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ORIGINAL ARTICLE

Association of a synonymous GAT3 polymorphism with antiepileptic drug pharmacoresistance

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It would be likely that the genetic variants of the *GTA3* gene encoding GAT-3, an astrocytic GABA transporter, may alter gamma-aminobutyric acid (GABA) neurotransmission in the synaptic cleft in the epileptic brain and cause antiepileptic drugs (AEDs) pharmacoresistance. A candidate gene association analysis with fine mapping was performed to dissect the genetic contributions of *GAT3* to AEDs pharmacoresistance. Two independent case sample sets were recruited (Samples 1 and 2), and each set was divided into two groups (drug-resistant and drug-responsive) according to the treatment outcomes with AEDs. Sample1 (n=400) was used for the initial exploratory stage of the study and sample 2 (n=435) was used for confirmation of the genetic association in the replication stage of the study. A *GAT3* polymorphism (*GAT3* c.1572 C>T, rs2272400) was nominally associated with AEDs pharmacoresistance (P_{CC} vs $P_{CT/TT}$=0.012, $P_{allelic}$=0.01). The odds ratio (OR) for AED pharmacoresistance was 1.6 (95% confidence interval (CI), 1.11–2.24; P=0.01) in the additive models of inheritance. The statistical significance remained after we adjusted for a confounding factor, the etiology of epilepsy, at 0.012 (adjusted OR: 1.73, 95% CI: 1.13–2.67) and used Bonferroni's correction for multiple comparisons at 0.048. Importantly, the positive association of c.1572 T was reproduced in the replication stage ($P_{allelic}$=0.037, joint P-value of the replication=0.001). The results suggest that *GAT3* c.1572T may be one of the contributing factors with a modest effect on AEDs pharmacoresistance in the epileptic brain, shed light on a better understanding of the underlying mechanisms and serve as an impetus for new avenues of treatment for AEDs pharmacoresistance.
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INTRODUCTION

Although there have been numerous developments in antiepileptic drug (AED) therapy, epilepsy remains uncontrolled in a significant proportion of patients.[1] Even with the advent of several new AEDs, hopes of rendering such patients seizure-free remain unlikely. This illustrates the need for novel AEDs with mechanisms of action that differ from the mechanisms of action of the currently available AEDs.[2]

The biological basis of AED pharmacoresistance is poorly understood, but just as epilepsy is a heterogeneous condition with multiple etiologies, it is likely that the pathogenesis of AED pharmacoresistance is multifactorial and may involve both genetic and environmental factors.[3,4] To date, two concepts have been advanced to explain the development of pharmacoresistance. The transporter hypothesis contends that the expression or function of multidrug transporters in the brain is augmented, leading to impaired access of AEDs to the central nervous system targets, which has been the center of much debate regarding association of genetic variants of the gene encoding a multidrug transporter with AED resistance.[5,6] The target hypothesis holds that epilepsy-related changes in the properties of the drug targets may result in reduced drug sensitivity.[7] Indeed, a reduction in drug target sensitivity in chronic human and experimental epilepsy has been suggested for the voltage-gated sodium channel and the $GABA_A$ receptor.[8]

Gamma-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the adult brain and has a critical role in the regulation of excitability of neuronal networks.[9] The modulatory effect of GABA on neuronal excitability in the normal brain is exerted not only by direct activation of the $GABA_A$ receptor but also by a more indirect method of interacting with and metabolism of GABA released from synapses. Any neurotransmission system is dependent upon an

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effective mechanism by which the action of the transmitter can be inactivated. In the case of GABA, the mechanisms include GABA transaminase, a catabolic enzyme of GABA and GABA transporters to which the function most frequently ascribed is the termination of synaptic transmission.[10] However, it is clear that GABA transaminase is unlikely to be responsible for the effective inactivation of GABA because GABA transaminase is located intracellularly in the inner membrane of the mitochondria.[11,12] In contrast, GABAergic neurotransmission is dependent upon optimal function of astrocytic transport processes.[10] Thus, a clear inference is that dysfunction of astrocytic GABA transport processes leads to alteration of the inhibitory activity of GABA in the normal brain, which has implications for neuronal excitability.

