Scientific, Inc., Waltham, MA, USA). The homogenate was then centrifuged at 49,000 x *g* for 15 minutes at 4℃, and the pellet was suspended in 100 volumes of Tris-HCl buffer.

The platelet membranes were finally adjusted to 0.1 mg protein/mL with assay buffer (50 mM Tris-HCl, pH 7.4). The protein content was determined with the Lowry technique.

Binding Assay

The binding of [^{3}H] PK 11195, a specific ligand of TSPO, to platelets was assayed with a method described previously[13]. Tissue (0.8 mL, 0.08 mg protein) was incubated with a radioligand (0.1 mL) and a cold ligand (or assay buffer; 0.1 mL) in an incubation volume of 1 mL (0℃–4℃) for 60 minutes. The reaction was terminated by rapid filtration over GF/B glass microfiber filters (FP-100, Whatman, GE Healthcare, Little Chalfont, UK) that had been soaked in poly-L-lysine solution (Sigma-Aldrich, St. Louis, MO, USA) using a cell harvester (M-24, Brandel, Gaithersburg, MD, USA), with 5 washes with 5 mL of ice-cold buffer.

The specific binding of [^{3}H] PK 11195 was defined as the difference in binding obtained in the presence and the absence of PK 11195 (10 μM, Research Biochemicals International, Natick, MA, USA). The radioactivity retained by the filters placed in a 24-well microplate (PicoPlate-24, PerkinElmer, Inc., Waltham, MA, USA) was measured with a microplate scintillation counter (Top Count, Packard Instrument Co., Meriden, CT, USA), using 500 μL of a scintillant (MicroScint-20, Packard Instrument Co.). [^{3}H] PK 11195 (86.0 Ci/mmol) was purchased from Daiichi Pure Chemical Company (Tokyo, Japan).

The dissociation constant (Kd) and the receptor density (Bmax) were determined with least-squares regression. Unless otherwise stated, the statistical data are presented as the mean and S.D.

Genomic Analysis

The 485G>A polymorphism of the TSPO gene was examined as described previously[12]. Genomic DNA was prepared from peripheral blood lymphocytes with a DNA extraction kit (Stratagene, La Jolla, CA, USA). The fragments including exon 4 of the TSPO gene were amplified with the polymerase chain reaction (PCR), and direct sequencing was performed. Sequence variations of the TSPO gene were analyzed within exon 4.

The PCR amplifications were performed in a 20-mL reaction mixture containing 100 ng of genomic DNA, 15 pM of each primer, 1.5 mM of $MgCl_2$, and 1 U Ex Taq polymerase (Takara, Tokyo, Japan).

The coding region in exon 4 of the TSPO gene was screened with direct sequencing, using the primer sets. Sequencing was performed on both strands with a sequencing kit (Big Dye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA, USA) and a sequencer (ABI 3700, Applied Biosystems).

The SNPs were scored with custom genotyping products (TaqMan Assays-by-Design SNP Genotyping Service, Applied Biosystems) based on the TaqMan assay method[14]. Genotypes were determined with a sequence detection system instrument (ABI 7900, Applied Biosystems) and analysis software (SDS v2.0, Applied Biosystems).

Statistical Analysis

Pearson product-moment correlation and analysis of variance were used to identify associations among Bmax values and STAI scores. The allelic distributions were compared between patients and control subjects by means of chi-square statistics and Fisher's exact test. All differences were considered significant at $p<0.05$. Statistical analysis was performed with the Prism software program (version 4.0) for Macintosh (GraphPad Software, San Diego, CA, USA).

Results

The subjects were 52 patients with AD (30 male and 22 female) and 163 healthy volunteers (89 male and 74 female). The STAI scores were significantly higher in patients with AD, especially male patients, than in healthy subjects (**Fig. 1**). In male patients, both state and trait anxiety scores were significantly higher, whereas in female patients, only trait anxiety scores were significantly higher.

The expression of platelet TSPO, as determined

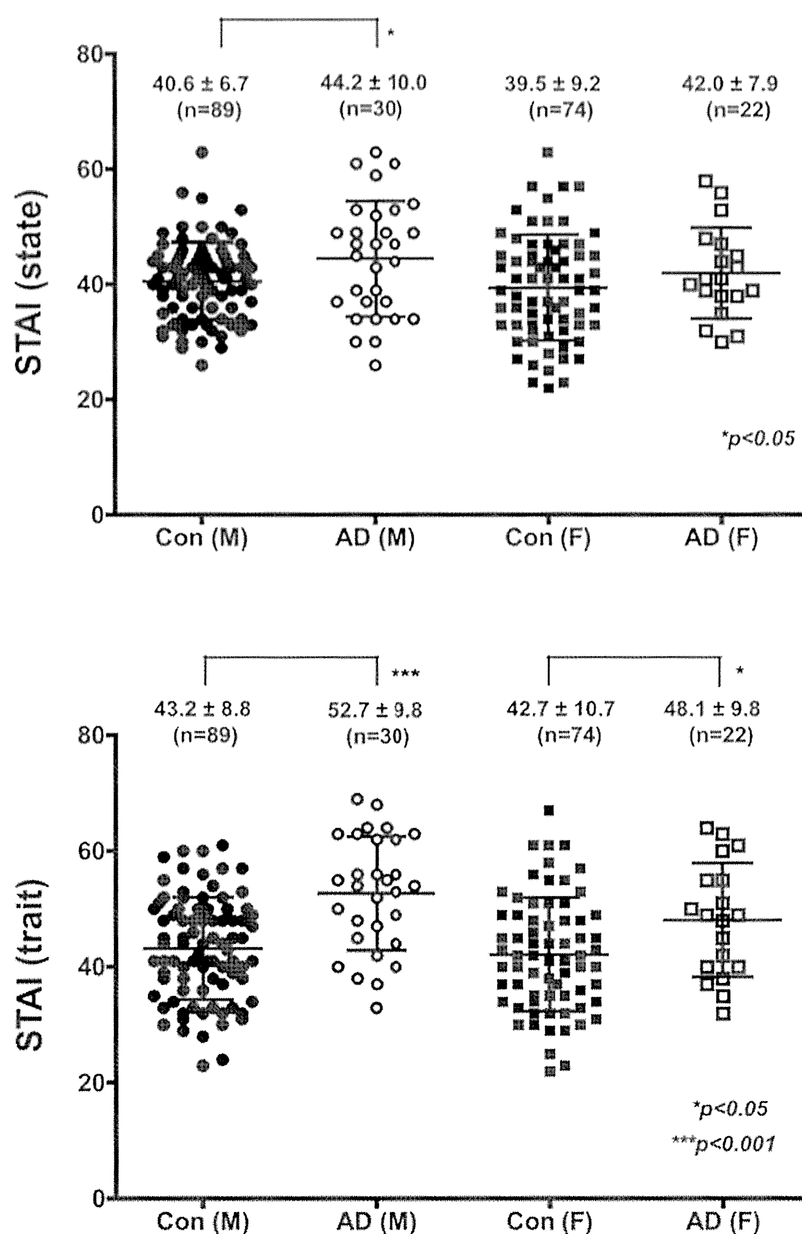

Fig. 1 Comparison of STAI scores of patients with atopic dermatitis and control subjects
Scores of the state anxiety (upper) and the trait anxiety (lower) are further compared between male (M) and female (F) subjects. There were significant differences in either state or trait anxiety between patients with atopic dermatitis (AD) and control subjects (Con), except for state anxiety in female subjects. The data are presented as means and S.D.

with a binding assay with [^{3}H] PK11195 in terms of Bmax, was also significantly higher in patients with AD than in healthy control subjects. The increase was greater in male patients (by 62% on average) than in female patients (by 22% on average).

Genomic analysis of the 485G>A polymorphism of the human TSPO gene in exon 4 showed, contrary to our expectation, that the G/G genotype was less frequent and the G/A and possibly A/A genotypes were more frequent in patients with AD than in control subjects (**Table 1**). The difference in the frequency distribution was significant in male

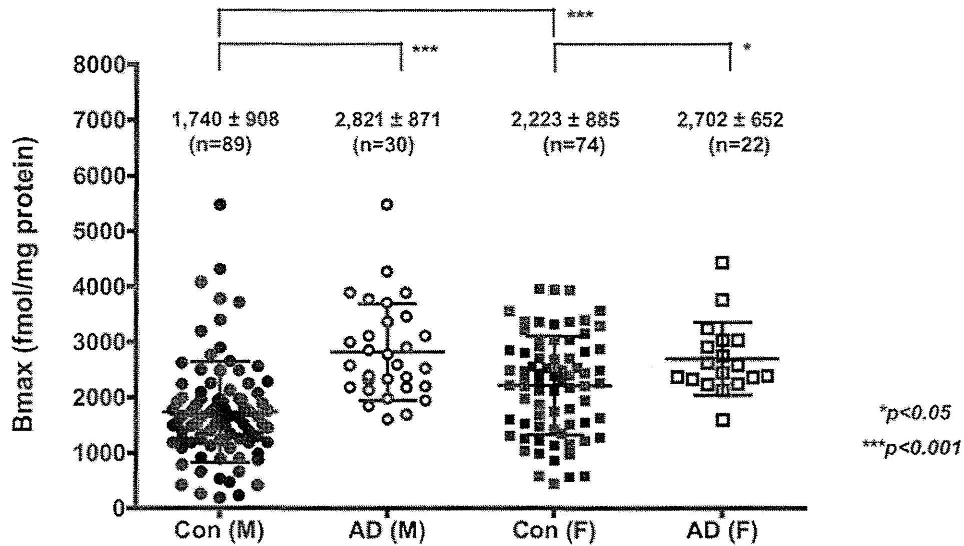

Fig. 2 Comparison of the platelet TSPO expression (Bmax) of patients with atopic dermatitis with control subjects
The Bmax values are further compared between male (M) and female (F) subjects. There were significant differences between patients with atopic dermatitis (AD) and control subjects (Con) in both male and female subjects. The data are presented as means and S.D.

Table 1 Genotypic and allelic distribution of the 485G>A polymorphism of the TSPO gene in patients with atopic dermatitis and control subjects

		Genotype (%)				Allele (%)		
	N	G/G	G/A	A/A		G	A	
Atopic Dermatitis	52	23 (44.2)	26 (50.0)	3 (5.8)		72 (69.2)	32 (30.8)	
Control	163	99 (60.7)	53 (32.5)	11 (6.8)		251 (77.0)	75 (23.0)	
Atopic Dermatitis (M)	30	9 (30.0)	18 (60.0)	3 (10.0)	*	36 (60.0)	24 (40.0)	*
Control (M)	89	55 (61.8)	29 (32.6)	5 (5.6)		139 (78.1)	39 (21.9)	
Atopic Dermatitis (F)	22	14 (63.6)	8 (36.4)	0 (0.0)		36 (81.8)	8 (18.2)	
Control (F)	74	44 (59.5)	24 (32.4)	6 (8.1)		112 (75.7)	36 (24.3)	

**P=0.0104 (genotype), 0.0105 (allele)*
There was a significant difference between male patients with atopic dermatitis patients and male control subjects.

subjects, but not in female subjects.

The severity of AD, as determined with the SCORAD index, appeared to be associated with TSPO expression (**Fig. 3**). Male patients showed a positive correlation between AD severity and TSPO expression, in which the relation was significant for the G/A genotype. In contrast, female patients showed no such correlations.

Discussion

In the present study, we found that AD symptoms are related to an individual's stress response, as reflected by the expression and genetic variation of TSPO. To the best of our knowledge, this is the first study showing a definite correlation between AD and TSPO.

