表2　これまで解析がされてきているトランスポーターのデータシート

遺伝子		染色体	mRNA (kb)	アミノ酸数	組織プロフィール	抗癌剤（基質／耐性）	その他の薬剤（基質）	機能（生理的基質）	関連疾患
ABCトランスポーター									
MDR1	*ABCB1*	7q21.1	～4.9	1,280	消化器系上皮 血液脳関門	ビンカアルカロイド アンスラサイクリン パクリタキセル エトポシド	ジゴキシン ローダミン サイクロスポリン	ステロイド？ pH制御	潰瘍性大腸炎 パーキンソン病
MDR3	*ABCB4*	7q21.1	～5.7	1,279	肝			ホスファチジルコリン トランスロカーゼ	胆汁うっ滞
MRP1	*ABCC1*	16q13.1	～6.0	1,531	肺 末梢血	ドキソルビシン	アルセナイト アンチモン	葉酸 ロイコトルエンC4	
MRP2	*ABCC2*	10q24	～4.9	1,545	肝	シスプラチン ドキソリビシン ビンクリスチン ガンプトテシン メトトレキセート		有機イオン 胆汁	デュビン・ジョンソン症候群
MRP3	*ABCC3*	17q21	～4.8	1,528	胃，小腸	エトポシド メトトレキセート			
MRP4	*ABCC4*	13q32	～5.9	1,325	広範囲		ガンシクロビル	プロスタグランジン 還元型グルタチオン ステロイド	
MRP5	*ABCC5*	3q27	～5.9	1,437	肝	シスプラチン		サイクリックヌクレオチド	
MRP6	*ABCC6*	16p13.1	～4.5	1,503	肝，腎	アンスラサイクリン		グルタチオン抱合体	弾性線維偽黄色腫
MRP7	*ABCC7*	7q31.2	～6.1	1,480	腎，肺			チャネル機能	嚢胞性線維症
MRP8	*ABCC11*	16q21.1	～4.5	1,344	肝，乳腺	エトポシド ドキソルビシン			
MRP9	*ABCC12*	16q21.1	～5.1	1,342	乳癌	5-FU ddC PMEA			
BCRP	*ABCG2*	4q22	～2.5	655	乳癌 胎盤，小腸	マイトサントロン カンプトテシン ビンクリスチン パクリタクセル		ハーフトランスポーター 胆汁酸	
BSEP	*ABCB11*	2q24	～4.3	1,321	肝				家族性肝内胆汁うっ滞
その他									
ATP7B		13q14	～6.7	1,465	肝	シスプラチン		銅，亜鉛 P型 ATPase	ウィルソン病
ATP6	複合サブユニット				広範囲	シスプラチン		pH制御 （V-ATPase：プロトンポンプ）	骨疾患（大理石病）

とATP6がある．前者はP型ATPase，後者は非P型V-ATPaseであり，両者ともにシスプラチン※2耐性に関与する．V-ATPaseは細胞内pHの制御分子である．シスプラチンは酸性下でDNAによく結合するが，シスプラチン耐性細胞ではV-ATPaseが高発現し細胞外へプロトンを排出することにより細胞内はアルカリ化しているため，シスプラチンがDNAに結合できないと考えられる[3]．

2 ABCトランスポーター発現制御からみた耐性研究

癌患者が自然耐性や獲得耐性のため化学療法の治療効果が望めないことは大きな問題であり，その耐性形質発現の分子的背景を明らかにすることは重要な研究である．特に化学療法後の再発癌では高頻度にP-糖タンパクが発現し，次の抗癌剤に抵抗性を示すことがよく知られている．なぜ抗癌剤による治療でP-糖タンパクが発現してくるのであろうか？ われわれは*MDR1*遺伝子をモデルとして，その発現制御の解析から転写因子であるY-box結合タンパク1（YB-1）を見出した．これまで多くの研究グループが*MDR1*遺伝子発現調節の研究を進め，YB-1以外にもさまざまな転写因子が関与することがわかってきた．しかし，それらの転写因子と*MDR1*の発現について明確な相関を臨床試料で示した報告は全くない．一方，われわれの見出したYB-1は転写因子であり，その標的*MDR1*遺伝子発現とYB-1発現が臨床試料でこれほど相関を示しているものはない．YB-1発現とP-糖タンパク発

※2　シスプラチン

シスプラチンの主な細胞内標的はDNAであり，cisGGあるいはcisAGでDNA架橋を起こし，転写や複製障害などにより細胞死を誘導する．シスプラチンはDNA損傷ストレス以外に酸化ストレスや小胞体（ER）ストレスを引き起こすことも知られている．

表3　臨床腫瘍におけるYB-1の発現とその意義

報告者	癌組織	年代	要旨	発表論文
Bargou	乳癌	1997	YB-1の核内発現はP-糖タンパク発現と相関がある	4
小田	骨肉種	1998	YB-1の核内発現はP-糖タンパク発現と相関がある	5
柴尾	大腸癌	1999	YB-1の発現はトポイソメラーゼⅡ1αやPCNA遺伝子発現と相関する	6
嘉村	卵巣癌	1999	YB-1の核内発現は予後と相関し，化学療法感受性に影響を与える	7
Del Valle	神経膠芽腫	2000	YB-1はJCV遺伝子発現を活性化して神経膠芽腫を再発させる	8
Carobbio	乳癌	2001	シスプラチンやパクリタキセル耐性細胞ではYB-1の核内集積がある	9
Gù	肺癌	2001	YB-1の発現はトポイソメラーゼⅡ1αやPCNA遺伝子発現と相関する	10
Janz	乳癌	2002	YB-1の高発現は化学療法が無効で予後不良である	11
八幡	乳癌	2002	シスプラチン耐性細胞ではYB-1の核内集積がある	12
小田	滑膜肉腫	2003	YB-1の核内発現は予後と相関し，P-糖タンパクおよびトポイソメラーゼⅡα発現と相関がある	13
佐治	乳癌	2003	YB-1の核内発現はP-糖タンパク発現と相関がある	14
Gimenez-Bonafe	前立腺癌	2004	YB-1の高発現はP-糖タンパクの活性を高める	15
Huang	卵巣癌	2004	YB-1の核内発現はP-糖タンパク発現と相関がある	16

現および予後などを臨床試料で解析した報告を表3にまとめた[4)~16)]．YB-1は転写因子としての機能だけでなく多機能タンパクであることも知られている[17)]．非ストレス下ではYB-1の多くは細胞質に局在しているが，抗癌剤や紫外線処理，熱ショックなどのストレスにより核に移行し，DNA修復や転写制御に関与する．YB-1の発現上昇はそれだけでシスプラチンなどDNA損傷を惹起する薬剤に耐性を示す[18)]．さらに，YB-1の発現は細胞増殖にも密接に関連しており，YB-1のノックアウトマウスは胎生致死である．YB-1ヘテロノックアウトのマウスES細胞もDNA損傷薬剤に感受性を示すことがわかっている[19)]．一方でDNA損傷に伴うゲノム応答の1つとしてYB-1のDNA修復への関与が示唆されている．特に，YB-1自身がシスプラチン架橋DNAを認識して結合すること[20)]，またPCNAやエンドヌクレアーゼと分子会合することが報告されている．ここで大切な点として，薬剤耐性へのp53の関与があげられる．すなわち，p53もトランスポーターの発現制御に関与している．p53はYB-1とも分子会合し*MDR1*遺伝子の転写を抑制する[21)]．p53の変異は薬剤耐性と密接に相関することはよく知られた事実であり，最近は*p53*の一塩基多型（SNP）も活性化されたp53の細胞内局在の変化やアポトーシス誘導に大きく影響することが示されている[22) 23)]．

3 ゲノムワイドの薬剤感受性／耐性研究の現状

薬剤感受性／耐性に関与する遺伝子を同定する一般的な方法として，細胞増殖抑制やアポトーシスなどの薬剤反応性プロファイルと発現プロファイルの相関を検討することがあげられる（図1）．これまでの研究成果としては，Scherfらによる60種のヒト癌細胞株についての118種の薬剤感受性プロファイルと発現プロファイルを比較したデータベースが有名である[24)]．また同様の報告として，55種の抗癌剤感受性データと39種のヒト癌細胞株の発現プロファイルの解析もあげられる[25)]．また，Zembutsuらは85種のヌードマウス移植ヒト癌細胞の薬剤感受性と発現プロファイルの解析について報告している[26)]．細胞株ではなく胃癌の臨床試料から，4種の薬剤感受性プロファイルと発現プロファイルを解析した報告もされている[27)]．一方，薬剤処理前後に，発現誘導される遺伝子の発現プロファイルを比較検討した報告もされている[28) 29)]．一過性の薬剤処理による遺伝子発現プロファイルの変化から薬剤感受性／耐性に関連する遺伝子を同定していく方法と，薬剤を毎週処理して経時的な発現プロファイルの変化から，より*in vivo*に近い耐性獲得を模倣して同定していく方法が試みられている[30)]．また，*in vitro*で樹立した耐性細胞株と親株との発現プロファイルの比較から耐性関連遺伝子を同定していく方法も報告されている[31) 32)]．これらの解析で同定される耐性関連遺伝子群の中には，生存シグナルもしくは抗アポトーシスに関与するものが含まれると考えられる．われわれはシスプラチンによるDNA損傷で発現が誘導され，耐性細胞株でも高発現している遺伝子の同定を進め，その中でも転写因子に着目して解析を行った（図2）．

