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Replication of *ZNF804A* gene variant associations with risk of heroin addiction

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Abstract

Heroin addiction is heritable, but few specific genetic variants have been reproducibly associated with this disease. The zinc finger protein 804A (*ZNF804A*) gene is a biologically plausible susceptibility gene for heroin addiction, given its function as a transcription factor in human brain. Novel associations of two common *ZNF804A* single nucleotide polymorphisms (SNPs), rs7597593 and rs1344706, with heroin addiction have been reported in Han Chinese. Both SNPs have also been implicated for regulating *ZNF804A* expression in human brain, including the addiction-relevant dorsolateral prefrontal cortex. In this independent replication study, we tested the rs7597593 and rs1344706 SNP genotypes and their corresponding haplotypes for association with heroin addiction using cases drawn from the Urban Health Study and population controls: total N=10,757 (7,095 European Americans and 3,662 African Americans). We independently replicated both *ZNF804A* SNP associations in European Americans: the rs7597593-T (P=0.016) and rs1344706-A (P=0.029) alleles both being associated with increased risk of heroin addiction, consistent with the prior report. Neither SNP was associated in African Americans alone, but meta-analysis across both ancestry groups resulted in significant associations for rs1344706-A (P=0.016, odds ratio [95% confidence interval] = 1.13 [1.02–1.25]) and its haplotype with rs7597593-T (P=0.0067, odds ratio [95% confidence interval] = 1.16 [1.04–1.29]). By demonstrating consistent associations across independent studies and diverse ancestry groups, our

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study provides evidence that these two *ZNF804A* SNPs and their risk haplotype are among the few replicable genetic associations with heroin addiction.

Keywords

ancestry; genetic association study; haplotype; heroin; opioid; replication; rs1344706; rs7597593; Urban Health Study; ZNF804A

Introduction

Addiction to heroin and other opioid drugs is a growing public health concern (Substance Abuse and Mental Health Service Administration, 2013) with profound economic consequences (Mark *et al.*, 2001). Heroin addiction is heritable (Mistry *et al.*, 2014), and although a few single nucleotide polymorphisms (SNPs) in the opioid receptor genes *OPRM1* (Hancock *et al.*, in press) and *OPRD1* (Nelson *et al.*, 2014) have been reproducibly associated, the specific genetic variants contributing to heroin addiction remain largely unknown.

Novel associations between SNPs in the zinc finger protein 804A (*ZNF804A*) gene and heroin addiction (Sun *et al.*, in press) have suggested that this gene may exert pleiotropic effects influencing multiple psychiatric-related phenotypes. *ZNF804A* was the first genome-wide significant finding reported for schizophrenia (O'Donovan *et al.*, 2008), and since then, *ZNF804A* SNP associations have been widely implicated for schizophrenia and other psychiatric disorders in populations of European and Asian ancestry (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013, International Schizophrenia Consortium *et al.*, 2009, Riley *et al.*, 2010, Schwab *et al.*, 2013, Steinberg *et al.*, 2011, Williams *et al.*, 2011, Xiao *et al.*, 2011). *ZNF804A* is abundantly expressed in human brain (Tao *et al.*, 2014) and is predicted to encode a transcription factor that directly interacts with genes related to dopaminergic transmission (Girgenti *et al.*, 2012), a neural mechanism known to drive both schizophrenia (Nieratschker *et al.*, 2010) and addiction (Hyman *et al.*, 2006). In extending *ZNF804A* SNP associations to heroin addiction, Sun *et al.* (in press) tested six *ZNF804A* SNPs in Han Chinese (N=3,922) and found that two of the most robust SNPs for psychosis were associated with heroin addiction, rs7597593 and rs1344706 (lowest uncorrected P=0.023 for single SNP tests and 0.0035 for their haplotype test). Both of these SNPs have also been implicated for regulating *ZNF804A* expression in postmortem human brain (Guella *et al.*, 2014, Riley *et al.*, 2010, Zhang *et al.*, 2011), with rs1344706 being implicated specifically in the dorsolateral prefrontal cortex—a highly relevant brain region for addiction (Goldstein & Volkow, 2011). Our study focused on these two regulatory SNPs and tested their associations for independent replication with heroin addiction in 7,095 European Americans and 3,662 African Americans.

