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Association of *RELN* promoter SNPs with schizophrenia in the Chinese population

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Abstract: Previous research on gene expression analysis and association tests have suggested that *RELN* is a risk gene for schizophrenia in world populations. Based on the reported down-regulation of RELN in schizophrenia patients compared with normal subjects, we speculated that variants in the *RELN* promoter region may confer risk for schizophrenia. In this study, we investigated the associations of three SNPs in the promoter region of *RELN* with schizophrenia in a case-control sample from southwestern China (940 cases and 1369 controls). The results suggested that none of the SNPs showed significant associations in our sample, indicating the risk variants for schizophrenia in *RELN* may not be located in the promoter region. We also performed meta-analysis by combining our data with previously reported data on the Chinese population with a total sample size of 2843 individuals, and the result remained non-significant. Collectively, our results suggested variants in the *RELN* promoter may not harbor risk SNPs associated with schizophrenia in the Chinese population.

Key words: RELN; Schizophrenia; Promoter; SNP; Chinese population

RELN 基因启动子区 SNP 位点与精神分裂症的相关性分析

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摘要： 目前有很多证据证明 *RELN* 基因在世界人群中是一个精神分裂症的致病基因。基于之前报道过的 *RELN* 基因在精神分裂症患者中表达下降的事实, 可以推测在 *RELN* 基因启动子区可能包含影响精神分裂症发生的多态位点。该研究分析了中国西南地区病例——对照人群中（940 位患者和 1369 位正常人）*RELN* 基因启动子区的 3 个单核苷酸多态性位点与精神分裂症的相关性。研究结果显示, 这些多态位点都不与精神分裂症相关, 表明 *RELN* 基因的致病位点并不在其启动子区。将前人研究结果与该研究结果进行综合分析（共 2843 个样本）, 结果仍不显著。因此, 该研究表明, *RELN* 基因启动子区的单核苷酸多态性位点在中国人群中并不与精神分裂症相关。

关键词： *RELN*; 精神分裂症; 启动子; 单核苷酸多态性位点; 中国人群
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Schizophrenia is a severe psychiatric disorder with high heritability. Many susceptibility genes have been proposed by linkage analyses, candidate gene studies, and genome wide association (GWA) analyses, including DISC1, NRG1, TCF4, NRGN, and ZNF804A (Thomson et al, 2007; Williams et al, 2004; O'Donovan et al, 2008). However, many cannot be successfully replicated among different populations. A recent GWA study on Ashkenazi Jews identified that a common variant (rs7341475) of *RELN* was significantly associated with

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schizophrenia in females (Shifman et al, 2008). This association was further supported by replications in a UK study and another Ashkenazi Jew study (Shifman et al, 2008; Liu et al, 2010), but was unable to be replicated in USA and Chinese populations (Shifman et al, 2008; Need et al, 2009), indicating potential genetic heterogeneity in *RELN* for schizophrenia between different world populations.

We previously identified several *RELN* SNPs and haplotypes associated with schizophrenia in the Chinese population (Li et al, 2011), which is consistent with reported associations in European populations and suggests *RELN* is likely a common risk gene for schizophrenia in populations worldwide, though the risk variants differ between reported associations (Kahler et al, 2008; Wedenoja et al, 2008, 2009).

Down-regulation of *RELN* mRNA and protein in the brain of schizophrenia patients has been reported previously, but the underlying mechanism remains unknown (Impagnatiello et al, 1998). Hypermethylation of *RELN* promoter in schizophrenic patients is a likely cause; however, negative findings have also been reported (Grayson et al, 2005; Tochigi et al, 2008). One reasonable explanation is that mutations in the *RELN* promoter may affect gene expression and function and influence neurodevelopment, leading to schizophrenia susceptibility. Interestingly, short tandem repeats in the promoter region influence *RELN* promoter activity, but the association tests in schizophrenia show negative results (Akahane et al, 2002). Additionally, an investigated *RELN* promoter SNP has shown marginal significance with schizophrenia in a small case-control Chinese sample (P=0.08) (Chen et al, 2002), suggesting the *RELN* promoter may harbor risk variants for schizophrenia. In this study, we examined the association between the RELN promoter SNPs and schizophrenia in a Chinese case-control sample.