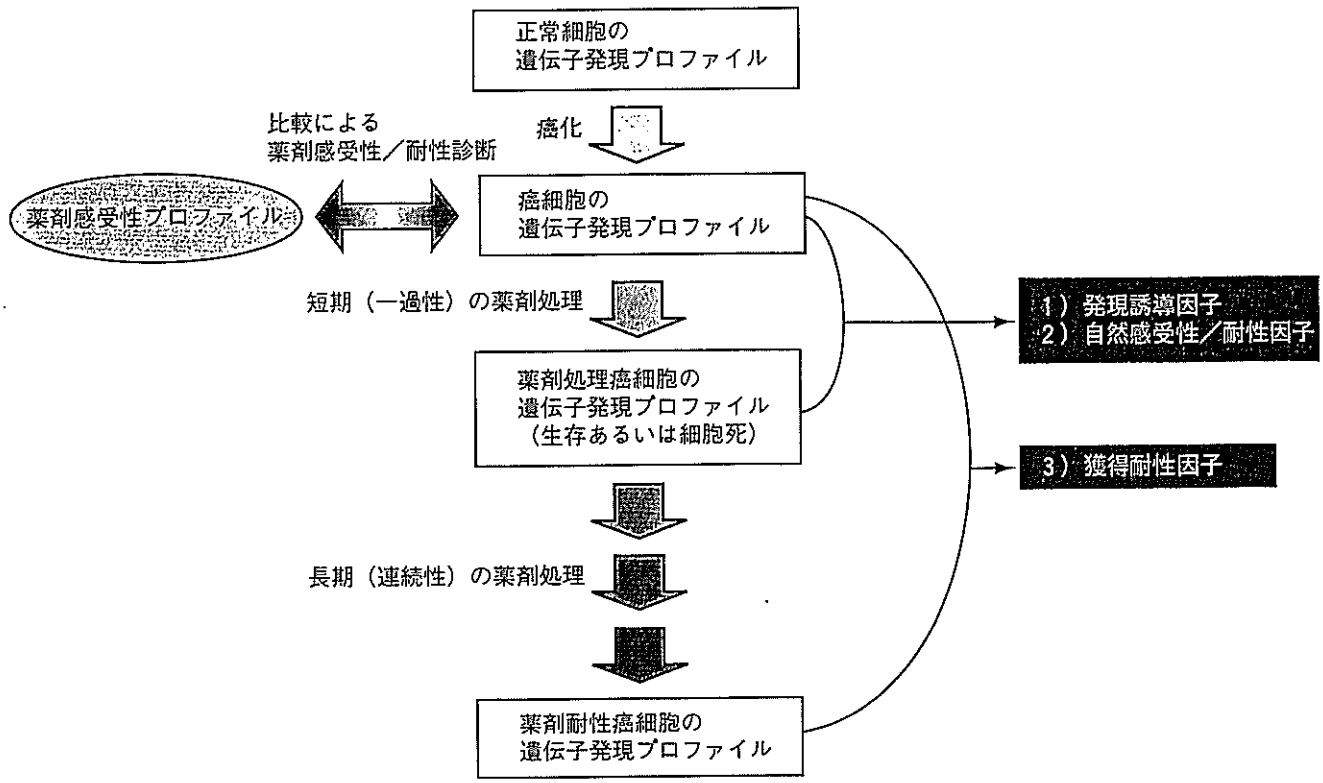

図1　癌細胞遺伝子発現プロファイル比較解析と耐性研究
各種癌細胞の遺伝子発現プロファイルを各種薬剤感受性プロファイルと比較することで自然耐性の診断が可能となる．一方，薬剤処理前後での遺伝子発現プロファイルまたは感受性細胞と耐性細胞の遺伝子発現プロファイルの比較から図中1）～3）にあげる因子が同定される．これらの情報から新規薬剤の作用を推測したり，創薬に結びつく有力な分子標的が見出されてくる

方法としては，まずシスプラチンにより発現誘導される遺伝子を同定し，その中から耐性細胞株においても高発現している遺伝子を選別した．次にその遺伝子のプロモーター解析から，発現誘導にかかわる転写因子の同定を行った．その結果，シスプラチン耐性に直接的および間接的に関与する3つの転写因子を見出している[33)～35)]．われわれは，さらにこれらの転写因子が核内で薬剤処理前後もしくは親株と耐性細胞株との間で会合分子プロファイルを変化させ，異なった機能をするのではないかと考え，会合分子のプロテオーム解析を試行している．このような研究から有力な創薬の対象となる分子標的が見出される可能性が高い．薬剤感受性／耐性の診断をするためには同定された遺伝子が直接的に薬剤感受性／耐性に関与するかどうかは問題ではないが，より少ない因子で効率よく診断するためには，直接耐性に関与するかどうか詳細に研究することも必須となる．個々の患者についてより簡便で迅速な診断方法の開発が現在模索されている．生体内にある癌細胞などの遺伝子発現プロファイルを正確に評価するためには，マイクロダイセクション法とDNAマイクロアレイ法を組合わせることが理想である．しかし，診断に用いる遺伝子／タンパクが限定され，その数が少なければ，抗体を用いた免疫組織染色で診断する方法が臨床に応用されやすいことが予想される．

4 その他のゲノムワイドの解析と薬剤感受性／耐性研究

薬剤感受性は癌細胞のもつゲノム不安性に基づくさまざまなゲノム構造の変化にも影響される．そこで，発現プロファイルのみならずその分子基盤である遺伝子そのものに目を向けた研究も始まっている．その方法は，ゲノム構造に起こる変化の解析とエピジェネティックな変化の解析の大きく2つに分類される．前者には，遺伝子の増幅／欠失解析や一塩基多型（SNP）解析などが含まれる．後者にはDNAメチル化とDNase I感受性領域の同定などの解析が含まれる[36) 37)]．ま

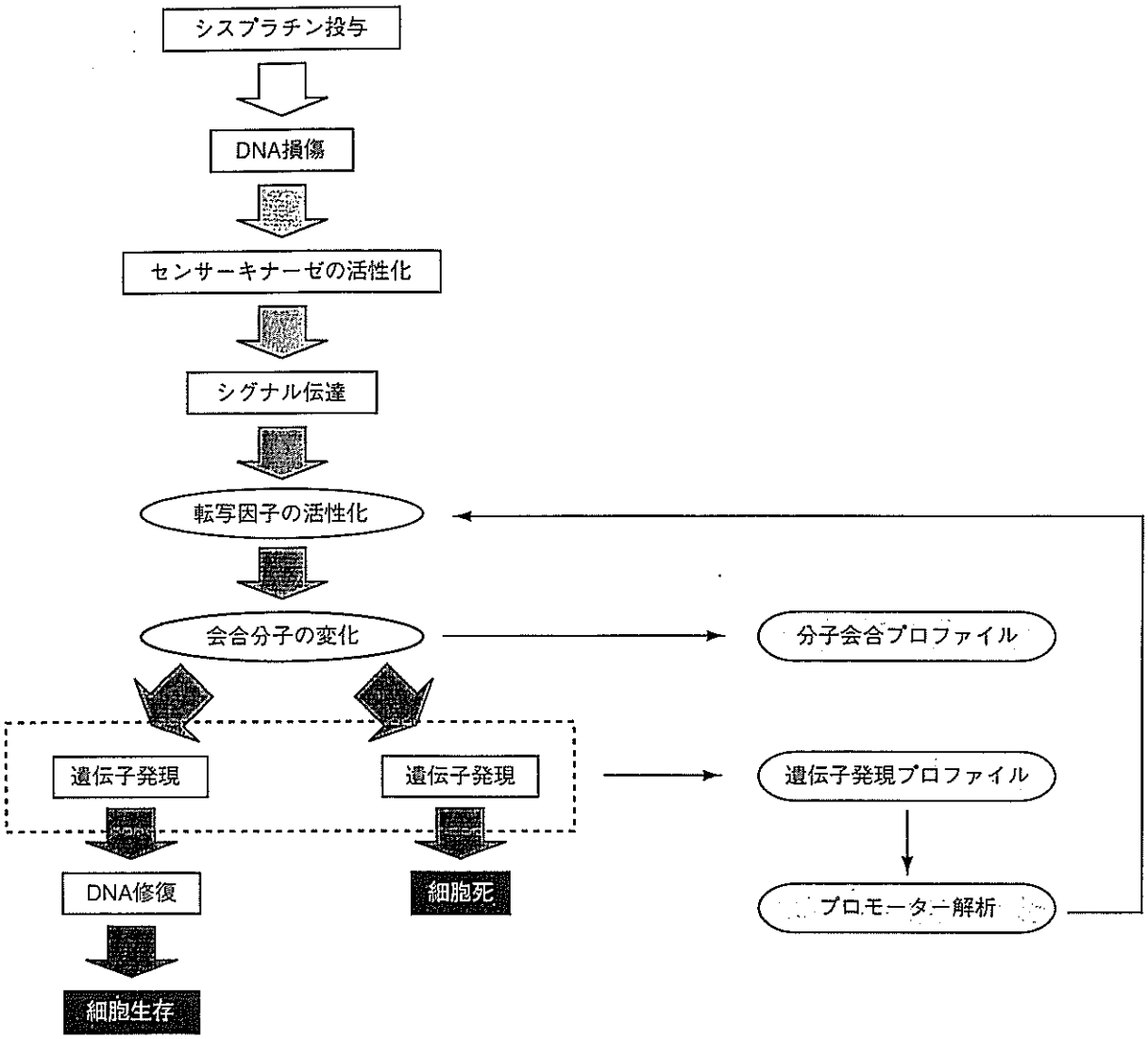

図2　シスプラチンによるストレス応答と転写因子
シスプラチンにより惹起されるDNA損傷ストレスはセンサーキナーゼの活性化から始まるシグナル伝達により核内の転写因子を活性化させて標的遺伝子の発現を変化させる．DNA損傷の程度により活性化された転写因子による遺伝子発現の差異が細胞生存と細胞死という細胞の運命を決定するのであろう．このとき，損傷DNAの認識やDNA修復にも転写因子は関与する．このゲノムストレス応答の主役と考えられる転写因子はさまざまな分子と会合し，ストレスに対応する．この会合プロファイルの変化や標的遺伝子のプロモーター解析からストレス応答に対する新しい転写因子が同定できる

た，薬剤に対する反応性の個人差は薬剤の体内動態を制御する因子や薬剤感受性／耐性を制御する因子の発現量や機能レベルを規定する遺伝子多型に基づくと考えられる．ABCトランスポーターだけでなく，薬剤代謝に関与するp450などの遺伝子についてもその発現とSNP解析が大切となってくる．さらにこのような研究をタンパクレベルで行うプロテオミクスが有望視されている．この場合，質的および量的解析，すなわち発現プロテオミクスと機能プロテオミクスの両方を考慮していくことが大切である．

おわりに

複雑な薬剤感受性／耐性関連遺伝子の解析にはゲノムワイドな解析手法が有力であるだけでなく，これらの解析により新しい薬剤の分子標的の探索や創薬に多くの情報を供給することができる．今後は癌患者に対する治療の臨床効果や副作用の評価にも必須な手法となると予想される．また個々の候補因子を*in vitro*および*in vivo*の癌細胞のみで評価するのではなく，その周辺を含む癌組織にも注意を払って評価することが

より大切となる．薬剤耐性も同様に癌細胞自身の薬剤感受性／耐性因子だけでなく，生体での薬物動態制御因子，また個々人のゲノムレベルやエピジェネティックレベルの変化にまで配慮していくことが究極の個別化治療につながると考えられる．

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Mini review

Functional SNPs of the breast cancer resistance protein - therapeutic effects and inhibitor development

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Abstract

Breast cancer resistance protein (BCRP) is a half-molecule ATP-binding cassette transporter that pumps out various anticancer agents such as 7-ethyl-10-hydroxycamptothecin, topotecan and mitoxantrone. We have previously identified three polymorphisms within the *BCRP* gene, G34A (substituting Met for Val-12), C376T (substituting a stop codon for Gln-126) and C421A (substituting Lys for Gln-141). C421A *BCRP*-transfected murine fibroblast PA317 cells showed markedly decreased protein expression and low-level drug resistance when compared with wild-type *BCRP*-transfected cells. In contrast, G34A *BCRP*-transfected PA317 cells showed a similar protein expression and drug resistance profile to wild-type. The C376T polymorphism would be expected to have a considerable impact as active BCRP protein will not be expressed from a T376 allele. Hence, people with C376T and/or C421A polymorphisms may express low levels of BCRP, resulting in hypersensitivity of normal cells to BCRP-substrate anticancer agents.

