Results After olfactory stimulation, significant activations were observed in the PFC of patients with MCS on both the right and left sides compared with controls. The activations were specifically strong in the orbitofrontal cortex (OFC). Compared with controls, autonomic perception and feelings identification were poorer in patients with MCS. OFC is associated with stimuli response and the representation of preferences.
Conclusions These results suggest that a past strong exposure to hazardous chemicals activates the PFC during olfactory stimuli in patients with MCS, and a strong activation in the OFC remains after the stimuli.

Keywords Cerebral blood flow · Multiple chemical sensitivity · Near-infrared spectroscopy · Olfactory stimulation · Orbitofrontal cortex · Recovery

Introduction

Multiple chemical sensitivity (MCS) is a chronic acquired disorder characterized by non-specific and recurrent multisystem symptoms associated with exposure to common odorous chemicals such as organic solvents, pesticides, cleaning products, perfumes, environmental tobacco smoke, or combustion products [1–3]. According to population-based surveys, the prevalence of MCS is estimated to range from 8 to 33 % [4–9]. Thus, MCS has become a large public health concern during the past two decades, particularly in industrialized countries. The symptoms of MCS can be mild to disabling, and they are triggered by multiple chemicals. These symptoms are reactions to previous chemical exposure that recur on subsequent exposure to the same or structurally unrelated chemicals at levels below those established as having harmful effects in the general population [2]. Central nervous system (CNS) symptoms such as headaches, dizziness, extreme fatigue, and concentration difficulties are common; airway and gastro-intestinal tract symptoms are also frequently reported [2, 10–12]. Diagnosis of MCS can be difficult because of the inability to assess the causal relation between exposure and symptoms [3, 13]. No standardized objective measures to identify MCS and no precise definition of this disorder have been established. Most definitions of MCS are qualitative, relying on subjective reports from patients and clinicians of distressing symptoms and environmental exposure [14].

We previously conducted a near-infrared spectroscopy (NIRS) activation study on olfactory stimulation in patients with MCS [14]. Activation was defined as a significant increase in the regional cerebral blood flow (rCBF) following odorant stimulation. Changes in the blood flow and oxygenation to the brain are closely linked to neural activity [15]. NIRS has been commonly applied in studies of prefrontal activity [16, 17] and is suitable for detecting oxygenation changes in higher cortical regions. Our previous study identified acute activation in the prefrontal cortex (PFC) during olfactory stimulation with several different odorants in patients with MCS [14]. The prefrontal area connects to the anterior cingulate cortex (ACC), an area of odorant-related activation in patients with MCS [18]. The results of challenge tests by exposure to odorous chemicals indicated a neuro-cognitive impairment in patients with MCS, and using single photon-emission computed tomography, brain dysfunction was found particularly in odor-processing areas, thereby suggesting a neurogenic origin of MCS [19]. One possibility is that patients with MCS may have an enhanced top–down regulation of odor response via the cingulate cortex. These findings also suggest that prefrontal information processing associated with the odor-processing neuronal circuits and memory from a past experience of chemical exposure may play significant roles in the pathology of this disorder.

Our previous study also showed that the patients with MCS adequately distinguished non-odorant in 10 odor repetitions during the early stage but not in the late stage of the olfactory stimulation test when the olfactory stimulation test was continuously repeated 10 times. Repeated or prolonged exposure to an odorant typically leads to a stimulus-specific decrease in olfactory sensitivity to that odorant, but sensitivity recovers over time in the absence of further exposure [20]. Thus, we postulate that prefrontal information processing in patients with MCS is activated by an emotional response to a repeated olfactory stimulation in the late stage of the test, and that the processing system in the PFC cannot respond adequately. Further, the sensory recovery of the olfactory system in patients with MCS may process odors differently from healthy subjects after olfactory stimulation. Although recovery is generally evident after short olfactory stimulation on the several tens of second time scale [21, 22], the recovery process of patients with MCS may differ from that of healthy subjects. In this study, we examined the recovery process after short olfactory stimulation in patients with MCS, using NIRS imaging.

Methods

Patients

Patients with MCS were diagnosed in the outpatient department for people with chemical sensitivities in the Hyakumanben Clinic (Outpatient Department of Sick House Syndrome) between October 2009 and January 2013. The same case definitions for MCS (inclusion and

exclusion criteria), as in our previous study [14], were applied in this study. MCS was diagnosed according to the 1999 consensus criteria [23]. Patients diagnosed with chronic fatigue syndrome, fibromyalgia syndrome, or mental health disorders were excluded from the study. Patients who had hyperpiesia, hyperlipidemia, diabetes, and/or allergic rhinitis were also excluded. Recruitment for this study was conducted 3 months prior to the olfactory stimulation test using NIRS. The MCS condition of all patients was confirmed by the clinic physician during recruitment. Controls were recruited and selected to match the patients by age and sex at the group level. The same inclusion and exclusion criteria were applied for all patients and controls as those in our previous study [14]. Inclusion was based on the scores of the Quick Environmental Exposure and Sensitivity Inventory (QEESI), whereas exclusion criteria included abnormal hematological examinations, smoking, drug or alcohol abuse, medications, pregnancy, and severe nasal stuffiness.

This study was approved by the ethical committee for human research at the Hyakumanben Clinic (99642-61) and the Louis Pasteur Centre for Medical Research (LPC.11) and was performed according to the guidelines of the Declaration of Helsinki (1975). All patients provided written informed consent and received the equivalent of 5000 JPY for their participation. This study was conducted from November 2012 to March 2013.

Olfactory stimulation

The same card-type olfactory identification test kit (Open Essence; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and odorants [mandarin orange (MO), Japanese cypress (JC), menthol (Mt), and perfume (Pf)] as those in our previous study were used for the olfactory stimulation test [14]. Perception of these odors was tested by placing the card at approximately 30 mm from the noses of both patients with MCS and controls.

Experimental procedure

In the present study, we followed the same experimental procedure as that in our previous study [14] and added a recovery period after the olfactory stimuli. Interviews were conducted immediately before the olfactory stimulation test and the assessments of health and nasal symptoms. The test room was maintained at a temperature of approximately 23 °C. Patients sat in a comfortable chair and remained in the test room long enough to feel comfortable before being exposed to the odorants. During the experiments, the patients closed their eyes and slowly repeated the Japanese alphabet in an undertone to establish a stable rCBF prior to the olfactory stimulation. They stopped repeating the Japanese alphabet and closed their eyes during the olfactory stimulation, which lasted for 10 s. Olfactory stimulation was performed after a 30-s pre-rest period to establish the baseline level (Fig. 1).

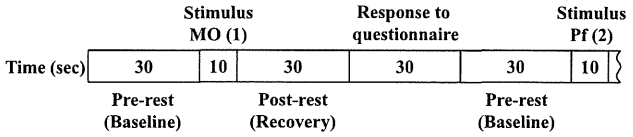

Fig. 1 Experimental protocol. First, the subjects had a 30-s pre-rest. Then, the subjects were given an olfactory stimulus for 10 s, followed by a 30-s post-rest and 30-s self-reporting to a questionnaire on irritating and hedonic scales, respectively. The 100-s cycles per odorant were repeated 10 times in a row

The questionnaire on irritating and hedonic scales was completed immediately after a 30-s rest period (post-rest) to allow recovery after the olfactory stimulation. The response time for the questionnaire was secured for 30 s. After that, the same process was repeated for an additional 9 olfactory stimuli. Irritation was evaluated on a visual analogue scale, with responses ranging from "not at all" to "strong". Hedonic responses were rated on a 5-point Likert scale ranging from pleasant (1) to unpleasant (5).

Olfactory stimuli were applied in the following order: MO, Pf, non-odorant (NO), JC, Mt, Pf, JC, NO, Mt, and MO. The order of the first block was as follows: MO (1), Pf (2), NO (3), JC (4), and Mt (5). The order of the second block was permuted from the first block as follows: Pf (6), JC (7), NO (8), Mt (9), and MO (10). The 100-s cycles were repeated 10 times in a row. Thus, the order of 10 repetitions (1–10) was as follows: MO (1), Pf (2), NO (3), JC (4), Mt (5), Pf (6), JC (7), NO (8), Mt (9), and MO (10).

NIRS data acquisition

NIRS works on the principle that near-infrared light is absorbed by oxygenated (oxyHb) and deoxygenated (deoxyHb) hemoglobin (Hb) but not by other tissues. Changes in oxyHb concentration in the PFC were measured using the functional NIRS topography system OMM-3000 Optical Multi-channel Monitor (Shimadzu Corporation, Kyoto, Japan). These changes reflect neuronal activity as their levels correlate with evoked changes in rCBF [15, 24, 25]. When neurons become active, local blood flow to the relevant brain regions increases and oxygenated blood displaces deoxygenated blood. Measurement of oxyHb concentrations is most useful because changes in oxyHb are the most sensitive indicators of changes in rCBF among the three NIRS parameters (oxyHb, deoxyHb, and totalHb) [26, 27]. Pairs of illuminators and detectors were set 3 cm apart in a 3 × 9 lattice pattern to form 42 channels through a holder set in the PFC. Changes in the oxyHb concentration were recorded every 130 ms using the NIRS

system. Optical data were analyzed on the basis of the modified Beer–Lambert Law and signals reflecting the oxyHb concentration changes in an arbitrary unit were calculated (millimolar–millimeter) [14].

Questionnaire on physical and psychological status

Patients completed a self-report questionnaire for the assessment of physical and psychological parameters, which included the Chemical Sensitivity Scale for Sensory Hyper-reactivity (CSS-SHR) [28], the Somato-Sensory Amplification Scale (SSAS) [29], the Autonomic Perception Questionnaire (APQ) [30], the Tellegen Absorption Scale (TAS) [31], the Marlowe–Crowne Social Desirability Scale [32], the Taylor Manifest Anxiety Scale (TMAS) [33], the Negative Affectivity Scale (NAS) [34], and the Toronto Alexithymia Scale (TAS-20) that evaluates the total score and the scores of the three subscales, which assess difficulties in identifying feelings (DIF), difficulties in describing feelings (DDF), and externally-oriented thinking (EOT) [35].

