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Functional expression and characterization in *Xenopus laevis* oocytes of the ABCG2 transporter derived from A549 human lung adenocarcinoma cells

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Abstract. We cloned the ATP-binding cassette sub-family G member 2 (ABCG2) transporter, the most recently identified among several major human multidrug-resistance pumps, from A549 human lung adenocarcinoma cells in order to characterize its function and substrate specificity. In a previous report, we confirmed that a stem cell-like side population of A549 cells highly expressed the ABCG2 gene and had a unique ability to resist the anticancer drug methotrexate (MTX). In this study, ABCG2 cDNA was cloned by RT-PCR and converted into cRNA by an *in vitro* transcription system for expression in *Xenopus laevis* (*X. laevis)* oocytes. The transcribed cRNA of the ABCG2 gene was injected into the oocytes under the absence of cofactors or heterologous partner proteins or some lipids from the media. A high expression of ABCG2 was observed on the oocyte surface by immunofluorescence and confocal laser microscopy. We tested the functional effect of ABCG2 expression on drug efflux by directly injecting MTX into *X. laevis* oocytes. The drug concentration within the oocytes was quantified with LC-MS/MS; the analysis showed that the accumulation of MTX was significantly decreased in the *X. laevis* oocytes expressing ABCG2 compared with the control oocytes not expressing ABCG2. These findings show that the ABCG2 protein has an important role in the efflux of MTX through the cell membrane of *X. laevis* oocytes. Therefore, it might be that ABCG2, abundantly expressed in the stem cell population of A549 cells, can modulate resistance to MTX in lung cancer therapy.

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Introduction

Cells can become resistant to chemotherapy by overexpressing ATP-binding cassette (ABC) transporters, which use the energy from ATP hydrolysis to transport a wide variety of substrates across the cell membrane. ABC transporters have seven subfamilies, A to G, which consist of several transmembrane proteins that use an energy source for the active extrusion of various drugs, endogenous materials, carcinogens and other xenobiotics across the cellular plasma membrane (1). Over the past 30 years, the functions of several ABC transporters were identified and include the absorption, distribution and excretion of drugs. These efflux transporters have a significant influence on drug pharmacokinetics and pharmacodynamics. Three human ABC transporters are most often associated with multidrug resistance phenomena in cancer cells: P-glycoprotein (P-gp)/MDR1/ABCB1, MRP1/ABCC1 and BCRP/MXR/ABCG2. P-gp (ABCB1) and MRP1 (ABCC1) (2-5), two of the most extensively studied ABC transporters, convey resistance to a wide variety of structurally unrelated cytotoxic agents (6,7). However, several studies had difficulty confirming the MDR hypothesis in clinical trials (8). Despite the wealth of information collected about their biochemistry, the investigation of ABC transporter substrate specificity and efflux function in cancer cells has proven to be unexpectedly difficult (9).

The breast cancer resistance protein, BCRP/ABCG2, is the most recently identified half-molecule multidrug-resistance ATP-binding cassette transporter, however, it is not understood how this transporter affects drug resistance in a clinical setting (10). The human ABCG2 gene, containing 16 exons, 15 introns and spanning over 66 kb, is located on chromosome 4q22 (11). The ABCG2 transporter is a 655-amino acid peptide with an ability to extrude a wide variety of biochemical compounds from cells (12). With high normal tissue expression in the brain endothelium, gastrointestinal tract, and placenta, ABCG2 is regarded as important in the protection from xenobiotics, in the regulation of oral bioavailability, and in the formation of part of the blood-brain barrier, the blood-testis barrier, and the maternal-fetal barrier. It can also be found in stem cells and some cancer cells (12). In multidrug resistant cancer

cells, ABCG2 has been known to efflux potential anti-cancer drugs, such as methotrexate (MTX) and mitoxantrone (MX) (11,13,14). In addition, ABCG2 has been linked to the stemness properties of cancer cells (15). A previous research study has shown that ABCG2-dependent drug efflux is associated with multidrug resistance in many cancers (16), with higher levels of DNA repair and lower levels of apoptosis (17).

