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Association between human opioid receptor genes polymorphisms and pressure pain sensitivity in females*

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Summary

This study examined the association between pressure pain sensitivity and various single nucleotide polymorphisms (SNPs) of human μ-, κ-, and δ-opioid receptor (i.e. *OPRM1*, *OPRK1*, and *OPRD1*) genes in 72 healthy adult Taiwanese women of Han Chinese race. Pressure pain threshold and tolerance were measured by an algometer and polymorphisms of the opioid receptor genes determined from blood samples. Our data revealed that pressure pain threshold, but not tolerance, in subjects with the minor allele (termed 'GA') genotype of the IVS2+31G>A polymorphism of the *OPRM1* gene was significantly higher than those with major allele (termed 'GG') genotype. Neither pressure pain threshold nor tolerance between major and minor alleles of other SNPs of the *OPRM1*, *OPRK1*, and *OPRD1* genes were significantly different. These data suggest an association between the IVS2+31G>A SNP of the *OPRM1* gene and pressure pain sensitivity in healthy adult females.

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We have previously shown that pre-operative pressure pain sensitivity, especially pressure pain tolerance, predicts postoperative pain and analgesic consumption in female patients [1]. However, the mechanisms that underlie the substantial inter-individual variability in pressure pain sensitivity in females remain to be elucidated.

The human μ-opioid receptor mediates the analgesic effects of endogenous opioid peptides and exogenous opioid agents [2]. The function of the μ-receptor is under the influence of single nucleotide polymorphisms (SNPs) of the human μ-opioid receptor (*OPRM1*) gene [3]. With an incidence of 10–15%, the 118A>G SNP is one of the most widely studied SNPs of the *OPRM1* gene [4]. In the terminology, '118' refers to the position of this SNP in the genome (i.e. position 118 in the exon) and 'A>G' represents a possible substitution of the nucleotide adenine, A, by guanine, G [4]. The genotype of individuals in relation to this SNP can therefore be homozygous GG, or – where a substitution occurs – homozygous AA, or heterozygous AG. As the incidence of AA is significantly higher than that of GG, the genotype AA is referred to as the 'major homozygous genotype' and the genotype GG is referred to as the 'minor homozygous genotype' of this SNP [4].

Fillingim et al. reported that the 118A>G SNP of the *OPRM1* gene was associated with pressure pain sensitivity in healthy adults: individuals with heterozygous (AG) and minor homozygous (GG) genotypes of this SNP were found to have higher pressure pain threshold than individuals with major homozygous (AA) genotype [5]. However, when data from men and women were analysed separately this association of genotype with response was only significant in men [5]. Since substantial inter-individual variability in pressure pain sensitivity also exists in females, these data suggest that the 118A>G SNP of the *OPRM1* gene may not be the mechanism that

helps explain this variability, so some other gene association may be responsible.

To elucidate further, we sought to examine whether other SNPs of the human opioid receptor genes might modulate pressure pain sensitivity in females. In addition to the 118A>G SNP, there appear to be four other common SNPs (i.e. those that have an incidence >5% in the population) of the *OPRM1* gene: 17C>T, −172G>T, IVS2+31G>A, and IVS2+691G>C [4, 6]. Again, in the terminology, '17' refers to the position 17 in the exon, '−172' refers to the position 172 in the 5′ un-translated region of the exon, 'C>T' refers to a possible substitution of the nucleotide cytosine, C, by thymine, T, and 'G>T' refers to a possible substitution of the nucleotide G by T [4, 6]. Moreover, 'IVS2+'refers to the intron 2 of the genome, '31' and '691' refer to the position 31 and position 691 in the intron 2, 'G>A' and 'C>G' represent a possible substitution of the nucleotide G by A and the nucleotide C by G, respectively [4, 6]. We therefore planned to examine these SNPs for possible association with pressure pain sensitivity.

