Cholinergic Receptor Nicotinic Beta 3 Subunit Polymorphisms and Smoking in Male Chinese Patients with Schizophrenia

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Abstract

Objective: The aim of this study was to examine if cholinergic receptor nicotinic beta 3 subunit (*CHRNB3*) was a common genetic basis for both nicotine dependence and schizophrenia.

Methods: Two *CHRNB3* promoter single nucleotide polymorphisms (SNPs) were genotyped in 773 patients with schizophrenia and 302 healthy volunteers. Associations between smoking, schizophrenia, smoking+schizophrenia and *CHRNB3* were analyzed. The mRNA expression of *CHRNB3* in human brains was examined, and the expression correlations between *CHRNB3* and dopaminergic and GABAergic receptor genes were evaluated.

Results: The association between *CHRNB3* and smoking was significant in the total sample, less significant in the smoking with schizophrenia and suggestive in the smoking without schizophrenia. *CHRNB3* had significant mRNA expression that was correlated with dopaminergic or GABAergic receptor expression in human brains. The two *CHRNB3* SNPs had significant cis-acting regulatory effects on *CHRNB3* mRNA expression.

Conclusion: Smoking was associated with *CHRNB3*. *CHRNB mRNA3* is abundant in human brain and could play important role in the pathogenesis of smoking.

Keywords: Cigarette; Smoking; CHRNB3; Polymorphism; Schizophrenia

Abbreviations

nAChR: Nicotinic Acetylcholine Receptor; ND: Nicotine Dependence; CHRNA: Cholinergic Receptor Nicotinic Alpha Subunit; CHRNB: Cholinergic Receptor Nicotinic Beta Subunit; SNP: Single Nucleotide Polymorphism; GWAS: Genome-Wide Association Study; CHRN: Nicotinic Acetylcholine Receptor; DSM-IV: Diagnostic and Statistical Manual of Mental Disorders, 4th Edition; CPD: Cigarettes Smoked Per Day; PCR: Polymerase Chain Reaction; PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism; SZ+SM+: Schizophrenia Patients with Smoking Behavior; SZ+SM−: Schizophrenia Patients without Smoking Behavior; SZ−SM+: Controls with Smoking Behavior;

SZ⁻SM⁻: Controls without Smoking Behavior; SZ⁺: Total Schizophrenia Patients with or without Smoking Behavior; SZ⁻: Total Controls with or without Smoking Behavior; SM⁺: Total Smoking Subjects with or without Schizophrenia; SM⁻: Total Non-Smoking Subjects with or without Schizophrenia;

Introduction

It has been inferred that the nicotinic acetylcholine receptors (nAChRs) play an important role in the pathogenesis of nicotine dependence (ND). Zuo., *et al.* [1] reviewed that cholinergic receptor nicotinic alpha subunit (*CHRNA*) genes 2 - 10 and beta subunit (*CHRNB*) genes 2 - 4 encoded α2-α10 and β2-β4 subunits that were included by neuronal nAChRs in humans [2]. Although most of the distinct nicotinic acetylcholine receptors (*CHRNs*) have been associated with ND [2], the associations replicated by different studies at single-point level only occurred at *CHRNB3-A6*, *CHRNA5-A3-B4* and *CHRNA4* loci, mostly (79%) at *CHRNB3-A6* locus [3,4].

The *CHRNB3* gene was strongly associated with ND [5], subjective response to tobacco use [6], as well as alcohol and cocaine dependence [7,8]. At least 15 variants of single nucleotide polymorphisms (SNPs) in or near *CHRNB3* region were highly associated with ND in the populations of African-Americans, European-Americans and Asians [3,9], replicated across four genome-wide association studies (GWAS) [10,11] and at least seven candidate gene studies [12,13].

More than 4,900 SNPs have been identified in the *CHRNB3* gene region so far. The SNPs associated with ND are predominantly located within or close to the promoter region of *CHRNB3* [11]. Two SNPs, rs6474413 and rs13277254, the tagSNPs in this promoter region are particularly of interest [14]. The rs6474413 accounted for one of the strongest signals for ND in an analysis of 3713 SNPs targeting 348 candidate genes [15]. It was a risk variant for ND, replicated across at least four independent studies including a GWAS [10] and three candidate gene studies [16] (Table 1). The rs13277254 appeared to be one of the top SNPs related to the development of ND in a comprehensive GWAS with 31,960 selected SNPs [17]. It was also a replicated risk variant for ND across at least five independent studies [14,16] (Table 1). Both SNPs were followed up in this study.

