

ABCB8 Mediates Doxorubicin Resistance in Melanoma Cells by Protecting the Mitochondrial Genome

Aaron M. Elliott and Muhammad A. Al-Hajj

Novartis Institute of Functional Genomics, San Diego, California

Abstract

Despite their initial effectiveness in the treatment of melanoma, chemotherapeutic agents are ultimately futile against this most aggressive form of skin cancer, and patients inevitably succumb to the disease. One of the mechanisms by which residual melanoma cells become chemoresistant is via the decreased efficiency of chemotherapeutics through the action of ATP-binding cassette (ABC) proteins that are variably expressed by the tumor cells. The clinical relevance of the ABC transporters in the context of cancer is paramount. Inhibitors of these transporters have been shown to increase the efficacy of standard therapy in experimental systems. Their clinical application requires better understanding of the role individual transporters play in the mechanism and the development of more specific inhibitors with minimal off target effects. ABC transporters in tumor cells have been shown to confer multidrug resistance in many solid tumors. However, their role in melanomas is far from clear. Here, we prospectively identify ABCB8 as a specific and major player in the chemoresistance of several melanoma cell lines. ABCB8 knockdown with shRNA reduced doxorubicin resistance ~3- to 4-fold in these cells. Furthermore, we show that this reversal is specific to doxorubicin and not to other commonly used chemotherapeutics. Our results also provide evidence that ABCB8 conferred resistance through the protection of mitochondrial DNA from doxorubicin-induced DNA damage. (Mol Cancer Res 2009;7(1):79–87)

Introduction

Chemotherapeutic resistance is a major impediment to the successful treatment of cancer. It is estimated that over 40% of human tumors acquire multidrug resistance, and oftentimes, a combination of toxic anticancer drugs are administered, each with various side effects (1). Chemotherapy resistance emerges as a result of changes in cellular mechanisms that can affect drug sensitivity, including alterations in cell cycle, reduced

apoptosis, altered metabolism, increased DNA damage repair, and most notably, increased efflux of drugs (2). The ability to actively pump toxic drugs out of the cell is the most commonly encountered mechanism of drug resistance and is mediated by the ATP-binding cassette (ABC) transporters. Of the 48 known human ABC transporters, the most studied and targeted mediators of drug resistance are ABCB1 (MDR1), ABCC1 (MRP1), and ABCG2 (MXR). However, these transporters do not function in every cancer type and therefore cannot account for all ABC transporter mediated resistance. Human malignant melanoma, which accounts for 10,000 US deaths annually, is notoriously resistant to chemotherapeutics (3, 4). Doxorubicin (Adriamycin), a widely used antineoplastic agent, is one of the most effective drugs ever developed but is virtually ineffective against melanoma cells as they frequently develop resistance (5-7). The roles of ABCB1 and ABCG2 (most widely known ABC transporters) in melanoma chemoresistance seem to be limited (8, 9). There is little if any expression of ABCB1 in melanoma cells and its expression is not induced by chemotherapeutics known to induce its expression in other tumor types (8, 10, 11). Identifying elements within the ABC family that are responsible for doxorubicin resistance in melanoma cells is critical, but unfortunately, it has been very challenging. However, recently it was discovered that inhibition of ABCB5, the structural paralog of ABCB1 and highly expressed in melanoma cells, could sensitize melanoma cells to doxorubicin (12, 13). Identifying the repertoire of ABC transporters that promote doxorubicin resistance in melanoma cells and how they function is essential to understanding the complex mechanisms of chemoresistance.

The human half-molecule ABC transporter ABCB8 (mABC1) is localized to the inner mitochondrial membrane (14). Although the physiologic function of ABCB8 remains unknown, mitochondrial transporters are thought to be involved in the transport of heme, phospholipids, or peptides (15). The knockdown of ABCB8 by siRNA in neonatal rat cardiomyocytes resulted in an increase in oxidative stress, suggesting that ABCB8 plays a cardioprotective role in these cells (16). Evidence suggests that ABCB8 may also play a role in chemoresistance. Microarray analysis comparing doxorubicin-resistant human T-lymphoblastoid leukemic cells and the doxorubicin-sensitive parental line revealed the differential expression of a variety of different ABC transporters including ABCB8 (17). Due to the lack of protective histones, mitochondrial DNA is easily damaged by doxorubicin, resulting in cell death (18). We sought to analyze the role ABC transporters play in melanoma resistance to chemotherapy, and to identify and characterize unique transporters that specifically play a role in the resistance of doxorubicin. In this study, we prospectively identify ABCB8 as a transporter that

Received 5/16/08; revised 9/18/08; accepted 9/19/08.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Muhammad A. Al-Hajj, MedImmune (AstraZeneca Biologics Unit), One MedImmune Way, Gaithersburg, MD 20878. Phone: 301-398-5710; Fax: 301-398-8710. E-mail: selfrenew@gmail.com

Copyright © 2009 American Association for Cancer Research.

doi:10.1158/1541-7786.MCR-08-0235

specifically contributes to the chemoresistance of melanoma cells to doxorubicin. Furthermore, our functional analysis indicated that ABCB8 could exert its function by protecting the mitochondrial DNA of cells from doxorubicin induced damage.

Results

Doxorubicin Sensitivity of Human Melanoma Cells and Expression of ABC Transporters

To determine the cytotoxicity of doxorubicin on melanoma cells, we did a dose-response curve by treating melanoma cell lines of various phases (i.e., radial growth phase, vertical growth phase, or metastasis) with increasing concentrations of doxorubicin for 24 hours (Table 1; Fig. 1A). The resulting IC_{50} values illustrate the varying degrees of sensitivity to doxorubicin between cell lines (WM793B, 0.69 $\mu\text{mol/L}$; WM1552C, 3.6 $\mu\text{mol/L}$; WM39, 0.69 $\mu\text{mol/L}$; 451Lu, 5.02 $\mu\text{mol/L}$; MDA-MB-435, 5.2 $\mu\text{mol/L}$). Using these data, we were able to determine an effective dose of doxorubicin to use for each cell line in the ABC transporter shRNA screen.

We choose five ABC transporters for the shRNA screen that we believe would be good candidates for regulating doxorubicin resistance in melanoma cells. ABCB8 and ABCB10 are both half-molecule transporters located on the mitochondrial membrane and are part of the cancer cell genome (19). Moreover, ABCB8 is overexpressed in doxorubicin-resistant leukemic cells (17). ABCC1, ABCC2, and ABCF2 are often expressed in epithelial malignant tumors and are up-regulated in cells selected for doxorubicin resistance (4, 17, 20-22). For a positive control, we selected ABCB5, which was recently shown to mediate doxorubicin resistance in melanoma cells (13). ABCG2 was selected as a negative control as previous studies have shown no correlation between chemoresistance in melanoma cells and ABCG2 expression (9).