Patients with AD, especially male patients, had significantly higher STAI scores, The expression of

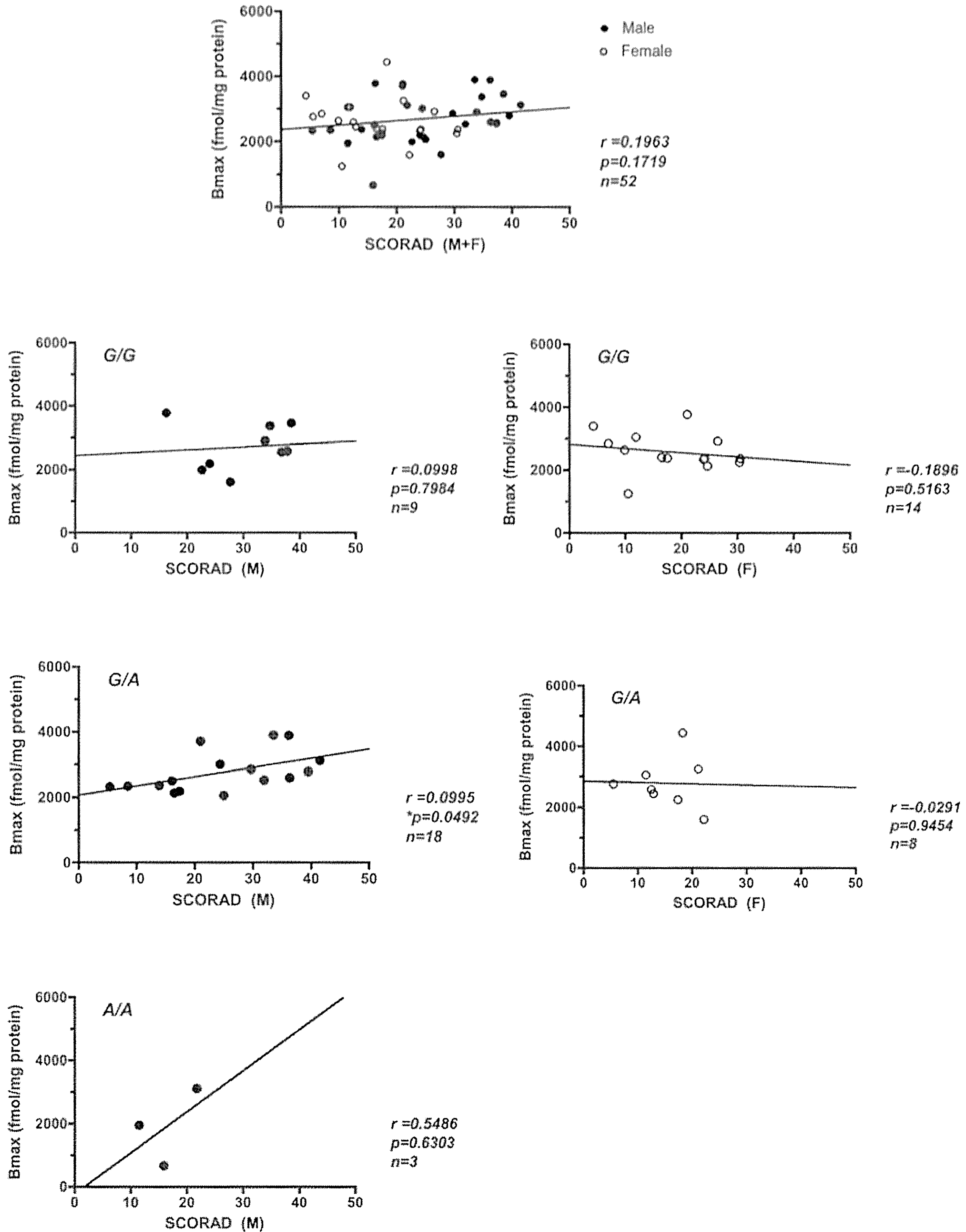

Fig. 3 Relation of Scoring of Atopic Dermatitis index to TSPO expression in atopic dermatitis
The graph at the top shows the relation of the Scoring of Atopic Dermatitis (SCORAD) index and TSPO expression (Bmax) in both male and female patients. The graphs in the second and third rows and at the bottom show patients with G/G, G/A, and A/A genotypes, respectively. The graphs on the left side show male subjects, and those on the right side show female subjects.
Male patients showed a positive correlation, in which the relation was significant for the G/A genotype. In contrast, female patients showed no such correlations.

platelet TSPO was also significantly higher in patients with AD, indicating that AD is a stress-responsive disease as reported previously[4,5].

Genomic analysis with lymphocytes for an SNP of the human TSPO gene in exon 4 (485G>A), in which the G/G genotype is presumably associated with individual's stress sensitivity[12], showed, contrary to our expectations, significantly lower and higher frequencies of the G/G and G/A genotypes, respectively, in male patients with AD, but not in female patients.

The severity of AD, as determined with the SCORAD index, was correlated with TSPO expression in male patients but not in female patients. The sex difference remains to be clarified, but a possible explanation is that hormonal changes during the menstrual cycle in female patients affects the function of TSPO.

Structure and Function of TSPO

The involvement of TSPO in steroidogenesis, apoptosis, and immunomodulation has attracted much interest[8-10]. TSPO may constitute a biomarker of brain inflammation and reactive gliosis and has been used as a therapeutic target in neurological and psychiatric disorders[15].

TSPO appears to be a heteromeric complex of at least 3 different subunits, including an isoquinoline-binding subunit (18 kDa), a voltage-dependent anion channel (VDAC, 32 kDa), and an adenine nucleotide translocase (ANT, 30 kDa)[15]. Isoquinolines, such as PK 11195, that bind specifically to TSPO interact specifically with the 18-kDa subunit, whereas TSPO-specific benzodiazepines, such as Ro 5-4864, bind to a site consisting of both VDAC and the 18-kDa subunits.

The 18-kDa subunit, recently referred to as "translocator protein (18 kDa)"[10], is involved in the regulation of cholesterol transport from the outer to the inner mitochondrial membrane (the rate-determining step in steroidogenesis). The protein is highly expressed in steroidogenic tissues. In the brain, the 18-kDa protein is primarily expressed in ependymal and glial cells.

The 18-kDa subunit is a small, highly conserved protein that is predominantly localized on the outer membrane of mitochondria, where it is associated with VDAC and ANT to form the mitochondrial permeability transition pore (MPTP)[16]. Consistent with its localization in the outer mitochondrial membrane and its association with the MPTP, the 18-kDa subunit plays a role in the regulation of apoptosis.

The full-length complementary DNA for the 18-kDa subunit has been cloned from humans[8]. The human TSPO gene (approximately 13 kbp) is located in the 22q13.31 band on chromosome 22 and is composed of 4 exons, with the first exon and half of the fourth exon being untranslated. The protein domain of exon 2 has been linked to the isoquinoline-binding site and part of the TSPO-specific benzodiazepine-binding site. In addition, a cholesterol-interaction site has been characterized at the carboxyl end of the protein.

The 18-kDa TSPO subunit is highly conserved in the 4 species cloned (*i.e.*, human, cow, rat, and mouse). As far as exons 2–4 are concerned, this gene is well conserved even in bacteria, implying that the functions of this gene must be fundamental for the cell[8].

Stress Response of TSPO

The TSPO is involved in the regulation of several major stress systems: 1) the hypothalamic-pituitary-adrenal axis, 2) the sympathetic nervous system, 3) the renin-angiotensin axis, and 4) the neuroendocrine-immune axis[8].

The sensitivities of TSPO to stress have been demonstrated in both animal and human studies[8]. Drugan *et al* (1986), using inescapable tail shocks in an animal model of stress, were the first to show the involvement of TSPO in the physiological response to stress[17]. Exposure of rats to 5 shocks induced a significant increase in the expression of renal TSPO.

Studies in humans have also shown that the expression of TSPO in platelets is sensitive to stress and anxiety. Increased platelet TSPO density was detected in resident physicians exposed to "examination stress." However, changes in the expression of TSPO were not accompanied by changes in "stress hormones" (cortisol, prolactin, and growth hormone), which can be explained either by

the fact that the study dealt with prolonged stress or by the fact that the hormonal peak release occurred earlier, during the examination. In conclusion, alterations in the expression of TSPO seem to be a sensitive indicator of stress.

Polymorphism of the TSPO Gene in Relation to Stress

In our recent studies, expression of platelet TSPO was significantly correlated with the trait anxiety score in healthy human subjects[11]. The evidence for TSPO as a promising biological marker of stress has prompted us to investigate the stress response of TSPO at the genomic level[12].

Nakamura *et al* (2006) have detected a novel missense variant in exon 4 of the TSPO gene, derived from the nucleotide transition in codon 162 (CGT —> CAT: 485G>A), resulting in an arginine-to-histidine (Arg —> His) change. Genotypic and allelic analyses of the 485G>A polymorphism have revealed significant differences between the patients with PD and healthy control subjects[12].

Unlike the present study, the study of Nakamura *et al* found that the G/G genotype was significantly more frequent in subjects with PD than in control subjects. Patients with PD had a nearly twofold higher rate of the G allele than did control subjects. Before the onset of PD, individuals with the G/G genotype showed high anxiety sensitivity and an increase in TSPO. These results suggest that individuals with the G/G genotype are at increased risk for PD.

The present study of the 485G>A polymorphism of patients with AD stands in contrast to the previous study in patients with PD. The STAI and TSPO expression suggest that patients with AD are under stress. Therefore, the G/G genotype is likely responsible for the pathogenesis of AD. If this is the case, then the G/G genotype should be more frequent in subjects with AD. Contrary to this expectation, the G/G genotype was less frequently observed, and the G/A and possibly A/A genotypes were more frequently observed in patients with AD. To explain this result, factors other than stress sensitivity should be considered.

Immune Factors in Relation to AD and TSPO

Because various immune and nonimmune factors participate in the pathogenesis of AD[18-20], anxiety and stress sensitivity cannot be attributed solely to increased disease activity. The most likely psychological substrate in anxiogenic stimuli is nerve growth factor (NGF)[21,22]. In addition, NGF is considered an important mediator lowering the itching threshold. The epidermis of lesions from patients with AD shows a higher expression of NGF than does the epidermis of healthy control subjects[23].

Genes associated with skin-barrier formation and adaptive immunity have been implicated in the development of AD. For example, filaggrin is essential for the maintenance of the skin-barrier function. Genetic mutations in filaggrin are significantly associated with the risk of AD and elevated immunoglobulin E levels[2].

On the other hand, TSPO is an attractive drug target for controlling inflammation. For example, administration of the TSPO ligand etifoxine modulates macrophage activation and blunts the production of inflammatory cytokines after peripheral nerve injury. This anti-inflammatory effect of etifoxine likely involves TSPO because the selective TSPO ligands PK 11195 and Ro5-4864 have also been shown to inhibit inflammatory responses[15,24].

The G allele in the 485G>A polymorphism of the TSPO gene might facilitate such an anti-inflammatory effect, and, accordingly, the G/G genotype would be less frequently observed in patients with AD. The relation of the phenotype variation of TSPO (Arg162His) to the anti-inflammatory effect remains to be clarified.

In conclusion, the present study provides new evidence that variation in the TSPO gene affects susceptibility to AD.

