Genetic polymorphisms in *CYP3A4* are associated with withdrawal symptoms and adverse reactions in methadone maintenance patients

Aim: Methadone maintenance therapy is one of the standard treatments for heroin addiction. The isozyme CYP3A4 of the CYP system is one of the metabolic enzymes, as well as CYP2B6, responsible for the metabolism of methadone. The aim of the present study is to evaluate the potential use of genetic polymorphisms in *CYP3A4* as biomarkers for the prediction of methadone treatment responses. **Materials & methods:** A total of 366 Han Chinese methadone maintenance treatment patients in Taiwan were recruited in this study. Main clinical assessments included the clinical opioid withdrawal scale (COWS), the treatment emergent symptom scale (TESS) and the plasma concentrations of methadone and its metabolites. Genetic associations of six SNPs in the *CYP3A4* gene were calculated using a general linear model. **Results:** Genotypes and allele types of rs4646440 and rs2242480 were found to be significantly associated with the severity of withdrawal symptoms rated by COWS (p = 0.012, 0.0096, 0.017 and 0.012, respectively) as well as the side effects rated by TESS (p = 0.0089, 0.028, 0.0027 and 0.0085, respectively). The allele types associated with more severe withdrawal symptoms are also associated with more severe side effects and less betel nut (*Areca catechu*) use (p = 0.009 for rs4646440, p = 0.0063 for rs2242480). Further analyses on specific withdrawal symptoms in COWS showed that the genetic variants in rs4646440 are significantly associated with heart rate (allele type p = 0.0019). **Conclusion:** These results suggested that genetic variants in the *CYP3A4* gene may be useful indicators for the severity of side effects and withdrawal symptoms for methadone treatment.

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KEYWORDS: betel nut ■ clinical opioid withdrawal scale ■ CYP3A4 ■ heroin addiction ■ methadone ■ SNP

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Methadone is currently one of the major treatment regimens for opioid maintenance therapy in many countries, including Taiwan [1]. Methadone maintenance treatment has been shown to successfully prevent withdrawal symptoms, reduce other illicit drug use and reduce the risk of spreading HIV [2].

The chemical characteristic of methadone includes a chiral center which may produce *R*-form and *S*-form enantiomers [3]. Methadone is usually administered in a racemic mixture of (*R*)- and (*S*)-enantiomers, where the (*R*)-enantiomer is responsible for most of the opioid effects [4]. In an analgesic activity study, the *R*-form of methadone demonstrated better major analgesic effects than its *S*-form isomer [5]. The metabolism of methadone is carried out through the CYP3A4, CYP2B6 [6,7], CYP2C19 and CYP2D6 isoenzymes of the CYP system in the liver [8–10]. Through the metabolic process, it produces an inactive metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) [11]. It has been estimated that approximately 50% of all clinical therapeutic drugs are metabolized by CYP3A4 [12,13]. The subfamily of CYP3A enzymes are responsible for 30% of drug metabolism in adults [12]; its activity and regulatory mechanism may have an impact on methadone disposition.

The genetic locus of *CYP3A4* is located in chromosome 7q21.1. Although there is a T–C transition in *CYP3A4*18* (rs28371759) located in exon 10 that has been reported to be associated with increased CYP3A4 enzyme activity [13], the minor allele frequency in this polymorphism is very low in the population of Taiwan [14]. Therefore, it is crucial to identify other SNPs residing in this genetic region that may be useful for prediction of individual responses to treatment. Few studies have focused on pharmacogenetics of the CYP system and methadone [8]. It has been reported that the genetic polymorphisms in *CYP3A4* and *2B6* genes have no influence on the response to treatment but a small influence on the dose requirement of methadone [4]. However, the numbers of subjects included in these studies were small, hence it warrants further investigation.

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The *Areca* nut, sometimes called the betel nut, is the seed of the palm tree *Areca catechu*, which is the fourth most commonly used psychoactive substance, after caffeine, nicotine and alcohol in Taiwan. It is estimated that 600 million people use betel nut worldwide [15]. The prevalence of betel nut chewing in the male population in Taiwan was estimated as high as 14.3% in a population-based survey performed during 1999–2001 [16]. Betel nuts can cause increased heart rate, euphoria, gastrointestinal discomfort and sweating. Some of these effects are similar to those caused by methadone. It is a common substance used in methadone maintenance treatment (MMT) patients in Taiwan. However, there were no reported data in regard to the influence of betel nut on the effects or usage of methadone.