Statistical analyses

To assess the recovery status after the olfactory stimulation, the oxyHb concentrations between the 30-s rest period after the olfactory stimulation and the baseline during the pre-rest period were compared in each channel. Because raw data of NIRS provided only relative values and could not be averaged directly across patients or compared among channels, raw data from each channel were converted into z-scores [14, 36–38]. In the present study, we used the same statistical analyses as that in our previous study [14]. The t test was used to compare the brain activity obtained from NIRS imaging for each channel between cases and controls. The non-parametric Mann–Whitney U test was used to analyze the results of the olfactory stimulation questionnaire and to quantify the differences between patients with MCS and controls. The t test was applied to analyze the results of the physical and psychological scales to determine differences between patients with MCS and controls at baseline. All data analyses were performed using the SPSS statistics software, version 22.

Results

Participants

Participants included 10 patients with MCS (age, 28–64 years; mean, 51.0 ± 10.6 years; all females) and six controls (age, 36–58 years; mean, 45.7 ± 8.3 years; all females). Three patients with MCS did not fulfill the inclusion criteria of QEESI. The fourth patient with MCS had severe allergenic reactions to allergens of cedar pollen, mites, cats, dogs, and fungi, and showed a high value of immunoglobulin E type on hematological examinations. The remaining six non-smoking patients with MCS (age, 49–64 years; mean, 54.5 ± 5.9 years; all females) and six non-smoking controls (age, 36–58 years; mean, 45.7 ± 8.3 years; all females) passed all criteria and were included in the analyses. All six patients with MCS had participated in our previous study [14]. Of the six controls, two controls had participated in our previous study [14] and the remaining four controls were participating for the first time in the present study. All patients with MCS tried to avoid the exposure to odorous chemicals as much as possible. These patients were homemakers or pensioners and their occupational histories showed that previous occupations included a clerical employee (office or retail store), a fabric tinter, and a supermarket baker. Two controls also tried to avoid exposure to odorous chemicals as much as possible. Their occupations were teacher. Four controls did not consciously try to avoid exposure to odorous chemicals, and their occupations were as follows: teacher, office worker, child welfare volunteer, and the fourth was a homemaker whose previous occupation was in sales for a general insurance company.

NIRS imaging and subjective evaluation of odors

Time-course of average z-scores of all channels for oxyHb in the patients with MCS group and controls during pre-rest, stimulus, and post-rest are shown in Fig. 2. Results of the t test in terms of the average of all channels (1–42) comparing z-scores for oxyHb concentrations between patients with MCS and controls are shown in Table 1. The first olfactory stimulation with MO (1) lead to increased rCBF levels in the PFC, which was not significantly different between patients with MCS and controls.

Increases in rCBF levels in patients with MCS were suppressed after exposure to the non-odorant tests NO (3) and NO (8) on the third repetition. There was no difference between patients with MCS and controls in the PFC responses. Significant differences in the PFC responses were observed between patients with MCS and controls after Pf (2), JC (4), Mt (5), Pf (6), Mt (9), and MO (10) olfactory stimuli. The increases in the PFC response after the olfactory stimulation were significant in patients with MCS. No significant difference was observed in the JC (7) test, but as shown in Fig. 2, MCS patients had increases in rCBF levels during 10 s after the stimulus, which were not observed in the control group.

Table 2 shows the correlation coefficient between rCBF after the first and second exposure to the same odor in terms of z-scores for all channels (1–42). Comparing the

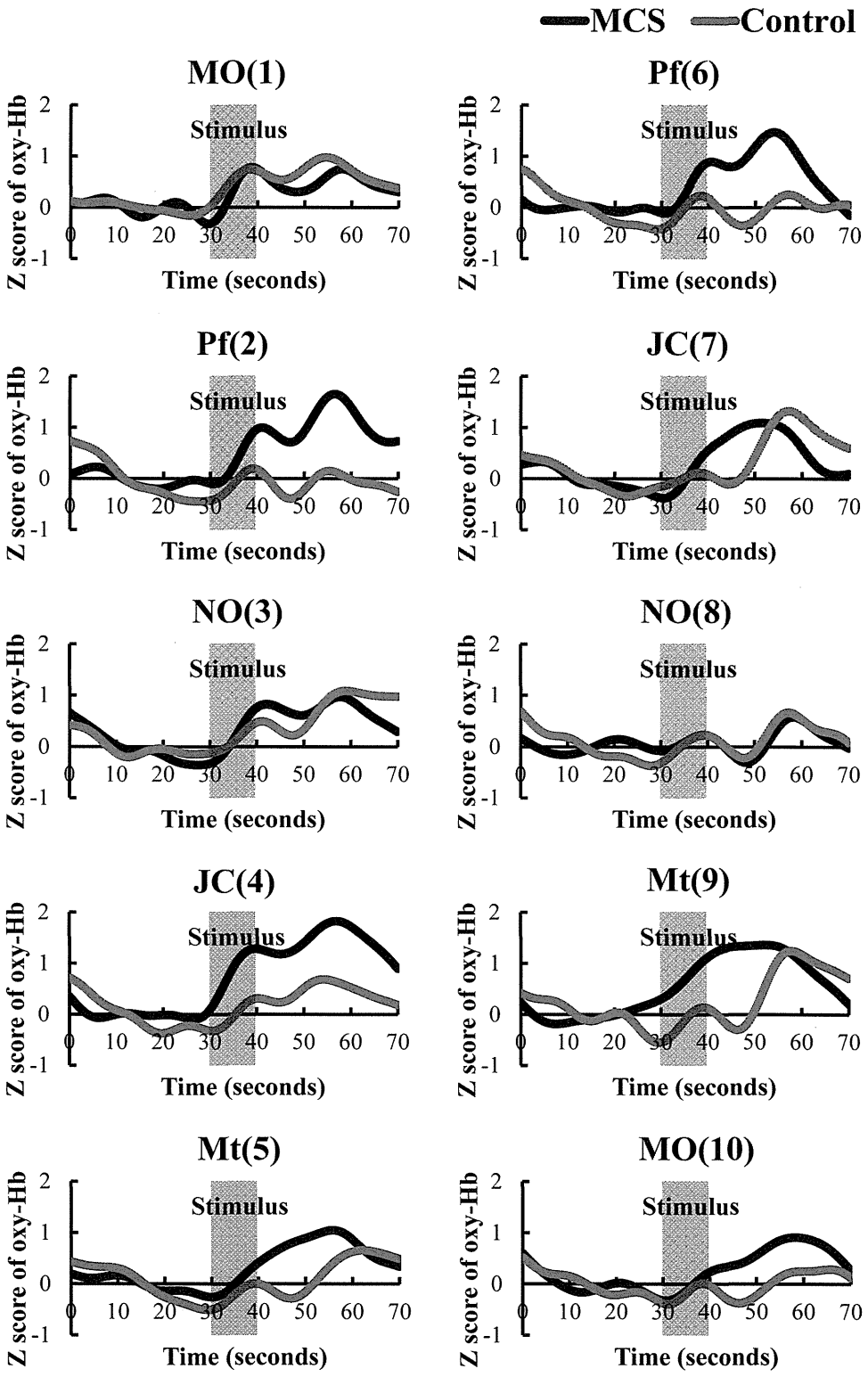

Fig. 2 Time-course of average *z*-scores of all channels for oxyHb in patients with MCS ($n = 6$) and controls ($n = 6$) during pre-rest (baseline, 10–30 s), stimulus (30–40 s), and post-rest (recovery, 40–70 s). *Y*- and *X*-axes represent *z*-scored oxyHb values and times. Law and signals reflecting the oxyHb concentration changes in an arbitrary unit were calculated (millimolar–millimeter). Data of the signal were adjusted by an FFT (Fast Fourier Transform) filter smoothing technique (OriginPro 9.1 software of OriginLab Corporation). The cutoff frequency was determined at thirty-five points. MCS group is indicated as a *black line* and control is indicated as *gray line*. *MO* mandarin orange, *Pf* perfume, *NO* non-odorant, *JC* Japanese cypress, *Mt* menthol. *Numbers in parentheses* indicate the orders of the 10 repetitions (1–10)

rCBF between the first and second exposures revealed significant correlations in both patients with MCS and controls for all stimuli, with the exception of MO and NO in patients with MCS. The correlation coefficients of patients with MCS were lower overall than those of controls. As in our previous study [14], the variation within the MCS group was larger than in the control group. In the subjective evaluation, both patients with MCS and controls

Table 1 The *t* test results in terms of average values for all channels (1–42) comparing *z*-scores for oxyHb between patients with MCS and controls

Test	MCS ($n = 6$)	Controls ($n = 6$)	p value
MO (1)	0.47 (2.40)	0.66 (2.05)	0.336
Pf (2)	1.05 (2.57)	−0.11 (1.33)	<0.001*
NO (3)	0.70 (2.47)	0.73 (1.48)	0.895
JC (4)	1.41 (3.37)	0.40 (1.35)	<0.001*
Mt (5)	0.73 (3.02)	0.23 (1.08)	0.013*
Pf (6)	0.82 (3.13)	−0.03 (1.02)	<0.001*
JC (7)	0.66 (3.11)	0.63 (2.63)	0.905
NO (8)	0.13 (1.15)	0.23 (1.34)	0.407
Mt (9)	1.06 (2.93)	0.56 (2.50)	0.038*
MO (10)	0.61 (1.59)	0.01 (0.94)	<0.001*

Values are expressed as means (±standard deviations). Numbers in parentheses in column 1 indicate the order of the 10 repetitions (1–10)

MO mandarin orange, *Pf* perfume, *NO* non-odorant, *JC* Japanese cypress, *Mt* menthol

* Significant at $p < 0.05$

Table 2 Correlation coefficient (*r*) between rCBF after the first and second exposures to the odor in terms of *z*-scores for all channels (1–42)

