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ORIGINAL ARTICLE

Lack of Association between NeuroD1/D6 Gene Polymorphism and Heroin Dependence in Han-Chinese Male Population

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Objectives: Heroin dependence (HD) is a chronic brain disease with genetic contribution. NeuroD1/D6 are neurogenic differentiation factors that play a role in development and differentiation of the brain. In this study, we intended to investigate the association of NeuroD1/D6 gene polymorphism and the occurrence of HD, and the role of this genetic variability in specific personality traits of HD patients. **Materials and Methods:** A total of 694 individuals were recruited in this study (313 HD patients and 381 normal controls). Patients with HD were further classified into four clinical subgroups to reduce heterogeneity. Genetic variants of selected single-nucleotide polymorphisms in NeuroD1 (rs1801262, rs16867467, and rs2583016) and NeuroD6 (rs2233404) were genotyped. We used the Chinese version of tridimensional personality questionnaire to assess specific personality traits in subjects. **Results:** For allele and genotype frequency analysis, we found a weak association between NeuroD1 rs16867467 and patients with HD ($P < 0.05$). Nonetheless, the association findings could not remain significant after Bonferroni's correction. For specific personality traits, NS score was negatively correlated with age in both study groups ($P < 0.05$); however, the NeuroD SNPs were not associated with the novelty-seeking/harm avoidance scores in patients with HD. **Conclusions:** Our study suggests that NeuroD1/D6 gene polymorphism may neither be associated with the occurrence of HD nor did these polymorphisms influence the personality traits in patients with HD.

Key words: Heroin dependence, NeuroD1/D6, single nucleotide polymorphism, Han Chinese male population

INTRODUCTION

Heroin is one of the major abused drugs in many countries.[1] According to the United Nations Office on Drugs and Crime, there are estimated 32.4 million opioid users in the adult population of the world.[2] Heroin dependence (HD) cause global burden regarding human immunodeficiency virus and hepatitis C virus transmission, healthcare costs, criminal activity, and lost productivity.[3] Lin *et al*. estimated the total economic cost of HD in Taiwan to be US$ 18,310 per person-year.[4] HD is a chronic complex disease of the brain with genetic involvement. According to family-based linkage and association studies, the genetic contribution to develop heroin addiction is about 40%–60%,[5] suggesting that the genetic factor play a major role in the development of HD.[6]

Neurobiological advances provided new insight into the pathophysiology of heroin addiction;[7] that HD is gradually thought of as a complex behavior, catalyzed by biological and psychosocial factors.[8] Actually, brain abnormalities are underlying causes of HD,[9] with activation of the different neurobiological circuit in different stages of the addiction cycle.[10] HD is also associated with neurogenesis of the brain, including development and differentiation.[11] For example, postmortem human brain studies demonstrated decreased number of neural precursor cells in heroin addicts, which demonstrates the relation of heroin addiction and brain development.[12]

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The NeuroD1 and NeuroD6 are neurogenic differentiation factors and belong to the basic helix-loop-helix transcription factors.[13] The NeuroD1/D6 is known as their differentiation-inducing property, displaying overlapping patterns of expression during brain development.[14] NeuroD1 could regulate differentiation in many neuronal regions of the central nervous system.[15,16] For instance, overexpression of NeuroD1 promotes neural differentiation in adult rats brain, whereas deletion of NeuroD1 results in decreased survival and maturation;[17] regulation of the NeuroD1 pathways would further interfere with contextual memory retention and the stability of dendritic spines.[18-20] On the other hand, NeuroD6 involves in neuronal development/differentiation, and the switch from pro-apoptotic to antiapoptotic pathways in differentiating neural cells.[14] NeuroD6 expression is also associated with enhanced mitochondrial biogenesis.[21] Based on the major role as important neurogenic factors in the adult brain, the NeuroD1 and NeuroD6 are potential candidates involved in HD.

