for a replication study analyzing a single marker, owing to the relatively high MAF in our populations (MAF in controls of present

TABLE I. Results of the Case–Control Association Analyses in Each Population and the Meta-Analysis

	Korea				Japan				Taiwan				Meta-analysis	
	MAF				MAF				MAF					
SNP, minor allele	Cases (N = 352)	Controls (N = 349)	*P*-value	OR (95% CI)	Cases (N = 860)	Controls (N = 895)	*P*-value	OR (95% CI)	Cases (N = 1,000)	Controls (N = 1,000)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)
rs1938526, G	0.411	0.384	0.310	1.12 (0.90–1.38)	0.453	0.438	0.359	1.06 (0.93–1.22)	0.314	0.292	0.130	1.11 (0.97–1.27)	0.048*	1.09 (1.00–1.19)
rs10994336, T	0.280	0.297	0.489	0.92 (0.73–1.16)	0.357	0.355	0.918	1.01 (0.88–1.16)	0.201	0.185	0.214	1.10 (0.94–1.29)	0.591*	1.03 (0.93–1.13)

CI, confidential interval; MAF, minor allele frequency; OR, odds ratio; SNP, single-nucleotide polymorphism.
*Test for heterogeneity; $I^2 = 0\%$.

study, 0.3642 for rs1938526 and 0.2699 for rs10994336; MAF in controls of Ferreira et al., 0.056 for rs1938526 and 0.053 for rs10994336). Although significant difference in allele frequencies was not detected in the analyses using each individual population, they showed similar ORs, indicating insufficient statistical power rather than truly negative association as a source of the lack of significance. These results collectively suggest this SNP as a risk marker common across ethnicities.

On the other hand, we could not observe association between rs10994336 and BD. This discrepancy between two SNPs in our populations, both of which showed genome-wide significant association in Ferreira et al., may be explained by the different degree of LD among populations, particularly low LD in Korean and Japanese. The D′ value between two SNPs was 0.86 in Caucasian (from HapMap data), 0.84 in Han-Chinese in Taiwan, 0.76 in Korean, and 0.71 in Japanese (from our data). In accordance with different degree of LD, ORs of rs1938526 and rs10994336 in our data were similar in Han-Chinese in Taiwan (1.11 and 1.10) and slightly different in Korean (1.12 and 0.92) and Japanese (1.06 and 1.01).

The OR of rs1938526 estimated in this study was quite lower than that detected in previous Caucasian studies. Previous studies for *ANK3* in BD suggested the presence of allelic heterogeneity in this gene [Schulze et al., 2009; Smith et al., 2009]. In the case of Parkinson's disease, association signals in GWASs were detected in genes whose rare functional mutations have already known to be disease causing [Satake et al., 2009; Simon-Sanchez et al., 2009; Edwards et al., 2010]. In the case of hypertriglyceridemia, excess of rare mutations in patients was observed in genes identified by GWASs was reported [Johansen et al., 2010]. Therefore, it should be worthwhile to resequence the locus around rs1938526 seeking for rare functional mutations.

In conclusion, the results of this study as well as the findings in previous GWASs and its replication study supported the association of *ANK3* with BD. Especially, the genomic region around rs1938526 is a valuable target for a rare mutation search by resequencing.

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Genome Biology

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Exome sequencing identifies a novel missense variant in *RRM2B* associated with autosomal recessive progressive external ophthalmoplegia

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Abstract

Background: Whole-exome sequencing using next-generation technologies has been previously demonstrated to be able to detect rare disease-causing variants. Progressive external ophthalmoplegia (PEO) is an inherited mitochondrial disease that follows either autosomal dominant or recessive forms of inheritance (adPEO or arPEO). AdPEO is a genetically heterogeneous disease and several genes, including *POLG1* and *C10orf2*/Twinkle, have been identified as responsible genes. On the other hand, *POLG1* was the only established gene causing arPEO with mitochondrial DNA deletions. We previously reported a case of PEO with unidentified genetic etiology. The patient was born of a first-cousin marriage. Therefore, the recessive form of inheritance was suspected.

Results: To identify the disease-causing variant in this patient, we subjected the patient's DNA to whole-exome sequencing and narrowed down the candidate variants using public data and runs of homozygosity analysis. A total of 35 novel, putatively functional variants were detected in the homozygous segments. When we sorted these variants by the conservation score, a novel missense variant in *RRM2B*, whose heterozygous rare variant had been known to cause adPEO, was ranked at the top. The list of novel, putatively functional variants did not contain any other variant in genes encoding mitochondrial proteins registered in MitoCarta.

Conclusions: Exome sequencing efficiently and effectively identified a novel, homozygous missense variant in *RRM2B*, which was strongly suggested to be causative for arPEO. The findings in this study indicate arPEO to be a genetically heterogeneous disorder, as is the case for adPEO.

Background

Massively parallel sequencing, also known as next generation-sequencing, is a revolutionary technology that enables us to obtain large amounts of genomic sequence information in an incomparably more rapid and less expensive manner than before [1]. This technology is applicable for various investigations, including resequencing of full genomes or more targeted parts thereof for discovery of genomic variations, genome-wide mapping of structural rearrangements, transcriptome sequencing, genome-wide epigenetic analysis, metagenomic sequencing, and so on [2]. Whole-genome and whole-exome (sequences of all protein-coding regions) resequencing aiming at identification of causative variants for rare, inherited diseases is one of these applications, and have demonstrated their efficiency and effectiveness (reviewed in [3]).

Previously, we reported a patient who had been born of a first-cousin marriage and was suspected to be affected by inherited progressive external ophthalmoplegia (PEO) [4]. Inherited PEO is a form of mitochondrial disease that follows either autosomal dominant or recessive forms of inheritance (adPEO (MIM 157640; 609283; 609286; 610131, 613077) or arPEO (MIM 258450)). The characteristic findings of inherited PEOs are multiple mitochondrial DNA (mtDNA) deletions and ragged red fibers in the muscle biopsy [5]. Typical clinical symptoms are bilateral ptosis and paralysis of the extraocular muscle. Other symptoms include exercise intolerance, cataracts, hearing loss, sensory axonal neuropathy, optic atrophy,

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ataxia, depression, hypogonadism, and Parkinsonism [6-10].

In the present case, the recessive form of inheritance was suspected because of the patient's family history. However, no pathogenic variant in *POLG1* (MIM 174763), which encodes a mitochondrial DNA polymerase and was the only established gene whose variants were known to cause arPEO so far, was identified [4].

The proband in this study was the only child and the available genetic information from family members was limited. Therefore, it was almost impossible to identify the causative variant using linkage analysis. On the other hand, exome sequencing using a next-generation sequencer has demonstrated its utility to detect causative variants of rare disease using a small number of samples, especially in the case of consanguineous family. Here, we performed exome sequencing in combination with runs of homozygosity (ROH) analysis in order to identify the causative variant in this patient.

Results

Exome sequencing identifies a novel, homozygous missense variant in *RRM2B*

A total of 3.2 Gb of sequence was generated from one lane of sequencing using the Illumina Genome Analyzer II (Illumina, San Diego, CA, USA). The proportion of the targeted exome covered at 1×, 5× and 10× was 96.3%, 88.0% and 78.3%, respectively. The mean coverage was 37.2×. A total of 19, 215 variants were detected in the coding regions defined by RefSeq Gene [11] and their flanking splice sites. The number of detected coding variants does not deviated greatly from that in previous reports [3,12]. After removing variants registered on the public database of sequence variants (dbSNP, build 130) or found in eight exomes of HapMap individuals [12] or the exome of a single, healthy, unrelated Japanese individual, which was analyzed in the same run of Illumina Genome Analyzer II sequencing, 1, 336 variants remained. Among these, 592 variants, including 141 homozygous ones, were functional (nonsense, missense, frameshift or splice site). Next, we performed ROH analysis to narrow down the candidate regions, using the base calling data on single nucleotide variants in this patient. To enhance the accuracy of the variant calling used for this analysis, 1) only the data of single nucleotide variants were used and insertion/deletion variants were excluded because of lower reliability of the detection of insertion/deletion variants [13], 2) variants called with coverage less than 8× were excluded, 3) variants called with a coverage of more than 100× were excluded because genomic regions that are known to be duplicated or have similar sequences such as pseudogenes tend to be read with high coverage. Because the primary aim of this analysis was not to evaluate ROH segments precisely, but to narrow down the list of candidate variants without overlooking the causative variant, we used relaxed criteria of ROH segments. The total size of ROH regions was 992 Mb (about 32% of the genome), which was significantly larger than the expected total size of ROH segments in an offspring born from a first cousin marriage (one-eighth of the genome). A total of 35 novel and functional variants in 33 genes were identified in ROH segments. A summary of the filtering strategy is given in Table 1.

When we sorted these listed variants by a conservation score (phyloP score) to identify those that were most likely to be functional, a novel missense variant in *RRM2B* (g.341G > A, p.P33S), whose rare, heterozygous variant had been known to cause adPEO, was ranked at the top (Table 2).