Estrogens, estrone and 17β-estradiol, were previously found to restore drug sensitivity levels in *BCRP*-transduced cells by increasing the cellular accumulation of anticancer agents. BCRP transports sulfated estrogens but not free estrogens and in a series of screening experiments for synthesized and natural estrogenic compounds, several tamoxifen derivatives and phytoestrogens/flavonoids were identified that effectively circumvent BCRP-mediated drug resistance. The kinase inhibitors gefitinib and imatinib mesylate also interact with BCRP. Gefitinib, an inhibitor of epidermal growth factor receptor-tyrosine kinase, inhibits its transporter function and reverses BCRP-mediated drug resistance both *in vitro* and *in vivo*. *BCRP*-transfected human epidermoid carcinoma A431 cells and *BCRP*-transfected human non-small cell lung cancer PC-9 cells show gefitinib resistance. Imatinib, an inhibitor of BCR-ABL tyrosine kinase, also inhibits BCRP-mediated drug transport. Hence, both functional SNPs and inhibitors of BCRP reduce its transporter function and thus modulate substrate pharmacokinetics and pharmacodynamics.

Keywords: ABCG2; Drug resistance; ABC transporter

1. Introduction

P-glycoprotein, MRP1 and BCRP are members of the ABC transporters that are involved in multidrug resistance [1–6]. These factors pump out various structurally unrelated chemotherapeutic agents in an energy-dependent manner and reduce their cytotoxic effects. BCRP, also known an ABCG2, is a half-

Abbreviations MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; ABC, ATP-binding cassette; SN-38, 7-ethyl-10-hydroxycamptothecin; MXR, mitoxantrone; SNPs, single-nucleotide polymorphisms.

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molecule ABC transporter containing 655 amino acids that has an ATP-binding domain and a transmembrane domain [4–6]. BCRP functions as a homodimer and confers resistance to anticancer agents such as SN-38, topotecan and MXR [7–11]. BCRP is widely expressed in normal cells and tissues, such as capillary endothelial cells, hematopoietic stem cells, the maternal-fetal barrier of the placenta and the blood-brain barrier [12–14]. This suggests that BCRP plays a protective role against xenobiotics and their metabolites. The apical localization of BCRP in the intestinal epithelium and the bile canalicular membrane indicates that it plays an important role in preventing intestinal absorption and in mediating hepatobiliary excretion of its substrates [12]. In this way, BCRP restricts the bioavailability of orally administered BCRP-substrate agents. A dual inhibitor of BCRP and P-glycoprotein, GF120918, has been shown to increase the oral bioavailability of topotecan through the inhibition of BCRP function [15,16]. In addition, a recent report has shown that BCRP is highly expressed in the mammary glands of mice, cows and humans during lactation and that it is responsible for the active secretion of various substrates [17].

Recently, the study of SNPs has progressed rapidly and generated remarkable findings and some SNPs have been shown to affect both the expression and function of their gene products. In particular, SNPs of drug-metabolizing enzymes and drug transporters have been studied extensively and some have been shown to affect the pharmacokinetics and pharmacodynamics of anticancer agents. Cytochrome P450 (CYP) 2C8 is the principal enzyme responsible for the metabolism of the anticancer drug paclitaxel. A *CYP2C8*3* variant, containing the two amino acid substitutions R139K and K399R, in exons 3 and 8, was previously shown to be defective in paclitaxel metabolism [18]. SN-38 is detoxified by conjugation to SN-38-glucuronide by the UDP-glucronosyltransferase (UGT) 1A1 enzyme [19]. Significantly, a *UGT1A1*28* variant, containing a 2-bp insertion (TA) in the TATA box within the gene promoter, was found to be significantly related to the reduced expression of UGT1A1 and the increased bioavailability of SN-38 [20]. A C3435T SNP in exon 26 of the *MDR1* P-glycoprotein gene was elucidated as the first functional polymorphism of its type in ABC transporters and shown to be closely associated with low expression levels of P-glycoprotein and high plasma levels of digoxin [21]. Another SNP within the *MDR1* gene, C1236T, has also been associated with increased exposure to SN-38 and its prodrug irinotecan [22]. In the following chapter, we describe the functional SNPs within the *BCRP* gene that have been identified by our laboratory and by other groups.

2. The effects of SNPs on BCRP expression and function

2.1. C421A (Q141K) BCRP SNP

We have previously identified three variant *BCRP* cDNAs, containing the substitutions G34A (V12M), C421A (Q141K) and a 944-949 deletion lacking Ala-315 and Thr-316 (Δ315-6) [23]. The G34A and C421A substitutions are SNPs whereas the 944–949 deletion is a splicing variant. We have subsequently found that C421A *BCRP*-transfected murine fibroblast PA317 (PA/Q141K) cells show markedly decreased exogenous protein expression and also a low-level of drug resistance when normalized to wild-type *BCRP*-transfected (PA/WT) cells (Fig. 1 and Table 1). In addition, both G34A *BCRP*-transfected PA317 (PA/V12M) cells and 944–949-deleted *BCRP*-transfected PA317 (PA/Δ315-6) cells showed either similar or marginally lower protein expression and drug resistance levels compared to PA/WT cells (Fig. 1 and Table 1). We had already shown in our previous study that the intracellular topotecan accumulation in PA/Q141K cells was higher than in other *BCRP* transfectants [23].

Kondo et al. have also reported low Q141K-BCRP protein expression levels using adenovirus-mediated gene transfection [24]. In addition, Kobayashi et al. examined BCRP protein expression in Japanese placental samples, and found that its levels were significantly lower in A421 homozygotes than in samples containing wild-type C421 alleles and that heterozygotes had intermediate levels of expression [25]. In contrast however, Zamber et al. reported that no significant correlation between the C421A variant and BCRP expression was observed in human intestinal samples [26]. The possible significance of the C421A-*BCRP* SNP on the pharmacokinetics of diflomotecan, a new camptothecin derivative anticancer agent, has also been evaluated in a phase I study [27]. In this trial, five patients who were heterozygous for the A421 allele,

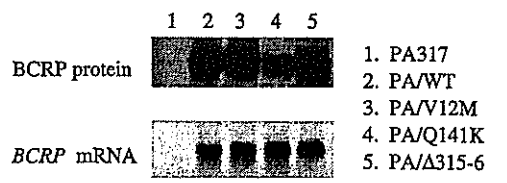

Fig. 1. Expression of BCRP protein and *BCRP* mRNA in PA317 cells transfected with *BCRP* variants.

Table 1
Drug sensitivities of *BCRP*-transfected PA317 cells

Cells	IC_{50} (ng/ml)		
	SN-38	Topotecan	MXR
PA317	2.5	0.060	17
PA/WT	98	0.58	>200
PA/V12M	98	0.63	>200
PA/Q141K	30	0.25	100
PA/Δ315-6	55	0.42	190

Cells were cultured for 5 days in the absence or presence of increasing concentrations of the indicated anticancer agents. Cell numbers were determined with a Coulter counter and IC_{50} values were calculated.

showed plasma levels of diflomotecan, after intravenous administration, that were 299% ($P=0.015$) of the levels in 15 patients who were homozygous wild-type, with mean values of 138 ng h/mL mg^{-1} versus 46.1 ng h/mL mg^{-1}, respectively [27]. The findings of this clinical study also support our hypothesis that the expression levels and subsequent functions of the A421-*BCRP* allele are disrupted when compared to the wild-type C421 gene.

The C421A SNP occurs in the functionally important ATP-binding region between the Walker A and B motifs and results in substitution of the positively charged Lys residue for a neutral Gln residue. This may be associated with a greater susceptibility of the resulting BCRP protein (Q141K) to degradation [23]. In addition, Mizuarai et al. have also reported that ATPase activity levels in the membrane of C421A *BCRP*-transduced insect Sf9 cells were 1.3-fold lower than wild-type cells [28].

We previously examined the frequency of the C421A SNP in a normal Japanese population and found that 57/124 samples carried the A421 allele and that nine of these were homozygous for this polymorphism [23], indicating that some individuals possess the C421A polymorphic *BCRP* gene and express low amounts of the Q141K BCRP. C421A is therefore an important SNP because the allelic frequency of this variant differs greatly between diverse populations (Table 2). The C421A variant also appears to be very common in Asian populations, with reported allele frequencies of between 27 and 34% [23,25,29]. In contrast, this variant is rare in sub-Sahara African and in African American populations, with a frequency of <5% [25,29]. The frequency in Caucasian populations is approximately 10% [25,28–30].

2.2. C376T (Q126stop)-BCRP SNP

Another of the *BCRP* gene SNPs, C376T, substitutes a stop codon for Gln-126 (Q126stop), and was found in 3/124 of our general Japanese samples as a heterozygosity [23]. C376T was also detected previously in another report in 2/120 Japanese placental samples, again as a heterozygosity [25]. Although the frequency of the T376 allele is low, this variant would be expected to have a high impact as no active BCRP protein could be expressed from this gene. Stop SNPs have been reported in the *MRP2* gene, which is responsible for the hyperbilirubinemia of Dubin-Johnson syndrome, but are relatively rare and would usually be classified as naturally occurring base changes [31,32]. This C371T *BCRP* SNP is thus an important variant because of relatively high frequency (~1% in Japanese) as a stop SNP. Individuals with either the C376T and/or C421A SNPs may express low amounts of BCRP and this may result in hypersensitivity of normal cells to anticancer agents.

2.3. Additional BCRP SNPs

BCRP SNPs are summarized in Table 3 and include G34A, G151T, C376T, C421A, C458T, C496G, A616C, T623C, T742C, G1000T, T1291C, T1465C, A1768T and G1858A, which all cause amino acid changes. Among these, in addition to C376T and C421A, only a few have been examined in association with protein expression levels and the function of BCRP. Mizuarai et al. reported that the G34A variant exhibits reduced drug resistance in polarized porcine kidney epithelial LLC-PK1 cells along with increased intracellular drug accumulation [28]. However, in our

Table 2
Frequency of the C421A *BCRP* allele among different ethnic populations

Population	N	Genotype		Allele frequency[a] (%)	Ref.
		C/A	A/A		
Asian (Japanese)	124	48	9	27	[23]
	120	45	14	30	[25]
(Han Chinese)	95	43	11	34	[29]
Caucasian	150	25	4	11	[25]
	150	22	2	9	[28]
(American)	88	19	1	12	[29]
(European)	84	14	2	11	[29]
(Swedish)	60	10	1	10	[30]
African (sub-Saharan)	938	14	1	1	[29]
African-American	94	8	1	5	[29]
	150	5	1	2	[25]

Abbreviations: *N*, number of patients studied; C/A, heterozygous frequency; A/A, homozygous variant frequency; Ref., reference.
[a] Data are given as the relative frequency of variant alleles.

