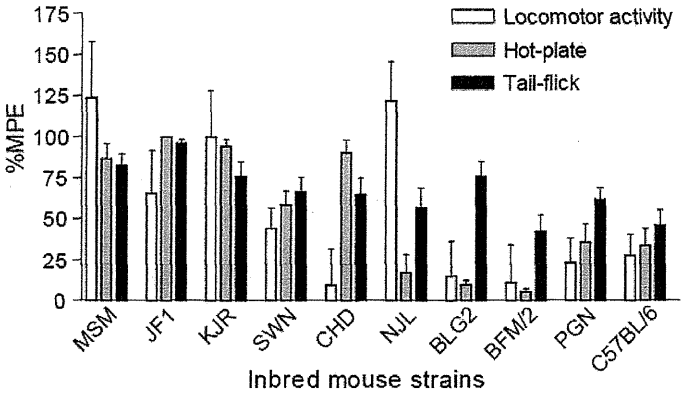

Figure 7. Comparison of morphine effects in 10 inbred mouse strains including wild-derived strains. Data on tests for open-field, hot-plate, and tail-flick were taken from a previous paper (Shigeta et al., 2008).

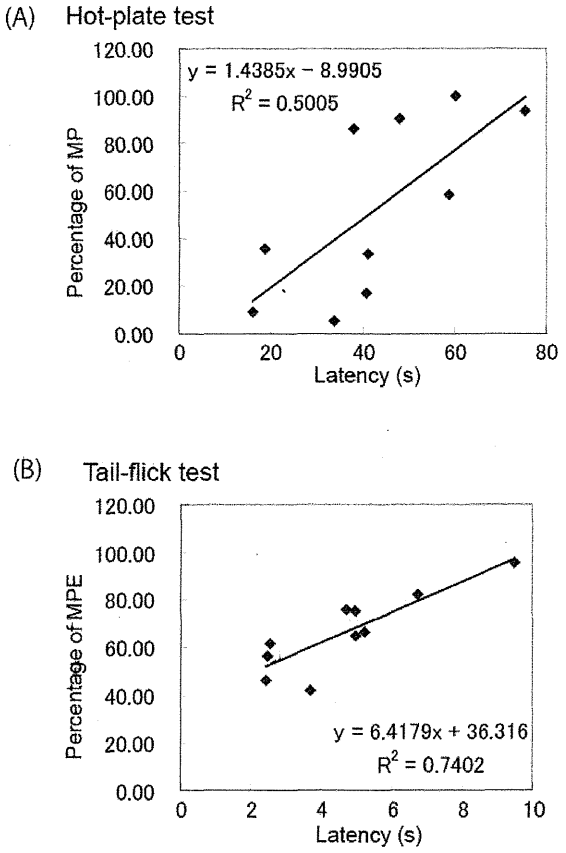

Figure 8. Correlation between pain perception and morphine effect. Strain comparison of sensitivities in (A) hot-plate test, and (B) tail-flick test. Correlation analyses of different pain sensitivities. Data on morphine effects in the hot-plate and tail-flick tests were taken from a previous paper (Shigeta et al., 2008).

These results suggested that there is a functional association of the opioid-dependent analgesic pathway to pain perception in these mouse strains. Therefore, investigating the genetic basis for different pain sensitivities will lead to greater understanding of the underlying mechanisms for different pain sensitivities and opioid-dependent analgesic pathways. This in turn may lead to the development of new antinociceptive drugs.

GENETIC MAPPING OF LOCI RELATED TO PAIN SENSITIVITY

As we explained above, a variety of wild derived strains are a powerful resource for research into the genetic basis of diversity in pain sensitivity-related phenomena. By adding wild derived strains to the list of mouse strains to investigate, we were able to detect differences in pain-related phenotypes. In particular, two strains, B6 and MSM, have proven to be a highly useful resource to study these phenotypes as the two strains exhibit differences.

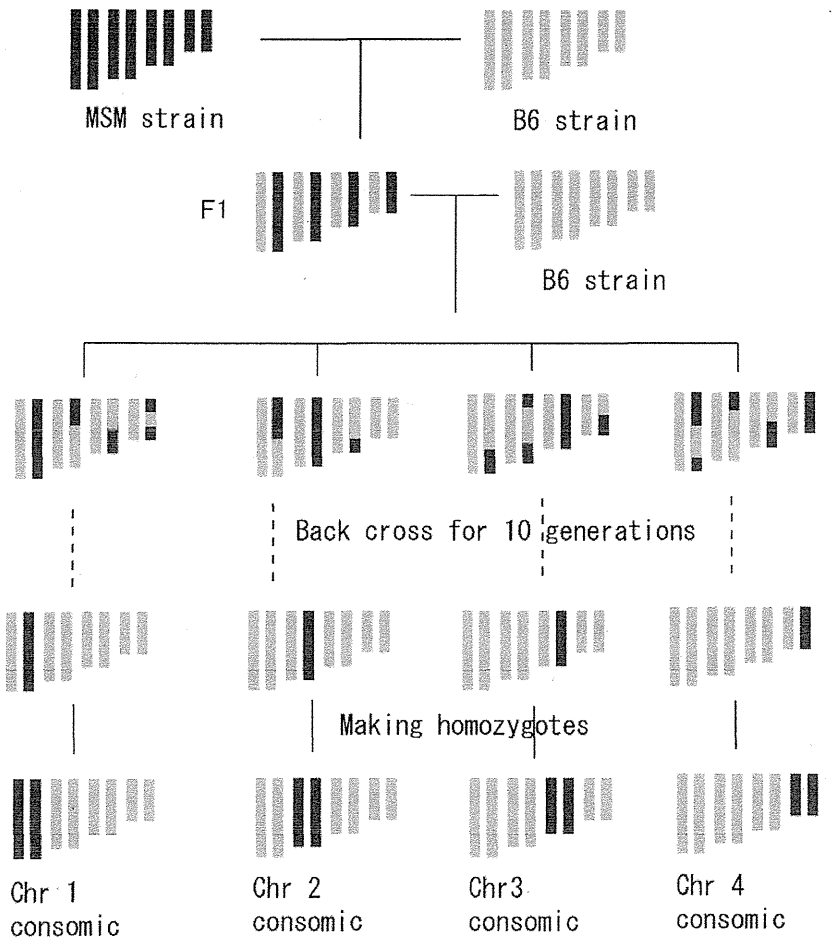

Figure 9. Breeding scheme for establishing a panel of consomic strains.

In order to investigate the genetic basis of the phenotypic difference, QTL studies have been conducted on mice and rats by using F2 intercross, N2 backcross, recombinant inbred strains, and heterozygote stocks (Flint, 2002; Flint, 2003; Valdar et al., 2006). Flint and colleagues surveyed previously reported QTL studies and found that most QTLs have just a small effect, contributing approximately 6% on average of the total phenotypic variance for behavioral and physiological phenotypes (Flint et al., 2005). In addition, they also conducted genome-wide high-resolution mapping using heterogeneous stock mice and revealed 843 QTLs for a variety of phenotypes, including behaviors. Among these QTLs, only 10 had effect sizes of higher than 5% and 109 QTLs of less than 2% (Valdar et al., 2006). Because of this small effect of each QTL, it is proposed that an enormous amount of effort will be required to identify the quantitative trait gene (QTG) for behavior.

In order to study QTLs more systematically, new mouse resources have been developed. Consomic strains, also known as chromosome substitution strains, are a favored resources for approaching the QTL because genotyping to map the chromosome is unnecessary, results are reproducible, QTL detection is statistically significant, and creating subconsomic strains is efficient (Belknap, 2003; Nadeau et al., 2000). Shiroishi and colleagues have established a new panel of consomic strains, dubbed B6-ChrN^{MSM}, in which each chromosome of B6 was substituted with a corresponding set of chromosomes from MSM (Fig. 9) (Takada et al., 2008). Recently, systematic QTL mapping was conducted for a series of behavioral tests related to emotionality and incidence of hydrocephalus; multiple loci were successfully mapped for these phenotypes on many chromosomes in the genome (Takahashi et al., 2008a; Takahashi, 2008b). Thus, we also attempted to map genetic loci related to pain sensitivity using hot-plate and tail-flick tests (unpublished data). In the following paragraphs, we report on the results of these tests using a panel of consomic strains and subsequent further mapping using subconsomic strains established from one of the consomic strains.

MAPPING THE CHROMOSOMES RELATED TO PAIN SENSITIVITY

We first identified those chromosomes related to pain sensitivity by comparing each consomic strain with B6 in hot-plate (Fig. 10A) and tail-flick (Fig. 10B) tests.

In the hot-plate test, two-way ANOVA revealed a significant strain effect [$F(22, 726)=24.345$, $P<0.001$] and sex-strain interaction [$F(21,726)=1.737$, $P<0.05$]. Females of B6-Chr2T^{MSM}, B6-Chr8^{MSM}, B6-Chr14^{MSM}, and B6-Chr17^{MSM}, and males of B6-Chr2T^{MSM}, B6-Chr8^{MSM} and B6-Chr9^{MSM} showed reduced pain sensitivity in the hot-plate test. Although only females of B6-Chr17^{MSM} showed significantly reduced sensitivity in the hot-plate test, the levels of sensitivity were similar between males and females. In contrast, females of B6-Chr14^{MSM} clearly had reduced sensitivity compared to males. Thus, Chr14 reduces hot-plate sensitivity only in females. In the tail-flick test, two-way ANOVA showed a significant effect of strain [$F(22, 739)=25.48$, $P<0.001$], but no significant sex-strain interaction. Thus, data from both sexes were combined for the tail-flick test and are shown in Figure 10. Fifteen consomic strains for Chrs 1, 2C, 2T, 3, 4, 7T, 8, 11, 12C, 12T, 14, 15, 16, 17 and Y showed reduced sensitivity compared to B6. Our results suggested a larger number of chromosomes related to different pain sensitivities to the tail-flick test than the hot-plate test. We found consomic strains for Chrs 1, 2C, 3, 4, 7T, 11, 12C, 12T, 15, 16 and Y related to reduced

sensitivity to only the tail-flick test, but not the hot-plate test. Chrs 2T and 9 related to increased sensitivity in the hot-plate test. It is interesting that Chr2T related to increased sensitivity in the hot-plate test, but reduced sensitivity in the tail-flick test. It is known that these two pain-sensitivity tests reflect distinct biological pathways; the tail-flick test for spinal reflex pain and the hot-plate test for centrally mediated acute pain reflexes (Crawley, 2007). Our results indicated that there are some common genetic loci for pain perception, and also many test-specific QTLs related to the spinal reflex reaction.

