A PHARMACOGENETICS STUDY ON ACUTE PAIN PERCEPTION AMONG PATIENTS ON METHADONE MAINTENANCE THERAPY (MMT)

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By

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TABLE OF CONTENTS

		Page
Acknowledgements		ii
Table of Contents		v
List of Tables		ix
List of Figures		xiii
List of Plates		xv
List of Abbreviations		xvi
Abstrak		xxi
Abstract		xxiii
CHAPTER 1 INTRODUCTION AND REVIEW OF LITERATURE		
1.1	Methadone Maintenance Therapy (MMT) and Pain Complaint	1
1.2	Hyperalgesia in Opioid-Dependent Patients on Methadone Maintenance Therapy (MMT)	3
1.3	Mu-Type Opioid Receptor Gene (*OPRM1*)	4
1.4	*OPRM1* Polymorphisms	7
1.5	*OPRM1* Polymorphisms and Pain Sensitivity	15
1.6	*OPRM1* Polymorphisms and Inter-Individual Variations in the Response to Opioids	19
1.7	ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 1 gene (*ABCB1*)	21
1.8	*ABCB1* Polymorphisms	23
1.9	*ABCB1* Polymorphisms and Pain Sensitivity	25
1.10	*ABCB1* Polymorphisms and Inter-Individual Variations in the Response to Methadone Maintenance Therapy (MMT)	26
1.11	Study Hypothesis and Objectives	31
1.11.1	Hypothesis	31
1.11.2	General Objective	31
1.11.3	Specific Objectives	31
CHAPTER 2 MATERIALS AND METHODS		
2.1	Introduction	33
2.2	Clinical Methods	34
2.2.1	Sample Size Calculation	34
2.2.2	Study Design	35
2.2.3	Enrolment and Study Population	37
2.2.4	Data Collection and Assessment of Subjects	39
2.2.5	Assessment Tools	45
2.3	Laboratory Methods	48
2.3.1	Polymerase Chain Reaction (PCR) Method for Detection of *OPRM1* Polymorphisms	48
2.3.2	Agarose Gel Electrophoresis	68
2.3.3	Allelic Discrimination of *ABCB1* Polymorphisms by Real-Time PCR Genotyping	70

2.3.4 Enzyme-Linked Immunosorbent Assay (ELISA) for Determination of Serum Methadone Concentration (SMC) 93
2.4 Statistical Methods 107

CHAPTER 3 RESULTS
3.1 Demographic and Clinical Characteristics 110
3.1.1 Demographic Data 110
3.1.2 Family History of Illicit Drug Use among Opioid-Dependent Patients 114
3.1.3 Past Drug History among Opioid-Dependent Patients 115
3.1.4 Methadone Maintenance Therapy (MMT) History 117
3.2 Cold Pressor Test (CPT) Responses 118
3.2.1 CPT Responses among Opioid-Naive Subjects 118
3.2.2 CPT Responses among Opioid-Dependent Patients 120
3.2.3 Comparisons of CPT Responses between Opioid-Naive Subjects and Opioid-Dependent Patients 125
3.3 Isolation of Total Genomic DNA 130
3.3.1 DNA Extraction 130
3.3.2 DNA Concentration and Purity 130
3.3.3 DNA Integrity 130
3.4 PCR Genotyping of *OPRM1* and *CYP2B6* 132
3.4.1 PCR Genotyping of OPRM1 118A>G, IVS2+31G>A, and IVS2+691G>C and CYP2B6 64C>T (**2*) and 15631G>T (**9*) 132
3.4.2 Validation of PCR Reactions 137
3.5 Allelic Discrimination Real-Time PCR Genotyping of *ABCB1* 140
3.5.1 Allelic Discrimination Real-Time PCR Genotyping of ABCB1 1236C>T (rs1128503) and 3435C>T (rs1045642) 140
3.5.2 Allelic Discrimination Real-Time PCR Genotyping of ABCB1 2677G>T/A (rs2032582) 143
3.5.3 Validation of Allelic Discrimination Real-Time PCR Reactions 148
3.6 *OPRM1* Genotype, Allele, Haplotype, and Diplotype Frequencies 149
3.6.1 *OPRM1* Polymorphisms among Opioid-Naive Subjects 149
3.6.2 *OPRM1* Polymorphisms among Opioid-Dependent Patients 154
3.7 *ABCB1* Genotype, Allele, Haplotype, and Diplotype Frequencies 158
3.7.1 *ABCB1* Polymorphisms among Opioid-Naive Subjects 159
3.7.2 *ABCB1* Polymorphisms among Opioid-Dependent Patients 164
3.8 Genetic Polymorphisms and Cold Pressor Test (CPT) Responses 168
3.8.1 *OPRM1* and CPT Responses among Opioid-Naive Subjects 168
3.8.2 *OPRM1* and CPT Responses among Opioid-Dependent Patients 178
3.8.3 *ABCB1* and CPT Responses among Opioid-Naive Subjects 190
3.8.4 *ABCB1* and CPT Responses among Opioid-Dependent Patients 202
3.9 Determination of Serum Methadone Concentration (SMC) Using the Enzyme-Linked Immunosorbent Assay (ELISA) 216
3.9.1 Blood Samples Collection among Opioid-Dependent Patients 216
3.9.2 Serum Methadone Concentration (SMC) in Opioid-Dependent Patients 216
3.10 *ABCB1* and Serum Methadone Concentration (SMC) among Opioid-

Dependent Patients 219
3.11 Serum Methadone Concentration (SMC) and Cold Pressor Test (CPT) Responses among Opioid-Dependent Patients 223

CHAPTER 4 DISCUSSION
4.1 Comparison of Pain Sensitivity between Opioid-Dependent Patients on Methadone Maintenance Therapy (MMT) against Opioid-Naive Individuals 226
4.2 *OPRM1* and Pain Sensitivity among Opioid-Naive Individuals 236
4.3 *OPRM1* and Pain Sensitivity among Opioid-Dependent Patients on Methadone Maintenance Therapy (MMT) 239
4.4 *ABCB1* and Pain Sensitivity among Opioid-Naive Individuals 246
4.5 *ABCB1* and Pain Sensitivity among Opioid-Dependent Patients on Methadone Maintenance Therapy (MMT) 255
4.6 *ABCB1* and Serum Methadone Concentration (SMC) among Opioid-Dependent Patients on Methadone Maintenance Therapy (MMT) 263
4.7 Serum Methadone Concentration (SMC) and and Pain Sensitivity among Opioid-Dependent Patients on Methadone Maintenance Therapy (MMT) 266
4.8 Implication of the Study in Clinical Practice 269
4.9 Limitations of the Study and Recommendation for Future Research 273
4.9.1 Selection of Subjects 273
4.9.2 Selection of Experimental Pain Model 274
4.9.3 Other Limitations 275

CHAPTER 5 CONCLUSIONS 279

REFERENCES 282

APPENDICES

Appendix A
Letter of Ethical of Approval from the Human Research Ethics Committee USM (HREC), Universiti Sains Malaysia in Kelantan, Malaysia and the Medical Research & Ethics Committee (MREC), Ministry of Health (MOH), Malaysia
Appendix B
Informed Consent Form
Appendix C
Instruments for DNA Extraction and PCR Genotyping
Reagents and Chemicals Used in DNA Extraction
Appendix D
The Protocols for Preparation of Solutions for DNA Extraction
The Protocols for Preparation of Solutions and Materials for PCR Genotyping
Appendix E
The Protocols for DNA Extraction and Purification

Appendix F
The Protocol for Determination of DNA Yield, Concentration, and Purity
Appendix G
The Protocol for Reconstitution of Primers
Appendix H
PCR Conditions for Allele-Specific Multiplex PCR of OPRM1 118A>G, IVS2+31G>A, and IVS2+691G>C and CYP2B6 64C>T (*2) and 15631G>T (*9)
Appendix I
Preparation of Agarose Gel and Running of the Gel
Appendix J
Instruments Used in Real-Time PCR Genotyping
Reagents and Chemicals Used in Real-Time PCR Genotyping
Appendix K
Allelic Discrimination Real-Time PCR of *ABCB1* Polymorphisms
Appendix L
Methadone Maintenance Therapy (MMT)-Related Information among Opioid-Dependent Patients
Appendix M
Comparison of Distribution of *OPRM1* Genotype, Allele, Haplotype, and Diplotype between Opioid-Naive Subjects and Opioid-Dependent Patients
Appendix N
Comparison of Distribution of *ABCB1* Genotype, Allele, Haplotype, and Diplotype between Opioid-Naive Subjects and Opioid-Dependent Patients
Appendix O
Figure S2: Profile Plot of Mean (SE) Serum Methadone Concentration (SMC) at 0, 0.5, 1, 2, 4, 8, 12, and 24 Hours in the Opioid-Dependent Patients
Appendix P
Figure S3: Profile Plot of Mean (SE) Dose-Adjusted Serum Methadone Concentration (SMC) (ng ml^{-1} mg^{-1}) of Opioid-Dependent Patients in Carriers and Non-carriers of CGC/TTT Diplotypes
Appendix Q
Figure S4: Profile Plot of Mean (SE) Pain Threshold in the Opioid-Dependent Patients with Serum Methadone Concentration (SMC) at 24 hours of < 400 ng/ml and ≥ 400 ng/ml