To analyze the expression levels of the selected ABC transporters, we did quantitative real-time PCR on 5 melanoma cell lines. Overall, we found relatively low expression levels of ABCB5, ABCC1, and ABCG2; moderate expression levels of ABCB8, ABCB10, and ABCC2; and high expression of ABCF2 (Fig. 1B). ABCB8 was ubiquitously expressed in all cell types tested, and its expression seems to be independent of cancer type (Fig. 1C). Similar ABC transporter expression data for melanoma cells has been reported previously (10). Interestingly, a recent study found ABCB8 to be highly expressed in both melanoma cells and normal melanocytes, suggesting its physiologic function may be more tissue specific than cancer specific (10).

Effect of ABC Transporter Knockdown on Doxorubicin Sensitivity in Melanoma Cells

The knockdown efficiency of five shRNA expression vectors was tested for each ABC transporter by quantitative real-time PCR. The shRNAs that displayed >80% knockdown of mRNA levels compared with the nontarget control were used in the study. For example, using the WM793B cell line shRNA knockdown of the following ABC transporters resulted in <20% endogenous gene expression: ABCB5 (99.3% knockdown; 0.41 expression units), ABCB8 (88.1% knockdown;

19.2 expression units), ABCB10 (83.2% knockdown; 26.6 expression units), ABCC1 (86.2% knockdown; 16.1 expression units), ABCC2 (96.2% knockdown; 4.8 expression units), ABCF2 (84.7% knockdown; 35.3 expression units), and ABCG2 (92.2% knockdown; 9.8 expression units; Fig. 2A). To determine which selected ABC transporters can mediate resistance against doxorubicin, ABC transporter expression levels were knocked down in three different melanoma cell lines by lentiviral transduction. After 3 days of puromycin selection, cells were exposed to effective doses of doxorubicin for 24 hours. Analyzing cell viability 24 hours after drug removal, we found several ABC transporters that when knocked down could sensitize melanoma cells to doxorubicin (Figs. 2B and 3A). Compared with the nontarget control, knockdown of ABCC1 significantly reduced cell survival after doxorubicin treatment in all 3 melanoma cell lines tested (MDA-MB-435, 40.8% \pm 3.2% versus 1.9% \pm 0.74%; WM793B, 41.8% \pm 1.69% versus 10.1% \pm 1.71%; WM1552C, 46.1% \pm 1.2% versus 11.8% \pm 0.99%). We found a similar effect with ABCC2 knockdown (WM793B, 45.8% \pm 1.3% versus 11.2% \pm 0.52%; WM1552C, 46.1% \pm 1.2% versus 20.1% \pm 2.6%; WM39, 61.8% \pm 3.2% versus 48% \pm 5.2%). Interestingly, knockdown of ABCB5 had an effect on doxorubicin response in only 1 of the 3 melanoma cell lines tested (WM793B, 46.4% \pm 0.81% versus 15.6% \pm 1.9%). We did not observe any significant effect on cell viability after exposure to doxorubicin compared with the control with the knockdown of ABCB10, ABCF2, or ABCG2. Thus, our data shows that ABCC1, ABCC2, and ABCB5 contribute to doxorubicin resistance in melanoma cells, whereas ABCB10, ABCF2, and ABCG2 are dispensable.

Reversal of Doxorubicin Resistance in Human Melanoma Cells by Knockdown of the Mitochondrial Transporter ABCB8

To determine if ABCB8 functions in doxorubicin resistance, we initially tested the effect of ABCB8 knockdown on three melanoma cell lines. We found that knockdown of ABCB8 was able to significantly reverse doxorubicin resistance in these lines (MDA-MB-435, 41.0% \pm 2.2% versus 10.7% \pm 2.6%; WM793B, 40.3% \pm 1.7% versus 8.3% \pm 0.57%; WM1552C, 44.2% \pm 1.9% versus 17.6% \pm 2.4%; Fig. 3A and C). Knockdown of ABCB8 on 2 additional melanoma cell lines also showed a significant effect on doxorubicin response (WM39, 59.2% \pm 5.4% versus 27.9% \pm 2.1%; 451Lu, 72.8% \pm 6.9% versus 49.9% \pm 2.7%; Fig. 3A). To determine if ABCB8-mediated chemoresistance is specific to melanoma cells, we furthered our study to include epithelial cancers of ovarian and breast origin. Doxorubicin dose response curves were created for the breast cancer and ovarian cancer cells were analyzed and used to determine effective doses for treatment (data not shown). Although not as effective as observed in melanoma cells, knockdown of ABCB8 could sensitize some of the ovarian (TOV-21G, 77.2% \pm 2.9% versus 62.7 \pm 2.0%) and breast (MCF-7, 41.4% \pm 8.0% versus 26.7% \pm 3.3%; Hs578T, 55.7% \pm 5.4% versus 43% \pm 4.7%) cancer cell lines to doxorubicin (Fig. 3A). Similar results were found when using a second shRNA, with high knockdown efficiency, targeting a different

Table 1. Summary of Melanoma Cell Lines used in the Study

Cell lines	Phase of melanoma progression	Source	Reference
WM793B	Vertical growth phase	Male/37 y/skin	(37)
WM1552C	Radial growth phase	Male/72 y/skin	(37)
WM39	Vertical growth phase	Male/unknown/skin	(37)
451Lu	Metastasis	Male/21 y/lung	(37)
MDA-MB-435	Metastasis	Female/31 y/pleural effusion	(38, 39)

region of the ABCB8 transcript. To determine if loss of ABCB8 or exposure to puromycin could reduce cell viability during the experiment, we compared puromycin-selected ABCB8 knock-down cells with no doxorubicin treatment to the scrambled shRNA control. We did not observe any significant decrease in cell viability in puromycin-selected ABCB8 knockdown cells

not exposed to doxorubicin (Fig. 3A). These results provide a role for ABCB8 in doxorubicin resistance in melanoma cells and to a lesser extent in breast and ovarian cancers.

