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ABCC2 haplotype is not associated with drug-resistant epilepsy

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Abstract

Several studies have investigated the association between the *ABCB1* polymorphism and drug-resistant epilepsy. However, the effect of *ABCC2* polymorphisms on anti-epileptic drug (AED) responsiveness remains unknown. The *ABCC2* polymorphisms have been genotyped in 279 Japanese epileptic patients treated with AEDs. The association between the AED responsiveness and the polymorphisms was estimated by a haplotype-based analysis. No genotype or haplotype was associated with drug-resistant epilepsy. On the other hand, the delGCGC haplotype at G-1774delG, C-24T, G1249A and C3972T was over represented among the epileptic patients with a complication of mental retardation in comparison with those without (32.4% vs 22.0%; $P=0.009$); and the G-1774delG allele was also associated with mental retardation ($P=0.03$). No association between the *ABCC2* genotypes or haplotypes, and the responsiveness of AEDs was observed, although this finding was inconclusive because of the small sample size.

Introduction

Multidrug resistance has been acknowledged to be one of the most important clinical problems in the treatment of epilepsy, and resistance to anti-epileptic drugs (AEDs) affects approximately 30% of patients, despite sufficient plasma levels of AEDs (Kwan & Brodie 2000; Schmidt & Löscher 2005; Remy & Beck 2006). In drug-resistant epilepsy, the up-regulation of drug efflux transporters, such as P-glycoprotein (MDR1, ABCB1) and multidrug resistance-associated protein 2 (MRP2, ABCC2), in the blood–brain barrier (BBB) has been discussed as an important mechanism underlying resistance to AEDs (Tishler et al 1995; Dombrowski et al 2001; Schmidt & Löscher 2005; Kubota et al 2006). More importantly, several previous studies have shown the *ABCB1* polymorphisms to be associated with epilepsy per se, and that they were therefore useful for distinguishing patients with drug-resistant epilepsy from those with drug-sensitive epilepsy or normal controls (Siddiqui et al 2003; Zimprich et al 2004; Hung et al 2005; Ebid et al 2007).

On the other hand, no clinical study has yet investigated the association between drug-resistant epilepsy and polymorphisms of *ABCC2*, which is another putative transporter (Dombrowski et al 2001; Potschka et al 2003; Kubota et al 2006), except for a report of dysembryoplastic neuroepithelial tumours causing intractable epilepsy (Vogelgesang et al 2004). Recent studies have reported a significant association between *ABCC2* polymorphisms of G-1774delG, C-24T and C3972T or these haplotypes and cancer or toxic liver injury (Naesens et al 2006; Rau et al 2006; Choi et al 2007; de Jong et al 2007; Haenisch et al 2007). Therefore a linkage disequilibrium analysis was performed to investigate the association between the *ABCC2* genotypes of G-1774delG, C-24T, G1249A and C3972T or these haplotypes and clarify the responsiveness to the AED therapy among epileptic patients.

Materials and Methods

Subjects

This study included 279 Japanese epileptic patients (156 males, 55.9%), who had been prescribed anti-epileptic drugs (AEDs) for longer than two years at Kumamoto Saishunso National

Hospital, which is specialized for the management of patients with drug-resistant epilepsy and mental retardation. The mean age, body weight and duration of treatment were 20.9 ± 10.1 years, 47.9 ±19.4 kg and 9.1 ± 4.5 years, respectively. Eighty patients (28.7%) had generalized seizures, 190 patients (68.1%) had partial seizures, and nine patients (3.2%) had unclassified seizures. Patients with idiopathic, cryptogenic or symptomatic epilepsy were 50 (17.9%), 116 (41.6%) or 113 (40.5%), respectively. One hundred and eighty-five patients had complications of mental retardation.

According to the guidelines, the appropriate AEDs were chosen and the classification of either the seizure types or epileptic syndromes was diagnosed (Seo et al 2006). In brief, the patients were treated with a single drug whenever possible. This treatment was changed to another drug if the seizures remained uncontrolled, if drug-precipitated seizures were suspected, or if the patient had any intolerable adverse drug reactions. A combination of AEDs was used in patients whose epilepsy remained uncontrolled despite treatments with a single AED of more than two drugs. Compliance was monitored by both therapeutic drug monitoring and interviews, since poor compliance is a common cause of treatment failure in patients with epilepsy. Any patients who persistently did not comply with the treatment regimen were excluded from the study.

