ABCC5 Gene Variant c.1146A>G Reduces MRP5 Expression and Can be a Potential Marker to Manage Cilostazol Induced Headache

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Abstract

Background: Patients taking cilostazol, a representative phosphodiesterase type III inhibitor used for vasodilation, occasionally complain of headaches. Cerebral arteriolar relaxation, due to an increase in intracellular cAMP or cGMP, is believed to be associated with a severe form of cilostazol-induced headaches. Multidrug resistance protein 5 (MRP5) is an important regulator of cAMP and cGMP could be a regulatory protein of this cilostazol induced headache.

Methods: The response to cilostazol on the basis of MRP5 genetic variations was studied in phase-I clinical trial including 101 healthy Korean individuals. Quantitative real time PCR, Western blot, confocal analysis and drug transporter assay was performed to detect and evaluate the activity of MRP5. Statistical analysis was performed by using SPSS 18.0 and GraphPad Prism 4.0 software.

Results: Population genetic and pharmacokinetic analyses indicated that a group of MRP5 genetic variations in linkage disequilibrium with c.1146A>G (rs7636910) is associated with the no or mild forms of cilostazol-induced headaches, but did not affect the systemic distribution of cilostazol or its metabolites. Quantitative real-time PCR and pyrosequencing assays of blood cells revealed that individuals with the c.1146G allele, a protective allele against cilostazol-induced headaches, had a 0.68-fold lower mRNA expression of MRP5 than that of individuals with c.1146A allele. In addition, in a gene transfection experiment using MDCKII cells, the G variant was found to be reduced the mRNA and protein expression of MRP5.

Conclusion: These results suggest that MRP5 has an important role in the regulation of cilostazol-induced chronic headache and the c.1146A>G variation could be a potential marker to manage/treat cilostazol-induced headaches.

Trial registration: The trial is registered at www.clinicaltrials.gov on (October 20, 2011) and the number is NCT01455558.

Background

Cilostazol is a representative phosphodiesterase (PDE) type III inhibitor that is used widely to mitigate ischemic symptoms in patients with intermittent claudication and as an antiplatelet agent for stroke prevention [1, 2]. The post-marketing surveillance has identified several adverse drug reactions (ADRs) of cilostazol that occur in the cardiovascular, digestive, nervous, respiratory, urinary systems, and in skin. Additionally, headaches were the most frequently reported reason for cilostazol withdrawal [1, 3]. Several studies reported that cilostazol induced a migraine-like headache. Those reports suggested the mechanism of headache was due to accumulation of cyclic adenosine monophosphate (cAMP) in brain cells that induced by cilostazol [4-6]. The PDE III enzymes catalyzes the degradation of both cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Therefore, cilostazol, an inhibitor of PDE III, can increase the intracellular levels of cAMP and cGMP. Both of cGMP and cAMP, which are synthesized in the nitric oxide (NO)-guanylate cyclase pathway and adenylate cyclase pathway,

respectively, can cause headaches resulting from cerebral vascular dilation through regulating the intracellular status of cyclic nucleotides in the vascular smooth muscle [7-9].

The ATP-binding cassette (ABC) transporter superfamily is a group of transport proteins located in the cell membrane that mediate the extracellular transport of various endogenous and exogenous substances. Thus, ABC transporters are considered as one of the main causes of drug resistance mechanism as they alter the intracellular pharmacokinetics of substrate drugs [10]. Multidrug resistance proteins (MRPs) are members of the ABC transporters family. Of the transporters, MRP4 and 5 have been shown to mediate ATP-dependent cGMP efflux in the cerebral arteries and peripheral tissues [8, 11]. In fact, it was reported that overexpression of MRP4 and 5 caused resistance to nucleoside analog drugs in cancer chemotherapy and antiviral treatment [12, 13]. If this is the case, it can be hypothesized that the MRP4 and 5 proteins may also be involved in headaches associated with the nucleoside analog-mediated mechanism. Although cilostazol is not a nucleoside analog, metabolism and disposition of this drug and its metabolites are partly regulated by MRP4 and thus also can be by MRP5 [14]. Therefore, the function or expression of MRP4 and 5 is likely to affect the occurrence or extent of cilostazol-induced headaches. However, the genetic polymorphisms in genes encoding nucleoside analog drug transporters are a known cause of individual variation associated with the development of headaches, there is no report yet regarding these proteins related occurrence of headaches [15, 16]. In the present study, the association of MRP5 genetic variations with the occurrence of headache induced by cilostazol treatment is evaluated in 101 healthy Korean participants. The study was also investigated a predictable marker causing cilostazol induced headaches, which may help the physician to take decision about treatment strategy.

Methods

The study plan was reviewed and approved by the institutional review board of Severance Hospital in the Yonsei University Health System, Seoul, Korea and the trial was registered at www.clinicaltrials.gov (the clinical trial registry number is NCT01455558).

