Journal of Human Genetics (2013) 58, 84–90

www.nature.com/jhg

ORIGINAL ARTICLE

OPRM1 genetic polymorphisms are associated with the plasma nicotine metabolite cotinine concentration in methadone maintenance patients: a cross sectional study

Yu-Ting Chen[1], Hsiao-Hui Tsou[2], Hsiang-Wei Kuo[1], Chiu-Ping Fang[1], Sheng-Chang Wang[1], Ing-Kang Ho[1,3,4], Yao-Sheng Chang[1], Chia-Hui Chen[1], Chin-Fu Hsiao[2], Hsiao-Yu Wu[2], Keh-Ming Lin[1], Andrew CH Chen[5], Jyy-Jih Tsai-Wu[6] and Yu-Li Liu[1,7,8]

Majority of the heroin-dependent patients smoke cigarettes. Although it has been reported that the *OPRM1* genetic polymorphism is associated with the brain mu-opioid receptor binding potential in cigarette smokers, there is no direct evidence showing the impact of plasma cotinine, a nicotine metabolite, on treatment responses to methadone maintenance treatment (MMT). In this study, we aimed to test the hypothesis that the genetic polymorphisms in the *OPRM1* are associated with the methadone treatment responses and the severity of cigarette smoking directly measured by the plasma concentration of cotinine in a Taiwanese MMT cohort. Fifteen *OPRM1* single-nucleotide polymorphisms (SNPs) were selected and genotyped on DNA samples of 366 MMT patients. Plasma concentrations of cotinine were measured by cotinine enzyme-linked immunosorbent assay. The plasma cotinine concentration had positive correlation with concentrations of methadone ($P=0.042$) and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine ($P=0.037$). Methadone treatment non-responders, defined by a positive urine morphine test, had a higher plasma concentration of cotinine ($P=0.005$), but a lower plasma concentration-to-dose ratio of both R- and S-methadone ($P=0.001$ and 0.012, respectively) than the responders. *OPRM1* genetic variants, rs1074287, rs6912029, rs1799971, rs12209447, rs510769, rs3798676, rs553202, rs7748401, rs495491, rs10457090, rs589046, rs3778152 and rs563649, were significantly associated with the plasma concentration of cotinine when using recessive model for genotypes (general linear model (GLM), $P<0.038$; false discovery rate (FDR)<0.035) and additive model for allele types (GLM, $P<0.03$; FDR<0.049) in association analyses. The G allele carriers of SNP rs1799971 (A118G) on exon 1 of *OPRM1* gene had a lower plasma cotinine concentration than the A allele carriers (GLM, $P=0.029$). *OPRM1* genetic polymorphisms are associated with the plasma concentration of cotinine in a Taiwanese MMT cohort. Carriers with the major allele of SNP rs1799971 had a higher plasma cotinine concentration.
Journal of Human Genetics (2013) **58,** 84–90; doi:10.1038/jhg.2012.139; published online 6 December 2012

Keywords: cotinine concentration; heroin dependence; methadone; opioid receptor; OPRM1; SNP

INTRODUCTION

Polysubstance abuse is common in subjects with heroin dependence, and nicotine is reported to be the most prevalent substance. In all, 77–93% patients under methadone maintenance treatment (MMT) were reported to smoke cigarettes.[1–4] It has been shown that tobacco consumption is positively correlated with concurrent administration of mu-opioid receptor (MOR) agonists, heroin, methadone and a MOR partial agonist buprenorphine.[5–7] In addition, a previous study indicated that an increase in the dose of methadone could be associated with a stronger craving for nicotine in methadone maintenance patients.[8] These data suggest that cigarette smoking may have an important role in the treatment responses to methadone.

[1]Center for Neuropsychiatric Research, National Health Research Institutes, Zhunan, Miaoli County, Taiwan; [2]Division of Biostatistics and Bioinformatics, Institute of Population Health Sciences, National Health Research Institutes, Zhunan, Miaoli County, Taiwan; [3]Center for Drug Abuse and Addiction, China Medical University Hospital, Taichung, Taiwan; [4]Graduate Institute of Clinical Medical Science, China Medical University, Taichung, Taiwan; [5]Department of Psychiatry, College of Physicians and Surgeons, Columbia University, New York, NY, USA; [6]Department of Medical Research, National Taiwan University Hospital, Taipei, Taiwan; [7]Graduate Institute of Drug Safety, School of Pharmacy, China Medical University, Taichung, Taiwan and [8]Department of Psychiatry, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan
Correspondence: Dr Y-L Liu, Center for Neuropsychiatric Research, National Health Research Institutes, 35 Keyan Road, Zhunan, Miaoli County, 350, Taiwan.
E-mail: ylliou@nhri.org.tw
Received 5 June 2012; revised 22 October 2012; accepted 5 November 2012; published online 6 December 2012

The mechanisms underlying the high co-occurrence of opioid and nicotine dependence are not clear. MORs may be involved because methadone is a full μ-opioid receptor agonist.[9] Several studies have reported the associations between smoking and opioid receptors.[10–15] For example, Li *et al.*[16] reported that the prevalence of cigarette smoking is significantly increased in heroin-dependent patients. However, these studies were primarily based on self-report. Recall bias from self-report is a systematic bias, which often occurs when the answer of a question is based on a subject's memory. To avoid recall bias, it is important to find a reliable biomarker for nicotine exposure.[17] Cotinine is a metabolite derived from the 5′-hydroxylation metabolic pathway of nicotine[18] and has been reported by the Society for Research on Nicotine and Tobacco Subcommittee on Biochemical Verification as a reliable biomarker in estimating an average nicotine exposure over an extended period of time.[17] Plasma cotinine concentration offers a window of 5–7 days for detection of nicotine exposure. Thus, we used plasma cotinine concentration in this study in an attempt to estimate the severity of cigarette smoking more accurately in MMT patients.

