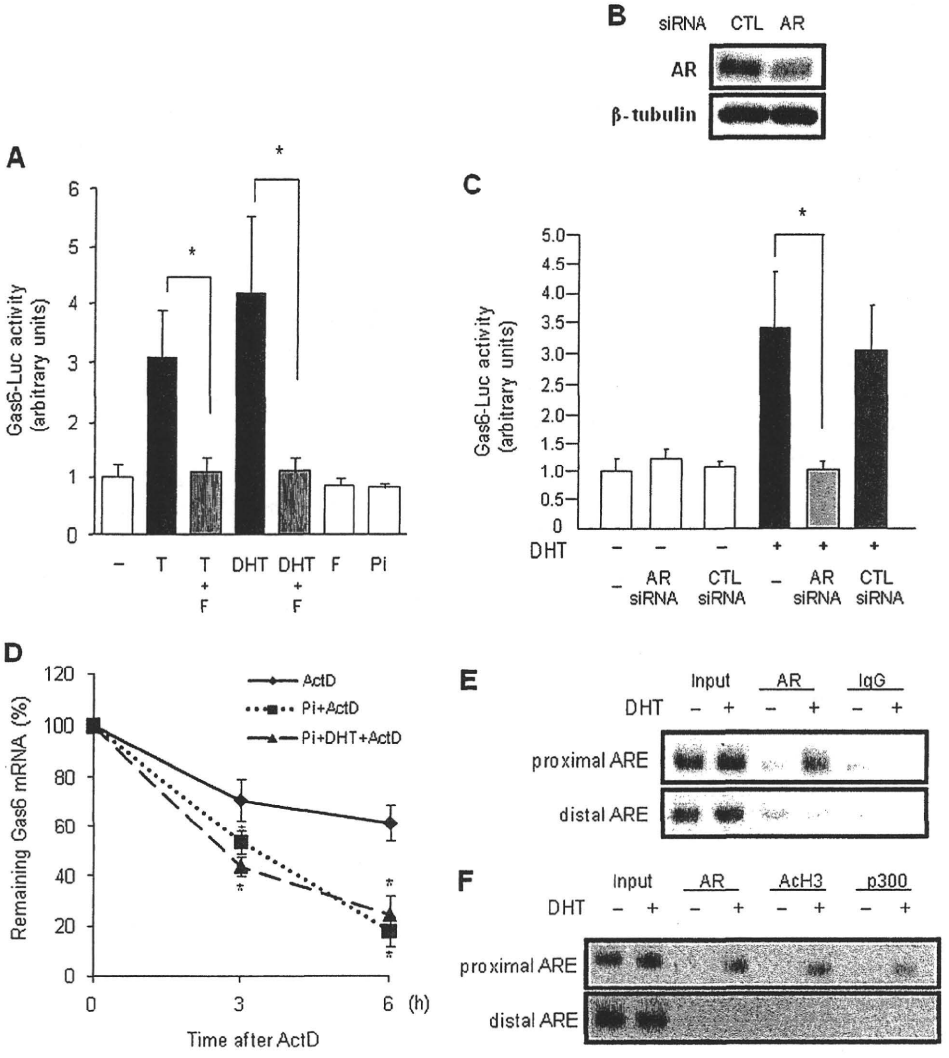

FIGURE 5. **Interaction of the AR with the proximal ARE is essential for transactivation of Gas6 gene by androgen.** *A*, HASMC were transfected with the Gas6-luc construct containing the proximal ARE. Twenty-four hours after transfection, testosterone (*T*, 100 nM), DHT (100 nM), P_i (*Pi*, 2.6 mM), or flutamide (*F*, 10 μM) was added, and the cells were incubated for an additional 24 h. *, $p < 0.05$ by Fisher's test. *B*, HASMC were transfected with AR or control (*CTL*) siRNA (100 nM). The AR protein was efficiently decreased by AR siRNA at 48 h after transfection. *C*, HASMC were transfected with 0.8 μg of Gas6 proximal ARE together with AR siRNA or nonspecific (*CTL*) siRNA (100 nM). Twenty-four hours later, DHT (100 nM) or vehicle was added. After a further 24 h, luciferase activity was assayed. *D*, serum-starved HASMC were incubated with Act D (5 μg/ml) in the presence of 2.6 mM P_i after 12 h of DHT (100 nM) treatment. The remaining Gas6 mRNA was determined at 0, 3, and 6 h after Act D treatment by real-time PCR analysis. Values of Gas6 mRNA with P_i (*dotted line with squares*), with P_i and DHT (*dashed line with triangles*), or without P_i (*solid line*) in the presence of Act D were normalized to that of β-actin mRNA at each time point. Gas6 mRNA level at time 0 was expressed as a percentage of the maximum value. The results are the average of three separate experiments. *, $p < 0.05$ *versus* Act D by Fisher's test. *E*, chromatin extracts were obtained from HASMC after treatment with or without 100 nM DHT for 12 h, and the ChIP assay was performed using an antibody against AR or control IgG. DNA fragments were extracted from immunoprecipitates. The Gas6 promoter region containing proximal ARE was amplified, but distal ARE was not. *F*, a ChIP assay was performed using an antibody against AR, acetylhistone H3 (*AcH3*), or p300 with chromatin extracts with or without treatment with 100 nM DHT for 24 h. Relative promoter activities are expressed as the mean ± S.E. of quadruplicate samples. Similar results were obtained from four independent experiments. *, $p < 0.05$ by Fisher's test.

presence of DHT (Fig. 5*C*). These findings suggest that Gas6 transactivation by androgens was dependent on the AR.

Because P_i did not affect Gas6 transcriptional activity, we further explored the effect of P_i on Gas6 regulation at the post-transcriptional level. The stability of Gas6 mRNA was examined in the presence or absence of Act D. We found that Gas6 mRNA was significantly more degraded in the presence of P_i than in its absence after Act D treatment (Fig. 5*D*). DHT did not have an effect on mRNA degradation (Fig. 5*D*). These findings suggest that P_i down-regulated Gas6 expression by increasing the mRNA degradation rate and not by decreasing transcriptional activity.

To confirm a direct association of the AR with the proximal ARE in the Gas6 gene, we performed a ChIP assay in HASMC. After 12 h of DHT treatment, a polyclonal antibody against the AR could efficiently precipitate the androgen-responsive region of Gas6, showing that the AR directly binds to the Gas6 gene promoter region containing the proximal ARE site in HASMC (Fig. 5*E*). We did not observe binding of the AR to the distal ARE site in the Gas6 gene (Fig. 5*E*). Furthermore, we attempted a characterization of the promoter interactions with an AR-containing transcriptional complex. Histone acetyltransferase, such as p300, is a well established coactivator of the AR, and acetylation of histone H3 is an important determinant of AR action, possibly mediated by p300 (19). We performed a ChIP assay with antibodies against acetylhistone H3 and p300. When the AR binds to the proximal ARE site of the Gas6 gene, acetylhistone H3 and p300 also bind to this site as coactivators (Fig. 5*E*). We did not

FIGURE 4. **Androgens stimulate Gas6 promoter activity in HASMC.** *A*, shown is a schematic representation of the sequence for ARE sites in wild-type human Gas6 promoter and mutant construct. Site-directed mutagenesis was used to alter the ARE sites within the Gas6 construct. The sequences of the consensus ARE site, Gas6 ARE sites, and the mutated ARE sites with altered bases *underlined* are shown. *B*, 24 h after transfection of 0.8 μg of Gas6-luc construct containing only the proximal ARE or the construct containing both the proximal and distal AREs, androgens (testosterone (*T*) and DHT, 100 nM) were added, and the cells were incubated for an additional 24 h. *, $p < 0.05$ *versus* androgens (−) by Fisher's test. *C*, HASMC were treated with DHT (100 nM) or vehicle for 24 h after transfection of the Gas6-luc constructs containing both proximal and distal AREs or mutants. *, $p < 0.05$ *versus* DHT (+) wild-type Gas6 by Fisher's test. *D*, HASMC were transfected with wild-type or two proximal ARE mutants. Twenty-four hours after transfection, DHT (100 nM) was added for an additional 24 h. Luciferase activity was normalized to that of the DHT-free wild-type Gas6 construct. *, $p < 0.05$ *versus* DHT(+) wild-type Gas6 by Fisher's test. Relative promoter activities are expressed as the mean ± S.E. of quadruplicate samples. Similar results were obtained from five independent experiments.

observe any binding of the AR, acetylhistone H3, or p300 to the distal ARE site in the Gas6 gene (Fig. 5*F*).

DISCUSSION

The effect of testosterone replacement therapy on atherosclerosis is controversial (21–25), although testosterone deficiency is known to be associated with cardiovascular disease in men (26–30). We and others have shown that a low testosterone level is associated with markers of atherosclerosis such as impaired endothelial vasomotor function (27), increased carotid intima-media thickness (28), and aortic calcification (9). Recently, testosterone has also been reported to inhibit VSMC proliferation and neointima formation (7), suggesting a direct action of testosterone on the vasculature. In this *in vitro* study we examined the effect of androgens on P_i-induced VSMC calcification and found that androgens at physiological concentrations exhibited inhibitory effects on VSMC calcification. In contrast to the present study, it has been reported that androgens induced vascular calcification in apolipoprotein E knockout mice (31). This discrepancy may derive from the complex *in vivo* effects of testosterone. Further work is required to define the role of androgens in vascular calcification.

Androgens act mainly through transcriptional control of target genes mediated by the nuclear AR (11, 32). In the present study we found that the AR was expressed predominantly in the nucleus of VSMC and had transcriptional activity. Recently, it was demonstrated that the AR-dependent action of androgens protects against angiotensin II-induced vascular remodeling (33). Consistent with this, our results showed that the inhibitory effect of androgens on VSMC calcification was mediated by the AR and not by estrogen receptor.

Recently, we demonstrated that apoptosis plays a central role in the process of P_i-induced VSMC calcification through down-regulation of the Gas6-mediated survival pathway (16, 17). In the present study we found that androgens prevented VSMC apoptosis and restored Gas6 expression and Akt survival signaling. These inhibitory effects of androgens on apoptosis and calcification were eliminated by flutamide and Gas6 siRNA. Our findings indicate that AR-dependent restoration of Gas6 by androgens contributes to the inhibition of apoptosis and VSMC calcification.

