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Principal expression of two mRNA isoforms (*ABCB5 α* and *ABCB5 β*) of the ATP-binding cassette transporter gene *ABCB5* in melanoma cells and melanocytes

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Summary

ATP-binding cassette (ABC) transporters play a pivotal role in physiology and pathology. We identified and cloned two novel mRNA isoforms (*ABCB5 α* and *ABCB5 β*) of the ABC transporter *ABCB5* in human melanoma cells. The deduced *ABCB5 α* protein appears to be an altered splice variant containing only a putative ABC, whereas the *ABCB5 β* isoform shares approximately 70% similarity with ABCB1 (MDR1) and has a deduced topological arrangement similar to that of the whole carboxyl terminal half of the ABCB1 gene product, P-glycoprotein, including an intact ABC. Northern blot, real-time PCR, and conventional RT-PCR were used to verify the expression profiles of *ABCB5 α/β* . We found that the melanomas included among the NCI-60 panel of cell lines preferentially expressed both *ABCB5 α* and *ABCB5 β* . However, *ABCB5 α/β* expression was undetectable in two amelanotic melanomas (M14 and LOX-IMVI). The expression profile of *ABCB5 α/β* in all of the other melanomas of the panel was confirmed both by RT-PCR and by sequencing. Neither *ABCB5 α* nor *ABCB5 β* expression was found in normal tissues such as liver, spleen, thymus, kidney, lung, colon, small intestines or placenta. *ABCB5 α/β* mRNAs were also expressed in normal melanocytes and in retinal pigment epithelial cells, suggesting that *ABCB5 α/β* expression is pigment cell-specific and might be involved in melanogenesis. Our findings indicate that expression of *ABCB5 α/β* might possibly provide two novel molecular markers for differential diagnosis of melanomas and constitute potential molecular targets for therapy of melanomas.

Keywords

ATP-binding cassette transporter; *ABCB5*; melanoma/melanocytes

Introduction

ATP-binding cassette (ABC) transporters are a super-family of integral membrane proteins, typically with multiple transmembrane domains (TMDs) and one or two nucleotide binding domains (NBDs). These membrane proteins participate in the ATP-dependent transmembrane transport of structurally diverse molecules ranging from small ions, sugars,

and peptides to more complex organic molecules (for reviews, see Borst and Elferink, 2002; Dean and Allikmets, 2001; Gottesman et al., 2002; Haimeur et al., 2004). At present, there are 48 human ABC transporter proteins that have been identified and classified into seven subfamilies from A to G (<http://nutrigene.4t.com/humanabc.htm>). However, the functions of the majority of these transporters remain to be determined.

Among all ABC transporters, *ABCB1* (*MDR1*)/P-glyco-protein (P-gp) represents a well-characterized multidrug-transporter and belongs to the ABCB subfamily (MDR-TAP), which includes 11 members that have different expression patterns presumably reflecting their distinct functions. The functions of several transporters of the ABCB subfamily (i.e. *ABCB5*, *ABCB8*, *ABCB9*, and *ABCB10*) are largely unknown. In the case of *ABCB5*, there is very limited information on either its gene structure, pattern of expression, or associated function. *ABCB5* was originally designated based on its sequence homology to other ABCB family members (Dean and Allikmets, 2001). To date, there are only two preliminary reports that describe the possible physiological role of ABCB 5 (the *ABCB5 β* isoform) in the regulation of progenitor cell fusion (Frank et al., 2003) and its possible association with drug sensitivity (Huang et al., 2004), and a recent report from our laboratory noting its expression in melanomas (Szakács et al., 2004).

To determine the expressed forms of *ABCB5* and its possible function(s) in normal tissues and in human melanomas, we screened a melanotic melanoma cDNA library, investigated the expression pattern of *ABCB5*, and examined the effects of cytotoxic drugs on expression of *ABCB5* by Northern blot, real-time RT-PCR, and conventional RT-PCR. We identified two isoforms of *ABCB5* mRNA (termed *ABCB5 α* and *ABCB5 β*). Both isoforms are preferentially expressed in melanomas and in other pigmented cells [melanocytes and retinal pigment epithelial cells (RPEs)], and may be associated with melanogenesis and/or drug response. Thus, both *ABCB5 α* and *ABCB5 β* could be molecular markers for diagnosis of melanomas and two potential molecular targets for therapy of melanomas.

