Association between mu opioid receptor gene polymorphisms and Chinese heroin addicts

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Mu opioid receptor (MOR) has been shown to be associated with alcoholism and opioid dependence. The present study examined the involvement of a polymorphism in A118G in exon 1 and C1031G in intron 2 of the MOR gene in 200 Chinese heroin-dependent and 97 control subjects. Results showed a significant association for both A118G and C1031G polymorphisms and opioid dependence. The G allele is more common in the heroin-dependent group (39.5% and 30.8% for A118G and C1031G polymorphisms, respectively) when compared to the controls (29.4% and 21.1% for A118G and C1031G polymorphisms, respectively). This study suggests that the variant G allele of both A118G and C1031G polymorphisms may contribute to the vulnerability to heroin dependence. *NeuroReport* 12:1103–1106 © 2001 Lippincott Williams & Wilkins.

Key words: Addiction; Gene polymorphism; Heroin; Mu opioid receptor

INTRODUCTION

Family, twins and adoption studies have shown that genetic factors may partially contribute to the vulnerability of substance abuse [1–3]. In particular, heroin was shown to have a larger genetic influence when compared to other substances of abuse [3]. Animal studies have shown that alcohol-preferring rats showed a significantly higher mu opioid receptor (MOR) density in the ventral tegmental area (VTA) than alcohol-avoiding rats [4]. Mice that lack the mu receptor exon 1, exon 2 or exon 2 and 3 displayed an absence of morphine antinociception [5]. Exon-2-disrupted MOR-deficient mice showed no induction of place preference after morphine conditioning and also showed a complete lack of somatic signs of withdrawal [6]. Therefore, it seemed that MOR might play an important role in addictive behavior. Berrettini *et al.* described two polymorphisms of the human MOR gene, namely G175T and C229T in exon 1 in cocaine- and/or opioid-dependent individuals with a trend towards a higher frequency of the 229 valine allele [7]. Bond *et al.* further showed that a functional single nucleotide polymorphism (SNP) was found at the position 118 in exon 1 of the MOR gene [8]. This SNP was also described earlier although no significant association of MOR genetic variation to alcohol dependence can be observed [9]. However, a significant association of this A118G variant was shown in heroin-dependent subjects [8] and that A118G variant receptor binds β-endorphin three times more tightly than the most common receptor. Agonist stimulation of the A118G variant receptor showed that β-endorphin is also approximately three times more potent at the A118G variant receptor than the most common receptor [8]. These authors suggested that the A118G SNP might confer a relative protection against opioid dependence. Furthermore, they showed that there was a difference in allele frequencies among African–Americans, Caucasians and Hispanics irrespective of opioid dependence status. However, it was noted that within the Hispanics, but not in African–Americans or Caucasians, the A118G variant allele was present in a significantly higher proportion of non-opioid-dependent subjects when compared to the opioid-dependent subjects, suggesting that ethnic variance exists for these mutations [8]. In view of this finding, we have attempted to screen DNA samples from 200 heroin-dependent as well as 97 control Chinese subjects for the A118G exon 1 polymorphism. This was done in order to establish whether such ethnic variance exists in the Chinese population. Nucleotide sequence information of intron 2 (NCBI Entrez NID: g2655102) revealed a C1031G polymorphism of the MOR gene. Since exon 2 mutant mice showed a lack of reward and withdrawal signs of addiction, the C1031G polymorphism of intron 2 may also be a reasonable target to search for such an association with heroin dependence.

MATERIALS AND METHODS

This study was approved by the Chinese University of Hong Kong Clinical Research Ethics Committee. Written

informed consent was obtained from all subjects participating in this study.

Study subjects: A total of 200 unrelated Hong Kong Chinese, heroin-dependent subjects (185 males, 15 females), were recruited. The mean age of the heroin-dependent subjects was 35.8 years. The subjects were derived from two sources: heroin-dependent individuals who sought treatment service at the Substance Abuse Clinic at the Prince of Wales Hospital ($n = 79$) and heroin-dependent volunteers identified by snowball recruitment ($n = 121$). All subjects were examined and assessed by a psychiatrist who specialized in substance abuse and all heroin-dependent subjects conformed to the criteria for opiate dependency as defined by DSM IV. Heroin-dependent subjects with a history of alcohol or substance dependence other than heroin were excluded from the study. Urine specimens obtained from each subject were analyzed (Triage Panel for Drug of Abuse, Biosite Diagnostics) to confirm heroin use and to exclude polysubstance abuse. All of the study subjects were interviewed by a trained interviewer using the Addiction Severity Index (ASI) questionnaire [10] that covers medical, employment, drug, legal, family history, family and social relationships and psychiatric status. A total of 97 control subjects (52 males, 45 females; mean age 32.8 years) were used in this study. They were healthy blood donors at the Prince of Wales Hospital. Those who had a history of drug or alcohol dependence, smoking, gambling and psychiatric illnesses were excluded.