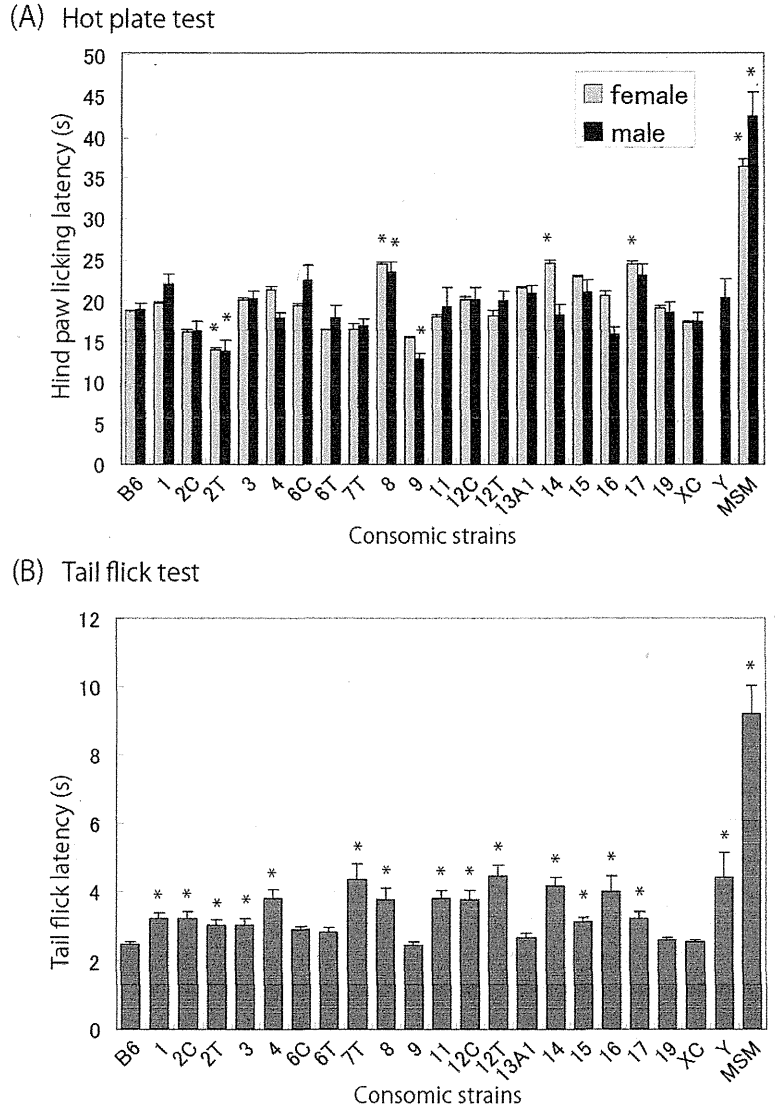

Figure 10. Results of chromosomal mapping for pain sensitivities.

Astarisks indicate significant difference between each consomic strain and B6.

Methods: Mouse strains, C57BL/6J (B6), MSM/Ms (MSM), and consomic strains were maintained in the animal facility of the National Institute of Genetics (NIG), Mishima, Japan. All strains were maintained at NIG in a controlled room at 23°C (±2°C) on a 12/12 light/dark cycle, light period starting at 8.00 h with food and water available ad libitum. Throughout this study, 9- to 13-week-old females were used for testing. Before the two pain sensitivity tests, hot-plate and tail-flick, all the animals were tested for home-cage activity and emotionality-related behavior (Takahashi et al., 2008a) in the same test order. All procedures were carried out with approval by our institutional animal care and use committee. The hot-plate test and tail flick test were conducted as follows:

Hot-plate test (HP)

The hot plate apparatus consisted of an acrylic resin cage (20 × 25 × 25 cm) and thermo-controlled aluminum plate (model MK-350B, Muromachi Kikai Co., Tokyo, Japan). The plate temperature was adjusted to 52 ± 0.2°C. Latency to the first licking of the hind limb was measured. When the mouse jumped on the plate before licking a hind limb, the test was stopped and excluded from the data.

Tail flick test (TF)

The tail flick apparatus consisted of a radiant heat source and a photo sensor to detect the tail flick (model MK-330B, Muromachi Kikai Co., Tokyo, Japan). Latency, being the median of three independent tests from the start of irradiation to the tail flick reaction, was measured. The cut-off time was 15 s in order to prevent tail damage.

Data analysis was performed using the SPSS version 14.0J software package. The significance for each consomic strain was determined by a t-test with a Bonferroni correction compared to B6 ($p=\alpha/m$: where $\alpha=0.05$ and $m=20$ for males and $m=19$ for females). In order to avoid an interactive influence from sex chromosomes on the effect of substituting chromosomes, we first performed t-tests for males and females independently. Sex-genotype interaction in consomic strains was analyzed by performing 2-way ANOVA in all consomic strains and B6. Because the tail flick test did not show strain-sex interaction, data for both females and males were combined and presented in Figure 10(B).

In some consomic pairs (B6-Chr2C^{MSM}/B6-Chr2T^{MSM}, B6-Chr6C^{MSM}/B6-Chr6T^{MSM} and B6-Chr12C^{MSM}/B6-Chr12T^{MSM}), different parts of a chromosome were substituted (Chr 2, 6 and 12), and the MSM region overlapped in the middle of the chromosome. Thus, to avoid overestimation, we considered it as one consomic strain even if both of them (C and T) showed significant behavioral differences from B6, as well as if one of those (C or T) showed a significant difference. A phenotypic effect was also calculated in one of a pair of the consomic strains which showed larger differences from B6.

A male-specific QTL on Chr 4 has been reported for the hot-plate test in F2 intercross mice between DBA and B6 (Mogil et al., 1997). However, our B6-Chr4^{MSM} did not show significant differences in the hot-plate test, but exhibited differences in the tail-flick test. This discrepancy may result from the strain differences between DBA and MSM.

Pain sensitivity can be affected by stress-induced analgesia (Imbe et al., 2006), which may reflect consomic mouse sensitivity toward stress (e.g. holding by experimenter). We previously investigated emotionality-related behavior using this panel of consomic strains (Takahashi et al., 2008a). The same animals used in the emotionality investigation were also used for these pain sensitivity analyses at the end of a series of behavioral tests. By using these data, genetic correlation analysis was conducted for emotionality-related indices and pain sensitivity. The results showed that there were moderate correlations between pain

sensitivity measurements and some emotionality-related behaviors (Table 2). Particularly, locomotion, leaning and rearing were negatively correlated, but stretching was positively correlated with sensitivity to the hot-plate test. Therefore, this indicates that pain sensitivity in the hot-plate test and an emotionality-related phenotype have genetically related bases. Further analysis is required to interpret these correlations between pain related indices and an emotionality-related phenotype.

Table 2. Correlation of pain sensitivity with behavioral parameters related to emotionality

		HP	TF
OF	Ambulation	-0.295	-0.100
	Center area	-0.136	0.069
	Center area %	0.236	0.217
	Center time	0.243	0.189
	Defecation	0.139	-0.262
	Locomotion	-.463(*)	-0.135
	Stretching	.595(**)	0.143
	Leaning	-.555(**)	-.480(*)
	Rearing	-.438(*)	0.316
	Grooming	0.276	0.127
	Face-wash	0.007	0.015
	Jumping	-0.191	-0.422
	Pausing	0.294	-0.130
L/D	Transition number	-0.385	-0.142
	Dark box duration	0.327	-0.070
	Latency to first transition	0.213	-0.097
EPM	Total distance (cm)	-0.380	-0.168
	Total number of arm entry	-0.415	-0.188
	Closed arm entry	-0.400	-0.037
	Open arm entry	-0.379	-0.345
	Open arm %	0.090	-.464(*)
	Open arm time	-0.075	-0.373

Genetic correlation was caluculated by using strain mean values of all consomic strains and B6 in all consomic strains and B6 using SPSS version 14.0J software packages. OF, Open-field test; L/D, Light-dark box test; EPM,Elevated plus maze test; HP, hot-plate test; TF, tail-flick test. Asterisks indicate a significant correlation (**, $P<0.01$; *, $P<0.05$) between emotionality related parameter and either hot-plate or tail-flick sensitivity.

FURTHER MAPPING USING SUBCONSOMIC STRAINS

In order to identify loci responsible for reduced pain sensitivity in the hot-plate test for B6-Chr17^{MSM}, we analyzed subconsomic strains encompassing the entire Chr 17 (Fig. 11). In the hot-plate test, C2 and C3 strains showed significantly prolonged latency for hind paw licking comparing to B6 (Dunnet's t-test: B6-C2, $p<0.05$; B6-C3, $p<0.01$) (Fig. 12). As a

result, we found that a region between a centromere to *D17Mit34* (10cM, 34.3Mb), had an effect of reducing the thermo-sensitivity at 52°C. Mogil and colleagues have previously reported that a congenic strain carrying a Chr17-proximal region of a *M.m.molossinus*-derived strain, MOLF/Ei, had an effect on reducing pain sensitivity (Mogil et al., 2006). This region, a spans of 24.5cM from the centromere, was named *Tpnr3* (*thermal pain response 3*) and partially overlapped with the region which we mapped in our present study. Thus, it is possible that these two studies have clarified the same locus related to pain sensitivity as both studies used *M.m.molossinus*-derived mice.

