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Efflux transporter mRNA expression profiles in differentiating JEG-3 human choriocarcinoma cells as a placental transport model

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The kinetics of drug transport across the trophoblast layer is determined by several factors. Human choriocarcinoma cell lines like BeWo and JEG-3 have been used as models of the trophoblast layer to examine the placental transport of drugs. Previously, the drugs examined in these models have been readily transported across the trophoblast layer via cellular gap junctions. These backgrounds enabled us to establish the differentiating JEG-3 cell (DJEG) layer model, which suppresses paracellular drug transport, as an evaluation system of placental drug transport. The efflux transporters on the trophoblast layer assume the meaningful role of protecting the fetus from xenobiotic substances. In order to clarify the usefulness of our DJEG placental drug transport model, this study examined the mRNA expression profiles of the efflux transporters MRPs, MDR1, and BCRP in JEG-3 cells and compared them with those of BeWo cells and their known placental expression. We suggest that the mRNA of efflux transporters MRP 1–8 and BCRP are expressed widely in JEG-3 cells; however, expression levels of MDR1 mRNA were undetectable. It was also indicated that polymorphisms of BCRP C421A in both the BeWo and JEG-3 cells are of the wild-type. We demonstrated the efflux transporters' expression profiles, as well as those of the BeWo cells, was demonstrated in the DJEG placental drug transport evaluating model as well as the BeWo cells, in the DJEG placental drug transport evaluation model. Based on these findings, we hope that the DJEG model will be adequate for use in evaluating placental drug transport in relation to the transporter proteins.

1. Introduction

Syncytiotrophoblasts in the chorionic villi exert a barrier function due to their differentiation from cytotrophoblasts. As the syncytiotrophoblast layer is exposed to maternal blood, it is thought that it may function to modulate the transfer of materials between maternal and fetal blood (Liu et al. 1997; Hemmings et al. 2001; Kitano et al. 2004; Settle et al. 2004; Bode et al. 2006). The kinetics of drug transport across the trophoblast layer is determined by several factors, including molecular weight, lipophilicity, carrier-mediated transport, and pH. Together with the teratogenic potency of a given drug, the placental transport ratio of drugs is one of the most important pharmaceutical factors that determines the pharmacokinetics and pharmacodynamics of medication in pregnancy. However, compared with the pharmacokinetics of other drugs or medication, little is known at present regarding placental drug transport and safety. To date, the safety of chemotherapy during pregnancy has been assessed largely using data obtained from animal experiments and limited clinical experience, and such treatment will continue to be problematic owing to ethical concerns regarding the fetus. Consequently, there is a critical need to accumulate data on the placental transport of several drugs *in vivo*, *in situ*, and *in vitro*, and to statistically evaluate their pharmacokinetics and dynamics.

Human choriocarcinoma cell lines like BeWo and JEG-3 have been used as models of the trophoblast layer for examination of placental transport of drugs (Utoguchi et al. 1999; Utoguchi and Audus 2000; Ushigome et al. 2001; Emoto et al. 2002; Jin and Audus 2005; Bode et al. 2006). In these models, the drugs under examination have been readily transported across the trophoblast layer via cellular gap junctions. These findings highlight the difficulty in forecasting carrier-mediated placental transport across the trophoblast layer using such models. These backgrounds have enabled us to establish the differentiating JEG-3 cell (DJEG) layer model cultured in CSC® medium kits [CSC® medium consists of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1) mixed medium (D/F) with acidic fibroblast growth factor (aFGF)] as an evaluation system of placental drug transport (Ikeda et al. 2011). This model suppresses paracellular drug transport across the trophoblast layer as compared with the normal cultured JEG-3 cell (NJEG) layer, and its features could indicate syncytiotrophoblast-like differentiating characters. The transepithelial electrical resistance (TER) of the DJEG layer increased to approximately 2-fold in comparison to that of the NJEG. We believed that the tight junctions and adherens junctions of the DJEG layer were formed adequately. The model in which the tight junction is formed adequately is needed to evaluate drug carrier-mediated transport, while