In our previous studies (18,19) we identified that ABCG2 expression was more than 2-fold higher in side population (SP) cells compared to non-side population cells, as determined by FACS with Hoechst 33342 staining. The ABCG2 transporter is known as a possible marker used to separate SP from non-SP cells, but it is not as well-studied compared with other ABC transporters (20). Therefore, we have cloned the ABCG2 cDNA from the A549 cell line and transformed it into the surface membrane of *Xenopus laevis (X. laevis)* oocytes to characterize the potential role of the ABCG2 efflux pump in multidrug resistance cancer cells.

Materials and methods

Materials. All chemicals and reagents were either molecular biology grade or immunohistochemistry grade. MTX was purchased from Sigma-Aldrich (St. Louis, MO, USA). The mouse monoclonal antibodies, BXP-21 and 34, were raised against the human BCRP and were produced by Abcam (Cambridge, England). Adult female *X. laevis* frogs were obtained from Xenopus 1, Inc. (Ann Arbor, MI, USA).

Preparation of human ABCG2 cDNA. The ABCG2 gene was isolated from A549 human lung adenocarcinoma cells by PCR with a single set of primers: 5'-TCC AGA TGG CTT CCA GTA AT-3' (forward) and 5'-GCA AGG GAA CAG AAA ACA AC-3' (reverse). The full-length ABCG2 cDNA was amplified with a one-step RT-PCR kit (Takara Bio, Shiga, Japan) and ligated into the pTARGET mammalian expression vector (Promega, Fitchburg, WI, USA). The amplified DNA was analyzed by gel electrophoresis and DNA sequencing. ABCG2 cDNA was also inserted by ligation (Toyobo, Osaka, Japan) into the pNBC 2.0 vector (Fig. 1A) for heterologous cRNA expression in a *X. laevis* oocytes system. All transformation and subcultivation procedures were performed with competent DH5-α *E. coli* cells (Real-Biotech Corp., Taipei, Taiwan). Plasmid DNA was prepared with the midi-prep kit (Qiagen, Hilden, Germany), and we checked the plasmid DNA with 1.0% agarose gel electrophoresis (Bio-Rad, Hercules, CA, USA) and DNA sequencing (Macrogen, Seoul, Korea).

cRNA microinjection and expression in X. laevis oocytes. The full-length ABCG2 cDNA was amplified with a one-step RT-PCR kit (Takara Bio) and it was utilized as an insert into the pTARGET mammalian expression vector. Plasmid DNA containing the desired ABCG2 cDNA insert was linearized by restriction enzyme (*Sph*I) digestion and used as a template for synthesizing cRNA using the mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA) and T7 RNA polymerase. Animal care and handling were carried out in accordance with the highest standards of institutional guidelines. *X. laevis* oocytes were prepared and maintained as previously described (27). Simultaneously, *X. laevis* oocytes were injected with 50 nl of water per oocyte (hereafter referred to as Control oocytes) or 50 nl of 1 μg/µl cRNA solution. The oocytes were cultivated for 2-3 days after cRNA injection at 18˚C in Barth's medium supplemented with 50 mg/l gentamicin (Sigma-Aldrich).

Western blot analysis. Groups of 24 oocytes were homogenized in 200 μl of ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40 and 0.25% sodium deoxycholate) per group in the presence of a protease inhibitor cocktail (Sigma-Aldrich) and were then sonicated for 5 sec at low power and vortexed. The homogenate was centrifuged at 1,000 x g at 4˚C for 10 min, and the supernatant was centrifuged again. The protein concentration of the supernatant was determined by the BCA method. A volume of supernatant containing 25 μg of extracted protein was subjected to 10% SDS-polyacrylamide gel electrophoresis and blotted by electrotransfer onto a polyvinylidene difluoride membrane. The blots were blocked with 5% non-fat dry milk and probed at 4˚C overnight with a 1:1,000 dilution of the BXP-21 monoclonal antibody to BCRP (Abcam). The blots were then incubated at room temperature for 1 h with a horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (Abcam) and were imaged with enhanced chemiluminescent detection (Amersham Biosciences, Uppsala, Sweden) using the Las-3000 (Fujifilm, Tokyo, Japan) analyzer and the Multi-Gauge software.

