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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.

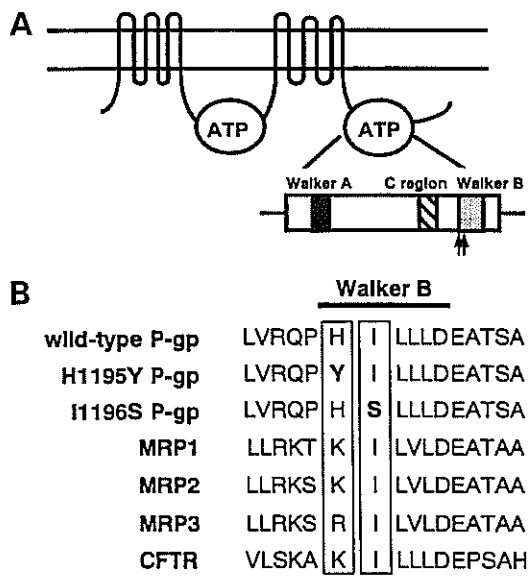

Figure 1. Map of specific mutations in P-gp. A, structure of P-gp. *Arrows*, location of the H1195Y and I1196S substitutions. B, alignment of various ATP-binding cassette transporter sequences close to the Walker B region of the second ATP-binding site. The His^{1195} and Ile^{1196} residues affected 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

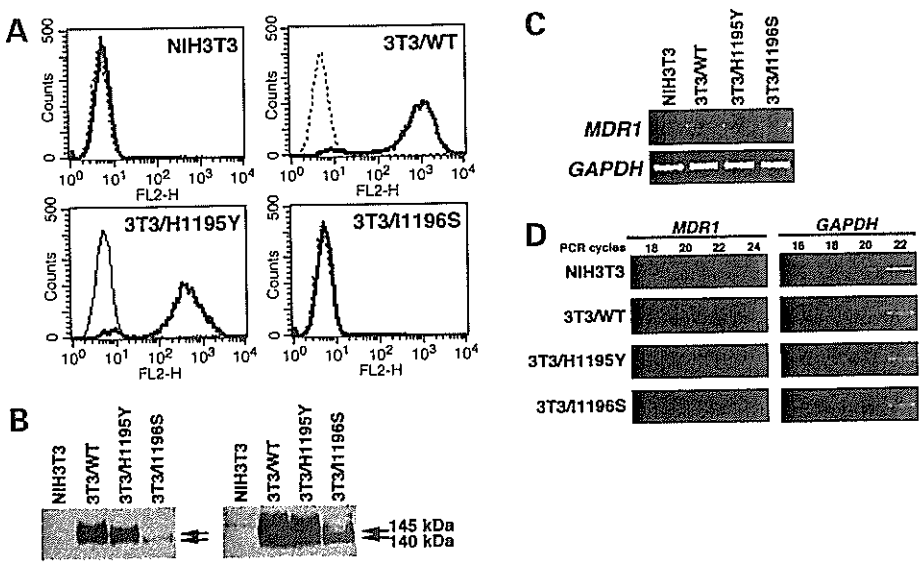

Figure 2. P-gp expression, *MDR1* cDNA integration, and *MDR1* mRNA expression in NIH3T3 transfectants. A, detection of cell surface expression of P-gp by FACS analysis. Parental NIH3T3 cells and the corresponding *MDR1* transfectants were harvested and then incubated with or without a biotinylated $F(ab')_2$ 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.

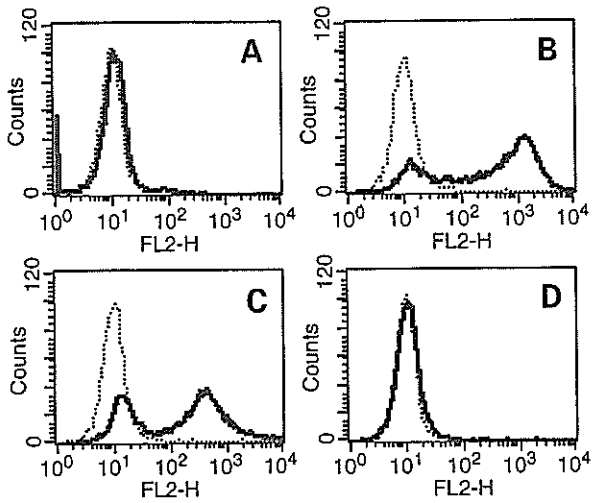

Figure 3. Cell surface expression of P-gp in retrovirally transduced cells. Cells were transduced with WT or mutant *MDR1* retroviruses, harvested, and incubated with or without a biotinylated $F(ab')_2$ fragment of MRK16 followed by treatment with R-phycoerythrin-conjugated streptavidin. After washing, 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