LIST OF PUBLICATIONS

LIST OF PRESENTATIONS

LIST OF AWARDS

LIST OF TABLES

		Page
Table 1.1	Characteristics and Positions of *OPRM1* Polymorphisms	11
Table 1.2	*OPRM1* Alleles Frequencies in Patients with Pain	13
Table 1.3	Association Studies of *OPRM1* Polymorphisms with Pain Sensitivity	18
Table 1.4	Characteristics and Positions of *ABCB1* Polymorphisms	24
Table 2.1 (a)	Participating Clinics in this Study	39
Table 2.1 (b)	Schedule of Study Procedures for Data Collection and Assessments of Opioid-Naive Subjects and Opioid-Dependent Patients	40
Table 2.2	*OPRM1* and *CYP2B6* Primers Used for Allele-Specific Multiplex PCR of OPRM1 118A>G, IVS2+31G>A, and IVS2+691G>C and CYP2B6 64C>T (**2*) and 15631G>T (**9*)	56
Table 2.3 (a)	Summary of the First PCR Method for *OPRM1* and *CYP2B6* Genotyping (Set A)	62
Table 2.3 (b)	Summary of Method for the Second PCR for *OPRM1* and *CYP2B6* Genotyping (Set 1)	65
Table 2.3 (c)	Summary of Method for the Second PCR for *OPRM1* and *CYP2B6* Genotyping (Set 2)	66
Table 2.3 (d)	Summary of Method for the Second PCR for *OPRM1* Genotyping (Set 3)	67
Table 2.4	Interpretation of PCR Amplification of Second PCR	69
Table 2.5	TaqMan® Drug Metabolism Genotyping Assays Used for Real-Time PCR Genotyping	76
Table 2.6	Summary of the Real-Time PCR Amplification Using C_7586662_10, C_11711720D_40, C_11711720C_30, and C_7586657_20	85

Table 2.7	Genotypes for Samples Run with C_7586662_10 and C_7586657_20 for the SNP rs1128503 and rs1045642, respectively	90
Table 2.8	Genotypes for Samples Run with Two Assays (C_11711720D_40 and C_11711720C_30) for the Tri-Allelic SNP rs2032582	92
Table 2.9	Instruments, Reagents, and Chemicals for Methadone ELISA Kit	97
Table 3.1 (a)	Demographic Data of Opioid-Naive Subjects	111
Table 3.1 (b)	Demographic Data of Opioid-Dependent Patients	113
Table 3.2	Family History of Illicit Drug Use among Opioid-Dependent Patients	114
Table 3.3	Past Drug History among Opioid-Dependent Patients	116
Table 3.4	Methadone Maintenance Therapy (MMT) History among Opioid-Dependent Patients	117
Table 3.5 (a)	Pain Threshold, Pain Tolerance, and Pain Intensity Scores among Opioid-Naive Subjects	119
Table 3.5 (b)	Pain Threshold, Pain Tolerance, and Pain Intensity Scores among Opioid-Dependent Patients	122
Table 3.5 (c)	Comparisons of Pain Thresholds among Opioid-Dependent Patients Based on Time	123
Table 3.5 (d)	Comparisons of Pain Tolerances among Opioid-Dependent Patients Based on Time	124
Table 3.5 (e)	Overall Mean Difference of Pain Responses between Opioid-Naive Subjects and Opioid-Dependent Patients	126
Table 3.5 (f)	Comparisons of Pain Tolerances between Opioid-Naive Subjects and Opioid-Dependent Patients Based on Time	128
Table 3.6	Results of *OPRM1* and *CYP2B6* Genotyping of Four DNA Samples Obtained from Opioid-Dependent Patients	137
Table 3.7	Genotype Frequencies of *OPRM1* and *CYP2B6* among 100 DNA Samples with Their Respective 95% Confidence Interval	138

Table 3.8	True Sample Genotypes for ABCB1 2677G>T/A (rs2032582) Polymorphism	147
Table 3.9	Genotype Frequencies of *ABCB1* among 100 DNA Samples with Their Respective 95% Confidence Interval	148
Table 3.10 (a)	Genotype and Allele Distributions for the Three Screened Polymorphisms of *OPRM1* among Opioid-Naive Subjects	151
Table 3.10 (b)	Haplotype and Diplotype Distributions for the Three Screened Polymorphisms of *OPRM1* among Opioid-Naive Subjects	153
Table 3.10 (c)	Genotype and Allele Distributions for the Three Screened Polymorphisms of *OPRM1* among Opioid-Dependent Patients	155
Table 3.10 (d)	Haplotype and Diplotype Distributions for the Three Screened Polymorphisms of *OPRM1* among Opioid-Dependent Patients	157
Table 3.10 (e)	Genotype and Allele Distributions for the Three Screened Polymorphisms of *ABCB1* among Opioid-Naive Subjects	161
Table 3.10 (f)	Haplotype and Diplotype Distributions for the Three Screened Polymorphisms of *ABCB1* among Opioid-Naive Subjects	163
Table 3.10 (g)	Genotype and Allele Distributions for the Three Screened Polymorphisms of *ABCB1* among Opioid-Dependent Patients	165
Table 3.10 (h)	Haplotype and Diplotype Distributions for the Three Screened Polymorphisms of *ABCB1* among Opioid-Dependent Patients	167
Table 3.11 (a)	Influences of 118A>G and IVS2+691G>C Polymorphisms on Pain Thresholds in Opioid-Naive Subjects	170
Table 3.11 (b)	Influences of 118A>G and IVS2+691G>C Polymorphisms on Pain Tolerances in Opioid-Naive Subjects	173
Table 3.11 (c)	Influences of 118A>G and IVS2+691G>C Polymorphisms on Pain Intensity Scores in Opioid-Naive Subjects	176
Table 3.11 (d)	Influences of 118A>G and IVS2+691G>C Polymorphisms on Pain Thresholds in Opioid-Dependent Patients	179
Table 3.11 (e)	Influences of 118A>G and IVS2+691G>C Polymorphisms on Pain Tolerances in Opioid-Dependent Patients	183

Table 3.11 (f)	Influences of 118A>G and IVS2+691G>C Polymorphisms on Pain Intensity Scores in Opioid-Dependent Patients	187
Table 3.11 (g)	Influences of *ABCB1* Polymorphisms on Pain Thresholds in Opioid-Naive Subjects	191
Table 3.11 (h)	Influences of *ABCB1* Polymorphisms on Pain Tolerances in Opioid-Naive Subjects	195
Table 3.11 (i)	Influences of *ABCB1* Polymorphisms on Pain Intensity Scores in Opioid-Naive Subjects	199
Table 3.11 (j)	Influences of *ABCB1* Polymorphisms on Pain Thresholds in Opioid-Dependent Patients	204
Table 3.11 (k)	Influences of *ABCB1* Polymorphisms on Pain Tolerances in Opioid-Dependent Patients	208
Table 3.11 (l)	Influences of *ABCB1* Polymorphisms on Pain Intensity Scores in Opioid-Dependent Patients	213
Table 3.12 (a)	Descriptive Statistics of Serum Methadone Concentration (SMC) During a Single 24-hour Inter-Dosing Interval in the Opioid-Dependent Patients	217
Table 3.12 (b)	Serum Methadone Concentration (SMC) among Opioid-Dependent Patients	218
Table 3.13	Influences of *ABCB1* Polymorphisms on Serum Methadone Concentration (SMC) among Opioid-Dependent Patients	220
Table 3.14 (a)	Demographic Characteristics for Opioid-Dependent Patients with Concentrations at 24 hours of < 400 ng/ml and ≥ 400 ng/ml	225
Table 3.14 (b)	Comparisons of Cold Pressor Test (CPT) Responses between Patients with Concentrations at 24 hours of < 400 ng/ml and ≥ 400 ng/ml	225
Table 4.1	Ethnic Distribution of Alleles Frequencies of *ABCB1* Polymorphisms among Healthy Individuals	248
Table 4.2	Ethnic Distribution of Alleles Frequencies of *ABCB1* Polymorphisms among Opioid-Dependent Patients	257