A major limitation of cancer treatment is the variety of serious side effects often associated with chemotherapeutics. These are often correlated with increasing concentrations of the

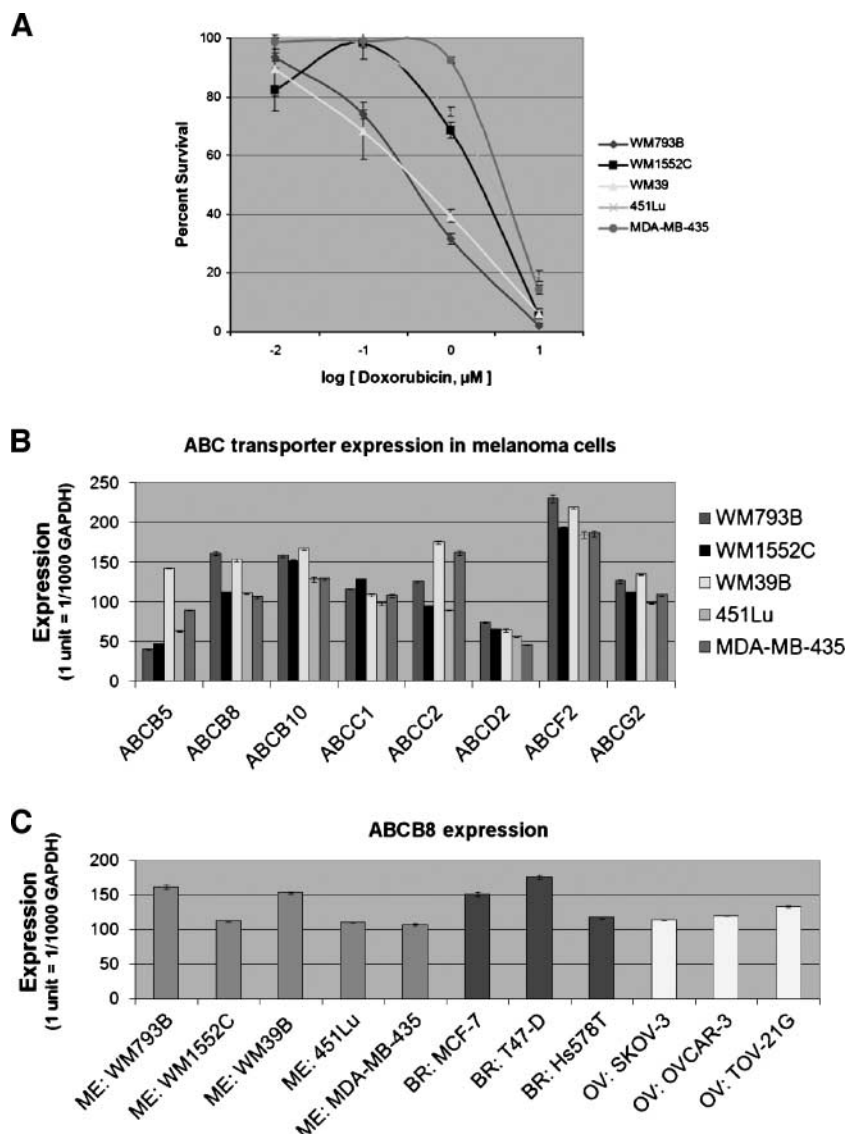


FIGURE 1. Dose-response curve for doxorubicin treatment and ABC transporter expression in human melanoma cells. **A.** Human melanoma cell lines were incubated with increasing concentrations of doxorubicin for 24 h. The CellTiter-Glo assay for cell viability was conducted 24 h after removal of doxorubicin. Points, mean percentage from three independent experiments; bars, SE. **B.** Differential expression of ABC transporters in melanoma cell lines determined by Taqman qRT-PCR. Columns, mean of quadruplicate Taqman qRT-PCR reactions; bars, SE. **C.** Differential expression of ABCB8 in cancer cell lines determined by Taqman qRT-PCR. ME, melanoma; BR, breast; OV, ovarian. Columns, mean of quadruplicate Taqman qRT-PCR reactions; bars, SE.

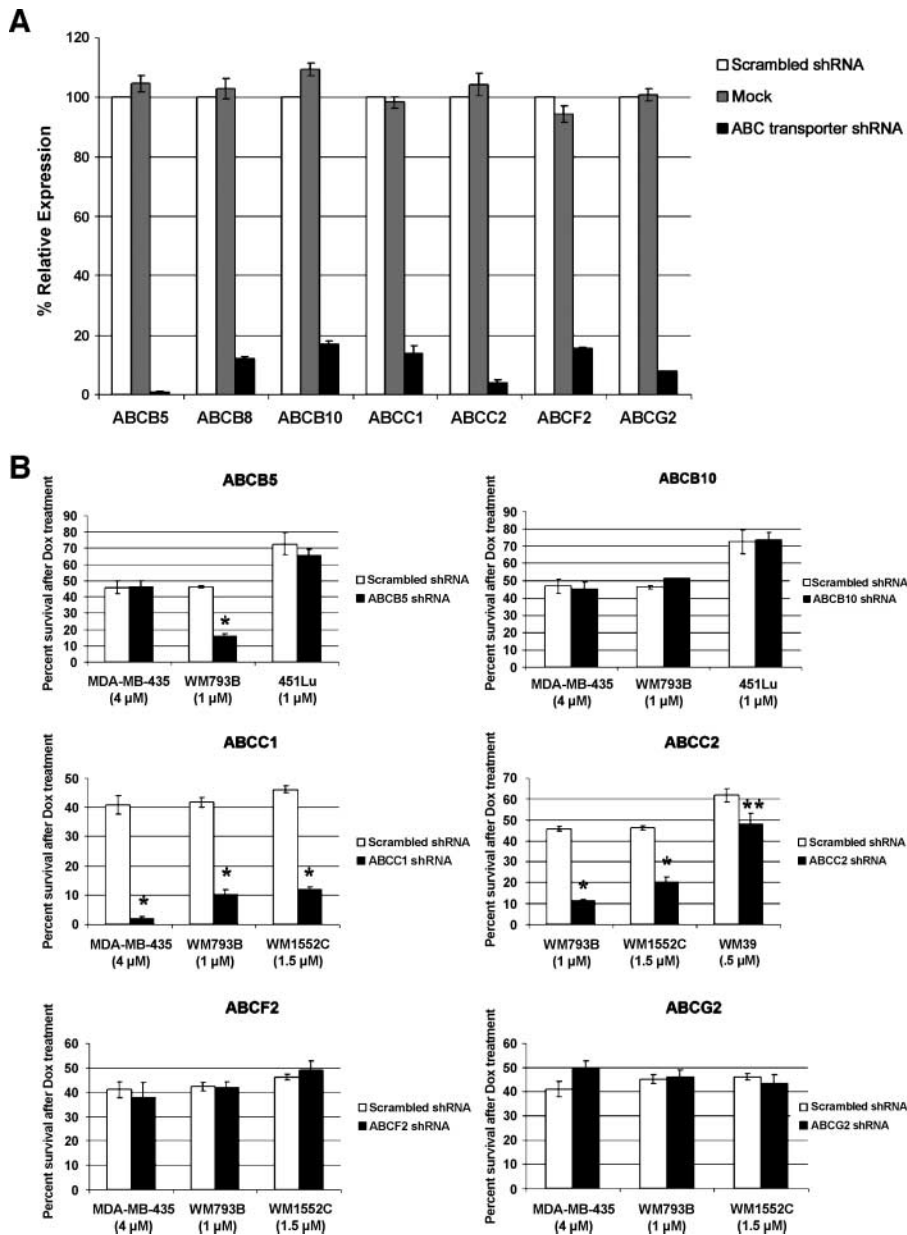


FIGURE 2. Functional screen for ABC transporters involved in doxorubicin resistance in human melanoma cells. **A.** Transient expression of shRNAs decrease ABC transporter mRNA levels in WM793B cells. mRNA knockdown levels were analyzed for 5 shRNA clones per gene, and only those clones with >80% knockdown efficiency were used and depicted in the graph. Values are relative to nontarget shRNA control. Columns, mean of triplicate Taqman real-time qRT-PCR reactions; bars, SE. **B.** Nontarget scrambled shRNA and specific ABC transporter shRNAs were expressed in 3 different melanoma cell lines and exposed to doxorubicin for 24 h. The CellTiter-Glo assay for cell viability was conducted 24 h after removal of doxorubicin. Columns, mean percentage from three independent experiments; bars, SE. *, $P < 0.01$; **, $P < 0.05$.