Although the involvement of other molecules such as protein kinase Cδ (7) and endothelial nitric-oxide synthase (33) in the vasoprotective actions of androgens is unclear, our data showed that Gas6 plays a pivotal role in the inhibitory effect of androgen on P_i-induced calcification. Several genes containing AREs and having AR-mediated actions have been identified (34, 35). However, little is known about transcriptional regulation and the target genes of the actions of the AR in the vascular system. In this study we identified two AREs in the promoter region of the Gas6 gene and characterized specific direct binding of the AR to the proximal ARE, in contrast to the nonfunctional distal ARE. Interestingly, Mo *et al.* (36) identified that an estrogen response (ER) element spanning −72 to −89 bp from the translation start site in Gas6 and ERα is recruited by estrogen-mediated stimulation of Gas6 gene expression in mouse mammary epithelial cells. In the human Gas6 promoter domain, we also found the existence of an estrogen response element at −243 to −251 bp. In clinical studies, a low serum estradiol level in women was correlated with increased arterial calcification (37), and estrogen replacement could reduce coronary calcification (38, 39). However, in experimental studies, estradiol treatment showed variable effects on vascular calcification with either inhibition (40, 41) or stimulation of calcification (42). Further studies are needed to elucidate the actions of estrogens in vascular calcification.

In summary, this study showed that Gas6 is a novel target that is directly and transcriptionally regulated by the AR, and direct interaction of the AR and Gas6 mediates the inhibitory effects of androgens on vascular calcification. This study provides a new mechanistic insight into the vascular protective action of androgens.

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Low testosterone level as a predictor of cardiovascular events in Japanese men with coronary risk factors

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ABSTRACT

Objective: Recent epidemiological studies have found that testosterone deficiency is associated with higher mortality largely due to cardiovascular (CV) disease in community-dwelling older men. We investigated whether a low plasma testosterone level could predict cardiovascular events in middle-aged Japanese men with coronary risk factors.

Methods: One hundred and seventy-one male outpatients (30–69 years old, mean ± SD = 48 ± 13 years) who had any coronary risk factor (hypertension, diabetes, dyslipidemia, smoking, and obesity) without a previous history of CV disease were followed up. At baseline, the subjects underwent examination of coronary risk factors, measurement of flow-mediated dilation (FMD) of the brachial artery as an indicator of vascular endothelial function and assays of plasma total testosterone, dehydroepiandrosterone-sulfate (DHEA-S), estradiol and cortisol.

Results: During the mean follow-up period of 77 months, a total of 20 CV events occurred. Kaplan–Meier survival analysis by tertile of plasma hormone levels revealed that the subjects with the lowest testosterone tertile were more likely to develop CV events than those with the highest tertile ($P < 0.01$ by log-rank test). Cox proportional hazards models showed that the subjects with the lowest tertile of plasma testosterone (<14.2 nmol/L) had an approximately 4-fold higher CV event risk compared to those with the higher testosterone tertiles after adjustment for coronary risk factors including medication and FMD (unadjusted hazard ratio, 3.61; 95% CI, 1.47–8.86: multivariate-adjusted hazard ratio, 4.61; 95% CI, 1.02–21.04). Multivariate analysis did not show any significant association of DHEA-S, estradiol or cortisol with CV events.

Conclusions: A low plasma testosterone level is associated with CV events in middle-aged Japanese men, independent of coronary risk factors and endothelial function. This is the first report to show the relationship between endogenous testosterone and CV events in Asian population.

1. Introduction

Plasma testosterone level declines with advancing age in men [1]. Testosterone deficiency is often associated with age-related diseases such as erectile dysfunction, osteoporosis, depressed mood, cognitive impairment and frailty [2,3]. Furthermore, a number of studies suggest that testosterone deficiency is related to cardiovascular (CV) disease and its risk factors in men. Inverse relations between testosterone level and coronary risk factors including obesity [4,5], hypertension [5,6], dyslipidemia [4,5], and diabetes [5,7] have been reported. In addition, we and others have shown that a low testosterone level is associated with markers of atherosclerosis such as impaired endothelial vasomotor function [8], increased carotid intima-media thickness [9] and aortic calcification [4]. Although these data do not indicate a causal relationship between endogenous testosterone and CV disease, recent epidemiological studies have demonstrated that community-dwelling older men with a low testosterone level are more likely to die [10–12], largely due to CV disease [11,12]. However, this issue remains unknown in Asian population.

Based on these backgrounds, we tested the hypothesis that a low testosterone level is an independent risk factor for CV disease even in middle-aged Japanese men with coronary risk factors. For this purpose, we conducted a survey of 171 male patients by using baseline clinical information and by measuring sex hormone levels in stored plasma.

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2. Methods

2.1. Subjects

Male subjects aged 30–69 years at baseline, who were referred to our department to check for CV disease and undergo examination of vasomotor function of the brachial artery in 1996–2000, and had any of the classical coronary risk factors including hypertension, dyslipidemia, diabetes mellitus and current smoking, were eligible. Hypertension, dyslipidemia and diabetes mellitus were defined according to diagnostic criteria [13–15] or if the subject was taking any medication for these diseases. Subjects with a history of CV disease, including stroke, coronary heart disease, congestive heart failure and peripheral arterial disease, were excluded. Malignancy, overt endocrine disease and use of steroid hormones were also excluded, because these conditions may have a significant influence on both plasma sex hormones and clinical course.

Of the 188 eligible subjects whose plasma was stored, written informed consent was obtained from 171 subjects; 1 subject refused and 16 subjects were lost to follow-up. Then, plasma hormone levels were measured and follow-up data were obtained in 171 subjects. The study protocol was approved by the ethics committee of the Graduate School of Medicine, The University of Tokyo. Each subject or a family member, if the subject had died, gave written informed consent for enrollment in this study.

2.2. Clinical measurements

Clinical information was collected at baseline when each patient attended our department. Blood sampling and measurement of height, weight, blood pressure and vasomotor function were performed in the morning after a 14-h overnight fast. Blood pressure was measured at least twice using an automated, digital electrosphygmomanometer (Omron Healthcare Co., Ltd., Kyoto, Japan) on the nondominant arm in a sitting position, and the average was used for analysis.

Serum total cholesterol and triglyceride concentrations were measured enzymatically, and serum high-density lipoprotein (HDL) cholesterol concentration was measured by the heparin-$Ca^{2+}Ni^{2+}$ precipitation method. Plasma glucose concentration was assayed by the glucose oxidase method, and hemoglobin A1c level was measured by high-performance liquid chromatography.

Plasma concentrations of total testosterone, dehydroepiandrosterone-sulfate (DHEA-S), estradiol and cortisol were determined using sensitive radioimmunoassays by a commercial laboratory (SRL, Inc., Tokyo, Japan). Because the plasma used for hormone assays was deep-frozen (−80 °C) for up to 7 years, we checked the change in titers using the stored samples, which had been measured at sampling 5–7 years before. Pearson's correlation coefficient between the two measurements was 0.965 for estradiol ($n=34$), 0.976 for testosterone ($n=20$), 0.991 for DHEA-S ($n=15$) and 0.937 for cortisol ($n=16$), indicating that there was no significant change in plasma titers in our frozen samples. The intra-assay coefficients of variation for the measurements were less than 5%.

Vasomotor function of the brachial artery was evaluated using an ultrasound machine according to the method described previously [16]. Briefly, endothelium-dependent flow-mediated vasodilation (%FMD) was measured as the maximal percent change in the vessel diameter after reactive hyperemia. Subsequently, endothelium-independent nitroglycerin-induced vasodilation was measured as the maximal percent change in the vessel diameter after sublingual administration of nitroglycerin spray (0.3 mg; Toa Eiyo Co., Tokyo). The same examiner (M.H.) performed the measurements of FMD throughout this study.

2.3. Follow-up

The subjects were followed in 2006–2007 by mail and/or visits to our clinic. Each subject or a family member completed the questionnaire on CV disease and health status. CV events analyzed as the endpoints of this study included stroke, coronary artery disease, sudden cardiac death, and peripheral arterial disease. If CV events were reported on the questionnaire, we attempted to confirm the diagnosis of each event by medical records and/or interview by research doctors who were unaware of the patient's plasma hormone levels. Finally, after thorough examination, 20 cases were determined as CV events. Eighteen cases were ascertained by medical records which included clinical course, physical examination, laboratory tests and imagings. Because medical records were not available on other two cases of self-reported ischemic stroke, they were diagnosed according to the phone interview to each patient.

2.4. Data analysis

Values are expressed as mean ± SD in the text unless otherwise stated. Differences between the groups were analyzed using ANOVA for continuous variables and Chi-squared test for categorical variables. Survival was analyzed using Kaplan–Meier plots and log-rank tests. Hazard ratios (HRs) for CV events were analyzed using Cox proportional hazards regression. A value of $P<0.05$ was considered statistically significant. Data were analyzed using SPSS (Ver. 17.0, SPSS Inc., Chicago, IL).

3. Results

3.1. Characteristics of subjects according to plasma testosterone level

Table 1 shows the baseline characteristics of the subjects by tertile of plasma testosterone. As reported previously [4–8], subjects with the lowest testosterone tertile tended to be obese, hypertensive, dyslipidemic, diabetic, and to have impaired endothelial vasomotor function compared to those with higher testosterone tertiles. Age and smoking status were not different between the groups.

3.2. CV events and hormones

During the mean follow-up period of 77 ± 46 months (median = 54 months), a total of 20 CV events occurred (Table 2). Eleven cases of coronary artery disease included three of myocardial infarction, three of medically treated angina pectoris, four of percutaneous coronary intervention, and one of coronary artery bypass grafting. All of the five cases of stroke were due to cerebral infarction.

As shown in Fig. 1, Kaplan–Meier survival analysis by tertile of plasma testosterone level revealed that low testosterone was associated with CV events. Cox proportional hazards models showed that the subjects with the lowest tertile of plasma testosterone, but not those with the middle tertile, had significantly increased risk for CV events compared to those with the highest tertile (Table 2). Adjustment for age and body mass index did not attenuate the effect.

Then, HRs for the lowest tertile of plasma testosterone vs. the higher (middle and highest) tertiles were analyzed. The subjects with the lowest tertile (<14.2 nmol/L) showed an unadjusted HR of 3.61 (95% CI, 1.47–8.86), and an adjusted HR of 4.24 (95% CI, 1.67–10.78) for age, body mass index, and current smoking. The HR was 4.61 (95% CI, 1.02–21.04) after adjustment for age, body mass index, current smoking, systolic blood pressure, HDL cholesterol, non-HDL cholesterol, hemoglobin A1c, %FMD,

Table 1
Baseline characteristics of subjects by tertile group of plasma testosterone.