Materials and Methods

Study participants

Heroin addiction cases were drawn from the Urban Health Study (UHS) of street-recruited people who reported past 30-day injection of an illicit drug (verified by signs of venipuncture) from the San Francisco Bay area between 1986 and 2005 (Kral *et al.*, 2001, Kral *et al.*, 2003). The current study focused on European Americans and African Americans who met the Office of National Drug Control Policy definition of heroin abuse, having injected 10+ times in the past 30 days (Morral *et al.*, 2000, Rhodes *et al.*, 2000). This level of heroin abuse is highly correlated with clinical dependence levels on the Severity of Dependence Scale (Gossop *et al.*, 1992, Strang *et al.*, 1999) and with DSM-IV heroin abuse/dependence (American Psychiatric Association, 1994) based on our analyses of the National Survey on Drug Use and Health data (Hancock *et al.*, in press). These UHS participants, henceforth referred to as heroin addiction cases, reported abusing heroin an average of 80.9 times in the past 30 days and thus were very likely dependent on heroin.

The UHS heroin addiction cases were compared to population controls assembled together from six study cohorts in the database of Genotypes and Phenotypes (dbGaP), as previously described (Hancock *et al.*, in press). Exclusions were made for DSM-IV opioid dependence, where the data were available in the two study cohorts ascertained for addiction. Controls from the other four cohorts were ascertained for phenotypes unrelated to addiction, where we expected minimal phenotype misclassification for heroin addiction.

All study protocols received Institutional Review Board approval at their respective sites, and all study participants provided informed consent.

Genotyping and Quality Control

As detailed elsewhere (Hancock *et al.*, in press, Johnson *et al.*, 2015), stored serum samples from the UHS were restored using the Illumina Formalin-Fixed Paraffin-Embedded kit to maximize the quality of genomic DNA and then genotyped on the Illumina Omni1-Quad BeadChip, Controls were genotyped on one of three Illumina platforms (Omni1-Quad, 1M-Duo, or Omni2.5). Both *ZNF804A* SNPs, rs7597593 located in intron 1 and rs1344706 located in intron 2, were assayed across these Illumina platforms and thus genotyped in all cases and controls. Both *ZNF804A* SNPs passed all quality control thresholds, thus having call rate ≥90%, minor allele frequency ≥1%, and Hardy Weinberg equilibrium $P \geq 1 \times 10^{-4}$.

The genotyped participants' ancestral proportions were determined using the STRUCTURE program (Pritchard *et al.*, 2000) with reference to HapMap populations. Following exclusions for European Americans having >25% African ancestry and African Americans having <25% African ancestry, the heroin addiction case and control datasets closely resembled the expected ancestral proportions (Figure 1). Participants were also excluded for call rate <90%, sample duplication, gender discordance, excessive homozygosity, and first-degree relatedness. Our final analysis dataset included 7,095 European Americans (711 cases and 6,384 controls) and 3,662 African Americans (1,293 cases and 2,369 controls), as shown in Table 1.

Statistical analyses

Additive SNP genotypes were tested for association with heroin addiction, separately by ancestry, using logistic regression models in SAS® software (SAS Institute, Cary, North Carolina) adjusted for sex and principal component eigenvectors. In each ancestry group, 10 eigenvectors were generated using EIGENSTRAT (Price *et al.*, 2006); among these, the three eigenvectors which together explained >90% of the variance in heroin addiction case/control status were included as covariates in our regression models to circumvent any potential bias due to population stratification.