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References

1. Palmer CN, Irvine AD, Terron-Kwiatkowski A, et al.: Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet 2006; 38: 441–446.
2. O'Regan GM, Sandilands A, McLean WH, Irvine AD: Filaggrin in atopic dermatitis. J Allergy Clin Immunol 2008; 122: 689–693.
3. Bender BG, Ballard R, Canono B, Murphy JR, Leung DY: Disease severity, scratching, and sleep quality in patients with atopic dermatitis. J Am Acad Dermatol 2008; 58: 415–420.
4. Buske-Kirschbaum A, Geiben A, Hellhammer D: Psychobiological aspects of atopic dermatitis: an overview. Psychother Psychosom 2001; 70: 6–16.
5. Howlett S: Emotional dysfunction, child-family relationships and childhood atopic dermatitis. Br J Dermatol 1999; 140: 381–384.
6. Linnet J, Jemec GB: Anxiety level and severity of skin condition predicts outcome of psychotherapy in atopic dermatitis patients. Int J Dermatol 2001; 40: 632–636.
7. Hashizume H, Takigawa M: Anxiety in allergy and atopic dermatitis. Curr Opin Allergy Clin Immunol 2006; 6: 335–339.
8. Gavish M, Bachman I, Shoukrun R, et al.: Enigma of the peripheral benzodiazepine receptor. Pharmacol Rev 1999; 51: 629–650.
9. Papadopoulos V, Lecanu L, Brown RC, et al.: Peripheral-type benzodiazepine receptor in neurosteroid biosynthesis, neuropathology and neurological disorders. Neuroscience 2006; 138: 749–756.
10. Papadopoulos V, Baraldi M, Guilarte TR, et al.: Translocator protein (18 kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol Sci 2006; 27: 402–409.
11. Nakamura K, Fukunishi I, Nakamoto Y, Iwahashi K, Yoshii M: Peripheral-type benzodiazepine receptors on platelets are correlated with the degrees of anxiety in normal human subjects. Psychopharmacology 2002; 162: 301–303.
12. Nakamura K, Yamada K, Iwayama Y, et al.: Evidence that variation in the peripheral benzodiazepine receptor (PBR) gene influences susceptibility to panic disorder. Am J Med Genet B Neuropsychiatr Genet 2006; 141B: 222–226.
13. Nakamoto Y, Watabe S, Shiotani T, Yoshii M: Peripheral-type benzodiazepine receptors in association with epileptic seizures in EL mice. Brain Res 1996; 717: 91–98.
14. Ranade K, Chang MS, Ting CT, et al.: High-throughput genotyping with single nucleotide polymorphisms. Genome Res 2001; 11: 1262–1268.
15. Rupprecht R, Papadopoulos V, Rammes G, et al.: Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. Nature Rev 2010; 9: 971–988.
16. Kinnally KW, Zorov DB, Antonenko YN, et al.: Mitochondrial benzodiazepine receptor linked to inner membrane ion channels by nanomolar actions of ligands. Proc Natl Acad Sci USA 1993; 90: 1374–1378.
17. Drugan RC, Basile AS, Crawly JN, et al.: Inescapable shock reduces [^{3}H]Ro 5-4864 binding to "peripheral-type" benzodiazepine receptors in the rat. Pharmacol Biochem Behav 1986; 24: 1673–1677.
18. Arck P, Paus R: From the brain-skin connection: the neuroendocrine-immune misalliance of stress and itch. Neuroimmunomodulation 2006; 13: 347–356.
19. Arndt J, Smith N, Tausk F: Stress and atopic dermatitis. Curr Allergy Asthma Rep 2008; 8: 312–317.
20. Wright RJ, Finn P, Contreras JP, et al.: Chronic caregiver stress and IgE expression, allergen-induced proliferation, and cytokine profiles in a birth cohort predisposed to atopy. J Allergy Clin Immunol 2004; 113: 1051–1057.
21. Alleva E, Petruzzi S, Cirulli F, Aloe L: NGF regulatory role in stress and coping of rodents and humans. Pharmacol Biochem Behav 1996; 54: 65–72.
22. Toyoda M, Nakamura M, Makino T, Hino T, Kagoura M, Morohashi M: Nerve growth factor and substance P are useful plasma markers of disease activity in atopic dermatitis. Br J Dermatol 2002; 147: 71–79.
23. Oh SH, Bae BG, Park CO, et al.: Association of stress with symptoms of atopic dermatitis. Acta Derm Venereol 2010; 90: 582–588.
24. Wilms H, Claasen J, Röhl C, et al.: Involvement of benzodiazepine receptors in neuroinflammatory and neurodegenerative diseases: evidence from activated microglial cells in vitro. Neurobiol Dis 2003; 14: 417–424.

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Haplotype Analysis of *GSK-3β* Gene Polymorphisms in Bipolar Disorder Lithium Responders and Nonresponders

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Abstract: The *GSK-3β* gene, *GSK3B,* codes for an enzyme that is a target for the action of mood stabilizers, lithium and possibly valproic acid.
In this study, the relationship between haplotypes consisting of single nucleotide polymorphisms (SNPs) of *GSK3B* −50T/C and −1727A/T and the effect of lithium was studied among Japanese bipolar disorder lithium nonresponders and responders.
The distributions of the *GSK3B* haplotypes (−50T/C and −1727A/T) showed a trend for significant difference between the lithium nonresponders and responders (global P=0.07074). Haplotype 1 (T-A) was associated with a higher lithium response (haplotype-specific P=0.03477), whereas haplotype 2 (C-A) was associated with a lower lithium response (haplotype-specific P=0.03443).
The pairwise D' and r^2 values between the 2 SNPs in this study were 1.0 and 0.097, respectively. The 2 SNPs showed weak linkage disequilibrium with each other.

Key Words: *GSK-3β*, bipolar disorder, lithium response

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Recent findings suggest that glycogen synthase kinase–3β (*GSK-3β*) may play a role in the pathophysiology and treatment of mood disorders. Mood stabilizers, lithium and valproic acid, have been used for the treatment of bipolar disorder, and their ability to inhibit *GSK-3β* has been implicated as the mechanism of action in bipolar disorder.[1] Various genetic studies have shown the association of genetic polymorphisms for *GSK-3β* with mood disorders.[2]

The *GSK-3β* gene, *GSK3B*, was mapped to 3q13.3,[3] and a linkage of regions on chromosomes 3q to not only schizophrenia but also bipolar disorder was suggested.[4] In addition, *GSK3B* has been known as one of the candidate genes for both schizophrenia and bipolar disorder.

Russ et al[5] detected 5 single nucleotide polymorphisms (SNPs) in the *GSK3B*. They identified 2 common SNPs at positions −50 C/T and −1727 A/T localized in the promoter region, with minor allele frequencies in white controls of 35% and 13%, respectively. It was reported that −50T/C of *GSK3B* influenced the long-term response to lithium salts in bipolar illness and that carriers of the mutant (C) allele (*GSK3B* C/C genotype) improved on lithium therapy.[6,7]

Because *GSK3B* −50T/C and −1727A/T were detected in the *GSK3B* promoter region,[8] the 2 SNPs were selected for the present study to determine their association with bipolar disorder (Fig. 1).

Brain-derived neurotrophic factor, which is modulated by antidepressants and produces antidepressivelike activity in preclinical behavioral models, is able to inhibit *GSK-3β*.[6] The *GSK-3β* substrate cyclic adenosine monophosphate regulatory element–binding protein transcription factor has been shown to modulate antidepressant activity.[5] A recent study revealed a genetic interaction between 2 functional SNPs in the *GSK-3β* gene and the microtube-associated protein τ H1/H1 haplotype, suggesting a possible combinative role of τ and *GSK-3β* in Parkinson disease and/or Alzheimer disease pathology.[9,10]

In this study, we hypothesized that genetic variants of the *GSK-3β* gene could partially underlie the response susceptibility to lithium treatment in bipolar disorder. In this study, we examined the possible association of the 2 previously studied *GSK3B* polymorphisms, −50T/C (rs334558) and −1727A/T (rs3755557), with bipolar disorder in Japanese lithium-treated patients, using an update of a previous study on the *GSK3B* haplotype undertaken in our laboratory.

METHODS

The relationship between haplotypes consisting of SNPs of *GSK3B* −50T/C and −1727A/T and the effect of lithium was studied for lithium responders and nonresponders among Japanese patients affected by bipolar disorder (*Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*).

The subjects had received lithium treatment for at least 24 months. The lithium treatment efficacy was evaluated by calculating the difference between the symptoms before and during lithium treatment, using a structured clinical rating scale, namely, the Young Mania Rating Scale.[11] Responder analysis revealed that 64% of the patients showed a reduction of 50% or more from baseline to endpoint in the Young Mania Rating Scale score (responder).

Genomic DNA samples were obtained from 42 patients (responders, 27 [11 men and 16 women]; nonresponders, 15 [4 men and 11 women]; mean [SD] age, 35.8 [8.8] years) after written informed consent had been obtained. The *GSK3B* −50T/C and −1727A/T genotyping was performed by the polymerase chain reaction method.[5,6]

The Hardy-Weinberg disequilibrium was assessed by the χ^2 test.

For statistical analysis of *GSK3B* haplotypes, gPLINK (http://pngu.mgh.harvard.edu/~purcell/plink/) and Haploview (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview) were used.[12,13]

RESULTS

As shown in Table 1, the distributions of the *GSK3B* haplotypes (−50T/C and −1727A/T) showed a trend with significant

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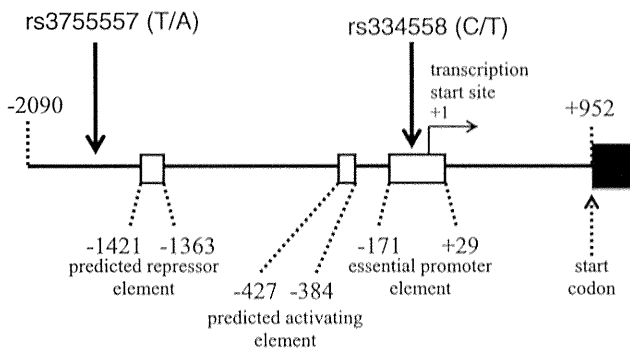

FIGURE 1. SNPs in the promoter region of the *GSK-3β* gene. Regulatory *cis*- elements for transcription are indicated according to the report of Lau et al[8] (1999).

difference between the lithium nonresponders and responders (global P=0.07074; empirical global P=0.1305). Haplotype 1 (T-A) was associated with a higher lithium response (haplotype-specific P=0.03477; empirical P=0.0673), whereas haplotype 2 (C-A) was associated with a lower lithium response (haplotype-specific P=0.03443; empirical P=0.079).

Pairwise D' and r^2 values between the 2 SNPs in this study were 1.0 and 0.097, respectively. The 2 SNPs showed weak linkage disequilibrium with each other.

Hardy-Weinberg equilibrium was tested by means of the goodness-of-fit χ^2 test. The 2 SNPs, *GSK3B* −50T/C and −1727A/T, were polymorphic, and their minor allele frequencies were 31% and 18%. There was no evidence of deviation from Hardy-Weinberg equilibrium for both SNPs.

There was no significant difference in genotypic or allelic frequencies of single SNPs (rs334558 and rs3755557) between the responder and nonresponder groups (Table 2). Genotypic and allelic frequencies of −50T/C polymorphism observed in this study were consistent with the genotypic (χ^2=0.8577, P=0.6512, df=2) and allelic frequency (χ^2=0.8432, P=0.3585, df=1) observed in the HapMap Japanese population; however, those of −1727A/T were not (χ^2=46.0482, $P < 0.01$, df=2; χ^2=68.6213, $P < 0.01$, df=1).

DISCUSSION

The results of the present study reinforce the association between *GSK-3β* and bipolar illness because *GSK3B* haplotype 1 (T-A) was associated with a higher lithium response and haplotype 2 (C-A) was associated with a lower lithium response.

However, Benedetti et al[6,7] showed that carriers of the mutant (C) allele of −50T/C (rs334558) improved on lithium salt therapy in 88 bipolar type I patients, supporting the hypothesis that GSK is a target for the therapeutic action of lithium.