The aim of the present study is to evaluate the potential use of genetic polymorphisms in *CYP3A4* as biomarkers for the prediction of methadone treatment responses. We tested the hypothesis as to whether genetic variants of SNPs in *CYP3A4* were associated with the plasma concentrations of methadone and its metabolites, with the treatment response of the severity of withdrawal symptoms, and with the treatment emergent side effects in a Han Chinese MMT cohort in Taiwan.

Methods

Subjects

A total of 366 Han Chinese heroin-addicted patients in Taiwan undergoing outpatient MMT were recruited. This study was performed in accordance with the ethical standards of the Declaration of Helsinki. The study protocol was approved by the institutional review board of the National Health Research Institutes (Zhunan, Taiwan) and by the institutional review boards of the six participating hospitals in Taiwan. Written informed consent was obtained from all participants. The study project was registered with the National Institutes of Health Clinical Trial [101]. The key inclusion criteria were as follows: aged 18 years or above, undergoing MMT for at least 3 months with regular attendance in the past 7 days, and a methadone dosage adjustment of not more than 10 mg in the past 7 days. The absolute exclusion criteria were: any comorbidity with medical or psychiatric disorders that required immediate treatment and women who were pregnant.

Assessments

Demography, substance-use history and methadone treatment course, including the methadone dosage and treatment duration, and the treatment compliance over the previous week, were obtained from medical records. Information regarding current comedications was obtained either from the medical records or from the participants' reports. Interviewer-administered assessments, including urine morphine test as one of Treatment Outcomes Profile (TOP) [17], clinical opioid withdrawal scale (COWS) [18], and Treatment Emergent Symptoms Scale (TESS) [19] for adverse events related to methadone treatment, were conducted by research nurses before methadone was administered. The total days of betel nut usage within a month for each patient was included in TOP.

Tests for urine & blood samples

Urine samples were collected before administration of methadone for the measurement of the morphine and amphetamine levels via a fluorescence polarization immunoassay with an Integra 800 device (Roche Diagnostics, Basel, Switzerland). A total of 12 ml of blood was collected via venipuncture around 24 ± 2 h after the last time that the patients had taken methadone. This was most likely the time closest to when the plasma concentration of methadone was near the lowest level. Blood samples were then delivered to Taipei Institute of Pathology (Taipei, Taiwan) for analysis of liver function enzymes including, glutamate oxaloacetate transaminase, glutamic pyruvic transaminase and γ-glutamyl transpeptidase [20]. Serology profiles were performed for HIV antibodies, hepatitis C viral antibodies, surface antigen of the hepatitis-B-virus, HBsAg and HBsAb antibodies. Assays for levels of methadone and its metabolites and DNA genotyping were performed at the National Health Research Institutes (Zhunan, Taiwan).

Analysis of methadone & its metabolites in blood plasma

Plasma concentrations of *R*- and *S*-methadone and their EDDP metabolites were measured using HPLC with the settings described in our previous report [21]. The HPLC system consisted of a Waters 2795 Alliance solvent pump (Milford, MA, USA) and autosampler, a Waters 2998 photodiode array detector, and an Hewlett-Packard (CA, USA) computer recorder installed with Waters Empower software. A CHIRAL-AGP analytical column was used (5 µm, 100 × 3 mm; Chrom Tech, Cheshire, UK) with a CHIRAL-AGP guard column (10 × 3 mm) (Chrom Tech). The column oven was set at 35°C. The mobile phase comprised 5% isopropanol, 95% sodium phosphate buffer (10 mM), and triethylamine (1 ml/l of mobile phase), with a final pH of 6.0. The flow rate was 0.5 ml/min.

