

**Genetic variations of ABC transporters  
associated with adverse drug reactions to  
Valproic acid**

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Valproic acid**

Directed by Professor Min Goo Lee

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Ji Hyun Lee

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## 감사의 글

“인내는 쓰고 열매는 달다라는 말”을 삶의 좌우명으로 생각하며 석사과정을 보냈습니다. 실험은 마치 인생과 같았습니다. 기쁨과 슬픔, 절망과 희망, 기대와 낙심 등이 서로 교차하며 실험자인 저에게 매번 다양함을 안겨 주었습니다. 때론 포기하고 싶을 때도 많았고, 때론 가슴 벅차 오를 기대와 소망에 젖어 들 때도 있었습니다. 이러한 모든 과정 중에서 함께 해주신 모든 분들께 감사의 마음을 전합니다.

먼저 입학하기 전부터 지금까지 실험에 필요한 조언과 지식과 교육뿐만 아니라, 많은 것들을 도와주신 이민구 선생님께 감사를 드립니다. 오직 학문에 대한 열의만으로 연구의 길에 들어선 교수님은, 인류복지와 사회기여를 위하여 연구에 임하셨고, 그와 같은 정신이 저에게 큰 도움이 되었습니다. 항상 크고, 멀리 볼 수 있도록 이끌어 주셨던 김경환 선생님, 날카롭고 지혜로운 안목으로 도와주신 안영수 교수님, 약리학교실을 멋지게 세우시는 김동구 주임교수님, 따뜻하고 정이 많으신 김혜영 선생님, 항상 열심히 하시는 박경수 선생님, 지적이고 재미있으신 김철훈 선생님께 감사의 마음을 전합니다. 그리고 심사위원으로 제 연구에 꼭 필요한 부분들을 많이 도와주시고, 조언을 주신 김원주, 박수철 교수님께 감사 드립니다.

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Abstract

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Multidrug resistance protein, also referred as P-glycoprotein (P-gp, MDR1; ABCB1) and other ABC efflux transporters such as members of the multidrug resistance-associated protein (MRP) family and breast cancer resistance protein (BCRP) may alter responses to valproic acid. These transporters affect the distribution of drug from the blood to the brain. In particular, MRP2, a transporter of valproic acid, is noticeably induced by valproic acid and by the occurrence of seizure in brain endothelium cells, astrocytes and (rarely) neuronal cells. In this study, we investigated associations between polymorphisms of these genes and individual adverse drug reactions (ADR) to valproic acid. One hundred sixty-eight patients were divided into two groups: those who experienced an adverse drug reaction to anti-epileptic agents (ADR; n=41), and those who did not (non-ADR; n=127), according to dose-related ADR of the CNS (central nervous system). Among the polymorphisms, the MRP2 g.-1774delG variant showed a strong association with CNS ADR caused by valproic acid ( $P = 0.004$ ). There were functional differences in MRP2 promoter activity between g.-1774G and g.-1774del variants in

brain neuronal cells (SH-SY5Y) ( $P < 0.05$ ), suggesting a role for this region in transcriptional change.

These data suggest that polymorphisms in the human MRP2 gene are associated with adverse drug reactions of CNS system to valproic acid.

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Key words: genetic polymorphism, MRP2, adverse drug reaction, valproic acid, epilepsy.

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## **I. INTRODUCTION**

Various forms of epilepsy are among the most common serious brain disorders and present through convulsion, autonomic movement, impaired consciousness, and more<sup>1</sup>. These neurological disorders affect an estimated forty two million people of all ages worldwide<sup>2</sup>. The blood–brain barrier (BBB) has challenged the treatment of epilepsy, since many antiepileptic drugs (AED) are not effectively distributed to the vascular endothelium or, accordingly, to their targets in brain<sup>3,4</sup>. The BBB, thought to be the first line barrier to the disposition of drug from the blood to brain<sup>5</sup>, consists of the interplay of three major microvascular components. Tight junctions between the endothelial cells constitute the major permeability barrier. The overall

biology of the barrier is shaped by the interactions between the endothelium and the pericytes/smooth muscle cells and the astrocyte foot processes that cover most of the abluminal surface of the microvasculature<sup>6</sup>.