Odorant	MCS ($n = 6$)		Controls ($n = 6$)	
	r	p value	r	p value
MO	0.082	0.197	0.193	0.002*
Pf	0.321	<0.001*	0.548	<0.001*
NO	0.092	0.144	0.447	<0.001*
JC	0.250	<0.001*	0.479	<0.001*
Mt	0.302	<0.001*	0.400	<0.001*

Values are expressed as Pearson product-moment correlation coefficients

MO mandarin orange, *Pf* perfume, *NO* non-odorant, *JC* Japanese cypress, *Mt* menthol

* Significant at $p < 0.05$

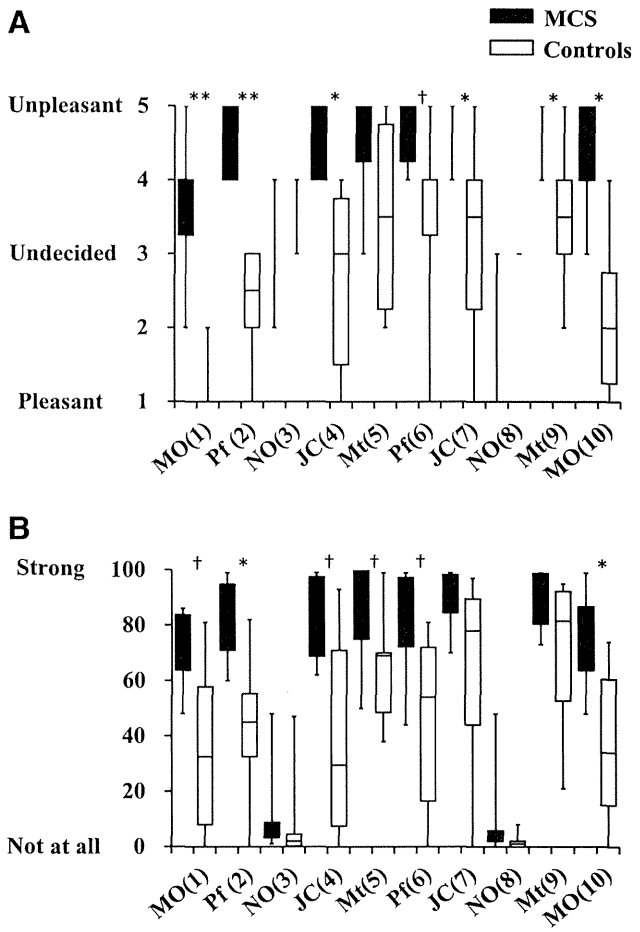

Fig. 3 Ratings of hedonic (**a**) and irritating (**b**) odors by patients with MCS ($n = 6$) and controls ($n = 6$) after the olfactory stimulation. *MO* mandarin orange, *Pf* perfume, *NO* non-odorant, *JC* Japanese cypress, *Mt* menthol. *Numbers in parentheses* indicate orders of the 10 repetitions (1 to 10). Statistically significant differences between groups are indicated. *$p < 0.05$, **$p < 0.01$. Significant tendencies are indicated: $^{\dagger}p < 0.10$

responded "not at all" on the irritation scale and "undecided" on the hedonic scale for NO (Fig. 3). The results of the hedonic scale indicated that scores of most patients with MCS were significantly higher than those of controls. The results of the irritation scale indicated that the Pf (2) and MO (10) scores of patients with MCS were significantly higher than those of controls. Scores for MO, Pf, JC, and Mt were also higher in the patients with MCS than in controls, but the differences were not statistically significant. Large ranges of scores in patients with MCS and controls were assumed to be causally related to the results.

Figure 4 provides the topographical maps of average *z*-scores for oxyHb in patients with MCS and controls. Figure 5 shows the average *t* values for each channel comparing the *z*-scores for oxyHb between patients with MCS and controls. Even after olfactory stimuli, significant activations were observed in the PFC of patients with MCS on both right and left sides (distinct from the center of the PFC) compared with controls. Activation was defined as a significant increase in rCBF due to olfactory stimulation. The activations were especially strong in the lateral orbitofrontal cortex (OFC), on both the right and left sides of the OFC in the PFC (Fig. 4). These remaining activations after the olfactory stimuli were stronger in the test for Pf (2) on the second repetition, for JC (4) on the fourth repetition, for Mt (5) on the fifth repetition, and for Pf (6) on the sixth repetition. After the test for Pf (6), the regional differences of the activation area between patients with MCS and controls were decreased. Our previous study suggested that the olfactory system in patients with MCS could not adequately process odors in the late stage of the

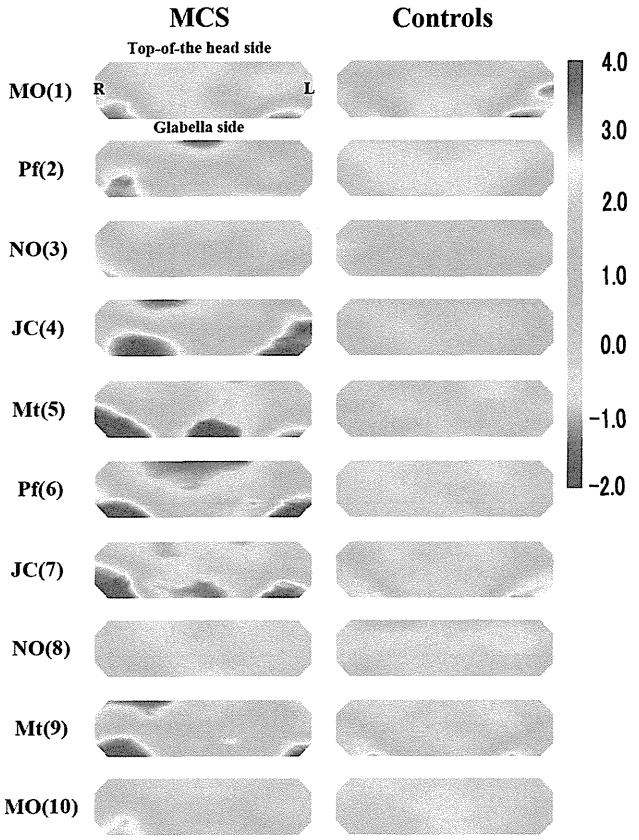

Fig. 4 Topographical maps of average *z*-scores for oxyHb between patients with MCS ($n = 6$) and controls ($n = 6$). *MO* mandarin orange, *Pf* perfume, *NO* non-odorant, *JC* Japanese cypress, *Mt* menthol. *Numbers in parentheses* indicate the order of the 10 repetitions (1–10)

olfactory stimulation test. And then, the NIRS imaging revealed that the CNS of patients with MCS may have been confused in the late stage of the olfactory stimulation test [14]. The results of the present study support this hypothesis.

Physical and psychological measurements

Table 3 shows the results of the *t* test for the physical and psychological scales. CSS-SHR scores were significantly higher of patients with MCS than of controls ($p < 0.001$). Thus, chemical sensitivity in patients with MCS was demonstrated not only by the results of QEESI but also by those of the CSS-SHR scale. In the psychological evaluations, the APQ ($p < 0.1$) and TAS-20 DIF ($p < 0.1$) scores showed a higher tendency only for patients with MCS compared with controls, probably because of the small sample size, but these differences were significant in our previous study [14]. No significant differences were observed in the SSAS, TAS, MCSD, TMAS, TAS-20 total, TAS-20 DDF, and TAS-20 EOT scores, similar to our previous study [14].

Discussion

As shown in Fig. 2, strong activation was observed during olfactory stimulation with several different odorants in patients with MCS compared with controls. All patients with MCS participating in this study had participated in our previous study [14]. Although the previous study had been conducted 1–2 years before this study, similar responses were observed in both.

The first olfactory stimulation with MO (1) lead to increased rCBF levels in the PFC, which was not significantly different between patients with MCS and controls. Because MO (1) was the first test, the patients may not have had the chance to get used to the olfactory stimuli and the response may have been caused by affective tension. After the first test, rCBF levels in controls remained almost stable until the end of the test involving MO (10). Thus, we did not superimpose the data of first and second block but showed all the data as the results.

In the subjective measurement by irritating and hedonic scales and physical and psychological measurements, the overall results were similar to our previous study [14] and were reproducible. In the present study, even after olfactory stimulation, significant activations were observed in the PFC of patients with MCS than in that of controls. The activations were especially strong on both right and left sides of OFC in the PFC. Further, the activations in both patients with MCS and controls were suppressed after the olfactory stimulation involving NO, and the differences were not significant. These results indicate that the olfactory system in patients with MCS adequately distinguishes the non-odorant. Comparing the rCBF between the first and second exposures revealed significant correlations in both patients with MCS and controls for all stimuli, with the exception of MO and NO in patients with MCS. The lack of correlation in the MO and NO test in patients with MCS may be due to the small sample size.