the expression ratios of the transporter proteins should be demonstrated for evaluation of pharmacokinetics. The efflux transporters on the trophoblast layer assume the meaningful role of protecting the fetus from xenobiotic substances (Utoguchi et al. 2000; Kitano et al. 2004; Pavek et al. 2004; Meyer et al. 2005; Ceckova et al. 2006; Staud et al. 2006; Merino et al. 2005). It was reported that the breast cancer resistance protein (BCRP) and the multidrug-resistant protein 1 (MDR1: P-glycoprotein) were expressed in the trophoblast-like cell lines BeWo and JEG-3. However, other than the report by Serrano et al. (2005) regarding MRP1–4 and 8, the expression profile of other efflux transporters, MRP1–8, in these cell lines is not well known.

Of these efflux transporters, BCRP is expressed mainly in the trophoblast layer; the effect of its polymorphisms on drug pharmacokinetics has been well studied. One of the polymorphisms affecting kinetics is the C421A/Q141K BCRP single-nucleotide polymorphism. It was reported that the C421A variant of BCRP may have the potential to alter the placental pharmacokinetics of glyburide used in pregnancy (Pollex et al. 2010), and that the BCRP C421A polymorphism may play an important role in the pharmacokinetics of rosuvastatin in healthy Chinese males (Zhang et al. 2006).

In order to clarify the usefulness of our DJEG layer placental drug transport model as a evaluation system for placental drug transport in relation to both simple diffusion and carrier-mediated transport, this study examined the mRNA expression profiles of the efflux transporters MRPs, MDR1, and BCRP in JEG-3 cells and compared them with those of BeWo cells and their known placental expression. Moreover, we investigated tight junction-associated proteins to describe the functional changes of DJEG. These tight junction-associated proteins include zonula occludence-1 (ZO-1), occludin (OCLN), and claudin 1 (CLDN1) (Sheth et al. 2000; Harada et al. 2007). Finally, the BCRP C421A polymorphism that defined one of the transport model features was identified in the JEG-3 and BeWo cells.

2. Investigations and results

2.1. mRNA Expression profiles of MRP1–8 and tight junction–associated protein in JEG-3 cells using multiplex RT-PCR

In MRP mRNA expression profiles, the expression of MRP3, MRP5 and MRP7 was induced slightly by DMEM, Ham's F-12, D/F+ aFGF, and CSC®; however, the expression of other mRNAs did not change markedly between several culture conditions. Nevertheless, mRNA expression of CLDN1 was markedly induced when cells were grown in DMEM, Ham's F-12, D/F + aFGF, and CSC®. The mRNA expressions of ZO-1 and OCLN among tight junction-associated proteins (CLDN1, ZO-1, and OCLN) were hardly changed, whereas that of hPL, a differentiating signal, was induced slightly by Ham's F-12, D/F + aFGF, and CSC®. The mRNA expression profiles in cultures grown in plastic culture plates and on Millicell® PET membranes were similar (Fig. 1).