	Tertile 1 <14.2 nmol/L (*n* = 57)	Tertile 2 14.2–19.4 nmol/L (*n* = 57)	Tertile 3 >19.4 nmol/L (*n* = 57)	*p* for trend
Testosterone (nmol/L)	11.0 ± 3.0	17.0 ± 1.6	24.0 ± 3.0	<0.001
(ng/dL)	(318 ± 86)	(490 ± 45)	(693 ± 86)	
DHEA-S (μmol/L)	4.94 ± 2.68	4.55 ± 2.25	4.83 ± 2.64	0.81
Estradiol (pmol/L)	115 ± 30	116 ± 31	133 ± 30	0.004
Cortisol (nmol/L)	386 ± 138	378 ± 142	361 ± 120	0.67
Age (years)	47 ± 13	45 ± 13	50 ± 14	0.24
Body mass index (kg/m^2)	27.6 ± 5.5	25.6 ± 4.3	24.1 ± 3.6	<0.001
Systolic blood pressure (mmHg)	131 ± 18	125 ± 16	123 ± 12	0.01
Diastolic blood pressure (mmHg)	79 ± 15	74 ± 11	74 ± 9	0.04
Non-HDL cholesterol (mmol/L)	4.19 ± 1.27	3.91 ± 1.06	3.74 ± 1.01	0.10
HDL cholesterol (mmol/L)	1.20 ± 0.36	1.23 ± 0.41	1.44 ± 0.48	0.005
Triglycerides (mmol/L)	2.04 ± 2.12	1.91 ± 1.85	1.46 ± 1.28	0.18
Fasting plasma glucose (mmol/L)	6.00 ± 1.18	5.73 ± 0.92	5.73 ± 1.28	0.34
Hemoglobin A1c (%)	5.9 ± 1.7	5.2 ± 0.8	5.5 ± 1.2	0.03
%FMD	4.2 ± 2.7	5.7 ± 4.2	6.1 ± 3.8	0.01
%NTG	12.8 ± 4.3	14.2 ± 5.4	13.2 ± 5.0	0.30
Hypertension, *n* (%)	30 (53)	20 (35)	20 (35)	0.09
Dyslipidemia, *n* (%)	33 (58)	35 (61)	24 (42)	0.09
Diabetes mellitus, *n* (%)	15 (26)	7 (12)	9 (16)	0.13
Current smoker, *n* (%)	28 (49)	25 (44)	29 (51)	0.74

DHEA-S, dehydroepiandrosterone-sulfate; HDL, high-density lipoprotein; %FMD, percent flow-mediated dilation of brachial artery; %NTG, percent nitroglycerine-induced dilation of brachial artery.
Values are expressed as mean ± SD. Continuous variables were compared by ANOVA and categorical variables by Chi-squared test.

Table 2
Cardiovascular events by tertile of plasma testosterone.

	Tertile 1 <14.2 nmol/L (*n* = 57)	Tertile 2 14.2–19.4 nmol/L (*n* = 57)	Tertile 3 >19.4 nmol/L (*n* = 57)	Total (*n* = 57)
Number of events				
Stroke	2	3	0	5
Coronary artery disease	7	2	2	11
Sudden cardiac death	2	0	0	2
Peripheral arterial disease	1	0	1	2
Total cardiovascular events	12	5	3	20
HRs (95% CI) for total cardiovascular events				
Unadjusted	4.82 (1.36, 17.12)	1.67 (0.40, 6.99)	1(Ref)	
Adjusted for age	6.36 (1.78, 22.80)	1.82 (0.43, 7.71)	1(Ref)	
Adjusted for age and BMI	7.01 (1.94, 25.34)	1.86 (0.44, 7.86)	1(Ref)	

BMI, body mass index. HRs (Hazard ratios) were analyzed using Cox proportional hazards regression.

medications (antihypertensives, statins, hypoglycemic agents and antiplatelet agents), estradiol and DHEA-S. In addition to testosterone, age (HR per year, 1.12; 95% CI, 1.05–1.20), %FMD (HR per 1% increase, 0.80; 95% CI, 0.64–0.99) and HDL cholesterol (HR per 1 mg/dL, 0.88; 95% CI, 0.81–0.95) were independently associated with CV events, but other variables were not in this final model. Further inclusion of other hormones and nitroglycerin-induced endothelium-independent vasodilation into the model did not influence the statistical results (data not shown).

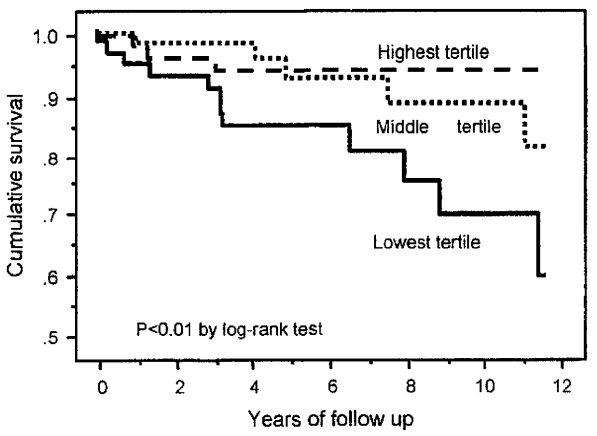

Fig. 1. Survival curves for cardiovascular events by tertile group of plasma concentration of testosterone. Cut-offs of the tertiles for testosterone were 14.2 and 19.4 nmol/L (410 and 560 ng/dL).

Two subjects with the lowest tertile of plasma testosterone suffered CV events within 6 months of follow-up; a case of sudden cardiac death and a case of coronary artery bypass grafting. Accordingly, similar statistical analyses were performed excluding these two cases. The results were essentially unchanged, although the HRs were slightly smaller (unadjusted HR, 3.06; 95% CI, 1.21–7.78; multivariate-adjusted HR, 3.80; 95% CI, 1.06–13.52).

Among other hormones examined, only DHEA-S was associated with increased risk for CV events, but was canceled by adjustment for age (data not shown). Further multivariate analysis did not show any significant association of DHEA-S, estradiol or cortisol with CV events.

4. Discussion

In this follow-up study of middle-aged Japanese men with coronary risk factors, a low plasma testosterone level was associated with CV events. Although the subjects with lower testosterone levels had worse profiles of coronary risk factors [4–7,11,12] and endothelial function [8] at baseline, as reported previously, adjustment for these confounders including age and cardiovascu-

lar medication indicated that low testosterone was an independent risk factor for CV events. In contrast, DHEA-S, estradiol and cortisol levels were not related to CV events.

A number of cross-sectional studies have shown an association between low testosterone level and CV disease [17,18], but have not provided evidence of a causal relationship between them. In recent years, longitudinal follow-up studies have demonstrated that community-dwelling older men (around 70 years on average) with lower testosterone levels are more likely to die from CV disease [11,12]. In contrast, a low testosterone level was not associated with CV deaths [19] or events [20] in community-dwelling middle-aged men (early 50s on average). These different findings might arise from the characteristics of the populations such as age and coronary risk factors, duration of follow-up and/or cut-off level of plasma testosterone at baseline. In any case, since all the above-mentioned studies were achieved in Caucasians, our study is the first to investigate the relationship between endogenous testosterone and CV events in Asians. Also, the present study showed a positive association between low testosterone level and CV events in middle-aged men with coronary risk factors, implying the clinical importance of measuring plasma testosterone in patients at risk, even if they are not old.

Unlike the previous reports showing an association of CV events with low levels of DHEA-S [21] and estradiol [22], and with a high cortisol:testosterone ratio [20], the present study did not show any significant association of CV events with estradiol, cortisol or cortisol:testosterone ratio (data not shown). The association between low DHEA-S and CV events was abolished by statistical adjustment for age, suggesting that the age-dependent decline of DHEA-S (Pearson's correlation coefficient between age and DHEA-S: −0.588; $P<0.001$) might have eliminated the association with CV events if present. Taking together with the Cox regression model including all hormones, it is suggested that testosterone is the strongest among four steroid hormones that could be predictive of CV events in this population.

There could be several mechanisms by which endogenous testosterone protects men from CV disease. Consistent with the present study, observational studies [4–8,11,12] suggest that testosterone might prevent risk factors such as obesity, hypertension, dyslipidemia, diabetes and endothelial dysfunction. Supplementary studies support the beneficial effects of testosterone on adiposity [23] and endothelial vasomotor function [24]. Based on these findings, risk markers and endothelial vasomotor function were entered into the multivariate models. Although statistical adjustment may have been insufficient to exclude the interaction between testosterone and these risk factors, testosterone remained a significant predictor of CV events in the present study. Testosterone has been reported to inhibit vascular smooth muscle cell proliferation and neointima formation [25], suggesting the direct action of testosterone on the vasculature. Also, the effects of testosterone on inflammation, hemostasis and cardiac ischemia [26] might be involved in the final process leading to CV events. The precise mechanisms, including the role of the androgen receptor and aromatization to estrogen, should be addressed in the future.

The finding of this study should not be extended to men without coronary risk factors. Our preliminary data of 47 middle-aged men without coronary risk factors showed that no subject suffered CV events during the mean follow-up period of 102 months, although a quarter of them had plasma testosterone level below the cut-off of this study (<14.2 nmol/L). Thus, the relationship between plasma testosterone and CV outcomes might be totally different in middle-aged Japanese men without coronary risk factors.

This study has several limitations. First, the number of CV events was too small to reach a clear conclusion with strong statistical power, due primarily to the small sample size and secondarily to the low incidence of CV events (approximately 2%/year). Second, the largely retrospective design (the protocol had been approved a few years before the final data collection) reduced the quality of the study and compelled us to lose many plasma samples and 16 subjects in the follow-up. Third, not all the CV events were confirmed by medical recordings. Two cases (a case in the lowest tertile and another in the middle tertile of plasma testosterone level) were determined according to the phone interview to each patient. Although the exclusion of these two cases did not significantly influence the statistical results (data not shown), self-reported outcomes limit the accuracy of this study. Fourth, the potential influence of medication on plasma testosterone level and on CV events cannot be excluded, although statistical adjustment for each class of drugs did not affect the results. For instance, beta-blockers have been reported to decrease plasma testosterone [27], but were taken by only nine subjects and were not related to testosterone level in our population (data not shown). Fifth, active forms of testosterone such as bioavailable and calculated free testosterone were not measured, because a direct assay of bioavailable testosterone or an assay of sex hormone binding globulin, which is necessary for free testosterone calculation, is not available in Japan. However, since previous longitudinal studies [11,12] have shown an association of total testosterone with CV mortality, the fundamental findings might not have differed if active forms of testosterone had been analyzed.

In summary, a low plasma testosterone level was associated with CV events in middle-aged Japanese men, independent of coronary risk factors and endothelial function. This study is the first to show the relationship between endogenous testosterone and CV events in Asian population, and provides evidence supporting the protective role of endogenous testosterone in the development of CV disease in men.