An odorant-related increase in activation in the ACC has been observed in patients with MCS [18]. The ACC is involved in adequate control of top–down or bottom–up modulation of stimuli and is connected to the PFC. Past exposures to hazardous chemicals are stored as memories in the PFC through olfactory nerve circuits, causing various physical or psychological responses such as emotional, visceral, or autonomic responses during the processing of top–down stimuli when exposed to odorants later in life [14]. In the present study, we found that recovery from activation in the PFC after an olfactory stimulation is delayed in patients with MCS compared with controls. These findings support the current understanding of the pathology of this disorder: compared to healthy subjects, patients with MCS strongly respond to odorants that they encounter in daily life, the repeated daily exposure to the odorants keeps

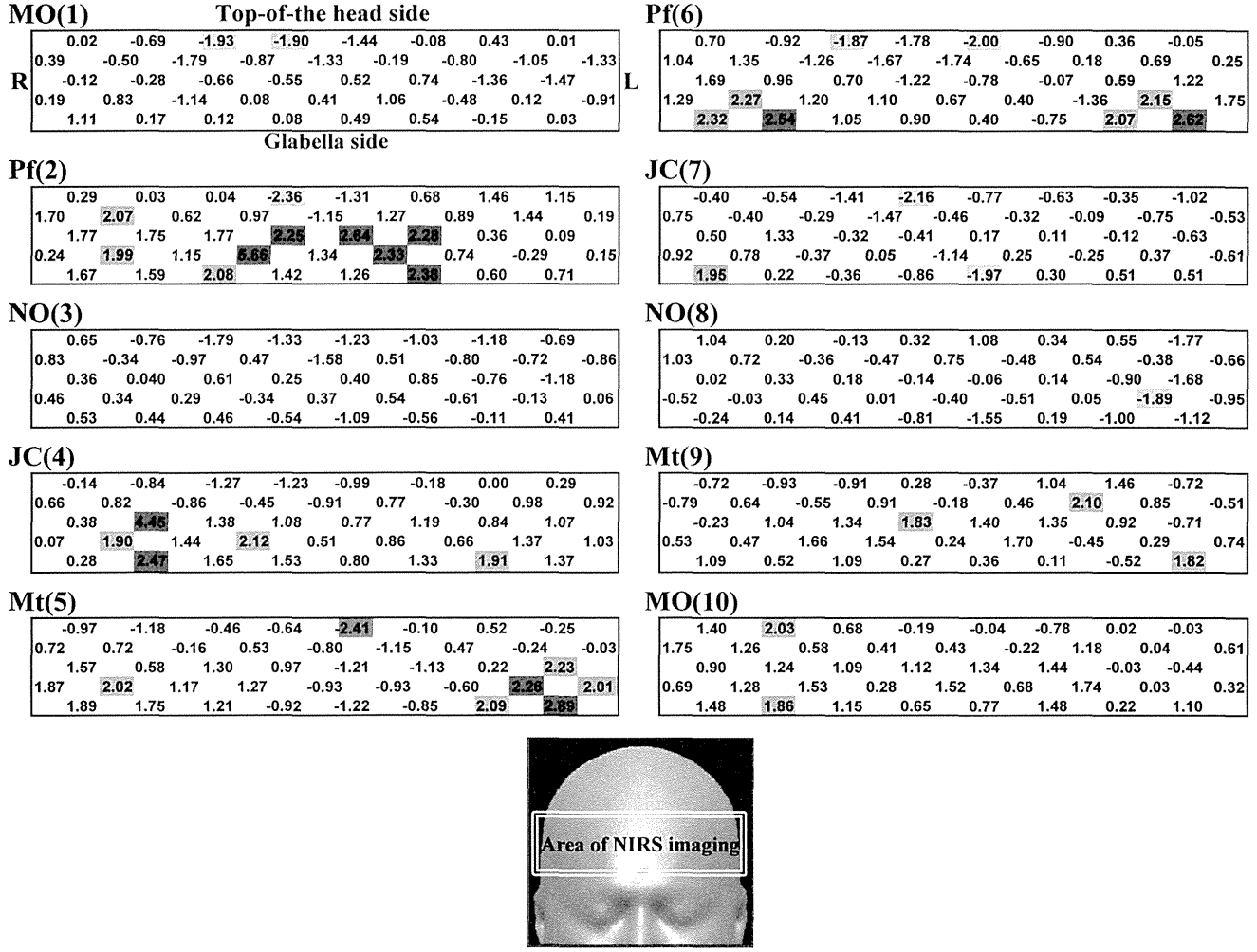

Fig. 5 Average t value of each channel comparing z-scores for oxyHb between patients with MCS ($n = 6$) and controls ($n = 6$). *Red rectangles* denote statistically significant positive correlations ($p < 0.05$), and *blue rectangles* denote statistically significant negative correlations ($p < 0.05$). *Yellow rectangles* denote positive correlations ($p < 0.10$), and *green rectangles* denote negative correlations ($p < 0.10$). The channels are located in the position shown by the *white double line rectangle* below

them in a reactive state. Due to their physical and psychological intolerance to odorants, the patients try to avoid exposure to the odorants. In this study, 4 patients with MCS had episodes of initial exposure to chemicals that triggered the first symptoms. These included organic solvents or incense at the workplace, exhaust gas from diesel machines in the neighborhood, odors from pesticides, or fragrance from a neighbor. Two patients had episodes of repeated exposure to solvents emitted from a neighboring industrial plant or a neighboring paint store, respectively. Patients with MCS complained about a chemical-sensitive condition thereafter. The psychological evaluations in our study support the theory of a strong response in patients with MCS.

In the recovery stage after the stimulation, the activation was especially strong in the OFC. The olfactory neuroanatomy is intertwined, via extensive reciprocal axonal connections, with primary emotion areas including the amygdala, hippocampus, and OFC [39, 40]. Olfactory stimulation directly activates amygdala neurons, innervating a region in the OFC. The olfactory sense has a unique intimacy with the emotion system, and the perception of smell is known to be dominated by emotion [41]. Strong activation in the OFC might remain as potent affective experiences following olfactory stimuli in patients with MCS compared with controls. In this study, lateral orbitofrontal regions were specifically activated in the patients with MCS. The valence of odors is represented in particular in the OFC [42]. Nearly all odors were evaluated as unpleasant by the MCS patients in the subjective evaluation after the stimulation. Pleasant odors preferentially activate medial orbitofrontal regions, whereas unpleasant odors activate more lateral regions [43–46]. The strong activations of the lateral OFC in the patients with

Table 3 Results of the *t* test for the physical and psychological scales

Scales	MCS ($n = 6$)	Controls ($n = 6$)	p value
QEESI (CI)	75.0 (19.2)	4.2 (5.3)	<0.001*
QEESI (OI)	33.2 (24.1)	6.7 (15.8)	0.048*
QEESI (SS)	72.7 (13.5)	5.2 (11.3)	<0.001*
CSS-SHR	51.7 (2.2)	32.5 (3.8)	<0.001*
SSAS	34.3 (8.7)	28.0 (5.2)	0.156
APQ	178.3 (39.4)	131.2 (42.6)	0.075
TAS	13.3 (7.7)	12.8 (10.4)	0.927
MCSD	16.0 (4.7)	20.5 (5.1)	0.141
TMAS	11.0 (4.1)	8.2 (1.5)	0.156
NAS	37.0 (17.0)	27.5 (8.6)	0.250
TAS-20 total	48.7 (16.2)	38.0 (4.0)	0.172
TAS-20 DIF	14.5 (5.4)	9.5 (3.0)	0.076
TAS-20 DDF	12.8 (4.8)	10.3 (1.2)	0.264
TAS-20 EOT	21.3 (7.2)	18.2 (4.0)	0.375

Values are expressed as means (±standard deviations)

CI chemical intolerance, *OI* other intolerance, *SS* symptom severity

* Significant at $p < 0.05$

MCS suggest that these odors were extremely unpleasant for the MCS patients.

Both the ACC and OFC are implicated in decision-making, emotion, and social behavior. Recent evidence suggests that the ACC and OFC make distinct contributions to each of these aspects of decision-making [47]. The OFC is involved in the cognitive processing of stimuli and the representation of preferences. The ACC may mediate the relationship between a past experience and the choice of the next action. Thus, our results suggest that a past strong exposure to hazardous chemicals activates the ACC (and the connected PFC) during olfactory stimuli in the patients with MCS, and a strong activation in the OFC remains after the stimuli. In particular, the lateral OFC is specifically activated when the odor is unpleasant for the patients with MCS. However, the OFC and ACC are anatomically interconnected, and their interaction stimulates decision-making. Their individual function independent of each other remains unclear. Further research is required to understand the recovery process in MCS and the pathology of this disorder.

The present study has some limitations. First, the very small sample size makes the results vulnerable to selection bias. This could be alleviated by including a larger study population. However, despite the small sample size of this study, differences between the patients with MCS and the controls in the NIRS imaging were evident. The results indicate that the evaluation combining NIRS imaging with olfactory stimulation tests is a valuable method for the objective evaluation of MCS. Second, to the best of our knowledge, this is the first case–control study evaluating changes in rCBF in the PFC using NIRS imaging after olfactory stimulation in patients with MCS. Further long-time evaluation after olfactory stimulation would provide valuable information for understanding the pathology of MCS. A third limitation of this study is the lack of standardized objective measures to identify and define MCS. Therefore, most definitions of MCS are entirely qualitative, relying on subjective reports of distressing symptoms and environmental exposure from patients and clinicians. Several individuals with self-reported MCS symptoms were excluded, at the discretion of the clinic physician, because of mental disorders or allergic symptoms.

In conclusion, despite the small sample size, this experimental study detected an activation that remained even after olfactory stimulation, specifically in the PFC of patients with MCS. We propose that recovery from such activation is delayed in patients with MCS and that their chemical-sensitive state remains due to the repeated daily exposure, leading them eventually to develop intolerance to these odorants. Our study demonstrates that NIRS imaging objectively reflects the status of patients with MCS.

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Conflict of interest The authors declare that no conflict of interests exists.

Ethical standard This study was approved by the ethical committee for human research at the Hyakumanben Clinic (99642-61) and the Louis Pasteur Center for Medical Research (LPC.11).

Informed consent This manuscript does not contain the personal medical information about an identifiable living individual. All patients provided written informed consent.

