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The Opposing Roles of IVS2+691 CC Genotype and AC/AG Diplotype of 118A>G and IVS2+691G>C of *OPRM1* Polymorphisms in Cold Pain Tolerance Among Opioid-Dependent Malay Males on Methadone Therapy

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ABSTRACT

Introduction: We recently reported that a majority of opioid-dependent Malay males on methadone therapy are cold pain sensitive. It is postulated that common *OPRM1* polymorphisms may be responsible. This study investigated the association between 118A>G (dbSNP rs1799971) and IVS2+691G>C (dbSNP rs2075572) variants on cold pain responses among opioid-dependent Malay males on methadone maintenance therapy.

Methods: Cold pain responses including pain threshold, pain tolerance, and pain intensity were measured using the cold pressor test. DNA was extracted from the venous blood before polymerase chain reaction genotyping.

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Repeated measures analysis of variance was used to compare the cold pain responses and *OPRM1* polymorphisms (118A>G and IVS2+691G>C) using models including genotype dominant and recessive models, allelic additive models, and analysis of haplotypes and diplotypes.

Results: A total of 148 participants were recruited. With the recessive model, those with IVS2+691 homozygous CC genotype had a shorter cold pain tolerance time than those without CC genotype (i.e., GG/GC genotype; 29.81 vs. 43.08 s, respectively, $P = 0.048$). On the other hand, with diplotype analysis, participants with combined homozygous 118 AA genotype and heterozygous IVS2+691 GC genotype (i.e., AC/AG diplotype) had a longer cold pain tolerance time than those without this diplotype (49.34 vs. 31.48 s, respectively, $P = 0.043$). Cold pain threshold was not associated with any of the 118A>G and IVS2+691G>C variations despite being analyzed using various models (all $P > 0.05$).

Conclusion: The IVS2+691 CC genotype and AC/AG diplotype of 118A>G and IVS2+691G>C seem to have opposing roles in pain tolerance among opioid-dependent Malay males on methadone therapy. Haplotypes of *OPRM1* may be associated with altered binding affinity.

Keywords: Cold pain; Malays; Methadone maintenance; Opioid dependence; Pain tolerance; Polymorphism

INTRODUCTION

The opioidergic neurotransmission system is an important processor of painful stimuli [1–4]. The μ-opioid receptor (OPRM1) is a primary target for clinically important opioid analgesics, including methadone. Studies among healthy subjects showed a possible role of the opioid receptor mu 1 gene (*OPRM1*) in altered pain sensitivity [5, 6]. Genetic factors that affect the density, function, and, consequently, the signaling efficacy of μ-opioid receptors may contribute to inter-individual variations in the pain response to opioids [7–9].

Southeast Asia is a highly populated and cultural-diverse region with ethnic Malays constituting the largest population group, mainly populating countries including Malaysia, Indonesia, and the southern part of the Philippines. Males who are ethnic Malays make up the majority of opioid-dependent patients on methadone treatment in Malaysia [10]. We have previously reported that a majority of opioid-dependent Malay males on methadone therapy are cold pain sensitive and that the pain is frequently associated with poor sleep quality [11]. One postulated mechanism for altered pain sensitivity in this susceptible population is polymorphisms in the OPRM1 gene.

Some of the most frequently studied polymorphisms of *OPRM1* include 118A>G (dbSNP rs1799971), IVS2+691G>C (dbSNP rs2075572), and IVS2+31G>A (dbSNP rs9479757). The association between 118A>G polymorphism and pressure pain was first reported by Fillingim et al. [6] and Lotsch et al. [5], but subsequent studies failed to replicate these preliminary reports in different experimental pain models and in different populations [12–14]. Similarly, the roles of other polymorphisms on pain sensitivity, including IVS2+691G>C and IVS2+31G>A, have been mixed. For example, the IVS2+691G>C polymorphism did not increase morphine requirements in patients with pain caused by malignant disease [7] and did not have significant association with 24-h post-operative opioid requirement [15]. To summarize, available studies have raised a

great deal of speculation over the role of *OPRM1* polymorphisms in pain sensitivity and one postulation is that this phenomenon may be gender and ethnic dependent [16].

