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Polymorphisms in the glutamate decarboxylase 1 gene associated with heroin dependence

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ABSTRACT

The GAD1 gene encodes the 67-kDa glutamic acid decarboxylase isoform (GAD67), the rate-limiting enzyme responsible for γ-aminobutyric acid (GABA) biosynthesis from glutamic acid, and may be involved in the development of drug dependence. To identify markers contributing to the genetic susceptibility to heroin dependence, this study examined the potential association between heroin dependence and 15 single nucleotide polymorphisms (SNPs, rs1978340, rs3762556, rs3791878, rs3749034, rs11542313, rs2241165, rs2241164, rs769407, rs3749033, rs16858977, rs701492, rs16858988, rs4668331, rs7578661, rs769395) of GAD1 gene using the MassARRAY system. Participants included 370 heroin-dependent subjects and 389 healthy controls. The allelic or genotypic frequencies of the rs1978340 (promoter region), rs3791878 (promoter region), and rs11542313 (exon 3) polymorphisms in heroin addicts were significantly different from those in healthy controls. Strong linkage disequilibrium was observed in two blocks ($D' > 0.9$). Significantly more C-C-C-C-A haplotypes ($p = 0.0053$ after Bonferroni correction) and significantly fewer T-C-A-C-A haplotypes ($p = 0.0003$ after Bonferroni correction) were found in heroin dependent subjects. These findings point to a role for GAD1 polymorphism in heroin dependence among Han Chinese, and may be informative for future genetic or neurobiological studies on heroin dependence.

1. Introduction

Heroin addiction is a chronic, relapsing brain disease that is characterized by drug dependence, tolerance, compulsive seeking, and use despite harmful consequences. As with other types of substance abuse, genetic predisposition has been shown as a potential risk factor in heroin dependence [1]. Family, adoption, and twin studies have consistently demonstrated a substantial genetic influence on the development of drug addiction, with inherited risk estimates in the range of 40–60% [2,3]. Other studies have suggested that polymorphisms in the glutamic acid decarboxylase gene 1 (GAD1) may relate to addiction to drugs including heroin [4,5].

Glutamic acid decarboxylase (GAD) is the rate-limiting enzyme in the conversion of glutamate to GABA (11). Two isoforms of GAD have been identified, GAD1 and GAD2, which previously were called GAD67 and GAD65, respectively. GAD1 is involved in cytosolic GABA synthesis and is responsible for maintaining basal GABA levels, whereas GAD2 is predominately involved in synaptosomal GABA release, and can be rapidly activated when there is high demand for GABA [6]. Rodent studies suggest that knockout of GAD1 (which is important in maintaining GABA levels in the brain) is usually lethal, while knockout of GAD2 has no effect on brain GABA levels [4,7,8], suggesting that GAD1 is the primary rate-limiting enzyme regulating GABA levels under normal conditions. Thus, regulation of GAD1 expression may exert a more profound effect on GABA homeostasis and, possibly, be more sensitive to exogenous agents. Several studies have shown that chronic administration of drugs of abuse, such as alcohol [9], methamphetamine [10], cocaine [11], nicotine [12], and amphetamine [13] alters the activity of GAD1 in the brain, suggesting that the GAD1 gene is an excellent candidate for addiction disorders.

Alcohol dependence and heroin dependence have been associated with polymorphisms in GAD1. Loh et al. examined the association of nine single nucleotide polymorphisms (SNPs) for GAD1 and three SNPs for GAD2 with alcoholism in 140 alcoholic cases and 146 controls in Han Taiwanese [14]. They found evidence of an association of alcohol dependence (AD) with GAD1 but not GAD2. We are aware of only one published report examining the association of GAD1 with heroin dependence in humans. Levran et al. have performed a case–control association analysis of 1350 variants of 130 genes and found experiment-wise significant asso-

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ciation between SNP (rs2058725) in intron 5 and heroin dependence in African Americans [5]. Therefore, the role of GAD1 genes in drug dependence requires further study.

In view of the crucial role of the GAD1 in addiction disorders, as well as the controversy in genetic association studies, the present study investigated more loci in a large case–control sample of the same ethnic origin to verify the putative association between GAD1 polymorphisms and heroin dependence.

