研究成果の刊行一覧表

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資　料

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Genetic variations in the WFS1 gene in Japanese with type 2 diabetes and bipolar disorder

Takanori Kawamoto,[a] Yukio Horikawa,[b,c,*] Takeshi Tanaka,[a] Noriko Kabe,[a] Jun Takeda,[b,c,d] and Masahiko Mikuni[a]

[a] *Department of Neuropsychiatry, Gunma University School of Medicine, Gunma, Japan*
[b] *Laboratory of Molecular Genetics, Department of Cell Biology, Institute for Molecular and Cellular Regulation, Gunma University, Gunma 371-8512, Japan*
[c] *Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation (JST), Kawaguchi, Japan*
[d] *Division of Bioregulatory Medicine, Department of Endocrinology, Diabetes and Rheumatology, Gifu University School of Medicine, Gifu, Japan*

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Abstract

Diabetic and psychiatric symptoms often appear in patients with Wolfram syndrome, and obligate carriers of *WFS1* have increased prevalence of type 2 diabetes and are more likely to require hospitalization for psychiatric illness including bipolar disorder. To identify the polymorphisms in Japanese, we examined a region of ~50 kb covering the entire *WFS1* gene, and evaluated the patterns of linkage disequilibrium. We found a total of 42 variations including 8 novel coding single nucleotide polymorphisms (A6T, A134A, N159N, T170T, E237K, R383C, V412L, and V503G), 14 novel non-coding polymorphisms, and 2 linkage disequilibrium blocks. We also performed association studies in patients with type 2 diabetes mellitus and patients with bipolar disorder. The haplotype comprising R456 and H611 was most associated with type 2 diabetes ($p = 0.013$) and the haplotype comprising g. −15503C/T and g. 16226G/A was most associated with bipolar disorder ($p = 0.006$), but neither reached significant difference after multiple adjustment. These genetic variations and linkage disequilibrium patterns in *WFS1* in Japanese should be useful in further investigation of genetic diversities of *WFS1* and various related disorders.

Keywords: Genetics; Mood disorder; Population study; Linkage disequilibrium; Single nucleotide polymorphism

Introduction

Wolfram syndrome, also known as DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness [OMIM 222300]), was first described by Wolfram and Wagener [1]. While only juvenile onset diabetes mellitus and progressive optic nerve atrophy are required for the diagnosis, many patients also develop diabetes insipidus, sensorineuronal hearing loss, ataxia, peripheral neuropathy, urinary tract atonia, and psychiatric illnesses such as psychosis, severe depression, and dementia [2].

Wolfram syndrome has been shown to have links with D4S432–D4S431 at chromosome 4p16 [3,4]. We earlier reported two siblings with Wolfram syndrome who demonstrated mood symptoms [5], and proceeded with a multi-institutional coordinated effort to discover the genetic etiology of the disease. Recently, mutations in the gene *WFS1/wolframin* were identified in patients with Wolfram syndrome [6,7]. The gene, which encodes a novel protein containing the predicted transmembrane domains, is expressed ubiquitously, with high expression in pancreatic islets and specific neurons (hippocampus CA1, amygdaloid areas, olfactory tubercles, and superficial layers of the allocortex). The subcellular localization of this protein has been determined to be primarily in the endoplasmic reticulum [8], but it's function is not established.

* Corresponding author. Fax: +81-27-220-8889.
E-mail address: yhorikaw@showa.gunma-u.ac.jp (Y. Horikawa).

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The prevalence of this autosomal recessive syndrome was estimated as ~1/770,000 in the United Kingdom [9]. While Wolfram syndrome is rare, obligate carriers have increased prevalence of type 2 diabetes mellitus [9,10], and heterozygous carriers are reported to be 26-fold more likely to require hospitalization for psychiatric illness [11].

Bipolar disorder, also called manic-depression, is characterized by mood swings between states of depression and elation, and genetic predisposition is thought to be an important factor. A linkage study of a large Scottish family provided a maximum LOD score of 4.8 in the region D4S431–D4S403 [12]. Other groups have presented supportive linkage evidence with markers in this region [13,14]. The psychiatric phenotypes observed in carriers and patients with Wolfram syndrome and the location of *WFS1* in the region linked to bipolar disorder have suggested a role of the gene in the development of the disease.

Evidence of abnormal glucose metabolism in psychiatric patients has been accumulating since the early 20th century [15]. Some studies report an increased prevalence of diabetes in hospitalized manic-depressives [16–19]. Gavard et al. carried out an evaluation of 20 studies of the diabetic personality, and found an increased prevalence of depression. A relationship between psychiatric disorder and diabetes mellitus has been suggested by mutations in *WFS1* that affect both diabetic and psychiatric phenotypes. Indeed, we estimated the LOD score for susceptibility to type 2 diabetes in one of the Wolfram pedigrees (WS-1) [6] using additional family information, and found suggestive linkage (M. Mikuni et al. unpublished).

In this study, we examined all of the regions of *WFS1* in Japanese to detect single nucleotide polymorphisms (SNPs)[1] as genetic markers, and evaluated the pattern of linkage disequilibrium (LD) to provide information on population diversity in this gene. We also performed association studies in Japanese patients with type 2 diabetes mellitus and patients with bipolar disorder.

Materials and methods

Subjects

One hundred and ninety two patients with type 2 diabetes mellitus (male/female, 114/78; age, 62.0 ± 11.2 years; age at diagnosis, 49.8 ± 11.0 years; postprandial glucose, 168.5 ± 69.0 mg/dl; hemoglobin (Hb) A_{1C}, 6.7 ± 1.1%; body mass index (BMI), 23.9 ± 3.5 kg/m^2) and 192 controls (male/female, 74/118; age, 67.6 ± 5.8; HbA_{1C}, 4.9 ± 0.3%; BMI, 22.9 ± 2.7 kg/m^2) were examined. Patients were diagnosed with type 2 diabetes by medical records or by 75 g oral glucose tolerance test according to the criteria of the Japan Diabetes Society. Control subjects were recruited on the following criteria: 60 or more years of age, no past history of diagnosis of diabetes, HbA_{1C} less than 5.6%, and no diabetes in family members or second degree relatives. Eighteen patients with bipolar I disorders (male/female, 9/9; age, 46.7 ± 12.4) and 29 patients with bipolar II disorders (male/female, 16/13; age, 53.6 ± 12.3) were recruited from Gunma University Hospital and local hospitals in Gunma prefecture, met DSM-IV diagnostic criteria (Diagnostic and Statistical Manual of Mental Disorders, 4th edition) [20], and were assessed by trained clinicians on the basis of unstructured interviews supplemented by case-note reviews. Ninety-six Japanese random controls (male/female, 39/57; age, 68.8 ± 5.6) were examined for comparisons of genetic variations. The study was approved by the Ethics Committee of Gunma University, and included the written informed consent of each subject.

Detection of polymorphisms in WFS1

Genomic DNA was extracted from samples of whole blood using QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Twelve of the random control samples (24 alleles) were used to detect single nucleotide polymorphisms (SNPs) in *WFS1*. Primers for PCR experiments were designed by Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) on the basis of the genomic contig sequence (GenBank Accession No. NT_006051) of the *WFS1* region. The mixture for the PCR was 20 μl in 10 ng template DNA, 0.5 mM each dNTP, 2.5 pmol each forward and reverse primer, 0.5U Ex*Taq* polymerase (Takara, Kyoto, Japan), and 2 μl of 10× PCR buffer. The reaction conditions were an initial denaturation step of 95 °C for 3 min, subsequent 40 cycle reactions at 94 °C for 30 s, 52–62 °C for 30 s, and 72 °C for 1 min, and a final extension step of 72 °C for 10 min. A 3 μl aliquot from each reaction was assayed on a 1% agarose gel to confirm the product, and the remainder was purified using MultiScreen Filtration System (MILLIPORE, Billerica, MA, USA) with Sephadex G-75 (Amersham Biosciences, Piscataway, NJ, USA). Each PCR product was subjected to cycle sequencing with BigDye terminator Cycle Sequencing FS (Applied Biosystems, Foster, CA, USA) using each forward and reverse primer. Reaction products were purified by ethanol precipitation, and sequenced by ABI PRISM 377 sequencer. Results were processed with Autoassembler, version 2.1 (Applied Biosystems, Foster, CA, USA) to compare sequences.