Recent studies indicate *OPRM1* polymorphisms are associated with side effects including changes in libido and insomnia [17], and also methadone-related deaths [18, 19]. On the other hand, the role of *OPRM1* polymorphisms on pain sensitivity among opioid-dependent patients on methadone is unclear because of limited evidence. Thus far, only one study has explored this [20] but unfortunately the low frequencies of variants in the study samples were a limitation. A better understanding of *OPRM1* polymorphisms in pain and opioid response of a specific population has implications for the treatment of pain and addictive disease, and clinical management of each in the presence of the other. In the present study, we aimed to investigate the influence of common *OPRM1* polymorphisms on pain responses among opioid-dependent Malay males on methadone maintenance therapy (MMT).

METHODS

Participants

Characteristics of the sampling population have been previously described [11]. Briefly, study participants were opioid-dependent males undergoing methadone treatment at Hospital Universiti Sains Malaysia (USM) and other MMT clinics (Kota Bharu, Pasir Mas, Pasir Puteh, and Bachok) in the state of Kelantan, Malaysia. Opioid dependency was defined according to the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) criteria [21]. They were sequentially recruited after informed consent. Study participants were included if

they were: (a) in the national MMT program with a duration of participation of more than 1 month; (b) more than 18 years of age; (c) free of regular use of alcohol; (d) free of intoxication; (e) able to understand study protocols and to follow simple study instructions; and (f) willing to sign written informed consent. Exclusion criteria included the following: (a) presence of acute medical, surgical, or psychiatric illnesses; (b) current intake of benzodiazepines, cannabinoids, and barbiturates; (c) on regular anticonvulsants, neuroleptics, or analgesics; (d) history of chronic or ongoing acute pain; (e) history of analgesics ingestion within 3 days before the cold pressor test (CPT); and (f) presence of severe cognitive impairment which might interfere with pain assessments and/or communication. Psychiatric illnesses were carefully evaluated during interview of participants, and the information verified with their medical records and also from their treating doctors. Participants also had to have two consecutive negative urine tests for morphine, tetrahydrocannabinol, amphetamines, and benzodiazepines before study inclusion.

This study was approved by the Human Research Ethics Committee (HREC), Universiti Sains Malaysia (USM) in Kelantan, Malaysia (Reference number: USMKK/PPP/JEPeM (253.3 (14))) and the Medical Research and Ethics Committee (MREC) at the Ministry of Health (MOH), Malaysia (Reference number: NMRR-13-524-16614).

Assessment of Cold Pain Threshold, Pain Tolerance, and Pain Intensity Using the Cold Pressor Test

The CPT method utilized in the current study has been previously described [11]. Briefly, a

48-quart cool box was filled with a mixture of two-thirds crushed ice and one-third tap water. A constant temperature of 0–2 °C was maintained by adding ice intermittently. The non-dominant hand and forearm of participant would be placed in the ice bath with their palm flat at the bottom of the box, with ice water covering the hand and approximately 10 cm of the forearm. The test was truncated at 300 s because the numbness associated with cold would reduce the pain.

Pain threshold was defined as the first experience of pain that can be identified, pain tolerance as the time elapsed when the participant had to withdraw his hand (i.e., the most severe pain that a subject was willing to tolerate), and pain intensity as the maximal pain experienced during test on a visual analog scale (VAS; 0–100). We examined the cold pressor responses six times over a 24-h period [i.e., at 0 h (at about 8.00 am), and at 2, 4, 8, 12, and 24 h after the first test] to reduce the diurnal variation in the cold pressor pain response. The cold pressor pain measurements at these six different time points were used for subsequent data analysis.

PCR Genotyping for *OPRM1* Polymorphisms

Venous blood samples (2.5 mL) were collected in tubes containing sodium citrate and were stored at −20 °C until further processing. Genomic DNA was extracted from the unclotted blood using QIAamp® DNA blood mini kit (Qiagen Gmbh, Hilden, Germany) according to the manufacturer's instructions. The quantity and quality of the extracted genomic DNA was determined on the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA) with measurements performed at 260 and 280 nm. A two-step polymerase chain reaction (PCR) method for simultaneous *OPRM1* and *CYP2B6* genotyping was developed and validated for reproducibility and specificity through direct sequencing [22]. All PCRs were performed in standard 0.2-mL Eppendorf PCR tubes and carried out in a volume of 25 μL comprising buffer [10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 0.1% Triton X-100, 50.0% glycerol (v/v)]. The reactions were performed on the Applied Biosystems® Veriti® 96-well thermal cycler (Applied Biosystems, Carlsbad, CA, USA).