Immunofluorescence and confocal laser microscopy. Oocytes injected with water or ABCG2 cRNA were incubated for 3 days, fixed in phosphate-buffered saline (PBS, pH 7.4) with 10% formaldehyde and immersed overnight in PBS with 30% sucrose. Groups of 3-4 oocytes were frozen in OCT compound (Tissue-Tek; Sakura Finetek Europe, Zoeterwoude, The Netherlands), and 25 μm sections were collected on gelatin-coated slideglasses. Sections on slideglasses were inverted onto 50 μl of primary antibody, BXP-34 (Abcam), diluted 1:50 in buffer A (PBS with 1% bovine serum albumin) for 1 h at room temperature in a moisture chamber and were rinsed three times with buffer A for 10 min. The sections were probed with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Abcam) diluted 1:100 in buffer A and were rinsed three times with buffer A for 10 min (21). Sections were mounted in aqueous anti-fade mounting medium (Biomeda Co., Foster City, CA, USA), and immunofluorescence was observed with a confocal scanning laser microscope (Olympus FV-1000, Tokyo, Japan) at x100 magnification. Using an initial section from an oocyte injected with ABCG2 cRNA, the background fluorescence was set to zero, and the brightest fluorescence was set to saturation. These parameter settings were used for all subsequent sections, and the autolevel was adjusted by Adobe Photoshop 7.0.

Determination of MTX efflux in X. laevis oocytes. ABCG2 cRNA-injected and water-injected *X. laevis* oocytes were cultivated in uptake buffer at room temperature for 3 days and then, 50 nl of 25 μM MTX was injected directly into each oocyte. Thereafter, 12 oocytes from each treatment group were transferred to 100 μl of uptake buffer and incubated for 2 h. After incubation, the oocytes were washed four times with ice-cold uptake buffer. Twelve oocytes from each group were transferred and homogenized in 100 μl of mobile phase

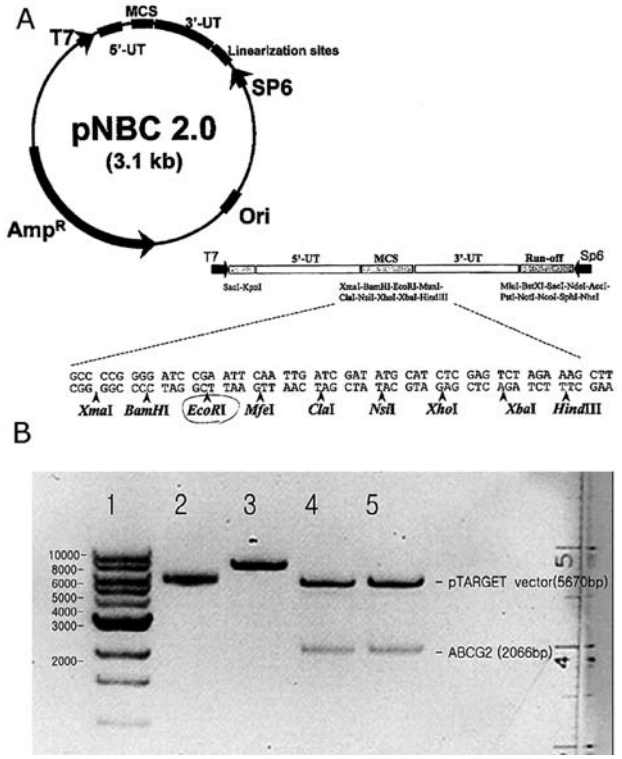

Figure 1. Plasmid construction of the full-length cDNA. (A) pNBC 2.0 *X. laevis* oocyte expression vector. (B) Lane 1, 1 kb DNA ladder (Takara Bio, Shiga, Japan); lane 2, pTARGET plasmid harboring ABCG2; lane 3, *Bam*HI digest of lane 2; lane 4, *Bam*HI-*Eco*RI double digest of lane 2; and lane 5, *Eco*RI digest of lane 2.