The NeuroD1 gene is located on chromosome 2q31.3, and the NeuroD6 gene is located on chromosome 7p14.3. The previous studies have focused on whether the NeuroD1/D6 gene variant confer susceptibility to human diseases,[13,22-27] but none dealing with drug addiction. We postulated that HD may be associated with NeuroD1/D6 gene variants by regulation of neurogenesis of the brain. The aims of this study are to examine whether NeuroD1/D6 gene variants are associated with the development of HD among Han-Chinese male population and explore the role of specific personality trait in the relation between NeuroD1/D6 genetic variability and HD.

MATERIALS AND METHODS

In this study, the patients with HD were recruited from different medical service in Taiwan. To reduce heterogeneity and prevent gender bias, we did not include female participants in this study. Each patient was interviewed by a psychiatrist for initial evaluation and subsequently interviewed by a psychologist with a Chinese version of the modified Schedule for Affective Disorders and Schizophrenia-Lifetime (SADS-L) and confirmed the diagnosis of HD by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision. Patients were further classified into clinical subgroups to reduce heterogeneity: HD with early onset (25 years old or younger at the onset of initial HD), HD with late onset (onset after the age of 25 years), HD with short duration (5 years or less since the onset of HD), and HD with long duration (more than 5 years after the onset of HD).

The healthy controls were volunteers recruited from different communities. The Chinese version of SADS-L was used to exclude psychiatric problems. The control subjects were free of past or present major and minor mental illnesses including substance use disorders. There was also no family history of psychiatric disorder or substance use disorder in the first-degree relatives of the control subjects.

The Chinese version of tridimensional personality questionnaire (TPQ) was used for assessing specific personality traits in the individual. Cloninger's TPQ is a self-report questionnaire that takes 20–30 min to complete that measures three distinct personality dimensions: novelty seeking (NS), harm avoidance (HA), and reward dependence (RD).[28] We only analyzed NS and HA dimensions in our study, because of low inter-reliability for RD dimension among the Han Chinese population in Taiwan.[29]

Genotyping methods

Genomic DNA was extracted from peripheral blood leukocytes using a commercial kit (DNAzol; Invitrogen, Carlsbad, CA, USA). The study variants of NeuroD1/D6 were selected on the basis of National Center for Biotechnology Information Single Nucleotide Polymorphism (SNP) database (www.ncbi.nlm.nih.gov/projects/SNP) and an available TaqMan assay from Applied Biosystems. Genetic variants of rs1801262, rs16867467, rs2583016 on NeuroD1, and rs2233404 on NeuroD6 were genotyped using TaqMan assays (Applied Biosystems, Foster City, CA, USA) employing FAM™ and VIC® dyes. We used the Applied Biosystems STEPONE™ software and STEPONEPLUS™ real-time PCR systems for thermocycling and data collection.

Statistical analysis

An independent samples *t*-test was used to determine the difference in mean age, age of onset of HD, duration of HD, NS, and HA scores between each group. Allele and genotype frequencies for each individual polymorphism were compared between each group using a two-tailed Pearson Chi-square test (with Fisher's exact test when the sample size was smaller than expected). A logistic regression was conducted to assess the influence of age and NeuroD1/D6 variants on the HD. A linear regression analysis was used to examine the relation between NeuroD1/D6 gene polymorphisms and novelty-seeking/harm avoidance (NS/HA) scores in patients with HD.

SPSS (version 20, SPSS Inc., Chicago, IL, USA) statistical software was used for all analyses and $P < 0.05$ was considered statistically significant. The linkage disequilibrium (LD) coefficients (D′), haplotype frequency, haplotype block, haplotype association and Hardy–Weinberg equilibrium for each variant were assessed using HAPLOVIEW software (version 4.2, Broad Institute, Cambridge, MA, USA). We defined a haplotype block

as a set of contiguous SNPs with an average D′ >0.8. All tests were two-tailed and α was set at 0.05.