To infer the haplotypes occurring between the two *ZNF804A* SNPs, we phased the study genotypes in each ancestry group using the ShapeIT (version 2) program (Howie *et al.*, 2012) with all 1000 Genomes reference haplotype panels, 500 conditioning states, recommended effective population sizes of 15,000 for African Americans and 11,418 for European Americans, and default settings for all other program options specified. Logistic regression models were used to test the two-SNP haplotypes (coded as dummy variables) for association with heroin addiction, adjusted for sex and eigenvectors. Ancestry-specific SNP and haplotype association results were combined using fixed-effects, inverse variance-weighted meta-analysis, the most commonly used approach for large-scale genetic association meta-analysis (Panagiotou *et al.*, 2013). Fixed-effects methods, which optimize power for discovery analyses (Pereira *et al.*, 2009), assume that heterogeneity across study-specific results are due to random error rather than true population differences; the inverse variance weighting involves taking the inverse of each study-specific standard error estimate and accounting for the direction of association (i.e., sign of the β) to generate combined regression coefficients.

Results

The *ZNF804A* SNP association results are shown in Table 2. Rs7597593-T was the minor allele in European Americans (frequency=38.9%, similar to the 39.5% observed in Han Chinese [Sun *et al.*, in press]) but the major allele in African Americans (frequency=63.8%). Rs1344706-A was the major allele in both the European Americans (frequency=61.1%) and African Americans (frequency=91.5%), compared to its 48.4% frequency in the Han Chinese (Sun *et al.*, in press). Both SNPs were associated at $P<0.05$ in European Americans with heroin addiction cases being 1.15 and 1.14 times as likely to carry the rs7597593-T and rs1344706-A alleles, respectively, compared with controls. Neither allele was significantly associated in the African Americans. However, consistent directions of association were observed across both ancestry groups for rs1344706-A, and it was the only SNP that was significantly associated in the multiancestry meta-analysis ($P=0.016$).

Of the 10,757 participants in our dataset, 45.8% were female (Table 1). We tested both SNPs for interaction with sex, given prior suggestive evidence of such interaction involving rs7597593 and schizophrenia (Zhang *et al.*, 2011). We found no evidence for SNP-by-sex interaction in either the ancestry-specific analyses or meta-analysis (results not shown).

High D′ values between rs7597593 and rs1344706 indicated strong linkage disequilibrium (D′=1 in the African [denoted AFR] and D′=0.96 in the European [denoted EUR] panels

from 1000 Genomes). Weaker r^2 values between the two SNPs (r^2=0.12 in AFR and r^2=0.34 in EUR) were constrained by their differing allele frequencies.

The two-SNP haplotype associations with heroin addiction are presented in Table 3. Haplotypes carrying the non-risk rs1344706-C allele were collapsed, due to the low frequency of rs1344706-C/rs7597593-T (<1%) in both ancestry groups, and used as the reference. The haplotype carrying both risk alleles (rs1344706-A/rs7597593-T) was significantly associated with increased risk of heroin addiction in the European Americans-specific analysis (*P*=0.0064) and the multiancestry meta-analysis (*P*=0.0066), while the haplotype carrying rs1344706-A without rs7597593-T was not associated.

Table 1 shows that a large portion of our heroin addiction cases also reported abuse (using 10 or more times in the past month) of other drugs, predominantly cocaine. We repeated the haplotype association analyses for heroin addiction after excluding the cases with comorbid cocaine abuse, leaving 490 EA cases and 703 AA cases for comparison to the population control sets. With this reduced sample, the rs1344706-A/rs7597593-T haplotype remained associated in the European Americans (*P*=0.014, odds ratio [95% confidence interval] = 1.21 [1.04–1.41]); its multiancestry meta-analysis had P=0.065 and a similar magnitude of association (odds ratio [95% confidence interval] = 1.12 [0.9–1.27]) in comparison to the full sample (Table 3).

