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Research Article

SINGLE NUCLEOTIDE POLYMORPHISM (SNPS) ANALYSIS OF MU-OPIOID RECEPTORS (*OPRM*1) USING DENATURING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (DHPLC) AMONG THE INTRAVENOUS DRUG USERS

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ABSTRACT

Objectives: The genetic polymorphisms of *OPRM*1 among the intravenous drug users (IVDUs) and healthy controls were investigated and the risk of addiction in relation to *OPRM*1 was predicted.

Methods: PCR-denaturing high performance liquid chromatography (DHPLC) method was developed to investigate SNPs in the coding regions of *OPRM*1 in 93 IVDUs and 100 healthy controls. Subjects were confirmed to be drug addicts and their personality was studied using validated Tridimensional Personality Questionnaires (TPQ).

Results: Based on the results obtained, seven SNPs were detected; two of them were previously associated with addiction. Homozygous *OPRM*1:c.118GG and heterozygous *OPRM*1:c.118AG variants were found to have higher frequencies among the IVDUs and healthy controls. In addition, carriers of *OPRM*1:c.118G allele scored higher for novelty seeking (NS) and harm avoidance (HA) with more explorative, neurotic and uninhibited personalities. We identified a new variant of *OPRM*1:c.77C>G which is located at the N-terminus of the G-coupled protein receptor and possibly decreases the binding affinity of its ligands among the IVDUs.

Conclusion: In conclusion, DHPLC allows the detection of new and existing variants of *OPRM*1. Genotyping of *OPRM*1:c.118A>G and assessment of personalities using TPQ provide valuable tools for determination of addiction risk.

Keywords: Mu-opiod receptor, *OPRM*1, intravenous drug users (IVDUs), Denaturing high performance liquid chromatography (DHPLC)

INTRODUCTION

Drug addiction is a chronic, relapsing disorder in which compulsive drug-seeking and drug-taking behavior persists despite serious negative consequences [1]. Narcotic production and drug trafficking have been associated with corruption, criminal activities and violence [2, 3]. In addition, the risk of HIV and AIDS infections has been associated with intravenous drug use and unprotected sexual activities among the addicts [4, 5]. Earlier studies conducted in twins, family and adopted siblings shed light on the possible role of genetic variations in association of substance abuse, dependence and addiction. The vulnerability to opioid dependence has also been associated with the individual genetic background. This was further supported by findings of an *in vivo* animal study which suggested the association of opioid dependence with a locus on mouse chromosome 10 [6]. This subsequently led to the interest of many researchers to explore the roles of human Mu-opioid receptor (gene locus *OPRM1*) as the candidate gene causes opioid dependence. Recent studies reported significant association between genetic polymorphism of *OPRM1* and addiction [7-10]. The association principally was raised due to the interactions of opioidergic pathways with the dopaminergic system and other associated pathways that mediate reward [1].

Drug addiction causes huge social and medical problems and addicts are seen as burden to the development of human race in many countries. In order to provide treatment options to reduce risk and negative impact of addiction, data on the demographic, injecting habit, severity, personality, genetic profiles and risk association are desirable. The endogenous opioid system is centrally important in the responses to addictive opiate drugs such as morphine, codeine, and heroin, as well as to synthetic opioid narcotics such as fentanyl [1]. The receptors mediate both the analgesic and rewarding properties of opioid compounds and opioid effects on the hypothalamic-pituitary adrenal (HPA) stress-responsive axis, respiratory and pulmonary function, gastrointestinal motility, immune responses, and other functions [11].

Binding assays with selective ligands classified the opioid receptors into three general classes: (i) the Mu-Opioid receptor (MOR) which has endogenous ligands, the enkephalins and β-endorphin, (ii) the Delta-Opioid receptor (DOR) which selectively binds enkephalins, and (iii) the Kappa-Opioid receptor (KOR) for which dynorphins are the endogenous ligands. Binding assays using cells expressing cloned receptors indicate that there exists considerable cross-selectivity for some of the peptide ligands [1, 12, 13].

The genetic approach therefore highlights Mu-Opioid receptors as convergent molecular switches, which mediate reinforcement following direct or indirect activation by non-opioid drugs of abuse [10]. Single-nucleotide polymorphisms of the gene encoding the Mu-opioid receptor, located on chromosome 6q24 correlates with an increased likelihood of heroin abuse [1, 14].

