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The Evidence for the Contribution of the Autism Susceptibility Candidate 2 (AUTS2) Gene in Heroin Dependence Susceptibility

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Abstract The single-nucleotide polymorphisms (SNP) rs6943555 in autism susceptibility candidate 2 (AUTS2) has been reported to be significantly associated with alcohol consumption in Europeans. In this study, we identified the SNP in AUTS2 contributing to the genetic susceptibility to heroin dependence. The potential association between heroin dependence and 21 SNPs (rs2270162, rs2851510, rs513150, rs595681, rs210606, rs10237984, rs13228123, rs10235781, rs6969375, rs6943555, rs10251416, rs17141963, rs12669427, rs723340, rs2293507, rs2293508, rs6960426, rs9886351, rs2293501, rs10277450, rs1918425) of AUTS2 was examined in a Chinese Han population using the MassARRAY system. The participants included 426 patients with heroin dependence and 416 healthy controls. Single SNP association, haplotype association, and clinical phenotype association were analyzed. Single SNP association revealed that AA homozygotes of rs6943555 were significantly overrepresented in the patients with heroin dependence compared with the control subjects (P=0.0019). The patients with heroin dependence had a significantly higher frequency of the A allele (P=0.0003, odd ratio (OR)=1.429, 95 % confidence interval (CI)=1.175–1.738). Strong linkage disequilibrium (LD) was observed in five blocks (D'>0.9). In block 2, significantly more A-A haplotypes (P=0.006 after Bonferroni corrections) and significantly fewer T-A haplotypes (P=0.040) were found in the patients with heroin dependence. The genotype and clinical phenotype correlation study of the rs6943555 carriers showed that the amount of heroin self-injection was lower in the patients with the AA genotype relative to AT + TT genotypes (P<0.01). Our results confirmed that, in addition to heroin consumption, the SNP rs6943555 of AUTS2 may also play an important role in the etiology of heroin dependence.

Keywords Autism susceptibility candidate 2 · Heroin dependence · Single-nucleotide polymorphisms · Han population

Introduction

Heroin dependence, a chronic, relapsing brain disease, is characterized by drug dependence, tolerance, and compulsive seeking and use despite of harmful consequences. In heroin dependence, genetic predisposition is a potential risk factor (van den Bree et al. 1998). The substantial genetic influence on the development of drug addiction has been demonstrated, with inherited risk estimates in the range of 40–60 % (Uhl 2004; Uhl et al. 2008). Identification of vulnerability genes of heroin dependence not only helps elucidate the pathogenesis of heroin dependence, but also helps prevent its occurrence and relapse.

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The polymorphisms in the autism susceptibility candidate 2 (AUTS2) gene may be associated with drug dependence, including alcohol and heroin dependence (Chen et al. 2013). AUTS2 has been reported to be associated with autism spectrum disorders (ASD) (Sultana et al. 2002), mental retardation (FitzPatrick et al. 2007), epilepsy (Mefford et al. 2010), dyslexia (Girirajan et al. 2011), and attention deficit hyperactivity disorder (ADHD) (Elia et al. 2010). Furthermore, AUTS2 is highly expressed in the developing cerebral cortex, hippocampus, and amygdala regions often affected by neuropathological changes in drug dependence (Bedogni et al. 2010; Baik 2013; Mark et al. 2011). AUTS2 expression in the brain has been confirmed to be associated with the alcohol preference in mice (Schumann et al. 2011), whereas downregulating the expression of the *Drosophila* AUTS2 homologue dAUTS2/tay gene reduced the alcohol sensitivity of *Drosophila* (Schumann et al. 2011). AUTS2 expression was found to be significantly associated with nicotine dependence, cannabis dependence, and antisocial personality disorder, whereas these small-scale studies need to be confirmed by larger cohorts (Philibert et al. 2007; Oksenberg and Ahituv 2013). Chen et al. recently confirmed that AUTS2 gene expression in lymphoblastoid cell lines (LCL) was significantly reduced in 124 male patients with heroin dependence compared with 116 healthy male controls (Chen et al. 2013). Altogether, these data indicate that the AUTS2 gene may be associated with drug dependence.

