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Influence of gender on *ABCC2* expression in peripheral blood mononuclear cells

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ABSTRACT. It is known that several factors, including gender, may influence the expression of multidrug resistance associated proteins 2 (*MRP2*/*ABCC2*) in the peripheral blood mononuclear cells (PBMCs). This study aims to compare *ABCC2* gene expression in PBMCs of healthy males and females. PBMCs were extracted from 48 females and 44 males, and gene expression was measured using real-time quantitative polymerase chain reaction (real-time QPCR). Multiple housekeeping genes (*Actin-β*, *β2-M, GAPDH*) were utilized as endogenous controls. The stability of housekeeping genes was verified using the Excel-based Bestkeeper® program. Our results showed that expression level of *ABCC2* in PBMCs was 1.2-1.4 fold higher in males compared to that in females, depending on the endogenous control(s) used. However, this difference was not statistically significant. When considering using a single endogenous control gene, *GAPDH* and *Actin-β* were found to be more suitable than *β2-M*. Moreover, *GAPDH* + *Actin-β*, or the combination of all three housekeeping gene as

endogenous control(s) showed greater stability than other endogenous control genes for normalization of *ABCC2* expression in PBMCs. This study suggests that *ABCC2* expression in PBMCs may be, in part, influenced by gender, and that at least two endogenous control genes should be utilized for gene expression normalization.

Key words: *ABCC2* expression; Peripheral blood mononuclear cells; Gender

INTRODUCTION

Peripheral blood mononuclear cells express a number of membrane-bound ATP-dependent drug efflux transporters, including the ATP binding cassette transporter isoform C2 (ABCC2) (Albermann et al., 2005; Fardel et al., 2005), also known as the multidrug resistance associated proteins 2 (MRP2). This protein is a pharmacological target of a number of drugs (Ito et al., 2001); substrates of ABCC2 include various anticancer drugs, immunosuppressants, antibiotics and antiviral drugs targeted for peripheral blood mononuclear cells (PBMCs). The ABCC2 transporter functions as a drug efflux pump, and subsequently prevent accumulation of various substrates in the cells (Gerk and Vore, 2002). Therefore, differential expression of ABCC2 in PBMCs, including T lymphocytes, macrophages, and monocytes, leads to variability in cellular accumulation of drugs and thus has an effect on therapeutic outcomes (Suzuki and Sugiyama, 2002; Hoffmann and Kroemer, 2004; Fardel et al., 2005; Cascorbi, 2006; Jedlitschky et al., 2006).

Several factors may exert influence on the inter-individual variability of *ABCC2* expression, including gestational age (Meyer et al., 2005), differences in cell cycle stages (van Der Kolk et al., 2001), ethnicity (Mor-Cohen et al. 2012), single nucleotide polymorphisms (SNPs) (van Der Kolk et al., 2001; Meyer et al., 2005; Casobi, 2006; Deo et al., 2012) as well as gender (Rost et al., 2005.; Simon et al., 2006; Suzuki et al., 2006; MacLean et al., 2008). Studies have shown that expression of ABCC2 protein was 1.5 fold higher in female rat hepatocytes compared to male counterparts as testosterone may reduce ABCC2 protein expression (Rost et al., 2005; Suzuki et al., 2006). On the other hand, expression of ABCC2 protein in renal cells was significantly higher in males as compared to females (Wang et al., 2012). Other studies examining ABCC2 protein expression in human liver cells (Deo et al., 2012) and PBMCs (Paintsil et al., 2011) found no effect of gender on its expression. These studies suggested that expression of *ABCC2* may be dependent on the tissue/organ in which it is expressed. Currently only limited studies have examined the effect of gender on the expression of *ABCC2* in PBMCs with proper endogenous controls used, especially in the Thai population. Therefore, the aim of the present study is to compare the expression levels of *ABCC2* in PBMCs between healthy males and females.

MATERIAL AND METHODS

Subjects

All of 92 (48 females, 44 males) healthy Thai volunteers with ranging between18-30 years of age were enrolled in the study. This study was approved by the Institutional Review Boards at Naresuan University, Phitsanulok, Thailand. Individual written consent was obtained from all subjects.

Specimen collection and preparation

Blood was drawn (16 cc) and collected at designated study times in a CPT® cell preparation tube. PBMCs were isolated by centrifugation at 1700 *g* for 20 min at room temperature. The isolated PBMCs were immediately processed upon collection, and "flash frozen" using liquid nitrogen. All samples were stored at -70°C until analysis.