Methadone, EDDP and amitriptyline as an internal standard (40 ng) were extracted from the plasma samples using a C18-E 100 mg/ml capacity Strata solid-phase extraction column (Phenomenex, Torrance, CA, USA). Following the conditioning of the column on a vacuum manifold (Waters), 800 µl aliquots of each plasma sample and 40 ng of the amitriptyline internal standard were added. The column was then washed and the retained compounds were eluted with 1 ml of ammonium phosphate (monobasic)/methanol (0.01 g/100 ml). The collected eluent was then evaporated and the remaining residue was dissolved in 100 µl of mobile phase. A total sample volume of 50 µl was then chromatographed.

The retention time for *R*- and *S*-EDDP, *R*- and *S*-methadone, and internal standard amitriptyline were 6.76, 7.72, 10.72, 14.46 and 21.43 min, respectively. The intraday and interday coefficients of variation (CV) were 3.3 and 6.6% for *R*-methadone, 2.5 and 5.6% for *S*-methadone, 1.6 and 3.9% for *R*-EDDP, and 2.8 and 5.5% for *S*-EDDP.

CYP3A4 SNP selection & genotyping

DNA samples from subjects were extracted from the whole blood lymphocyte pellets using a Puregene kit (Gentra Systems, Minneapolis, MN, USA). *CYP3A4* SNPs were selected based on three main considerations: literature reports of polymorphisms in this gene that are related to heroin addiction in ethnic groups from Asia [22]; tagSNPs within the *CYP3A4* gene found on HapMap [102] with minor allele frequencies above 0.1 and a r^2 cutoff of 0.8; and functional SNPs predicted by FastSNP [23]. All SNPs were genotyped using the method of MALDI-TOF MS [24]. 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, USA) according to the manufacturer's instructions.

After removal of the unincorporated dNTPs and inactivation of the shrimp alkaline phosphatase from the PCR reaction, primer extension was performed via the addition of the appropriate probe, Thermo Sequenase (Amersham Pharmacia, Piscataway, NJ, USA) and a ddNTP/dNTP mixture. The reaction conditions were 55 cycles of denaturizing at 94°C for 5 s, annealing at 52°C for 5 s, and extension at 72°C for 5 s. The various extension products were differentiated by MALDI-TOF analysis. This genotyping method has been applied in a broad variety of clinical applications because of its accuracy of SNP detection, sufficient sensitivity to score SNPs from small amounts of template, flexibility of the procedure and cost–effectiveness [25].

Statistics

All statistical analyses were conducted using SAS software, Version 9.1 (SAS Institute, Inc., Cary, NC). Since the plasma methadone concentration is not normally distributed, its association analyses between the urine morphine test and plasma methadone concentration were analyzed by nonparametric Mann–Whitney U test. Association analyses between genotype or allele type, and COWS, TESS or betel nut use were calculated using a nonparametric permutation test. It corrected multiple testing by calculation of adjusted p-values corresponding to a nominal type I error of 5% with the MULTTEST procedure. For power analyses, the PROC GLMPOWER procedure was used. The other sample mean was fitted by the approximate normality distribution definition of Central Limit Theorem for a large sample size [26]. The relations between the total score of COWS and methadone dose or plasma concentrations were calculated by Pearson product-moment correlation analysis. The internal consistencies of the clinical assessments for COWS, COWS with heart rate, TESS and the betel nut use were calculated by Cronbach's α for the coefficient of reliability [27]. The following analyses were calculated by parametric statistical methods. The relationships between methadone and its metabolite enantiomers were assessed by Pearson's correlation. The Hardy–Weinberg equilibrium test and haplotype (95% CIs) association analyses were performed using HAPLOVIEW version 4.1 [28]. The haplotypes and their frequencies were determined by PHASE 2.1 [29]. The associations between haplotype and the plasma drug concentrations were assessed using a linear regression model with PROC GENMOD. The global p-value was calculated via a mixed model with the PROC MIXED procedure. p-values < 0.05 were considered as statistically significant threshold.

Results

Patient clinical characteristics & methadone metabolism

Complete clinical and genotyping data for a total of 366 MMT subjects were analyzed (Supplementary Table 1; www.futuremedicine.com/doi/suppl/10.2217/pgs.11.103) [103]. Almost all patients were cigarette smokers. In terms of HIV infection, three individuals were currently on

antiretroviral medication treatment. The average dose of methadone was 54.7 ± 28.1 mg/day in the study cohort and the average treatment duration was 65 weeks. Ten patients had coadministered medications that could potentially interfere the pharmacokinetics of methadone (fluoxetine, n = 2; sertraline, n = 1; ritonavir, n = 2; atazanavir, n = 2; oxcarbazepine, n = 1; ciprofloxacine, n = 1; cimetidine, n = 1; and prednisolone, n = 1) (Supplementary Table 2) [104,105]. The Cronbach's α was 0.6 for 11 items of COWS, 0.61 for 12 items of COWS with heart rate, 0.79 for 34 items of TESS, and 0.99 for four items of the betel nut use.