1 Methods and Materials

1.1 Samples

We recruited 940 unrelated schizophrenia patients (481 females and 459 males, mean age=37.4 a, SD=9.1) and 1 369 unrelated normal controls (732 females and 637 males, mean age=36.7 a, SD=6.8) from southwestern China. The patients were all from Yunnan Mental Health Hospital and The Second People's Hospital of Yuxi City and were diagnosed with having schizophrenia according to DSM-IV and ICD-10 criterion. Patients who had history of alcoholism, substance induced psychotic disorders, epilepsy, neurological diseases, or other symptomatic psychoses were excluded from this study. Control subjects were recruited from the local general populations. All individuals were asked to provide detailed information about medical and family psychiatric histories, and those who had a history of mental disorders, drug abuse, or alcohol dependence were excluded. All patient and control subjects were of Han Chinese origin from the Yunnan province of southwestern China. All individuals were provided with written informed consents for participation, and the research protocol was approved by the internal review board of the Kunming Institute of Zoology, Chinese Academy of Sciences.

1.2 SNP selection

As no SNPs were shown in the -2 kb region upstream from the transcription start site (TSS) of *RELN* in Han Chinese from Beijing (CHB) data obtained from HapMap database, we used bi-directional sequencing to search for potential SNPs in the promoter region of *RELN* on 100 schizophrenic and 100 healthy randomly selected subjects. Primers used for PCR amplification of the RELN promoter were 5′- *AGCCCAGAAGCAATGAA TAAC*-3′ (forward) and 5′-*TCCCAACTTGTGACTCCATT C*-3′(reverse). The PCR program started with an initial incubation at 95 ^{o}C for 5 min, followed by 40 cycles of 95 ^{o}C for 30 s, 60 ^{o}C for 40 sec and 72 ^{o}C for 1 min, and then held at 72 ^{o}C for 10 min. Two SNPs were identified (g.-847G>A and g.-888G>C), with g.-888G>C having been investigated previously (Chen et al, 2002). We further carried out case-control association analysis on these SNPs (plus another *RELN* promoter SNP rs6951875, shown in HapMap data) in our samples.

1.3 SNP genotyping

Venous blood was collected from all participants, and genomic DNA was extracted from the blood sample using the phenol-chloroform method. The DNA samples of the cases and controls were randomly distributed in the case-control DNA plates.

All selected SNPs were genotyped by SNaPShot as described in our previous study (Luo et al. 2008). In brief, genomic fragments which contained selected SNPs were amplified by PCR with a total volume of 25 μL (including 10 ng of genomic DNA) in 96-well plates. Amplified fragments were purified and specific genotyping primers were used to amplify the target site. After one base extension, the reaction was terminated

and the products were loaded on an ABI 3130 automatic sequencer (Applied Biosystems). Primer sequences for SnaPShot analysis were 5′-*TTTTTATGAGGTATTCTGACACTGGATGAAGAATAATTAT*-3′(rs6951875), 5′-*TTTTTTTTTTTTTTGCGGGAGGGACAGGGGGCCTGGGTGGGAAGGGAGC*-3′ (g.-847G>A) and 5′-*TTTTTTTTTTTTTTTTTTTATGAGGCTCTGTCGCTGCCGCGAGGGGCCGGGCGG*-3′(g.-888G>C). The SNP genotype callings were automatically performed using ABI GeneMapper 4.0 and verified manually. To ensure accuracy of genotyping, we used bi-directional sequencing on 100 randomly selected individuals. No genotyping errors were found.