2. Materials and methods

2.1. Subjects

A total of 370 unrelated subjects with heroin dependence (aged 18 years and older; mean age of 36.6 ± 6.5) were recruited from the Methadone Maintenance Treatment (MMT) Program of the Xi'an Mental Health Center. Participants were daily or nearly daily users of heroin for a minimum of 1 year prior to assessment. Their addiction status was assessed by a psychiatrist from the Xi'an Mental Health Center, and each subject exhibited behaviors that fulfilled the DSM-IV diagnostic criteria for opioid dependence. The diagnosis of opioid addiction was based on DSM-IV criteria, medical history, urine test results, and interview responses. A case vignette was made to assist with diagnosis, using a semistructured interview with questions on (a) the age at initiation and duration of heroin use, (b) quantity of drug used over this period, (c) route of administration (nasal inhalation or injection), (d) whether other substances were used or abused, and (e) comorbidity for any other psychiatric disorder. Major central nervous system (CNS) diseases and psychoses were evaluated by a senior psychiatrist at the beginning of the methadone management program. Participants were excluded if they: met DSM-IV criteria for an additional Axis I disorder; had a history of alcohol, cigarette, amphetamine, barbiturate, benzodiazepine, or marijuana dependence according to DSM-IV; were taking other prescribed medications that could affect the central nervous system; had a history of seizures, hematological diseases, or severe liver or kidney impairment; or were pregnant. The study complied with the guidelines of our local Medical Ethical Committee, and all participants recruited in this study provided written informed consent.

In all, 389 healthy blood donors (mean age of 37.1 ± 5.8) were recruited at the First Hospital Affiliated to the Medical College of Xi'an Jiaotong University. Subjects who had substance abuse, participated in other studies, or suffered from chronic brain diseases were excluded. All participants were Han Chinese from Shanxi Province and not genetically related. Written informed consent was obtained from all participants. The study protocol was approved by the Ethical Committee of Xi'an Mental Health Center, Xi'an, China.

2.2. SNPs selection

SNPs in the promoter region, untranslated regions (UTRs), exons, and introns of GAD1 were systematically screened. Fifteen SNPs with minor allele frequencies (MAF) greater than 0.05 were selected from the GAD1 and nearby regions based on a review of published literature and a search of HapMap and dbSNP (public databases that contain information about the Han Chinese population). These SNPs were further analyzed in an association study. The positions of the SNPs in the GAD1 gene are shown in Fig. 1.

2.3. Genotyping

Three to five milliliters of peripheral blood were collected in tubes coated with EDTA. Genomic DNA was extracted from blood leukocytes using the EZNA™ Blood DNA Midi Kit (Omega Bio-Tek, Norcross, GA, USA), according to the manufacturer's protocol. SNP genotyping was performed using matrix assisted laser desorption ionization-time of flight (MALDI-TOF; MassARRAY system, Sequenom Inc., San Diego, CA, USA) mass spectrometry. Primers were designed using Sequenom software, and the extension reaction produced allele-specific products with masses differing by 30 Da, or approximately one single nucleotide. Primer extension and PCR were performed according to the manufacturer's instructions, using iPLEX enzyme (Sequenom) and HotStarTaq DNA polymerase (Qiagen, Hilden, Germany). The completed genotyping reactions were spotted onto a 384-well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser (Sequenom) and the molecular weights of the products were determined using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. Genotype calling was performed in real time using MassARRAY RT software version 3.0.0.4 and data analysis was performed using MassARRAY Typer software version 3.4 (Sequenom).

2.4. Statistical analysis

Allele and genotype frequencies for each individual polymorphism and Hardy–Weinberg equilibrium were evaluated by the Chi-square test. Associations between the case–control status and each polymorphism were assessed by the Fisher's Exact test or the Pearson Chi-square test. Unconditional logistic regression was used to calculate the odds ratio (OR) and 95% confidence interval (CI) of independent association between each locus and the presence of heroin dependence. Bonferroni correction was used in multiple testing, and the p-value was divided by the total number of loci or haplotypes. All statistical analyses were carried out using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA). Pair-wise linkage disequilibrium (LD) statistics (D' and r^2) and haplotype frequency were computed using Haploview 4.0 to construct haplotype blocks.