SNP	Gene	p	Ref.	p	Ref.	p	Ref.	p	Ref.	p	Ref.
rs13277254	*CHRNB3-A6*	4.0×10^{-3}	[1]	4.0×10^{-5}	[2]	7.8×10^{-4}	[3]	6.3×10^{-4}	[4]	6.5×10^{-5}	[5]
rs6474413	*CHRNB3-A6*	$\underline{3.6 \times 10^{-8}}$	[6]	6.3×10^{-5}	[2]	9.3×10^{-4}	[3]	9.4×10^{-5}	[7]		

Table 1: *Replicated associations between CHRNB3 and nicotine dependence. [p. p-value; Ref. reference. The associations identified by GWASs were* underlined *References: [1] Johnson et al. Addiction. 2010;105(11):2014-2022; [2] Saccone et al. Am J Med Genet B Neuropsychiatr Genet. 2009;150B(4):453-466; [3] Saccone et al. Genes Brain Behav. 2010;9(7):741-750; [4] Hoft et al. Neuropsychopharmacology. 2009;34(3):698-706; [5] Bierut et al. Hum Mol Genet. 2007;16(1):24-35; [6] Rice et al. Addiction. 2012;107(11):2019-2028; [7] Saccone et al. Hum Mol Genet. 2007;16(1):36-49.].*

To date, the association between SNPs of *CHRNB3* and ND has been primarily found in the general populations free of psychiatric mental disorders [9,11]. However, clinically, ND rarely occurs alone (i.e., not comorbid with any other phenotype); it usually co-occurs with other mental illnesses, including alcoholism, drug dependence, depression, schizophrenia, etc., among which, schizophrenia is of particular interest in this study. It has been shown that smoking and schizophrenia are tightly linked [18]. Smoking prevalence in patients with schizophrenia is higher than that in the general population [19,20]. Over 60% of patients with schizophrenia are smokers [20,21]. Furthermore, associations between schizophrenia and *CHRNs* have been reported. Studies have already reported that the reduced expression of receptor α7 (*CHRNA7*) or α4β2 (*CHRNB4* and *CHRNA2*) was closely related to schizophrenia [22,23]. In addition, some polymorphisms of *CHRNA4*, *CHRNA2* and *CHRNB2* were associated with schizophrenia [24,25]. α5 or β3 subunit could modify the channel properties [26,27], and generate symptoms common in patients with ND and schizophrenia. Chromosome 8p where *CHRNB3* is located was a potential hub for developmental neuropsychiatric disorders, including schizophrenia [28], implying that variants in *CHRNB3* could also be related to schizophrenic symptoms. Additionally, *CHRN* genes are expressed in many human brain regions [2,3]. Many of these brain areas

are important for the development of schizophrenia. For example, *CHRNB3* is abundant in striatum [3]. Striatum receives dopaminergic input to the GABAergic medium spiny neurons [3,29]; schizophrenia is widely hypothesized to be related to the dopamine [30] and GABA [31] release from dopaminergic and GABAergic terminals in striatum. Then, does schizophrenia confound the ND-*CHRNB3* associations? Is the comorbidity of smoking and schizophrenia attributed to the common genetic basis? To answer these questions, we explored the possible associations between these two SNPs of *CHRNB3* and smoking and schizophrenia in a Chinese population.

Materials and Methods

Subjects

773 patients with schizophrenia (567 smokers and 206 non-smokers) with 45 ± 11 years of age and 302 healthy volunteers (168 smokers and 134 non-smokers) with 43 ± 15 years of age were enrolled in this study. Patients were recruited from Hui-Long-Guan Psychiatric Hospital (Beijing) and Rong-Jun Hospital (Hebei) during the period from July 2006 to October 2007. Healthy volunteers were recruited from Changping and Tongzhou districts in Beijing. Male patients met the criteria for chronic schizophrenia of Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) [32]. Patients with schizoaffective psychosis, mental disorders caused by alcohol consumption, organic brain diseases and physical diseases, and other diseases were excluded from the patient group. Participants with a history of mental illness, epilepsy, nervous system disorders, severe physical diseases, and drug abuse or dependence were excluded from the non-schizophrenia group.