The power analysis was performed using G*POWER 3.1 software (Franz Faul, University of Kiel, Kiel, Germany). Our total sample size (n = 694) had a power of approximately 0.65 to detect small effects (effect size = 0.1), and 1.00 to detect medium (effect size = 0.3) and large effects (effect size = 0.5) of genotype distributions; In the allele frequencies of these 3 polymorphisms, this study had a power of 0.75 to detect small effects, and 1.00 to detect medium and large effects. For linear regression analysis to analyze our HD samples with TPQ scores (n = 265), the study had a power of 0.46 to detect small effects (effect size = 0.02) and 1.00 to detect a medium effects (effect size = 0.15) and a large effects (effect size = 0.35).

RESULTS

Our study initially included 705 unrelated Han Chinese male subjects, but 11 were excluded due to incomplete genotype data. As a result, there are 694 subjects for further genetic analysis, including 313 patients with HD and 381 normal controls. Table 1 demonstrated the demographic data of the including subjects. There was no significant difference in mean age between patients with HD and normal controls (P > 0.05). Mean NS score was higher in patients with HD than in normal controls (P < 0.05), but this difference remained significant only when comparing normal controls to patient with HD of early onset and patient with HD of long duration. For harm avoidance score, either total patients with HD or patients within each subgroup had higher mean score than normal controls.

We analyzed the allele and genotype frequencies of the selected SNPs (rs1801262, rs16867467, and rs2583016 on NeuroD1; rs2233404 on NeuroD6) among patients with HD and normal controls [Table 2]. All variants were in Hardy–Weinberg equilibrium in both patients and controls, according to Chi-square goodness-of-fit test. For allele frequency analysis of these SNPs, no significant difference was found between HD group and control group, except for NeuroD1 rs16867467; on NeuroD1 rs16867467, patients were likely to have more propensity of minor allele than controls (P = 0.047). The genotype frequency analysis also revealed a weak association between the NeuroD1 rs16867467 polymorphism and HD (P = 0.025), especially with patients in the early-onset subgroup (P = 0.015). However, all above associations could not pass the significance threshold following *post hoc* test using the Bonferroni's correction. On the other hand, the genotype analysis showed no influence of the other SNPs on total HD, nor did the influence on the development of HD in the early-onset subgroup, late-onset subgroup, short-duration subgroup or long-duration subgroup.

We further investigated the association of NeuroD variants and HD by haplotype analysis. Figure 1 presented the generation of an LD map and block structure for all variants of the studied neuroD polymorphisms, as well as the D′ values. The haplotype block (rs1801262–rs16867467-rs2583016) includes 3 SNPs in NeuroD1. The descriptive results of haplotype analysis between groups are showed in Table 3. After 10,000 permutation procedures for multiple comparison, there was no significant difference between HD patients and normal controls in the haplotype frequencies (P > 0.05). When further analysis in other HD subgroup, there was also no significant difference between each subgroup of HD and control group (P > 0.05).

For the HD group (n = 313) and control group (n = 381), 265 patients and 182 controls completed the personality assessment with the Chinese version of the TPQ. Initially, we found a negative correlation between age and NS scores in both controls and patients (P < 0.05), but no correlation was found between age and HA scores in both groups (P > 0.05). We then used the linear regression analysis, with age and duration

Table 1: Demographic characteristics in patients with heroin dependence and healthy controls

Variable	Group						P^b	P^c	P^d	P^e	P^f
	NC (n=381)	Total HD (n=313)	Early onset HD (n=119)	Late onset HD (n=194)	HD duration ≤5 (n=91)	HD duration >5 (n=222)					
Age	39.7±11.6	41.1±9.2	37.9±9.5	43.1±8.4	35.2±7.6	43.6±8.7	0.078	0.116	<0.001	<0.001	<0.001
Onset	NA	28.7±8.1	20.9±3.2	33.5±6.2	32.9±7.7	27.0±7.6	NA	NA	NA	NA	NA
Duration	NA	12.4±9.2	16.9±9.8	9.6±7.5	2.3±1.7	16.5±7.7	NA	NA	NA	NA	NA
NS score[a]	13.0±4.1	14.1±4.7	15.0±4.4	13.6±4.8	13.1±4.9	14.5±4.5	0.008	<0.001	0.228	0.835	0.001
HA score[a]	10.6±4.9	12.5±5.2	12.4±5.0	12.6±5.3	12.6±5.2	12.5±5.2	<0.001	0.002	<0.001	0.004	<0.001

