Original article

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The association between *CCL2* polymorphisms and drug-resistant epilepsy in Chinese children

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ABSTRACT – The treatment of drug-resistant epilepsy remains a major challenge, affecting approximately 30% of epilepsy patients. More recently, immunity and inflammation are considered to be key elements of epilepsy. Targeting brain inflammation may represent a novel therapeutic strategy for epilepsy and refractory epilepsy. In this study, we investigated the association of a tag SNP of the *CCL2* gene, rs1024611 (originally designated as -2578G>A or -2518G>A) with drug-resistant epilepsy in Chinese children with epilepsy. We enrolled 484 epilepsy patients, including 98 drug-resistant patients and 386 drug-responsive patients. The rs1024611 was genotyped by PCR-RPLP. The rs1024611 AA genotype was associated with a greater susceptibility to drug-resistant epilepsy (p=0.008; OR=2.51, 95% CI: 1.33-4.72), adjusted for age, sex, and seizure type, and the association remained significant after Bonferroni correction for multiple testing (p<0.05). Our results demonstrate that the *CCL2* genetic polymorphism is associated with drug-resistant epilepsy in Chinese paediatric patients.

Key words: drug-resistant epilepsy, *CCL2*, polymorphism

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Epilepsy is the most common chronic neurological condition, affecting at least 50 million people worldwide (Duncan *et al*., 2006). Despite novel antiepileptic drugs (AEDs), about 20~30% epilepsy patients do not respond adequately to appropriate AED treatments. Drug-resistant epilepsy is considered a complex and multifactorial phenomenon and multiple mechanisms probably contribute to the clinical phenotype. Two hypotheses have received extensive attention: the transporter hypothesis and target hypothesis. Recently, inflammation and blood brain barrier (BBB) dysfunction have been suspected to play important roles in the pathophysiology of epilepsy and refractory epilepsy. There is accumulating evidence that inflammatory mediators, such as interleukin (IL)-1β,

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Toll-like receptors (TLRs), high-mobility group box protein 1 (HMGB1), cyclooxygenase-2, and chemokine C-C motif ligand 2 (CCL2) are involved in the development of epilepsy and pharmacoresistant epilepsy in experimental animal models and patients (Wu *et al*., 2008; Foresti *et al*., 2009; Walker and Sills, 2012; Lorigados *et al*., 2013). Furthermore, inflammation and the antiepileptic effects of anti-inflammation have been observed in both experimental animal models and humans (Maroso *et al*., 2010; Vezzani *et al*., 2010; Marchi *et al*., 2011). Therefore, inflammatory pathway genes could play a major role in pharmacoresistant epilepsy.

Chemokines are a family of chemoattractant cytokines characterised by a role in recruiting and activating a variety of cell types. Chemokines have been shown to be involved in many important biological and pathological processes, including angiogenesis, central nervous system development, autoimmune diseases, and nervous system inflammation. CCL2, an important chemokine, directs the migration and infiltration of cells expressing chemokine C-C motif receptor 2 (CCR2), such as monocytes, T-lymphocytes, and natural killer cells, to regions of inflammation. In humans, CCL2 and CCR2 have been observed to be expressed in multiple brain regions, and the expression of CCL2 was up-regulated in animals with status epilepticus and patients with intractable epilepsy (Foresti *et al*., 2009; Walker and Sills, 2012). Recently, an integrative analysis of published studies involving large-scale gene expression profiling of brain tissues from epilepsy surgery has shown that genes involved in neuroinflammation were most prominently differentially expressed, including *CCL2*, *CCL3*, *CCL4*, *CCL28*, *CCL3L1*, *CX3CL1* and *CXCL14*, the chemokine receptor *CXCR4*, as well as transforming growth factor-β superfamily member inhibin β-A (Mirza *et al*., 2011). Among them, differential *CCL2* gene expression was most consistent and up-regulated in four of nine large-scale genome-wide gene expression profiling studies (Mirza *et al*., 2011).

However, whether *CCL2* genetic variants are correlated with pharmacoresistant epilepsy is not reported. The *CCL2* gene is located on chromosome 17q11.2 in humans, including three exons, encoding 99 amino acids. The disturbance of BBB plays a key role, not only in epilepsy pathology, but also in the development of drug resistance. We hypothesized that the over-expression of *CCL2*, working together with other inflammatory mediators, results in impaired recruitment of monocytes and lymphocytes during epileptic activity, leading to a disturbance of BBB integrity. In this study, we genotyped a functional tag SNP corresponding to this gene in order to examine whether this SNP is associated with pharmacoresistant epilepsy.