All subjects were unrelated. The frequency of smoking and the number of cigarettes smoked per day (CPD) is widely used to index the severity of ND. Smoking with high frequency of smoking and high number of CPD has been shown to carry high genetic loading [33]. Thus, smoking, instead of ND *per se*, served as the study target of this study. The subjects who had smoked one or more cigarettes per day and at least 5 days every week in the most recent year were considered as current smoking subjects (i.e. smokers), and those never or smoked less than one cigarette per day in 5 days every week within the last year were considered as non-smoking subjects (i.e. non-smokers) [34].

The ethnicity of all subjects was Chinese Han in northern China whose admixture degree is relatively low. There was no significant difference in the average ages between patients with schizophrenia and healthy volunteers and between smokers and non-smokers (*p* > 0.05). Written informed consent was obtained from each subject or their immediate relatives. This study was approved by the Institutional Review Board of Beijing Hui-Long-Guan Hospital.

DNA extraction and PCR-RFLP analysis

Genomic DNA was extracted from peripheral blood collected with ethylenediaminetetraacetic acid as previously described [35]. The above two *CHRNB3* SNPs were genotyped using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

The primers for rs6474413 and rs13277254 were designed using PRIMER PREMIER 5.0. The primer pairs for specific locus and the predicted DNA bands of PCR-RFLP are shown in the supplementary table S1. The PCR amplification was carried out in a 15 µl reaction solution containing 1× PCR buffer, 0.2 mM dNTPs, 2.5 pM of each primer, 1U of Taq polymerase (Roche, Branchburg, NJ), and 30 - 50 ng genomic template DNA. PCR reactions were initially subjected to the denaturation at 95ºC for 5 min, followed by 35 cycles of 95ºC for 30s, 52ºC for 50 s and 72ºC for 30s and a final extension at 72ºC for 10 min before cooling to 15ºC. The PCR products for rs6474413 and rs13277254 were digested with 5 U*RsaI* and *HinfI* restriction enzymes (New England Biolabs, Beverly, MA), respectively. Digested PCR products were separated in 3% agarose gel and stained with ethidium bromide. The predicted fragments for rs6474413 and rs13277254 variants are shown in table S1. 10 - 15% of the genotyping was duplicated randomly to confirm the results of genotyping.

Locus	Location	Forward primer	Reverse primer	Predicted bands of PCR-RFLP
rs6474413 (C/T)	Promoter	5'-CAGCCTGGA GGATACAAG AC-3'	5'-GAACGT TGTGG GAATGCAGG-3'	CC: 316bp CT: 132bp, 184bp, 316bp TT: 132bp, 184bp
rs13277254 (A/G)	5'-flank region	5'-CAGAACCAAC CACAATGCTCC-3'	5'-CAAGACCACTT ACAGTCCATG-3'	AA: 330bp AG: 127bp, 203bp, 330bp GG: 127bp, 203bp

Table S1: *Genomic location, primers, and predicted DNA bands of PCR-RFLP for selected SNPs.*

Association analysis

The allele, genotype and haplotype frequencies were derived using the program SHEsis [36]. Hardy-Weinberg equilibrium of genotype frequencies in each subgroup was tested. Study power was calculated using Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) [37]. Multiple comparisons for association analyses were corrected using Bonferroni's correction.

Three types of associations were analyzed: (1) To test the associations between smoking and *CHRNB3*, the allele, genotype, and haplotype frequencies were respectively compared between smoking group (i.e. total smoking subjects with or without schizophrenia; SM^+) and non-smoking group (i.e. total non-smoking subjects with or without schizophrenia; SM^-). To further control for the confounding effects of comorbid schizophrenia on the smoking-*CHRNB3* associations, the total sample was stratified into schizophrenia and non-schizophrenia subgroups based on the diagnosis, and then the comparisons were conducted between smoking schizophrenia group (SZ^+SM^+) and non-smoking schizophrenia group (SZ^+SM^-), and between smoking non-schizophrenia group (SZ^-SM^+) and non-smoking non-schizophrenia group (SZ^-SM^-), respectively. (2) To test the associations between schizophrenia and *CHRNB3*, the allele, genotype, and haplotype frequencies were respectively compared between schizophrenia group (i.e. total schizophrenia patients with or without smoking behavior; SZ^+) and non-schizophrenia group (i.e. total control subjects with or without smoking behavior; SZ^-). To further control for the confounding effects of comorbid smoking on these schizophrenia-*CHRNB3* associations, the total sample was stratified into smoking and non-smoking subgroups and then the comparisons were conducted between schizophrenia smoking group (SZ^+SM^+) and non-schizophrenia smoking group (SZ^-SM^+), and between schizophrenia non-smoking group (SZ^+SM^-) and non-schizophrenia non-smoking group (SZ^-SM^-), respectively. (3) To test the associations between schizophrenia with smoking and *CHRNB3*, the allele, genotype, and haplotype frequencies were respectively compared between smoking+schizophrenia group (SZ^+SM^+) and non-smoking non-schizophrenia group (SZ^-SM^-). Age and socio-economic status served as covariates in all of these association analyses.