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KIBRA Genetic Polymorphism Influences Episodic Memory in Alzheimer's Disease, but Does Not Show Association with Disease in a Japanese Cohort

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Key Words

Alzheimer's disease · Episodic memory · Genetics · Neuropsychological assessment · *KIBRA* gene · Rivermead Behavioral Memory Test

Abstract

Background/Aims: A single-nucleotide polymorphism (SNP) in the *KIBRA* gene, rs17070145, was reported to be significantly associated with episodic memory in cognitively normal cohorts. This observation has expanded genetic studies on *KIBRA* to Alzheimer's disease (AD). Importantly, the association between *KIBRA* and episodic memory in AD has never been addressed. In this study, we investigated whether the *KIBRA* rs17070145 SNP influences AD episodic memory and the disease in a Japanese cohort. ***Methods:*** Blood samples from 346 AD patients and 375 normal cognitive controls were collected and genotyped for rs17070145. Episodic memory was measured in 32 AD patients, diagnosed for the first time, by use of the Rivermead Behavioral Memory Test (RBMT). ***Results:*** We found that *KIBRA* C allele carriers scored significantly lower than *KIBRA* non-C carriers on both RBMT total profile score ($p = 0.042$, effect size = 0.84) and RBMT total screening score ($p < 0.001$, effect size = 1.42). The *KIBRA* gene did not show association with AD in our Japanese cohort. ***Conclusion:*** Our results evidence a strong association between the *KIBRA* gene and episodic memory impairment in AD, but show no influence on AD in our Japanese cohort. We propose that *KIBRA* might have an effect similar to cognitive reserve.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by a progressive deterioration of cognitive abilities and memory loss. For the famil-

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ial occurrence of the disease (early-onset), the existence of familial AD-responsible genes has been demonstrated, with mutations in 3 genes, *APP*, *PSEN1* and *PSEN2*, consistently reported. However, the genetic component that underlies sporadic AD (late-onset), which accounts for over 95% of all AD cases, is still poorly understood.

KIBRA (also known as WW and C2 domain-containing protein 1) is a protein mainly expressed in the brain and kidney [1], whose functions are still being characterized, but that, importantly, has been shown to be involved in the control of synaptic plasticity in the brain [2]. Recently, it was reported that KIBRA regulated the Salvador/Hippo/Warts network which restricted tissue size [3]. In 2005, a *KIBRA* gene single-nucleotide polymorphism (SNP), rs17070145, was reported to be significantly associated with episodic memory in 3 independent cognitively normal cohorts from Switzerland and the USA [4]. This result was later confirmed in a German sample of healthy individuals [5], a Japanese sample of healthy individuals [6] and in a cohort in which nearly 50% of individuals had a diagnosis of mild cognitive impairment [7].

These observations led to studies of the *KIBRA* rs17070145 SNP in AD, whose core feature is dysfunction of episodic memory [8]. Recently, Corneveaux et al. [9] reported an association of the *KIBRA* CC genotype (*KIBRA* CC carriers) with increased risk for late-onset AD (n = 702). Conversely, the *KIBRA* T allele (*KIBRA* CT and *KIBRA* TT carriers) was shown to be associated with an increased risk for AD in a Spanish cohort [10]. Despite the available information, *KIBRA* has not yet been established as an AD risk gene, and, importantly, no studies have ever addressed the association between *KIBRA* and episodic memory in AD.

Therefore, in this study, we investigated whether the *KIBRA* SNP rs17070145 influences AD episodic memory and AD in a Japanese cohort.

Methods

Subjects

We collected blood samples from 346 consecutive AD patients who visited Osaka University Hospital between July 27, 2001, and June 10, 2010, and from 375 cognitively normal controls, who were population-based elderly subjects (Suita City, Japan) tested by a questionnaire including the date, orientation and history. Blood samples were collected after written informed consent had been obtained from subjects and/or representatives. This study was approved by the genome ethical committee of the Osaka University Graduate School of Medicine. AD patients met the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association criteria for probable AD [11].

We also have a research-oriented clinic for patients with cognitive impairment in the Department of Neuropsychiatry of the Osaka University Medical Hospital. It is also a clinic for early identification of dementia. In this clinic, all patients were examined comprehensively by specialists of geriatric psychiatry, and they underwent standard neuropsychological examinations including the Mini Mental State Examination (MMSE), routine laboratory tests, electroencephalography, cranial magnetic resonance imaging and radionuclear neuroimaging studies. Blood drawing for the genome study was not routine in this clinic. Eighty first-time diagnosed AD patients agreed to it, and 32 out of them agreed to an additional visit for the memory examination by use of the Rivermead Behavioral Memory Test (RBMT) between September 30, 2002, and May 23, 2007 (RBMT-AD specialized clinic subjects). RBMT-AD specialized clinic subjects were excluded from the study if they (1) had the complication of other neurological diseases, (2) had any evidence of focal brain lesions on magnetic resonance images or of cerebral arterial occlusive lesions on magnetic resonance angiography, or (3) did not have a caregiving family member familiar with their everyday life.

Rivermead Behavioral Memory Test

The RBMT, developed by Wilson et al. [12, 13], is a standardized, validated and reliable test for everyday memory, including personal events, name of persons, newspaper articles, places visited, routes followed, schedules and appointments. It is difficult to assess everyday memory with traditional memory tests [14], but the RBMT differs from conventional tests in that each of its 12 items is an analog of an everyday task, rather than a test based on experimental material, such as paired associates or list of words. The Japanese version of the RBMT was developed by Watamori et al. [15], and its reliability and validity have been previously confirmed [16–19]. Concretely, the authors reported that the RBMT can distinguish AD from both mild cognitive impairment and normal control, and strongly correlates with objective memory tests, such as the Everyday Memory Checklist caregiver rating and Clinical Dementia Rating (CDR) memory domain.

Although the RBMT has 4 parallel forms (A, B, C and D) for repeated uses, only the RBMT-A form was administered to subjects in this study. The subtests of the RBMT are (1) remembering a first name and a surname with a facial portrait, (2) remembering to ask for a personal item belonging to the subject, (3) remembering to ask about an appointment, (4) picture recognition, (5) remembering a short story (immediate), (6) remembering a short story (delayed), (7) face recognition, (8) remembering a new route (immediate), (9) remembering a new route (delayed), (10) remembering to deliver a message (immediate and delayed), (11) orientation for time, place and persons, and (12) date. In 8 of the subtests, i.e. points 1–4, 6, 7, 9 and 10 (delayed), the subjects were instructed to remember the information that they were about to be given. The subtests were then conducted 5–30 min after the information had been given. Subtests 2, 3 and 10 are tests of prospective memory. In subtest 2, the subjects were asked to hand in a personal item at the start of the session and instructed to ask for it at the conclusion of the session. The item was then placed out of sight. In subtest 3, subjects were instructed at the beginning of the test session to remember to ask for their next appointment when they heard a buzzer 20 min later. In subtest 10, they had to remember to de-

Table 1. Characterization of the *KIBRA* C carriers and non-C carriers of the RBMT-AD specialized clinic subjects

	CC/CT (n = 12)	TT (n = 20)	p
Mean age ± SD, years	68.5 ± 10.0	72.2 ± 8.1	0.267
Mean age of first abnormal memory loss episode reported by caregivers ± SD, years	63.8 ± 2.6	69.3 ± 2.0[1]	0.104
Male/female, n	5/7	8/12	1.000
APOE ε4+/−, n	10/2	12/8	0.248
CDR score 0.5/1/2, n	1/9/2	3/14/2	1.000
Mean MMSE score ± SD	17.8 ± 2.7	20.4 ± 4.6	0.093
Mean ADAS score ± SD	20.0 ± 5.8	17.6 ± 7.1[1]	0.335
Years of education			
Median	12	10	0.151
IQR	10.25–15	9–14[1]	

p values assessed by t test (continuous variables) and Fisher's exact test (categorical variables). IQR = Interquartile range (Q1–Q3).

[1] One datum was missed.

liver a message in the course of retracing a route around the room. For each subtest of the RBMT, a raw score was given. Then, two kinds of score were produced, a simple pass/fail or screening score ranging in each case from 0 to 1, and a standardized profile score ranging in each case from 0 to 2. A total screening score ranging from 0 to 12 and a total profile score ranging from 0 to 24 were used as indices of overall everyday memory status of the subjects.

Genotyping

Genotyping of *KIBRA* rs17070145 polymorphism was performed by the Taq-Man SNP assay and ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, Calif., USA) as previously described [20–23]. The apolipoprotein E *(APOE)* genotype was determined by the PCR-RFLP method [20–23].

Statistical Analysis

Baseline characteristics are presented as means ± standard deviation, medians or interquartile ranges for continuous variables, and frequencies for categorical variables. Comparisons for continuous variables and categorical variables were performed with the t test and χ^2 test or Fisher's exact test, respectively. The analysis of covariance model was used to investigate the effect of treatment on the RBMT scores with the following covariate: presence of the *KIBRA* SNP C allele (*KIBRA* CT and *KIBRA* CC), *APOE* ε4, age, the age of first abnormal memory loss episode reported by caregivers, gender, CDR stage, MMSE score, Alzheimer's Disease Assessment Scale for Japanese cognitive subscale (ADAS-Jcog) and/or years of education. The best set of covariates was selected by using Akaike's information criterion [24]. All tests were two-sided, and the statistical significance level was set at 5%. Statistical analysis was performed with SAS software version 9.02 (SAS Institute, Cary, N.C., USA), and all p values and confidence intervals (CI) presented are the original and were not corrected for multiple testing. Meta-analysis of *KIBRA* CC AD odds ratio and 95% CI was performed by the Der-Simonian-Laird method.

Results

From the RBMT-AD specialized clinic subjects, we found 1 patient with *KIBRA* CC, 11 patients with *KIBRA* CT and 20 patients with *KIBRA* TT (*KIBRA* non-C carriers). *KIBRA* CC and CT groups (*KIBRA* C carriers) were combined because there was only 1 *KIBRA* CC patient and that patient displayed memory performance similar to that of the *KIBRA* CT group (total profile score was 2, total screening score was 0). A lower frequency of the *KIBRA* C allele was observed, which was in accordance with the National Center for Biotechnology Information database of genetic variation (dbSNP) for the Asian population. Most of the patients were in an early stage of dementia (table 1). No significant differences in age, gender, *APOE* ε4, CDR, MMSE score, ADAS score and years of education were found between *KIBRA* C and *KIBRA* non-C carriers.

When analyzing the RBMT scores of the two groups, we found that C carriers scored significantly lower than non-C carriers on both the profile score ($p = 0.042$, effect size = 0.84) and screening score ($p < 0.001$, effect size = 1.42; table 2), evidencing an association of *KIBRA* rs17070145 polymorphism with episodic memory impairment in our Japanese AD cohort. We then assigned RBMT total scores as dependent variables and *KIBRA* C, age, age of first abnormal memory loss episode reported by caregivers, gender, *APOE* ε4, CDR stage, MMSE score, ADAS-Jcog score and/or years of education as independent variables and performed multiple linear regression analysis. For all the different combinations, we selected the appropriate models to which Akaike's information criteria were the smallest [24]. Model 1 was appropriate for total profile score and model 2 for total screening score. *KIBRA* C was found to be significantly associated with both total profile and screening scores after adjustment with the models shown in table 2.