Results

Molecular cloning of a novel isoform (*ABCB5 α*) of the human *ABCB5* gene

The human *ABCB5* gene was initially annotated based on the presence of two linked sequences (XM_166496) homologous to the NBDs of the ABCB subfamily members. *ABCB5* was mapped at approximately 7p15.3 in the human genome (National Center for Biotechnology Information). However, expression of a full-length *ABCB5* gene with two NBDs and 12 TMDs has not been reported, although genomic sequences are capable of encoding such a protein. We designed a specific *ABCB5* probe (B5P102S, Table 1), according to the known sequences (XM_166496) to screen a melanoma cDNA library. We obtained more than ten positive clones which were sequenced. Sequence analysis revealed that the majority of clones were homologous to *ABCB1* (*MDR1*) and similar to *ABCB5* sequences (XM_166496, BC044248) in GenBank. No full-length *ABCB5* was found in this melanotic cDNA library. As these sequences represent the major isoform of *ABCB5* in the cDNA library, we designated this isoform as *ABCB5 α* (Figure 1). *ABCB5 α* has seven exons (exon 1–7) and contains both a 5' untranslated region (UTR) (exon 1 to exon 4) and a 3' UTR (exon 7). The deduced peptide sequence has a molecular weight of 15 KDa and contains an ABC signature motif and a Walker B consensus found in other ABC transporters. *ABCB5 α* has no Walker A consensus sequence, and shows 60 to 70% homology with *ABCB1* (Figure 1B, C).

Mapping the 5' untranslated regions (5' UTR) of *ABCB5α* in melanomas

The evidence from several isolated cDNA clones indicates the presence of a 5' UTR, as indicated in Figure 2B. Further experiments using the primers (Int-1F and Ex1F, Figure 2B) confirmed the initiation of the 5' UTR in *ABCB5α* mRNAs (Figure 2: C1, C2). RT-PCR also confirmed that *ABCB5α* mRNA was predominantly expressed in melanomas, but not in normal tissue controls (uterus, lung, and placenta) (Figure 2C, lanes 11–13). Of note, multiple RT-PCR bands are observed in melanomas (Figure 2: C3, C4), suggesting alternative mRNA splicing events occurred. Nonetheless, the desired RT-PCR bands were subcloned and confirmed to be *ABCB5α* sequences by DNA sequencing.

Real-time PCR analysis of *ABCB5α* expression in the NCI-60 panel

We designed specific primers for *ABCB5α* (Table 1), based on the sequences in exon 7, to examine the *ABCB5α* expression profile in the NCI-60 cancer cell lines. *ABCB5α* was preferentially expressed in the melanoma cancer cell lines (among the NCI-60 panel cell lines) and in the two breast cancer cell lines (MDA-MB-435, MDA-N) that are believed to be of melanoma origin according to their molecular signatures and specific gene expression for melanocytes (Ellison et al., 2002; Ross et al., 2000). Lower *ABCB5α* expression was also found in other non-melanoma tumors such as two glioblastoma cell lines (SNB19 and SNB75) and leukemias (SR and CCRF-CEM). Interestingly, *ABCB5α* was not expressed in two amelanotic melanomas (M14 and LOXI-IMVI) (Figure 3, columns 38 and 39). In addition, *ABCB5α* was undetectable in the doxorubicin-selected multi-drug resistant (MDR) cell line (MCF-7/ADR) (Figure 3, column 2).

RT-PCR cloning of the *ABCB5β* isoform of the human *ABCB5* gene

ABCB5α only contains seven exons, without an intact set of domain structures required for a functional ABC transporter, suggesting that *ABCB5α* alone may be nonfunctional. To verify the presence of an mRNA for a putative half-transporter (*ABCB5β*) of *ABCB5*, we designed specific primers to amplify the putative *ABCB5β* (Figure 4A). The expected size of the cDNA fragment in melanomas or melanocytes was 2.9-kilo-base (kb) and was homologous to the C-terminal half of ABCB1 (Figure 4B–D). Sequence analysis revealed that *ABCB5β* was similar to the recently published *ABCB5* sequence (Frank et al., 2003), which possesses 20 exons that have a domain arrangement similar to the C-terminal half of P-gp. Moreover, *ABCB5β* has an additional ABC signature motif and a Walker B consensus at its N-terminus (Figure 4B). RT-PCR confirmed that *ABCB5β* mRNA was preferentially expressed in melanomas, but not in normal tissue controls (uterus, lung, and placenta) (Figure 4E, lanes 11–13), which was similar to the expression pattern of *ABCB5α*. Of note, SK-MEL-2 cells did not express the full-length *ABCB5β* (Figure 4E: lane 6). Furthermore, the expected bands from two melanoma cell lines (MALME-M3 and UACC-257) were subcloned and confirmed to be *ABCB5β* by DNA sequencing.