Materials and methods

The population of our study comprised 484 paediatric patients with epilepsy, including 98 patients with pharmacoresistant epilepsy, from the Department of Neurology, Wuhan Children's Hospital, China. Seizures were classified as partial and generalised, according to the guidelines of the International Classification of Seizures. Aetiology of epilepsy was classified as idiopathic, symptomatic, or non-classifiable, based on medical history, physical and neurological examination, electroencephalography, computer tomography, and magnetic resonance imaging. Drug responsiveness was determined from clinical records. A drug response was defined as seizure freedom or 50% or more reduction in seizure frequency for at least one year during treatment with AEDs. Drug resistance was defined as no change or less than 50% reduction in seizure frequency for at least one year with two or more appropriate AEDs at maximally tolerated therapeutic doses. This study was approved by the hospital's Medical Ethical Committee. Those patients with poor compliance with AEDs, an unreliable or unclear record of seizure frequency, and severe adverse drug reactions were excluded. An informed consent was obtained from the parents of each subject. Genomic DNA was extracted from peripheral blood samples. Tagger software in Haploview was used to identify tag SNPs and represent all of the polymorphisms in the *CCL2* gene. The tag SNP was selected from between 3,000 bp 5′ upstream to 1,000 bp 3′ downstream of the *CCL2* gene (chr17: 29603482, 29608958; NCBI B36 assembly) in the Han Chinese Beijing Panel of the International HapMap project phase III. In this database, for the genotypes of 10 SNPs; rs1024611, rs3917883, rs1024610, rs3760399, rs2857655, rs3760396, rs2857656, rs4586, and rs13900 were available, and only the rs1024611 (originally designated as -2578G>A or -2518G>A) was selected as tag SNP using aggressive tagging with minor allele frequency (0.15) and a pairwise correlation coefficient r^2 of 0.8 for this region. Linkage disequilibrium was calculated by using the Haploview 4.2 software.

The rs1024611 was genotyped using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis. The genomic DNA containing this polymorphic site was amplified by using the following primers: F1 5′-AGG TTT GTG CCA GAG CCT AA-3′ and R1 5′-TTG GCC TTT GCA TAT ATC AGA-3′. Following amplification, PCR products were digested by restriction enzyme PvuII and the digested PCR products were analysed by electrophoresis on 2-4% agarose gels, stained with ethidium bromide.

Statistical analysis was carried out using the statistical software SPSS Windows version 13.0 (SPSS,

Chicago, IL). Genotypic and allelic frequencies were calculated and compared between populations using the chi-square test. A *p* value of less than 0.05 was considered to be statistically significant. The strengths of the associations were described as odds ratios (OR), accompanied by their 95% confidence intervals (CI).

Results

Patient characteristics

The demographic characteristics of our subjects are shown in *table 1*. There were no significant differences in age and sex distribution between drug-responsive and drug-resistant patients. Overall, the most common seizure type was partial epilepsy (59.9%). Seizure type was not significantly different between the two groups, whereas the underlying mechanism of epilepsy determined relative response to medication; patients with idiopathic or cryptogenic epilepsy were more prone to drug responsiveness (43.3% *vs*. 24.5%) while patients with symptomatic epilepsy were more prone to drug resistance (10.4% *vs*. 39.8%). The spectrum of AEDs used in these patients included carbamazepine, oxcarbazepine, valproic acid, phenobarbital, clonazepam, nitrazepam, levetiracetam, lamotrigine, and topiramate. First-line monotherapy was tried before polytherapy. In our study, at the last follow-up visit, 78.0% drug-responsive patients achieved control of seizures with monotherapy.

Linkage disequilibrium and tagged-haplotype analysis

By using Haploview and HapMap Chinese Beijing (CHB) to analyse the *CCL2* region (chr17: 29603482, 29608958), the haploblock was constructed and consisted of four SNPs, two SNPs (rs1024611[G>A] and rs1024610[A>T]) in the promoter region, one synonymous SNP in exon 2 (rs4586[C>T]), and one SNP in the 3′ UTR (rs13900[T>C]) (*figure 1*). The rs1024611 was identified as the tag SNP and was in perfect linkage disequilibrium with rs1024610 (r^2=1.0) and almost linkage disequilibrium with rs4586 and rs13900 (r^2>=0.95). Three major haplotypes, GACT(0.619), AATC (0.321), and ATTC(0.048) were identified with frequencies in excess of 0.01.