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Association of Sick Building Syndrome with Neuropathy Target Esterase (NTE) Activity in Japanese

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ABSTRACT: Sick building syndrome (SBS) is a set of several clinically recognizable symptoms reported by occupants of a building without a clear cause. Neuropathy target esterase (NTE) is a membrane bound serine esterase and its reaction with organophosphates (OPs) can lead to OP-induced delayed neuropathy (OPIDN) and nerve axon degeneration. The aim of our study was to determine whether there was a difference in NTE activity in the peripheral blood mononuclear cells (PBMCs) of Japanese patients with SBS and healthy controls and whether *PNPLA6* (alias *NTE)* gene polymorphisms were associated with SBS. We found that the enzymatic activity of NTE was significantly higher ($P < 0.0005$) in SBS patients compared with controls. Moreover, population with an AA genotype of a single nucleotide polymorphism (SNP), rs480208, in intron 21 of the *PNPLA6* gene strongly reduced the activity of NTE. Fifty-eight SNP markers within the *PNPLA6* gene were tested for association in a case–control study of 188 affected individuals and 401 age-matched controls. Only one SNP, rs480208, was statistically different in genotype distribution ($P = 0.005$) and allele frequency ($P = 0.006$) between the cases and controls (uncorrected for testing multiple SNP sites), but these were not significant by multiple corrections. The findings of the

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association between the enzymatic activity of NTE and SBS in Japanese show for the first time that NTE activity might be involved with SBS. Environ Toxicol 00: 000–000, 2013.
Keywords: sick building syndrome; NTE; microsatellite; SNPs; *PNPLA6*

INTRODUCTION

Sick building syndrome (SBS) is a term coined for a set of clinically recognizable symptoms, which characterized by nonspecific complaints such as headaches, fatigue, lethargy, nausea, eye and throat irritation, dizziness, back and joint pain, and sleep disturbances, without a clear cause reported by occupants of a building (Iseaeli and Pardo, 2011). It is hypothesized that SBS could be caused by a number of factors at working or living space, including diverse indoor environmental pollutants, such as formaldehyde, toluene, xylene, and organophosphates (Chang, 1994; Hodgson, 2000). The hallmark of SBS is a hypersensitivity to low levels of absorbed environmental pollutants. However, the mechanism by which these pollutants induce host sensitization remains unclear.

Neuropathy target esterase (NTE), officially designated as *PNPLA6*, is a neuronal, membrane bound protein with serine esterase activity mostly exposed on the cytoplasmic face of the membranes, and is a member of the serine hydrolase patatin-like phospholipase (*PNPLA*) family (Glynn et al., 1998; Li et al., 2003; Wilson et al., 2006). NTE hydrolyzes the endogenous substrates lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) (van Tienhoven et al., 2002; Quistad et al., 2003; Zaccheo O et al., 2004), and also monitor humans and animals exposed to insecticides and warfare chemical agents (Lotti et al., 1986). NTE protein is encoded by the *patatin-like phospholipase domain containing 6 (PNPLA6)* gene (NCBI GeneID 10908; aliases *NTE, SPG39*) that consists of 35 exons and spans over 27 kb of genomic sequence located on chromosome 19 at position 19p13.2. NTE was originally identified as the target protein for OPs, an insecticide that cause OPIDN in the adult human and some other vertebrates, with more than 70,000 human cases recorded in the last 70 years (Glynn, 2003, 2006; Vose et al., 2007). OPIDN is characterized by paralysis of the lower limbs and degeneration of long axons in the spinal cord and peripheral nerves (Jokanovic et al., 2002), initiated by the organophosphorylation of NTE with a subsequent specific modification (aging) of the inhibited enzyme (Johnson, 1990; Glynn, 2003). Mice heterozygous for the *Nte* mutation (*Nte*+/-) that had approximately 40% lower enzymatic activity of *Nte* in the brain or wild-type mice that were treated with the *Nte*-inhibiting compound ethyl octylphophonofluoridate (EOPF) both had elevated motor activity (Winrow et al., 2003). These suggest that even a minor reduction of NTE activity can lead to neuronal hyperactivity.

On the basis of these previous findings, we hypothesized that NTE enzymatic activity and SNPs within its gene (*PNPLA6, alias NTE)* coding or noncoding regions might be involved in SBS, even though such an association had not been previously reported. In this study, we investigated the association between (1) NTE enzymatic activity in PBMCs and SBS and (2) *PNLPLA6* gene polymorphisms and SBS in Japanese. In addition, we determined the *PNLPLA6* gene expression tissue distribution by RT-PCR using 34 different types of human tissue and mapped the transcription initiation sites for *PNPLA6* expressed by different tissues.

MATERIALS AND METHODS

Subjects

A total of 188 unrelated Japanese patients with SBS and age- and sex-matched 401 healthy controls by average age and sex ratio were investigated in this study (Table I). We used diagnostic criteria of SBS established by World Health Organization (World Health Organization 1983, Molina et al. 1989). The mean age of the 188 case individuals with SBS was 37.8 ± 16.5 years old. Most case subjects were female (70.7%). Control subjects were comprised of 67.8% females and had a mean age of 37.3 ± 12.1 years old. Informed consent was obtained from the cases and controls by explaining the details of this study prior to collection of peripheral blood. The Institutional Review Board, Tokai University School of Medicine and Research Ethics Committee, The Kitasato Institute approved the present study and all participants provided written informed consent.

NTE Esterase Assays

PBMCs were isolated from 10 mL of heparinized blood at room temperature within the time period of 8 h. Ten milliliters of anti-coagulated peripheral blood was layered onto 5 mL of Mono-Poly™ Resolving Medium (MP Bio Japan K.K., Tokyo) and centrifuged at 400 × *g* at *RT* for 30 min.

TABLE I. Characteristics between SBS case and control

Cohort	Case	Control
No. samples	188	401
Age	37.8 ± 16.5	37.3 ± 12.1
Sex[a]	55 (0.293) / 133 (0.707)	129 (0.322) / 272 (0.678)

[a] Male/Female

The plasma and mononuclear (lymphocytes) and polymorphonuclear leukocyte layers were separated from each other. The mononuclear fraction was washed with PBS and centrifuged at 260 × *g* at *RT* for 7 min. After repeating the washing step, the PBMCs pellet was resuspended in TE buffer (50 mM Tris–HCl, 1 mM EDTA, pH 8.0) and the NTE activity assayed as described previously (Johnson, 1977). NTE activity was determined in lymphocytes as the difference between the determination of the phenol liberated from the paraoxon-resistant (40 mM) and mipafox-sensitive (50 mM) hydrolysis of phenyl valerate.

Analysis of Microsatellite Polymorphism in the *PNPLA6* Gene Region

To determine the number of repeat units of the microsatellite loci that exhibit polymorphisms in the *PNPLA6* gene, unilateral primers were synthesized by labeling at the 5′ end with the fluorescent reagent, 6-FAM (PE Biosystems, Foster City, CA). Two PCR primer sets used for the amplification of two different microsatellite loci were (a) NTE_MS(att)F: 6-FAM-TAACTTCCCTGTTCCCGTTG and NTE_MS(att)R:AACTAGCTGCATGTGGTGACTCG CGC, and (b) NTE_MS(tttg)F: 6-FAM-CAATAGTCCTGC AATGGCAGTC and NTE_MS(tttg)R:AGGCGGACGGTA CACAAGGTCAAG. PCR amplification and GenScan detection of the microsatellite polymorphisms were carried out as previously described (Matsuzaka et al., 2000).

Screening and Typing for SNPs Within the Region of the *PNPLA6* Gene

To study the *PNPLA6* gene region on human chromosome 19p13.2, we extracted and evaluated 106 SNPs located within the genomic region of the sequence registered as NM_006702 in National Center for Biotechnology Information (NCBI) database. To identify SNPs with a minor allele frequency (MAF) > 0.2, we carried out direct sequencing analysis of the SNP loci within the *PNPLA6* gene using 15 pairs of oligonucleotide PCR primers (Supporting Information Table SI). The reaction mixture (20 μL) contained 2 μL of dNTP (2.5 mM each), genomic DNA (5 μL; 2 ng/μL), 2 μL of 10 × buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 20 pmol of forward and reverse primers and 0.5 U rTaq polymerase (TaKARa Co. Japan). PCR amplification was performed in the automated thermal cycler, GeneAmp PCR system 9600 (Applied Biosystems Japan Co.). PCR reaction conditions consisted of an initial denaturation for 5 min at 96°C, annealing for 1 min at various temperatures depending on the primers used (Supporting Information Table SI) and an extension for 1 min at 72°C. This was followed by 30 amplification cycles of 45 s at 96°C, 45 s at the temperatures for annealing, and 1 min at 72°C, with a final extension of 7 min at 72°C. Each PCR product was purified by exonuclease I and then sequenced using an ABI 3100 automated sequencer (Applied Biosystems Japan Co.).

Plasmid DNA Purification and Sequencing

Plasmid DNA was purified using the QIAprep mini columns (Qiagen) and the entire cloned insert within the plasmid was subjected to nucleotide sequence determination. Double-stranded DNA was sequenced by dideoxy sequencing using the BigDyeTerminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 377 DNA sequencer.

Construction of an Oligo-Capped cDNA Library and Mapping of the mRNA Start Sites onto the Genomic Sequences

An oligo-capped cDNA library was constructed from poly (A) RNA for each human tissues and cells using the First-ChoiceTM RLM-RACE kit according to the user manual (Ambion, TX, USA) (Matsuzaka et al., 2004). The cDNA was then subjected to PCR using 5′ RACE Outer Primer: 5′-GCTGATGGCGATGAATGAACACTG-3′, and GSP-oc1: 5′- CCATAGAAGAGCACTTTGTCCCTCT-3. The PCR reaction was the same as described earlier for screening and typing SNPs. Purification, cloning, and sequencing of PCR products were the same as described above. The cDNA sequence for *PNPLA6* thus determined was perfectly matched with the corresponding genomic sequence.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PC{R was performed as previously described (Matsuzaka et al., 2002). The gene-specific primers (Amplicon_R: 5′-CTTCTTGGCAATGTTGAGCA-3′, Amplicon_1F: 5′-ATTGTAATCCTCAGAATCTCAGTAAC-3′, Amplicon_2F: 5′-CAGCTGGAATCAACCGATG-3′, Amplicon_3F: 5′-CAT TCTGCAGATGGGGACAT-3′) were used.

