RESEARCH ARTICLE

Combined effect between two functional polymorphisms of *SLC6A12* gene is associated with temporal lobe epilepsy

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Abstract

Temporal lobe epilepsy (TLE) is the most common epilepsy subtype with complex genetic structure. A recent study in four populations (Ireland, UK, Australia and Finland) reported an allelic association between betaine/GABA transporter-1 (*BGT-1* or *SLC6A12*) and mesial temporal lobe epilepsy with hippocampal sclerosis. To demonstrate the association between *SLC6A12* gene polymorphisms and TLE, TaqMan method was used to genotype five single-nucleotide polymorphisms of *SLC6A12* gene in 358 TLE patients and 596 nonepileptic control subjects of Chinese Han origin. Real-time PCR was used to detect the effects of variations on gene expression associated with TLE. Though, the single-marker analysis did not demonstrate allelic association with TLE, rs542736–rs557881 interaction showed significant association. The *SLC6A12* expression levels in peripheral blood mononuclear cells were significantly higher in TLE patients than in control subjects and were correlated to rs542736 G–rs557881 A haplotypes. Our preliminary results suggested combined effect of two common polymorphisms on *SLC6A12* gene may be associated with TLE, but the precise mechanism needs further investigation.

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Introduction

Temporal lobe epilepsy (TLE) is the most common subtype of epilepsy and is characterized by a highly heterogeneous pathogenesis. The onset of this disease tends to be during adolescence and it is generally believed to be the cause of brain dysfunction (Manford *et al.* 1992; Korn *et al.* 2005; Seifert *et al.* 2006). Nowadays, TLE carries a better prognosis through a treatment of antiepileptic drugs and surgery (Labate *et al.* 2011; Wiebe and Jette 2012). TLE has its own clinical characteristics of neurological or psychiatric symptoms with some special feelings as simple partial seizure, accompanied by complex partial seizure like the month-digestion automatism sometimes, and electroencephalogram shows an ictal onset zone in the temporal structures (Cataldi *et al.* 2013). Genetic factors together with environmental factors are believed to influence the occurrence, development and prognosis of TLE at several aspects including seizure onset, clinical endophenotype, antiepileptic drug response and drug resistance.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. Discrepancy of previous studies on the expression of GABA was conducted in many neurological disorders, notably epilepsy (Manyam *et al*. 1980; During *et al*. 1995; Weiner *et al*. 1996). GABA transporters serve to reuptake GABA from synaptic cleft and maintain the extracellular GABA concentration (Amara and Arriza 1993). A recent study in four populations (Ireland, UK, Australia and Finland) reported an allelic association between betaine/GABA transporter-1 (*BGT-1* or *SLC6A12*) and mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) (Cavalleri *et al.* 2007). *SLC6A12* is a subtype of GABA transporters which is observed in the cortex and CA fields of hippocampus (Zhu and Ong 2004b). Although the role of *SLC6A12* in controlling neuronal excitability was unknown, the cumulating evidences suggested that *SLC6A12* may be

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involved in the occurrence of epilepsy, and the expression of *SLC6A12* has been reported to be increased in kainic acid-induced status epilepticus (Zhu and Ong 2004a); *EF1502* [N-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-(methylamino)-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol] was found to play a vital role in the control of partial epilepsy by selectively inhibiting *SLC6A12* in animal models (White *et al.* 2005; Madsen *et al.* 2009).

Accordingly, the present study was undertaken to replicate the association of variations in *SLC6A12* gene with TLE in Chinese population, and study the influence of the interaction of single-nucleotide polymorphism (SNPs) on *SLC6A12* gene.

Methods

Subjects

Three hundred and fifty-eight TLE patients and 596 non-epileptic control subjects of Chinese Han origin were recruited for this study. TLE patients were diagnosed and recruited from March 2004 to December 2008 by consultant physicians in Department of Neurology, Xuanwu Hospital, Capital Medical University, according to the 'Classification of Epilepsies and Epileptic Syndromes' by Commission on Classification and Terminology of the International League Against Epilepsy (ILAE 1989). The TLE diagnosis was done by comprehensive evaluation of seizure symptoms, neurological examination, magnetic resonance imaging (MRI) and video electroencephalography (EEG) showing typical ictal activity or interictal discharges. Patients were excluded when: (i) MRI showed the evidences of brain tumour, vascular abnormalities, brain parasite; (ii) obvious familial temporal lobe epilepsy pattern in three first-degree relatives. Control subjects were enrolled from a consecutive unrelated group without genetic neurological disorder. All subjects were given written informed consent. This study was approved by the Ethics Committee of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences.