Our study thus aims to develop a method to investigate the genetic polymorphisms of the gene (*OPRM*1) encoding the Mu-opioid receptor and the possible risks associated with addiction. DHPLC was chosen as the screening tool as it is capable of detecting new and existing allelic types at a much lesser cost. We also understand that genetic traits are important determinant for addiction but element of environmental interaction should be taken into account for the addictive disorders.

MATERIALS AND METHODS

Chemical reagents used in this study include GoPhorIT™ *Taq* DNA Ploymerase (detergent free buffer; concentration 5 U/μL) (Mbiotech, Inc., Seoul, Korea) and DHPLC buffers: Buffer A (100 mM TEAA to pH 7.0, 0.1 mM EDTA); Buffer B (100 mM TEAA to pH 7.0, 0.1 mM EDTA and 25%v/v acetonitrile) (Variant Inc., San Jose, California, USA).

Standard controls are *PUC 18* DNA *Hae III* Digest (SIGMA-ALDRICH, USA) and Wave Low Range Mutation Control Standard (Transgenomic, USA). Other chemicals are purchased from respective companies and are of molecular grade.

Subjects

The study protocol was approved by the local research ethics committee. The participant comprised of 93 intravenous drug users (IVDUs) and 100 healthy controls were unrelated individuals of Malay ethnicity up to three generations. All the recruited subjects were aged 18 to 55 years old during the time of study. The IVDUs were recruited from an out-patients clinic attending methadone maintenance therapy while the healthy volunteers were recruited from healthy unrelated individuals who were healthy blood donors.

The IVDUs have abused heroine intravenously before they were on methadone maintenance therapy. Interviews were carried out based on Diagnostic and Statistical Manual of Mental Disorders edition four (DSM-IV) [15] to confirm their addiction status [15. 16]. The healthy controls were also assessed using the DSM-IV and confirmed to be independent of substance abuse. Data on the demographic and pattern of abuse was collected using the Drug Addiction Study Questionnaire (WHO) [11] and Tridimensional Personality Questionnaires [12]. The IVDUs were excluded if they have severe cognitive impairment or mental retardation. Only male subjects were recruited for this study; as most of the IVDUs were males. Written informed consents were obtained from all participants.

The Tridimensional Personality Questionnaire (TPQ) consists of 100 questions scored using true/false answers. Three dimensions of personality which include novelty seeking (NS), harm avoidance (HA) and reward dependence (RD) were evaluated in relation to dopamine, serotonin, and norepinephrine activities respectively [17]. On the other hand, Drug Addiction Study Questionnaire was a semi- structured clinical or research interview developed in 1980 by McLellan et al. [18]. The interview assesses seven areas including medical, employment, drug and alcohol use, legal, family/social and psychological of an individual during the past 30 days.

The interview was conducted in a quiet room with no access from unknown individuals to protect privacy and confidentiality of the subjects. The questionnaire used in this study was translated into the local language *"Bahasa Melayu"* and validated to ensure that the subjects did understand and answer the questions correctly.

DNA Samples

The whole blood samples from 93 IVDUs and 100 healthy controls were extracted for DNA using the standard lysis method as described in Teh *et al*. [19].

PCR Amplification

The complete sequence was located with the accession number of AY587764. Primers were designed to flank the intronic regions of the gene to amplify exons 1, 2 and 3. Primer sequences were examined using a primer analysis program, Primer3® (*http://biotools.umassmed.edu/bioapps/primr3_www.cgi*). The primers were then evaluated for their specificities using the BLAST search engine (*http://www.ncbi.nlm.nih.gov/blast/blast.cgi).* Forward and reverse primers flanking the intron regions of exon 1, 2 and 3 of *OPRM*1 were designed [MU EX1 FW 5'-GAAAAGTCTCGGTGCTCCTG-3', MU EX1 RV 5'-CAATCACTGTCCGTGGTCTC-3', MU EX2 FW 5'-GCTGATACTGGAAAACAA-3', MU EX2 RV 5'-TAAGACAATGGGGCACTC-3', MU EX3 FW 5'-ATCAATGAGCAAAATGGC-3' and MU EX3 RV 5'-CAAACCTCTTCTCTAACAC -3']. The sets of primers produce amplicons with the sizes of 500 bp for exon 1, 590 bp for exon 2 and 758 bp for exon 3. Exon 4 was not included in the study because it is very short fragment and the lack of significant mutation site.