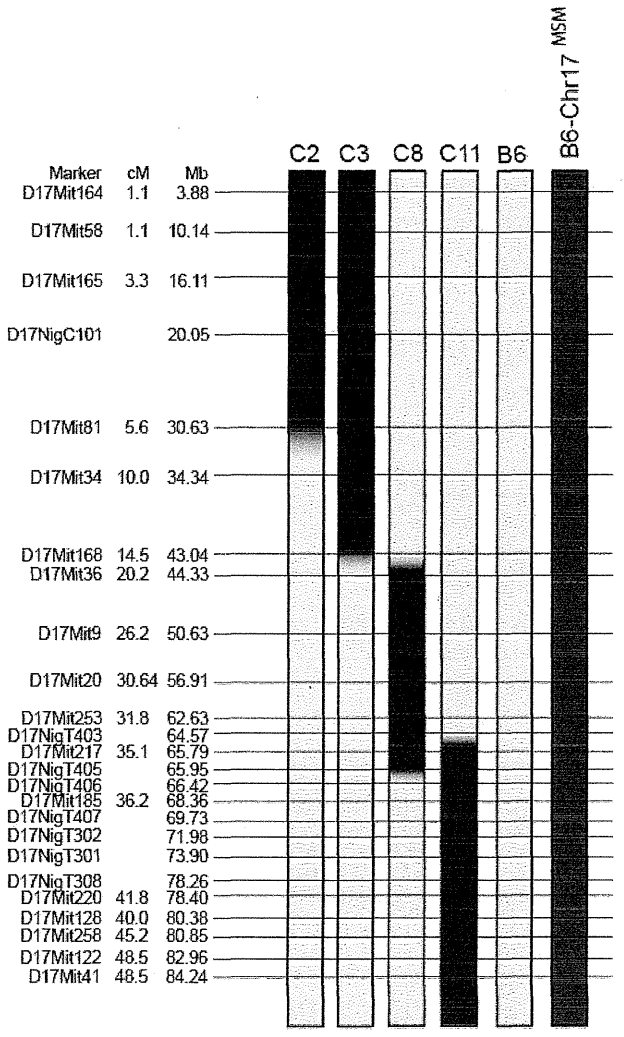

Figure 11. Genetic mapping of reduced hot-plate sensitivity in MSM-derived Chr17. Diagram of subconsomic strains used for analysis. Substituted chromosomal regions from MSM are black. Names of subconsomic strains are as follows: B6.MSM-(*D17Mit164-D17Mit81*)/Ms (C2), B6.MSM-(*D17Mit164-D17Mit168*)/Ms (C3), B6.MSM-(*D17Mit36-D17NigT405*)/Ms (C8), B6.MSM-(*D17Mit217-D17Mit123*)/Ms (C11).

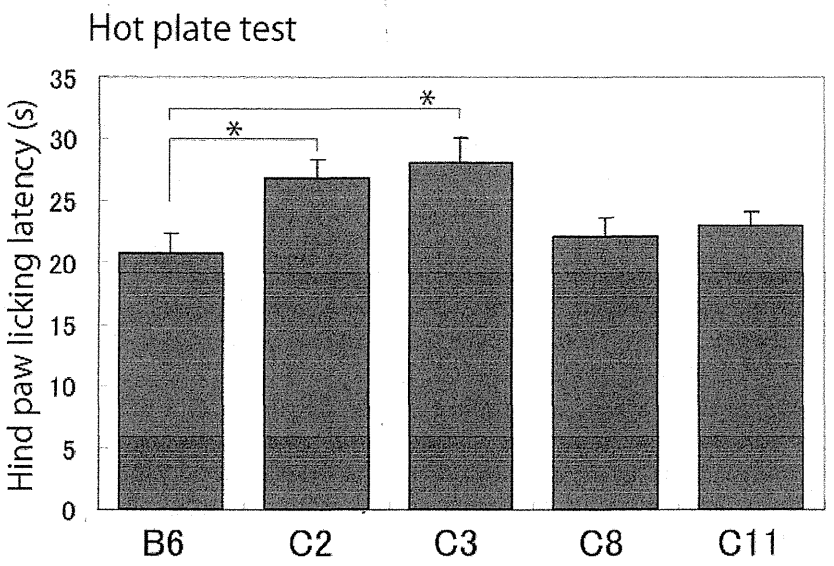

Figure 12. Hot-plate sensitivity in subconsomic strains. Mean latency for hind-paw licking in each subconsomic strain is shown. Error bars represent SEM. Asterisks show $P<0.05$ with Dunnet's t-test compared with B6.

CONCLUSION

This review clearly showed that consomic strains are a powerful resource for addressing pain sensitivity differences among mouse strains. We successfully identified one of the loci related to reduced sensitivity in the hot-plate test. The screening of a panel of consomic strains showed a large number of chromosomes are related to different levels of heat sensitivity. Therefore, systematic analysis of consomic strains and subsequent analysis of subconsomic strains will give further information about the complex genetic basis regulating different heat sensitivity levels in mice.

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REFERENCES

Abe, K., Noguchi, H., Tagawa, K., Yuzuriha, M., Toyoda, A., Kojima, T., Ezawa, K., Saitou, N., Hattori, M., Sakaki, Y., Moriwaki, K., Shiroishi, T. (2004) Contribution of Asian mouse subspecies Mus musculus molossinus to genomic constitution of strain C57BL/6J, as defined by BAC-end sequence-SNP analysis. *Genome Research* 14, 2439-2447.

Belknap, J.K. (2003) Chromosome substitution strains: some quantitative considerations for genome and fine mapping. *Mammalian Genome* 14, 723-732

Bonhomme, F. & Guénet J.-L. (1996) The laboratory mouse and its wild relatives. In M.F. Lyon, S. Rastan, S.D.M. Brown, (Eds.) *Genetic variants and strains of the laboratory mouse.* (pp 1577-1596) Oxford: Oxford University Press.

Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I., Julius, D. (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 288, 306-313.

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A., Julius, D. (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* 398, 436-441.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., Julius, D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389, 816-824.

Crawley, J.N., ed. (2007) *What's wrong with my mouse?* (second edition) Hoboken, New Jersey: John Wiley & Sons, Inc.

Crawley, J.N., Belknap, J.K., Collins, A., Crabbe, J.C., Frankel, W., Henderson, N., Hitzemann, R.J., Maxson, S.C., Miner, L.L., Silva, A.J., Wehner, J.M., Wynshaw-Boris, A., Paylor, R. (1997) Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. *Psychopharmacology* 132, 107-124.

Ferris, S.D., Sage, R.D., Wilson, A.C. (1982) Evidence from mtDNA sequences that common laboratory strains of inbred mice are decended from a single female. *Nature* 295, 163-165.

Flint, J. (2002) Genetic effects on an animal model of anxiety. *FEBS Letters* 529, 131-134.

Flint, J. (2003) Analysis of quantitative trait loci that influence animal behavior. *J Neurobiol* 54, 46-77.

Flint, J., Valdar, W., Shifman, S., Mott, R. (2005) Strategies for mapping and cloning quantitative trait genes in rodents. *Nature Reviews Genetics* 6, 271-286.

Furuse, T., Blizard, D.A., Moriwaki, K., Miura, Y., Yagasaki, K., Shiroishi, T., Koide, T. (2002) Genetic diversity underlying capsaicin intake in the Mishima battery of mouse strains. *Brain Research Bulletin* 57, 49-55.

Gregorová, S., Forejt, J. (2000) PWD/Ph and PWK/Ph inbred mouse strains of Mus m. musculus subspecies--a valuable resource of phenotypic variations and genomic polymorphisms. *Folia Biol (Praha)* 46, 31-41.

Imbe, H., Iwai-Liao, Y., Senba, E. (2006) Stress-induced hyperalgesia: animal models and putative mechanisms. *Frontiers in Bioscience* 11, 2179-2192.

Koide, T., Moriwaki, K., Ikeda, K., Niki, H., Shiroishi, T. (2000) Multi-phenotype behavioral characterization of inbred strains derived from wild stocks of Mus musculus. *Mammalian Genome* 11, 664-670.

Koide, T., Moriwaki, K., Uchida, K., Mita, A., Sagai, T., Yonekawa, H., Katoh, H., Miyashita, N., Tsuchiya, K., Nielsen, T.J., Shiroishi, T. (1998) A new inbred strain JF1 established from Japanese fancy mouse carrying the classic piebald allele. *Mammalian Genome* 9, 15-19.

Lariviere, W.R., Wilson, S.G., Laughlin, T.M., Kokayeff, A., West, E.E., Adhikari, S.M., Wan, Y., Mogil, J.S. (2002) Heritability of nociception. III. Genetic relationships among commonly used assays of nociception and hypersensitivity. *Pain* 97, 75-86.

Liu, Y.-H., Takahashi, A., Kitano, T., Koide, T., Shiroishi, T., Moriwaki, K., Saitou, N. (2008) Mosaic genealogy of the Mus musculus genome revealed by 21 nuclear genes from its three subspecies. *Genes Genetic Systems* 83, 77-88.

Mogil, J.S. (1999) The genetic mediation of individual differences in sensitivity to pain and its inhibition. *Proc. Natl. Acad. Sci. USA* 96, 7744-7751.

Mogil, J.S., Richards, S.P., O'Toole, L.A., Helms, M.L., Mitchell, S.R., Belknap, J.K. (1997) Genetic sensitivity to hot-plate nociception in DBA/2J and C57BL/6J inbred mouse strains: possible sex-specific mediation by δ2-opioid receptors. *Pain* 70, 267-277.

Mogil, J.S., Ritchie, J., Sotocinal, S.G., Smith, S.B., Croteau, S., Levitin, D.J., Naumova, A.K. (2006) Screening for pain phenotypes: Analysis of three congenic mouse strains on a battery of nine nociceptive assays. *Pain* 126, 24-34.

Moriwaki, K., Shiroishi, T., Yonekawa, H., eds. (1994) *Genetics in wild mice*. Tokyo/Basel: Japan Scientific Societies Press/Karger.

Nadeau, J.H., Singer, J.B., Martin, A., Lander, E.S. (2000) Analysing complex genetic traits with chromosome substitution strains. *Nature Genetics* 24, 221-225.

Ogasawara, M., Imanishi, T., Moriwaki, K., Gaudieri, S., Tsuda, H., Hashimoto, H., Shiroishi, T., Gojobori, T., Koide, T. (2005) Length variation of CAG/CAA triplet repeats in 50 genes among 16 inbred strains. *Gene* 349, 107-119.

Shigeta, Y., Kasai, S., Han, W., Hata, H., Nishi, A., Takamatsu, Y., Hagino, Y., Yamamoto, H., Koide, T., Shiroishi, T., Kasai, K., Tsunashima, K., Kato, N., Ikeda, K. (2008) Association of morphine-induced antinociception with variations in the 5' flanking and 3' untranslated regions of the mu opioid receptor gene in 10 inbred mouse strains. *Pharmacogenetics and Genomics* 18, 927-936.

Silver, L.M. (1995) *Mouse Genetics*. New York: Oxford University Press.

Takada, T., Mita, A., Maeno, A., Sakai, T., Shitara, H., Kikkawa, Y., Moriwaki, K., Yonekawa, H., Shiroishi, T. (2008) Mouse inter-subspecific consomic strains for genetic dissection of quantitative complex traits. *Genome Research* 18, 500-5008.