MORs has been reported to be crucial to several biological functions, for instance, reward, analgesia and stress responses,[19,20] as well as the development of addiction,[21] including nicotine dependence.[22–25] MOR mRNA and protein in brain regions have been reported to be upregulated by nicotine. This process may stimulate endogenous opioid release and subsequently activate the MOR.[26–28] The human gene encoding the MOR, *OPRM1,* is located at the chromosome 6q24-q25 region. MOR is broadly expressed throughout the brain.[29] Several single-nucleotide polymorphisms (SNPs) in *OPRM1* gene have been identified to be associated with a range of substance dependence. The SNP rs1799971 is one of the most studied polymorphisms in the gene. It has been reported to be related to the risk of addictions[30] and different types of pain perception.[31–33] This A-to-G SNP is a non-synonymous polymorphism, which changes the amino acid from asparagine to aspartic acid (Asn40Asp) at a glycosylation site in the extracellular terminus of *OPRM1*.[34] In our previous study, we found that patients with the G allele required higher a methadone dose than those with the A allele.[35] Furthermore, rs1799971 has also been reported to be significantly associated with long-term smoking cessation in both males and females,[36] with nicotine reinforcement in women, and with the brain μ-opioid receptor binding potential in smokers.[11,15] However, the results are not consistent. For example, Perkins *et al.*[13] reported that rs1799971 was associated with smoking reward, but Zhang *et al.*[10] addressed that rs1799971 was not associated with smoking initiation or nicotine dependence. Of note, none of these studies used nicotine or its metabolite concentration to measure the severity of cigarette smoking. Thus, this study was aimed to test the hypothesis that the genetic polymorphisms in the *OPRM1* are associated with severity of smoking by direct measure of the cotinine concentration in a Taiwanese MMT cohort.

SUBJECTS AND METHODS

Subjects

This study was approved by the institutional review boards of the National Health Research Institutes (Zhunan, Taiwan) and the six participating hospitals. Written informed consent was obtained from all participants. The project has also been registered with the National Institutes of Health Clinical Trial (http://www.clinicaltrial.gov/ct/show/NCT01059747). A total of 366 subjects with heroin dependence undergoing MMT as outpatients were recruited. Briefly, the inclusion criteria were an age of 18 or above, undergoing MMT for at least 3 months with regular attendance for the past 7 days, and a methadone dosage adjustment for no >10 mg in the past 7 days. Exclusion criteria were comorbidity with physical and mental disorders that require immediate treatment or pregnancy.

Clinical assessments

Demographics, clinical characteristics and methadone treatment courses, including the dose and treatment duration, and the treatment adherence over the previous week, were obtained from the medical records. Information about other medications in the previous week was obtained from medical records or the subjects' reports. Interviewer-administered assessments, including treatment outcomes profile[37] for the amount and frequency of alcohol and other illicit substance use in the past 28 days, clinical opioid withdrawal scale for the severity of 11 opioid withdrawal symptoms[38] were conducted before methadone was administered.

Methadone-related adverse events were assessed by research nurses using treatment emergent symptoms scale.[39] The treatment emergent symptoms scale was composed of 43 treatment emergent symptoms. If the symptom occurred only after the initiation of MMT, it was counted as adverse event related to methadone. Severity of each symptom was rated on three-point Likert scale ranging from mild, moderate to severe. In this study, only the adverse events with a frequency $>15\%$ were included for further analysis.

Urine drug test

Urine specimens were collected before administration of methadone on the study day. The morphine levels were assayed via a kinetic interaction of microparticles method on an Integra 800 device (Roche Diagnostics, Basel, Switzerland). Urine morphine level $>300\ \mathrm{ng\,ml^{-1}}$ was considered positive. The coefficients of variation of the morphine standards for positive and negative controls were 5.7% and 3.8%, respectively. In this study, the urine morphine test was used as a surrogate measurement for the methadone treatment outcome.

Plasma cotinine assay

The level of nicotine metabolite cotinine in plasma was measured by the cotinine enzyme-linked immunosorbent assay kit (Neogen, Lansing, MI, USA). A total of 20 μl of plasma sample or different concentrations of cotinine standards were added along with 100 μl of cotinine enzyme conjugate into the well of a plate coated with cotinine antibody for competitive immunoassay. The mixture was incubated at room temperature on a plate shaker for 30 min. In all, 300 μl wash buffer was added into each well after removing the mixture. In total, 150 μl substrate K-blue was then immediately added into each well of the plate and the plate was shaken for 15 min. In all, 150 μl stop solution was added into each well to stop the immunological reaction. The optical density was measured by a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm wavelength. Patient plasma cotinine level was extrapolated from the inversely logarithmic linear regression curve built by the cotinine standards. The intra-plate and inter-plate coefficients of variation were approximately $1.4-4.3\%$ and $8.4-11.6\%$, respectively.

OPRM1 SNP selection and genotyping

The *OPRM1* SNPs were selected according to the literature reports,[40–42] a minor allele frequency above 0.1 on the HapMap of Chinese ethnic group (http://hapmap.ncbi.nlm.nih.gov/index.html.en), and the functions predicted by the bioinformatics FastSNP.[43] Fifteen SNPs were selected from this process for genotyping.

Genomic DNA was extracted from the buffy coat of 6 ml whole blood lymphocyte pellets using the Puregene Blood kit C (QIAGEN Sciences, Germantown, MD, USA), and was used for genotyping for all selected 15 SNPs on *OPRM1*. The genotypes of these 15 SNPs were identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry.[44] Primers and probes flanking the SNPs were designed using SpectroDESIGNER software (Sequenom, San Diego, CA, USA). DNA fragments (100–300 bp) encompassing each SNP site were amplified by PCR (GeneAmp 9700 thermocycler, Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions.

After removal of the un-incorporated dNTPs and inactivation with 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 dNTP mixture. The reaction conditions were 45 cycles of denaturation at 94 °C for 5 s, annealing at 56 °C for 5 s and extension at 72 °C for 5 s. The various extension products were differentiated by matrix-assisted laser desorption/ionization-time of flight analysis. This genotyping method has been applied in a broad variety of clinical applications because of the accuracy of SNP detection, sufficient sensitivity to score SNPs from small amounts of template, flexibility of the procedure and cost-effectiveness.[45] DNA sample of a subject were genotyped twice to evaluate the consistency of the results. The call rate and consistency were 100% for all 15 SNPs.

Quantitative real-time polymerase chain reactions for *OPRM1*

Human brain total RNAs in 10 brain regions were purchased from Clontech (Palo Alto, CA, USA). The brain regions included caudate nucleus, cerebellum, cerebral cortex, frontal lobe, hippocampus, insula, medulla oblongata, nucleus accumbens, substantial nigra and the temporal lobe. Total RNAs of these regions were converted to complementary DNA with random hexamers of Fermentas (Hanover, MD, USA).

mRNA level of *OPRM1* was measured by the TaqMan Gene Expression real-time PCR assay (Hs01053957_m1) purchased from Applied Biosystems. In all, 200 ng of complementary DNA was quantified in duplicates at OPRM1 probe with TATA-box-binding protein (TBP; Hs00920497_m1) as internal control probe. Real-time PCR amplification was conducted on a StepOnePlus Real-Time PCR System (Applied Biosystems). Gene expression was quantified relative to TBP expression using StepOne Software v2.1 (Applied Biosystems) and the relative quantification method. The relative expression level of *OPRM1* compared with that of TBP was defined as $-\Delta CT = -(CT_{OPRM1} - CT_{TBP})$. The *OPRM1* mRNA/TBP mRNA ratio was calculated from $2^{-\Delta CT} \times K$, in which K is a constant.