LIST OF FIGURES

		Page
Figure 1.1	Mu-Type Opioid Receptor Gene (*OPRM1*) Structure and Polymorphisms Studied.	6
Figure 1.2	ABCB1 Protein and Gene Structure Showing the Polymorphisms Studied	22
Figure 2.1	Flow Chart of the Study Design	36
Figure 2.2	Summary of Cold Pressor Test (CPT) Procedures	47
Figure 2.3	Procedure for Polymerase Chain Reaction (PCR) Method for Detection of *OPRM1* Polymorphisms	50
Figure 2.4	OPRM1 Gene Structure and Variant Alleles Studied	61
Figure 2.5	Procedure for Allelic Discrimination of *ABCB1* Polymorphisms by Real-Time PCR Genotyping	71
Figure 2.6	The Fluorogenic 5 -Nuclease Assay for Detection of a Specific PCR Product Using TaqMan® Drug Metabolism Genotyping Assay and TaqMan® Genotyping Master Mix	79
Figure 2.7	Allelic Discrimination Plot for Allelic Discrimination (AD) Post-Read Run	87
Figure 2.8	Allelic Discrimination Plot for 93 Samples and Three No Template Controls Using 20X Taqman® Drug Metabolism Genotyping Assay (C_11711720D_40) and 2X Taqman® Genotyping Master Mix	89
Figure 2.9	Procedure for Enzyme-Linked Immunosorbent Assay (ELISA) for Determination of Serum Methadone Concentration (SMC)	95
Figure 2.10	Labelling Grid for Enzyme-Linked Immunosorbent Assay (ELISA)	100
Figure 2.11	Optical Density Values of Calibrators and Calibration Curve of the Percentage of Maximal Optical Density	106
Figure 3.1	Profile Plot of Mean (± 95% confidence interval) Pain Tolerance for Both Opioid-Naive Subjects and Opioid-Dependent Patients	129

Figure 3.2	DNA Sequences of the PCR Products	139
Figure 3.3 (a)	C_7586662_10 (A/G) Assay Allelic Discrimination Plot for Assignments of ABCB1 1236C>T (rs1128503) Genotypes	141
Figure 3.3 (b)	C_7586657_20 (A/G) Assay Allelic Discrimination Plot for Assignments of ABCB1 3435C>T (rs1045642) Genotypes	142
Figure 3.3 (c)	C_11711720C_30 (C/A) Assay Allelic Discrimination Plot for Assignments of ABCB1 2677G>T Genotypes	143
Figure 3.3 (d)	C_11711720D_40 (C/T) Assay Allelic Discrimination Plot for Assignments of ABCB1 2677G>A Genotypes	144
Figure 3.4	Component Plot from the Amplification Data	146

LIST OF PLATES

		Page
Plate 3.1	Agarose Gel Electrophoresis of the Genomic DNA Samples Extracted from Seventeen Subjects	131
Plate 3.2 (a)	First PCR Products of Set A for Ten Samples	133
Plate 3.2 (b)	Second PCR Products of Set 1 for Four Samples	134
Plate 3.2 (c)	Second PCR Products of Set 2 for Four Samples	135
Plate 3.2 (d)	Second PCR Products of Set 3 for Four Samples	136

LIST OF ABBREVIATIONS

%	Percent
°C	Celsius centigrade
µg	Microgram
µl	Microlitre
µM	Micromolar
1st PCR	First PCR
2nd PCR	Second PCR
A_{260}	Absorbance at a wavelength of 260 nm
A_{280}	Absorbance at a wavelength of 280 nm
ABCB1	ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 1
ABCB1	ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 1 gene
AD	Allelic discrimination
ANOVA	Analysis of variance
BBB	Blood-brain barrier
BLAST	Basic Local Alignment Search Tool
BMI	Body mass index
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CNS	Central nervous system
COMT	Catechol-O-methyltransferase gene

CPT	Cold pressor test
CTU	Clinical Trial Unit
CYP	Cytochrome P450
CYP3A4	Cytochrome P450 family 3 subfamily A member 4
CYP2B6	Cytochrome P450 family 2 subfamily B member 6
CYP2B6	Cytochrome P450 family 2 subfamily B member 6 gene
CYP2D6	Cytochrome P450 family 2 subfamily D member 6
CYP2D6	Cytochrome P450 family 2 subfamily D member 6 gene
Da	Dalton
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ES	Effect size
EX	Exon
FW	Forward
g	Gram
GABA	Gamma-aminobutyric acid
HAART	Highly active antiretroviral therapy
H_2O	Water

HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HREC	Human Research Ethics Committee
HRP	Horseradish peroxidase
HUSM	Hospital Universiti Sains Malaysia
HWE	Hardy-Weinberg equilibrium
INFORMM	Institute for Research in Molecular Medicine
INT	Intron
IVDU	Intravenous drug user
kb	Kilo base pairs
KCl	Potassium chloride
kDa	Kilo Dalton
LD	Linkage disequilibrium
LFT	Liver Function Test
LOQ	Limit of quantification
mg	milligram
MGB	Minor Groove Binder
$MgCl_2$	Magnesium chloride
ml	millilitre
mM	millimolar
MMT	Methadone Maintenance Therapy
MOH	Ministry of Health
MREC	Medical Research & Ethics Committee

mRNA	Messenger ribonucleic acid
mt	Mutant-type
N	Number of subject
NCBI	National Center for Biotechnology Information
NFQ	Non-Fluorescent Quencher
ng	Nanogram
NIH	National Institutes of Health
nm	Nanometer
NMRR	National Medical Research Register
NTC	No Template Controls
OD	Optical density
OPRD1	Opioid receptor delta 1 gene
OPRK1	Opioid receptor kappa 1 gene
OPRM1	Mu-type opioid receptor
OPRM1	Mu-type opioid receptor gene
PCR	Polymerase Chain Reaction
P-gp	P-glycoprotein
pM	Picomolar
pmol	Picomole
RFT	Renal Function Test
RM	Ringgit Malaysia
RM-ANCOVA	Repeated-measures analysis of covariance
RM-ANOVA	Repeated-measures analysis of variance

Rn	Normalized reporter
rpm	Rotations per minute
RV	Reverse
SD	Standard deviation
SDS	Sequence Detection Software
SE	Standard error of mean
SMC	Serum methadone concentration
SNP	Single nucleotide polymorphism
SPO_2	Blood Oxygen Saturation
TBE	Tris, Borate, EDTA
T_m	Melting temperature
TMB	Tetramethylbenzidine
U	Unit
USA	United States of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
V	Volt
VAS	Visual Analogue Scale
wt	Wild-type
X^2	Chi-square

KAJIAN FARMAKOGENETIK TENTANG PERSEPSI KESAKITAN AKUT DI KALANGAN PESAKIT YANG DIRAWAT DENGAN TERAPI GANTIAN METADON (MMT)

ABSTRAK

OPRM1 dan ABCB1 terlibat dalam modulasi kesakitan dan kesan terhadap rawatan analgesik. Polimorfisme gen OPRM1 dan ABCB1 merupakan antara sebab terdapatnya perbezaan dalam respons terhadap ujikaji kesakitan di antara individu. Objektif kajian ini adalah untuk mengkaji pengaruh faktor farmakogenetik terhadap kesakitan ke atas pesakit terapi gantian metadon (MMT) dan individu yang opioid naif. Protokol kajian telah mendapat kelulusan Jawatankuasa Etika Penyelidikan Manusia, USM, Kelantan dan Jawatankuasa Etika & Penyelidikan Perubatan, Kementerian Kesihatan Malaysia, Malaysia. Kajian berbentuk keratan rentas ini bermula Mac hingga Oktober 2013 dan melibatkan 148 orang pesakit penagih dadah yang menerima rawatan metadon dari klinik MMT di Kelantan dan juga 152 orang lelaki Melayu sihat yang opioid naif. Individu yang mempunyai keputusan ujian air kencing yang positif dadah, mengalami kesakitan kronik dan akut, dan mengalami keadaan lain yang boleh mempengaruhi kesakitan atau keputusan ujian perendaman tangan dalam air sejuk (CPT) tidak dipilih untuk kajian. Penerangan yang lengkap tentang kajian diberikan terlebih dahulu kepada subjek sebelum mendapatkan persetujuan mereka secara bertulis. Respons terhadap kesakitan kesejukan termasuk tahap permulaan kesakitan (*pain threshold*), toleransi kesakitan (*pain tolerance*) dan intensiti kesakitan (*pain intensity*) diukur menggunakan CPT. DNA diekstrak daripada darah dan digunakan untuk mengesan genotip *OPRM1*