Plasma concentrations of methadone and EDDP were measured at 193.07 ± 121.76 (mean ± standard deviation), 142.20 ± 99.09 ng/ml for *R*- and *S*-methadone, and 13.70 ± 15.13 and 14.64 ± 12.98 ng/ml for *R*- and *S*-EDDP, respectively. After correcting for the methadone dosage, the concentration:dosage ratio (C:D ratio) of both *R*- and *S*-methadone were found to be significantly higher in the urine morphine test negative group (n = 178 vs 185 for urine morphine test negative vs positive patients) (4.03 ± 1.82 ng/ml/mg vs 3.70 ± 2.71 ng/ml/mg for R-methadone, Mann–Whitney U test, p = 0.001, and 2.98 ± 1.66 ng/ml/mg vs 2.58 ± 1.45 ng/ml/mg for S-methadone, Mann–Whitney U test, p = 0.012).

SNPs in *CYP3A4*

The chromosome position, functions, roles, allelic types and minor allele frequencies of six selected SNPs in *CYP3A4* were presented in Table 1. All six polymorphisms are in Hardy–Weinberg's equilibrium. A haplotype was constructed between the SNPs of rs2242480 and rs4646437 (linkage disequilibrium D′ = 0.95) with haplotype frequencies of 70.5% for C-G, 16.8% for T-A, 12.2% for T-G and 0.55% for C-A. The haplotype did not show further significant association with any clinical characteristics.

CYP3A4 is associated with opioid withdrawal symptoms

The severity of opioid withdrawal symptoms in MMT patients rated by the total score of COWS has a median of 1 (range from 1–13). The total scores of COWS had no significant differences between urine morphine test positive (median = 1, ranged from 0 to 13) and negative (median = 1, ranged from 0 to 12) patients. It had significant correlations with the methadone dose (r = 0.24, p = 0.0013), and the plasma *R*- and *S*-methadone concentrations (r = 0.26, p = 0.0005 and r = 0.16, p = 0.038) in urine test negative patients.

The total score of COWS showed significant associations with several selected SNPs in *CYP3A4*. The genotype and allelic types of the four SNPs, rs3735451 (intron 12), rs4646440 (intron 10), rs2242480 (intron 10), and rs2246709 (intron 7), were associated with the total score of COWS (Table 2). Further analysis of the subgroups of COWS demonstrated that rs3735451, rs4646440 and rs2242480 were significantly associated with heart rate (beats/min), which indicated a more severe withdrawal symptom. The C allele in rs3735451, A allele in rs4646440, T allele in rs2242480 and AA genotype in rs4646440 had higher heart rates (p = 0.036, 0.0019, 0.014 and 0.02, respectively). The genotype and allelic type of the SNP rs2246709 in intron 7 were associated with runny nose and tearing withdrawal symptom in COWS. AA genotype and A allele were both associated with a less severe withdrawal symptom (p = 0.0016 and 0.0033, respectively).

CYP3A4 is associated with methadone side effects

The four most common methadone-related adverse events were constipation (67.8%), sedation (47.0%), changes in libido (30.3%) and dry mouth (27.6%) in subjects recruited in this study. The side effect was rated by the total score of TESS had a median of 5 (range from 0–37).

Table 1. SNPs in the *CYP3A4* gene selected in this study.