Statistical analyses

All statistical analyses were conducted using SAS software, version 9.1 (SAS Institute, Cary, NC, USA). The demographics and plasma methadone concentrations were analyzed using a non-parametric Wilcoxon two-sample test or χ^2 test between the treatment responders, who were defined as negative for the urine morphine test, versus the non-responders. The Spearman correlation coefficient was used to test the associations between the plasma cotinine concentration and clinical characteristics. Association analyses between SNPs in *OPRM1* (genotype or allele type) and plasma cotinine concentration were calculated using general linear model (GLM) with the GLM procedure. In addition, the false discovery rate (FDR) with the MULTTEST procedure was used to correct for multiple comparisons[46] including multiple genotype models correction. A dominant model was defined when the genotypes with the major allele were grouped to compare with the genotypes without the major allele. Conversely, a recessive model was defined when the genotypes with the minor allele were grouped for comparison. The Hardy–Weinberg equilibrium tests and haplotype block association analyses were performed using HAPLOVIEW version 4.2.[47] A haplotype block was constructed according to 95% confidence bounds on D prime (D′). GENMOD procedure was used to test the association between haplotypes and plasma cotinine concentrations, and to calculate global *P*-values. Power analyses were calculated by GLMPOWER procedure under the assumption of independent observation, normality in the distribution of residuals, and homogeneity of variance. The statistical significant level was designated with *P*-values of <0.05.

RESULTS

Clinical characteristics and methadone treatment outcome

A total of 366 Han Chinese patients on MMT (297 males and 69 females) were recruited. The average age was 38.17 years and the average body mass index was 23.58 kg m^{-2}. In all, 364 out of 366 subjects (99.5%) on MMT smoked cigarettes. The average plasma concentration of cotinine was 397.1 ± 189.2 ng ml^{-1} (Table 1). Methadone treatment responders were defined as MMT patients who were tested negative in the urine morphine test, whereas the non-responders were tested positive in the urine morphine test. Responders had a lower plasma concentration of cotinine than the non-responders (368.1 ± 171.2 ng ml^{-1} vs 427.1 ± 202.0 ng ml^{-1}, Wilcoxon two-sample test $P=0.005$), but a higher plasma concentration-to-dose ratio of both R- and S-methadone than the non-responders.

We found that the plasma cotinine concentration was significantly and positively correlated with age ($r^2=0.12$, $P=0.025$), S-methadone ($r^2=0.13$, $P=0.013$), S-2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (S-EDDP; $r^2=0.12$, $P=0.023$), (R + S)-methadone ($r^2=0.11$, $P=0.042$) and (R + S)-EDDP ($r^2=0.11$, $P=0.037$).[48] It also showed a significantly negative correlation with the total clinical opioid withdrawal scale score ($r^2=-0.11$, $P=0.036$), tremor ($r^2=-0.10$, $P=0.049$), malaise ($r^2=-0.44$, $P=0.021$) and weight loss ($r^2=-0.45$, $P=0.048$; Supplementary Table 1).

Details of this study cohort, including demographics, MMT duration, methadone daily dose, plasma concentrations of methadone and its metabolites, the methadone treatment response, and its side effects, were reported in our previous report.[35]

Single locus and haplotype of *OPRM1*

All 15 selected SNPs were spanning for 63 195 base pairs (bps) across the *OPRM1* genetic region with an average distance of 4213 bps

Table 1 Demographics and clinical characteristics of methadone maintenance subjects (*N* = 366)

	Overall	*Response to MMT*		P-*value*
		Responder	*Non-responder*	
Age (years)	38.17 ± 7.72	37.87 ± 7.46	38.37 ± 7.96	0.568[a]
Male, *N* (%)	297 (81.15)	142 (79.78)	152 (82.16)	0.562[b]
BMI (kg m^{-2})	23.58 ± 3.52	23.59 ± 3.49	23.62 ± 3.57	0.892[a]
Cotinine concentration (ng ml^{-1})	397.11 ± 189.16	368.07 ± 171.16	427.08 ± 201.95	**0.005**[a]
Methadone current dose (mg day^{-1})	54.67 ± 28.12	55.32 ± 30.13	54.53 ± 26.07	0.882[a]
R-methadone/methadone dose ratio	3.86 ± 2.32	4.03 ± 1.82	3.70 ± 2.71	**0.001**[a]
S-methadone/methadone dose ratio	2.77 ± 1.57	2.98 ± 1.66	2.58 ± 1.45	**0.012**[a]
R-EDDP/methadone dose ratio	0.31 ± 0.50	0.33 ± 0.54	0.26 ± 0.33	0.258[a]
S-EDDP/methadone dose ratio	0.33 ± 0.49	0.33 ± 0.58	0.31 ± 0.38	0.954[a]

Abbreviations: BMI, body mass index; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine; MMT, methadone maintenance treatment.
Values are shown as mean ± s.d. Bold *P*-value: $P<0.05$.
[a]*P*-value of Wilcoxon two-sample test.
[b]*P*-value of χ^2 test.

between two selected SNPs (Supplementary Table 2). The locations of these selected SNPs include the promoter, the exon and the intron. Six SNPs are tag SNPs and seven other SNPs are intronic enhancers in those selected 15 SNPs. All these SNPs were in Hardy–Weinberg equilibrium distribution with minor allele frequencies >0.059. A single haplotype block composed of the 14 SNPs of *OPRM1* from rs1074287 (promoter) to rs563649 (intron 1; Ref. 35) was created using the default algorithm[15] of HAPLOVIEW.[47]

OPRM1 is associated with plasma concentration of cotinine

The plasma concentration of cotinine was significantly associated with those selected SNPs of *OPRM1* using additive model of analyses in allele types ($P<0.03$ after adjusted with age, gender and body mass index; Table 2), except rs499796. After controlled for multiple testing by the FDR, the results remained statistically significant for the allele types (FDR$\leqslant$0.049), except rs2075572. Carriers with the allele type of rs1074287 (G), rs6912029 (T), rs1799971 (A), rs12209447 (T), rs510769 (A), rs3798676 (T), rs553202 (T), rs7748401 (G), rs495491 (C), rs10457090 (G), rs589046 (A), rs3778152 (G), rs563649 (A) and rs2075572 (G) had higher plasma concentrations of cotinine than their counter allele type carriers. All these SNPs were located from promoter to intron 2 regions in *OPRM1*.