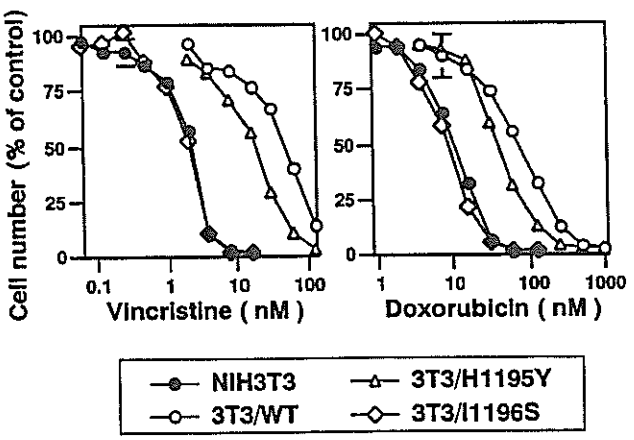

Figure 4. Drug resistance of the mutant *MDR1* transfectants. NIH3T3 (●), 3T3/WT (○), 3T3/H1195Y (△), and 3T3/I1196S (◇) cells were cultured for 5 d with various concentrations of vincristine or doxorubicin. Cell numbers were determined using a cell counter. *Points*, mean of triplicate experiments;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

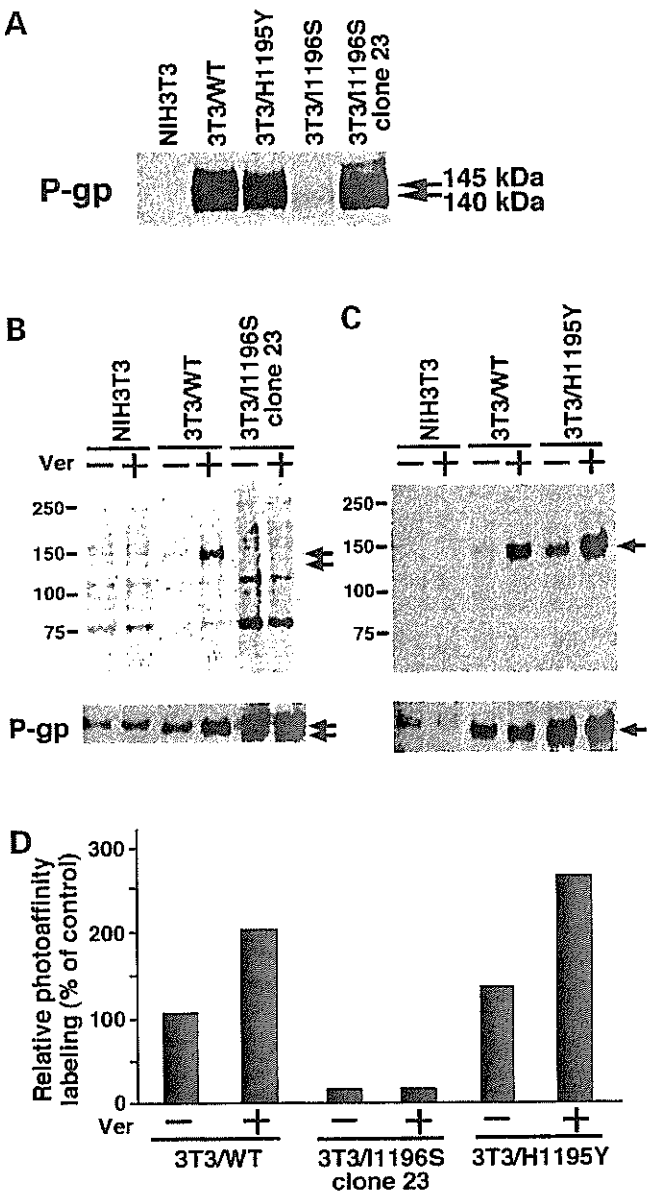

Figure 5. ATP-binding activities in the mutant *MDR1* transfectants. **A**, P-gp expression levels in the transfectants. Protein (20 μg) was loaded in each lane and subjected to Western blotting analysis using the anti-P-gp monoclonal antibody C219. **B**, ATP-binding activity of I1196S P-gp. Plasma 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.