[1] *Abbreviations used:* bp, base pair; SNPs, single nucleotide polymorphisms; LD, linkage disequilibrium; PCR, polymerase chain reaction; cSNPs, coding single nucleotide polymorphisms.

Mutation screening and genotyping of frequent polymorphisms in WFS1

We examined the coding region of *WFS1* and genotyped sixteen frequent SNPs in the 47 bipolar patients and 96 control subjects. All exons were examined in the 192 type 2 diabetic patients and 192 controls.

Estimation of haplotype frequencies and evaluation of pattern of linkage disequilibrium

Haplotypes were inferred by the expectation-maximization method by Arlequin Software (http://anthro.unige.ch/arlequin). The coefficient for LD, D', and r^2 value was estimated by GOLD software (http://www.well.ox.ac.uk/asthma/GOLD).

Statistical analyses

Statistical difference in allele frequencies between bipolar disorder or diabetes and control groups was assessed by χ^2 test (including Fisher's test when one sample number was less than five for a corresponding 2×2 table). Statistical analysis was performed with StatView 5.0 software (SAS Institute, Cary, NC).

Results

Identification of polymorphisms in WFS1

Twelve of the random controls were examined to detect genetic variations in the entire region of *WFS1*, and a total of 42 polymorphisms were identified in this study as shown in Fig. 1 and Table 1. Comparing our data with the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html), 22 of the SNPs are novel. The distribution of polymorphisms was approximately 1/1000 bp in the 49.2 kb of DNA examined.

Evaluation of the pattern of linkage disequilibrium

As shown in Fig. 2, 16 SNPs were used to define haplotypes and to evaluate the pattern of LD. The other SNPs were excluded because of the rarity of minor alleles. As shown in Fig. 2, there are two LD blocks in this region, one ranging from position g. −15503 to g. 14909 and the other from position g. 16226 to g. 25103. The two SNPs at position g. 16226 and g. 16568, and the four SNPs at position g. 19460, g. 20758, g. 23707, and g. 25103 are in complete linkage disequilibrium.

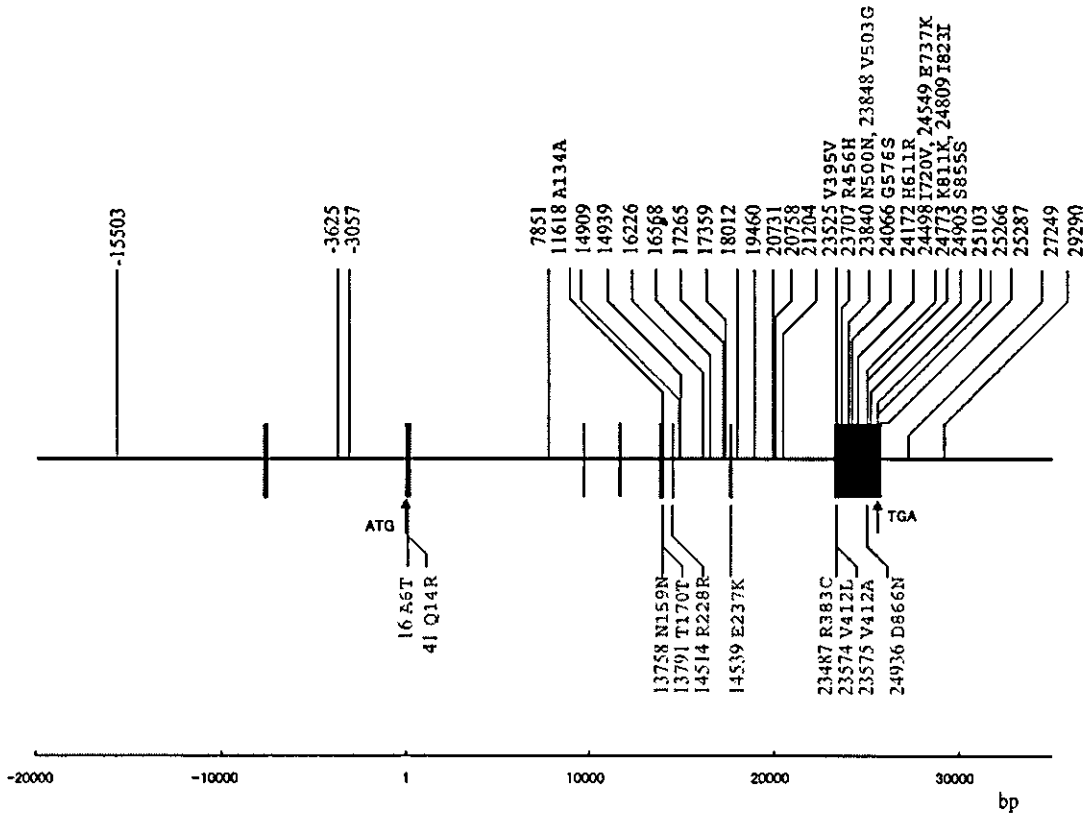

Fig. 1. Polymorphisms of *WFS1* identified in this study. The locations of the polymorphisms described in the text are shown. The nucleotide indicates the location of the SNP relative to the A of the ATG of the initiator Met of *WFS1* (GenBank No. NT_006051). The cSNPs shown in red are observed only in Type 2 diabetic patients. The cSNP shown in blue is observed only in patients with bipolar disorder.

Table 1
Polymorphisms identified in *WFS1* region in this study

Position genome	AA change	Variation	Location	Frequency of minor allele
−15503		C>T*	5′ flanking	0.42
−3625		C>T*	Intron 1	0.21
−3057		G>A	Intron 1	0.46
16	A6T	G>A*	Exon 2	—
41	Q14R	A>G	Exon 2	0.0026
7851		A>G	Intron 2	0.29
11618	A134A	G>A*	Exon 4	0.010
13758	N159N	C>T*	Exon 5	—
13791	T170T	C>G*	Exon 5	0.0079
14514	R228R	G>C	Exon 6	0.010
14539	E237K	G>A*	Exon 7	—
14909		G>A*	Intron 6	0.29
14939		T>C*	Intron 6	0.083
16226		G>A*	Intron 6	0.13
16568		G>A	Intron 6	0.13
17265		G>T*	Intron 6	0.13
17359		C>T*	Intron 6	0.042
18012		G>A*	Intron 7	0.13
19460		G>A*	Intron 7	0.13
20731		C>T	Intron 7	0.29
20758		T>C*	Intron 7	0.13
21204		delCTCA*	Intron 7	0.083
23487	R383C	C>T*	Exon 8	—
23525	V395V	T>C	Exon 8	0.010
23574	V412L	G>C*	Exon 8	0.0026
23575	V412A	T>C	Exon 8	0.0026
23707	R456H	G>A	Exon 8	0.078
23840	N500N	T>C	Exon 8	0.010
23848	V503G	T>G*	Exon 8	—
24066	G576S	G>A	Exon 8	0.12
24172	H611R	A>G	Exon 8	0.094
24498	I720V	A>G	Exon 8	0.063
24549	E737K	G>A	Exon 8	0.047
24773	K811K	A>G	Exon 8	0.010
24809	I823I	C>T	Exon 8	0.005
24905	S855S	G>A	Exon 8	0.010
24936	D866N	G>A	Exon 8	0.0052
25103		G>A*	3′ UTR	0.13
25266		G>A	3′ UTR	0.042
25287		GA	3′ UTR	0.042
27249		delCT*	3′ flanking	0.042
29290		C>T*	3′ flanking	0.13

The nucleotide indicates the location of the SNP relative to the A of the ATG of the initiator Met of *WFS1* (GenBank No. NT_006051). The frequencies of minor alleles of non-coding SNPs shown in this table are observed in random control samples. The frequencies of minor alleles of coding SNPs are observed in 192 non diabetic controls. Asterisk indicates a novel polymorphism.