AUTS2 is located on 7q11.22 and has 19 exons with intron-exon junctions conforming to splice-site consensus sequences. The first 6 exons are separated by very large introns, whereas the last 13 exons are closely clustered. Recently, a single-nucleotide polymorphism (SNP) rs6943555 in AUTS2 has been significantly associated ($P<10^{-6}$) with alcohol consumption, based on a genome-wide study including 12 population samples of European ancestry comprising 26,316 individuals with replication genotyping in additional 21,185 subjects (Schumann et al. 2011). Only one study reported that rs6943555 in AUTS2 was significantly associated with heroin dependence in 546 male heroin-dependent individuals and 373 male control subjects (Chen et al. 2013). The major limitation is that the study only analyzed one locus, and several important SNPs, such as rs2270162 (promoter), rs3735260 (5′-untranslated region), rs2293507 (exon), and rs2293508 (exon) were omitted. The limited SNPs could not effectively capture the true causative SNPs in AUTS2 due to the weak linkage disequilibrium between them. Thus, more strict studies are needed to perform.

In this study, to identify the SNP in AUST2 contributing to the genetic susceptibility to heroin dependence, 21 loci were analyzed, in a large case–control sample of the same ethnic origin.

Materials and Methods

Subjects

Four hundred and twenty-six adult subjects (mean age±SD 36.2±6.8 years, 367 males and 59 females) were enrolled in the present study. They all received rehabilitation therapies in the methadone maintenance treatment (MMT) program provided by the Xi'an Mental Health Center (Xi'an, China). The dependence status of each subject was assessed by a psychiatrist at the beginning of the methadone management program, and opioid dependence was diagnosed based on the medical history, urine tests, and interview according to the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria major neurological and psychological presentations were evaluated at the beginning of rehabilitation and reappraised once every year. To assist the diagnosis, a case vignette was adopted, which included a semi-structured interview with questions on (i) the age of initiation of heroin use and duration of heroin use, (ii) quantity of heroin used over this period, (iii) route of administration (nasal inhalation or injection), (iv) whether other substances were used or abused, and (v) comorbidity of other psychiatric disorders. The exclusion criteria are as follows: age less than 18 years, accompanied by a major central nervous system disease, psychosis, and using drugs other than heroin as the primary choice. All subjects had been on the MMT for at least 6 months at the time of the current study. The control group consisted of 416 unrelated healthy subjects (mean age±SD 37.13±5.23, 355 males, 61 females) who underwent health examinations in the Medical Examination Center of the First Hospital Affiliated to the Medical College of Xi'an Jiaotong University (Xi'an, China). The subjects who had substance abuse, participated in other studies or suffered from chronic brain diseases were excluded. All participants completed a family history questionnaire and were self-identified as Han Chinese from Shaanxi province for three generations. Participants were excluded from the study if they had a relative in this study or had a mixed ancestry. Written informed consent was obtained from all participants. The study protocol was approved by the Ethical Committee of the Medical College, Xi'an Jiaotong University (Xi'an, China).

Selection of Polymorphisms

The principal hypothesis is that there is one or more common SNPs in the AUTS2 gene that are associated with altered risk of heroin dependence. Therefore, we aimed to identify a set of tagging SNPs that efficiently tag all the known common variants and are likely to tag most of the unknown common variants. Marker selection was based on previous studies (Chen et al. 2013; Chojnicka et al. 2013), and preliminary analysis was performed using the HapMap data. We examined

tagSNPs in the Haploview software v4.2, using the Chinese Han in Beijing (CHB) population and a minor allele frequency (MAF) cutoff ≥5 % (the HapMap data release 27). As a first screen of the most common SNPs in the northwestern Han Chinese heroin dependence sample, a MAF ≥20 % with pairwise tagging and $r^2 \geq 0.8$ (de Bakker et al. 2005) was used as the cutoff when choosing tagSNPs. The LD pattern of this gene was determined in the Chinese population using the preliminary data from HapMap. In addition, we gave higher priority to prior association analysis in a Japanese population given that both Japanese and Han Chinese are Asian. A total of twenty one SNPs were selected for genotyping. The relative genomic locations of the AUTS2 gene and selected SNPs are shown in Fig. 1. SNPs including rs2270162 are located in promoter region; rs2851510, rs513150, rs595681, rs210606, and rs10237984 are located in intron 1; rs13228123 is located in intron 3; rs10235781, rs6969375, rs6943555, and rs10251416 are located in intron 4; rs17141963 and rs6960426 are located in intron 5; rs723340 is located in intron 6; rs2293507 and rs2293508 are located in exon 7; rs6960426 is located in intron 8; rs9886351 is located in intron 15; rs2293501 is located in intron 17; and rs10277450 and rs1918425 are located in 3′ near. These SNPs were further analyzed in an association study.