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Estrogen-mediated post transcriptional down-regulation of P-glycoprotein in *MDR1*-transduced human breast cancer cells

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The human multidrug resistance gene 1 (*MDR1*) encodes the plasma membrane P-glycoprotein (P-gp/ABCB1) that functions as an efflux pump for various anticancer agents. We recently reported that estrogens down-regulate the expression of breast cancer resistance protein (BCRP/ABCG2). In our present study we demonstrate that estrogens also down-regulate P-gp expression in the *MDR1*-transduced, estrogen receptor α (ER-α)-positive human breast cancer cells, MCF-7/MDR and T-47D/MDR. The P-gp expression levels in MCF-7/MDR cells treated with 100 pM estradiol were found to be 10–20-fold lower than the levels in these same cells that were cultured without estradiol. In contrast, estradiol did not affect the P-gp expression levels in the ER-α-negative cancer cells, MDA-MB-231/MDR and NCI/ADR-RES. Estrone and diethylstilbestrol were also found to down-regulate P-gp in MCF-7/MDR cells, but progesterone treatment did not produce this effect. Tamoxifen reversed the estradiol-mediated down-regulation of P-gp in MCF-7/MDR cells, suggesting that ER-α activity is necessary for the effects of estradiol upon P-gp. However, estradiol was found not to alter the *MDR1* transcript levels in either MCF-7/MDR and T-47D/MDR cells, suggesting that post-transcriptional mechanisms underlie its effects upon P-gp down-regulation. MCF-7/MDR cells also showed eight-fold higher sensitivity to vincristine when treated with 100 pM estradiol, than when treated with 1 pM estradiol. These results may serve to provide a better understanding of the expression control of ABC transporters, and possibly allow for the establishment of new cancer chemotherapy strategies that would control P-gp expression in breast cancer cells and thereby increase their sensitivity to *MDR1*-related anticancer agents. (*Cancer Sci* 2006; 97: 1198–1204)

P-glycoprotein (P-gp), also known as ABCB1, is a 170–180 kDa transmembrane glycoprotein encoded by the human *multidrug resistance gene 1* (*MDR1*). P-gp is also a member of the ATP-binding cassette (ABC) transporter family and functions as an efflux pump for various structurally unrelated anticancer agents such as the *vinca* alkaloids, anthracyclines and taxans.[1–4] Cancer cells transfected with *MDR1* cDNA have been shown to have significantly higher resistance to vinblastine and doxorubicin than their parental cell controls.[5] Moreover, the *MDR1* gene expression levels in acute myelogenous leukemic cells have been correlated with a poor response to induction chemotherapy.[6] *MDR1* transcript levels have been shown to be generally high in untreated, intrinsically drug-resistant tumors of the colon, kidney, liver and pancreas, and are elevated in some cancers following a relapse after chemotherapy.[7] Hence, P-gp expression is one of the most important determinants of the effectiveness of chemotherapeutic agents in the treatment of cancer.

To circumvent P-gp-mediated drug resistance in human malignancies, various inhibitors that competitively inhibit the P-gp-mediated efflux of anticancer agents have been studied.[8,9] Another possible strategy for circumventing P-gp-mediated multidrug resistance would be the use of compounds that reduce its expression in cancer cells. However such compounds have not yet been reported. P-gp is also widely expressed in normal cells and tissues, such as the liver, kidney, adrenal, intestine, placenta, and the endothelial cells at both blood–brain and blood–testis barriers.[10,11] This suggests that P-gp functions in the protection of these tissues from cytotoxic agents and xenobiotics. Therefore, the expression levels and activity of P-gp in these tissues would be expected to have an impact on the blood and tissue levels of such compounds. Indeed, clinical studies of P-gp inhibitors have now shown that they increase the plasma concentrations of P-gp substrate anticancer agents.[12,13] Hence, the suppression of P-gp may also affect the pharmacokinetics of its chemotherapeutic substrates.

We previously reported that both estrogens[14] and antiestrogens[15] inhibit breast cancer resistance protein (BCRP)-mediated drug resistance and subsequently showed that sulfated estrogens are physiological substrates of BCRP.[16,17] In the course of analyzing the interaction of estrogens with BCRP, we also found that the physiological levels of estradiol (E_2) down-regulate both endogenous and exogenous BCRP expression in estrogen receptor α (ER-α)-positive cells by post-transcriptional mechanisms.[18] Therefore, the two effects of estrogens on BCRP, i.e. the inhibition of drug efflux and the down-regulation of protein expression, occur by different mechanisms.[18,19] In this study, we examined the effect of estrogens in *MDR1*-transduced, ER-α-positive human breast cancer cells and found that they down-regulate P-gp expression in these cells.