SNP_ID[†]	Chromosome[‡]	Function	Role	Allele[§]	MAF	HWP	n
rs3735451	99193911	Tag SNP/intronic with no known function	Intron 12	T/C	0.31	0.83	366
rs4646440	99198806	Intronic enhancer	Intron 10	G/A	0.24	0.78	366
rs2242480	99199402	Tag SNP/intronic with no known function	Intron 10	C/T	0.29	0.81	366
rs28371759	99199562	**18*, missense (conservative); splicing regulation	Exon 10	A/G	0.031	1	366
rs4646437	99203019	Tag SNP/intronic enhancer	Intron 7	G/A	0.17	0.56	366
rs2246709	99203655	Tag SNP/intronic with no known function	Intron 7	A/G	0.43	0.31	366

[†]As listed in the dbSNP database.
[‡]Chromosome position determined by NCBI Human Genome Build 36.
[§]The minor allele is indicated after the slash.
HWP: p-values of Hardy–Weinberg's equilibrium test; MAF: Minor allele frequency; n: Subject number.

Table 2. Association analyses between the SNPs in *CYP3A4* and the severity of withdrawal symptoms rated by clinical opioid withdrawal scale.

SNP_ID	Genotype	n†	Median	Range	p-value	Allele	n‡	Median	Range	Permutation test p-value
rs3735451	CC	34	1.5	0–13	0.0046	C	228	1	0–13	0.0034
	CT	160	1	0–12		T	504	1	0–12	
	TT	172	1	0–7						
rs4646440	AA	23	2	0–13	0.012	A	178	1	0–13	0.0096
	GA	132	1	0–10		G	554	1	0–12	
	GG	211	1	0–12						
rs2242480	CC	186	1	0–12	0.017	C	520	1	0–12	0.012
	CT	148	1	0–10		T	212	1	0–13	
	TT	32	1.5	0–13						
rs2246709	AA	114	1	0–8	0.010	A	418	1	0–12	0.0089
	GA	190	1	0–12		G	314	1	0–13	
	GG	62	1	0–13						

†*Subject number.*
‡*Allelic number.*

There were no significant differences between urine morphine test positive (median = 5, ranged from 0 to 37) and negative (median = 4, ranged from 0 to 36) patients.

SNPs rs3735451, rs4646440, rs2242480, rs4646437 and rs2246709 of *CYP3A4* showed significant associations with the side effects of methadone rated by the total sore of TESS (Table 3). Further analysis of specific symptom of TESS showed that SNP rs4646440, and rs4646437 are significantly associated with the sedation side effect caused by methadone. The A allele carriers for both rs4646440 and rs4646437 were significantly associated with severe sedation rated by TESS (p = 0.039 and 0.0024, respectively).

CYP3A4 is associated with total betel nut use

SNPs rs4646440 and rs2242480 in intron 10, and rs4646437 in intron 7 showed significant associations with total betel nut use in methadone maintenance patients (Table 4). Overall, those SNPs found to be significantly associated with the frequency of betel nut use in the past 4 weeks were distributed mainly between the intron 6 and the intron 12 of the *CYP3A4* gene (Figure 1).

CYP3A4 & other clinical characters

We did not find associations between all six selected SNPs in *CYP3A4* and other clinical characters including use of other drugs use (e.g., amphetamine, morphine, heroin and alcohol), HIV status, liver function indicators (GOT, GPT and GGT), methadone dosage, plasma levels of methadone and its metabolite EDDP, C:D ratios, and apparent clearances of methadone and its metabolite EDDP.

Discussion

To our knowledge, this is the first study focusing on the association between *CYP3A4* polymorphisms and withdrawal symptoms of methadone in MMT patients. The clinical assessments of withdrawal symptoms rated by COWS, COWS with heart rate, side effects rated by TESS, and the betel nut use were within acceptable (Cronbach's α above 0.6) internal consistencies. We demonstrated that there were significant associations of several allele types and genotypes with the severity of withdrawal symptoms and the side effects. We also noted that the allele types associated with more severe withdrawal symptoms were also associated with more severe side effects, and with less frequent in betel nut use. The significant association results were persisted even though the exclusion of ten patients who had taken medicine interacting with CYP3A4 (Supplementary Table 2).

Most SNPs that were selected in previously published studies regarding genetics of *CYP3A4* are located at the exon regions. *CYP3A4*18* is a good example where there is a T–C transition in exon 10 [13]. The C allele represents an increase in the enzymatic activity. However, the minor allele frequency of this SNP in the population in Taiwan is very low, that is 1.6% [14]. We therefore selected SNPs through the HapMap, in consideration of the tagSNP in the Asian population and SNPs with potential functional roles.