Takahashi, A., Nishi, A., Ishii, A., Shiroishi, T., Koide, T. (2008a) Systematic analysis of emotionality in consomic mouse strains established from C57BL/6J and wild-derived MSM/Ms. *Genes, Brain and Behavior* online open.

Takahashi, A., Shiroishi, T., Koide, T. (2008b) Multigenic factors associated with a hydrocephalus-like phenotype found in inter-subspecific consomic mouse strains. *Mammalian Genome* 19, 333-338.

Valdar, W., Solberg, L.C., Gauguier, D., Burnett, S., Klenerman, P., Cookson, W.O., Taylor, M.S., Rawlins, J.N.P., Mott, R., Flint, J. (2006) Genome-wide genetic association of complex traits in heterogeneous stock mice. *Nature Genetics* 38, 879-887.

Yonekawa, H., Moriwaki, K., Gotoh, O., Miyashita, N., Matsushima, Y., Shi, L., Cho, W.S., Zhen, X.-L., Tagashira, Y. (1988) Hybrid origin of Japanese mice "Mus musculus

molossinus": evidence from restriction analysis of mitochondrial DNA. *Mol. Biol. Evol.* 5, 63-78.

Yonekawa, H., Moriwaki, K., Gotoh, O., Watanabe, J., Hayashi, J.-i., Miyashita, N., Petras, M.L., Tagashira, Y. (1980) Relationship between laboratory mice and the subspecies Mus musculus domesticus based on restriction endonuclease cleavage patterns of mitochondrial DNA. *Japan.J.Genet.* 55, 289-296.

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Association between *OPRM1* gene polymorphisms and fentanyl sensitivity in patients undergoing painful cosmetic surgery

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ABSTRACT

Individual differences in sensitivity to fentanyl, a widely used opioid analgesic, can hamper effective pain treatment. Still controversial is whether the single nucleotide polymorphisms (SNPs) of the human *OPRM1* gene encoding the μ-opioid receptor influence the analgesic effects of opioids. We examined associations between fentanyl sensitivity and the two SNPs, A118G and IVS3+A8449G, in the human *OPRM1* gene in 280 Japanese patients undergoing painful orofacial cosmetic surgery, including bone dissection. Regarding the A118G SNP in exon 1, in a cold pressor-induced pain test before surgery, less analgesic effects of fentanyl were shown in subjects carrying the minor G allele of the A118G SNP (median of difference between pain perception latencies before and after fentanyl injection [PPLpost–PPLpre]: 12 s) compared with subjects not carrying this allele (PPLpost–PPLpre: 15 s, $p = 0.046$). Furthermore, the IVS3+A8449G SNP in intron 3, which represents a complete linkage disequilibrium block with more than 30 SNPs from intron 3 to the 3′ untranslated region, was associated with 24-h postoperative fentanyl requirements. Subjects carrying the minor G allele of the IVS3+A8449G SNP required significantly less fentanyl for 24-h postoperative pain control (median: 1.5 μg/kg) compared with subjects not carrying this allele (median: 2.5 μg/kg, $p = 0.010$). Although further validation is needed, the present findings shed light on the involvement of *OPRM1* 3′ untranslated region polymorphisms in fentanyl sensitivity in addition to the A118G SNP and open new avenues for personalized pain treatment with fentanyl.

1. Introduction

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 [21]. Individual differences may be related to a variety of genetic and nongenetic factors, including gender, age, ethnic origin, hepatic or renal function, and mental status [10]. Several studies using mice lacking the μ-opioid receptor (MOP) [29,37,38] or CXBK mice possessing DNA insertion in the MOP 3′ untranslated region (UTR) [15,22] have shown that analgesia produced by opioids depends crucially on the level of MOP expression. Individual variation in MOP expression resulting from single nucleotide polymorphisms (SNPs) of the *OPRM1* gene encoding MOP has also been recognized in humans [39]. Therefore, some SNPs in the *OPRM1* gene may lead to differences in the analgesic efficacy of opioids.

More than 250 SNPs have been identified in the *OPRM1* gene [19–21]. The A118G SNP in exon 1 leads to an amino acid substitution that changes the putative *N*-glycosylation site [3]. Several recent studies investigating the association between the A118G SNP and morphine requirements in patients with cancer pain or postoperative pain have suggested that this SNP has significant associations with opioid requirements for analgesia [7,8,16,27], although other studies have failed to find such associations [10,24]. Interestingly, the 3′UTR nucleotide sequence difference in the *Oprm1* gene

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influences opioid sensitivity in CXBK mice [15,22], and more than 30 SNPs from intron 3 to the 3′UTR in the *OPRM1* gene show complete linkage disequilibrium [20]. Therefore, in addition to the A118G SNP, the SNPs in the complete linkage disequilibrium block may influence the analgesic effects of opioids.

In contrast to animal studies using standardized pain tests, the analgesic effects of opioids in humans are usually evaluated in patients with actual pain, particularly with cancer pain or acute postoperative pain [7,8,10,24,27]. Deriving gene-opioid effect relationships from data sampled from patients with cancer pain is difficult because the mechanism, severity, and nature of cancer pain can differ substantially from patient to patient [27]. Patients with acute postoperative pain following standardized surgical procedures may be more optimal subjects for investigating such relationships [21]. Furthermore, studies in human subjects without actual pain employing a standardized pain test and studies in patients undergoing uniform surgical procedures may be ideal for evaluating the analgesic effects of opioids [21].

Fentanyl is one of the most commonly used opioid analgesics. However, associations between genetic polymorphisms and fentanyl sensitivity have not been studied in patients treated only with fentanyl. Although equivalent fentanyl doses can be calculated from the doses of other analgesics [16], the conversion may not reflect precise fentanyl doses because of the varying properties of different analgesics.

The present study examined whether the A118G SNP and IVS3+A8449G SNP representing the complete linkage disequilibrium block could affect the analgesic effects of fentanyl evaluated by a standardized pain test in healthy Japanese subjects and whether these *OPRM1* SNPs could influence 24-h postoperative fentanyl requirements for analgesia in patients undergoing standardized painful surgery.

2. Methods

2.1. Patients

Enrolled in the study were 280 healthy patients (American Society of Anesthesiologists Physical Status I, age 15–50 years, 97 males and 183 females) 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. The study protocol was approved by the Institutional Review Board, Tokyo Dental College, Chiba, Japan, and the Institutional Review Board, Tokyo Institute of Psychiatry, Tokyo, Japan. Written informed consent was obtained from all of the patients and also from parents if required.

2.2. Preoperative cold pressor-induced pain test

Patients were premedicated with oral diazepam, 5 mg, and oral famotidine, 150 mg, 90 min before induction of anesthesia. Patients had an intravenous (i.v.) line on the forearm of 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 i.v. bolus injection of fentanyl, 2 μg/kg, as previously described [2,31]. Briefly, crushed ice cubes and cold water were blended 15 min before testing in a 5-L isolated tank, and the mixture was stirred immediately before each test to ensure uniform distribution of temperature (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 patients had the test conducted by the same investigator. Baseline latency to pain perception, defined as the time of immersion of the hand in the ice water, before 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. 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).

2.3. Anesthesia and surgery

After the cold pressor-induced pain test ended, general anesthesia was induced with target-controlled infusion (TCI) of propofol using a TCI pump (TE-371, Terumo, Tokyo, Japan). Vecuronium, 0.1 mg/kg, was administered to facilitate nasotracheal intubation. After induction of anesthesia, 10 ml of venous blood was sampled for preparation of DNA specimens. General anesthesia was maintained with propofol at a target blood concentration of 4–6 μg/ml. Vecuronium was administered at a rate of 0.08 mg/kg/h. Lungs were ventilated with oxygen-enriched air. Local anesthesia was performed on the right side of the surgical field with 8 ml of 2% lidocaine containing epinephrine, 12.5 μg/ml, and right mandibular ramus osteotomy was conducted. 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.

2.4. Postoperative pain management

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, droperidol, 1.25 mg, was administered i.v. to prevent nausea/vomiting, and i.v. patient-controlled analgesia (PCA) with a fentanyl-droperidol combination (2 mg of fentanyl and 5 mg of 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 on demand and a lockout time were set at 40 μg and 10 min, respectively. Continuous background infusion was not employed. Droperidol was coadministered with fentanyl to prevent nausea/vomiting because our preliminary study showed a high incidence (up to 30%) of nausea/vomiting with PCA fentanyl in young females. Patient-controlled analgesia was continued for 24-h postoperatively. In case of treatment-refractory adverse effects or inadequate analgesia, PCA was discontinued, and rectal diclofenac sodium, 50 mg, was prescribed as a rescue analgesic as required. 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 with body weight. Additionally, perioperative fentanyl use was calculated as the sum of intraoperative fentanyl use and postoperative fentanyl use because the analgesic effect of the intermediate-acting opioid fentanyl, administered pre- and intraoperatively, could outlast the duration of surgery and thus affect postoperative fentanyl use, especially in patients who received large doses of fentanyl intraoperatively. Therefore, in the present study, we considered perioperative fenta-

nyl use an appropriate indicator of fentanyl analgesia in addition to postoperative fentanyl use. Furthermore, 1 mg/kg of diclofenac sodium was converted to the fentanyl equivalent dose of 1 μg/kg according to a dose conversion described previously [16]. Because the potent analgesic diclofenac sodium was administered at a uniform dose (50 mg/body) across all patients, and individual differences in the weight-adjusted dose of diclofenac sodium could affect postoperative fentanyl use, total perioperative analgesic use was calculated as the sum of perioperative fentanyl use and the fentanyl equivalent dose of diclofenac sodium.

2.5. Genotyping procedures

DNA was extracted from whole-blood samples by the conventional phenol–chloroform method or by using a QIAamp DNA Mini Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions.

For genotyping the two selected SNPs in the *OPRM1* gene, direct sequencing was adopted. To perform direct sequencing, primers were designed to cover each polymorphic site within the *OPRM1* gene on the basis of the reference genomic contig sequence in the National Center of Biotechnology Information database (GenBank Accession No. NT-025741). All of the primers used for genotyping are shown in Table 1. Polymerase chain reaction (PCR) was performed with forward and reverse primers for each region shown in Table 1. Following the cycle sequencing reaction with a BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems Japan, Ltd., Tokyo, Japan) according to the manufacturer's instructions and purification of the PCR products, DNA sequences of the fragments were determined using the automated sequencer ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems Japan, Ltd., Tokyo, Japan). The genotyping results for the A118G SNP were confirmed by recently developed "modified MPEX" and "SSPCE-FCS" methods described in detail previously [23]. Allele-specific PCR was performed to confirm incongruous results for the A118G SNP. To perform allele-specific PCR, two forward primers (whose 3′ ends were specified for detecting the A or G allele at the A118G polymorphic site) and a reverse primer were used. The sequences of the forward primers specific for the A and G alleles were 5′-ACTTGTCCCA CTTAGATGGCA-3′ and 5′-ACTTGTCCCACTTAGATGGCG-3′, respectively, and the P2R primer was used as the reverse primer (Table 1). Afterward, the DNA fragments amplified with both primer pairs were analyzed by electrophoresis using 1–2% agarose gel and ethidium bromide staining for visualization under ultraviolet illumination to detect allele-specific PCR products.