Material and chemicals

QIAamp RNA Blood Mini Kit was purchased from QIAGEN®, and cDNA synthesis kits were obtained from Stratagene®. EXPRESS SYBR®GreenER™qPCR SuperMixes, Two-step qRT-PCR kits and all gene primers were obtained from Invitrogen®.

RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from 800 µL PBMCs according to the manufacturer protocol (Qiagen®, Valencia, CA, USA). All RNA samples were eluted in a final volume of 50 µL RNase-Free water (Qiagen®). Quantification of total RNA concentrations and the purity of all samples were determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop® Technologies). Isolated RNA samples were stored at -70°C until analysis.

Reverse transcription was performed with 100 ng total RNA using a cDNA synthesis kits (Stratagene®, Germany), according to manufacturer instructions. First-strand cDNA was synthesized in a 50-µL reaction mixture containing 10 µL cDNA synthesis master mix (2X), 3 µL oligo (dT) primer, and 1 µL AffinityScript RT/RNase Block enzyme mixture. The PCR cycling protocol is as follows: 25°C for 5 min, 42°C for 45 min, and 95°C for 5 min. The reaction tubes were stored at -20°C for long-term storage until analysis.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (qPCR) was carried out with the Bio-Rad iCycler® (Bio-Rad®, USA). Expression of each target gene including *ABCC2*, *GAPDH*, *actin-β*, and *β-2M* in PBMCs was determined. A 20-µL reaction volume containing 10 µL qPCR mastermix, 0.5 µL target gene forward primers, 0.5 µL target gene reverse primers (Table 1), 1 µL undiluted cDNA, and 8 µL diethyl pyrocarbonate (DEPC) treated water was used. The machine was warmed at 50°C for 2 min and the PCR cycling parameters are as follows: denaturation at 95°C for 10 min, 45 cycles of amplification at 95°C for 10 s, and annealing at 58°C for 10 s. PCR cycles were followed by a dissociation curve analysis to confirm product specificity by increasing temperature in increments of 0.5°C every 5 s. No-template controls were included for each primer pair of each target gene. Each PCR was performed in triplicate. The qPCR products were stored at -20°C and ran on a 2.5% agarose gel to confirm product lengths.

Data were generated with the BIO-RADCFX96 (BIO-RAD®, USA). Cycle threshold (Ct) values were calculated for all samples using the BIO-RADCFX Software (BIO-RAD®, USA). To ensure reliable normalization, three housekeeping genes including *actin-β*, *GAPDH*, and *β-2M* were tested as endogenous control(s).

Table 1. Primer sequences and amplicon size.

Gene	Sequence (5'-3')	Amplicon length (base pair)
Actin-ß	Forward: CTG GAA CGG TGA AGG TGA CA Reverse: AAG GGA CTT CCT GTA ACA ATG CA	140
GAPDH	Forward: TGC ACC ACC AAC TGC TTA GC Reverse: GGC ATG GAC TGT GGT CAT GAG	87
ß2-M	Forward: CTC CGT GGC CTT AGC TGT G Reverse: TTT GGA GTA CGC TGG ATA GCC T	69
ABCC2	Forward: ACATACAGGCCCTGAAGAGG Reverse: TCAGCCCATAGTCATCGTCT	71

β2-M: Beta-2-microglobulin *GAPDH*: Glyceraldehyde-3- phosphate dehydrogenase *Actin-β*: Beta-actin.

Evaluation of housekeeping genes

The Excel-based BestKeeper® program (Pfaffl et al., 2004) was utilized to evaluate the stabilities of all endogenous control(s), and to determine the most suitable housekeeping genes for normalization of *ABCC2* gene expression. Individual genes (*GAPDH*, *actin-β*, *β-2M*) as well as the combination of 2 or 3 genes such as *GAPDH* + *actin-β*, *GAPDH* + *β-2M*, *actin-β* + *β*-2*M*, and *GAPDH* + a*ctin-β* + *β-2M,* were tested. Stabilities of the individual housekeeping genes were defined by the highest correlation coefficient (r) when compared to the BestKeeeper® index, and had standard deviation (SD) < 1.

Data and statistical analysis

Descriptive statistical analysis was performed for numerical data. Delta-delta Ct method was used to calculate expression level of *ABCC2* relative to no-template control. Housekeeping genes were used for normalization of *ABCC2* expression. The Student *t*-test was used to compare the means of the gene expression between males and females. One way analysis of variance (ANOVA) was utilized to compare the differences in *ABCC2* expression among the various endogenous controls(s) used. Univariate analysis was performed to determine the effect of endogenous control(s) and gender on *ABCC2* expression. A P value < 0.05 was considered to be statistically significant.