Discussion

Our study independently replicated the associations of the *ZNF804A* SNPs rs7597593 and rs1344706 and their haplotype with heroin addiction. Such replicable genetic associations with heroin addiction are limited. This replication may also be viewed as a generalization, as our results showed that the associations initially reported in Han Chinese extend to other ancestries, particularly European ancestry. Sun *et al.* (in press) first identified these heroin addiction associations in a Han Chinese dataset (N=1,035 DSM-IV defined cases and 2,887 community-based controls). Consistent with the prior report, both the rs7597593-T and rs1344706-A alleles and their corresponding haplotype were associated with increased risk of heroin addiction in our European American dataset. No statistically significant SNP or haplotype associations were observed in our African American dataset, but rs1344706-A and its risk haplotype with rs7597593-T presented consistent directions across the ancestry groups and remained associated at *P*<0.05 in our multiancestry meta-analyses. Similar magnitudes of rs1344706-A association have now been observed across diverse ancestries: odds ratios of 1.16 in Han Chinese (Sun *et al.*, in press) and 1.13 in our meta-analysis of European Americans and African Americans.

The mechanism underlying ZNF804A function in human brain and heroin addiction risk is not known. However, rs1344706 has strong potential for influencing the *ZNF804A* gene, and its further characterization may help to elucidate the disease-relevant mechanism. The rs1344706-A risk allele maintains the predicted binding sites for two transcription factors expressed in the brain (Myt1L and POU3F1/Oct-6), and it has been associated with increased *ZNF804A* mRNA expression in postmortem dorsolateral prefrontal cortex, a highly relevant brain region for addiction (Goldstein & Volkow, 2011), from psychiatrically

normal controls (Riley *et al.*, 2010). An allelic specific expression study further suggested that rs1344706 directly affects *ZNF804A* expression in this brain region (Guella *et al.*, 2014). ZNF804A functions as a transcription factor that regulates genes in the dopaminergic pathway (Girgenti *et al.*, 2012), so increased *ZNF804A* mRNA expression would be expected to alter downstream effects on dopamine release and synthesis and contribute to heroin addiction risk. Additionally, *ZNF804A* is most highly expressed in human brain during the fetal period, and rs1344706 has been shown to affect expression of a novel splice variant of *ZNF804A* that is specific to fetal brain (Tao *et al.*, 2014). This splicing mechanism could underlie the rs1344706 association with heroin addiction.

Rs1344706 may also influence structure of addiction-relevant brain regions. In reporting the rs1344706 association with heroin addiction, Sun *et al.* (in press) showed that its risk allele and haplotype were associated with greater gray matter volume across several human brain regions; they detected an interaction with heroin addiction specifically in the left sensorimotor cortex, a region that is relevant for developing and preserving addiction, whereby the risk haplotype was associated with greater gray matter volume in heroin abusers but lower grey matter volume in controls. This association involving rs1344706 and grey matter by disease state is consistent with prior findings in healthy individuals (Voineskos *et al.*, 2011) and schizophrenia cases and controls (Nenadic *et al.*, 2015). These studies further linked rs1344706-A to reduced cognitive function (Sun *et al.*, in press, Voineskos *et al.*, 2011).

Rs7597593 has also been implicated for influencing *ZNF804A* mRNA expression, but the association was found only in females (Zhang *et al.*, 2011). We did not find any evidence for sex-by-genotype interactions in our study. Overall, we replicated the previously observed rs7597593 association with heroin addiction in European Americans, but not in African Americans. Rs7597593 may tag a different causal variant with varying linkage disequilibrium across the ancestral groups, which may be identified by fine mapping this region using a large sample of African Americans.

Our results support *ZNF804A* as one of the few susceptibility genes for heroin addiction with replicable associations and provide further evidence that this gene has pleiotropic effects on multiple psychiatric diseases. We found that the two-SNP haplotype association with heroin addiction remained after excluding cases with comorbid cocaine abuse, albeit to a lesser extent with the reduced sample size. Given that heroin and cocaine abuse were highly comorbid in the UHS, follow-up in other cohorts will be needed to assess the pleiotropic or independent associations of ZNF804A SNPs with addiction to heroin and other drugs. Taken together with prior work showing that these disease-associated SNPs influence *ZNF804A* gene regulation in human brain, the *ZNF804A* gene region merits future study to establish the underlying mechanism that confers risk on heroin addiction.

