

Interindividual Variability in the Prevalence of *OPRM1* and *CYP2B6* Gene Variations May Identify Drug-Susceptible Populations

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Abstract

Methadone is used worldwide for the treatment of heroin addiction; however, fatal poisonings are increasingly reported. The prevalence of *CYP2B6* and μ -opioid receptor (*OPRM1*) gene variations were examined between a postmortem population where the deaths were associated with methadone and a live non-drug-using control population using Taqman™ SNP Genotyping assays. The *CYP2B6**6 allele was higher in the postmortem population, but the difference was not significant ($P = 0.92$). The *CYP2B6* T750C promoter variation was similar in frequency for both populations. Linkage between T750C and *CYP2B6**6 was identified for both populations ($P < 0.01$). The prevalence of the *OPRM1* A118G variation was significantly higher in the control population ($P = 0.0046$), which might indicate a protective mechanism against opioid toxicity. Individual susceptibility to methadone may be determined by screening for *CYP2B6**6.

Introduction

The application of pharmacogenomics for the interpretation of drug toxicity and individual drug susceptibility is steadily increasing and several studies indicate that the genotyping of single nucleotide polymorphisms (SNPs) may be a useful forensic tool (1–3). Most functionally important SNPs are located within genetic coding regions (exonic domains) and are ideal candidates to examine interindividual drug responses (4). Gene variations in the Cytochrome P450 (CYP) en-

zymes can alter individual drug metabolizer status (3,5–7). Similarly linkage between receptor variations and drug response has been reported, with the μ opioid receptor gene (*OPRM1*) recognized as a key variable in individual sensitivity to opioids (8). A number of significant SNPs have also been identified within critical non-coding regions (i.e., gene promoter regions) (9).

Screening for SNPs involved in methadone action in vivo could identify individuals susceptible to methadone toxicity in addition to improving toxicological interpretation. *CYP2B6* is one of the cytochrome P450 enzymes responsible for methadone metabolism. The T750C variant is located in the proximal promoter region and has been linked with decreased gene expression. The influence of T750C on methadone susceptibility is currently unknown. The *CYP2B6**6/*6 genotype results from the combination of the G516T and A785G SNPs and has been associated with poor metabolism. *CYP2B6**6/*6 is present at a frequency of about 6% in Caucasian populations (7,10,11). In living subjects, the frequency of *CYP2B6**1/*6 has previously been reported at 24–26% (12,13) with *CYP2B6**1/*4 at 26% (12). However, earlier work reported higher frequencies in postmortem subjects whose deaths were attributed to methadone at 40% and 37.5%, respectively (3). This may be indicative of *CYP2B6* involvement in methadone toxicity. The work also documented a significant association between *CYP2B6* variants and increased postmortem methadone concentrations (3).

OPRM1 is of special interest as it is the preferential binding target of methadone. The association between SNP frequencies in the *OPRM1* gene and drug response have been examined in Hispanic (14), African-American (15), Caucasian (16), and Japanese populations (17). The A118G SNP has been reported at frequencies ranging between 2 and 48.5% across different populations (14,15,18,19).

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The purpose of this study was to explore the joint effects of *OPRM1* and *CYP2B6* genes in predicting individual susceptibility to methadone. Using SNP genotyping the prevalence of the *OPRM1* A118G variation, the *CYP2B6* T750C promoter variation and *CYP2B6**4, *9, and *6 alleles were studied in 84 methadone associated fatalities comparing the gene frequencies against a non-methadone-using control population.

Methods

Case subjects and controls

A population of 84 subjects whose deaths were associated with methadone in the East of Scotland was selected. The control population consisted of 100 healthy non-methadone-using volunteers from Bournemouth University. This study was approved by the Bournemouth University Ethics Committee.

DNA extraction and quantitation

Genomic DNA for the postmortem subjects was isolated from leukocytes (sodium fluoride anticoagulated blood) using the DNeasy Blood and Tissue Kit (Qiagen, Crawley, U.K.). For the control group buccal cells were collected using the Epicentre Catch-All Swab and extracted in 150 μ L of MasterAmp™ buccal extraction fluid (Epicentre Biotechnologies, Madison, WI). All DNA samples were quantified using the Human Quantifiler® Kit (Applied Biosystems, Warrington, U.K.) in accordance with the manufacturer's instructions.