We also analyzed 346 AD patients and 375 cognitively normal controls. As expected, we found significant differences in gender and *APOE* ε4 allele frequencies (table 3). *KIBRA* rs17070145 genotype and allele distribution in control and AD groups are shown in table 4. The genotype frequencies were in accordance with the Hardy-

Table 2. RBMT scores (total profile score and total screening score) between *KIBRA* C carriers and non-C carriers of the RBMT-AD specialized clinic subjects

	CC/CT	TT	p	Effect size
Total profile score (not adjusted)	2.17, 0.60–3.17	4.26, 3.01–5.51	0.042	0.84
Total profile score (model 1)	1.88, 0.42–3.34	4.22, 3.16–5.29	0.012	1.07
Total profile score (model 2)	1.79, 0.31–3.27	4.28, 3.20–5.35	0.010	1.13
Total screening score (not adjusted)	0.10, 0.00–0.36	0.93, 0.58–1.39	<0.001	1.42
Total screening score (model 1)	0.07, 0.00–0.35	0.91, 0.56–1.37	<0.001	1.54
Total screening score (model 2)	0.05, 0.00–0.31	0.93, 0.58–1.40	<0.001	1.66

Scores are expressed as mean estimates, followed by 95% CI. p values assessed by ANCOVA; model 1: adjusted for *APOE* ε4, years of education and ADAS score (this model is appropriate for total profile score); model 2: adjusted for *APOE* ε4, years of education, ADAS score and age (this model is appropriate for total screening score).

Table 3. Characterization of cognitively normal controls (NC) and AD patients

	NC (n = 375)	AD (n = 346)	p
Mean age ± SD, years	75.5 ± 4.9	75.2 ± 8.6	0.600
Male/female, n	170/205	110/236	<0.001
APOE ε4+/−, n	60/315	172/174	<0.001

p values assessed by t test (continuous variable) and Fisher's exact test (categorical variables).

Table 4. rs17070145 genotype and allele distribution in cognitively normal controls (NC) and AD patients

	CC	CT	TT	p^a	p^b	p^c
NC	13 (3.5)	128 (34.1)	234 (62.4)	0.673	0.414	0.694
AD	16 (4.6)	104 (30.1)	226 (65.3)	0.669		

Results are numbers, with percentages in parentheses.
[a] p for Hardy-Weinberg equilibrium tests (Pearson χ^2 test).
[b] p for genotype distribution (Fisher's exact test).
[c] p for allele distribution (Fisher's exact test).

Weinberg equilibrium. The *KIBRA* SNP did not show any association with AD in our Japanese cohort (table 4), even after adjustment for age, gender and *APOE* ε4 (data not shown).

Figure 1 shows KIBRA CC AD odds ratio and 95% CI in our Japanese cohort and previously reported cohorts. Our cohort's KIBRA CC AD odds ratio was 1.35 (95% CI = 0.64–2.85). Meta-analysis of them was not significant (OR = 1.10, 95% CI = 0.92–1.30).

Discussion

Despite numerous reports evidencing association of *KIBRA* with episodic memory, the relevance of *KIBRA* to AD still remains elusive. In our study, we addressed for the first time whether *KIBRA* genetic variation is associated with episodic memory impairment in AD. Our results evidence a strong association between the *KIBRA* gene and episodic memory impairment in AD and suggest a role for *KIBRA* similar to cognitive reserve, with no impact on diagnosis of AD.

There are several memory test batteries available, such as the Auditory-Verbal Learning Test (AVLT) [25], the Revised Wechsler Memory Scale Logical Memory Test [26], the Rey-Osterrieth complex figure [27] and the Takeda Three Colors Combination Test [28]. Association of *KIBRA* rs17070145 with episodic memory was shown for the first time by AVLT in 3 independent cognitively normal cohorts [3], and it has been recently confirmed in a Scottish cohort study (n = 2,091) [29]. In addition, the latter reported no association of the *KIBRA* SNP with the Revised Wechsler Memory Scale Logical Memory Test that rewards relational coding (Lothian Barth cohort, n = 542) [29], suggesting that *KIBRA* is not specific for complex episodic memory such as the Revised Wechsler Memory Scale Logical Memory Test but for simple episodic memory such as the AVLT instead. In our study, we

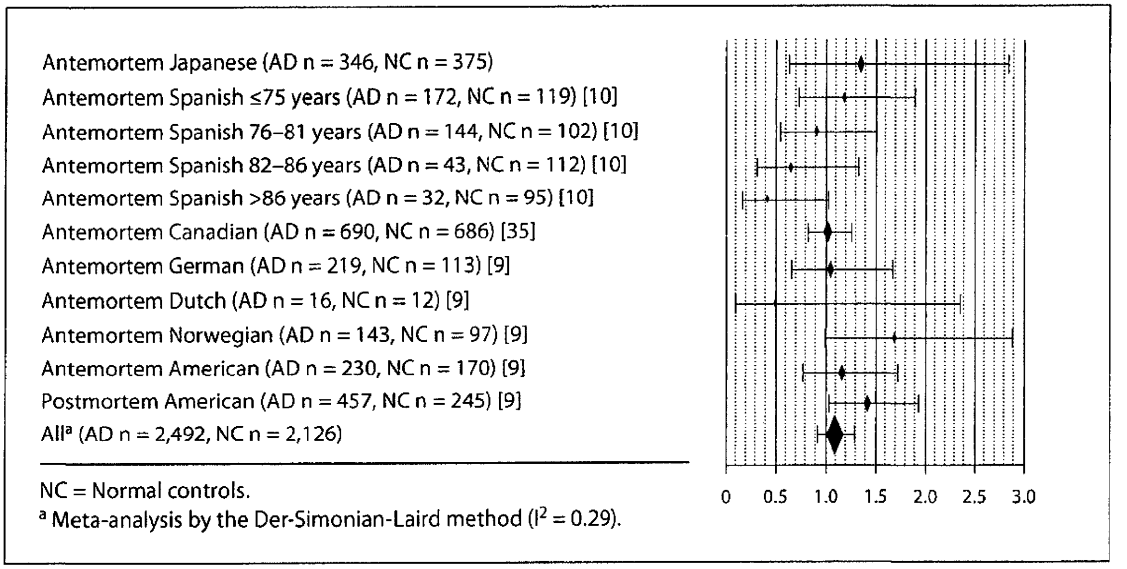

Fig. 1. *KIBRA* CC AD odds ratio and 95% CI in our Japanese cohort and previously reported cohorts.

used the RBMT, which assesses multidimensional aspects of everyday memory such as orientation, prose recall, visual recognition, prospective memory and so on. It also includes both complex episodic memory and simple episodic memory. Our results show that the *KIBRA* rs17070145 polymorphism is strongly associated with episodic memory impairment in our specialized clinic Japanese AD cohort (table 2). MMSE and ADAS-cog showed no differences between *KIBRA* C and non-C carriers (table 1). These results suggest that the *KIBRA* gene specifically seems to affect memory functions but not global cognitive status.

This association remained significant after adjustment for covariant components, which indicates that the *KIBRA* SNP might be an independent risk factor for episodic memory impairment.

Whereas an association of *KIBRA* with episodic memory has been repeatedly reported [4, 5, 7, 29], the impact of *KIBRA* on AD is still controversial (fig. 1). In a Spanish cohort, *KIBRA* CC AD odds ratio decreased continuously with age. *KIBRA* CC AD patients perhaps had earlier onsets and died soon. Hence, we tested the association between *KIBRA* and age of onset and course of the disease in RBMT-AD specialized clinic subjects. We defined the age of first abnormal memory loss episode reported by caregivers as onset age of AD. Although we found no significant differences between *KIBRA* C carriers and non-C carriers, the age of first abnormal memory loss episode reported by caregivers tended to be later in *KIBRA* non-C carriers (table 1). It is possible that the presence of the *KIBRA* T allele delays diagnosis of some AD clinical symptomatology. This effect could be similar to the well-reported effect of cognitive reserve, reflected in years of education. Highly educated individuals have better cognitive performance and, thus, tend to be judged as cognitively normal, albeit AD neuropathology is already present [30–32]. On the other hand, it appears that AD symptomatology progresses faster in people with higher education once AD is diagnosed [33]. Incidentally, in our cohort, duration from first memory loss episode to AD diagnosis was significantly shorter in the *KIBRA* TT group (2.6 ± 1.7 vs. 4.7 ± 1.2 years, $p = 0.001$). We propose that *KIBRA* might have an effect similar to cognitive reserve, particularly in simple word recall.

The impact of *APOE*, an established AD risk gene that accelerates AD brain pathology, on episodic memory was also examined. A recent study reported no differences, suggesting that *APOE* ε4 does not influence episodic memory (AVLT delayed recall) in cognitively normal individuals under 60 years of age [34]. In accordance, our results evidenced no significant differences in both RBMT total profile score (*APOE* ε4–: 3.40 ± 2.12 vs. *APOE* ε4+: 3.64 ± 3.16; $p = 0.831$) and RBMT total screening score (*APOE* ε4–: 0.80 ± 0.63 vs. *APOE* ε4+: 0.86 ± 1.17; $p = 0.873$) between *APOE* ε4 carriers and non-*APOE*-ε4 carriers. This lack of correlation between *APOE* and the episodic memory is intriguing and is in contrast with our findings for *KIBRA*, which seems to

have a less certain effect on AD but a more significant impact on episodic memory in young [4] and elderly subjects [4, 5, 7, 29] and even in mild AD patients, as our study shows (table 2). It is possible that *KIBRA* does not have a direct impact on AD neuropathology but could have an effect on the clinical diagnosis of AD, in a manner similar to cognitive reserve.

Compared to many reports based on Caucasian samples, our cohort evidenced lower frequencies of *KIBRA* CC. Thus, comparison of our results with those based on Caucasian samples must be carried out with caution. As our research-oriented clinic is specialized in the early identification of dementia, we should take selection bias into consideration. Further studies with larger samples, including cognitive functional and pathological data, will be carried out in the future in order to clarify the importance of the *KIBRA* SNP for episodic memory and AD pathology.

Acknowledgements

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Research report

Frontal cortex activation associated with speeded processing of visuospatial working memory revealed by multichannel near-infrared spectroscopy during Advanced Trail Making Test performance

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ABSTRACT

Although visuospatial working memory (VSWM) is commonly used during speeded and unconscious memory processing in daily life, most neuroimaging studies on VSWM use tasks that impose motor restrictions onto the examinees to avoid movement-related artifacts. Multichannel near-infrared spectroscopy (NIRS), however, can measure cortical activation during cognitive processing without interfering with task procedure. The purpose of this study is to determine whether multichannel NIRS can detect VSWM-induced frontal cortex activation similar to that seen in VSWM performance in daily-life activity. Using NIRS, we measured relative changes in the concentration of oxygenated hemoglobin as an index of frontal activation in 52 measurement points (channels) on the frontal area during the Advanced Trail Making Test (ATMT), a tool used to assess VSWM. The ATMT consists of two tasks, R and F, with the former assessing motor factors and the latter relating to both motor and cognitive factors involved in speeded and unconscious VSWM operations. Twenty-six healthy volunteers were enrolled in this study. Channel activation during Task F performance was observed bilaterally over the dorsolateral and ventrolateral prefrontal cortex. This distribution may reflect central executive function of working memory. Channel activation during Task R was circumscribed to part of the left ventrolateral prefrontal cortex partially overlapping with areas active during Task F performance, likely representing task-related motor factor activation. Our findings suggest that multichannel NIRS during ATMT performance is an appropriate means of measuring cortical activation induced by VSWM operations during daily activity.