TABLE 2. Allelic and Genotypic Distribution According to Lithium Therapeutic Response

Variant	Responder	Nonresponder	χ^2	*df*	*P*
GSK-3β −50T/C (rs334558)					
Genotypes					
CC	12	10	2.5	2	0.28
CT	9	5			
TT	6	0			
Alleles					
C	33	25	3.48	1	0.06
T	21	5			
GSK-3β −1727A/T (rs3755557)					
Genotypes					
AA	19	11	0.31	2	0.86
AT	6	3			
TT	2	1			
Alleles					
A	44	25	0.77	1	0.93
T	10	5			

There is a significant racial difference in the *GSK3B* polymorphisms between Japanese and white populations. A significantly lower frequency of the T allele of −50T/C (rs334558) and a significantly higher frequency of the C allele of −50T/C (rs334558) were found in the Japanese patients than those reported for white populations. Benedetti et al[6,7] showed that the genotype frequencies were T/T 38%, T/C 45%, and C/C 15% and that the allele frequencies were T 60.5% and C 39.5% for Italian bipolar type I patients. In this study, the observed genotype frequencies T/T 14.3%, T/C 33.3%, and C/C 52.4% and the allele frequencies T 31.0% and C 69.0% for the Japanese bipolar disorder patients were not significantly different from those for Japanese healthy subjects (19%,63%,18%; 49%:51%).[14] There was a significant difference in the genotype frequency of -50T/C between the Italian and Japanese patients. As for −1727A/T, the allele frequencies (A 87% and T 13%) for white healthy subjects shown by Russ et al were not significantly different from those for the Japanese healthy subjects (A 81% and T 19%). They identified 2 common SNPs at positions −50T/C and −1727A/T localized in the promoter region of the gene, with minor allele frequencies in white controls of 35% (C) and 13% (T), respectively, and we identified in the Japanese controls 49% (T) and 19% (T), respectively.[5,14] In this study, genotypic and allelic frequencies of −1727A/T polymorphism observed were inconsistent with the genotypic and allelic frequencies observed in the HapMap Japanese population. However, the frequencies observed in our data are consistent with previous studies conducted in an Asian population,

TABLE 1. Haplotype Frequencies in Lithium Responders and Nonresponders

Haplotype	−50T/C	−1727A/T	Frequency: Responder	Frequency: Nonresponder	χ^2	*P*	Empirical *P*
1	T	A	0.3889	0.1667	4.456	0.03477	0.0673
2	C	A	0.4259	0.6667	4.473	0.03443	0.079
3	C	T	0.1852	0.1667	0.04509	0.8318	0.851

The haplotype distributions showed a trend with significant difference between the lithium nonresponders and responders (global P=0.07074; empirical global P=0.1305). Haplotype 1 (T-A) was associated with a higher lithium response (haplotype-specific P=0.03477; empirical P=0.0673), whereas haplotype 2 (C-A) was associated with a lower lithium response (haplotype-specific P=0.03443; empirical P=0.079).

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and the frequencies of −1727 A/A genotype in our Japanese control subjects (64%) were similar to those in Korean subjects (70%–73%).[14–16]

In previous genetic and functional studies on *GSK-3β*, it was revealed that the major physiological mechanism that regulates the activity of GSK3 is the phosphorylation of the N-terminal serine of GSK3.[17] It was shown that the T allele of −50T/C (rs334558) *GSK3B* polymorphism gives greater transcriptional activity, which can be associated with the hyperphosphorylation of τ, resulting in neurodegeneration.[9] In addition, Benedetti et al[6,7] reported that, in humans, the promoter variant (rs334558*C) was associated with reduced activity and better antidepressant response. Furthermore, lithium has been used for the treatment of bipolar disorder, and its ability to inhibit *GSK-3β* has been implicated as the mechanism of action in bipolar disorder.[1]

Therefore, the *GSK3B* transcriptional activity regulation by lithium may also be associated with the susceptibility to lithium treatment in bipolar disorder. Our finding that *GSK3B* haplotype 1 (T-A) was associated with a higher lithium response may suggest that patients with the T allele of −50T/C (rs334558), which gives greater transcriptional activity, are more affected by lithium, which inhibits *GSK-3β* activity.

Initially, GSK-3 was identified as a phosphorylating and inactivating glycogen synthase that is critical to the regulation of glucose storage.[18] It was recently discovered that GSK3 is a serine/threonine-specific protein and that it plays an important role in regulating neuronal plasticity, gene expression, and cell survival.[19]

The importance of *GSK-3β* and τ protein seen not only in Parkinson disease and/or Alzheimer disease[9,10] pathology but also in bipolar illness[6] has already been documented. On the other hand, Yoona and Kima[20] suggested that 2 promoter polymorphisms of the *GSK-3β* gene may not be related to the pathogenesis of major depression disorder and the risk for suicidal behavior in Korean depressive patients.

The sample size in this study including Japanese bipolar disorder lithium responders and nonresponders was not large enough for clinical situation, even if this study is a pilot study for personalized medicine (tailor-made therapy) for bipolar disorder. Therefore, larger-scale comparison is needed to confirm the actual relationship between susceptibility to lithium and *GSK-3β* haplotypes among bipolar disorder patients.

REFERENCES

1. Gould TD, Manji HK. Glycogen synthase kinase-3: a putative molecular target for lithium mimetic drugs. *Neuropsychopharmacology* 2005;30:1223–1237.
2. Saus E, Soria V, Escaramís G, et al. A haplotype of glycogen synthase kinase 3β is associated with early onset of unipolar major depression. *Genes Brain Behav* 2010;9:799–807.
3. Shaw PC, Davies AF, Lau KF, et al. Isolation and chromosomal mapping of human glycogen synthase kinase-3 alpha and -3 beta encoding genes. *Genome* 1998;41:720–727.
4. Bailer U, Leisch F, Meszaros K, et al. Genome scan for susceptibility loci for schizophrenia and bipolar disorder. *Biol Psychiatry* 2002;52:40–52.
5. Russ C, Lovestone S, Powell JF. Identification of sequence variants and analysis of the role of the glycogen synthase kinase 3 beta gene and promoter in late onset Alzheimer's disease. *Mol Psychiatry* 2001;6:320–324.
6. Benedetti F, Serretti A, Pontiggia A, et al. Long-term response to lithium salts in bipolar illness is influenced by the glycogen synthase kinase 3-beta -50T/C SNP. *Neurosci Lett* 2005;376:51–55.
7. Benedetti F, Dallaspezia S, Lorenzi C, et al. Gene-gene interaction of glycogen synthase kinase 3-β and serotonin transporter on human antidepressant response to sleep deprivation. *J Affect Disord* 2012;136:514–519.
8. Lau KF, Miller CC, Anderton BH et al. Molecular cloning and characterization of the human glycogen synthase kinase-3beta promoter. *Genomics* 1999;60:121–128.
9. Kwok JB, Hallupp M, Loy CT, et al. *GSK3B* polymorphisms alter transcription and splicing in Parkinson's disease. *Ann Neurol* 2005;58:829–839.
10. Kalinderi K, Fidani L, Katsarou Z, et al. GSK3β polymorphisms, MAPT H1 haplotype and Parkinson's disease in a Greek cohort. *Neurobiol Aging* 2011;32:546–545.
11. Young RC, Biggs JT, Ziegler VE, et al. Rating scale for mania: reliability, validity, and sensitivity. *Br J Psychiatry* 1978;133:429–435.
12. Barrett JC, Fry B, Maller J, et al. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263–265.
13. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007;81:559–575.
14. Numajiri M, Nishizawa D, Ikeda K, et al. The relationship between glycogen synthase kinase − 3beta -1727A/T × -50T/C genetic polymorphisms and nicotine dependence. *Nihon Arukoru Yakubutsu Igakkai Zasshi* 2013;48:293–299.
15. Lee KY, Ahn YM, Joo EJ, et al. No association of two common SNPs at position -1727 A/T, -50 C/T of GSK-3 beta polymorphisms with schizophrenia and bipolar disorder of Korean population. *Neurosci Lett* 2006;395:175–178.
16. Yoon HK, Kim YK. Association between glycogen synthase kinase-3beta gene polymorphisms and major depression and suicidal behavior in a Korean population. *Prog Neuropsychopharmacol Biol Psychiatry* 2010;34:331–334.
17. Frame F, Cohen P, Biondi RM. A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. *Mol Cell* 2001;7:1321–1327.
18. Embi N, Rylatt DB, Cohen P. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur J Biochem* 1980;107:519–527.
19. Jope RS, Johnson GV. The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* 2004;29:95–102.
20. Yoona HK, Kima YK. Association between glycogen synthase kinase-3β gene polymorphisms and major depression and suicidal behavior in a Korean population. *Prog Neuropsychopharmacol Biol Psychiatry* 2010;34:331–334.

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MOLECULAR PAIN

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Haplotypes of *P2RX7* gene polymorphisms are associated with both cold pain sensitivity and analgesic effect of fentanyl

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Abstract

Background: The $P2X_7$ receptor is a member of the P2X family of adenosine 5′-triphosphate-gated cation channels. Several recent studies have demonstrated that this receptor is involved in mechanisms related to pain and inflammation. However, unknown is whether polymorphisms of the *P2RX7* gene that encodes the human $P2X_7$ receptor influence pain sensitivity and analgesic effects of opioids. The *P2RX7* gene is known to be highly polymorphic. Thus, the present study examined associations between fentanyl sensitivity and polymorphisms in the *P2RX7* gene in 355 Japanese patients who underwent painful orofacial cosmetic surgery.

Results: We first conducted linkage disequilibrium (LD) analyses for 55 reported single-nucleotide polymorphisms (SNPs) in the region within and around the *P2RX7* gene using genomic samples from 100 patients. In our samples, 42 SNPs were polymorphic, and a total of five LD blocks with six Tag SNPs (rs2708092, rs1180012, rs1718125, rs208293, rs1718136, and rs7132846) were observed. Thus, we further analyzed associations between genotypes/haplotypes of these Tag SNPs and clinical data using a total of 355 samples. In the genotype-based association study, only the rs1718125 G > A SNP tended to be associated with higher pain scores on a visual analog scale 24 h after surgery (VAS24). The haplotype-based association study showed that subjects with homozygous haplotype No.3 (GTAAAC; estimated frequency: 15.0%) exhibited significantly higher cold pain sensitivity and lower analgesic effects of fentanyl for acute cold pain in the cold pressor test. Conversely, subjects who carried haplotype No.1 (ACGGAC; estimated frequency: 24.5%) tended to exhibit lower cold pain sensitivity and higher analgesic effects of fentanyl. Furthermore, subjects with homozygous haplotype No.2 (GCGGAC; estimated frequency: 22.9%) exhibited significantly lower VAS24 scores.

Conclusions: Cold pain sensitivity and analgesic effects of fentanyl were related to the SNP and haplotypes of the *P2RX7* gene. The patients with the rs1718125 G>A SNP tended to show higher VAS24 scores. Moreover, the combination of polymorphisms from the 5′-flanking region to exon 5 recessively affected cold pain sensitivity and analgesic effects of opioids for acute cold pain. The present findings shed light on the involvement of *P2RX7* gene polymorphisms in naive cold pain sensitivity and analgesic effects of fentanyl.

Keywords: $P2X_7$ receptor, ATP, Purinergic receptor, Single-nucleotide polymorphism, Pain, Fentanyl, Cold pain, Haplotype analysis, Perioperative analgesia

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Introduction

Extracellular adenosine 5′-triphosphate (ATP) has been recognized as a neurotransmitter and/or neuromodulater in the nervous system that specifically acts on P2 purinergic receptors on the cell surface. P2 purinergic receptors are divided into two classes. P2X receptors are ATP-gated cation channels and subdivided into seven subtypes ($P2X_{1-7}$). P2Y receptors are heptahelical G-protein-coupled receptors and subdivided into eight subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$). Both P2X and P2Y receptors are widely expressed in the sensory nerve system and exert various effects on neuronal and glial cells [1]. Recent studies revealed that ATP and its receptors are involved in peripheral and central nociceptive transmission, including mechanisms involved in neuropathic pain [2,3].

