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GRK5 Is Associated with the Regulation of Methadone Dosage in Heroin Dependence

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Abstract

Background: There is no countable biomarker for opioid dependence treatment responses thus far. In this study, we recruited Taiwanese methadone maintenance treatment patients to search for genes involving the regulatory mechanisms of methadone dose by genome-wide association analyses.
Methods: A total of 344 Taiwanese methadone maintenance treatment patients were included in a genome-wide association study. The involvement of *GRK5* in opioid dependence was then further confirmed by gene expression study on lymphoblastoid cell lines derived from 3 independent age- and gender-matched groups: methadone maintenance treatment patients, medication-free former heroin abusers, and normal controls.
Results: The results indicated that *GRK5*, the gene encoding an enzyme related to μ-opioid receptor desensitization, is associated with methadone dose by additive model of gene-based association analysis ($P = 6.76 \times 10^{-5}$). We found that 6 of the 55 single nucleotide polymorphisms from the genome-wide genotype platform and 2 single nucleotide polymorphisms from the 29 additionally selected single nucleotide polymorphisms were significantly associated with methadone maintenance dose in both genotype and allele type ($P \leq .006$), especially in patients who tested negative in the urine morphine test. The levels of *GRK5* gene expression were similar between methadone maintenance treatment patients and medication-free former heroin abusers. However, the normal controls showed a significantly lower level of *GRK5* gene expression than the other groups ($P = .019$).
Conclusions: The results suggested an important role for *GRK5* in the regulatory mechanisms of methadone dose and course of heroin dependence.

Keywords: *GRK5*, haplotype, methadone maintenance dose, MMT, SNP

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Significance Statement

There is no countable biomarker for opioid dependence and its treatment responses thus far. In this study, we first recruited 344 Taiwanese methadone maintenance treatment (MMT) patients to search for genes involved in the regulatory mechanisms of methadone dose by genome-wide association analyses. The results indicated that *GRK5*, the gene encoding an enzyme related to μ-opioid receptor desensitization, is associated with methadone dose. The involvement of *GRK5* in opioid dependence was then further confirmed by gene expression study on cell lines derived from 3 independent age- and gender-matched groups: MMT patients, medication-free former heroin abusers, and normal controls. Our findings provide novel evidence suggesting that *GRK5* could be a useful indicator for the severity of opioid dependence and a biomarker for methadone dosage. This study is the first clinical research supporting *GRK5* as a candidate gene involving the regulation of methadone dosage in opioid dependence.

Introduction

Methadone is a well-established medication for treatment of heroin dependence (Mattick et al., 2009). Methadone maintenance therapy (MMT) has shown superior treatment effectiveness particularly in terms of patient retention rate and reduction in heroin use compared with no treatment (Mattick et al., 2002, 2003, 2009). However, determination of an optimal methadone dosage remains a challenge for clinicians because of several serious potential adverse effects. The optimal methadone dosage is affected by various physiological and psychological statuses of patients (Hiltunen and Eklund, 2002; Murray et al., 2008; Sullivan et al., 2014; Mouly et al., 2015). A steady-state methadone concentration in the blood is usually achieved when patients receive daily treatment of methadone for longer than 5 times the half-life (Chan and Sim, 2015). A steady-state dosage is influenced by both individual metabolic activity and receptor-coupled mechanisms of actions toward the treatment responses. Thus, it would be a better sample to use MMT patients who had reached a steady dosage of treatment in search of genes related to dosage regulation.