Using recessive model of analyses, the genotypes of all *OPRM1* SNPs were significantly associated with the plasma concentrations of cotinine ($P<0.038$ after adjusted with age, gender and body mass index), except rs499796 and rs2075572. After multiple model correction, the recessive model showed a higher significant level than the additive model. However, there were no statistically significant associations between these SNPs and the plasma cotinine concentrations using dominant model of analyses ($P\geqslant0.059$ after adjusted with age, gender and body mass index; data not shown).

Analysis of OPRM1 haplotype and plasma concentrations of cotinine

Although the tetradeca-nucleotide single haplotype block (rs1074287-rs6912029-rs1799971- rs12209447-rs510769-rs3798676-rs553202-rs499796-rs7748401-rs495491-rs10457090-rs589046-rs3778152-rs563649) did not show significant associations with the plasma concentrations of cotinine (global $P=0.241$; data not shown), subjects with the 5′-AGGCGCCTTTAGAG-3′ haplotype were found to have a significantly lower plasma cotinine concentration than other haplotypes ($P=0.013$) (Table 3). Furthermore, higher plasma cotinine concentrations were shown in those with the 5′-GTATATCTGCGAGA-3′ haplotype ($P=0.010$). However, it did not reach a statistical significance after controlled by the FDR.

Table 2 Association analysis of the *OPRM1* and the cotinine concentration using the genotype recessive model and the allelic additive model

SNP ID		N	*Mean± s.d.*	P-*value*	*FDR*	*Power*		N	*Mean± s.d.*	P-*value*	*FDR*	*Power*
rs1074287	AA	251	378.37±181.81	**0.004**	**0.015**	0.80	**A**	606	387.88±185.39	**0.002**	**0.011**	0.82
	AG+GG	115	438.00±199.03				**G**	126	441.45±200.57			
rs6912029	GG	289	384.95±185.18	**0.014**	**0.018**	0.69	**G**	648	391.02±186.77	**0.010**	**0.012**	0.74
	GT+TT	76	445.22±198.15				**T**	82	448.72±200.80			
rs1799971	AA	151	422.91±197.58	**0.024**	**0.035**	0.59	**A**	472	408.99±193.31	**0.029**	**0.026**	0.63
	GA+GG	215	378.98±181.29				**G**	260	375.54±179.36			
rs12209447	CC	289	384.22±185.29	**0.013**	**0.015**	0.71	**C**	649	390.48±186.77	**0.009**	**0.012**	0.75
	TC+TT	77	445.45±196.85				**T**	83	448.89±199.58			
rs510769	GG	254	377.97±180.72	**0.002**	**0.015**	0.83	**G**	609	387.67±184.93	**0.001**	**0.010**	0.85
	AG+AA	112	440.51±201.20				**A**	123	443.82±202.53			
rs3798676	CC	294	384.35±185.24	**0.010**	**0.015**	0.74	**C**	654	390.49±186.73	**0.007**	**0.012**	0.78
	CT+TT	72	449.17±197.30				**T**	78	452.54±200.10			
rs553202	CC	324	390.07±184.78	**0.038**	0.068	0.47	**C**	689	393.59±187.06	**0.020**	**0.049**	0.52
	CT+TT	41	449.18±217.03				**T**	43	453.47±212.71			
rs499796	TT	325	393.43±186.48	0.173	0.306	0.18	**T**	689	394.91±187.85	0.101	0.205	0.24
	CT+CC	41	426.25±209.40				**C**	43	432.24±206.21			
rs7748401	TT	293	384.27±184.61	**0.009**	**0.015**	0.74	**T**	653	390.46±186.46	**0.006**	**0.011**	0.78
	GT+GG	73	448.63±199.52				**G**	79	452.00±202.09			
rs495491	TT	255	377.65±180.44	**0.001**	**0.015**	0.85	**T**	610	387.52±184.82	**0.001**	**0.010**	0.87
	TC+CC	111	441.81±201.64				**C**	122	445.04±202.92			
rs10457090	AA	279	388.98±185.95	**0.006**	**0.015**	0.79	**A**	621	395.72±187.74	**0.004**	**0.011**	0.83
	AG+GG	68	459.80±200.33				**G**	73	463.55±204.03			
rs589046	GG	255	377.65±180.44	**0.001**	**0.015**	0.85	**G**	610	387.52±184.82	**0.001**	**0.010**	0.87
	AG+AA	111	441.81±201.64				**A**	122	445.04±202.92			
rs3778152	AA	293	384.27±184.61	**0.009**	**0.015**	0.74	**A**	653	390.46±186.46	**0.006**	**0.011**	0.78
	GA+GG	73	448.63±199.52				**G**	79	452.00±202.09			
rs563649	GG	294	384.47±184.33	**0.009**	**0.015**	0.73	**G**	654	390.55±186.33	**0.006**	**0.011**	0.78
	AG+AA	72	448.70±200.92				**A**	78	452.10±203.39			
rs2075572	CC	216	384.23±187.14	0.077	0.126	0.34	**C**	563	389.60±186.62	**0.030**	0.053	0.50
	GC+GG	150	415.65±191.13				**G**	169	422.12±195.33			

Abbreviations: BMI, body mass index; FDR, false discovery rate; *N*, subject number; SNP, single-nucleotide polymorphism.
P-value, general linear model of *P*-value adjusted age, gender and BMI. Bold *P*-value: $P<0.05$.
FDR, the FDR analog of the *P*-value.
Power, calculated from the sample size of the study.

Table 3 Association between individual haplotypes for *OPRM1* block and the cotinine concentration

Phenotypes	*Haplotypes*	*Frequency*	*Observation*	N	*Mean ± s.d.*	P-*value*[a]	*FDR*	P-*value*[b]
Cotinine concentration, ng ml^{-1}	5′-AGGCGCCTTTAGAG-3′	0.343	2	43	360.27 ± 174.24	0.013	0.098	0.204
			1	165	379.92 ± 183.74			
			0	158	425.08 ± 195.79			
	5′-GTATATCTGCGAGA-3′	0.101	2	6	493.00 ± 248.37	0.010	0.098	
			1	62	444.14 ± 197.32			
			0	298	385.39 ± 184.81			

Abbreviations: FDR, false discovery rate; MMT, methadone maintenance treatment; *N*, subject number.
Frequency, the percentage of specific haplotype among the MMT cohort.
Observation, numbers of the specific haplotype that a patient had.
FDR, the FDR analog of the *P*-value.
[a]General linear model of *P*-value.
[b]The global *P*-value.