DNA extraction: A 6 ml blood sample was drawn from a peripheral vein and anticoagulated with EDTA. DNA was extracted from peripheral blood samples with the QIAamp DNA Blood Mini kit (Qiagen, Germany).

A118G genotyping: Purified DNA (50 ng) was diluted into the PCR reaction mix consisting of PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each primer and 1 U AmpliTaq Gold polymerase (Perkin Elmer) in a total volume of 50 µl. A fragment containing MOR exon 1 was amplified using a forward primer: 5′-TCAGTACCATGGA CAGCAGC-3′ and the reverse primer: 5′-GCACACGATG GAGTAGAGGG-3′. PCR was carried out in a thermal cycler (Perkin Elmer, USA). The amplification steps were: denaturation for 13 min at 94°C, 35 cycles for 45 s at 94°C, annealing for 30 s at 62°C, synthesis for 30 s at 72°C and final elongation for 7 min at 72°C. The PCR products were then loaded into a 96-well plate and put into the WAVE DNA fragment analysis system (Transgenomic, Inc., San Jose, CA) for genotyping. Denaturing high performance liquid chromatography (dHPLC) is a semi-automated method for detecting unknown DNA sequence variants [11]. The base mismatch of a heteroduplex in a heterozygote sample formed a linear region of the single-stranded DNA that has been used to separate the two species and predict the mutation. Genotyping was carried out without knowledge of drug status.

C1031G polymorphism genotyping: Purified DNA (20 ng) was diluted into the PCR reaction mix consisting of PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each primer and 1 U Taq polymerase (MBI Fermemtas, USA) in a total volume of 25 µl. The mismatch PCR method was used with a forward primer: 5′-GCTCTGGTCAAGGC TAAGAAT-3′ and a reverse primer: 5′-GTAAGAGAG TAGGTTGGACCA-3′. PCR was carried out in a thermal cycler. The amplification steps were: denaturation for 5 min at 94°C, 35 cycles for 45 s at 94°C, annealing for 30 s at 62°C, synthesis for 45 s at 72°C and final elongation for 7 min at 72°C. PCR product of 145 bp was cut by *Hinf*I restriction enzyme. Genotype CC and GG can be identified by the presence of a 124 bp and 145 bp fragment, respectively. Both bands can be found in the heterozygote CG. The restriction-digested products were resolved on a 4% agarose gel stained with ethidium bromide.

Statistical analysis: The χ^2 test was applied for comparing genotype, allelic frequency distribution, odd ratios and the combined genotypes between the controls and heroin-dependent subjects. Logistic regression analysis was used to compute the combined genotypes upon the odds of zero, single or double dose effects of the susceptible allele being associated with heroin dependence [12]. Statistical significance level was set at $p < 0.05$.

RESULTS

Genotype and allele frequencies of the A118G polymorphism in control and heroin-dependent subjects are shown in Table 1. DNA analysis showed a significant difference ($\chi^2_{(2)} = 6.176$, $p = 0.046$) in genotype frequency between the heroin-dependent and control subjects (37.5% *vs* 52.6% for the AA homozygous; 16.5% *vs* 11.3% for the GG homozygous; 46% *vs* 36.1% for heterozygous AG; Table 1).

A higher proportion of the G allele was observed in the heroin-dependent subjects than in the controls (39.5% *vs* 29.4%; Table 1). The χ^2 test revealed a significant difference ($\chi^2_{(1)} = 5.792$; $p = 0.016$) in this allelic frequency. The higher proportion of G was due to an increase in GG homozygotes. Their frequency was 16.5% in the heroin-dependent subjects *vs* 11.3% in the controls. Notably, the proportion of heterozygotes among the heroin-dependent subjects was also higher than in the control population.

Table 1. Genotype and allelic frequency distribution of the A118G polymorphism in heroin-dependent and control subjects.

Group	Genotype frequency*			Total	Allele frequency#		Total
	AA	AG	GG		A	G	
Controls	51 (52.6%)	35 (36.1%)	11 (11.3%)	97	137 (70.6%)	57 (29.4%)	194
Heroin-dependent subjects	75 (37.5%)	92 (46.0%)	33 (16.5%)	200	242 (60.5%)	158 (39.5%)	400

The absolute number of cases and the percentage of total (in brackets) are shown.
For the comparison of genotype frequency: $^*\chi^2_{(2)} = 6.176$, $p = 0.046$; for the comparison of allelic frequency: $^\#\chi^2_{(1)} = 5.792$; $p = 0.016$.