Briefly, the first-step PCR ('Set A') was performed using specifically designed primers (see Table S1 in the supplementary material) to isolate regions of interest that contain the relevant *OPRM1* polymorphisms (118A>G and IVS2+691G>C). These were later used for the second allele-specific PCR to avoid amplifications of similar sequences in the human genome that may be located outside the gene. PCR mixture for Set A contained 1.0 U of Biotool® DNA *Taq* polymerase (Biotools Biotechnological and Medical Laboratories, SA, Madrid, Spain), 2.0 mM MgCl$_2$, 0.2 mM dNTPs (Biotools Biotechnological and Medical Laboratories SA), and 0.10–0.25 μM of the primers (Invitrogen, Waltham, MA, USA). The cycling conditions were optimized for Set A. Ten microlitres of the first PCR products of Set A was analyzed using 2.0% agarose gel (Promega Corporation, Madison, WI, USA) and 1 × TBE (Tris, Borate, EDTA) at 100 V for 60 min. Two microlitres of the diluted first-step PCR products of Set A was used as template for detection of wild-type or mutant-type alleles in the next-step PCR. Two secondary PCRs (Set 1 and 2) were then carried out using identical reaction mixtures described for the first-step PCR, with the exceptions of primer concentrations shown in Table S1 in the supplementary material. The

cycling conditions were again optimized and 10 μl of the second PCR products were again analyzed using 2.0% agarose gel and 1 × TBE at 100 V for 60 min.

Statistical Analysis

Genotyping data were analyzed using the population genetic data analytical program, Golden Helix SNP and Variation Suite 7 (SVS 7, version 7.3.1; Golden Helix Inc., Bozeman, MT, USA) based on an expectation–maximization (EM) algorithm for the following procedures: (a) the calculation of *OPRM1* alleles and genotype frequencies; (b) the estimation of heterozygosity in each polymorphism in Hardy–Weinberg proportion; and (c) the estimation of maximum-likelihood haplotype frequency. Repeated measures analysis of variance (RM-ANOVA) was used to compare mean differences of cold pain responses between *OPRM1* polymorphisms (118A>G and IVS2+691G>C) according to their genotypes and allelic additive models, genotype dominant and recessive models, haplotypes and also diplotypes (frequency less than 10.0% were pooled). Statistical analysis was performed using SPSS/Win software (version 22; SPSS, Inc., Chicago, IL, USA). There was no correction for multiple testing since only one gene was tested [23]. All confidence intervals (CIs) were computed at the 95% level. A *P* value <0.05 was considered significant.

RESULTS

Characteristics of Study Participants

From March to October 2013, 169 patients were screened: 148 completed the study and 21 failed the screening. Twelve patients were excluded for health reasons, 3 for scheduling conflicts,

and 6 for miscellaneous reasons. The mean age of the study participants was 36.86 [standard deviation (SD) 6.13, range 25–55] years. The majority of the participants used more than one illicit drug in their lifetime with marijuana and amphetamines being the most widely used. Ten (6.8%) participants reported morphine and related substances as their past illicit drug use. The mean duration in the MMT program was 2.82 (SD 2.02, range 0.33–9.00) years. The mean daily methadone dose was 72.70 (SD 28.25, range 20–160) mg/day. There was no correlation between daily methadone dose and duration in the MMT program ($r = 0.06$, $P = 0.485$).