OPRM1 expressions in different areas of the human brain

Levels of the total *OPRM1* mRNA expression measured by quantitative real-time polymerase chain reaction were higher in the cerebellum and nucleus accumbens areas (Supplementary Figure 1).

DISCUSSION

In this study, we have demonstrated the first time that the *OPRM1* genetic polymorphisms are strongly associated with the severity of co-occurring nicotine dependence by direct measurement of plasma cotinine concentrations in a MMT cohort. Although several studies have suggested that *OPRM1* may be associated with the propensity of tobacco smoking and nicotine dependence,[10–15] most of these reports did not use nicotine or its metabolite as a marker. Fourteen SNPs selected from the promoter to intron 2 of *OPRM1* of both genotypes recessive model and allele types showed significant associations with the plasma concentrations of cotinine. The trideca-nucleotide haplotype block identified in this study was similar to the haplotype block reported in a study on European Americans,[49] and it showed a borderline significant association with the plasma concentrations of cotinine. However, it did not reach a statistical significance after adjustment for multiple testing. This suggested that SNP may be a better predictor for the severity of co-occurring nicotine dependence than haplotype in *OPRM1* gene.

Our results suggested that cigarette smoking may be a mechanism for patients to cope with the low efficacy of methadone treatment: when using urine morphine test as a surrogate outcome measure for subgrouping the MMT patients into the treatment responders and non-responders, the non-responders had a higher plasma cotinine concentration than the responders. As the responders had higher plasma levels of both S- and R-methadone concentration-to-dose ratios than the non-responders, it suggested that the cigarette smoking may be a physiological compensation for the low efficacy of methadone treatment. Similarly, Fonseca *et al.*[50] have reported the non-responders smoked more cigarettes per day than responders in a Spanish MMT program.

Cigarette smoking is highly prevalent (77–93%) in MMT patients.[1–4] A previous study has shown that an increase in the dose of methadone could cause more craving for nicotine.[8] In this study, our analyses of the coefficients of variation for the plasma cotinine concentrations were similar to those reported in other studies.[51] We found that plasma cotinine concentration may reflect the metabolism of methadone as positive correlations were found between plasma cotinine concentration and the plasma S-methadone, S-EDDP, total (R + S)-methadone and total (R + S)-EDDP concentrations. Furthermore, the plasma cotinine concentration was negatively correlated with the total opioid withdrawal score rated by clinical opioid withdrawal scale, the tremor score, the malaise score and the weight loss score. The higher the plasma cotinine concentration, the lower the withdrawal symptoms and less side effect symptoms were found in the MMT patients. These results support the finding that cigarette smoking may enhance the effect of methadone on the decrease in the opioid withdrawal symptom scores.[52] This may in part explain a high cigarette smoking rate in patients undergoing MMT.

rs1799971, located in exon 1, is the most investigated SNP in the *OPRM1* in which an adenine to guanine substitution (A118G) causes an amino-acid change, which in turn may reduce the MOR binding potentials,[11] and alter the downstream signaling.[30] The minor allele frequencies of rs1799971 are 15–30% in European descendants, 40–50% in Asian ancestry and 1–3% in African American and Hispanic populations, respectively.[53–55] In this study, we found that the G allele carrier of rs1799971 had a lower plasma cotinine concentration than the AA homozygote. This result suggested that the G allele in rs1799971 had a protective effect against cigarette smoking in patients under MMT.

In this study, the *OPRM1* SNPs that were significantly associated with plasma cotinine concentrations were also significantly associated with change in libido side effect in our previous report.[35] Minor allele carriers, who had higher change in libido scores, had higher plasma cotinine concentration than their counter allele in our MMT cohort. Without the involvement of *OPRM1* genetic polymorphism, our database did not show a directly significant association between the change in libido score and the plasma cotinine concentration (Kruskal–Wallis test $P = 0.701$; data not shown). It has been reported that an intermediate dose of isolated nicotine would significantly reduce erectile response in healthy, young, nonsmoking men.[56] Impairment of sexual performance were commonly reported by men undergoing MMT.[57] Previous studies suggested that nicotine may be the primary pharmacological agent responsible for genital hemodynamic disruption by acting on the nervous system centrally or peripherally. Our results provided further evidence that the *OPRM1* gene may be involved in nicotine-induced pathological process in change of libido.

We also found that those *OPRM1* SNPs that were significantly associated with the plasma cotinine concentrations in recessive model were also significantly associated with insomnia side effect in recessive model in our previous report,[35] except SNPs rs1799971 and rs553202. Insomnia is a relatively uncommon side effect in heroin-dependent patients under methadone treatment.[58,59] In this study, there were 18% MMT patients that reported to have sleep disturbance. Our

results indicated that the genotype associated with lower insomnia scores, which indicated a better sleep quality, were also associated with higher plasma cotinine concentrations in our MMT cohort. This result was contradicted to the finding in healthy adolescents and adults who had been reported with sleep disturbances after tobacco exposure.[60–64] There was no significant correlations between the insomnia score and the plasma cotinine concentration (Kruskal–Wallis test $P = 0.173$; data not shown) in the cohort unless the involvement of *OPRM1* genetic polymorphism. We therefore consider that the plasma cotinine concentration may be helpful in reducing the methadone-induced insomnia side effect through OPRM1 receptor.

Neuroimaging studies have been reported that a few brain regions of activation were associated with cigarette craving when subjects were presented with some smoking-related cues.[65] For example, left anterior cingulate cortex, medial prefrontal cortex, left middle cingulate gyrus, bilateral posterior cingulate gyrus and bilateral precuneus, were areas associated with attention, decision-making and episodic memory, and also associated with cigarette craving in nicotine-dependent subjects.[66] Using quantitative real-time PCR, we also found that *OPRM1* expressed across all areas in the brain. The expression levels were relatively higher in the cerebellum and the nucleus accumbens, the regions that have been reported to be associated with substance abuse.[67]

Nevertheless, some limitations of this study should be considered. First, this study did not examine the metabolic influence of the genetic polymorphisms of the genes encoding liver cytochrome P-450 isozymes on nicotine and cotinine. Second, the majority of participants were male. Also, this study was a cross-sectional design. A longitudinal study would warrant in order to clarify any cause and effect relation. In addition, other genetic factors, such as genetic variants in other opioid receptor genes would, warrant further studies.