Moreover, the increase in GG homozygotes was accompanied by a decrease in AA homozygotes. The odds ratio for AG *vs* AA genotypes was shown to be 1.79 (1.02–3.14, $p = 0.03$) and 2.04 (0.89–4.74, $p = 0.067$) for GG *vs* AA genotypes. The χ^2 test for trend showed a significance of $p = 0.02$ ($\chi^2_{(2)} = 5.373$).

Nucleotide sequence information revealed a C1031G polymorphism in intron 2 of the MOR gene (Fig. 1). χ^2 analysis revealed a statistical significant difference in allelic frequency ($\chi^2_{(1)} = 4.739$; $p = 0.014$; Table 2) and not genotype frequency ($\chi^2_{(2)} = 4.407$; $p = 0.094$; Table 2) between the heroin-dependent subjects and the controls. The C allele was the most common allele in both controls (78.9%) and heroin-dependent subjects (69.3%). Significantly, a higher prevalence of the G allele was reported in the heroin-dependent subjects than in the controls (30.8% *vs* 21.1%). The higher proportion of G allele seems to be the result of a near two-fold increase in the number of GG homozygotes in the heroin-dependent subject compared with the controls (15.5% *vs* 8.3%). The odds ratio for CG *vs* CC genotypes was 1.45 (0.8–2.63, $p = 0.1956$) and 2.30 (0.94–5.8, $p = 0.0476$) for GG *vs* CC genotypes. The χ^2 test for trend showed a significance of $p = 0.02978$ ($\chi^2_{(2)} = 4.722$).

Logistic regression analysis was therefore used to examine whether the combined genotypes upon the odds of zero, single or double dose effects of the susceptible G allele may be associated with heroin dependence. Table 3 shows the genotypes distribution of the combined A118G and C1031G polymorphisms of MOR the gene in the control and heroin-dependent subjects. Individuals who carried the 0, 1 or 2 genotype corresponded to those who had zero, single or double dose of the susceptibility genotypes. Subjects with the 0 *vs* 1, 1 *vs* 2 and 0 *vs* 2 genotypes were compared. The χ^2 test showed that statistical significance was found in the 0 *vs* 1 and 0 *vs* 2 genotypes, with the highest relative odds (RO) found when the 0 *vs* 2 genotypes was compared between controls and heroin-dependent subjects (RO = 3.396, $p = 0.001$).

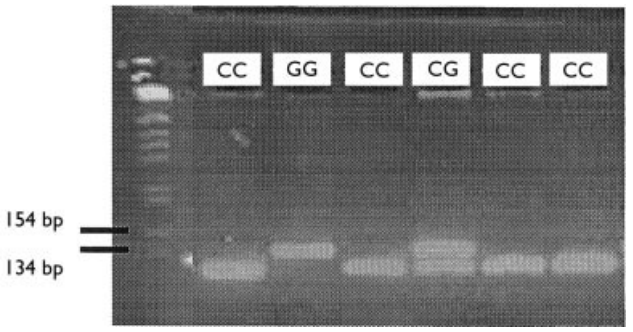

Fig. 1. Genotyping of the C1031G polymorphism of intron 2 of MOR gene by restriction analysis of the 145 bp PCR product. The PCR product was subjected to *Hinf*I digestion and the resulting fragments were resolved on a 4% agarose gel, which is shown after staining with ethidium bromide. Genotype CC and GG can be identified by the presence of a 124 bp and 145 bp band respectively. Both bands can be found in the heterozygote CG.

Table 3. Combined genotype of the A118G and C1031G polymorphisms in controls and heroin-dependent subjects.

Group	Genotype		
	0	1	2
Controls ($n = 97$)	32	51	14
Heroin-dependent subjects ($n = 200$)	35	113	52

0 *vs* 1, $\chi^2_{(1)} = 5.738$, $p = 0.017$; 1 *vs* 2, $\chi^2_{(1)} = 2.268$, $p = 0.132$, 0 *vs* 2, $\chi^2_{(1)} = 10.358$, $p = 0.001$.
Subjects who had the AA of A118G and CC of C1031G were scored as the 0 genotype, this means they had a zero dose of the susceptibility genes. Subjects who had the AA, AG of A118G or the CC, CG of C1031G were labeled as the 1 genotype, this means they had a single dose of the susceptibility genes. Subjects who carried the AG, GG of A118G and CG, GG of C1031G were scored as the 2 genotype, indicating that they had a double dose of the susceptibility genes.