All of the patients and/or their parents gave written informed consent to participate in the study. The protocol was approved by the ethics committees of Kumamoto Saishunso National Hospital and the Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University.

Definitions

Based on the definition of previous reports (Kwan & Brodie 2000; Seo et al 2006), the patients were considered to be free of seizure (drug-responsive) if they had not experienced any type of seizures for at least one year, otherwise they were considered to be drug-resistant. Every case considered to have drug-resistant epilepsy demonstrated a treatment failure with at least two drugs because of adverse reactions and/or a lack of efficacy at maximum tolerated dose, thus confirming the blood concentrations to have reached appropriate levels. However, the disease remission in drug-responsive patients and disease progression in drug-resistant patients could not be clearly differentiated, and therefore these factors might be potential confounders. The seizure control was assessed at the time of the last clinical examination.

Genotyping

Genomic DNA was prepared from whole blood using the DNA Extractor WB kit (Wako Pure Chemical Industries Ltd, Osaka, Japan) and/or from buccal cells using a protocol modified from Richards et al (1993).

ABCC2 polymorphisms at C-24T (rs717620), G1249A (rs2273697) and C3972T (rs3740066) were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism as reported by Naesens et al (2006) and Rau et al (2006). *ABCC2* polymorphism at G-1774delG was analysed by PCR and the pyrosequencing method. PCR primers (forward, 5′-CCTTGCCAGCACTTATCTTGT-3′; reverse, 5′-biotin-AATGGCCAACAGGTATATGACA-3′) and sequencing primer (forward, 5′-TTGCCAGCACTTATCTT-3′) were designed using the Pyrosequencing Assay Design Software program (Biotage AB, Uppsala, Sweden). For PCR amplification, after initial denaturation (95°C, 5 min), a thermal cycler protocol (45 cycles) was employed cycling 30 s at 94°C, 30 s at the annealing temperature of 56°C, followed by 30 s extension at 72°C. The pyrosequencing was performed with a PSQ 96MA system (Biotage AB, Uppsala, Sweden) (Rohrbacher et al 2006). The deoxynucleotide triphosphate (dNTP) dispensation order was CGTTGTTGTGATA.

Statistical analysis

Student's *t*-test or analysis of variance and Fisher's exact test were used for comparisons of the continuous data and categorical variables, respectively. The odds ratios (OR) and 95% confidence interval (CI) were obtained with a logistic regression analysis. P values of <0.05 were considered to be statistically significant (SPSS software version 15.0; SPSS Inc, Chicago, IL).

Based on the genotype data of the *ABCC2* polymorphisms, a linkage disequilibrium analysis, haplotype inference and a haplotype-based case–control study were performed using the expectation-maximization algorithm (Nakajima et al 2002), with the SNPAlyze software program, version 6.0 (Dynacom Co. Ltd, Chiba, Japan). The P values for haplotype analyses were presented after 10000 permutation tests.

Results

One hundred and thirty-three (47.7%) patients were classified as having drug-resistant epilepsy and 146 patients (52.3%) as having drug-responsive epilepsy. The -1774delG, -24T, 1249A and 3972T allele frequencies in patients were 30.0%, 20.4%, 14.6% and 21.8%, respectively. The demographic characteristics and the genotype frequencies are shown in Tables 1 and 2, respectively. The age at the onset of epilepsy, mental retardation and aetiology of epilepsy were significantly associated with the responsiveness to AEDs, and drug-resistant patients were prescribed more AEDs (Table 1) while the distribution of all the genotypes were similar between drug-resistant and drug-responsive groups (Table 2). On the other hand, a significant linkage disequilibrium was detected among G-1774delG, C-24T, G1249A and C3972T (each $|D'| > 0.75$; $P < 0.0001$). Out of the sixteen possible haplotypes only four were encountered with frequencies of above 5% (GCGC: 32.7%, delGCGC: 28.8%, GTGT: 18.2%, GCAC: 14.4%). The frequency of the delGCGC haplotype was higher for drug-resistant patients than for drug-responsive patients (32.7% vs 25.5%; $P = 0.049$; Table 2). However, the delGCGC haplotype was more closely associated with mental retardation, which is a confounding factor for drug-resistant epilepsy. The incidence of drug-resistant epilepsy was higher in patients with mental retardation than in those without (61.1% vs 21.3%; $P < 0.001$) and a stratified analysis on mental retardation showed no significant association between the delGCGC haplotype and drug-responsiveness ($P = 0.23$ for mental retardation and $P = 0.83$ for non-mental retardation).