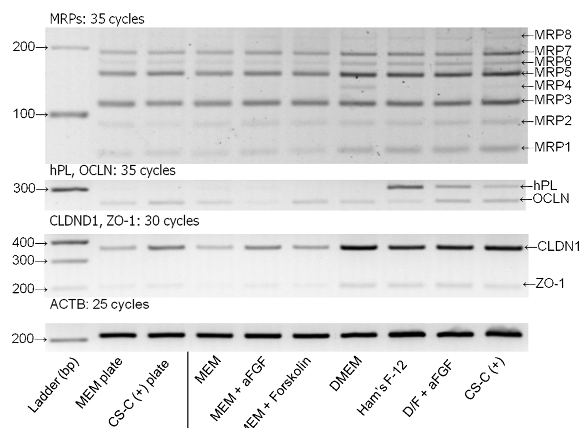


Fig. 1: Multiplex RT-PCR mRNA expression profiles of MRPs, hPL, OCLN, CLDN1, ZO-1, and ACTB from the JEG-3 cells cultured under several culture conditions in 24-well plates (left; 2 lanes) and on Millicell® 24-well hanging cell culture inserts (right; 7 lanes). Cells were cultured under each condition for 5 d after initial culture in MEM for 3 d. PCR conditions are described in the Experimental section. Numbers (left) indicate the size of the PCR products (bp). Letters (right) indicate the position of each target gene. Data shown are representative of triplicate experiments. ACTB was investigated as a control for total mRNA amounts

2.2. TaqMan® Real-time RT-PCR of CLDN1 in JEG-3 cells

The results of multiplex RT-PCR demonstrated that mRNA expression of CLDN1 was induced in JEG-3 cells cultured and differentiated with DMEM, Ham's F-12, D/F + aFGF, and CSC®. The TaqMan® real-time RT-PCR method was used for a detailed study of induction. The mRNA expression of CLDN1 was induced by forskolin, DMEM, and CSC® (Fig. 2).

2.3. Efflux transporter mRNAs expression profiles in Caco-2, BeWo, and JEG-3 cells using multiplex RT-PCR

The efflux transporter mRNA expression profiles were indicated in BeWo and JEG-3 cells as compared with those of Caco-2 cells, in which the expression of efflux transporters is well studied (Ikeda et al. 2008), which were used as a positive control. The BCRP and MDR1 expression profiles between the Caco-2 cells and choriocarcinoma cells, BeWo and JEG-3, were contradictory. However, the profiles between the BeWo and JEG-3 cells were similar. The MRPs expression profiles between the BeWo and JEG-3 cells were also similar, which suggested that the JEG-3 model was syncytiotrophoblast-like, as were the BeWo cells (Fig. 3).

2.4. Polymorphism of BCRP C421A in BeWo and JEG-3 cells

The C421A polymorphism of BCRP was determined in the BeWo and JEG-3 cells using TaqMan® genotyping and gene

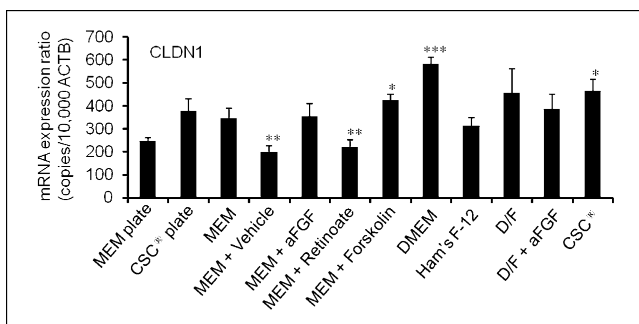


Fig. 2: Comparison of CLDN1 mRNA expression ratios under different JEG-3 culture conditions determined using TaqMan® real-time RT-PCR. JEG-3 cells were cultured on Millicell® 24-well hanging cell culture inserts for 5 d after initial culture in MEM for 3 d. TaqMan® real-time RT-PCR method is described in the Experimental section. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ [Student's t-test vs. MEM (retinoic acid and forskolin, vs. MEM + vehicle), n = 4]