ABCB5α/β mRNA expression in normal tissues and cancer cells

Sequence analyses indicate that both *ABCB5α* and *ABCB5β* share common exonic regions (exon 1 to exon 6). Thus, we utilized a specific oligonucleotide probe (B5P102S) for the detection of *ABCB5α/β* by Northern blotting with a human tissue mRNA-blot. The hybridization results revealed that *ABCB5α/β* mRNA was overexpressed in the melanoma cell line UACC-257 and were lower in amount in SK-MEL-5, consistent with the results from RT-PCR (Figures 2 and 4). There are several *ABCB5*-related transcripts (7.5-, 4.5-, 2.9-, and 2.4-kb) in UACC-257 cells. Moreover, the transcripts ranging from 2.4–2.9 kb represent the predominant species and the bands are somewhat smeared, suggesting that they might be differentially transcribed or spliced *ABCB5α/β* mRNAs. The bands at approximately 4.5 and 7.5 kb could represent immature splicing variants (Figure 5A).

However, we cannot rule out the existence of a full-length *ABCB5* transcript in this melanoma cell line. Surprisingly, little or no expression of *ABCB5* α/β was found in normal tissues such as brain, colon, small intestine, thymus, spleen, kidney, liver, placenta, lung, and peripheral blood leukocytes, and results have been confirmed by RT-PCR (data not shown). There is a weak and uncertain expression of *ABCB5* α/β in both heart and skeletal muscles (Figure 5A, lanes 3 and 4). No detectable expression of *ABCB5* α/β was found in the cancer cell lines HL-60, HeLa S3, K562, MOLT 4, Raji (Burkitt's lymphoma), SW480 (colorectal adenocarcinoma), A549, or G-361 cells (data not shown).

Confirmation of *ABCB5* α/β expression in normal pigment cells

To determine whether *ABCB5* α/β activation is acquired during malignant transformation or expressed during differentiation, we examined normal pigment cells (i.e. melanocytes and RPEs). Our results reveal that *ABCB5* α/β is also overexpressed in melanocytes, comparable with the levels seen in the melanoma control (UACC-257) (Figure 6A, B). We also detected the overexpression of *ABCB5* β mRNA (exon 2–exon 7) in RPEs. However, expression of the mRNA fragment from exon 7 to exon 20 in RPEs was altered, with a weaker band of slightly slower mobility compared with normal melanocytes, suggesting that RPEs may have alternatively spliced *ABCB5* β transcripts. Nonetheless, these data suggest that *ABCB5* α/β expression is pigment cell-type specific.

ABCB5 α/β expression and drug response

We examined *ABCB5* α/β expression in the 26 cell lines from the Cancer Line Profiling Array, in which 15 out of the 26 (58%) cell lines overlap with the NCI-60 cell line panel. We found that the *ABCB5* α/β expression pattern was consistent with the previous experiments shown in Figures 2–5, in that only the melanoma cell lines (SK-MEL-28 and SK-MEL-5) expressed *ABCB5* α/β (Figure 7A, columns 16 and 17, right panel). The MDA-MB-435 associated cell line MDA-MB-435S showed only weak expression after drug treatment (Figure 7A, column 9, right panel). *ABCB5* α/β expression responded to different drug or stress treatments in these two melanoma cell lines. Several drugs [desferrioxamine, doxorubicin, PALA, 5-fluorouracil, actinomycin D, cycloheximide, and etoposide] down-regulated *ABCB5* α/β mRNA expression, whereas eight drugs (L-mimosine, mitomycin, cisplatin, paclitaxel, demecolcine, amethopterin, thiotepa, and aphidicoline) up-regulated *ABCB5* α/β mRNA levels in SK-MEL-5 cells, which originally had very low endogenous *ABCB5* α/β mRNA expression (Figure 7A, column 17, right panel). However, the drug response pattern in SK-MEL-28 was somewhat different from that of SK-MEL-5, as detailed in Figure 7B and C. Nevertheless, three drugs (5-fluorouracil, PALA, and doxorubicin) had a similar effect on both SK-MEL-28 and SK-MEL-5. These drugs suppressed *ABCB5* α/β expression, whereas three other drugs (L-mimosine, mitomycin, and cisplatin) up-regulated *ABCB5* α/β expression.