Genotype and allele frequencies of *CCL2* polymorphism and drug responsiveness

The genotype distribution of *CCL2* rs1024611 was compatible with the Hardy-Weinberg equilibrium in our epileptic patients. The genotype and allele frequency distributions in the drug-responsive and drug-resistant group are shown in *table 2*. The A allele frequency was slightly higher in the drug-resistant group compared to that in the drug-responsive group (50.5% *vs*. 48.0%; *p*=0.020). At the genotype level, the AA genotype was associated with drug-resistant epilepsy (*p*=0.006; OR=2.37, 95%CI: 1.27-4.42) and the association remained statistically significant after adjustment for age, sex, and seizure type (*p*=0.008; OR=2.51, 95%CI: 1.33-4.72). After Bonferroni correction for multiple testing, the association between AA genotype and drug-resistant epilepsy remained significant ($p<0.05$). After stratifying our patients by seizure type, no significant difference was found, probably due to the small sample size.

Discussion

In this study, we used linkage analysis to identify a tag SNP rs1024611 for the entire *CCL2* gene and examined

Table 1. Demographic and clinical characteristics of epileptic patients.

Subgroups/categories	Drug-responsive	Drug-resistant	*p* value
Sex (M/F)	262/124	66/32	0.920
Age (years)	7.79±3.45	6.51±4.56	0.356
Seizure type			
Partial	233 (60.4%)	57 (58.2%)	0.692
Generalised	153 (39.6%)	41 (41.8%)	
Aetiology			
Idiopathic or cryptogenic	167 (43.3%)	24 (24.5%)	<0.0001
Symptomatic	40 (10.4%)	39 (39.8%)	
Non-classifiable	179 (46.4%)	35 (35.7%)	
Antiepileptic drugs at the last visit			
monotherapy	301 (78.0%)	0	<0.0001
2 drugs	85 (22.0%)	73(74.5%)	
3 drugs	0	25 (25.5%)	

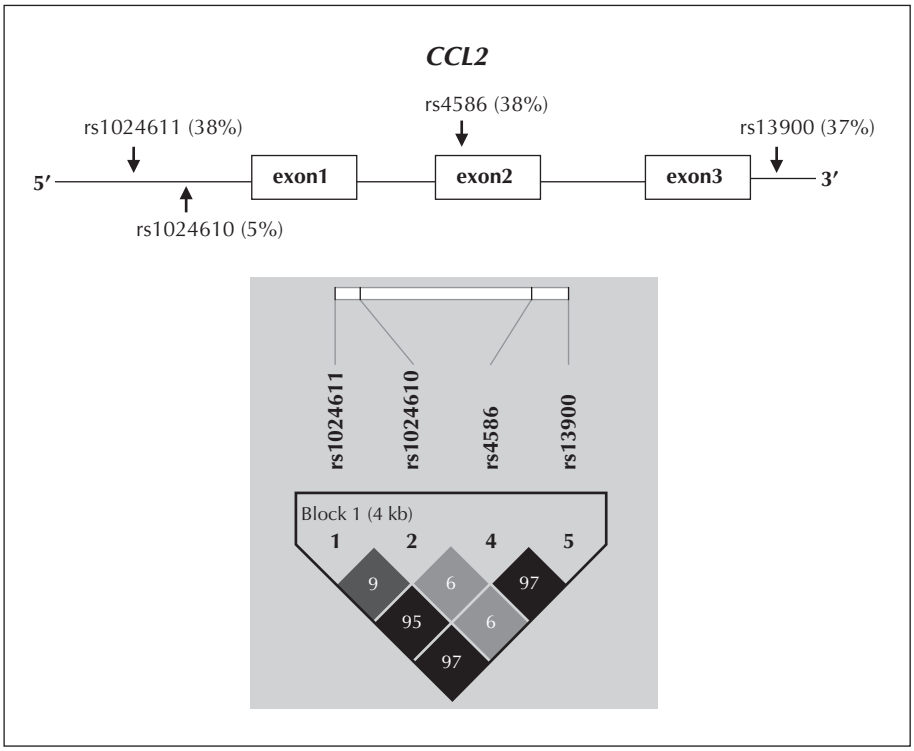

Figure 1. The location of *CCL2* gene polymorphisms and their pairwise linkage disequilibrium (r2) plots in the Chinese Beijing population based on data from the HapMap Phase 3 release.
The percentages in parentheses are the minor allele frequencies in the Chinese Beijing population.