Genotyping

Three to five milliliters of peripheral blood were collected in tubes coated with EDTA. Genomic DNA was extracted from leukocytes in the blood using the EZNA™ Blood DNA Midi Kit (Omega Bio-Tek, Norcross, GA, USA), according to the manufacturer's protocol. Primers for PCR and single-base extension were designed by using the Assay Designer software package (Sequenom Inc., San Diego, CA, USA). SNP genotyping was performed by Shanghai Benegene Biotechnology Co., Ltd (Shanghai, China) using the MassARRAY system (Sequenom) by means of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry method according to the manufacturer's instructions. Briefly, the extracted DNA to be queried was diluted to 5 ng/μl, and then 1 μl of diluted DNA was mixed with 0.95 μl of water, 0.625 μl of PCR buffer containing 15 mM $MgCl_2$, 1 μl of 2.5 mM dNTP, 0.325 μl of 25 mM $MgCl_2$, 1 μl of PCR primers, and 0.1 μl of 5 units/μl HotStar *Taq* (Qiagen). The PCR reaction was incubated at 94 °C for 15 min, followed by 45 cycles at 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 1 min, and final incubation at 72 °C for 3 min. After PCR amplification, remaining dNTPs were dephosphorylated by adding 1.53 μl of water, 0.17 μl of SAP buffer, and 0.3 units of shrimp alkaline phosphatase (Sequenom). The reaction was placed at 37 °C for 40 min, and the enzyme was inactivated by heating for 5 min at 85 °C. After the treatment of shrimp alkaline phosphatase, the single primer extension over the SNP was combined with 0.75 μl of water, 0.2 μl of 10X iPLEX buffer, 0.2 μl of termination mix, 0.041 μl of iPLEX enzyme (Sequenom), and 0.804 μl of 10 μM extension primer. The single-base extension reaction was carried out at 94 °C for 30 s and then 94 °C for 5 s, followed by 5 cycles of 52 °C for 5 s and 80 °C for 5 s, total 40 cycles, then 72 °C for 3 min. The reaction mixture was desalted by adding 6 mg of cation exchange resin (Sequenom). The completed genotyping reactions were spotted onto a 384-well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser (Sequenom) and determined by the matrix-assisted laser desorption ionization time-of-flight mass spectrometer. Genotype calling was performed in real time with the MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer software version 3.4 (Sequenom).

Statistical Analysis

All statistical tests were conducted with the SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). The Hardy–Weinberg equilibrium (HWE) for each SNP was assessed using the GENEPOP version 4.0 (Rousset and Raymond 1995). Associations between polymorphisms and heroin dependence were

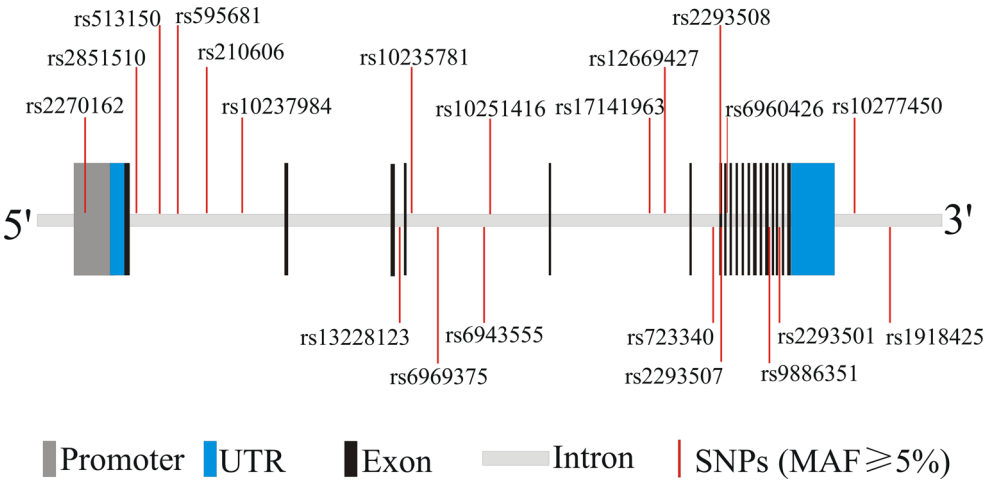

Fig. 1 Gene structure of human AUTS2 and the relative positions of the 21 variations used in our study

Table 1 Genotypic and allelic frequencies of AUTS2 polymorphisms in the controls and patients with heroin dependence