ABCB5 β is not co-activated in MDR cellular models

Sequence analyses showed that *ABCB5* α/β have approximately 60–70% homology with *ABCB1* (*MDR1*) sequences. We examined whether *ABCB5* α/β mRNA sequences were also expressed in MDR cell models. *ABCB5* β was not expressed in two MDR cell lines (KB-V1 and MES-SA/Dx5) selected by doxorubicin or vinblastine respectively in KB-3-1 cells and MES-SA sarcoma cells (Figure 7D). Indeed, *ABCB5* β did not respond to a typical *MDR1* substrate (vinblastine) stimulation in UACC-257 cells, in contrast to *ABCB1* (*MDR1*), which demonstrated a dose-dependent increase in expression in the presence of vinblastine (Figure 7E).

Discussion

As an approach to understanding the role of the transporter ABCB5, which has a large degree of predicted homology to the known multidrug transporter ABCB1, we examined the expression pattern of *ABCB5* in both normal tissues and cancer cells. We demonstrate specific expression of two mRNA species, *ABCB5 α* and *ABCB5 β* in pigmented cells including melanocytes, melanomas, and RPEs. Expression of *ABCB5* is regulated in melanoma cells by exposure to several different cytotoxic drugs, suggesting a possible role in drug resistance in melanoma.

Tissue distribution, cellular compartments, and function

In general, the expression pattern and localization of ABC transporters help define their function. For example, *ABCA1*, identified in mouse and human genomes and associated with Tangier disease (Bodzioch et al., 1999), has a broad tissue distribution and controls the transport of membrane phospholipids and cholesterol toward specific extracellular acceptors (Dean et al., 2001). In contrast, *ABCA4* is exclusively localized in photoreceptors, where it apparently transports retinal/vita-min A from the outer segment of the photoreceptor disks into the cytoplasm (Allikmets et al., 1997).

In the case of the ABCB subfamily, there are 11 members. The best characterized, *ABCB1* (*MDR1*)/P-gp, is a multidrug transporter that is highly expressed in some normal tissues including the biliary tract of the liver, proximal tubules of the kidney, colon, small bowel, adrenal glands, the blood–brain and blood–testis barriers, and hematopoietic stem cells (Chaudhary and Roninson, 1991; Cordon-Cardo et al., 1990; Fojo et al., 1987; Thiebaut et al., 1987), where it plays an important role in detoxification by actively extruding its substrates. In contrast to *ABCB1*, which has a wide range of tissue expression, both *ABCB4* (*MDR3*) and *ABCB11* (sPGP) are principally expressed in the bile canalicular membranes of the liver, where they are believed to be involved in phosphatidylcholine translocation from the inner to the outer leaflet of the membrane and in the secretion of bile salts, respectively (Borst and Elferink, 2002; Childs et al., 1995; Gerloff et al., 1988). Moreover, *ABCB6* (MTABC3), an 80-kDa protein that is highly expressed in mitochondria of heart and skeletal muscles, is thought to regulate iron homeostasis by transport of Fe/S clusters from mitochondria to the cytoplasm, thereby helping to prevent iron accumulation and DNA damage to that organelle (Mitsuhashi et al., 2000). However, there is only one publication in the literature that addresses the possible physiological role of *ABCB5 β* in the regulation of progenitor cell fusion (Frank et al., 2003). One additional study has also addressed *ABCB5 α* expression in some normal tissues, but not in melanocytes, melanomas, or other cancer cells. The authors of this study found that *ABCB5 α* was mainly expressed in the testes and uterus (Langmann et al., 2003). Moreover, we have recently reported expression of *ABCB5 α* in some melanomas (Szakács et al., 2004). Thus far, there has been no systematic study to examine the *ABCB5 α/β* expression pattern in normal human tissues and in cancer cells.

We utilized multiple methods, including Northern blot, real-time RT-PCR and conventional RT-PCR, to examine the expression patterns of *ABCB5* and its expression profile. A full-length *ABCB5* has not been reported in GenBank. However, *ABCB5* might be expressed as different isoforms or partial transporters. Thus, we have identified two mRNA isoforms termed *ABCB5 α* and *ABCB5 β* , in which *ABCB5 α* lacks the intact domain for a half-transporter and would therefore encode a protein non-functional in transport. Nonetheless, this form represents the most abundant isoform in the melanoma cDNA library. The small predicted protein product could regulate transport, or the small RNA product could play a role by interaction with either mRNA or proteins (Gottesman, 2004). Whether *ABCB5 α* possesses such a regulatory role for either *ABCB5* or other related ABC transporters has not been determined. In contrast, for *ABCB5 β* , the protein topology predicts a half-transporter

or more than a half-transporter, with approximately 70% similarity to ABCB1/P-gp (Figure 4).