Of the four types of GABA transporters, which have been cloned, GAT-3 (human SLC6A11) is a representative GABA transporter, expressed primarily in the glia of the mammalian brain,[13] indicating that the GAT-3 transporter may be a candidate for the regulation of paracrine GABA, and therefore a potentially important AED target.

In the present study, we hypothesized that the genetic variants of the *GAT3* gene encoding GAT-3 may alter GABA neurotransmission in the synaptic cleft in the epileptic brain and cause AED pharmacoresistance. On the basis of the hypothesis, a candidate gene association analysis with fine mapping was performed to dissect the genetic contributions of the *GAT3* gene to AED pharmacoresistance.

MATERIALS AND METHODS

Subjects

Two independent case sample sets (samples 1 and 2) were recruited. Each sample set was comprised of patients with an established clinical diagnosis of epilepsy, as defined by international guidelines.[14,15] Sample 1 (n=400) was recruited from the epilepsy clinics at three tertiary hospitals that cover three large provinces in Korea. Sample 2 (n=435) was recruited from two tertiary epilepsy centers located in the capital city of Seoul and Busan, the second largest city in Korea. Table 1 summarizes the characteristics of the sample sets. Sample1 was used for the initial exploratory stage of the study and sample 2 was used for confirmation of the genetic association in the replication stage of the study. All the recruited cases are ethnically Korean.

Each sample set was divided into two groups (drug-resistant (DR)) and drug-responsive (DS)) according to the treatment outcomes with AEDs.[5] Drug resistance was defined as the occurrence of at least four unprovoked seizures in the course of 1 year before recruitment with trials of >2 appropriate AEDs at maximal tolerated doses, which were established based on the occurrence of clinical side effects at supramaximal doses. Patients who underwent surgical treatment for DR epilepsy were classified as having DR epilepsy, regardless of the surgical outcome. However, patients who were frequently not compliant with AED therapy and those who had reported seizures with a questionable phenotype were excluded from the present study at the recruitment stage. Drug-responsiveness was defined as complete freedom from seizures for at least 1 year, up to the date of the last follow-up visit. In total, 207 healthy Korean volunteers without a family history of epilepsy were recruited in order to characterize the haplotype block structure of the *GAT3* gene. The study was approved by the Ethics Committees and the Institutional Review Boards at each of the five hospitals, and informed consent was obtained from all study participants.

Study strategy

The present study used a three-stage strategy. First, in the exploratory stage, we developed the coding single-nucleotide polymorphisms (SNPs) of the *GAT3* gene, using pooled DNA from 200 healthy Korean controls, and explored the SNPs' genetic association with AED pharmacoresistance. Second, we based the extension stage on the positive association from the exploratory stage, fine mapping to characterize the haplotype structure, tagging SNPs of the *GAT3* gene and performing an extended association analysis. Finally, in the confirmation stage, we applied replication for an independent statistical confirmation.

Selection of SNPs for genotyping

In the exploratory stage, we amplified all coding regions of the *GAT3* gene, including the exon–intron boundary sequences, via PCR, using pooled DNA from the control group, as described elsewhere,[16] and performed direct sequencing of both strands and visual analysis of the electropherograms, to discover coding SNPs of the *GAT3* gene (Supplementary Methods). We performed genotyping on all the coding SNPs, from the exploratory stage, for individual control DNAs and selected coding SNPs, with a minor allele frequency (MAF) >0.1 only, as candidate SNPs for the genetic association analysis. This is compatible with the common disease/common variant hypothesis.

For the extension stage, *GAT3* genotype information for a region covering from exon 1 to exon 13 was downloaded from dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). SNPs for genotyping were selected on the basis of the following criteria: (1) one common SNP with a frequency >5% for every 5000 bp; and (2) all coding SNPs with a frequency >5%. Allele frequency information refers to the HapMap project for Chinese and Japanese under the assumption that the Chinese or Japanese data are similar to the Korean data because the Korean HapMap project is incomplete. In selecting between different SNPs, the substitution of adenine for thymidine or guanine for cytosine, or *vice versa*, was avoided as much as possible for accuracy in genotyping.