Table 3
SNPs within the *BCRP* gene

Variation	Region	Effect	Domain
A-1379G	5′-flanking (promoter)	–	
Δ-654–651	5′-flanking (promoter)	–	
G-286C	5′-flanking (promoter)	–	
T-476C	Exon 1 (5′-UTR)	–	
Δ-235A	Exon 1 (5′-UTR)	–	
A-113G	Exon 1 (5′-UTR)	–	
A-29G	Exon 1 (5′-UTR)	–	
G34A	Exon 2	V12M	N-terminal
T114C	Exon 2	No change	N-terminal
G151T	Exon 2	G51C	N-terminal
C369T	Exon 4	No change	NBD
C376T	Exon 4	Q126stop	NBD
C421A	Exon 5	Q141K	NBD
C458T	Exon 5	T153M	NBD
C474T	Exon 5	No change	NBD
C496G	Exon 5	Q166E	NBD
A564G	Exon 6	No change	NBD
A616C	Exon 6	I206L	NBD
T623C	Exon 6	F208S	NBD
T742C	Exon 7	S248P	Linker
G1000T	Exon 9	E334stop	Linker
G1098A	Exon 9	No change	Linker
T1291C	Exon 11	F431L	TMD
A1425G	Exon 12	No change	TMD
T1465C	Exon 12	F489L	TMD
A1768T	Exon 15	N590Y	TMD
G1858A	Exon 16	D620N	TMD
G2237T	Exon 16 (3′-UTR)	–	
G2393T	Exon 16 (3′-UTR)	–	

Abbreviations: UTR, untranslated region; NBD, nucleotide-binding domain; TMD, transmembrane domain.

previous study we showed a different outcome [23]. Further studies of *BCRP* SNPs and their roles in the expression and/or function of this protein would provide a fuller picture of its genetic regulation. In addition, these findings may be essential for our complete understanding of the pharmacological activities and pharmacokinetic profiles of BCRP-substrate anticancer agents.

3. BCRP inhibitors

BCRP inhibitors have two important clinical implications. First, they may overcome BCRP-mediated drug resistance in tumor cells. Second, they may modulate the pharmacokinetics and pharmacodynamics of BCRP-substrate agents in normal tissues and consequently increase the toxicity of specific anticancer agents. Various compounds have been found to reverse drug resistance through the inhibition of BCRP function [33–36]. In this chapter, we summarize the BCRP inhibitors identified by our laboratory and also by other groups (Table 4). The chemical structures of some of these inhibitors are shown in Fig. 2.

3.1. Estrogens and their metabolites

We were the first laboratory to identify estrogens as BCRP inhibitors and show that estrone and 17β-estradiol can restore drug sensitivity in *BCRP*-transduced human myelogenous leukemia K562 (K562/BCRP) cells [37]. These agents showed only a marginal growth-inhibitory effect on either K562/BCRP or parental K562 cells and increased the cellular accumulation of topotecan in K562/BCRP cells, but not in K562 cells. BCRP is highly expressed in the syncytiotrophoblasts of the placenta that synthesize and secrete these estrogens [12]. Therefore, we first

Table 4
BCRP inhibitors and substrates

Compound	Reference
1. Anticancer agents	
SN-38[a]	[8,11]
Topotecan[a]	[8,9]
MXR[a]	[5,6]
Flavopiridol[a]	[53]
Gefitinib	[46–49]
Imatinib mesylate	[50,51]
CI1033	[52]
Methotrexate polyglutamate[a]	[39,54]
2. Steroid hormones	
Estrone, Estradiol, Estriol	[37]
3. Sulfated steroids	
Estrone sulfate[a], Estradiol sulfate[a]	[38–40]
Dehydroepiandrosterone sulfate[a]	[40]
4. Synthesized estrogens	
Diethylstilbestrol	[41]
5. Anti-estrogens	
Tamoxifen, Toremifene, TAG-139	[41]
6. Flavonoids	
Genistein[a], Naringenin, Acacetin, Kaempferol, Naringenin-7-glucoside	[42]
7. Others	
Hoechst 33342[a]	[55]
Fumitremorgin C	[34]
GF120918	[33]
Novobiocin	[35,36]

[a] Compounds that are transported by BCRP (BCRP substrates).

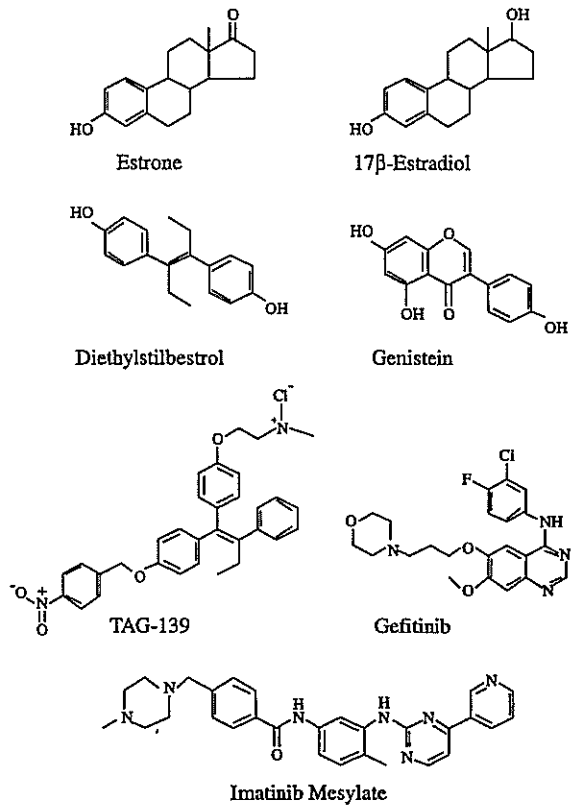

Fig. 2. Chemical structures of BCRP inhibitors.

hypothesized that these estrogens would be physiological substrates of BCRP and be transported by BCRP. To clarify this, we performed a transcellular transport assay using *BCRP*-transduced LLC-PK1 (LLC/BCRP) cells, in which exogenous BCRP is expressed in the apical membranes [38]. In this assay, excretion of ^{3}H-labeled estrone and 17β-estradiol was high and reabsorption was low in BCRP-expressing cells. However, thin layer chromatography analysis demonstrated an increased excretion of estrone sulfate and 17β-estradiol sulfate, but not estrone or 17β-estradiol, in LLC/BCRP cells. Fumitremorgin C completely inhibited the increased excretion of sulfated estrogens across the apical membrane. Moreover, the conversion of estrogens into their sulfate conjugates was similar between LLC/BCRP and LLC-PK1 cells, suggesting that the increased excretion of estrone sulfate was attributable to BCRP-mediated transport. The BCRP- and ATP-dependent uptake of ^{3}H-labeled estrone sulfate, but neither estrone nor 17β-estradiol, was also observed in membrane vesicles from K562/BCRP cells. Additionally, SN-38 and fumitremorgin C both suppressed the transport of estrone sulfate in membrane vesicles from K562/BCRP cells. Our findings thus suggest that BCRP does not transport either free estrone or 17β-estradiol but exports the corresponding sulfate conjugates of these estrogens [38–40].

3.2. Estrogen agonists and antagonists

Estrogen agonists, antagonists and their derivatives have been also evaluated in this laboratory for potential BCRP-reversing activities [41]. Among the commercially available compounds that we tested, diethylstilbestrol showed the strongest BCRP-reversing activity and was found to increase the cellular accumulation of topotecan and reverse resistance to SN-38 and MXR in K562/BCRP cells, but show either marginal or no effects in parental K562 cells [41]. In contrast, neither tamoxifen nor toremifene have much effect on increasing topotecan uptake in K562/BCRP cells. In our screening with various tamoxifen derivatives for BCRP inhibitors, TAG-139 was identified as a strong candidate [41]. Reversal of SN-38 and MXR resistance in K562/BCRP cells by TAG-139 was 5-fold stronger than estrone. Interestingly, the dose-dependent characteristics of drug resistance reversal by TAG-139 and estrone were very similar, suggesting that tamoxifen derivatives and estrone interact with the same binding site of BCRP [41].

3.3. Phytoestrogens/flavonoids

Some flavonoids that show weak estrogenic activities are called phytoestrogens. We have shown that these phytoestrogens/flavonoids, such as genistein, naringenin, acacetin and kaempferol, potentiate the cytotoxicity of SN-38 and MXR in K562/BCRP cells [42]. Some glycosylated flavonoids, such as naringenin-7-glucoside, are also effective inhibitors of BCRP and showed marginal effects on the drug sensitivity of K562 cells. Genistein and naringenin did not reverse either P-glycoprotein-mediated vincristine resistance or MRP1-mediated etoposide resistance but increased the cellular accumulation of topotecan in K562/BCRP cells. K562/BCRP cells also accumulated less ^{3}H-labeled genistein than K562 cells. In addition, the excretion of ^{3}H-labeled genistein was greater in LLC/BCRP cells than that in parental LLC-PK1 cells. Fumitremorgin C abolished the increased excretion of ^{3}H-labeled genistein in LLC/BCRP cells and thin layer chromatography analysis revealed that genistein is transported in its native form but not in its metabolized form. These results suggest that genistein is among the natural substrates of BCRP and competitively inhibits the BCRP-mediated drug efflux [42].

3.4. Kinase inhibitors

Gefitinib is an orally active, selective epidermal growth factor receptor-tyrosine kinase inhibitor that is currently used in the treatment of patients with advanced non-small cell lung cancer [43,44]. Recently, the possible interaction of gefitinib with BCRP has been evaluated by this laboratory and others [45–49]. Gefitinib was found to reverse SN-38 resistance in K562/BCRP cells and *BCRP*-transduced murine lymphocytic leukemia P388 cells, but not in the parental cells [46]. Furthermore, gefitinib increases the intracellular accumulation of topotecan in K562/BCRP cells and also suppresses the ATP-dependent transport of estrone sulfate in membrane vesicles from these cells. Additionally, the combination of gefitinib with irinotecan was shown to result in the markedly enhanced antitumor activity of irinotecan in multiple tumor models [46]. These results suggest that gefitinib inhibits the transporter function of BCRP and reverses BCRP-mediated drug resistance both in vitro and in vivo. Stewart et al. have also indicated that oral dosing of gefitinib significantly increases the oral bioavailability of irinotecan [47]. Furthermore, *BCRP*-transduced human epidermoid carcinoma A431 cells and *BCRP*-transduced human non-small cell lung cancer PC-9 cells acquired cellular resistance to gefitinib [46]. Elkind et al. have also reported that the expression of functional BCRP protects the A431 cells from the cytotoxic effects of gefitinib [49]. These findings strongly suggest that BCRP is one of the important determinants of gefitinib sensitivity.

Imatinib mesylate, an inhibitor of BCR-ABL tyrosine kinase, has been reported to reverse BCRP-mediated drug resistance [50,51]. Houghton et al. reported that imatinib significantly increase the accumulation of topotecan in the human osteosarcoma Saos2 cells expressing functional BCRP [50]. However, the overexpression of BCRP did not confer resistance to imatinib and the accumulation of ^{14}C-labeled imatinib was similar in Saos2 cells expressing either functional or non-functional BCRP. These results suggest that imatinib binds to BCRP and inhibits its function but that it is not a BCRP substrate [50]. In contrast, Burger et al. have reported that imatinib is in fact a substrate for BCRP [51] by demonstrating that the accumulation of imatinib is low in a BCRP-overexpressing subline, MCF7/MR. They also showed that Ko-143, a specific inhibitor of BCRP, increased the accumulation of imatinib in MCF7/MR cells [51]. Another potent tyrosine kinase inhibitor, CI1033, has also been shown to enhance the uptake and cytotoxicity of SN-38 and topotecan in *BCRP*-transfected cells [52]. CI1033 accumulation was diminished in BCRP-expressing cells, suggesting that it may be transported by BCRP [52].