Genotyping Frequencies of *OPRM1* Polymorphisms

The 118A/G and IVS2+691G/C alleles of the OPRM1 gene were successfully amplified from 148 subjects. Their genotype and allele frequencies are shown in Table S2 in the supplementary material. One participant carried the homozygous wild-type (IVS2+691 GG) genotype of the IVS2+691G>C polymorphism. The distributions of genotypes for the above polymorphisms followed the Hardy–Weinberg equilibrium ($P > 0.078$). Assuming a mutant allele was a high-risk allele, genotype frequencies under dominant and recessive model were determined for these polymorphisms (see Table S2 in the supplementary material). The most likely haplotype pair (i.e., diplotype) for each genotype in each individual was estimated and the haplotype frequency distributions were obtained with an expectation-maximum (EM) algorithm haplotypes. Their estimated haplotype and diplotype frequencies are as shown in Table S3 in the supplementary material. On the basis of the genotyping data

of subjects included in the study, the standardized linkage disequilibrium value (Levontin's D') and correlation coefficient (r^2) between 118A>G and IVS2+691G>C polymorphisms were 0.473 and 0.031, respectively [x^2 (df) = 9.176 (1), $P = 0.0026$].

Association Between Cold Pain Parameters and Polymorphisms of *OPRM1*

Cold pain threshold was not associated with any of the 118A>G and IVS2+691G>C variations despite being analyzed using various models including dominant and recessive models, allelic additive models, as well as haplotype and diplotypes analyses (all $P > 0.05$; Table 1). Likewise, none of 118A>G and IVS2+691G>C variations were associated with cold pain intensity despite various models (all $P > 0.05$; Table 2). Table 3 shows the relationship between variants of 118A>G and IVS2+691G>C with cold pain tolerance time. With the recessive model, those with IVS2+691 homozygous CC genotype had a shorter cold pain tolerance time than those without CC genotype (GG/GC genotype; 29.81 s vs. 43.08 s, respectively, $P = 0.048$). Variations in the 118A>G polymorphism were not associated with cold pain tolerance time (all $P > 0.05$; Table 3). However, with diplotype analysis, participants with combined homozygous 118 AA genotype and heterozygous IVS2+691 GC genotype (AC/AG diplotype) had a longer cold pain tolerance time than those without this diplotype (49.34 s vs. 31.48 s, respectively, $P = 0.043$).

DISCUSSION

The *118G* allele frequency of 40.2% in our study is in good agreement with previous smaller scale studies of this polymorphism among Malay

drug addicts in Malaysia [24] and heroin-dependent Malays in Singapore [25], although ours was much higher compared to intravenous drug users on MMT [26]. Likewise, frequency of variant IVS2+691*C* allele of 83.1% in our subjects is similar to those reports from heroin-dependent Malays in Singapore [25]. The reported frequency in our study seems to have contrasted with those of the Chinese (73.1%) and Indian (75%) Singaporeans [25], as well as the Chinese (69.3%) in Hong Kong [27]. The above reports indicate that variability in frequency of *118G* and IVS2+691*C* allele in populations on methadone treatment may be an ethnic-dependent phenomena.

In the current study, there was no association seen between 118A>G variations with cold pain threshold and pain tolerance. Instead, IVS2+691G>C polymorphisms using different models were shown to affect cold pain tolerance, but not pain threshold and pain intensity. These include homozygous CC genotype (a significant shorter pain tolerance time in CC vs. GG/GC) and combined homozygous 118 AA genotype and heterozygous IVS2+691 GC genotype (AC/AG diplotype; a significant longer pain tolerance time with this diplotype vs. without this diplotype). We have previously reported that a majority of opioid-dependent Malay males on methadone therapy are cold pain sensitive [11] and *OPRM1* polymorphisms might be responsible for this altered sensitivity based upon our study results. What are the implications of this? Any inter-individual variations in opioid-induced hyperalgesia among methadone users may weaken or strengthen their determination to abstain [28], and the knowledge of *OPRM1* polymorphisms in such a circumstance may help to provide a prediction. In addition, this knowledge on *OPRM1* polymorphisms may help to guide the

Table 1 Relationship between 118A>G and IVS2+691G>C polymorphisms and pain threshold in opioid-dependent patients

