Genetic variation of MRP1 associated with the treatment response in the depressive disorder

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Directed by Professor Min Goo Lee

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ABSTRACT

Genetic variation of MRP1 associated with the treatment response in the depressive disorder

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(Directed by Professor Min Goo Lee)

Multidrug resistant protein 1(MRP1/ABCC1), a member of ATP binding cassette(ABC) transporter superfamily, is known as a xenobiotics efflux pump. It is expressed highly in the blood brain barrier(BBB) and choroid plexus of blood cerebrospinal fluid barrier(BCSFB) and has been implicated in altering the treatment response to psychotropic drugs such as antidepressants. This study aimed to identify the association between genetic variations in MRP1/ABCC1 and the therapeutic response to the antidepressant citalopram in the depressive disorder. One hundred and twenty three patients who had been administered citalopram monotherapy to control their major depressive disorder were recruited and genotype data from sixty four patients who had completed their eight-week follow up were evaluated together with those from one hundred controls. Nine MRP1 single nucleotide polymorphisms (SNPs) showing more than 5% allele frequency in

the Korean population were analyzed. The c.4002G>A, a synonymous SNP in exon 28, showed a strong association with the remission state at 8 weeks (P=0.005, O.R. 4.7, 95% C.I. 1.5~14.7). The c.4002G>A forms a linkage disequilibrium(LD) block with three other SNPs including c.5462T>A in the 3' untranslated region. Accordingly, the haplotype showed a significant association with the remission state (P=0.014). Subsequent molecular studies were conducted to support the association between these MRP1 polymorphisms and the citalopram response. Thus kinetic studies using MRP1-enriched membrane vesicles revealed that citalopram is a substrate of MRP1 (K_m=1.99 μM, V_{max}=137 pmol/min/mg protein). In addition, individuals with A allele of c.4002G>A or c.5462T>A polymorphisms showed higher MRP1 mRNA levels in peripheral blood cells. The cause of the difference in mRNA expressions was assumed from the RNA structural changes according to the c.5462T>A which forms a LD block with c.4002G>A. These results suggest that MRP1 polymorphisms may be a predictive marker of citalopram treatment in major depression.

Keywords: MRP1/ABCC1, citalopram, remission, major depressive disorder, ABC transporter

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I. INTRODUCTION

Until now, there have been no definite factor to predict the individual pharmacological and adverse responses to antidepressants which have 30 to 40 % failure rate regardless of its properties.[1,2] Identification of the factors is a critical task for prescribing appropriate agents for patients to control their major depressive disorder. Factors that affect pharmacokinetic parameters such as polymorphisms in the drug metabolizing cytochrome P450 (CYP) genes have been considered as strong candidates for influencing the individual efficacy of antidepressant drugs.[3,4] Recently, drug transporters such as the organic anion transporters and the ATP-binding cassette (ABC) transporters have emerged as a major interest in pharmacogenetics and tailored medicine, especially in the neuropsychiatric field, because these transporters can control the drug levels at target sites in the central nervous system.[5-7]

Multidrug resistance protein 1 (MRP1, *ABCC1*), a member of the ABC transporter superfamily, is one of the major drug transporters that can affect drug levels in the brain.[8,9] MRP1 was originally cloned from a lung cancer cell line as a transporter mediating drug resistance against anticancer chemotherapy similarly to P-glycoprotein (MDR1, *ABCB1*).[8] In addition to many tumors, MRP1 is expressed in a wide variety of normal human tissues.[9-12] Considering its abundant expression in the blood brain barrier (BBB) and brain cerebrospinal fluid barrier (BCSFB), MRP1 has been implicated in affecting the responses to, and side effects of, psychotropic drugs.[11,13] This 190 kDa ATP-dependent transporter is generally considered to protect tissues where it is expressed by pumping out intracellular xenobiotics and their metabolites, as verified by studies using *Mrp1*$^{(-/-)}$ knockout mice.[13,14] However, contrary to other ABC transporters such as MDR1 which are apically located, MRP1 can potentiate the effects of psychotropic drugs by increasing drug levels in the cerebrospinal fluid (CSF) or interstitial fluid (ISF) because of its basolateral membrane location at the BBB.[7,15,16] Recently, it was shown that point mutations in MRP1 can affect the transport function and substrate specificity of MRP1.[17-19] In addition, a naturally occurring mutation in MRP1 has been shown to be associated with altered drug response and resistance.[20] Therefore, it is highly conceivable that genetic variations in MRP1 including common polymorphisms may affect the individual drug response to substrate drug molecules. Furthermore, the results from some polymorphism studies have suggested that the inter-ethnic diversity of drug reaction could be existed according to the differences in their populations.

Citalopram is an antidepressant drug that belongs to a class of drugs known as selective serotonin reuptake inhibitors (SSRIs).[21] Citalopram has

been widely used in psychiatric researches for its simplicity in receptor profiles and the lower activity of its metabolites compared to those of the parent drug.[22] Citalopram is reported to be a substrate of MDR1/ABCB1, although there is some controversy.[23] Recently, one study suggested that several single nucleotide polymorphisms (SNPs) in the MDR1/ABCB1 gene were associated with the treatment response to citalopram.[24] However, no information is presently available as to whether citalopram is a substrate of any transporter belonging to the "C" branch of the ABC transporter superfamily (ABCC) transporters such as MRP1 (ABCC1). Thus, the goal of the current study was to investigate the association between genetic variations of ABCC transporters and therapeutic responses to citalopram.

In an initial screen, a possible association between the genetic variations in the MRP1 gene and citalopram response was observed (data not shown) using several SNP markers of ABCC transporters known to be expressed in the brain, including MRP1, and its homologs MRP4 (ABCC4), and MRP5 (ABCC5).[25] The association between MRP1/ABCC1 polymorphisms and citalopram response has been thoroughly investigated through an integrated molecular genetic approach using full panels of MRP1 SNP markers.

II. MATERIALS AND METHODS

1. Subjects

This study was approved by the Institutional Review Board of Korea University Medical Center, Seoul, Korea and Yonsei Medical Center, Seoul, Korea. All blood samples were acquired from each subject after obtaining a written informed consent. Initially, a total of 123 subjects were recruited by the Pharmacogenomic Research Center for Psychotropic Drugs in the Department of Psychiatry, Korea University College of Medicine between January 2004 and December 2005. Trained psychiatrists examined all subjects with the Structured Clinical Interview for DSM-IV and the K-DIGS (Korean version of the Diagnostic Interview for Genetic Studies). The severity of depressive symptoms was assessed by the 21-item Hamilton Depression Rating Scale (HDRS). Patients with a minimum score of 17 on the HDRS and who were at least 18 years of age were enrolled in this study. Patients with primary or co-morbid diagnoses of schizophrenia, schizoaffective disorder, rapid cycling bipolar disorder, alcohol- or substance-related disorders, dementia and mental disorders due to a general medical condition based on DSM-IV criteria within the past 6 months were not included in this study. Patients with serious or uncontrolled medical illnesses were also excluded from the study. A 2-week washout period was applied prior to the beginning of the study when the patients had received previous medications. During the treatment period of eight weeks, all subjects took citalopram daily (10–60 mg) and were not allowed to take other antidepressants. Their clinical symptoms were evaluated regularly at baseline (0), 1, 2, 4, and 8 weeks after treatment. Physicians were allowed to adjust the dosing of citalopram according to the degree of patients' depressive symptoms at each visit. Finally, a total of 64 patients who had

completed their 8-week follow up were included in the analysis. The reasons for dropout are summarized in Table 1. Remission was defined as a state with the HDRS scores lower than 8. In addition, side effects suspected to be associated with citalopram use were checked at relevant time points.