dan *ABCB1*. Kajian ini mendapati bahawa pesakit yang menerima MMT adalah lebih sensitif kepada kesakitan (*hyperalgesia*) seperti yang ditunjukkan oleh tahap permulaan kesakitan dan toleransi kesakitan yang lebih cepat. Di kalangan individu yang opioid naif, terdapat kaitan yang signifikan antara polimorfisme 2677G>T/A dengan tahap permulaan kesakitan dan toleransi kesakitan. Selain itu, individu yang mempunyai alel *3435T* (genotip 3435 CT dan TT) menunjukkan intensiti kesakitan yang lebih tinggi berbanding individu yang mempunyai genotip 3435 CC. Diplotip 1236 CC/2677 GG/ 3435 CC didapati mempunyai kaitan yang signifikan dengan tahap permulaan kesakitan dan toleransi kesakitan yang lebih tinggi. Individu yang mempunyai diplotip 1236 TT/2677 TT/ 3435 TT menunjukkan intensiti kesakitan yang lebih tinggi berbanding individu tanpa diplotip ini. Walaubagaimanapun, polimorfisme *OPRM1* didapati tidak mempunyai kaitan yang signifikan dengan respons terhadap CPT. Di kalangan pesakit, genotip IVS2+691 CC didapati mempunyai kaitan yang signifikan dengan toleransi kesakitan yang lebih rendah, tetapi diplotip AC/AG bagi polimorfisme *OPRM1* didapati mempunyai kaitan yang signifikan dengan toleransi kesakitan yang lebih tinggi. Alel *2677G* didapati mempunyai kaitan yang signifikan dengan tahap permulaan kesakitan dan toleransi kesakitan yang lebih rendah, dan alel *2677G* atau haplotip CGC bagi polimorfisme *ABCB1* didapati mempunyai kaitan yang signifikan dengan toleransi kesakitan yang lebih tinggi. Diplotip CGC/TTT didapati mempunyai kaitan yang signifikan dengan kepekatan metadon yang lebih tinggi. Keputusan kajian ini boleh menjadi asas bagi pemahaman berkaitan pengaruh faktor genetik kepada respons terhadap ujikaji kesakitan di kalangan pesakit yang dirawat dengan MMT dan individu yang opioid naif. Polimorfisme *ABCB1* boleh menjadi faktor peramal kepada kesan terhadap rawatan metadon.

A PHARMACOGENETICS STUDY ON ACUTE PAIN PERCEPTION AMONG PATIENTS ON METHADONE MAINTENANCE THERAPY (MMT)

ABSTRACT

OPRM1 and ABCB1 are involved in pain modulation and analgesic responses. It is possible that *OPRM1* and *ABCB1* polymorphisms contribute to inter-individual differences in experimental pain responses. The objectives of this study were to investigate the pharmacogenetic factors that influence pain responses in patients on methadone maintenance therapy (MMT) and opioid-naive individuals. The protocol for the study was approved by the Human Research Ethics Committee (HREC), USM in Kelantan, and the Medical Research & Ethics Committee (MREC), at the MOH, Malaysia. This cross-sectional study involved Malay males opioid-dependent patients receiving MMT from MMT clinics in Kelantan (n = 148) and healthy opioid-naive individuals from the local population (n = 152), recruited from March to October, 2013. Excluded were individuals with a positive result of urine screening for drug test, chronic or ongoing acute pain, and other conditions that may affect pain or cold pressor test (CPT). Written informed consent was obtained from the subjects after full explanation of the study procedure. Cold pain responses including pain threshold, pain tolerance, and pain intensity were measured using the CPT. DNA was extracted from whole blood and genotyped for *OPRM1* and *ABCB1* polymorphisms. This study revealed hyperalgesia among opioid-dependent patients, as manifested by their quicker detection of pain and quicker hand withdrawal. In healthy opioid-naive individuals, the 2677G>T/A polymorphism of *ABCB1* was associated with pain threshold and pain tolerance. In

addition, carriers of *3435T* allele (3435 CT and TT genotypes) exhibited significantly higher pain intensity scores than carriers of the 3435 CC genotype. The 1236 CC/2677 GG/ 3435 CC diplotype was associated with a higher cold pain threshold and also pain tolerance. Individuals with 1236 TT/2677 TT/ 3435 TT diplotype exhibited higher pain intensity scores compared to those without this diplotype. However, *OPRM1* polymorphisms were not associated with cold pain responses in opioid-naive individuals. In the opioid-dependent patients, the IVS2+691 CC genotype was associated with a lower pain tolerance, but AC/AG diplotype of 118A>G and IVS2+691G>C polymorphisms of *OPRM1* were associated with a higher pain tolerance. The *2677G* allele for 2677G>T/A polymorphism was associated with lower pain threshold and pain tolerance, and *2677G* allele or CGC haplotype for the 1236C>T, 2677G>T/A, and 3435C>T polymorphisms of *ABCB1* were associated with a higher pain intensity scores. The CGC/TTT diplotypes was associated with a higher serum methadone concentration. These findings may become the foundation for understanding of genetic contributions to experimental pain responses in opioid-dependent patients on MMT and opioid-naive individuals. *ABCB1* polymorphisms may be a predictor for the treatment outcomes of opioid-dependent patients on MMT.

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Methadone Maintenance Therapy (MMT) and Pain Complaint

Methadone is well known and effective in the treatment of opioid dependence. Maintenance pharmacological treatments are effective in retaining patients in treatment and suppressing drug use (Amato *et al.*, 2011). Research evaluating the healthcare costs associated with treatment of opioid dependence with medications found that patients who received medication had lower hospital utilization and total costs than patients who did not receive pharmacologic therapy (Baser *et al.*, 2011). Many patients with opioid dependence are now receiving methadone maintenance therapy (MMT) in Malaysia. Drug use is common in Malaysia (Chemi *et al.*, 2014). Among patients on MMT in Malaysia, the starting age for drug use was between 14 to 35 years and the mean age at enrolment into MMT was 42 years (Manan *et al.*, 2013). This is a productive and active age group. It is therefore likely that clinicians will more frequently encounter patients on MMT for management of pain due to trauma, acute medical illness and chronic diseases, and surgery (Eyler, 2013). Opioid maintenance therapy may however alter sensitivity to pain (Compton *et al.*, 2000; Compton *et al.*, 2001; Doverty *et al.*, 2001a; Doverty *et al.*, 2001b; Athanasos *et al.*, 2006; Hay *et al.*, 2009; Compton *et al.*, 2012; Krishnan *et al.*, 2012). Unfortunately, clinicians often underestimate the pain complaints in this patient population (Alford *et al.*, 2006; Eyler, 2013).

Patients on MMT therefore often receive undertreatment for pain. There is a lack of awareness among physician about treatment of chronic and acute pain in this patient population. For instance, patients receive inadequate doses of opioid analgesic for their pain (Scimeca *et al.*, 2000; Alford *et al.*, 2006; Hines *et al.*, 2008; Eyler, 2013). On the other hand, poor pain management may be a risk factor for relapse for individuals with addiction in remission (Tsui *et al.*, 2010; Chang and Compton, 2013), and may contribute to discontinuation of MMT. The consequent continued use of illicit opiates poses challenges in the treatment of patients with opiate dependence (Eyler, 2013).

In addition to medical provider barriers, patient factors may also contribute to poor pain management in opioid dependence. Opioid-dependent patients frequently report increased pain sensitivity (Compton *et al.*, 2000; Compton *et al.*, 2001; Doverty *et al.*, 2001a; Doverty *et al.*, 2001b; Athanasos *et al.*, 2006; Pud *et al.*, 2006; Hay *et al.*, 2009; Compton *et al.*, 2012; Krishnan *et al.*, 2012). Cross-tolerance to opioids may also be present (Doverty *et al.*, 2001a; Athanasos *et al.*, 2006), and they may need more analgesia. Indeed, evidence has shown that opioid-dependent patients require higher than normal doses of opioid analgesics. Despite significantly greater plasma morphine concentrations, methadone patients experienced minimal anti-nociception in comparison with controls (Doverty *et al.*, 2001a). Higher morphine doses may achieve some pain relief, but this may be at the cost of unacceptable respiratory depression (Athanasos *et al.*, 2006). It is thus important for clinicians to understand pain sensitivity among patients on MMT for more effective pain management.

The present study investigated pain sensitivity using cold pressor test (CPT) among and between opioid-naive subjects and opioid-dependent patients on MMT, against a hypothesis that there are significant differences in pain sensitivity within groups, and between this patient population and the general population.

1.2 Hyperalgesia in Opioid-Dependent Patients on Methadone Maintenance Therapy (MMT)

Several studies on pain sensitivity among opioid-dependent patients on MMT have been undertaken previously in several populations in California, USA (Compton *et al.*, 2000; Compton *et al.*, 2001; Compton *et al.*, 2008; Compton *et al.*, 2010; Compton *et al.*, 2012), in Israel (Pud *et al.*, 2006; Peles *et al.*, 2011), and in Australia (Doverty *et al.*, 2001a; Doverty *et al.*, 2001b; Athanasos *et al.*, 2006; Hay *et al.*, 2009; Krishnan *et al.*, 2012). Studies found that heightened pain sensitivity was frequently reported in opioid-dependent patients on MMT. They also found that their opioid-dependent patients on MMT were more pain sensitive compared to controls.