[a]265 HD patients and 182 healthy controls completed the TPQ; [b]Healthy controls versus total patients with HD; [c]Healthy controls versus patients with early onset HD; [d]Healthy controls versus patients with late onset HD; [e]Healthy controls versus patients with HD duration ≤5; [f]Healthy controls versus patients with HD duration >5. NC=Normal controls; HD=Heroin dependence; NS=Novel seeking; HA=Harm avoidance; NA=Not available; TPQ=Tridimensional personality questionnaire

Table 2: Gene location, allele, and genotype frequencies of the investigated NeuroD 1/D 6 gene polymorphisms among patients with heroin dependence and controls

NeuroD family	Variants	Location in neuroD	Position reference dbSNP	MAF		P^a	Allele[b]		P^c	Controls (n=381)			P^c	Total HD (n=313)			P^c
				HD	NC					Genotype, n (%)				Genotype, n (%)			
							1	2		1/1	1/2	2/2		1/1	1/2	2/2	
NeuroD1	rs1801262	Exon	182543455	0.078	0.070	0.536	*T*	C		3 (0.8)	47 (12.3)	331 (86.9)		0 (0.0)	49 (15.7)	264 (84.3)	0.154
	rs16867467	Intron	182544889	0.240	0.196	0.047*	*T*	C		20 (5.2)	109 (28.6)	252 (66.1)		15 (4.8)	120 (38.3)	178 (56.9)	0.025*
	rs2583016	5'UTR	182545218	0.080	0.070	0.466	*C*	T		2 (0.5)	49 (12.9)	330 (86.6)		0 (0.0)	50 (16.0)	263 (84.0)	0.250
NeuroD6	rs2233404	Intron	31378933	0.297	0.274	0.348	*G*	A		23 (6.0)	163 (42.8)	195 (51.2)		30 (9.6)	126 (40.3)	157 (50.2)	0.209
Variants	Early onset HD (n=119)			P^c	Late onset HD (n=194)			P^c	HD duration ≤5 (n=91)			P^c	HD duration >5 (n=222)			P^c	
	Genotype, n (%)				Genotype, n (%)				Genotype, n (%)				Genotype, n (%)				
	1/1	1/2	2/2		1/1	1/2	2/2		1/1	1/2	2/2		1/1	1/2	2/2		
rs1801262	0 (0.0)	18 (15.1)	101 (84.9)	0.518	0 (0.0)	31 (16.0)	163 (84.0)	0.248	0 (0.0)	16 (17.6)	75 (82.4)	0.333	0 (0.0)	33 (14.9)	189 (85.1)	0.339	
rs16867467	5 (4.2)	51 (42.9)	63 (52.9)	0.015*	10 (5.2)	69 (35.6)	115 (59.3)	0.227	3 (3.3)	36 (39.6)	52 (57.1)	0.122	12 (5.4)	84 (37.8)	126 (56.8)	0.058	
rs2583016	0 (0.0)	18 (15.1)	101 (84.9)	0.733	0 (0.0)	32 (16.5)	162 (83.5)	0.339	0 (0.0)	17 (18.7)	74 (81.3)	0.309	0 (0.0)	33 (14.9)	189 (85.1)	0.531	
rs2233404	10 (8.4)	54 (45.4)	55 (46.2)	0.507	20 (10.3)	72 (37.1)	102 (52.6)	0.123	5 (5.5)	31 (34.1)	55 (60.4)	0.275	25 (11.3)	95 (42.8)	102 (45.9)	0.061	