Variation selection and genotyping

Five SNPs in the *SLC6A12* locus were selected including two exonic SNPs (rs542736 and rs557881) and three intronic SNPs (rs2284329, rs216243 and rs9783494), of which four tag SNPs (rs542736, rs2284329, rs216243 and rs9783494) were from HapMap database (http://hapmap.ncbi.nlm.nih.gov/) when determinant coefficient (r^2) and minor allele frequency (MAF) thresholds were set at 0.8 and 0.1, respectively, and rs557881 was a missense SNP with MAF > 0.1 in Chinese Han Beijing (CHB).

Genomic DNA was extracted from peripheral blood by QIAmp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). Five SNPs were all genotyped by TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, USA). Amplification reactions were carried out in a volume of 10 μL by using Bio-Rad iQ5 Multicolor RT-PCR detection system (Biorad, Hercules, USA). Sanger sequencing was used to confirm the genotyping results in 5% randomly selected samples.

Gene expression by quantitative RT-PCR

Forty-five patients with TLE and 90 healthy control subjects were selected for genotyping of two exonic SNPs (rs542736 and rs557881). Eighteen TLE and 41 control samples carrying GG and AA genotypes of rs557881 were analysed for expression of *SLC6A12*.

Total RNA was isolated from peripheral blood mononuclear cells (PBMC) with RNAprep pure Blood Kit (Tiangen, Beijing, China). The purity and integrity of total RNA were detected by ultraviolet spectrometric measurements and agarose gel electrophoresis, respectively. Complementary DNA (cDNA) was synthesized in 20 μL reaction volume from 1 μg total RNA using Quantscript RT Kit (Cwbio, Beijing, China). Expression of *SLC6A12* messenger RNA (mRNA) was measured by TaqMan® relative quantitative analysis using Bio-Rad iQ5 Multicolor RT-PCR detection system (Biorad). The primers and probe for amplification of *SLC6A12* cDNA were

5′-CACAGAGCATTGCACGGACTT-3′ (forward primer),
5′-CCATGACAGGTGAGGTAAAATTCTC-3′ (reverse primer) and
FAM-TGAACCACTCAGGAGCCGGCACA-BHQ (probe).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control gene, and the primers and probe used for amplification of GAPDH cDNA were 5′-TCTGACTTCAACAGCGACAC- 3′ (forward primer), 5′-CAAATTCGTTGTCATACCAG-3′ (reverse primer) and FAM-CACTCCTCCACCTTTGACGCT-BHQ (probe).

The detailed RT-PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate. Relative expression level of *SLC6A12* gene was normalized to $2^{-(\text{SLC6A12 Ct–GAPDH Ct})}$.

Statistical analyses

χ^2 goodness-of-fit test was applied to access the genotypic distributions of SNPs for Hardy–Weinberg equilibrium (HWE). Differences in allele and genotype frequencies were performed with 2×2 and 2×3 contingency tables, respectively. Linkage disequilibrium (LD) between SNPs was calculated by SHEsis (Shi and He 2005). Haplotypes of each subject were analysed using PHASE program (ver. 2.1). Comparison of a difference in *SLC6A12* mRNA was done by Kruskal–Wallis or Mann–Whitney tests. The Bonferroni correction was applied to correct a P value in multiple comparisons. Statistical significance was considered as $P < 0.05$ (two-sided). All the above analyses were conducted using SAS9.1 (SAS, Cary, USA).

Table 1. Demographic and clinical variables in TLE patients and nonepileptic control subjects.

Variables	TLE ($n = 358$)	Control ($n = 496$)
Age, years (mean, SD)*	32.7 ± 12.6	33.6 ± 7.4
Gender*		
Male (%)	190 (53.1)	252 (50.8)
Female (%)	168 (46.9)	244 (49.2)
Age at onset, years (mean, SD)	20.2 ± 12.6	–
Family history of epilepsy (%)	20 (5.6)	–
Antecedent FC (%)	26 (7.3)	–
Antecedent brain trauma or encephalitis (%)	33 (9.2)	–
Aura (%)	227 (63.4)	–
MRI		
HS (%)	84 (23.5)	–
Other (%)	43 (12.0)	–
Normal (%)	231 (64.5)	–
EEG		
Temporal spikes and rhythmic activity (%)	187 (52.2)	–
Others (%)	171 (47.8)	–

TLE, temporal lobe epilepsy; FC, febrile seizure; MRI, magnetic resonance imaging; HS, hippocampal sclerosis; EEG, electroencephalography; SD, standard deviations.*$P > 0.05$.