drug. Therefore, lowering the dose-limiting toxicity of a drug without compromising effectiveness would increase the therapeutic window of treatment. To examine this, we selected two melanoma cell lines expressing ABCB8 shRNA or nontarget control shRNA and exposed them to increasing concentrations of doxorubicin for 24 hours. We found that knockdown of ABCB8 resulted in a 75% reduction in IC_{50} from 0.75 to 0.19 μ mol/L in WM793B cells and a 64% reduction in IC_{50} from 4.8 to 1.75 μ mol/L in MDA-MB-435 cells (Fig. 3B). Thus, inhibiting ABCB8 in melanoma cells before doxorubicin treatment results in a significant reversal of chemoresistance and a decrease in the IC_{50} . Therapeutically, these low concentrations could eliminate potential side effects commonly seen with high doses of doxorubicin.

ABCB8-Mediated Chemoresistance in Melanoma Cells Is Specific to Doxorubicin

A common feature of ABC transporters is their ability to confer cross-resistance to a variety of structurally unrelated drugs (2). To determine if ABCB8 could sensitize melanoma cells to a broad range of chemotherapeutics, we treated two melanoma cell lines expressing ABCB8 shRNA or nontarget control shRNA with commonly used anticancer drugs from various drug classes. We found that ABCB8-mediated drug resistance was specific to doxorubicin. We did not observe any differences in IC_{50} values in melanoma cells expressing ABCB8 shRNA compared with the control when treated with Taxol, 5-fluorouracil, or cisplatin (Table 2). These results suggest that ABCB8 does not confer resistance to a wide

variety of compounds and may be specific to a limited structurally related class of drugs, including doxorubicin.

Loss of ABCB8 Results in an Increase in Doxorubicin-Induced Mitochondrial DNA Damage

The mitochondrial genome is extremely susceptible to DNA damaging agents, such as doxorubicin, due to the lack of protective histones (18). Because ABCB8 is localized to the mitochondrial membrane we envisioned that ABCB8 could be providing a protective role by preventing damage to the mitochondrial genome. To test this possibility, we treated the WM793B melanoma cell line expressing ABCB8 shRNA or nontarget control shRNA with increasing concentrations of doxorubicin for 4 hours. High molecular weight DNA was

isolated 24 hours later for QPCR. QPCR is a quantitative PCR technique commonly used to measure mitochondrial DNA damage (23). This technique is based on the premise that lesions present in the mitochondrial DNA will prevent the progression of the polymerase, resulting in a decrease in the amplification of damaged templates compared with nondamaged DNA. Therefore, amplification is inversely proportional to DNA damage. We found that knockdown of ABCB8 results in a significant increase in mitochondrial DNA damage (0.84 lesions/10 Kb \pm 0.05) compared with cells expressing the nontarget control (0.44 lesions/10 Kb \pm 0.03) when treated with 1 μ mol/L of doxorubicin (Fig. 4A, B, and C). Compared with the control, the effect of ABCB8 knockdown on mitochondrial DNA damage was more pronounced when cells were treated with 5 μ mol/L doxorubicin (0.68 lesions/10 Kb \pm