Furthermore, in addition to the μ-opioid receptor, the analgesic effects of endogenous opioid peptides and exogenous opioid agents are also under the regulation of the κ- and δ-opioid receptors [2]. In turn, the function of these opioid receptors is also under the influence of SNPs of κ- and δ-opioid receptor genes (*OPRK1* and *OPRD1*) [7, 8]. Both the 36G>T SNP (a possible substitution of the nucleotide G by T at the position 36 in the exon) of the *OPRK1* gene [7] and the 921T>C SNP (a possible substitution of the nucleotide T by C at the position 921 in the exon) of the *OPRD1* gene [8] have also been reported to have an incidence >5% (and thus conventionally regarded as common).

We wished to extend the previous work of Fillingim et al. and examine possible genotype associations with pain sensitivity in females. Our main (null) hypothesis was that the 118A>G SNP of the *OPRM1* gene is not associated with pressure pain sensitivity in females. Therefore, we also planned to examine the common SNPs of the *OPRM1, OPRK1* and *OPRD1* genes and their possible association with pressure pain sensitivity in females.

Methods

The study (MMH-I-S-195) was approved by the Institutional Review Board of the Mackay Memorial Hospital, Taipei, Taiwan. All participants gave written informed consent.

Subjects

Study subjects were females, aged between 20 and 50, recruited via advertisement. Individuals had to be healthy, with additional exclusion criteria being anaemia (haematocrit ≤ 20%), obesity (body-mass index ≥ 30 $kg.m^{-2}$), abnormal hepatic and renal function tests, hypertension, substance abuse, chronic analgesic use, psychiatric disease, and intake of any analgesic drug < 1 week before the study.

Sample size determination was based on findings reported by Fillingim et al. [5] and the study by Chou et al. [9]. Effect size was calculated by computing the Cohen's *d*, which is defined as the difference between two means divided by the pooled standard deviation for those means [10]. In Fillingim's study, the effect size for the 118A>G SNP of the *OPRM1* genotype effect on pressure pain threshold was 0.89 for men ($n = 71$) and 0.38 for women ($n = 96$), respectively [5]. In Chou's study on post-hysterectomy Taiwanese women, morphine consumption was significantly different between those patients with the minor homozygous genotype (GG) and those with the major homozygous genotype (AA) of the 118A>G SNP (33 ± 10 vs 27 ± 10 mg, effect size = 0.6, p = 0.02) [9]. These data indicate likely effect sizes that can be obtained were between 0.38 and 0.89. We therefore assumed an effect size of 0.6, a significance level at 0.05, and a power of 0.8 for sample size calculation. For a one-sided independent *t*-test, 35 subjects per group were needed. A total of 72 healthy adult Taiwanese women of Han Chinese race were thus included to ensure at least this power for the study.

Anxiety assessment

It is well-established that pain perception is influenced by anxiety [11]. To control for this factor, the anxiety level of individual subject was evaluated by a Chinese version of the Beck Anxiety Inventory (BAI) questionnaire (The Psychological Corp., San Antonio, TX, USA), a 21 item self-report inventory measuring symptoms of depression [12]. Any individuals with BAI score >16 were excluded.

Pressure pain threshold and tolerance assessment

The pressure pain threshold and pressure pain tolerance were measured by the same investigator using an electronic pressure algometer (Somedic AB, Solletuna, Sweden), as previously described [1]. In brief, a probe with surface area 1 cm^2 was applied to the pulp of ring finger of the right hand, and pressure increased by 30 $kPa.s^{-1}$. Subjects pressed a button when they started to feel pain (i.e. pressure pain threshold) and again when they could no longer withstand the pain (i.e. pressure pain tolerance). A training session was performed to allow familiarisation, after which formal assessments were made three times with a 5-min interval between each. The average of the three assessments was used.

Genotyping

Upon the completion of pressure pain threshold and tolerance assessment, 5 ml blood was withdrawn into EDTA tubes. Genomic DNA was isolated from 200 μl EDTA blood on a Liquid Chromatograph (LC) using the MagNA Pure LC DNA Isolation Kit (Roche Diagnostics Scandinavia AB, Bromma, Sweden). Purified genomic DNA was eluted in 100 μl elution buffer and stored at −20 °C. Primers for polymerase chain reaction (PCR) amplification of the polymorphisms in the *OPRM1*, *OPRK1*, and *OPRD1* genes were adapted from previous reports [7, 13, 14]. The conditions for PCR and the primer sequences are shown in Table 1. Polymorphisms were then verified by direct DNA sequencing. Sequencing reactions were carried out with 6 ml of purified PCR product and 3.2 pmol primer with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer Inc., Waltham, MA, USA) on a Perkin-Elmer GenAmp PCR system 9700 (PerkinElmer). The sequencing primers were the same as those used in the PCR amplification. Residual dideoxy terminators were then removed by ethanol precipitation and the sequences were then analysed on an Applied Biosystems 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analyses

Hardy–Weinberg equilibrium was assessed for each SNP by χ^2 analysis.