A few genes encoding drug transporters, P-glycoprotein transporters, have been reported to be associated with methadone dose, for example, *ABCB1* (ATP binding cassette subfamily B member 1) gene in a Jewish (Levran et al., 2008) and an Australian cohort (Coller et al., 2006) respectively. The gene encoding liver metabolic enzyme cytochrome P-450 (CYP) 2B6 has also been reported to be associated with methadone dose in an Israeli study (Levran et al., 2013a). Most studies reported a set of genes that are involved with both metabolic and receptor coupled pathways. For example, *ABCB1* and mu-opioid receptor *OPRM1* genes in Australian methadone MMT patients (Barratt et al., 2012); *CYPs* and *ABCB1* genes in Caucasian patients (Fonseca et al., 2011); *BDNF*, *NTRK2*, *OPRM1*, *DRD2*, and *ANKK1* genes in Israeli patients (Levran et al., 2013b); and *ABCB1*, *CYP2B6*, *OPRM1*, *ANKK1*, and *DRD2* genes in Taiwanese patients (Hung et al., 2011). These reports suggest that more than one single gene is involved with the biological mechanisms underlying the regulation of methadone dosage.

In our previous studies in a Taiwanese MMT patient cohort, we found that *CYP2C19* (Wang et al., 2013b), *2B6*, *2C19*, *3A4* (Tsai et al., 2014), and *OPRM1* genes (Wang et al., 2012) had demonstrated marginally significant associations with methadone dose. In the present study, we performed a genome-wide association study with 344 MMT patients and analyzed genes associated with steady-state methadone dose. The gene encoding guanine nucleotide-binding protein (G protein)-coupled receptor kinase 5 (*GRK5*) was identified as a gene significantly associated with methadone dosage. Hence, more SNPs in the significant genetic regions in *GRK5* were further selected and genotyped. GRK5 is an enzyme that belongs to the G protein-coupled receptor kinase subfamily of the Ser/Thr protein kinase family (Pitcher et al., 1998). It has been reported that GRK5 is responsible for desensitization of mu-opioid receptor (Mann et al., 2015). We speculate that this may be one of the mechanisms through which methadone dosage is regulated.

Methods

All methods in this study were performed in accordance with the guidelines and regulations of the institutional review committees. The study protocol was approved by the institutional review boards of the National Health Research Institutes (EC0970504, Zhunan, Taiwan) and the 7 participating hospitals: Tao-Yuan Mental Hospital, En-Chu-Kong Hospital, Far-Eastern Memorial Hospital, Taipei City Hospital Song-De and Yang-Ming Branches, China Medical University Hospital, and Wei-Gong Memorial Hospital (Wang et al., 2013a). Written informed consent was obtained from all participants. The project has also been registered with the National Institutes of Health (NIH) Clinical Trial (https://clinicaltrials.gov/ct2/show/NCT01059747 for 344 MMT patients). The inclusion criteria were aged ≥18 years, receipt of MMT for at least 3 months with regular attendance for the past 7 days, and a methadone dosage adjustment of no more than 10 mg in the past 7 days. Exclusion criteria were co-morbidity with medical or other mental disorders requiring immediate treatment and pregnancy.

The genome-wide genotype data were obtained from a cross-sectional study recruited from year 2008 to 2009. A total of 344 MMT patients had passed the genome-wide association study (GWAS) quality controls with available detailed data regarding plasma methadone concentrations and urine morphine test results (Yang et al., 2016). To further verify the role of *GRK5* in heroin addiction and methadone treatment, additional age- and gender-matched normal controls (n = 23) who did not have any psychiatric or substance abuse history, and former heroin abusers (n = 23) who were medication-free and abstinent for average 2 years were included in the gene expression study. The gene expression study protocol was approved by the institutional review board of the National Health Research Institutes (EC0980209-R5, Zhunan, Taiwan). Written informed consents were also obtained from all participants. The former heroin abusers were recruited mainly from nongovernment organization-affiliated, medication-free therapeutic communities. Subjects in the medication-free former-heroin abuser group met the following criteria: (1) lifetime DSM-IV diagnosis of opioid dependence; and (2) no illicit drug use other than opioid or alcohol intoxication in the last 4 weeks. The normal control subjects were recruited from referrals, advertisements, and posted notice, and they met the following criteria: (1) no lifetime

DSM-IV diagnosis of opioid abuse or dependence; and (2) no any illicit drug use nor alcohol intoxication in the past 28 days (https://clinicaltrials.gov/ct2/show/results/NCT01668706 for former heroin abusers and normal controls of NIH registration). All these subjects were included for comparison in the gene expression study. Urine specimens were collected for screening of illicit drug use. Venous blood was taken for routine biochemical examinations and viral infectious disease profile.