Association study of genetic variations of WFS1 in patients with type-2 diabetes

All exons were examined in 192 type 2 diabetic patients. We found a total of 21 cSNPs, ten silent mutations and eleven missense mutations, of which seven are novel cSNPs (A6T, A134A, N159N, T170T, E237K, R383C, and V412L). As shown in Table 2, minor alleles H456 and R611were present more frequently in type 2 diabetic patients than in control subjects ($p=0.091$ and $p=0.050$, respectively). Because these two cSNPs are in strong linkage disequilibrium, as shown in Fig. 2, the haplotype defined by these SNPs was investigated for association with type 2 diabetes mellitus. The R456–H611 haplotype was less frequent in type 2 diabetic patients than in control subjects (Table 3, $p=0.013$, $1-\beta \approx 0.4$), but when we compared the two groups with and without this haplotype, there were no significant differences in age, BMI, fasting and postprandial glucose, or HbA_{1C} (data not shown).

Association study of genetic variations of WFS1 in patients with bipolar disorder

Mutation screening of *WFS1* in 47 patients with bipolar disorders revealed twelve coding SNPs. The allelic frequencies in patients and controls are shown in Table 4. One SNP (c. 402G>A, A134A) was located in exon 4

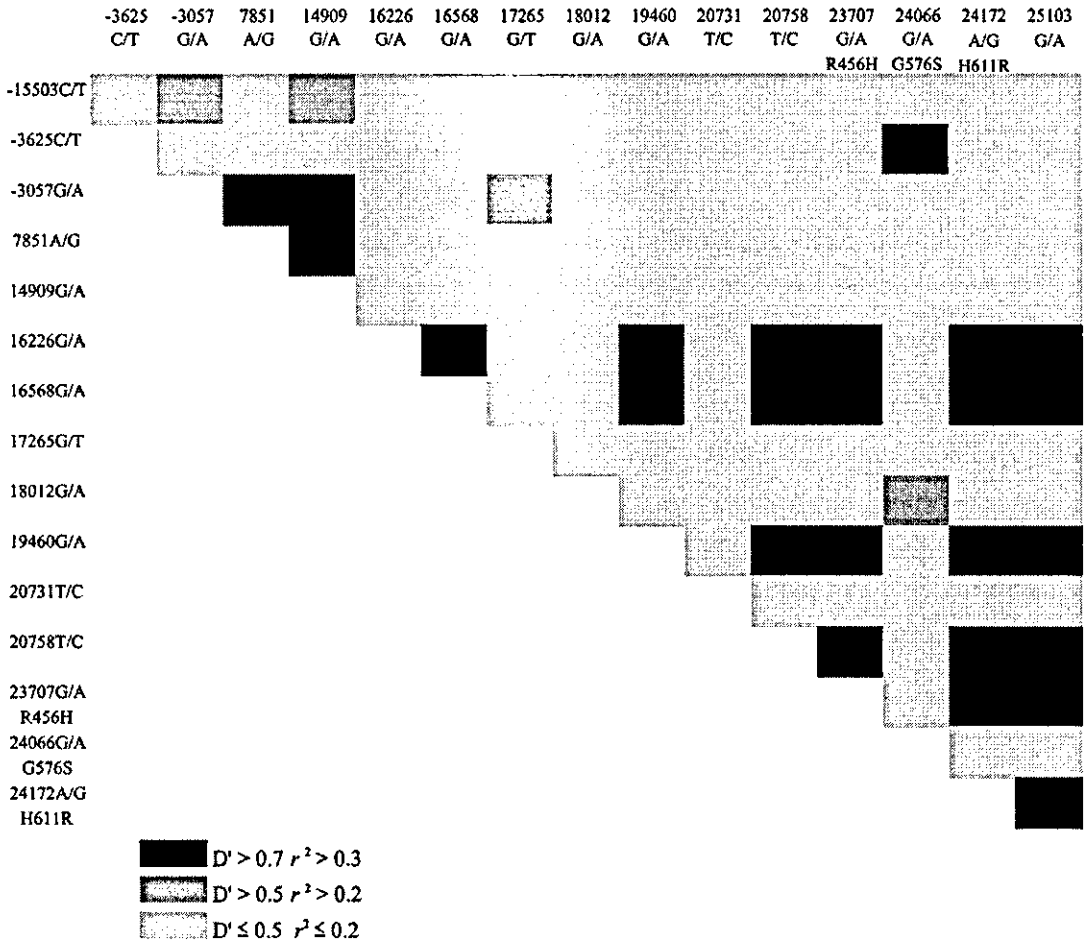

Fig. 2. Pairwise linkage disequilibrium in *WFS1* evaluated by D' and r^2. Extent of pairwise LD of *WFS1*, measured by two distinct coefficients, D' and r^2. Pairwise combinations are classified into three categories based on the degree of the observed LD. Pairwise combination with LD of $D' > 0.7$ and $r^2 > 0.3$, $D' > 0.5$ and $r^2 > 0.2$, and $D' \leqslant 0.5$ and $r^2 \leqslant 0.2$ is shown with black, dark grey, and grey box, respectively. The nucleotide indicates the location of the SNP relative to the A of the ATG of the initiator Met of *WFS1* (GenBank No. NT_006051).

and the others in exon 8. Of the cSNPs identified in this study, two (c. 402G>A, A134A; c. 1508T>G, V503G) were novel and not registered in the NCBI dbSNP database. None of the cSNPs were associated with bipolar disorder, but a novel cSNP (V503G) including four reported cSNPs (V395V, N500N, K811K, and S855S) was observed only in patients with bipolar disorder in a heterozygous state. Pairwise haplotype analysis was performed with combinations of eleven SNPs based on LD pattern (Fig. 3). The haplotype comprising g. −15503C/T and g. 16226G/A is most associated with bipolar disorder ($p = 0.006$), but does not reach significance after multiple adjustment Fig. 3. Association study with an increased number of samples is required.

Discussion

While Wolfram syndrome is rare, obligate carriers show increased prevalence of type 2 diabetes mellitus [9,10], and heterozygous carriers are reported to be 26-fold more likely to require hospitalization for psychiatric illness [11]. A relationship between psychiatric disorder and diabetes mellitus is suggested by mutations in *WFS1* that are observed in both diabetic and psychiatric phenotypes.

We estimated the LOD score for susceptibility to type 2 diabetes in one of the Wolfram pedigrees available and obtained suggestive maximum scores 1.20 and 2.67 at $\theta = 0$ for the dominant and the nonparametric model, respectively (unpublished), leading us to examine all exons of *WFS1* in type 2 diabetes. Ten cSNPs (A6T, Q14R, N159N, T170T, R228R, E237K, R383C, V412L, V412A, and D866N) were found only in patients with type 2 diabetes and not in those with bipolar disorder. Of these, seven cSNPs (A6T, A134A, N159N, T170T, E237K, R383C, and V412L) have not been reported previously [21]. This study shows that the minor alleles H456 and R611 are present more frequently in type 2 diabetic patients than in control subjects, while the

Table 2
Frequencies of coding SNPs in *WFS1* in patients with type 2 diabetes and controls

SNP	Amino acid change	Frequencies of minor allele		*P* value
		Patients ($n = 384$)	Controls ($n = 384$)	
g. 16 G>A	A6T*	0.0027	—	0.49
g. 41 A>G	Q14R*	0.0027	0.0026	>0.99
g. 11618 G>A	A134A*	0.019	0.0086	0.34
g. 13758 C>T	N159N*	0.0027	—	0.49
g. 13791 C>G	T170T*	0.013	0.0079	0.50
g. 14514 G>C	R228R	0.019	0.010	0.38
g. 14539 G>A	E237K*	0.0053	—	0.25
g. 23487 C>T	R383C*	0.0027	—	0.49
g. 23525 T>C	V395V	0.0054	0.0079	>0.99
g. 23574 G>C	V412L*	0.0081	0.0026	0.37
g. 23575 T>C	V412A	0.0054	0.0026	0.62
g. 23707 G>A	R456H	0.12	0.080	0.091
g. 23840 T>C	N500N	0.017	0.0079	0.33
g. 24066 G>A	G576S	0.087	0.11	>0.99
g. 24172 A>G	H611R	0.15	0.10	0.050
g. 24498 A>G	I720V	0.063	0.060	0.87
g. 24549 G>A	E737K	0.049	0.065	0.35
g. 24773 A>G	K811K	0.020	0.0079	0.21
g. 24809 C>T	I823I	0.0085	0.0026	0.73
g. 24905 G>A	S855S	0.017	0.0026	0.53
g. 24936 G>A	D866N	0.011	0.0052	0.44

The nucleotide indicates the location of the SNP relative to the A of the ATG of the initiator Met of *WFS1* (GenBank No. NT_006051). Asterisk indicates a novel polymorphism.