Pharmacokinetics

Six separate clinical trials comparing the PK profiles between newly developed sustained release (SR) formulations and an immediate release (IR) formulation of cilostazol in healthy Korean participants were completed at the Yonsei University Severance Hospital (Seoul, Korea) from August 2008 to August 2009, however the data only from IR formulation were used in this study [17]. In brief, each participant received two IR (100 mg × two tablets, BID) formulations of cilostazol (PLETAAL), with one tablet administered every 12 h. Venous blood samples were collected into heparin tubes at 0, 1, 2, 3, 4, 6, 8, 10, 12, 13, 14, 15, 16, 18, 20, 22, 24, 36, 48, and 72 h after drug administration. The plasma concentrations of cilostazol and its metabolites (OPC-13015 and OPC-13213) were measured by using liquid chromatography tandem mass spectrometry (LC-MS/MS), and PK profiles, including the parameters C_{max} , AUC_{inf} , and $t_{1/2}$, were computed by using Phoenix WinNonlin version 5.3 (Pharsight Corporation, Mountain View, CA).

Participants

The volunteers who had participated in this clinical trials were healthy Korean males or females between 19 and 55 years of age, within 20% of their ideal body weight, and without congenital abnormalities or chronic diseases (17). Informed consent for the analysis of their genetic information was obtained from all 101 participants. In total, 33 volunteers that gave consent for MRP5 mRNA analysis were newly recruited, in accordance with the same criteria as those of clinical trials, because this study using clinical trial data was planned retrospectively.

Adverse Drug Reactions

Basic vital signs (blood pressure, body temperature, and pulse rate), a personal symptom interview, electrocardiograms (ECGs), pregnancy tests (human chorionic gonadotropin in blood) for females, and laboratory tests (hematology, blood chemistry, and urinalysis) were performed at appropriate times. An ADR was defined as any unfavorable result in the checked information and was recorded on the case-report forms [17]. All participants who had enrolled in the clinical trials were questioned about headaches by using the 10-point VRS during a personal interview. The headache intensity was categorized into four groups, no headache (0), mild headache (1–3), moderate headache (4–6), and severe headache (7–10) according to the numeric rating scale (NRS) used in an earlier report (18). For statistical analysis, the four groups were re-categorized into two groups: no or mild headache (0–3), and moderate or severe headache (4–10).

gDNA, RNA extraction, and cDNA synthesis

gDNA was extracted from fresh human whole blood by using ExgeneTM Blood SV mini kit (GeneAll, Seoul, Korea) in accordance with the manufacturer's instructions. Leukocytes were obtained from fresh human whole blood using erythrocyte lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). Total RNA was extracted from leukocytes by using TRIzol Reagent (Invitrogen, Carlsbad, CA), and RNA was converted into cDNA using an AccuScript High Fidelity 1st-Strand cDNA Synthesis Kit (Stratagene, Santa Clara, CA) in accordance with the manufacturer's instructions.

Gene scanning and genotyping

A promoter region of –2.0 kb from the downstream transcription start site, all exons in their entirety, and the flanking intronic sequences of the MRP4 and MRP5 genes were scanned by a DNA direct sequencing method using the ABI Prism 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. Mutations were analyzed using Phred, Phrap, Consed, and Polyphred 5.04 software (<http://droog.mbt.washington.edu/PolyPhred.html>). Haplotype and LD structure were constructed by using the Haploview software package (version 4.0; Broad Institute of MIT and Harvard, Cambridge, MA). SNPs were genotyped with the SNaPshot assay using the ABI Prism 3730xl Genetic Analyzer, and the results were analyzed by using GeneScan analysis software (Applied Biosystems).

Allele quantification for c.1146A>G of MRP5

The allele quantification ratio was compared between the A and G alleles at c.1146A>G of MRP5 gDNA and cDNA using the PyroMark Q24 system (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions (sequencing primer for gDNA: 5'-CATTTTCTCAGAGTGTTC-3', for cDNA: 5'-CTCCTCGCGGATTTT-3').

The following primers were used for PCR: Forward primer for gDNA, 5'-ATCAAAATGTATGCCTGGGTCAA-3', reverse primer for gDNA (5' biotinylated): 5'-AAAGAATCCAAGGTCCTTCTACCA-3'; forward primer for cDNA (5' biotinylated): 5'-TGCCTGGGTCAAAGCATTTT-3', reverse primer for cDNA: 5'-ATCGAAGCCCAGGGTCAT-3'. PCR was conducted using the PyroMark PCR Kit (Qiagen) by following cycling conditions: 15 min at 95°C; 45 cycles of 30 s at 94°C; 30 s at 56°C; and 30 s at 72°C; followed by a final cycle at 72°C for 10 min.

Cell management

HEK cells were maintained at MEM (LM007-07, Welgene) with 10% FBS (16000044, Gibco) and 1% Penicillin-streptomycin solution (LS202-02, Welgene) in the humidified 5% CO₂ incubator.