DNA samples from 100 healthy control subjects were randomly selected from the DNA bank of Yonsei Clinical Research Center, Seoul, Korea. The health status of the control subjects was evaluated by routine physical examination, laboratory tests, and radiographic chest postero-anterior (PA) images under 0.02 mSv exposure. To measure the MRP1 mRNA levels of blood cells, fresh peripheral blood samples were taken from an additional 8 control and 19 patient subjects who were treated with citalopram at Yonsei Medical Center, Seoul, Korea. All subjects enrolled in this study gave a written informed consent and had an East-Asian ethnic background of Korean origin.

Table 1. Reasons for dropout during the 8-week follow-up.

Reason	n (%)
Poor compliance[a]	38 (64.4)
Quit medication due to rapid improvement[b]	10 (16.9)
Quit medication due to other medical illness	4 (6.8)
Change medication due to severe adverse drug reaction	4 (6.8)
Change medication due to acute aggravation of depressive symptoms	3 (5.1)
Total	**59 (100.0)**

[a]Any failure in medication more than 48 h was regarded as poor compliance. [b]For ethical reasons, patients were allowed to quit or change medications during the 8-week follow-up period.

2. MRP1 genotyping

DNA was extracted from peripheral blood samples using a purification kit (Qiagen, Hilden, Germany). Genetic variations of

MRP1/ABCC1 in the control Korean population were determined using the DNA direct sequencing method. Fragments covering the entire coding region and flanking intronic sequences of MRP1/ABCC1 were amplified by PCR. A 2.3 kb region upstream from the translation initiation site was also included in the PCR amplification to identify polymorphisms in the promoter region. The PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit and an ABI 3730xl automated DNA analyzer (Applied Biosystems, Foster City, CA). Mutation analyses were performed using Polyphred 5.04 software (http://droog.mbt.washington.edu/PolyPhred.html). Positions of the genetic variations are given according to the recommendations for a nomenclature system for human gene mutations.[26]

Genotype screening of each locus in the control and depression patient subjects was performed by the single base primer extension assay using the SNaPShot Multiplex kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, the genomic DNA flanking the SNP was amplified with each primer pair and standard PCR reagents. One μl of the purified amplification products was added to a SNaPshot Multiplex Ready reaction mixture containing 0.15 pM of each genotyping primer (Table 2.) for the primer extension reaction. The primer extension reaction was carried out for 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. The reaction products were treated with 1 unit of shrimp alkaline phosphatase at 37 °C for 1 h and 72 °C 15 min to remove excess fluorescent dye terminators. One μl of the final reaction samples containing the extension products was added to 9 μl of Hi-Di formamide (Applied Biosystems). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analyzed by electrophoresis in an ABI Prism 3730xl DNA analyzer. Results were analyzed using the GeneScan analysis software (Applied Biosystems).

Table 2. Primer Sequences used in genotyping.

	SNP (Position)	SNP(Location)	rs	Sense primer	Anti-sense primer	Annealing Tm (°C)
1	promoter	g.-1989A>G	rs12929977	CAGGAAACAGCTATGACC	TGTAAAACGACGGCCAGT	60
2	promoter	g.-1841G>A	rs4148330	CAGGAAACAGCTATGACC	TGTAAAACGACGGCCAGT	60
3	exon9	c.1062T>C (N353N)	rs35587	CAGGAAACAGCTATGACC	TGTAAAACGACGGCCAGT	65
4	exon17	c.2168G>A (R723Q)	rs4148356	CAGGAAACAGCTATGACC	TGTAAAACGACGGCCAGT	60
5	intron18	IVS18-30G>C	rs2074087	CAGGAAACAGCTATGACC	TGTAAAACGACGGCCAGT	60
6	exon28	c.4002G>A (S1334S)	rs2239330	CAGGAAACAGCTATGACC	TGTAAAACGACGGCCAGT	65
7	intron28	IVS28-45G>A	rs212087	CAGGAAACAGCTATGACC	TGTAAAACGACGGCCAGT	60
8	intron29	IVS29-13delT	rs4148379	CAGGAAACAGCTATGACC	TGTAAAACGACGGCCAGT	65
9	exon31	c.5462T>A	rs212090	ACCTGGACCTGGAACTGTA	GGTTGCTCACTCTCAGTCTC	60

3. Statistical analysis and haplotype construction

Hardy-Weinberg equilibrium tests were conducted between the patient and control groups and between the remitted and non-remitted groups of patients. The Haploview software package (version 4.0) was used to construct linkage disequilibrium (LD) mapping and haplotype construction.[27] The chi-square test or Fisher's exact test was applied to analyze the categorical values including demographic data and genotype frequencies. An odds ratio (OR) with a 95% confidence interval (CI) was calculated to estimate the effect of a significant allele with clinical data by binary logistic regression method. The cutoff probability value used in this study was set at 0.05. All statistical analyses were performed using SPSS (version 15.0 for Microsoft Windows).

4. MRP1 membrane vesicle assay for citalopram transport

Citalopram and [^{3}H]citalopram were purchased from Sigma (St. Louis, MO) and Amersham (Piscataway, NJ), respectively. MRP1 transport assay using plasma membrane vesicles was performed as described

previously.[28] Briefly, pcDNA3.1-hMRP1 was transfected into HEK293T cells using the calcium phosphate transfection kit (Invitrogen, Carlsbad, CA), and inside-out vesicles were made from the harvested cells 72 h after transfection. Ten μg vesicular protein was used per time point and ATP-dependent uptake of [^{3}H]citalopram (50 nCi per time point) into the membrane vesicles at 37 °C was measured by the rapid filtration system using a FH225V manifold filtration system (Hoefer, San Francisco, CA) and 25 mm Ø glass microfiber filters (Whatman, Maidstone, UK). The filters were pre-soaked with the non-radiolabeled citalopram. Uptake values obtained in the presence of AMP were subtracted by those in the presence of ATP to calculate ATP-dependent uptake. Kinetic parameters of citalopram were determined by measuring the initial rate of [^{3}H]citalopram uptake at three different substrate concentrations. For each substrate concentration, ATP-dependent uptake was measured at 1-min. The K_m and V_{max} values were determined from a Lineweaver Burk plot and V_{max} was then corrected for the amount of total protein in the vesicles (pmol/min/mg protein). The MRP1 inhibitor MK571 (Calbiochem, San Diego, CA) was used to verify the specificity of MRP1-mediated citalopram transport.

5. Real-time PCR for the quantification of MRP1 mRNA

Red blood cells (RBC) were removed from fresh whole blood using the RBC lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). From the remaining white blood cells, total RNA was extracted using a RNA extraction kit (Qiagen), and then RNA was converted into cDNA using a first strand cDNA synthesis kit (Stratagene, La Jolla, CA). Quantitative real-time PCR was conducted with the ABI 7300 system using the ready-made

TaqMan probes according to the manufacturer's protocol (Applied Biosystems). The TaqMan® probe for MRP1 expression (Hs00219905_m1, Applied Biosystems) was located between exon 24-25 and levels of the housekeeping gene β_2 microglobulin (Hs99999907_m1, Applied Biosystems) were used for normalization.[29] The amount of MRP1 mRNA was normalized to that of β_2 microglobulin using the -ΔCT (CT, threshold cycle) method, where $\Delta CT = CT_{\beta 2\ microglobulin} - CT_{MRP1}$. The Kruskal-Wallis test followed by the Mann-Whitney test was applied to analyze the real time PCR results between the genotype groups.