High-resolution DNA melting analysis

DNA was extracted using a standard protocol and purified using a kit procedure (Qiagen, Hilden, Germany). The real-time PCR using high-resolution melting (HRM) analysis was mainly used in genotyping. HRM analysis was performed using Rotor-Gene 6000 (Corbett Life Science, Brisbane, Australia) according to the manufacturer's protocol. Direct sequencing was used to achieve an exact genotype data.

The HRM process consists of performing the PCR in the presence of the DNA binding dye, SYTO 9 green (Invitrogen, Carlsbad, CA, USA), monitoring the progressive changes in fluorescence caused by release of the dye from a DNA duplex as it is denatured with increasing temperature, collecting a HRM curve, and identifying the samples with melting curve aberrations indicative of

Table 1 Characteristics by case–control status

	Sample1			*Sample2*		
	DS (n=*200*)	*DR* (n=*200*)	P-*value*	*DS* (n=*269*)	*DR* (n=*166*)	P-*value*
Gender, man (%)	101 (50.5)	97 (48.5)	0.764	140 (52.0)	91 (54.8)	0.642
Age of seizure onset (years)	17.78 ± 16.0	12.72 ± 11.31	0.001	NA	NA	NA
Etiology of epilepsy (%)						
Idiopathic (generalized/focal)	37 (19/18)	5 (5/0)	<0.001	34 (32/2)	4 (3/1)	<0.001
Symptomatic/cryptogenic	163 (89/74)	195 (121/74)		235 (86/149)	162 (108/54)	

Abbreviations: DR, drug-resistant group; DS, drug-responsive group; NA, not available.

the presence of a sequence variant. Fluorescence intensity as a function of temperature monitored by the LightScanner instrument (Idaho Technology, Salt Lake City, UT, USA) can reveal very small changes in the melting curve shape when analyzed with the LightScanner software using the 'Scanning' mode (Idaho Technology).

PCR was performed in 10 μl reactions containing 40 ng of template DNA, 1.5 mM $MgCl_2$, 0.5 mM dNTP, 400 nM forward and reverse primers, 0.8× SYTO 9 green, 0.5 U of Ampli*Taq* Polymerase Gold and 1× PCR buffer (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions for PCR reactions were as follows: initial denaturation at 95.0 °C for 2 min with 40 cycles at 95.0 °C for 5 s and annealing/extension at 58.0–63.0 °C for 10 s. HRM was carried out by fluorescence acquisition during a temperature increase from a minimum of 68.0 °C to a maximum of 90.0 °C with increments of 0.1 °C and holding steps of 10 s (Supplementary Table 1).

For the substitution of adenine for guanine or thymidine for cytosine, or *vice versa*, the accuracy of the HRM analysis was 99.5% according to duplicate direct sequencing analysis of, on average, 5% of the total genotype. The substitution of adenine for thymidine or guanine for cytosine, or *vice versa*, are the most difficult to genotype using HRM analysis because of a very small T_M melt curve shift, thus direct sequencing was used to achieve an exact genotype data. For both genotyping platforms, the overall genotype call rate was >99%.

Characterization of haplotype and tSNPs

Haploview 4.1 (Broad Institute, Cambridge, MA, USA) was used to infer haplotypes and tSNPs. Linkage disequilibrium (LD) was calculated as D'. For defining haplotype blocks, the default algorithm is obtained from elsewhere.[17] The 95% confidence bounds on D' were generated, and each comparison was classified as 'strong LD,' 'uninformative' or a 'strong recombination.' A block was created when 95% of the informative comparisons showed a 'strong LD.' This method, by default, ignores markers with a MAF<0.05. Haplotypes were estimated using an accelerated EM algorithm similar to the partition/ligation method described elsewhere.[18] This creates a highly accurate population frequency estimate of the phased haplotypes based on the maximum likelihood, as determined from the unphased input. Haplotype tSNPs were selected by default using the block-by-block tags menu in Haploview. Individuals with >50% missing genotypes were excluded from the analysis. It is not possible in Haploview to visualize the probabilities of each individual's estimated haplotypes. Therefore, HAPSTAT 3.0 (University of North Carolina, Chapel Hill, NC, USA) was used to assign probabilities to each individual's estimated haplotype.