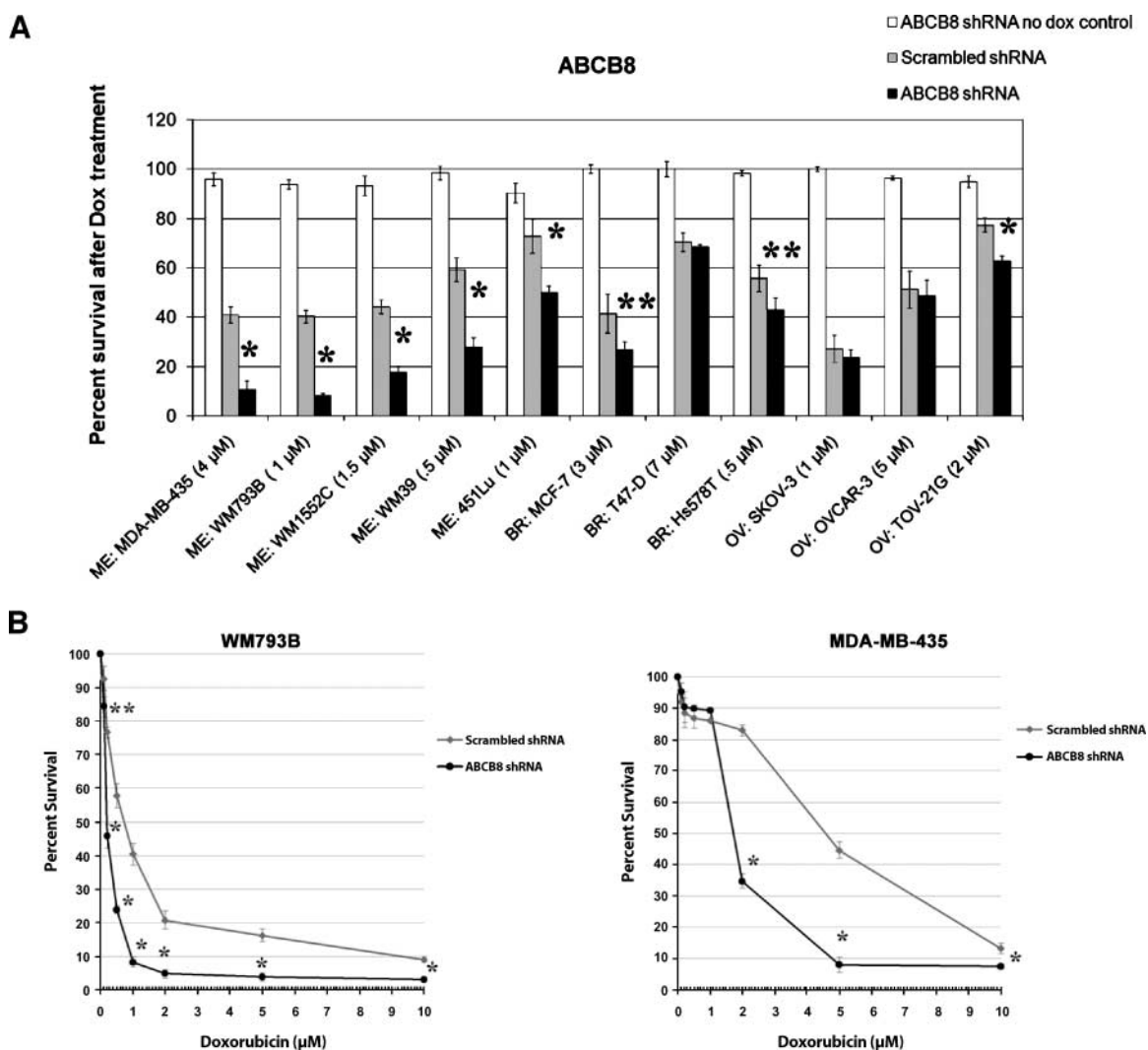


FIGURE 3. Reversal of doxorubicin resistance by ABCB8 shRNA expression. **A.** Nontarget scrambled shRNA and ABCB8 shRNA were expressed in cancer cell lines and exposed to doxorubicin for 24 h. The CellTiter-Glo assay for cell viability was conducted 24 h after removal of doxorubicin. Percent survival was determined by the formula: shRNA infected (doxorubicin treated)/shRNA infected (untreated). Dox, doxorubicin. Puromycin-selected ABCB8 knockdown cells not exposed to doxorubicin were used as a control and viability compared with scrambled shRNA control. Columns, mean percentage from three independent experiments; bars, SE. *, $P < 0.01$; **, $P < 0.05$. **B.** Melanoma cell lines WM793B and MDA-MB-435 expressing nontarget scrambled shRNA or ABCB8 shRNA were incubated with increasing concentrations of doxorubicin for 24 h and cell survival calculated as above 24 h after drug removal. The results are representative of at least three independent experiments; bars, SE. *, $P < 0.01$; **, $P < 0.05$.

Table 2. The IC₅₀ Values of Various Chemotherapeutics in NonTarget Scrambled shRNA and ABCB8 shRNA Transduced Human Melanoma Cell Lines

Chemotherapeutics	WM793B	WM793B	MDA-MB-435	MDA-MB-435
	Scrambled shRNA (μmol/L)	ABCB8 shRNA (μmol/L)	Scrambled shRNA (μmol/L)	ABCB8 shRNA (μmol/L)
Taxol	0.92 ± 0.11	0.89 ± 0.10	0.013 ± 0.006	0.015 ± 0.004
5-Fluorouracil	24.4 ± 3.6	25.2 ± 4.2	37.6 ± 5.7	38.1 ± 3.8
Cisplatin	10.8 ± 0.72	10.6 ± 0.44	26.7 ± 4.8	27.1 ± 5.2
Doxorubicin	0.75 ± 0.07	0.19 ± 0.01	4.8 ± 0.40	1.75 ± 0.12

NOTE: Values represented as IC₅₀ mean ± SD.

0.07 versus 2.1 lesions/10 Kb ± 0.14) and 10 μmol/L doxorubicin (0.86 lesions/10 Kb ± 0.05 versus 2.9 lesions/10 Kb ± 0.21). Our results show that knockdown of ABCB8 results in an increase in doxorubicin-induced mitochondrial DNA damage. We conclude that ABCB8 functions in melanoma chemoresistance by protecting the mitochondrial genome from damage.

Discussion

Malignant melanoma is notoriously resistant to doxorubicin, one of the most commonly used chemotherapeutic agents to treat cancer. This resistance is mediated by a variety of mechanisms, most importantly, the action of the proteins that are part of the ABC transporter family. Although the expression of these transporters is often responsible for mediating chemotherapeutic resistance in many cancer types, there is little data available on which ABC transporter(s) is responsible for conferring drug resistance in melanomas. In this study, we identified a member of the ABC family, ABCB8, which displayed a role in mediating specifically doxorubicin resistance in human melanoma cells. Using shRNA to knockdown-specific ABC transporters, we found that loss of ABCB8 could sensitize cells to doxorubicin. Furthermore, our analysis of the mechanism of action of this transporter suggests that it infers doxorubicin resistance by preventing mitochondrial DNA damage.

The role of the mitochondria in cancer development and chemoresistance has been studied for decades. It is well-established that cancer cells possess alterations in mitochondrial functions compared with normal cells allowing them to adapt to the microenvironment of the tumor niche, where selective pressures results in changes in the energy metabolism of these cells (24). Interestingly, there is also evidence that a metabolic mechanism may be a mean by which a cancer cell becomes drug resistant. Recent reports have showed that drug-resistant tumor cells have low mitochondrial membrane potential, and these cells use inefficient high-rate glycolysis to protect against reactive oxygen species (25). Because several of the commonly used chemotherapeutics in the clinical target mitochondrial DNA, it is thought that resistance to many of these anticancer agents might be due to alteration in the mitochondrial function. The chemotherapeutic 5-fluorouracil activates the mitochondrial protein Romo1, which can in turn increase the levels of antioxidant enzymes resulting in drug resistance (26). A novel strategy to investigate the role of mitochondria in doxorubicin resistance was used by Singh et al (18). Using a cancer cell line

lacking mitochondria, they discovered these cells were extremely resistant to doxorubicin compared with the parental cell line containing mitochondria. Moreover, the treatment of HeLa cells with doxorubicin resulted in fragmentation of mitochondrial DNA. To date, there are four known human mitochondrial ABC transporters: ABCB6, ABCB7, ABCB8, and ABCB10. Although the functions of the human transporters remain elusive, studies conducted on the yeast orthologs suggest they are involved in iron metabolism and the transport of heme precursors, phospholipids, or peptides (14, 15, 27). Bacteria are also known to express a variety of ABC transporters, which can mediate resistance to various compounds including anticancer agents and antibiotics (28). Given the link of the origin of mitochondria from bacteria, it would not be illogical if the ABC transporters located on the human mitochondrial membrane maintained a similar function.

Although the function of mitochondria have been implicated in resistance to numerous chemotherapeutics, our data suggests that ABCB8 does not confer cross-resistance to a variety of structurally unrelated drugs and may be specific to a particular class of drugs, which includes doxorubicin. Moreover, it does not seem that ABCB8 mediates protection against all chemotherapeutics that target mitochondrial DNA. Cisplatin is known to cause mitochondrial DNA damage and has been shown to bind 50 times more to mitochondrial DNA than chromosomal DNA in human melanoma cells (29). However, in our studies, loss of ABCB8 was not able to sensitize melanoma cells to cisplatin. It would be interesting to determine if other ABC transporters located on the mitochondria are able to regulate chemoresistance against cisplatin or other anticancer drugs known to target mitochondrial DNA.