Clinical Assessments

Demographics, clinical characteristics, and methadone treatment courses, including the methadone dose, treatment duration, and treatment adherence in the previous week, were obtained from medical records. Several interviewer-administered assessments including the Treatment Outcomes Profile (Marsden et al., 2008), which measures the amount and frequency of alcohol and other illicit substance use in the past 28 days, and the Clinical Opioid Withdrawal Scale (COWS), which measures the severity of 11 opioid withdrawal symptoms (Wesson and Ling, 2003), were conducted before methadone was administered on the test day. A COWS score >5 is considered as significant withdrawal symptoms (Tompkins et al., 2009). Anti-hepatitis C virus (HCV) antibody was measured by electrochemiluminescence immunoassay on Cobas e601 (Roche Diagnostics, Basel, Switzerland), and anti-HIV antibody was measured using Abbott chemiluminescence immunoassay (Abbott Diagnostics, Chicago, IL). The levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with Kinetic UV method. The level of gamma-glutamyl transpeptidase (γ-GT) was measured with enzymatic colorimetric assay. These assays were performed using the Integra 800 device (Roche Diagnostics).

Urine Morphine Test

Urine specimens were collected prior to administration of methadone on the study day. In our present analyses and previous reports, the urine morphine test was used as a surrogate measurement for the methadone treatment outcome.

Genome-Wide SNP Genotyping

Genomic DNA was extracted from the buffy coat of 6 mL whole blood. Genotyping was performed with the Axiom Genome-Wide CHB 1 Array, which was population-optimized to have a better genomic coverage of common alleles (minor allele frequency >5%) of the Han Chinese genome. All sample call rates were >98.8%, and the mean individual sample call rate was 99.7 ± 0.18%. Details of quality control and raw data can be accessed in Gene Expression Omnibus (GEO accession number: GSE78098; see also supplementary Methods) (Yang et al., 2016).

GRK5 SNP Selection and Genotyping

Genotypes of the additionally selected 29 SNPs in *GRK5* were identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Rodi et al., 2002). In brief, the primers and probes flanking the SNPs were designed using SpectroDESIGNER software (Sequenom, San Diego, CA). DNA fragments (100–300 bp) encompassing each SNP site were amplified by PCR (GeneAmp 9700 thermocycler, Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. After the unincorporated dNTPs were removed and the PCR reaction was inactivated by shrimp alkaline phosphatase, primer extension was performed by adding appropriate probes to the PCR reaction. Various nucleotide extension products were differentiated by matrix-assisted laser desorption/ionization-time of flight analysis (Tost and Gut, 2005).

EBV-Transformed Lymphoblastoid Cell Culture

Lymphocytes from subjects were transfected with Epstein-Barr virus (EBV) then cultured as lymphoblastoid cell line for gene expression assay (see also supplementary Methods). Total RNA of EBV-transformed lymphoblastoid cells was extracted by TRIzol Reagent (Life Technologies, Inc., Gaithersburg, MD).

GRK5 Gene Expression

A total of 63 MMT patients selected from the 344 MMT patients in consideration of minor genotype carriers, 23 age- and gender-matched normal controls, and 23 medication-free abstinent former heroin abusers were included in the gene expression study (see also supplementary Methods). Gene expression was quantified relatively to *TBP* expression, the relative quantification method, using ABI StepOne Plus Software. The relative expression level of *GRK5* compared with that of *TBP* was defined as $-\Delta CT = -[CT_{GRK5} - CT_{TBP}]$, where CT was the cycle threshold. The *GRK5* mRNA/*TBP* mRNA ratio was calculated from $2^{-\Delta CT} \times K$, in which K was a constant.