3. Results

The distribution frequencies of 15 genotyped SNPs were in agreement with Hardy–Weinberg equilibrium. Linkage disequilibrium (LD) analyses of the patient and control data revealed that the

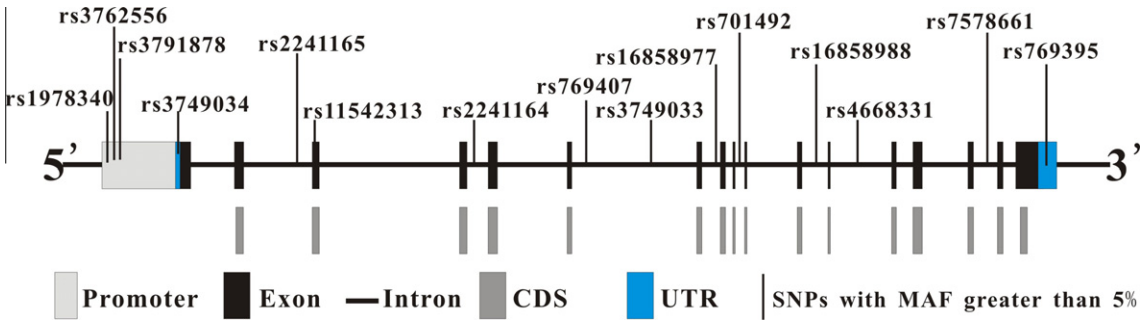

Fig. 1. Gene structure of human GAD1, showing the relative positions of the 15 SNPs used in our study.

five SNPs (rs1978340, rs3762556, rs3791878, rs3749034, rs2241165) and eight SNPs (rs769407, rs3749033, rs16858977, rs701492, rs16858988, rs4668331, rs7578661, rs769395) are located in haplotype block 1 and block 2 ($D' > 0.9$, Fig. 2). The genotype distributions, allelic frequencies, and haplotypes in control and patient groups, together with the results of statistical analysis are listed in Tables 1–3.

The difference in the distribution of genotype frequencies of rs1978340 between heroin-dependent subjects and healthy controls was significant ($p = 0.0021$). Heroin-dependent subjects had a significantly lower frequency of the T allele ($\chi^2 = 11.575$, $p = 0.0007$, OR = 0.663, 95% CI = 0.523–0.841). The analysis revealed a strong association between the rs3791878 genotype distribution and heroin dependence ($p = 0.0005$). Heroin-dependent subjects had a significantly higher frequency of the C allele ($\chi^2 = 10.594$, $p = 0.0011$, OR = 1.465, 95% CI = 1.163–1.844). There was a significant between-group difference in the genotype distribution of rs11542313 ($p = 0.0050$). Heroin-dependent subjects had a significantly lower frequency of the T allele of rs11542313 ($\chi^2 = 11.351$, $p = 0.0008$, OR = 0.706, 95% CI = 0.576–0.865).

C-C-C-C-A haplotype (block 1) occurred significantly more frequently ($\chi^2 = 7.7734$, $p = 0.0053$, OR = 1.3799, 95% CI = 1.0999–1.7311) and T-C-A-C-A haplotypes occurred significantly less frequently ($\chi^2 = 17.2546$, $p = 0.0003$, OR = 0.5988, 95% CI = 0.4695–0.7637) in heroin-dependent subjects. These differences retained statistical significance after Bonferroni correction.

4. Discussion

Most prior studies of the relation between GAD1 gene and substance addictive behaviors have used animal models. Mice and rat studies have shown altered GAD1 genes expression during alcohol dependence or withdrawal [9], chronic exposure to high-dose methamphetamine [10], and repeated cocaine use [13]. Thus, regulation of GAD1 expression may profoundly affect GABA homeostasis and be sensitive to exogenous agents. The present study examined the association of GAD1 genes with heroin dependence in humans.

In this case–control association study, the T alleles of GAD1 rs1978340 and rs11542313 were strongly associated with decreased risk of heroin dependence while the C allele of GAD1 rs3791878 was associated with increased risk of heroin dependence. To the best of our knowledge, this is the first report to show a significant association of GAD1 rs1978340, rs3791878, and rs11542313 with heroin dependence. Similarly, a previous study showed significant differences in genotype and allele distributions for SNP rs11542313 between alcohol-dependent and control subjects [15]. Earlier studies have also revealed associations of rs1978340 and rs3791878 in the GAD1 promoter with bipolar affective disorder [16], schizophrenia [17], and autism spectrum disorder [18] as well as associations of other variants in the GAD1 promoter with depression [19]. Markers rs1978340 and rs3791878 showed effects in alcohol dependent males, with the