Targeting the ABC transporters therapeutically has been challenging. To date, there are no available ABC transporter inhibitors in the clinic. Although some clinical trials with ABCB1 inhibitors have been promising, their effectiveness has been limited by their lack of specificity and toxic side effects (2). Determining which ABC transporters regulate chemoresistance to various anticancer drugs in specific cancers should provide better therapeutic targets. Moreover, the recent solving of the ABC transporter crystal structure will allow us to better understand the mechanism of transport and provide a template for designing specific inhibitors (30). Our results suggest that mitochondrial transporters, specifically ABCB8, may be good targets for reversing chemoresistance. In this study, we show that ABCB8 functions in doxorubicin chemoresistance in melanoma cells. However, circumstantial evidence suggests ABCB8 may also function in other cancers. ABCB8 is

significantly overexpressed in doxorubicin-resistant leukemic cells and is one of the most commonly mutated genes in breast cancer (17, 19). We observed a modest response to doxorubicin after ABCB8 knockdown in two breast cancer cell lines and one ovarian cancer cell line. Therefore, unlike melanoma cells, other ABC transporters must be able to compensate for ABCB8 loss in breast and ovarian cancers. Interestingly, we observed a gradual decrease in melanoma cell survival after ABCB8 knockdown after prolonged periods in culture, which was not seen in breast or ovarian cancer cell lines (data not shown). This suggests that in addition to regulating doxorubicin resistance, ABCB8 may play a specific physiologic role in melanoma that could be targeted therapeutically.

One of the major hurdles drug companies must overcome when developing chemotherapeutics is drug toxicity. The majority of anticancer compounds fail to reach the clinic because of life-threatening side effects, which often increase at higher drug concentrations. Developing specific ABC transporter inhibitors and administering them with particular drugs could lower the effective dose of chemotherapeutic and therefore reduce the severity of side effects. We found that with doxorubicin treatment, we could lower the IC_{50} in melanoma cells ~3- to 4-fold after loss of ABCB8. Previous studies have shown that low levels of chemoresistance (2- to 4-fold) can hamper the effectiveness of treatment (12, 13, 31-33). Thus, knockdown of ABCB8 before doxorubicin treatment should not only lower the effective dose needed for sensitization but also reduce the severity of side effects often seen with doxorubicin treatment. However, it seems that it may not be this simplistic as treatment with an ABC inhibitor and chemotherapeutic may kill the cancer cells but could also compromise the ability of healthy cells to protect themselves against the drug. In accordance, our data along with previous studies suggests that ABCB8 may be responsible for protecting cardiac cells against doxorubicin induced damage.

A common side effect associated with doxorubicin treatment and the biggest limitation of its use in the clinic is cardiomyopathy, which results in congestive heart failure that is refractory to conventional treatments (34). Studies have shown that doxorubicin treatment causes mitochondrial DNA damage and results in mitochondrial dysfunction in rat cardiac cells (18, 35). Recently, it was shown in neonatal rat cardiomyocytes that adenoviral overexpression of ABCB8 reduced the mitochondrial membrane potential loss due to hydrogen peroxide treatment (16). This indicates that ABCB8 plays a cardioprotective role. Moreover, ABCB8 is highly expressed in the heart, probably due to higher numbers of mitochondria in cardiac cells (36). These studies along with our data showing that loss of ABCB8 results in an increase in mitochondrial DNA damage after doxorubicin treatment suggests that ABCB8 is important in protecting cardiac cells against doxorubicin-induced damage. Therefore, when developing inhibitors of ABC transporters to reverse chemoresistance, it is important to realize that these transporters also have physiologic functions that could compromise the ability of normal cells to efflux toxic compounds. Determining the physiologic functions of the ABC transporters in specific cell types and the mechanisms cells use to confer drug resistance will enable us to develop new strategies to improve cancer treatment.

Materials and Methods

Cell Culture

The human malignant melanoma cell lines WM793B, WM1552C, WM39, 451Lu, and MDA-MB-435 were purchased from American Type Culture Collection. WM793B, WM1552C, WM39, and 451Lu were cultured in 4:1 mixture MCDB 153 (Sigma) with 1.5 g/L sodium bicarbonate (HyClone) and Leibovitz's L-15 medium (Invitrogen) with 2 mmol/L L-glutamine supplemented with 2% fetal bovine serum

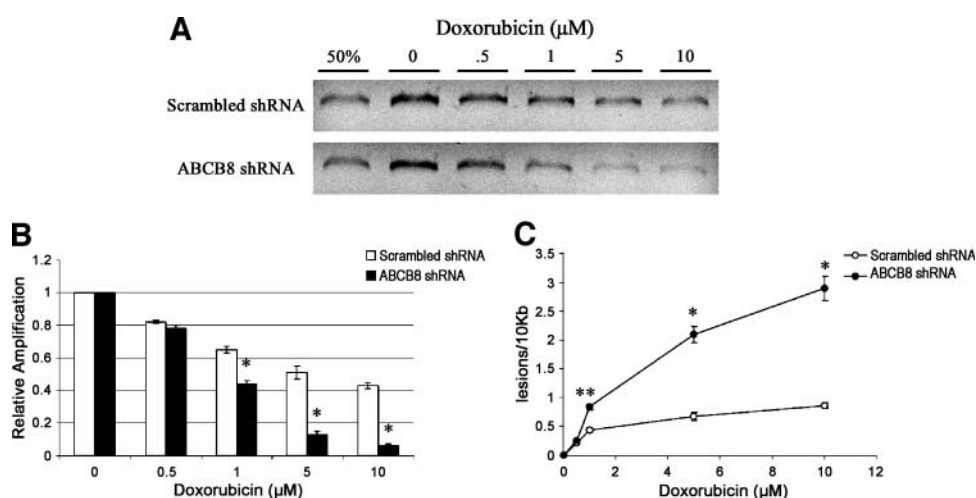


FIGURE 4. Loss of ABCB8 results in an increase in doxorubicin-induced mitochondrial DNA damage. **A.** QPCR amplification of an 8.9-kb fragment of the mitochondrial genome in WM793B melanoma cells expressing nontarget scrambled shRNA or ABCB8 shRNA treated with increasing concentrations of doxorubicin for 4 h. Total DNA was extracted 24 h later and QPCR was done using mitochondrial specific primers. Amplified DNA was analyzed on 0.8% agarose gel. **B.** The amount of amplification associated with each QPCR product relative to the nontreated control is plotted as a function of doxorubicin concentration. Columns, mean of at least three independent experiments; bars, SE. *, $P < 0.01$. **C.** Relative amplification values were converted to mitochondrial DNA lesion frequency using the Poisson equation described. Columns, mean of at least three independent experiments; bars, SE. *, $P < 0.01$; **, $P < 0.05$.