Statistical analysis

Genotype frequencies at each locus were tested for the Hardy–Weinberg equilibrium. The strength of the association between single SNPs or haplotypes and AED pharmacoresistance was evaluated as an odds ratio (OR), according to the mode of inheritance of a causal allele or haplotype obtained with SPSS 17.0 (Chicago, IL, USA) or HAPSTAT 3.0. The mode of inheritance can be additive, dominant or recessive. Under the additive model, two copies of a causal allele or haplotype have twice the effect on the trait as compared with a single copy. Under the dominant model, having one or two copies has the same effect. Under the recessive model, having only two copies of the causal allele or haplotype will affect the trait.

RESULTS

Initial association analysis

We examined five SNPs from the coding region of the *GAT3* gene (rs2304725 in exon 5, rs2272394 and rs2272395 in exon 9, rs2272400 in exon 12 and rs2245532 in exon 13). Of these, we excluded the SNP rs2245532 from the exploratory stage analysis, because it has a low MAF. Of the four coding SNPs with MAF>0.1 from the exploratory stage, we found rs2272400 (*GAT3* c.1572 C>T) was associated with AED pharmacoresistance (Table 2). The DR group was more likely to have the CT or TT genotype than the DS group was. The crude OR of CT or TT vs CC was 1.7 (P=0.012, 95% confidence interval (CI): 1.12–2.57). This statistical significance remained after adjusting for a confounding factor, the etiology of epilepsy, at 0.012 (adjusted OR: 1.73, 95% CI: 1.13–2.67) and after using Bonferroni's correction for multiple comparisons, at 0.048.

Characterization of haplotype structure and tagging SNPs

It is plausible, judging from exploratory stage result, that the SNP rs2272400 may be a causal variant or may merely exist in LD, with unknown functional variants existing around it. Therefore, we undertook fine mapping to characterize the LD block around rs2272400 and to search for the possible, unknown functional variant.

On the basis of the SNP selection criteria in extension stage, a total of 42 SNPs covering the *GAT3* gene (about 120 kb) were selected from the dbSNP (Supplementary Table 1). Genotyping was performed for the individuals of the control group; 6 of the 42 SNPs displayed a low call rate or a MAF<0.05, and were therefore excluded from defining haplotype blocks and tSNPs. Of the remaining 36 SNPs, 5 were in the coding region, 1 was in the 5′-untranslated region and 30 were in the intronic sequences of the *GAT3* gene. The overall genotype call rate for the 36 SNPs was 99.6%. Genotype distributions at all loci, except rs966030, were consistent with the Hardy–Weinberg equilibrium (each P>0.05) and rs966030 was also excluded from the analysis (Supplementary Table 2).

Seven haplotype blocks, 1–27 kb in size, and four recombination hot spots of the *GAT3* gene were created, and 13 tSNPs corresponding to the haplotype blocks were defined (Figure 1 and Table 3) using Haploview. Genotyping of the tSNPs was carried out for the individuals of the DR and DS groups and the overall genotype call rate for the 13 tSNPs was 99.8%. These 13 tSNPs were used to infer the individual's haplotypes.

Table 2 Association of the coding single-nucleotide polymorphisms (SNPs) of the *GAT3* gene with pharmacoresistance

SNP	*Allele*	*Protein residue*	*MAF*	*Genotype*	*DS, %*	*DR, %*	*COR*	*95% CI*	P-*value*	*AOR*[a]	*95% CI*[a]	P-*value*[a]
rs2304725	T/**C**	Ser-Ser	0.48	TT	0.320	0.365	1			1		
				CT/CC	0.680	0.635	0.82	0.54–1.24	0.116	0.70	0.47–1.13	0.156
rs2272394	G/**A**	Ala-Ala	0.15	GG	0.785	0.735	1			1		
				GA/AA	0.215	0.265	1.32	0.83–2.09	0.242	1.27	0.79–2.05	0.321
rs2272395	T/**C**	Pro-Pro	0.10	TT	0.790	0.820	1			1		
				CT/CC	0.210	0.180	0.83	0.50–1.36	0.449	0.77	0.46–1.28	0.307
rs2272400	C/**T**	Cys-Cys	0.22	CC	0.705	0.585	1			1		
				CT/TT	0.295	0.415	1.70	1.12–2.57	0.012[b]	1.73	1.13–2.67	0.012[b]

Abbreviations: AOR, adjusted odds ratio; CI, confidence interval; COR, crude odds ratio; DR, drug-resistant group; DS, drug-responsive group; MAF, minor allele frequency.
[a]Adjusted for etiology of epilepsy, Bold characters denote minor allele.
[b]P=0.048 after using Bonferroni's correction for multiple comparisons.