Immunoblotting

HEK293 cells (1×10^6 cells) were seeded in 60 mm plates at the day before transfection and then transfected for 36 h by using Lipofectamine 2000. The cells were washed with ice-cold phosphate-buffered saline (PBS), lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing 0.5% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM Tris-hydrochloride (HCl, pH 7.4), and protease inhibitors. The protein concentration was determined by using Bio-Rad protein assay dye reagent. The proteins were separated by using SDS-polyacrylamide gel electrophoresis (PAGE) in a 4%–12% gradient gel (Invitrogen) and transferred to PVDF membranes (Amersham Biosciences). Non-specific binding of protein to the membranes was blocked by 5% nonfat skim milk for 1 h incubation at 37°C. The membranes were washed three times (each of 10 min) with tris-buffered-saline (TBS) containing tween 20 (0.1 %) and probed with anti-MRP5 (1:500, sc-20769, Santa Cruz Biotechnology), anti-beta actin antibodies (1:10,000, A5441, Sigma) for 30 min at 37°C. The membranes were washed three times again as mentioned before and incubated with horseradish-peroxidase-conjugated anti-mouse IgG secondary antibody (1:2,000, Santa Cruz) for 2 h at room temperature. The membranes were then exposed to a medical x-ray film using the enhanced chemiluminescence (ECL) system (Santa Cruz).

Immunocytochemistry

HEK293 cells (1×10^6 cells) were seeded in 60 mm plates containing two microscope cover glasses at the day before transfection. The cells were then transfected with cDNA constructed for wild type (A allele) and mutant (G allele) MRP5 gene by using Lipofectamine 2000. The cells were incubated for 36 h, then culture medium was removed and the cells were fixed with 4% paraformaldehyde in PBS at 4°C for 15

min. After that, paraformaldehyde was removed the cells were incubated with blocking solution (3% BSA and 1% normal horse serum in PBS) for 1 h at 37°C. The cells were then incubated with rabbit polyclonal anti-human MRP5 (1:100, sc-20769, Santa Cruz) and mouse monoclonal anti-human P-cadherin (1:100, ab6528, Abcam) in blocking solution at 37°C for 30 min. After removal of the solution containing the primary antibodies, the cells were incubated with anti-rabbit FITC (1:200, sc-2012, Santa Cruz) and anti-mouse Alexa Fluor 546 (1:200, A11003, Life Technologies) in blocking solution at room temperature for 1 h. After washing in PBS, the cells were mounted on slides and were visualized under a confocal microscope (LSM700, Carl Zeiss, Germany).

Statistical analysis

SPSS 18.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA) software packages were used for statistical analyses. All genotyped SNPs underwent Hardy-Weinberg equilibrium testing before case-control analysis. Categorical data were evaluated by using the chi-square and Fisher's exact tests, whereas ANOVA, *t*-test, and Kruskal-Wallis test were applied to continuous data. A significant effect of a certain genotype on the clinical phenotype was estimated as an OR with a 95% CI by using a binary logistic regression method. The fundamental standard *P* value of significance was based on two-sided comparisons and was selected as 0.05. However, the *P* value was adjusted with Bonferroni's correction when multiple statistical analyses were performed at once.

Results

Demographic and clinical characteristics of healthy volunteers

A 10-point verbal rating scale (VRS) was used to evaluate the severity of headaches. In total, 101 healthy human volunteers who had received a standard preparation of cilostazol (PLETAAL) as participants of bioequivalence trials of new cilostazol regimens were divided into two groups: 1) those who experienced no or mild headaches (*n* = 68, VRS score 0–3); and 2) those who experienced moderate to severe headaches (*n* = 33, VRS score 4–10). The baseline demographic characteristics were not statistically different between the two groups (Table 1). The mean age of the volunteers was 24.8 years, and 84 (83.2%) were male. The mean height and weight were 173.2 cm and 67.3 kg, respectively. The number of participants those regularly smoked and consumed alcohol were 27 (26.7%) and 71 (70.3%), respectively.

MRP5 variations associated with cilostazol-induced headaches

Although almost 1360 single nucleotide polymorphisms (SNPs) have been identified in the human *ABCC5* gene (the gene that codes for the MRP5 protein), the variant selected in this study was yet to find any association with cilostazol induced headache (www.genecards.org). Thirteen genetic variations in the promoter and coding regions of MRP5 were found in our 101 clinical trial participants by gene scanning (Fig. 1). Of these 13, three SNPs (c.222G>A, c.2714T>G, c.2847C>T) were excluded from the study because of their low frequency (minor allele frequency <5%). The c.4896G>A and c.5557A>G SNPs located in the 3' untranslated region had perfect linkage disequilibrium (LD) in the Korean population (D'

and $r^2 = 1$); c.4896G>A was used as a tag SNP. The genotype frequencies of each polymorphism are shown in Table 2. The genotype distributions at all loci were consistent with Hardy-Weinberg equilibrium (each $P > 0.05$). Of the nine SNPs tested, four SNPs (c.1146A>G, c.1147+94G>A, c.3624C>T, and c.4896G>A) showed a strong association with headaches in individuals administered cilostazol ($P = 0.005, 0.004, 0.001$, and 0.007 , respectively, in the co-dominant model). Of these, the values for three SNPs, c.1146A>G, c.1147+94G>A, and c.3624C>T, which were in LD (Fig. 1), remained significant after conservative Bonferroni correction for multiple testing ($\alpha/\text{number of comparison} = 0.05/9$).