Polymorphism	N	Mean[a]	95% CI		F statistic (df)[b]	P value[c]
			Lower limit	Upper limit		
118A>G						
Genotype (N = 146)						
AA	53	24.06	16.43	31.70	0.24 (2)	0.785
AG	69	27.33	20.64	34.02		
GG	24	24.11	12.76	35.46		
Allele (N = 292)						
A	175	25.35	21.19	29.51	0.04 (1)	0.844
G	117	26.01	20.92	31.10		
Dominant model						
AA	53	24.06	16.45	31.68	0.25 (1)	0.615
AG + GG	93	26.50	20.75	32.25		
Recessive model						
AA + AG	122	25.91	20.89	30.93	0.08 (1)	0.774
GG	24	24.11	12.79	35.43		
IVS2+691G>C						
Genotype (N = 146)						
GG	1	20.41	−35.01	75.82	0.68 (2)	0.510
GC	47	29.51	21.43	37.60		
CC	98	23.80	18.20	29.40		
Allele (N = 292)						
G	49	29.14	21.29	36.99	0.94 (1)	0.333
C	243	24.90	21.38	28.43		
Dominant model						
GG	1	20.41	−35.07	75.88	0.03 (1)	0.853
GC + CC	145	25.65	21.04	30.26		
Recessive model						
GG + GC	48	29.32	21.35	37.30	1.26 (1)	0.264
CC	98	23.80	18.22	29.38		
Haplotype (N = 292)[d]						
AC	131	24.04	19.22	28.86	0.40 (3)	0.751
GC	112	25.91	20.70	31.12		

Table 1 continued

Polymorphism	N	Mean[a]	95% CI		F statistic (df)[b]	P value[c]
			Lower limit	Upper limit		
AG	44	29.26	20.94	37.57		
GG	5	28.12	3.46	52.78		
AC	131	24.04	19.23	28.85	0.60 (2)	0.547
GC	112	25.91	20.71	31.11		
Combined AG and GG	49	29.14	21.28	37.00		
AC	131	24.04	19.24	28.84	0.75 (1)	0.386
Not AC	161	26.90	22.56	31.23		
GC	112	25.91	20.71	31.12	0.02 (1)	0.886
Not GC	180	25.43	21.32	29.53		
AG	44	29.26	20.97	37.54	0.88 (1)	0.349
Not AG	248	24.97	21.48	28.46		
Diplotype $(N = 146)$						
AC/GC	47	24.88	16.73	33.03	0.41 (5)	0.839
AC/AC	31	22.72	12.68	32.75		
AC/AG	22	25.96	14.05	37.87		
GC/AG	21	33.13	20.94	45.32		
GC/GC	20	22.92	10.43	35.42		
Others[e]	5	28.12	3.13	53.11		
AC/GC	47	24.88	16.79	32.97	0.05 (1)	0.828
Not AC/GC	99	25.96	20.39	31.54		
AC/AC	31	22.72	12.77	32.67	0.42 (1)	0.518
Not AC/AC	115	26.39	21.23	31.56		
AC/AG	22	25.96	14.13	37.79	0.00 (1)	0.950
Not AC/AG	124	25.55	20.57	30.54		
GC/AG	21	33.13	21.10	45.16	1.78 (1)	0.184
Not GC/AG	125	24.35	19.42	29.28		
GC/GC	20	22.92	10.52	35.32	0.21 (1)	0.645

Table 1 continued

Polymorphism	N	Mean[a]	95% CI		F statistic (df)[b]	P value[c]
			Lower limit	Upper limit		
Not GC/GC	126	26.04	21.10	30.98		

ANOVA analysis of variance, *CI* confidence interval, *N* number of subject/allele/haplotype/diplotype

[a] Mean for pain threshold (seconds)

[b] Repeated measured ANOVA between group analysis was applied

[c] *P* value is significant at <0.05

[d] Haplotype patterns were constructed from the two polymorphisms of *OPRM1* (118A>G and IVS2+691G>C)

[e] Diplotype with frequency less than 10.0% was pooled under 'others' (included AG/GG and GC/GG)

methadone dosages and, therefore, avoid adverse drug reactions.

Studies by Wang et al. [17] indicated that IVS2+691G>C polymorphisms might affect the side effects of methadone including change in libido and insomnia. Our study suggests that variations in IVS2+691G>C might also affect pain tolerance. The actual functions of IVS2+691G>C are still under investigations but available data support its role in affinity of transcriptional regulatory factors for the intronic DNA sequence [29–31]. The DNA intronic sequence could involve in alternative DNA splicing and this results in creations of different isoforms of human OPRM1 gene [30, 32]. It may also directly alter mRNA levels and, therefore, affect expression of OPRM1 gene [29–31]. Based on our results, we hypothesized the increased expression of IVS2+691 CC variant resulted in a higher OPRM1 availability and because of chronic sensitization by prolonged opioid use, it may then facilitate opioid tolerance, hyperalgesia and subsequently lower the pain tolerance. However, tolerance normally indicates a rightward shift in a dose–response curve. Since participants have been treated with methadone anywhere from 0.3 to 9 years, sensitization may, therefore, also be quite variable or absent. It is possible that those participants in the program with a shorter

stay were different than those with longer treatment times.