Genotyping

The procedure for *OPRM1* A118G, *CYP2B6**4, *9, and *6 genotyping was described previously (3). For the *CYP2B6* T750 promoter SNP samples were amplified by PCR to identify homozygous wild type and heterozygous controls using primer C2B6PrF (forward: 5' CAGGTTCAAGT-GATTCTCTTG) and primer C2B6PrR (reverse: 5' CATGTTCAAACTGAGAGGCT). PCRs were performed with a reaction volume of 50 μ L, including 27.5 μ L PCR grade water, 10 μ L of 5 \times GoTaq™ buffer, 3.0 mM MgCl₂, 1 μ L of 10 mM deoxynucleoside triphosphates (dNTPs) (final concentration of 0.25 mM), 1 μ L Forward and Reverse primers, 10 ng/ μ L DNA, and 0.5 μ L GoTaq DNA polymerase. PCR reactions were performed with a Primus 96 advanced machine (Alpha Laboratories, Eastleigh, U.K.). The cycling conditions were as follows: initial denaturation at 95°C for 5 min; subsequent denaturation at 95°C for 1 min; annealing at 65°C for 30 s; primer extension at 72°C for 2 min, repeated for 30 cycles, followed by final extension at 72°C for 5 min. PCR products were visualized by electrophoresis with a 2% Cyber Green-agarose gel on blue light. Samples were purified using

the QIAquick PCR Purification Kit (Qiagen) in accordance with the manufacturer's instructions. DNA sequencing was conducted by COGENICS (COGENICS, Essex, U.K.). Samples were then genotyped for the T750C SNP using the validated TaqMan® SNP Genotyping assay (Applied Biosystems, product no. 4362691)

Statistical analysis

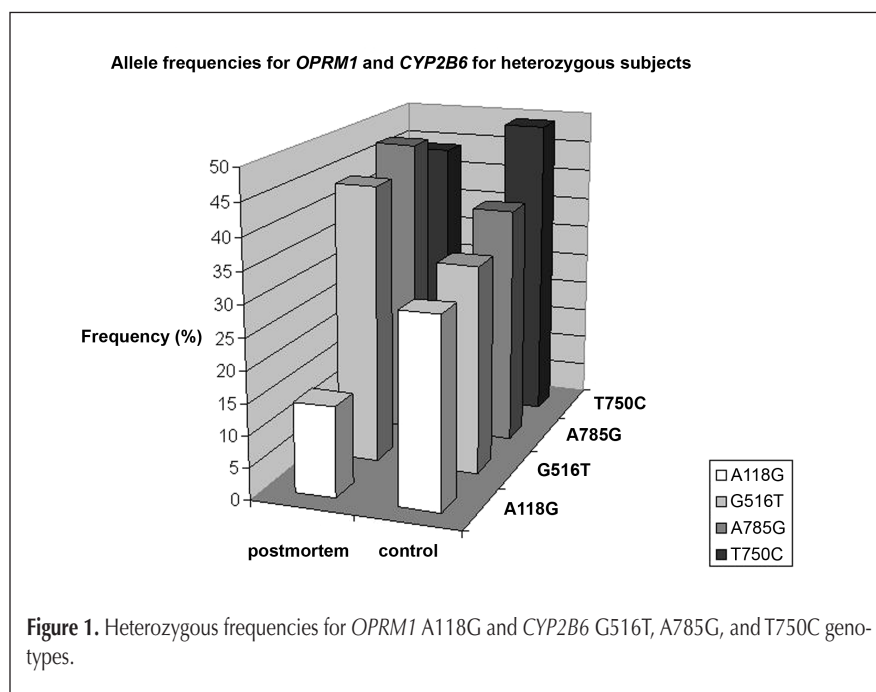
Data are presented as means \pm SE of the mean. A Binary Logistic Regression model was used to examine the effects of *OPRM1* A118G, *CYP2B6**4, *9, *6, age, sex, and race variables between the control and postmortem population (95% confidence intervals). T-Test and Fisher's exact test were used to examine the difference between genotype frequencies for the control and postmortem populations. Binary correlations were used to examine the association between the *CYP2B6* and *OPRM1* SNPs. A *P* value of ≤ 0.05 was considered to indicate statistical significance. All analyses were performed with SPSS Software (version 14.0).

Results

The 84 postmortem cases where methadone had been implicated in the cause of death included 62 men and 22 women, the majority of whom were Caucasian (98%). The mean age of the case subjects was 33.2 ± 1.12 years (range 17–60). The 100 control subjects included 45 men and 55 women, the majority of whom were Caucasian (97%). The mean age of the control subjects was 24 ± 0.72 years (range 18–55).