In the event that only a relatively small proportion of our subjects would have heterozygous genotype or minor homozygous genotype, we planned to use the method adopted by Fillingim et al. to group subjects [5]. Subjects with the major homozygous genotype were classified as the 'major allele' genotype group. Subjects with the heterozygous and the minor homozygous genotypes were combined to form the 'minor allele' genotype group. Statistical significance of differences of demographic data and pain sensitivity data between the two groups was assessed using Student's *t*-test for normally distributed variables and the Mann–Whitney *U*-test for variables that were not normally distributed. We also calculated Cohen's *d* to determine effect size [10], a relatively conservative test that avoids exaggerated significance for small absolute differences. Positive values for effect size would indicate greater pain threshold and/or tolerance in the minor allele group, whereas negative values would indicate greater pain threshold and tolerance in the major allele group. An effect size of 0.2–0.5 was classified as 'small', 0.5–0.8 as 'medium', >0.8 as 'large'. A medium effect size was considered as a sizeable group difference. Data were presented as mean (SD). The significance level was set at 0.05. A commercial software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

Results

Genotype frequency

The genotype frequency (i.e. incidence) of each SNP of the *OPRM1*, *OPRK1*, and *OPRD1* genes is shown in Table 2. Genotyping analysis revealed that none of our subjects possessed polymorphism in the 17 and the IVS2+691 loci of the *OPRM1* gene: i.e. the genotypes of both loci in our subjects were of the major homologous type (i.e. CC and GG, respectively; Table 2). Moreover, 4 of our subjects (5.6%) possessed the heterozygous (GA) genotype of the IVS2+31G>A SNP of the *OPRM1* gene while none of our subjects possessed the minor homozygous (AA) genotype of this SNP (Table 2).

Table 1 The primer sequences and conditions for polymerase chain reaction of the various single nucleotide polymorphisms (SNPs) of the human μ-, κ-, and δ-opioid receptor (*OPRM1*, *OPRK1*, and *OPRD1*) genes.

Gene	SNP	Primer sequences	Denaturation	Annealing	Extension	Cycles
OPRM1	−172G>T	Forward: 5′-CACAGAAGAGTGCCCAGTGAA-3′ Reverse: 5′-AGCCAGGAGCACCGAGACT-3′	95 °C, 15 s	60 °C, 7 s	72 °C, 12 s	40
	17C>T	Forward: 5′-TCGGTGCTCCTGGCTACCT-3′ Reverse: 5′-GTTGCCATCTAAGTGGGACAAGTT-3′	95 °C, 30 s	60 °C, 60 s	72 °C, 30 s	40
	118A>G	Forward: 5′-GCTTGGAACCCGAAAAGTCT-3′ Reverse: 5′-GTAGAGGGCCATGATCGTGAT-3′	95 °C, 15 s	60 °C, 7 s	72 °C, 12 s	40
	IVS2+31G>A	Forward: 5′-CAACATCTACATTTTCAACC-3′ Reverse: 5′-AATTCCTATTTTTAAGTATTCAAAG-3′	95 °C, 30 s	57 °C, 30 s	72 °C, 60 s	35
	IVS2+691G>C	Forward: 5′-CTTAATGTGATCGAAGTGGACT-3′ Reverse: 5′-GTAATGATGAGCACTGGCAT-3′	95 °C, 15 s	59 °C, 7 s	72 °C, 14 s	40
OPRK1	36G>T	Forward: 5′-CGGAAAGGCAGCGAGAAGT-3′ Reverse: 5′-CTTGCCCTGCGCATAGAGTT-3′	94 °C, 30 s	58 °C, 30 s	72 °C, 90 s	35
OPRD1	921T>C	Forward: 5′-GCTGCGCTGCACCTGG-3′ Reverse: 5′-TGAAGTTCTCGTCGAGGAAAGC-3′	95 °C, 30 s	59 °C, 30 s	72 °C, 20 s	35