Table 3
Frequencies of haplotypes comprising R456H and H611R in patients with type 2 diabetes and controls

Haplotype	DM	Controls	χ^2	*P* value
R–H	0.83	0.89	6.206	0.013
R–R	0.04	0.03	1.334	0.248
H–H	0.01	0.00	—	0.069
H–R	0.12	0.08	2.207	0.137
	—	—	8.658	0.034

R–H in haplotype column is R456–H611 haplotype.

Table 4
Frequencies of coding-SNPs of *WFS1* in patients with bipolar disorder and in controls

Position genome	Position cDNA	Nucleotide change	Amino acid change	Exon	Frequencies of rare allele		*P* value
					Patients ($n = 94$)	Controls ($n = 192$)	
11618	402	G>A	A134A*	4	0.01	0.01	>0.999
23525	1185	T>C	V395V	8	0.01	0.00	0.33
23707	1367	G>A	R456H	8	0.07	0.08	0.91
23840	1500	T>C	N500N	8	0.01	0.00	0.33
23848	1508	T>G	V503G*	8	0.01	0.00	0.33
24066	1726	G>A	G576S	8	0.13	0.12	0.85
24172	1832	A>G	H611R	8	0.04	0.09	0.16
24498	2158	A>G	I720V	8	0.03	0.06	0.40
24549	2209	G>A	E737K	8	0.03	0.05	0.76
24773	2433	A>G	K811K	8	0.01	0.00	0.33
24809	2469	C>T	I823I	8	0.01	0.01	0.55
24905	2565	G>A	S855S	8	0.01	0.00	0.33

The nucleotide indicates the location of the SNP relative to the A of the ATG of the initiator Met of *WFS1* (GenBank No. NT_006051 for genome, AF 084481 for cDNA); asterisk indicates a novel polymorphism.

R456–H611 haplotype is significantly less frequent and the H456–R611 is more frequent in patients with type 2 diabetes. In the previous study, 370 Japanese patients with type 1 diabetes and 760 control subjects were analyzed, and H456 and R611 were found more frequently in patients than in controls. Preliminary studies in

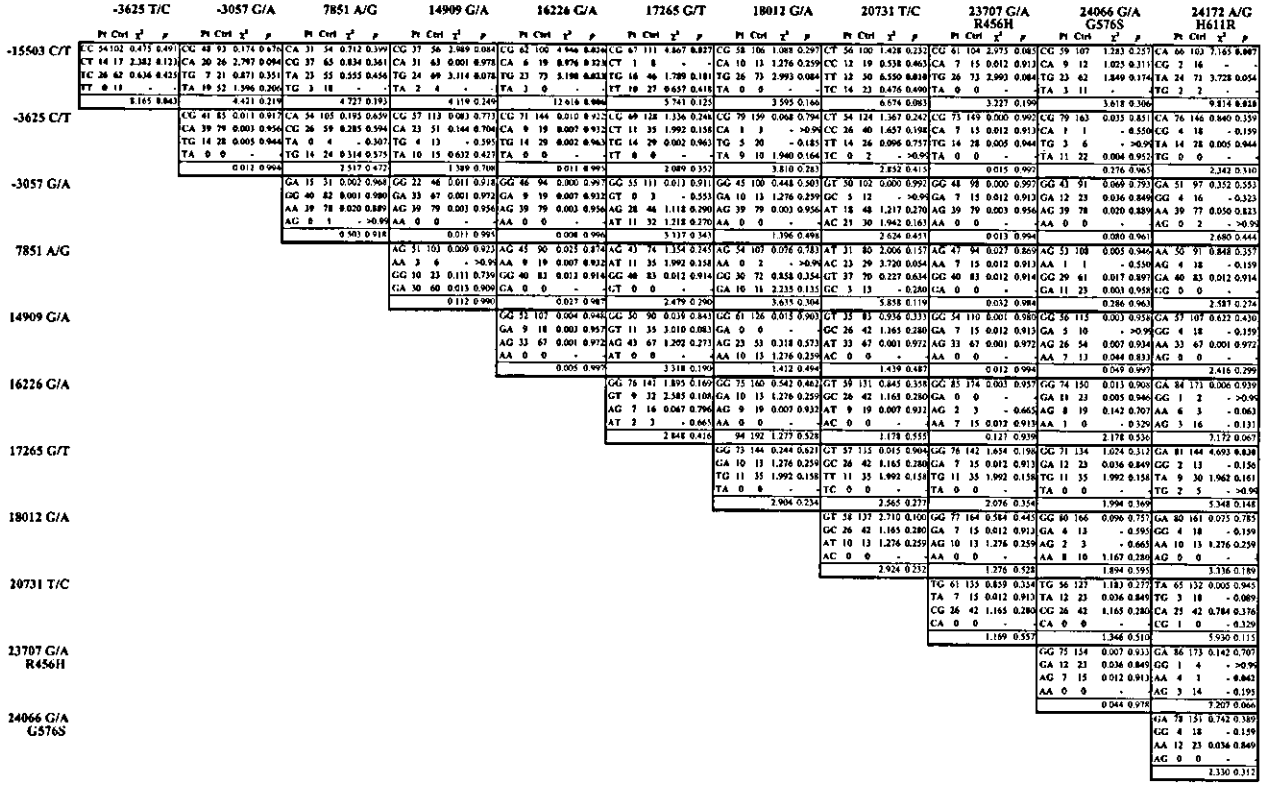

Fig. 3. Association study using pairwise haplotype frequencies in patients with bipolar disorder versus controls. In each cell, P values in the first through fourth row are based on 2×2 χ^2 test, and the P value in the last row is based on 2×4 χ^2 test. P value < 0.05 is indicated in bold.

patients with type 2 diabetes by the same group also showed a significant increase in H456 in type 2 diabetic patients compared to control subjects [22]. These results suggest the protective role of the H456, R611 and their haplotype in the pathogenesis of diabetes. However, the R456–H611 haplotype was found more frequently in type 2 diabetes in the UK [23], suggesting the presence of genetic heterogeneity among populations.

WFS1 is also a candidate for genetic predisposition to bipolar disorder because of the psychiatric phenotypes observed in both carriers and patients with Wolfram syndrome [2]. In addition, *WFS1* is located in a region that has been linked to bipolar disorder [12–14]. Ohtsuki et al. [24] examined exon 8 of *WFS1*, and found no association with bipolar disorder in Japanese. Kato et al. [25] examined four cSNPs (A559T, G576S, A602V, and H611R), and found no association with the disease in Japanese. In this study, we first performed mutation screening of all exons of *WFS1* in 47 Japanese patients with bipolar disorders, and identified 12 coding SNPs (Table 4). Two (c. 402G>A, A134A; c. 1508T>G, V503G) of these were novel, but none were significantly associated with bipolar disorder. To detect all of the polymorphisms in the *WFS1* gene, we examined about 50,000 bp covering the entire region of the gene in 12 Japanese subjects, and identified a total of 42 genetic variations. 18 SNPs and two insertion/deletion polymorphisms were identified in non-coding regions, and 22 SNPs were identified in coding regions. Pairwise haplotype analysis then was performed with combinations of eleven SNPs based on the LD pattern estimated using the 16 frequent SNPs (Fig. 2).

As shown in Fig. 3, we defined haplotypes by all possible pairs of 11 SNPs, and examined the associations with bipolar disorder. The haplotype comprising g. −15503C/T and g. 16226G/A was most associated with bipolar disease ($p = 0.006$), but does not reach significant difference after multiple adjustments. One possible reason for not finding a significant association with bipolar disorder is that bipolar sample in the present study consists mostly of bipolar II patients ($n = 29$) rather than bipolar I patients ($n = 18$). Most studies of genetic linkage in bipolar disorder have samples that are predominantly bipolar I as this syndrome is more well defined. Further study with an increased number of samples is required to determine the contribution of this haplotype to the disease.