The existence of the *RRM2B* variant in the patient's DNA was confirmed by Sanger sequencing (Figure 1a). As expected, each of the parents had this variant in the heterozygous state. This variant changes an amino acid residue that is highly conserved across 44 vertebrates (Figure 1b). Among 359 control subjects (718 chromosomes) of Japanese origin, one subject carried this variant in the heterozygous state.

Exclusion of other variants that could cause PEO

In the list of 35 novel and functional variants in the ROH segments, no other variants in genes encoding mitochondrial proteins were registered in Human MitoCarta [14]. We could not find any pathogenic mutations in other genes known to cause mitochondrial diseases with multiple mtDNA deletions (*POLG1*, *POLG2* (MIM 604983), *C10orf2* (MIM 606075), *SLC25A4* (MIM 103220), *OPA1* (MIM 605290), *TYMP* (MIM 131222) and *WFS1* (MIM 606201)) in exome analysis, as was observed in a previous study using Sanger sequencing [4]. Although the mtDNA sequence was not targeted by the SureSelect Human All Exon Kit (Agilent, Santa Clara, CA, USA), 16, 558 of 16, 568 (99.9%) bases in mtDNA were read four or more times due to its higher copy number than nuclear DNA, and no known pathogenic variant was found. Because of the family history of the patient, we suspected that his disease was caused by a recessive mutation. However, there was another possibility that *de novo* variants affect him in a dominant manner. To test this possibility, we investigated whether he had *de novo* variants that could explain his symptoms. In the list of 592 novel and putatively functional variants, there were 26 heterozygous variants in genes registered in MitoCarta. Among them, five variants were not found in dbSNP132 or 1000 Genome Project data [15] (SNP calls released in June 2011), and were located at conserved base positions (phyloP score > 2). By performing Sanger sequencing, we confirmed that all of these variants were not *de novo*, but inherited from either of his healthy parents or found as a false positive (Table 3).

Table 1 Summary of the filtering to narrow down the candidates for the causal variant

Criteria for the filtering	Number of remaining variants
Coding variants	19, 215
Not in dbSNP130	2, 015
Not in eight HapMap exomes [12]	1, 833
Not in in-house data of a healthy Japanese individual	1, 336
Functional (missense, nonsense, frameshift and splice site)	592
In run-of-homozygosity regions	35 (in 33 genes)

The filtering was performed using the listed criteria in descending order.

Evaluation of the amount of mtDNA

The mtDNA copy number relative to nuclear DNA in the patient's skeletal muscle was not decreased, but rather increased (Figure 2). As expected, the *ND4/RNaseP* ratio was lower than the *ND1/RNaseP* ratio in the patient, which suggests increased levels of mtDNA deletions that include the *ND4* region, such as the 4, 977-bp common mtDNA deletion [16]. This result indicated that the clinical manifestation in the present patient was not due to mtDNA depletion.

Discussion

In this study, we subjected DNA from a PEO patient with unidentified genetic etiology to exome sequencing and detected a novel, homozygous missense variant in *RRM2B*. *RRM2B* encodes p53-inducible ribonucleotide reductase small subunit 2-like protein (p53R2) and this protein plays an essential role in the maintenance of mtDNA by reducing ribonucleotides in the cytosol [17], as is indicated by the fact that rare variants in this gene cause various forms of mitochondrial diseases characterized by mtDNA depletion and deletions. To our knowledge, 15 cases of mitochondrial depletion syndrome (MIM 612075) from 11 families [18-22] and one sporadic case of mitochondrial neurogastrointestinal encephalopathy [23] (MIM 603041) associated with homozygous or compound heterozygous rare variants in *RRM2B* have been reported. More recently, two families with adPEO due to a heterozygous nonsense variant were described [24]. In the screening of *RRM2B* variants in 50 mitochondrial disease patients without causative variants in *POLG1* and *C10orf2*, one Kearns-Sayre syndrome (MIM 530000) patient who carried two different novel missense variants and one PEO patient who carried an in-frame deletion were identified [25].

The clinical symptoms and findings in the muscle biopsy of our case were typical for Mendelian-inherited PEO. No members of his maternal family have shown any neuromuscular symptoms, suggesting that the mtDNA deletions of the patient were not maternally inherited. Real-time quantitative PCR analysis revealed that there was no mtDNA depletion. We did not observe gastrointestinal dysmotility, cardiac conduction abnormalities, pancreatic dysfunction and sensory ataxic neuropathy, which are characteristic symptoms for other mitochondrial diseases associated with mtDNA deletions, namely mitochondrial neurogastrointestinal encephalopathy, Kearns-Sayre syndrome, Pearson syndrome, and

Table 2 List of novel and functional variants in run-of-homozygosity regions

Chromosome	Position	Reference allele	Variant allele	Variant calling/coverage	Gene	Amino acid change	PhyloP score
8	103313660	G	A	58/58	*RRM2B*	Pro33Ser	6.741
1	39620317	G	A	5/7*	*MACF1*	Arg2523Gln; Arg3025Gln	5.329
4	107449465	A	C	63/63	*MGC16169*	Asn34Lys	5.199
22	15980313	C	T	5/5*	*LOC100287323*	Val569Ile	4.997
11	64117795	G	A	4/4*	*SLC22A12*	Trp37Stp; Trp258Stp	4.945
10	29010439	G	C	24/24	*BAMBI*	Gly108Ala	4.878
20	49482400	G	A	4/4*	*NFATC2*	Ala778Val	4.437
1	238437608	C	T	10/12	*FMN2*	Pro1101Leu	3.804
1	85362528	T	-	65/69	*WDR63*	Splice site	3.503
3	99094433	A	G	24/34	*DKFZp667G2110*	Lys546Glu	3.299
3	336547	T	G	23/23	*CHL1*	Ser30Ala	3.014
3	46595758	C	G	27/40*	*LRRC2*	Arg41Gly	2.522
4	169335658	A	C	9/13*	*ANXA10*	Thr193Pro	2.257
5	140538797	C	T	127/127	*PCDHB8*	Thr333Ile	2.011

Variants with PhyloP score > 2 are listed. Asterisks indicate variants with coverage < 8× or a variant calling/coverage ratio < 0.7; the reliability of these variant calls is generally lower than that of the others.

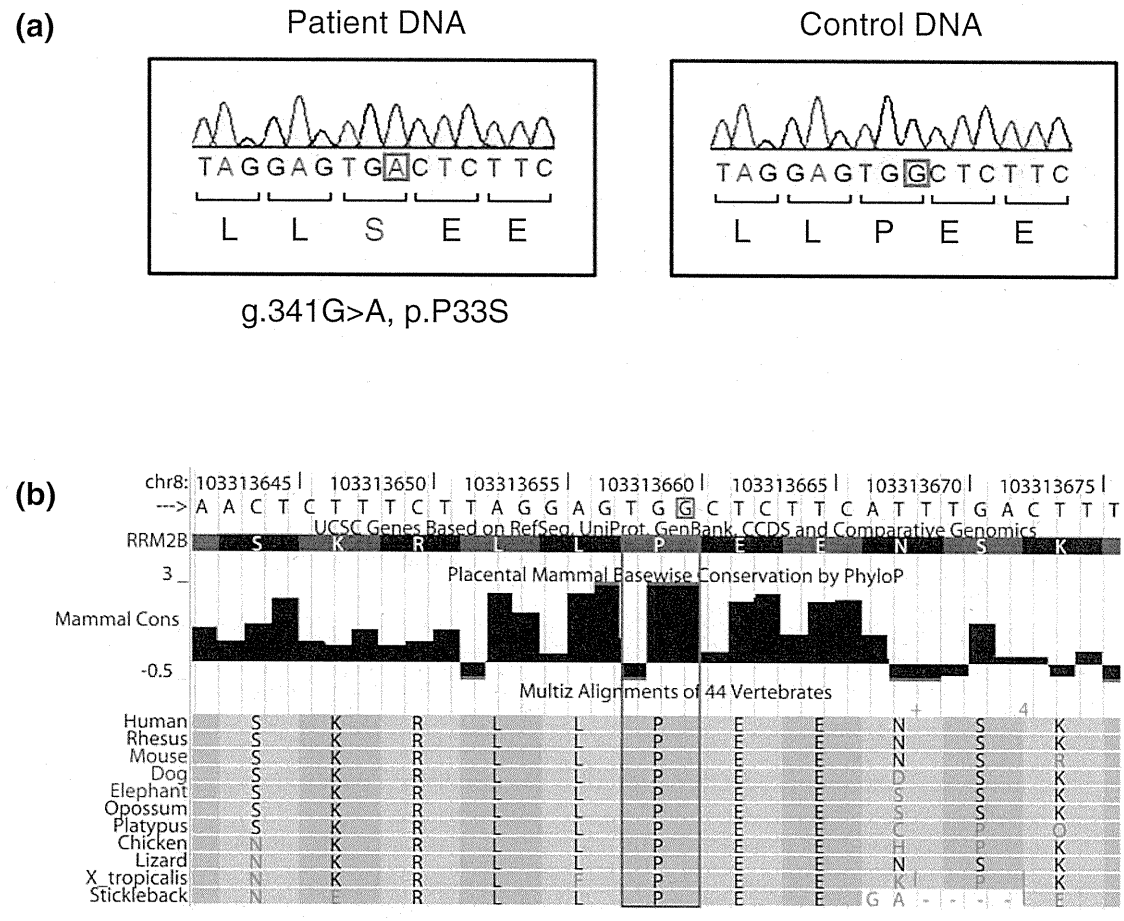

Figure 1 The identified disease-associated variant in *RRM2B*. **(a)** Partial sequence of *RRM2B* in the patient's DNA (left) and control DNA (right). Red squares indicate the base position of the g.341G > A, p.P33S variant. **(b)** The substituted amino acid residue (red box) is highly conserved across 44 vertebrate species (from the UCSC genome browser [31]).

sensory ataxic neuropathy, dysarthria, and ophthalmoparesis (MIM 607459), respectively. Therefore, this patient was diagnosed as having arPEO caused by a homozygous missense variant of *RRM2B*.