Drug pharmacokinetics are controlled by drug transporters which regulate drug absorption, distribution and excretion. Drug transporters are considered a second line barrier for limiting drug disposition from the blood to the brain<sup>7</sup>. In brain capillary endothelial cells, MDR1 (multidrug resistance protein 1), MRP1 (multidrug resistance-associated protein 1), 2, 4 and 5 and BCRP (breast cancer resistance protein) are among the many drug transporters that might be expressed at the luminal side of the membrane<sup>5, 8, 9</sup>. MRP2, in particular, was over-expressed (about 225% greater) at the drug refractory temporal lobe epileptic patients with hippocampal sclerosis (HS)<sup>5, 10-12</sup>. Drug reactions can be different between individuals with the same AED blood concentrations and therapeutic levels because drug distribution is influenced by variable levels of drug transporters. MRP2 is thought to play a more important role in the distribution of AEDs than other transporters in the epileptic brain<sup>13</sup>. MRP2 has been demonstrated to transport many AEDs including valproate (VPA), carbamazepine (CBZ), phenytoin (PHT) and more<sup>9, 14-17</sup>. Valproic acid has become established as one of the most widely used AEDs in the treatment of both generalized and partial seizures in adults and children, and is considered to be a substrate of MRP2<sup>9, 14-17</sup>. Probenecid, an inhibitor of MRPs, has been shown to increase the concentration of valproic acid in the brain<sup>18</sup>. In animal models, TR<sup>-</sup> rats (MRP2 knockout) had lower VPA and VPA-glucuronide in their bile than control Wistar rats (which intrinsically expressed MRP2)<sup>19</sup>.

Functional polymorphisms in genes encoding drug transporters can alter AED uptake, cerebral distribution and efflux, resulting in individual differences in AED concentration and effectiveness and/or the occurrence of adverse drug reactions (ADRs). Many functional single

nucleotide polymorphisms (SNPs) have been reported. For example, mRNA expression of the c.1446CG MRP2 genotype was recently revealed to be higher than the c.1446CC genotype in the liver<sup>20</sup>. In addition, the c.2302C>T (exon 18,Arg768Trp) mutation is responsible for Dubin-Johnson syndrome (DJS)<sup>21, 22</sup>. The c.2302C>T and c.4348G>A genotypes correlated with significantly lower MRP2 protein expression levels compared to wild-type and V417I.<sup>21</sup> The c.1249G>A mutation significantly reduced the amount of MRP2 mRNA in human preterm placentas<sup>23</sup>. The g.-1774delG polymorphism has been linked with toxic hepatitis by our group<sup>24</sup>. In the present study, we investigated the association between the g.-1774delG MRP2 genotype and ADR of the central nervous system (CNS) in VPA treatment groups.

## II. Materials and Methods

### 1. Subjects.

This retrospective study included 168 epileptic Korean patients who received VPA at Sinchon and Yong-dong Severance Hospitals. Forty-one patients demonstrated VPA dose-related ADR in the central nervous system (dizziness, headache, somnolence, diplopia, dysarthria, tremor, etc), while the remainder (n=127) did not. Patients diagnosed with chronic active epilepsy, West syndrome, Lennox-Gastaut syndrome, progressive myoclonic epilepsy (PME), tuberous sclerosis, Sturge-Weber syndrome, hamatoma or brain tumor were excluded. There were no statistical differences in age, sex, response/non-response and sclerosis between the two groups.

Demographic characteristics of the epileptic patients are presented in Table 1. DNA from control subjects (n=110) was randomly selected from the DNA bank of the Korea Pharmacogenomics Research Network at Seoul National University. Blood samples were collected from each subject and DNA was extracted using a QIAamp DNA blood mini kit (Qiagen GmbH, Hilden, Germany).

### 2. Genetic analysis.

Polymorphisms of the MDR1, MRPs and BCRP genes in the Korean population were discovered by DGGE (denaturing gradient gel electrophoresis), TDGS (Two-Dimensional Gene Scanning) and direct polymerase chain reaction (PCR) using methods similar to those described in a previous paper<sup>24</sup>. Genotype screening of each locus in control and epileptic patients was performed by the SNaPshot or SNaPIT method (Applied Biosystems, Lincoln Centre Drive Foster City, CA), according to the protocols supplied by the manufacturer.

### 3. Statistical analysis.

Haploview software (version 3.2) was used to design MRP2 haplotype constructs and analyze major or minor haplotypes based on a standard expectation-maximization algorithm. Allele and genotype frequencies of transporter polymorphisms were assessed using Chi-square tests (SPSS software version 11.5 for Windows).

### 4. Logistic regression.

The strength of the association between dose-related CNS ADR patients and the presence of the G allele at the g.-1774 region was evaluated as an odds ratio (OR) obtained with logistic regression analysis (SPSS version 11.5). ORs were adjusted for gender, age, hippocampal sclerosis, use of AEDs (Larmotrigene, Carbamazepine, Phenytoine and Topiramate) and the presence of the G allele at the g.-1774 promoter region.