Table 2. Genotype and allele frequencies of *CCL2* rs1024611 in drug-responsive and drug-resistant patients.

Genotype/allele	Drug-responsive *n* (%)	Drug-resistant *n* (%)	OR	95%CI	*p* value
GG	141 (36.5)	25 (25.5)	Reference		
GA	183 (47.4)	47 (48.0)	1.45	0.85-2.47	0.171
			1.65[a]	0.94-2.88[a]	0.080
AA	62 (16.1)	26 (26.5)	2.37	1.27-4.42	0.006
			2.51[a]	1.33-4.72[a]	0.008
GA+AA	245 (63.5)	73 (74.5)	1.68	1.02-2.77	0.040
			1.56[a]	1.05-3.12[a]	0.044
G	465 (52.0)	97 (49.5)	Reference		
A	327 (48.0)	99 (50.5)	1.45	1.06-1.99	0.020

OR: odds ratio; CI=confidence interval.
[a]Adjusted for age, sex, and seizure type.

the association between rs1024611 and drug-resistant epilepsy. Our results indicate that the genetic variant of this gene might confer susceptibility of drug-resistant epilepsy in our Chinese paediatric patients, with AA genotype being positively associated with drug-resistant epilepsy following Bonferroni multiple test correction. To the best of our knowledge, the present study is the first study to report an association between genetic variance in cytokine genes, rather than drug transporter or metabolic enzyme genes, and drug-resistant epilepsy.

The rs1024611 polymorphism within the *CCL2* distal promoter was associated with higher levels of transcription, resulting in a higher level of CCL2 production, due to a consensus binding site for the transcriptional regulator Prep1 (Pham *et al*., 2012; Rovin *et al*., 1999), and was associated with increased CCL2 expression *in vitro*. Furthermore, it was demonstrated that the rs1024611G allele is associated with increased CCL2 expression in serum, plasma, urine, and CSF in normal as well as pathological conditions (Fenoglio *et al*., 2004; Letendre *et al*., 2004; McDermott *et al*., 2005).

The genotype-phenotype relationship for epilepsy and drug resistant epilepsy is complex and studies have suggested that genetic factors may influence drug response in epilepsy, such as variants in genes encoding drug target, drug transport, and drug metabolizing enzymes (Siddiqui *et al*., 2003; Dong *et al*., 2011; Lakhan *et al*., 2011), however, these studies have not provided unifying conclusions. Our previous study, including 152 patients who received oxcarbazepine (OXC) monotherapy, did not show any significant difference between drug-responsive and drug-resistant groups with regards to ABCB1 allele or genotype frequencies for three common SNPs (C3435T, G2677T/A and C1236T), or plasma concentration of 10,11-dihydro-10-hydroxy-carbazepine, the clinically relevant metabolite of OXC, among three ABCB1 genotypes (unpublished data).

Increased BBB permeability has been linked to seizure genesis and drug resistance (Miller *et al*., 2008; Marchi *et al*., 2010; Ghosh *et al*., 2010). CCL2 has been reported to be associated with compromised BBB (Stamatovic *et al*., 2005; Stamatovic *et al*., 2006; Yao and Tsirka, 2011). Other than affecting BBB, increased CCL2 may enhance the inflammatory response in the brain during epileptic activity and result in reduced drug sensitivity. Besides rs1024611, we also genotyped rs4586(C>T) in our study and found that it was in almost complete linkage disequilibrium with rs1024611 (data not shown), which further confirmed that the rs1024611 is representative for this genomic region in the Chinese population.

In conclusion, we have demonstrated an association between the rs1024611 polymorphism in the *CCL2* gene and drug-resistant epilepsy in Chinese children with epilepsy. The *CCL2* gene, far from being involved in drug transport, metabolism, and elimination, was reported to influence BBB permeability, suggesting that chemokines, along with other proinflammatory mediators, may be important contributing factors to the clinical phenomenon of resistance to AEDs. Therefore, our study provides a rationale to target inflammation which could be a novel therapeutic strategy in epilepsy, and the addition of anti-inflammatory agents could be beneficial for patients with intractable epilepsy. However, this study has several limitations, including the examination of only one tag SNP in one gene, a lack of data corresponding to CCL2 expression in serum, plasma, or CSF, a relatively small sample size, and the sample size disparity between drug-responsive and drug-resistant patients, conducted only in Chinese children. Thus, further studies with larger samples and/or in different ethnic groups, conducted with a larger number genetic variants, is warranted.

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None of the authors has any conflict of interest to disclose.

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