Before this study, *POLG1* had been the only established gene responsible for arPEO, while adPEO is a genetically heterogeneous disease, caused by rare variants in *POLG1, POLG2, C10orf2, SLC25A4, OPA1* and *RRM2B*. The results of this study identifying the second responsible gene for arPEO indicate that arPEO is also a genetically heterogeneous disease, as is the case for adPEO.

The symptoms observed in this patient included major depressive episodes. Frequent comorbidity of mood disorders in patients of mitochondrial disease has been generally recognized [26] and several lines of evidences have supported the possible involvement of mitochondrial dysfunctions in the pathophysiology of mood

Table 3 List of novel, putatively functional and heterozygous variants in mitochondrial genes

Chromosome	Position	Reference allele	Variant allele	Variant calling/ coverage	Gene	Amino acid change	PhyloP score	Inheritance
7	30615756	G	C	36/69	*GARS*	Asp256His	6.494	Paternally inherited
10	104476790	T	T	14/30	*SFXN2*	Leu73Pro	4.906	Maternally inherited
7	100670236	C	C	20/51	*FIS1*	Ala90Pro	3.824	Maternally inherited
11	47620527	A	A	3/8	*MTCH2*	Tyr23His	3.680	Not confirmed in Sanger sequencing
1	10286026	C	G	22/46	*KIF1B*	Ile732Met	3.092	Maternally inherited

Variants with PhyloP score > 2 are listed.

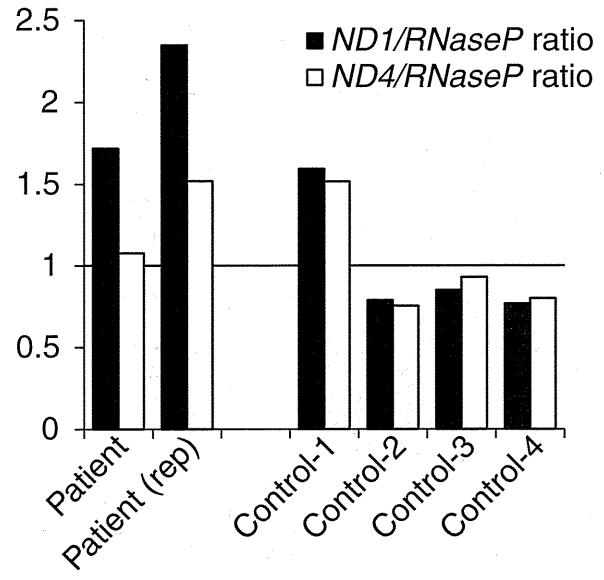

Figure 2 Relative amounts of mtDNA in skeletal muscle tissues from the patient and four control subjects. *ND1/RNaseP* and *ND4/RNaseP* ratios calculated by real-time quantitative PCR were used to evaluate mtDNA levels. The mtDNA level in the patient was comparable to those of controls. Values are relative to the average of all four controls.

disorders [27]. So far, rare variants of *POLG1*, *C10orf2* and *SLC25A4* have been reported in inherited PEO pedigrees with frequent comorbidity of mood disorders [28]. Given the typical symptoms of major depressive disorder in the present case, *RRM2B* should be added to the list of genes causal for PEO associated with mood disorders.

The identified P33S variant changes an amino acid residue highly conserved among vertebrates. The amino-terminal region of p53R2, in which this altered amino acid is located, is suggested to be crucial for interaction with p21 protein. p53R2 may contribute to DNA repair in cooperation with p21 [29]. In its amino-terminal region, the homozygous p.R41P variant was detected in a mitochondrial depletion syndrome case [21]. On the other hand, other pathogenic missense variants have been located in various sites of p53R2, including those involved in iron-binding [18,20], those putatively crucial for homodimerization of p53R2 [21,23] or heterotetramerization with the RRM1 (ribonucleoside-diphosphate reductase large subunit) homodimer [18,22], and so on. The relationships between clinical phenotypes and the properties of variants, as well as their underlying mechanisms, should be the subject of further investigations.

Conclusions

In this study, we describe a homozygous missense variant in *RRM2B* that is strongly suggested to cause arPEO. We were not only able to identify the disease-associated variant, but could also exclude other candidates (that is, variants in known PEO-related genes such as *POLG1*, other mitochondrial genes in nucleic DNA and mtDNA) using data from single exome sequencing. This result further demonstrates the efficiency and effectiveness of exome sequencing to detect causative variants of rare, inherited, and genetically heterogeneous diseases.

Materials and methods

Clinical information of the patient

The detailed clinical history, family history and laboratory data of the studied subject are described elsewhere [4]. Briefly, a 43-year-old man presented with hearing loss, bilateral ptosis, external ophthalmoplegia and muscle weakness. Examinations revealed the existence of pigmentary degeneration of the retina and gonadal atrophy. The initial symptom of progressive hearing loss began at age 16 years. Depressive mood, anxiety and hypochondriacal complaints were observed in his clinical course. His parents were first cousins, he had no siblings, and no other member of his family has a known history of neurological illness. In the muscle biopsy, marked variation of muscle fiber size, ragged red fibers, COX-negative fibers and multiple mtDNA deletions were detected. According to his clinical history, family history and laboratory data, arPEO was suspected.

The present study conformed to the Declaration of Helsinki, and was approved by the RIKEN Wako Institute Ethics Committee I, as well as the ethics committees of Kagoshima University Graduate School of Medical and Dental Sciences and other participating institutes. Written informed consent was obtained from every subject.

Exome sequencing and data analysis

Total DNA was obtained from peripheral blood of the patient using standard protocols. Total DNA (3 μg) was sheared into approximately 300-bp fragments using a Covaris sonicator (Covaris, Woburn, MA, USA). A paired-end exome library for Illumina sequencing was prepared using the SureSelect Human All Exon Kit (Agilent) following the manufacturer's instructions. Massively parallel sequencing was performed using one lane of the Genome Analyzer II (Illumina) at RIKEN Omics Science Center by the Life Science Accelerator system. Base calling was performed by the Illumina pipeline with default parameters. Obtained reads were mapped against the human reference genome (UCSC hg18/GRCh36) using CLC Genomics Workbench v4.0.2 software (CLC Bio, Aarhus, Denmark) with default parameters. Variant calling was performed using the SNP and DIP detection tools in CLC Genomics Workbench v4.0.2 with default parameters. Analysis of ROH was performed using PLINK software v1.0.7 [30]. The primary aim of this

analysis was not to evaluate ROH segments precisely, but to narrow down the list of candidate variants without overlooking the causative variant. Therefore, we used relatively small (1, 000 kb) sliding windows for ROH segments, did not consider local blocks of linkage disequilibrium in the Japanese population, and did not exclude the data of variants whose frequency was not registered in dbSNP; those variants might not be polymorphic in the Japanese population and possibly contributed to extend the length of ROH. Conservation information for the variants among 44 vertebrate species (phyloP score) was collected from the UCSC genome browser [31].

Sanger sequencing

Sanger sequencing of PCR amplicons was performed to confirm the detected disease-associated variant using a 3730 × L DNA Analyser (Applied Biosystems, Foster City, CA, USA). The primers used were: forward, 5'-AGGCAGACAGGCTCTCAAAC-3'; reverse, 5'-GGCAGAATTAGATGCCATTG-3'.

Real-time quantitative PCR

The amount of nuclear DNA and mtDNA in the skeletal muscle of the patient and four age- and sex-matched controls (all males aged 39 to 48 years) was evaluated by real-time quantitative PCR analysis according to the previously validated methods [32]. Briefly, copy numbers of *RNaseP* (for nuclear DNA), *ND1* and *ND4* (for mtDNA) were evaluated using the TaqMan method (Applied Biosystems). Analysis of the patient's tissue was performed in two independent reactions, and each experiment was triplicated. *ND1*/*RNaseP* and *ND4*/*RNaseP* ratios were calculated as $2^{[Ct(RNaseP)-Ct(\text{each gene})]}$.

Data accessibility

The sequence data from this study have been submitted to dbGaP [33] (study accession [phs000392.v1.p1]).