ID/bp	Location	MAF	Controls (n=416)		Heroin dependence (n=426)		P value[a]	Logistic regression OR, 95 % CI
			No.	%	No.	%		
rs2270162/69,061,966	Promoter	0.224					0.411	
GG			250	60.1	274	64.3	0.206	0.835, 0.632–1.104
GC			146	35.1	136	31.9	0.325	1.155, 0.867–1.538
CC			20	4.8	16	3.8	0.462	1.287, 0.657–2.522
Per C allele			186	22.4	168	19.7	0.184	0.853, 0.675–1.079
rs2851510/69,080,200	Intron 1	0.224					0.284	
CC			250	60.1	278	65.3	0.120	0.801, 0.606–1.060
TC			146	35.1	132	31.0	0.200	1.207, 0.905–1.609
TT			20	4.8	16	3.8	0.462	1.287, 0.657–2.522
Per T allele			186	22.4	164	19.2	0.116	0.828, 0.654–1.048
rs513150/69,175,653	Intron 1	0.377					0.067	
TT			166	39.9	162	38.0	0.576	1.082, 0.820–1.428
TC			186	44.7	218	51.2	0.061	0.772, 0.588–1.012
CC			64	15.4	46	10.8	0.050	1.502, 1.000–2.256
Per C allele			314	37.7	310	36.4	0.565	0.994, 0.774–1.150
rs595681/69,190,837	Intron 1	0.387					0.112	
AA			154	37.1	142	33.3	0.267	1.174, 0.884–1.559
AG			202	48.6	200	46.9	0.634	1.068, 0.815–1.400
GG			60	14.4	84	19.7	0.042	0.686, 0.477–0.987
Per G allele			322	38.7	368	43.2	0.061	1.204, 0.991–1.463
rs210606/69,234,928	Intron 1	0.394					0.277	
GG			154	37.0	142	33.3	0.267	1.174, 0.884–1.559
GA			196	47.1	200	46.9	0.955	1.008, 0.769–1.322
AA			154	37.0	84	19.7	0.145	0.768, 0.538–1.095
Per A allele			328	39.4	368	43.2	0.116	1.168, 0.962–1.419
rs10237984/69,298,131	Intron 1	0.392					0.214	
TT			154	37.0	142	33.3	0.267	1.174, 0.884–1.559
TG			198	47.6	200	46.9	0.847	1.027, 0.783–1.347
GG			64	15.4	84	19.7	0.100	0.741, 0.518–1.060
Per G allele			326	39.2	368	43.2	0.095	1.180, 0.972–1.433
rs13228123/69,592,701	Intron 3	0.397					0.285	
CC			154	37.0	142	33.3	0.267	1.174, 0.884–1.559
AC			194	46.6	198	46.5	0.959	1.007, 0.768–1.321
AA			68	16.3	86	20.2	0.151	0.773, 0.554–1.099
Per A allele			330	39.7	370	43.4	0.117	1.168, 0.962–1.418
rs10235781/69,606,936	Intron 4	0.361					0.781	
TT			172	42.8	172	42.6	0.805	0.966, 0.736–1.268
CT			170	42.3	178	44.1	0.428	0.896, 0.683–1.176
CC			60	14.9	54	13.4	0.974	0.994, 0.698–1.196
Per C allele			290	36.1	286	35.4	0.778	0.971, 0.792–1.191
rs6969375/69,721,267	Intron 4	0.362					0.763	
AA			170	41.1	164	38.5	0.395	1.127, 0.855–1.486
AG			188	45.4	200	47.0	0.705	0.949, 0.723–1.245
GG			56	13.5	62	14.6	0.799	0.951, 0.646–1.401
Per G allele							0.446	1.080, 0.886–1.317
rs6943555/69,806,023	Intron 4	0.358					0.0019	
TT			172	41.4	134	31.5	0.003	1.533, 1.155–2.035

Table 1 (continued)