Results

Five SNPs were successfully genotyped in all patients (358) and controls (596). There were no significant differences in age and gender ratio between two groups ($P > 0.05$). The demographic and clinical variables of the subjects are summarized in table 1.

The genotype distributions of five SNPs did not deviate from HWE. The single-marker analysis of five SNPs in *SLC6A12* gene did not show allelic association with TLE (table 2).

The pairwise LD patterns for five SNPs were examined and no obvious LD was found between each other (table 3). Interestingly, after sequencing 40 samples, we

Table 2. Genotype distributions and allele frequencies of SNPs in *SLC6A12* gene.

SNP	Genotype and allele	TLE, *n* (%) (358)	Control, *n* (%) (496)	χ^2	*P*	OR (95% CI)
rs542736	GG	61 (17.0)	71 (14.3)	3.286	0.194	–
	GA	179 (50.0)	233 (47.0)			
	AA	118 (33.0)	192 (38.7)			
	G	301 (42.0)	375 (37.8)	3.121	0.0773	1.19 (0.98–1.46)
	A	415 (58.0)	617 (62.2)			
rs2284329	GG	60 (16.8)	85 (17.1)	0.926	0.630	–
	GT	191 (53.4)	249 (50.2)			
	TT	107 (29.8)	162 (32.7)			
	G	311 (43.4)	419 (42.2)	0.244	0.621	1.05 (0.86–1.28)
	T	405 (56.6)	573 (57.8)			
rs216243	GG	39 (10.9)	55 (11.1)	1.408	0.495	–
	GA	173 (48.3)	220 (44.4)			
	AA	146 (40.8)	221 (44.5)			
	G	251 (35.1)	330 (33.3)	0.593	0.441	1.08 (0.88–1.33)
	A	465 (64.9)	662 (66.7)			
rs9783494	CC	22 (6.1)	18 (3.6)	2.954	0.228	–
	CG	126 (35.2)	178 (35.9)			
	GG	210 (58.7)	300 (60.5)			
	C	170 (23.7)	214 (21.6)	1.124	0.289	1.32 (0.90–1.42)
	G	546 (76.3)	778 (78.4)			
rs557881	GG	43 (12.0)	53 (10.7)	0.368	0.832	–
	GA	163 (45.5)	230 (46.4)			
	AA	152 (42.5)	213 (42.9)			
	G	249 (34.8)	336 (33.9)	0.151	0.697	1.04 (0.85–1.28)
	A	467 (65.2)	656 (66.1)			

Table 3. LD for SNP in *SLC6A12* gene.

SNPs	rs2284329	rs216243	rs9783494	rs557881
	D′ (r^2)			
rs542736	0.281 (0.069)	0.187 (0.028)	0.396 (0.069)	0.459 (0.168)
rs2284329	–	0.799 (0.441)	0.516 (0.103)	0.514 (0.184)
rs216243	–	–	0.303 (0.052)	0.437 (0.189)
rs9783494	–	–	–	0.977 (0.532)

found rs526690, a synonymous SNP, was nearby and completely in LD with rs557881, which was not reported in HapMap database (http://hapmap.ncbi.nlm.nih.gov/). Haplotype analysis for five SNPs indicated that the frequency of haplotype G-T-A-G-A (rs542736- rs2284329- rs216243- rs9783494- rs557881) was higher in TLE patients than in controls (14.1 vs 9.7%, corrected $P = 0.03$) and rs542736 played a major effect (table 4). Therefore, as conditioning on rs542736, only rs542736–rs557881 haplotype was associated with TLE, in which the frequency of G–A haplotype was significantly higher in TLE patients compared to controls (18.2 vs 12.1%, corrected $P = 0.002$) and the frequency of A–A haplotype was lower (47.1 vs 54.0%, corrected $P = 0.02$), but G–G and A–G haplotypes had no effect (table 4).