CONCLUSIONS

In summary, we found that several *OPRM1* genetic variants were significantly associated with the plasma concentrations of cotinine in MMT patients when using recessive model for genotypes and additive model for allele types of analyses. Methadone treatment non-responders had a higher plasma cotinine level, and lower plasma concentration-to-dose ratio of both R- and S-methadone than the responders. It appears that the SNPs of *OPRM1* are better indicators for heavy smoking than haplotype combinations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the physicians and nursing staff from the six participating hospitals, Sun-Yuan Chou, Yen-Feng Lin, Kai-Chi Fang, Chieh-Liang Huang, Lien-Wen Su, Yong-Chun Fang, Ming-Lun Liu, Ming-Chu Tseng, Pei-Feng Li, Shu-Chuan Ting, Yu-Ching Lin, Miao-Feng Lee, Chi-Yun Huang and Yu-Hun Tsai, for conducting recruitments and interviews of the patients. We also thank Yu-Huei Shih for assistance with the preparation of the manuscript, and the Clinical Trial Information Management System (CTIMeS) at NHRI for data collection. We thank the National Center for Genome Medicine at Academia Sinica, Taiwan, for genotyping/technical support. This Center was supported by grants from the National Core Facility Program for Biotechnology of National Science Council, Taiwan. We also acknowledge the significant contributions of the Tao-yuan Psychiatric Center, En-Chu-Kong Hospital, Far-Eastern Memorial Hospital, Taipei City Hospital Song-De Campus, China Medical University and Hospital, and Taipei City Hospital Yang-Ming Campus. This study was supported by grants from the National Research Program for Genomic Medicine (NSC 98-3112-B-400-011, NSC 99-3112-B-400-003 and NSC 100-3112-B-400-015), the National Science Council (NSC 97-2314-B-400-001-MY3 and NSC 100-2314-B-400-002-MY3) and the National Health Research Institutes, Taiwan (PH-098-PP-41, PH-098-PP-46, PH-098-PP-38, PH-098, 99-PP-42, PH-098～100-SP-11, PH-100-PP-37, NHRI-101A1-PDCO-1312141).

Author contributions: Y-TC carried out statistical analyses and drafted the manuscript. H-HT participated in statistical analyses. H-WK carried out sample pretreatment, RNA extraction, and the SNP analyses. C-PF carried out the real-time PCR analyses. S-CW recruited patients and carried out the clinical assessments. I-KH participated in study design and persuaded clinicians from different hospitals to join this study. C-HC participated in patient recruitment and study design. C-FH participated in Clinical Trial Information Management System (CTIMeS). H-YW and Y-SC participated in statistical analyses. K-ML participated in study design. ACHC participated in the manuscript editing. J-JTW participated in result discussion. Y-LL carried out the experimental design, the clinical association study, and correspondence for the manuscript.

1 Best, D., Lehmann, P., Gossop, M., Harris, J., Noble, A. & Strang, J. Eating too little, smoking and drinking too much: wider lifestyle problems among methadone maintenance patients. *Addict. Res. Theory* **6,** 489–498 (1998).
2 Richter, K. P., Gibson, C. A., Ahluwalia, J. S. & Schmelzle, K. H. Tobacco use and quit attempts among methadone maintenance clients. *Am. J. Public Health* **91,** 296–299 (2001).
3 Nahvi, S., Richter, K., Li, X., Modali, L. & Arnsten, J. Cigarette smoking and interest in quitting in methadone maintenance patients. *Addict. Behav* **31,** 2127–2134 (2006).
4 Bien, T. H. & Burge, R. Smoking and drinking: a review of the literature. *Int. J. Addict.* **25,** 1429–1454 (1990).
5 Chait, L. D. & Griffiths, R. R. Effects of methadone on human cigarette smoking and subjective ratings. *J. Pharmacol. Exp. Ther.* **229,** 636–640 (1984).
6 Henningfield, J. E., Clayton, R. & Pollin, W. Involvement of tobacco in alcoholism and illicit drug use. *Br. J. Addict.* **85,** 279–291 (1990).
7 Mello, N. K., Lukas, S. E. & Mendelson, J. H. Buprenorphine effects on cigarette smoking. *Psychopharmacology (Berl)* **86,** 417–425 (1985).
8 Story, J. & Stark, M. J. Treating cigarette smoking in methadone maintenance clients. *J. Psychoactive Drugs* **23,** 203–215 (1991).
9 Bond, C., LaForge, K. S., Tian, M., Melia, D., Zhang, S., Borg, L. *et al.* Single-nucleotide polymorphism in the human mu opioid receptor gene alters beta-endorphin binding and activity: possible implications for opiate addiction. *Proc. Natl Acad. Sci. USA* **95,** 9608–9613 (1998).
10 Zhang, L., Kendler, K. S. & Chen, X. The mu-opioid receptor gene and smoking initiation and nicotine dependence. *Behav. Brain Funct.* **2,** 28 (2006).
11 Ray, R., Ruparel, K., Newberg, A., Wileyto, E. P., Loughead, J. W., Divgi, C. *et al.* Human Mu Opioid Receptor (OPRM1 A118G) polymorphism is associated with brain mu-opioid receptor binding potential in smokers. *Proc. Natl Acad. Sci. USA* **108,** 9268–9273 (2011).
12 Hardin, J., He, Y., Javitz, H. S., Wessel, J., Krasnow, R. E., Tildesley, E. *et al.* Nicotine withdrawal sensitivity, linkage to chr6q26, and association of OPRM1 SNPs in the SMOking in FAMilies (SMOFAM) sample. *Cancer Epidemiol. Biomarkers Prev.* **18,** 3399–3406 (2009).
13 Perkins, K. A., Lerman, C., Grottenthaler, A., Ciccocioppo, M. M., Milanak, M., Conklin, C. A. *et al.* Dopamine and opioid gene variants are associated with increased smoking reward and reinforcement owing to negative mood. *Behav. Pharmacol.* **19,** 641–649 (2008).
14 Crystal, H. A., Hamon, S., Randesi, M., Cook, J., Anastos, K., Lazar, J. *et al.* A C17T polymorphism in the mu opiate receptor is associated with quantitative measures of drug use in African American women. *Addict. Biol.* **17,** 181–191 (2012).
15 Ray, R., Jepson, C., Patterson, F., Strasser, A., Rukstalis, M., Perkins, K. *et al.* Association of OPRM1 A118G variant with the relative reinforcing value of nicotine. *Psychopharmacology (Berl)* **188,** 355–363 (2006).
16 Li, L., Liu, Y., Zhang, Y., Beveridge, T. J. & Zhou, W. Temporal changes of smoking status and motivation among Chinese heroin-dependent, methadone-maintained smokers. *Addict. Behav.* **35,** 861–865 (2010).
17 Benowitz, N. L., Jacob, III P., Ahijevych, K., Jarvis, M. J., Hall, S., LeHouezec, J. *et al.* Biochemical verification of tobacco use and cessation. *Nicotine Tob. Res.* **4,** 149–159 (2002).
18 Hecht, S. S., Hochalter, J. B., Villalta, P. W. & Murphy, S. E. 2'-Hydroxylation of nicotine by cytochrome P450 2A6 and human liver microsomes: formation of a lung carcinogen precursor. *Proc. Natl Acad. Sci. USA* **97,** 12493–12497 (2000).
19 Kreek, M. J. & Koob, G. F. Drug dependence: stress and dysregulation of brain reward pathways. *Drug Alcohol Depend.* **51,** 23–47 (1998).
20 Vaccarino, A. L. & Kastin, A. J. Endogenous opiates: 1999. *Peptides* **21,** 1975–2034 (2000).
21 Mague, S. D. & Blendy, J. A. OPRM1 SNP (A118G): involvement in disease development, treatment response, and animal models. *Drug Alcohol Depend.* **108,** 172–182 (2010).