In the present study, we have identified a total of 42 polymorphisms as well as the precise LD pattern in *WFS1*. While some of the haplotypes of *WFS1* may be associated with type 2 diabetes or bipolar disorder, the data are inconclusive because of the small number in the study sample. Although the functional properties of the genetic variations in *WFS1* that might affect susceptibility to type 2 diabetes or bipolar disorder are not

known, the genetic variations and linkage disequilibrium patterns reported in this study should be useful in the investigation of the genetic associations between *WFS1* and various diseases, especially in Japanese.

Acknowledgments

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Sex and age dependencies of cerebral blood volume changes during cognitive activation: a multichannel near-infrared spectroscopy study

Masaki Kameyama, Masato Fukuda,* Toru Uehara, and Masahiko Mikuni

Department of Psychiatry and Human Behavior, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan

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In this study, we measured the change in cerebral hemoglobin concentrations during a cognitive task using multichannel near-infrared spectroscopy (NIRS), and investigated the relationship between regional cerebral blood volume and sex, age, and task performance. Thirty-nine healthy volunteers (24 males and 15 females; mean age, 33.0 years) participated after giving their informed consent and performed a word fluency task. The relative oxy-hemoglobin concentration ([oxy-Hb]) was measured using frontal and temporal probes with two sets of 24-channel NIRS machines. The effects of sex, age, and task performance on [oxy-Hb] changes were analyzed using analysis of covariance: with sex, age, and task performance as independent variables, and [oxy-Hb] changes as dependent variables, and years of education as covariates. The effects on [oxy-Hb] increase were significant in many channels in the frontal and temporal probes for sex, that is the most prominent effect, and in a few frontal channels for age: [oxy-Hb] increases were larger in males than in females, and in the young than in the middle-aged. The effects on [oxy-Hb] increase were not significant for task performance, but [oxy-Hb] increases in subjects with low performance tended to be larger than those in subjects with high performance. The results demonstrated that multichannel NIRS could detect cerebral activation during cognitive tasks and clarify sex- and age-dependent differences in such cerebral activation. Sex- and age-dependent differences in cerebral activation, as demonstrated in the present study, should be considered when interpreting cerebral blood volume, cerebral blood flow, and cerebral glucose metabolism data.

Keywords: Near-infrared spectroscopy; Cerebral blood volume; Sex differences; Aging; Performance; Word fluency test

Introduction

Recent advances in neuroimaging methodologies have enabled their application to the assessment of cognitive brain function in healthy subjects and diagnostic examination of neurological and psychiatric disorders. In interpreting obtained results, effects of demographic parameters such as sex and age should be incorporated in data analyses.

A number of neuroimaging studies investigated the effects of sex and age on cognitive brain function. The greater lateralization of brain activation in males than in females was reported in language tasks using functional magnetic resonance imaging (fMRI) (Shaywitz et al., 1995; Vikingstad et al., 2000) and positron emission tomography (PET) (Jaeger et al., 1998). An age-dependent decline of brain activation was also well reported in cognitive tasks such as a calculation task (NIRS) (Hock et al., 1995), WAIS-III (NIRS and fMRI) (Kwee and Nakada, 2003), and a color-word-stroop task (NIRS) (Schroeter et al., 2003).

The effects of sex and age on cognitive function could interact with the performance of the task employed. Sex and age dependencies of cognitive functions have been well established: females perform better in verbal tasks, whereas males excel in spatial tasks (Collins and Kimura, 1997; Halpern, 2000; Silverman et al., 1996); the elderly perform more poorly than younger subjects in various cognitive tasks (Grady and Craik, 2000). However, as far as the authors surveyed, sex and age effects on brain function in relation to their interaction with task performance have not been fully clarified. This can cause problems in the interpretation of sex and age effect results: sex- or age-related differences in brain function could be the direct effects of sex or age, or the indirect effects of different task performances due to sex and age.

Near-infrared spectroscopy (NIRS) is a recently developed neuroimaging methodology. Its noninvasiveness (Ito et al., 2000), portability, natural setting of examination, and low running cost have enabled the examination of male and female subjects over a wide age range, from infancy to old age while performing cognitive tasks. Therefore, the effects of sex, age, and task performance should be studied in more detail using NIRS data interpretation.

NIRS can detect changes in the concentration of cerebral blood hemoglobins, such as oxy-hemoglobin ([oxy-Hb]), deoxy-hemoglobin ([deoxy-Hb]) and total-hemoglobin ([total-Hb]), which is the sum of [oxy-Hb] and [deoxy-Hb]. The principle of NIRS is based on the modified Lambert-Beer Law stating that the absorption of near-infrared light by oxy- and deoxy-hemoglobin varies with wavelength. Both [oxy-Hb] increases and [deoxy-Hb] decreases in NIRS are interpreted to reflect cortical activation

* Corresponding author. Department of Psychiatry and Human Behavior, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan. Fax: +81-27-220-8192.
E-mail address: fkdpsy@med.gunma-u.ac.jp (M. Fukuda).
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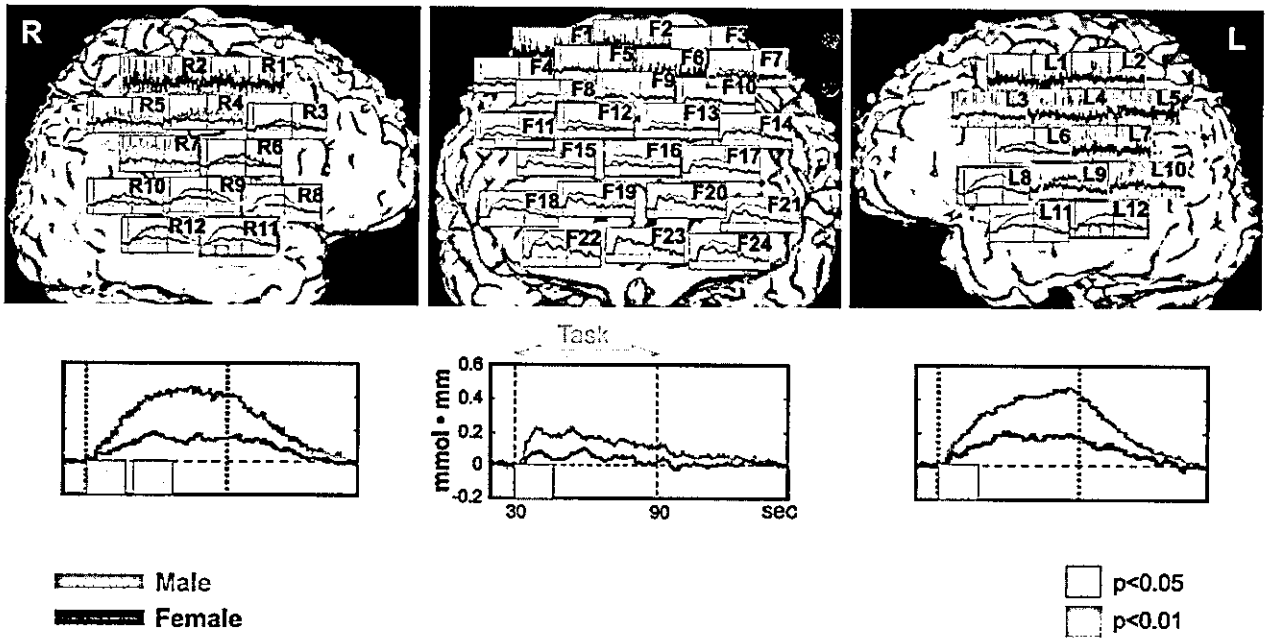

Fig. 1. Male group vs. female group. The upper figures show the grand average waveforms of oxy-hemoglobin changes obtained by NIRS in male (red line) and female subjects (blue line), superimposed on a reconstructed cerebral cortex image. The measurement channels with significant differences are displayed with thick red border lines and the time segments with significant differences in the channels are marked with red squares (filled squares, $P < 0.01$; unfilled squares, $P < 0.05$). Three representative channels (circled in yellow) are enlarged below.

because they have been shown to correspond to regional cerebral blood flow (CBF) in fMRI studies (Kleinschmidt et al., 1996; Toronov et al., 2001). Correlations of hemoglobin data in NIRS with CBF data simultaneously obtained using other methodologies have been shown to be stronger for [oxy-Hb] than for [deoxy-Hb] in a fMRI study (Strangman et al., 2002b) and a laser-Doppler study (Malonek et al., 1997). The differences are interpreted to be due to poorer contrast-to-noise ratios in the measurements of [deoxy-Hb] than in those of [oxy-Hb] (Strangman et al., 2002b). The technological principle of NIRS was reviewed in detail by Koizumi et al. (1999), Strangman et al. (2002a), and Obrig and Villringer (2003).