6. mRNA structure modeling and searching the microRNA binding site of MRP1 sequences

MRP1 mRNA structures were constructed using the RNAstructure version 4.5 software.[30] MicroRNA and its binding site in the 3' untranslated region (UTR) of MRP1 were searched at miRBase (http://microrna.sanger.ac.uk/sequences/).[31]

III. RESULTS

1. Demographic and clinical characteristics

The 64 patients who completed the 8-week follow-up were divided into remitted (n=35, HDRS score lower than 8) and non-remitted (n=29, HDRS score over or equal to 8) groups according to the HDRS scores at 8 weeks. Patients in the two groups showed no differences in sex, current age, age at onset, education level, number of previous episodes, or dose of citalopram at 8 weeks (Table 3). Only initial HDRS scores showed a difference between the two groups and the non-remitted group had higher initial HDRS scores by an average of 3.4 (*P*=0.02).

Table 3. Demographic and clinical characteristics of patients.

Characteristics	Overall (N=64)	Remitted (N=35)	Non-Remitted (N=29)	Statistics	*P*
Sex	**No.(frequency)**		**No.(frequency)**		
male	17(0.27)	8(0.23)	9(0.31)	$\chi 2$=0.544	0.461
female	47(0.73)	27(0.77)	20(0.69)		
	mean(SD)[range]		**mean(SD)**		
Age (years)	52.52 (±15.79)[22-76]	51.60 (±16.26)	53.62(±15.40)	t=0.51	0.61
Age at Onset (years)	45.65 (±16.45)[15-75]	45.83(17.14)	45.43(±15.86)	t=0.10	0.93
Education (years)	8.6 (±5.96)[0-18]	9.18(±5.85)	7.93(±6.11)	t=0.82	0.41
No. of Past Episode	0.47 (±1.22)[0-7]	0.29(±0.67)	0.69(±1.65)	t=1.24	0.22
Initial HDRS score	23.09 (±5.60)[17-42]	21.54(±4.10)	24.97(±6.60)	t=2.43	§0.02
Dose of citalopram at 8 weeks (mg)	24.67(±9.11)[10-40]	22.73(±8.01)	27.04(±9.93)	t=1.82	0.08

$\chi 2$, chi square; **SD**, standard deviation; **HDRS**, Hamilton Depression Rating Scale;**p**, p value for differences between remitted and non remitted groups; **§**, p value <0.05

2. Genotype and haplotype comparisons

Although almost 1600 SNPs have been identified to date in the

human MRP1/ABCC1 gene (www.genecards.org), a total of 16 genetic variations in the promoter and coding regions of MRP1/ABCC1 were found by gene scanning in our control population comprised of 100 healthy Koreans. Out of the 16 polymorphisms and mutations, 9 SNPs that showed greater than 5% allele frequency were included in this study for statistical comparisons. The genotype and allele frequencies of the 9 SNPs in the control and patient populations are shown in Table 4. All SNPs were in Hardy-Weinberg equilibrium and there were no significant differences in the genotype frequencies between the control and patient groups. However, in the genotype frequency comparisons between the remitted and the non-remitted patient groups, c.4002G>A showed a significant association with remission state at 8 weeks (P=0.038 in the co-dominant model, P=0.026 in the minor allele dominant model by the Fisher's Exact test). In the allelic analysis, the 'A' allele of this SNP was strongly associated with the remitted group (P=0.005 by chi-square test) and this value reached the conservative Bonferroni significance (α/number of comparison =0.05/9). The statistical power (1-β) for this comparison was more than 0.8 (G*Power version 3.0.5 software package),[32] and the permutation test also showed a statistical significance (P=0.047, Haploview version 4.0 software package). In addition to c.4002G>A, the c.5462T>A polymorphism in the 3'UTR also showed a possible association with remission state in the allelic analysis (P=0.035 by chi-square test).

The pairwise LD analysis and haplotype construction revealed two LD blocks in the MRP1 gene (Figure 1). The two SNPs in the 5' flanking region, g.-1989A>G and g.-1841G>A form the first LD block (LD1), and the four SNPs of c.4002G>A, IVS28-45G>A, IVS29-13delT and c.5462T>A form the second block (LD2). The frequency comparisons among haplotypes

Table 4. Genotype and allele analysis of SNPs in control and patients.

SNP (rs#) position		Control vs. Patients				Remitted vs. Non-Remitted								
		Genotype				Genotype				Allele				
		CTL	PT	χ2	*P*	R	NR	χ2	*P*		R	NR	χ2	*P*
g.-1989A>G	**AA**	17	18	3.13	0.209	10	8	0.03	0.986	**A**	36	29	0.03	0.872
(rs12929977)	**AG**	56	29			16	13		(0.930)					
promoter	**GG**	25	17			9	8			**G**	34	29		
g.-1841G>A	**GG**	21	19	2.93	0.231	11	8	0.28	0.868	**G**	36	30	0.10	0.753
(rs4148330)	**GA**	58	28			14	14		(0.624)					
promoter	**AA**	20	15			8	7			**A**	30	28		
c.1062T>C(N354N)	**CC**	27	20	1.83	0.400	11	9	1.94	0.378	**C**	40	29	0.65	0.420
(rs35587)	**CT**	39	29			18	11		(0.973)					
exon 9	**TT**	33	15			6	9			**T**	30	29		
c.2168G>A(R723Q)	**GG**	91	53	3.42	*0.142	30	23	1.72	*0.491	**G**	64	52	0.12	0.732
(rs4148356)	**GA**	7	10			4	6		(0.499)					
exon 17	**AA**	1	1			1	0			**A**	6	6		
IVS18-30G>C	**GG**	53	39	0.90	*0.676	22	17	0.40	*0.897	**G**	56	45	0.11	0.739
(rs2074087)	**GC**	42	23			12	11		(0.729)					
intron 18	**CC**	4	2			1	1			**C**	14	13		
c.4002G>A(S1334S)	**GG**	78	46	2.26	*0.328	21	25	6.01	***§**0.038	**G**	52	54	7.89	**§**0.005
(rs2239330)	**GA**	19	14			10	4		***§**(0.026)					
exon 28	**AA**	2	4			4	0			**A**	18	4		
IVS28-45G>A	**GG**	67	38	0.91	0.633	18	20	3.38	*0.194	**G**	46	48	3.77	0.052
(rs212087)	**GA**	23	18			10	8		(0.195)					
intron 28	**AA**	9	7			6	1			**A**	22	10		
IVS29-13delT	**TT**	38	21	0.42	0.810	15	6	3.53	0.171	**T**	44	28	2.73	0.099
(rs4148379)	**T/d**	43	30			14	16		(0.060)					
intron 29	**d/d**	17	11			5	6			**d**	24	28		
c.5462T>A(3'UTR)	**TT**	71	42	0.80	*0.671	20	22	4.14	*0.133	**T**	51	51	4.45	**§**0.035
(rs212090)	**TA**	22	18			11	7		(0.117)					
exon 31	**AA**	7	4			4	0			**A**	19	7		

CTL, control; **PT,** patients with citalopram treatment; **R,** remitted group; **NR,** non-remitted group; **χ2,** chi-square; ***P***, p value; *, Fisher's Exact Test was done; The p values were calculated using the co-dominant model. Data in the '( )' represent the p values by the minor allele dominant model. **§** p<0.05