Materials and Methods

Antibodies. The anti-P-gp antibody C219,[20] anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody and anti-ER-α antibody (NCL-ER--6F11) were obtained from Cencor (Malvern, PA), Chemicon (Temecula, CA) and Novocastra (Newcastle, UK), respectively. The peroxidase-conjugated sheep antimouse secondary antibody was purchased from Amersham (Buckinghamshire, UK). The biotinylated $F(ab')_2$ fragment of the anti-P-gp antibody, MRK16, was prepared in our laboratory as described previously.[21]

Cell cultures. The estrogen-free basal medium used in this study consists of phenol red free-DMEM (Dulbecco's modified Eagle medium) and 7% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT). The human cancer cell lines MCF-7, T-47D, MDA-MB-231 and NCI/ADR-RES were obtained from the 60 cell line panel of the National Cancer Institute (NCI, Bethesda, MD) and maintained in basal medium supplemented with 10 pM E_2 at 37°C in a humidified incubator with 5% CO_2. NCI/ADR-RES was used as a control cell line that overexpresses

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endogenous P-gp in the NCI cell line panel, but lacks the expression of ER-α.[22] The MCF-7, T-47D and MDA-MB-231 cell lines were transduced with HaMDR retrovirus[23–25] and selected in 4 ng/mL vincristine for seven days. The resulting mixed populations of the transduced cells that stably expressed P-gp were designated MCF-7/MDR, T-47D/MDR and MDA-MB-231/MDR, respectively.

Western blot analyzes of P-gp and ER-α. Cells were cultured in basal medium in the absence or presence of various concentrations of E_2 for four days. For the Western blot analysis of P-gp, the cells were harvested and the resulting cell pellets were resuspended in lysis buffer (10 mM Tris-HCl [pH 8.0], 0.1% Triton X-100, 10 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM 4-[2-aminoethyl]-benzenesulfonylfluoride, 1% aprotinin, 1 mM dithiothreitol [DTT]). The cell lysates were obtained after freeze-thawing and brief centrifugation, and then solubilized in sample buffer A (2% SDS, 50 mM Tris-HCl [pH 8.0], 0.2% bromophenol blue, 5% 2-mercaptoethanol). The solubilized proteins were separated by SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were incubated with the anti-P-gp antibody C219 (1 μg/mL) and anti-GAPDH antibody (0.44 μg/mL), followed by a treatment with the peroxidase-conjugated sheep antimouse secondary antibody. Membranes were subsequently developed with an Enhanced Chemiluminescence Plus detection kit (Amersham).

For the Western blot analysis of ER-α, cell pellets were directly solubilized in sample buffer B (2% SDS, 62 mM Tris-HCl, 10% glycerol). The solubilized proteins from 1.5×10^5 cells were separated by SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were incubated with the anti-ER-α antibody NCL-ER--6F11 (1:30 dilution) and anti-GAPDH antibody (0.44 μg/mL). The membranes were then processed as described above.

Fluorescence-activated cell sorting (FACS) analysis of P-gp expression levels. To determine the expression levels of P-gp on the surface of E_2-treated cells, the human-specific monoclonal antibody, MRK16, that reacts with a cell surface epitope of P-gp was employed. Cells were incubated in basal medium in the absence or presence of various concentrations of E_2 for four days. The cells (5×10^5 per test) were then incubated with or without the biotinylated $F(ab')_2$ fragment of MRK16 (100 μg/mL) and then washed and incubated with R-phycoerythrin-conjugated streptavidin (400 μg/mL; BD Biosciences, Franklin Lakes, NJ).[25] The fluorescence staining levels were detected using FACS Calibur (BD Biosciences).

Reverse transcription polymerase chain reaction analysis of *MDR1* mRNA. *MDR1* mRNA levels were examined by reverse transcription polymerase chain reaction (RT-PCR) analysis. Cells were incubated in basal medium in the absence or presence of various concentrations of E_2 for four days. The extraction of total RNA and subsequent RT-PCR reactions were performed using an RNeasy kit (Qiagen, Valencia, CA) and an RNA LA PCR kit (Takara, Ohtsu, Japan), respectively, according to the manufacturer's instructions. First-strand *MDR1* cDNA was synthesized with 0.3 μg of total RNA, and a 702 bp fragment of *MDR1* cDNA was subsequently amplified with the following primers; forward 5′-GATATCAATGATACAGGGTT-3′ and reverse 5′-TGTCC-AATAGAATATTCCCC-3′. As an internal control, amplification of *GAPDH* cDNA (551 bp fragment) was carried out with the following primers; forward 5′-ATCACCATCTTCCAGGAGCGA-3′ and reverse 5′-GCTTCACCACCTTCTTGATGT-3′. The PCR conditions were as follows: 95°C for 5 min; the indicated cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension step for 7 min at 72°C.