Table 2 Occurrence of various single nucleotide polymorphism (SNP) genotypes of the human μ-, κ-, and δ-opioid receptor (*OPRM1*, *OPRK1*, and *OPRD1*) genes in our sample population. As only a relatively small proportion of our subjects had heterozygous genotype or minor homozygous genotype, we thus combined the heterozygous and the minor homozygous genotypes to form the 'minor allele' genotype group and subjects with the major homozygous genotype were classified as the 'major allele' genotype group to facilitate further analysis.

		Genotype incidence (%)		
Gene	**SNP**	**Major homozygous**	**Heterozygous**	**Minor homozygous**
OPRM1	118A>G	65.2% (*n* = 47)	5.6% (*n* = 4)	29.2% (*n* = 21)
	17C>T	100% (*n* = 72)	– (*n* = 0)	– (*n* = 0)
	−172G>T	77.8% (*n* = 56)	9.7% (*n* = 7)	12.5% (*n* = 9)
	IVS2+31G>A	94.4% (*n* = 68)	5.6% (*n* = 4)	– (*n* = 0)
	IVS2+691G>C	100% (*n* = 72)	– (*n* = 0)	– (*n* = 0)
OPRK1	36G>T	70.8% (*n* = 51)	12.5% (*n* = 9)	16.7% (*n* = 12)
OPRD1	921T>C	55.6% (*n* = 40)	38.8% (*n* = 28)	5.6% (*n* = 4)

Genotype distributions of the IVS2+31G>A SNP of the *OPRM1* gene and the 921T>C SNP of the *OPRD1* gene were in accordance with Hardy–Weinberg equilibrium (both $p > 0.05$) while the other SNPs included in this study showed significant deviation (all $p < 0.0001$).

Demographic data

As previously stated, we classed subjects with the major homozygous genotype were classified as the 'major allele' group and we combined subjects with the heterozygous and with the minor homozygous genotypes into a 'minor allele' group. These two groups were matched with respect to demographic data (age, body weight, body height, heart rate, systolic blood pressure, diastolic blood pressure, mean blood pressure, and the BAI score) across all the SNPs (Tables 3 and 4).

Genotype and pain sensitivity

The differences in both pressure pain threshold and pressure pain tolerance between major allele and minor allele genotypes of the 118A>G or the −172G>T SNP of the *OPRM1* gene examined by the *t*-test were not statistically significant (Table 5). The pressure pain threshold and pressure pain tolerance in subjects with major allele and minor allele genotypes of the 36G>T SNP of the *OPRK1* gene or the 921T>C SNP of the *OPRD1* gene were also not significantly different (Table 5).

The pressure pain tolerance in subjects with major allele and minor allele genotypes of the IVS2+31G>A SNP of the *OPRM1* gene examined by the *t*-test were not significantly different. However, the pressure pain threshold in subjects with minor allele genotype (GA) of the IVS2+31G>A SNP of the *OPRM1* gene examined by the *t*-test was significantly higher than that in subjects with major allele (GG) genotype ($p = 0.036$, Table 5).

Since we had determined a Cohen's *d* of 0.2–0.5 as 'small', 0.5–0.8 as 'medium', and >0.8 as 'large', our data revealed that the effect sizes of the 118A>G and the −172G>T SNP of the *OPRM1* gene, of the 36G>T SNP of the *OPRK1* gene and of the 921T>C SNP of the *OPRD1* gene were all small (Table 5). The effect size of

Table 3 Demographic data by single nucleotide polymorphism (SNP) genotypes of the human μ-, κ-, and δ-opioid receptor (*OPRM1*, *OPRK1*, and *OPRD1*) genes. Subjects with the major homozygous genotype were classified as the 'major allele' genotype group. Subjects with the heterozygous and the minor homozygous genotypes were combined to form the 'minor allele' genotype group. Data are mean (SD).