STATISTICAL ANALYSES

Differences in enzymatic activity between the cases and controls were analyzed by the Mann–Whitney *U*-test. Allele and genotype frequencies were determined by direct counting. The significance of differences in the distribution of alleles and genotypes between the patients and controls was tested using a case-control design by the Fisher's exact probability test (*P*-value test). Finding of $Pc < 0.05$ is significant by corrections for multiple testing, such as the Bonferroni's method, $P*L*(n-1)$ for all allelic and genotypic frequencies. *P*, *L*, and *n* were *P*-value, number of locus

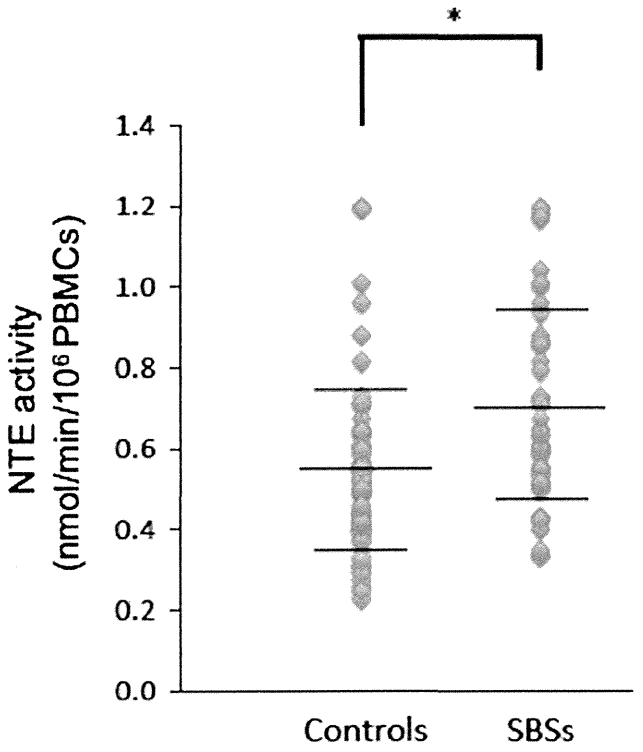

Fig. 1. NTE activity in PBMCs of SBS patients (n = 42) and controls (n = 52). Each diamonds represent individuals, and the average values and SD are shown by the long and short horizontal bars, respectively. *: $P < 0.0005$ (Mann–Whitney U-test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

examined, and number of allele examined, respectively. The odds ratio and 95% CI (Confidence Interval) were also calculated for all SNPs. The program Haploview (MIT/Harvard Broad Institute, MA, USA) was used to estimate Pairwise linkage disequilibrium (LD) and haplotype frequency (Barrett et al., 2005). The Genetic Power Calculator was used to calculate a genetic power for association study (http://pngu.mgh.harvard.edu/~purcell/gpc/).

RESULTS

Increased Enzymatic Activity of NTE in Sick Building Syndrome Patients Group Compared to Healthy Control Group

To test our hypothesis that NTE has a role in SBS, we assessed the enzymatic activity of NTE in the PBMCs between the SBS patient group and the healthy control group. We observed a statistically significant ($P < 0.0005$, Fig. 1) higher enzymatic activity of NTE in the SBS patient group (average enzymatic activity plus minus SD was 0.70 ± 0.25 nmol/min/10^6 PBMCs) than the healthy control groups (average enzymatic activity plus minus SD was 0.54 ± 0.22 nmol/min/10^6 PBMCs).

Association Study of a Microsatellite Marker Within the *PNPLA6* Gene with Sick Building Syndrome

To investigate the genetic variations in the *PNPLA6* gene with SBS, a total of 180 Japanese patients with SBS and 370 healthy controls were enrolled for the association analysis using two potential microsatellite markers, one within intron 6 and the other located within the 3′ flanking region of the *PNPLA6* gene (Supporting Information Fig. S1). The microsatellite with the trinucleotide repeat (ATT)n positioned 1,893 bp downstream of the stop codon in the exon 35 was found to have eleven alleles with the number of repeats for allele 1 to allele 11 ranging from 9 to 19 repeats

TABLE II. Association test using microsatellite repeat polymorphism in the 3′ flanking region of PNPLA6 gene with SBS patients

Allele	PCR product length	Repeat	Patients ($2n$ = 360)	Contorls ($2n$ = 740)	OR (90% CI)[a]	P-value[b]	Pc-value[c]
Allele 1	284	9	0	0.001	–	0.485	1.000
Allele 2	287	10	0.169	0.185	0.90 (0.64 1.25)	0.525	1.000
Allele 3	290	11	0.003	0.005	0.51 (0.06 4.42)	0.543	1.000
Allele 4	293	12	0.069	0.061	1.15 (0.70 1.91)	0.582	1.000
Allele 5	296	13	0.067	0.061	1.05 (0.63 1.75)	0.842	1.000
Allele 6	299	14	0.072	0.070	1.03 (0.63 1.68)	0.906	1.000
Allele 7	302	15	0.394	0.380	1.06 (0.82 1.38)	0.638	1.000
Allele 8	305	16	0.192	0.174	1.12 (0.81 1.55)	0.482	1.000
Allele 9	308	17	0.028	0.055	0.49 (0.24 0.97)	0.041	0.409
Allele 10	311	18	0.006	0.003	2.06 (0.30 14.09)	0.461	1.000
Allele 11	314	19	0	0.001	–	0.485	1.000

[a] OR; odds ratio, CI; Confidence interval.
[b] Determined by Fisher's exact test.
[c] Determined by Bonferroni's adjustment for multiple tests.

TABLE III. Allele and genotype frequencies of SNPs in the *PNPLA6* gene and its associations for sick building syndrome

	db SNP Accession No.	Location	Allele (A/B)[a]	Patients				Controls				MAF				
				AA	AB	BB	total	AA	AB	BB	total	Patients	Controls	OR (95% CI)	*P* value	*Pc* value[c]
1	rs560849	Promoter	T/c	40	55	34	129	108	210	77	395	0.477	0.461	0.94 (0.71–1.24)	0.655	1,000
2	rs540516	intron 1	C/t	46	60	23	129	156	179	54	389	0.411	0.369	0.84 (0.63–1.12)	0.229	1,000
3	rs604959	exon 2	A/c	54	83	27	164	157	191	53	401	0.418	0.370	0.82 (0.63–1.07)	0.137	1,000
4	rs541271	intron 6	G/a	53	85	31	169	121	156	48	325	0.435	0.388	0.82 (0.63–1.07)	0.151	1,000
5	rs526411	intron 6	C/t	53	86	30	169	120	155	50	325	0.432	0.392	0.85 (0.65–1.11)	0.229	1,000
6	rs654059	intron 6	T/c	43	83	43	169	79	165	77	321	0.500	0.497	0.99 (0.76–1.29)	0.926	1,000
7	rs522750	intron 6	G/a	37	52	32	121	86	145	102	333	0.479	0.476	0.84 (0.62–1.12)	0.234	1,000
8	rs488886	intron 6	C/t	46	64	27	137	76	97	25	198	0.431	0.371	0.78 (0.57–1.07)	0.122	1,000
9	rs491518	intron 6	C/a	35	67	35	137	49	112	39	200	0.500	0.475	0.90 (0.67–1.23)	0.524	1,000
10	rs591040	exon 9	C/t	167	10	0	177	363	13	0	376	0.028	0.017	0.61 (0.26–1.38)	0.233	1,000
11	rs492092	intron 12	C/g	43	72	26	141	122	209	73	404	0.440	0.439	1.00 (0.76–1.32)	0.992	1,000
12	rs620744	intron 14	C/t	39	68	32	139	121	205	73	399	0.475	0.440	0.87 (0.66–1.14)	0.313	1,000
13	rs586551	intron 16	C/t	50	59	30	139	95	109	36	240	0.428	0.377	0.81 (0.60–1.09)	0.167	1,000
14	rs577219	intron 19	G/t	46	62	27	135	83	85	34	202	0.430	0.379	0.81 (0.59–1.11)	0.186	1,000
15	-	intron 19	DEL/a	47	63	26	136	81	85	36	202	0.423	0.389	0.87 (0.63–1.19)	0.374	1,000
16	rs473899	intron 21	A/g	60	46	30	136	95	124	43	262	0.390	0.401	0.95 (0.71–1.29)	0.762	1,000
17	rs2303177	intron 21	A/g	89	35	6	130	166	86	10	262	0.181	0.202	0.87 (0.59–1.27)	0.474	1,000
18	rs661825	intron 21	G/c	55	52	23	130	102	118	40	260	0.377	0.381	0.98 (0.72–1.34)	0.917	1,000
19	rs496380	intron 21	A/g	54	54	22	130	102	118	40	260	0.377	0.381	0.98 (0.72–1.34)	0.917	1,000
20	rs557596	intron 21	T/c	52	56	22	130	103	117	40	260	0.385	0.379	0.98 (0.72–1.33)	0.876	1,000
21	rs2432110	intron 21	T/c	40	29	22	91	57	63	31	151	0.401	0.414	0.95 (0.65–1.38)	0.781	1,000
22	rs793864	intron 21	C/g	53	26	11	90	76	53	22	151	0.267	0.321	0.77 (0.51–1.16)	0.207	1,000
23	rs1645799	intron 21	G/a	34	36	22	92	49	69	33	151	0.435	0.447	0.95 (0.66–1.38)	0.792	1,000
24	-	intron 21	C/g	21	27	20	68	51	69	32	152	0.493	0.438	0.80 (0.53–1.20)	0.283	1,000
25	-	intron 21	T/a	21	27	20	68	51	69	32	152	0.493	0.438	0.80 (0.53–1.20)	0.283	1,000
26	-	intron 21	C/g	21	27	20	68	55	65	32	152	0.493	0.424	0.76 (0.51–1.14)	0.183	1,000
27	-	intron 21	G/t	21	27	20	68	51	69	32	152	0.493	0.438	0.80 (0.53–1.20)	0.283	1,000
28	rs688348	intron 21	A/c	23	25	20	68	53	69	30	152	0.478	0.424	0.81 (0.54–1.21)	0.295	1,000
29	rs480208	intron 21	A/g	59	85	44	188	174	152	75	401	0.460	0.377	0.71 (0.55–0.91)	0.006	0,348
30	rs581698	intron 21	G/c	51	67	46	164	137	183	76	396	0.485	0.423	0.78 (0.60–1.01)	0.058	1,000
31	rs582611	intron 21	A/g	27	26	17	70	67	89	39	195	0.429	0.429	1.00 (0.68–1.48)	0.994	1,000
32	rs534758	intron 21	G/t	49	46	7	102	118	79	17	214	0.294	0.264	1.16 (0.80–1.68)	0.428	1,000
33	rs583984	intron 21	T/c	34	52	24	110	59	97	38	194	0.455	0.456	0.97 (0.69–1.35)	0.836	1,000
34	rs50874	intron 21	A/g	32	51	21	104	56	96	41	193	0.447	0.461	0.94 (0.67–1.33)	0.743	1,000
35	rs534464	intron 23	G/c	83	49	18	150	155	127	36	318	0.283	0.313	0.87 (0.64–1.17)	0.359	1,000
36	rs563266	intron 23	T/c	56	65	29	150	117	154	47	318	0.410	0.390	0.92 (0.70–1.22)	0.558	1,000
37	rs597582	intron 23	C/t	83	51	16	150	158	128	32	318	0.277	0.302	0.88 (0.65–1.20)	0.429	1,000
38	rs598023	intron 23	A/g	81	52	16	149	158	128	32	318	0.282	0.302	0.91 (0.67–1.23)	0.532	1,000