$P2X_7$ receptors exhibit unique pharmacological characteristics compared with other P2X receptor subtypes. A high concentration of ATP (i.e., >100 μM) is required for $P2X_7$ receptor activation [4]. In addition to acting as ATP-gated Ca^{2+}-permeable cation channels, $P2X_7$ receptors induce the formation of large nonselective pores with a 900 Da cut-off [4,5]. Many studies have shown the involvement of $P2X_7$ receptors in pain. $P2X_7$ receptor knockout mice have been shown to exhibit a reduction of thermal and mechanical hypersensitivity in a partial sciatic nerve ligation model [6]. Recent developments in selective inhibitors of $P2X_7$ receptors also showed that $P2X_7$ receptor blockade reduced nociceptive behavior in several animal models of neuropathic and inflammatory pain [7-10]. Although these studies revealed an important role for $P2X_7$ receptors in neuropathic and inflammatory pain development in animal models, the involvement of $P2X_7$ receptors in the modulation of naive pain sensitivity and efficacy of analgesics in humans is still unclear.

Opioid analgesics, such as fentanyl and morphine, are widely used for the treatment of moderate to severe pain. However, the analgesic efficacy of opioids is well known to vary widely among individuals [11]. Individual differences may be related to various genetic and nongenetic factors, including gender, age, ethnic origin, hepatic or renal function, and mental status [12]. Several studies that used mice that lack the μ-opioid receptor (MOP) [13-15] have shown that analgesia produced by opioids crucially depends on the level of MOP expression. Furthermore, several single-nucleotide polymorphisms (SNPs) in the *OPRM1* gene, which encodes the human MOP protein, have been reported to lead to differences in the analgesic efficacy of opioids [16]. Several gene-association studies have also reported that the analgesic efficacy of opioids could be affected by other molecules [17-21].

Many gene polymorphisms, most of which are SNPs, reportedly exist in the genes that encode P2X and P2Y receptors. The gene that encodes the human $P2X_7$ receptor (*P2RX7*) is known to be highly polymorphic. Some SNPs in the *P2RX7* gene have been shown to cause changes in receptor function [22-24]. Only a few studies have tested associations with human pain sensitivity [24], and whether genetic polymorphisms in the *P2RX7* gene exhibit associations with pain sensitivity or opioid analgesia is still unclear. In contrast to animal studies that use standardized pain tests, the analgesic effects of opioids in humans are usually evaluated in patients with actual pain, particularly cancer pain or acute postoperative pain [16]. Patients with acute postoperative pain following standardized surgical procedures may be more optimal subjects for investigating gene-opioid effect relationships [11,17,25]. Furthermore, because subjects prior to cosmetic orthognathic surgery have no spontaneous pain, the analgesic effects of opioids in humans can be evaluated under more optimal conditions. Therefore, the present study examined whether SNPs and haplotypes in the *P2RX7* gene affect cold pain sensitivity and the analgesic effects of fentanyl, one of the most commonly used opioid analgesics, evaluated by a standardized pain test and fentanyl requirements in healthy Japanese subjects who underwent uniform surgical procedures.

Materials and methods

Ethics statement

The study protocol was approved by the Institutional Review Board, Tokyo Dental College, Chiba, Japan, and the Institutional Review Board, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan. Written informed consent was obtained from all of the patients and also from parents if required.

Patients

Enrolled in the study were 355 healthy patients (American Society of Anesthesiologists Physical Status I, age 15–52 years, 125 males and 230 females [the same patients who served as subjects in our previous report] [17]) who were scheduled to undergo cosmetic orthognathic surgery (mandibular sagittal split ramus osteotomy) for mandibular prognathism at Tokyo Dental College Suidoubashi Hospital. Patients with chronic pain, those taking pain medication, and those who had experienced Raynaud's phenomenon were excluded.

Preoperative cold pressor-induced pain test

The patients were premedicated with oral diazepam, 5 mg, and oral famotidine, 150 mg, 90 min before the induction of anesthesia. The patients had an intravenous (i.v.) line on the forearm on their nondominant side. The temperature in the operating room was maintained at 26°C. The cold pressor-induced pain test was then

performed before and 3 min after an i.v. bolus injection of fentanyl, 2 μg/kg, as previously described [25,26]. Briefly, crushed ice cubes and cold water were blended 15 min before the test in a 1 L isolated tank, and the mixture was stirred immediately before each test to ensure uniform temperature distribution (0°C) within the tank. The dominant hand was immersed up to the wrist. Patients were instructed to keep the hand calm in the ice-cold water and withdraw it as soon as they perceived any pain. All of the patients were administered the test by the same investigator. The baseline latency to pain perception, defined as the time of immersion of the hand in the ice water, before an i.v. injection of fentanyl (PPLpre) was recorded. A cut-off point of 150 s was set to avoid tissue damage. The hand was warmed with a hair dryer as soon as it was withdrawn from the ice water until the sensation of cold was completely abolished. The patients then received i.v. fentanyl, 2 μg/kg. Three minutes after the injection, the pain perception latency of the dominant hand (PPLpost) was measured again. The analgesic effect of fentanyl in the preoperative cold pressor-induced pain test was evaluated simply as the difference between PPLpost and PPLpre (PPLpost - PPLpre).

Anesthesia, surgery, and postoperative pain management

Anesthesia, surgery, and postoperative pain management were performed as previously described [17]. Briefly, after the cold pressor-induced pain test ended, general anesthesia was induced with a target-controlled infusion (TCI) of propofol. After the induction of anesthesia, 10 ml of venous blood was sampled for the preparation of DNA specimens. Local anesthesia was performed on the right side of the surgical field with 8 ml of 2% lidocaine that contained epinephrine, 12.5 μg/ml, and right mandibular ramus osteotomy was performed. Local anesthesia was then performed on the left side, and left mandibular ramus osteotomy was performed. The bilateral mandibular bone segments were fixed in appropriate positions. Whenever systolic blood pressure or heart rate exceeded +20% of the preinduction value during surgery, i.v. fentanyl, 1 μg/kg, was administered. At the end of the surgery, rectal diclofenac sodium, 50 mg, and i.v. dexamethasone, 8 mg, were administered at the request of surgeons to prevent postoperative orofacial edema/swelling. After emergence from anesthesia and tracheal extubation, i.v. patient-controlled analgesia (PCA) with a fentanyl-droperidol combination (2 mg fentanyl and 5 mg droperidol diluted in normal saline in a total volume of 50 ml) commenced using a CADD-Legacy PCA pump (Smiths Medical Japan, Tokyo, Japan). A bolus dose of fentanyl, 20 μg, on demand and a lockout time of 10 min were set. Patient-controlled analgesia continued for 24 h postoperatively. The intensity of spontaneous pain was assessed 3 and 24 h postoperatively using a 100-mm visual analog scale (VAS), with 0 mm indicating no pain and 100 mm indicating the worst pain imaginable. Intraoperative fentanyl use and postoperative PCA fentanyl use during the first 24 h postoperative period were recorded. Doses of fentanyl administered intraoperatively and postoperatively were normalized to body weight. Additionally, perioperative fentanyl use was calculated as the sum of intraoperative fentanyl use and postoperative fentanyl use.

Genotyping procedures and linkage disequilibrium analysis

Genomic DNA was extracted from whole-blood samples using standard procedures. The extracted DNA was dissolved in TE buffer (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid, pH 8.0). The DNA concentration was adjusted to 5–50 ng/μl for genotyping individual SNPs or 100 ng/μl for whole-genome genotyping using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

For the analysis of SNPs within and around the *P2RX7* gene region, genotype data from whole-genome genotyping were used. Briefly, whole-genome genotyping was performed using Infinium assay II and an iScan system (Illumina, San Diego, CA) according to the manufacturer's instructions. Five kinds of BeadChips were used to genotype 40, 67, 6, 119, and 123 samples, respectively: HumanHap 300 (total markers: 317,503), HumanHap300-Duo (total markers: 318,237), Human610-Quad v1 (total markers: 620,901), Human1M v1.0 (total markers: 1,072,820), and Human 1 M-Duo v3 (total markers: 1,199,187). Some BeadChips included a number of probes specific to copy number variation markers, but most were for SNP markers on the human autosome or sex chromosome. Approximately 300,000 SNP markers were commonly included in all of the BeadChips. After the whole-genome genotyping, the data for genotyped samples were analyzed using BeadStudio or GenomeStudio with the Genotyping module v3.3.7 (Illumina) to evaluate the quality of the results, and the genotype data for all of the SNPs with *P2RX7* gene annotation were extracted. In the data-cleaning process, markers that had "Cluster sep" values (i.e., an index of genotype cluster separation) <0.4 and were separated from any of the three genotype clusters were excluded from the subsequent association study.

Single-nucleotide polymorphisms for the association studies were selected based on recently advanced tagging strategies [27-29]. To identify relationships between the SNPs used in the study, linkage disequilibrium (LD) analysis was performed in 55 SNPs that were in the approximately 108 kbp region that contained the *P2RX7* gene among 1,072,820 markers in the Human 1 M v1.0 BeadChip for 100 samples using Haploview v.4.2 [30].

For the estimation of LD strength between the SNPs, the commonly used D' and r^2 values were pairwise calculated using the genotype dataset of each SNP. Linkage disequilibrium blocks were defined among the SNPs that showed "strong LD," based on the default algorithm of Gabriel et al. [31], in which the upper and lower 95% confidence limits on D' for strong LD were set at 0.98 and 0.7, respectively. Tag SNPs in the LD block were consequently determined using Tagger software with default settings, which is incorporated in Haploview and has been detailed in a previous report [29].

Statistical analysis

Parametric and nonparametric data are expressed as mean ± SD and median [interquartile range], respectively. The statistical analysis was performed using IBM SPSS statistics v.20.0.0 (IBM, Tokyo, Japan). In the present study, none of the clinically measured endpoints that were related to pain sensitivity (i.e., PPLpre) or fentanyl analgesia (i.e., analgesia measured with the preoperative cold pressor test, perioperative fentanyl use, and VAS scores at 3 and 24 h postoperatively) were normally distributed. Therefore, nonparametric analyses, including the Mann–Whitney *U*-test or Kruskal-Wallis test (with Steel-Dwass multiple comparison tests), were used to detect possible associations between any of the clinical or genomic parameters (e.g., sex and genotypes of the Tag SNP) and clinical endpoints related to pain sensitivity or the analgesic effects of fentanyl. The sample size of the present nonparametric data was higher than the estimated size that possesses statistical power (1 minus type II error probability) of 99% for the Cohen's conventional "medium" effect size of 0.25, when power analysis was performed for analysis of variance with three genotype groups using G*Power v.3.1.3 [32]. Haplotype analyses were performed using HPlus v.3.2 software with default settings (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) that employs expectation-maximization with a modified progressive ligation computational algorithm to infer haplotypes [33].

Results

To identify the LD blocks in the approximately 108 kbp region that contains the *P2RX7* gene, 55 SNPs among 1,072,820 markers that were included in the whole-genome genotyping (Human 1 M v1.0 BeadChip) were tested using genomic samples of 100 patients who were randomly selected from all 355 Japanese patients. In these Japanese samples, 42 SNPs were found to be polymorphic. A total of five LD blocks (LD1-5) were observed within and around the *P2RX7* gene region (the exon, intron, and approximately 45 kbp 5′-flanking region and 10 kbp 3′-flanking region of the *P2RX7* gene [approximately 53 kbp]), and six representative Tag SNPs (rs2708092, rs1180012, rs1718125, rs208293, rs1718136, and rs7132846) were selected in this region (Table 1, Additional file 1). Thus, we further examined associations between clinical endpoints and these six representative Tag SNPs using genomic samples from all 355 Japanese patients.

Of the 355 Japanese patients who enrolled in the study, 353 completed the study. The distributions of six representative Tag SNP genotypes are shown in Table 2. No observed genotype frequencies were significantly different from Hardy-Weinberg equilibrium.