Detection of mRNA expression of CHRNB3 in human brains

The expression in brain would help us to know if *CHRNB3* could have the possibility to play functional roles in brain disorders and thus it would help to significantly reduce the false positive rates of the statistical *CHRNB3*-disease associations. We examined its mRNA expression in the human brains of two independent cohorts free of neurodegenerative disorders. The first cohort included ten human postmortem brain tissues extracted from 134 Europeans with ages at death between 16 and 102 years (mean 59 ± 25 years) [UK Brain Expression Consortium (UKBEC) [38]]. mRNA expression in this cohort was examined using Affymetrix Human ST 1.0 exon arrays (validated by qPCR) and could be downloaded for analysis from www.braineac.org. The expression levels with normalized intensity > 36, i.e. log2(normalized intensity) > 5.17, were taken as "expressed". The second cohort included 13 human brain tissues extracted from 173 Americans with ages at death between 21 and 70 years (mean 41±14 years) [The Genotype-Tissue Expression (GTEx) project [39]]. mRNA expression in this cohort was examined using RNA-Seq (validated by qPCR) and could be downloaded for analysis from https://www.gtexportal.org/. The expression levels with TPM values > 1 were taken as "expressed".

Expression correlation analysis in human brain

CHRNB3 is mainly expressed in striatum enriched with GABAergic neurons and midbrain enriched with dopaminergic neurons according to literature [3]. Both GABAergic neurons and dopaminergic neurons are two main neurotransmission systems that have been demonstrated to be related to *CHRNs* [3]. Using Affymetrix Human ST 1.0 exon arrays, the mRNA expression levels of *CHRNB3* and the dopaminergic and GABAergic receptors/enzymes were evaluated in two independent brain tissue cohorts free of neurodegenerative diseases and were validated by qPCR. The first cohort included ten human brain tissues extracted from 134 Europeans mentioned above. The second cohort included frontal cortical tissues extracted from 93 Europeans, and mRNA expression data were obtained from GEO datasets (accession number: GSE30453) [40]. We tested the relationship between 25 dopaminergic and GABAergic receptor/enzyme genes and the expression of *CHRNB3*. Pearson correlation analysis was used for the first cohort and for the second cohort generalized linear model (GLM) analysis was used. Age, sex and postmortem interval were corrected in correlation analysis. The 25 GABAergic and dopaminergic genes were *GABRD, GABRR1-3, GABRG1-3, GABRA1-6, GABRB1-3, GABRE, GABRP, GABRQ, DRD1-5* and *TH.* Signs of correlation coefficient (r) or regression coefficient (β) are used to indicate the direction of the correlation. As 10 brain regions and 25 dopaminergic and GABAergic genes were assessed in the first cohort, α was set at 2.0×10^{-4}. As 12,114 transcripts in the array were evaluated in the second cohort, α was set at 4.1×10^{-6}.

cis-acting genetic regulation of expression analysis in human brain tissues

A *cis*-acting expression of quantitative locus (*cis*-eQTL) analysis was performed to test relationships between two *CHRNB3* variants and *CHRNB3* mRNA expression levels. Genotype data of the two *CHRNB3* SNPs and expression data of *CHRNB3* mRNA in the above first cohort (i.e. 134 Europeans) were assessed. The Wilcoxon Trend Test was used to compare the difference of the expression levels among genotypes of each SNP. If significant regulatory effects of *CHRNB3* variants were identified, it would help to significantly reduce the false positive rates of the statistical *CHRNB3*-disease associations.

Results

Power analysis, Hardy-Weinberg Equilibrium test and quality check of genotyping

The four subgroups have 80% of statistical power to significantly (α = 0.05) differentiate an allele frequency difference down to 0.059 (for smoking schizophrenia subgroup; SZ^+SM^+), 0.097 (non-smoking schizophrenia subgroup; SZ^+SM^-), 0.107 (for smoking non-schizophrenia subgroup; SZ^-SM^+), and 0.119 (for non-smoking non-schizophrenia subgroup; SZ^-SM^-), respectively. All subjects in the four subgroups showed no departure from Hardy-Weinberg Equilibrium on the genotype frequency distributions of these two polymorphisms (p > 0.05). Whole sample genotyping was duplicated and no obscure results were found.