Logistic regression analysis with clinical variables

The G allele of c.1146A>G was associated with a lower chance of headaches than the A allele. The relative effect of MRP5 genetic variation on cilostazol-induced headaches was quantified by using logistic regression analysis that integrated all available clinical variables (Table 3). The presence of the G allele at the c.1146 locus was the only variable that significantly affected the incidence of cilostazol-induced headaches ($P = 0.003$, odds ratio (OR) of G allele vs. A allele: 0.36, 95% confidence interval (CI): 0.18–0.71). When other factors were considered together with the presence of G allele, they did not affect the cilostazol-induced headaches.

The A and G alleles of c.1146A>G contributed differently to MRP5 mRNA expression

As three SNPs showed statistically positive associations with headaches, real-time PCR was performed to identify relevant functional variations by using RNA samples from fresh peripheral blood cells. As shown in Figure 2, the MRP5 mRNA expression of three SNPs (c.1147+94G>A, c.3624C>T, and c.4896G>A) was not statistically different by genotype. However, for the c.1146A>G variation, a difference in MRP5 mRNA expression was observed in accordance with the genotype. Individuals with c.1146GG genotype had a 32% decrease in MRP5 mRNA expression compared to the individuals with c.1146AA genotype (mean Δ_{Ct} against GAPDH: -5.94 vs. -5.38 ; $P = 0.0046$). Next, the expression of the A and G alleles of c.1146A>G was analyzed in genomic DNA (gDNA) and complementary DNA (cDNA) by using a pyrosequencing-based method to measure the expression of MRP5 mRNA according to the allele (Fig. 3). Samples from the 11 heterozygous individuals (AG genotype) were used to analyze the allelic effect on MRP5, and samples from five AA or GG volunteers were used as controls. Unexpectedly, the expression of MRP5 mRNA (cDNA) with c.1146G was lower than that of c.1146A (59.5% vs. 40.5%, $P < 0.0001$), although the gDNA levels were comparable (49.9% vs. 50.5%, $P = 0.769$).

The MRP5 gene with the G allele at c.1146A>G has lower mRNA and protein expression than that of MRP5 gene with the A allele

Plasmid cDNA of the MRP5 gene with the A and G alleles at the location of c.1146 was produced for transfection into HEK cells. After transfection, MRP5 mRNA expression was confirmed by RT-PCR analysis, MRP5 protein expression was confirmed through immunoblotting, and the expression and location of MRP5 protein was confirmed through immunocytochemistry. In the control vector, low MRP5 mRNA and protein expression was found, with high expression in the wild-type (A allele), and lower

expression with the G allele compared with the wild-type (Fig. 4A and B). In immunocytochemistry, MRP5 protein expression was found along with the cell membrane (in the same position as the membrane marker P-cadherin) of wild type (A allele) MRP5 gene expressing HEK cells. Whereas, the MRP5 protein expression was found to be very low in mutant (G allele) type of HEK cells (Fig. 4C).

Discussion

Cilostazol was administered as a reference drug to the study participants in six separate trials. The data from the volunteers who reported moderate or severe headaches after cilostazol administration were combined for analysis. Before the clinical trial started, the medical history of the participants was confirmed from a personal interview. The headache severity was evaluated by using a 10-point VRS and the participants were divided into two groups: those with no or mild headaches were assigned to the ADR-No group, and those with moderate or severe headaches were assigned to the ADR-Yes group. Participants with mild headaches were classified into the ADR-No group was because they may have felt that they had a mild headache when questioned about headaches during the personal interview. Moreover, people who are not taking any medications and with no known disease can experience mild headaches due to environmental factors such as stress, alcohol and caffeine withdrawal, neck stiffness, and light sensitivity [19]. Therefore, participants with mild headaches were separated from those with moderate or severe headaches to ensure that the headaches were due to cilostazol intake only.

In the genetic association test, cilostazol-induced headaches were found to be significantly associated with three SNPs after conservative Bonferroni correction for multiple testing (Table 2). However, only the c.1146A>G SNP was found to be positively associated with MRP5 mRNA expression in vivo (Fig. 2). Therefore, the relationship between these SNPs was examined to interpret the results. D' and R-square were used most often to measure LD and were computed by using the Haploview program [20, 21].

As shown in Figure 1, three SNPs were considerably connected to each other, although not in perfect linkage. Indeed, the D' value for all three SNPs was 1.0. The r^2 values were 0.28 between c.1146A>G (rs7636910) and c.1147+94G>A (rs55695073), 0.44 between c.1147+94G>A (rs55695073) and c.3624C>T (rs3749442), and 0.65 between c.1146A>G (rs7636910) and c.3624C>T (rs3749442). In the haplotype analysis of the three loci, the frequency of the GGC haplotype was 41.1%, showing an association with the no or mild headaches group, whereas the frequency of the AAT haplotype was 29.0%, and showed an association with the moderate or severe headaches group (Supplementary Table 1). However, the frequencies of the AGC and AGT haplotypes were 20.3% and 9.7%, respectively. These two haplotypes showed no association with cilostazol-associated headaches, indicating that crossover would have had to occur in a region between c.1146A>G (rs7636910) and c.1147+94G>A (rs55695073). According to the results, the synonymous c.1146A>G SNP might have a practical function of the regulating MRP5 mRNA expression, and other SNPs might be connected to c.1146A>G but did not have this function.