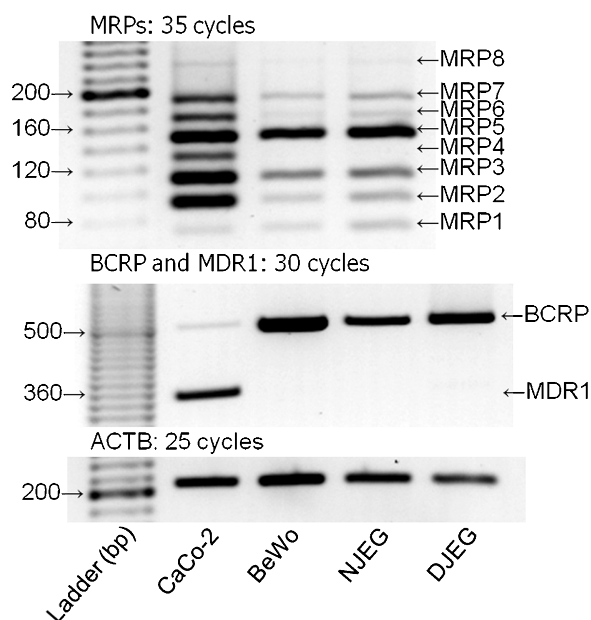
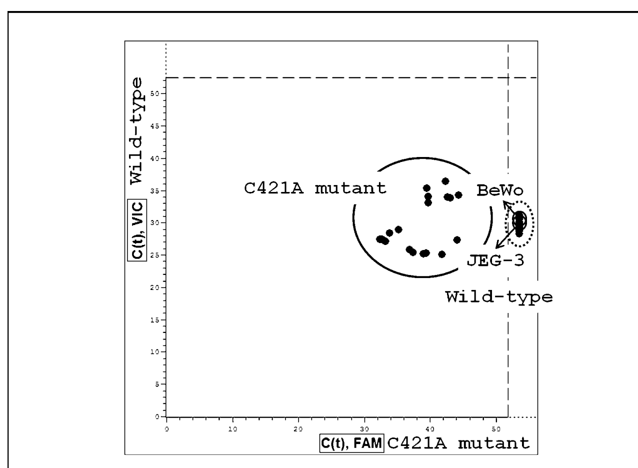
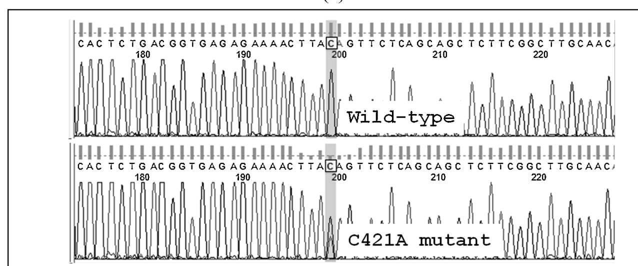


Fig. 3: Multiplex RT-PCR mRNA expression profiles of MRPs, BCRP, MDR1, and ACTB from Caco-2 (positive control), BeWo, and JEG-3 cells cultured in 6-well plates. Caco-2, BeWo, and NJEG cells were cultured in DMEM, F-12K, and MEM for 14 d, respectively. DJEGs were cultured under the CSC® condition for 10 d after initial culture in MEM for 3 d. PCR conditions are described in the Experimental section. Numbers (left) indicate the size of the PCR products (bp). Letters (right) indicate the position of each target gene. Data shown are representative of triplicate experiments. ACTB was investigated as a control for total mRNA amounts

sequencing. The results of polymorphisms detected using the two methods including those of the control samples provided by 13 volunteers. As a result, the BCRP C421A variant in the BeWo and JEG-3 cells was defined as ‘wild type’ (Fig. 4).



(a)



(b)

Fig. 4: Examination of BCRP C421A polymorphism between BeWo and JEG-3 cells. The genomic partial region containing the polymorphism was amplified and the C421A polymorphism investigated using TaqMan® genotyping (A) and targeted genomic DNA sequencing (B). X-axis: fluorescence intensity (FAM) of the BCRP C421A mutant; detection over 50 cycles indicates the C421A mutant negativity. Y-axis: fluorescence intensity (VIC) of the wild type variant; detection over 50 cycles indicates the wild-type negativity