Significant associations between smoking and alleles, genotypes and haplotypes (Table 2)

The frequencies of allele T and genotype T/T of rs6474413, and haplotype T-A of rs6474413-rs13277254 in total smoking group [SM^+] (f = 0.733 for T, 0.539 for T/T, and 0.683 for T-A) were significantly (p < α) lower than total non-smoking group [SM^-] (f = 0.784 for T, 0.605 for T/T, and 0.758 for T-A) (SM^+ vs. SM^-: p = 0.012 for T, 6.5×10^{-5} for T/T, and 3.8×10^{-4} for T-A; Table 2). Conversely, the frequencies of allele C and genotype C/C of rs6474413, and haplotypes C-A and T-G of rs6474413-rs13277254 in total smoking group (f = 0.267 for C, 0.073 for C/C, 0.104 for C-A, and 0.052 for T-G) were significantly (p < α) or suggestively (p close to α) higher than total non-smoking group (f = 0.216 for C, 0.037 for C/C, 0.061 for C-A, and 0.026 for T-G) (SM^+ vs. SM^-: p = 0.012 for C, 0.029 for C/C, 1.2×10^{-3} for C-A, and 8.7×10^{-3} for T-G; Table 2).

When stratified the total smoking and non-smoking groups by the diagnosis of disease status, the frequencies of allele T of rs6474413 and haplotype T-A of rs6474413-rs13277254 in smoking comorbid with schizophrenia group (f = 0.731 for T and 0.689 for T-A) remained suggestively or significantly lower than non-smoking schizophrenia group (f = 0.784 for T and 0.765 for T-A) (SZ$^+$SM$^+$ vs. SZ$^+$SM$^-$: p = 0.035 for T and 3.7×10^{-3} for T-A; Table 2). Conversely, the frequencies of allele C of rs6474413, and haplotypes C-A and T-G of rs6474413-rs13277254 in smoking schizophrenia group (f = 0.267 for C, 0.109 for C-A, and 0.044 for T-G) remained suggestively higher than non-smoking schizophrenia group (f = 0.216 for C, 0.070 for C-A, and 0.017 for T-G) (SZ$^+$SM$^+$ vs. SZ$^+$SM$^-$: p = 0.035 for C, 0.026 for C-A, and 0.014 for T-G; Table 2). Furthermore, the frequency of haplotype T-A in non-schizophrenia smoking group (f = 0.661) remained suggestively lower than non-schizophrenia non-smoking group (f = 0.746) (SZ$^-$SM$^+$ vs. SZ$^-$SM$^-$: p = 0.025; Table 2). Other afore-mentioned *CHRNB3*-smoking associations did not remain significant.

	Frequencies								p-values (χ²) [OR (95%CI)] .		
	SZ$^+$SM$^+$	SZ$^+$SM$^-$	SZ$^-$SM$^+$	SZ$^-$SM$^-$	SZ$^+$	SZ$^-$	SM$^+$	SM$^-$	SM$^+$ vs. SM$^-$	SZ$^+$SM$^+$ vs. SZ$^+$SM$^-$	SZ$^-$SM$^+$ vs. SZ$^-$SM$^-$
	(n = 567)	(n = 206)	(n = 168)	(n = 134)	(n = 773)	(n = 302)	(n = 735)	(n = 340)			
rs6474413											
C	0.269	0.216	0.260	0.216	0.255	0.241	0.267	0.216	**0.012** (6.3) [1.3(1.1-1.6)]	**0.035** (4.2) [1.3(1.0-1.8)]	0.250 (1.6) [1.3(0.9-1.9)]
T	0.731	0.784	0.740	0.784	0.745	0.759	0.733	0.784			
C/C	0.073	0.042	0.072	0.030	0.065	0.054	0.073	0.037	**0.029**	0.186	0.124
C/T	0.391	0.349	0.377	0.371	0.380	0.375	0.388	0.358	0.345	0.315	0.973
T/T	0.536	0.609	0.551	0.598	0.555	0.572	0.539	0.605	**6.5×10^{-5}**	0.086	0.483
Overall									**0.035** (6.7)	0.119 (4.3)	0.262 (2.7)
rs13277254											
A	0.800	0.835	0.752	0.798	0.809	0.772	0.789	0.820			
G	0.200	0.165	0.248	0.202	0.191	0.228	0.211	0.180	0.090 (2.9) [0.8(0.7-1.0)]	0.125 (2.3) [0.8(0.6-1.1)]	0.204 (1.8) [0.8(0.5-1.1)]
A/A	0.641	0.706	0.570	0.656	0.658	0.608	0.624	0.686	0.055	0.086	0.154
A/G	0.319	0.259	0.364	0.282	0.330	0.328	0.329	0.268	0.047	0.111	0.175
G/G	0.041	0.036	0.067	0.061	0.039	0.064	0.047	0.046	0.972	0.834	0.837
Overall									0.123 (4.2)	0.257 (2.7)	0.300 (2.4)
Haplotype											
C-A	0.109	0.070	0.089	0.048	0.099	0.071	0.104	0.061	**1.2×10^{-3}** (10.4) [1.8(1.3-2.5)]	**0.026** (4.7) [1.6(1.0-2.5)]	0.057 (3.6) [1.9(0.9-3.8)]
C-G	0.158	0.148	0.173	0.165	0.155	0.169	0.161	0.155	0.704 (0.2) [1.1(0.8-1.4)]	0.691 (0.2) [1.1(0.8-1.5)]	0.827 (0.1) [1.1(0.7-1.6)]