Pain is however complex. Environments, both internal and external may play a role in both pain experience and pain control. Malaysia is a multi-ethnic country. Previous studies have indicated that there occurred large inter-ethnic and intra-ethnic pharmacologic differences among the Malaysian population. Pain sensitivity data are largely unavailable in Malaysia. Experimental pain studies may provide important information regarding pain sensitivity in these patients. This study sought to fill this gap.

1.3 Mu-Type Opioid Receptor Gene (*OPRM1*)

Several studies in Malaysia have suggested that pharmacogenetics variability may be an important factor in pharmacologic variability in the Malaysian population (Zahari *et al.*, 2009; Haerian *et al.*, 2011; Zahari *et al.*, 2011; Teh *et al.*, 2012; Zahari and Ismail, 2014; Wei *et al.*, 2015). The genetically polymorphic mu-type opioid receptor (MOR-1) or μ-opioid receptor (hMOP) is one of the major types of opioid receptors (Chen *et al.*, 1993). Others include kappa (κ-opioid receptor) and delta-type opioid receptors (δ-opioid receptor). These receptors are also known as MOR, KOR, and DOR; or more recently OP_3, OP_2, and OP_1, respectively. All the three major types of opioid receptors belong to the G protein-coupled receptor superfamily (Chen *et al.*, 1993; Min *et al.*, 1994; Mansour *et al.*, 1995; Bond *et al.*, 1998; Burford *et al.*, 2000). It is located on presynaptic or postsynaptic neurons depending upon cell types (Olive *et al.*, 1997; Pennock and Hentges, 2011). It is mainly expressed in the central nervous system (CNS) including the brainstem (periaqueductal gray, PAG), thalamus, cortex, and spinal cord (Mansour *et al.*, 1995). It is also expressed in peripheral tissues such as in intestinal tract (Bagnol *et al.*, 1997) and immune cells (Mousa *et al.*, 2001).

It has been suggested that the efficacy of most of the commonly used opioids is associated with its affinity for μ-opioid receptor. The μ-opioid receptor-binding efficacy of morphine and related opioid analgesics is highly correlated with their ability to modulate pain. Genetic factors that affect the density and function, consequently the signaling efficacy of μ-opioid receptor, may contribute to inter-individual variations in

the response to opioids (Befort *et al.*, 2001; Klepstad *et al.*, 2004; Ravindranathan *et al.*, 2009).

The μ-opioid receptor gene (*OPRM1*) is mapped to chromosome 6q24−q25 (Wang *et al.*, 1994) (Figure 1.1). The *OPRM1* has 400 amino acids, 236 371 bp's and has molecular weight of 44 779 Da and four coding exons that are separated by three introns (GenBank accession number NC_000006).

Receptor cloning has identified more than 17 isoforms (splice variants) of human μ-opioid receptor such as isoform 2 (MOR1A or MOR-1A) (Bare *et al.*, 1994), isoform 5 (MOR-1C or MOR-1O), and isoform 3 (MOR-1R or MOR-1X) (Pan *et al.*, 2003). The difference between these variants is in the tip of the intracellular C-terminus, far from the binding pocket of the receptor protein (Choi *et al.*, 2006; Pasternak, 2010).

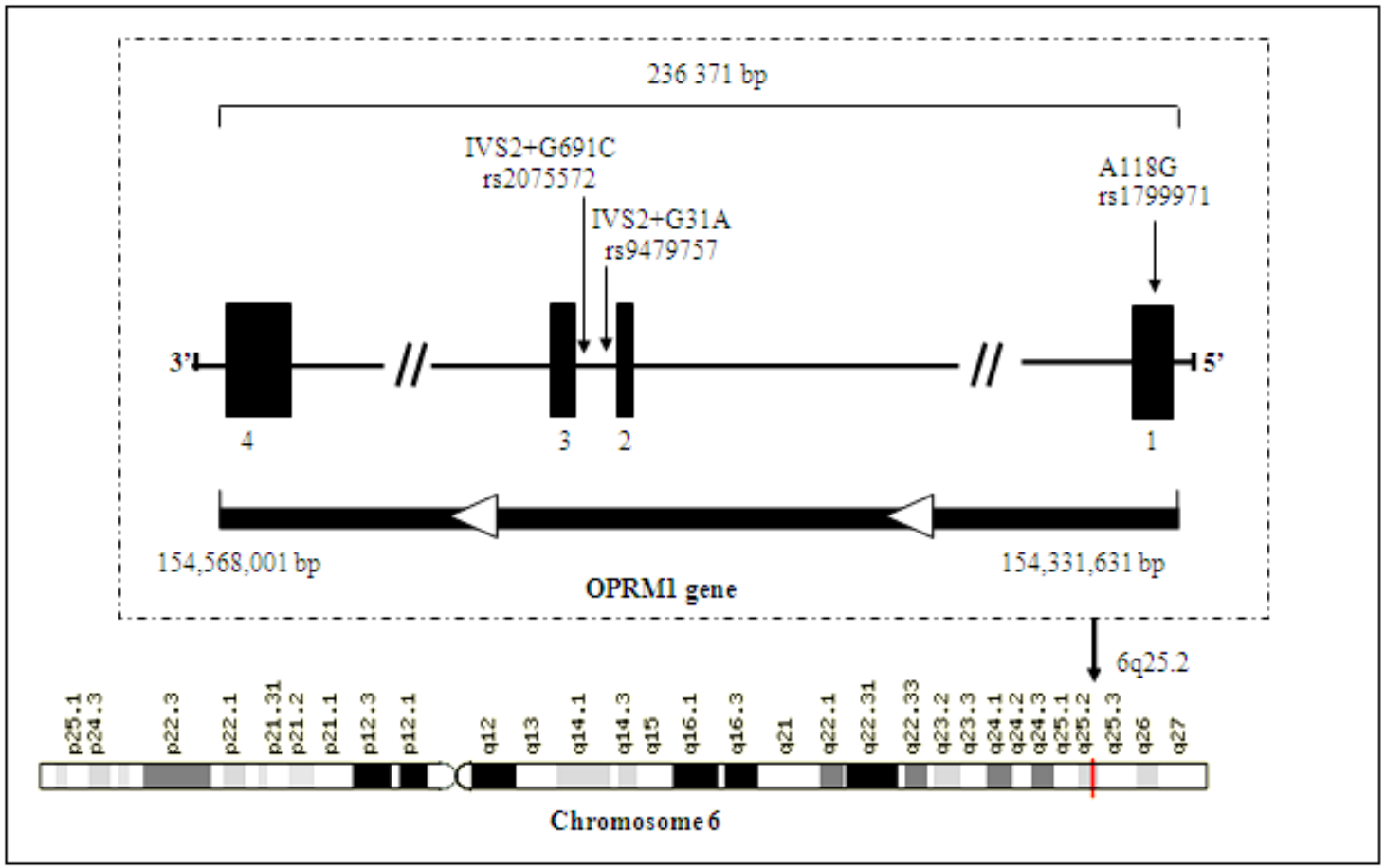

Figure 1.1 Mu-Type Opioid Receptor Gene (*OPRM1*) Structure and Polymorphisms Studied.

Boxes represent exons; horizontal lines connecting boxes represent introns, promoter and untranslated regions; arrows indicate relative locations of the polymorphisms. This figure is build based on UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly which is available at http://genome.ucsc.edu.

1.4 *OPRM1* Polymorphisms

At present, more than 2757 polymorphisms of *OPRM1* have been identified (http://www.ncbi.nlm.nih.gov/projects/SNP/ and http://www.genecards.org) including 118A>G (dbSNP rs1799971), IVS2+31G>A (dbSNP rs9479757), and IVS2+691G>C (dbSNP rs2075572) (Smolka *et al.*, 1999; Hoehe *et al.*, 2000; Shi *et al.*, 2002; Klepstad *et al.*, 2004; Lotsch and Geisslinger, 2006; Olsen *et al.*, 2012).

In a study by Bond *et al.* (1998), they detected five variants, all single nucleotide polymorphisms (SNPs) in the μ-opioid receptor including A118G, G24A, G779A, and G942A among 113 former heroin addicts on methadone maintenance and 39 individuals with no history of drug or alcohol abuse or dependence. The most prevalent SNP was the A118G (also known as 118A>G, dbSNP rs1799971 and Asn40Asp) that cause an amino acid exchange in the receptor protein. In exon 1, a substitution of aspartate (D) for asparagine (N) was found at codon 40, Asn40Asp, which corresponds to the N-terminal region of the receptor in the extracellular space. It is predicted to result in the removal of a putative N-glycosylation site and therefore might be expected to affect the μ-opioid receptor N-glycosylation and reduced stability of the receptor in cell cultures (Huang *et al.*, 2012). N-glycosylation plays a part in many cellular processes like receptor folding, sorting, expression, and ligand binding.