ID/bp	Location	MAF	Controls (n=416)		Heroin dependence (n=426)		P value[a]	Logistic regression OR, 95 % CI
			No.	%	No.	%		
AT			190	45.7	206	48.4	0.440	0.859, 0.685–1.178
AA			54	13.0	86	20.2	0.006	0.591, 0.408–0.857
Per A allele			298	35.8	378	44.4	0.0003	1.429, 1.175–1.738
rs10251416/69,806,314	Intron 4	0.471					0.202	
AA			112	26.9	132	31.0	0.190	0.819, 0.607–1.104
AG			216	51.9	222	52.1	0.959	0.993, 0.757–1.301
GG			88	21.2	72	16.9	0.115	1.321, 0.935–1.867
Per G allele			392	47.1	366	43.0	0.086	0.845, 0.698–1.024
rs17141963/70,125,709	Intron 5	0.488					0.203	
GG			106	25.6	108	25.5	0.967	1.007, 0.739–1.371
GT			212	51.2	196	46.2	0.143	1.224, 0.934–1.606
TT			96	23.2	120	28.3	0.090	0.765, 0.561–1.042
Per T allele			404	48.8	436	51.4	0.283	1.111, 0.917–1.345
rs12669427/70,132,947	Intron 5	0.488					0.285	
TT			106	25.5	128	30.1	0.134	0.793, 0.596–1.074
CT			214	51.4	212	49.8	0.614	1.072, 0.818–1.405
CC			96	23.1	86	20.2	0.309	1.186, 0.854–1.648
Per C allele			406	48.8	384	45.1	0.125	0.861, 0.711–1.043
rs723340/70,209,637	Intron 6	0.394					0.943	
CC			152	36.5	158	37.1	0.884	0.972, 0.734–1.288
CT			200	48.1	206	48.4	0.957	0.993, 0.757–1.302
TT			64	15.4	62	14.6	0.733	1.068, 0.731–1.560
Per T allele			328	39.4	330	38.7	0.771	0.971, 0.799–1.181
rs2293507/70,228,020	Exon 7	0.341					0.954	
CC			180	43.3	182	42.7	0.891	1.019, 0.775–1.340
CA			188	45.2	192	45.1	0.958	1.007, 0.768–1.322
AA			48	11.5	52	12.2	0.771	0.940, 0.619–1.428
Per A allele			284	34.1	296	34.7	0.793	1.027, 0.840–1.256
rs2293508/70,228,139	Exon 7	0.341					0.954	
GG			180	43.3	182	42.7	0.771	0.940, 0.619–1.428
AG			188	45.2	192	45.1	0.958	1.007, 0.768–1.322
AA			48	11.5	52	12.2	0.891	1.019, 0.775–1.340
Per A allele			284	34.1	296	34.7	0.793	1.027, 0.840–1.256
rs6960426/70,230,794	Intron 8	0.341					0.186	
GG			176	42.3	154	36.2	0.646	0.911, 0.614–1.354
AG			186	44.7	212	49.8	0.146	0.818, 0.624–1.073
AA			54	13.0	60	14.1	0.070	1.292, 0.979–1.706
Per A allele			294	35.3	332	39.0	0.123	1.168, 0.959–1.424
rs9886351/70,248,469	Intron 15	0.385					0.060	
AA			159	38.5	137	32.2	0.057	1.317, 0.991–1.749
AG			192	46.2	211	49.5	0.325	0.873, 0.666–1.144
GG			65	15.4	78	18.3	0.266	0.814, 0.566–1.170
Per G allele			320	38.5	367	43.1	0.054	1.211, 0.997–1.471
rs2293501/70,251,923	Intron 17	0.337					0.956	
GG			180	43.3	180	42.3	0.766	1.042, 0.793–1.370
AG			192	46.2	200	47.0	0.820	0.969, 0.739–1.271
AA			44	10.6	46	10.8	0.913	0.976, 0.630–1.511

Table 1 (continued)

ID/bp	Location	MAF	Controls (n=416)		Heroin dependence (n=426)		P value[a]	Logistic regression OR, 95 % CI
			No.	%	No.	%		
Per A allele			280	33.7	292	34.3	0.789	1.028, 0.840–1.258
rs10277450/70,363,783	3′near	0.449					0.163	
TT			129	31.2	118	27.8	0.254	1.188, 0.884–1.597
GT			198	47.8	224	52.7	0.062	0.773, 0.589–1.013
GG			87	21.0	83	19.5	0.225	1.234, 0.878–1.735
Per G allele			372	44.9	390	45.9	0.695	1.039, 0.858–1.260
rs1918425/70,371,194	3′near	0.454					0.101	
TT			131	31.5	117	25.3	0.196	1.217, 0.904–1.637
TC			192	46.2	228	49.4	0.031	0.742, 0.566–0.973
CC			93	22.4	81	19.0	0.229	1.228, 0.879–1.716
Per C allele			378	45.4	390	45.8	0.888	1.014, 0.837–1.228

MAF minor allele frequency in controls

[a] P value was calculated by 2×3 and 2×2 chi-squared tests based on codominant, dominant for the rare allele, heterosis, and recessive for the rare allele models of inheritance

Alpha value is adjusted by Bonferroni correction and statistically significant results (P<0.003)

assessed by the Fisher's exact test or the Pearson's chi-squared test. The odds ratios (OR) and the 95 % confidence intervals (95 % CI) were calculated to estimate the strength of association by unconditional logistic regression analysis. The Bonferroni correction was used to adjust the test level when multiple comparisons were conducted, and the P value was divided by the total number of loci or haplotypes. The D' for each SNP pair was plotted. Haplotype blocks were defined according to the "solid spine of LD" approach. Haplotype blocks were defined according to the criteria of Gabriel et al. (Gabriel et al. 2002; Barrett et al. 2005). Haplotypes with a frequency of less than 5 % were excluded. To ensure that the LD blocks most closely reflect the population level LD patterns, definition of the blocks were based on the control samples alone. The significance of any haplotypic association was evaluated using a likelihood ratio test, followed by permutation testing that compared estimated haplotype frequencies in cases and controls.