CYP2B6 G516T, A785G, and T750C frequency data

Of the 84 postmortem samples screened, 37 were genotyped



as heterozygous carriers of G516T (allele *9), a case percentage of 44% and 2 were genotyped as homozygous carriers, a case percentage of 2.4%. For the control group, 34% were genotyped as being heterozygous (a difference of 10%) and 3% were homozygous for the G516T variant (Figures 1 and 2, Table I). This difference was not statistically significant with a P value of 0.92 (Fishers exact test). Heterozygous carriers of A785G (allele * 4) for the postmortem group were identified in 40 cases (Table I), a frequency of 47.6% and 2.4% were homozygous for the A785G variant, which is noticeably higher than other reports from Caucasian populations (11–13,20).

For the control group, 38% of subjects were heterozygous for A785G. The difference between the postmortem and control frequencies was 9.6%, this result was not statistically significant ($P = 0.90$). For the methadone-attributed deaths allele *6 was identified in 39 subjects, an allelic frequency of 46.4%, which was higher than the control group where allele *6 was identified in 36% of subjects ($P = 0.92$).

The T750C promoter region SNP has been linked with alterations in gene expression (10). In the postmortem population 37 subjects were heterozygous for T750C, a case frequency of 44%, a similar frequency to that identified in the control population (49%). Sixteen postmortem subjects were homozygous for the CC genotype (19%) again a similar frequency to the control population (17%) (Figure 2, Table I).

Linkage between the T750C, G516T, and A785G gene variants was identified in both populations (Table II). In the postmortem population the T750C, G516T, and A785G variants were identified together in 17 cases (20%) and in the control population the SNPs were found in 17% of cases. Interestingly, wherever the variant homozygote for T750C was identified, the G516T variant was not present. For the postmortem population, the variant T750C homozygote was found in 16 subjects (19%), and for the control population, it was found in 17 subjects. Only one of the nine postmortem subjects also had the A785G variant, and for the control group, only three subjects also had the A785G variant.

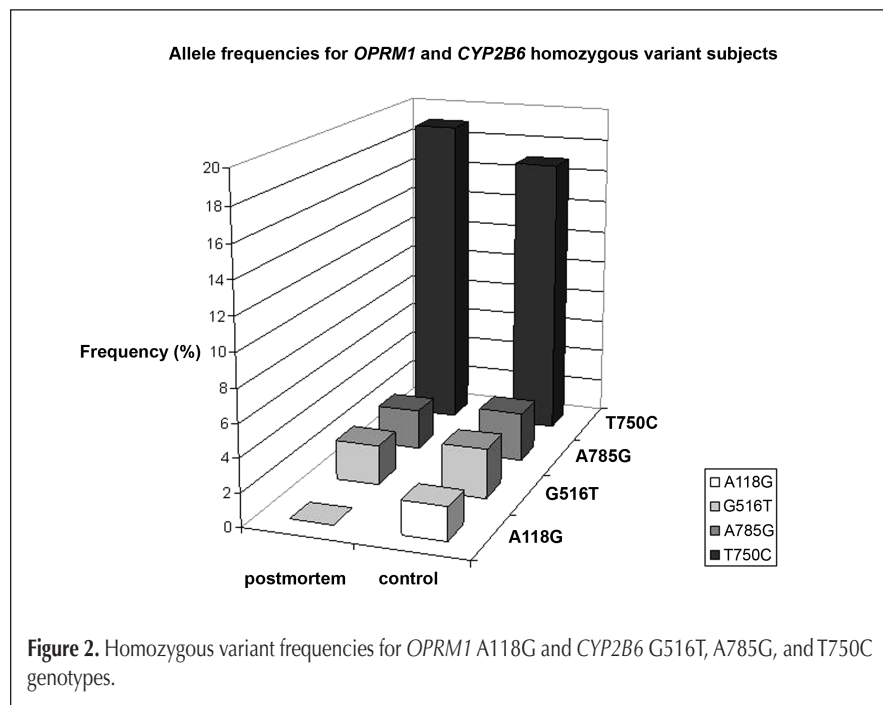


Figure 2. Homozygous variant frequencies for *OPRM1* A118G and *CYP2B6* G516T, A785G, and T750C genotypes.