In DHPLC, misincorporations of taqs, excess of dNTPs, primer-dimers would create a separate heteroduplex peak. If large enough, these peaks would be difficult to be distinguished from actual sample peaks. Therefore, PCR was carried out with the optimum condition to ensure amplification of the specific target gene of interest without non-specificity.

PCR master mixtures include 0.6 unit of GoPhorIT™ *Taq* DNA polymerase (Mbiotech, Inc., Seoul, Korea), 1 X reaction buffer, 2 mM of $MgCl_2$, approximately 200 ng of genomic DNA, 0.2 mM of dNTPs and 0.2 µM of each forward and reverse primers. After an initial denaturation at 95°C for 2 minutes; Touch-Down PCR cycling was performed for 9 cycles as follows: 94°C for 20 seconds, 63°C to 55°C for 20 seconds (with decrement of 1.0°C each cycle), 72°C for 20 seconds; and 30 cycles of 2 steps PCR with 94°C for 20 seconds, 55°C for 45 seconds. PCR products were evaluated by 1.5 % of agarose gel stained with ethidium bromide.

Amplification was done by using a thermal cycler (9700 Applied Biosystems, Foster City, CA, USA). The mixtures were made on ice to avoid artifacts formation. They were also handled within a sterilized working environment to avoid contaminations to the samples.

Positive controls were generated by first amplifying DNA samples archived at integrative Pharmacogenomics Institute (iPROMISE), Faculty of Pharmacy for the respective exons of (*OPRM*1). Samples were then sent for sequencing and the normal wild-type sample was chosen to be the positive control for the respective exons of *OPRM*1 gene.

The PCR products were then validated by running them onto a 1.5% agarose gel that contained ethidium bromide (EtBr). DNA bands were then visualized under an ultraviolet trans-illumination dock. Samples that were undetectable under the ultraviolet were considered unsuccessful and re-amplifications were done. All the samples were however successfully amplified after second attempt.

Post PCR Denaturing / Reannealing Step

Eight microliters of unknown samples were mixed with eight µl of the homogygous wild type positive control samples. A heteroduplex-formation step was carried out before subjecting the PCR product for heteroduplex analysis by DHPLC.

The condition was 30 cycles of Touch-Down PCR starting from 95 ºC for 3 minutes, at a rate of – 0.1 ºC every cycle until a temperature of 65 ºC was reached.

DHPLC Analysis and Sequence Determination

Varian® Helix System consisting of the following instruments: Varian 210 Solvent Delivery Modules, Varian 340 UV/Vis Detector, Varian 430 Autosampler, Varian 520 Oven and a Helix Column (Variant Inc., San Jose, California, US) was used for the DHPLC analysis.

Optimization of the DHPLC melting temperatures was determined using Stanford University's Melt Calculator (*http://insertion.stanford.edu/melt.html*). The sequences of exons 1, 2 and 3 of the *OPRM*1 gene were submitted to the DHPLC Melt Program. A list of suggested temperatures and buffer compositions specifically for each exon was generated by the program.

Further optimization of the melting temperature for each exon was also done to obtain a good chromatographic resolution for the variant peaks. Samples were initially run at temperatures 2 °C higher and lower of the recommended temperature for each exon (Table 1). Denaturation of the DNA was visualized with the peak pattern moving towards the injection peak, until it either disappeared or merged with the injection peak.

Restriction enzymes of *pUC 18 HaeIII* (SIGMA-ALDRICH, USA) and Wave Low Range Mutation Control Standard (Transgenomic, USA) were used in each run to confirm the size of the samples and validate the condition of the oven respectively. A sensitivity test was done to determine the lowest concentration of dsDNA that was able to be detected by the UV detector.

Direct Sequencing

Results obtained from DHPLC analysis were validated by direct DNA sequencing using the same primers as for DHPLC. The DNA samples were purified using QIAquickR PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced on ABI 3700 using BigDyeR Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The DNA sequences were aligned and compared to the gene sequence (accession no. AY587764) from NCBI Gen Bank (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi).