On the other hand, the AC/AG diplotype of 118 and IVS2+691 was associated with a longer cold pain tolerance time. Opioid-related adverse effects such as hyperalgesia were probably less likely to occur in patients with AC/AG diplotype at 118 and IVS2+691 given that the mechanisms of opioid-induced adverse events involve *OPRM1*. Thus, although 118A>G polymorphism alone does not influence cold pain tolerance, the 118 variant may play an indirect role in pain modulation. A strong linkage disequilibrium (LD) between studied polymorphisms and unstudied polymorphisms has been shown to exist [15, 33], and resulted in altered binding affinity between endogenous (and/or exogenous) opioids and OPRM1. As a consequence, there was less hyperalgesia and, therefore, an increase in cold pain tolerance.

There were associations, although not significant, where variants of *118G* allele and GC haplotype of 118 and IVS2+691 caused a higher cold pain intensity, but 118 AA genotype resulted in a lower cold pain intensity. At this juncture, it is difficult to explain the differential effects on pain intensity with these polymorphisms and further studies with larger samples are needed. Besides individual polymorphisms, haplotype and diplotype

Table 2 Relationship between 118A>G and IVS2+691G>C polymorphisms and pain-intensity scores in opioid-dependent patients

Polymorphism	N	Mean[a]	95% CI		F statistic (df)[b]	P value[c]
			Lower limit	Upper limit		
118A>G						
Genotype (N = 146)						
AA	53	62.23	58.21	66.25	1.94 (2)	0.147
AG	69	66.97	63.45	70.49		
GG	24	67.88	61.91	73.85		
Allele (N = 292)						
A	175	64.10	61.90	66.30	3.37 (1)	0.067
G	117	67.34	64.65	70.04		
Dominant model						
AA	53	62.23	58.23	66.23	3.84 (1)	0.052
AG + GG	93	67.21	64.18	70.23		
Recessive model						
AA + AG	122	64.91	62.24	67.58	0.80 (1)	0.373
GG	24	67.88	61.87	73.89		
IVS2+691G>C						
Genotype (N = 146)						
GG	1	73.33	43.88	102.79	0.92 (2)	0.401
GC	47	63.12	58.82	67.41		
CC	98	66.41	63.44	69.39		
Allele (N = 292)						
G	49	63.53	59.35	67.71	0.93 (1)	0.336
C	243	65.78	63.90	67.65		
Dominant model						
GG	1	73.33	43.83	102.84	0.28 (1)	0.595
GC + CC	145	65.34	62.89	67.80		
Recessive model						
GG + GC	48	63.33	59.09	67.57	1.38 (1)	0.241
CC	98	66.41	63.44	69.38		
Haplotype (N = 292)[d]						
AC	131	64.39	61.84	66.94	1.19 (3)	0.312
GC	112	67.40	64.64	70.16		