RESULTS

Housekeeping gene stability

The stabilities of endogenous controls (*GAPDH*, *actin-β* and *β-2M*) used to normalize *ABCC2* expression level in PBMCs were tested by the Bestkeeper® program (Table 2). When considering the stability of a single endogenous control, both *actin-β* (r = 0.968, SD = 1.50) and *GAPDH* (r = 0.865, SD = 1.45) were found to be more stable than *β-2M* (r = 0.867, SD = 1.89). Interestingly, the values of Bestkeeper index® was increased when 2 or 3 endogenous controls were used for normalization (r = 0.991, -1, SD = 1.45, -1.56). Among these genes, *actin-β* expression was highly correlated with that of *GAPDH* (r = 0.874, P = 0.001) and *β-2M* (r = 0.756, P = 0.001). On the other hand, expression of *GAPDH* only showed medium correlation with that of β-*2M* (r = 0.520, P = 0.001).

Table 2. Stability of Housekeeping gene(s) tested by BestKeeper®[34].

N = 92	*β2-M*	*GAPDH*	*Actin-β*	*β2-M +GAPDH*	*β2-M +Actin-β*	*GAPDH +Actin-β*	*β2-M +GAPDH +Actin-β*	Best-Keeper® Index
Geometric mean Ct	24.90	25.28	23.89	25.12	24.41	24.59	24.71	24.70
Arithmetic Ct	25.02	25.35	23.96	25.18	24.49	24.65	24.77	24.76
Min Ct	19.77	21.89	20.31	21.86	20.04	21.50	21.45	21.40
Max Ct	29.94	31.13	29.25	30.54	29.60	30.19	30.11	30.10
S.D.(±Ct)	1.89	1.45	1.50	1.56	1.56	1.45	1.45	1.45
C.V.(%)	7.55	5.72	6.26	6.38	6.38	5.88	5.85	5.87
Coeff.of corr. [r]*	0.867	0.865	0.968	0.991	0.972	0.947	1.00	-

Ct: Cycle threshold; Min: Minimum; Max: Maximum; S.D.: Standard deviation C.V.: coefficient of variation; Coeff. of corr: coefficient of correlation. *The housekeeping gene(s) is correlated with the BestKeeper® Index determining by correlation coefficient test; P value = 0.001 *β2-M*: Beta-2-microglobulin *GAPDH*: Glyceraldehyde-3- phosphate dehydrogenase *Actin-β*: Beta-actin.

Gender difference of the level of *ABCC2* expression in PBMCs

As illustrated in Table 3, *ABCC2* expression in PBMCs was dependent on the endogenous controls used for gene expression normalization. Both analysis of variance (ANOVA) and *post hoc* analysis using Tukey's test showed that the mean levels of *ABCC2* expression differed between each normalization methods (P < 0.005). High inter-individuals variability of *ABCC2* expression in PBMCs was observed in both male and female volunteers. The degree of variability was also dependent on the types of endogenous control used. However, males mostly showed higher variability in *ABCC2* expression (% C.V. = 109.00- 315.83) as compared with females (% C.V. = 130.79- 179.83), except when utilizing *GAPDH* as an endogenous control. When comparing the mean levels of *ABCC2* expression using independent sample *t*-tests, males showed higher expression of *ABCC2* as compared to females with a male to female ratio of 1.195-1.447 depending on the types of endogenous controls used. However, the results were not statistically significant (P > 0.05). Interestingly, univariate analysis showed that gender was correlated with *ABCC2* expression level (P = 0.006) while types of endogenous control(s) had no influence on the level of *ABCC2* in PBMCs (P > 0.05).

Table 3. Relative levels of ABCC2 expression normalized to each housekeeping gene.

Parameters	Folds (Percentage)						
	Actin-β	*GAPDH*	*β2-M*	*GAPDH +Actin-β*	*β2-M +Actin-β*	*β2-M +GAPDH*	*β2-M +GAPDH +Actin-β*
Overall (N = 92)							
Mean*	-181.01	-49.76	-142.99	-87.97	-152.83	-70.77	-94.57
S.D.	407.98	62.15	330.13	136.90	356.10	128.03	179.02
%C.V.	-225.39	-124.91	-230.87	-155.63	-233.00	-180.91	-189.30
Females (N = 48)							
Mean#	-198.32	-56.36	-177.62	-100.86	-179.44	-87.17	-112.80
S.D.	305.72	73.71	347.49	142.96	322.70	147.83	186.52
%C.V.	-154.15	-130.79	-195.63	-141.75	-179.83	-169.58	-165.35
Males (N = 44)							
Mean#	-162.12	-42.37	-105.22	-73.91	-123.80	-52.88	-74.68
S.D.	499.38	46.18	309.62	130.14	391.00	100.88	170.36
%C.V.	-308.03	-109.00	-294.26	-176.09	-315.83	-190.78	-228.12
Ratio M/F+	1.195	1.329	1.447	1.261	1.261	1.272	1.250