Statistical Analysis

Results were presented in percentage frequency with 95% confidence interval (CI). Chi-square (χ^2) test and Fisher exact tests were used for comparisons between two groups. Differences were considered significant if p value is less than 0.01; as indicated by stars (*).Data was analyzed by SPSS for Windows release 11.0.0 (SPSS, Inc., USA).

Genotype frequencies were predicted using Hardy-Weinberg equilibrium, $p^2 + 2(pq) + q^2 = 1$. The observed frequencies were compared with the frequencies of the predicted genotypes.

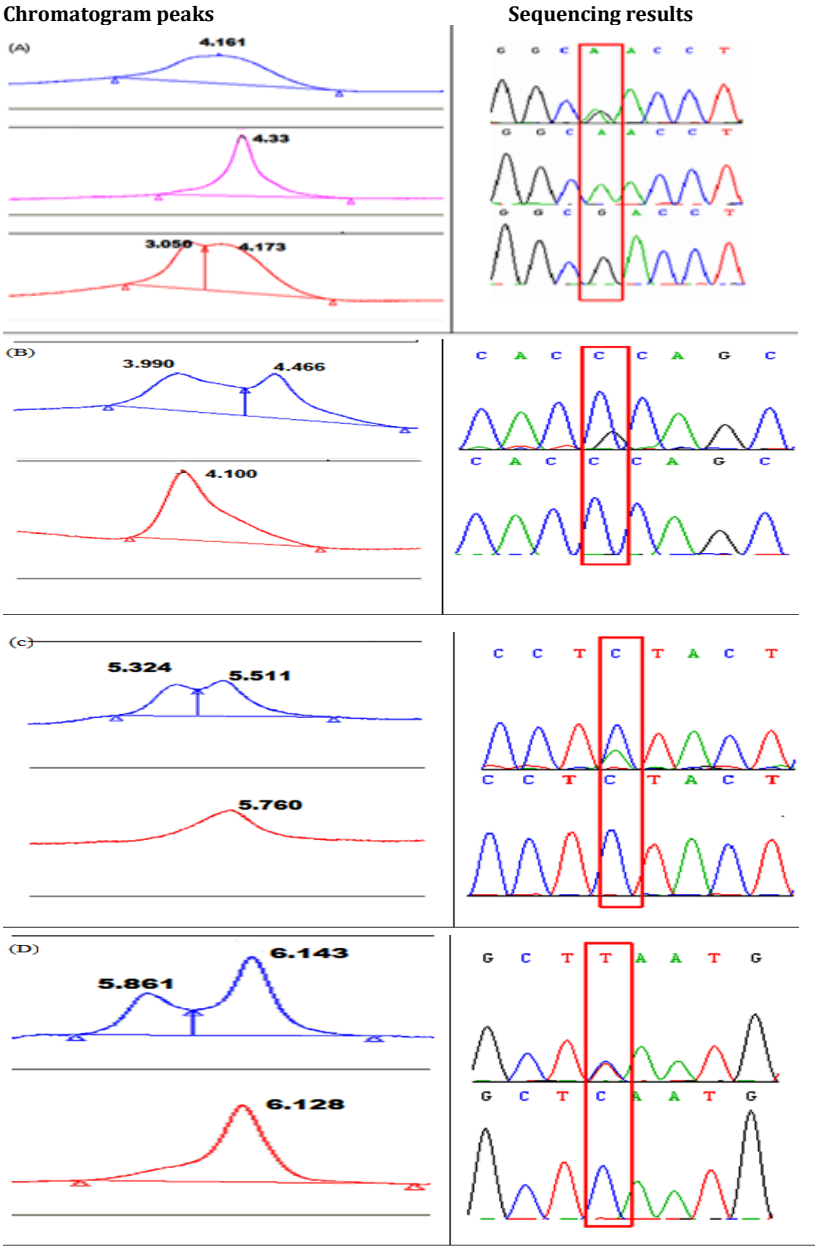
Odd ratio (OR) was calculated by dividing the risk factor of the IVDUs to the risk factor of the healthy volunteers to estimate the relative risk of a genetic variant in association with addiction.