For human cognitive function, a number of NIRS studies have been published since 1993 (Chance et al., 1993; Hoshi and Tamura, 1993; Kato et al., 1993; Villringer et al., 1993) using various tasks: a confrontational writing task (Yamamoto et al., 1999), a speech listening task (Sato et al., 1999; Sakai et al., 2001), a calculation task (Hock et al., 1995), WAIS-III (Kwee and Nakada, 2003), and a word fluency (generation) task (Fallgatter et al., 1997; Herrmann et al., 2003; Hock et al., 1997; Matsuo et al., 2000, 2002; Suto et al., 2004; Watanabe et al., 1998). Some of these studies, using only one or two channels over a limited frontal area, investigated the effect of age, but not that of sex, on brain activation (Hock et al., 1995; Kwee and Nakada, 2003).

In the present study, we measured the changes in cerebral hemoglobin concentration using multichannel NIRS machines during a cognitive task, and investigated the relationship of regional cerebral blood volume (CBV) with sex, age, and task performance. The purpose of this study was to elucidate the effects of sex, age, and task performance on cerebral activation over larger brain areas along the task time course.

Materials and methods

Subjects

The subjects were 39 healthy volunteers (24 males and 15 females; mean age, 33.0 years; male mean age, 33.5 years ranging from 25 to 47; female mean age, 32.1 years ranging from 23 to 52; mean years of education, 16.8 years ranging from 12 to 22). Written informed consent was obtained from the subjects before the start of the investigation. All the subjects were right-handed as assessed by the Edinburgh Handedness Inventory (Oldfield, 1970). The handedness scores were mean 0.99, SD 0.05 for men and mean 0.96, SD 0.08 for women. None of the subjects had pre-existing neurological or psychiatric disorders. The present study was approved by the Institutional Review Board of Gunma University Graduate School of Medicine.

Task procedure

The subjects performed a word fluency task, which consisted of a 30-s pretask baseline period, a 60-s task period, and a 60-s post-task baseline period. The subjects were instructed in detail, in the instruction period before the NIRS measurements, to generate as many words as possible in the task period until they fully understood the task requirements. The initial syllables assigned were changed every 20 s (/a/, /ka/ and /sa/, respectively) during the 60-s task period. The words generated were monitored for correct and incorrect responses, and the number of correct words generated was determined as the subjects' task performance.

In the pretask and the post-task baseline periods, the subjects were instructed to repeat a train of syllables "/a/, /i/, /u/, /e/, /o/". The subjects were seated in a comfortable chair with their eyes

open throughout the measurements. The timing of syllable changes was delivered to the subjects by the examiners' cue. The subjects were asked to avoid body movements such as neck movements, strong biting, and blinking during the NIRS measurements because they had been identified as most influential in the preliminary artifact-evoking study. The movements of the subjects were monitored throughout the examination.

NIRS measurements

NIRS machine

Relative [oxy-Hb], [deoxy-Hb], and [total-Hb] were measured with two sets of 24-channel NIRS machines (Hitachi ETG-100). The machine uses two wavelengths of the near-infrared light (780 and 830 nm), whose difference in the absorption spectrum enables the measurement of [oxy-Hb] and [deoxy-Hb] (Maki et al., 1995). The distance between pairs of emission and detector probes was set at 3.0 cm, which enabled cerebral blood volume measurements at a 2- to 3-cm depth from the skin of the head, that is, the surface of cerebral cortices (Hock et al., 1997; Toronov et al., 2001).

Probe positions and measurement points

The probes of the NIRS machines were fixed with thermoplastic shells and placed on the subject's frontal and bilateral temporal areas. The 16 probes on the subject's frontal area can measure the relative concentrations of hemoglobins at 24 measurement points in a 9 × 9 cm^2 area. The lowest probes in the frontal shell were positioned along the Fp_1–Fp_2 line according to the international 10/20 system used in electroencephalography. Each of the nine probes on the subject's bilateral temporal areas can measure the relative concentrations of hemoglobins at 12 measurement points in a 6 × 6 cm^2 area. The central probe in each temporal shell was positioned at the midpoint between the vertex and external ear hole. The measurement points were superimposed on a magnetic resonance image of a three-dimensionally reconstructed cerebral cortex of a subject. Measurement points were labeled as F1–F24 for the frontal channels, L1–L12 for the left temporal channels, and R1–R12 for the right temporal channels, from top to bottom.

Measurement parameters

The rate of data sampling was 0.1 s. Obtained data were analyzed using the "integral mode": the pretask baseline was determined as the mean over a 10-s period just before the task period, the post-task baseline was determined as the mean 5 s, 50 s after the task period, and linear fitting between the pre- and the post-task baselines was applied to data between the two baselines. The moving average method was adapted for analyzed data to remove short-term motion artifacts (moving average window: 1 s).

Data analyses

Data contaminated with artifacts due to body movements were excluded from further analyses. For data analyses, the task and the post-task periods were divided into six time segments along the time course: the task period into three segments ("task 1", "task 2", and "task 3" segments for the first, the second, and the third 20-s periods, respectively) and the post-task period into three segments ("post-task 1", "post-task 2", and "post-task 3" segments for the first 20-s, the second 20-s, and the third 15-s period, respectively). Obtained [oxy-Hb] data were averaged for each subject over these six time segments. Data from the channels positioned over a hair-covered area often showed a low signal-to-noise ratio because of the paucity of near-infrared light detected, hence were excluded from the analyses when the standard deviations of [oxy-Hb] during the pretask period exceeded 0.035: four channels in the frontal shell (F1, F2, F3, and F6) and seven left channels (L1, L2, L3, L4, L5, L7, and L10) and five right channels (R1, R2, R4, R5, and R7) in the temporal shells.

For the remaining 32 channels with a sufficient signal-to-noise ratio, the effects of sex, age and task performance on [oxy-Hb] changes were analyzed over the six time segments using the three-way analysis of covariance (ANCOVA): with sex (24 males and 15 females), age (19 young and 20 middle-aged subjects, divided by the median age of the subjects, 29.0 years; the young group: mean age 26.3, SD 1.6 years; the middle-aged group: mean age 39.4, SD 6.7 years), and task performance (19 subjects with low performance and 20 subjects with high performance, divided by the median performance of 17 words) as independent variables, and [oxy-Hb] changes during the six time segments as dependent variables, and years of education as covariates. Statistical analysis was performed using SPSS 11.0J software (Tokyo, Japan).

In addition, the effects of the language nature of the task, which was employed in the present study, were examined for the left–right hemisphere asymmetry of [oxy-Hb] changes by comparing the channel pairs of mirror-imaged positions using a *t* test.