Bond *et al.* (1998) also found that the A118G variant receptor bind β-endorphin approximately three times stronger than the wild-type receptor. However, no differences in binding affinities for most opioid peptides and alkaloids tested were observed

suggesting that the A118G polymorphism did not change the overall binding properties of the receptor. The predicted amino acid change as a result of the A118G SNP is a single residue substitution in the N-terminal region in the extracellular space and is unlikely to drastically affect the overall tertiary structure of the receptor. At the molecular level, Zhang *et al.* (2005) found a 1.5 to 2.5-fold reduced mRNA expression of the μ-opioid receptor in human brain autopsy tissues of *118G* carriers and a further 10-fold reduction in protein levels has been found in cell cultures.

The *118G* allele has been found to be associated with reduced analgesic efficacy to opioids agents, higher opioid requirements or less pain relief (Klepstad *et al.*, 2004; Romberg *et al.*, 2005; Chou *et al.*, 2006a; Chou *et al.*, 2006b; Oertel *et al.*, 2006; Reyes-Gibby *et al.*, 2007; Campa *et al.*, 2008; Hayashida *et al.*, 2008; Sia *et al.*, 2008; Fukuda *et al.*, 2009; Ginosar *et al.*, 2009; Tan *et al.*, 2009; Wu *et al.*, 2009; Zhang *et al.*, 2010; Zwisler *et al.*, 2010; Liu and Wang, 2012). The 118A>G polymorphism were also reported to be associated with specific phenotypes such as alcohol dependence (Town *et al.*, 1999; Kim *et al.*, 2004), opioid addiction (Szeto *et al.*, 2001; Tan *et al.*, 2003; Nagaya *et al.*, 2012), substance dependence (Schinka *et al.*, 2002), pain sensitivity (Fillingim *et al.*, 2005; Oertel *et al.*, 2006), and obsessive compulsive disorder (Urraca *et al.*, 2004). Evidences are however inconsistent (Bergen *et al.*, 1997; Sander *et al.*, 1998; Gelernter *et al.*, 1999; Hoehe *et al.*, 2000; Li *et al.*, 2000; Franke *et al.*, 2001; Shi *et al.*, 2002; Crowley *et al.*, 2003; Ross *et al.*, 2005; Coulbault *et al.*, 2006; Janicki *et al.*, 2006; Huang *et al.*, 2008; Landau *et al.*, 2008; Lötsch *et al.*, 2009; Wong *et al.*, 2010; Klepstad *et al.*, 2011; Camorcia *et al.*, 2012).

Polymorphisms that do not cause amino acid exchanges but are frequent or have also been proposed to have functional consequences include IVS2+31G>A and IVS2+691G>C SNPs found in intron 2. Substitution of Adenine (A) for Guanine (G) at 31 bp downstream of exon 2 (IVS2+31G>A) and substitution of Cytosine (C) for Guanine (G) at 691 bp downstream of exon 2 (IVS2+691G>C) located within intron 2 (Hoehe *et al.*, 2000; Xin and Wang, 2002; Lotsch and Geisslinger, 2006), may however change the affinity of the transcriptional regulatory factors for the intronic DNA sequence and directly alter mRNA levels, and therefore may change the regulation of the expression of OPRM1 gene. Additionally, intronic sequence can be involved in alternative DNA splicing, resulting in different isoforms of human μ-opioid receptor (Wendel and Hoehe, 1998; Hoehe *et al.*, 2000).

IVS2+31G>A polymorphism have been found to be associated with higher pressure pain threshold in healthy adult females resulting in lower pressure pain sensitivity than those without this SNP (Huang *et al.*, 2008). However, IVS2+31G>A polymorphism showed no influence on the efficacy of morphine in cancer pain patients (Klepstad *et al.*, 2004). The *IVS2+31A* carrier, especially subjects carrying both the IVS2+31G>A and the A118G polymorphisms also had higher level of addiction to heroin resulting in higher heroin intake dosages than non-carriers of those mutations (Shi *et al.*, 2002). The mechanism for this is unknown.

The IVS2+691G>C polymorphism however did not increase morphine requirements in patients with pain caused by malignant disease (Klepstad *et al.*, 2004) and did not have significant association with 24-h post-operative opioid requirement

(Hayashida *et al.*, 2008). Association between C1031G (IVS2+691G>C) polymorphism and heroin dependence was also reported in Chinese subjects (Szeto *et al.*, 2001) but Li *et al.* (2000) and Tan *et al.* (2003) failed to show an association between this polymorphism and heroin abuse. Self-reported positive responses on first use of heroin such as euphoria were also found not to be associated with this polymorphism or A118G polymorphism (Zhang *et al.*, 2007). Furthermore, results from Bergen *et al.* (1997) did not support a role of this polymorphism in susceptibility to alcohol dependence.

Details on characteristic and position of *OPRM1* polymorphisms are shown in Table 1.1 and *OPRM1* alleles frequencies in patients with pain are shown in Table 1.2.

Table 1.1 Characteristics and Positions of *OPRM1* Polymorphisms

Polymorphism	Characteristic mutation (s)	Amino acid location	Synonyms
118A>G (dbSNP rs1799971)	At nucleotide 118 in exon 1, an Adenine (A) is changed to a Guanine (G)	N40D (Asn40Asp)	*OPRM1:c.118A>G* SNP, dbSNP rs61596185, dbSNP rs17181017, dbSNP rs52818856, g.154360797A>G, g.154039662A>G, g.34162A>G, c.118A>G, c.397A>G, c.-11+28644A>G, c.47+29103A>G, p.Asn40Asp, p.Asn133Asp, A118G, 304A/G, ASN40ASP, Asn40Asp, N40D, OPRM1 118
IVS2+31G>A (dbSNP rs9479757)	Guanine (G) to Adenine (A) substitution at 31 bp downstream of exon 2 (Intron 2)	-	dbSNP rs60522300, dbSNP rs17174808, g.154411344G>A, g.154090209G>A, g.84709G>A, c.343+31G>A, c.400+31G>A, c.643+31G>A, c.922+31G>A, IVS2+G31A, IVS2 +31G→A, OPRM1 31, OPRM1 50665

Table 1.1 ……Continued

Polymorphism	Characteristic mutation (s)	Amino acid location	Synonyms
IVS2+691G>C (dbSNP rs2075572)	Guanine (G) to Cytosine (C) substitution at nucleotide +691 in intron 2	-	dbSNP rs56680128, dbSNP rs17210094, dbSNP rs17174815, g.154412004G>C, g.154090869G>C, g.85369G>C, c.644-83G>C, c.923-83G>C, c.344-83G>C, c.401-83G>C, IVS2+G691C, C1031G, OPRM1 691, OPRM1 51325

Table 1.2 *OPRM1* Alleles Frequencies in Patients with Pain

References	Ethnicity	Number of subject, N	Frequency (%)
***118G* allele**			
Chronic cancer pain			
Klepstad *et al.* (2004)	Caucasian	206	10.4
Ross *et al.* (2005)	Caucasian	156	15.1
Campa *et al.* (2008)	Italian Caucasian	138	15.2
Liu and Wang (2012)	Chinese	96	39.6
Labor pain			
Landau *et al.* (2008)	1. Caucasian	213	17.8
	2. Asian	10	40.0
	3. All cases	223	18.8
Camorcia *et al.*(2012)	Italian Caucasian	77	16.2
Wong *et al.* (2010)	Mixed (White/Caucasian, African-American, Asian and Hispanic)	190	15.3
Post-operative pain			
Tan *et al.* (2009)	1. Chinese	620	33.9
	2. Malays	241	49.0
	3. Indian	137	44.1
Zhang *et al.* (2010)	Chinese	174	31.3
Sia *et al.* (2008)	Chinese Singaporean	585	33.6
Wu *et al.* (2009)	Han Chinese	189	30.2
Hayashida *et al.* (2008)	Japanese	138	44.9
Fukuda *et al.* (2009)	Japanese	280	43.8
Chou *et al.*(2006a)	Taiwanese	80	34.4
Chou *et al.* (2006b)	Taiwanese	120	24.6
Tsai *et al.* (2010)	Taiwanese	212	32.6

Table 1.2 ……Continued

References	Ethnicity	Number of subject, N	Frequency (%)
***118G* allele**			
Post-operative pain			
Janicki *et al.* (2006)	American	101	15.8
Kolesnikov *et al.*(2011)	Caucasian	102	11.3
Coulbault *et al.* (2006)	French Caucasian	74	12.8
Bruehl *et al.*(2006)	Mixed White Non-Hispanic, African-American and Other	48	12.5
Wong *et al.* (2010)	Mixed (White/Caucasian, African-American, Asian and Hispanic)	103	13.6
Chronic pain			
Janicki *et al.* (2006)	American	131	7.9
Menon *et al.*(2012)	Australian Caucasian	153	11.8
Lötsch *et al.* (2009)	Caucasian	352	14.4
***IVS2+31A* allele**			
Chronic cancer pain			
Klepstad *et al.* (2004)	Caucasians	206	9.7
Ross *et al.* (2005)	Caucasians	156	8.7
***IVS2+691C* allele**			
Chronic cancer pain			
Klepstad *et al.* (2004)	Caucasians	206	61.2
Ross *et al.* (2005)	Caucasians	156	55.1
Post-operative pain			
Hayashida *et al.* (2008)	Japanese	138	79.3