		Age (years)		Height (cm)		Weight (kg)		BAI scores	
Gene	**SNP**	**Major allele**	**Minor allele**	**Major allele**	**Minor allele**	**Major allele**	**Minor allele**	**Major allele**	**Minor allele**
OPRM1	118A>G	37.5 (6.2)	38.6 (6.7)	158.9 (4.8)	158.1 (4.2)	53.6 (7.0)	51.9 (4.6)	4.3 (5.6)	5.1 (4.5)
	−172G>T	37.4 (6.6)	39.3 (5.5)	159.3 (4.4)	156.3 (4.6)	53.3 (6.0)	51.9 (7.4)	5.1 (5.3)	3.1 (4.5)
	IVS2+31G>A	38.0 (6.3)	35.8 (7.2)	158.6 (4.5)	159.8 (6.6)	52.6 (5.7)	60.5 (11.5)	4.7 (5.3)	3.0 (3.6)
OPRK1	36G>T	38.3 (6.4)	36.8 (6.2)	158.6 (4.1)	158.7 (5.7)	52.9 (5.4)	53.4 (8.2)	4.0 (4.6)	6.2 (6.1)
OPRD1	921T>C	38.2 (6.7)	37.7 (6.0)	158.5 (5.3)	158.8 (3.4)	51.8 (4.4)	55.1 (5.4)	4.5 (5.1)	4.9 (5.4)

BAI, Beck anxiety inventory score.

Table 4 Haemodynamic data by single nucleotide polymorphism (SNP) genotypes of the human μ-, κ-, and δ-opioid receptor (*OPRM1*, *OPRK1*, and *OPRD1*) genes. Subjects with the major homozygous genotype were classified as the 'major allele' genotype group. Subjects with the heterozygous and the minor homozygous genotypes were combined to form the 'minor allele' genotype group. Data are mean (SD).

		HR (beats.min^{-1})		SBP (mmHg)		DBP (mmHg)		MBP (mmHg)	
Gene	SNP	Major allele	Minor allele	Major allele	Minor allele	Major allele	Minor allele	Major allele	Minor allele
OPRM1	118A>G	76.9 (10.1)	77.8 (9.7)	114.3 (14.0)	115.2 (13.0)	65.6 (12.7)	67.4 (12.7)	81.8 (11.8)	83.3 (12.2)
	−172G>T	77.3 (9.7)	76.8 (10.9)	114.4 (14.0)	115.5 (12.3)	66.5 (12.3)	65.3 (13.9)	82.4 (11.9)	82.0 (12.2)
	IVS2+31G>A	77.0 (10.1)	79.8 (6.0)	114.3 (13.8)	119.5 (3.8)	66.6 (12.2)	59.8 (19.1)	82.5 (11.9)	79.7 (12.8)
OPRK1	36G>T	76.7 (10.2)	78.3 (9.1)	114.7 (14.7)	114.5 (10.4)	65.4 (13.4)	68.2 (10.6)	81.8 (12.7)	83.7 (9.8)
OPRD1	921T>C	75.9 (9.3)	79.0 (8.0)	116.2 (14.5)	112.4 (11.9)	67.9 (13.8)	63.7 (10.3)	83.8 (13.0)	79.9 (10.6)

HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure.

Table 5 Pressure pain threshold and tolerance data by various single nucleotide polymorphism (SNP) genotypes of the human μ-, κ-, or δ-opioid receptor (*OPRM1*, *OPRK1*, and *OPRD1*) genes. Subjects with the major homozygous genotype were classified as the 'major allele' genotype group. Subjects with the heterozygous and the minor homozygous genotypes were combined to form the 'minor allele' genotype group. Data are mean (SD).