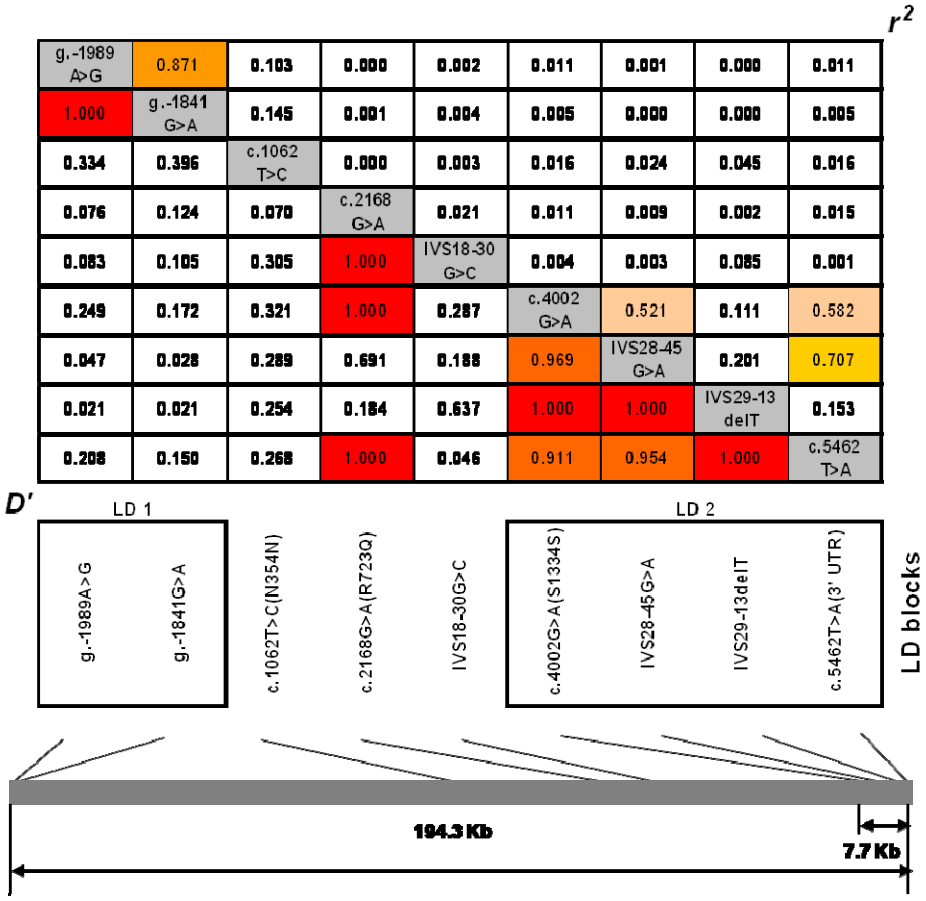

Figure 1. Linkage disequilibrium analysis of the 9 MRP1/ABCC1 SNPs.

Linkage disequilibrium (LD) analysis was performed using the Haploview 4.0 software package. Pairwise LD is demonstrated as r^2 and *D'*. The darker colors of *D'*s, lower left side, represent the closer linkage. The same rule applies to r^2 (upper right side). The LD blocks determined by the Gabriel's rule (confidence intervals) are presented in black boxes. The diagram below the chart indicates the distances between SNPs in the *MRP1/ABCC1* gene.

occurring in the two LD blocks are shown in Table 5. Similar to the results of the genotype analysis, there were no differences in the haplotype frequencies between the control and patient groups. In addition, the two major haplotypes formed in the LD1 block showed no significant differences in frequency between the remitted and the non-remitted patient groups. In the LD2 block, three major haplotypes accounted for approximately 90% of the total population in the control and patient groups. Notably, the third haplotype of 'AATA', which contains both c.4002A and c.5462A, showed an association with remission (*P*=0.014 by chi-square test, *P*=0.033 by permutation test).

Table 5. Haplotype frequencies and analysis.

	Haploytpes							Control vs. Patients				Remitted vs. Non-Remitted			
	LD1		LD2												
	• g.-1989A>G	• g.-1841G>A	• c.4002G>A	• IVS28-45G>A	• IVS29-13delT	• c.5462T>A		CTL (N=100)	PT (N=64)	χ^2	*P*	R (N=35)	NR (N=29)	χ^2	*P*
1	G	A						49.5%	47.5%	0.12	0.725	47.0%	48.3%	0.02	0.886
2	A	G						45.9%	50.8%	0.74	0.391	51.4%	50.0%	0.03	0.872
3			G	G	d	T		39.4%	42.4%	0.26	0.608	35.9%	50.3%	2.67	0.102
4			G	G	T	T		39.3%	30.7%	2.38	0.123	29.2%	32.4%	0.16	0.688
5			A	A	T	A		11.0%	15.6%	1.38	0.240	22.8%	6.9%	6.11	§0.014

Abbrebiation: LD, linkage disequilibrium; **CTL**, controls; **PT**, patients; **χ2**, chi square; **R**, remitted group; **NR**, non remitted group; **P**, p value; **§** p<0.05

The association between the adverse drug reactions of citalopram and MRP1 genotypes was also analyzed. As shown in Table 6, the patients with the 'A' allele of c.4002G>A or c.5462T>A were observed to have experienced fewer side effects from citalopram use (*P*=0.02 and *P*=0.04, respectively).

Table 6. Adverse drug reactions according to the c.4002G>A and c.5462T>A genotypes.

c.4002G>A	with SE	without SE	OR (95% CI)	*P* value
GG	23 (0.89)	22 (0.61)		
GA, AA	3 (0.11)	14 (0.39)	0.21 (0.05 ~ 0.81)	*0.02
c.5462T>A				
TT	21 (0.81)	20 (0.56)		
TA, AA	5 (0.19)	16 (0.44)	0.30 (0.09 ~ 0.97)	§0.04

Sixty two patients who completed the interview on the side effects (SE) of citalopram were included in this analysis. Side effects related to citalopram use were somnolence, headache, inattentiveness/agitation, spasticity, forgetfulness, weight loss/gain, apathy, irregular menstruation and libido decreased/anorgasmia. Patients who reported one or more of the above symptoms at relevant periods were classified as the group 'with SE'. *****, Fisher's exact test was done. §, Chi-square test was done.

3. Logistic regression analysis with clinical data

The relative effect of MRP1/ABCC1 genetic variation on citalopram response was quantified using a logistic regression analysis integrating all available clinical variables (Table 7). These include the patient's age, sex, initial HDRS score, presence of previous episodes, onset age, dose of citalopram at 8 weeks, and education level. Some of these factors have been shown previously to be associated with the outcome of antidepressant therapy.[1] Interestingly, the presence of the 'A' allele at the c.4002G>A locus was found to be the only parameter associated with citalopram response, and none of the clinical variables affected the outcome of citalopram treatment in the present analysis. Patients with the 'A' allele including heterozygotes were 5.8 times more likely to experience remission at 8 weeks (*P*=0.019, 95% CI 1.3~24.9) than the patients with the GG genotype.

Table 7. Logistic regression analysis for remission at 8 weeks.

	OR	95% CI	*P*
Age	0.96	0.905 - 1.011	0.112
Sex	3.57	0.798 - 15.928	0.096
Initial HDRS	0.87	0.747 - 1.022	0.091
Past Episode	0.76	0.168 - 3.448	0.723
Onset age	3.25	0.698 - 15.157	0.133
Citalopram at 8 wks	0.42	0.083 - 2.122	0.294
Education	0.62	0.132 - 2.871	0.536
A allele of c.4002G>A	5.77	1.335 - 24.903	§0.019

OR, odds ratio; **CI**, confidence interval; ***P*** , p value; Age and HDRS analyzed were in continous values. The other values were analyzed with categorical values. The positive categories are: presence of past episode, onset age between 20 and 50 years old, citalopram dose over 20 mg/day at 8 week, education levels higher or equal to college degree, hetero- or homozygote of A allele at c.4002.; **§**, $p<0.05$

4. Membrane vesicular transport assay

In order to identify the molecular mechanisms underlying the association between the MRP1 genetic variation and citalopram response, First, the ability of MRP to transport citalopram was measured. It has been shown previously that P-glycoprotein (MDR1/ABCB1) transports citalopram. However, until now, no data on MRP1 have been available. Figure 2a shows the ATP-dependent and MRP1-mediated citalopram uptake in inside-out membrane vesicles at a fixed citalopram concentration (2 μM), and Figure 2b shows the initial uptake velocities (V_0) at different concentrations of citalopram (0, 1, 2, and 10 μM). Inhibition of more than 90% of citalopram transport by MK571 (100 μM) confirmed the specificity of MRP1-mediated transport (Fig. 2a). The major parameters were determined by Lineweaver-