1. Introduction

Visuospatial working memory (VSWM) is a cognitive function involved in temporal storage and effective manipulation of optical information related to object identification and spatial location [7,35,41]. It has been reported that several brain regions within the frontal lobe such as the dorsolateral prefrontal cortex (DLPFC), the ventrolateral prefrontal cortex (VLPFC), the frontal eye field (FEF) cortex, and the supplementary motor area (SMA) are involved in VSWM processing, with variation depending on task-specific features and modes of reaction [1,12,14,15,20,22,29,30,31,39,40,41]. The majority of these data stem from studies using static and conscious memory-demanding tasks, such as the delayed response task and the n-back task. Clinically convenient, these tasks use controlled stimuli and require little space and time, but they impose motor restrictions onto the examinees to avoid movement-related neuroimaging artifacts. This is an important limitation, as in daily activity, memory representation is continuously updated during fast and unconscious VSWM operations. It is therefore unclear whether aforementioned brain areas truly reflect those activated during VSWM operations in daily activity.

The Advanced Trail Making Test (ATMT) is a derivative from the Trail Making Test Part A (TMT-A), which is a simple and standardized neuropsychological test that is widely used in clinical practice [3,42]. In the TMT-A the subject is asked to connect on paper the encircled numbers 1 through 25 in ascending order by drawing a line with a pencil as rapidly as possible. In speeded memory processing, this task requires the subject to use motor factors, including visual search and visuomotor coordination as well as cognitive factors, especially VSWM processing, to memorize locations

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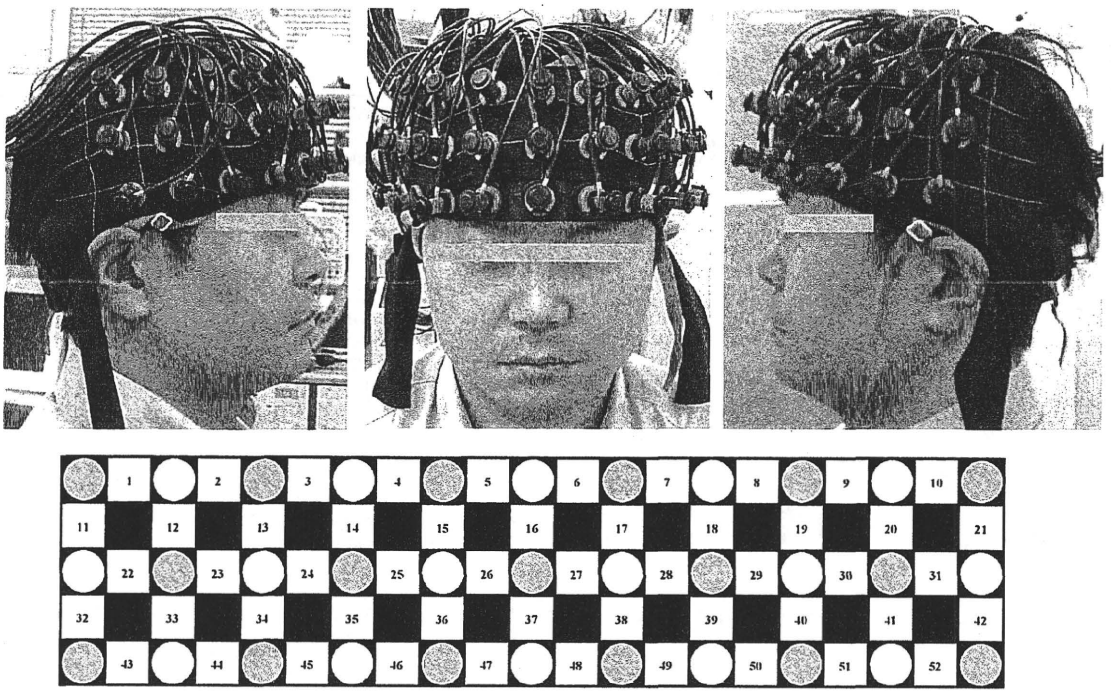

Fig. 1. Location of optical diodes and measurement points (channels). Channels 1 through 52 are depicted as white squares between laser diodes (emitters, gray circles) and photodiodes (detectors, white circles).

of sequential numbers during a visual search [2,11,33,46,49,51]. The TMT-A therefore targets speeded and unconscious VSWM operations representative of daily activity. The ATMT is highly similar to the TMT-A, but contains the following adjustments. The ATMT consists of two tasks, the Fixed Task (Task F) and Random Task (Task R). Task F and TMT-A procedures are very much alike, with the exception of Task F being administered by button pressing on a touch-sensitive panel, which allows the subjects to continue the task performance to induce enough cortical activation. A critical difference between the Task F and Task R is the latter's design to elicit activation related exclusively to motor factors to perform Task F [37,50].

Unlike other neuroimaging techniques such as MRI and PET, multichannel near-infrared spectroscopy (NIRS) is a non-invasive brain functional imaging method that does not restrict subject movement and is therefore a good choice for the ATMT. In addition, NIRS is not affected by electromagnetic noise from electric devices [34]. Near-infrared light is a powerful tool for biological analyses as it can not only penetrate deeply into tissues, but it is also differentially absorbed by hemoglobin (Hb) depending on the oxygenation state of the latter and the optical path length in the tissue (modified Beer–Lambert Law). The law is demonstrated as $A = \varepsilon CL + S$, in which A is the absorbance, ε is the molar absorption coefficient, C is the chromophore concentration, L is the optical path length between the emitter and the detector of the NIRS probe, and S is the optical attenuation related to scattering. This relationship is used in NIRS to measure relative changes in the concentration of oxygenated hemoglobin ([oxy-Hb]) and deoxygenated hemoglobin ([deoxy-Hb]) by emitting near-infrared light at several different wavelengths into brain tissue and detecting its remnant [18,24,28,47]. Areas of high neuronal activation show increased oxygen consumption and enhanced blood supply to ensure provision of oxygenated hemoglobin [19,23]. In other words, neural activation is measured by relative changes of regional cerebral blood volumes.

Several NIRS studies have reported frontal activation during TMT-A performance [33,46,52,53]. The cognitive factors measured by this task are similar to those assessed with Task F of the ATMT. The above-mentioned studies reported bilateral activation, primarily in the prefrontal cortex, although the measuring range in these experiments may have been too restricted. In the here-presented work, multichannel NIRS was used to provide wide coverage of the frontal area during the ATMT in an effort to detect VSWM-induced cortical subregions within the frontal cortex that show an activation pattern consistent with VSWM performance in daily-life activity. To confirm that Task F activation was indeed related to VSWM processing, we additionally measured activation during Task R, which is assumed to elicit exclusively motor factor-related activation. We hypothesized that the DLPFC and the VLPFC activation associated with speeded and unconscious VSWM processing in a paradigm mimicking VSWM performance in daily-life activity can be detected by using multichannel NIRS during ATMT performance.

2. Materials and methods

2.1. Subjects

Twenty-eight healthy volunteers were recruited for this study. Two were excluded due to left-handedness and excessive motion artifacts, leaving a total of 26 right-handed subjects (14 males and 12 females, age 27.2 ± 6.8 yrs; range, 19–40 years). None of the participants had a history of psychiatric or neurological disorders. This research was approved by the ethics committee of Osaka University Graduate School of Medicine and all procedures and methods were in keeping with the policies and principles contained in the Declaration of Helsinki. All subjects gave written informed consent prior to the experiments.

2.2. NIRS measurements

We measured relative changes in the concentration of oxygenated hemoglobin ([oxy-Hb]), deoxygenated hemoglobin ([deoxy-Hb]) and calculated total hemoglobin by combining the two former based on NIRS data (ETG-4000; Hitachi Medical Corporation, Tokyo, Japan) during ATMT performance. The ETG-4000 uses two kinds of near-infrared light, 695 nm and 830 nm. Seventeen laser diodes (emitter) and 16 photodiodes (detector) were mounted reciprocally at 3-cm intervals on a piece of elastic rubber headwear attached to the frontal area with adjustable straps. Approximate detection depth was 2–3 cm below the skin surface in 52 separate regions (approx. 6 cm high × 30 cm wide). The lowest center

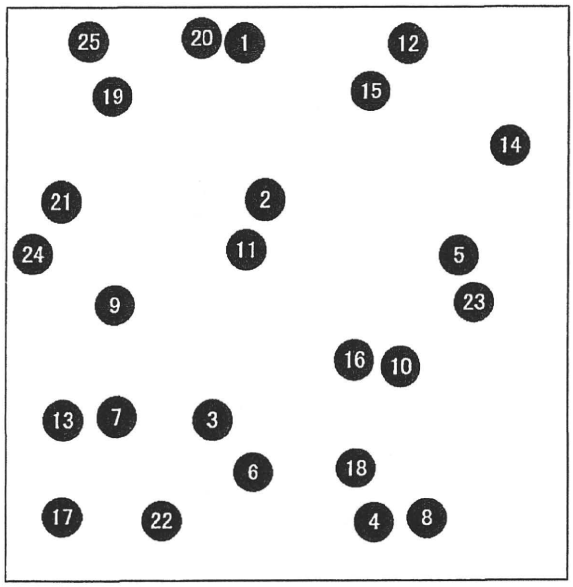

Fig. 2. An example of the Advanced Trail Making Test (ATMT) window on the touch-sensitive panel.

photodiode was located at Fpz according to the international 10/20 system for electroencephalography (EEG). The channels at the posterior edges corresponded to the T3-Cz-T4 line of the international 10/20 system or slightly anterior to this line (Fig. 1).

2.3. Tasks

Brain activation was measured with the Advanced Trail Making Test (ATMT). The ATMT was administered on a touch-sensitive panel (CV515PJ; Totoku Electric, Tokyo, Japan) connected to a personal computer. The subjects pressed 25 numbered black circles (buttons) displayed randomly on an 18 × 18 cm screen in numerical order with their dominant hand. Once a button is pressed, it disappears, and immediately a +25 numbered button appears on the screen (Fig. 2). The time lag between pressing a target button and the preceding button push was deemed reaction time. The participants were instructed to press the buttons as quickly as possible in numeric order from number 11 to the highest possible number within 90 s. Buttons corresponding to numbers 1 through 10 were pressed as part of a practice trial to ensure comprehension of the procedure prior to ATMT execution; data for buttons 1–11 were therefore excluded from data analysis. Before and after ATMT execution, the subjects gazed at the center of the screen and tapped their right index finger on this point at a uniform pace. This was considered as the baseline task.