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Controls for comparison to the Urban Health Study heroin addiction cases were drawn from the following six cohorts in dbGaP. (1) Funding support for the Study of Addiction: Genetics and Environment (SAGE) was provided through the National Institutes of Health (NIH) Genes, Environment and Health Initiative (GEI) (U01 HG004422). SAGE is one of the genome-wide association studies (GWAS) funded as part of the Gene Environment Association Studies (GENEVA) under GEI. Assistance with phenotype harmonization and genotype cleaning, as well as with general study coordination, was provided by the GENEVA Coordinating Center (U01 HG004446). Assistance with data cleaning was provided by the NCBI. Support for collection of datasets and samples was provided by the Collaborative Study on the Genetics of Alcoholism (COGA; U10 AA008401), the Collaborative Genetic Study of Nicotine Dependence (COGEND; P01 CA089392), and the Family Study of Cocaine Dependence (FSCD; R01 DA013423). Funding support for genotyping, which was performed at the Johns Hopkins University Center for Inherited Disease Research (CIDR), was provided by the NIH GEI (U01 HG004438), the National Institute on Alcohol Abuse and Alcoholism, National Institute on Drug Abuse, and the NIH contract "High throughput genotyping for studying the genetic contributions to human disease" (HHSN268200782096C). The datasets used for the analyses described in this manuscript were obtained via dbGaP accession number phs000092.v1.p1.

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(5) This work utilized in part data from the NINDS dbGaP database from the CIDR NeuroGenetics Research Consortium (NGRC) Parkinson's disease study (accession number phs000196.v2.p1).

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LBJ and the spouse of NLS are listed as inventors on Issued U.S. Patent 8,080,371, "Markers for Addiction," covering the use of certain SNPs in determining the diagnosis, prognosis, and treatment of addiction. Otherwise, the authors have no conflicts of interest.

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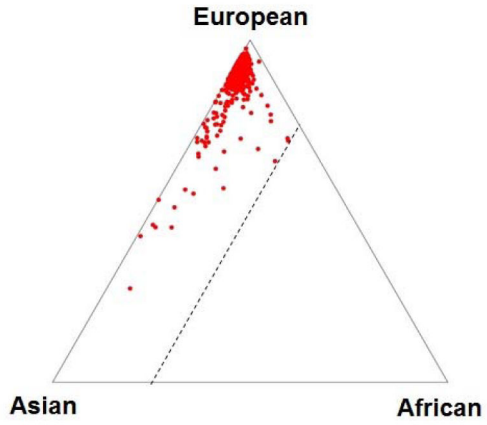
(A)



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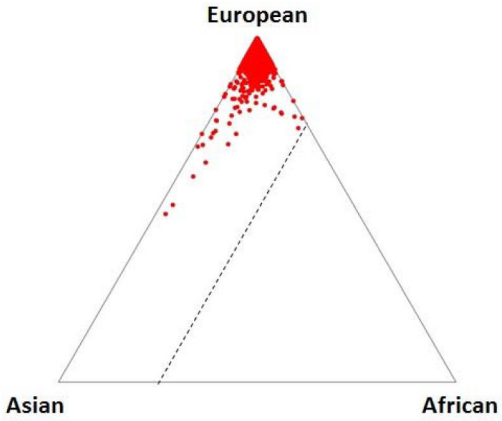

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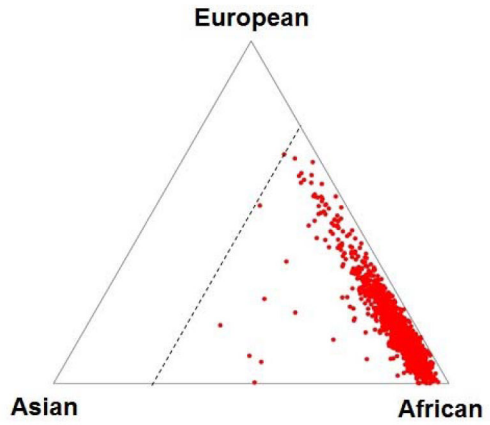