1.5 *OPRM1* Polymorphisms and Pain Sensitivity

Genetic and environmental factors interactions may further influence pain sensitivity (Young *et al.*, 2012). Polymorphisms in genes involved in pain processing predict variation in pain sensitivity (Govoni *et al.*, 2008; Lind and Gordh, 2009; Lotsch *et al.*, 2009; Miaskowski, 2009; Kim and Schwartz, 2010; Kasai and Ikeda, 2011; Shipton, 2011). It is known that opioidergic mechanisms are involved in the responses to nociceptive stimuli (Holden *et al.*, 2005; Sprenger *et al.*, 2006; Eippert *et al.*, 2009; Schoell *et al.*, 2010). The mu-type opioid receptor (OPRM1) is the primary binding site for endogenous opioid peptides β-endorphin and endomorphin (Mizoguchi *et al.*, 2000) and exogenous opioids, including methadone and morphine (Saidak *et al.*, 2006). Studies showed that the activation of the OPRM1 system is associated with reductions in the sensory and affective ratings of the pain experience (Zubieta *et al.*, 2001).

Variability in the modulation of pain or pain sensitivity and large inter-individual differences in treatment outcomes with opioid analgesic therapy suggest varied sensitivity to endogenous and exogenous opioids, and potential variability in the OPRM1 receptor protein and gene (Govoni *et al.*, 2008; Lind and Gordh, 2009; Lotsch *et al.*, 2009; Miaskowski, 2009; Kim and Schwartz, 2010; Kasai and Ikeda, 2011; Shipton, 2011; Young *et al.*, 2012). A previous study in healthy males has shown that striatal OPRM1 availability predicted the cold pressor pain threshold. They found that healthy males with low OPRM1 binding potential in the striatum are associated with a low cold pain threshold (Hagelberg *et al.*, 2012). They hypothesised that individuals with low OPRM1 binding potential have low receptor density, and consequently, low

level of OPRM1-mediated suppression of pain pathways, leading to increased sensitivity to experimental pain (Hagelberg *et al.*, 2012).

Martin *et al.*(2003) observed that a low endogenous opioid tone in the regulation of physiological pain in opioid receptor knockout mice was associated with increased nociceptive responsiveness. The A118G polymorphism is a candidate mutation with potential importance for nociceptive responsiveness because it has been found to increase the receptor affinity of β-endorphin by a factor of three (Bond *et al.*, 1998). This may be hypothesised to increase the activity in the endogenous opioid system. A high endogenous opioid tone in the regulation of physiological pain may be associated with a decreased response to nociceptive stimulation.

Previous studies have also suggested an influence of polymorphisms of the OPRM1 gene (*OPRM1*) on pain phenotypes in healthy subjects (Fillingim *et al.*, 2005; Lotsch *et al.*, 2006; Huang *et al.*, 2008). A genetic study indicated that 118A>G polymorphism of the OPRM1 gene also influenced experimental pain responses in healthy subjects (Fillingim *et al.*, 2005; Lotsch *et al.*, 2006). Individuals with *118G* allele exhibited lower sensitivity to pressure pain (higher pressure pain thresholds) compared to those with wild-type allele (Fillingim *et al.*, 2005). A relationship between 118A>G polymorphism and heat pain perception has also been previously described in healthy individuals, and its direction was dependent on gender, where the *118G* allele was associated with lower pain ratings among males but higher pain ratings among females (Fillingim *et al.*, 2005). As the *118G* allele has been found to increase the receptor affinity of β-endorphin (Bond *et al.*, 1998), and consequently, increase the

activity in the endogenous opioid system, these findings supported the hypothesis that the 118A>G polymorphism of the OPRM1 gene was associated with increased endogenous opioid tone (Fillingim *et al.*, 2005).

The IVS2+31G>A polymorphism was also found to be associated with pressure pain sensitivity in healthy adult females. Pressure pain threshold in subjects with the major allele (termed 'GG') genotype was significantly lower than those with minor allele (termed 'GA') genotype (Huang *et al.*, 2008). However, evidence of a possible role of IVS2+691G>C polymorphism in the risk of altered pain sensitivity among healthy individuals is limited.

Table 1.3 summarises the association studies of *OPRM1* polymorphisms with pain sensitivity. In total, the studies have raised a great number of questions over the potential role of the *OPRM1* polymorphisms in pain sensitivity but with no clear resolution. Thus, the association between pain sensitivity and *OPRM1* polymorphisms remains controversial. Further studies are necessary to evaluate the results of these association studies between *OPRM1* polymorphisms and pain sensitivity.

In addition to this lack of concordance in the literature, it is not known whether the inter-individual variations in pain responses are influenced by *OPRM1* polymorphisms in the healthy Malay male population. This study was designed to clarify the position in the literature, and to determine whether healthy Malay male variations in cold pressor pain responses are influenced by *OPRM1* polymorphisms.

Table 1.3 Association Studies of *OPRM1* Polymorphisms with Pain Sensitivity

References	Ethnicity	Number of subject, N	Frequency (%)	Type of pain	Result
***118G* allele**					
Fillingim *et al.*(2005)	American	167	11.4	Thermal, mechanical, and ischemic pain	G-allele carriers had lower pressure pain sensitivity (higher pressure pain thresholds) than AA subjects. No association with heat and ischemic pain sensitivity.
Lötsch *et al.*(2006)	White	45	10.0	Corticol responses to trigeminal pain stimuli	G-allele carriers had lower sensitivity to nociceptive stimuli [lower N1 event-related potential (ERP) response to CO_2] than AA subjects.
Zhang *et al.*(2010)	Chinese	174	31.3	Electrical stimulation pain	G-allele carriers had higher electrical stimulation pain sensitivity (lower pain tolerance thresholds) than AA subjects.
Huang *et al.*(2008)	Taiwanese	72	31.9	Mechanical pain	No association.
Fukuda *et al.*(2009)	Japanese	280	43.8	Cold pressor-induced pain	No association.
***IVS2+31A* allele**					
Huang et al.(2008)	Taiwanese	72	2.8	Mechanical pain	GA genotype carriers had lower pressure pain sensitivity (higher pressure pain thresholds) than GG subjects.
***IVS2+691C* allele**					
Huang *et al.*(2008)	Taiwanese	72	0.0	Mechanical pain	Not analysed.

1.6 *OPRM1* Polymorphisms and Inter-Individual Variations in the Response to Opioids

The μ-opioid receptor (OPRM1) is a primary target for the clinically important opioid analgesics, including morphine, fentanyl, and methadone. It has been suggested that the efficacy and side-effects of most of the commonly used opioids is associated with its affinity for μ-opioid receptor. In fact, the μ-opioid receptor-binding efficacy of morphine and related opioid analgesics is highly correlated with their ability to modulate pain. Genetic factors that affect the density and function and consequently the signaling efficacy of μ-opioid receptor may contribute to inter-individual variations in the response to opioids (Befort *et al.*, 2001; Klepstad *et al.*, 2004; Ravindranathan *et al.*, 2009).

In total, the studies have raised a great deal of speculation over the potential role of the *OPRM1* polymorphisms in lowered pain sensitivity, increased opioid dose requirements, reduced opioid analgesia, and reduced risk of side-effects such as nausea, vomiting and pruritus but with no clear resolution (Zahari and Ismail, 2013).

Opioid-dependent patients on MMT exhibit abnormal pain sensitivity called opioid-induced hyperalgesia (i.e. increased pain sensitivity after opioid administration). The finding of shorter pain tolerance times (withdrawal latencies) in response to pain tests among opioid maintained addicts compared to healthy controls has been shown consistently in many studies providing further evidence that opioid-dependent patients are more sensitive to painful stimuli than are others (Compton *et al.*, 2000; Compton *et*

al., 2001; Pud *et al.*, 2006; Hay *et al.*, 2009; Krishnan *et al.*, 2012). There is inter-individual variation in opioid-induced hyperalgesia among methadone users and hyperalgesia may weaken their determination to abstain (Eyler, 2013).

Several studies have also reported on the association between methadone treatment and *OPRM1* polymorphisms (Compton *et al.*, 2003; Bunten *et al.*, 2010; Fonseca *et al.*, 2010; Bunten *et al.*, 2011; Wang *et al.*, 2012). Although evidence shows that *OPRM1* polymorphisms are associated with changes in libido and insomnia, and methadone-related deaths, the relationship of *OPRM1* polymorphisms with abnormal pain sensitivity among opioid-dependent patients on methadone therapy is not fully understood. Thus far, one study explored the possibility that the *OPRM1* polymorphisms could provide a possible explanation for the noted pain intolerance of opioid addicted individuals (Compton *et al.*, 2003). Unfortunately, this showed that the role of the polymorphisms remains uncertain due to the low frequencies of the variants in the study samples.