We found that several SNPs in the *CYP3A4* gene were significantly associated with withdrawal symptoms in methadone maintenance patients. A total of four SNPs, rs3735451 (intron 12), rs4646440 (intron 10), rs2242480

Table 3. Association analyses between the SNPs in *CYP3A4* and the side effect of methadone rated by the treatment emergent symptom scale.

SNP_ID	Genotype	n†	Median	Range	Permutation test p-value	Allele	n‡	Median	Range	Permutation test p-value
rs3735451	CC	34	6	0–36	0.0071	C	228	5	0–36	0.029
	CT	160	5	0–29		T	504	4	0–37	
	TT	172	4	0–37						
rs4646440	AA	23	5	0–36	0.0089	A	178	5	0–36	0.028
	GA	132	5	0–29		G	554	4	0–37	
	GG	211	4	0–37						
rs2242480	CC	186	4	0–37	0.0027	C	520	4	0–37	0.0085
	CT	148	5	0–29		T	212	5	0–36	
	TT	32	6	0–36						
rs4646437	AA	13	7	0–36	0.037	A	127	5	0–36	0.037
	GA	101	5	0–29		G	605	4	0–37	
	GG	252	4	0–37						
rs2246709	AA	114	4	0–33	0.011	A	418	4	0–36	0.017
	GA	190	5	0–36		G	314	5	0–37	
	GG	62	6	0–37						

†*Subject number.*
‡*Allelic number.*

(intron 10), and rs2246709 (intron 7), in the intron regions of *CYP3A4* showed significant associations with the severity of withdrawal symptoms. Among these four SNPs, carriers with the C allele of rs3735451, the A allele of rs4646440, the T allele of rs2242480 and the G allele of rs2246709 had more severe withdrawal symptoms than their counter allele carriers. When we further analyzed the association with the specific withdrawal symptoms, we found that SNPs rs3735451, rs4646440 and rs2242480 in intron 12 were significantly associated with heart rate, and the allelic type of SNP rs2246709 in intron 7 was associated with the runny nose or tearing withdrawal symptom. As inducers of CYP3A4 enzyme may decrease methadone blood concentrations, which in turn may result in more severe withdrawal symptoms [30–33], it is possible that patients with the alleles in these four SNPs that were associated with more severe withdrawal symptoms may have higher CYP3A4 enzyme activities. Although the metabolic ratio of EDDP:methadone is higher in the severe COWS patients, it did not reach a statistically significant level. These results suggest that other CYP enzymes [9] may contribute more in the process of metabolism of methadone.

We also found in this study that the genetic variants of *CYP3A4* were significantly associated with the side effects of methadone. SNPs rs3735451, rs4646440, rs2242480, rs4646437 and rs2246709 showed significant associations with the severity of side effects of methadone rated by the total score of TESS. In further side-effect subgroup analysis, SNPs rs4646440 and rs4646437 showed significant associations with sedation side effect. Although it has been reported that drug–drug interaction may be related to some side effects of methadone [34,35], it is less likely that the methadone side effects observed in this study resulted from drug–drug interaction as concomitant use of other medications was well controlled in this study. However, we could not rule out the possibility that some side effects may be caused by other substances of abuse, for example, alcohol, amphetamine, heroin, nicotine and so on.

Combining these results, subjects with more severe opioid withdrawal symptoms may also have more severe side effects caused by methadone. SNPs rs3735451, rs4646440, rs2242480 and rs2246709 showed significant associations with both the severity of withdrawal symptoms and methadone side effects. Carriers with the C allele in rs3735451, the A allele in rs4646440, the T allele in rs2242480, and the G allele in rs2246709 had more severe withdrawal symptoms, and also showed higher methadone side effects than their counter allele carriers. The correlation between the withdrawal symptoms rated by COWS and the side effects rated by TESS was not very strong, but it was statistically significant ($r = 0.21$, $p < 0.0001$). The significant associations between the SNPs of *CYP3A4* and the COWS, the TESS or the frequency of betel nut use were contributed mainly from urine morphine test positive patients. As the symptoms between the COWS and the TESS were different, the possible

Table 4. Association analyses between the SNPs in *CYP3A4* and the frequency of betel nut use.