### 5. Cell culture.

BB19 cells, human brain capillary endothelial cells immortalized with the E6E7 genes of human papillomavirus, were kindly provided by Dr. Rebecca Ruhl at the Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR. Cells were cultured in human Endo-SFM medium (#11111-044, Gibco, Carlsbad, CA) containing 10% Human AB-serum (# H-4522, Sigma Aldrich, St. Louis, MO), 100 units penicillin, 100 µg streptomycin, 292 µg L-glutamine (#10378-016 Gibco), and 37 µg/mL endothelial cell growth supplement (# CB-40006B, Fisher, Litho, USA) at 37°C with 5% humidified CO<sub>2</sub>. Cells were cultured in tissue culture treated T-75 flasks (Fisher).

SH-SY5Y (ATCC, Manassas, VA, USA), a human brain neuronal cell line originated from a neuroblastoma, was a gift from Dr. In Suk Kim, Yonsei University College of Medicine, Seoul,

Korea. SH-SY5Y cells were maintained with DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% PS (100units penicillin, 100 µg streptomycin, Gibco) and were cultured under preconfluent monolayer conditions in 100-mm diameter poly-d-lysine coated culture dishes at 37 °C with 5% humidified CO<sub>2</sub>.

#### 6. Plasmid preparation of MRP2 promoter.

A pGL3 basic vector containing the human MRP2 promoter region (about 2.3 kb base pairs - bp -2314 to +348 relative to the translation initiation site-) was constructed by our group in a previous study from homozygotic persons with MRP2 haplotypes 1, 2 and 3.<sup>24</sup> The g.-1774delG polymorphism is located in haplotype 1 and the g.-24C>T polymorphism in haplotype 3. The reporter vector of a minor haplotype variant containing only the g.-1549G>A variation was constructed using mutagenic primers introducing a -24 T→C change to the plasmid of haplotype 3 containing both g.-1549G>A and g.-24C>T variations. Haplotype 2 includes no polymorphism (wild type). In short, a total of five clones were prepared: pGL3 basic, haplotypes 1,2, and 3 and g.-1549A alone.

#### 7. Dual luciferase assay

The pGL3 basic vector containing WT MRP2 promoter variants was transfected to SH-SY5Y and BB19 cells to measure the activity of the MRP2 promoter with and without VPA (Sigma Aldrich P4543, St. Louis, MO). The day before transfection,  $0.2 \times 10^6$  SH-SY5Y cells were seeded to a 12-well plate in order to reach 90-95% confluence at the time of transfection. 1.6 µg of pGL3-MRP2, including the variants and mock, were added to wells with Lipofectamin 2000 (Invitrogen). Vector-containing Renilla luciferase was co-transfected in order to confirm the transfection efficiency of the pGL3-MRP2 vector. After a 3 to 5 hr incubation, cells were

washed with phosphate buffered saline (PBS, Invitrogen) and incubated at 37°C with 5% humidified CO<sub>2</sub> for 24 hours. Reporter activity was measured using the Dual Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA). After removing the growth medium, cells were washed with PBS and the plates were placed on a rocking platform shaker with 250 µL of 1X passive lysis buffer per well for 15 minutes. Lysates were transferred to an e-tube and luciferase binding was performed with 50 µL LARII and 10-50 µL cell lysate in a 96-well assay plate (Costar, Corning incorporation, USA). After 10 seconds, light emission was measured using a luminometer (Berthold Technologies, Wildbad, Germany) and Stop & Glo was added to each well to stop firefly luciferase activity and evoke Renilla luciferase activity.

### III. Results

#### 1. SNP association with drug response or resistance.

Drug resistance was defined as the occurrence of at least four seizures over the year before recruitment, with trials of more than three appropriate antiepileptic drugs, at maximal tolerated doses, based on the occurrence of clinical side effects at supra-maximal doses<sup>25</sup>. The patients were classified as either responsive or resistant to drug therapy. We did not find any significant associations between the selected drug transporter SNPs and either group.