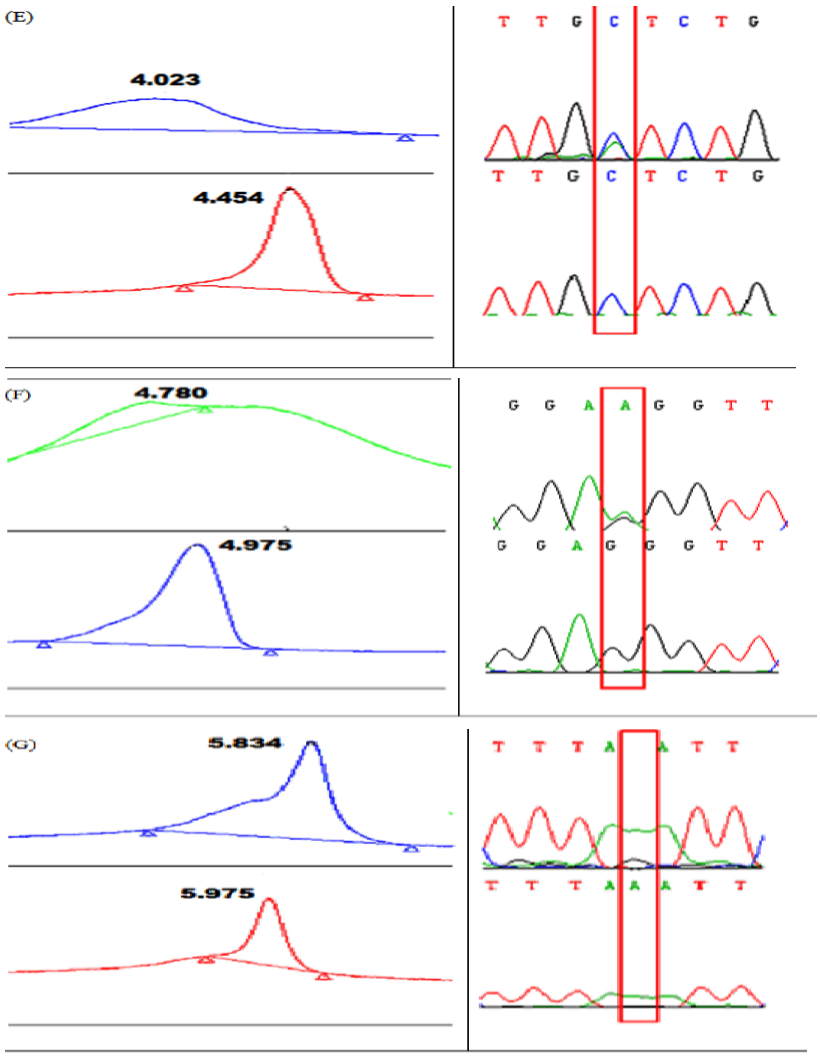
RESULTS AND DISCUSSION

The optimised melting temperature for each exon (Table 1) was able to detect variations that were observed by the changes in the peak profiles. Mismatches were detected by the appearance of one to three additional peaks (Figure 1 A to G). The peaks were also verified using Star Reviewer® software. All samples were tested, and samples that gave different profiles were confirmed with direct sequencing.

Table 1: Recommended DHPLC conditions by melt program for exons 1, 2 and 3 of the Mu-opioid receptor gene (OPRM1)

Exon and nucleotide sequence	Fragment size (bp)	Optimized Melting temperatures	Buffer flow rate (ml/min)
OPRM1 exon 1	500	59°C	0.45
OPRM1 exon 2	590	54°C and 58°C	0.45
OPRM1 exon 3	758	58°C and 59°C	0.45





Heterozygous OPRM1:c.338 CA
Homozygous OPRM1:c.338 CC wild type

Heterozygous OPRM1:c.IVS2+31 GA
Homozygous OPRM1:c.IVS2+31 GG

OPRM1:c.IVS2+759delA
OPRM1:c.IVS2+759delA wild type

A: OPRM1:c.118 AG, B: OPRM1:c.77 CG, C: OPRM1:c.226 CA, D: OPRM1:c.IVS1+79CT, E: OPRM1:c.338 CA, F: OPRM1:c.IVS2+31 GA, G: OPRM1:c.IVS2+759delA

Fig. 1: Chromatogram of peak profiles and direct sequencing results for OPRM1

In total, seven variants were detected. Five of them were newly reported which include *OPRM*1:c.77C>G, *OPRM*1:c.226C>A, *OPRM*1:c.IVS1+79C>T, *OPRM*1:c.338C>A, *OPRM*1:c.IVS2+759*del*A. The frequency of occurrence for each variant is as shown in Table 2.