Both *ABCB5α* and *ABCB5β* were preferentially expressed in melanomas. In contrast, *ABCB5α/β* expression was undetectable in two amelanotic melanomas (M14 and LOXI-IMVI). The expression profile of *ABCB5α/β* in all of the other melanomas of the NCI-60 panel were confirmed by both RT-PCR and sequencing. Neither *ABCB5α* nor *ABCB5β* expression was found in normal tissues other than pigmented cells such as normal melanocytes (derived from neural crest) and RPEs (derived from neural ectoderm), suggesting that *ABCB5α/β* expression is not neural crest-specific, but rather is pigment cell-specific (Figure 5B). Thus, the physiological role of *ABCB5α/β* might be associated with melanogenesis, the steps needed to produce melanin within melanosomes. It has been shown that several proteins are specifically involved in melanogenesis, which include enzymatic and structural proteins such as tyrosinase, TRP1/gp75, silver/gp100, MART1/melan-A, and TRP2/DCT. These melanosomal proteins have been used as molecular targets for inducing cellular and humoral responses to both normal melanocytes and melanomas (Sakai et al., 1997; Virador et al., 2001). Thus, expression of specific *ABCB5* isoforms (such as *ABCB5α/β*) could be used as molecular markers for differential diagnosis or constitute potential targets for therapy of pigment cell-related lesions such as melanomas.

***ABCB5α/β* in cancer drug resistance**

Clearly, several ABC transporters, including *ABCB1* (*MDR1*), *ABCC1* (*MRP1*) and *ABCG2* (*BCRP/MXR*), can confer multidrug resistance MDR to cancer cells in vitro. Gain of function of these transporters in cancer cells could cause clinical multidrug resistance. Indeed, both *MDR1* and *MRP1* are expressed in many human cancers, including leukemias and some solid tumors (Borst and Elferink, 2002; Gottesman et al., 2002; Haimeur et al., 2004). It has been shown that an siRNA directed at *ABCB5β* can reduce *ABCB5β* mRNA levels and induce drug sensitivity to several drugs including camptothecin 10-OH, 5-FU, and mitoxantrone, implying that elevated *ABCB5* expression may confer drug resistance to these agents (Huang et al., 2004). Nevertheless, there is no transfection data available to establish the role of *ABCB5β* in mediating multidrug resistance. In addition, *ABCB5β* is not expressed in two multidrug resistant cell lines (KB-V1 and MES-SA/Dx5), and has a distinct pattern of response to cytotoxic drugs compared with *ABCB1* (*MDR1*)/P-gp, suggesting that *ABCB5β* might have a distinct transport function (compared with that of P-gp) that has not yet been determined. Finally, testing uptake of pigment-regulating (thiols, heterocyclics) or melanin-binding (metal ions, heterocyclics) drugs could be used as a way of finding a transporter function for the *ABCB5* isoforms, given that they are associated with pigmented cells.

In conclusion, expression of both *ABCB5α* and *ABCB5β* is pigment cell-type specific, thereby likely providing additional molecular markers for the differential diagnosis of melanomas. This specific expression might allow development of *ABCB5α/β* gene products as molecular targets for therapy of melanomas.

Materials and methods

Cells and cell culture

The NCI-60 panel has been previously described (Scherf et al., 2000). The melanoma cell lines were grown in RPMI 1640 medium (Invitrogen Inc., San Diego, CA, USA) containing 10% fetal calf serum and antibiotics at 37°C in a humidified atmosphere of 5% CO₂. The cells were washed once with phosphate-buffered saline, pH 7.4, frozen in liquid nitrogen, and stored at -80°C prior to total RNA isolation. Two drug-selected multidrug resistant

(MDR) cell lines (KB-V1 and MES-SA/Dx5) were also used in this study. The development of KB-V1 (a vinblastine-selected KB-3-1 cell line) has been previously documented (Shen et al., 1986). MES-SA/Dx5 (a doxorubicin-selected uterine sarcoma cell line, Harker and Sikic, 1985) was obtained from Dr B.I. Sikic (Stanford University). Vinblastine, doxorubicin, and other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Melanocyte cultures

Human primary melanocytes derived from the foreskins of two normal donors (kindly provided by Dr Zalfa Abdel-Malek) were cultured at 37°C with 5% CO₂ in medium 154 (Cascade Biologics, Portland, OR, USA) supplemented with Human Melanocyte Growth Supplement containing bovine pituitary extract, fetal bovine serum (FBS), bovine insulin, bovine transferrin, fibroblast growth factor, hydrocortisone, heparin, and phorbol 12-myristate 13-acetate (TPA) (Cascade Biologics) and 50 ng/ml of gentamycin/amphotericin B (Biowhittaker, Walkersville, MD, USA).