The Mann–Whitney *U*-test revealed that the VAS score at 24 h was significantly lower ($p = 0.019$; Table 3) in subjects who did not carry the minor A allele of the rs1718125 SNP than subjects who carried this allele, whereas the rs1718125 SNP had no significant association with PPLpre, PPLpost-PPLpre, 24-h postoperative fentanyl use, perioperative fentanyl use, total perioperative analgesic use, and the VAS score at 3 h. The unpaired *t*-test, Fisher's exact test, and Mann–Whitney *U*-test revealed no significant differences in age, sex, duration of surgery, and duration of anesthesia between subjects who carried the minor A allele and subjects who did not carry this allele (p =0.079 [age: Table 3], $p = 0.740$ [sex: Table 3], p =0.430 [duration of surgery: AA + AG, 106 min (93, 128); GG, 105 min (92, 122)], and $p = 0.384$ [duration of anesthesia: AA + AG, 173 min (160, 195); GG, 173 min (157, 193)], respectively). Therefore, we did not conduct additional multivariate covariate analyses. The other five SNPs did not show any associations with any of the clinical endpoints (Table 3). When multiple-testing corrections (i.e., the standard Bonferroni correction for the number of SNPs) were applied, no significant association was found between the genotype of the rs1718125 SNP and VAS score at 24 h.

We further analyzed the haplotype-based associations of the six *P2RX7* gene Tag SNPs with clinical endpoints. Of the 26 estimated haplotypes, 10 haplotypes (estimated frequency >1%) are listed in Table 4. Haplotype No.1 (ACGGAC) was significantly associated with both higher PPLpre score and higher PPLpost-PPLpre score in the linear regression analysis that used the recessive model (Table 5) and log-additive model (PPLpre: coefficient = 4.532 [confidence interval (CI): 0.048, 9.017], z-score = 1.981, p =0.048; PPLpost-PPLpre: coefficient = 8.735 [CI: 1.829, 15.640], z-score = 2.479, $p = 0.013$) and associated with higher PPLpost-PPLpre score using the dominant model (coefficient =8.802 [CI: 0.601, 17.004], z-score = 2.104, $p = 0.035$), although the adjusted p values of these associations with haplotype No.1 after multiple-testing corrections (i.e., the standard Bonferroni correction for the number of clinical endpoints) were not significant. The linear regression analyses that used

Table 1 Allelic frequencies of SNPs in five LD blocks around the *P2RX7* gene of Japanese subjects

rs number of SNPs	Position		Major allele	Minor allele	Frequency	LD block
rs12819523	Flanking_5UTR	−45261	G	A	7.5%	
rs12819930	Flanking_5UTR	−45087	G	A	10.7%	1
rs10492051	Flanking_5UTR	−45012	G	-	0.0%	
*_**rs2708092**_	Flanking_5UTR	−44792	A	G	49.0%	1
*_**rs1180012**_	Flanking_5UTR	−40230	C	T	38.8%	1
rs2516210	Flanking_5UTR	−35256	T	C	15.9%	1
rs1796421	Flanking_5UTR	−30332	T	C	15.9%	1
rs1796412	Flanking_5UTR	−28966	T	C	18.2%	1
rs7298521	Flanking_5UTR	−28847	C	T	22.9%	1
rs1794899	Flanking_5UTR	−27705	G	A	18.0%	1
rs1796415	Flanking_5UTR	−27667	T	-	0.0%	
rs3892756	Flanking_5UTR	−16010	T	-	0.0%	
rs10849846	Flanking_5UTR	−12255	T	C	21.8%	1
rs208277	Flanking_5UTR	−10097	A	G	41.1%	1
rs12314721	Flanking_5UTR	−4921	C	T	14.9%	1
rs9805004	Flanking_5UTR	−4808	C	T	18.2%	1
rs670541	Flanking_5UTR	−1044	C	T	8.9%	1
rs591874	Intron1	IVS1 + 567	A	C	40.9%	1
rs7959194	Intron1	IVS1 + 5089	G	-	0.0%	
rs11065450	Intron1	IVS1 + 8759	C	A	28.7%	
rs568531	Intron1	IVS1 + 8775	C	T	7.5%	2
rs607094	Intron1	IVS1 + 9566	T	C	6.4%	2
rs208286	Intron1	IVS1-5688	G	-	0.0%	
rs17525809	Exon2	T227C[V76A]	T	C	8.9%	
*_**rs1718125**_	Intron2	IVS2 + 263	G	A	28.3%	3
rs10849851	Intron3	IVS3-2061	A	G	21.0%	3
rs1653583	Intron3	IVS3-53	C	T	7.2%	3
*_**rs208293**_	Intron4	IVS4-47	G	A	41.1%	3
rs208294	Exon5	T463C[Y155H]	T	C	43.5%	3
rs1186055	Intron5	IVS5 + 206	G	T	39.5%	
rs208296	Intron5	IVS5 + 630	C	T	36.1%	
rs11065464	Intron5	IVS5-1025	C	A	24.3%	
rs208298	Intron5	IVS5-922	G	A	16.6%	4
rs654856	Intron5	IVS5-38	C	A	13.4%	4
rs2857589	Intron7	IVS7 + 486	C	A	5.1%	4
rs503720	Intron7	IVS7-217	G	A	16.1%	4
rs16950860	Exon8	C808T[R270C]	C	-	0.0%	
rs7958311	Exon8	G809A[R270H]	G	A	35.6%	
rs7958316	Exon8	G827A[R276H]	G	-	0.0%	
*_**rs1718136**_	Intron8	IVS8-3583	A	G	15.0%	5
rs7137542	Intron8	IVS8-545	G	-	0.0%	
*_**rs7132846**_	Intron9	IVS9-16	C	T	12.9%	5
rs1718119	Exon11	G1040A[A348T]	G	A	17.5%	5

Table 1 Allelic frequencies of SNPs in five LD blocks around the *P2RX7* gene of Japanese subjects *(Continued)*

rs1653598	Intron11	IVS11 + 34	A	G	14.9%	5
rs2567998	Intron11	IVS11-16	A	-	0.0%	
rs10160951	Exon12	C1289G[P430R]	C	-	0.0%	
rs12829218	Intron12	IVS12 + 133	A	-	0.0%	
rs891781	Intron12	IVS12 + 180	C	T	17.5%	5
rs7312642	Intron12	IVS12-1921	G	T	12.1%	
rs12815078	Intron12	IVS12-101	A	G	6.1%	
rs2230912	Exon13	A1379G[Q460R]	A	-	0.0%	
rs3751144	Exon13	C1422T	C	T	12.9%	
rs3751143	Exon13	A1487C[E496A]	A	C	32.0%	
rs1718161	Flanking_3UTR	+3582	A	G	6.5%	
rs2686365	Flanking_3UTR	+9907	C	-	0.0%	

*Tag SNPs in the LD blocks (selected using Tagger software with default settings; $r^2 > 0.8$).

the recessive model revealed that haplotype No.3 (GTAAAC) was significantly associated with both lower PPLpre score and lower PPLpost-PPLpre score, even after multiple-testing corrections (Table 5). These significant associations were not evident when analyzed using either a dominant or log-additive model. Furthermore, linear regression analyses that used the recessive model also revealed that haplotype No.2 (GCGGAC) was significantly associated with lower VAS scores at 24 h, even after multiple-testing corrections (Table 5). This significant association was also not evident when analyzed using either the dominant or log-additive model.

Discussion

We studied patients who underwent mandibular sagittal split ramus osteotomy. Subjects who undergo this cosmetic surgery are usually young and healthy. The operation causes considerable perioperative pain that arises from the dissected mandibular bone, and the surgical technique is highly standardized at our institute. We conducted a standardized pain test before the induction of general anesthesia in opioid-naive subjects without pain. Using these ideal subjects and methods, we found that PPLpre and the analgesic effects of fentanyl (PPLpost-PPLpre) evaluated in the cold pressor test were significantly lower in subjects who carried homozygous haplotype No.3 (GTAAAC) in the *P2RX7* gene compared with the other subjects. Subjects who carried haplotype No.1 (ACGGAC), which has a different allele in the Tag SNP on the LD1-3 regions from haplotype No.3, tended to have higher PPLpre scores and higher analgesic effects of fentanyl. These results suggest that the combination of polymorphisms from the 5′-flanking region to exon 5 (from LD1 to LD3 regions) in the *P2RX7* gene could recessively affect cold pain sensitivity and the analgesic effects of opioids for acute cold pain. Furthermore, VAS scores at 24 h tended to be lower in subjects who carried homozygously the major G allele of the rs1718125 SNP (3rd Tag SNP) than subjects who carried the minor A allele. We also found that VAS scores at 24 h were significantly lower in subjects who carried homozygous haplotype No.2 (GCGGAC) in the *P2RX7* gene compared with the other subjects. Thus, haplotype No.2 could recessively affect the postoperative analgesic effects of opioids. Although we analyzed five types of BeadChips that were merged together in the present study because the sample sizes were small for each of the five datasets and they all presumably had Japanese ancestry, performing a meta-analysis is usually better than merging. Thus, further studies that have a greater number of samples might be required to reveal the influences of these haplotypic effects in the *P2RX7* gene. Twenty-four hour postoperative fentanyl use,

Table 2 Distribution of six Tag SNP genotypes examined in the *P2RX7* gene

rs2708092	AA:	99	(28.0%)	/	AG:	177	(50.0%)	/	GG:	79	(22.0%)
rs1180012	CC:	128	(36.1%)	/	CT:	169	(47.6%)	/	TT:	58	(16.3%)
rs1718125	GG:	180	(50.7%)	/	GA:	149	(42.0%)	/	AA:	26	(7.3%)
rs208293	GG:	111	(31.3%)	/	GA:	177	(49.9%)	/	AA:	67	(18.9%)
rs1718136	AA:	247	(69.6%)	/	AG:	97	(27.3%)	/	GG:	11	(3.1%)
rs7132846	CC:	274	(77.2%)	/	CT:	75	(21.1%)	/	TT:	6	(1.7%)

The data are expressed as number (%) of subjects.