		Pain threshold (kPa)				Pain tolerance (kPa)			
Gene	SNP	Major allele	Minor allele	Effect size (Cohen's *d*)	p value	Major allele	Minor allele	Effect size (Cohen's *d*)	p value
OPRM1	118A>G	267 (69)	271 (61)	0.06	0.813	455 (124)	501 (183)	0.29	0.347
	−172G>T	265 (62)	283 (79)	0.26	0.339	472 (159)	466 (102)	−0.05	0.887
	IVS2+31G>A	265 (65)	334 (49)*	1.20	0.036*	468 (150)	515 (92)	0.38	0.308
OPRK1	36G>T	262 (64)	285 (69)	0.35	0.180	464 (152)	488 (138)	0.23	0.534
OPRD1	921T>C	264 (70)	275 (61)	0.16	0.486	460 (162)	487 (125)	0.19	0.441

*$p < 0.05$ for comparison between the minor allele genotype group vs the major allele genotype group.

the IVS2+31G>A SNP of the *OPRM1* gene was small for pressure pain tolerance, but was large for pressure pain threshold (Cohen's $d = 1.20$, Table 5).

Discussion

Our main (null) hypothesis was that 118A>G SNP of the *OPRM1* gene is not associated with pressure pain sensitivity in females. Our data were consistent with this null hypothesis with respect to this SNP. Furthermore, extending our investigation to other common SNPs of other opioid receptor genes (including the *OPRM1*, *OPRK1*, and *OPRD1*), we discovered that the IVS2+31G>A SNP of the *OPRM1* gene was associated with pressure pain sensitivity. This confirmed the speculation that SNPs other than 118A>G may be involved in pressure pain sensitivity in females.

The clinical relevance of this novel finding is unclear, but we can speculate on some possibilities based on previous work. Although the existence of the IVS2+31G>A SNP in the *OPRM1* is well-established [15], very few data are available to indicate its clinical relevance. Some reports have suggested that this SNP may be associated with narcotic addiction [16]. Heroin-addicted individuals with heterozygous (GA) genotype of the IVS2+31G>A SNP tend to have higher heroin daily intakes than individuals with major homozygous (GG) genotype [16], suggesting that this SNP may participate in the regulation of the function of the μ-opioid receptor. How this may be directly related to pressure pain sensitivity is unclear. It is well-established that, in general, the function of the μ-opioid receptor is under the influence of the polymorphisms of the *OPRM1* gene [3]. For instance, cell cultures with the 'variant' μ-opioid receptor (i.e. heterozygous GA genotype of the 118A>G SNP of the *OPRM1* gene) had three times greater binding affinity for β-endorphin than cell cultures with the 'common' μ-opioid receptor (i.e. with the major homozygous GG genotype of this SNP) [3]. So one possibility is that the IVS2+31G>A SNP influences the binding affinity between endogenous (and/or exogenous) opioid agents and the μ-opioid receptor.

The established influence of gender difference on the association between the 118A>G SNP of the OPRM1 gene and pain sensitivity [5] motivated our current study. A gender difference in opioid analgesic efficacy has also

been reported [17, 18]. For instance, morphine has been shown to be more potent in females than in males [18]. Sex hormones have been suggested to contribute to these gender differences in opioid analgesic efficacy [19, 20]. For instance, gonadectomy-induced increases in morphine analgesic efficacy were significantly higher in female rats than in male rats [19]. In addition, progesterone has been reported to increase the density of the opioid-receptors in brain in ovariectomized female rats [20]. In addition to analgesic efficacy, previous data also illustrated a gender difference in opioid-induced side effects, such as nausea, vomiting and respiratory depression [21, 22]. This raises the possibility that sex hormones might also contribute to the opioid-induced side effects. Thus, gender differences in pain sensitivity, opiate analgesic efficacy, and opiate side effects together suggest that there may be common (genetic) mechanisms underlying these effects. Our novel finding of the association between the IVS2+31G>A SNP of the *OPRM1* gene and pressure pain threshold in females raises the further possibility that this SNP in some way underlies mechanisms that explain the gender differences reported in analgesic sensitivity and/or opiate side-effects. However, our study only examined females: a similar association of the SNP with pain sensitivity in males would invalidate this hypothesis (but might in turn establish the IVS2+31G>A SNP as an important SNP in pain-related gene study).