(Invitrogen), 5 µg/mL bovine insulin, 1.68 mmol/L CaCl₂, and 100 IU/mL penicillin/streptomycin (Sigma). MDA-MB-435 cells were cultured in Leibovitz's L-15 medium (Invitrogen) with 2 mmol/L L-glutamine supplemented with 10% fetal bovine serum (Invitrogen), 10 µg/mL bovine insulin, and 100 IU/mL penicillin/streptomycin (Sigma).

Isolation of RNA and Quantitative Real-time PCR

Total RNA was prepared using the Absolutely RNA Miniprep kit (Stratagene). RNA was incubated with DNase I and converted into cDNA using the High Capacity cDNA Archive kit (Applied Biosystems). All Taqman probes were pre-designed and ordered from Applied Biosystems. The ABCB5 probe is specific for the functional β isoform. Probes were mixed with Taqman Universal PCR Master Mix (Applied Biosystems) and amplified in triplicate on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase. To detect ABC transporter expression levels in melanoma cell lines cDNA was amplified using the Taqman PreAmp Master Mix kit (Applied Biosystems). Samples were run in quadruplicate on a BioMark Dynamic Array chip (Fluidigm).

Doxorubicin Dose Response Curve

Melanoma cell lines were seeded in 96-well plates (10,000 cells per well). Doxorubicin (Sigma) was added at increasing concentrations for 24 h. The CellTiter-Glo Luminescent Cell Viability Assay (Promega) was conducted 24 h after doxorubicin removal. Percent survival was determined by the formula: doxorubicin treated/doxorubicin untreated. All experiments were conducted in triplicate.

shRNA Experiments

shRNA clones were from the MISSION TRC-Hs 1.0 library (Sigma). Clones with >80% knockdown efficiency were selected for further experiments. These included ABCB5β (Mission shRNA TRCN0000060192; CCGGGCTGGAAAGAT-AGCAACTGAACTCGAGTTCAGTTGCTATCTTTCCAG-CTTTTTG), ABCB8 (Mission shRNA TRCN0000059874; CCGGCTTTGAGTACATGGCCCTGAACTCGAGTTCAGGGC-CATGTACTCAAAGTTTTT), ABCB10 (Mission shRNA TRCN0000060177; CCGGCGTGGATTCTCAGCATG-TCTCGAGACATCGTGAGAAATCCAACCGTTTTT), ABCC1 (Mission shRNA TRCN0000059366; CCGG-CCTCTCAGTGTCTTACTCATTCTCGAGAATGAGTAA-GACTGAGAGGTTTTT), ABCC2 (Mission shRNA TRCN0000059303; CCGGCGTGATAAATCCAGGAC-CAACTCGAGTTGGTCTCGGATTTATACACGTTTTT), ABCF2 (Mission shRNA TRCN0000059335; CCGGCCTGC-ATCTACAATAATCTACTCGAGTAGATTATTGTAGATG-CAAGGTTTTT), and ABCG2 (Mission shRNA TRCN-0000059802; CCGGCCTGCCAATTTCAAATGTAATCTC-GAGATTACATTTGAAATTGGCAGGTTTTT). Controls used were MISSION TurboGFP Control (SHC003) and MISSION Nontarget shRNA Control (SHC002; CCGGCAA-CAAGATGAAGAGACCAACTCGAGTTGGTGCTCTT-CATCTTGTTGTTTT). To produce shRNA lentivirus, Mission

shRNA plasmid, VSV-G plasmid, and Δ8.9 were cotransfected into 293T cells using Lipofectamine (Invitrogen). After 3 d, viral supernatants were isolated and passed through a 0.45-µm cellulose acetate filter. For determination of knockdown efficiency, WM793B cells were transduced and selected for 3 d in 1 µg/mL puromycin (Sigma) and the total RNA isolated. Samples were processed for real-time PCR as described above. Expression levels were calculated as percent reduction compared with shRNA nontarget control. For all viability assays, cells were seeded in 96-well plates (3,000 cells per well) and transduced with a specific shRNA lentivirus. After 3 d of puromycin selection, cells were treated with specific concentrations of doxorubicin for 24 h. The CellTiter-Glo assay was conducted 24 h after doxorubicin removal. Percent survival was determined by the formula: shRNA infected (doxorubicin treated)/shRNA infected (doxorubicin untreated). Experiments using Taxol (Sigma), 5-fluorouracil (InvivoGen), and cisplatin (Sigma) were carried out as above except the cell exposure period for 5-fluorouracil was extended to 72 h. All IC₅₀ values were calculated using GraphPad Prism 5.0 software. All experiments were done in triplicate. The Independent Groups *t* test was used for statistical analysis.

Mitochondrial DNA QPCR

WM793B cells were seeded in 6 well plates (50,000 cells per well) and transduced with nontarget shRNA control or ABCB8 shRNA lentivirus. After 3 d of puromycin selection, the cells were exposed to different concentrations of doxorubicin for 4 h. Cells were washed in PBS and incubated 24 h in normal media. Cells were harvested using 0.05% trypsin (HyClone) and high-molecular weight DNA was isolated using the Blood and Cell Culture DNA Mini kit (Qiagen). DNA isolated using this technique is ideal for QPCR (23). The DNA was quantified using a ND-1000 spectrophotometer (Nano-Drop). The DNA samples were diluted to a concentration of 3 ng/µL. QPCR was done as described (23). Briefly, using the GeneAmp XL PCR kit (Applied Biosystems), 15 ng of DNA were mixed with 1× buffer, 100 ng/µL bovine serum albumin (Invitrogen), 200 µmol/L deoxynucleotide triphosphates, 1.3 mmol/L Mg²⁺, 20 pmol of each primer, and 1 U of polymerase. The primer sequences for the 8.9-kb fragment of the mitochondrial genome were 5'-TTTCATCATGCGGA-GATGTTGGATGG-3' and 5'-TCTAAGCCTCCTTATTC-GAGCCGA-3'.