Burk plot analysis (Figure 2b, inset) and V_{max} was corrected for the total MRP1 protein levels in the vesicles. The results revealed that citalopram is a robust substrate of MRP1 with K_m and V_{max} values of 1.99 μM and 137 pmol/min/mg protein, respectively. These parameters suggest that citalopram can be transported more efficiently than other known MRP1 substrates such as daunorubicin.[33]

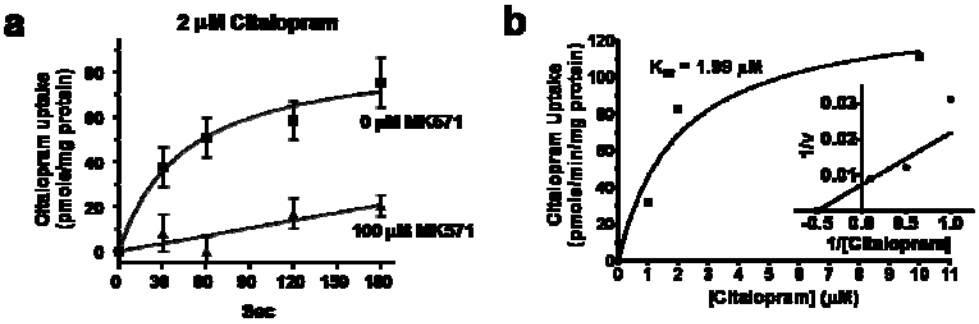

Figure 2. Membrane vesicular transport assay of citalopram.

Inside-out membrane vesicles were prepared from HEK293T cells transfected with pcDNA3.1-hMRP1 and mock (empty pcDNA3.1) plasmids, and ATP-dependent [^{3}H]citalopram uptake was measured. Values obtained with mock transfected cells were subtracted from those with pcDNA3.1-hMRP1 transfected cells to measure MRP1-mediated citalopram transport. **(a)** Time course of MRP1-mediated citalopram uptake at a fixed substrate concentration (2 μM). The MRP1 inhibitor MK571 (100 μM) inhibits 90.4% of MRP1-mediated citalopram transport measured at 30 sec. Data presented are means ± standard deviation (n=8 for 0 μM MK571, n=5 for 100 μM MK571). **(b)** The dose response curve was calculated using V_0s measured with 0, 1, 2, and 10 μM citalopram. All V_0s were calculated based on the time course curve measured at 0, 30, 60, 120 and 180 s using the Graphpad Prism version 4.0 software package. The inset figure represents the Lineweaver-Burk plot transformed from the dose-response curve of arithmetic scales. The estimated K_m and V_{max} values were 1.99 μM and 137 pmol/min/mg protein, respectively.

5. Real-time PCR for quantification of MRP1 expression

Because neither c.4002G>A nor c.5462T>A alter the amino acid sequence of MRP1, It was investigated next whether these polymorphisms could affect MRP1 mRNA transcript levels using a real-time PCR assay (Figure 3). RNA samples were prepared from fresh peripheral blood cells taken from a group of control and patient subjects as detailed in Materials and Methods. A prolonged exposure of cells to xenobiotics can alter the expression levels of drug metabolizing enzymes and transporters.[25] Therefore, the effect of citalopram exposure on MRP1 mRNA levels was analyzed initially. The results in Figure 3a show that there were no differences in MRP1 mRNA levels between the control and patient subjects treated with citalopram. In a subset of patients, blood samples were taken immediately before and 2-weeks after the citalopram treatment, and the MRP1 mRNA levels were compared. Again, citalopram treatment did not significantly affect MRP1 mRNA levels (Figure 3b). Results were then pooled and analyzed according to the genotypes. Interestingly, the presence of the 'A' allele at the c.4002G>A or c.5462T>A locus significantly increased MRP1 mRNA levels (Figure 3c and 3d). The minor allele homozygotes of these two SNPs had approximately two times more MRP1 mRNA than the major allele homozygotes.

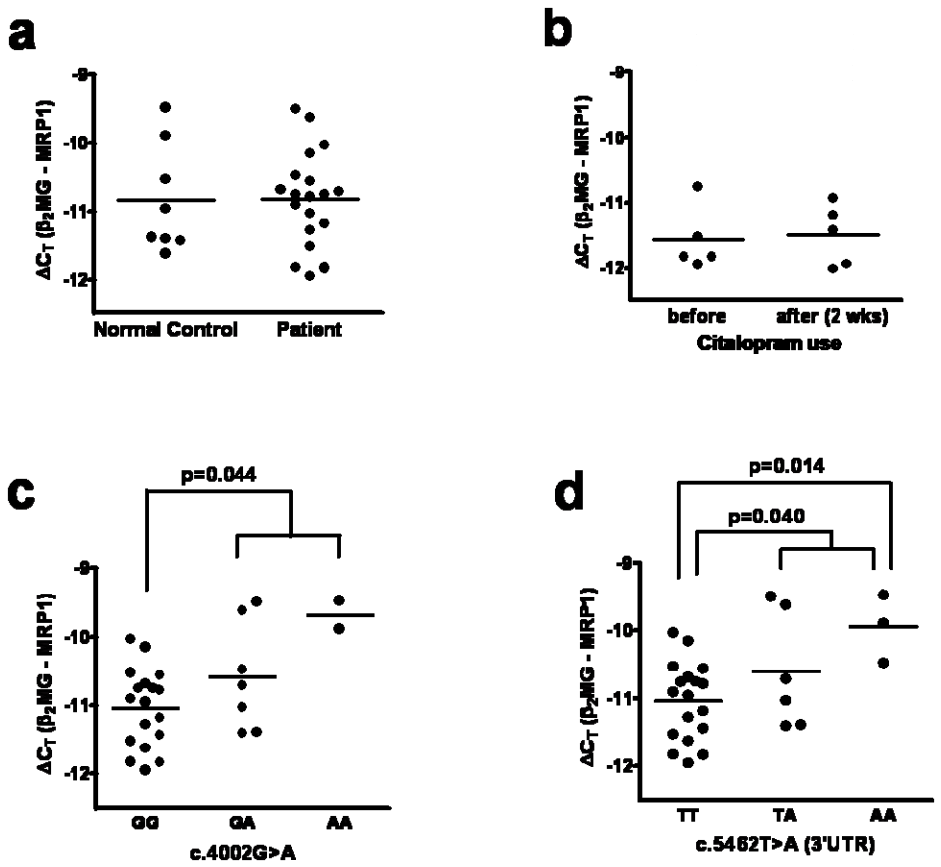

Figure 3. Real-time PCR analysis of MRP1/ABCC1.

RNA samples were prepared from fresh peripheral blood cells, and the amounts of MRP1 and β_2-microglobulin (β_2MG) mRNAs were quantified by real-time PCR using the TaqMan probes. The threshold cycle (C_T) of MRP1 mRNA was normalized to that of β_2-microglobulin. **(a)** MRP1 mRNA levels in the control (n=8) and patient subjects treated with citalopram (n=19). **(b)** In a subset of patients (n=5), MRP1 mRNA levels were compared immediately before and 2 weeks after citalopram treatment. **(c)** MRP1 mRNA levels according to the c.4002G>A genotype. **(d)** MRP1 mRNA levels according to the c.5462T>A genotype. The Kruskal-Wallis test followed by the Mann-Whitney test was applied to analyze real-time PCR results between genotypes.