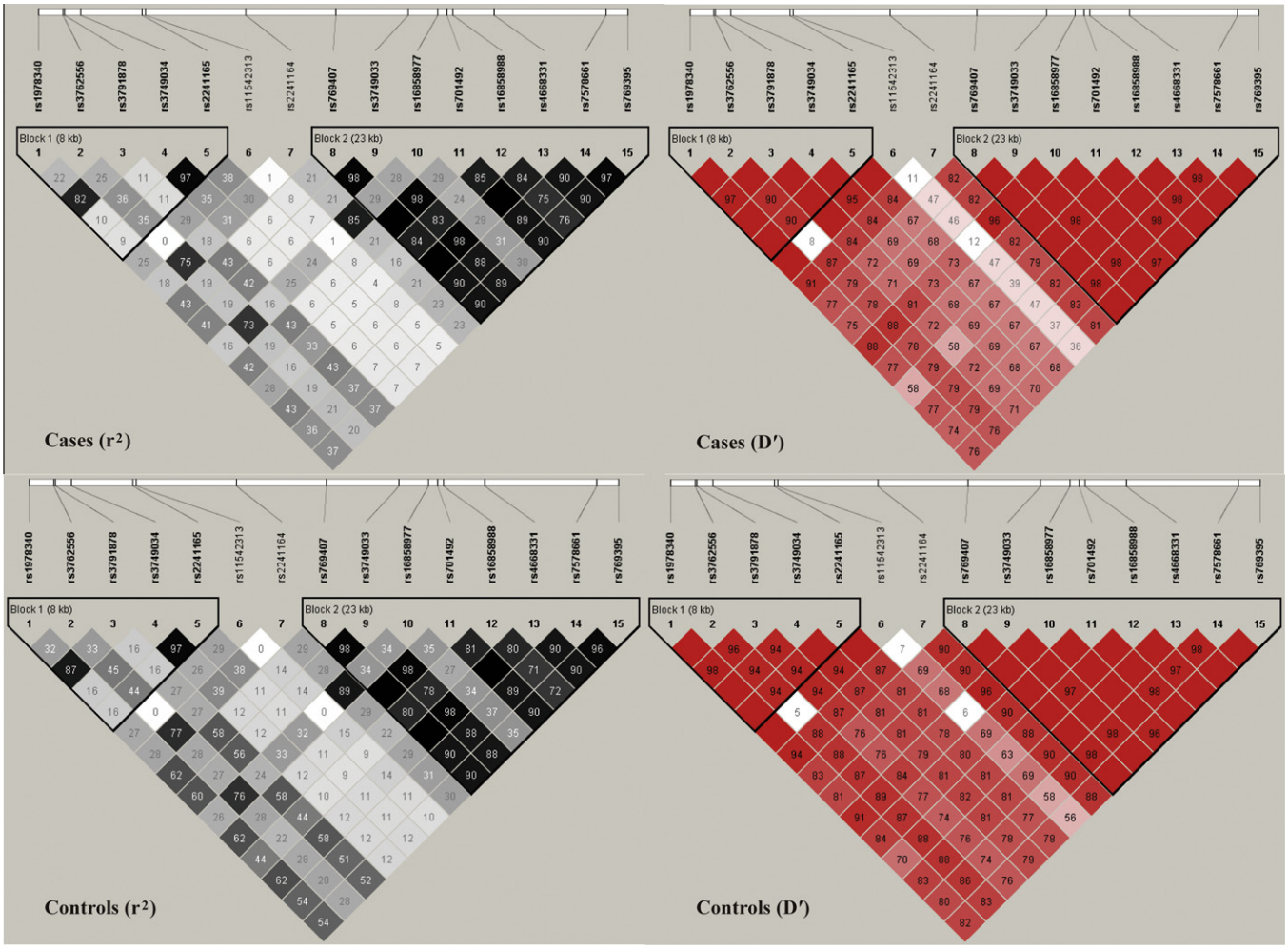

Fig. 2. LD plot of the 15 SNPs in GAD1 gene in cases (above) and controls (below). Values in squares are the pair-wise calculation of r^2 (left) or D' (right). Black squares indicate $r^2 = 1$ (i.e. perfect LD between a pair of SNPs). Empty squares indicate $D' = 1$ (i.e. complete LD between a pair of SNPs).

Table 1
Genotype and allele frequencies of the GAD1 gene polymorphisms in cases (n = 370) and controls (n = 389) and the results of their associations with risk of heroin dependence.