Intracellular accumulation of rhodamine 123 and vincristine. To examine rhodamine 123 accumulation, MCF-7 and MCF-7/MDR cells were treated with either 1 or 100 pM E_2 for four days and then trypsinized, harvested and resuspended in basal medium. These cells (1×10^6 per test) were then incubated with 100 nM rhodamine 123 in basal medium supplemented with the same concentrations of E_2 for 20 min at 37°C, washed twice with ice-cold PBS, and subjected to FACS analysis using FACS Calibur.[26]

To examine vincristine accumulation, MCF-7 and MCF-7/MDR cells were seeded on 12-well plates and cultured in basal medium in the presence of either 1 or 100 pM E_2 for four days. The cells were then washed and incubated with 50 nM [^{3}H]vincristine (5.4 Ci/mmol, Amersham) for 0.5, 10, and 30 min at 37°C in basal medium supplemented with the same concentration of E_2. At specified times the cells were washed three times with ice-cold PBS and then solubilized by the addition of 1 N NaOH. The cell extract was neutralized and the radioactivity levels were determined in a liquid scintillation counter (Aloka, Tokyo, Japan).

Drug sensitivity assay. The sensitivity of MCF-7/MDR cells to anticancer agents was evaluated by cell growth inhibition assays. MCF-7/MDR cells were pretreated with the indicated concentrations of E_2 for two days and then treated with various concentrations of vincristine in the presence of the same concentrations of E_2. After four days of vincristine treatment, the cell numbers were determined with a cell counter (Sysmex, Kobe, Japan). Data are represented as the mean ± SD from triplicate determinations. In instances where a horizontal bar is not shown, the SD is low and is contained within the symbol.

Results

Establishment of *MDR1* transduced cells. In a previous study we demonstrated that estrogens down-regulate both endogenous and exogenous BCRP expression in human breast cancer cells. In this study we initially screened the 60 cell line panel of the NCI for ER-α and P-gp expression. However, most of these established human breast cancer cells are estrogen-independent and do not express ER-α. Two estrogen-dependent human breast cancer cell lines, MCF-7 and T-47D cells, expressed ER-α (Fig. 1b) but were found not to express appreciable amounts of P-gp (Fig. 1a). We

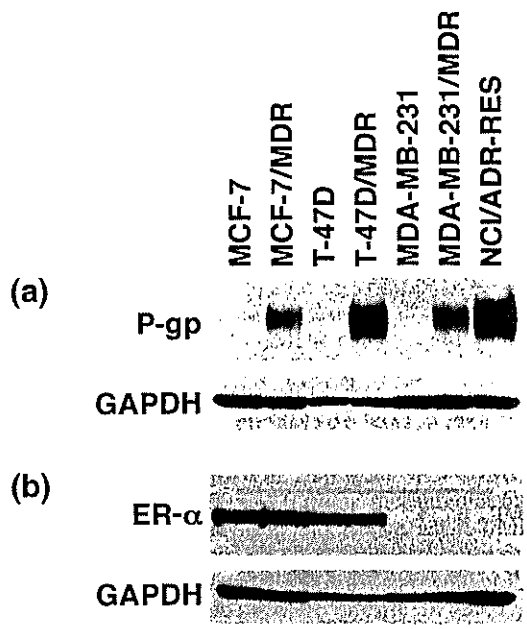

Fig. 1. P-glycoprotein (P-gp) and estrogen receptor α (ER-α) expression levels in cancer cell lines. For the detection of P-gp, cell lysates (40 μg) were subjected to western blot analysis with the anti-P-gp monoclonal antibody, C219. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were assessed as a loading control using the anti-GAPDH monoclonal antibody. For the detection of ER-α, whole cell lysates were subjected to western blot analysis with the anti-ER-α monoclonal antibody, NCL-ER--6F11, and anti-GAPDH monoclonal antibody.

therefore established stable *MDR1*-transduced human breast cancer cell lines to examine the effects of estrogens upon the expression levels of P-gp. In addition, we used two ER-α-negative cancer cells as controls. MDA-MB-231, which does not express P-gp, and NCI/ADR-RES, a doxorubicin-selected MDR cell line[22,27] that expresses endogenous P-gp (Fig. 1).