TABLE III. *(Continued)*

	db SNP Accession No.	Location	Allele (A/B)[a]	Patients				Controls				MAF		OR (95% CI)	*P* value	*Pc* value[c]
				AA	AB	BB	total	AA	AB	BB	total	Patients	Controls			
39	rs598028	intron 23	C/g	82	52	16	150	157	129	32	318	0.280	0.303	0.89 (0.66–1.21)	0.463	1,000
40	rs599328	intron 24	C/g	59	46	14	119	104	74	18	196	0.311	0.281	0.86 (0.61–1.23)	0.417	1,000
41	rs599330	intron 24	C/t	58	46	15	119	98	76	22	196	0.319	0.306	0.94 (0.66–1.33)	0.729	1,000
42	rs524530	intron 24	A/g	64	43	12	119	106	72	19	197	0.282	0.279	0.99 (0.69–1.41)	0.950	1,000
43	rs539887	intron 24	A/c	60	50	9	119	103	77	17	197	0.286	0.282	0.98 (0.69–0.98)	0.914	1,000
44	rs563826	intron 25	A/g	53	39	10	102	104	73	20	197	0.289	0.287	0.99 (0.68–1.44)	0.951	1,000
45	rs2446176	intron 29	G/a	67	49	22	138	100	74	34	208	0.337	0.341	0.98 (0.71- 1.35)	0.905	1,000
46	re1645800	intron 30	G/a	56	58	23	137	73	102	38	213	0.380	0.418	0.85 (0.62–1.16)	0.314	1,000
47	rs503336	intron 30	A/g	58	59	23	140	73	102	38	213	0.375	0.418	0.84 (0.61–1.14)	0.256	1,000
48	rs518321	intron 30	T/c	58	60	22	140	74	102	37	213	0.371	0.413	0.84 (0.62–1.14)	0.268	1,000
49	rs504934	intron 30	C/t	56	61	23	140	72	103	38	213	0.382	0.420	0.85 (0.63–1.16)	0.314	1,000
50	rs7245592	intron 30	G/a	26	29	15	70	56	66	30	152	0.421	0.414	0.97 (0.65–1.46)	0.890	1,000
51	rs521032	intron 30	T/c	27	28	16	71	61	61	30	152	0.423	0.398	0.90 (0.60–1.35)	0.623	1,000
52	rs506104	intron 30	C/a	28	28	15	71	61	63	28	152	0.408	0.391	0.93 (0.62–1.40)	0.732	1,000
53	rs538850	intron 31	C/t	50	50	20	120	69	97	33	199	0.375	0.410	0.87 (0.62–1.20)	0.388	1,000
54	rs538852	intron 31	G/a	61	50	9	120	111	75	13	199	0.283	0.254	0.86 (0.60–1.23)	0.412	1,000
55	rs577029	intron 31	T/c	49	51	20	120	67	97	34	198	0.379	0.417	0.86 (0.62–1.19)	0.350	1,000
56	rs577145	intron 31	T/c	49	51	20	120	67	96	33	196	0.379	0.413	0.87 (0.62–1.21)	0.396	1,000
57	rs541600	intron 31	C/t	49	51	20	120	67	94	33	194	0.379	0.412	0.87 (0.63–1.21)	0.409	1,000
58	rs89621	intron 31	C/t	49	51	20	120	67	94	33	194	0.379	0.412	0.87 (0.63–1.21)	0.409	1,000

[a] (A/B) is Major or Minor allele.
[b] MAF: Minor allele frequencies
[c] determined by Bonferroni's adjustment for multiple tests.

TABLE IV. Genotype association between SNP (rs480208) in the *PNPLA6* gene and SBS

Genotype	Genotype frequency: Patients (n = 188)	Genotype frequency: Controls (n = 401)	OR (90% CI)[a]	P-value[b]	Pc-value[c]
A/A	0.313	0.434	0.60 (0.41 0.86)	0.005	0.580
A/G	0.452	0.379	1.35 (0.95 1.92)	0.092	1.000
G/G	0.234	0.187	1.33 (0.87 2.02)	0.185	1.000

[a] OR; odds ratio
[b] Determined by Fisher's exact test.
[c] Determined by Bonferroni's adjustment for multiple tests.

(Table II). As seen in Table II, no statistically significant association was found between SBS and the controls after a statistical correction by the Bonferroni's method. The other microsatellite marker with the tetranucleotide repeat (TTTG)n and positioned 1,092 bp downstream of the exon 6 was not polymorphic (data not shown).

SNPs Within the *PNPLA6* Gene

To address the possibility that *PNPLA6* is involved in the pathogenesis of SBS, we screened for SNPs in the *PNPLA6* gene. To search for genetic polymorphisms in the genomic sequences of the *PNPLA6* gene, PCR products separately amplified from the respective genomic regions were obtained from 10 patients with SBS and 10 healthy controls, and their nucleotide sequences were determined by direct sequencing. As a result, a total of 57 SNPs with MAFs > 0.2 were obtained in *PNPLA6* genomic region (Table III). Of them, only one SNP, rs604959, in exon 2, was located in the coding region of *PNPLA6* gene and it is not involved in an amino acid change. Also, MAF of a SNP, rs491518, in exon 9 was lower than 0.2.

Association Study of *PNPLA6* Gene Polymorphisms with Sick Building Syndrome

The association of the 58 polymorphisms in the *PNPLA6* gene was investigated in 188 patients with SBS and 401 controls subjects. Although only one SNP, rs480208 in intron 21 of the *PNPLA6* gene, was significantly associated with SBS (P = 0.006, Table III) without correction, the association between rs480208 and SBS showed no significance if the Bonferroni's adjustment is made for 58 tests (Table III). Furthermore, the AA genotype frequency of the rs480208 indicated a significant difference between SBS and controls (P = 0.005, Table IV) with the higher SNP frequency in the controls without correction. However, the association of the AA genotype with SBS is not significant if the Bonferroni's adjustment is made for 116 tests (Table IV).

Genotype-Specific Differences in the NTE Enzymatic Activity

To evaluate the functional impact of the rs480208 with respect to NTE enzymatic activity, we analyzed the relationship between the genotype of the rs480208 and the enzymatic activity of NTE. The average enzymatic activity of NTE in the people with the rs480208 homozygous AA genotype was significantly (P < 0.01) lower than in the people with the AG + GG genotypes (Fig. 2).

Genome Organization and Tissue Expression Profile of the *PNPLA6* Gene

To localize the transcription initiation site(s) of the *PNPLA6* gene, which has some transcripts with different 5′ region, a mixed oligo-capped cDNA library was constructed from various human tissues. By clustering the nucleotide sequences of the cDNAs obtained by PCR screening of the library, the 5′ ends of the *PNPLA6* mRNAs were identified in nine types of transcripts [Fig. 3(a)]. The expression of *PNPLA6* gene was

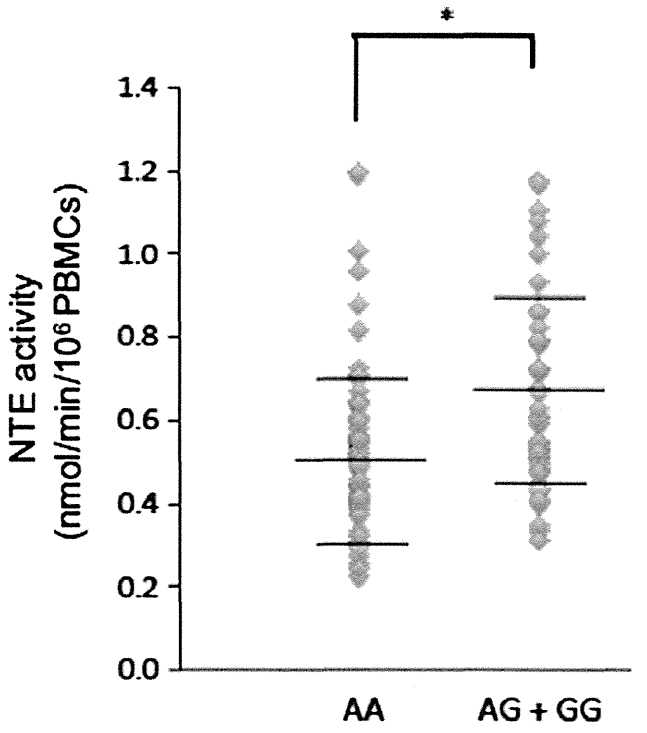

Fig. 2. NTE activity in PBMCs for the genotypes AA (n = 52) and AG + GG (n = 41) of rs480208. Each circle represents an individual, and the average values and SD are shown by the long and short horizontal bars, respectively. *: P < 0.01 (Mann–Whitney U-test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