Quantitative RT-PCR analysis demonstrated the expression levels of *SLC6A12* gene in PBMCs were significantly higher in patients with TLE (0.008 ± 0.005) than in control subjects (0.002 ± 0.0009) ($P < 0.0001$) (figure 1a). The rs542736 GG genotype had a tendency of higher *SLC6A12* gene expression than AA or GA genotype in the control group ($P = 0.07$). As well, the interaction of rs542736 and rs557881 on *SLC6A12* gene expression was found in the control group (figure 1c) but not in patient group (figure 1b). Among rs557881 AA genotype carriers, expression of *SLC6A12* gene significantly increased in risk rs542736 GG homozygote carriers ($P < 0.05$). Whereas, in the rs557881 GG genotype group, no significant effect was detected among rs542736 GG, GA and AA genotype carriers.

Discussion

The present study provides support for a role of *SLC6A12* in the pathogenesis of TLE. We found that haplotypes rs542736 G – rs557881 A may be associated with the increased risk of TLE through affecting *SLC6A12* gene expression and rs542736 A – rs557881 A may be associated with the decreased risk of illness, however, other two haplotypes had no effect.

The SNP rs542736 is located in the 3′ untranslated region (UTR) of *SLC6A12* gene. Our finding suggests that rs542736 G allele may be involved in the occurrence of TLE through *cis*-acting element of *SLC6A12*. SNPs in the 3′UTR may affect gene expression by interfering with transcriptional or posttranscriptional activity, including protein binding, polyadenylation and microRNA binding (Chen *et al.* 2006; Mishra *et al.* 2008; Chatterjee and Pal 2009). We noticed that two SNPs (rs1051104 and rs2075228) in the 3′UTR of *SLC6A12* gene were in complete LD with rs542736 (from 82 CHB genotyping data in HapMap). It is likely that one of them or combination of them may have a functional

Table 4. Haplotypic association of *SLC6A12* SNPs with TLE.

Haplotype[a]	TLE (%)	Control (%)	χ^2	P/P_c^*	OR (95% CI)
ATAGA	233 (32.5)	379 (38.2)	5.80	0.02/0.12	0.78 (0.63–0.96)
GTAGA	101 (14.1)	96 (9.7)	7.99	**0.005/0.03**	1.53 (1.13–2.09)
GGGCG	66 (9.2)	73 (7.4)	2.10	0.15/–	1.30 (0.91–1.84)
AGGGA	62 (8.7)	113 (11.4)	3.21	0.07/–	0.74 (0.53–1.03)
GGGGG	44 (6.1)	75 (7.6)	1.28	0.26/–	0.80 (0.53–1.20)
GGACG	30 (4.2)	54 (5.4)	1.40	0.24/–	0.76 (0.47–1.23)
Others[b]	180 (25.2)	202 (20.3)	–	–	–
AXXXA	337 (47.1)	536 (54.0)	8.07	**0.005/0.02**	0.76 (0.62–0.92)
GXXXG	171 (23.9)	255 (25.7)	0.74	0.390/–	0.91 (0.72–1.14)
GXXXA	130 (18.2)	120 (12.1)	12.21	**0.0005/0.002**	1.61 (1.22–2.13)
AXXXG	78 (10.8)	81 (8.2)	3.66	0.0556	1.38 (0.98–1.93)

[a]Haplotypes of *SLC6A12* gene: rs542736, rs2284329, rs216243, rs9783494, rs557881.
[b]Frequencies of haplotypes less than 5% were pooled and not analysed.
*P_c was by Bonferroni multiple comparison correction; P<0.05 are in bold character.