| T-A | 0.689 | 0.765 | 0.661 | 0.746 | 0.709 | 0.699 | 0.683 | 0.758 | **3.8×10^{-4}** (12.6) [0.7(0.6-0.9)] | **3.7×10^{-3}** (7.7) [0.7(0.5-0.9)] | **0.025** (5.0) [0.7(0.5-0.9)] |
| T-G | 0.044 | 0.017 | 0.077 | 0.041 | 0.037 | 0.061 | 0.052 | 0.026 | **8.7×10^{-3}** (7.1) [2.0(1.2-3.4)] | **0.014** (5.5) [2.6(1.1-6.0)] | 0.087 (3.3) [2.0(0.9-4.1)] |

Table 2: *The associations between smoking and alleles, genotypes and haplotypes.*

[SZ⁺SM⁺, Schizophrenia patients with smoking behavior; SZ⁺SM⁻, Schizophrenia patients without smoking behavior; SZ⁻SM⁺, Controls with smoking behavior; SZ⁻SM⁻, Controls without smoking behavior; SZ⁺, total schizophrenia patients with or without smoking behavior; SZ⁻, total controls with or without smoking behavior; SM⁺, total smoking subjects with or without schizophrenia; SM⁻, total non-smoking subjects with or without schizophrenia. vs., comparing between two groups. Overall, the overall genotype frequency distributions. The p-values remaining <u>significant (p<α)</u> or suggestive (p close to α) after Bonferroni Correction are bold; other non-significant comparisons are omitted in this table.

For SM⁺ vs. SM⁻: α = 0.05, 0.017 and 0.0125 for allele, genotype and haplotype analyses, respectively.

For SZ⁺SM⁺ vs. SZ⁺SM⁻ and SZ⁻SM⁺ vs. SZ⁻SM⁻: α = 0.025, 0.0085 and 0.0063 for allele, genotype and haplotype analyses, respectively].

Finally, neither *CHRNB3*-schizophrenia nor *CHRNB3*-(schizophrenia+smoking) associations were statistically significant, including SZ⁺ vs. SZ⁻, SZ⁺SM⁺ vs. SZ⁻SM⁺, SZ⁺SM⁻ vs. SZ⁻SM⁻, and SZ⁺SM⁺ vs. SZ⁻SM⁻ (data not shown).

CHRNB3 mRNA was significantly expressed, and related to the expression of dopaminergic or GABAergic receptors in the brain

CHRNB3 had significant mRNA expression in three human brain regions in the two independent cohorts (Table S2). Specifically, in the first UK European cohort, *CHRNB3* was significantly expressed in the SNIG [log2(normalized intensity) = 6.48], and had low expression in frontal cortex, occipital cortex (specifically primary visual cortex), hippocampus, temporal cortex, putamen, and intralobular white matter as well (data not shown). In the American cohort, *CHRNB3* was significantly expressed in cerebellar hemisphere and cerebellum (1.3 ≤ TPM ≤ 2.1).