#### 2. Clinical characteristics of epilepsy patients

A total of 41 (24%) epileptic patients had ADR of the CNS and 127 (76%) epileptic patients did not (non-ADR). The demographic characteristics are listed in Table 1. The gender frequency, drug response and the average age of drug use between the two groups were similar. ( $P>0.05$ ) Subjects without ADR used fewer drugs than those with ADR ( $P=0.039$ ). The most frequent symptom out of 14 types of ADR to VPA use was tremors (78.0%). Based on logistic regression, ADRs were more likely to occur in patients with the g.-1774delG (presence of G allele), which had the highest OR (9.10) among the considered factors. ( $P<0.05$ )

Table 1. Demographic characteristics of epilepsy patients

Total 168 patients	ADR <sup>1</sup> of CNS <sup>2</sup> (n=41) <sup>3</sup>	non-ADR of CNS (n=126) <sup>4</sup>	p-value
Gender			
Male	18 (43.9)	65 (51.2)	0.423
Female	23 (56.1)	62 (48.8)	
Age (years)	37.8 ± 12.3	36.7 ± 14.5	0.655
Drug Response			
resistant epilepsy	14 (34.1)	57 (44.9)	0.276
responsive epilepsy	27 (65.9)	70 (55.1)	
The number of used drugs	3.76 ± 0.51	3.22 ± 1.42	0.039 <sup>+</sup>
HS <sup>5</sup>	16 (51.6)	56 (59.6)	0.53

<sup>1</sup>ADR: Adverse drug reaction, <sup>2</sup>CNS: Central nervous system, <sup>3</sup> These patients experienced ADRs of the CNS caused by valproic acid, <sup>4</sup>These patients did not experience any ADR, <sup>5</sup>Hippocampal sclerosis, <sup>+</sup>*P*<0.05, chi-square test.

### 3. Genetic polymorphisms of MRP2 in the Korean population

MRP polymorphisms covering all MRP2 exons, exon-intron junctions and the promoter region up to -2.3 Kb from the translation initiation site have been described in the Korean population<sup>24</sup>. We investigated 11 of the polymorphisms from previous studies, in 110 healthy control people and 168 epileptic patients. We found four promoter, four exonic and three intronic polymorphisms among them.

### 4. Relationship between genotypes of MRP2 and CNS ADR to VPA.

Alleles frequencies of the MRP2 polymorphisms are shown in Table 2. Non-ADR patients were more likely to have the deletion allele instead of the G allele at g.-1774 when compared with patients with CNS ADR (*P*=0.0057; ADR=20.7%, non-ADR=37.3%). The frequency of

the T allele at g.-24 was higher in patients with CNS ADR than those without ( $P=0.0274$ ; ADR=34.1%, non-ADR=22.0%). The difference between the groups based on g.-24C>T frequencies was not significant after Bonferroni's correction, while the difference based on g.-1774delG frequencies remained significant.

Table 2. Alleles frequencies of MRP2 polymorphisms

Alleles	Control (n=110)	ADR of CNS (n=41)	non-ADR of CNS (n=127)	p-value
g.-1774delG				
G	146 (66.4)	65 (79.3)	158 (62.7)	0.0057*
del	74 (33.6)	17 (20.7)	94 (37.7) <sup>+</sup>	
g.-1549G>A				
G	174 (79.1)	59 (72.0)	199 (80.2)	0.1151
A	46 (20.6)	23 (28.0)	49 (19.8)	
g.-24C>T				
C	182 (72.7)	54 (65.9)	198 (78.0)	0.0278
T	38 (17.3)	28 (34.1)	56 (22.0)	
g.-23G>A				
G	215 (97.7)	82 (100)	253 (99.6)	0.5693
A	5 (2.3)	0	1 (0.4)	
c.1249G>A				
G	199 (90.5)	73 (89.0)	235 (92.5)	0.3194
A	21 (9.5)	9 (11.0)	19 (7.3)	
c.1457C>T				
C	217 (98.6)	82 (100)	254 (100)	.
T	3 (1.4)	0	0	
c.2620+3A>G				
A	220 (100)	82 (100)	254 (100)	.
G	0	0	0	
c.2934G>A				
G	209 (95.0)	79 (96.3)	244 (96.1)	0.9095
A	11 (5.0)	3 (3.7)	10 (3.9)	
c.3972C>T				
C	169 (76.8)	53 (64.6)	192 (75.6)	0.0522
T	51 (23.2)	29 (35.4)	62 (24.4)	
c.4147-35G>A				
G	217 (98.6)	80 (97.6)	253 (99.6)	0.0869
A	3 (1.4)	2 (2.4)	1 (0.4)	
c.4508+12G>A				
G	218 (99.1)	82 (100)	254 (100)	.
A	2 (0.9)	0	0	

Data are number of patients (%),<sup>+</sup>Chi square

The genotype frequencies of each polymorphism are shown in Table 3. Genotype frequency distributions were consistent with Hardy-Weinberg equilibrium (each  $P>0.5$ ). The ADR group

patients were more likely to have a GG genotype than a deldel genotype at g.-1774delG compared to the non-ADR group ( $P=0.023$ , homo GG and deldel  $P=0.0146$ ). The TT and CT genotype frequencies at g.-24C>T were higher in patients with CNS ADR than non-ADR patients. ( $P=0.035$ , hetero CT TT  $P=0.019$ ).