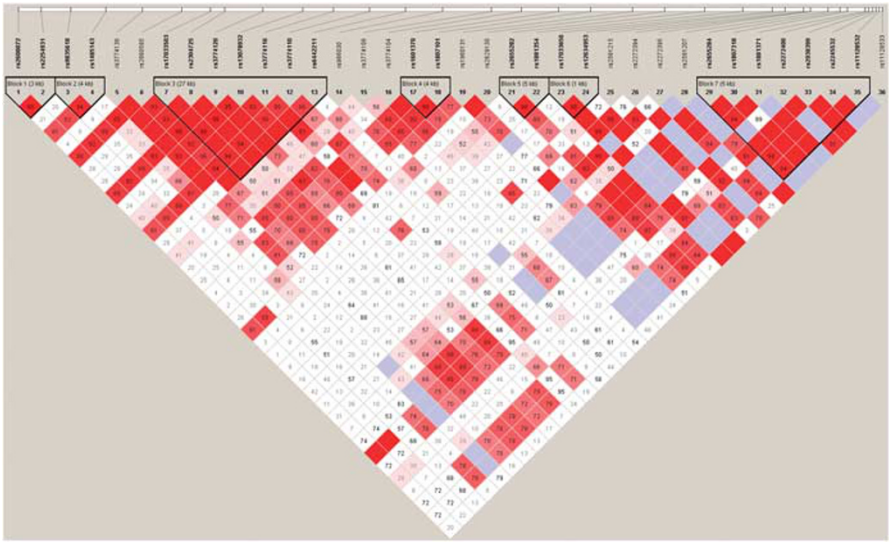

Figure 1 Haploblock structure of the *GAT3* gene according to Haploview analysis in Korean. Seven haplotype blocks, 1–27 kb in size, and four recombination hot spots of the *GAT3* gene were defined.

Table 3 Association of *GAT3* haplotypes with antiepileptic drug pharmacoresistance

	Frequency		*Additive model*				*Dominant model*				*Recessive model*			
Haplotype	*DS*	*DR*	*OR*	*95% CI*		P-*value*	*OR*	*95% CI*		P-*value*	*OR*	*95% CI*		P-*value*
Block 2; rs9835618 and rs1485143														
H1: **AT**	0.38	0.40	1	—	—	—	1	—	—	—	1	—	—	—
H2: **GT**	0.36	0.31	0.8	0.59	1.13	0.217	0.9	0.62	1.30	0.577	0.7	0.39	1.18	0.165
H3: **TT**	0.26	0.28	1.0	0.72	1.45	0.903	1.2	0.79	1.69	0.453	1.0	0.53	1.78	0.915
Block 3; rs17033503, rs2304725, rs3774120, rs13078932, rs3774116, rs3774110 and rs6442211														
H1: AT**G**AT**CC**	0.41	0.43	1	—	—	—	1.0	—	—	—	1.0	—	—	—
H2: G**C**AG**TAC**	0.27	0.24	0.8	0.59	1.19	0.322	0.7	0.47	1.04	0.080	1.2	0.65	2.15	0.594
H3: AT**G**AT**CT**	0.15	0.17	1.0	0.66	1.53	0.977	1.0	0.61	1.50	0.842	1.0	0.39	2.63	0.991
H4: G**C**GG**CCC**	0.13	0.17	1.2	0.80	1.86	0.355	1.1	0.71	1.70	0.678	1.7	0.75	4.05	0.195
Block 5; rs2655282 and rs1881354														
H1: **CA**	0.58	0.60	1	—	—	—	1	—	—	—	1	—	—	—
H2: **TG**	0.31	0.28	0.9	0.64	1.20	0.428	0.8	0.58	1.21	0.340	1.0	0.55	1.71	0.928
H3: **TA**	0.10	0.12	1.2	0.76	1.91	0.426	1.3	0.78	2.05	0.334	0.8	0.20	3.61	0.816
Block 7; rs2655284, rs1807318, rs1881371, rs2272400, rs2938399, rs2245532 and rs11128532														
H1: **AGGCT**AC	0.44	0.40	1	—	—	—	1	—	—	—	1	—	—	—
H2: **AGGCC**AC	0.32	0.31	1.0	0.75	1.44	0.815	0.8	0.57	1.20	0.317	1.3	0.75	2.09	0.382
H3: **AGGTT**AC	0.16	0.23	1.6	1.09	2.33	0.017*	1.4	0.86	2.30	0.099	1.7	0.88	3.37	0.111
H4: **GA**T**CC**GT	0.07	0.05	0.9	0.47	1.57	0.614	0.7	0.36	1.26	0.213	3.1	0.69	14.1	0.141