2.6. Statistical analysis

Parametric and nonparametric data are expressed as mean ± SD and median [interquartile range], respectively. Statistical analysis was performed using SPSS v.12.0 for Windows (SPSS Japan, Inc., Tokyo, Japan). The genotype of each of the two screened SNPs was dichotomized into the major allele homozygote (AA) and the combination of the heterozygote and minor allele homozygote (AG + GG). This dichotomization was employed because, in the present study, the number of subjects carrying only the minor G allele of the A8449G SNP was only one, and also because statistical power was not sufficiently high (less than 60%) if the genotype of the A118G SNP was trichotomized and compared between the AA and GG genotypes. 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, total perioperative analgesic use, and VAS scores at 3 and 24-h postoperatively) were normally distributed. Therefore, nonparametric analyses, including the Mann–Whitney *U*-test, Kruskal–Wallis test, or Spearman's rank correlation test, were used to detect possible associations between any of the clinical or genomic parameters (e.g., sex, age, and genotypes of the two screened SNPs) and clinical endpoints related to pain sensitivity or the analgesic effects of fentanyl. Nonparametric analyses were also used to detect possible associations between analgesia in the cold pressor-induced pain test and postoperative endpoints of fentanyl analgesia. Although multiple factors other than the genotypes of the two screened SNPs (e.g., age and sex) may also affect fentanyl analgesia, thus suggesting the use of multivariate covariate analyses, the nonparametric distributions of most of our data precluded the application of such parametric techniques. Therefore, when a significant association between a genotype and a clinical endpoint was found, factors other than genotype were compared between genotypes using unpaired *t*-test, Fisher's exact test, or Mann–Whitney *U*-test according to the types of data to evaluate whether the genotype groups were controlled for other factors that might affect pain sensitivity, fentanyl analgesia, or fentanyl requirements, including age, sex, duration of surgery, and duration of anesthesia. Values of $p < 0.05$ were considered statistically significant. 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 90% for the Cohen's conventional "medium" effect size of 0.3. Power analyses were performed using G*Power v.3.0.5 [11].

3. Results

All 280 Japanese patients who enrolled in the study completed the study. The patients' clinical data are summarized in Table 2. Rescue analgesics were required in none of the patients. In the preoperative cold pressor-induced pain test, fentanyl (2 μg/kg) increased pain perception latency (PPLpost *vs.* PPLpre, $p < 0.0001$, Table 2). Genotype distributions of the two SNPs in the patients are shown in Table 3. These genotype frequencies were in Hardy–Weinberg equilibrium.

Mann–Whitney *U*-test revealed that although sex had no significant association with PPLpre (Fig. 1A) or VAS at 3 or 24 h (data not shown), the analgesic effect of fentanyl in the cold pressor-induced

Table 1
Primers used for genotyping the *OPRM1* SNPs.

Target SNP	Region	Sequence (5′ > 3′)	Forward/reverse	Primer No.
A118G	Exon 1	CTC CCT TCC AGC CTC CGA ATC C	Forward	P1F
		CTC TTT CAT CCT CCC GCC CAA CA	Reverse	P2R
		ACT TGT CCC ACT TAG ATG GCA	Forward	P3F
		ACT TGT CCC ACT TAG ATG GCG	Forward	P4F
		CAA TCA CTG TCC GTG GTC TCC	Reverse	P5R
		GGA GAA TGT CAG ATG CTC AGC	Forward	P6F
IVS3+A8449G	Intron 3	GGG AGG CTA GAA ACA AGA TTC	Forward	P7F
		CGT CTA TGA CTT CTA CTC TAC TGC	Forward	P8F
		GCC TAT ATT TAT TTG GTA TCT GAT	Reverse	P9R

Table 2
Patients' demographic and clinical data.

	All patients		A118G (rs1799971)		IVS3+A8449G (rs9384179)	
			AA	AG + GG	AA	AG + GG
Age (years)	25.8 ± 7.4	(15–50)	25.6 ± 7.3	25.9 ± 7.5	25.9 ± 7.4	25.5 ± 8.1
Male/female	97/183		34/52	63/131	71/148	26/35
Body weight (kg)	57.9 ± 11.1	(38–128)	59.8 ± 10.8	57.1 ± 11.2	57.2 ± 10.8	60.5 ± 11.8
PPLpre (s)	14 [9, 23]	(2–150)	15 [10, 27]	14 [8, 22] (*)	14 [9, 23]	15 [9, 25]
PPLpost (s)	29 [16, 57]	(4–150)	34 [18, 89]	28 [16, 49] (*)	30 [16, 57]	28 [18, 85]
Analgesic effect (PPLpost–PPLpre) (s)	13 [5, 37]	(−17 to +143)	15 [6, 52]	12 [5, 33]*	13 [5, 36]	15 [4, 48]
Duration of anesthesia (min)	170 [157, 186]	(101–286)	171 [157, 189]	169 [157, 185]	170 [156, 186]	170 [162, 185]
Duration of surgery (min)	104 [91, 120]	(66–211)	105 [89, 119]	103 [91, 121]	104 [91, 121]	105 [89, 117]
Total propofol dose (mg/kg)	25.0 [22.0, 27.9]	(5–42)	25.3 [22.4, 27.8]	25.0 [22.0, 28.2]	25.0 [22.0, 27.8]	25.5 [22.5, 28.7]
Preoperative fentanyl use (μg/kg)	2		2	2	2	2
Intraoperative fentanyl use (μg/kg)	3.9 [2.9, 5.3]	(0–13.6)	3.8 [2.9, 5.3]	3.9 [2.9, 5.3]	3.9 [2.9, 5.2]	3.8 [3.0, 5.6]
24-h postoperative fentanyl use (μg/kg)	2.3 [1.1, 4.1]	(0–13.8)	2.3 [1.1, 3.7]	2.3 [1.1, 4.3]	2.5 [1.3, 4.3]	1.5 [0.8, 3.4]*
Perioperative fentanyl use (μg/kg)	6.6 [5.0, 8.6]	(0.8–24.0)	6.2 [5.0, 8.1]	6.6 [5.0, 8.7]	6.7 [5.2, 8.6]	6.0 [4.6, 8.3] (*)
Total perioperative analgesic use (μg/kg)	7.5 [5.9, 9.5]	(1.8–25.3)	7.1 [5.8, 9.0]	7.6 [6.0, 9.6]	7.6 [6.1, 9.6]	6.9 [5.4, 8.9] (*)
VAS pain score at 3 h (mm)	25 [13, 48]	(0–90)	25 [15, 50]	26 [12, 46]	26 [13.5, 50]	24 [10, 36]
VAS pain score at 24 h (mm)	25 [10, 40.5]	(0–83)	25 [9, 49]	25 [10, 40]	25 [10, 44.5]	25 [8, 36]

Data are expressed as numbers, mean ± SD (range), or median [interquartile range].
(*) $p < 0.1$, compared with subjects not carrying minor allele.
* $p < 0.05$, compared with subjects not carrying minor allele.

pain test (PPLpost–PPLpre) was significantly greater in males than in females ($p = 0.009$, Fig. 1B), and 24-h postoperative fentanyl use, perioperative fentanyl use, and total perioperative analgesic use were significantly less in males than in females ($p = 0.039$, Fig. 1C; $p = 0.002$, data not shown; $p < 0.001$, Supplementary Fig. 1A; respectively). Spearman's rank correlation test revealed that age had no significant association with any clinical endpoints (data not shown).

Mann–Whitney *U*-test revealed that PPLpre tended to be less ($p = 0.064$, Fig. 2A and Table 2), PPLpost–PPLpre was significantly less ($p = 0.046$, Fig. 2B) in subjects carrying the minor G allele of the A118G SNP than those not carrying this allele, whereas the A118G SNP had no significant association with 24-h postoperative fentanyl use (Fig. 2C), perioperative fentanyl use, total perioperative analgesic use and VAS at 3 or 24 h (total perioperative analgesic use, Supplementary Fig. 1B; others, Table 2). Unpaired *t*-test, Fisher's exact test, and Mann–Whitney *U*-test revealed no significant differences in age, sex, duration of surgery, or duration of anesthesia between subjects carrying the minor G allele and those not carrying this allele (Table 2). The statistical power was not sufficiently high (less than 60%) if the genotype of the A118G SNP was trichotomized, and the Kruskal–Wallis test failed to show significant differences in PPLpre, PPLpost–PPLpre, or perioperative fentanyl use between genotypes (Supplementary Fig. 2A–C).

Mann–Whitney *U*-test revealed that 24-h postoperative fentanyl use were significantly less and both perioperative fentanyl use and total perioperative analgesic use tended to be less in subjects carrying the minor G allele of the IVS3+A8449G SNP than in those not carrying this allele ($p = 0.010$, Fig. 3C; $p = 0.073$, Table 2; $p = 0.064$, Supplementary Fig. 1C; respectively). The IVS3+A8449G SNP had no significant association with PPLpre (Fig. 3A), PPLpost–PPLpre (Fig. 3B), or VAS at 3 or 24 h (Table 2). Unpaired *t*-test, Fisher's exact test, and Mann–Whitney *U*-test revealed no significant differences in age, sex, duration of surgery, or duration of anesthesia between subjects carrying the minor G allele and those not carrying this allele (Table 2).

Table 3
Genotype distribution for the two screened SNPs.

A118G	AA: 86 (30.7%)/AG: 143 (51.1%)/GG: 51 (18.2%)
IVS3+A8449G	AA: 219 (78.2%)/AG: 60 (21.4%)/GG: 1 (0.4%)

Data are expressed as the number (%) of subjects.