Table 3 Patients' demographic and clinical data

	rs2708092		rs1180012		rs1718125	
	AA	AG + GG	CC	CT + TT	GG	GA + AA
Age (years)	26.1 ± 7.0	25.8 ± 7.8	27.3 ± 7.7	25.1 ± 7.5	26.6 ± 7.7	25.2 ± 7.5
Male/Female	32/67	92/163	51/77	74/153	65/115	60/115
PPLpre (s)	15 [10,21]	14 [9,23]	14 [9,23]	14 [9, 23]	14 [9, 22]	15 [9, 23]
PPLpost (s)	29 [18, 52]	27 [16, 54]	28 [16, 49]	28 [16, 54]	27 [16, 49]	30 [16, 56]
Analgesic effect (PPLpost-PPLpre) (s)	15 [5, 35]	11 [4, 35]	10 [4, 30]	13 [4, 36]	10 [4, 30]	14 [5, 37]
24-h postoperative fentanyl use (μg/kg)	2.2 [1.2, 4.7]	2.3 [1.0, 4.1]	2.3 [0.9, 4.1]	2.3 [1.1, 4.3]	2.1 [0.9, 3.9]	2.4 [1.3, 4.4]
Perioperative fentanyl use (μg/kg)	7.6 [5.8, 9.2]	6.7 [5.1, 9.1]	6.8 [5.0, 9.1]	7.1 [5.3, 9.1]	6.8 [5.0, 8.6]	7.1 [5.5, 9.5]
Total perioperative analgesic use (μg/kg)	8.5 [6.7, 10.2]	7.6 [6.0, 10.1]	7.7 [5.8, 10.2]	8.0 [6.2, 10.0]	7.8 [5.8, 9.6]	8.0 [6.3, 10.5]
VAS pain score at 3 h (mm)	27 [15, 47]	27 [15, 50]	27 [15, 50]	30 [15, 50]	27 [15, 50]	30 [16, 50]
VAS pain score at 24 h (mm)	25 [10, 38]	25 [10, 45]	22 [8, 40]	26 [11, 43]	23 [8, 40]	28 [14, 46]*
	rs208293		rs1718136		rs7132846	
	GG	GA + AA	AA	AG + GG	CC	CT + TT
Age (years)	27.5 ± 7.9	25.2 ± 7.4	26.2 ± 7.9	25.4 ± 6.9	25.7 ± 7.3	26.8 ± 8.8
Male/Female	41/70	84/160	95/152	30/78	104/170	21/60
PPLpre (s)	13 [9, 22]	15 [9, 24]	14 [9, 24]	15 [9, 21]	14 [9, 23]	14 [9, 23]
PPLpost (s)	27 [16, 51]	28 [16, 54]	29 [16, 56]	25 [15, 48]	28 [16, 53]	28 [16, 53]
Analgesic effect (PPLpost-PPLpre) (s)	10 [3, 34]	13 [5, 35]	13 [5, 36]	11 [4, 30]	12 [4, 35]	12 [4, 35]
24-h postoperative fentanyl use (μg/kg)	2.3 [1.1, 4.0]	2.3 [1.0, 4.3]	2.3 [1.1, 4.3]	2.0 [1.0, 4.1]	2.3 [1.1, 4.2]	2.3 [1.0, 4.2]
Perioperative fentanyl use (μg/kg)	6.8 [5.1, 9.0]	7.1 [5.3, 9.2]	7.0 [5.2, 9.2]	6.7 [5.2, 8.9]	6.9 [5.2, 9.1]	6.9 [5.2, 9.1]
Total perioperative analgesic use (μg/kg)	7.7 [5.9, 10.1]	8.0 [6.2, 10.1]	8.0 [6.1, 10.1]	7.7 [6.1, 9.9]	8.0 [6.1, 10.1]	8.0 [6.1, 10.1]
VAS pain score at 3 h (mm)	27 [16, 50]	27 [15, 49]	27 [15, 50]	26 [15, 49]	28 [15, 50]	27 [15, 50]
VAS pain score at 24 h (mm)	22 [10, 39]	26 [11, 45]	25 [10, 42]	25 [8, 42]	25 [10, 42]	25 [10, 42]

The data are expressed as numbers, mean ± SD (range), or median [interquartile range]. *p <0.05, compared with subjects not carrying minor allele.

perioperative fentanyl use, and total perioperative analgesic use were not associated with haplotypes in the *P2RX7* gene. Although further validation is needed, the analgesic/opioid requirements for postoperative pain management might not be associated with genetic polymorphisms in the *P2RX7* gene.

Table 4 Estimated *P2RX7* gene Tag SNP haplotypes and their frequencies

	Estimated haplotypes	Frequency	(SE)
1	ACGGAC	0.245	(0.016)
2	GCGGAC	0.229	(0.019)
3	GTAAAC	0.150	(0.014)
4	ATGAGC	0.103	(0.011)
5	GTAAAT	0.051	(0.010)
6	ACGGAT	0.048	(0.011)
7	ATAAGC	0.046	(0.009)
8	ACGAAC	0.041	(0.008)
9	ATAAAC	0.018	(0.014)
10	GTGGAC	0.016	(0.006)

In the present study, subjects who carried homozygous haplotype No.3 (GTAAAC) in the *P2RX7* gene exhibited higher cold pain sensitivity and lower analgesic effects of fentanyl for acute cold pain in the cold pressor test, but still unknown is whether this combination of SNPs alters gene function or expression, which may be an important limitation of the present study. Many SNPs have been identified in the *P2RX7* gene, and some of the SNPs in the *P2RX7* gene have been shown to cause changes in receptor function [22-24]. Although our present study did not focus on these reported functional SNPs, some of them are located on the present LD blocks. The rs208294 T/C SNP (Tyr^{155} to His), which was described as the C489T SNP in previous reports with non-Japanese subjects, is located in the LD3 region in the present study. This SNP showed high LD (D' =1.0) with the present 4th Tag SNP, rs208293 G/A SNP. In the previous report, functional analyses in recombinant cells that expressed $P2X_7$ receptors with the mutation of the rs208294 SNP using $[Ca^{2+}]_i$ influx and ethidium uptake experiments revealed that the T > C mutation in this position caused a loss of function of $P2X_7$

Table 5 Associations between *P2RX7* gene Tag SNP haplotypes and clinical data

	Haplotypes	PPLpre				
		Coefficient	SE	CI	z-score	*p* value
1	ACGGAC	13.245	6.576	(0.357, 26.134)	2.014	0.044
2	GCGGAC	−0.23	4.252	(−8.563, 8.103)	−0.05	0.957
3	GTAAAC	−7.275	2.34	(−11.860, −2.689)	−3.11	0.002
		Analgesic effect (PPLpost-PPLpre)				
		Coefficient	SE	CI	z-score	*p* value
1	ACGGAC	19.452	9.34	(1.146, 37.759)	2.083	0.037
2	GCGGAC	−2.966	6.87	(−16.431, 10.499)	−0.43	0.666
3	GTAAAC	−14.97	4.585	(−23.957, −5.983)	−3.27	0.001
		VAS pain score at 24 h				
		Coefficient	SE	CI	z-score	*p* value
1	ACGGAC	7.878	4.067	(−0.094, 15.849)	1.937	0.053
2	GCGGAC	−10.799	3.523	(−17.705, −3.894)	−3.07	0.002
3	GTAAAC	7.434	8.88	(−9.971, 24.839)	0.837	0.402

Haplotype-based associations were tested using linear regression analyses with a recessive model.

receptors [34]. Although the rs208293 G/A SNP alone did not show any association with cold pain sensitivity and the analgesic effects of fentanyl, the rs208294 T/C SNP might be one of the mutations in LD1-3 that caused the present results. Interestingly, $P2X_7$ receptors have been reported to contain several alternative splicing variants ($P2X7_{a\text{-}k}$), some of which presented altered function [35-38]. Because the splicing difference in more than half of these isoforms occurs from the LD1 to LD3 regions (from exon 1 to exon 5) in the *P2RX7* gene, polymorphisms in these regions might affect this splicing mechanism and induce a functional change in neuronal transmission via $P2X_7$ receptors. Further studies that focus on gene polymorphisms from LD1 to LD3 in the *P2RX7* gene may reveal the functional mechanisms that affect pain sensitivity and clinical efficacy of opioids. A recent report showed a genetic association between the hypofunctional rs7958311 G/A SNP (Arg^{270} to His) and lower pain intensity in two cohort studies of human patients with chronic pain [24]. The rs7958311 G/A SNP is located in the gap between the present LD4 and LD5 regions. Including this SNP, some other nonsynonymous SNPs (e.g., rs3751143 A/C SNP [Glu^{496} to Ala] [22]) are located in the gap among the present LD regions and were not evaluated in the present study. Further association analyses of these SNPs in the *P2RX7* gene may be necessary to reveal the role and mechanisms of $P2X_7$ receptors in naive pain sensitivity and the efficacy of analgesics.

The potential of $P2X_7$ receptors as a therapeutic target in the management of neuropathic pain and inflammation has been the subject of intensive recent investigations [2,3,39], but the involvement of $P2X_7$ receptors in the modulation of naive pain sensitivity and efficacy of analgesics is still unclear. $P2X_7$ receptors are expressed predominantly on immune cells, and are speculated to contribute to the hyperexcitability of nociceptive neurons through the release of both interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) [40-42]. These cytokines are well known to play important roles in the generation and maintenance of pain, and especially in chronic pain states [39,42]. $P2X_7$ receptors are also known to act not only as cation channels following brief activation by extracellular ATP but also as large nonselective pores following prolonged or repeated activation. Sorge et al. reported that mice that carried a mutation in the *P2RX7* gene that causes impaired pore formation showed less hypersensitivity in neuropathic and inflammatory pain states [24]. These previous data might indicate that the pore formation of $P2X_7$ receptors closely affects chronic pain states [43]. In the present study, haplotype No.2 (GCGGAC) and the rs1718125 SNP in the *P2RX7* gene showed associations with VAS scores at 24 h but not with VAS scores at 3 h or the analgesic effects of fentanyl in the cold pressor test. Immediate pain transmission in the cold pressor test and the acute phase of postoperative pain (reflected by VAS score at 3 h) might have different pain-modulated mechanisms from subacute postoperative pain (VAS score at 24 h), and the latter might be affected by the pore formation of $P2X_7$ receptors. Further validation of the relationship between these *P2RX7* gene polymorphisms and time-dependent changes in postoperative pain states is needed to clarify this issue.

The present study revealed that PPLpre and PPLpost-PPLpre in the cold pressor test were associated with haplotypes of the SNPs (haplotype No.1 and No.3) in the *P2RX7* gene, suggesting that $P2X_7$ receptors are involved in cold pain transmission and the analgesic effects of opioids for cold pain stimuli under naive conditions. However, these haplotypes showed no significant association with clinical pain conditions (VAS scores) or fentanyl requirements. The pain stimulus in the cold pressor test may be mediated by the activation of transient receptor potential ankyrin 1 (TRPA1) channels in primary sensory neurons [44]. Postoperative pain may be mediated mainly by the activation of transient receptor potential vanilloid 1 (TRPV1) channels subsequent to incision and inflammation and not by the activation of TRPA1 channels [45]. $P2X_7$ receptors are well known to act as ATP-gated Ca^{2+}-permeable cation channels. Furthermore, the function of TRP channels might be regulated by external Ca^{2+} [46]. Thus, together with our present results, TRPA1 channels may be affected by the channel function of $P2X_7$ receptors, and TRPV1 channels may be affected by the pore formation of $P2X_7$ receptors. Although further investigation and validation are needed to confirm this hypothesis, $P2X_7$ receptors are suggested to play differential roles in pain transmission, depending on the type of pain stimulus. Furthermore, the mechanisms of $P2X_7$ receptor-induced alterations in the analgesic effects of opioids are still unclear. Only a few reports have shown that the blockade of $P2X_7$ receptors with specific antagonists or targeting small interfering RNA (siRNA) enhanced the analgesic effects of morphine in chronic morphine-treated (i.e., morphine-tolerant) rats, although these effects were not evident in naive rats [47,48]. Thus, neuronal modulation via $P2X_7$ receptors may affect the analgesic effects of opioids, but these modulatory effects may depend on the type of pain stimulus or pain expression (i.e., acute or chronic).

Conclusions

In Japanese subjects, naive cold pain sensitivity and the analgesic effect of fentanyl were related to an SNP and haplotypes in the *P2RX7* gene. The rs1718125 G > A SNP tended to be associated with higher VAS scores at 24 h after mandibular sagittal split ramus osteotomy. Furthermore, subjects with homozygous haplotype No.3 (GTAAAC) exhibited higher cold pain sensitivity and lower analgesic effects of fentanyl for acute cold pain in the cold pressor test. The combination of polymorphisms from the 5′-flanking region to exon 5 in the *P2RX7* gene could recessively affect cold pain sensitivity and the analgesic effects of opioids for acute cold pain. Subjects with homozygous haplotype No.2 (GCGGAC) had lower VAS scores at 24 h after surgery. Although further validation is needed, our data may provide valuable information about the role of $P2X_7$ receptors in pain pathways and pain treatment.

Additional file

Additional file 1: State of linkage disequilibrium (LD) between the SNPs in the *P2RX7* gene. The numbers in squares represent percentages of the D' values. Squares without numbers represent $D' = 1$. The color scheme is presented according to the "Standard Color Scheme" of Haploview v.4.2 software: bright red (Lod ≥ 2, $D' = 1$), shades of pink/red (Lod ≥ 2, $D' < 1$), blue (Lod < 2, $D' = 1$), white (Lod < 2, $D' < 1$). Blue lines indicate the exon regions of the *P2RX7* gene.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The study was conceived and the experiments were designed by SI, DN, and KI. SI and DN performed the experiments, performed the statistical analyses, and wrote the manuscript. Clinical surgery and the experiments were performed by KF and MH. Genotyping procedures were performed by SK and JH. MM and KI supervised the experiments and finalized the manuscript. All of the authors contributed to writing the manuscript, and all of the authors read and approved the final manuscript.