Table I. *CYP2B6* T750C SNP and *CYP2B6**4, *9, and *6 Alleles and *OPRM1* A118G SNP Frequencies for the Postmortem and Control Populations

Gene Variants			Postmortem Population (84 subjects)	Control Population (100 subjects)
<i>CYP2B6</i>	T750C (promoter region)	TT	31 (37%)	37 (37%)
		TC	37 (44%)	44 (44%)
		CC	16 (19%)	19 (19%)
	A785G (allele * 4)	AA	42 (50%)	59 (59%)
		AG	40 (47.6%)	38 (38%)
		GG	2 (2.4%)	3 (3%)
	G516T (allele * 9)	GG	45 (53.6%)	63 (63%)
		GT	37 (44%)	34 (34%)
		TT	2 (2.4%)	3 (3%)
	Allele * 6		39 (46.4%)	36 (36%)
<i>OPRM1</i>	A118G (exon 1)	AA	72 (85.7%)	68 (68%)
		AG	12 (14.3%)	30 (30%)
		GG	0	2 (2%)

OPRM1 A118G frequency data

In the postmortem group the A118G SNP was found in 12 subjects a frequency of 14.3% (Figure 1, Table I) which is a similar result to previous studies (3,8,21). No homozygote for the G allele was identified. In the control group 30 subjects had the AG genotype and 2 subjects were homozygous for the G allele. The difference in frequency between the groups (15.7%) was statistically significant with a P value of 0.0046.

No linkage was found between A118G and T750C, G516T, and A785G in the control population. Interestingly, linkage was identified between A118G and G516T and A785G in the postmortem population (Table II).

Age, sex, and race effects

Sex correlated with T750C in the postmortem population ($-0.301 R^2$, P value = 0.005); however, the R^2 value indicates

only a modest correlation with the value < 0.5 . This was not repeated in the control population ($0.039 R^2$, P value = 0.699). No SNP correlation with age was observed in the postmortem population, but both G516T and A785G were associated with

age in the control population ($0.256 R^2$, P value = 0.010; $0.293 R^2$, P value = 0.003, respectively), although the R^2 values again indicate a modest correlation of < 0.5 . Age and sex were also correlated with population ($0.395 R^2$, P value = 0.001; $-0.375 R^2$, P value = 0.001, respectively). Sex and age information for both the postmortem and control populations are provided in Table III.

Table II. Genotype Prevalence Between the Non-Drug-Using Control Population and the Postmortem Population whose Deaths Have Been Associated with Methadone

Population	Gene Variants	R^2 Value	P Value
Postmortem	T750C & G516T	-0.442*	0.001
	T750C & A785G	-0.389*	0.001
	T750C & A118G	-0.123	0.265
	A118G & G516T	0.485*	0.001
	A118G & A785G	0.495*	0.001
	A785G & G516T	0.931*	0.001
Control	T750C & G516T	-0.365*	0.001
	T750C & A785G	-0.274*	0.006
	T750C & A118G	0.072	0.479
	A118G & G516T	0.058	0.572
	A118G & A785G	0.072	0.479
	A785G & G516T	0.903*	0.001

* Correlation is significant at the < 0.01 level (two-tailed).

Discussion

The importance of *CYP2B6* and *OPRM1* variations for individual susceptibility to methadone was reported previously, where subjects with the *CYP2B6**4 (0.95 mg/L vs. 0.56 mg/L), *9 (0.96 mg/L vs. 0.58 mg/L), and *6 (0.96 mg/L vs. 0.56 mg/L) alleles were all associated with significantly higher postmortem blood methadone concentrations compared with *CYP2B6**1 (3). Therefore, the identification of methadone risk factors will improve the toxicological interpretation of methadone-related deaths at autopsy while assisting in the prevention of methadone toxicity, particularly during the drug induction phase.

The present study examined the prevalence of *CYP2B6* and *OPRM1* SNPs in a postmortem population where the deaths had been associated with methadone compared with a healthy control population of non-methadone-using living subjects. Earlier work noted higher incidences of the *CYP2B6* G516T

Table III. *CYP2B6* T750C, G516T, A785G and *OPRM1* A118G Sex and Age Information for the Postmortem and Control Populations