IV. DISCUSSION

This report is the first to show that MRP1/ABCC1 polymorphisms can affect the outcome of citalopram treatment and that citalopram is a substrate of MRP1. Previously, it has been suggested that polymorphisms in the MDR1/ABCB1 gene can affect the citalopram response.[23,34] In addition, there is some evidence that citalopram can be exported by MDR1/ABCB1 at the BBB and BCSFB.[35] In the case of MDR1/ABCB1, numerous studies have reported the association between genetic polymorphisms and the response of antidepressants. For example, c.3435C>T and c.2677G>T/A in MDR1/ABCB1 were reported to be associated with the clinical responses to paroxetine.[36] Most of the studies have inferred that polymorphisms in MDR1/ABCB1 may decrease treatment response by potentiating an adverse drug reaction or by lowering drug levels at the target site. Of interest, our present results integrating the population genetic data with functional data measuring MRP1 mRNA levels indicate that increased MRP1/ABCC1 expression by genetic polymorphisms would be associated with positive citalopram response.

The location of transporters at the BBB and BCSFB is a critical factor for determining their physiological roles and estimating the effects of transporter polymorphisms on drug responses. It is widely believed that MDR1/ABCB1 is expressed in the apical membrane of endothelial cells at the BBB. In contrast, the most recent studies using immunostaining of brain tissues and primary cultures indicate that MRP1 is expressed in the basolateral membrane of human and rat BBB cells.[7,15] Based on the assumption that MRP1 is located at the basolateral side of BBB endothelial cells, increased MRP1 expression would be expected to increase citalopram levels in the brain, especially in the ISF where serotonin transporters, the

target of citalopram, exist (Figure 4). Serotonin is metabolized to 5-hydroxylindoleacetic acid (5-HIAA) in presynaptic cells after reuptake by serotonin transporters. It has been reported that decreased 5-HIAA in CSF correlates with a positive clinical outcome after treatment with antidepressants such as citalopram.[35] Moreover, a high citalopram level in CSF is associated with both a decreased 5-HIAA level in CSF and a positive treatment response,[35] suggesting that the quantity of citalopram in the target region determines the drug's ability to effectively inhibit the serotonin reuptake pump.

In other point of view, increased citalopram levels in the brain may be associated with adverse drug reactions. However, the patients with the 'A' allele of c.4002G>A or c.5462T>A experienced fewer side effects from citalopram use (Table 6). It has been reported that MRP1 is found in astrocytes and neuronal cells,[37,38] and that citalopram can induce cellular apoptosis.[39,40] Therefore, a higher expression of MRP1 may potentially protect neurons and astrocytes by lowering intracellular citalopram levels (Figure 4). In this case, the experience of fewer side effects can help the patients tolerate the citalopram treatment and continue their medication for up to eight weeks for a beneficial outcome. However, there was no significant correlation between the remission state and the side effects experienced in this study (r^2 = 0.186, P=0.148 by Pearson Correlation Analysis). Therefore, the positive citalopram response appears to have been an independent phenotype rather than being secondary to the experience of fewer side effects. It is also possible that MRP1 polymorphisms may affect the blood concentration of citalopram due to its wide tissue distribution, especially in pharmacokinetically important organs such as kidney.[25] Future studies examining the pharmacokinetic profiles of citalopram based on

MRP1 genotype will greatly help to elucidate underlying mechanisms of MRP1 polymorphism-associated citalopram response and address some of the limitations of the present study, which neither determined the blood concentrations of citalopram nor measured expression levels of MRP1 in brain cells.

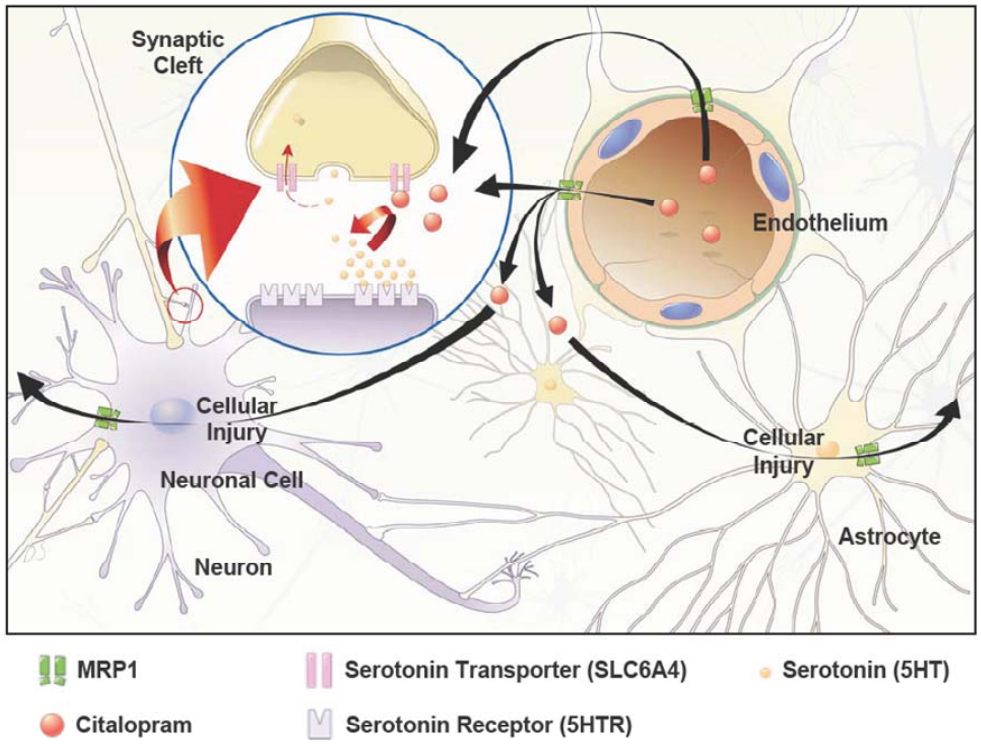

Figure 4. Putative functions of MRP1 during citalopram transport in the brain.

In the endothelial cells of the blood brain barrier (BBB), MRP1 transports citalopram from plasma to interstitial fluid (ISF) and maintains appropriate drug levels at the target site where serotonin transporters exist. On the other hand, MRP1 exports citalopram from neurons and astrocytes to reduce drug-induced cytotoxicities.

The frequencies of the 4 SNPs, which constitute the LD2 block, show differences among ethnic groups and their minor allele frequencies are reported to be lower in Asians than in Caucasians.[41,42] Even in the same East Asian groups, the minor allele frequencies show considerable variation. For example, the 'A' allele of c.4002G>A was found more frequently in the Korean population (0.14) than in the Chinese (0.11) and less frequently than in the Japanese (0.20) population.[43] Therefore, it would be of interest to investigate the association between the citalopram response and MRP1/ABCC1 genetic variations in other populations. In this study, the 'A' allele of c.4002G>A was associated with a higher MRP1 mRNA level (Figure 3). This result does not agree with a previous report that several MRP1 SNPs, including c.4002G>A, do not affect the MRP1 mRNA levels in a Caucasian population of German origin.[42] The reasons for this discrepancy are currently unknown although different haplotype structures between the Korean and German populations may be involved. The haplotype associated with the positive citalopram response contains two other minor allele variants, IVS28-45G>A and c.5462T>A, in addition to c.4002G>A (Table 5, LD2 haplotype 'AATA'). Because SNPs in LD2 are not in perfect linkage, it is possible that c.4002G>A may link with different alleles at these loci in the German population, and the proportion of the functional haplotypes responsible for the positive citalopram response would be different from the Korean population.