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References

1. Burnstock G, Knight GE: **Cellular distribution and functions of P2 receptor subtypes in different systems.** *Int Rev Cytol* 2004, **240**:31–304.
2. Burnstock G: **Purinergic mechanisms and pain–an update.** *Eur J Pharmacol* 2013, **716**:24–40.
3. Tsuda M, Tozaki-Saitoh H, Inoue K: **Pain and purinergic signaling.** *Brain Res Rev* 2010, **63**:222–232.
4. North RA: **Molecular physiology of P2X receptors.** *Physiol Rev* 2002, **82**:1013–1067.
5. Locovei S, Scemes E, Qiu F, Spray DC, Dahl G: **Pannexin1 is part of the pore forming unit of the P2X(7) receptor death complex.** *FEBS Lett* 2007, **581**:483–488.
6. Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J, Murfin M, Richardson J, Peck WL, Grahames CB, Casula MA, Yiangou Y, Birch R, Anand P, Buell GN: **Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain.** *Pain* 2005, **114**:386–396.
7. Honore P, Donnelly-Roberts D, Namovic MT, Hsieh G, Zhu CZ, Mikusa JP, Hernandez G, Zhong C, Gauvin DM, Chandran P, Harris R, Medrano AP,

Carroll W, Marsh K, Sullivan JP, Faltynek CR, Jarvis MF: A-740003 [N-(1-{[(cyanoimino) (5-quinolinylamino) methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide], a novel and selective P2X7 receptor antagonist, dose-dependently reduces neuropathic pain in the rat. *J Pharmacol Exp Ther* 2006, 319:1376–1385.

8. McGaraughty S, Chu KL, Namovic MT, Donnelly-Roberts DL, Harris RR, Zhang XF, Shieh CC, Wismer CT, Zhu CZ, Gauvin DM, Fabiyi AC, Honore P, Gregg RJ, Kort ME, Nelson DW, Carroll WA, Marsh K, Faltynek CR, Jarvis MF: P2X7-related modulation of pathological nociception in rats. *Neuroscience* 2007, 146:1817–1828.
9. Hughes JP, Hatcher JP, Chessell IP: The role of P2X(7) in pain and inflammation. *Purinergic Signal* 2007, 3:163–169.
10. Carroll WA, Donnelly-Roberts D, Jarvis MF: Selective P2X(7) receptor antagonists for chronic inflammation and pain. *Purinergic Signal* 2009, 5:63–73.
11. Ikeda K, Ide S, Han W, Hayashida M, Uhl GR, Sora I: How individual sensitivity to opiates can be predicted by gene analyses. *Trends Pharmacol Sci* 2005, 26:311–317.
12. Coulbault L, Beaussier M, Verstuyft C, Weickmans H, Dubert L, Tregouet D, Descot C, Parc Y, Lienhart A, Jaillon P, Becquemont L: Environmental and genetic factors associated with morphine response in the postoperative period. *Clin Pharmacol Ther* 2006, 79:316–324.
13. Loh HH, Liu HC, Cavalli A, Yang W, Chen YF, Wei LN: μ opioid receptor knockout in mice: effects on ligand-induced analgesia and morphine lethality. *Brain Res Mol Brain Res* 1998, 54:321–326.
14. Sora I, Elmer G, Funada M, Pieper J, Li XF, Hall FS, Uhl GR: μ opiate receptor gene dose effects on different morphine actions: evidence for differential in vivo μ receptor reserve. *Neuropsychopharmacology* 2001, 25:41–54.
15. Sora I, Takahashi N, Funada M, Ujike H, Revay RS, Donovan DM, Miner LL, Uhl GR: Opiate receptor knockout mice define μ receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc Natl Acad Sci U S A* 1997, 94:1544–1549.
16. Kasai S, Ikeda K: Pharmacogenomics of the human μ-opioid receptor. *Pharmacogenomics* 2011, 12:1305–1320.
17. Ide S, Nishizawa D, Fukuda K, Kasai S, Hasegawa J, Hayashida M, Minami M, Ikeda K: Association between genetic polymorphisms in Ca(v)2.3 (R-type) Ca2+ channels and fentanyl sensitivity in patients undergoing painful cosmetic surgery. *PLoS One* 2013, 8:e70694.
18. Aoki Y, Nishizawa D, Kasai S, Fukuda K, Ichinohe T, Yamashita S, Ikeda K: Association between the variable number of tandem repeat polymorphism in the third exon of the dopamine D4 receptor gene and sensitivity to analgesics and pain in patients undergoing painful cosmetic surgery. *Neurosci Lett* 2013, 542:1–4.
19. Moriyama A, Nishizawa D, Kasai S, Hasegawa J, Fukuda K, Nagashima M, Katoh R, Ikeda K: Association between genetic polymorphisms of the beta1-adrenergic receptor and sensitivity to pain and fentanyl in patients undergoing painful cosmetic surgery. *J Pharmacol Sci* 2013, 121:48–57.
20. Nishizawa D, Fukuda K, Kasai S, Hasegawa J, Aoki Y, Nishi A, Saita N, Koukita Y, Nagashima M, Katoh R, Satoh Y, Tagami M, Higuchi S, Ujike H, Ozaki N, Inada T, Iwata N, Sora I, Iyo M, Kondo N, Won MJ, Naruse N, Uehara-Aoyama K, Itokawa M, Koga M, Arinami T, Kaneko Y, Hayashida M, Ikeda K: Genome-wide association study identifies a potent locus associated with human opioid sensitivity. *Mol Psychiatry* 2014, 19:55–62.
21. Zwisler ST, Enggaard TP, Noehr-Jensen L, Mikkelsen S, Verstuyft C, Becquemont L, Sindrup SH, Brosen K: The antinociceptive effect and adverse drug reactions of oxycodone in human experimental pain in relation to genetic variations in the *OPRM1* and *ABCB1* genes. *Fundam Clin Pharmacol* 2010, 24:517–524.
22. Fuller SJ, Stokes L, Skarratt KK, Gu BJ, Wiley JS: Genetics of the P2X7 receptor and human disease. *Purinergic Signal* 2009, 5:257–262.
23. Bradley HJ, Baldwin JM, Goli GR, Johnson B, Zou J, Sivaprasadarao A, Baldwin SA, Jiang LH: Residues 155 and 348 contribute to the determination of P2X7 receptor function via distinct mechanisms revealed by single-nucleotide polymorphisms. *J Biol Chem* 2011, 286:8176–8187.
24. Sorge RE, Trang T, Dorfman R, Smith SB, Beggs S, Ritchie J, Austin JS, Zaykin DV, Vander Meulen H, Costigan M, Herbert TA, Yarkoni-Abitbul M, Tichauer D, Livneh J, Gershon E, Zheng M, Tan K, John SL, Slade GD, Jordan J, Woolf CJ, Peltz G, Maixner W, Diatchenko L, Seltzer Z, Salter MW, Mogil JS: Genetically determined P2X7 receptor pore formation regulates variability in chronic pain sensitivity. *Nat Med* 2012, 18:595–599.
25. Fukuda K, Hayashida M, Ide S, Saita N, Kokita Y, Kasai S, Nishizawa D, Ogai Y, Hasegawa J, Nagashima M, Tagami M, Komatsu H, Sora I, Koga H, Kaneko Y, Ikeda K: Association between *OPRM1* gene polymorphisms and fentanyl sensitivity in patients undergoing painful cosmetic surgery. *Pain* 2009, 147:194–201.
26. Bisgaard T, Klarskov B, Rosenberg J, Kehlet H: Characteristics and prediction of early pain after laparoscopic cholecystectomy. *Pain* 2001, 90:261–269.
27. Carlson CS, Eberle MA, Rieder MJ, Smith JD, Kruglyak L, Nickerson DA: Additional SNPs and linkage-disequilibrium analyses are necessary for whole-genome association studies in humans. *Nat Genet* 2003, 33:518–521.
28. Carlson CS, Eberle MA, Rieder MJ, Yi Q, Kruglyak L, Nickerson DA: Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. *Am J Hum Genet* 2004, 74:106–120.
29. de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D: Efficiency and power in genetic association studies. *Nat Genet* 2005, 37:1217–1223.
30. Barrett JC, Fry B, Maller J, Daly MJ: Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005, 21:263–265.
31. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D: The structure of haplotype blocks in the human genome. *Science* 2002, 296:2225–2229.
32. Faul F, Erdfelder E, Lang AG, Buchner A: G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods* 2007, 39:175–191.
33. Li SS, Khalid N, Carlson C, Zhao LP: Estimating haplotype frequencies and standard errors for multiple single nucleotide polymorphisms. *Biostatistics* 2003, 4:513–522.
34. Cabrini G, Falzoni S, Forchap SL, Pellegatti P, Balboni A, Agostini P, Cuneo A, Castoldi G, Baricordi OR, Di Virgilio F: A His-155 to Tyr polymorphism confers gain-of-function to the human P2X7 receptor of human leukemic lymphocytes. *J Immunol* 2005, 175:82–89.
35. Feng YH, Li X, Wang L, Zhou L, Gorodeski GI: A truncated P2X7 receptor variant (P2X7-j) endogenously expressed in cervical cancer cells antagonizes the full-length P2X7 receptor through hetero-oligomerization. *J Biol Chem* 2006, 281:17228–17237.
36. Cheewatrakoolpong B, Gilchrest H, Anthes JC, Greenfeder S: Identification and characterization of splice variants of the human P2X7 ATP channel. *Biochem Biophys Res Commun* 2005, 332:17–27.
37. Georgiou JG, Skarratt KK, Fuller SJ, Martin CJ, Christopherson RI, Wiley JS, Sluyter R: Human epidermal and monocyte-derived langerhans cells express functional P2X receptors. *J Invest Dermatol* 2005, 125:482–490.
38. Xu XJ, Boumechache M, Robinson LE, Marschall V, Gorecki DC, Masin M, Murrell-Lagnado RD: Splice variants of the P2X7 receptor reveal differential agonist dependence and functional coupling with pannexin-1. *J Cell Sci* 2012, 125:3776–3789.
39. Alves LA, Bezerra RJ, Faria RX, Ferreira LG, da Silva FV: Physiological roles and potential therapeutic applications of the P2X7 receptor in inflammation and pain. *Molecules* 2013, 18:10953–10972.
40. Clark AK, Staniland AA, Marchand F, Kaan TK, McMahon SB, Malcangio M: P2X7-dependent release of interleukin-1β and nociception in the spinal cord following lipopolysaccharide. *J Neurosci* 2010, 30:573–582.
41. Suzuki T, Hide I, Ido K, Kohsaka S, Inoue K, Nakata Y: Production and release of neuroprotective tumor necrosis factor by P2X7 receptor-activated microglia. *J Neurosci* 2004, 24:1–7.
42. Inoue K: P2 receptors and chronic pain. *Purinergic Signal* 2007, 3:135–144.
43. Falk S, Uldall M, Heegaard AM: The role of purinergic receptors in cancer-induced bone pain. *J Osteoporos* 2012, 2012:758181.
44. Sawada Y, Hosokawa H, Hori A, Matsumura K, Kobayashi S: Cold sensitivity of recombinant TRPA1 channels. *Brain Res* 2007, 1160:39–46.
45. Barabas ME, Stucky CL: TRPV1, but not TRPA1, in primary sensory neurons contributes to cutaneous incision-mediated hypersensitivity. *Mol Pain* 2013, 9:9.
46. Banke TG, Chaplan SR, Wickenden AD: Dynamic changes in the TRPA1 selectivity filter lead to progressive but reversible pore dilation. *Am J Physiol Cell Physiol* 2010, 298:C1457–C1468.