Table 2 AUTS2 haplotype in block 1–4 frequencies and the results of their associations with risk of heroin dependence

Block	ID	Haplotype[a]	Cases (n, %)	Controls (n, %)	Statistics			
					χ^2	P	OR	95% CI
1	HAP1	G-C-T-G-A-G-A-T-A	174 (40.845)	156 (37.500)	0.998	0.320	1.151	0.872–1.518
	HAP2	G-C-C-A-G-T-C-C-G	137 (32.160)	131 (31.490)	0.043	0.835	1.031	0.772–1.378
	HAP3	C-T-T-A-G-T-C-T-A	77 (18.075)	91 (21.875)	1.903	0.168	0.788	0.561–1.106
2	HAP1	A-A	188 (44.131)	145 (34.856)	7.574	0.006*	1.476	1.118–1.949
	HAP2	T-A	55 (12.911)	75 (18.029)	4.223	0.040	0.674	0.462–0.983
	HAP3	T-G	182 (42.723)	192 (46.154)	1.003	0.316	0.870	0.663–1.142
3	HAP1	T-T-C-C-A-G	204 (47.887)	201 (48.317)	0.016	0.901	0.983	0.750–1.288
	HAP2	G-C-T-A-G-A	144 (33.803)	135 (56.490)	0.173	0.677	1.063	0.798–1.416
	HAP3	G-C-C-C-A-G	29 (6.808)	39 (9.375)	1.869	0.172	0.706	0.428–1.165
4	HAP1	A-A	142 (33.333)	137 (32.932)	0.015	0.902	1.018	0.764–1.357
	HAP2	A-G	100 (23.474)	119 (28.606)	2.880	0.090	0.766	0.562–1.043
	HAP3	G-G	180 (42.254)	157 (37.740)	1.786	0.181	1.207	0.916–1.591
5	HAP1	G-C	193 (45.305)	186 (44.712)	0.030	0.863	1.024	0.781–1.344
	HAP2	T-T	231 (54.225)	225 (54.087)	0.002	0.968	1.006	0.767–1.319

*P value is adjusted by Bonferroni correction and statistically significant results (P<0.006 in block 1; P<0.025 in blocks 2, 4, and 5; P<0.008 in block 3)

[a] Haplotypes with frequency <0.05 were excluded

Results

The genotype distributions, allelic frequencies, and haplotypes in patients with heroin dependence and the healthy controls are shown in Tables 1 and 2. There was no significant deviation from Hardy–Weinberg equilibrium for any SNP in patients with heroin dependence and the healthy controls ($P>0.05$, Table 1). Linkage disequilibrium (LD) analyses revealed 5 haplotypes in controls and also showed that 9 SNPs (rs2270162, rs2851510, rs513150, rs595681, rs210606, rs10237984, rs13228123, rs10235781, and rs6969375) were located in block 1, 2 SNPs (rs6943555 and rs10251416) in block 2, 6 SNPs (rs17141963, rs12669427, rs723340, rs2293507, rs2293508, and rs6960426) in block 3, 2 SNPs (rs9886351 and rs2293501) in block 4, and 2 SNPs (rs10277450 and rs1918425) in block 5 (Fig. 2).

Compared with the healthy controls, the AA homozygotes of rs6943555 were significantly over-represented in the patients with heroin dependence and the healthy controls ($P=0.0019$). The patients with heroin dependence had a significantly higher frequency of the A allele ($P=0.0003$, OR = 1.429, 95 % CI = 1.175–1.738), whereas other 20 AUTS2 SNPs gave negative results (Table 1).

Furthermore, strong LD was observed in rs6943555–rs10251416 (block 2, D'>0.9). The patients with heroin dependence showed significantly more A-A haplotypes ($P=0.006$ after Bonferroni corrections) and a significantly lower frequency of the T-A haplotypes ($P=0.040$) (the frequency did not pass the threshold value (threshold significance P value was set at 0.025 in block 2, Table 2)).

The genotypes of rs6943555 in the patients with heroin dependence were classified into two sub-groups: AA genotype ($n=86$) and AT + TT genotype ($n=340$). The results showed novel difference in the AUTS2 gene rs6943555 genotype subjects, regarding the amount of intravenous heroin use (Table 3). The patients with AA genotype had decreased amount of intravenous heroin use, compared with those with AT + TT genotype ($P<0.01$).