Table 2 continued

Polymorphism	N	Mean[a]	95% CI		F statistic (df)[b]	P value[c]
			Lower limit	Upper limit		
AG	44	63.23	58.83	67.64		
GG	5	66.17	53.09	79.24		
AC	131	64.39	61.84	66.94	1.71 (2)	0.183
GC	112	67.40	64.64	70.16		
Combined AG and GG	49	63.53	59.36	67.70		
AC	131	64.39	61.83	66.95	1.10 (1)	0.296
Not AC	161	66.22	63.92	68.53		
GC	112	67.40	64.64	70.15	3.31 (1)	0.070
Not GC	180	64.16	61.98	66.33		
AG	44	63.23	58.82	67.65	1.10 (1)	0.295
Not AG	248	65.78	63.93	67.64		
Diplotype (N = 146)						
AC/GC	47	68.09	63.79	72.38	1.01 (5)	0.417
AC/AC	31	62.47	57.19	67.76		
AC/AG	22	61.89	55.61	68.16		
GC/AG	21	64.17	57.74	70.59		
GC/GC	20	68.58	62.00	75.17		
Others[e]	5	66.17	53.00	79.33		
AC/GC	47	68.09	63.81	72.36	2.28 (1)	0.133
Not AC/GC	99	64.12	61.18	67.07		
AC/AC	31	62.47	57.20	67.75	1.53 (1)	0.219
Not AC/AC	115	66.19	63.45	68.93		
AC/AG	22	61.89	55.62	68.15	1.45 (1)	0.231
Not AC/AG	124	66.02	63.38	68.66		
GC/AG	21	64.17	57.73	70.61	0.17 (1)	0.683
Not GC/AG	125	65.61	62.97	68.25		
GC/GC	20	68.58	62.00	75.16	1.06 (1)	0.305

Table 2 continued

Polymorphism	N	Mean[a]	95% CI		F statistic (*df*)[b]	P value[c]
			Lower limit	Upper limit		
Not GC/GC	126	64.89	62.27	67.52		

ANOVA analysis of variance, *CI* confidence interval, *N* number of subject/allele/haplotype/diplotype

[a] Mean for pain threshold (seconds)

[b] Repeated measured ANOVA between group analysis was applied

[c] *P* value is significant at <0.05

[d] Haplotype patterns were constructed from the two polymorphisms of *OPRM1* (118A>G and IVS2+691G>C)

[e] Diplotype with frequency less than 10.0% was pooled under 'others' (included AG/GG and GC/GG)

analyses are also necessary to clarify the influence of *OPRM1* on inter-individual variations in pain sensitivity among opioid-dependent patients on methadone therapy. There is also the possibility that heightened pain sensitivity is a result of the prolonged opioid use itself or opioid-induced hyperalgesia. The heightened pain sensitivity is a form of latent hyperalgesia to chronic opioid misuse, and subsequent methadone therapy does not exacerbate hyperalgesia but does not make it worse either [34, 35].

Some limitations to this study need to be highlighted. There are a number of *OPRM1* polymorphisms (such as rs1074287, rs6912029, rs12209447, rs510769, rs3798676, rs7748401, rs495491, rs10457090, rs589046, rs3778152, and rs563649) but we only studied two common variants. Only Malay males were included but we aimed to reduce the confounding effects of gender and ethnicity on cold pain response [36–44]. Furthermore, Malay males are the majority of opioid-dependent patients on methadone treatment in Malaysia. There were other confounding factors such as induction of tolerance to methadone effects, methadone clearance and genetic variations of other pain-related genes and genes related to pharmacokinetics and pharmacodynamics of

methadone, which may be important. Only a tonic pain model using cold pain was studied here and phasic pain may differ qualitatively, neurologically, and functionally from tonic pain stimuli [45]. However, CPT has been shown to be the better pain-induction technique for methadone studies [20, 46, 47]. Lastly, our analyses did not correct for multiple testing because of single gene being studied. There was a significant LD between loci and it was 47.3% of the theoretical maximum but there was a weak correlation between single nucleotide polymorphisms [$D' = 0.473$, $r^2 = 0.031$, x^2 (df) = 9.176 (1), $P = 0.0026$]. In the view that correction was not performed for multiple tests, these two findings would be considered nominally significant. It is possible that our result might be a false-positive association partly because of limitation in sample size. Therefore, our study may be considered explorative in nature and it would require replication by other investigators. Nevertheless, our results can be considered as a basis for further larger scale study.

CONCLUSIONS

In summary, our study indicates that the IVS2+691 CC genotype was associated with a shorter cold pain tolerance time but AC/AG

Table 3 Relationship between 118A>G and IVS2+691G>C polymorphisms and pain tolerance in opioid-dependent patients