With regard to the molecular mechanism of the c.1146A>G SNP, this variation is a synonymous SNP at position 382. However, c.1146A>G is located at the second to last nucleotide of the eighth exon within the U1 small nuclear RNA (snRNA) binding site [22]. Therefore, one possibility is that if the binding site of the U1 snRNA is changed by the mutation, the splice variant of MRP5 will be produced during the process of converting pre-mRNA to mature RNA. To confirm this, RT-PCR was performed by using cDNA samples synthesized from the mRNA of fresh human blood. However, the length of the splicing product was identical from the AA, AG, and GG genotypes, and a new splice variant of MRP5 was not identified (Supplementary Fig. 1). The other possibility was that synonymous variations in the coding regions can alter mRNA expression. Therefore, we performed a pyrosequencing assay of samples from heterozygous individuals. This is an allele-specific expression analysis method that is much more sensitive to relatively small changes in expression, whereas across-sample comparisons include much more noise (biological variation between samples and technical noise from normalizing to a housekeeping gene) and can only detect larger differences in expression. Thus, for the allelic expression differences in MRP5 an evaluation of whether the c.1146A>G SNP was associated with allelic expression differences was performed. It was found that c.1146G resulted in significantly lower MRP5 mRNA expression than c.1146A (Fig. 3). The differences in the two allele-specific transcripts were as large as ~1.5-fold.

It was necessary to confirm MRP5 mediated transportation of cilostazol to conclude whether the pharmacokinetics of cilostazol affected headaches or not. Studies were performed to examine whether cilostazol could be transported via MRP5 in MDCKII-MRP5 cells. The protein expression of MRP5 in MDCKII-MRP5 cells was found to be increased depending on the total protein loaded, and MRP5 was found to be expressed in along with the cell membrane (Supplementary Fig. 2A and B). These results showed that in vitro test system was capable of detecting substrates of MRP5. To confirm the behavior of transient MDCKII-MRP5 cell systems, the efflux ratio (P_{app} basal to apical/ P_{app} A to B) of carboxy dichlorofluorescein (1 μ M CDF, probe substrate for MRP5) in MDR5-expressing cells was determined and was found to be 2.0 fold higher than that of apical to basal ratio (Supplementary Fig. 2C). The presence of 100 μ M MK-571 (a reference inhibitor for MRP5) significantly decreased the B to A transport of CDF from 264.5×10^{-6} to 92.2×10^{-6} cm/s ($p < 0.01$). Both A to B and B to A direction transport rates of cilostazol across MDCKII-MRP5 cells were increased in a concentration-dependent manner. As shown in Supplementary Figure 2D and Supplementary Table 2, the efflux ratio (P_{app} B to A/ P_{app} A to B) for 10, 30, 60, and 100 μ M cilostazol was 0.8, 1.1, 0.9, and 1.4, respectively. According to the US FDA guidance for industry, to be a substrate of efflux transporter protein, the efflux ratio cut off value (P_{app} B to A/ P_{app} A to B) should be above 2 [23]. So, the results in this experiment indicated that the cilostazol is not a substrate of MRP5 transporter.

Accordingly, the concentration of cilostazol and its active metabolites in blood were compared for each genotype of c.1146G>A (Supplementary Fig. 3). Various PK parameters of cilostazol and its active metabolites, including the maximum observed plasma concentration (C_{max}), the area under the plasma concentration-time curve from time zero to infinity (AUC_{inf}), and the terminal elimination half-life ($t_{1/2}$),

were not so different, suggesting that the MRP5 polymorphism was not related to the systemic distribution of cilostazol or its metabolites (Supplementary Table 3).

At the beginning of this study, the correlation between MRP4 as well as MRP5 genetic variants and cilostazol-induced headaches was identified. The MRP4 gene was analyzed for 17 genetic variants present in Koreans. However, no genetic variation was found to be significantly related with headache (Supplementary Table 4).

NO is a key molecule in primary headaches, and the vasodilation effect of NO is the main cause of headaches [7, 24]. The role of NO in the regulation of vascular tone can be categorized into two major mechanisms [25]. Several studies have suggested that NO regulates vascular tone without affecting the NO/cGMP pathway [26-29]. However, other research suggested that NO/cGMP pathway can regulate the mechanism of headache, and our study focused on a cGMP transporting protein as the factor controlling headaches [30, 31]. Xu et al. reported that vessel diameters were increased to a smaller extent in MRP5-knockdown rats than in control rats when using the NO donor S-nitroso-N-acetylpenicillamine (SNAP) in native conditions [32]. An increase in cAMP reduced SNAP-induced vasodilation in MRP5 knockdown rats [8]. These data are consistent with our results showing that participants receiving cilostazol with the c.1146A>G mutant had fewer headaches and lower MRP5 mRNA expression than participants with the wild-type allele. In MRP5-knockdown rats, the reduction in cGMP-induced vasodilatation may be due to increased PDE5 activity and increased cAMP levels, although the exact mechanisms require further investigation. A similar analogy may explain the protective effect of the c.1146G allele on cilostazol-induced headaches. Cilostazol is a specific PDE III inhibitor. PDE III is referred to as a “cAMP-preferring” enzyme and is most highly expressed in vascular smooth muscle cells [33]. A role for cAMP in the promotion of NO/cGMP-dependent vasodilation was proposed, in which cAMP mainly inhibited cGMP transportation to the outside of cells through MRP5 and also enhanced the PDE5-mediated cGMP breakdown to 5'-GMP [8]. Therefore, participants with the c.1146A allele show normal expression of the cGMP efflux protein MRP5, and the efflux blocking effect predominates over the PDE-stimulating effect. However, participants with the c.1146G allele showed a loss of sensitivity related to the role of cAMP in blocking cGMP transport; this was a result of the reduced MRP5 expression that was attributable to the c.1146A>G SNP, thereby revealing the ability of cAMP to enhance PDE5 activity. As a result, reduced cGMP levels in vascular smooth muscle cells can be accompanied by attenuation of the vasodilatory response, implying that the cilostazol-induced cAMP increment can reduce the probability of headaches in individuals with the c.1146G mutant allele. However, we also need to investigate the influence of additional factors, such as MRP5-mediated cAMP extrusion and alternative pathways for cGMP efflux to develop a clear understanding of this phenomenon.