An important remaining question is the molecular mechanism underlying the increased MRP1 mRNA levels in the LD2 'AATA' haplotype. The lack of the amino acid change by the synonymous SNP c4002G>A (S1334S) prompted a reevaluation of other functional variations linked with c4002G>A. First, SNPs located around the exon-intron junctional boundary

or the branch point site are potent candidates for causing alterations in mRNA levels by alternative splicing or other mechanisms. However, IVS28-45G>A at the exon 28-29 splice junction does not appear to be a functional variation, because IVS28-45G>A did not cause a splicing variant in the reverse-transcription PCR assay that can detect splicing events between exons 26 and 31 (data not shown). In addition, the -45 G/A locus from the starting nucleotide of exon 29 is far from the putative splice branch site at the exon 28-29 splice junction, and IVS28-45G>A did not show a significant association with citalopram response according to an analysis of the single locus (Table 4).

The second possibility is that genetic variations in the 3'UTR region could alter mRNA levels by affecting mRNA stability or by modulating interactions with microRNAs. Indeed, an *in silico* analysis using RNAstructure software package (version 4.5)[30] revealed that c.5462T>A, but not c.4002G>A, could greatly alter the MRP1 mRNA secondary structure (Figure 5). The calculated free energy level of total MRP1 mRNA with the 'A' allele at c.5462 was approximately 1.3 Kcal/mol lower than that with the 'T' allele. On the other hand, c.4002G>A did not induce a change in the mRNA structure or free energy levels. In general, mRNAs that have an extended half-life with substantial stability are expected to produce more protein during the turnover of mRNAs.[44] Thus, a more stable mRNA structure with the c.5462A allele may explain the functional association with the citalopram response.

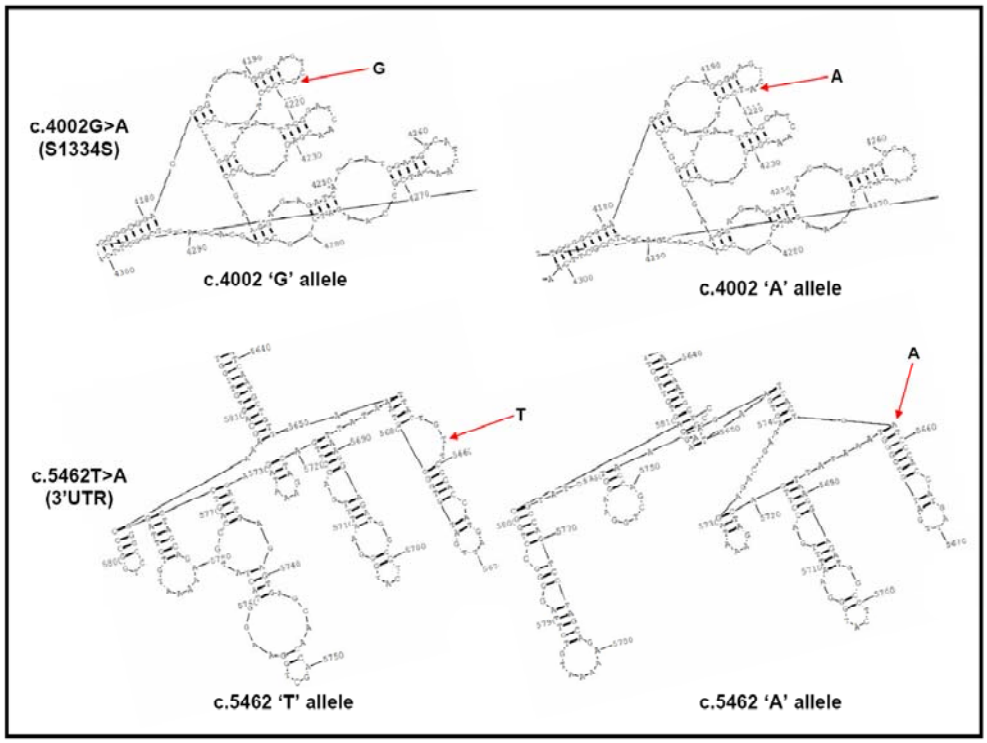

Figure 5. mRNA structures according to the c.4002G>A and c.5462T>A genotypes.

RNA structures were predicted by the *RNAstructure version 4.5* software package. All mRNA sequences were included in the structure construction, but only structures around the designated SNPs are presented. The c.4002G>A variation did not cause a change in the calculated free energy levels. The c.5462T>A variation lowered free energy levels from -2332.3 Kcal/mol to -2333.6 Kcal/mol.

Recently, there has been growing interest on the role of microRNA affecting the 3'UTR.[45] At present, more than 500 microRNAs have been identified in the human genome. These are ~22 nucleotides in length and represent conserved homologous sequences across several species.[46,47] Interestingly, the hsa-miR-541 microRNA (HGNC:MIRN541, miRBase, http://microrna.sanger.ac.uk/) has a partial complementary sequence to regions in flanking c.5462 (from c.5449 to c.5470, Figure 7). The seed pairing between this microRNA and the MRP1 3'UTR was perfect, and the estimated free energy was -16.7 Kcal/mol in the c.5462T allele and -14.4 Kcal/mol in the c.5462A allele. Considering the critical free energy level for binding between the template RNA and microRNA is -15 Kcal/mol, the possible low association of hsa-miR-541 with c.5462A may inhibit the degradation of MRP1 mRNA by the microRNA-associated complex.[48,49] This possibility is currently under investigation.

One of the largest cohorts examined for citalopram response is described in the Sequenced Treatment Alternatives for Depression (STAR*D) study.[50] Many genetic variations associated with citalopram response, including several SNPs in the genes that influence pharmacokinetics such as ABCB1, CYP2D6, and CYP2C19, were identified in the STAR*D cohort using a candidate gene approach.[51] In addition, a recent genome-wide association study using samples in the STAR*D cohort suggested that SNPs in the genes encoding papilin and interleukin receptor are associated with citalopram response.[52] However, drawing pharmacogenetic conclusions from the STAR*D study also has some limitations, including the multiethnicity of the cohort and the failure to evaluate patient compliance.[51] In addition, because minimal exclusion criteria were applied, the STAR*D cohort consisted of individuals with concomitant psychotropic medications or

individuals who were not drug naïve. To minimize these problems in the present study, it was needed to establish a cohort consisting of ethnically homogenous Korean descendents and drug naïve patients with strict exclusion criteria. Patients were also treated with single agent citalopram and compliance was carefully monitored.