SNP_ID	Genotype	n†	Median (days/4 weeks)	Range	Permutation test p-value	Allele	n‡	Median (days/4 weeks)	Range	Permutation test p-value
rs4646440	AA	23	0	0–4	0.030	A	178	0	0–28	0.0090
	GA	132	0	0–28		G	554	0	0–28	
	GG	211	0	0–28						
rs2242480	CC	186	0	0–28	0.015	C	520	0	0–28	0.0063
	CT	148	0	0–28		T	212	0	0–28	
	TT	32	0	0–4						
rs4646437	AA	13	0	0–4	0.17	A	127	0	0–28	0.034
	GA	101	0	0–28		G	605	0	0–28	
	GG	252	0	0–28						

†Subject number.
‡Allelic number.

explanation for this finding was that patients who were sensitive to methadone medication, a low dosage of methadone may be sufficient to produce measurable side effects.

In this study, we found an association of SNP rs4646440, rs2242480 and rs4646437 in *CYP3A4* with total betel nut use in methadone maintenance patients in Taiwan. Among these three SNPs, subjects who carried the G allele of rs4646440, the C allele of rs2242480, and the G allele of rs4646437 had a higher average intake amount of total betel nut than their counter allele carriers. Subjects with these allele types were protected from more severe forms of the withdrawal symptoms and from some side effects caused by methadone. We did not find any association with alcohol or other illicit drug usage, so this association is less likely to be between polymorphisms and substance abuse but only specific to betel nut. We postulated that subjects with the allele types that are associated with more severe withdrawal symptoms and with more severe side effects caused by methadone may have higher CYP3A4 enzymatic activities, and that the reason why they take less amount of total betel nut may be contributed in part by the individual difference in the enzymatic activities of CYP3A4. To our knowledge, this is the first report indicating that the *CYP3A4* genetic polymorphisms may be indicators for use of betel nut, a stimulant-like substance commonly used in rural areas of Taiwan and some Southern and Southeastern Asia. This result was found from our statistical analyses. No literature has been reported that the betel nut may be metabolized by CYP3A4. The biological mechanisms underlying the CYP3A4 activities and use of betel nut warrant further investigation.

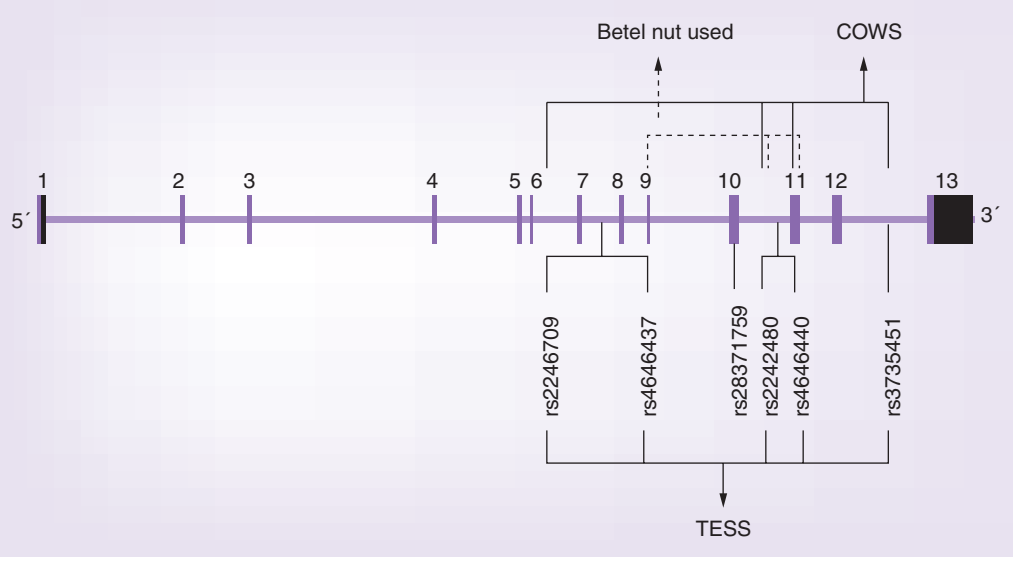

Figure 1. Significant associations between the SNPs in *CYP3A4* and the withdrawal symptoms, the side effects and the total betel nut use in patients under methadone maintenance treatment.
COWS: Clinical opioid withdrawal scale; TESS: Treatment emergent symptom scale.