In a variation of Task F where the location of the buttons remained unchanged when a correct number button was pressed, Task R rearranged the numbers randomly once the correct number was pushed. Task F allowed the subjects to memorize the locations of the buttons which have been found during the visual search, thereby enabling the subjects to shorten the reaction time compared to Task R by using VSWM operations [37,50].

2.4. Procedure

During data collection, the participant sat on a chair in front of a table in a silent room. The tasks including the first baseline task (30 s), the ATMT (90 s) and the second baseline task (60 s) were executed for each Task R and Task F, which were presented in counter-balanced order to each half of the subjects. Instructions for changing the tasks were given orally.

2.5. Data analysis

Changes in [oxy-Hb] and [deoxy-Hb] are expressed as the product of concentration of hemoglobin and optical path length, which produces a measurement of mM mm. In this study, we focused on changes in [oxy-Hb] (temporal resolution of 100 ms) because [oxy-Hb] is reported to be most sensitive to changes in regional cerebral blood volume, and strongly correlates with BOLD signal of fMRI, while the direction of changes in [deoxy-Hb] is determined by changes in venous blood oxygenation and volume [23,24,44,48].

The ETG-4000 analysis software was used in the integral mode. In this configuration, mean changes in [oxy-Hb] during baseline states 10 s just before the beginning of the activation task period or after 50 s from the end of the activation task period were corrected to 0 mM mm by linear fitting. The activation task period itself was set at 90 s, and the recovery period from the end of activation task until baseline stabilization was 50 s. To smooth out short-term motion artifacts we employed the moving average method with a 5 s window.

For behavioral data, we compared the mean reaction time for Task R and Task F using a paired, two-tailed Student's t-test, with $p = 0.05$ set as the significance threshold. Mean changes in [oxy-Hb] during the ATMT for each channel were calculated for each subject, followed by a two-tailed single-sample t-test which is equal to a paired t-test against zero (mean changes in [oxy-Hb] during baseline periods), for Task R and Task F respectively. Since the single-sample t-test was used for all 52

Table 1
The individual channel values in Task R of the ATMT.

Right channel	Activation	t	p	α	r
CH1	0.061 ± 0.092	2.052	0.051	0.002	0.175
CH2	0.031 ± 0.110	0.019	0.985	0.002	0.201
CH3	0.009 ± 0.110	<0.001	>0.999	0.002	0.159
CH4	0.028 ± 0.125	0.001	0.999	0.002	0.198
CH5	0.037 ± 0.110	0.003	0.997	<0.001	−0.012
CH11	0.066 ± 0.156	0.632	0.533	0.003	0.272
CH12	0.096 ± 0.184	1.320	0.199	0.004	0.381
CH13	0.051 ± 0.119	0.711	0.484	0.003	0.302
CH14	0.037 ± 0.101	0.263	0.795	0.003	0.238
CH15	0.045 ± 0.071	2.150	0.041	0.004	0.331
CH22	0.116 ± 0.201	1.858	0.075	0.005	0.391
CH23	0.153 ± 0.263	1.893	0.070	0.005	0.409
CH24	0.100 ± 0.146	2.183	0.039	0.003	0.246
CH25	0.067 ± 0.092	2.624	0.015	0.003	0.320
CH26	0.054 ± 0.134	0.823	0.418	0.005	0.398
CH32	0.105 ± 0.256	0.758	0.455	0.004	0.364
CH33	0.168 ± 0.284	1.973	0.060	0.005	0.412
CH34	0.160 ± 0.254	2.078	0.048	0.004	0.364
CH35	0.097 ± 0.143	2.322	0.029	0.004	0.323
CH36	0.062 ± 0.140	0.929	0.362	0.004	0.351
CH43	0.139 ± 0.302	1.081	0.290	0.004	0.361
CH44	0.170 ± 0.267	2.168	0.040	0.004	0.340
CH45	0.179 ± 0.210	3.253	0.003	0.003	0.299
CH46	0.127 ± 0.180	2.466	0.021	0.002	0.228
CH47	0.068 ± 0.142	1.124	0.272	0.004	0.328
Left channel	**Activation**	t	p	α	r
CH6	0.015 ± 0.08	<0.001	>0.999	0.002	0.106
CH7	0.002 ± 0.162	<0.001	>0.999	<0.001	−0.012
CH8	0.001 ± 0.200	<0.001	>0.999	0.001	0.081
CH9	−0.030 ± 0.189	<0.001	>0.999	0.001	0.100
CH10	0.018 ± 0.171	<0.001	>0.999	0.003	0.279
CH17	0.028 ± 0.148	<0.001	>0.999	0.002	0.210
CH18	0.020 ± 0.215	<0.001	>0.999	0.001	0.022
CH19	0.077 ± 0.167	0.382	0.890	0.003	0.274
CH20	0.023 ± 0.244	<0.001	>0.999	0.004	0.342
CH21	0.098 ± 0.197	1.300	0.206	0.004	0.357
CH27	0.045 ± 0.179	0.026	0.980	0.003	0.290
CH28	0.065 ± 0.146	0.703	0.489	0.002	0.226
CH29	0.113 ± 0.165	2.358	0.027	0.004	0.343
CH30	0.162 ± 0.295	1.557	0.132	0.004	0.331
CH31	0.119 ± 0.296	0.608	0.549	0.003	0.316
CH38	0.070 ± 0.127	1.627	0.116	0.004	0.338
CH39	0.121 ± 0.190	2.250	0.033	0.005	0.386
CH40	0.197 ± 0.246	3.124	0.004*	0.005	0.390
CH41	0.211 ± 0.334	2.287	0.031	0.005	0.406
CH42	0.149 ± 0.267	1.679	0.106	0.004	0.364
CH48	0.111 ± 0.155	2.707	0.012	0.004	0.369
CH49	0.134 ± 0.151	3.576	0.001*	0.004	0.377
CH50	0.226 ± 0.253	3.724	0.001*	0.006	0.457
CH51	0.189 ± 0.331	1.817	0.081	0.004	0.370
CH52	0.184 ± 0.344	1.536	0.137	0.004	0.367
Middle channel	**Activation**	t	p	α	r
CH16	0.031 ± 0.143	0.002	0.998	0.003	0.244
CH37	0.051 ± 0.209	0.027	0.979	0.003	0.317

Activation: the average across 26 participant's mean changes in [oxy-Hb] ± standard deviation (mM mm) during Task R of the ATMT in each channel, t and p: the values derived by comparing the 26 participant's mean changes in [oxy-Hb] during Task R with those during the baseline periods using the two-tailed single-sample t-test, which were corrected for multiple comparisons by the Dubey/Armitage-Parmar procedure, α: the adjusted critical α-level by the above procedure, r: the mean value of Pearson's correlation coefficients between the channel and the other 51 channels.

* Significant p values.

Table 2
The individual channel values in Task F of the ATMT.

Right channel	Activation	t	p	α	r
CH1	0.006 ± 0.181	<0.001	>0.999	0.003	0.294
CH2	−0.019 ± 0.164	<0.001	>0.999	0.002	0.201
CH3	−0.051 ± 0.269	<0.001	>0.999	0.001	0.069
CH4	0.023 ± 0.184	<0.001	>0.999	0.003	0.244
CH5	0.022 ± 0.168	<0.001	>0.999	0.003	0.245
CH11	0.031 ± 0.151	0.003	0.997	0.003	0.295
CH12	0.120 ± 0.177	2.434	0.022	0.005	0.386
CH13	0.025 ± 0.194	<0.001	>0.999	0.002	0.178
CH14	0.025 ± 0.159	<0.001	>0.999	0.004	0.324
CH15	0.044 ± 0.121	0.429	0.672	0.004	0.327
CH22	0.144 ± 0.169	3.321	0.003*	0.004	0.349
CH23	0.236 ± 0.194	5.131	<0.001*	0.003	0.301
CH24	0.146 ± 0.163	3.465	0.002*	0.003	0.276
CH25	0.069 ± 0.142	1.288	0.209	0.004	0.366
CH26	0.086 ± 0.156	1.503	0.145	0.003	0.277
CH32	0.159 ± 0.193	3.024	0.006	0.003	0.260
CH33	0.217 ± 0.211	4.252	<0.001*	0.004	0.361
CH34	0.268 ± 0.245	4.380	<0.001*	0.002	0.213
CH35	0.156 ± 0.188	3.161	0.004	0.003	0.309
CH36	0.083 ± 0.190	0.784	0.440	0.003	0.294
CH43	0.213 ± 0.261	3.097	0.005	0.003	0.315
CH44	0.292 ± 0.259	4.678	<0.001*	0.003	0.308
CH45	0.321 ± 0.283	4.640	<0.001*	0.003	0.257
CH46	0.176 ± 0.200	3.620	0.001*	0.005	0.435
CH47	0.091 ± 0.209	1.003	0.325	0.005	0.392
Left channel	**Activation**	t	p	α	r
CH6	0.008 ± 0.107	<0.001	>0.999	0.004	0.380
CH7	0.002 ± 0.215	<0.001	>0.999	0.002	0.216
CH8	0.035 ± 0.243	<0.001	>0.999	0.002	0.227
CH9	0.057 ± 0.155	0.340	0.736	0.003	0.267
CH10	0.033 ± 0.261	<0.001	>0.999	0.002	0.151
CH17	0.045 ± 0.202	0.004	0.997	0.003	0.259
CH18	0.062 ± 0.309	0.001	0.999	0.003	0.249
CH19	0.144 ± 0.212	2.273	0.032	0.003	0.296
CH20	0.068 ± 0.244	0.021	0.983	0.002	0.216
CH21	0.110 ± 0.211	0.964	0.344	0.002	0.121
CH27	0.083 ± 0.180	0.963	0.345	0.003	0.307
CH28	0.113 ± 0.191	1.762	0.090	0.003	0.303
CH29	0.207 ± 0.246	3.325	0.003*	0.004	0.374
CH30	0.280 ± 0.226	5.211	<0.001*	0.003	0.277
CH31	0.220 ± 0.245	3.250	0.003	0.002	0.142
CH38	0.131 ± 0.143	3.758	<0.001*	0.005	0.395
CH39	0.195 ± 0.215	3.570	0.001*	0.003	0.313
CH40	0.267 ± 0.284	3.712	0.001*	0.003	0.291
CH41	0.294 ± 0.264	4.662	<0.001*	0.004	0.335
CH42	0.260 ± 0.223	4.665	<0.001*	0.002	0.163
CH48	0.159 ± 0.209	2.758	0.011	0.005	0.399
CH49	0.211 ± 0.191	4.604	<0.001*	0.004	0.335
CH50	0.341 ± 0.211	7.194	<0.001*	0.005	0.397
CH51	0.334 ± 0.328	4.154	<0.001*	0.003	0.317
CH52	0.314 ± 0.303	4.226	<0.001*	0.003	0.309
Middle channel	**Activation**	t	p	α	r
CH16	0.038 ± 0.154	0.012	0.991	0.003	0.260
CH37	0.112 ± 0.207	1.484	1.150	0.003	0.295

Activation: the average across 26 participant's mean changes in [oxy-Hb] ± standard deviation (mM mm) during Task F of the ATMT in each channel, t and p: the values derived by comparing the 26 participant's mean changes in [oxy-Hb] during Task F with those during the baseline periods using the two-tailed single-sample t-test, which were corrected for multiple comparisons by the Dubey/Armitage-Parmar procedure, α: the adjusted critical α-level by the above procedure, r: the mean value of Pearson's correlation coefficients between the channel and the other 51 channels.
* Significant p values.

channels, we corrected for multiple comparisons using the Dubey/Armitage-Parmar procedure which takes into account spatial correlations among the cortical regions and has been used in a comparable fashion in previous work using multichannel NIRS [16,43,45] (see Tables 1 and 2). Additionally, the number of activated channels in Task R and Task F were compared using a two-tailed Chi-square test with a significance level of $p < 0.05$. Statistical analyses were performed with SPSS software (version 12.0J).