We also analyzed the joint effects of the two SNPs on both 24-h postoperative fentanyl use and total perioperative analgesic use. Mann–Whitney *U*-test revealed that significantly less 24-h postoperative fentanyl use and total perioperative analgesic use were required in subjects not carrying the minor G allele of the A118G SNP and carrying the minor G allele of the IVS3+A8449G SNP ($n = 30$; median [interquartile range] = 1.3 [0.7, 3.1] and 6.7 [4.8, 8.5], respectively) compared with subjects carrying other genotype combinations ($n = 250$; median [interquartile range] = 2.3 [1.2, 4.1] and 7.6 [6.0, 9.5], respectively; $p = 0.031$ and $p = 0.027$, respectively).

Spearman's rank correlation test revealed that PPLpost–PPLpre was significantly associated with VAS at 3 h ($p = 0.024$, Fig. 4), indicating that patients preoperatively showing reduced fentanyl analgesia suffered from more intense pain at 3 h. PPLpost–PPLpre was not associated with VAS at 24 h, perioperative fentanyl use, or total perioperative analgesic use (data not shown).

4. Discussion

We studied patients undergoing mandibular sagittal split ramus osteotomy. Subjects who undergo this cosmetic surgery are usually young and healthy. The operation causes considerable perioperative pain arising from dissected mandibular bone, and the surgical technique is highly standardized at our institute. We conducted a standardized pain test before induction of general anesthesia in opioid-naive subjects without pain. Using these ideal subjects and methods, we found that (i) PPLpre was slightly reduced (i.e., sensitivity to cold pressor-induced pain tended to increase) in subjects carrying the minor G allele of the A118G SNP compared with subjects not carrying this allele, (ii) the analgesic effects of fentanyl evaluated with the cold pressor were reduced in subjects carrying the minor G allele of the A118G SNP compared with subjects not carrying this allele, (iii) 24-h postoperative fentanyl use decreased in subjects carrying the minor G allele of the IVS3+A8449G SNP compared with subjects not carrying this allele, and (iv) no significant association was found between the genotypes of the two screened SNPs and VAS scores, indicating that comparable levels of postoperative analgesia were achieved with PCA fentanyl in our patients regardless of genotypes.

We found that the analgesic effect of fentanyl was significantly reduced in subjects carrying the G allele of the A118G SNP (Fig. 2B). Several recent studies have investigated possible associations be-

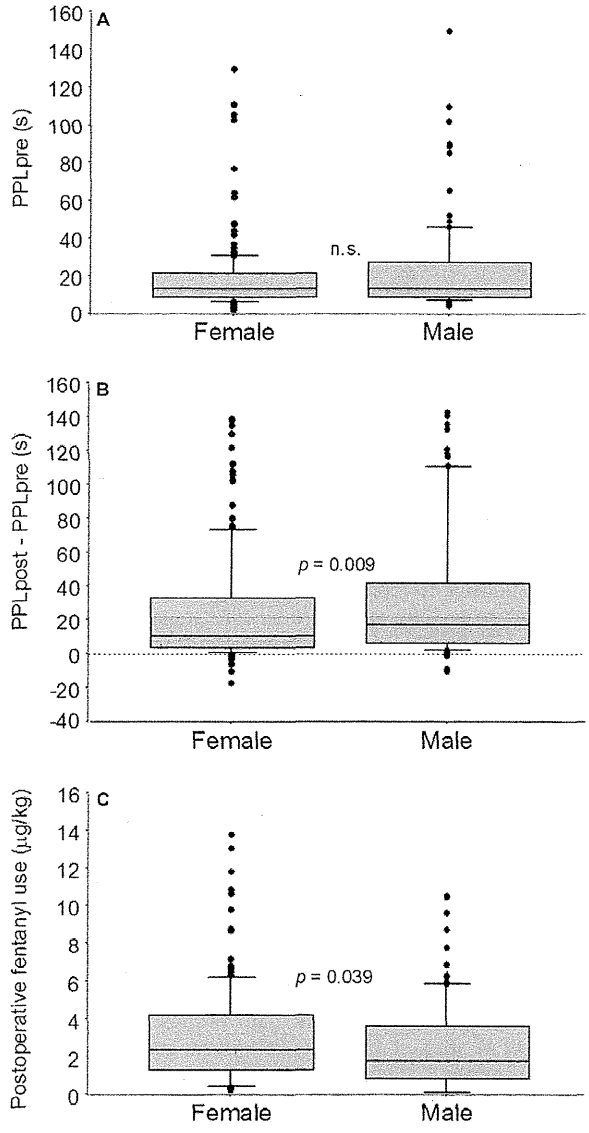

Fig. 1. Associations between sex (male, $n = 97$; female, $n = 183$) and (A) baseline pain perception latency (PPLpre), (B) the analgesic effect of fentanyl in the cold pressor-induced pain test (PPLpost–PPLpre), and (C) 24-h postoperative fentanyl use. Data are expressed by box and whisker plots. Upper and lower ends of boxes represent the 75th and 25th percentiles. Whiskers represent the 90th and 10th percentiles, and filled circles represent outliers. The median is depicted by a solid line in the box.

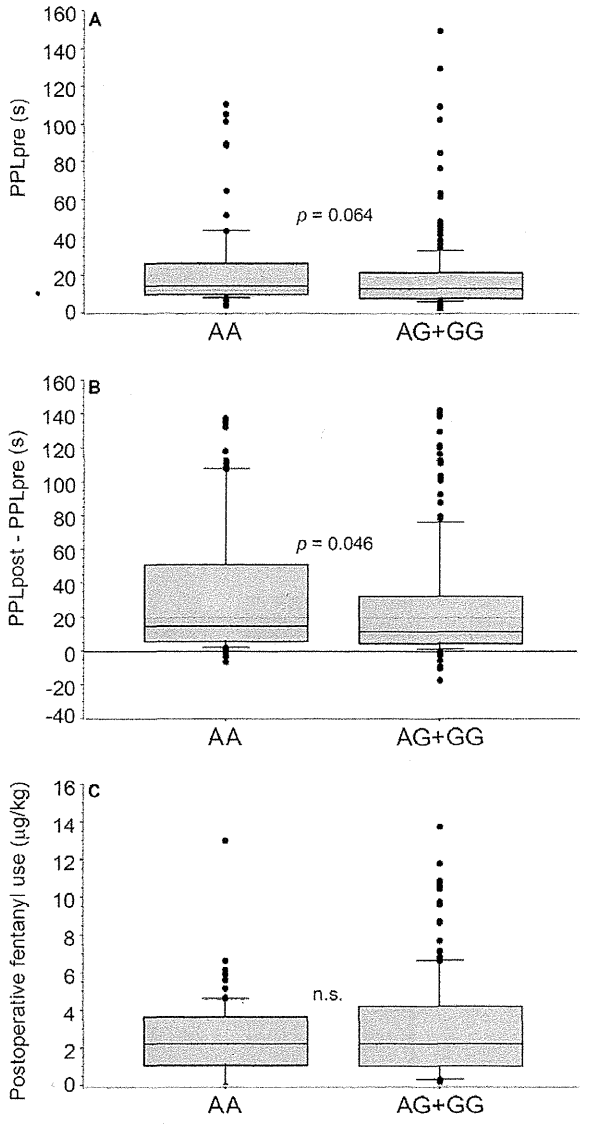

Fig. 2. Associations between genotypes of the A118G SNP (AA, $n = 86$; AG + GG, $n = 194$) and (A) baseline pain perception latency (PPLpre), (B) the analgesic effect of fentanyl in the cold pressor-induced pain test (PPLpost–PPLpre), and (C) 24-h postoperative fentanyl use. Data are expressed by box and whisker plots. Upper and lower ends of boxes represent the 75th and 25th percentiles. Whiskers represent the 90th and 10th percentiles, and filled circles represent outliers. The median is depicted by a solid line in the box.

tween the A118G SNP and postoperative analgesics requirements, including morphine, but these studies have yielded inconsistent results [7,8,10,16,24,36]. Chou et al. found that the G allele was associated with significantly more morphine requirements after total abdominal hysterectomy and total knee arthroplasty in 80 and 120 Taiwanese patients, respectively [7,8]. Sia et al. also found that the G allele was associated with significantly more morphine requirements for postcesarean analgesia in 588 Singaporean women of Han Chinese descent [36]. We also previously found that the G allele was associated with significantly more analgesic requirements after major open abdominal surgery in 138 Japanese patients [16]. These results are generally consistent with the present study. However, other studies of French or American patients have failed to find significant associations between the A118G SNP and postoperative morphine requirements [10,24]. The discrepancies might be related to ethnic differences in the frequency of the A118G variant. The frequency of the 118G allele ranges from 5% to 16% in Caucasian–American and African–American populations and from 25% (Taiwanese) to 47% (Indian) in Asian populations [21]. The discrepancy between these studies might be attributable to the higher G allelic frequency in the A118G SNP in the four studies by Chou et al. [7,8], Sia et al. [36], and Hayashida et al. [16] (34%, 25%, 34%, and 45%, respectively) than in the studies by Coulbault et al. and Janicki et al. (12% and 16%, respectively) [10,24]. Chou et al., Sia et al., and Hayashida et al. probably could have conducted more reliable statistical analyses of the influence of the A118G SNP to reveal such associations. In the present study in a Japanese population, the G allelic frequency (estimated to be 43.8%) was higher compared with the previous studies. Such genotype distributions may have enabled us to conduct a more reliable statistical analysis on the influence of the A118G SNP than any of the previous studies.