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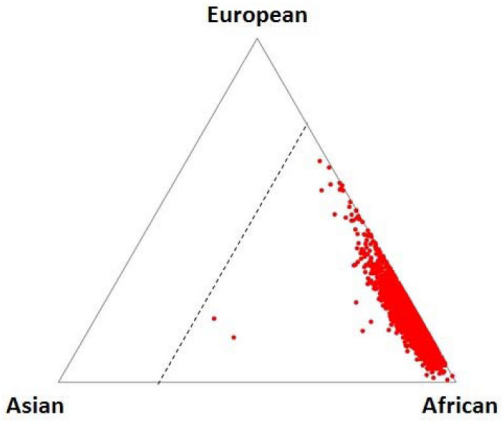

Figure 1.

Ancestral proportions estimated using the STRUCTURE program with reference to HapMap populations and 10,000 randomly selected HapMap phase III SNPs. Ancestral proportions, as used for participant-level quality control, are shown for (A) European American heroin addiction cases, (B) European American controls, (C) African American heroin addiction cases, and (D) African American controls. Triangle vertices represent West Africans (denoted YRI in HapMap), European Americans (CEU), and East Asians (CHB), and triangle edges indicate the ancestral proportions with the dotted line representing 25% African ancestry. (A)

Table 1

Characteristics of the heroin addiction cases and controls, by ancestry.

Ancestry group	Heroin addiction	No. genotyped participants passing quality control	No. (%)female	*No. (%) cases reporting past 30-day abuse of other drug(s) in addition to heroin*			
				Any other drug	Specific drugs*		
					Cocaine	Marijuana	Amphetamines and other stimulants
European Americans	Cases	711	135 (19.0)	264 (37.1)	221 (31.1)	4 (0.6)	64 (9.0)
	Controls	6,384	3,505 (54.9)	NA	NA	NA	NA
African Americans	Cases	1,293	372 (28.8)	611 (47.3)	590 (45.6)	24 (1.9)	36 (2.8)
	Controls	2,369	913 (38.5)	NA	NA	NA	NA

NA, not applicable.

*Abuse (10 or more times in the past month) of different drug categories was not mutually exclusive.

Table 2

ZNF804A SNP associations with heroin addiction.

SNP	Coded allele	Position on chromosome 2 (NCBI build 37)	*European Americans (N=711 cases and 6,384 controls)*			*African Americans (N=1,293 cases and 2,369 controls)*			*Meta-analysis*	
			Coded allele frequency (%)	*P*	OR (95% CI)	Coded allele frequency (%)	*P*	OR (95% CI)	*P*	OR (95% CI)
rs7597593	T*	185,533,580	38.9	0.016	1.15 (1.03–1.29)	63.8	0.67	0.98 (0.89–1.08)	0.088	1.05 (0.97–1.13)
rs1344706	A	185,778,428	61.1	0.029	1.14 (1.01–1.29)	91.5	0.27	1.10 (0.93–1.32)	0.016	1.13 (1.02–1.25)

CI, confidence interval; OR, odds ratio

*T is the complementary allele to the A allele reported by Sun *et al.* (in press).

Table 3

ZNF804A two-SNP haplotype associations with heroin addiction.

	European Americans (N=711 cases and 6,384 controls)			*African Americans (N=1,293 cases and 2,369 controls)*			*Meta-analysis*	
rs7597593- rs1344706 haplotype	**Frequency (%)**	***P***	**OR (95% CI)**	**Frequency (%)**	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**
T-A	38.3	0.0064	1.20 (1.05–1.37)	63.5	0.39	1.08 (0.90–1.30)	0.0066	1.16 (1.04–1.29)
C-A	22.9	0.55	1.05 (0.90–1.22)	28.1	0.14	1.16 (0.95–1.40)	0.16	1.09 (0.97–1.23)
C-C or T-C	38.8	Reference		8.5	Reference		Reference	

CI, confidence interval; OR, odds ratio