3. Discussion

This study allowed us to demonstrate MRP mRNA expression profiles in JEG-3 cells for the first time. The expression of MRP3, MRP5 and MRP7 mRNA was induced in the DJEG. The mRNA profiles of these MRPs were in agreement with reports of those regarding the syncytiotrophoblast-like BeWo cell line (Serrano et al. 2005) and *in vivo* placental cells (Klaassen et al. 2010). The expressions of hPL and CLDN1 mRNAs were also induced in the DJEGs. The induced expression of hPL is a signal of trophoblast differentiation. The induction of CLDN1 mRNA expression indicates adequate formation of the tight junction in the DJEGs. The BeWo cells have been useful in evaluation of their functional placental effects *in vitro* because of their syncytiotrophoblast-like features. However, study of the layer function of BeWo cells for evaluating placental transport has proven rather difficult. Our previous study indicated that the barrier function of DJEGs was higher compared with that of NJEG and BeWo cells. The findings in this study suggest that DJEGs induce a differentiation signal, hPL, and demonstrate an efflux transporter mRNA expression profile that is in agreement with that of the syncytiotrophoblast-like cells.

In a study using a primary culture of cytotrophoblasts, gene expression of BCRP was induced after the cytotrophoblasts were cultured for more than 3 days, and a primary culture cytotrophoblast layer cultured for more than 3 days would approximate the syncytiotrophoblast layer, whereas gene expresion of MDR1 was reduced (Evseenko et al. 2006). It is well known that the Caco-2 cells express mainly MDR1 mRNA. These results indicate that the expression of MDR1 in the BeWo and JEG-3 cells was not detected compared with that of Caco-2 cells, although expression of BCRP was induced. The expression levels of BCRP mRNA in the BeWo and JEG-3 cells exceeded that of MDR1 in the Caco-2 cells, which were investigated for their effect on the pharmacokinetics of several drugs. Moreover, we demonstrated that the BCRP C421A polymorphism in both the BeWo and JEG-3 cells was of the wild type. These findings on MRP expression profiles and BCRP polymorphisms could be useful for analysis of the pharmacokinetics of placental drug transport across the DJEG model.

In a previous study, DJEGs exhibited high TER values and induction of differentiation signals (Ikeda et al. 2011). However, although TER values were low in the presence of forskolin, the mRNA expressions of the differentiation signals were comparable to those of the DJEGs. In this study, the expression of CLDN1 was induced with TaqMan® gene expression analysis in the presence of forskolin. It was believed that forskolin induced differentiation of the JEG-3 cells; however, the barrier function of the differentiating cells had not been formed solidly.

We found no significant change in other MRP mRNA expression profiles in JEG-3 cells under differing culture conditions, although the DJEGs did have a tendency to increase the expression of MRP3, MRP5, and MRP7. However, BCRP was induced in the DJEGs. The induction of BCRP has previously been reported in primary cytotrophoblast differentiation, and it was suggested that this differentiating trophoblast model of JEG-3 cells would approximate the *in vivo* condition, as would the BeWo cells. Changes in the expression profiles of efflux transporter mRNAs were demonstrated in the differentiated JEG-3 cells; however, further investigations will need to be conducted in order to clarify the relationship between these findings, the protein expressions, and actual drug transport function in these cell models. Several substrates of MDR1, MRPs, and BCRP

Table: Positions and amplified fragment length of the primer sequences used for multiplex RT-PCR

Gene		Sequence (Position)		NCBI GenBank Accession No.	Product Size (bp)
hPL	Sense	259	AGCTGGCCATTGACACCTAC	NM_001317	301
	Antisense	559	TGGATGCCTTCCTCTAGGTC		
OCLN	Sense	215	CCGAGTTTCAGGTGAATTGG	NM_002538	248
	Antisense	462	GGACTTTCAAGAGGCCTGGA		
CLDN1	Sense	419	GGCAGATCCAGTGCAAAGTC	NM 021101.4	350
	Antisense	768	AAGGCAGAGAGAAGCAGCAG		
ZO-1	sense	648	ATGTGCTGAAAGGAGGACCA	NM_003257	198
	Antisense	845	CTGGTTCAGGATCAGGACGA		