Abbreviations

adPEO: autosomal dominant progressive external ophthalmoplegia; arPEO: autosomal recessive progressive external ophthalmoplegia; mtDNA: mitochondrial DNA; PEO: progressive external ophthalmoplegia; ROH: runs of homozygosity.

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Authors' contributions

AT and TK designed the study and drafted the manuscript. AT performed data analysis and molecular experiments. MK, MN and AS performed clinical assessment. MK, MN, TY and AS provided materials for experiments. TY, SK, AS and TK coordinated the study and performed critical revision of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Glucose tolerance status and risk of dementia in the community

The Hisayama Study

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ABSTRACT

Objective: We investigated the association between glucose tolerance status defined by a 75-g oral glucose tolerance test (OGTT) and the development of dementia.

Methods: A total of 1,017 community-dwelling dementia-free subjects aged ≥60 years who underwent the OGTT were followed up for 15 years. Outcome measure was clinically diagnosed dementia.

Results: The age- and sex-adjusted incidence of all-cause dementia, Alzheimer disease (AD), and vascular dementia (VaD) were significantly higher in subjects with diabetes than in those with normal glucose tolerance. These associations remained robust even after adjustment for confounding factors for all-cause dementia and AD, but not for VaD (all-cause dementia: adjusted hazard ratio [HR] = 1.74, 95% confidence interval [CI] = 1.19 to 2.53, $p = 0.004$; AD: adjusted HR = 2.05, 95% CI = 1.18 to 3.57, $p = 0.01$; VaD: adjusted HR = 1.82, 95% CI = 0.89 to 3.71, $p = 0.09$). Moreover, the risks of developing all-cause dementia, AD, and VaD significantly increased with elevated 2-hour postload glucose (PG) levels even after adjustment for covariates, but no such associations were observed for fasting plasma glucose (FPG) levels: compared with those with 2-hour PG levels of <6.7 mmol/L, the multivariable-adjusted HRs of all-cause dementia and AD significantly increased in subjects with 2-hour PG levels of 7.8 to 11.0 mmol/L or over, and the risk of VaD was significantly higher in subjects with levels of ≥11.1 mmol/L.

Conclusions: Our findings suggest that diabetes is a significant risk factor for all-cause dementia, AD, and probably VaD. Moreover, 2-hour PG levels, but not FPG levels, are closely associated with increased risk of all-cause dementia, AD, and VaD. *Neurology*® 2011;77:1126-1134

GLOSSARY

AD = Alzheimer disease; **CI** = confidence interval; ***DSM-III-R*** = *Diagnostic and Statistical Manual of Mental Disorders*, 3rd edition, revised; **FPG** = fasting plasma glucose; **HR** = hazard ratio; **IFG** = impaired fasting glycemia; **IGT** = impaired glucose tolerance; **NGT** = normal glucose tolerance; **OGTT** = oral glucose tolerance test; **PG** = postload glucose; **VaD** = vascular dementia.

Diabetes mellitus is one of the most common metabolic disorders, and its prevalence has risen globally in recent years. Some epidemiologic studies have reported that diabetes is independently implicated in the development of dementia.[1–3] However, these findings are inconsistent for its subtypes; one study found an association between diabetes and the risk of both Alzheimer disease (AD) and vascular dementia (VaD),[1] whereas other studies found an association with only AD[2,3] or only VaD,[4–7] and still others showed no association between diabetes and either condition.[8,9] These conflicting results may have been related to differences in the study designs, including the defined criteria for diabetes and dementia subtypes, as well as in the regional characteristics and ethnicities of the settings and subjects. Thus, accurate definitions of diabetes and dementia subtypes are needed to ascertain the true associations between the two, and a 75-g oral glucose tolerance test (OGTT) and morphologic examination of the brain may meet this requirement. However, to date, very few cohort studies have had enough quality data to allow reliable diagnosis using these methods.

Supplemental data at www.neurology.org

Supplemental Data

From the Departments of Environmental Medicine (T.O., Y.D., T.N., Y.H., J.H., Y.K.), Neuropsychiatry (T.O., S.K.), Medicine and Clinical Science (Y.D., T.N., Y.H., J.H.), and Neuropathology (T.I.), Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

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To resolve these issues, we performed a prospective cohort study of dementia in a Japanese community-dwelling population, all members of which underwent the OGTT. The most important feature of this study is that the subtypes of dementia were verified by detailed neurologic and morphologic examination, including neuroimaging and autopsy. Using data from this cohort study, we investigated the association between glucose tolerance levels defined by the OGTT and the development of dementia and its subtypes.

METHODS

Study population. A population-based prospective study of cerebro-cardiovascular diseases was begun in 1961 in the town of Hisayama, a suburb of the Fukuoka metropolitan area of Kyushu Island in Japan. In addition, comprehensive surveys of cognitive impairment in the elderly of this town have been conducted since 1985. In 1988, a total of 1,228 residents aged ≥60 years (91.1% of the total population in this age group) participated in a screening examination for the present study. After exclusion of 33 subjects who had dementia, 90 who had already had breakfast, 5 who were on insulin therapy, and 81 who could not complete the OGTT, a total of 1,019 subjects without dementia underwent the OGTT. From a total of 1,019 subjects, 2 who died before starting follow-up were excluded, and the remaining 1,017 subjects (437 men and 580 women) were enrolled in this study.

Follow-up survey. The subjects were followed up prospectively for 15 years, from December 1988 to November 2003 (mean 10.9 years; SD 4.1 years). A complete description of the follow-up survey is provided in appendix e-1 on the *Neurology*® Web site at www.neurology.org.

Diagnosis of dementia. The diagnosis of dementia was made based on the guidelines of the *DSM-III-R*.[10] Subjects diagnosed with AD met the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association criteria[11] and subjects diagnosed with VaD met the National Institute of Neurological Disorders and Stroke–Association Internationale pour la Recherche et l'Enseignement en Neurosciences criteria.[12] Possible or probable dementia subtypes were diagnosed with clinical information including neuroimaging. Definite dementia subtypes were also determined on the basis of clinical and neuropathologic information. The diagnostic procedure for autopsy cases was reported previously.[13] A neuropathologic diagnosis of AD was made following the National Institute on Aging–Reagan Institute criteria,[14] where the frequency of neuritic plaques and neurofibrillary tangles was evaluated using the Consortium to Establish a Registry for Alzheimer's Disease criteria[15] and Braak stage.[16] Definite VaD cases were confirmed with causative stroke or cerebrovascular change and no neuropathologic evidence of other forms of dementia. Every dementia case was adjudicated by expert psychiatrists.

During the follow-up, 232 subjects (79 men and 153 women) developed dementia. Of these, 201 (86.6%) were evaluated by brain imaging, and 118 (50.9%) underwent brain autopsy; in 110, both were performed. Thus, 209 subjects in all (90.1%) had some kind of morphologic examination. Among the 118 autopsy cases, the clinical diagnosis of 42 cases (35.6%) was changed by the neuropathologic findings. Among all dementia cases, 18 AD cases and 11 VaD cases had other coexisting subtypes of dementia. These cases were counted as events in the analysis for other dementia. In all, 105 cases were categorized as AD, 65 as VaD, and 62 as other dementia.

Risk factor measurement. At the baseline examination, we performed the OGTT after an at least 12-hour overnight fast. Plasma glucose levels were determined by the glucose-oxidase method. Glucose tolerance status was defined by the 1998 WHO criteria[17]: normal glucose tolerance (NGT), fasting plasma glucose (FPG) <6.1 and 2-hour postload glucose (PG) <7.8; impaired fasting glycemia (IFG), FPG 6.1 to 6.9 and 2-hour PG <7.8; impaired glucose tolerance (IGT), FPG <7.0 and 2-hour PG 7.8 to 11.0; and diabetes, FPG ≥7.0 mmol/L or 2-hour PG ≥11.1 mmol/L. Each of the FPG and 2-hour PG level was also divided into 4 categories (FPG: <5.6, 5.6 to 6.0, 6.1 to 6.9, and ≥7.0 mmol/L; 2-hour PG: <6.7, 6.7 to 7.7, 7.8 to 11.0, and ≥11.1 mmol/L).

In order to assess the independent effects of glucose tolerance levels on dementia occurrence, the following baseline factors in addition to age and sex were used as confounding factors: 1) information on smoking habits, alcohol intake, and physical activity was obtained by means of a questionnaire administered to each subject; 2) a low education level was defined as ≤6 years of formal education; 3) history of stroke was determined on the basis of all clinical data available in the Hisayama Study; 4) hypertension was defined as blood pressure levels ≥140/90 mm Hg or current treatment with antihypertensive agents; 5) EKG abnormalities were defined as left ventricular hypertrophy (Minnesota Code 3-1), ST depression (4-1, 2, or 3) or atrial fibrillation (8-3); 6) serum total cholesterol levels were measured enzymatically; and 7) body mass index (kg/m^2) and waist to hip ratio were used as indicators of obesity.