Results

The three-way ANCOVA demonstrated significant main effects of two independent variables, sex, and age (Table 1). Significant main effects of sex were obtained in four frontal channels (F7, F11, F12, F16) out of 20 channels analyzed, in three left temporal channels (L8, L9, L12) out of five channels analyzed, and two right temporal channels (R8 and R11) out of seven channels analyzed (F = 4.4–8.9, df = 1, P = 0.046–0.006) (Fig. 1). In all the channels, [oxy-Hb] increases were larger in male than in female subjects. Time segments with significant differences were obtained only for task 1 in the frontal channels and from task 1 to task 3 in the temporal channels. Significant main effects of age were obtained only in four frontal channels (F11, F14, F15, F19) out of 20 channels analyzed (F = 4.7–6.2, df = 1, P = 0.037–0.018) (Fig. 2). In these four channels, [oxy-Hb] increases were larger in the young than in the middle-aged subjects. The time segment with a significant difference in [oxy-Hb] changes was confined only to the task 1 segment. No significant main effect of task performance was obtained, but the mean [oxy-Hb] increases tended to be larger in the subjects with low performance than in the subjects with high performance in the two frontal (F19: post-task 1, F21: post-task 2), one left temporal (L9: post-task 1), and two right temporal (R3: task 1, R11: task 3) channels (F = 2.9–3.8, df = 1, P = 0.098–0.062) (Fig. 3). Significant interactions among independent variables were obtained in two frontal and four temporal channels: the interaction of sex with age in the task 3 and post-task 1 segments in one frontal channel (F20; F = 5.1 and 4.9, df = 1, P = 0.032 and 0.035, respectively). The interaction of age with task performance in the task 1 and task 3 segments in one frontal channel (F5) and in the task 1 segment in one right temporal channel (R8; F = 4.4, df = 1, P = 0.045). The interaction of sex with task performance in one frontal (F5), one left (L9) and two right (R10 and R12) temporal channels (F = 4.2–6.5, df = 1, P = 0.048–0.016) from task 1 to task 3 segments and the interaction of sex with age and task

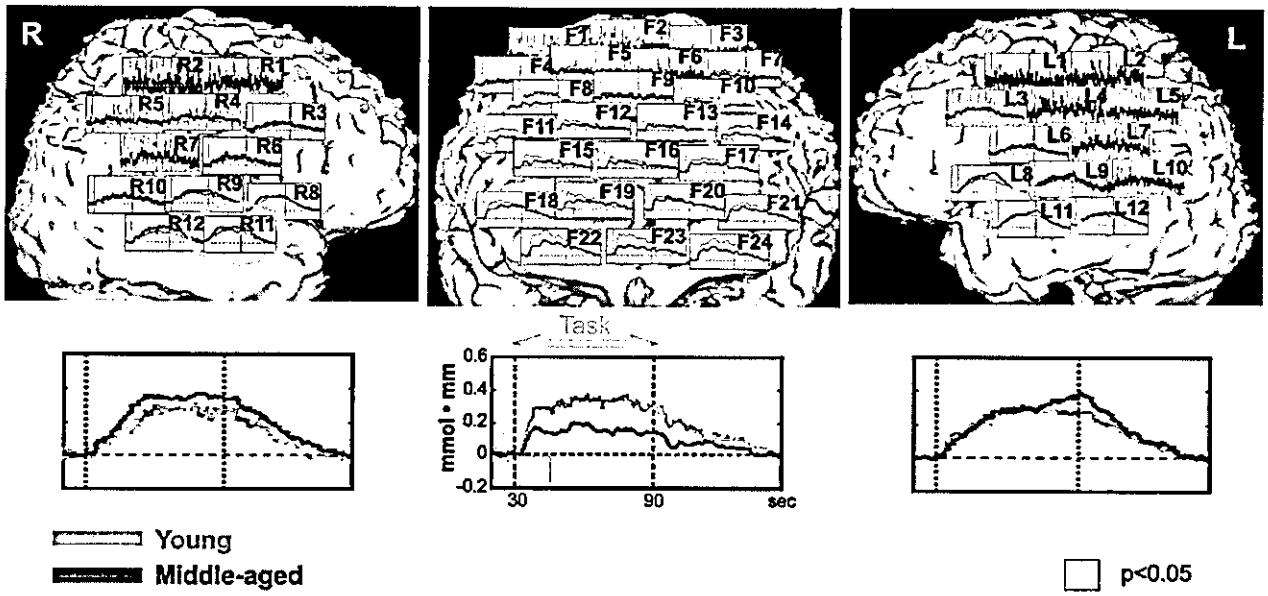

Fig. 2. Young group vs. middle-aged group. The upper figures show the grand average waveforms of oxy-hemoglobin changes obtained by NIRS in young (red line) and middle-aged subjects (blue line), superimposed on a reconstructed cerebral cortex image. The measurement channels with significant differences are displayed with thick red border lines and the time segments with significant differences in the channels are marked with red squares ($P < 0.05$). Three representative channels (circled in yellow) are enlarged below.

performance in the post-task 1 and post-task 2 segments in one right temporal channel (R8; $F = 5.0$ and 5.7, $df = 1$, $P = 0.034$ and 0.023, respectively). Covariate effects of the years of education were significant in eight frontal channels (F4, F5, F15, F18, F19, F20, F21, F23) and three left (L8, L11, L12) and six right (R6, R8, R9, R10, R11, R12) temporal channels mainly in post-task period (from task 3 to post-task 3) for positive correlations with [oxy-Hb] increases ($F = 4.2–26.0$, $df = 1$, $P = 0.049–0.000$).

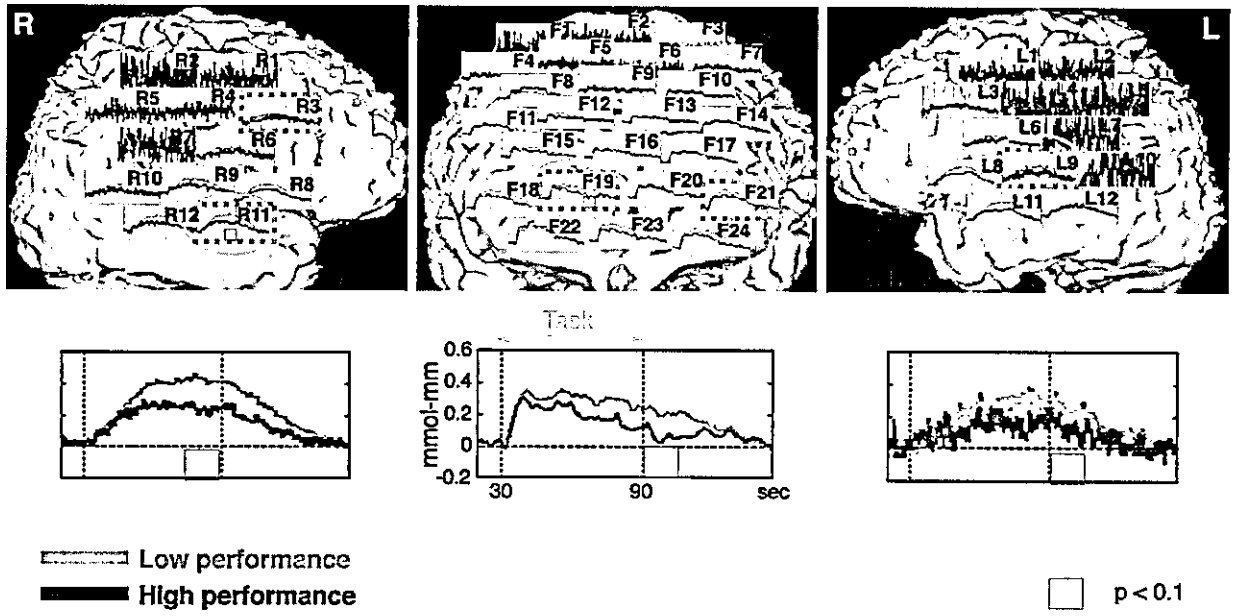

Fig. 3. Group with low performance vs. group with high performance. The upper figures show the grand average waveforms of oxy-hemoglobin changes obtained by NIRS in subjects with low performance (red line) and high performance (blue line), superimposed on a reconstructed cerebral cortex image. The measurement channels in which the mean [oxy-Hb] increases tended to be larger in the subjects with low performance than in the subjects with high performance are displayed with dotted red border lines and the time segments with differences in the channels are marked with red squares ($P = 0.1$). Three representative channels (circled in yellow) are enlarged below.