4. Conclusions

There is great variation in the response of patients to cancer chemotherapy, in terms of both treatment efficacy and host toxicity. BCRP confers resistance to agents such as irinotecan, topotecan and MXR that are used in practical chemotherapy for a wide variety of cancers. BCRP expression in the normal tissues of cancer patients may also serve to reduce the adverse effects of these agents, such as hematological toxicity and digestive disorders. C376T and C421A SNPs within the *BCRP* gene were shown to be associated with low protein expression and increased sensitivity to BCRP-substrate anticancer agents. Hence, individuals with these SNPs may demonstrate a different bioavailability of irinotecan due to its decreased excretion, and consequent increased intracellular and plasma levels. Consequently, C376T and C421A SNPs might also be implicated in the side effects of irinotecan. Screening for such functional SNPs in cancer patients prior to chemotherapy may thus be useful for the prevention of serious side effects of anticancer agents.

BCRP was also shown to be associated with the excretion of sulfated estrogens and other sulfated compounds. Because BCRP is responsible for the excretion of several anticancer agents, the inhibition of its function may lead to the increased plasma levels of orally administered agents. It is possible that BCRP inhibitors alter the bioavailability of BCRP substrates. These effects might be manipulated to the advantage of clinicians by improving several aspects of chemotherapy such as reduction of the variability in exposure to orally administered topotecan and potentiation of the cytotoxic activity of irinotecan. In addition, unintentional side effects may be caused by modulation of the bioavailability of chemotherapeutics by BCRP inhibitors.

These findings indicate that functional SNPs and inhibitors of BCRP result in similar effects on the pharmacokinetics of BCRP-substrate agents. In clinical studies of BCRP-substrate anticancer agents (e.g. diflomotecan) and BCRP inhibitors (e.g. flavonoids), patients should therefore be evaluated according to their *BCRP* genotypes. Also, treatment regimens should be modified according to the SNP status of the patients to minimize unintentional side effects.

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A T3587G germ-line mutation of the *MDR1* gene encodes a nonfunctional P-glycoprotein

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Abstract

The human *multidrug resistance gene 1* (*MDR1*) encodes a plasma membrane P-glycoprotein (P-gp) that functions as an efflux pump for various structurally unrelated anticancer agents. We have identified two nonsynonymous germ-line mutations of the *MDR1* gene, C3583T *MDR1* and T3587G *MDR1*, in peripheral blood cell samples from Japanese cancer patients. Two patients carried the C3583T *MDR1* allele that encodes H1195Y P-gp, whereas a further two carried T3587G *MDR1* that encodes I1196S P-gp. Murine NIH3T3 cells were transfected with pCAL-MDR-IRES-ZEO constructs carrying either wild-type (WT), C3583T, or T3587G *MDR1* cDNA and selected with zeocin. The resulting zeocin-resistant mixed populations of transfected cells were designated as 3T3/WT, 3T3/H1195Y, and 3T3/I1196S, respectively. The cell surface expression of I1196S P-gp in 3T3/I1196S cells could not be detected by fluorescence-activated cell sorting, although low expression of I1196S P-gp was found by Western blotting. H1195Y P-gp expression levels in 3T3/H1195Y cells were slightly lower than the corresponding WT P-gp levels in 3T3/WT cells. By immunoblotting analysis, both WT P-gp and H1195Y P-gp were detectable as a 145-kDa protein, whereas I1196S P-gp was visualized as a 140-kDa protein. 3T3/I1196S cells did not show any drug resistance unlike 3T3/H1195Y cells. Moreover, a vanadate-trap assay showed that the I1196S P-gp species lacks ATP-binding activity. Taken together, we conclude from these data that T3587G *MDR1* expresses a nonfunctional P-gp and this is therefore the first description of such a germ-line mutation. We contend that the T3587G *MDR1* mutation may affect the pharmacokinetics of *MDR1*-related anticancer agents in patients carrying this allele. [Mol Cancer Ther 2006; 5(4):877–84]

Introduction

P-glycoprotein (P-gp), also known as ABCB1, is a 170- to 180-kDa transmembrane glycoprotein that functions as an efflux pump for various structurally unrelated anticancer drugs, such as the *Vinca* alkaloids, anthracyclines, and taxanes (1–4). P-gp is expressed in a variety of normal human tissues and cells, such as the small and large intestine, adrenal gland, kidney, liver, placenta, and the capillary endothelial cells of the brain and testes (5, 6). P-gp also mediates the excretion of its substrates from the intestine and therefore inhibits their intestinal absorption (7). In addition, P-gp mediates the biliary excretion and renal tubular secretion of its substrates (8, 9). Moreover, the coadministration of P-gp substrate anticancer agents and P-gp inhibitors, such as verapamil, increases both the plasma concentration and the area under the concentration-time curve of these substrate agents (10, 11). Mice lacking *multidrug resistance gene 1* (*MDR1*)-type P-gps (*mdr1a/mdr1b*−/− mice) display large changes in the pharmacokinetics of digoxin and other drugs (12, 13). Hence, the low expression of P-gp in normal cells/tissues alters the pharmacokinetics of its substrate anticancer agents.

Recently, single nucleotide polymorphisms (SNP) have been extensively investigated, as several of them have been shown to alter mRNA and/or protein expression levels. As P-gp determines the pharmacokinetics of several anticancer drugs, *MDR1* SNPs that affect P-gp expression and function have been of particular interest. A synonymous SNP in the *MDR1* gene, C3435T, which does not cause an amino acid substitution, was reported to be associated with low intestinal P-gp expression, low P-gp activity, and high digoxin absorption in individuals carrying this allele (14–16). Furthermore, our haplotype analysis has now further revealed that a *MDR1**2 haplotype with a linkage of C1236T *MDR1* (synonymous), G2677T *MDR1* (A893S P-gp), and C3435T *MDR1* is associated with a reduced renal excretion of irinotecan in Japanese cancer patients possibly due to a reduced P-gp function (17). However, the molecular mechanisms underlying the low renal excretion of irinotecan in this instance are still unclear.

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We have also reported previously the identification of a T3587G *MDR1* germ-line mutation in a Japanese patient, which confers a serine substitution for Ile^{1196} in P-gp (I1196S P-gp; ref. 17). We subsequently attempted to evaluate the possible functional alterations that may be caused by this substitution by analyzing the renal clearance of irinotecan in this individual who was heterozygous for the T3587G *MDR1*. There was an indication that the T3587G *MDR1* may be associated with high renal clearance of SN-38, but this observation was too preliminary to draw any firm conclusions as only one heterozygous patient was analyzed. This finding, however, prompted us to functionally characterize the Ser^{1196} substitution using *MDR1* cDNA-transfected cells and to further analyze additional Japanese subjects for the presence of other *MDR1* SNPs. We were subsequently able to identify a novel germ-line mutation in the *MDR1* gene, C3583T *MDR1*, which causes a substitution of tyrosine for His^{1195} in the P-gp (H1195Y P-gp). In our current study, we have established T3587G *MDR1* and C3583T *MDR1* cDNA transfectants and examined both expression levels and functional properties of I1196S P-gp and H1195Y P-gp. Our findings show that the T3587G *MDR1* cDNA encodes a nonfunctional P-gp and that the C3583T *MDR1* cDNA encodes a functional P-gp.

Materials and Methods

Sequence Analysis of the *MDR1* Gene

Peripheral blood nucleated cells were obtained from both healthy volunteers and cancer patients of Japanese nationality, after obtaining written informed consent, to undertake genetic analysis from each of these individuals. Exon 27 of the *MDR1* gene, which incorporates nucleotides 3,490 to 3,636 from the first ATG codon of the mRNA, was amplified by PCR from genomic DNA samples using the forward and reverse primers: 5′-CTTTACTTTCAGTTCTACTTTCA-3′ and 5′-GAGAATACAGCATTTTTAAGGA-3′, respectively. The resulting PCR products were directly sequenced using the primer 5′-CAGTTCTACTTTCATAACAACA-3′.

MDR1 Vectors

For the transfection of *MDR1* cDNA, we generated pCAL-MDR-IRES-ZEO bicistronic constructs, in which either wild-type (WT) or mutant *MDR1* cDNA insert was cloned upstream of the internal ribosome entry site (IRES) of the encephalomyocarditis virus. In the resulting transfectants, a single bicistronic mRNA species is transcribed under the control of the CAG promoter consisting of a cytomegalovirus immediate-early enhancer, a chicken β-actin transcription start site, and a rabbit β-globin intron (18). The upstream *MDR1* cDNA is translated in a cap-dependent manner, and the downstream *zeocin resistance gene* (*ZEO*) is translated under the control of the IRES.

For the retrovirus-mediated transfer of *MDR1* cDNAs, we constructed pHa-MDR-IRES-DHFR bicistronic retroviral vector plasmids, in which either WT or mutant *MDR1* cDNA insert was cloned upstream of the IRES.

Establishment of Mutant *MDR1* Transfectants

Murine fibroblast NIH3T3 cells were cultured in DMEM supplemented with 7% fetal bovine serum at 37°C in a humidified 5% CO_2 environment. For the establishment of WT or mutant *MDR1* transfectants, NIH3T3 cells were transfected with pCAL-MDR-IRES-ZEO containing either WT *MDR1*, C3583T *MDR1*, or T3587G *MDR1* cDNA. The cells were selected with 50 μg/mL zeocin and the resulting zeocin-resistant colonies were mixed. The zeocin-resistant mixed populations of the transfected cells were designated as 3T3/WT, 3T3/H1195Y, and 3T3/I1196S, respectively. Because 3T3/I1196S cells expressed only a small amount of P-gp, we isolated 30 T3587G *MDR1* cDNA transfectant clones by limiting dilution and tested for P-gp expression. A clone with the highest I1196S P-gp expression, designated as 3T3/I1196S clone 23, was used in the evaluation of ATP-binding activity of mutant P-gps.

The anticancer agent resistance levels in parental NIH3T3 cells and in the various *MDR1* transfectants were evaluated by cell growth inhibition assays after incubation of the cells for 5 days at 37°C in the absence or presence of various concentrations of vincristine or doxorubicin. Cell numbers were determined with a cell counter (Sysmex, Kobe, Japan).

Retrovirus-Mediated Mutant *MDR1* Gene Transfer

For retrovirus-mediated transfer of *MDR1* cDNAs, PA317 amphotropic retrovirus packaging cells were transfected with the pHa-MDR-IRES-DHFR plasmid containing either WT *MDR1*, C3583T *MDR1*, or T3587G *MDR1* cDNA insert using a calcium phosphate coprecipitation method. The transfectants were then selected by exposure to 120 ng/mL methotrexate and Ha-MDR-IRES-DHFR retrovirus-containing supernatants were harvested. NIH3T3 cells were then transduced with each of the Ha-MDR-IRES-DHFR retrovirus preparations following centrifugation at 2,800 rpm for 2 hours in the presence of polybrene (6 μg/mL) and cultured further in medium without retrovirus.