Statistical Analyses

Summary statistics such as means and SDs were used to describe the samples. Means among different cohorts were compared using the Kruskal-Wallis test for 3-group comparison or Mann-Whitney U-test for 2-group. Categorical variables among cohorts were compared using a standard chi-square test. Spearman correlation coefficient was used to test the associations between the methadone dosage and total COWS score. The aforementioned analyses were conducted using SAS software, Version 9.4 (SAS Institute, Inc., Cary, NC).

Gene-based analyses were performed using the Knowledge-based mining system for Genome-wide Genetic studies (KGG) (Li et al., 2012). Single-SNP association *P* values were first calculated using PLINK (Purcell et al., 2007). In KGG, the SNPs were divided into linkage disequilibrium blocks, and a score based on the extended Sime's test (Li et al., 2011) was calculated for each block using the single-SNP *P* values. The scores for blocks within a gene were combined into a scaled chi-square statistic as a hybrid set-based test. KGG also produced Q-Q plots for the gene-based *P* values, single-SNP *P* values for SNPs in genes, and single-SNP *P* values for SNPs outside genes. We then calculated the genomic inflation factor, lambda value (Devlin and Roeder, 1999), for the gene-based *P* values.

To further evaluate the effects of SNPs in a candidate gene identified by KGG, single SNP association tests were performed for SNPs that were more densely genotyped in that gene. The genotypic or allelic models for testing associations of SNPs with the treatment outcomes were performed using the Correlation/Trend test provided in SNP & Variation Suite, Version 8.4.0 (Golden Helix, Inc., Bozeman, MT). We also performed haplotype association tests in these densely genotyped SNPs. Global *P* values of the haplotype association tests for methadone dose were calculated using the generalized estimating equation model. The haplotype analysis was also conducted using SAS software, Version 9.4 (SAS Institute, Inc). Mann-Whitney U tests were

performed to compare the difference in gene expression levels between groups. The 2D dot plot and the Manhattan plot, which presents the –log10 of genome-wide *P* values, were calculated and plotted by GraphPad Prism 5 (GraphPad Software, San Diego, CA). The regional visualization of association plots was plotted by LocusZoom (Pruim et al., 2010).

False discovery rate was employed to correct for multiple comparisons, that is, an adjusted *P* value is defined as the smallest significance level for which the given hypothesis would be rejected when the entire family of tests is considered (Storey, 2003). Hardy-Weinberg equilibrium tests were performed using HAPLOVIEW version 4.2 (Barrett et al., 2005). A statistically significant level was based on a 2-sided test and assigned for $P < .05$.

RESULTS

Clinical Characteristics and Methadone Treatment Outcomes

A total of 344 MMT patients passed the quality controls (Yang et al., 2016) with an average age around 38 years, and the percentage of males was 82 (Table 1). All patients had taken methadone daily in the previous 1 week. The average total withdrawal symptom scores rated by COWS >5 (n = 23) were 6.6 ± 2.3 in the 344 MMT patients. The methadone dose did not show correlations with the total COWS score >5 (Spearman correlation coefficient, n = 23, r = 0.34, *P* = .12 in MMT patients). The clinical characteristics and single gene observations from this cohort were well characterized for the recruitment and treatment responses in our previous studies (Wang et al., 2013a).

Identification of Genes Associated with the Methadone Dose Using Gene-Based Models in the GWAS

PLINK linear regression was employed for the 344 subjects and 615 216 SNPs. KGG was employed for the gene-based model analyses. Total of 15 553 genes were tested for associations with methadone dose in the gene-based tests using the additive model. The methadone dose was most statistically significant associated with the *GRK5* gene in the gene-based test using the additive test (supplementary Figure 1A; supplementary Table 1). The genomic inflation factor, lambda value, was 0.892, suggesting that there were no population stratification and systematic genotyping errors in our sample. The Q-Q plots in supplementary Figure 1B showed that the *P* values for gene-based tests and for SNPs within and outside genes mostly fell within the 95% CIs of the expected values, again suggesting that there was no inflation of type I error rates in our tests.