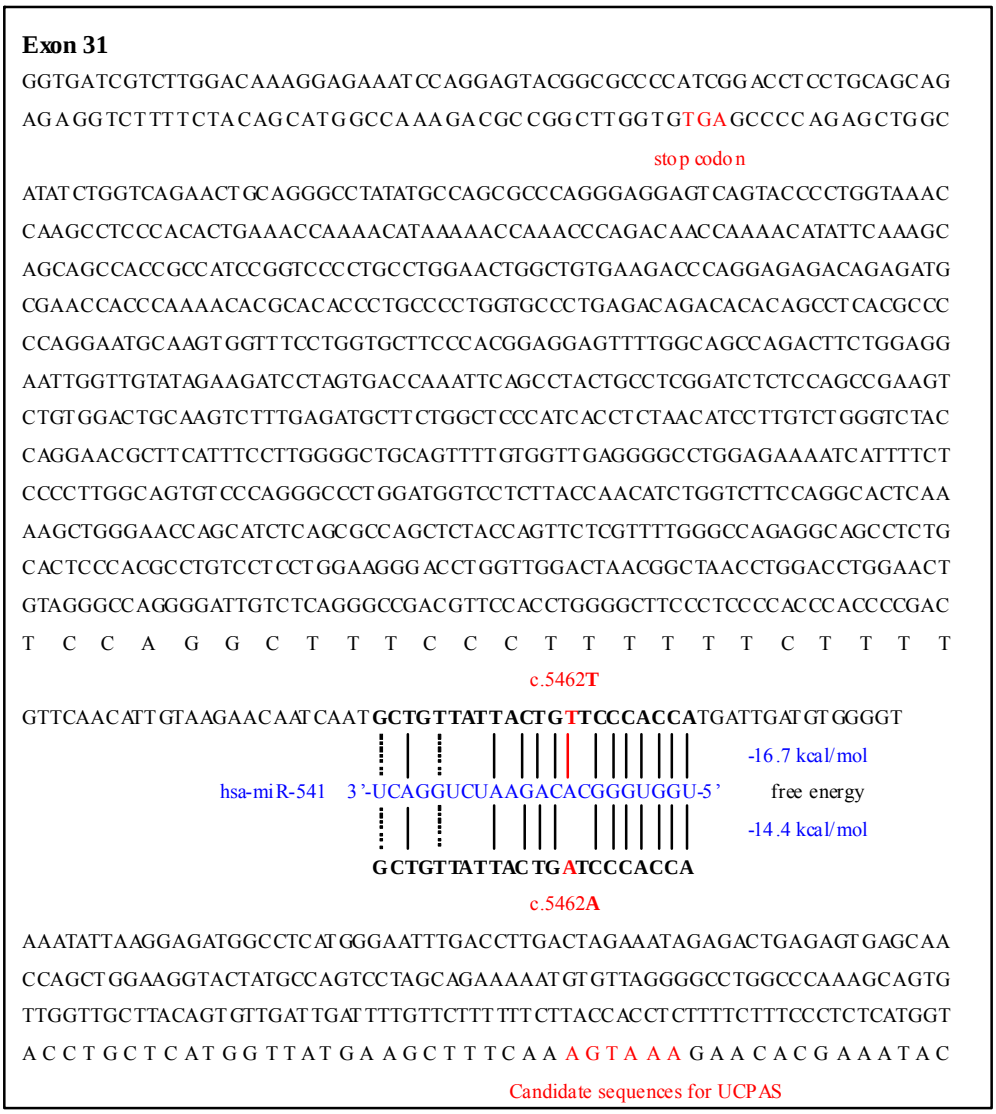

Figure 6. MRP1 (ABCC1) 3' UTR sequences (NM_004996) and the predicted microRNA binding site.

UCPAS, upstream core polyadenylation signal. The free energies were calculated using the *RNAstructure version 4.5* software package. The vertical bars presented indicate complementary relationships between 3'UTR and the has-miR-541 microRNA.

Some subgroup analyses were also performed. For example, among the 59 dropout patients, 7 patients stopped citalopram treatment due to acute aggravation of depressive symptoms or severe adverse drug reactions, whereas 10 patients quit citalopram medication before 8 weeks because of rapid improvement of depressive symptoms (Table 6). These patients could be categorized into non-remitted and remitted, respectively, and thus a genetic association analysis performed. Again, the result suggested that c.4002G>A is associated with citalopram response. In addition, the association analysis using the interim data measured at 4 weeks after citalopram treatment showed the results similar to that after of 8 weeks treatment. However, since multiple analyses increase the risk of false positives (type I error), only the results of 8-week follow-up were used to minimize as initially intended. Although statistical values in this study satisfied significances corrected for multiple testing such as the Bonferroni test, permutation test, and logistic regression analysis, the small sample size of this study may still be a limitation to generalizing the current association result. Nevertheless, the present study is strengthened by the fact an integrated approach was employed that identified possible disease-associated variations from the population data and then verified the function of their gene products at the molecular level. It is believed that this kind of method is an effective approach to assess the clinical relevance of gene variations and to overcome statistical limitations.

V. CONCLUSION

Context : MRP1, as a xenobiotics efflux pump is expressed highly in the BBB and BCSFB and has been implicated in altering the treatment response to psychotropic drugs such as antidepressants.

Objective : The aim of this study is to investigate the association between MRP1 polymorphisms and treatment responses of citalopram, an antidepressant, in the depressive disorder.

Design : MRP1 gene was screened to find significant variations in Korean population, then selected 9 SNPs with frequencies more than 5 %. These SNPs were analyzed in the 123 population based patients who had been administered citalopram for 8 weeks to treat their major depressive disorder. The integrated molecular genetic approaches were used to support the association between MRP1 polymorphisms and citalopram response.

Results : 1. c.4002G>A, located on exon 28**,** showed a significant association with the remission state at 8 weeks. 2. Haplotype analysis of LD block including c.4002G>A and c.5462T>A demonstrated statistical significance. 3. MRP1 enriched vesicular membrane assay showed citalopram is a substrate of MRP1. 4. The mRNA expressions of MRP1 were changed according to the genotypes of c.4002G>A and c.5462T>A. 5. The LD block formed with c.4002G>A and c.5462T>A could change mRNA structural stability of MRP1 and affect its expression.

Conclusion : It is suggested that the different expression of MRP1 from its genetic variation in the BBB and BCSFB could alter the individual responses of antidepressant in the depressive disorder.

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ABSTRACT (IN KOREAN)

우울병의 치료반응과 MRP1 유전 변이와의 연관성

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이성희

MRP1(multidrug resistance protein 1, ABCC1)은 ABC(ATP Binding Cassette) 수송체 계열의 일종으로 생체이물(xenobiotics)을 수송하며, 이를 세포 외로 배출하는 역할을 주로 하는 것으로 알려져 있다. MRP1은 다양한 종류의 세포막에 존재하지만 혈액뇌장벽(BBB, blood brain barrier)과 혈액뇌척수액장벽(BCSFB, blood cerebrospinal fluid barrier)에도 위치하여 항우울제와 같은 정신질환 약물의 치료반응에 영향을 줄 것으로 여겨져 왔다. 이 번 연구는 MRP1의 유전적 다형성이 우울병 치료에 사용되는 항우울제 citalopram의 반응과의 연관성을 규명하는 데 있다.

123 명의 우울증 환자를 대상으로 citalopram 단일 약물 치료를 한 후 이중 8주간의 추적 관찰과 약물 치료를 완료한 64명을 대상으로 정상대조군 100명과 비교 분석 하였다. 한국인 인구집단에서 빈도가 5% 이상인 9 개의 SNP(single nucleotide

polymorphism)을 이미 조사하고 선별하여 정상대조군과 8주 치료를 완료한 64명을 대상으로 유전자 분석을 하였다. 이 후 관련 분자생물학적 연구를 진행하였다.

결과로서, 1. MRP1 유전자의 28번째 exon에 위치한 SNP c.4002G>A가 citalopram 8주 치료 후의 관해(remission) 상태와 강한 연관성을 보였다(P=0.005, O.R. 4.7, 95% C.I. 1.5~14.7). 2. SNP c.4002G>A 는 c.5462T>A (3' UTR에 위치)를 포함한 3개의 SNP와 더불어 LD(linkage disequilibrium) block을 형성하였고 8주 치료 후 관해 상태와 연관성을 분석하였을 때 통계적으로 유의한 결과를 보여주었다(P=0.014). 이후 진행한 분자생물학적 연구에서, 3. MRP1을 과발현 시킨 inside-out membrane vesicle을 이용한 수송 역학(kinetic) 실험을 통해, citalopram이 MRP1의 기질임을 밝혔다(K_m=1.99 μM, V_{max}=137 pmol/min/mg protein). 4. MRP1 mRNA의 양이 c.4002G>A와 c.5462T>A SNP의 유전형에 따라 말초혈액 세포에서 다르게 나타남을 확인하였다. c.4002G>A와 LD를 이루는 c.5462T>A의 유전적 변이에 의해 mRNA의 구조적 변화와 이에 의한 안정성의 차이로 양적인 변화가 생기는 것을 이해할 수 있었다.

본 연구를 통해 BBB와 BCSFB에 존재하는 citalopram 수송단백인 MRP1의 유전적 다형성으로 인해 우울병의 약물치료 반응을 예측할 수 있음을 제시 하였다.

핵심 되는 말: MRP1/ABCC1, citalopram, 관해, 우울병, ABC 수송단백

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