Statistical analysis. The SAS software package, version 9.2 (SAS Institute, Cary, NC), was used to perform all statistical analyses. Age- and sex-adjusted mean values of possible risk factors were calculated by the analysis of covariance method. Frequencies of risk factors were adjusted for age and sex by the direct method. The differences in the mean values and frequencies of risk factors between NGT and other glucose tolerance levels were tested using Fisher least significant difference method and logistic regression analysis, respectively. The incidence of dementia was calculated by the person-years method and was adjusted for age and sex by the direct method using 5-year age groups of the overall study population; the differences among glucose tolerance levels and trends across FPG and 2-hour PG levels were tested using Cox proportional hazards model. The adjusted hazard ratios (HRs) and their 95% confidence intervals (CIs) were also calculated using the Cox proportional hazards model. Missing values of waist to hip ratio (n = 27) and education (n = 12) were replaced with the means in the multivariate analysis. The population attributable fraction of combined category of IGT and diabetes for dementia was calculated using the following equation with the observed multivariate-adjusted HR of the combined category and its frequency in event cases (Pe)[18]:

$$PAF = Pe\,(HR - 1)/HR$$

Two-sided $p < 0.05$ was considered statistically significant in all analyses.

Standard protocol approvals, registrations, and patient consents. This study was conducted with the approval of the Kyushu University Institutional Review Board for Clinical Re-

Table 1 **Age- and sex-adjusted mean values or frequencies of potential risk factors for dementia according to the 1998 WHO criteria: The Hisayama Study, 1988**[a]

	Normal glucose tolerance (n = 559)	Impaired fasting glycemia (n = 73)	Impaired glucose tolerance (n = 235)	Diabetes (n = 150)	No. of missing values
Age, y, mean (SD)	68 (6)	70 (6)[b]	69 (6)	69 (6)	0
Men, %	40.8	52.1	43.8	45.3	0
Fasting plasma glucose, mmol/L, mean (SD)	5.3 (0.9)	6.4 (0.9)[c]	5.8 (0.9)[c]	7.7 (0.9)[c]	0
Two-hour postload glucose, mmol/L, mean (SD)	5.9 (2.2)	5.9 (2.2)	8.9 (2.2)[c]	14.9 (2.2)[c]	0
Systolic blood pressure, mm Hg, mean (SD)	133 (21)	141 (21)[c]	143 (21)[c]	145 (21)[c]	0
Diastolic blood pressure, mm Hg, mean (SD)	75 (10)	76 (10)	78 (10)[c]	77 (10)[b]	0
Hypertension, %[d]	43.8	66.7[c]	63.2[c]	62.2[c]	0
Electrocardiogram abnormalities, %	20.6	31.7	18.8	21.6	0
Body mass index, kg/m^2, mean (SD)	21.8 (3.0)	22.2 (3.0)	23.2 (3.0)[c]	23.2 (3.0)[c]	0
Waist to hip ratio, cm/cm, mean (SD)	0.91 (0.07)	0.93 (0.07)[b]	0.93 (0.07)[c]	0.94 (0.07)[c]	27
Total cholesterol, mmol/L, mean (SD)	5.3 (1.1)	5.5 (1.1)	5.4 (1.1)	5.7 (1.1)[c]	0
History of stroke at entry, %	3.3	3.5	5.9	6.3	0
Education ≤6 y, %	10.3	12.5	13.9	11.3	12
Smoking, %	23.5	23.8	23.5	22.7	0
Alcohol intake, %	23.4	29.0	27.7	34.8[c]	0
Physical activity, %	20.2	22.8	16.8	14.7	0

[a] Mean age was sex adjusted. Percentage of men was age adjusted. Electrocardiogram abnormalities were defined as Minnesota Code 3-1, 4-1, 4-2, 4-3, or 8-3.
[b] $p < 0.05$ vs normal glucose tolerance.
[c] $p < 0.01$ vs normal glucose tolerance.
[d] Hypertension: blood pressure ≥140/90 mm Hg or current use of antihypertensive agents.

search, and written informed consent was obtained from the participants.

RESULTS Table 1 shows the age- and sex-adjusted mean values or frequencies of risk factors for dementia by the WHO criteria at baseline. Compared with those with NGT, the mean values of systolic and diastolic blood pressures, body mass index, waist to hip ratio, and total cholesterol, and the frequencies of hypertension and alcohol intake, were higher in subjects with IFG, IGT; or diabetes.

The age- and sex-adjusted incidences and adjusted HRs of all-cause dementia and its subtypes according to glucose tolerance status defined by the WHO criteria are shown in table 2. Compared with those with NGT, the age- and sex-adjusted incidence and HR of all-cause dementia were significantly higher in subjects with IGT as well as those with diabetes. This association remained unchanged in subjects with diabetes even after adjustment for age, sex, hypertension, EKG abnormalities, body mass index, waist to hip ratio, total cholesterol, history of stroke at entry, education, smoking habits, alcohol intake, and physical activity. In regard to subtypes of dementia, the age- and sex-adjusted incidence and adjusted HRs of AD were significantly higher in subjects with diabetes than in those with NGT. The age- and sex-adjusted incidence and HR of VaD were significantly increased in subjects with IGT or diabetes compared with those with NGT; however, these associations were not significant after multivariable adjustment. No significant associations were observed between glucose tolerance levels and the risk of other dementia. When IGT and diabetes were brought together in one category, this category also had the significantly higher risks of all-cause dementia, AD, and VaD in the age- and sex-adjusted analysis, and these associations remained significant for all-cause dementia and AD even after adjustment for other possible risk factors. The population attributable fraction of this combined category was 14.6% for all-cause dementia, 20.1% for AD, and 17.0% for VaD.

Table 3 presents the associations between FPG levels and adjusted risks of all-cause dementia and its subtypes. The age- and sex-adjusted incidences and HRs of all-cause dementia and any of the dementia subtypes did not differ among FPG levels. This tendency was unchanged even in the multivariate analysis. Conversely, as shown in table 4, the age- and

Table 2 **Age- and sex-adjusted incidence and adjusted hazard ratios and their 95% confidence intervals for the development of all-cause dementia and its subtypes according to glucose tolerance status defined by WHO criteria**

Glucose tolerance level	Person-years at risk, n	No. of events, n	Age- and sex-adjusted incidence	Crude HR (95% CI)	p	Age- and sex-adjusted HR (95% CI)	p	Multivariable-adjusted[a] HR (95% CI)	p
All-cause dementia									
Normal	6,658	115	20.1	1 (referent)		1 (referent)		1 (referent)	
IFG	854	13	16.0	0.89 (0.50-1.58)	0.70	0.74 (0.42-1.31)	0.30	0.63 (0.35-1.13)	0.12
IGT	2,611	63	24.9	1.46 (1.07-1.99)	0.02	1.40 (1.03-1.91)	0.03	1.35 (0.98-1.86)	0.07
DM	1,544	41	29.3	1.62 (1.14-2.32)	0.008	1.71 (1.19-2.44)	0.003	1.74 (1.19-2.53)	0.004
IGT + DM	4,155	104	26.3	1.52 (1.17-1.98)	0.002	1.51 (1.16-1.97)	0.002	1.46 (1.10-1.92)	0.008
Alzheimer disease									
Normal	6,658	51	8.6	1 (referent)		1 (referent)		1 (referent)	
IFG	854	5	6.6	0.77 (0.31-1.94)	0.58	0.63 (0.25-1.57)	0.32	0.61 (0.24-1.55)	0.29
IGT	2,611	29	11.7	1.53 (0.97-2.41)	0.07	1.46 (0.92-2.30)	0.11	1.60 (0.99-2.59)	0.05
DM	1,544	20	14.2	1.81 (1.08-3.03)	0.03	1.94 (1.16-3.26)	0.01	2.05 (1.18-3.57)	0.01
IGT + DM	4,155	49	12.5	1.63 (1.10-2.41)	0.01	1.62 (1.10-2.40)	0.02	1.73 (1.15-2.60)	0.009
Vascular dementia									
Normal	6,658	27	5.1	1 (referent)		1 (referent)		1 (referent)	
IFG	854	6	7.1	1.76 (0.73-4.26)	0.21	1.40 (0.58-3.41)	0.46	1.01 (0.41-2.52)	0.98
IGT	2,611	20	7.8	1.95 (1.09-3.47)	0.02	1.86 (1.05-3.32)	0.04	1.39 (0.76-2.54)	0.29
DM	1,544	12	8.7	2.00 (1.01-3.95)	0.04	2.07 (1.05-4.09)	0.04	1.82 (0.89-3.71)	0.09
IGT + DM	4,155	32	7.9	1.97 (1.18-3.29)	0.01	1.94 (1.16-3.23)	0.01	1.54 (0.90-2.63)	0.11
Other dementia									
Normal	6,658	37	6.4	1 (referent)		1 (referent)		1 (referent)	
IFG	854	2	2.2	0.42 (0.10-1.75)	0.23	0.36 (0.09-1.51)	0.16	0.34 (0.08-1.44)	0.14
IGT	2,611	14	5.5	0.99 (0.54-1.84)	0.99	0.96 (0.52-1.78)	0.90	0.94 (0.49-1.78)	0.84
DM	1,544	9	6.5	1.08 (0.52-2.24)	0.83	1.10 (0.53-2.28)	0.80	1.19 (0.56-2.52)	0.66
IGT + DM	4,155	23	5.8	1.03 (0.61-1.73)	0.92	1.01 (0.60-1.70)	0.97	0.97 (0.57-1.67)	0.91