have been utilized in order to obtain pharmacokinetic estimates (McNamara et al. 1992; Gerk et al. 2001; Terasaki et al. 2003; Pollex et al. 2008; Gedeon et al. 2008), and studies on the transport of these substrates using this model could provide evidence for the carrier-mediated transport of drugs across the placenta. Moreover, the mRNAs of tight junction-associated proteins, i.e. ZO-1, OCLN, and CLDN1, under several culture conditions were investigated. Among these, CLDN1 was induced in the DJEGs and this suggested adequate formation of the tight junction of the DJEGs.

BCRP, an important efflux transporter, is expressed strongly in the placenta. It has been found that the pharmacokinetics of some drugs was altered by BCRP polymorphisms, of which the BCRP C421A polymorphism is one. The polymorphism should be made known for evaluation of the placental drug transport model. It was indicated that the analytical findings that will be investigated using the DJEG placental drug transport model involved the wild-type BCRP C421A.

In conclusion, we suggest that the mRNA of the efflux transporters MRP 1–8 and BCRP are expressed widely in JEG-3 cells; however, the expression level of MDR1 mRNA is undetectable. These results are in agreement with those of the BeWo and *in vivo* study. It was indicated that polymorphisms of BCRP C421A are wild type in both the BeWo and JEG-3 cells. The DJEG placental drug transport evaluation model described previously (Ikeda et al. 2011) possesses the syncytiotrophoblast-like features, as do the BeWo cells, as well as adequate tight junction formation, and could be applicable to modelling of inclusive drug transport across the trophoblast layer *in vitro*. Based on these findings, we hope that our DJEG model will be of adequate use as a factor for evaluating placental drug transport in relation to the transporter proteins.

4. Experimental

4.1. Materials

JEG-3 cells (lot no. 04E018), fetal bovine serum, and CSC® medium kits (lot no. 54005) optimized for culturing human umbilical vein endothelial cells (HUVECs) were obtained from Dainippon Sumitomo Pharma Co. Ltd. (Osaka, Japan). Specific oligonucleotide primers were obtained from Invitrogen Japan KK. (Tokyo, Japan). TaqMan® gene expression master mix, TaqMan® genotyping master mix, TaqMan® gene expression assays, and TaqMan® human β-actin (ACTB) endogenous controls (MGB probes) were obtained from Applied Biosystems, Ltd., Japan (Osaka, Japan). DMEM was purchased from Sigma-Aldrich (St. Louis, MO). An RNeasy® Protect mini kit and QIAamp DNA Blood mini kit were obtained from Qiagen Inc. (Valencia, CA), and a 100-bp EZ Load™ molecular ruler and an iScript™ cDNA synthesis kit were obtained from Bio-Rad Laboratories, Inc. (Tokyo, Japan). Cell culture plates and flasks were purchased from Becton Dickinson and Company (Franklin Lakes, NJ). A multiplex PCR assay kit® was purchased from TaKaRa Bio Inc. (Shiga, Japan). Cellmatrix® (collagen type I-A) was obtained from Nitta Gelatin Inc. (Osaka, Japan). Millicell® 24-well hanging cell culture inserts (pore size, 1 μm; polyethylene terephthalate (PET) membrane, 0.33 cm^2) were purchased from Millipore Co. (Billerica, MA). All other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