Abbreviations: CI, confidence interval; DR, drug-resistant group; DS, drug-responsive group; OR, odds ratio.
Blocks 1, 4 and 6 can be tagged by only one SNP. * Statistically significant value. Bold characters denote tagging SNPs.

Association of rs2272400 with AED pharmacoresistance

Of the sevev haplotype blocks of the *GAT3* gene, three blocks were tagged by only one SNP (block 1 by rs2600072, block 4 by rs1601370 and block 6 by rs17033650), and no association was found between them and pharmacoresistance. The probabilities of each individual's haplotypes were estimated using the corresponding tSNPs to four blocks that present >2 haplotypes with a frequency >0.05, and the frequency of the estimated haplotypes of each block were compared between the DR and DS groups. Three haplotypes with a frequency >0.05 were in Blocks 2 and 5 and four haplotypes in Blocks 3 and 7. Of these 14 haplotypes in the four blocks, only haplotype 3 (H3: AGGTTAC in Table 3) in Block 7, which includes the SNP, rs2272400, was associated with AED pharmacoresistance. When considering the most frequent haplotype as a reference, the OR for AED pharmacoresistance was 1.6 (95% CI, 1.09–2.33; P=0.017) in the additive model of inheritance.

The associated LD block (Block 7), about 7.5 kb in size, included 35 SNPs with MAF >0.05 (http://www.ncbi.nlm.nih.gov/SNP/).

Table 4 Association of *GAT3* c.1572 T with pharmacoresistance according to mode of inheritance in each sample sets

	MAF (%)			*Additive model*				*Dominant model*				*Recessive model*			
SNP ID	*DS*	*DR*	P-*value*	*OR*	*95% CI*		P-*value*	*OR*	*95% CI*		P-*value*	*OR*	*95% CI*		P-*value*
Sample1	0.165	0.238	0.010	1.6	1.11	2.24	0.010	1.5	1.03	2.23	0.036	1.7	0.86	3.27	NS
Sample2	0.180	0.241	0.037	1.4	1.03	2.02	0.031	1.5	1.02	2.19	0.041	1.4	0.67	2.91	NS
Joint analysis	0.174	0.239	0.001	1.5	1.18	1.90	0.001	1.5	1.14	1.96	0.004	1.5	0.94	2.51	NS

Abbreviations: CI, confidence interval; DR, drug-resistant group; DS, drug-responsive group; MAF, minor allele frequency; NS, statistically not significant; OR, odds ratio.

However, all the SNPs, except rs2272400, occupied intronic sequences in the *GAT3* gene, and none seemed to effect gene function or phenotype, as would be expected in coding SNPs or splice site variants.