22 Berrendero, F., Kieffer, B. L. & Maldonado, R. Attenuation of nicotine-induced antinociception, rewarding effects, and dependence in mu-opioid receptor knock-out mice. *J. Neurosci.* **22,** 10935–10940 (2002).
23 Trigo, J. M., Martin-Garcia, E., Berrendero, F., Robledo, P. & Maldonado, R. The endogenous opioid system: a common substrate in drug addiction. *Drug Alcohol Depend.* **108,** 183–194 (2010).
24 Walters, C. L., Cleck, J. N., Kuo, Y. C. & Blendy, J. A. Mu-opioid receptor and CREB activation are required for nicotine reward. *Neuron* **46,** 933–943 (2005).
25 Xue, Y. & Domino, E. F. Tobacco/nicotine and endogenous brain opioids. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **32,** 1131–1138 (2008).
26 Boyadjieva, N. I. & Sarkar, D. K. The secretory response of hypothalamic beta-endorphin neurons to acute and chronic nicotine treatments and following nicotine withdrawal. *Life Sci.* **61,** PL59–PL66 (1997).
27 Davenport, K. E., Houdi, A. A. & Van Loon, G. R. Nicotine protects against mu-opioid receptor antagonism by beta-funaltrexamine: evidence for nicotine-induced release of endogenous opioids in brain. *Neurosci. Lett.* **113,** 40–46 (1990).
28 Contet, C., Kieffer, B. L. & Befort, K. Mu opioid receptor: a gateway to drug addiction. *Curr. Opin. Neurobiol.* **14,** 370–378 (2004).
29 Le Merrer, J., Becker, J. A., Befort, K. & Kieffer, B. L. Reward processing by the opioid system in the brain. *Physiol. Rev.* **89,** 1379–1412 (2009).
30 Deb, I., Chakraborty, J., Gangopadhyay, P. K., Choudhury, S. R. & Das, S. Single-nucleotide polymorphism (A118G) in exon 1 of OPRM1 gene causes alteration in downstream signaling by mu-opioid receptor and may contribute to the genetic risk for addiction. *J. Neurochem.* **112,** 486–496 (2010).
31 Fillingim, R. B., Kaplan, L., Staud, R., Ness, T. J., Glover, T. L., Campbell, C. M. *et al.* The A118G single nucleotide polymorphism of the mu-opioid receptor gene (OPRM1) is associated with pressure pain sensitivity in humans. *J. Pain* **6,** 159–167 (2005).
32 Oertel, B. G., Kettner, M., Scholich, K., Renne, C., Roskam, B., Geisslinger, G. *et al.* A common human micro-opioid receptor genetic variant diminishes the receptor signaling efficacy in brain regions processing the sensory information of pain. *J. Biol. Chem.* **284,** 6530–6535 (2009).
33 Way, B. M., Taylor, S. E. & Eisenberger, N. I. Variation in the mu-opioid receptor gene (OPRM1) is associated with dispositional and neural sensitivity to social rejection. *Proc. Natl Acad. Sci. USA* **106,** 15079–15084 (2009).
34 Nikolov, M. A., Beltcheva, O., Galabova, A., Ljubenova, A., Jankova, E., Gergov, G. *et al.* No evidence of association between 118A>G OPRM1 polymorphism and heroin dependence in a large Bulgarian case-control sample. *Drug Alcohol Depend.* **117,** 62–65 (2011).
35 Wang, S. C., Tsou, H. H., Chen, C. H., Chen, Y. T., Ho, I. K., Hsiao, C. F. *et al.* Genetic polymorphisms in the opioid receptor mu1 gene are associated with changes in libido and insomnia in methadone maintenance patients. *Eur. Neuropsychopharmacol.* **22,** 695–703 (2012).
36 Munafo, M. R., Elliot, K. M., Murphy, M. F., Walton, R. T. & Johnstone, E. C. Association of the mu-opioid receptor gene with smoking cessation. *Pharmacogenomics J.* **7,** 353–361 (2007).
37 Marsden, J., Farrell, M., Bradbury, C., Dale-Perera, A., Eastwood, B., Roxburgh, M. *et al.* Development of the Treatment Outcomes Profile. *Addiction* **103,** 1450–1460 (2008).
38 Wesson, D. R. & Ling, W. The clinical opiate withdrawal scale (COWS). *J. Psychoactive Drugs* **35,** 253–259 (2003).
39 Guy, W. *ECDEU Assessment Manual for Psychopharmacology.* Revised edn (U.S. Dept. of Health, Education, and Welfare, Public Health Service, Alcohol, Drug Abuse, and Mental Health Administration, National Institute of Mental Health, Psychopharmacology Research Branch, Division of Extramural Research Programs, Rockville, MD, USA, 1976).
40 Shi, J., Hui, L., Xu, Y., Wang, F., Huang, W. & Hu, G. Sequence variations in the mu opioid receptor gene (OPRM1) associated with human addiction to heroin. *Hum. Mutat.* **19,** 459–460 (2002).
41 Hayashida, M., Nagashima, M., Satoh, Y., Katoh, R., Tagami, M., Ide, S. *et al.* Analgesic requirements after major abdominal surgery are associated with OPRM1 gene polymorphism genotype and haplotype. *Pharmacogenomics* **9,** 1605–1616 (2008).
42 Compton, P., Geschwind, D. H. & Alarcon, M. Association between human mu-opioid receptor gene polymorphism, pain tolerance, and opioid addiction. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* **121B,** 76–82 (2003).
43 Yuan, H., Chiou, J., Tseng, W., Liu, C., Liu, C., Lin, Y. *et al.* FASTSNP: an always up-to-date and extendable service for SNP function analysis and prioritization. *Nucleic Acids Res.* **34,** W635–W641 (2006).