Table 3. Genotype frequencies of MRP2 polymorphisms

	Control (n=110)	ADR of CNS (n=41)	non-ADR of CNS (n=127)	p-value
g.-1774DelG <sup>1</sup>				
GG	50	25	50	
Gdel	46	15	58	
deldel	14	1	18	0.023 <sup>+</sup>
g.-1549G>A				
GG	66	21	82	
GA	42	17	35	
AA	2	3	7	0.228
g.-24C>T <sup>2</sup>				
CC	74	17	79	
CT	34	20	40	
TT	2	4	8	0.066
g.-23G>A				
GG	105	41	126	
GA	5	0	1	
AA	0	0	0	>0.999
c.1249G>A				
GG	92	32	109	
GA	15	9	17	
AA	3	0	1	0.365
c.1457C>T				
CC	107	41	127	
CT	3	0	0	.
TT	0	0	0	
c.2620+3A>G				
AA	110	41	127	.
AG	0	0	0	
GG	0	0	0	
c.2934G>A				
GG	100	38	117	
GA	9	3	10	
AA	1	0	0	>0.999
c.3972C>T				
CC	61	17	74	
CT	47	19	44	
TT	2	5	9	0.156
c.4147-35G>A				
GG	107	39	126	
GA	3	2	1	
AA	0	0	0	0.148
c.4508+12G>A				
GG	108	41	127	
GA	2	0	0	
AA	0	0	0	.

<sup>1</sup>Homo GG & homo deldel  $P=0.015$ , hetero Gdel homo GG  $P=0.038$ , hetero Gdel homo deldel  $P=0.017$ ,<sup>2</sup>Hetero CT TT  $P=0.02$ ., <sup>+</sup> $P<0.05$ .

The frequencies of haplotypes present in at least 10% of the population are presented in Table 4. Haplotype frequencies of the MRP2 polymorphisms at all site. The frequency of haplotype 1 was significantly higher in patients without CNS ADR (32.3%) than those with ADR (15.8%) ( $P=0.0039$ ). The frequency of haplotype 3 was higher in patients with CNS ADR than those without ( $P=0.0139$ ), but this difference was no longer significant after Bonferroni's correction was applied.

Table 4. Haplotype frequencies of the MRP2 polymorphisms at all site

Haplotype	Group				Chi-Square	p-value
	Freq.	Control N (%)	ADR N (%)	non-ADR N (%)		
c.4508+12G>A c.4147-35G>A c.3972C>T c.2934G>A c.2620+3A>G c.1457C>T c.1249G>A g.-23G>A g.-24C>T g.-1549G>A g.-1774DelG						
1 - G C G G C A G C G G	0.283	71 (32.6)	13 (15.8)	82 (32.3)	8.345	0.0039 <sup>+</sup>
2 G G C G G C A G C G G	0.265	51 (23.1)	24 (29.0)	65 (25.7)	0.348	0.5555
3 G A T G G C A G T G G	0.141	31 (14.5)	18 (22.3)	29 (11.4)	6.057	0.0139 <sup>*</sup>
4 G G T G A C A G C G G	0.100	2 (0.7)	9 (10.6)	25 (9.8)	0.004	0.8338
5 G G C G A C A G C G G	0.071	19 (8.7)	8 (0.09)	17 (0.06)	0.639	0.4241
-----	-	-	-			
Total		220	82	254		

Data are number of patients (%). Haplotype was inferred computationally (Haploview v. 3.32). <sup>+</sup> $P<0.01$ , <sup>\*</sup> $P<0.05$ .