Table 1. Demography of the MMT Patients

Variable	n	Mean ± SD
Age	344	38.16 ± 7.69
Gender [male (%)]	281	(81.69)
BMI (kg/m^2)	341	23.64 ± 3.52
Methadone dosage (mg/d)	344	55.22 ± 28.47
Sum of COWS	344	1.51 ± 1.87
Urine morphine (+) (%)	173	(50.58)
HCV (+) (%)	313	(94.56)
HIV (+) (%)	76	(22.55)
Liver function test		
AST (U/L)	326	52.42 ± 57.06
ALT (U/L)	332	61.13 ± 75.30
γ-GT (U/L)	310	63.38 ± 104.68

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; COWS, Clinical Opioid Withdrawal Scale; MMT, methadone maintenance treatment; γ-GT, gamma-glutamyl transpeptidase.

SNPs in *GRK5* Were Associated with the Methadone Maintenance Dose

GRK5 gene is located at the 10q26.11 chromosome region, spans approximately 248 kb pairs, and contains 16 exons. A total of 55 SNPs within the *GRK5* genetic region were placed in the genome-wide genotyping platform in which 6 (rs4752269, rs2061078, rs12415832, rs4752300, rs10787966, rs1889432) of the 55 SNPs (supplementary Table 2) showed significant associations with methadone dose. To confirm the genetic association results with methadone dose, 29 additional SNPs were selected according to the minor allele frequency >0.1 on the HapMap of Chinese ethnic group (http://hapmap.ncbi.nlm.nih.gov), the functions predicted by the bioinformatics FastSNP (Yuan et al., 2006), and reports in the literature (Jakobsdottir et al., 2005; Arawaka et al., 2006; Tarantino et al., 2011). Six (rs871196, rs11819686, rs12780837, rs10886472, rs11198907, and rs1537576) of the 29 SNPs showed significant associations with methadone dose. All SNPs were in Hardy-Weinberg equilibrium. Eight SNPs (rs4752269, rs2061078, rs12415832, rs10886472, rs11198907, rs4752300, rs10787966, and rs1889432) were significantly associated with methadone dose in MMT patients (Figure 1). The minor allele type carriers of these 8 SNPs had a higher methadone dose than the major allele type carriers in all MMT patients. SNP rs10886472 showed the highest level of significance in association with the methadone dose in all MMT patients (supplementary Table 3). The significant associations were mainly contributed from the urine morphine test-negative MMT patients (supplementary Table 4). In addition, it remains statistically significant after adjusting for HCV infection rate. The allele frequencies of these SNPs are similar to those in the Han Chinese in Beijing, China population in the National Center for Biotechnology Information (supplementary Table 5).

GRK5 Haplotypes Were Associated with Methadone Dose

Two haplotype blocks were created by Haploview from the 8 SNPs in *GRK5* (supplementary Figure 2). They are block 1 located at the intron 3–4 consisting of 3 SNPs (rs10886472-rs11198907-rs4752300), and block 2 located at the intron 4–6 consisting of 2 SNPs (rs10787966-rs1889432) (supplementary Table 6A). The major haplotypes (CGG haplotype frequency 76% in block 1, and GA haplotype frequency 77% in block 2) within each block were significantly associated with methadone dose. Carriers with the major haplotype had a lower methadone dose than those with the minor haplotype. The haplotype blocks that showed significant associations with methadone dose were mainly observed in the urine morphine test-negative patients (supplementary Table 6B).