Gene Variants	Postmortem Population				Control Population			
	Male age range	Male mean age	Female age range	Female mean age	Male age range	Male mean age	Female age range	Female mean age
<i>CYP2B6</i>								
T750C								
TT	18–51 (n = 17)	31	17–60 (n = 14)	35	19–47 (n = 18)	26	19–41 (n = 16)	25
TC	18–54 (n = 31)*	31	29–57 (n = 6)*	41	18–44 (n = 27)	26	19–55 (n = 22)	24
CC	21–56 (n = 14)*	33	30–34 (n = 2)*	32	18–34 (n = 10)	23	20–23 (n = 7)	21
A785G								
AA	18–56 (n = 33)	34	17–57 (n = 9)	41	18–39 (n = 35)	24	18–36 (n = 24)	21
GA	20–54 (n = 28)	31	20–60 (n = 12)	30	20–44 (n = 19)	27	19–55 (n = 19)	26
GG	32 (n = 1)	32	37 (n = 1)	37	47 (n = 1)	47	22–24 (n = 2)	23
G516T								
GG	18–56 (n = 36)	33	17–57 (n = 9)	45	18–43 (n = 38)	25	18–30 (n = 25)	21
GT	20–54 (n = 25)	30	20–60 (n = 12)	28	20–44 (n = 16)	27	19–55 (n = 18)	24
TT	32 (n = 1)	32	37 (n = 1)	37	47 (n = 1)	47	22–24 (n = 2)	23
<i>OPRM1</i>								
A118G								
AA	18–56 (n = 58)	32	17–60 (n = 14)	40	18–34 (n = 37)	24	19–55 (n = 31)	25
GA	20–54 (n = 4)	34	20–49 (n = 8)	31	20–47 (n = 16)	29	18–25 (n = 14)	20
GG	(n = 0)	0	(n = 0)	0	21–25 (n = 2)	23	(n = 0)	0

* Indicates a sex correlation where $P \leq 0.5$.

and A785G variations in postmortem subjects (3) when compared with previous studies sampling live subjects (11–13). Similarly the results from this study report higher frequencies of G516T and A785G in the postmortem population than the control group (Figure 1, Table I). However, this difference was not statistically significant ($P = 0.92$, $P = 0.90$, respectively) and may be indicative of a trend that has been limited by sample size. A larger number of postmortem subjects would elucidate the association between G516T and A785G prevalence and methadone susceptibility.

The T750C variant had a similar distribution across both populations sampled suggesting that the presence of this variant is not a suitable risk factor for methadone susceptibility (Table I). However, when examining linkage between T750C and G516T and T750C and A785G a significant association was discovered (Table II). T750C has been reported to decrease *CYP2B6* expression (10). The work discussed previously revealed the importance of the G516T and A785G variants for methadone susceptibility with the *CYP2B6**6 allele clearly correlating with higher postmortem methadone concentrations (3,7,11). It is therefore logical to postulate that, in a normal population, subjects with this genotype will experience higher methadone accumulation and are thus at greater risk of methadone toxicity (3). To our knowledge, none of these subjects were deliberate methadone overdose cases. Subjects with all three *CYP2B6* variants could therefore experience reduced expression of an already poorly functioning gene. The resulting effect on metabolism would likely involve methadone accumulation and toxicity. The association of T750C, G516T, and A785G was significant for both the postmortem population and the control population suggesting the possibility of a haplotype between these three *CYP2B6* SNPs. Further investigation into the T750C SNP and gene expression is required to determine the effects of both the 750 TC and 750 CC genotypes for *CYP2B6* expression. Furthermore, examination of T750C, G516T, and A785G in a larger population is required to fully determine whether this variation is linked with methadone susceptibility. The results from the current study revealed that T750C presence is constant between the postmortem and control groups indicating that this variation is not a suitable risk factor for methadone susceptibility.

Identification of genetic mutations that alter the functional activity of *OPRM1* may also explain interindividual responses to methadone (22). A118G is located in the N-terminal region of the receptor resulting in the loss of a putative N-glycosylation site which could result in alterations to *OPRM1* expression (23). The frequency of the A118G SNP was significantly higher in the control group, with a 15.7% difference between populations for heterozygous subjects ($P = 0.0046$, Table I). Several studies have reported that A118G may confer protection from opioid toxicity (8,14,24). A118G has been associated with reductions in cell surface receptors limiting the availability of drug binding sites and as a result drug response (23). However, it was previously reported that the A118G SNP may be associated with the lethal interaction between methadone and benzodiazepines, although this may be a consequence of benzodiazepine induced μ -receptor up-regulation (3). A118G effects on gene expression have been well documented (23,25). A study on

mRNA expression in postmortem brain tissue reported a 10-fold reduction in protein levels for subjects with the G allele (23). Furthermore, lower surface receptor expression and decreased forskolin-induced receptor activation has been identified in cell systems expressing the G allele (26). It seems logical therefore that receptor function will be reduced in subjects with the G allele. This may confer protection from opioid toxicity at therapeutic levels. However, these subjects will have a reduced sensitivity of the μ -receptor, which could lead to higher methadone concentrations in vivo before toxic effects appear. As methadone has a long mean elimination half-life of approximately 55 h (27), this could be especially pronounced in drug abusing subjects, limiting any possible protective function rendered by the G allele.