Because rs2272400 had an association with AED pharmacoresistance, this SNP was re-genotyped in an independent set of Sample 2 and the association was reevaluated. Importantly, results in the replication stage also had a significant association between *GAT3* c.1572 C>T and AED pharmacoresistance ($P_{allelic}$=0.037; OR, 1.4; 95% CI, 1.03–2.02; P=0.031 in the additive model; and OR, 1.5, 95% CI, 1.02–2.19; P=0.041 in the dominant model of inheritance; Table 3). Moreover, the joint analysis of the exploratory and replication studies, including 469 DS and 266 DR patients, displayed a much more significant association (joint $P_{allelic}$=0.001; OR, 1.5; 95% CI, 1.18–1.90; P=0.001 in the additive model; and OR, 1.5, 95% CI, 1.14–1.96; P=0.004 in the dominant model of inheritance; Table 4). However, such an association did not exist in comparing the epilepsy group (DS+DR groups, n=400) with a non-epileptic control group (n=200) (details not shown).

DISCUSSION

In the present study, the association between genetic variances in *GAT3* and AED pharmacoresistance has been investigated for the first time. Our results suggest that the T allele of the SNP rs2272400 (*GAT3* c.1572 C>T) may increase the risk of AED pharmacoresistance by at least 1.5-fold in the epileptic brain, with an additive effect. The SNP, rs2272400, is located in the coding region of *GAT3* (exon 12), but confers no amino acid change (synonymous SNP). Previously, silent SNPs were largely assumed to have no effect on gene function and phenotype; however, recent findings have shown that this might not be the case. They may influence promoter activity (gene expression) or can lead to the synthesis of protein products with the same amino acid sequence, but different structural and functional properties.[19,20] It has been shown that silent polymorphisms, in particular C3435T in *MDR1*, can alter P-glycoprotein conformation and protein activity/substrate specificity.[19]

As GABAergic neurotransmission is terminated by uptake into the neuron or surrounding glial cells, inhibition of the GABA transporters responsible for uptake would prolong the GABAergic signal in a use-dependent manner, thereby counteracting GABA hypoactivity.[10] Thus, inhibition of GABA transport has gained much attention as an anticonvulsive strategy. The fate of GABA may be completely different depending on whether the site of uptake is neuronal or astrocytic. GABA taken up into the nerve ending is likely to be re-utilized as a transmitter by inclusion in synaptic vesicles, whereas GABA taken up by astroglial cells may be lost by metabolism.[21] It has recently been shown that although a clear correlation exists between anticonvulsant activity of GABA transport inhibitors and the ability to selectively inhibit astroglial GABA uptake, no such correlation appears to exist for anticonvulsant activity and inhibition of neuronal GABA uptake.[22] This suggests that glial cell uptake of GABA has a functionally important role in regulating the availability of GABA in the synapse. Among the GABA transporters, the GAT-1 transporter appears to be the most abundantly expressed, having a preferential neuronal location, whereas the GAT-3 transporter appears to be primarily expressed in astrocytes.[10] Therefore, the GAT-3 transporter can be a more attractive target for anticonvulsive strategy rather than the GAT-1 transporter.

However, only one compound with this mechanism has been approved for the treatment of epileptic disorders, that is, a selective inhibitor of the GAT-1 transporter, tiagabine.[23,24] Furthermore, recent work by Wu *et al*.[25] suggests that the release of GABA via GABA transporter reversal may be integral in maintaining GABA levels responsible for activating tonic inhibitory effect on surrounding neurons in the hippocampus, indicating that the GABA transporter has a much more dynamic role in control of brain excitability than has previously been recognized. Indeed, it has been demonstrated that GAT-3 antagonism by SNAP-5114, a selective antagonist of the GAT-3 transporter, inhibits non-vesicular carrier-mediated GABA release, especially in response to depolarization.[26] In addition, this transporter-mediated GABA release by reversal of the GABA transporter is widely thought to occur only under pathological conditions.[27] Taken together, these results postulate that the GABA transporter is a major determinant of the level of tonic inhibition, and an important source of GABA release during seizures.[26,28] Therefore, dysfunction of the GAT-3 transporter leads to a block in GABA transporter reversal, resulting in a reduction in ambient GABA levels, especially in the response to electrical seizure activities in the epileptic brain.[25,26,28]

Although the exact role of the T allele of the SNP, rs2272400, in the modulation of gene function or protein expression causing AED pharmacoresistance remains to be elucidated at this point, a plausible explanation inferred from the recent work is that the silent T allele may alter structural and functional properties of the GAT-3 transporter, as is in the case of *MDR1* C3435T.[19,20] Thus, the alteration may prevent the GAT-3 transporter from reversal to release GABA into synaptic pool, resulting in a decreased GABAergic tone protecting against neuronal hyperexcitability during seizures in the epileptic brain, which causes AED pharmacoresistance.