Functional SNP Estimation in *GRK5*

rs11198907, rs4752300, and rs10787966 in the intron 4, among the 8 SNPs, showed significant associations with the level of *GRK5* gene expression in the genotype recessive model (supplementary Table 7). The significant level of associations was higher

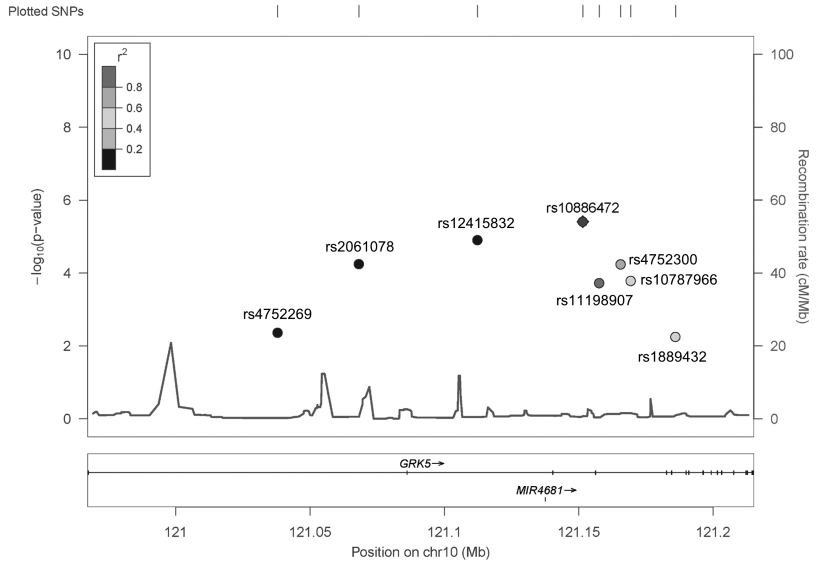

Figure 1. Correlation/trend association of the 8 single nucleotide polymorphisms (SNPs) in guanine nucleotide-binding protein (G protein)-coupled receptor kinase 5 (*GRK5*) gene-differentiated treatment response according to methadone dosage in 344 methadone maintenance treatment (MMT) patients using the regional visualization of association plots in LocusZoom.

in the MMT patients who tested negative in the urine morphine test (Mann-Whitney U test, $P = .048$, .031, and .041, respectively, in all patients vs .021, .021, and .035, respectively, in the MMT patients with a negative urine morphine test) (supplementary Figure 3).

MMT Patients and Medication-Free Former Heroin Abusers Showed Higher Level of *GRK5* Gene Expression Than Normal Controls

To further verify the role of *GRK5* gene in opioid dependence, we recruited additional former heroin abusers (n = 23) who had been medication-free and abstinent from opioid use for at least 2 years and normal controls (n = 23) who had never used any illicit substance. They were compared with 63 age- and gender-matched subjects from the 344 MMT patients (Table 2). MMT patients showed a similar level of *GRK5* gene expression compared with medication-free former heroin users. However, MMT patients showed a significantly higher level of *GRK5* gene expression in culture lymphoblastoids than normal controls (Mann-Whitney U test, $P = .019$) (Figure 2).

Discussion

The present study was designed to identify genes associated with methadone treatment dose for opioid dependence by GWAS. *GRK5* gene expression assays were further tested in medication-free abstinent former heroin abusers, normal controls, and patients under MMT to confirm the involvement of *GRK5* in the course of opioid addiction and pharmacodynamics of methadone.

The activity of mu-opioid receptor in processing consequent signal transduction is regulated by several G protein-coupled receptor kinases (Mann et al., 2015). Phosphorylation of the mu-opioid receptor by GRK5 could initiate further hierarchical phosphorylation cascade. The results in this study support the role of *GRK5* in the regulatory mechanism of methadone dosage. We found in the current GWAS study that a genetic polymorphism, rs10886472 (intron3), within the 8 SNPs located from introns 1 to 6 of *GRK5* gene showed the highest significant level in association with methadone maintenance dose. The significant associations were mainly contributed from the urine morphine test-negative MMT patients, which represent a subgroup of patients who had a better methadone treatment outcome. On the contrary, association between *GRK5* and methadone dose was not found in those MMT patients with a positive urine morphine test. The haplotype blocks constructed by the 8 SNPs in *GRK5* also demonstrated strong significant associations with methadone dose. In the *GRK5* gene expression tendency analyses of rs11198907, rs4752300, and rs10787966 located within intron 4 (supplementary Figure 3), major genotype carriers showed a higher level of *GRK5* expression and a lower methadone dose than the minor genotype carriers. In an animal study, the *Grk5* knockout mice lost the manifestation of morphine reward and dependence (Glück et al., 2014). These observations all suggest crucial roles of GRK5 in the regulation of methadone dosage and course of heroin addiction. The possible effects of *GRK5* gene expression on the decreased reward sensation in the heroin-addictive patients under MMT warrant further investigation.