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Score = 1239 bits (3207),  Expect = 0.0
Identities = 650/655 (99%), Positives = 653/655 (99%), Gaps = 0/655 (0%)
Frame = +1

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             M+SSNVEVFIPVSQGNTNGFPATASNDLKAFTEGAVLSFHNICYRVKLKSGFLPCRKPVE
Sbjct  1     MSSSNVEVFIPVSQGNTNGFPATASNDLKAFTEGAVLSFHNICYRVKLKSGFLPCRKPVE  60

Query  181   KEILSNINGIMKPGLNAILGPTGGGKSSLLDVLAARKDPSGLSGDVLINGAPRPANFKCN  360
             KEILSNINGIMKPGLNAILGPTGGGKSSLLDVLAARKDPSGLSGDVLINGAPRPANFKCN
Sbjct  61    KEILSNINGIMKPGLNAILGPTGGGKSSLLDVLAARKDPSGLSGDVLINGAPRPANFKCN  120

Query  361   SGYVVQDDVVMGTLTVRENLQFSAALRLATTMTNHEKNERINRVIQELGLDKAADSKVGT  540
             SGYVVQDDVVMGTLTVRENLQFSAALRLATTMTNHEKNERINRVIQELGLDK ADSKVGT
Sbjct  121   SGYVVQDDVVMGTLTVRENLQFSAALRLATTMTNHEKNERINRVIQELGLDKVADSKVGT  180

Query  541   QFIRGVSGGERKRTSIGMELITDPSILFLDEPTTGLDSSTANAVLLLLKRMSKQGRTIIF  720
             QFIRGVSGGERKRTSIGMELITDPSILFLDEPTTGLDSSTANAVLLLLKRMSKQGRTIIF
Sbjct  181   QFIRGVSGGERKRTSIGMELITDPSILFLDEPTTGLDSSTANAVLLLLKRMSKQGRTIIF  240

Query  721   SIHQPRYSIFKLFDSLTLLASGRLMFHGPAQEALGYFESAGYHCEAYNNPADFFLDIING  900
             SIHQPRYSIFKLFDSLTLLASGRLMFHGPAQEALGYFESAGYHCEAYNNPADFFLDIING
Sbjct  241   SIHQPRYSIFKLFDSLTLLASGRLMFHGPAQEALGYFESAGYHCEAYNNPADFFLDIING  300

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Sbjct  301   DSTAVALNREEDFKATEIIEPSKQDKPLIEKLAEIYVNSSFYKETKAELHQLSGGEKRKK  360

Query  1081  ITVFKEISYTTSFCHQLRWVSKRSFKNLLGNPQASIAQiivtvvlglvigaiYFGLKDDS  1260
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Sbjct  361   ITVFKEISYTTSFCHQLRWVSKRSFKNLLGNPQASIAQIIVTVVLGLVIGAIYFGLKNDS  420

Query  1261  TGIQNRAGVLFFLTTNQCFSSVSAVELFVVEKKLFIHEYISGYHRVSSYFLGKLLSDLLP  1440
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Sbjct  421   TGIQNRAGVLFFLTTNQCFSSVSAVELFVVEKKLFIHEYISGYYRVSSYFLGKLLSDLLP  480

Query  1441  MRMLPSIIFTCIVYFMLGLKPKADAFFVMMFTLMMVAYSASSMALAIAAGQSVVSVATLL  1620
             MRMLPSIIFTCIVYFMLGLKPKADAFFVMMFTLMMVAYSASSMALAIAAGQSVVSVATLL
Sbjct  481   MRMLPSIIFTCIVYFMLGLKPKADAFFVMMFTLMMVAYSASSMALAIAAGQSVVSVATLL  540

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Sbjct  601   NPCNYATCTGEEYLVKQGIDLSPWGLWKNHVALACMIVIFLTIAYLKLLFLKKYS  655
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Figure 2. BLASTx analysis of the ABCG2-pTARGET plasmid. The confirmed nucleotide sequence of ABCG2 was translated into its amino acid sequence (upper line). The amino acid sequence exactly matched the human ABCG2 amino acid sequence (accession; NM_004827).

solution, a mixture with an acetonitrile: ammonium formate ratio of 18:82 containing 0.1% formic acid, and the homogenate was centrifuged at 4˚C for 20 min at 10,000 rpm. The resulting supernatant (100 μl) and 100 μl of uptake buffer were combined, filtered and subjected to LC-MS/MS (liquid chromatography with tandem mass spectrometry) analysis.