Fluorescence-Activated Cell Sorting Analysis of P-gp Expression

The expression levels of human P-gp on the cell surfaces of various *MDR1* transfectants were examined by fluorescence-activated cell sorting (FACS) analysis using a human-specific monoclonal antibody MRK16, which reacts with a cell surface epitope of P-gp. The cells were incubated with or without a biotinylated $F(ab')_2$ fragment of MRK16 (100 μg/mL) followed by washing and incubation with R-phycoerythrin-conjugated streptavidin (400 μg/mL; BD Biosciences, Franklin Lakes, NJ; ref. 19). Fluorescence staining levels were measured using FACSCalibur (BD Biosciences).

Western Blotting

Cell lysates of the *MDR1* transfectants were separated by SDS-PAGE and then electrotransferred onto a nitrocellulose membrane. The membrane was incubated with 1 μg/mL anti-P-gp monoclonal antibody C219 (Cencor, Malvern, PA; ref. 20) followed by washing and treatment with peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham, Buckinghamshire, United Kingdom). The membrane-bound antibody was visualized with Enhanced Chemiluminescence Plus Detection kit (Amersham).

Genomic PCR and Reverse Transcription-PCR

Genomic DNA was extracted from each of the transfectants with a DNeasy Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. *MDR1* cDNA (3,561 bp) was then amplified by PCR using the forward and reverse primers, 5′-CACGTGGTTGGAAGCTAACC-3′ and 5′-GAAGGCCAGAGCATAAGATGC-3′, respectively. As an internal control, the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene (551-bp fragment) was amplified with the forward and reverse primers, 5′-ATCACCATCTTCCAGGAGCGA-3′ and 5′-GCTTCACCACCTTCTTGATGT-3′, respectively. The PCR conditions for *MDR1* amplification were as follows: 95°C for 5 minutes followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 3 minutes and a final extension at 72°C for 7 minutes. The *GAPDH* control amplification conditions were as follows: 95°C for 5 minutes followed by 20 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and a final extension at 72°C for 7 minutes.

The isolation of total RNA and subsequent reverse transcription-PCR was done using a RNeasy kit (Qiagen) and a RNA LA PCR kit (Takara, Ohtsu, Japan), each according to the manufacturer's instructions. First-strand *MDR1* cDNA was synthesized from 0.3 μg total RNA and a 702-bp *MDR1* cDNA fragment was amplified by PCR with the forward and reverse primers, 5′-GATATCAATGATACAGGGTT-3′ and 5′-TGTCCAATAGAATATTCCCC-3′, respectively. The PCR conditions were as follows: 95°C for 5 minutes followed by 18 to 24 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and a final extension at 72°C for 7 minutes. As an internal control, the amplification of *GAPDH* cDNA (551-bp fragment) was carried out as described above.

Vanadate-Induced Nucleotide Trapping in P-gp with 8-Azido-[α-32P]ATP

The ATP-binding activity of P-gp was examined by vanadate-induced nucleotide trapping analysis as described previously (21). Briefly, membrane fractions (5-20 μg) were prepared from *MDR1* transfectants and incubated with 10 μL buffer containing 10 μmol/L 8-azido-[α-^{32}P]ATP, 200 μmol/L orthovanadate, 3 mmol/L $MgCl_2$, 2 mmol/L ouabain, 0.1 mmol/L EGTA, and 40 mmol/L Tris-HCl (pH 7.5) in the absence or presence of 50 μmol/L verapamil for 10 minutes at 37°C. The reactions were stopped by the addition of 500 μL ice-cold TE buffer [40 mmol/L Tris-HCl (pH 7.5), 0.1 mmol/L EGTA]. The supernatants containing unbound ATP were removed from the membrane pellet after centrifugation (15,000 × *g*, 5 minutes, 4°C), and this procedure was repeated once more. The pellets were then resuspended in 8 μL TE buffer and irradiated for 5 minutes (at 254 nm, 8.2 mW/cm^2) on ice. The samples were then electrophoresed on a 7% SDS-polyacrylamide gel, electrotransferred to polyvinylidene difluoride membranes, and analyzed by autoradiography using a radioimaging analyzer (BAS2500, Fuji Photo Film Co., Tokyo, Japan). The polyvinylidene difluoride membranes were further analyzed by Western blotting with the anti-P-gp antibody C219. The P-gp expression levels were quantified using Scion Image software (Scion, Frederick, MD). The quantities of trapped 8-azido-[α-^{32}P]ATP in the WT and mutant P-gps, expressed as RI intensities in BAS2500, were normalized to the P-gp expression levels, and the relative photoaffinity labeling of each was then plotted. Two independent experiments were done, and the average of these analyses is shown.

Results

Frequency of the C3583T *MDR1* and T3587G *MDR1*

We identified previously a germ-line mutation of the *MDR1* gene, T3587G (17), in a Japanese cancer patient who was heterozygous for this allele and have now identified another germ-line mutation of the *MDR1* gene, C3583T, in a normal Japanese population. The C3583T *MDR1* and T3587G *MDR1* alleles encode H1195Y P-gp and I1196S P-gp, respectively, and both of the His^{1195} and Ile^{1196} residues are located in the Walker B region of the second ATP-binding site of P-gp (Fig. 1A). To examine the frequencies of occurrence for these mutations, we analyzed the genomic sequences of exon 27 of the *MDR1* gene, which incorporates the nucleotide region 3,490 to 3,636 of the mRNA. Of the 605 samples that we examined, two individuals were found to be heterozygous for the C3583T allele and an additional two subjects were found to be T3587G heterozygotes. Because of their low frequencies (<1%), C3583T *MDR1* and T3587G *MDR1* germ-line mutations would therefore be called naturally occurring base changes and not SNPs. We have not thus far identified any individuals who are homozygous for either of these mutations, nor have we observed individuals who are heterozygous for a combination of the C3583T and T3587G alleles.

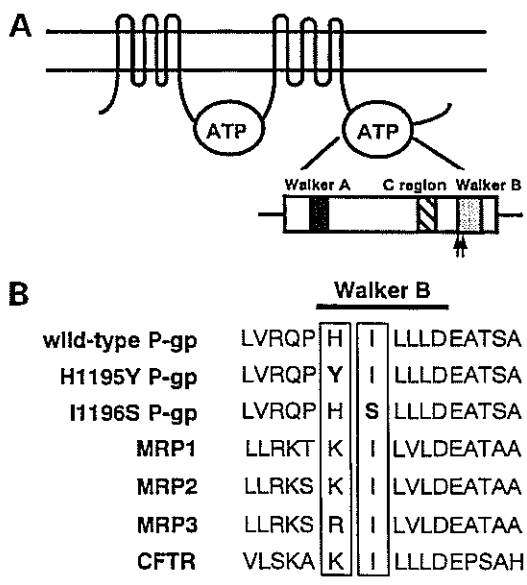

Figure 1. Map of specific mutations in P-gp. A, structure of P-gp. *Arrows*, location of the H1195Y and I1196S substitutions. B, alignment of various ATP-binding cassette transporter sequences close to the Walker B region of the second ATP-binding site. The His^{1195} and Ile^{1196} residues affected by the C3583T and T3587G mutations, together with the corresponding amino acids of other transporters, are boxed.

P-gp Expression Levels in the *MDR1* Transfectants

To investigate the molecular functions of the H1195Y mutant P-gp and I1196S mutant P-gp, we generated 3T3/WT, 3T3/H1195Y, and 3T3/I1196S cells, which were stably transfected with WT *MDR1*, C3583T *MDR1*, and T3587G *MDR1* cDNA, respectively. The P-gp expression levels on the cell surfaces of these transfectants were subsequently examined by FACS analysis using the MRK16 antibody, which recognizes a cell surface epitope of human P-gp. Both 3T3/WT and 3T3/H1195Y cells express P-gp on their cell surface, although these expression levels in 3T3/H1195Y cells (mean channel, 510) were slightly lower than in 3T3/WT cells (mean channel, 980; Fig. 2A). Surprisingly, the 3T3/I1196S cells did not express P-gp on their cell surface (Fig. 2A). We then examined the P-gp expression levels in the NIH3T3 cells and *MDR1* transfectants by Western blotting. In parental NIH3T3 cells, endogenous P-gp is expressed at very low levels (Fig. 2B). Moreover, both WT P-gp and H1195Y P-gp were detectable as a 145-kDa protein, whereas I1196S P-gp was observed as a 140-kDa protein (Fig. 2B). In addition, the expression levels of I1196S P-gp in 3T3/I1196S cells were at significantly lower levels than the other P-gp species.

As the expression levels of I1196S P-gp were very low in 3T3/I1196S cells, we examined the copy number of exogenous *MDR1* cDNA and the expression level of *MDR1* mRNA in these transfectants. A 3,561-bp human *MDR1* cDNA fragment, which is close to the full-length open reading frame, was amplified from genomic DNA isolates of the various *MDR1* cDNA transfectants. Each of the transfectants was found to have similar copy numbers of *MDR1* cDNA (Fig. 2C). We next did semiquantitative reverse transcription-PCR of *MDR1* mRNA in the transfectants. As shown in Fig. 2D, each of the *MDR1* transfectants also express similar levels of *MDR1* transcripts.

We then did retrovirus-mediated transfer of *MDR1* cDNAs to confirm the differences that we had observed in the expression levels of mutant P-gps. Amphotropic retrovirus was prepared from PA317 cells transfected with the pHa-MDR-IRES-DHFR vectors carrying either WT or mutant *MDR1* cDNA insert. NIH3T3 cells were then transduced with these *MDR1* retroviral preparations and the cells were cultured for 2 days and analyzed by FACS. As shown in Fig. 3, P-gp expression was observed in NIH3T3 cells transduced with both WT and H1195Y *MDR1* retroviruses but not in cells transduced with I1196S *MDR1* retrovirus. Transduction efficiencies were 70% and 60% for WT and H1195Y *MDR1* retroviruses, respectively. P-gp expression in cells transduced with H1195Y *MDR1* retrovirus was again found to be at a slightly lower levels than in cells transduced with WT *MDR1* retrovirus (Fig. 3B and C).