RPE cultures

Human RPE cells obtained from two donor eyes were cultured as previously described (Naginei et al., 1994). Briefly, RPE cultures were initiated from RPE-choroid explants in minimum essential medium (MEM) containing 20% FBS. The RPE cells were selected and further propagated in MEM containing 10% FBS, nonessential amino acids, and antibiotic-antimycotic mixture. The homogeneity of the cell population was confirmed by a monoclonal antibody against cytokeratin (an epithelial cell-specific cytoskeletal protein). RPE cultures at passage 7 were used for total RNA isolation.

Melanoma cDNA library screening

The SMART cDNA library derived from a melanotic melanoma patient was obtained from BD Clontech Laboratories, Inc. (Palo Alto, CA, USA) and used to examine the presence or absence of the putative *ABCB5* sequences. Screening of the cDNA library was performed using a DNA hybridization protocol that was previously described (Brown, 1993). The B5P102S probe (Table 1) designed according to the GenBank sequence (XM_166496) was synthesized and gel purified (Lofstrand, Inc., Gaithersburg, MD, USA), [³²P]-labeled, and hybridized to the HybondTM-NX nylon membrane (Amer-sham Pharmacia Biotech UK Limited, Buckinghamshire, England) containing cDNA library clones. The positive clones were isolated, purified, and sequenced by the Pan Sequencing Facility (National Cancer Institute, Bethesda, MD, USA).

RNA isolation and cDNA synthesis

Total RNA was purified from the cells or cell lines described above by the RNeasy kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. RNA aliquots were stored at -70°C. The quality (purity and integrity) of the RNA samples was assessed by an Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip reagent set (Agilent Technologies, Palo Alto, CA, USA), and by assessing the ribosomal RNA bands on a native agarose gel. The RNA was quantified using a spectrophotometer. cDNA was synthesized using SuperScriptTM reverse transcriptase (Invitrogen, Inc.). Human Marathon-ReadyTM cDNAs from normal tissues (i.e. lung, uterus, and placenta) were obtained from Clontech Laboratories, Inc.

Oligonucleotide synthesis

Oligonucleotides were synthesized and purified by Lofstrand, Inc. The amplimers were designed from the predicted sequence using the GenScan program (<http://genes.mit.edu/>)

GENSCAN.html) and sequences were obtained from the cDNA library screening. All *ABCB5* specific primers are summarized in Table 1.

Northern blot

A human tissue mRNA blot (the Human MTN blot, Clontech Inc., see <http://www.bdbiosciences.com> for additional information) containing approximately 1 μ g poly A⁺ RNA each from different normal tissues and cancer cell lines was used to examine the tissue distribution of *ABCB5* α/β . Total RNAs from the two melanoma cell lines (UACC-257 and SK-MEL-5) were used as positive controls for *ABCB5* mRNA expression. The RNAs were separated by electrophoresis through 1% formaldehyde/agarose gels (~25 μ g/lane) and transferred onto a Hybond-NTM nylon membrane (Amersham Pharmacia Biotech, Braunschweig, Germany) by capillary transfer. The mRNA probe was derived by reverse transcription of both the oligo-nucleotide probe (B5P102S, Table 1) and the actin probe (Clontech Laboratories, Inc.). The mRNA probe was [³²P]-labeled and hybridized to the membrane. The membranes were washed and exposed to the film.

Conventional RT-PCR

A conventional RT-PCR method was used to verify the size, sequence authenticity, expression pattern, and alternatively spliced transcripts of *ABCB5* in the melanoma cell lines. Total cDNAs were subjected to denaturation at 94°C for 30 s followed by 35–40 cycles of amplification as programmed: 30 s at 94°C, 30 s at 55°C, and 2–3 min at 72°C. The PCR products were subcloned in the TOPO cloning vector (Invitrogen, Inc.). Plasmids containing the desired inserts were sequenced.

Real-time RT-PCR

Expression levels of *ABCB5* α mRNA in the NCI-60 cell panel were measured by real-time RT-PCR using the LightCyclerTM RNA Amplification SYBR Green kit and the LightCyclerTM machine (Roche Biochemicals, Indianapolis, IN, USA). The RT-PCR was carried out on 150 ng of total RNAs, in the presence of specific primers (250 nM) designed within exon 7 of *ABCB5* α (Table 1). The data were normalized to the mean expression of *ABCB5* α across the NCI-60 cell lines (Szakács et al., 2004).