The MCF-7/MDR, T-47D/MDR and MDA-MB-231/MDR cell lines were established after transduction of the corresponding parental cells with a HaMDR retrovirus and subsequent selection of the transduced cells with vincristine. Mixed populations of the transduced cells were found to express P-gp (Fig. 1a). In addition, the P-gp expression levels in the transduced cells were found to be stable for at least several months.

MCF-7, MCF-7/MDR, T-47D and T-47D/MDR cells express ER-α (Fig. 1b), indicating that *MDR1* transduction did not affect the expression levels of this gene in these cells. In contrast, MDA-MB-231, MDA-MB-231/MDR and NCI/ADR-RES cells do not express ER-α (Fig. 1b). Furthermore, MCF-7/MDR, T-47D/MDR and MDA-MB-231/MDR cells showed significantly higher resistance to vincristine and doxorubicin than their parental cells (Table 1).

Estradiol down-regulates P-gp expression in *MDR1*-transduced cells. We next examined whether estrogens would down-regulate P-gp in the MCF-7/MDR, T-47D/MDR, MDA-MB-231/MDR and NCI/ADR-RES cells. As shown in Fig. 2, exogenous P-gp expression was predictably decreased in MCF-7/MDR and T-47D/MDR cells in a dose-dependent manner, following treatment with 10 pM-10 nM E_2. MCF-7/MDR or T-47D/MDR cells that had been cultured in the absence of E_2, and those treated with 1 pM E_2, show similar levels of P-gp expression, suggesting that this very low dose of E_2 does not affect the expression levels of P-gp. The P-gp expression levels in MCF-7/MDR cells treated with 100 pM E_2 were between 10- and 20-fold less than those in the same cells that were cultured without E_2. In contrast, E_2 did not alter the expression levels of P-gp in either the MDA-MB-231/MDR or NCI/ADR-RES cells (Fig. 2). These results suggest that ER-α expression may be required for the E_2-mediated down-regulation of P-gp. Furthermore, since it is exogenous P-gp that is suppressed by E_2, this down-regulation is likely to be independent of the suppression of the endogenous *MDR1* promoter.

The effects of estradiol on the cell surface expression of P-gp. Because Western blot analysis revealed that E_2 suppresses the expression of P-gp in a dose-dependent manner (Fig. 2), we further examined its effects on the cell surface expression levels of P-gp, also by FACS analysis. Our subsequent findings demonstrated that the expression levels of P-gp on the cell surface were considerably decreased in MCF-7/MDR cells in a dose-dependent manner following treatment with 10 pM-10 nM E_2 (Fig. 3). It is noteworthy

Table 1. Drug resistance levels in *MDR1*-transduced cells

Cell line	Degree of resistance†		Estrogen receptor α expression
	Vincristine	Doxorubicin	
MCF-7/MDR	149 ± 2.1	18 ± 1.8	+
T-47D/MDR	87 ± 2.1	26 ± 2.4	+
MDA-MB-231/MDR	220 ± 1.8	24 ± 0.58	–

†The degree of drug resistance is calculated as the IC_{50} value of the resistant cells, divided by this measurement in the parental cells. The data shown are the mean values ± SD from tripricate determinations.

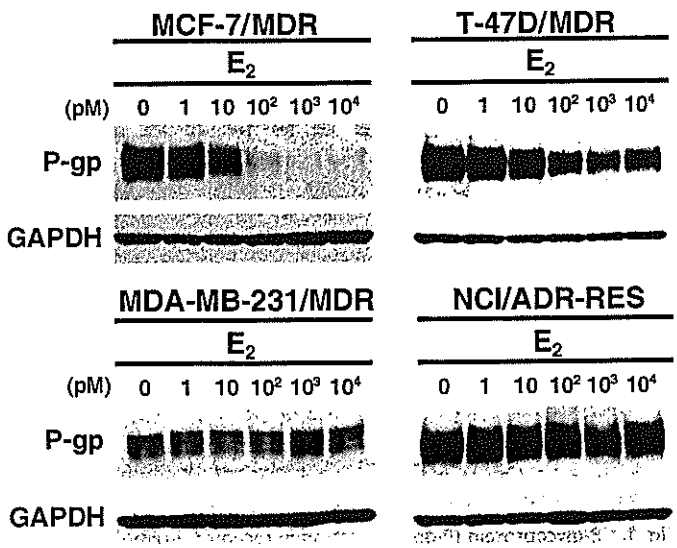

Fig. 2. The effects of E_2 on P-glycoprotein (P-gp) expression. The indicated cells were cultured in basal medium in the absence or presence of the indicated concentrations of E_2 for 4 days. Cell lysates (40 μg) were then subjected to western blot analysis with the anti-P-gp monoclonal antibody, C219. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were assessed as a loading control using the anti-GAPDH monoclonal antibody.