3. Results

The mean reaction time ± standard deviation for button pressing during ATMT performance was 2919 ± 631 ms in Task R and 2043 ± 416 ms in Task F; the difference was statistically significant (d.f. = 25, $t = -10.33$, $p < 0.001$). We next generated the grand-average waveform corresponding to [oxy-Hb] changes across subjects for each channel (Figs. 3 and 4 for Task R and F, respectively). To detect activation, the mean changes in [oxy-Hb] during the ATMT were compared to those during the baseline task in all 52 channels, which indicated three channels (5.8%) for Task R (Table 1, Fig. 3) and 19 channels (36.5%) for Task F (Table 2, Fig. 4). The channels activated by Task R were also activated during performance of Task F. Chi-square test analysis demonstrated that Task F activated significantly more channels than Task R ($\chi^2 = 14.758$, d.f. = 1, $p < 0.001$).

4. Discussion

In the present study, frontal activation during Task F and Task R of the ATMT was measured by multichannel NIRS with 52 channels covering the entire frontal cortex. A larger number of channels were activated during Task F compared to Task R, primarily over the bilateral DLPFC and VLPFC. This suggests that these prefrontal areas are engaged during VSWM even when the limitation of subject's motor restrictions during speeded processing tasks is taken into account. Thus, this activation pattern is consistent with VSWM performance in daily activity. Because this study's Task F was very similar to the TMT-A, direct comparison to other work using that same test is possible, despite the difference in the number of channels used. Our findings are consistent with previous NIRS work that indicated VSWM-induced activation throughout the frontal area [33,46,52,53]. The TMT-A is sensitive to both cognitive and motor factors required for task execution, albeit motor factors to a lesser degree [51]. Task R, on the other hand, elicited activation only in cortical regions involved in the processing of motor factors. One may speculate that the cognitive factors engaged specifically during Task F but not Task R performance are closely related to VSWM [37,50].

Based on an anatomical craniocerebral correlation using the international 10/20 electrode placement system for EEG reported by Okamoto et al. [38], we can infer that regions activated by cognitive factors critical to Task F performance in this study correspond to the bilateral DLPFC and VLPFC (Fig. 4). Recent studies have provided substantial evidence that the lateral prefrontal cortex is part of the neural substrate of VSWM [1,12,20,22,39,40], which is considered a core cognitive factor assessed by Task F of the ATMT. Our findings are supported by those data, specifically prefrontal activation associated with VSWM processing. However, despite reports that the FEF and the SMA are also involved in VSWM [14,15,29,30,31,39,41], no significant activation was found in the superior channels overlying these cortical areas in our analysis.

The channels activated by Task R were also activated during Task F performance, which were restricted to part of the left VLPFC. This activation induced by Task R and Task F performance in our study might reflect motor factors involved in ATMT performance. This assumption is supported by recent reports indicating that the VLPFC is associated with motor functions such as complex hand movements and associative sensorimotor learning [8,9].

Based on the conceptual model of working memory suggested by Baddeley [6,7] and Hitch [5] proposing that the central executive administers three slave components (i.e., visuospatial sketchpad, phonological loop, and episodic buffer) to act together for executive function, it appears likely that the activation of DLPFC and VLPFC

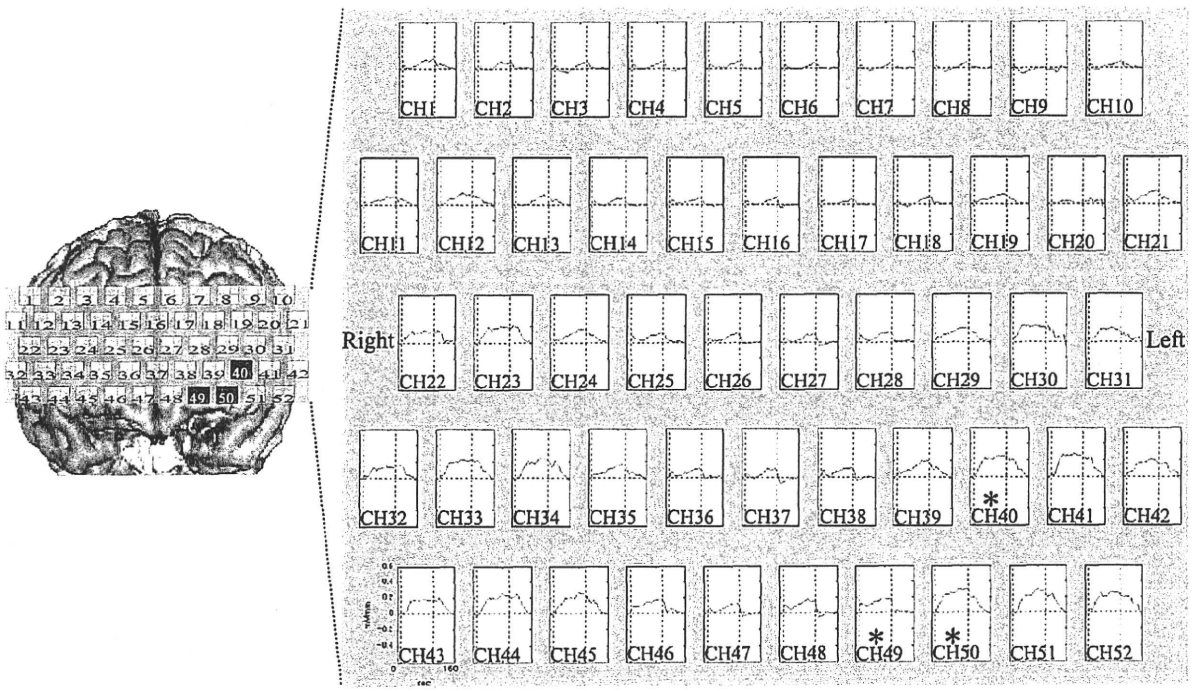

Fig. 3. Grand-average waveforms of changes in oxy-Hb concentration during Task R for all channels plotted as changes in [oxy-Hb] (mM mm) versus time (s). For each channel, the horizontal scale covers time from 0 to 160 s with the vertical scale indicating activation in between −0.6 and 0.6 mM mm. The task period is marked by two dashed lines at 10 and 100 s. The numbers superimposed on the standard brain surface indicate the channel numbers. The positions of the activated channels are indicated by black squares. CH: channel, *: channel with significant activation.

during Task F in our study was mainly associated with the central executive component. This argument is supported by the fact that the central executive is related to (1) retrieval of the location of the next number, (2) divided attention in the dual-task performance during the visual search, (3) active maintenance of rules and strategies related to simultaneous searching and memorizing, (4) continuous updating of memory representations [1,13,25,32,40]. The visuospatial sketchpad is thought to be associated with encoding and maintenance of memory representations of subsequent numbers and locations. Thus, its role may have been considerably small in our study as memory representations in the ATMT are only double-digit numbers with unified standard and those locations in a two-dimensional surface, which is approximately twice larger than the visual attention field [37,50].

The present study has several limitations. First, changes in [oxy-Hb] are relative values compared to the baseline state, and it is

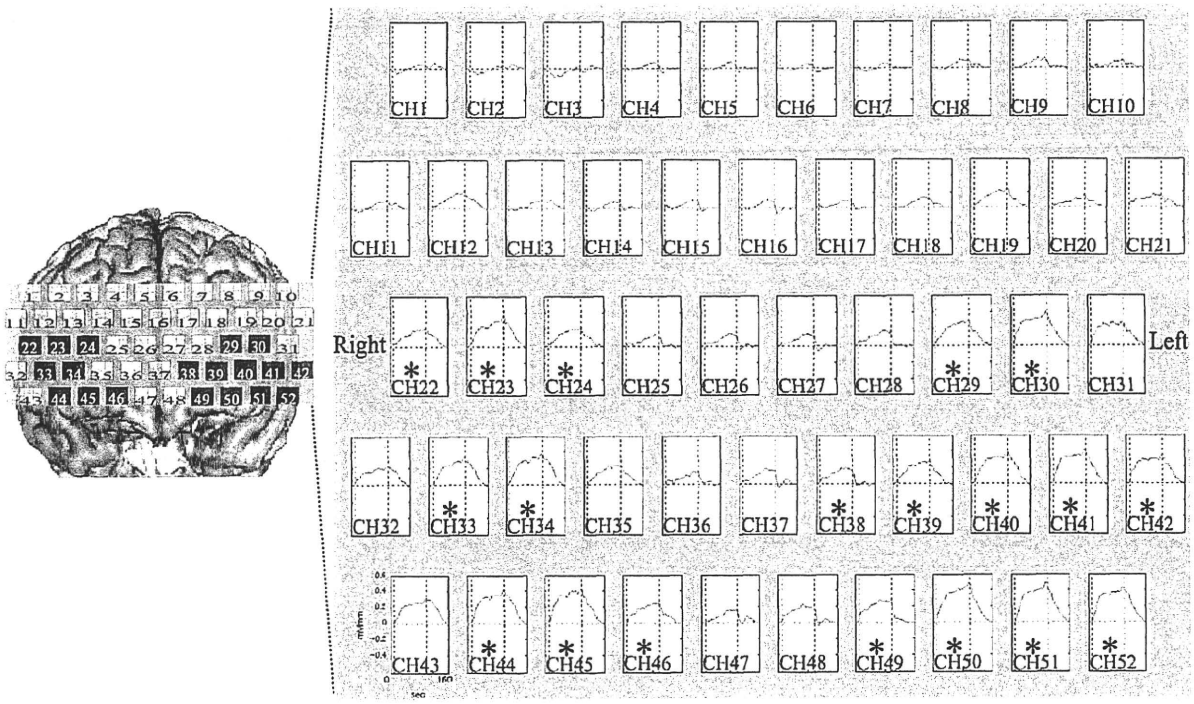

Fig. 4. Grand-average waveforms of changes in oxy-Hb concentration during Task F for all channels plotted as changes in [oxy-Hb] versus time. For details, see Fig. 3 legend.