Interestingly, an association of the T allele of the SNP, rs2272400, with epilepsy was not demonstrated in the present study, even though decreased GABAergic tone is a common mechanism underlying both AED pharmacoresistance and epileptogenesis. This result suggests the possibility that the SNP, rs2272400, has no effect on *GAT3* gene function in the normal adult brain, but has a crucial role in blocking the transporter-mediated GABA release in response to seizures in the epileptic brain, which is supported with the results of Kinney's work,[26] in which GAT-3 transporter antagonism by SNAP-5114 occurred only in an action potential-dependent manner.

A particular strength of this study was that our results provide genetic evidence in support of a physiological role for GABA

transporter reversal, which has been supported by elegant electrophysiological or pharmacological studies.[25,26,28] Moreover, the results were successfully reproduced with a different cohort. Our results can be used to propose a better understanding of the mechanisms of AED pharmacoresistance and new avenues for treatment of DR epilepsies via optimal functional studies to define how the SNP, rs2272400, has a role in blocking transporter-mediated GABA release in response to seizures in the epileptic brain. Nevertheless, this study suffers from a few limitations that should be addressed.

First, the sample sets recruited in the present study do not cover various ethnic populations worldwide. Undoubtedly, AED pharmacoresistance is a universal phenomenon with currently available AEDs, whereas populations differ in the frequency distribution of alleles at the same loci. Therefore, although the ethnic homogeneity in the sample sets of the present study was advantageous in replication of the original data, it may not be optimal to generalize the data to other ethnic populations.

Second, statistical power to detect association in the present study was not strong enough to present our results as definite predictors. As mentioned above, the pathogenesis of AED pharmacoresistance is multifactorial in which epistasis among genetic or environmental factors, or both, with a modest effect is believed to have an important role.[29–33] Therefore, it can be postulated that the likelihood of detecting an association for each of the multiple causal factors with a modest effect should be weak. Fortunately, the weak association in the initial exploratory stage in the present study was successfully replicated with an independent sample set, which has been utilized as a means of verification for the results from association analyses.[34] However, a larger, adequately powered study needs to be undertaken to test and confirm our hypothesis.

Third, the sample sets were stratified by epilepsy etiology. The mechanisms underlying AED pharmacoresistance might be distinct from each other according to different etiologies of epilepsy, as suggested by Semah *et al.*;[35] acquired epilepsies, such as those following stroke or due to the development of a vascular malformation or tumor, seem to be much more treatment-responsive than those associated with cortical dysgenesis, hippocampal sclerosis or dual pathologies. In the present study, subdivision of samples according to lesion etiologies was not attempted in an effort to avoid decreasing statistical power due to the small sample size. Fortunately, the statistical power of the initial association remained significant after adjusting for the confounding factor, the etiology of epilepsy. In addition, given that the injuries that cause acquired epilepsy vary but share a common molecular mechanism for producing brain damage resulting in epileptogenesis,[36,37] the imbalance between excitatory and inhibitory neurotransmission due to an impaired GABA transporter reversal may not be lesion-specific, but rather a common mechanism underlying AED pharmacoresistance.

Finally, we suspended further study to seek a possible functional variant that would associate more strongly with AED pharmacoresistance than rs2272400, because, in the associated LD block, we found no coding SNPs with MAF$>$0.1 and assumed intronic SNPs largely had no effect on gene function and phenotype. However, future studies should address the effects of the rare coding SNPs, with MAF$<$0.1, and/or the intronic sequence on gene function and phenotype.

In conclusion, our results suggest the T allele of the SNP rs2272400 (*GAT3* c.1572 C$>$T) may slightly increase the risk of AED pharmacoresistance in the epileptic brain. Our data, although it should be replicated with a larger cohort of Korean descent and other large cohorts of different descent and confirmed via optimal functional studies, have shed light on a better understanding of the mechanisms of and new avenues for treatment of AED pharmacoresistance.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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