## 5. Measurement of MRP2 promoter activity

VPA stimulated MRP2 promoter activity about 10-fold. The epileptic patients with ADR of the CNS had less haplotype 1 (containing g.-1774del) than the epileptic patients with no ADR. By inference, the g.-1774G allele, a major allele of the MRP2 promoter sequence, was associated with ADR of the CNS in patients treated with VPA. Epileptic patients with g.-1774del instead of g.-1774G were more likely to be resistant to VPA-related ADR, probably because the g.-1774del allele produced higher MRP2 promoter activity than g.-1774G, leading to more MRP2 proteins in the brain tissue after VPA treatment. The presence of more MRP2 affected the distribution of AEDs (especially VPA) from the blood to the brain, resulting in the appearance of VPA-related ADR in patients with g.-1774G rather than g.-1774del. Our luciferase assay data supported this hypothesis. There was no difference in luciferase activity between g.-1774del and the others (g.-1774G, g.-24C>T, g.-1549G>A) with non-VPA treatment. Luciferase activity was different, however, following 3-mM VPA treatment; g.-1774del promoter (haplotype 1) activity was 134% higher than g.-1774G promoter (haplotype 2) activity (n=5;  $P<0.05$  by t-test). Further, the g.-1774del MRP2 promoter increased 1014% with 3 mM VPA treatment compared to non-VPA treatment. However, the g.-1774G MRP2 promoter also increased 816% with 3-mM VPA treatment compared to non-VPA treatment. The change of g.-1774 G del increased the expression of MRP2 in neuronal SH-SY5Y cells treated with 3 mM VPA. The effect of g.-1774 G del will be further investigated in brain capillary endothelial cells (BB19).

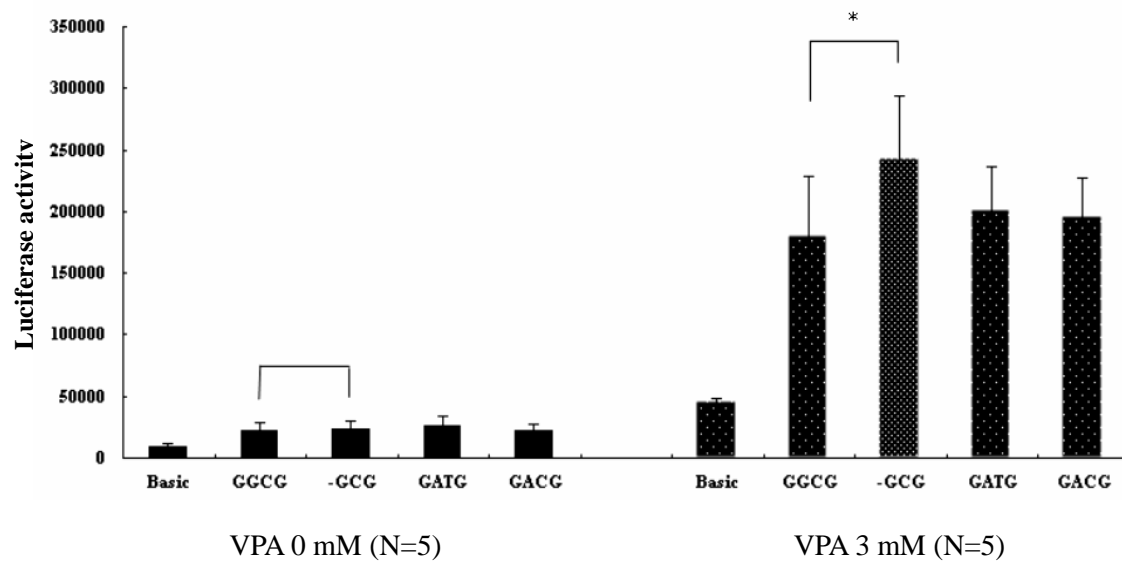


Fig 1. Luciferase activity of MRP2 promoter variants. Valproic acid (VPA) stimulated MRP2 promoter activity. The g.-1774del (-GCG) variant was more activated than g.-1774G (GGCG) in cells treated with 3mM VPA. \*  $P < 0.05$ .

## 6. Factors influencing ADR to VPA

Many factors, including gender, age, treatment with carbamazepine, lamotrigine, phenytoine, or topiramate and hippocampal sclerosis, did not influence ADR. The presence of the genotype G/G at the g.-1774 region of the MRP2 promoter influenced ADR to VPA in epileptic patients. Logistic regression showed that the G/G was independently correlated with ADR to VPA ( $P < 0.05$ , ORs=9.102, 95% confidence interval = 1.111-73.378). The del/del allele was the reference category (Table 5). The G/G genotype at g.-1774 might influence lower expression of MRP2 than del/del in the brain, leading to ADR.