Table 1 Demographic characteristics of the drug-responsive and -resistant epilepsy

	Responsive (n = 146)	Resistant (n = 133)	*P* value
Male	79 (54.1%)	77 (57.9%)	0.55
Age (years)	20.7 ± 11.0	21.1 ± 9.1	0.69
Body weight (kg)	49.1 ± 18.5	46.4 ± 20.4	0.25
Onset of epilepsy (years)	5.8 ± 5.0	3.7 ± 4.6	< 0.001
Duration of therapy (years)	8.7 ± 4.4	9.6 ± 4.5	0.09
Mental retardation	72 (49.3%)	113 (85.0%)	< 0.001
Seizure type[a]			
Partial	95 (65.1%)	95 (71.4%)	0.38
Generalized	47 (32.2%)	33 (24.8%)	
Aetiology			
Idiopathic	42 (28.8%)	8 (6.0%)	< 0.001
Cryptogenic	66 (45.2%)	50 (37.6%)	
Symptomatic	38 (26.0%)	75 (56.4%)	
Prescribed AEDs[b]			
Carbamazepine	64 (43.8%)	76 (57.1%)	0.031
Phenytoin	6 (4.1%)	19 (14.3%)	0.003
Phenobarbital	14 (9.6%)	24 (18.0%)	0.054
Valproic acid	38 (26.0%)	47 (35.3%)	0.12
Zonisamide	12 (8.2%)	22 (16.5%)	0.043
Clobazam	17 (11.6%)	44 (33.1%)	< 0.001

Presented are mean ± s.d. or number of patients (percentages of patients in the each group). [a]The nine patients had unclassified seizures. [b]Prescribed AEDs at the last visit: monotherapy (n = 139); polytherapy of two drugs (n = 86); polytherapy of more than three drugs (n = 54). AED, anti-epileptic drug.

Table 2 Frequency of *ABCC2* genotypes and haplotypes in drug-responsive and -resistant epilepsy

Variant	Responsive (n = 146)	Resistant (n = 133)	*P* value[a]
G-1774delG			
G/G	77 (58.8%)	54 (41.2%)	0.08
G/delG	58 (45.0%)	71 (55.0%)	
delG/delG	11 (57.9%)	8 (42.1%)	
C-24T			
C/C	93 (53.1%)	82 (46.9%)	0.81
C/T	47 (50.0%)	47 (50.0%)	
T/T	6 (60.0%)	4 (40.0%)	
G1249A			
G/G	104 (50.5%)	102 (49.5%)	0.25
G/A	35 (54.7%)	29 (45.3%)	
A/A	7 (77.8%)	2 (22.2%)	
C3972T			
C/C	89 (52.0%)	82 (48.0%)	0.66
C/T	48 (51.1%)	46 (48.9%)	
T/T	9 (64.3%)	5 (35.7%)	
Haplotypes[b]			
GCGC	33.2%	32.3%	0.83
delGCGC	25.5%	32.7%	0.049
GTGT	17.7%	18.8%	0.73
GCAC	16.2%	12.0%	0.19
Other[c]	7.4%	4.2%	–

[a]*P* values were determined by Fisher's exact test for genotype frequencies and 10 000 permutation test for haplotype frequencies. [b]Haplotype configuration was defined as G-1774delG, C-24T, G1249A and C3972T. [c]Haplotypes with frequency below 5%.