PCR was initiated at 75°C with a hot-start addition of the polymerase. PCR was carried out on a PTC-200 DNA Engine Thermal Cycler (Bio-Rad). Thermocycler variables used were as follows 94°C for 1 min followed by 19 cycles of 94°C for 15 s and 60°C for 12 min. A final extension of 72°C was done for 10 min. An untreated control containing 50% of the template was included to ensure quantitative conditions. PCR products were resolved on a 0.8% Tris-borate EDTA agarose gel containing 1 µg/mL ethidium bromide (Sigma). PCR amplified products were quantitated using AlphaImager HP (Alpha Innotech). Relative amplification was calculated by dividing the amplification values of the doxorubicin treated samples (A_D) by the untreated control (A_O). Assuming that DNA lesions are randomly distributed and using the Poisson equation

the average lesion frequency per DNA fragment at a particular doxorubicin dose can be determined. Therefore, lesions/fragment at dose $D = -\ln(A_D)/(A_0)$. The Independent Groups t test was used for statistical analysis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

- Higgins CF. Multiple molecular mechanisms for multidrug resistance transporters. *Nat Rev* 2007;446:749–57.
- Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* 2006;5:219–34.
- American Cancer Society. Cancer facts and figures 2007. Atlanta (GA): American Cancer Society; 2007.
- Helmbach H, Rossmann E, Kern MA. Drug-resistance in human melanoma. *Int J Cancer* 2001;93:617–22.
- Panneerselvam M, Bredehorst R, Vogel GW. Resistance of human melanoma cells against cytotoxic and complement-enhancing activities of doxorubicin. *Cancer Res* 1987;47:4601–9.
- Vorobiof DA, Rapoport BL, Mahomed R, et al. Phase II study of pegylated liposomal doxorubicin in patients with metastatic malignant melanoma failing standard chemotherapy treatment. *Melanoma Res* 2003;13:201–3.
- Smylie MG, Wong R, Michalcioiu C, et al. A phase II, open label, monotherapy study of liposomal doxorubicin in patients with metastatic malignant melanoma. *Invest New Drugs* 2007;25:155–9.
- Goldstein LJ, Galski H, Fojo A, et al. Expression of a multidrug resistance gene in human cancers. *J Natl Cancer Inst* 1989;81:116–24.
- Deichmann M, Thome M, Egner U. The chemoresistance gene ABCG2 (MXR/BCRP1/ABCP1) is not expressed in melanomas but in single neuroendocrine carcinomas of the skin. *J Cutan Pathol* 2005;32:467–73.
- Heimerl S, Bosserhoff AK, Langmann T. Mapping ATP-binding cassette transporter gene expression profiles in melanocytes and melanoma cells. *Melanoma Res* 2007;17:265–73.
- Schadendorf D, Herfordt R, Czarnetzki BM. P-glycoprotein expression in primary and metastatic malignant melanoma. *Br J Dermatol* 1995;132:551–5.
- Szakacs G, Annereau JP, Lababidi S, et al. Predicting drug sensitivity and resistance: profiling ABC transporter genes in cancer cells. *Cancer Cell* 2004;6:129–37.
- Frank NY, Margaryan A, Huang Y, et al. ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma. *Cancer Res* 2005;65:4320–33.
- Hogue DL, Liu L, Ling V. Identification and characterization of a mammalian mitochondrial ATP-binding cassette membrane protein. *J Mol Biol* 1999;285:379–89.
- Burke MA, Ardehali M. Mitochondrial ATP-binding cassette proteins. *Transl Res* 2007;150:73–80.
- Ardehali H, O'Rourke B, Marban E. Cardioprotective role of the mitochondrial ATP-binding cassette protein 1. *Circ Res* 2005;97:740–2.
- Gillet J, Efferth T, Steinbach D, et al. Microarray-based detection of multidrug resistance in human tumor cells by expression profiling of ATP-binding cassette transporter genes. *Cancer Res* 2004;64:8987–93.
- Singh KK, Russell J, Sigala B, Zhang Y, Williams J, Keshav KF. Mitochondrial DNA determines the cellular response to cancer therapeutic agents. *Oncogene* 1999;18:6641–6.
- Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. *Science* 2006;314:268–74.
- Cole SP, Bhardwaj G, Gerlach JH, et al. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 1992;258:1650–4.
- Cui Y, Konig J, Buchholz JK, Spring H, Leier I, Keppler D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Mol Pharmacol* 1999;55:929–37.
- Mallory JC, Crudden G, Oliva A, Saunders C, Stromberg A, Craven RJ. A novel group of genes regulates susceptibility to antineoplastic drugs in highly tumorigenic breast cancer cells. *Mol Pharmacol* 2005;65:1747–56.
- Santos JH, Mandavilli BS, Van Houten B. Measuring oxidative mtDNA damage and repair using quantitative PCR. In: Copeland WC, editor. *Methods in molecular biology*, vol. 197: mitochondrial DNA: methods and protocols. Totowa (NJ): Humana Press Inc; 2002. p. 159–76.
- Fantin VR, Leder P. Mitochondriotoxic compounds for cancer therapy. *Oncogene* 2006;25:4787–97.
- Harper M, Antoniou A, Villalobos-Menuet E, et al. Characterization of a novel metabolic strategy used by drug-resistant tumor cells. *FASEB J* 2002;16:1550–7.
- Hwang IT, Chung YM, Kim JJ, et al. Drug resistance to 5-FU linked to reactive oxygen species modulator 1. *Biochem Biophys Res Commun* 2007;359:304–10.
- Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res* 2001;11:1156–66.
- Lage H. ABC-transporters: implications on drug resistance from microorganisms to human cancers. *Int J Antimicrob Agents* 2003;22:188–99.
- Murata T, Hibasami H, Maekawa S, Taqawa T, Nakashima K. Preferential binding of cisplatin to mitochondrial DNA and suppression of ATP generation in human malignant melanoma cells. *Biochem Int* 1990;20:949–55.
- Hollenstein K, Frei DC, Locher KP. Structure of an ABC transporter in complex with its binding protein. *Nature* 2007;446:213–6.
- Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002;2:48–58.
- Li L, Xu J, Min T, et al. Reversal of MDR1 gene-dependent multidrug resistance using low concentration of endonuclease-prepared small interference RNA. *Eur J Pharmacol* 2006;536:93–7.
- Materna V, Stege A, Surowiak P, et al. RNA interference-triggered reversal of ABCC2-dependent cisplatin resistance in human cancer cells. *Biochem Biophys Res Commun* 2006;348:153–7.
- Takemura G, Fujiwara H. Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. *Prog Cardiovasc Dis* 2007;49:330–52.
- Zhou S, Starkov A, Froberg MK, Leino RL, Wallace KB. Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin. *Cancer Res* 2001;61:771–7.
- Melaine N, Satie AP, Lassurquere J. Molecular cloning of several rat ABC transporters including a new ABC transporter, Abcb8, and their expression in rat testis. *Int J Androl* 2006;29:392–9.
- Hsu MY, Elder DE, Herlyn M. Melanoma: The Wistar Melanoma (WM) Cell Lines. In: Masters JRW, Palsson B, editors. *Human Cell Culture*, vol 1: Great Britain: Kluwer Academic Publishers; 1999. p. 259–74.
- Ellison G, Klinowska T, Westwood RF, et al. Further evidence to support the melanocytic origin of MDA-MB-435. *Mol Pathol* 2002;55:294–9.
- Cailleau R, Olive M, Crueiger QVJ. Long term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro* 1978;14:911–5.