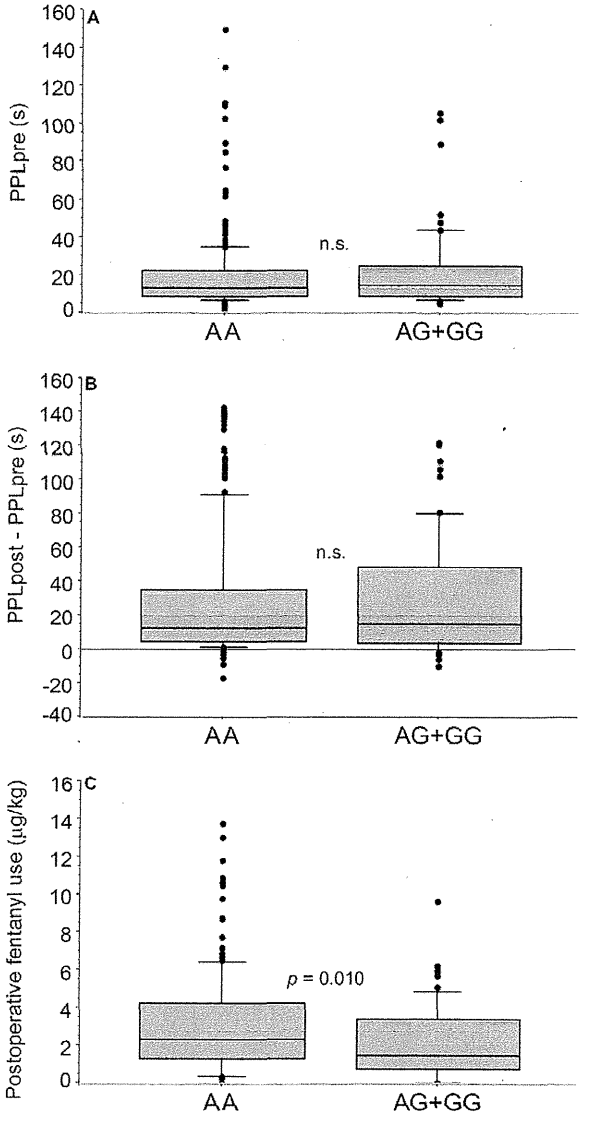

Fig. 3. Associations between genotypes of the IVS3+A8449G SNP (AA, $n = 219$; AG + GG, $n = 61$) and (A) baseline pain perception latency (PPLpre), (B) the analgesic effect of fentanyl in the cold pressor-induced pain test (PPLpost–PPLpre), and (C) 24-h postoperative fentanyl use. Data are expressed by box and whisker plots. Upper and lower ends of boxes represent the 75th and 25th percentiles. Whiskers represent the 90th and 10th percentiles, and filled circles represent outliers. The median is depicted by a solid line in the box.

The A118G substitution leads to an amino acid substitution of asparagine at position 40 with aspartate (Asn40Asp), which is the putative *N*-glycosylation site in the human MOP protein. This 40Asp reportedly causes changes in the levels of binding capacity of several opioid analgesics *in vitro* [3,28]. Although some contradictory data have been reported [1], such alterations in ligand binding capacity may result in alterations in the effects of opioids. Furthermore, Zhang et al. showed that in human autopsy brain tissues, *OPRM1* mRNA expression from the 118G allele was 1.5- to 2.5-fold less abundant than that from the 118A allele [39]. They also showed that only the 118G allele was associated with 1.5-fold lower mRNA levels and more than 10-fold lower MOP protein levels in hamster ovary cells transfected with the coding region of the *OPRM1* gene carrying A, G, C, and T at position 118. Such deleterious effects of the 118G variant on both mRNA and protein yield may explain why subjects with the G allele of the A118G SNP were

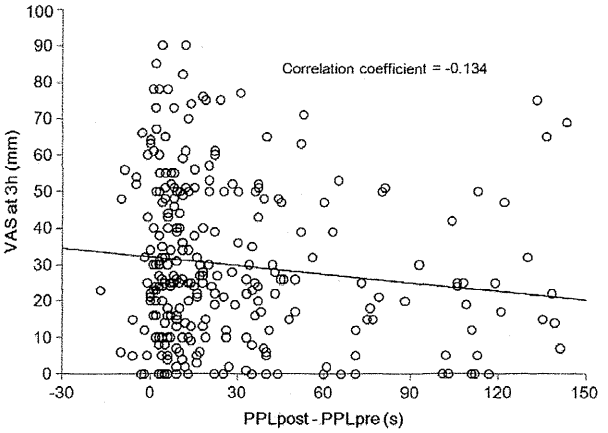

Fig. 4. A scatter plot showing the association between the analgesic effect of fentanyl in the cold pressor-induced pain test (PPLpost–PPLpre) and VAS at 3 h. Each point represents an individual patient.

less responsive to fentanyl. Our observations, together with those of Zhang et al. [39], appear to be consistent with observations in mice in which reduced expression levels of MOP mRNA and protein were associated with decreases in morphine-induced analgesia [21].

We also found that sensitivity to cold pressor-induced pain tended to be higher in subjects carrying the minor G allele of the A118G SNP (Fig. 2A). The number of studies investigating associations between the A118G SNP and sensitivity to experimental pain is limited, and the results have been inconsistent. Fillingim et al. investigated possible associations between the A118G SNP and thermal, mechanical, and ischemic pain sensitivity in 167 healthy American volunteers and reported that subjects with the minor G allele had significantly higher pressure pain thresholds (i.e., lower sensitivity to pressure pain) than those not carrying this allele, and that the G allele was associated with lower heat pain ratings at 49 °C among men but higher pain ratings among women [12]. Lötsch et al. studied 45 White volunteers and found that subjects with the minor G allele showed lower amplitudes of event-related potentials in the cerebral cortex induced by nociceptive stimuli (40% and 60% CO_2) applied to the nasal mucosa, suggesting lower sensitivity to nociceptive stimuli in subjects with the minor G allele [30]. Huang et al. studied 72 adult Taiwanese women and failed to find a significant association between the A118G SNP and mechanical pain sensitivity [17]. The discrepancy might be related to (i) differences in experimental pain models (mechanical, thermal, or chemical pain *vs.* cold pressor-induced pain), (ii) differences in the frequency of the A118G variant (22.6%, 10.0%, 30.6%, and 43.8% in Fillingim et al., Lötsch et al., Huang et al., and the present study, respectively), (iii) differences in the number of subjects recruited in the studies (45, 72, and 280 in Lötsch et al., Huang et al., and the present study, respectively), (iv) ethnic differences that may reveal different genotypic associations, or (v) whether subjects were premedicated with centrally acting drugs, which might affect their responses to noxious stimuli.

In the present study, the analgesic effects of fentanyl were reduced, and 24-h postoperative fentanyl use increased in females compared with males (Fig. 1). In rat models, opioids consistently produce greater analgesia in males than in females [9,26]. Our study was consistent with these previous findings. However, several human studies have reported conflicting results, such as no sex differences in opioid analgesia [14,33,35], greater opioid-induced analgesia in females than males [4,6,13,14,32,34], or greater analgesia in males than females [5,32]. These discrepant results might be related to differences in the cultural, ethnic, psychosocial,

or genetic backgrounds of the subjects, opioid analgesics used, pain models employed, or the number of enrolled subjects. Further studies employing greater numbers of more controlled samples might be required to reveal the influences of sex.

We also found that 24-h postoperative fentanyl use was reduced in subjects with the G allele of the IVS3+A8449G SNP (Fig. 3C). These results suggest that in addition to the A118G SNP in exon 1, the IVS3+A8449G SNP may also affect the analgesic effects of opioids. The IVS3+A8449G SNP represents a complete linkage disequilibrium block with 30 polymorphisms in intron 3 and the 3′ flanking region of the *OPRM1* gene [20]. Interestingly, CXBK mice showing reduced sensitivity to opioids possess a sequence of more than 5000 nucleotide bases of a transposon intracisternal A particle (IAP) in the 3′UTR of the *Oprm1* gene [15]. Numerous regulatory elements, such as adenylate/uridylate-rich elements, and transcription factor-binding motifs exist in the 3′UTR of the *Oprm1* gene. The IAP insertion might disrupt the stability of *Oprm1* mRNA or might reduce the transcription of *Oprm1* mRNA by separating these elements [25]. Considering the significant sequence homology between the human *OPRM1* 3′UTR and the mouse *Oprm1* 3′UTR [18], human *OPRM1* mRNA levels might be influenced by differences in the *OPRM1* 3′UTR sequence via mechanisms similar to those underlying reduced *Oprm1* mRNA levels in CXBK mice. Moreover, significant joint effects of the two SNPs on fentanyl requirements suggest the interaction of the *Oprm1* 3′UTR and A118G SNP.

In conclusion, in Japanese patients who underwent sagittal split ramus osteotomy, the analgesic effects of fentanyl were related to genotypes of the *OPRM1* gene. Subjects with the G allele of the *OPRM1* A118G SNP were less sensitive to fentanyl and tended to be more sensitive to cold pressor-induced pain. Additionally, subjects with the G allele of the IVS3+A8449G SNP representing the complete disequilibrium block, including the 3′UTR of the *OPRM1* gene, required less fentanyl for postoperative analgesia. Females were also less sensitive to fentanyl than males. Although further validation is needed, our data provide valuable information for appropriate individualization of fentanyl doses to achieve adequate pain control and open new avenues for personalized pain treatment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pain.2009.09.004.