Discussion

AUST2 plays a potential role in neuronal development, and multiple neurological diseases, including drug addiction (Chen et al. 2013; Philibert et al. 2007; Oksenberg and Ahituv

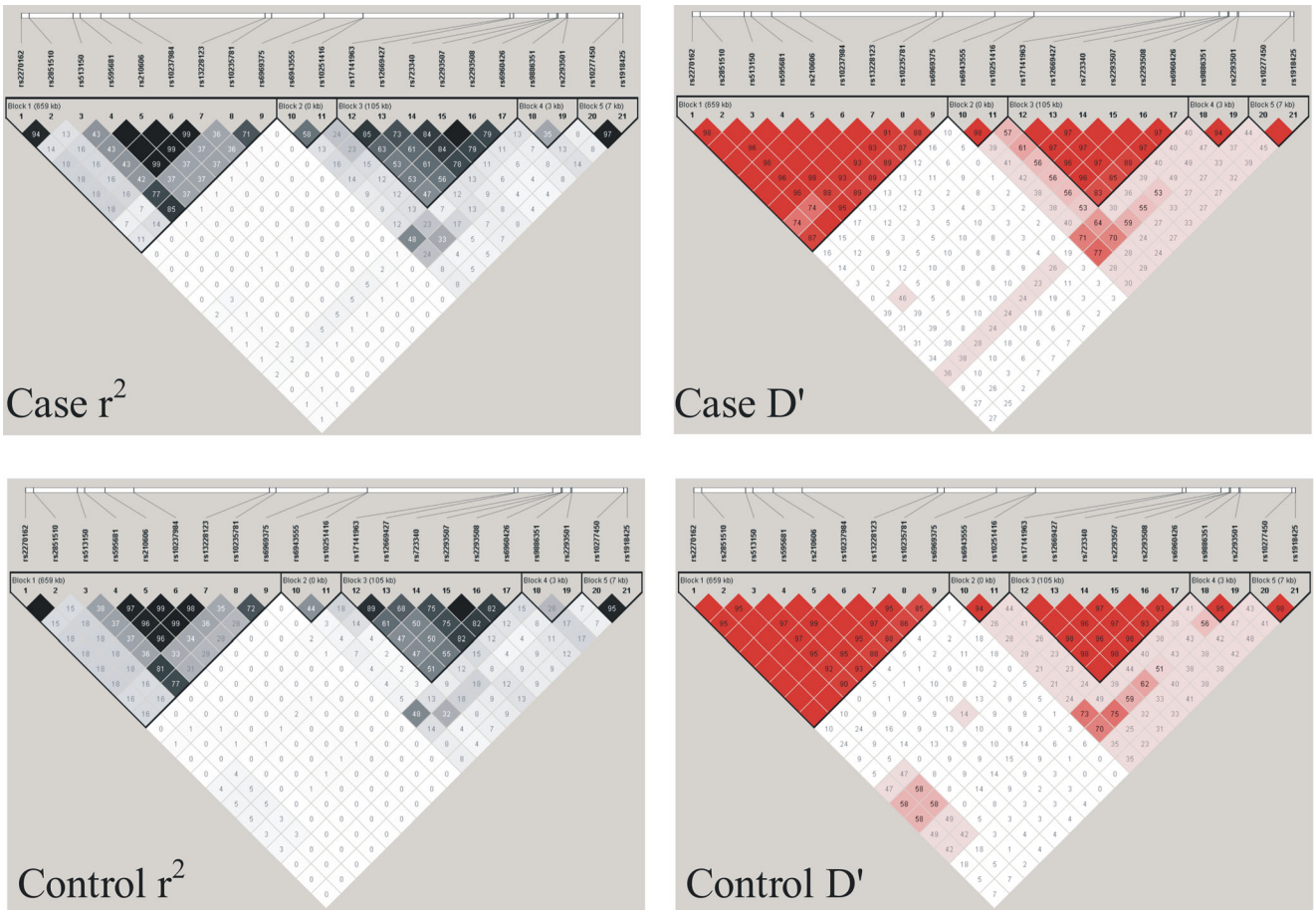

Fig. 2 LD plot of the 21 SNPs in AUTS2 gene in cases (*above*) and controls (*below*). Values in squares are the pair-wise calculation of r^2 (*left*) or D' (*right*). *Black squares* indicate $r^2=1$ (i.e., perfect LD between a pair of SNPs). *Empty squares* indicate D'=1 (i.e., complete LD between a pair of SNPs)