Table 5. Odds Ratios of factors

		B	P-value	95.0% C.I.for		
				EXP(B)		
				Odd Ratio	Lower	Upper
MRP2	SEX <sup>1</sup>	-.293	.444	.746	.352	1.581
	AGE	.010	.489	1.010	.982	1.038
	Carbanazepine <sup>2</sup>	-.319	.413	.727	.339	1.560
	Lamotrigine <sup>3</sup>	.163	.732	1.177	.464	2.985
	Phenytoine <sup>4</sup>	.290	.571	1.337	.489	3.654
	Topiramate <sup>5</sup>	-.555	.170	.574	.260	1.268
	Presence of G/del <sup>6</sup>	1.603	.138	4.967	.598	41.232
	Presence of G/G <sup>7</sup>	2.200	.040*	9.029	1.111	73.378
	HS <sup>8</sup>	.329	.406	.720	.332	1.563
	Constant	2.812	.033	.060		

<sup>1</sup>Reference category: Female (0), <sup>2-5</sup>Reference category: non-use of each drug,

<sup>6,7</sup>Reference category: del/del, <sup>8</sup>Hippocampal sclerosis was shown at patients (1), \*P<0.05.

#### IV. Discussion

The selective cellular barrier controlling drug delivery into the brain has been identified by the over-expression of MRP2 in capillary endothelia, astrocytes and neuronal cells in previous studies<sup>5, 26, 27</sup>. In this study, we found that the g.-1774del genotype in the promoter region up-regulated the expression of MRP2. Patients with g.-1774del showed resistance to ADR by increasing the expression of MRP2, followed by a decrease of drug concentration in the brain. Our data supported this hypothesis and verified an association between g.-1774delG and ADR.

The difference of luciferase activity between g.-1774G and g.-1774del in neuronal SH-SY5Y cells was not large. However, they showed extensive difference by statistical analysis ( $P < 0.05$  by t-test). Because expression level of MRP2 is lower in brain neuronal tissue than in hepatic tissue, even a little change is thought to be important. The luciferase assay of MRP2 promoter activity has not been conducted in brain capillary endothelial cells yet. In this cell line, SNP function is thought to be more effective, since MRP2 is more highly expressed in endothelial cells than in neuronal cells<sup>28</sup>.

In reality, if MRP2 and other transporters are over-expressed in the brain, most patients should be not only non-ADR but also resistant to drug therapy. However, allele and genotype frequencies of g.-1774delG were not different between drug resistant and drug responsive patients ( $P > 0.05$ ). The absence of a strict definition of drug resistant epilepsy may have weakened the statistic analysis. The selected grouping was only a foundation for retrospective chart review. If the definition was stricter, for example, at least one seizure per year or non-seizure over the year, the different frequencies of g.-1774delG within each group would have been more significant. An accurate study design and definitions are a key part of effective pharmacogenomic studies<sup>2</sup>. Standards for association studies in pharmacogenomics have been presented in previous studies<sup>2</sup>.

In spite of the retrospective study design, an association with g.-1774delG was noticeably significant. This SNP was reported previously in our group in relation to toxic hepatitis.<sup>24</sup> In that study, the g.-1774del genotype caused lower expression of MRP2 than g.-1774G in a human hepatocyte originated cell line (HepG2). This finding is contrary to the results of the present study. There are different known transcription factors in hepatocyte, neuronal and endothelial cells. The promoter activity in the cells transfected with a g.-1774G (haplotype 2) clone was about 100 times stronger than those transfected with the control vector in hepatic cells. A strong transcription activator is present in hepatocytes. However, the difference in promoter activity between g.-1774G and mock control was lower in neuronal SH-SY5Y cell lines. Neuronal transcription factors are expected to be weaker than those in hepatocytes. The binding sequence is “AAAAACAACAAGATAA” (the underlined C is the anti-sense sequence at g.-1774G). Transcription factors known to bind this sequence are GATA-1, Evi-1, FOXO, HNF3, and more (Genomatix software GmbH). Each tissue specific transcription factor binds this sequence and induces different levels of MRP2 expression. For example, HNF3, a common activator in hepatocytes, was thought to activate MRP2 expression through binding with g.-1774G rather than g.-1774del. Consequently, the expression of MRP2 of g.-1774del would be lower than g.-1774G. In contrast, if neuronal cell transcription activators were more likely to bind g.-1774del over g.-1774G, neuronal expression of MRP2 of g.-1774del would be higher than g.-1774G. The factors that bind to this region have yet to be determined. Previous studies have referred to the different binding factors of this region in human coronary artery smooth muscle cells without specific identification.