Abbreviations: CI = confidence interval; DM = diabetes mellitus; HR = hazard ratio; IFG = impaired fasting glycemia; IGT = impaired glucose tolerance.
[a] Multivariate adjustment was made for age, sex, hypertension, electrocardiogram abnormalities, body mass index, waist to hip ratio, total cholesterol, history of stroke at entry, education, smoking habits, alcohol intake, and physical activity.

sex-adjusted incidences and HRs of all-cause dementia, AD, and VaD significantly increased with rising 2-hour PG levels. Compared with those with 2-hour PG levels of <6.7 mmol/L, the age- and sex-adjusted incidences and HRs of all-cause dementia, AD, and VaD were marginally or significantly higher in subjects with 2-hour PG levels of 7.8 to 11.0 mmol/L and significantly higher in subjects with 2-hour PG levels of ≥11.1 mmol/L. These associations remained robust even after multivariable adjustment; the risks of all-cause dementia and AD were significantly increased in subjects with 2-hour PG levels of 7.8 to 11.0 mmol/L and over, and the risk of VaD was significantly higher in those with 2-hour PG levels of ≥11.1 mmol/L.

Sensitivity analysis in which only definite cases of dementia determined by brain autopsy were used as event cases did not make any material difference in these findings, except with respect to VaD, for which the significant association disappeared, probably due to the few event cases (table 5). When only clinical diagnoses were used for cases with both clinical and neuropathologic diagnoses, the findings were substantially unchanged, though the HRs became slightly lower probably due to the decreased accuracy of diagnosis (tables e-1, e-2, and e-3).

DISCUSSION In a long-term prospective study of an elderly Japanese population, we demonstrated that diabetes that was assessed 15 years earlier was a significant risk factor for the development of all-cause dementia, AD, and VaD. Moreover, the risks of developing all-cause dementia and its sub-

Table 3 Age- and sex-adjusted incidence and adjusted hazard ratios and their 95% confidence intervals for the development of all-cause dementia and its subtypes according to fasting plasma glucose levels

Fasting plasma glucose levels	Person-years at risk, n	No. of events, n	Age- and sex-adjusted incidence	Crude HR (95% CI)	p	Age- and sex-adjusted HR (95% CI)	p	Multivariable-adjusted[a] HR (95% CI)	p
All-cause dementia									
<5.6	5,589	101	20.7	1 (referent)		1 (referent)		1 (referent)	
5.6-6.0	3,286	71	25.1	1.24 (0.91-1.68)	0.17	1.21 (0.89-1.64)	0.22	1.18 (0.86-1.61)	0.31
6.1-6.9	1,724	39	21.6	1.13 (0.91-1.91)	0.14	1.13 (0.78-1.64)	0.52	0.96 (0.65-1.41)	0.82
≥7.0	1,067	21	22.3	1.21 (0.70-1.79)	0.64	1.14 (0.71-1.82)	0.60	1.21 (0.75-1.96)	0.44
				p for trend: 0.23		*p* for trend: 0.42		*p* for trend: 0.63	
Alzheimer disease									
<5.6	5,589	48	10.1	1 (referent)		1 (referent)		1 (referent)	
5.6-6.0	3,286	30	10.3	1.11 (0.70-1.74)	0.67	1.14 (0.72-1.80)	0.58	1.11 (0.69-1.77)	0.68
6.1-6.9	1,724	16	9.1	1.15 (0.65-2.02)	0.64	1.00 (0.57-1.77)	0.99	0.99 (0.49-1.64)	0.72
≥7.0	1,067	11	11.9	1.23 (0.64-2.37)	0.53	1.29 (0.67-2.48)	0.45	1.41 (0.72-2.76)	0.32
				p for trend: 0.47		*p* for trend: 0.56		*p* for trend: 0.58	
Vascular dementia									
<5.6	5,589	24	4.9	1 (referent)		1 (referent)		1 (referent)	
5.6-6.0	3,286	19	6.7	1.38 (0.76-2.52)	0.29	1.29 (0.71-2.36)	0.41	1.19 (0.64-2.19)	0.58
6.1-6.9	1,724	17	8.7	2.40 (1.29-4.47)	0.006	1.93 (1.03-3.61)	0.04	1.48 (0.77-2.84)	0.24
≥7.0	1,067	5	5.2	1.12 (0.43-2.93)	0.82	1.10 (0.42-2.89)	0.84	0.99 (0.37-2.69)	0.99
				p for trend: 0.10		*p* for trend: 0.19		*p* for trend: 0.49	
Other dementia									
<5.6	5,589	29	5.7	1 (referent)		1 (referent)		1 (referent)	
5.6-6.0	3,286	22	8.1	1.33 (0.76-2.31)	0.32	1.27 (0.73-2.21)	0.40	1.21 (0.68-2.16)	0.51
6.1-6.9	1,724	6	3.8	0.69 (0.29-1.67)	0.42	0.60 (0.25-1.45)	0.26	0.53 (0.22-1.31)	0.17
≥7.0	1,067	5	5.2	0.92 (0.36-2.37)	0.86	0.91 (0.35-2.36)	0.85	1.02 (0.39-2.67)	0.97
				p for trend: 0.68		*p* for trend: 0.53		*p* for trend: 0.52	

Abbreviations: CI = confidence interval; HR = hazard ratio.
[a] Multivariate adjustment was made for age, sex, hypertension, electrocardiogram abnormalities, body mass index, waist to hip ratio, total cholesterol, history of stroke at entry, education, smoking habits, alcohol intake, and physical activity.

types progressively increased with elevating 2-hour PG levels.

In prior prospective epidemiologic studies, there have been conflicting results regarding the associations between diabetes and incidences of all-cause dementia and AD, while the influence of diabetes on the risk of VaD has been positive in most studies.[1,4–7] Cohort studies in which diabetes was defined by nonfasting blood glucose levels or clinical information did not reveal clear associations of diabetes with the development of all-cause dementia and AD,[4–8] while the risks of dementia and its subtypes significantly increased in diabetes in some studies, most of which defined diabetes using the OGTT.[1–3] The latter findings were in accord with ours. This fact suggests that differences in the methods used to define diabetes lead to a discrepancy in the association between diabetes and the risk of dementia, especially AD, and that an OGTT is essential for the definition of diabetes in epidemiologic studies on the diabetes–dementia association.

In our study, the incidence of VaD was significantly higher in subjects with IGT or diabetes than in those with NGT, but this association disappeared after adjustment for other covariates. This might occur due to the few VaD cases. In addition, since other known cardiovascular risk factors, such as hypertension, obesity, and dyslipidemia, accumulate under a prediabetic or diabetic state, as shown in our data (table 1), IGT and diabetes seem to increase the risk of VaD through mediation of these risk factors, especially hypertension.

In the present study, increased 2-hour PG levels including a prediabetic range were significantly

Table 4 **Age- and sex-adjusted incidence and adjusted hazard ratios and their 95% confidence intervals for the development of all-cause dementia and its subtypes according to 2-hour postload glucose levels**

2-Hour postload glucose levels	Person-years at risk, n	No. of events, n	Age- and sex-adjusted incidence	Crude HR (95% CI)	p	Age- and sex-adjusted HR (95% CI)	p	Multivariable-adjusted[a] HR (95% CI)	p
All-cause dementia									
<6.7	5,354	85	17.6	1 (referent)		1 (referent)		1 (referent)	
6.7–7.7	2,277	44	20.9	1.20 (0.84–1.73)	0.32	1.25 (0.87–1.80)	0.24	1.16 (0.78–1.71)	0.47
7.8–11.0	2,844	67	24.7	1.53 (1.11–2.11)	0.009	1.54 (1.12–2.12)	0.009	1.50 (1.07–2.11)	0.02
≥11.1	1,192	36	32.8	2.08 (1.41–3.07)	<0.001	2.32 (1.57–3.44)	<0.001	2.47 (1.62–3.77)	<0.001
				p for trend: <0.001		*p* for trend: <0.001		*p* for trend: <0.001	
Alzheimer disease									
<6.7	5,354	37	7.6	1 (referent)		1 (referent)		1 (referent)	
6.7–7.7	2,277	20	8.8	1.25 (0.73–2.16)	0.41	1.23 (0.71–2.12)	0.46	1.49 (0.83–2.67)	0.17
7.8–11.0	2,844	30	11.3	1.59 (0.98–2.57)	0.06	1.56 (0.96–2.53)	0.07	1.87 (1.13–3.12)	0.02
≥11.1	1,192	18	15.8	2.44 (1.39–4.29)	0.002	2.75 (1.56–4.85)	<0.001	3.42 (1.83–6.40)	<0.001
				p for trend: 0.002		*p* for trend: <0.001		*p* for trend: <0.001	
Vascular dementia									
<6.7	5,354	21	4.6	1 (referent)		1 (referent)		1 (referent)	
6.7–7.7	2,277	12	6.3	1.33 (0.65–2.70)	0.43	1.49 (0.73–3.04)	0.27	1.14 (0.54–2.41)	0.73
7.8–11.0	2,844	20	7.2	1.83 (0.99–3.38)	0.05	1.87 (1.01–3.45)	0.04	1.38 (0.72–2.64)	0.34
≥11.1	1,192	12	11.2	2.75 (1.35–5.60)	0.005	3.15 (1.55–6.43)	0.002	2.66 (1.24–5.70)	0.01
				p for trend: 0.004		*p* for trend: 0.002		*p* for trend: 0.02	
Other dementia									
<6.7	5,354	27	5.4	1 (referent)		1 (referent)		1 (referent)	
6.7–7.7	2,277	12	5.8	1.04 (0.52–2.04)	0.92	1.08 (0.55–2.15)	0.82	0.86 (0.40–1.84)	0.70
7.8–11.0	2,844	17	6.2	1.21 (0.66–2.23)	0.53	1.21 (0.66–2.23)	0.53	1.14 (0.60–2.16)	0.69
≥11.1	1,192	6	5.8	1.05 (0.44–2.55)	0.91	1.12 (0.46–2.71)	0.81	1.21 (0.48–3.04)	0.69
				p for trend: 0.65		*p* for trend: 0.59		*p* for trend: 0.59	