Table 3 Dependence characteristic by genotype of AUTS2 gene rs6943555

Variable	AUTS2 rs6943555	
	AA	AT + TT
Age (year)	37.42±3.24	37.16±4.08
Gender (%)		
Male	83.72	81.18
Female	16.28	18.82
Occupation (%)		
Employed	27.91	24.71
Unemployed	72.09	75.29
Marital status (%)		
Married	44.19	42.06
Unmarried	55.81	57.94
History of addiction (year)	11.28±4.42	11.65±4.38
Withdrawal times	7.82±6.36	8.32±5.35
Pattern of addiction (%)		
Oral suck (*n*)	15.12	17.06
Intravenous injection (*n*)	84.88	82.94
Amount of addiction (g)		
Oral suck	0.56±0.36	0.60±0.28
Intravenous injection[a]	1.02±0.31	1.39±0.38

[a] Associated with AT + TT of AUTS2, $P<0.01$

2013). In this study, 21 SNPs genotyped spanned the coding and non-coding regions of the AUTS2 gene. Our results provide direct evidence that a genetic change in AUTS2 is linked to heroin dependence in humans, and extend the list of variants that may affect the development of heroin dependence (Chen et al. 2013).

In a relative small-scale study, Chen et al. found that AUTS2 was downregulated in the LCL by 3.01-fold in the patients with heroin dependence compared with healthy controls (Liao et al. 2014). A subsequent large-scale study achieved the same conclusion (Chen et al. 2013). The study also achieves the following conclusions (Chen et al. 2013): (1) the genetic association analysis showed that A allele of rs6943555 were significantly over-represented in the patients with heroin dependence compared with the control subjects, and AA carriers had significantly lower AUTS2 mRNA levels in their LCL compared with TT carriers and AT carriers. (2) Significant differences were also found in the distribution of allele frequencies of rs6943555 gene between the patients with heroin dependence and healthy controls. The frequency of the A allele of rs6943555 in the patients with heroin dependence was significantly higher than that in the controls. 3. Further genotype and clinical phenotype correlation study of the rs6943555 carriers showed that the amount of heroin self-injection was lower in patients with the AA genotype relative to AT + TT genotypes. Thus, all the studies suggest that the AUTS2 gene might be associated with heroin dependence and reduced AUTS2 gene expression might confer increased susceptibility to heroin dependence. Interestingly, in a genome-wide association study of alcohol consumption in Europeans, Schumann and colleagues discovered that the minor ancestral frequency allele A of the rs6943555 in AUST2 was associated with 5.5 % lower alcohol consumption (Schumann et al. 2011). They also reported reduced expression of the *Drosophila* homologue AUTS2 gene (dAUTS2/tay) resulted in reduced sensitivity to alcohol in *Drosophila*, while reduced sensitivity to alcohol has been reported to be a risk of alcohol drinking disorders (Schumann et al. 2011; Schuckit and Smith 2011). These results suggested that reduced expression of AUTS2 gene might confer increasing susceptibility to alcohol drinking disorders in humans. Consistently, our studies also suggested that reduced AUTS2 gene expression might confer increasing susceptibility to heroin dependence. It is noteworthy that, in our study, the patients with heroin dependence carrying allele A were found to have decreased amount of heroin intravenous injections. Altogether, these data indicate that the AUTS2 gene might be closely associated with drug dependence.

We further investigated the interaction among polymorphisms and observed strong LD. Haplotype analysis revealed that the A-A (block 2) haplotypes of the AUTS2 gene displayed a risk effect. There were significant point-wise associations of these variants with heroin dependence. These results indicated that people with this haplotypes of the AUTS2 gene were more prone to heroin dependence. To some extent, this finding further supports work showing the association of AUTS2 polymorphisms with heroin dependence (Baik 2013).

The limitations of this study are that the AUTS2 gene expression was not analyzed in the brain. Hence, the interpretation of the results of this study needs to be cautious. Further independent research is necessary to verify our findings in this study. All the known common coding SNPs, 3′UTR SNPs and 5′UTR SNPs were tagged by our selected panel of tSNPs with $r^2>0.80$ and were not associated with disease. However, we cannot exclude the possibility that unidentified variants exist in the promoter or regulatory region or intron-exon boundaries, which affect the transcription of AUTS2 and are tagged by the two tSNPs for which we find association.

In conclusion, the present study identified a strong association between rs6943555 polymorphism (the A allele of rs6943555) of the AUTS2 with heroin dependence. These findings encourage future efforts in searching for functional polymorphisms within and close to the AUTS2 gene using a systematic approach in a larger sample population.

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