Polymorphism	N	Mean[a]	95% CI		F statistic (df)[b]	P value[c]
			Lower limit	Upper limit		
118A>G						
Genotype (N = 146)						
AA	53	36.92	26.48	47.36	0.22 (2)	0.806
AG	69	32.40	23.26	41.55		
GG	24	33.19	17.68	48.69		
Allele (N = 292)						
A	175	35.14	29.45	40.82	0.28 (1)	0.597
G	117	32.72	25.77	39.68		
Dominant model						
AA	53	36.92	26.52	47.32	0.43 (1)	0.514
AG + GG	93	32.60	24.75	40.45		
Recessive model						
AA + AG	122	34.36	27.50	41.23	0.02 (1)	0.891
GG	24	33.19	17.71	48.66		
IVS2+691G>C						
Genotype (N = 146)						
GG	1	25.07	−49.93	100.07	2.09 (2)	0.128
GC	47	43.46	32.52	54.40		
CC	98	29.81	22.23	37.39		
Allele (N = 292)						
G	49	42.71	32.02	53.40	2.97 (1)	0.086
C	243	32.45	27.65	37.25		
Dominant model						
GG	1	25.07	−50.73	100.87	0.06 (1)	0.812
GC + CC	145	34.23	27.94	40.53		
Recessive model						
GG + GC	48	43.08	32.28	53.87	3.96 (1)	0.048
CC	98	29.81	22.25	37.36		
Haplotype[d] (N = 292)						
AC	131	32.38	25.82	38.94	1.02 (3)	0.382
GC	112	32.53	25.43	39.63		

Table 3 continued

Polymorphism	N	Mean[a]	95% CI		F statistic (df)[b]	P value[c]
			Lower limit	Upper limit		
AG	44	43.35	32.03	54.68		
GG	5	37.04	3.46	70.63		
AC	131	32.38	25.83	38.93	1.48 (2)	0.229
GC	112	32.53	25.45	39.62		
Combined AG and GG	49	42.71	32.00	53.42		
AC	131	32.38	25.81	38.95	0.52 (1)	0.470
Not AC	161	35.63	29.70	41.55		
GC	112	32.53	25.43	39.64	0.33 (1)	0.563
Not GC	180	35.19	29.59	40.80		
AG	44	43.35	32.07	54.64	3.02 (1)	0.083
Not AG	248	32.54	27.79	37.29		
Diplotype (N = 146)						
AC/GC	47	30.08	19.06	41.10	1.02 (5)	0.409
AC/AC	31	28.10	14.53	41.67		
AC/AG	22	49.34	33.24	65.45		
GC/AG	21	37.95	21.47	54.44		
GC/GC	20	31.82	14.92	48.71		
Others[e]	5	37.04	3.26	70.83		
AC/GC	47	30.08	19.05	41.11	0.79 (1)	0.375
Not AC/GC	99	36.11	28.51	43.71		
AC/AC	31	28.10	14.53	41.68	0.99 (1)	0.321
Not AC/AC	115	35.81	28.76	42.85		
AC/AG	22	49.34	33.41	65.28	4.17 (1)	0.043
Not AC/AG	124	31.48	24.77	38.19		
GC/AG	21	37.95	21.42	54.48	0.24 (1)	0.626
Not GC/AG	125	33.54	26.76	40.31		
GC/GC	20	31.82	14.87	48.77	0.09 (1)	0.768

Table 3 continued

Polymorphism	N	Mean[a]	95% CI		F statistic (df)[b]	P value[c]
			Lower limit	Upper limit		
Not GC/GC	126	34.54	27.79	41.30		

ANOVA analysis of variance, *CI* confidence interval, *N* number of subject/allele/haplotype/diplotype
[a] Mean for pain threshold (seconds)
[b] Repeated measured ANOVA between group analysis was applied
[c] *P* value is significant at <0.05
[d] Haplotype patterns were constructed from the two polymorphisms of *OPRM1* (118A>G and IVS2+691G>C)
[e] Diplotype with frequency less than 10.0% was pooled under 'others' (included AG/GG and GC/GG)

diplotype of 118A>G and IVS2+691G>C was associated with a longer cold pain tolerance time in opioid-dependent Malay males on methadone therapy. It can be postulated that haplotypes of *OPRM1* may be associated with altered binding affinity. The *OPRM1* polymorphisms may serve as an important variable for developing directed interventions and to guide management of pain among opioid-dependent patients on MMT with genetic risk for suffering pain. Personalized medicine based on pharmacogenetics may be able to maximize the benefits of methadone pharmacotherapy and minimize their harms.

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