Cancer line profiling array and cDNA dot blot

The Cancer Line Profiling Array was purchased from BD Clontech, Inc. This array includes normalized BD SMART cDNAs from 26 different cell lines, each treated with 26 different conditions (such as chemotherapeutic agents, oxidative stress inducers, heat-shock, and radiation). The properties of chemotherapeutic agents and additional information about the treatments are available at <http://www.bioinfo.clontech.com/dparray>. Briefly, heat-shock was performed by incubating the cell suspension at 44°C in a water bath. An ultraviolet transilluminator (with a power output 1,000 μ W/cm²) was used to irradiate cells using 365-nm light from a distance of 15 cm. Gamma-irradiation was performed using a ⁶⁰Co source to receive an overall dose of 2 Gy. The cells were treated with a dose that would produce 50% cell growth inhibition (IC₅₀) for 48 hr, at 37°C, in a 5% CO₂ atmosphere. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Chen et al., 1994). The cDNA dot blot was used to examine *ABCB5* α/β mRNA expression and drug response patterns in this array, which was performed according to the manufacturer's instructions. The cDNA probe used in this experiment was B5-103AS on exon 2 of *ABCB5* α/β (Table 1).

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Figure 1. Molecular cloning of *ABCB5α* (accession number: AY851364). (A) Genomic organization of the exons of *ABCB5* on chromosome 7p; (B) *ABCB5α* domain structure analysis; (C) Sequence analysis of *ABCB5α*, with deduced amino acids indicated: UTR, untranslated region; S, ABC signature.

Figure 2. *ABCB5* expression in melanomas. (A) Amplimer locations for RT-PCR are diagrammed. The sequences for the amplimers are summarized in Table 1. (B) The 5' genomic sequences of *ABCB5* are shown. The putative transcription initiation site (+1) was obtained by examining the 5' sequences of several clones isolated from the melanoma cDNA library. The amplimers were designed downstream or upstream of the putative transcription initiation site. (C) RT-PCR analyses of *ABCB5* expression using the different sets of amplimers were performed as described in *Materials and methods*. Forty cycles of RT-PCR were used for *ABCB5* and 25 cycles for ribosomal cDNA (loading controls). The expected products are indicated by arrowheads and the asterisk-labeled bands were excised, subcloned, and sequenced.

Figure 3.

Real-time RT-PCR analysis of *ABCB5α* expression in the NCI-60 cell panel. RT-PCR was performed using the primer sets *ABCB5α*-Ex7F1 and *ABCB5α*-Ex7R2, as described in Table 1. *ABCB5α* expression was normalized to the mean expression of *ABCB5α* across the NCI-60 cell lines. The positive values on the Y-axis indicate elevated expression of ABCB5 and vice versa.

Figure 4.

ABCB5 expression in melanomas. (A, B) The predicted *ABCB5* gene structure with a domain arrangement similar to that of the C-terminal half of P-gp. The amplicon locations for RT-PCR are diagrammed. The first ATG for protein translation is indicated. The N-terminal Walker A motif was untranscribed in this deduced structure. (C) RT-PCR analyses of the expression of full-length *ABCB5* (2.9 kb) in melanoma cells (UACC-257) and in normal melanocytes. (D) The existence of the full-length *ABCB5* was also verified in the two melanoma cDNA libraries (SMART-MEL and NCI-GAP) and in the melanomas from the NCI-60 cell panel. (E) Forty cycles of RT-PCR were used for *ABCB5* and 25 cycles for ribosomal cDNA. The expected products are indicated by arrowheads and the asterisk-labeled bands were excised, subcloned, and sequenced (accession number: AY851365).

Figure 5. *ABCB5α/β* expression in both normal tissue and cancer cells. (A) Northern blotting analysis of *ABCB5α/β* expression. Upper panel: human tissue mRNA blot containing approximately 1 μ g poly A⁺ RNA from both heart and skeletal muscles (lanes 3–4). Total RNAs from two melanoma cell lines (SK-MEL-5 and UACC-257, lanes 1 and 2) were used as positive controls. The cDNA probe B5P102S was reverse transcribed, [³²P]-labeled, and hybridized to the membrane. The membranes were washed and exposed to the film for 4 d. Lower panel: The membrane was reprobbed with a [³²P]-labeled Human β -Actin cDNA Probe and exposed to the film for 2 h. (B) A schematic presentation of the expression of *ABCB5α/β* in various normal tissues derived from three germ layers (ectoderm, mesoderm, and endoderm). The highlighted tissues are *ABCB5α/β* mRNA positive, whereas the question mark (?) indicates uncertain expression of *ABCB5α/β*.

Figure 6.