Conclusion

In total, 101 volunteers who had completed clinical trials of cilostazol were analyzed to determine whether headaches had occurred or not. Statistical significance of the c.1146G SNP of the MRP5 gene was detected in individuals with no or mild headaches, and the c.1146G mutant allele resulted in lower

MRP5 mRNA and protein expression than the wild-type allele, which has a protective effect on headaches in volunteers taking cilostazol. Therefore, the synonymous polymorphism c.1146A>G (p.Q382Q, rs7636910) may be a biomarker for headaches induced by cilostazol.

Abbreviations

MRP5: Multidrug resistance protein 5; cAMP: Cyclin adenosine monophosphate; cGMP: Cyclic guanosine monophosphate; PDE: Phosphodiesterase; ADRs: Adverse drug reactions; NO: Nitric oxide; ABC: ATP binding cassette transporter; MRP4: Multidrug resistance protein 4; ECG: Electrocardiogram; PBS: Phosphate buffered saline; LD: Linkage disequilibrium; SNP: Single nucleotide polymorphism; PK: Pharmacokinetic.

Declarations

Ethics approval and consent to participate

The study protocol was reviewed by the Ethics Committee of Severance Hospital in the Yonsei University Health System, Seoul, Korea and the study was conducted according to the ethical principles of the Declaration of Helsinki. Additionally, at the beginning of the study, after describing the aims and possible risks or benefits of the trial, all participants provided written informed consent.

Consent for publication

All authors consented.

Availability of data and materials

The datasets generated and/or analyzed during this study are not publicly available. All of the relevant data are included in this article and its Supplemental information files.

Competing interests The Author's declare that they have no competing interests.

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Authors' contributions

DHW performed clinical trials, drafted the manuscript. WDB analyzed data, drafted the manuscript. MH interpreted data, drafted the manuscript. SMK performed the experiments. DJK analyzed statistical data. JhP performed the experiments. KsP supervised clinical trials. MGL designed the study. SJL interpreted data. JHL interpreted data. SWK designed the study, supervised this study. All authors read and approved the final manuscript.

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References

1. Pratt CM (2001) Analysis of the cilostazol safety database. *Am J Cardiol* 87(12A):28D-33D.
2. Toyoda K, Uchiyama S, Yamaguchi T, Easton JD, Kimura K, Hoshino H, et al (2019) Dual antiplatelet therapy using cilostazol for secondary prevention in patients with high-risk ischaemic stroke in Japan: a multicentre, open-label, randomised controlled trial. *Lancet Neurol* 18(6):539-48.
3. https://www.accessdata.fda.gov/drugsatfda_docs/label/2007/020863s021lbl.pdf.
4. Guo S, Olesen J, Ashina M (2014) Phosphodiesterase 3 inhibitor cilostazol induces migraine-like attacks via cyclic AMP increase. *Brain* 137(Pt 11):2951-9.
5. Khan S, Deen M, Hougaard A, Amin FM, Ashina M (2018) Reproducibility of migraine-like attacks induced by phosphodiesterase-3-inhibitor cilostazol. *Cephalalgia* 38(5):892-903.
6. Butt JH, Rostrup E, Hansen AS, Lambertsen KL, Kruuse C (2018) Induction of migraine-like headache, but not aura, by cilostazol in patients with migraine with aura. *Brain* 141(10):2943-51.
7. Thomsen LL, Olesen J (2001) Nitric oxide in primary headaches. *Curr Opin Neurol* 14(3):315-21.
8. Xu HL, Wolde HM, Gavrilyuk V, Baughman VL, Pelligrino DA (2004) cAMP modulates cGMP-mediated cerebral arteriolar relaxation in vivo. *Am J Physiol Heart Circ Physiol* 287(6):H2501-9.
9. Zaccolo M, Movsesian MA (2007) cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. *Circ Res* 100(11):1569-78.
10. Robey RW, Pluchino KM, Hall MD, Fojo AT, Bates SE, Gottesman MM (2018) Revisiting the role of ABC transporters in multidrug-resistant cancer. *Nat Rev Cancer* 18(7):452-64.
11. Jedlitschky G, Burchell B, Keppler D (2000) The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. *J Biol Chem* 275(39):30069-74.
12. Adema AD, Floor K, Smid K, Honeywell RJ, Scheffer GL, Jansen G, Peters GJ (2014) Overexpression of MRP4 (ABCC4) and MRP5 (ABCC5) confer resistance to the nucleoside analogs cytarabine and troxacitabine, but not gemcitabine. *Springerplus* 3:732.
13. Adachi M, Sampath J, Lan LB, Sun D, Hargrove P, Flatley R, et al (2002) Expression of MRP4 confers resistance to ganciclovir and compromises bystander cell killing. *J Biol Chem* 277(41):38998-9004.
14. Cheepala SB, Pitre A, Fukuda Y, Takenaka K, Zhang Y, Wang Y, et al (2015) The ABCC4 membrane transporter modulates platelet aggregation. *Blood* 126(20):2307-19.
15. Rongen GA, Smits P, Ver Donck K, Willemsen JJ, De Abreu RA, Van Belle H, Thien T (1995) Hemodynamic and neurohumoral effects of various grades of selective adenosine transport inhibition in humans. Implications for its future role in cardioprotection. *J Clin Invest* 95(2):658-68.
16. Benarroch EE (2008) Adenosine and its receptors: multiple modulatory functions and potential therapeutic targets for neurologic disease. *Neurology* 70(3):231-6.