There were some limitations of this study. The withdrawal symptoms rated by COWS were correlated with the methadone dose and plasma concentrations mainly in urine morphine test negative patients. This indicated that the withdrawal symptoms could be explained by methadone dose or plasma concentration in patients who no longer take heroin. It did not find associations between the SNP and the methadone dose or plasma concentration. This may be owing to the only SNP located at exon 10 being rs28371759 with minor allele frequency of 3%. It is possible that the minor allele is too low to observe its impact on plasma concentration. As most of the other SNPs were located at the intron region and transcription factors may bind preferentially to specific allele type (MZF1 binds the G allele of rs4646440 and GHF1 binds the T allele of rs2242480), their influence on the CYP3A4 expression is essential to be verified by biological experiments. Another reason is that the CYP3A4 is not a major metabolic enzyme for methadone, the intron SNP may be contributed to interaction with gene, such as *CYP2B6* which has higher impact on plasma methadone concentration [36]. The perceived response difference to methadone we found in subjects may be caused by factors other than plasma concentration. The doses of methadone in some of our samples appear to be relatively low. This could confound the interpretation of study findings. Other limitations include that possible drug–drug interactions were not taken into consideration; the design was a cross-sectional study, instead of a longitudinal study. We did not control the possible functional insufficiency of the hepatic microsomal system, which may interfere with the pharmacokinetics of methadone. Nevertheless, we had controlled the potential confounding effects of noncompliance with treatment by the recruitment process where only subjects who had been stabilized on methadone treatment and showed regular attendance were recruited.

In summary, the results of our current study demonstrated that SNPs in *CYP3A4* rs3735451(C allele), rs4646440 (A allele), rs2242480 (T allele) and rs2246709 (G allele) are associated with more severe withdrawal symptoms in MMT patients. rs4646440 (A allele) and rs4646437 (A allele) are associated with a higher sedation side effect caused by methadone. rs4646440 (G allele), rs2242480 (C allele) and rs4646437 (G allele) are associated with a higher total amount of consumption of betel nut. Subjects with alleles that are associated with more severe withdrawal symptoms also had more severe side effects caused by methadone. Whether genetic variants in the *CYP3A4* gene would be useful indicators for the severity of side effects and withdrawal symptoms for methadone treatment requires additional work to confirm these findings.

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Executive summary

Patient clinical characteristics & methadone metabolism

- The average methadone dose was 55 mg/day among 366 methadone maintenance patients whose average steady-state plasma methadone concentrations were 193 and 142 ng/ml for *R*- and *S*-methadone enantiomers.

***SNPs in* CYP3A4**

- The SNPs of *CYP3A4* were selected mainly from the intron regions owing to the lack of polymorphisms in the exon region of our ethnic group.

CYP3A4 *is associated with opioid withdrawal symptoms*

- Three SNPs including rs3735451 (intron 12), rs4646440 (intron 10) and rs2246709 (intron 7) demonstrated strong allelic associations (permutation, $p < 0.0097$) with the total scores of withdrawal symptoms rated by the clinical opioid withdrawal scale, especially the symptom of heart rate, which was assessed as an item within 11 items of the clinical opioid withdrawal scale total score.

CYP3A4 *is associated with methadone side effects*

- These SNPs showed a significant association with withdrawal symptoms, but they also had significant associations with methadone treatment side effects rated by the treatment emergent symptoms scales, specifically the sedation side effects.

CYP3A4 *is associated with total betel nut (*Areca catechu*) use*

- The SNPs of rs4646440 and rs2242480 at intron 10, and rs4646437 at intron 7 of those allele carriers who had higher withdrawal symptoms and side effects showed a significantly less use of frequenttotal betel nut in the past 4 weeks.

CYP3A4 *& other clinical characters*

- No further significant association results were found between the SNPs of *CYP3A4* and comedications, HIV status, liver function parameters, methadone dose or plasma concentrations.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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- **Using the same subjects of this study, we found that *CYP2B6* influences the plasma concentrations of methadone.**

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