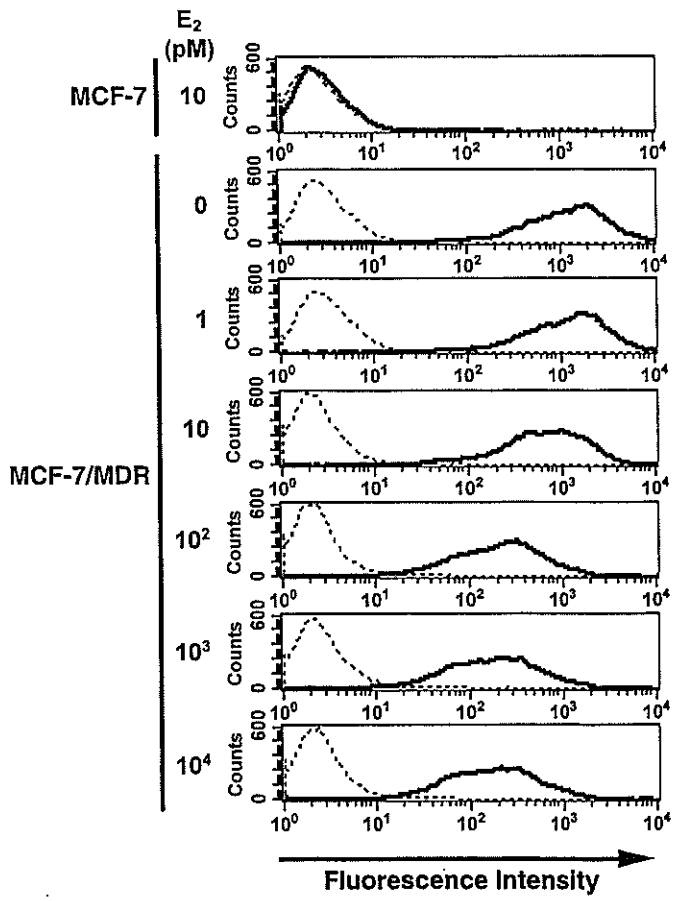

Fig. 3. Analysis of the cell surface expression of P-glycoprotein (P-gp) using fluorescence-activated cell sorting (FACS) analysis. MCF-7/MDR cells were cultured in basal medium in the absence or presence of the indicated concentrations of E_2 for 4 days. MCF-7/MDR cells were then harvested, incubated with or without the biotinylated $F(ab')_2$ fragment of the anti-P-gp monoclonal antibody, MRK16, and then incubated with R-phycoerythrin-conjugated streptavidin. After washing the cells, the fluorescence intensities were determined using FACSCalibur. The bold and dotted lines indicate that the cells were incubated with and without MRK16, respectively.

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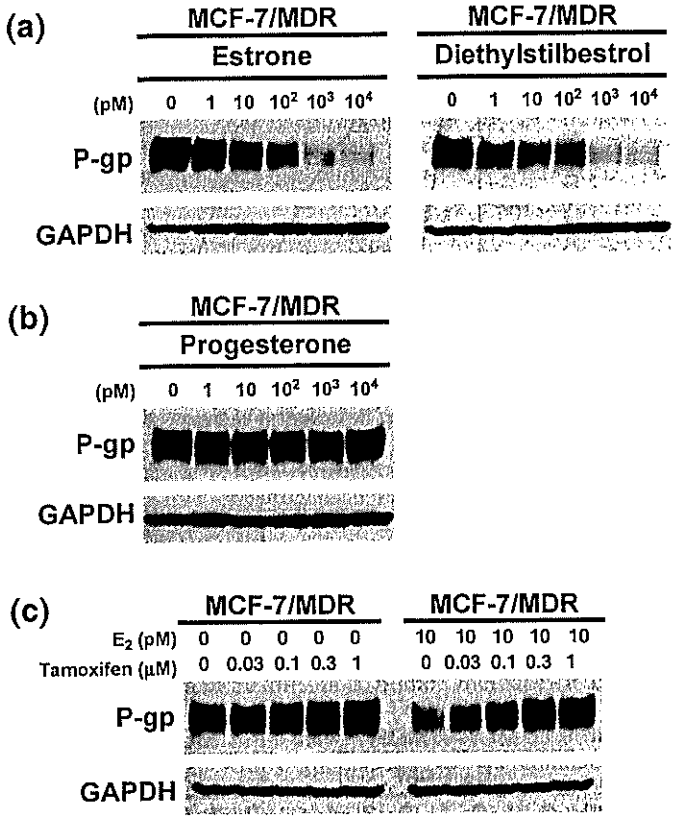