References

[1] Beyer A, Koch T, Schroder H, Schulz S, Hollt V. Effect of the A118G polymorphism on binding affinity, potency and agonist-mediated endocytosis, desensitization, and resensitization of the human μ-opioid receptor. J Neurochem 2004;89:553–60.
[2] Bisgaard T, Klarskov B, Rosenberg J, Kehlet H. Characteristics and prediction of early pain after laparoscopic cholecystectomy. Pain 2001;90:261–9.
[3] Bond C, LaForge KS, Tian M, Melia D, Zhang S, Borg L, Gong J, Schluger J, Strong JA, Leal SM, Tischfield JA, Kreek MJ, Yu L. Single-nucleotide polymorphism in the human mu opioid receptor gene alters β-endorphin binding and activity: possible implications for opiate addiction. Proc Natl Acad Sci USA 1998;95:9608–13.
[4] Burns JW, Hodsman NB, McLintock TT, Gillies GW, Kenny GN, McArdle CS. The influence of patient characteristics on the requirements for postoperative analgesia. A reassessment using patient-controlled analgesia. Anaesthesia 1989;44:2–6.
[5] Cepeda MS, Carr DB. Women experience more pain and require more morphine than men to achieve a similar degree of analgesia. Anesth Analg 2003;97:1464–8.
[6] Chia YY, Chow LH, Hung CC, Liu K, Ger LP, Wang PN. Gender and pain upon movement are associated with the requirements for postoperative patient-controlled iv analgesia: a prospective survey of 2,298 Chinese patients. Can J Anaesth 2002;49:249–55.
[7] Chou WY, Wang CH, Liu PH, Liu CC, Tseng CC, Jawan B. Human opioid receptor A118G polymorphism affects intravenous patient-controlled analgesia morphine consumption after total abdominal hysterectomy. Anesthesiology 2006;105:334–7.
[8] Chou WY, Yang LC, Lu HF, Ko JY, Wang CH, Lin SH, Lee TH, Concejero A, Hsu CJ. Association of μ-opioid receptor gene polymorphism (A118G) with variations in morphine consumption for analgesia after total knee arthroplasty. Acta Anaesthesiol Scand 2006;50:787–92.
[9] Cicero TJ, Nock B, Meyer ER. Sex-related differences in morphine's antinociceptive activity: relationship to serum and brain morphine concentrations. J Pharmacol Exp Ther 1997;282:939–44.
[10] 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;79:316–24.
[11] 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;39:175–91.
[12] Fillingim RB, Kaplan L, Staud R, Ness TJ, Glover TL, Campbell CM, Mogil JS, Wallace MR. The A118G single nucleotide polymorphism of the μ-opioid receptor gene (OPRM1) is associated with pressure pain sensitivity in humans. J Pain 2005;6:159–67.
[13] Gear RW, Miaskowski C, Gordon NC, Paul SM, Heller PH, Levine JD. Kappa-opioids produce significantly greater analgesia in women than in men. Nat Med 1996;2:1248–50.
[14] Gordon NC, Gear RW, Heller PH, Paul S, Miaskowski C, Levine JD. Enhancement of morphine analgesia by the GABAB agonist baclofen. Neuroscience 1995;69:345–9.
[15] Han W, Kasai S, Hata H, Takahashi T, Takamatsu Y, Yamamoto H, Uhl GR, Sora I, Ikeda K. Intracisternal A-particle element in the 3′ noncoding region of the μ-opioid receptor gene in CXBK mice: a new genetic mechanism underlying differences in opioid sensitivity. Pharmacogenet Genomics 2006;16:451–60.
[16] Hayashida M, Nagashima M, Satoh Y, Katoh R, Tagami M, Ide S, Kasai S, Nishizawa D, Ogai Y, Hasegawa J, Komatsu H, Sora I, Fukuda K, Koga H, Hanaoka K, Ikeda K. Analgesic requirements after major abdominal surgery are associated with OPRM1 gene polymorphism genotype and haplotype. Pharmacogenomics 2008;9:1605–16.
[17] Huang CJ, Liu HF, Su NY, Hsu YW, Yang CH, Chen CC, Tsai PS. Association between human opioid receptor genes polymorphisms and pressure pain sensitivity in females . Anaesthesia 2008;63:1288–95.
[18] Ide S, Han W, Kasai S, Hata H, Sora I, Ikeda K. Characterization of the 3′ untranslated region of the human μ-opioid receptor (MOR-1) mRNA. Gene 2005;364:139–45.
[19] Ide S, Kobayashi H, Tanaka K, Ujike H, Sekine Y, Ozaki N, Inada T, Harano M, Komiyama T, Yamada M, Iyo M, Ikeda K, Sora I. Gene polymorphisms of the μ opioid receptor in methamphetamine abusers. Ann NY Acad Sci 2004;1025:316–24.
[20] Ide S, Kobayashi H, Ujike H, Ozaki N, Sekine Y, Inada T, Harano M, Komiyama T, Yamada M, Iyo M, Iwata N, Tanaka K, Shen H, Iwahashi K, Itokawa M, Minami M, Satoh M, Ikeda K, Sora I. Linkage disequilibrium and association with methamphetamine dependence/psychosis of μ-opioid receptor gene polymorphisms. Pharmacogenomics J 2006;6:179–88.
[21] 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;26:311–7.
[22] Ikeda K, Kobayashi T, Ichikawa T, Kumanishi T, Niki H, Yano R. The untranslated region of μ-opioid receptor mRNA contributes to reduced opioid sensitivity in CXBK mice. J Neurosci 2001;21:1334–9.
[23] Imai K, Ogai Y, Nishizawa D, Kasai S, Ikeda K, Koga H. A novel SNP detection technique utilizing a multiple primer extension (MPEX) on a phospholipid polymer-coated surface. Mol Biosyst 2007;3:547–53.
[24] Janicki PK, Schuler G, Francis D, Bohr A, Gordin V, Jarzembowski T, Ruiz-Velasco V, Mets B. A genetic association study of the functional A118G polymorphism of the human μ-opioid receptor gene in patients with acute and chronic pain. Anesth Analg 2006;103:1011–7.
[25] Kasai S, Han W, Ide S, Hata H, Takamatsu Y, Yamamoto H, Uhl GR, Sora I, Ikeda K. Involvement of the 3′ non-coding region of the μ opioid receptor gene in morphine-induced analgesia. Psychiatry Clin Neurosci 2006;60:11–7.
[26] Kepler KL, Standifer KM, Paul D, Kest B, Pasternak GW, Bodnar RJ. Gender effects and central opioid analgesia. Pain 1991;45:87–94.
[27] Klepstad P, Rakvag TT, Kaasa S, Holthe M, Dale O, Borchgrevink PC, Baar C, Vikan T, Krokan HE, Skorpen F. The 118 A>G polymorphism in the human μ-

opioid receptor gene may increase morphine requirements in patients with pain caused by malignant disease. Acta Anaesthesiol Scand 2004;48:1232–9.
[28] Kroslak T, Laforge KS, Gianotti RJ, Ho A, Nielsen DA, Kreek MJ. The single nucleotide polymorphism A118G alters functional properties of the human μ opioid receptor. J Neurochem 2007;103:77–87.
[29] 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;54:321–6.
[30] Lötsch J, Stuck B, Hummel T. The human μ-opioid receptor gene polymorphism 118A>G decreases cortical activation in response to specific nociceptive stimulation. Behav Neurosci 2006;120:1218–24.
[31] Martikainen IK, Narhi MV, Pertovaara A. Spatial integration of cold pressor pain sensation in humans. Neurosci Lett 2004;361:140–3.
[32] Miller PL, Ernst AA. Sex differences in analgesia: a randomized trial of μ versus κ opioid agonists. South Med J 2004;97:35–41.
[33] Olofsen E, Romberg R, Bijl H, Mooren R, Engbers F, Kest B, Dahan A. Alfentanil and placebo analgesia: no sex differences detected in models of experimental pain. Anesthesiology 2005;103:130–9.
[34] Risch N. Searching for genes in complex diseases: lessons from systemic lupus erythematosus. J Clin Invest 2000;105:1503–6.
[35] Romberg R, Olofsen E, Sarton E, den Hartigh J, Taschner PE, Dahan A. Pharmacokinetic–pharmacodynamic modeling of morphine-6-glucuronide-induced analgesia in healthy volunteers: absence of sex differences. Anesthesiology 2004;100:120–33.
[36] Sia AT, Lim Y, Lim EC, Goh RW, Law HY, Landau R, Teo YY, Tan EC. A118G single nucleotide polymorphism of human μ-opioid receptor gene influences pain perception and patient-controlled intravenous morphine consumption after intrathecal morphine for postcesarean analgesia. Anesthesiology 2008;109:520–6.
[37] Sora I, Elmer G, Funada M, Pieper J, Li XF, Hall FS, Uhl GR. Mu opiate receptor gene dose effects on different morphine actions: evidence for differential in vivo μ receptor reserve. Neuropsychopharmacology 2001;25:41–54.
[38] 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 USA 1997;94:1544–9.
[39] Zhang Y, Wang D, Johnson AD, Papp AC, Sadee W. Allelic expression imbalance of human μ opioid receptor (OPRM1) caused by variant A118G. J Biol Chem 2005;280:32618–24.

Association between *KCNJ6* (*GIRK2*) Gene Polymorphisms and Postoperative Analgesic Requirements after Major Abdominal Surgery

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Abstract

Opioids are commonly used as effective analgesics for the treatment of acute and chronic pain. However, considerable individual differences have been widely observed in sensitivity to opioid analgesics. We focused on a G-protein-activated inwardly rectifying potassium (GIRK) channel subunit, GIRK2, that is an important molecule in opioid transmission. In our initial polymorphism search, a total of nine single-nucleotide polymorphisms (SNPs) were identified in the whole exon, 5′-flanking, and exon-intron boundary regions of the *KCNJ6* gene encoding GIRK2. Among them, G-1250A and A1032G were selected as representative SNPs for further association studies. In an association study of 129 subjects who underwent major open abdominal surgery, the A/A genotype in the A1032G SNP and -1250G/1032A haplotype were significantly associated with increased postoperative analgesic requirements compared with other genotypes and haplotypes. The total dose (mean±SEM) of rescue analgesics converted to equivalent oral morphine doses was 20.45±9.27 mg, 10.84±2.24 mg, and 13.07±2.39 mg for the A/A, A/G, and G/G genotypes in the A1032G SNP, respectively. Additionally, *KCNJ6* gene expression levels in the 1032A/A subjects were significantly decreased compared with the 1032A/G and 1032G/G subjects in a real-time quantitative PCR analysis using human brain tissues, suggesting that the 1032A/A subjects required more analgesics because of lower *KCNJ6* gene expression levels and consequently insufficient analgesic effects. The results indicate that the A1032G SNP and G-1250A/A1032G haplotype could serve as markers that predict increased analgesic requirements. Our findings will provide valuable information for achieving satisfactory pain control and open new avenues for personalized pain treatment.

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Introduction

Opioids are commonly used as effective analgesics for the treatment of acute and chronic pain. However, sensitivity to opioid analgesics is well known to vary widely among individual subjects [1]. Individual differences can be attributed to both genetic and environmental factors, although the relative influence of each of these factors can be diverse [2]. Genetic variations in opioid-related genes involved in opioid pharmacokinetics and pharmacodynamics might lead to individual differences in phenotypes related to pharmacological actions of opioid analgesics.

Numerous molecules are involved in the pharmacological effects of opioids. Opioid ligands bind to opioid receptors, and the signal is transmitted to a variety of effectors (e.g., adenylate cyclase, calcium ion channels, and G-protein-activated inwardly rectifying potassium [GIRK] channels), thereby resulting in analgesic effects [3]. The genes encoding these molecules are candidates for researching the relationships between genetic variations and individual differences in phenotypes related to opioid actions.

Among opioid-related genes, GIRK channels are attractive targets for the investigation of the relationship between genetic variations and sensitivity to opioid analgesics because they play a key role in opioid-induced analgesia [3]. Additionally, recent quantitative trait locus analysis and computational mapping have identified *Kcnj9* (mouse *Girk3*) as a candidate gene affecting variability in the analgesia induced by multiple drug classes [4]. GIRK channels are members of the inwardly rectifying potassium channel family, and four subtypes (GIRK1-GIRK4) have been identified in mammals [5]. GIRK channels are expressed in many tissues, including the heart [6], spinal cord [7,8], and various regions in the brain with different subunit compositions [9–11]. GIRK channel activation is triggered by activation of several $G_{i/o}$ protein-coupled receptors, including opioid receptors [12]. Several studies using knockout mice have shown that opioid-induced