44 Rodi, C., Darnhofer-Patel, B., Stanssens, P., Zabeau, M. & van den Boom, D. A strategy for the rapid discovery of disease markers using the MassARRAY system. *Biotechniques* **32,** S62–S69 (2002).
45 Tost, J. & Gut, I. G. Genotyping single nucleotide polymorphisms by MALDI mass spectrometry in clinical applications. *Clin. Biochem.* **38,** 335–350 (2005).
46 Storey, J. D. The positive false discovery rate: a bayesian interpretation and the q-Value. *Ann. Stat.* **31,** 2013–2035 (2003).
47 Barrett, J. C., Fry, B., Maller, J. & Daly, M. J. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21,** 263–265 (2005).
48 Wang, S. C., Ho, I. K., Wu, S. L., Liu, S. C., Kuo, H. W., Lin, K. M. *et al.* Development of a method to measure methadone enantiomers and its metabolites without enantiomer standard compounds for the plasma of methadone maintenance patients. *Biomed. Chromatogr.* **24,** 782–788 (2010).
49 Levran, O., Awolesi, O., Linzy, S., Adelson, M. & Kreek, M. J. Haplotype block structure of the genomic region of the mu opioid receptor gene. *J. Hum. Genet.* **56,** 147–155 (2011).
50 Fonseca, F., de la Torre, R., Diaz, L., Pastor, A., Cuyas, E., Pizarro, N. *et al.* Contribution of cytochrome P450 and ABCB1 genetic variability on methadone pharmacokinetics, dose requirements, and response. *PLoS One* **6,** e19527 (2011).
51 Wielkoszynski, T., Tyrpien, K. & Szumska, M. The enzyme-linked immunosorbent assay (ELISA) method for nicotine metabolites determination in biological fluids. *J. Pharm. Biomed. Anal.* **49,** 1256–1260 (2009).
52 Elkader, A. K., Brands, B., Selby, P. & Sproule, B. A. Methadone-nicotine interactions in methadone maintenance treatment patients. *J. Clin. Psychopharmacol.* **29,** 231–238 (2009).
53 Gelernter, J., Kranzler, H. & Cubells, J. Genetics of two mu opioid receptor gene (OPRM1) exon I polymorphisms: population studies, and allele frequencies in alcohol- and drug-dependent subjects. *Mol. Psychiatry* **4,** 476–483 (1999).
54 Bergen, A. W., Kokoszka, J., Peterson, R., Long, J. C., Virkkunen, M., Linnoila, M. *et al.* Mu opioid receptor gene variants: lack of association with alcohol dependence. *Mol. Psychiatry* **2,** 490–494 (1997).
55 Tan, E. C., Tan, C. H., Karupathivan, U. & Yap, E. P. Mu opioid receptor gene polymorphisms and heroin dependence in Asian populations. *Neuroreport* **14,** 569–572 (2003).
56 Harte, C. B. & Meston, C. M. Acute effects of nicotine on physiological and subjective sexual arousal in nonsmoking men: a randomized, double-blind, placebo-controlled trial. *J. Sex. Med.* **5,** 110–121 (2008).
57 Hanbury, R., Cohen, M. & Stimmel, B. Adequacy of sexual performance in men maintained on methadone. *Am. J. Drug Alcohol Abuse* **4,** 13–20 (1977).
58 Sharkey, K. M., Kurth, M. E., Anderson, B. J., Corso, R. P., Millman, R. P. & Stein, M. D. Assessing sleep in opioid dependence: a comparison of subjective ratings, sleep diaries, and home polysomnography in methadone maintenance patients. *Drug Alcohol Depend.* **113,** 245–248 (2011).
59 Kurth, M. E., Sharkey, K. M., Millman, R. P., Corso, R. P. & Stein, M. D. Insomnia among methadone-maintained individuals: the feasibility of collecting home polysomnographic recordings. *J. Addict. Dis.* **28,** 219–225 (2009).
60 Holmen, T. L., Barrett-Connor, E., Holmen, J. & Bjermer, L. Health problems in teenage daily smokers versus nonsmokers, Norway, 1995-1997: the Nord-Trondelag Health Study. *Am. J. Epidemiol.* **151,** 148–155 (2000).
61 Patten, C. A., Choi, W. S., Gillin, J. C. & Pierce, J. P. Depressive symptoms and cigarette smoking predict development and persistence of sleep problems in US adolescents. *Pediatrics* **106,** E23 (2000).
62 Phillips, B. A. & Danner, F. J. Cigarette smoking and sleep disturbance. *Arch. Intern. Med.* **155,** 734–737 (1995).
63 Wetter, D. W. & Young, T. B. The relation between cigarette smoking and sleep disturbance. *Prev. Med.* **23,** 328–334 (1994).
64 Zhang, L., Same, J., Caffo, B. & Punjabi, N. M. Cigarette smoking and nocturnal sleep architecture. *Am. J. Epidemiol.* **164,** 529–537 (2006).
65 Berkman, E. T., Falk, E. B. & Lieberman, M. D. In the trenches of real-world self-control: neural correlates of breaking the link between craving and smoking. *Psychol. Sci.* **22,** 498–506 (2011).
66 Hartwell, K. J., Johnson, K. A., Li, X., Myrick, H., Lematty, T., George, M. S. *et al.* Neural correlates of craving and resisting craving for tobacco in nicotine dependent smokers. *Addict. Biol.* **16,** 654–666 (2011).
67 Zubieta, J. K., Heitzeg, M. M., Xu, Y., Koeppe, R. A., Ni, L., Guthrie, S. *et al.* Regional cerebral blood flow responses to smoking in tobacco smokers after overnight abstinence. *Am. J. Psychiatry* **162,** 567–577 (2005).

Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)