Table 2: Comparison of the frequencies of genotypes between the IVDUs and the healthy controls with their respective 95% confidence intervals

	IVDUs				Healthy controls Genotype frequency (n/100)				χ^2 Test (Fisher Exact)
Genotypes	Genotype frequency (n/93)	95% confidence interval (CI) observed			Genotype frequency (n/100)	95% confidence interval (CI) predicted			
		Low	Frequency (%)	High		Low	Frequency (%)	High	P value
OPRM1:c.118GG	6	1.45	6.45	11.45	5	0.70	5.00	9.30	$\chi^2 = 0.189$, P = 0.664
OPRM1:c.118AG	15	8.62	16.12	23.62	8	2.70	8.00	13.30	$\chi^2 = 3.033$, P = 0.082
OPRM1:c.77CG	1	0.01	1.08	2.51	3	0.00	3.00	6.30	$\chi^2 = 0.880$, P = 0.174
OPRM1:c.226CA	0	-	-	-	3	0.00	3.00	6.30	NA
OPRM1:c.IVS1+ 79CT	0	-	-	-	3	0.00	3.00	6.30	NA
OPRM1:c.338CA	5	0.79	5.37	9.95	7	2.00	7.00	12.00	$\chi^2 = 0.218$, P = 0.640
OPRM1:c.IVS2+31GA	2	0.00	2.15	0.51	2	0.00	2.00	4.74	$\chi^2 = 0.005$, P = 0.944
OPRM1:c.IVS2+759 del A	0	-	-	-	1	0.00	1.00	2.95	NA

Legend: not detected; NA: not applicable Four SNPs were detected in exon - intron 1 region. The most common was nucleotide substitution of A to G at position 118. The substitution of amino acid has been reported to affect the binding affinity of the receptor [7]. Five (5.00%) of the healthy controls and 6 (6.45%) of the IVDUs were identified to be homozygous *OPRM*1:c.118GG. In total, 23 individuals from the population were detected to be heterozygous of *OPRM*1:c.118AG; 8.00% of healthy controls and 16.13% of the IVDUs respectively. The second variant detected in the current study which has been previously reported [16] was *OPRM*1:c.IVS2+31G>A. It was found to be located at exon - intron 2 of the receptor. Two DNA samples were detected to have this variant with the formation of heteroduplexes as shown in Figure 1 F.

Other variants were new. The *OPRM*1:c.77C>G SNP in exon 1 carries an amino acid substitution of proline to arginine at the position 26 of the amino acid chain. This was predicted using online program at *http://www.sciencelauncher.com/dna2 protein_calc.html*. A total of four (2.07%) DNA samples were found to have *OPRM*1:c.77C>G variation (one in the IVDUs and three in healthy controls). Substitution of C to A at nucleotide 226 of *OPRM*1 does not cause any amino acid change. Only three (3.00%) of the healthy controls were detected with this variant. Another variant that was detected in the healthy controls (3.00%) but none among the IVDUs was *OPRM*1:c.IVS1+79C>T in intron 1 of *OPRM*1. Heterozygous *OPRM*1:C.338CA was found to occur in quite high number in both healthy controls (7.00%) and IVDUs (5.37%).

The variant occurs in a total of 12 (6.21%) individuals; and was predicted to cause a substitution of alanine residue to aspartic acid (*http://www.sciencelauncher.com/dna2protein_calc.html*).

The least common variant that was found in this study was *OPRM*1:c.IVS2+759*del*A at intron 2. Only 1 individual was detected to carry this variant. Deletion of the single nucleotide at this position may cause a frame shift in the reading frame of *OPRM*1 gene.