Drug Resistance in *MDR1* Transfectants

We next examined the drug resistance levels in our *MDR1* transfectants. 3T3/WT cells showed a 22-fold higher resistance to vincristine and 7-fold higher resistance to doxorubicin than parental NIH3T3 cells (Fig. 4). 3T3/H1195Y cells also showed higher levels of resistance to these drugs compared with the parental cells, but these were at slightly lower levels than 3T3/WT cells (Fig. 4). These findings correlated with the expression levels of P-gp

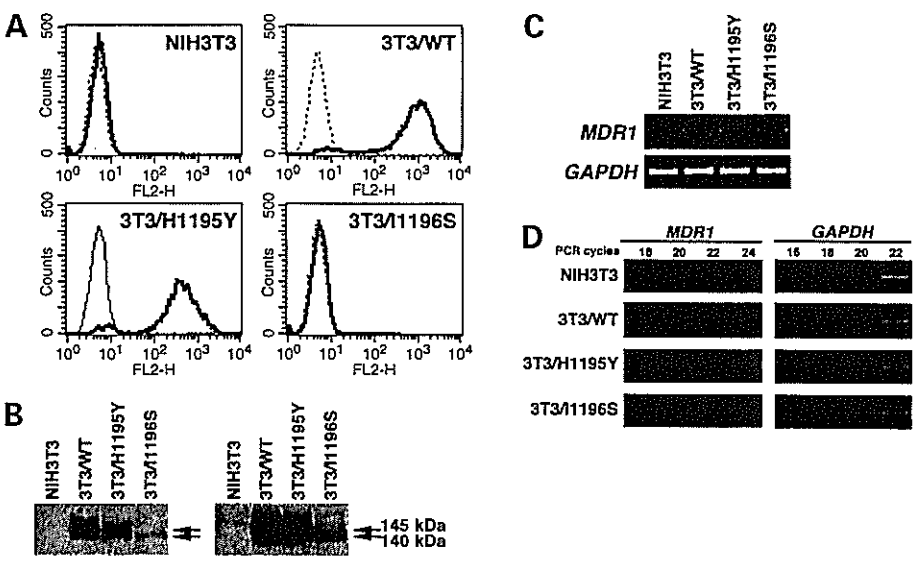

Figure 2. P-gp expression, *MDR1* cDNA integration, and *MDR1* mRNA expression in NIH3T3 transfectants. **A**, detection of cell surface expression of P-gp by FACS analysis. Parental NIH3T3 cells and the corresponding *MDR1* transfectants were harvested and then incubated with or without a biotinylated $F(ab')_2$ fragment of MRK16 followed by treatment with R-phycoerythrin-conjugated streptavidin. After washing, the fluorescence intensities were calculated using FACSCalibur. *Bold* and *dotted lines*, cells incubated with or without MRK16, respectively. **B**, Western blot analysis of P-gp in the *MDR1* transfectants. Protein extracts (20 μg) were subjected to Western immunoblotting analysis using the anti-P-gp monoclonal antibody C219 (1 μg/mL). *Left* and *right*, short (5 min) and long (15 min) exposures, respectively. **C**, genomic PCR analysis of exogenous *MDR1* cDNA in the *MDR1* transfectants. *MDR1* cDNA (3,561 bp) and *GAPDH* (551 bp) were amplified from genomic DNA preparations by PCR. *GAPDH* amplification was used as an internal control. **D**, reverse transcription-PCR analysis of *MDR1* transcripts in the NIH3T3 transfectants. *MDR1* (702 bp) and *GAPDH* (551 bp) transcripts were amplified by reverse transcription-PCR from 0.3 μg total RNA over the indicated number of cycles. *GAPDH* was again used as an internal control.

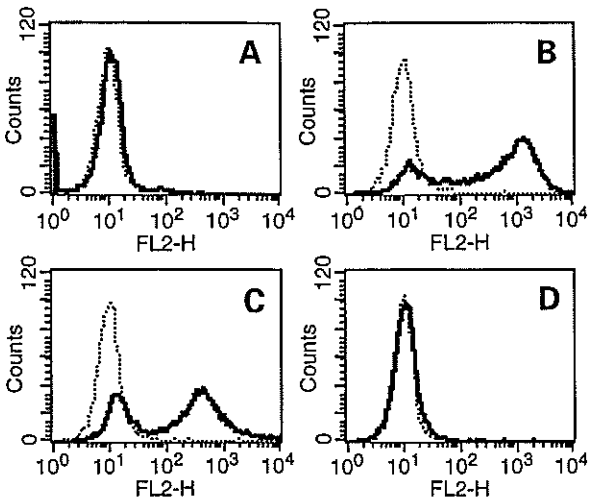

Figure 3. Cell surface expression of P-gp in retrovirally transduced cells. Cells were transduced with WT or mutant *MDR1* retroviruses, harvested, and incubated with or without a biotinylated $F(ab')_2$ fragment of MRK16 followed by treatment with R-phycoerythrin-conjugated streptavidin. After washing, the fluorescence intensities were calculated using FACSCalibur. *Bold* and *dotted lines*, cells incubated with or without MRK16, respectively. **A**, parental NIH3T3 cells. **B**, NIH3T3 cells transduced with WT *MDR1* retrovirus. **C**, NIH3T3 cells transduced with H1195Y *MDR1* retrovirus. **D**, NIH3T3 cells transduced with I1196S *MDR1* retrovirus.

in these cells and it is also significant that 3T3/I1196S cells showed no increased resistance to these chemotherapeutic agents when compared with the parental cells (Fig. 4), although I1196S P-gp was found to be expressed at low levels in 3T3/I1196S cells.

Loss of ATP-Binding Ability in I1196S P-gp

Because H1195Y P-gp and I1196S P-gp have amino acid substitutions in the second ATP-binding site of P-gp, we examined the ATP-binding activities of these variants. 3T3/I1196S clones were isolated and screened for higher P-gp expression, and clone 23 was found to contain the highest expression levels of I1196S P-gp. 3T3/I1196S clone 23 was thus used in these analyses (Fig. 5A). Because 3T3/I1196S clone 23 expressed ~25% of the WT P-gp levels, and 3T3/H1195Y cells expressed ~50% of the WT levels, we normalized these amounts in the relevant experiments (Fig. 5B and C). It was significant that the I1196S P-gp species showed no ATP-binding activity in either the absence or presence of 50 μmol/L verapamil (Fig. 5B and D). However, verapamil stimulated the nucleotide trapping of both WT P-gp and H1195Y P-gp, both of which showed similar levels of ATP-binding activity (Fig. 5C and D). These results suggest that I1196S P-gp lacks ATP-binding activity and therefore cannot function as an efflux pump.

Discussion

P-gp encoded by the *MDR1* gene is an important factor in the determination of the pharmacokinetics of its substrates, which include several anticancer drugs, as the coadministration of these agents and known P-gp inhibitors increases both the plasma concentration and the area under the concentration-time curve of these substrates (10, 11). C3435T *MDR1* was reported previously as a synonymous SNP that is associated with low intestinal P-gp expression, low P-gp activity, and high digoxin absorption (14). The association of a low level of P-gp activity was also observed with the *MDR1**2 haplotype containing C1236T *MDR1*, G2677T *MDR1*, and C3435T *MDR1* SNPs (17), but the details of the underlying mechanisms are still unknown. A *MDR1* SNP that causes a deficiency in P-gp function has not been reported previously.

In our previous and present studies, we have identified two nonsynonymous germ-line mutations, C3583T *MDR1* and T3587G *MDR1*. The C3583T *MDR1* substitutes a tyrosine for the His^{1195} residue of P-gp, whereas the T3587G *MDR1* results in a serine substitution for Ile^{1196}. Importantly, both of these residues are located in the Walker B region of the second ATP-binding site of P-gp (Fig. 1A). The Ile^{1196} residue in the P-gp is highly conserved among the members of ATP-binding cassette transporter superfamilies, but His^{1195} is not conserved among these proteins (refs. 22, 23; Fig. 1B). To examine the possible functional implications of these mutations, we established mutant *MDR1* cDNA transfectants and analyzed the biological consequences of the amino acid changes caused by these mutations.

Genetic variations have been known to affect mRNA expression and stability and also disrupt protein expression levels, turnover, and function. Because our study was designed to examine the possible effects of mutations in the coding region of the *MDR1* gene on protein expression levels, turnover, or function, we needed to establish WT or mutant *MDR1* transfectants that expressed similar amounts of *MDR1* mRNA. When standard two-promoter expression plasmid vectors are used for cDNA transfer, a high degree of variation in the expression of the transgene among transfectant clones may occur due to their different integration sites in the host genome and the possible effects of neighboring enhancers and/or silencers. We therefore

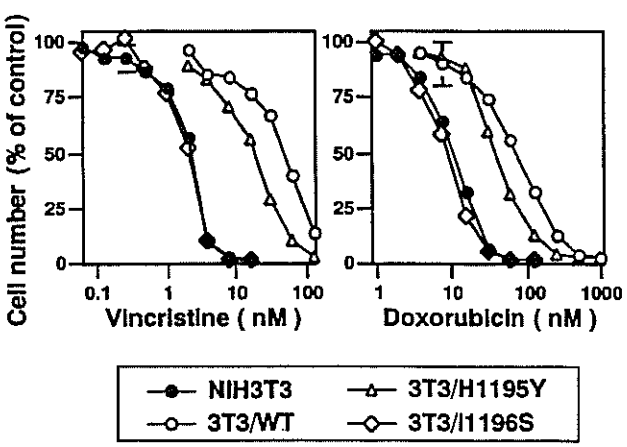

Figure 4. Drug resistance of the mutant *MDR1* transfectants. NIH3T3 (●), 3T3/WT (○), 3T3/H1195Y (△), and 3T3/I1196S (◇) cells were cultured for 5 d with various concentrations of vincristine or doxorubicin. Cell numbers were determined using a cell counter. *Points*, mean of triplicate experiments; *bars*, SD.