[a]MAF in patients with HD compared with the control group using Pearson χ^2 test, [b]Allele 1 that is italicized and bold indicates the minor allele, and only alleles with frequency higher than 5% are showed, [c]Genotype frequencies in patients with HD or its subgroups compared with the controls using Pearson χ^2 test, *A P<0.002 (0.05/24) was considered statistically significant after Bonferroni's correction. NC=Normal controls; HD=Heroin dependence; MAF=Minor allele frequency

Figure 1: The LD structure between three polymorphisms in NeuroD1 gene region. The upper panel shows the location of polymorphisms in NeuroD1 gene and the lower panel shows the output of HAPLOVIEW version 4.2. D′ value (left LD map) and r^2 value (right LD map) shown within each square represent a pairwise LD relationship between the two polymorphisms. Red squares indicate statistically significant LD between the pair of polymorphisms. Darker colors of red indicate higher values of D′ up to a maximum of 1 and white squares indicate pairwise D′ values with no statistically significant difference of LD. LD = Linkage disequilibrium

of HD as covariates, to evaluate the relationship between selected SNPs and specific personality traits in patients with HD [Table 4]. However, each NeuroD SNP was not associated with the NS/HA scores in patients with HD.

DISCUSSION

To evaluate the association between NeuroD1/D6 polymorphism and the development of HD, we performed this genetic association study with these SNPs in NeuroD1/D6 gene regions, combined with haplotype frequency analysis. We also performed a regression analysis to evaluate the role of neuroD1/D6 gene variants in TPQ scores in patients with HD. As previous statement, a series of studies provide evidence for association between neurogenesis of the brain and the pathogenesis of HD. As an essential role in the neural development and differentiation, NeuroD1/D6 may be involved in HD.

In our study, there was weak association between NeuroD1 rs16867467 and the occurrence of HD, but this association was still significant only in the early-onset subgroup. Nonetheless, the association findings could not remain significant after adjustment using *post hoc* Bonferroni's correction. For a number of tests were performed because of multiple SNPs involved, Bonferroni's correction was indicated to account for multiple comparisons. When further investigation by haplotype analysis, there was also no significant difference in

Table 3: Haplotype analysis of NeuroD1 gene in patients with heroin dependence and normal controls

Haplotype block (NeuroD1)			Frequency						P^a	P^b	P^c	P^d	P^e
rs1801262	rs16867467	rs2583016	Total NC	Total HD	Early onset HD	Late onset HD	HD duration ≤5	HD duration >5					
C	C	T	0.739	0.683	0.675	0.690	0.679	0.686	0.053	0.102	0.285	0.406	0.091
C	T	T	0.193	0.237	0.250	0.227	0.228	0.239	0.106	0.115	0.433	0.660	0.109
T	C	C	0.064	0.076	0.069	0.078	0.085	0.070	0.843	1.000	0.871	0.794	0.936

[a]Healthy control versus total patients with HD; [b]Healthy control versus patients with early-onset HD; [c]Healthy control versus patients with late-onset HD; [d]Healthy control versus patients with HD duration ≤5; [e]Healthy control versus patients with HD duration >5; Haplotype frequencies showed in table were >0.01, All P valves were corrected using 10,000 permutation procedures. NC=Normal controls; HD=Heroin dependence

Table 4: Linear regression analysis between NeuroD 1/D 6 gene polymorphisms and specific personality traits in patients with heroin dependence

NeuroDmember	Variants	Novel seeking[a]					
		β (SE)			P^b	P^c	P^d
		Genotype	Age	Duration			
NeuroD1	rs1801262	1.282±0.779	−0.125±0.040	0.104±0.040	0.101	0.002	0.010
	rs16867467	0.532±0.473	−0.121±0.040	0.106±0.040	0.262	0.003	0.009
	rs2583016	1.358±0.772	−0.125±0.040	0.103±0.040	0.080	0.002	0.011
NeuroD6	rs2233404	0.457±0.425	−0.118±0.040	0.107±0.040	0.284	0.003	0.008
NeuroDmember	Variants	Harm avoidance[a]					
		β (SE)			P^b	P^c	P^d
		Genotype	Age	Duration			
NeuroD1	rs1801262	0.535±0.883	0.043±0.045	0.007±0.045	0.545	0.339	0.883
	rs16867467	0.083±0.535	0.045±0.045	0.007±0.045	0.876	0.321	0.875
	rs2583016	0.590±0.874	0.043±0.045	0.006±0.045	0.501	0.339	0.889
NeuroD6	rs2233404	0.439±0.480	0.047±0.045	0.010±0.045	0.361	0.296	0.830