Table 1
Summary of the ANCOVA results for [oxy-Hb] changes

Channel	Time segment	Main effects			Interaction effects				Covariate
		sex (S)	age (A)	performance (P)	S × A	S × P	A × P	S × A × P	Education years
F5	task 1	1.9 (0.180)	2.5 (0.127)	0.2 (0.693)	0.2 (0.701)	4.4 (0.044)*	5.8 (0.023)*	1.5 (0.225)	1.7 (0.200)
	task 3	0.1 (0.784)	0.3 (0.603)	0.0 (0.996)	1.1 (0.292)	6.4 (0.017)*	5.1 (0.032)*	0.0 (0.937)	0.4 (0.526)
F7	task 1	8.9 (0.006)**	3.9 (0.059)	0.1 (0.733)	1.6 (0.215)	1.3 (0.263)	0.2 (0.655)	0.5 (0.480)	0.3 (0.592)
F11	task 1	5.0 (0.033)*	5.9 (0.022)*	0.1 (0.798)	1.5 (0.235)	0.6 (0.464)	0.4 (0.542)	0.0 (0.835)	0.1 (0.726)
F12	task 1	5.9 (0.021)*	3.3 (0.081)	1.5 (0.232)	1.9 (0.174)	1.2 (0.284)	0.1 (0.754)	0.5 (0.476)	0.0 (0.918)
F14	task 1	3.2 (0.083)	6.2 (0.018)*	0.2 (0.681)	0.2 (0.650)	0.1 (0.736)	0.1 (0.818)	0.0 (0.875)	0.6 (0.433)
F15	task 1	3.9 (0.058)	5.1 (0.031)*	0.1 (0.769)	0.4 (0.538)	0.9 (0.349)	0.0 (0.828)	0.0 (0.840)	0.0 (0.981)
F16	task 1	5.0 (0.033)*	3.0 (0.546)	0.1 (0.778)	2.3 (0.140)	0.1 (0.742)	0.0 (0.839)	0.1 (0.780)	0.0 (0.936)
F19	task 1	2.4 (0.133)	4.7 (0.037)*	0.2 (0.657)	0.2 (0.623)	0.0 (0.964)	0.3 (0.611)	0.1 (0.824)	0.1 (0.755)
F20	task 3	0.1 (0.724)	0.0 (0.898)	0.5 (0.498)	5.1 (0.032)*	1.3 (0.272)	0.9 (0.357)	0.2 (0.627)	1.5 (0.237)
	post-task 1	0.1 (0.802)	0.3 (0.586)	2.6 (0.116)	4.9 (0.035)*	0.1 (0.715)	0.4 (0.558)	0.1 (0.717)	1.7 (0.201)
L8	task 1	6.1 (0.020)*	0.0 (0.850)	0.6 (0.432)	0.0 (0.850)	0.9 (0.342)	0.0 (0.947)	0.0 (0.883)	0.1 (0.816)
L9	task 3	4.5 (0.043)*	0.1 (0.818)	2.6 (0.117)	0.7 (0.424)	4.3 (0.048)*	0.1 (0.741)	1.2 (0.288)	0.4 (0.550)
L12	task 2	4.4 (0.046)*	0.1 (0.706)	0.2 (0.655)	0.1 (0.742)	0.1 (0.750)	1.1 (0.292)	2.8 (0.104)	0.1 (0.966)
R8	task 1	4.4 (0.045)*	0.2 (0.630)	0.9 (0.348)	0.1 (0.777)	0.2 (0.660)	0.1 (0.809)	0.3 (0.566)	0.1 (0.817)
	task 2	4.8 (0.037)*	0.0 (0.986)	0.9 (0.345)	0.5 (0.466)	1.6 (0.212)	0.9 (0.339)	1.0 (0.337)	1.0 (0.328)
	post-task 1	1.4 (0.247)	0.2 (0.691)	0.9 (0.344)	0.5 (0.499)	2.5 (0.125)	4.4 (0.045)*	5.0 (0.034)*	13.3 (0.001)**
	post-task 2	0.0 (0.906)	1.3 (0.270)	2.0 (0.165)	0.1 (0.768)	0.6 (0.464)	3.3 (0.078)	5.7 (0.023)*	26.0 (0.000)**
R10	task 2	2.1 (0.156)	0.1 (0.760)	0.2 (0.649)	0.4 (0.527)	5.1 (0.031)*	0.0 (0.985)	1.3 (0.265)	0.1 (0.759)
	task 3	0.9 (0.343)	0.1 (0.815)	0.1 (0.709)	0.5 (0.480)	6.5 (0.016)*	1.1 (0.299)	0.8 (0.383)	2.9 (0.099)
R11	task 1	5.1 (0.031)*	0.0 (0.907)	0.6 (0.427)	0.1 (0.763)	0.3 (0.585)	1.3 (0.262)	0.0 (0.897)	0.2 (0.684)
	task 2	4.4 (0.045)*	0.2 (0.635)	1.5 (0.224)	0.1 (0.714)	2.7 (0.109)	2.9 (0.098)	0.3 (0.574)	3.6 (0.066)
R12	task 2	1.5 (0.228)	0.1 (0.753)	0.3 (0.575)	0.5 (0.490)	4.2 (0.048)*	0.5 (0.488)	0.6 (0.434)	2.9 (0.098)
	task 3	1.1 (0.302)	0.2 (0.645)	0.5 (0.480)	0.4 (0.510)	5.6 (0.024)*	1.3 (0.260)	0.6 (0.462)	7.2 (0.012)*

F and *P* values are shown only for the channels and time segments with statistical significance in main and interaction effects as '*F* (*P*)'.
*$P < 0.05$.
**$P < 0.01$.

All the *t* tests examining the left–right hemisphere asymmetry of [oxy-Hb] changes showed no significant differences.

Discussion

The present study examined the effects of sex, age, and task performance on brain activation during a cognitive task using multichannel NIRS. The statistically significant main effects on [oxy-Hb] increase were obtained for sex in many channels in the frontal and temporal probes and for age in a few frontal channels: [oxy-Hb] increases were larger in males than in females, and in the young than in the middle-aged. On the other hand, the main effect of task performance on [oxy-Hb] increases was not significant, although [oxy-Hb] increases in subjects with low performance tended to be larger than those in subjects with high performance.

The obtained differences in cerebral activation for sex during the cognitive task are consistent with a previous PET study by Buckner et al. (1995) that demonstrated a larger prefrontal activation in male than in female subjects during a verb generation task. They interpreted the result as being derived from the difference in difficulty performing the same task between male and female subjects based on the better performance of the female subjects: in general, female subjects perform language tasks more efficiently than male subjects (Halpern, 2000). However, this explanation is not relevant for the present study because the task performance was equal between the male and the female subjects.

The age-dependent decrease in cerebral activation during the cognitive task is consistent with previous NIRS studies using a calculation task (Hock et al., 1995), WAIS III (Kwee and Nakada, 2003), and a color-word-stroop task (Schroeter et al., 2003), and with a PET study using a serial verbal learning test (Hazlett et al., 1998). The authors of these previous reports assumed the decrease to be the consequence of structural cortical changes (decrease of region-specific cortical thickness and shrinkage of neuronal dendrites), functional cortical changes (decline of frontal activation), or the decline of working memory utilization due to aging. The rather low age range of the subjects in the present study (23–52 years old) and the absence of a significant difference in the task performance between the young and the middle-aged subjects could exclude the first and the third interpretations mentioned above, respectively.

Larger [oxy-Hb] increases in the subjects with high performance, although the differences did not reach statistical significance, are consistent with the findings of task performance dependencies of cerebral metabolism activation in a PET study using a verbal fluency test (Parks et al., 1988), and CBV changes in an NIRS study using the calculation task (Hoshi and Tamura, 1993). The finding could be interpreted as indicating greater effort and hence less efficient processing in poor performers.

As for the laterality of brain activation in the language task, previous brain imaging studies reported a left lateralized pattern with some inconsistencies: both in male and female subjects (Buckner et al., 1995; Frost et al., 1999; Schlosser et al., 1998) or only in male subjects (Shaywitz et al., 1995; Vikingstad et al., 2000). The absence of such a left lateralized pattern in the present study could be explained by the task characteristics employed. The change in [oxy-Hb] was determined as the difference between the syllable-repeating condition in the pretask baseline period and the word-fluency condition in the task period. Such a