However, the importance of the IVS2+31G>A SNP is somewhat limited by its relatively infrequent occurrence (just 5.6% of the group in our study). Since the recorded inter-individual variation in pressure pain sensitivity is greater than would be expected by ~5% of the female population conferring a different response, there are likely factors other than polymorphisms of the opioid receptor genes that explain the variability. One possibility, raised by previous investigation, is that the sympathetic nervous system is involved [23]. Another possibility is that other genes, perhaps unrelated to opioid receptor genes, may be involved.

Our data confirmed that genotype distribution of the IVS2+31G>A SNP of the *OPRM1* gene and 921T>C SNP of the *OPRD1* gene were in accordance with Hardy–Weinberg equilibrium, suggesting our novel finding with respect to the IVS2+31G>A SNP is probably robust. However, genotype distributions of the other five SNPs included in this study showed some deviation from Hardy–Weinberg equilibrium. One possible explanation is that our study included only a small group of female Han Chinese volunteers instead of an infinitively large population with random mating, as is assumed by the Hardy–Weinberg equilibrium [24]. Moreover, it is likely that migration might have caused some deviation from equilibrium. Finally, our data revealed that none of our subjects possessed polymorphism in the 17- and the IVS2+691 loci of the *OPRM1* gene, which is otherwise to be expected in non-Chinese populations. Shi et al. [16] also failed to find polymorphism in the 17-locus of the *OPRM1* gene in Han Chinese subjects. Thus the discrepancy between our data for allele frequencies and those previously reported [4, 6] may be due to ethnic population differences, and also a possible explanation for deviations in Hardy–Weinberg equilibrium that we observed.

As only a relatively small proportion of our subjects had heterozygous genotype or minor homozygous genotype, we chose to combine subjects with the heterozygous and the minor homozygous genotypes to form the minor allele genotype group to facilitate analysis. To explore the possibility that our results may have differed had we classified these into three groups (i.e. the major homozygous, the heterozygous, and the minor homozygous genotype groups), we subjected our data to analysis of variance (ANOVA), and this did not revealed statistically significant differences in pressure pain sensitivity (threshold and tolerance) among three genotypes of the 118A>G and the −172G>T SNP of the *OPRM1* gene (data not shown). The genotype differences in pressure pain sensitivity examined by the ANOVA were found neither in 36G>T SNP of the *OPRK1* gene nor in the 921T>C SNP of the *OPRD1* gene (data not shown).

It is important to appreciate several limitations in this study. First, it may not be appropriate to extrapolate our pressure pain model to other types of pain, especially post-operative pain. While our current findings may seem to have limited immediate clinical application, we have previously shown that pre-operative pressure pain sensitivity, especially pain tolerance, predicts post-operative pain and analgesic requirements in female patients [1]. Second, our result with the IVS2+31G>A SNP may represent a false positive (type 1) statistical error as we tested a large numbers of hypotheses simultaneously in this study. However, this was mitigated to some extent by our choice of Cohen's *d* for effect size, as a somewhat conservative statistical test (akin to adjusting the p value regarded as significant). Third, because only female subjects of Han Chinese race were studied, findings from this study may not apply to subjects of other races. Fourth, the sample size is relatively small for a genetic association study. Although the required sample size was calculated based on previous data of the 118A>G SNP of the *OPRM1*, the sample may have been underpowered with respect to other SNPs investigated. Specific statistical tools are available for use in gene association studies (e.g. genetic power calculator) [25], which take into account

the minor allele frequency of the SNPs to be investigated. However, these calculators are designed to investigate association of an SNP to disease, for which it is necessary to have knowledge of disease prevalence. This was not the case in our study, which examined association with pain sensitivity. Finally, it is likely that variations in other candidate genes (e.g. multidrug resistance transporters gene, catechol-o-methyltransferase gene, etc.) that regulate opioid efficacy and/or pain perceptions [26] may influence the study subjects' sensitivity to pressure pain, and we did not examine these.

In summary, our data may be considered a preliminary report of an association between the IVS2+31G>A SNP of the *OPRM1* gene and pressure pain threshold in healthy adult females. Future studies should properly focus on larger sample size, different populations and pain models, and to construct more detailed genetic models (e.g. haplotypes) [27] of the SNPs of the *OPRM1*, *OPRD1*, and *OPRK1* genes to elucidate their association with human pain sensitivity.

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