1.4 Statistical analysis

The Haploview program was applied to test the genotypic distribution of SNPs for Hardy-Weinberg equilibrium (HWE) between paired SNPs, and to define haplotype blocks (Barrett et al, 2005). Allelic and genotypic associations were accessed with PLINK (Purcell et al, 2007). To detect significant differences in association in female or male samples separately, we conducted statistical analysis with sex as a covariate using PLINK (Purcell et al, 2007). The 95% confidence intervals (CI) of odds ratio were calculated with the online tool (http://faculty.vassar.edu/lowry/odds2x2.html). Haplotype frequency estimation and association tests were performed using PLINK, and only those haplotypes with a frequency of >0.01 in cases and control subjects were considered (Purcell et al, 2007). Power analysis was performed using G*power program (Erdfelder et al, 1996). For meta-analysis, we used the Mantel-Haenszel method with a fixed-effects model. Analysis was conducted by RevMan manager (The Cochrane Collaboration, 2002).

2 Results

Due to genotyping failure of some samples, analyses were based on 2 230 samples for SNP g.-847G>A (861 cases and 1 369 controls), 2 307 samples for SNP g.-888G>C (940 cases and 1 367 controls), and 2 296 samples for SNP rs6951875 (940 cases and 1 356 controls). The overall genotype calling rate was 98.6%.

Genotype distributions of the three SNPs in both cases and controls were in HWE (P>0.05). The LD map of the tested SNPs in cases and control samples are shown in Fig. 1.

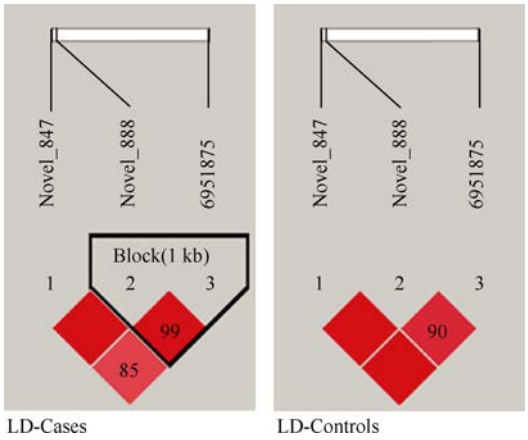

Fig. 1 The LD map of the tested SNPs in case and control samples

Linkage disequilibrium (LD) between the paired SNPs. The haplotype blocks were estimated by Haploview using the standard D' confidence interval (CI) algorithm.

None of the tested SNPs were significantly associated with schizophrenia (Tab.1 and Tab.2). We also investigated the association of these SNPs with schizophrenia in females and males separately, and no

Tab. 1 Allele frequencies and single SNP association (P-value) analysis

Sample	SNP	Minor allele	MAF		P-value	OR(95%CI)
			Case	Control		
All samples	g.-847G>A	A	0.2189	0.2213	0.851	0.99(0.85-1.14)
	g.-888G>C	C	0.0803	0.0877	0.371	0.91(0.73-1.12)
	rs6951875	T	0.0952	0.0867	0.319	1.11(0.90-1.36)
Female samples	g.-847G>A	A	0.2184	0.2316	0.463	0.93(0.76-1.13)
	g.-888G>C	C	0.0769	0.0840	0.532	0.91(0.67-1.23)
	rs6951875	T	0.0988	0.0815	0.144	1.24(0.93-1.64)
Male samples	g.-847G>A	A	0.2195	0.2096	0.585	1.06(0.86-1.31)
	g.-888G>C	C	0.0839	0.0921	0.503	0.90(0.67-1.22)
	rs6951875	T	0.0915	0.0926	0.933	0.99(0.74-1.33)

Abbreviations: MAF-minor allele frequency; OR-odds ratio; CI-confidence interval.

Tab. 2 Genotype counts and *p*-values of the tested SNPs

Sample	SNP ID	Genotype	Genotype Counts		*P*-value
			Cases	Controls	
All samples	g.-847G>A	GG/GA/AA	528/289/44	819/494/56	0.3046
	g.-888G>C	GG/GC/CC	794/141/5	1138/218/11	0.6012
	rs6951875	CC/CT/TT	766/169/5	1131/215/10	0.3495
Female samples	g.-847G>A	GG/GA/AA	265/150/20	425/275/32	0.5707
	g.-888G>C	GG/GC/CC	409/70/2	614/113/5	0.7742
	rs6951875	CC/CT/TT	389/89/3	611/108/5	0.2340
Male samples	g.-847G>A	GG/GA/AA	263/139/24	394/219/24	0.3341
	g.-888G>C	GG/GC/CC	385/71/3	524/105/6	0.7952
	rs6951875	CC/CT/TT	377/80/2	520/107/5	0.8258