Odd ratios were calculated to examine the relative risk of *OPRM*1 variants in association to the development of addictive behaviors. Odd ratios for variant alleles of *OPRM*1:c.118G, *OPRM*1:c.77G, *OPRM*1:c.338A and *OPRM*1:c.IVS2+31A are shown in Table 3. Variant allele of *OPRM*1:c.118G has an odd ratio of 1.72 which reflects the more likeliness of becoming an addict,

However it was not significant (OR 1.73; 95% CI: 0.92 – 3.25). *OPRM*1:c.IVA2+31A also showed the same pattern with an increased odd ratio of risk susceptibility (OR: 1.08; 95% CI: 0.15-7.71)

Table 3: Frequency variants among the IVDUs and healthy controls and their odd ratios

Allele	Allelic Frequency		Odds Ratio (95% CI)
	IVDU; N (%)	Healthy controls; N (%)	
OPRM1:c.118A	159 (85.49)	182 (91.00)	1.73 (0.92 - 3.25)
OPRM1:c.118G	27 (14.51)	18 (9.00)	
OPRM1:c.77C	185 (99.50)	197 (98.50)	0.36 (0.04 - 3.44)
OPRM1:c.77G	1 (0.50)	3 (1.50)	
OPRM1:c.338C	181 (97.31)	193 (96.50)	0.76 (0.24 - 2.44)
OPRM1:c.338A	5 (2.69)	7 (3.50)	
OPRM1:c.IVS2+31G	184 (98.92)	198 (99.00)	
OPRM1:c.IVS2+31A	2 (1.08)	2 (1.00)	1.08 (0.15 - 7.72)

wt: wild type allele

The IVDUs have higher scores (P<0.05) in novelty seeking and harm avoidance compared to the healthy controls (Table 4). The IVDUs were re-categorized according to their genotypes (heterozygous or homozygous) for *OPRM*1:c.118A>G. The behavioral patterns of the IVDUs were evaluated according to their respective genotypes. The novelty seeking scores for the IVDUs with *OPRM*1:c.118G variant allele (heterozygous and homozygous) was higher than IVDUs who were homozygous wild type *OPRM*1:c.118AA; however no significant difference was found (Table 5).

Table 4: Mean Scores in NS, HA and RD for IVDUs and the healthy controls

Scales	IVDUs		Healthy Controls		P value, t-test
	Mean	SD	Mean	SD	
Novelty-seeking (NS)	21.22	4.91	12.46	3.36	< 0.01*
Harm avoidance (HA)	18.24	5.02	14.36	5.20	< 0.01*
Reward dependence (RD)	13.75	3.70	17.60	2.40	> 0.01

* Statistically significant = P< 0.01

Table 5: Means scores of TPQ for the IVDUs with respect to OPRM1:c.118A>G variants

Genotypes	Sample No	Mean ± SD scores for TPQ		
	N	NS	HA	RD
Homozygous wild type OPRM1:c.118AA	72	16 ± 6.36	18 ± 1.83	14 ± 2.71
Homozygous OPRM1:c.118GG	6	20 ± 4.51	18 ± 3.49	16 ± 3.21
Heterozygous OPRM1:c.118AG	15	24 ± 5.55	16 ± 6.00	13 ± 4.11

Novelty seeking (NS), harm avoidance (HA) and reward dependence (RD)

It is very important to make sure that the PCR products were detergent free because detergent would bind permanently to the inside layer of the column and shorten the half life of the column. In this study, detergent free GoPhorlT™ *Taq* DNA Polymerase (MBioTech, Korea) was used. In addition, one type of PCR tubes was used throughout this study as PCR tube may contain particles that produce peaks that interferes with the peak profiles of samples.

One sample with a size of 500 bp which spanned exon 1 and intervening sequences in intron 1 was used as the template in this study to investigate the sensitivity of the dHPLC. Current method allows detection of the dsDNA as low as 13 ng/µL.

The DHPLC is an automated high throughput method with the advantage of a high sensitivity that can be used to screen novel mutation [20]. Very distinguishable peak profiles (Figure 1 A to G) was observed for the seven variants. In addition, DHPLC is cost and time efficient as each run only takes eight minutes for complete resolution of peak profiles. It was able to detect known and unknown variants in the *OPRM*1 gene. There were five new variants detected; which include *OPRM*1:c.77C>G, *OPRM*1:c.226C>A, *OPRM*1:c.IVS1+79C>T, *OPRM*1:c.338C>A, *OPRM*1:c.IVS2+759*del*A. The most common variants found in the IVDUs and also the healthy controls were *OPRM*1:c.118A>G and *OPRM*1:c.338C>A. Other variants observed to be at a much lower frequencies.