Detection and quantification of MTX by LC-MS/MS. The LC-MS/MS system was composed of an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) and an API2000 MS/MS system (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada) using electrospray ionization in positive ion scan mode. Chromatographic separation was achieved by using a mobile phase of acetonitrile: 1 mM ammonium formate containing 0.1% formic acid (18:82, v/v) (22) delivered at a flow rate of 0.2 ml/min through an analytical column (Waters, XTerra MS C_{18}, 5 μm particle size, 2.1x150 mm). The column temperature was maintained at 30˚C. The column effluent was monitored at the following transitions: MTX m/z 455.4→308.0 with a dwell time of 150 ms. The spray gas was nitrogen set at a pressure of 100 psi. The curtain gas and collision gas were both nitrogen and were both set at a pressure of 50 psi. The ion spray voltage was set at 5,500 V and the temperature was set to 350˚C.

Results

ABCG2 cDNA confirmation. Full-length ABCG2 cDNA (2,066 bp, which was modified to insert of Kozack sequence for high transfection efficacy as described in Materials and methods) was generated by RT-PCR and inserted into the pTARGET vector (5,670 bp). We utilized full-length ABCG2 cDNA for the competent DH5-α *E. coli* cell transformation. We confirmed the plasmid of ABCG2 using restriction enzymes (*Bam*HI, *Eco*RI) digestion, gel electrophoresis and UV scanning (Fig. 1B) as well as DNA sequencing. The sequencing result matched the wild-type sequence of ABCG2 (Fig. 2). The ABCG2 insert was separated from the pTARGET vector, inserted into the pNBC 2.0 vector and transformed in *X. laevis* oocytes.

cRNA synthesis and in vitro transcription. The pNBC 2.0 vector containing the ABCG2 insert was linearized by *Sph*I restriction enzyme digestion. The linearized plasmid was transcribed to cRNA by an *in vitro* transcription kit. The amount of cRNA was confirmed by gel electrophoresis.

ABCG2 expressed in X. laevis oocytes localizes to the surface membrane. *In vitro* transcribed cRNA was quantified prior to injection into *X. laevis* oocytes. One experimental group of 30 oocytes received an injection of water (control oocytes). The other experimental group of 30 oocytes received an injection of cRNA (ABCG2+pNBC oocytes). Four oocytes from each group were fixed and made into 25 μm cryosections as described in Materials and methods. Exposure of these sections to the BXP-34 monoclonal antibody, directed against ABCG2, resulted in immunofluorescence only in the cell membrane of oocytes expressing ABCG2 as detected by confocal fluorescence microscopy (Fig. 3A). Control oocytes showed no immunofluorescence. Western blot analysis of oocytes injected with ABCG2 cRNA at the time of the immunofluorescence

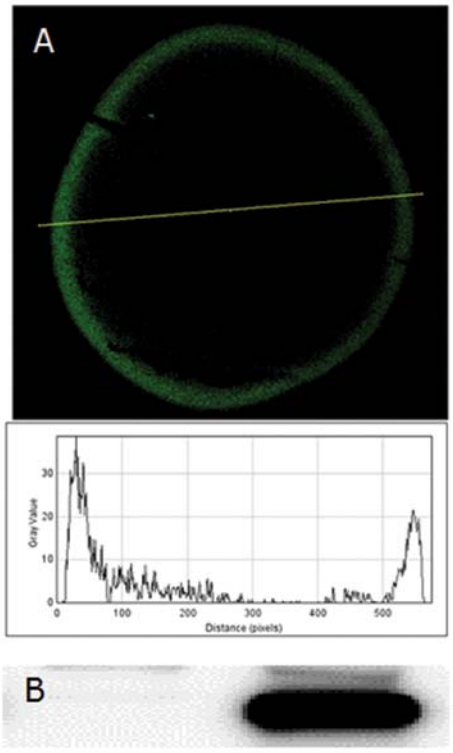

Figure 3. Confocal immunofluorescence microscopy of *X. laevis* oocyte injected with 50 nl of cRNA of ABCG2. (A) Distribution of ABCG2 at the oocyte surface. (B) Cell lysate (25 μg) from the oocytes injected with ABCG2 cRNA. After electrophoresis and blotting, the blots were probed with the BXP-21 antibody.