S.D.; Standard deviation, C.V.; Coefficient of variation *β2-M*: Beta-2-microglobulin *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase *Actin-β*: Beta actin. *The levels of ABCC2 expression were statistically different when normalized to different types of endogenous control (P < 0.001) using one way ANOVA. However, univariate analysis showed that gender, and not types of endogenous control, influenced the level of the expression, (P = 0.006). #The levels of ABCC2 expression between males and females were comparable using independent t-tests (P > 0.05).

DISCUSSION

Stability of endogenous control(s)

GAPDH, *Actin-β* and *β2-M* are common housekeeping genes that are widely used for gene expression normalization. A previous study examining the stability of 10 housekeeping genes in lymphocytes found that *β2-M* was more stable than *GAPDH* or *Actin-β* (Vandesompele et al., 2002). In contrast, the present study showed that *GAPDH* and *Actin-β* were more stable compared to *β2-M*, which is supported by our previous study (Sudchada et al., 2010). However, similar to the present study, previous studies (Vandesompele et al., 2002; Sudchada et al., 2010) suggested more than one gene should be used for normalization during gene expression analysis. These findings suggest the endogenous controls used to normalize target genes may affect interpretation of the results. Therefore, the endogenous controls should be validated prior to target gene analysis.

In the present study, we also found that determination of *ABCC2* expression using *β2-M* or *Actin-β* as a single endogenous control or combined with other housekeeping genes show higher variability between males and females, which is supported by our previous findings (Sudchada et al., 2010). To allow more accurate gene expression profiles, endogenous controls should be carefully considered and validated.

Comparing levels of *ABCC2* expression between males and females

Various factors may influence on the expression of *ABCC2*, including gender. Results from this study suggests that gender may affect *ABCC2* gene expression in PBMCs. Therefore, gender may potentially correlate with differences in drug response to PBMC-targeted ABCC2 substrates.

A study conducted in Sprague-Dawley (SD) rats using immunoblot analysis reported that female rats had 1.5 fold greater ABCC2 protein expression compared to males ($P < 0.001$). It is possible that testosterone can reduce ABCC2 protein expression ($P < 0.042$) (Rost et al., 2005). Similar results were found in another rat study using western blot analysis (Suzuki et al., 2006). Suzuki et al. (2006) showed that protein expression of ABCC2 was greater in female rats as compared to male rats, which was also reversely correlated with doxorubicin levels in liver cells. In contrast, another study investigating the correlation between plasma ofloxacin concentration and ABCC2 protein expression in renal cells found that ABCC2 expression was greater in male rats as compared with female rats ($P < 0.05$) (Wang et al., 2012). These results indicate that ABCC2 protein expression may vary between male and female rats in different organs.

A study in liver cells from the Human Liver Bank in University of Washington School of Pharmacy reported that gender had no influence on expression of *ABCC2* in hepatocytes (Deo et al., 2012). Similar finding was observed in a study on white blood cells extracted from 9 healthy volunteers (5 females, 4 males) using *GAPDH* as an endogenous control (Paintsil et al., 2011). The study also observed that *ABCC2* expression was not correlated with metabolite levels of ethynylstavudine and lamivudine, but was tentatively to be correlated with zidovudine ($P > 0.05$) (Paintsil et al., 2011). However, this study was conducted on a small number of subjects with limited statistical power (Paintsil et al., 2011). In contrast, the present study found that gender may potentially influence *ABCC2* expression in PBMCs, where males showed greater levels of *ABCC2* expression compared to females.

We found high variabilities in the level of *ABCC2* expression in this study (%CV = 124-233), which is supported by previous findings indicating that inter-individual variability was two

folds higher than the median *ABCC2* expression in PBMCs (Liptrott et al., 2008). Furthermore, we observed higher variability of *ABCC2* expression in males as compared to females. Similarly, a previous study found that males had greater variability of protein ABCC2 expression in liver than females (MacLean et al., 2008). On the other hand, another study found that females had greater variability in PBMC *ABCC2* expression than males (Paintsil et al., 2011).

In summary, this study provides evidence that gender may correlate expression level of *ABCC2* in PBMCs, which may partially account for the inter-variability of pharmacokinetics and pharmacodynamics of ABCC2 substrates in PBMCs. However, further studies on the protein expression in PBMCs and other potential factors such as SNPs are warranted.

Conflicts of interest

The authors declare no conflicts of interest.

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