The frequencies of the allelic and genotypic variations of *OPRM*1:c.118A>G along with *OPRM*1:c.226C>A and *OPRM*1:c.IVS1+79C>T were higher in the IVDUs compared to the healthy controls. The *OPRM*1:c.226C>A and *OPRM*1:c.IVS1+79C>T variants however do not carry any amino acid change. Thus, it is less likely to cause any alteration in the binding of ligands to the *OPRM*1. The substitution of A to G which causes an amino acid change from an asparagine residue to an aspartic residue in amino acid position 40 for variant *OPRM*1:c.118A>G has been implicated in an increased beta-endorphin binding affinity and a decreased potency of morphine-6-glucuronide [7]. This could lead to the increased consumption of opioid drugs to achieve physiological effects in individuals carrying this variant and eventually result in dependence and addiction. Our finding suggests the possible association between addiction and the *OPRM*1:c.118A>G variant itself which have been reported by other researchers [1, 6, 21].

While the difference in the occurrence of *OPRM*1:c.IVS2+31G>A variant was small between the healthy controls and the IVDUs (2.00% vs 2.15%), individuals with *OPRM*1:c.IVS2+31G>A variant were reported to have tendencies of taking 2.5-fold higher daily heroin intake in comparison to individuals without the variant [21]. This variant thus imposes a possible risk to drug addiction.

The polymorphism of *OPRM*1:c.77C>G among IVDUs was the first to be reported. In *Rhesus* this variant was in parallels with the functional effects of the *OPRM*1:c.118A>G polymorphism in humans [22]. Since the position of 77 in exon 1 is located at the N-terminus of the G-coupled protein receptor, it is possible that this SNP could possibly decrease the binding affinity of its ligands to *OPRM*1 in human. The lack of significance in the results could be due to the small sample size. As for the variant alleles of *OPRM*1:c.338A and *OPRM*1:c.77G, a negative association was indicated (0.76 and 0.36 respectively). On the other hand, *OPRM*1:c.IVS1+79C>T and *OPRM*1:c.IVS2+759*del*A was found at lower frequencies among the IVDUs. *OPRM*1:c.226C>A was not detected among the IVDUs. Whether these variants contribute to protective effect towards addiction however requires further study as variation at the intronic sites has been associated to exon skipping [23].

The association of *OPRM*1 variants and the risk of addiction was established with the odd ratios. There was a trend of positive association for *OPRM*1:c.118A>G and *OPRM*1:c.IVS2+31A in increasing risks of addiction even though it was not statistically significant. This could be also due to small sample size of the IVDUs with each particular variant type.

TPQ scales measure the different aspects of temperaments that were associated with distinct neurotransmitters (NS for dopamine; HA for serotonin; and RD for norepinephrine) [16]. The result of the assessment shows that the IVDUs could possibly have a higher level of activity for dopamine and serotonin while a lower level of activity for norepinephrine neurotransmitters. These neurotransmitters were derived from different monoaminergic-related genes [1]. Monoamine neurotransmitters especially dopamine, serotonin and norepinephrine regulates the activity of the HPA axis which is under the physiological control of *OPRM*1 [21]. This explains why the IVDUs are more likely to turn into addiction. All of the IVDUs with variant *OPRM*1:c.118A>G also took marijuana within 30 days before their first injection. In comparison to the IVDUs with homozygous wild type, 86% of them took marijuana within the 30 days. It is also noticeable that the IVDUs with variant alleles had their first injection at a younger age (11 to early 30s years old) then the wild type individuals (11 to late 40s years old). A further statistical analysis revealed that an individual with the *OPRM*1:c.118A>G variant have 62% chance of turning into an addict, while individuals without the variant would only have 45% chance of being an addict.

CONCLUSION

In conclusion, we have successfully developed a medium throughput DHPLC method for the detection of genetic variations in the coding regions of *OPRM*1. Five new variants were detected among the subjects. A trend of positive association for *OPRM*1:c.118A>G and *OPRM*1:c.IVA2+31A in increasing risks of addiction was found but not statistically significant due to small sample size of the IVDUs with each particular variant type. Carriers of *OPRM*1:c.118A>G and evaluation of personalities using TPQ could provide valuable information on an individual's risk in becoming an addict.

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