Abbreviations: CI = confidence interval; HR = hazard ratio.
[a] Multivariate adjustment was made for age, sex, hypertension, electrocardiogram abnormalities, body mass index, waist to hip ratio, total cholesterol, history of stroke at entry, education, smoking habits, alcohol intake, and physical activity.

linked to elevated risks of all-cause dementia, AD, and VaD, but no such associations were observed for FPG. The epidemiologic evidence from Asia has also indicated that 2-hour PG levels are better in detecting prediabetes and diabetes compared with FPG levels.[19] However, very few prospective studies have investigated the associations between FPG as well as 2-hour PG levels and the risks of dementia and its subtypes. Only the Uppsala Longitudinal Study of Adult Men evaluated the associations of FPG levels with the risks of developing AD and VaD,[20,21] and this study concluded that increased FPG levels were not risk factors for these subtypes of dementia. This is in good agreement with our findings. The Uppsala Study[21] and the Honolulu-Asia Aging Study[1] also found no clear associations between 2-hour PG levels and the risks of AD and VaD. These findings are inconsistent with ours. Our recent clinicopathologic study of deceased Hisayama residents revealed that higher levels of 2-hour PG but not of FPG were clearly associated with increased risk for formation of neuritic plaques even after adjustment for confounding factors.[22] This evidence together with the findings of the present study suggests that elevated 2-hour PG levels play an important role in the formation of neuritic plaques, and thereby in the development of AD. Meanwhile, it is well known that increased 2-hour PG levels are closely associated with the development of stroke, which is well established as a main cause of VaD. Thus, it is reasonable to postulate a close association between 2-hour PG levels and the risk of VaD.

Possible pathophysiologic mechanisms through which diabetes or elevated blood glucose levels might

Table 5 Age- and sex-adjusted hazard ratios and their 95% confidence intervals for the development of all-cause dementia and its subtypes determined by autopsy according to 2-hour postload glucose levels

2-Hour postload glucose levels	Person-years at risk, n	No. of events, n	Crude HR (95% CI)	p	Age- and sex-adjusted HR (95% CI)	p
All-cause dementia						
<6.7	5,354	47	1 (referent)		1 (referent)	
6.7–7.7	2,277	23	1.14 (0.69–1.88)	0.61	1.24 (0.75–2.05)	0.39
7.8–11.0	2,844	29	1.19 (0.75–1.89)	0.47	1.20 (0.76–1.91)	0.44
≥11.1	1,192	19	1.94 (1.14–3.31)	0.01	2.24 (1.31–3.83)	0.003
			p for trend: 0.04		*p* for trend: 0.02	
Alzheimer disease						
<6.7	5,354	12	1 (referent)		1 (referent)	
6.7–7.7	2,277	7	1.35 (0.53–3.44)	0.53	1.40 (0.55–3.56)	0.48
7.8–11.0	2,844	12	1.94 (0.87–4.33)	0.10	1.92 (0.86–4.26)	0.11
≥11.1	1,225	8	3.27 (1.34–8.00)	0.009	3.88 (1.58–9.53)	0.003
			p for trend: 0.009		*p* for trend: 0.005	
Vascular dementia						
<6.7	5,354	17	1 (referent)		1 (referent)	
6.7–7.7	2,277	8	1.09 (0.47–2.54)	0.83	1.23 (0.53–2.86)	0.63
7.8–11.0	2,844	8	0.90 (0.39–2.09)	0.81	0.92 (0.40–2.12)	0.84
≥11.1	1,192	7	1.98 (0.82–4.77)	0.13	2.32 (0.96–5.61)	0.06
			p for trend: 0.36		*p* for trend: 0.26	
Other dementia						
<6.7	5,354	18	1 (referent)		1 (referent)	
6.7–7.7	2,277	8	1.04 (0.45–2.39)	0.93	1.17 (0.51–2.70)	0.72
7.8–11.0	2,844	9	0.96 (0.43–2.14)	0.92	0.98 (0.44–2.19)	0.97
≥11.1	1,192	4	1.04 (0.35–3.07)	0.95	1.16 (0.39–3.43)	0.79
			p for trend: 0.99		*p* for trend: 0.88	

Abbreviations: CI = confidence interval; HR = hazard ratio.

affect the initiation and promotion of dementia have been extensively discussed in a number of studies.[23] A recent review summarized 4 major pathways for hyperglycemia-induced dementia: namely, atherosclerosis, microvascular disease, glucose toxicity leading to the accumulation of advanced protein glycation and increased oxidative stress, and changes in insulin metabolism resulting in an insulin-resistant state and distorted amyloid metabolism in the brain.[23] The former 2 pathways are considered to be involved in the development of VaD, while the latter 2 pathways may mainly contribute to the development of AD. Additionally, recent evidence has emerged to imply that vascular factors may be involved in AD.[23] It is reported that 2-hour PG values can be a good marker of oxidative stress levels arising from hyperglycemia[24,25] and correlate with insulin resistance.[26] Higher oxidative stress and insulin resistance may precede the accumulation of amyloid-β peptide and neurofibrillary tangles[23,27] and accelerate arteriosclerosis in the brain,[28] resulting in increased risk of AD and VaD. It is known that Asians have lower levels of insulin secretion compared with other ethnic groups[29] and can develop diabetes, insulin resistance, and metabolic syndrome with lower body mass index levels.[30] These findings suggest that hyperglycemia plays a larger role in the development of dementia compared with insulin resistance in Asians including Japanese. Further studies are needed to elucidate the pathogenesis of hyperglycemia and diabetes in the development of dementia.

The strengths of our study include its longitudinal population-based study design, use of OGTT for determination of glucose tolerance levels in all subjects, long duration of follow-up, perfect follow-up of subjects, and morphologic examination of the brains of most dementia cases with autopsy and neuroimaging. Several limitations of our study should be noted. First, the diagnosis of glucose tolerance status was based on a single measurement of glucose levels at baseline, as was the case in most other epidemiologic studies. During the follow-up, risk factor levels were changed due to modifications in lifestyle or medication especially in subjects with diabetes, and

misclassification of glucose tolerance categories was possible. This could have weakened the association found in this study, biasing the results toward the null hypothesis. Therefore, the true association may be stronger than that shown here. Second, some subjects (n = 33 to 65) did not participate in the follow-up surveys of cognitive function performed in 1992, 1998, and 2005, and their cognitive conditions were evaluated only by mail or telephone. This might have resulted in failure to detect dementia cases. However, we also collected information on the development of dementia in another way, namely through the daily monitoring system established in the town. Thus, we believe that we detected almost all dementia cases, and this bias did not affect our findings. Third, the diagnosis of dementia was verified by autopsy only in 50.9% of dementia cases, resulting in a certain degree of subtype misclassification; agreement rate between clinical diagnosis and neuropathologic diagnosis was not high (64.4%) in our autopsy cases of dementia. However, a sensitivity analysis using only definite cases of dementia determined by brain autopsy did not make any material difference in our findings.

Our findings emphasize the need to consider diabetes as a potential risk factor for all-cause dementia, AD, and probably VaD. The other main finding, that elevated 2-hour PG levels are closely associated with increased risks of all-cause dementia and its subtypes, supports the view that postprandial glucose regulation is critical to prevent future dementia. Further investigations are required to clarify the associations between 2-hour PG levels by the OGTT and subtypes of dementia in other ethnic populations.