Genotypic *p*-values were calculated using PLINK under additive model (Purcell et al, 2007).

significant results were observed (Tab. 1 and Tab. 2). Finally, we performed a meta-analysis by combining a previous study with a total sample size of 2 843 (Chen et al, 2002), but still failed to find a significant association between g.-888G>C and schizophrenia (*OR*=0.86, 95% *CI*=0.71–1.04, *P*=0.12) (Tab. 3).

We further performed a haplotype-based analysis of the three SNPs with schizophrenia in all samples, female samples and male samples. No haplotypes were significantly associated with schizophrenia (Tab. 4). Notably, the global *p*-value of haplotype analyses in the female samples reached significance (*P*=0.007), which is likely due to a false positive effect caused by the low frequency haplotypes.

Tab. 3 Meta-analysis of association between g.-888G>C and schizophrenia

Study	Ethnicity	Numbers of C allele		Numbers of G allele		OR(95%CI)
		Cases	Controls	Cases	Controls	
Chen et al, 2002	Chinese	38	50	520	460	0.67(0.43−1.04)
Li et at, 2011	Chinese	151	240	1729	2494	0.91(0.73−1.12)
Total		189	290	2249	2954	0.86(0.71−1.04)

For the 2 association studies, fixed effect model based pooled OR=0.86, 95% CI=0.71-1.04, Z=1.57, *P*=0.12; heterogeneity test: χ^2=1.45, *df* =1, *P* =0.23.

Tab. 4 Analysis of the 3 SNPs for haplotypic association with schizophrenia

Sample	Haplotype	fre_cases	fre_controls	CHISQ	*p*-value	Global p
All	GCT	0.0811	0.0808	0.001	0.97	0.98
	AGC	0.2215	0.2243	0.05	0.82	
	GGC	0.6974	0.6949	0.03	0.86	
Female	GCT	0.0771	0.0777	0.003	0.96	0.007
	AGC	0.2187	0.2321	0.59	0.44	
	GGC	0.6840	0.6850	0.003	0.96	
Male	GCT	0.0837	0.0840	0.001	0.97	0.93
	AGC	0.2199	0.2130	0.1485	0.70	
	GGC	0.6965	0.7030	0.1063	0.74	

We performed a power calculation using the G*power program. The present sample size revealed >99% power to detect a significant association (α<0.05) with an effect size index of 0.1 (corresponding to a ‘weak’ gene effect).

3 Discussion

It has been reported that SNP g.-888G>C shows an association tendency with schizophrenia (*P*=0.08) (Chen et al, 2002). Our current study, however, showed no significant associations, suggesting SNPs in the promoter region of *RELN* confer no risk for schizophrenia or that there is a potential genetic divergence among regional Han Chinese populations.

In our previous study, we observed multiple SNPs within *RELN* significantly associated with schizophrenia in Han Chinese. These SNPs were all located in the intron region and not causal SNPs (Li et al, 2011), however, suggesting the possibility of finding risk SNPs in the promoter region which had not been systematically screened before. We failed to find significant association between *RELN* promoter SNPs and schizophrenia, implying that the causal SNP was not located in the promoter region. However, there are several possibilities that could explain the negative results in this study.

Firstly, since we only sequenced 2 kb of the *RELN* promoter region, we could not detect other potential genetic variants risks not located in the -2 kb promoter region of *RELN*. Secondly, though our sample size was relatively large, it was smaller than the GWA study sample sizes and was unlikely to identify the risk variant with weak effect. Taken together, our findings indicate that the SNPs in the core promoter region (-2 kb upstream of TSS) of *RELN* were not likely associated with schizophrenia in the Chinese population. Further studies should focus on other regions, and a replication study with large sample size is needed.

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