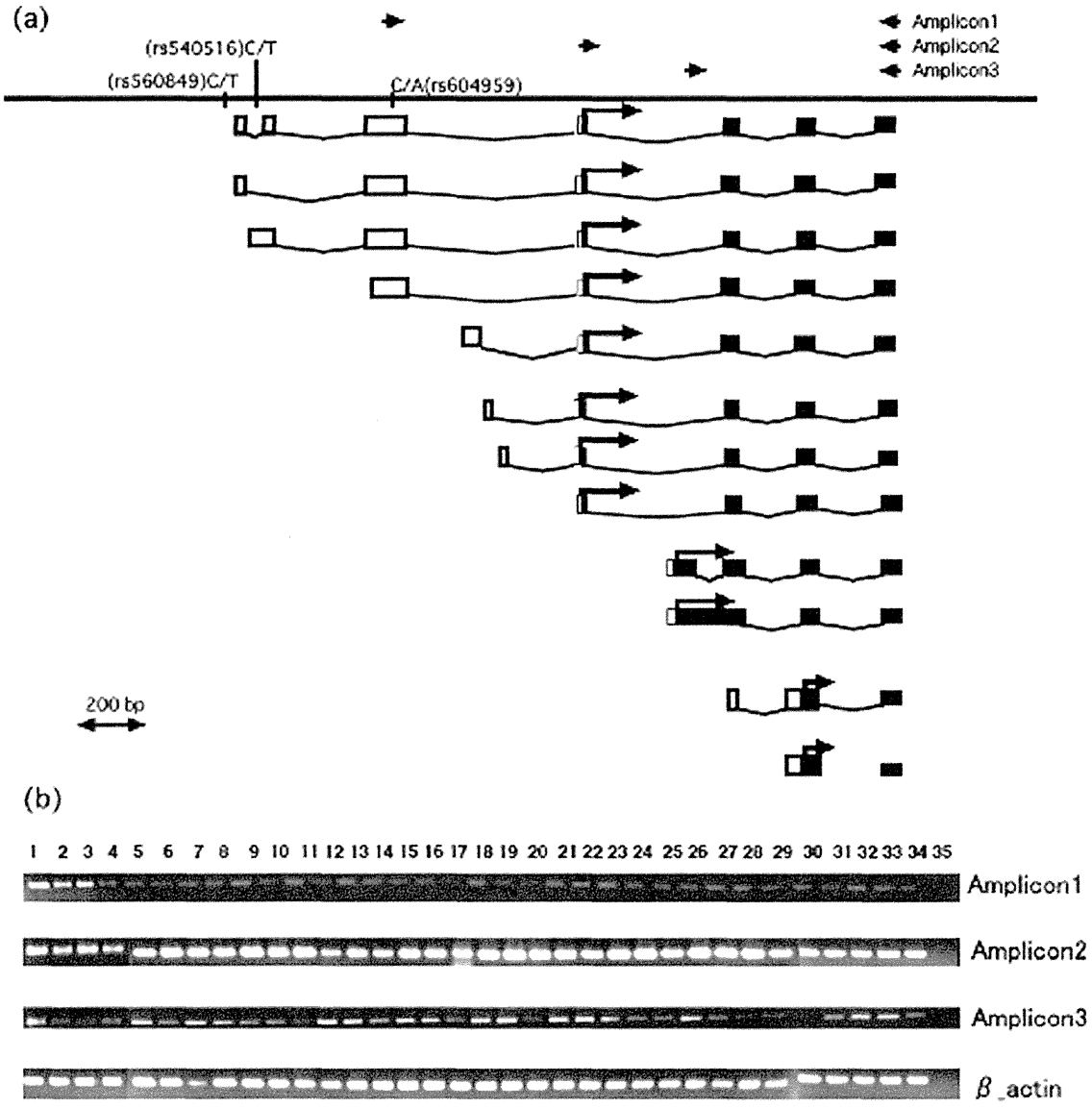

Fig. 3. The exon–intron structure around the 5′ end of the *PNPLA6* gene transcription initiation sites (a). The putative coding regions are in closed boxes and the putative non-coding region is open boxes. The deduced translational initiation methionines are shown by hooked arrows on the exons. The locations of SNP markers (registered rs No. provided) and oligonucleotide primers used for amplicon 1, 2, and 3 of RT-PCR are represented on upper line. *PNPLA6* mRNA expression (b). Each expressions in various human tissues were detected by RT-PCR. 1: amygdala, 2: cerebellum, 3: hypothalamus, 4: thalamus, 5: skin, 6: pooled skin, 7: fetal skin, 8: keratinocyte, 9: skin fibroblast, 10: dermal microvascular epidermal carcinoma cell line, 11: epidermal carcinoma cell line, 12: thymus, 13: fetal thymus, 14: bone marrow, 15: lymph node, 16: tonsil, 17: spleen, 18: periphery blood lymphocyte, 19: CD4 + activated T cells, 20: CD4 + resting T cells, 21: CD8 + activated T cells, 22: CD8 + resting T cells, 23: brain, 24: liver, 25: placenta, 26: skeletal muscle, 27: heart, 28: kidney, 29: pancreas, 30: lung, 31: stomach, 32: testis, 33: uterus, 34: cervix, and 35: negative control.

investigated by RT-PCR in various tissues. To control for the absence of genomic contamination, the primers were designed to span the introns [Fig. 3(a)]. After 40 cycles of amplification, RT-PCR generated the transcripts with the expected size in all of the 35 tissues examined [Fig. 3(b)].

DISCUSSION

The OP compounds are known to inhibit NTE activity in PBMCs by 40–80% by means of its reaction of serine residue of NTE (Lotti et al., 1986; Johnson, 1990). However, simple inhibition of NTE in PBMCs by the OP has not shown to contribute to OPIDN. Moreover, an abnormal accumulation of intrinsic substrate for NTE, PC or LPC impaires membrane integrity and disruption of phospholipid homeostasis (van Tienhoven et al., 2002; Zaccheo et al., 2004;). In our study, we found that NTE activity was significantly higher in the SBS than the control group (Fig. 1). It was also reported that NTE transcriptional activity was significantly up-regulated in the PBMCs of chronic fatigue syndrome (CFS) patients (Kaushik et al., 2005). The symptoms of CFS resemble more closely those of SBS in a lot of terms of hypersensitivity, headaches, fatigue, and sleep disturbance etc., (Zwarts et al., 2008) suggesting that SBS and CFS might have overlapping pathological causes and molecular mechanisms. Furthermore, we observed that NTE activity in the lymphocytes of Japanese with genotype AA of SNP rs480208 was significantly decreased compared to the genotypes AG+GG (Fig. 2). These results have two possibilities. One possibility is that rs480208 could directly regulate expression of *NTE* gene. Another is that mutation(s) closed to rs480208 could affect activity of NTE protein or expression of *NTE* gene. However, the AA genotype frequency of rs480208 showed no significant association with the risk of developing SBS by Bonferroni's corrections, but the frequency of the AA homozygous genotype in SBS was lower than the genotypes in the controls without the corrections for multiple testing (Table IV, $P = 0.005$, $Pc = 0.580$). It remains problematic that the statistical corrections using the Bonferroni's test may be too severe for sample size in our present study and that we may have missed biologically important genetic variant by loss of linkage disequilibrium (LD) between the disease variant and the genetic marker, which is important for the identification of susceptibility genes in association study based on common disease, common variant hypothesis. So, we evaluated the LD extension of approximately 25.4 kb of *NTE* genomic region from rs560849 to rs89621 (Supporting Information Fig. 1). We identified two LD blocks with a threshold of $\Delta = 0.7$. We numbered these blocks 1 to 2 from ATG to stop codon in *PNPLA6* gene. Blocks 1 and 2 spanned roughly 8.5 and 13.3 kb, respectively, and covered almost genomic region of *PNPLA6* gene. Furthermore, as for multifactorial and complex disease like SBS might be affected by a lot of kinds of environmental and multiple genetic factors, it is important to ensure sufficient genetic power and sample size for the detection of subtle genetic effect. So, we calculated the genetic power in our present association study. The genetic power for significant ($P = 0.001$) is 0.6042 and number of case for 80% power needs 532 of sample numbers (Supporting Information Table SII). In addtion to the by means of increasing the number of samples, the classification by environmental factors, such as OP and non-OPs, i.e. formaldehyde, toluene, xylene could overcome the problem of the detection of of susceptibility genes. Because SBS is multifactorial disease and a lot of kinds of indoor pollutants could trigger the development of this disease, it might be difficult to detect the contributions of each factors in small size of population of SBS. However, classification and combination of each molecular signaling leaded to development of SBS might be clear for each components and genetic and environmental factors of molecular mechanisms of SBS. Thus, these findings suggest that the AA homozygote might confer a degree of genetic protection or resistance in the development of SBS and a potentially important genetic connection between *PNPLA6* and SBS.

Previous studies of NTE have concentrated mostly on its sensitivity to OP and role in OPIDN (Glynn, 2003). More recent studies have investigated on the possible role of NTE in phospholipids membrane homeostasis (Vose et al., 2008). In addition, a genetic study has implicated NTE mutations with motor neuron disease (Rainier et al., 2008), whereas our study and those of others (Kaushik et al., 2005) point to a role of NTE in SBS and CFS. Futhermore, mice heterozygous *Nte* allele (*Nte*+/–) showed that a minor reduction in Nte activity can lead to a possible neuronal hyperactivity in mice (Winrow et al., 2003). However, it was reported that that reduction in NTE activity does not correlate with disease state in motor neuron disease in human tissue (Hein et al., 2010). These findings suggest that reduction of NTE enzymatic activity alone is insufficient to cause human motor neuron disease, and also other function(s) of NTE might affect the disease development. Because NTE function for a cell-signaling controlling a communication between neurons and glial cell in nervous development was proposed (Glynn, 1999, 2000). In summary, we found that NTE enzymatic activity in SBS group is significantly increased compared with controls, and that a SNP, rs480208, in *PNPLA6* gene might be directly or indirectly associated with NTE enzymatic activity. Overall, these results suggest that NTE enzymatic activity may become an important marker in the investigation of SBS. However, additional studies on different populations and genetic and environmental factors are required to further confirm its role of polymorphimsm of *PNPLA6* in the pathogenesis of SBS.

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