ABCB5 α/β expression in human pigment cells. (A, B) RT-PCR analyses of the expression of *ABCB5 α/β* in two normal human melanocytes (only one sample is shown) as described in Figures 2 and 4. (C) The existence of *ABCB5 β* was also verified by RT-PCR in the two human RPE samples (only one is shown). UACC-257 was used as a positive control for *ABCB5 α/β* . The asterisk indicates an altered *ABCB5 β* isoform.

Figure 7.

(A) cDNA dot blotting with the cDNA probe B5P103AS was performed to monitor *ABCB5* expression in human cancer cells exposed to 26 different stressful conditions (right panel). The same membrane of the right panel was stripped and reprobed with the ubiquitin probe (Clontech Laboratories, Inc.) to normalize the cDNA sample loading (left panel). (B, C) The results from A were quantitated by densitometry using an ALPHA Innotech image analyzer (San Leandro, CA, USA). *ABCB5* expression in both SK-MEL-5 and SK-MEL-28 cells were normalized to ubiquitin expression. (D) *ABCB5* expression in the MDR cellular models KB-V1 and MES-SA/Dx5: the MDA-MB-435 cell line was used as a negative control for *ABCB1* (*MDR1*) and a positive control for *ABCB5*.

MES-SA and KB-3-1 were the parental cells for MES-SA/Dx5 and KB-V1 respectively. (E) RT-PCR (35 cycles) analysis of *ABCB1* and *ABCB5* mRNA levels was performed in UACC-257 cells that were exposed to different concentrations of vinblastine (VBL) for 48 h. In lane 6, MES-SA/Dx5 cells were used as a positive control for *ABCB1* (*MDR1*) expression and a negative control for *ABCB5* expression. The cDNAs for MDA-MB-435S and MDA-MB-435 were prepared from independently isolated RNAs. 5-Fu, 5-fluorouracil; PALA, N-(phosphonacetyl)-L-aspartate.

Table 1Oligonucleotides used in amplification and detection of *ABCB5 α/β*

Isoform-amplifiers/probes ¹	Sequences
<i>ABCB5α/</i> β -Int-1F ²	5'-aaggatcc CAT GGG GTT TAT ACA TGG TCA GCA C-3'
<i>ABCB5α/</i> β -Ex1F	5'-aaggatcc ATT GCT TCT CGG CCT TTT GGC TAA G-3'
<i>ABCB5α/</i> β -Ex2F	the same sequence as B5P-102S
<i>ABCB5α/</i> β -Ex2R	5'-aactcgag GGG CTA TTG CGA AGG TTT CAA AGT G-3'
<i>ABCB5α-Ex7F1</i>	5'-TCT GGC CCC TCA AAC CTC ACC-3'
<i>ABCB5α-Ex7R1</i>	5'-TCA TAG GCA GGA GGT CAG G-3'
<i>ABCB5α-Ex7R2</i>	5'-TTT CAT ACC GCC ACT GCC AAC TC-3'
<i>ABCB5β-Ex7F</i>	5'-ggatcgat TGT GAC CCT AAA GGA TGG AAT tCT G-3'
<i>ABCB5β-Ex7R</i>	5'-ggatcgat CAG AAT tC CATC CTT TAG GGT CAC A-3
<i>ABCB5β-Ex20R</i>	5'-aactcgag TCA CTG CAC TGA CTG TGC ATT CAC T-3'
B5P-102S ³	5'-TCT TTA GTG TAA TCC ATA GCA GTT ATT GCA TTG GAG CAG CAG TCC CTC ACT TTG AAA CCT TCG CAA TAG CCC GAG GAG CTG CCT TTC ATA TTT TCC AGG TTA-3'
B5P-103AS ³	5'-TAA CCT GGA AAA TAT GAA AGG CAG CTC CTC GGG CTA TTG CGA AGG TTT CAA AGT GAG GGA CTG CTG CTC CAA TGC AAT AAC TGC TAT GGA TTA CAC TAA AGA-3'
Ribo 1F	5'-GAA AGA TGG TGA ACT ATG CC-3'
Ribo 2R	5'-TTA CCA AAA GTG GCC CAC TA-3'

¹ F, forward primer; R, reverse primer; S, DNA sense strand; AS, DNA antisense strand; Ribo, ribosomal 28S cDNA probe.

² DNA amplifiers of *ABCB5 α/β* were designated according to their locations on introns (Int) or exons (Ex). The endonuclease restriction sites (the underlined bases) were introduced to facilitate subcloning.

³ DNA probes were designed at exon 2 of both *ABCB5 α* and *ABCB5 β* . The probes were synthesized and gel purified by Lofstrand, Inc.