The delGCGC haplotype was overrepresented among the epileptic patients with mental retardation in comparison with those without (32.4% vs. 22.0%; $P = 0.009$; Table 3). In addition, the incidence of mental retardation with at least one delGCGC haplotype was significantly higher than those with the other haplotype combinations (OR: 2.0, 95% CI: 1.2–3.3, $P = 0.006$), while the OR was not significantly increased with an increase in the number of delGCGC alleles. The G-1774delG allele was also significantly associated with mental retardation, and the incidence of mental retardation among the delG allele carriers and non-carriers was 72.3% and 59.5%, respectively ($P = 0.03$). The G-1774delG genotype distribution in the patients with mental retardation showed an insignificant deviation from the Hardy–Weinberg equilibrium ($P = 0.07$) and the observed delG/delG genotype frequency (n = 14) was lower than the predicted frequency (n = 20), although the distribution in those without mental retardation was in Hardy–Weinberg equilibrium ($P = 1.0$).

No significant association was detected between either the *ABCC2* genotypes or haplotypes and the serum level of total bilirubin.

Discussion

In this study, the *ABCC2* genotypes or haplotypes did not have any impact on the responsiveness to AEDs, while significant associations between mental retardation and the G-1774delG polymorphism or delGCGC haplotype at G-1774delG, C-24T, G1249A and C3972T were observed.

Drug efflux transporters, such as ABCC2, located at the luminal membrane of endothelial cells of the BBB have been suggested to limit brain penetration of drugs by exporting their substrates back into the capillary lumen (Schmidt & Löscher 2005; Remy & Beck 2006). ABCC2 mRNA and protein were overexpressed in capillary endothelial cells and astrocytes in epileptogenic brain tissue from patients with intractable epilepsy (Dombrowski et al 2001; Kubota et al 2006). However, it was not clear whether the ABCC2 expression was constitutive or induced by intractable seizures, chronic treatment with AEDs, or other yet unknown factors (Schmidt & Loscher 2005). This study intended to verify the association between *ABCC2* polymorphisms that have been reported to be functional, and their responsiveness to AEDs, based on previous studies on *ABCB1* polymorphisms (Siddiqui et al 2003; Zimprich et al 2004; Hung et al 2005; Seo et al 2006). No association was observed, although this finding was inconclusive because of the small sample size. Phenytoin, carbamazepine and valproic acid were shown to be substrates for ABCC2 in studies using ABCCs inhibitors, ABCC2 transfected cells and/or ABCC2-deficient transport-deficient (TR(−)) rats (Potschka et al 2003; Schmidt & Loscher 2005), although recent reports have raised some objections to these results (Baltes et al 2007a, b). Baltes et al

Table 3 Frequency of *ABCC2* genotypes and haplotypes in mental retardation and non-mental retardation groups

Variant	Non-mental retardation (n = 94)	Mental retardation (n = 185)	*P* value[a]
G-1774delG[b]			
G/G	53 (40.5%)	78 (59.5%)	0.08
G/delG	36 (27.9%)	93 (72.1%)	
delG/delG	5 (26.3%)	14 (73.7%)	
C-24T			
C/C	58 (33.1%)	117 (66.9%)	0.55
C/T	31 (33.0%)	63 (67.0%)	
T/T	5 (50.0%)	5 (50.0%)	
G1249A			
G/G	66 (32.0%)	140 (68.0%)	0.54
G/A	25 (39.1%)	39 (60.9%)	
A/A	3 (33.3%)	6 (66.7%)	
C3972T			
C/C	58 (33.9%)	113 (66.1%)	0.13
C/T	28 (29.8%)	66 (70.2%)	
T/T	8 (57.1%)	6 (42.9%)	
Haplotypes[c]			
GCGC	35.5%	31.4%	0.34
delGCGC	22.0%	32.4%	0.009
GTGT	17.9%	18.3%	1.00
GCAC	15.2%	13.8%	0.61
Other[d]	9.4%	4.1%	—

[a]*P* values were determined by Fisher's exact test for genotype frequencies and 10 000 permutation test for haplotype frequencies. [b]delG carriers vs non-carriers, $P = 0.03$. [c]Haplotype configuration was defined as G-1774delG, C-24T, G1249A and C3972T. [d]Haplotypes with frequency below 5%.