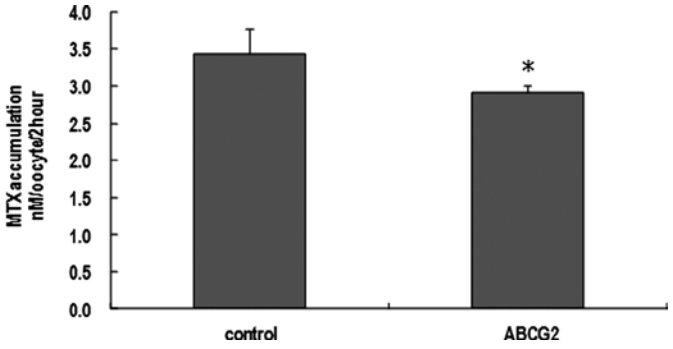

Figure 4. Uptake of MTX in control *X. laevis* oocytes and in ABCG2-expressing oocytes. MTX, 50 nl of 25 μM, was directly injected into each oocyte (n=12 oocytes, *P<0.05).

experiments confirmed robust expression of ABCG2 protein with a molecular mass of 70 kDa (Fig. 3B).

Accumulation and efflux of MTX in oocytes expressing ABCG2. To determine whether functional ABCG2 was expressed in *X. laevis* oocytes, we monitored the accumulation of MTX in control and in ABCG2-injected oocytes. Oocytes expressing ABCG2 showed a significant reduction in the accumulation of MTX over a 2 h period. After background subtraction, MTX accumulation in the ABCG2-expressing oocytes was ca. 80% of the control group as measured by LC-MS/MS (Fig. 4).

Discussion

This research extends previous functional protein analyses in SP cells in order to confirm the relationship between the up-regulation of specific genes and multidrug resistance. When conducting a functional study of upregulated genes, we performed our experiment with ABCG2 first because it was the most interesting gene of SP cells in our previous study. Though known as a potential marker of SP cells (20), we focused on ABCG2 as a cell-surface, ABC-protein family transporter that may mediate the efflux of anticancer agents, thereby reducing intracellular drug concentrations.

The ABCG2 gene spans over 66 kb and is made up of 16 exons and 15 introns; the resulting protein is 655 amino acids long and runs as a 72 kDa protein on an SDS-PAGE gel under reducing conditions (23). Fluorescence *in situ* hybridization studies with a bacterial artificial chromosome probe containing ABCG2 localized the gene to chromosome 4q21-4q22 in a cell with a normal chromosome 4 (24). In the past 10 years, many research articles and reviews have described experiments on the ABCG2/BCRP/MXR transporter. For example, physiological and pharmacological roles of ABCG2 were revealed by the study of ABCG2 knockout mice, including the study of their blood-brain, blood-testis and blood-fetal barriers (25). These functions indicate a primary biological role for ABCG2 in the protection of these cells from xenobiotics. However, several other physiological functions have been observed, including the extrusion of porphyrins and/or porphyrin conjugates from hematopoietic cells, liver and harderian glands (26) as well as the secretion of vitamin B_2 and other vitamins into breast milk (27). Various compounds have been tested as inhibitors of the ABCG2 transporter, and estrogenic compounds, including estrone, several tamoxifen derivatives, phytoestrogens and flavonoids, have been shown to reverse ABCG2-mediated drug resistance (28). Functional SNPs and inhibitory agents of BCRP modulate the *in vivo* pharmacokinetics and pharmacodynamics of BCRP substrates (29). Screening methods and 3D-QSAR (quantitative structure-activity relationship) allow the design of ABCG2 inhibitors and may provide promising drug candidates for clinical trials (30). However, the physiological significance of these processes and functional studies have been difficult to establish, indicating that there is still a lot to learn about this intriguing protein, ABCG2.

We cloned the ABCG2 gene, confirmed its sequence and transformed it using the mammalian expression vector pTARGET. We anticipated some point mutations within the ABCG2 gene because it has a relatively large portion of SP cells compared to other cell lines, but the sequence of ABCG2 in the A549 cells was the same as its wild-type sequence. After the transfection of ABCG2 into *X. laevis* oocytes, using immunofluorescence and confocal laser microscopy, we confirmed that the location of the ABCG2 protein was in the plasma membrane. The location of the ABCG2 protein was similar to that found previously (21). Furthermore, the efflux function of ABCG2 (31,32) was also confirmed by testing the accumulation of the substrate MTX. Therefore, we conclude that ABCG2 may modulate resistance to MTX in lung cancer therapy. We believe that the *X. laevis* oocyte expression system is an effective means of studying ABCG2 function and substrate specificity.

Acknowledgements

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