In the present study, a luciferase assay demonstrated that VPA stimulated the activity of the MRP2 promoter in SH-SY5Y cells. VPA is a well known HDAC (histone deacetylase) inhibitor that induces apoptosis in cancer cells<sup>29, 30</sup>. In normal cells, however, VPA activates

chromosomal transcription ability by inhibiting HDAC. Chromosomal DNA forms a compact nucleosome with histone proteins and needs to be unpacked in order to start transcription. HDAC removes the acetyl group in acetyl-lysine residues and biologically inhibits the activity of transcription<sup>31</sup>. In neuronal SH-SY5Y cells, VPA could activate the MRP2 promoter by inhibiting HDAC. MRP2 has FXR (farnesoid X receptor), RXR (retinoid X receptor) and PPAR (Peroxisome Proliferator-activated Receptor) / RXR binding regions. VPA interacts with these proteins and regulates the expression level of MRP2 protein<sup>32, 33</sup>

P-gp polymorphisms have repeatedly been associated with drug response in epilepsy<sup>25, 34-37</sup>. Up to now, however, the only functional studies of MRP2 have been conducted in a knockout animal model<sup>38</sup>. This report is the first investigation to show an association between MRP2 polymorphisms and ADR in epilepsy. These results may be useful in tailoring AED medication. The G allele of the MRP2 promoter was associated with ADR to VPA in Korean epileptic patients. The del allele of MRP2 protected the brain against adverse effects of VPA by over-expressing MRP2 in brain neuronal cells. These data will help design therapy to minimize ADR in epileptic patients and aid in the development of new AED.

## V. Conclusion

The present study identified a novel association between a MRP2 polymorphism and ADR to VPA, using statistical analysis and molecular biology approaches to conclude:

1. In patients treated with VPA, the g.-1774del of MRP2 (genotype, haplotype) was more likely to be found patients without ADR than those with ADR.
2. The g.-1774G del increased the activity of the MRP2 promoter, and VPA stimulated higher expression of MRP2 containing the del allele rather than the G allele in an in vitro model.
3. The g.-1774G allele was associated with ADR to VPA by decreasing the level of MRP2 expression in brain neuronal cells, but the g.-1774del allele was resistant to ADR to VPA.
4. Another polymorphism, g.-24C>T, was also weakly associated with ADR to VPA.

From these results, we conclude that MRP2 plays an important role in the distribution of AED medication in epileptic patients. Further, a polymorphism of MRP2 is associated with ADR in CNS patients.

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ABSTRACT (IN KOREA)

**ABC 수송 단백질의 유전적 변이와  
Valproic acid의 약물 이상 반응과의 상관성 연구**

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이지현

다중 약물 저항성 단백질 (MDR1) 과 MRP 계열의 다중 약물 저항성 단백질 (MRP1, MRP2, MRP4 and MRP5) 과 BCRP (breast cancer resistance protein)는 항 간질 약물의 반응 변동을 일으키는 후보자이다. 이들 단백질들은 혈관 뇌 장벽 (BBB)의 주요 구성요소인 혈관 내피 세포나 astrocyte에 발현하고 있기 때문에 혈관에서 뇌로의 약물 분포에 영향을 미친다. 특히, Valproic acid를 수송한다고 알려져 있는 MRP2는 Valproic acid와 seizure 자체에 의해서 그 발현이 유도된다고 알려져있다. 따라서 간질의 치료로 Valproic acid를 사용하는 환자들에 있어서, MRP2는 뇌조직의 약물농도에 영향을 주는 중요한 단백질로 생각된다. 본 연구는 위 단백질의 유전자들의 유전적 다형성과 개개인의 항 간질 약물 중 Valproic acid에 대한 약물 이상 반응과의 상관성에 관한 것이다. 총 168명의 간질 환자는 중추 신경계 약물 이상 반응을 보이는 간질 환자 그룹과 (n=41) 약물 이상 반응을 보이지 않는 간질 환자 그룹으로 (n=127) 나누었다. 약물 수송단백중 MRP2의 promoter지역에 존재하는 g.-1774delG 변이가 valproic acid로 인한 중추 신경계 약물 이상반응과 강한 상관성을 보이는 것을 알수 있었다 (P=0.004). 그리고 g.-1774G 와 g.-1774del 과의 MRP2 promoter

활성은 뇌 신경세포에서 차이를 볼수 있었다 ( $P<0.05$ )

본 자료는 인간의 MRP2 유전자의 다형성이 항 간질 약물인 Valproic acid의 약물 이상 반응과의 상관성을 제시한다.

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핵심되는 말 : 유전적 다형성, MRP2, 약물 이상 반응, valproic acid, 간질.