Cohort	Expression threshold	Methods	SNIG	CRHM	CRBL
UK Europeans (n = 134)	log2-transformed normalized intensity values > 5.17	Affymetrix Human ST 1.0 exon arrays	6.48		
Americans (n = 173)	TPM values > 1.0	RNA-Seq	1.3	2.1	1.4

Table S2: *The mRNA expression of CHRNB3 in human brains.*
[Abbr.: Substantia Nigra (SNIG), Cerebellar Hemisphere (CRHM), and Cerebellum (CRBL)].

The mRNA expression of *CHRNB3* was significantly related to the expression of dopaminergic or GABAergic receptor (p<α) in many brain areas (Table S3). The correlation coefficients (0.361 ≤ |r| ≤ 0.920), regression coefficients (0.008 ≤ |β| ≤ 0.202) and p values (3.9 × 10^{-42} ≤ p ≤ 3.3 × 10^{-5}) for these correlations are summarized in the supplementary table S3.

| | First cohort | | | | | | | | | | | | | Second cohort | |
| Gene | FCTX | | HIPP | | OCTX | | PUTM | | SNIG | | TCTX | | WHMT | | FCTX | |
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	β	p
DRD1		-		-		-		-	-.417	1.4×10⁻⁵		-		-	0.202	6.5×10⁻¹⁰
DRD2		-		-		-		-	.834	2.9×10⁻²⁷	.514	2.2×10⁻⁹		-	-0.088	6.4×10⁻⁷
DRD3	-.434	3.6×10⁻⁷		-		-		-		-		-		-		-
DRD4	-.361	3.0×10⁻⁵		-		-		-		-		-		-		-
DRD5		-		-		-		-		-		-	.390	4.2×10⁻⁶		-
TH		-		-		-		-	.920	3.9×10⁻⁴²	.555	5.5×10⁻¹¹	.401	2.1×10⁻⁶		-
GABRA1		-		-		-		-	.482	3.4×10⁻⁷		-		-	0.010	4.9×10⁻¹⁰
GABRA2		-		-	.418	8.0×10⁻⁷		-		-		-		-	0.027	2.7×10⁻⁸
GABRA3		-		-	.429	3.8×10⁻⁷		-	.590	8.1×10⁻¹¹		-		-		-
GABRA4	.399	3.5×10⁻⁶		-	.403	2.2×10⁻⁶		-	.622	3.9×10⁻¹²		-	.477	8.3×10⁻⁹	0.022	1.4×10⁻⁹
GABRA5		-		-	.457	5.3×10⁻⁸		-		-		-		-	0.028	7.1×10⁻¹⁰
GABRB1	.391	5.5×10⁻⁶		-	.379	9.5×10⁻⁶	-.361	2.6×10⁻⁵	.735	2.1×10⁻¹⁸		-		-		
GABRB2		-		-		-		-	.410	2.1×10⁻⁵		-		-	0.013	1.9×10⁻⁹
GABRB3		-		-	.429	3.9×10⁻⁷		-	.417	1.4×10⁻⁵		-		-	0.021	4.9×10⁻¹⁰
GABRD		-		-		-		-		-		-	.363	2.0×10⁻⁵		-
GABRG2		-		-	.368	1.8×10⁻⁵		-	.624	3.2×10⁻¹²		-	.373	1.2×10⁻⁵	0.008	6.7×10⁻¹⁰
GABRG3	.385	7.9×10⁻⁶		-		-		-		-		-		-	0.028	4.8×10⁻⁸
GABRP	-.394	4.6×10⁻⁶		-		-	.366	2.0×10⁻⁵		-		-		-		
GABRQ		-	-.378	1.7×10⁻⁵		-		-		-		-		-		-

Table S3: Expression correlation between CHRNB3 and dopaminergic and GABAergic receptor genes in human brains.

[For first cohort: The statistical significance level (α) = 2.0×10⁻⁴. r, Pearson correlation coefficient. p, p values. "-", p > α.

Second cohort: The statistical significance level (α) = 4.1×10⁻⁶. β, regression coefficient from generalized linear model (GLM).

Other three brain regions and seven genes were omitted in this table due to no p values less than α.

Abbr.: Frontal Cortex (FCTX), Hippocampus (HIPP), Occipital Cortex (specifically primary visual cortex, OCTX), Putamen (PUTM), Substantia Nigra (SNIG), Temporal Cortex (TCTX), and Intralobular White Matter (WHMT)].