[a]The association between NeuroD variants and TPQ subscore are corrected by age and duration of HD using linear regression analysis; [b]The relation between TPQ subscore and each NeuroD variant; [c]The relation between TPQ subscore and age; [d]The relation between TPQ subscore and duration of HD. TPQ=Tridimensional personality questionnaire; HD=Heroin dependence; SE=Standard error

haplotype frequencies between the experimental groups. As a result, our study may not support the hypothesis about the role of NeuroD1/NeuroD6 as mediators between neurogenesis of the brain and the development of HD. However, Bonferroni's correction may be too conservative to miss the effects of interest. Another point of view is the influence of other polymorphisms in the NeuroD1/NeuroD6 gene regions and the environmental factors to be important roles in the development of HD, which could interfere with the genetic study results. Further studies to replicate and confirm our results are warranted.

Literature had revealed that high novelty-seeking and harm avoidance personality trait may be a risk factor for the development of amphetamine dependence.[30] Similar finding had been demonstrated in alcohol patients with higher NS and HA traits.[31] Other experiments also found that higher NS score was associated with higher impulsivity, excitability, and behavioral disinhibition.[32] In this study, HD patients had higher NS and HA traits that were consistent with literature on patients with amphetamine or alcohol dependence.[30,31] However, in the linear regression analysis, these SNPs in NeuroD1/NeuroD6 gene regions have no significant influence on specific personality trait in patients with HD. These findings imply that the NeuroD1/D6 gene polymorphism may not play a role in the association between personality traits and HD.

There are several limitations regarding our study. First, the number of individuals included in each subgroup of HD may not be sufficient to detect the influence of NeuroD1/D6 polymorphism on the development of HD. In addition, only 265 of total 313 HD patients completed the TPQ assessment that would reduce the power to detect an association with specific personality traits. Second, this study excluded female patients with HD to reduce heterogeneity and prevent the confounding effects of gender. However, the gender differences could not be overlooked. For example, previous studies have revealed that women with HD were more likely to have family members using illicit substances and to report a child-raising burden and a

prior history of traumatic experiences than male heroin users,[33] whereas men with HD more often have other co-occurring substance use.[34] Whether NeuroD1/NeuroD6 gene variants associate with the development of HD in female population should be verified by further studies. Third, the patients with HD in this study were heterogeneous mix regarding severity that would interfere with the power of genetic association studies.[35] Subgroups of HD with severity profile may be more suitable in future research. Fourth, the environmental factors may be important in the etiology and pathogenesis of HD,[36] but our studies did not include of effect of possible environmental risk factors, which could confound with our study results. Fifth, the D' value between some contiguous markers was <0.8, these four markers may not provide complete coverage of the NeuroD1/D6 gene. Moreover, we excluded the heroin addicts with antisocial personality disorder, but HD with or without antisocial personality disorder may have different genetic predisposition;[37] further studies including these subtypes of HD patient may be need for more comprehensive investigation to patients with antisocial personality disorder.

CONCLUSIONS

Our study may not support the association between NeuroD1/NeuroD6 gene polymorphism and the development of HD in Han-Chinese male population. Patients with HD had higher NS and HA traits, but NeuroD1/D6 genes may not contribute to specific personality traits in HD patients. Further association studies of female, other populations or other study designs are needed to confirm our findings.

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Conflicts of interest

There are no conflicts of interest.

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