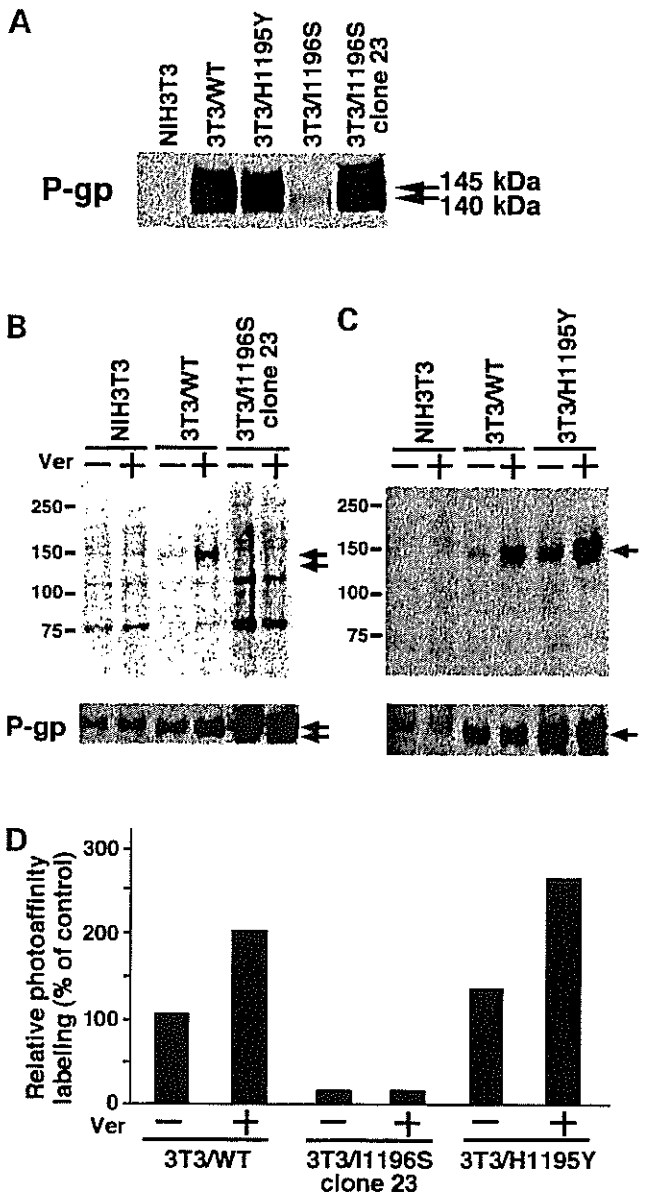

Figure 5. ATP-binding activities in the mutant *MDR1* transfectants. **A**, P-gp expression levels in the transfectants. Protein (20 μg) was loaded in each lane and subjected to Western blotting analysis using the anti-P-gp monoclonal antibody C219. **B**, ATP-binding activity of I1196S P-gp. Plasma membrane protein extracts of NIH3T3 (20 μg), 3T3/WT (5 μg), and 3T3/I1196S clone 23 (20 μg) cells were incubated with 10 μmol/L 8-azido-[α-^{32}P]ATP and 200 μmol/L vanadate in the absence (−) or presence (+) of 50 μmol/L verapamil for 10 min at 37°C. The proteins were then photoaffinity labeled by UV irradiation after the removal of unbound ligands and analyzed as described in Materials and Methods. *Top*, autoradiography using a radioimaging analyzer; *bottom*, Western blotting analysis of the same blot with the anti-P-gp antibody C219. *Arrows*, P-gps. **C**, ATP-binding activity of H1195Y P-gp. Plasma membrane protein extracts of NIH3T3 (20 μg), 3T3/WT (10 μg), and 3T3/H1195Y (20 μg) cells were analyzed as in **B**. *Top*, autoradiography using a radioimaging analyzer; *bottom*, Western blotting analysis of the same blot with the anti-P-gp antibody C219. *Arrows*, P-gps. **D**, relative ATP-binding activity of mutant P-gps. The trapped 8-azido-[α-^{32}P]ATP in the WT and mutant P-gps were quantified using BAS2500 imaging and normalized to the protein expression levels, and the relative photoaffinity labeling of each was then plotted. Two independent experiments were done, and the average of these analyses is shown.

used our previously reported flexible bicistronic vector system that uses an IRES to coexpress dominant drug-selectable markers, such as *dihydrofolate reductase* (*DHFR*) or *ZEO*, with the mutant *MDR1* gene (24, 25).

We reported previously the construction of bicistronic vectors in which the *MDR1* gene is coexpressed with herpes simplex virus-thymidine kinase (26–28), α-galactosidase A (28, 29), O^6-methylguanine DNA methyltransferase (30, 31), p47 of NADPH oxidase (32), and gp91 of NADPH oxidase (19, 33). We have further shown in this system that the drug treatments facilitated the enrichment or elimination of cells expressing the other nonselectable genes.

We next used this system to express mutant ATP-binding cassette transporters. We generated bicistronic pHa-BCRP-IRES-DHFR constructs to analyze the effects of *BCRP* coding SNPs on protein expression (34, 35). In the previous study, cells were transfected with pHa-BCRP-IRES-DHFR vectors containing either WT, G34A, C421A, or 944-949-deleted *BCRP* cDNA and then selected with methotrexate. In the resulting transfectants, a single mRNA is transcribed under control of a retrovirus long terminal repeat promoter, and two gene products are translated independently from a bicistronic mRNA. The upstream *BCRP* cDNA is translated cap-dependently, and the downstream *DHFR* cDNA is translated under a control of the IRES. Because only one mRNA species is transcribed, the cells expressing *DHFR* theoretically always coexpress the *BCRP* cDNA. We therefore combined all of the methotrexate-resistant colonies (>100) and used these mixed populations of methotrexate-resistant cells for further analysis. In this case, the expression of *BCRP* mRNA will reflect the mean levels for the transfectant clones and the mRNA levels within the mixed population will not be greatly affected by the expression levels of an individual clone. Indeed, we subsequently showed that four BCRP transfectants (mixed populations established after methotrexate selection) expressed similar levels of exogenous *BCRP* mRNA (34, 35). Additional FACS analysis then showed that almost all of the methotrexate-selected cells expressed BCRP on their cell surfaces. We then showed that BCRP expression from C421A *BCRP* cDNA is markedly lower than the WT.

In our present study, we constructed similar pCAL-MDR-IRES-ZEO bicistronic vectors that carry either WT or mutant *MDR1* cDNA insert. The transfectants were then selected with zeocin, and each of the resistant colonies (>100) were combined and used for further studies. As shown in Fig. 2A, most of the 3T3/WT and 3T3/H1195Y cells expressed cell surface P-gp. We also showed that the transfectants possess similar plasmid copy numbers (Fig. 2C) and similar levels of *MDR1* mRNA (Fig. 2D). To confirm our finding of a lower expression level of H1195Y P-gp, we did retrovirus-mediated gene transfer. Cells transduced with H1195Y *MDR1* retrovirus showed slightly lower P-gp expression levels than those transduced with WT *MDR1* retrovirus (Fig. 3). We therefore speculate that the difference in P-gp expression between 3T3/WT and 3T3/H1195Y cells is genuine and can be attributed to post-transcriptional events, such as protein maturation and/or stability.

Dubin-Johnson syndrome is an inherited disorder characterized by chronic conjugated hyperbilirubinemia due to the absence or dysfunction of the multidrug resistance-associated protein 2 (MRP2). Some Dubin-Johnson syndrome patients express mutant MRP2 proteins with amino acid substitutions or deletions (36–38). R768W MRP2, which has an amino acid substitution in signature C of the first ATP-binding site of the protein, is associated with relatively high serum bilirubin concentrations in affected patients (38) and this mutant protein is not properly glycosylated (36). Q1382R MRP2, a mutation that is located between the Walker A and the signature C regions of the second ATP-binding site, results in a lack of ATP hydrolysis activity (36). Moreover, the MRP2 mutant, which has a deletion in both its Arg^{1392} and Met^{1393} residues located between the Walker A and the signature C regions of the second ATP-binding site, is also a nonfunctional protein that shows impaired maturation and is sequestered in the endoplasmic reticulum (37). Hence, some MRP2 mutants that have mutations/deletions in the ATP-binding sites and lack ATP-hydrolyzing activity are underglycosylated, have not matured, and are unstable. We show in our current experiments that the I1196S P-gp also lacks ATP-binding activity and that its expression levels in 3T3/I1196S cells are markedly lower than in 3T3/WT cells. In addition, whereas the WT P-gp migrates as a 145-kDa protein, the I1196S P-gp migrates as a 140-kDa protein (Fig. 2B). The SDS-PAGE profile of I1196S P-gp is also very similar to the glycosylation-deficient P-gp that has the three amino acid substitutions, N91Q, N94Q, and N99Q (39). Taken together, these data suggest the possibility that I1196S P-gp does not undergo proper maturation, which results in low protein expression levels. Analyses of the biosynthesis and glycosylation status of I1196S P-gp are ongoing in our laboratory.

The conserved Asp^{1200} in the Walker B region of P-gp is required for the binding and hydrolysis of ATP (40, 41). Our present study also shows that substitution of serine for Ile^{1196} results in the loss of ATP-binding activity but that the substitution of tyrosine for His^{1195} does not affect P-gp function. It is not yet fully understood why mutant ATP-binding cassette transporters that lack ATP-binding activity are unstable, but defects in proper protein folding, particularly in ATP-binding sites, seem to be associated with protein degradation.

In our current study, we have also identified the T3587G and C3583T germ-line mutations in the *MDR1* gene in two individuals (0.3%) from a Japanese population of 605 individuals. In each case, however, these subjects were heterozygous for either the T3587G or C3583T allele. We contend, therefore, that there are two principal questions that arise from these findings: (*a*) the clinical significance of a homozygous T3587G *MDR1* genotype and (*b*) the clinical significance of a heterozygous T3587G *MDR1* genotype. Because the studies of *MDR1* double-knockout mice (*mdr1a/mdr1b−/−* mice) have shown that a *MDR1* deficiency causes large alterations in the pharmacokinetics of digoxin, vinblastine, and other drugs (12, 13), patients without P-gp function would also expected to show abnormal pharmacokinetics of P-gp substrate anticancer agents. Significantly, this may lead to potentially life-threatening side effects during cancer chemotherapy. Our present experiments have suggested the possible existence of a nonfunctional P-gp phenotype, but the extremely low allelic frequency of the T3587G *MDR1* mutation in our Japanese cohort makes it difficult to assess the relevance of a homozygous T3587G *MDR1* genotype in a clinical study. Hence, the existence of a subpopulation that has a high frequency of T3587G *MDR1* alleles would be necessary to detect homozygotes. It is likely that, in the absence of this, the prior genotype screening of homozygous T3587G *MDR1* patients undergoing cancer chemotherapy with P-gp substrate anticancer agents would be fruitless.

Another possible clinical study that could be undertaken would focus on T3587G *MDR1* heterozygous patients. We have identified heterozygous T3587G carriers in our Japanese population at a ratio of 1:300. In this regard, it is noteworthy that, in a previous report from our laboratory, a heterozygous T3587G *MDR1* patient treated with irinotecan showed the highest renal clearance of SN-38 among the group of irinotecan-treated patients in the study, although the renal clearances of irinotecan and SN-38 glucuronide in this individual were in the intermediate levels (17). However, it is not possible at this early stage to speculate on the effects of a heterozygous T3587G *MDR1* mutation from the results of only a single patient. To further clarify the consequences of a heterozygous T3587G allele, it will be necessary to further screen patients with T3587G *MDR1* mutation and examine whether they exhibit any aberrant kinetics or unusual toxicities as a result of treatments with *MDR1*-related anticancer agents. Such studies are currently ongoing in our laboratory and we wish to assess in the future whether the T3587G *MDR1* mutation would indeed be a candidate to be included in a putative SNP genotyping kit that would facilitate the screening of patients undergoing cancer therapy with P-gp substrates.

In a separate previous study from our laboratory, we identified the C376T *BCRP* SNP that encodes a Q126stop truncated BCRP (34, 35). The calculated frequency of homozygous C376T *BCRP* carriers was found to be 1.4 in 10,000, and we have not identified a homozygous carrier at this stage. Additionally, we have also reported that the C421A polymorphism in the *BCRP* gene, which substitutes lysine for the Gln^{141} residue of BCRP, is frequently observed in Japanese populations. Significantly, the Gln^{141} residue of BCRP lies between the Walker A and the signature C regions of its ATP-binding site. Moreover, Q141K BCRP-expressing cells show low levels of BCRP expression compared with WT BCRP-expressing cells (34, 35). This SNP may thus be important in the pharmacokinetics of irinotecan-related anticancer agents because cancer patients with the C421A allele show higher area under the concentration-time curve values after treatment with diflomotecan, an oral analogue of irinotecan, than patients harboring the WT allele (42). Hence, screening for SNPs that affect the expression of ATP-binding cassette and other transporters as well as drug-metabolizing enzymes are potentially very important for devising the appropriate treatments for cancer patients.