No linkage between the *OPRM1* A118G genotype and *CYP2B6* T750C, G516T, and A785G genotypes was observed for the control population in this study (Table II). However, linkage was identified between A118G and G516T and A118G and A785G in the postmortem population, this result is in agreement with earlier results which documented a significant association between A118G and G516T and A118G and A785G in postmortem subjects (3). In this study G516T, A785G, and A118G were found together in 8 of the postmortem cases (9.5%) and 11% of the control population. An association between A118G, G516T, and A785G would have significant impacts on methadone response. The *CYP2B6**6 allele associates with increased susceptibility to methadone fatality and A118G has been linked with increased heroin addiction (28). Therefore, this combination of gene variants could lead to subjects with increased tendency to drug addiction and a reduced ability to metabolize methadone (3).

The logistic regression model detected statistically significant age and sex interactions; however, the R^2 values indicate that this association could be limited (Table II). Furthermore, gender significantly correlated with *CYP2B6* T750C in the postmortem population ($P < 0.01$). As the frequency of this SNP remained constant across both the postmortem and control populations, it is not known if this variation is suitable as a risk factor for methadone susceptibility, and further investigation is required. In the control population, *CYP2B6* G516T and A785G significantly correlated with age. However, as the control group was made up of a student population, this may have influenced this result.

Both age and sex influences on the opioid system have previously been described (29,30). Increased μ -opioid receptor densities have previously been found to correlate with age, with older subjects having significantly more receptors (29). Gender influences could be linked with hormonal factors which can influence drug absorption, disposition, metabolism, and pharmacodynamics (31). This could contribute to differences in both drug action and response between males and females. It has been well documented that estrogen is involved in the enhancement of psycho-stimulants in females (32–35). Conversely, it has also been reported that estrogen may confer protection against opioid toxicity as interindividual variation in morphine potency is higher in males (30). A118G sex-specific associations (36) have been reported previously, but the functional significance of this is unclear (25).

Alternatively, sex trends in drug abuse could be an influential factor in the significance reported in this study. It has been well documented that drug abuse and mortalities from drug overdose occur more frequently in males than females (37,38), with reports of men being between two and three times more likely to have a drug abuse/dependence disorder (38). Furthermore, studies using animal models indicate that sex differences in drug dependence may be a result of sexually dimorphic development of the brain (33). Sociocultural and economic parameters can also not be excluded, as years of education, employment status, medical health status, and incidence of physical and sexual abuse are all underlying factors contributing to the development of drug abuse and dependence (31,39,40). The postmortem population examined in this study only reported 22 females (26%), which could explain the significance identified in this study for gender.

Conclusions

In summary, in this retrospective review of methadone-associated fatalities the *CYP2B6**6 allele may be linked with increased susceptibility to methadone particularly when in combination with the *CYP2B6* T750C promoter variation. It would be interesting to conduct future studies that take into account potential confounding factors such as the presence of all other concomitant drugs, the mode of drug intake, postmortem interval, prescription histories, medical histories, and case histories to strengthen our findings. The association between the prevalence of T750C, G516T, and A785G remained constant across both the postmortem and control population. The role of the *OPRM1* A118G variation for methadone susceptibility remains unclear. The higher prevalence of A118G in the control population is in support of the theory that A118G is protective against opioid toxicity (8,14,24). Although this protective function may work effectively with opioids such as morphine with a relatively short elimination half-life (27), for methadone, which has a significantly longer elimination half-life, this could result in high concentrations accumulating in vivo, leading to methadone toxicity and, in extreme cases, death. Furthermore, no linkage was identified in this study between A118G and the *CYP2B6* G516T and A785G variations within the control population. It is suggested that a larger postmortem sample size is required to effectively determine the role of A118G for methadone response. Genotyping *CYP2B6**6 homozygotes and heterozygotes may therefore assist in reducing adverse reactions to methadone during the drug induction phase while serving as a useful tool for certifying methadone toxicity at autopsy.

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