CHRNB3 SNPs had significant cis-acting regulatory effects on mRNA expression in the brain

Cis-eQTL analysis indicated that the two *CHRNB3* SNPs had significant *cis*-acting regulatory effects on the expression of *CHRNB3* mRNA in cerebellar cortex and thalamus ($p < 0.05$) (Table 3).

| | | p-values for *cis*-eQTL analysis | |
SNPs	Regulating Target	Cerebellar Cortex	Thalamus
rs13277254	*CHRNB3 mRNA*	0.021	0.031
rs6474413	*CHRNB3 mRNA*	0.022	0.030

Table 3: The significant cis-regulatory effects of two CHRNB3 SNPs on the CHRNB3 mRNA expression in two human brain regions.

[cis-eQTL, cis-acting expression of quantitative locus analysis. Data were extracted from UKBEC database. Sample size = 134 European postmortem brain tissues].

Discussion

As expected, smoking was associated with *CHRNB3* no matter being comorbid with or without schizophrenia. *CHRNB3* was abundant in human brain and might be biologically functional, which supported that the statistical *CHRNB3*-smoking association might be true. It could play important role in the pathogenesis of smoking. Our data provided the first insight into the association between *CHRNB3* polymorphisms and smoking in the male Chinese patients with schizophrenia.

We found smoking-*CHRNB3* associations most significantly in the total smoking group (SM⁺), but less significantly in the smoking subgroup with schizophrenia (SZ⁺SM⁺) and smoking subgroup without schizophrenia (SZ⁻SM⁺), which probably resulted from smaller sample sizes. In view of this interpretation, the findings in the subgroups actually replicated each other. Using similar analytic strategy, we did not find any significant association between schizophrenia and *CHRNB3* after controlling for the confounding effects of smoking. Finally, we did not find any significant association between schizophrenia+smoking (SZ⁺SM⁺) and *CHRNB3* when compared to non-schizophrenia non-smoking group (SZ⁻SM⁻), which is probably due to uncontrolled confounding effects of schizophrenia on the association analysis. Our findings indicated that the high comorbid rate of schizophrenia and smoking was not attributed to *CHRNB3*, but probably other factors; for example, it has been hypothesized that smoking is an attempt for self-medication by compensating for the underlying neurobiological deficits in patients with schizophrenia [18,41-43]; it has been also reported that smoking can relieve some extrapyramidal symptoms caused by antipsychotic treatments in patients with schizophrenia [44,45]. These other factors are worthy of further investigation.

β3 nAChR subunit, encoded by *CHRNB3,* is abundant in cerebellar hemisphere, midbrain, striatum, cerebellum and more other regions [3], and could play roles in brain disorders. Many of these regions have been reported to be associated with cognitive symptoms of ND; for example, frontal cortex is implicated in decision making and behavioral control, occipital cortex is implicated in cue response, thalamus is implicated in regulation of arousal and trans-cortical integration of information, and cerebellum is implicated in control of cognition. Several pathways in the midbrain, e.g. habenula-interpeduncular pathway and ventral tegmental area - nucleus accumbens pathway, are also critical to nicotine-induced reward responses [3]. Alternatively, β3 nAChR subunit may modulate dopamine and GABA release, and contribute to the reinforcing effect of nicotine.

We demonstrated that the two promoter SNPs regulated *CHRNB3* mRNA expression, indicating that these two SNPs were functional, consistent with the previous report that rs6474413 was linked with reduced β3 expression and reduced nicotine intake in mice [46]. These SNPs may influence risk for smoking via regulating the transcription of *CHRNB3* or via linkage disequilibrium with putative causal variants. Finally, we note that the findings in the present study are limited to male patients; and the investigation on female patients is warranted in the future, because there might exist sex-specificity in these findings.

Conclusion

Risk for smoking behavior was associated with CHRNB3. CHRNB3 mRNA is abundant in human brain and could play important role in the pathogenesis of smoking behavior.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board of Beijing Hui-Long-Guan Hospital and all participants volunteered to participate in the study and signed informed consent.

Consent for Publication

Informed consents to publish were obtained from the patients in this research.

Availability of Data and Materials

The materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality under the circumstances without conflicting to our further research.

Competing Interests

There was no any conflict of interest in our study.

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Authors' Contributions

JS, FW and NC appraised all potential studies and wrote and revised the draft manuscript. ZRW and FDY designed the study and revised the draft manuscript and subsequent manuscripts. SPT, JHT and HMA assisted with the presentation of findings and assisted with drafting and revising the manuscript. YLT, XPW and XGL conceived and designed the study, assisted with searches, appraised relevant studies and assisted with drafting and revising the manuscript. All authors read and approved the final manuscript.

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