(2007b) indicated that substrate recognition or transport efficacy by efflux transporters differed between man and mouse for certain AEDs. Such species-specific differences may explain, at least in part, the controversial data from different species. In addition, functional compensation by P-glycoprotein was observed in the brain microvessels of ABCC2-deficient TR(−) rats (Hoffmann & Löscher 2007). Therefore, the transport efficacy of ABCC2 may differ depending on species, tissues, and co-regulation of transporters. It is therefore still not clear whether the AEDs were substrates for ABCC2 and therefore whether ABCC2 influenced AED resistance.

A significant linkage was found among the C-24T, G1249A and C3972T polymorphisms of *ABCC2*, and the genotypes and haplotypes were reported to influence the expression of ABCC2 mRNA or protein, dispositions of the substrates, and adverse reactions (Naesens et al 2006; Rau et al 2006; de Jong et al 2007; Haenisch et al 2007). Recently, Choi et al (2007) reported that another G-1774delG polymorphism exhibited a decrease in the basal promoter activity and a defect in the bile acid-induced ABCC2 promoter activity, and consequently the haplotype containing the G-1774delG showed a strong association with cholestatic or mixed-type toxic hepatitis in man. Therefore these four polymorphisms and their haplotypes were investigated. In this study, all polymorphisms were found to be in significant linkage disequilibrium, and haplotype frequencies were similar to the Korean population (Choi et al 2007). The frequency of the delGCGC haplotype was higher for the drug-resistant patients than for the drug-responsive patients. However, after stratification by mental retardation, a confounding factor for drug-resistance, the delGCGC haplotype did not associate with drug-responsiveness anymore, although these results might have been due to the loss of statistical power inherent in the sub-classification scheme. Therefore, the significant associations between the *ABCC2* genotypes or haplotypes and drug-responsiveness were not observed. The serum level of total bilirubin was assessed as a surrogate marker for the activity of ABCC2, because conjugated-bilirubin is an established substrate of ABCC2 (Nies & Keppler 2007). However, the levels were not different among *ABCC2* genotypes or haplotypes. Carbamazepine and phenobarbital have been reported to induce ABCC2 (Giessmann et al 2004; Nies & Keppler 2007), therefore the AEDs could affect these results. However, the number of subjects investigated was too small to clarify the functional significance of the genotype or haplotype by the stratification of these drugs.

It was unexpected that the delGCGC haplotype and the G-1774delG allele were significantly associated with mental retardation. In addition, the observed frequency of homozygous deletion variant G-1774delG was less than the predicted frequency in mental retardation, though the distribution in non-mental retardations was in Hardy–Weinberg equilibrium. Although, no convincing explanation could be found for these results, and false positive results could occur because the sample size was small and because many variables were investigated, the delGCGC haplotype, especially the G-1774delG allele, may have been linked to other aetiological genes of mental retardation. This is because a hereditary deficiency of ABCC2, known as Dubin–Johnson syndrome, is not characterized by mental retardation and because the *ABCC2* gene is located at 10q24 and is close to the 10q subtelomere region, the locus of common polymorphisms associated with mental retardation (Irving et al 2003). Nevertheless, further studies are required to verify whether this haplotype is a genetic marker for mental retardation.

This study has several shortcomings. Firstly, due to the heterogeneous clinical nature of epilepsy and the potential influence of seizure pathophysiology on responsiveness to AEDs, it was crucial to define the types of epilepsy in pharmacogenomic studies (Ferraro et al 2006). However, this study was not stratified by epilepsy syndrome or seizure types, which are known to affect the AED responsiveness, because the number of subjects was too small to assess in each group. Secondly, many patients had mental retardation, which is known to affect the AED responsiveness (Callaghan et al 2007), therefore the population in this study was different from those in other studies. In addition, the aetiology and severity of the disease were not identified. Thirdly, the lack of a neurologically normal control group was a major limitation of this study and it hindered the accurate interpretation of the data regarding mental retardation. Finally, this study was investigated retrospectively, although the prospective trial is required for evidence-based clinical recommendations (Ferraro et al 2006; Szoeke et al 2006).

Conclusion

This study showed that *ABCC2* polymorphisms may not have influenced the AED responsiveness. However, this finding was inconclusive due to the small sample size and because the statistical power was lost by an additional sub-classification scheme. Further study would be necessary to evaluate the impact of the *ABCC2* polymorphisms on ABCC2 expression and activity in the BBB and on the incidence of AED-resistant epilepsy.

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