17. Lee D, Lim LA, Jang SB, Lee YJ, Chung JY, Choi JR, et al (2011) Pharmacokinetic comparison of sustained- and immediate-release oral formulations of cilostazol in healthy Korean subjects: a randomized, open-label, 3-part, sequential, 2-period, crossover, single-dose, food-effect, and multiple-dose study. *Clin Ther* 33(12):2038-53.
18. Jones KR, Vojir CP, Hutt E, Fink R (2007) Determining mild, moderate, and severe pain equivalency across pain-intensity tools in nursing home residents. *J Rehabil Res Dev* 44(2):305-14.
19. (2004) The International Classification of Headache Disorders: 2nd edition. *Cephalalgia* 24 Suppl 1:9-160.
20. Lewontin RC (1964) The Interaction of Selection and Linkage. I. General Considerations; Heterotic Models. *Genetics* 49(1):49-67.
21. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21(2):263-5.
22. Zhuang Y, Weiner AM (1986) A compensatory base change in U1 snRNA suppresses a 5' splice site mutation. *Cell* 46(6):827-35.
23. <http://www.portacelltec.de/pdf/Guidance-for-Industry.pdf>.
24. Olesen J (2010) Nitric oxide-related drug targets in headache. *Neurotherapeutics* 7(2):183-90.
25. Wanstall JC, Homer KL, Doggrell SA (2005) Evidence for, and importance of, cGMP-independent mechanisms with NO and NO donors on blood vessels and platelets. *Curr Vasc Pharmacol* 3(1):41-53.
26. Alonso-Galicia M, Hudetz AG, Shen H, Harder DR, Roman RJ (1999) Contribution of 20-HETE to vasodilator actions of nitric oxide in the cerebral microcirculation. *Stroke* 30(12):2727-34.
27. Homer KL, Wanstall JC (2000) Cyclic GMP-independent relaxation of rat pulmonary artery by spermine NONOate, a diazeniumdiolate nitric oxide donor. *Br J Pharmacol* 131(4):673-82.
28. Ellershaw DC, Greenwood IA, Large WA (2000) Dual modulation of swelling-activated chloride current by NO and NO donors in rabbit portal vein myocytes. *J Physiol* 528 Pt 1:15-24.
29. Pucovsky V, Gordienko DV, Bolton TB (2002) Effect of nitric oxide donors and noradrenaline on Ca²⁺ release sites and global intracellular Ca²⁺ in myocytes from guinea-pig small mesenteric arteries. *J Physiol* 539(Pt 1):25-39.
30. Sausbier M, Schubert R, Voigt V, Hirneiss C, Pfeifer A, Korth M, et al (2000) Mechanisms of NO/cGMP-dependent vasorelaxation. *Circ Res* 87(9):825-30.
31. Pimentel AM, Costa CA, Carvalho LC, Brandao RM, Rangel BM, Tano T, et al (2007) The role of NO-cGMP pathway and potassium channels on the relaxation induced by clonidine in the rat mesenteric arterial bed. *Vascul Pharmacol* 46(5):353-9.
32. Xu HL, Gavriluk V, Wolde HM, Baughman VL, Pelligrino DA (2004) Regulation of rat pial arteriolar smooth muscle relaxation in vivo through multidrug resistance protein 5-mediated cGMP efflux. *Am J Physiol Heart Circ Physiol* 286(5):H2020-7.

33. Schultz C, Vaskinn S, Kildalsen H, Sager G (1998) Cyclic AMP stimulates the cyclic GMP egression pump in human erythrocytes: effects of probenecid, verapamil, progesterone, theophylline, IBMX, forskolin, and cyclic AMP on cyclic GMP uptake and association to inside-out vesicles. *Biochemistry* 37(4):1161-6.