AUTHOR CONTRIBUTIONS

Tomoyuki Ohara contributed to the study concept, design, data collection, endpoint adjudication, interpretation of data, statistical analysis, and writing the manuscript. Yasufumi Doi contributed to the study concept, design, interpretation of data, statistical analysis, and writing the manuscript. Toshiharu Ninomiya contributed to the data collection, endpoint adjudication, interpretation of data, and statistical analysis. Yoichiro Hirakawa and Jun Hata contributed to data collection and interpretation of data. Toru Iwaki and Shigenobu Kanba contributed to endpoint adjudication and interpretation of data. Yutaka Kiyohara is a study coordinator and contributed to the study performance, obtaining supporting sources, study concept, design, endpoint adjudication, interpretation of data, and writing of manuscript. All authors critically reviewed the manuscript and approved final version.

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DISCLOSURE

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Association of Alzheimer disease pathology with abnormal lipid metabolism

The Hisayama Study

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ABSTRACT

Objective: The relationship between lipid profiles and Alzheimer disease (AD) pathology at the population level is unclear. We searched for evidence of AD-related pathologic risk of abnormal lipid metabolism.

Methods: This study included brain specimens from a series of 147 autopsies performed between 1998 and 2003 of residents in Hisayama town, Japan (76 men and 71 women), who underwent clinical examinations in 1988. Lipid profiles, such as total cholesterol (TC), triglycerides, and high-density lipoprotein cholesterol (HDLC), were measured in 1988. Low-density lipoprotein cholesterol (LDLC) was calculated using the Friedewald formula. Neuritic plaques (NPs) were assessed according to the Consortium to Establish a Registry for Alzheimer's Disease guidelines (CERAD) and neurofibrillary tangles (NFTs) were assessed according to Braak stage. Associations between each lipid profile and AD pathology were examined by analysis of covariance and logistic regression analyses.

Results: Adjusted means of TC, LDLC, TC/HDLC, LDLC/HDLC, and non-HDLC (defined as TC-HDLC) were significantly higher in subjects with NPs, even in sparse to moderate stages (CERAD = 1 or 2), compared to subjects without NPs in multivariate models including *APOE* ε4 carrier and other confounding factors. The subjects in the highest quartiles of these lipid profiles had significantly higher risks of NPs compared to subjects in the lower respective quartiles, which may suggest a threshold effect. Conversely, there was no relationship between any lipid profile and NFTs.

Conclusion: The results of this study suggest that dyslipidemia increases the risk of plaque-type pathology. ***Neurology***® **2011;77:1068-1075**

GLOSSARY

AD = Alzheimer disease; **CERAD** = Consortium to Establish a Registry for Alzheimer's Disease; **CI** = confidence interval; **HDLC** = high-density lipoprotein cholesterol; **LDLC** = low-density lipoprotein cholesterol; **NFT** = neurofibrillary tangle; **NP** = neuritic plaque; **OR** = odds ratio; **TC** = total cholesterol; **TG** = triglycerides.

To elucidate the association of lifestyle diseases with Alzheimer disease (AD) pathology, a large-scale, population-based clinicopathologic study is required. Since 1961, we have been conducting a long-term prospective cohort study of cerebro-cardiovascular diseases in the town of Hisayama, a suburb of Fukuoka City in Japan. Careful surveillance of cognitive impairment was started from 1985, which was carried out through a daily monitoring system established by the study team, local practitioners, and the town government. In a series of studies, we have reported the incidence and survival of dementia,[1] and trends in the prevalence of AD and vascular dementia.[2] These studies indicate that the prevalence of AD is increasing at an accelerating pace in parallel with an increase of metabolic disorders. Recently, we also reported that insulin

Supplemental data at www.neurology.org

Supplemental Data

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resistance is associated with the plaque-type pathology of AD,[3] even though there are some controversial findings.[4,5]

Along with insulin resistance and diabetes, dyslipidemia is an important metabolic disorder. In humans, however, there are few studies regarding the association between dyslipidemia and AD-related pathology.[6,7] In this study, to clarify the relationship between abnormal lipid metabolism and AD, we searched for evidence of AD-related pathologic risk by examining the associations between lipid profiles and the typical AD-related pathologic outcomes, neuritic plaques (NPs) and neurofibrillary tangles (NFTs).

METHODS

Subjects. The design of the Hisayama Study has been described in detail elsewhere.[3,8–10] In the present study, we examined a series of autopsy samples of Hisayama residents from October 1, 1998, to March 31, 2003. During this period, 290 residents in Hisayama died and 214 were autopsied (autopsy rate 73.8%). The clinical data for the present study were collected from a clinical examination performed in 1988, as described previously.[9] Briefly, of a total of 3,227 residents aged 40–79 years included in the study registry, 2,587 (participation rate, 80.2%) took part in a clinical examination in 1988. Of the 214 autopsy cases, we excluded 3 subjects whose brain specimens were inadequate for evaluation, and 64 subjects who did not complete the fasting blood protocol in 1988. Finally, 147 subjects who underwent both the fasting blood protocol and brain autopsy were included in the present study. None of the 147 subjects showed signs of dementia at the clinical examination in 1988. The study subjects mostly overlapped with those in our previous study, in which we reported the association of insulin resistance with the plaque-type pathology of AD.[3]

Standard protocol approvals, registrations, and patient consents. The study was approved by the Ethics Committee of the Faculty of Medicine, Kyushu University, and was performed in accordance with the ethical standards described in the 5th revision of the Declaration of Helsinki, 2000. Written informed consent was obtained from all study subjects.

Risk factors. In the clinical examination performed in 1988, blood samples were collected on the morning after an overnight fast. We used values of total cholesterol (TC), low-density lipoprotein cholesterol (LDLC), high-density lipoprotein cholesterol (HDLC), triglycerides (TG), TC/HDLC, LDLC/HDLC, and non-HDLC as lipid profiles. Levels of TC, HDLC, and TG were determined enzymatically. LDLC was calculated using the Friedewald formula (LDLC = TC − HDLC − TG/5).[11] Non-HDLC was defined as non-HDLC = TC − HDLC. Other risk factors were also measured as described previously.[3] *APOE* genotyping was determined by direct sequencing. The homozygous ϵ4 genotype was not found among these participants, and those who carried one copy of the ϵ4 allele were categorized as *APOE* ϵ4 carriers.

Assessment of neuropathologic changes. Brain specimens in each case included the middle frontal gyrus, superior and middle temporal gyri, inferior parietal lobule, anterior cingulated gyrus, amygdala, hippocampus with entorhinal and transentorhinal cortex, calcarine cortex, basal ganglia including the nucleus basalis of Meynert, thalamus, substantia nigra, locus ceruleus, and dorsal vagal nucleus. Sections were routinely stained using hematoxylin-eosin, Klüver-Barrera stain, and a modified Bielschowsky method. Specimens from each subject were immunostained with antibodies against phosphorylated tau (AT8, mouse monoclonal, 1:500; Innogenetics, Belgium). The assessment of AD pathology was conducted according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) guidelines[12] and to Braak stage.[13,14] For the pathologic assessment of cerebrovascular diseases, any type of cerebral infarction or hemorrhage was recorded according to gross examination and microscopic assessment, regardless of clinical features.

Statistical analyses. Mean or geometric mean values of continuous data among the NP or NFT groups were adjusted for age and sex and compared by analysis of covariance. Proportions of categorical data were adjusted for age and sex by direct method and compared by logistic regression analysis. We also used logistic regression analysis to determine relationships between risk factors and pathologic outcome, which are expressed as odds ratios (OR) and 95% confidence intervals (CI). Model 1 was adjusted for age and sex. Model 2 was adjusted for model 1 plus systolic blood pressure, fasting blood glucose levels, fasting insulin levels, body mass index, smoking habit, regular exercise, and cerebrovascular disease. Model 3 was adjusted for model 2 plus *APOE* ϵ4 carrier.

Each lipid profile was divided into 4 groups to compare the risk of NPs among quartiles. Missing values (2 for LDL cholesterol, 1 for fasting insulin levels, 7 for *APOE* ϵ4 carrier, and 1 for the grading of Braak stage) were excluded from the analysis. In addition, subjects were divided into high or low groups at the boundary of the most unfavorable quartile to compare the risk of NPs. Significance was defined as $p < 0.05$.

RESULTS

The demographic characteristics of the study subjects at clinical examination are described in table 1. The mean age at death was 76 years in subjects without NPs (CERAD = 0) and 83 years in those with NPs (CERAD = 1 to 3). There was no clear selection bias regardless of autopsy, according to a comparison of demographic characteristics between our study subjects and those who did not undergo autopsy (data not shown). After the clinical examination in 1988, 34.0% (n = 50) of subjects developed dementia; specifically, 17.7% (n = 26) were Alzheimer-type dementia, 13.6% (n = 20) were vascular dementia, and 2.0% (n = 3) were mixed-type dementia.

The frequencies of NPs were categorized into the following 4 groups by CERAD criteria: 32.0% (n = 47) for none (score 0), 15.7% (n = 23) for sparse (score 1), 15.0% (n = 22) for moderate (score 2), and 37.4% (n = 55) for frequent (score 3). The extent of NFTs was classified into the following 4 groups by Braak stage: 13.0% (n = 19) for stage 0, 17.8% (n = 26) for stage I to II, 43.8% (n = 64) for stage III to IV, and 25.3% (n = 37) for stage V to VI. Prevalence of cerebrovascular disease at autopsy