A recent genome-wide association study where the methadone doses were obtained by self-report from subjects identified a new significant locus located at the upstream of *OPRM1* gene (Smith et al., 2017). However, the most significant SNP, rs73568641, in the report showed a very low minor allele frequency (0.04) in Han Chinese in Beijing, China and JPT populations (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=73568641). We therefore examined a few other SNPs located nearby rs73568641 and found that *OPRM1* genetic loci showed only marginal significant associations with methadone dose in our subjects where the most significant *P* value was .004 in single SNP association analysis (supplementary Figure 4).

Elevated level of *GRK5* gene expression in the lymphoblastoids of MMT patients and medication-free former heroin abusers may be related to desensitization of mu-opioid receptor, which may indicate a more severe status of chronic opioid dependence. In this study, *GRK5* gene expression among the 3 age- and gender-matched cohorts showed a rank order of expression level as follows: The levels of gene expression are

Table 2. General Demography of the Subjects Recruited for the Verification Study: Patients under Methadone Treatment (MMT), Medication-Free Former Heroin Abusers, and Healthy Normal Controls

	MMT patients (n = 63)		Former heroin abusers (n = 23)		Normal Controls (n = 23)		
Variable	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	*P* value
Age	63	38.14 ± 6.13	23	38.04 ± 6.73	23	37.39 ± 6.65	.876[a]
Gender							.763[b]
Male	50	(79.37%)	19	(82.61%)	17	(73.91%)	
Female	13	(20.63%)	4	(17.39%)	6	(26.09%)	
BMI	63	23.91 ± 3.36	22	25.98 ± 3.25	23	24.17 ± 4.05	**.012**[a]
Cotinine (ng/mL)	63	350.72 ± 169.32	23	13.32 ± 48.35	23	39.69 ± 81.03	**<.001**[a]
Nonsmoking	2	(3.17%)	19	(82.61%)	16	(69.57%)	**<.001**[b]
Smoking	61	(96.83%)	4	(17.39%)	7	(30.43%)	
HIV							**.021**[b]
Negative	47	(77.05%)	20	(90.91%)	23	(100.00%)	
Positive	14	(22.95%)	2	(9.09%)	0	(0.00%)	
HCV							**<.001**[b]
Negative	5	(8.62%)	8	(36.36%)	23	(100.00%)	
Positive	53	(91.38%)	14	(63.64%)	0	(0.00%)	
Liver function test							
AST (U/L)	60	45.05 ± 34.13	22	48.91 ± 38.94	23	25.48 ± 10.24	**.001**[a]
ALT (U/L)	61	54.87 ± 48.55	22	77.55 ± 76.34	23	30.78 ± 20.44	**.012**[a]
γ-GT (U/L)	59	51.88 ± 95.77	22	40 ± 25.13	23	55.13 ± 63.62	.825[a]

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, gamma-glutamyl transpeptidase; HCV, Hepatitis C virus antibody; HIV, Human Immunodeficiency Virus.

[a] Kruskal-Wallis test.

[b] Chi-square test. *P* values < .05 were shown in bold font.