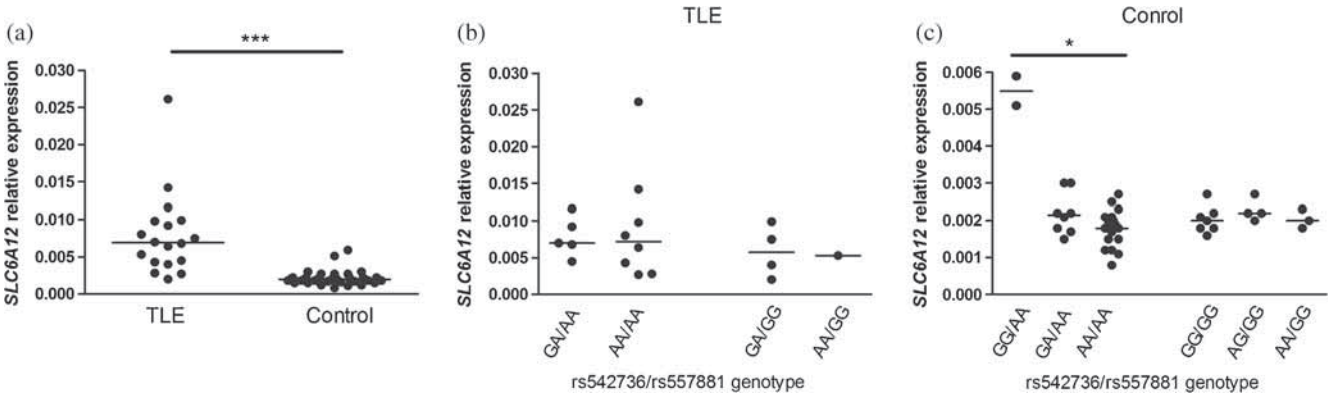

Figure 1. Expression levels of *SLC6A12* mRNA. (a) Difference expression between TLE patients and control subjects. (b, c) The relationships between individual genotypes and expression levels. $*P < 0.05$; $***P < 0.0001$.

role through *cis*-acting regulation of *SLC6A12* expression. However, we did not find that the other three SNPs located in typical conserved elements within 3′UTR influenced mRNA expression (Conne *et al.* 2000). Recently, several mRNAs have been reported to be altered in TLE models (Aronica *et al.* 2010; Liu *et al.* 2010; Song *et al.* 2011). Therefore, these SNPs might influence the stability of *SLC6A12* mRNA and thereby the development of TLE through modifying certain mRNA targeting activity.

We demonstrated that the combined effect of rs542736 and rs557881 might be a substantial risk factor for TLE. The SNP rs557881 (c.28A > G, p.Cys10Arg) is a missense variation and G allele is evolutionarily conserved. Our results showed that the presence of rs557881 G allele might serve as a compensatory variation to interact epistatically with deleterious rs542736 G allele to maintain normal phenotype. Although it is difficult to interpret the potential mechanism of this compensatory effect, we speculate that rs557881 may disrupt the *cis*-regulation of rs542736 by affecting its RNA stem–loop structure. In addition, the synonymous SNP rs526690 (c.42A >G, p.Ala14Ala), which has strong LD with rs557881 may also contribute to this compensatory effect.

The SNP rs216243, which is located in intron, was found to be associated with MTLE-HS in four populations (Ireland, UK, Australia and Finland) (Cavalleri *et al.* 2007). However, we failed to replicate this result. Ethnic background and endophenotype specificities of TLE cases (HS accounted for 23.5% in our study) may be the major reasons for the controversial results.

SLC6A12 gene is able to transport both organic osmolyte betaine and GABA, but the affinity for GABA is lower than other GABA transporters (Lopez-Corcuera *et al.* 1992; Liu *et al.* 1993). Therefore, some studies indicated that *SLC6A12* might regulate the levels of GABA/betaine in the neuronal extracellular space (ECS) and modulate the size of ECS through influencing the excitability and osmolarity of neuron (Zhu and Ong 2004a; Rowley *et al.* 2011). Astrocytic glial swelling caused by overexpression of *SLC6A12* gene might contribute to a higher excitability and lower threshold of epileptiform discharges after brain injury (Zhu and Ong 2004a). It was reported that kainic acid-induced status epilepticus presented an increased expression of *SLC6A12* gene. Consistently, the present study showed that the *SLC6A12* expression in PBMCs were significantly higher in patients with TLE than in control subjects. Thus, these data together support our genetic findings that polymorphisms may be associated with susceptibility to TLE through influencing *SLC6A12* gene expression. Further, we provide a genetic evidence for *SLC6A12* as targets for antiepileptic drugs such as EF1502.

In conclusion, our study demonstrated that combined effects of two functional SNPs (rs542736 and rs557881) on *SLC6A12* gene were associated with susceptibility to TLE, which might be induced by increased expression of *SLC6A12* gene. The functional relevance of the two SNPs merit further analyses and the identified association needs further validations in a larger cohort.

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