Tables

Table 1. Baseline characteristics of the participants.

Characteristics	All participants	Participants with no or mild headaches	Participants with moderate or severe headaches	<i>P</i> -value ^a
No. of participants [n (%)]	101 (100)	68 (67.3)	33 (32.7)	
Age [years; mean (SD)]	24.8 (3.50)	24.6 (3.4)	25.2 (3.6)	0.480
Sex [Male; n (%)]	84 (83.2)	59 (86.8)	25 (75.8)	0.166
Height [cm; mean (SD)]	173.2 (7.6)	173.9 (7.5)	171.9 (7.8)	0.209
Weight [kg; mean (SD)]	67.3 (8.8)	67.7 (8.6)	66.5 (9.3)	0.527
Smoking [Yes; n (%)]	27 (26.7)	22 (33.3)	5 (15.2)	0.056
Drinking [Yes; n (%)]	71 (70.3)	48 (72.7)	23 (69.7)	0.752

^a χ^2 or *t*-test used as appropriate.

Table 2. Genotype distribution of MRP5 polymorphisms in the participants.

SNP (rs number) Location (Amino acid)	Genotype	Participants with no or mild headaches	Participants with moderate or severe headaches	<i>P</i> -value ^a
c.-55-499A>C (rs79313368)	AA	43	27	0.145
Intron	AC	20	6	
	CC	4	0	
c.1000-119T>G (rs2872247)	TT	38	25	0.15
Intron	TG	21	6	
	GG	9	2	
c.1146A>G (rs7636910)	AA	19	20	0.005*
	AG	31	10	
Exon (Q382Q)	GG	18	3	0.002*,b
c.1147+94G>A (rs55695073)	GG	42	12	0.004*
	GA	23	12	
Intron	AA	3	8	0.023*,b
c.1185T>C (rs1132776)	TT	43	23	0.876
	TC	21	8	
Exon (A395A)	CC	4	2	
c.1782T>C (rs939336)	TT	46	23	0.873
	TC	19	8	
Exon (C594C)	CC	3	2	
c.1958+20T>C (rs3817403)	TT	61	30	>0.999
	TC	6	3	
Intron	CC	1	0	
c.3624C>T (rs3749442)	CC	35	8	0.001*
	CT	26	12	
Exon (L1208L)	TT	7	13	0.011*,b
c.4896G>A (rs3749445)	GG	28	4	0.007
	GA	27	16	
3'-UTR	AA	13	13	

^a*P* values were obtained with the χ^2 test or Fisher's exact test (expected cell value <5). Significant SNPs after Bonferroni correction for multiple testing are shown in bold.

^b*P* value was calculated in the minor allele dominant mode. **P* < 0.01.

UTR = untranslated region.

Table 3. Logistic regression analysis for severe headaches when taking cilostazol.

	Odds ratio	95% CI	<i>P</i>
Presence of G allele at c.1146	0.36	0.18–0.71	0.003*
Age	1.03	0.95–1.13	0.463
Sex ^a	0.86	0.28–2.62	0.791
Height	0.97	0.89–1.04	0.375
Weight	1.02	0.96–1.08	0.535
Smoking	0.45	0.2–1.02	0.057
Drinking	1.02	0.51–2.07	0.951

^aReference category: Male, **P* < 0.01.

CI = confidence interval.

Figures

Figure 1

Single nucleotide polymorphism (SNP) locations in the MRP5 gene identified in the 101 healthy Korean participants and their linkage disequilibrium (LD) status. The location of the SNPs within each exon was calculated exactly; however, the location within introns was not. The frequency of the c.222G>A, c.2714T>G, and c.2847C>T SNPs was less than 0.05. c.5557A>G showed a perfect LD structure with c.4896G>A (D' and r² = 1). UTR: untranslated region, CDS: coding sequence, LOD: logarithm of odd.

Figure 2

The mRNA expression of MRP5 in human peripheral blood was measured with real-time PCR and normalized to that of GAPDH. The Delta Ct value was used to present the mRNA expression. The MRP5 expression was compared by genotype of (A) c.1146A>G, (B) c.1147+94G>A, and (C) c.3624C>T. ANOVA and a t-test were used for statistical analyses.

Figure 3

The allele quantification ratio for c.1146A>G of the MRP5 gene was determined by using pyrosequencing. The expression ratio was mutually compared with the A and G alleles from (A, B) cDNA and (C) gDNA from human peripheral blood. (B, C) Heterozygotes were analyzed with vertical scatter plots.

Figure 4

mRNA and protein expression of MRP5 wild type and c.1146A>G. (A) MRP5 mRNA expression was compared with the MRP5 wild-type allele and c.1146A>G using RT-PCR in HEK293 cells. GAPDH was used as an internal control. (B) MRP5 protein expression was compared with the MRP5 wild-type allele and c.1146A>G by using immunoblotting in HEK293 cells. Beta-actin was used as an internal control. (C) The MRP5 protein expression pattern was compared with the MRP5 wild-type allele and c.1146A>G by using immunocytochemistry. Pan-cadherin was used as a membrane marker. Scale bar = 10 μ m.

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