4.2. Cell culture

JEG-3 cells were cultured in Eagle's minimum essential medium (MEM) supplemented with Earle's salts, 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, and 1% (v/v) nonessential amino acids. Caco-2 cells were cultured in DMEM supplemented with Earle's salts and 10% (v/v) fetal bovine serum. BeWo cells were cultured in F-12K supplemented with Earle's salts and 10% (v/v) fetal bovine serum. These cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The JEG-3 cells were seeded at approximately 40,000 cells/cm^2 on the PET membranes of the 24-well inserts and plates (Falcon®; BD, Franklin Lakes, NJ) (Ikeda et al. 1999; Ikeda et al. 2011). In the culture condition investigation, the seeded JEG-3 cells were cultured with MEM for 3 d, and then further cultured under each condition examined for 4–13 d. The cells were collected for semi-quantification of mRNA. Total RNAs were extracted for multiplex reverse transcription-polymerase chain reaction (RT-PCR) using an RNeasy® protect cell mini kit. Forskolin and retinoic acid were dissolved in DMSO and 100 μM and 10 μM, respectively, were added to MEM (1% DMSO). The genomic DNAs were extracted for determining BCRP C421A polymorphisms using a QIAamp DNA Blood mini kit.

4.3. Multiplex RT-PCR

Total RNA was extracted using an RNeasy® Protect cell mini kit, and an iScript™ cDNA synthesis kit was used for the generation of first-strand cDNA templates (50 μL) from 1 μg total RNA. Tight junction-associated proteins included ZO-1, OCLN, and CLDN1. JEG-3 cells express claudins 1 to 5, with CLDN1 being expressed at the lowest level (Harada et al. 2007). We selected CLDN1 to estimate the induction of this low level expression of claudin. The primer pairs used for the multiplex RT-PCR of hPL, OCLN, CLDN1, and ZO-1 are shown in the Table. Ikeda et al. (2008) previously described the primer pairs for the MRPs, BCRP, MDR1, and ACTB. Using a GeneAmp® PCR System 9700 (Applied Biosystems, CA), multiplex PCR was carried out with a multiplex PCR assay kit in a total volume of 50 μL, using 1 μL of each cDNA template. Multiplex PCR amplification consisted of 25 (ACTB), 30 (BCRP, MDR1, CLDN1, and ZO-1), or 35 cycles (MRPs, hPL, and OCLN) of denaturation at 94 °C for 30 s, annealing at 60 °C for 90 s, and extension at 72 °C for 90 s. Electrophoresis of MRPs was performed using 4% MetaPhor® agarose (TaKaRa Bio Inc., Shiga, Japan) gels; other PCR products were electrophoresed on 2% agarose gels. The gels were stained with ethidium bromide and visualised under UV light.

4.4. TaqMan® real-time RT-PCR for ACTB and CLDN1

Templates for first-strand cDNA as well as the multiplex RT-PCR were synthesized. The mRNA expression levels of CLDN1 and β-actin (encoded by the *ACTB* gene, the TaqMan® human endogenous control) were analyzed using a real-time PCR detector (Opticon®2; Bio-Rad). The PCR amplification comprised 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 60 s following an initial pre-activation at 50 °C for 2 min and at 95 °C for 10 min. The expression ratios of mRNA were normalized to that of the housekeeping gene *ACTB*.

4.5. Polymorphism of BCRP

The ethical committee of the Department of Pharmacy, Osaka Ohtani University approved this study (No. BE-003–10). Whole peripheral blood (20 μL) was added to 180 μL of PBS. This total volume (200 μL) and cell suspensions of BeWo and JEG-3 cells (200 μL each) were supplemented with 20 μL of protease. Genomic DNA was extracted from these samples using a QIAamp DNA Blood mini kit. The primers for DNA sequencing to detect the C421A BCRP variant were designed by Primer3, a PCR primer-designing program. The forward primer was 5′-GCAGGCTTTGCAGACATCTA-3′, the reverse was 5′-

TGCTGATCATGATGCTTTCAG-3′, and the product size was 367 bp. Sequencing was entrusted to Life Technologies Japan Sequence Service (Tokyo, Japan). The TaqMan® genotyping (No. C__15854163_70) was analyzed with genomic templates of approximately 5 ng/tube using a real-time PCR detector (Opticon®2; Bio-Rad). The PCR amplification comprised 50 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 60 s following an initial pre-activation at 95 °C for 10 min.

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