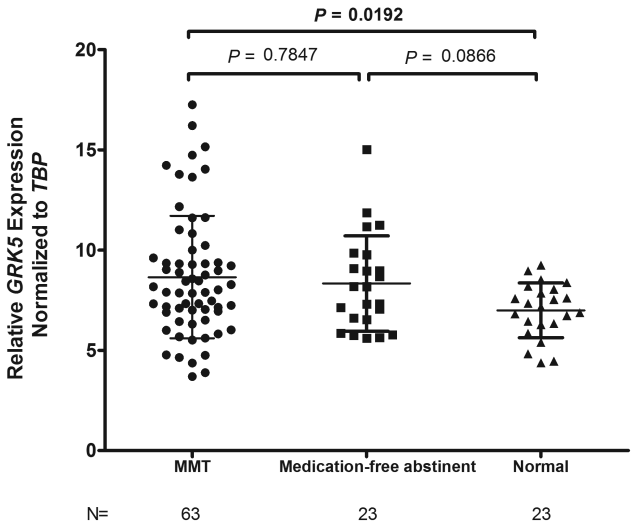

Figure 2. Levels of guanine nucleotide-binding protein (G protein)-coupled receptor kinase 5 (*GRK5*) gene expression among methadone maintenance treatment (MMT) patients, medication-free abstinent former heroin abusers (abstinent), and normal controls (normal). Data were presented as means ± SD. N: number of subject.

similar between MMT patients and medication-free abstinent former heroin abusers, but lower in the normal controls group. To our knowledge, our result is the first human study suggesting that MMT patients may potentially show a higher level of desensitization of mu-opioid receptor, as shown in the level of *GRK5* gene expression. As mentioned above, phosphorylation of receptor is one of the mechanisms through which *GRK5* influences desensitization of mu-opioid receptor (Mann et al., 2015). The results also support the hypothesis that *GRK5* is involved with the development of opioid dependence. An animal study reported increased gene expression of *Grk5* in some brain regions in rats that were in a state of morphine withdrawal precipitated by naloxone (Fan et al., 2002).

Many heroin-dependent patients are also infected with HCV (Batki et al., 2010; Wang et al., 2011). In this study, the HCV infection rate was 94.6% in 344 MMT patients (Table 1), 91% in the 63 selected age- and gender-matched MMT patients, 63.6% in the 23 former heroin abusers, and 0% in normal controls (Table 2). It is known that HCV infection may influence the serum levels of AST and ALT (Crofts et al., 1993; Zechini et al., 2004; Aragon and Younossi, 2010). This could explain why the levels of AST and ALT were higher in MMT patients and former heroin abusers than the normal controls in the gene expression study (Table 2).

Limitations of this study include that all the identified genes did not pass the statistically significant threshold set for GWAS ($P < 3.2 \times 10^{-6}$). This indicated that the sample size in this study remains small for GWAS, which is common in pharmacogenomics studies (Motsinger-Reif et al., 2013). Gene expression was analyzed indirectly from EBV-transformed lymphoblastoid. Also, neuropsychological measures, for example, brain imaging, etc., are not available in this study. This precluded further explanations for the consequence of neurological changes after *GRK5* expression alternations. Most subjects in this cohort were male, and 95% of these patients tested positive for HCV. In addition, the study was a cross-sectional design. However, participants in the study were recruited from a well-characterized, steady-state MMT cohort (Wang et al., 2013a). Medication compliance issues were well controlled by recruiting only subjects who had continuously undergone the MMT and shown regular attendance at the clinic. As the *GRK5* gene has been reported to interact with multiple receptors and signal transduction pathways (Watari

et al., 2014), future studies should be conducted in search of pathways involving the methadone dose. Studies attempting to replicate the results and explore neuronal mechanisms of *GRK5* for methadone treatment and opioid use are in need.

In summary, we identified that SNPs and haplotypes in *GRK5* were associated with methadone dose in this study. When the levels of *GRK5* gene expression were compared among 3 independent age- and gender-matched cohorts, MMT patients and medication-free former heroin abusers showed a higher level of gene expression than normal controls. These results suggested the involvement of *GRK5* with the regulatory mechanisms of methadone dose and course of heroin dependence.

Supplementary Materials

Supplementary data are available at: *International Journal of Neuropsychopharmacology* online.

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Statement of Interest

None.

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