Variable	Location	Group	Genotype (n, %)			Allele (n, %)		p^a	p^b	p^c
rs1978340	Promoter		CC	CT	TT	C	T	0.4902	0.0021	0.0007
		Cases	232 (62.7)	125 (33.8)	13 (3.5)	589 (79.6)	151 (20.4)			
		Control	205 (52.7)	151 (38.8)	33 (8.5)	561 (72.1)	217 (27.9)			
rs3762556	Promoter		CC	CG	GG	C	G	0.0530	0.8867	0.6238
		Cases	113 (30.5)	168 (45.4)	89 (24.1)	394 (53.2)	346 (46.8)			
		Control	125 (32.1)	174 (44.7)	90 (23.1)	424 (54.5)	354 (45.5)			
rs3791878	Promoter		AA	AC	CC	A	C	0.6970	0.0022	0.0011
		Cases	15 (4.1)	138 (37.3)	217 (58.6)	168 (22.7)	572 (77.3)			
		Control	38 (9.8)	158 (40.6)	193 (49.6)	234 (30.1)	544 (69.6)			
rs3749034	5′UTR		CC	CT	TT	C	T	0.9789	0.1468	0.3964
		Cases	183 (49.5)	167 (45.1)	20 (5.4)	533 (72.0)	207 (28.0)			
		Control	191 (49.1)	163 (41.9)	35 (9.0)	545 (70.1)	233 (29.9)			
rs2241165	Intron 2		AA	AG	GG	A	G	0.8639	0.1793	0.3875
		Cases	186 (50.3)	165 (44.6)	19 (5.1)	537 (72.6)	203 (27.4)			
		Control	193 (49.6)	163 (41.9)	33 (8.5)	549 (70.6)	229 (29.4)			
rs11542313	Exon 3		CC	CT	TT	C	T	0.2140	0.0050	0.0008
		Cases	99 (26.8)	173 (46.8)	98 (26.5)	371 (50.1)	369 (49.9)			
		Control	73 (18.8)	177 (45.5)	139 (35.7)	323 (41.5)	455 (58.5)			
rs2241164	Intron 4		GG	GA	AA	G	A	0.0681	0.8035	0.5181
		Cases	114 (30.8)	164 (44.3)	92 (24.9)	392 (53.0)	348 (47.0)			
		Control	125 (32.1)	175 (45.0)	89 (22.9)	425 (54.6)	353 (45.4)			
rs769407	Intron 6		GG	GC	CC	G	C	0.3988	0.0801	0.0844
		Cases	197 (53.2)	152 (41.1)	21 (5.7)	546 (73.8)	194 (26.2)			
		Control	193 (49.6)	157 (40.4)	39 (10.0)	543 (69.8)	235 (30.2)			
rs3749033	Intron 6		AA	AT	TT	A	T	0.2855	0.0820	0.0926
		Cases	199 (53.8)	150 (40.5)	21 (5.7)	548 (74.1)	192 (25.9)			
		Control	196 (50.4)	154 (39.6)	39 (10.0)	546 (70.2)	232 (29.8)			
rs16858977	Intron 7		GG	GC	CC	G	C	0.2133	0.9538	0.7549
		Cases	117 (31.6)	171 (46.2)	82 (22.2)	405 (54.7)	335 (45.3)			
		Control	126 (32.4)	180 (46.3)	83 (21.3)	432 (55.5)	346 (44.5)			
rs701492	Intron 9		CC	CT	TT	C	T	0.3184	0.0760	0.0848
		Cases	193 (53.2)	149 (41.0)	21 (5.8)	535 (73.7)	191 (26.3)			
		Control	188 (49.6)	152 (40.1)	39 (10.3)	528 (69.7)	230 (30.3)			
rs16858988	Intron 11		GG	GA	AA	G	A	0.5902	0.2313	0.2169
		Cases	215 (58.1)	139 (37.6)	16 (4.3)	569 (76.9)	171 (23.1)			
		Control	216 (55.5)	145 (37.3)	28 (7.2)	577 (74.2)	201 (25.8)			
rs4668331	Intron 12		AA	AT	TT	A	T	0.3988	0.0801	0.0844
		Cases	197 (53.2)	152 (41.1)	21 (5.7)	546 (73.8)	194 (26.2)			
		Control	193 (49.6)	157 (40.4)	39 (10.0)	543 (69.8)	235 (30.2)			
rs7578661	Intron 15		CC	CG	GG	C	G	0.2310	0.1178	0.0951
		Cases	191 (51.6)	153 (41.4)	26 (7.0)	535 (72.3)	205 (27.7)			
		Control	187 (48.1)	158 (40.6)	44 (11.3)	532 (68.4)	246 (31.6)			
rs769395	3′UTR		TT	TC	CC	T	C	0.1520	0.1060	0.0790
		Cases	189 (51.1)	153 (41.4)	28 (7.6)	531 (71.8)	209 (28.2)			
		Control	184 (47.3)	158 (40.6)	47 (12.1)	526 (67.6)	252 (32.4)			

[a] p Values for Hardy–Weinberg equilibrium in controls.
[b] p Values for genotype frequency difference.
[c] p Values for allele frequency difference.