DISCUSSION

The present study showed that a significant association in allelic and genotype frequencies of the prevalent A118G variant was also found in the Chinese population. However, when the allelic frequency of the Chinese population was compared with the results of the three different ethnic groups reported in Bond *et al.* [8], the A allele frequency of the control Chinese subjects (70.6%) was lower to that reported in the African–American (98.4%), Caucasian (88.5%) and Hispanic (85.8%) subjects. On the other hand, the 10.1% increase in G allelic frequency between the heroin-dependent and control subjects for the A118G variant observed in the present population is similar to that reported in other studies [8,9].

The C1031G polymorphism in the intron 2 of the MOR gene was chosen to determine whether variants other than A118G of the MOR gene also confer any relevant effects to heroin dependence. The present results showed that there was a significant difference in the allelic frequency between the heroin-dependent and control subjects. It seemed that the G allele might play a role in the predisposition of

Table 2. Genotype and allelic frequency distribution of the C1031G polymorphism of heroin-dependent and control subjects.

Group	Genotype frequency*			Total	Allele frequency#		Total
	CC	CG	GG		C	G	
Controls	64 (66.0%)	25 (25.8%)	8 (8.3%)	97	153 (78.9%)	41 (21.1%)	194
Heroin-dependent subjects	108 (54.0%)	61 (30.5%)	31 (15.5%)	200	277 (69.3%)	123 (30.8%)	400

The absolute number of cases and the percentage of total (in brackets) are shown.
For the comparison of genotype frequency: $^*\chi^2_{(2)} = 4.407$; $p = 0.094$; for the comparison of allelic frequency: $^\#\chi^2_{(1)} = 4.739$; $p = 0.014$.

heroin dependence since there is a significant increase in allelic frequency for the G allele in the heroin-dependent subjects as observed in both the A118G (10.1%) and C1031G (9.9%) polymorphisms. Thus, we speculate that the G allele may be in linkage disequilibrium with other functional mutations. The association between A118G polymorphism of exon 1 of the MOR gene showed that the A allele is more common than G allele in both the control and heroin-dependent subjects. However, more heroin-dependent subjects than controls were shown to carry the G allele (Table 1). It can be observed in the genotype frequencies that the increase in G allele was due to the increased in GG and AG genotypes with a decrease in the number of AA genotype. In addition to these findings, when the genotypes of AG and GG or CG and GG were examined for genotype association with AA or CC respectively, a significant trend difference was shown. When the combined genotypes of the two polymorphisms were examined, there was a significantly higher RO observed for the GG genotypes, further supporting a significant trend towards an association of the G allele to the susceptibility of heroin addiction. This suggested that the G allele may be a high-risk allele and that the mu receptor is probably a genetic risk factor associated with heroin addiction. In the A118G polymorphism, it was shown that the A118G variant of MOR binds β-endorphin three times more tightly than the wild type MOR [8]. Since β-endorphin can regulate MOR in the secretion of stress and reproductive hormones and pain perception, such genetic variance may confer some functional alteration to the MOR in its response to heroin or other substance of abuse. This is also in line with other evidence showing that β-endorphin blood plasma immunoreactivity is less in alcoholics than the controls [13] and a diminished endogenous hypothalamic–opioid activity was found among individuals from families with high incidence of alcoholism [14]. With regards to MOR exon 2, it is well known that MOR exon 2 knockout mice showed decreased morphine-induced analgesia and a lack of morphine-induced withdrawal and euphoria [6]. Recently, no evidence of ethanol-induced reinforcement was shown in these knockout mice [15]. As a result, it can be postulated that the variant MOR originally acts as a compensatory mechanism to cope with the defect of reduced endogenous opioid activity in order to balance the homeostasis in the body. Therefore, it remains to be determined whether MOR gene variance can alter the tertiary structure of the MOR or whether it is the expression of the gene that is accountable for the individual differences in their predisposition to heroin dependence.

It was also reported that there is an association between delta opioid receptor gene and heroin dependence [16]. MOR-deficient mice showed a reduction in delta-analgesia and the absence of delta-respiratory depression. This indicates that functional interactions may take place between mu receptors and central delta receptors in specific neuronal pathways [17]. In view of this, it is worth noting the interactive effects of different opioid receptor genes cannot be ignored.

CONCLUSION

The A118G polymorphism was shown in this study to also exist in the Chinese population and, in addition, the intron 2 C1031G polymorphism was reported for the first time to be associated with heroin dependence. Results showed that there is a trend that the G allele in intron 2 and G allele in exon 1 are both associated with heroin dependence. It seems that the mu receptor may be a genetic risk factor for heroin dependence. Further study on other nearby polymorphic sites is essential since the polymorphisms examined in this study may be in linkage disequilibrium with them.

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