A better understanding of the role of *OPRM1* polymorphisms in pain and opioid response 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 *OPRM1* polymorphisms on pain responses among opioid-dependent patients on methadone therapy in Malay patients.

1.7 ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 1 gene (*ABCB1*)

The ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 1 (ABCB1) gene [also known as multidrug resistance (MDR1) gene] encodes P-glycoprotein (P-gp), an efflux transporter that transports its substrates out of the cells (Kim, 2002). It is located on chromosomal region 7q21 (Fojo *et al.*, 1986) (Figure 1.2). Its cDNA spans about 4.5 kb with 28 exons, encoding a 1280-amino acid (multidrug resistance protein 1) with the size 170 kDa (Chen *et al.*, 1990). The P-gp is highly expressed in endothelial cells of the brain vasculature, and it is believed to affect the efflux of endogenous and exogenous substrates across the blood-brain barrier (BBB) (Schinkel *et al.*, 1996; King *et al.*, 2001; Kastin *et al.*, 2002). This causes a lower concentration of substrates such as morphine in the cell, and reduces the effectiveness of the substrates (Hamabe *et al.*, 2007).

The function of P-gp may be affected by *ABCB1* polymorphisms, with certain polymorphisms leading to decreased mRNA, and P-gp expression and activity (Hitzl *et al.*, 2001; Kim *et al.*, 2001; Sauer *et al.*, 2002; Meissner *et al.*, 2004; Leschziner *et al.*, 2007). However, the associations between *ABCB1* polymorphisms and *ABCB1* mRNA and protein expression, and function of P-gp have been inconclusive (Leschziner *et al.*, 2007).

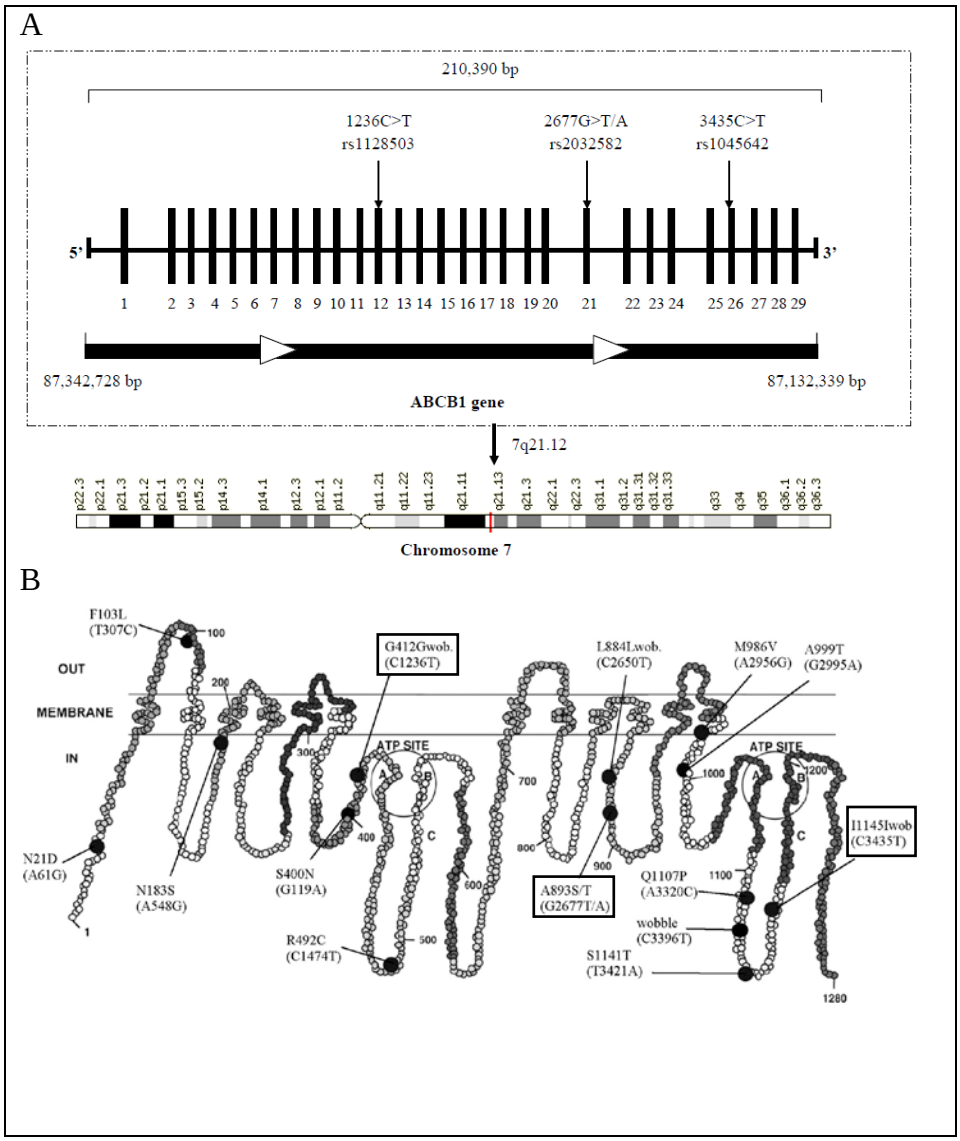

Figure 1.2 ABCB1 Protein and Gene Structure Showing the Polymorphisms Studied.

A. Gene structure of *ABCB1* on human chromosome 7q21.12 and polymorphisms studied. Boxes in the ABCB1 gene structure represent exons; horizontal lines connecting boxes represent introns, promoter and untranslated regions; arrows indicate relative locations of the polymorphisms. This figure is build based on the AceView genes as of January 2016 which is available at http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly. B. ABCB1 protein and known polymorphisms as reported by Meletiadis *et al.* (2006). The studied polymorphisms are highlighted in boxes.

1.8 *ABCB1* Polymorphisms

The most common polymorphisms found in *ABCB1* include 1236C>T (dbSNP rs1128503), 2677G>T/A (dbSNP rs2032582), and 3435C>T (dbSNP rs1045642). The silent 3435C>T polymorphism in exon 26 of *ABCB1* involves a transition of cytosine (C) to thymine (T) at nucleotide 3435. This synonymous polymorphism is found at residue 1145 in the second ATP binding domain, which is located at a cytoplasmic loop of P-gp. It does not result in an amino acid change (ATC isoleucine, ATT isoleucine; Ile1145Ile) (Ambudkar *et al.*, 1999; Fung and Gottesman, 2009). Other polymorphisms that has previously been reported in high-frequencies include 1236C>T and 2677G>T/A. The latter is a tri-allelic polymorphism at nucleotide 2677 in exon 21 of *ABCB1*. The non-synonymous polymorphism involves the transition of guanine (G) to thymine (T) or adenine (A) leading to one of two possible amino acid changes (GCT alanine, TCT serine, ACT threonine; Ala893Ser/Thr). It is found at residue 893 in a cytoplasmic loop of P-gp (Ambudkar *et al.*, 1999; Fung and Gottesman, 2009). The occurrence of 2677G>T (Ala893Ser) and 2677G>A (Ala893Thr) is not similar with the first far more frequent than the latter (Fung and Gottesman, 2009).

Another SNP, the 1236C>T polymorphism, is a silent polymorphism in exon 12 of *ABCB1*. The SNP involves transition of cytosine (C) to thymine (T) at nucleotide 1236. This does not result in an amino acid change (GGC glycine to GGT glycine; Gly412Gly) at residue 412 in a cytoplasmic loop (Ambudkar *et al.*, 1999; Fung and Gottesman, 2009). Details on characteristic and position of *ABCB1* polymorphisms are shown in Table 1.4.

Table 1.4 Characteristics and Positions of *ABCB1* Polymorphisms

Polymorphism	Characteristic mutation (s)	Amino acid location	Synonyms
1236C>T (dbSNP rs1128503)	At nucleotide 1236 in exon 12, an cytosine (C) is changed to a thymine (T)	Gly412Gly	g.87179601A>G g.87550285A>G g.167964T>C c.1236T>C p.Gly412=
2677G>T/A (dbSNP rs2032582)	Transition of guanine (G) to thymine (T) or adenine (A) at nucleotide 2677 in exon 21	Ala893Ser/Thr	g.87160618A>C g.87160618A>T g.87531302A>C g.87531302A>T g.186947T>A g.186947T>G c.2677T>A c.2677T>G p.Ser893Ala p.Ser893Thr
3435C>T (dbSNP rs1045642)	At nucleotide 3435 in exon 26, an cytosine (C) is changed to a thymine (T)	Ile1145Ile	g.87138645A>G g.87509329A>G g.208920T>C c.3435T>C p.Ile1145=