Table 2
GAD1 haplotype in block 1 frequencies and the results of their associations with risk of heroin dependence.

Haplotype[a]	Cases (n, %)	Controls (n, %)	Statistics			
			χ^2	p^b	OR	95% CI
C-G-C-T-G	192 (26.0)	219 (28.2)	0.9324	0.3343	0.8943	0.7128–1.1220
T-C-A-C-A	137 (18.5)	214 (27.5)	17.2546	0.0003[c]	0.5988	0.4695–0.7637
C-C-C-C-A	226 (30.5)	188 (24.1)	7.7734	0.0053[c]	1.3799	1.0999–1.7311
C-G-C-C-A	147 (19.8)	125 (16.1)	3.7197	0.0538	1.2950	0.9954–1.6847

[a] Haplotypes with frequency <0.05 were excluded.
[b] Alpha value is adjusted by Bonferroni correction and statistically significant results ($p < 0.01$).
[c] Continuity correction was applied.

minor allele being the risk allele and all cases being early onset [4]. In the present study, these two SNPs were not associated with heroin dependence. Differences of this kind may be correlated with alterations in hormone levels, neuronal system adaptations, the pharmacokinetics of substances of abuse [20], or with differences between ethnic groups. To some extent, this finding further supports a role of GAD1 promoter polymorphism in drug dependence.

These GAD gene variations can induce a change in mRNA secondary structure, which could in turn affect the stability, processing, or subcellular targeting of the mRNA transcript and thereby change splicing, transcription, and the efficiency of translation. In a recent study, homozygosity for the GAD1 alleles thought to confer risk for schizophrenia in a large clinical sample (T in rs1978340 and T for rs11542313) was associated with relatively increased

Table 3
GAD1 haplotype in block 2 frequencies and the results of their associations with risk of heroin dependence.

Haplotype[a]	Cases (*n*, %)	Controls (*n*, %)	Statistics			
			χ^2	p[b]	OR	95% CI
G-A-C-C-G-A-C-T	333 (45.0)	343 (44.1)	0.1279	0.7206	1.0376	0.8474–1.2705
C-T-G-T-A-T-G-C	167 (22.6)	195 (25.1)	1.3018	0.2539	0.8714	0.6877–1.1040
G-A-G-C-G-A-C-T	198 (26.8)	183 (23.5)	2.1112	0.1462	1.1878	0.9416–1.4983

[a] Haplotypes with frequency <0.05 were excluded.
[b] Alpha value is adjusted by Bonferroni correction and statistically significant results ($p < 0.00625$).

GABA/Cre [21,22]. Previous studies have demonstrated an association of heroin self-administration with decreased GABA efflux [23,24]. The SNPs rs11542313 and rs1978340 can change mRNA secondary structure and thereby protein synthesis level [25,26] and may be in LD with other causative SNPs; additionally, their haplotype could change the interaction with DNA methyltransferases or histones thought to regulate GAD1 expression [27,28]. Kuo et al. examined the association of nine SNPs for GAD1 in 575 alcoholic patients and 530 controls in an Irish population. Initial sensitivity was significantly associated with haplotype GAAC ($p = 0.01$) in females, which consisted of a minor allele of marker rs2241165 and common alleles of rs3828275, rs2058725, and rs701492. Again, the negative regression coefficient of this haplotype effect on initial sensitivity and tolerance/maximum drinking indicated fewer drinks were needed before the effect was noticeable [4]. In our study, haplotype analysis revealed significantly more C-C-C-C-A haplotype (block 1) in heroin-dependent patients than in control patients. Also, the point-wise associations of these variants with heroin dependence were significant. These results indicated that people with C-C-C-C-A haplotype of GAD1 gene are more prone to heroin dependence. Our study supports recent work showing the association of GAD1 polymorphisms with heroin dependence [5].

In conclusion, in a relatively large and homogeneous sample, GAD1 gene polymorphisms (rs1978340, rs3791878, and rs11542313) were associated with heroin dependence. These findings encourage future investigations into functional polymorphisms within and close to the GAD1 gene using a systemic approach in large sample populations.

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