Fig. 4. The effects of estrogens, progesterone and tamoxifen on P-glycoprotein (P-gp) expression levels. MCF-7/MDR cells were cultured in basal medium in the absence or presence of the indicated concentrations of each agent for 4 days. Cell lysates (40 μg) were then subjected to western blot analysis with the anti-P-gp monoclonal antibody, C219, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody to normalize for protein loading control. (a) Effects of estrogens on P-gp expression. (b) Effects of progesterone on P-gp expression. (c) Effects of tamoxifen on P-gp expression in the absence of E_2 (left panel) and the reversal effects of tamoxifen on the E_2-mediated down-regulation of P-gp (right panel).

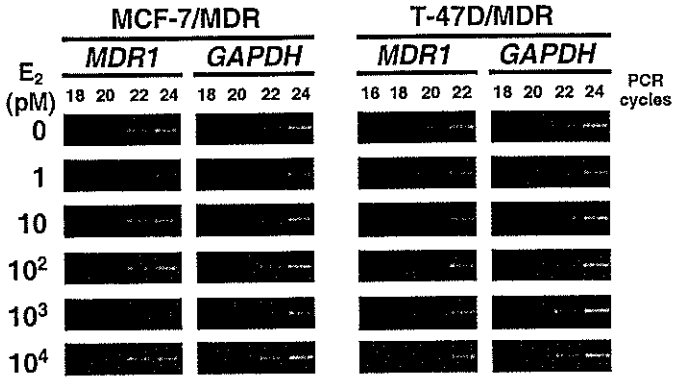

Fig. 5. Reverse transcription polymerase chain reaction (RT-PCR) analysis of the *MDR1* mRNA expression levels in both MCF-7/MDR and T-47D/MDR cells. Cells were cultured in basal medium in the absence or presence of the indicated concentrations of E_2 for 4 days. Exponentially growing cells were harvested and total RNA was extracted. First-strand cDNAs were synthesized with 0.3 μg of total RNA, and an *MDR1* cDNA fragment (702 bp) was subsequently amplified by PCR using the indicated cycles. The amplification of *GAPDH* mRNA (551 bp fragment) was performed as an internal control.

that the cells treated with 100 pM-10 nM E_2 in this experiment still expressed significant amounts of P-gp but we speculate that this may be due to the high sensitivity of the fluorescence signals when using a biotin-streptavidin amplification system.

The effects of estrogen and antiestrogen on P-gp expression. We examined the effects of other estrogenic compounds, and of progesterone, upon P-gp expression in MCF-7/MDR cells. The expression levels of P-gp in these cells were observed to have decreased in a dose-dependent manner following treatment with either estrone or diethylstilbestrol (Fig. 4a). However, slightly higher concentrations of estrone or diethylstilbestrol were required to achieve similar levels of P-gp down-regulation, compared to E_2 (Figs 2, 4a). On the other hand, progesterone did not show any suppressive effects on P-gp expression in MCF-7/MDR cells (Fig. 4b).

We next examined whether tamoxifen, an antiestrogen, could reverse the estrogen-mediated down-regulation of P-gp in MCF-7/MDR cells. In the absence of E_2, tamoxifen slightly increased P-gp expression. Significantly, in the presence of 10 pM E_2, tamoxifen was found to reverse the E_2-mediated down-regulation of P-gp in a dose-dependent manner (Fig. 4c). These data further suggest that estrogenic activity is required for the down-regulation of P-gp expression.

Estradiol does not alter *MDR1* mRNA levels. We next examined the effects of E_2 on *MDR1* mRNA levels. RT-PCR analyzes were carried out following the incubation of both MCF-7/MDR and T-47D/MDR cells in the absence or presence of various concentrations of E_2 for four days. Our subsequent results revealed that the *MDR1* mRNA levels were not affected by E_2-treatment in either cell type (Fig. 5). This indicates that the E_2-mediated down-regulation of P-gp may depend on either translational or post-translational processes.

The effects of estradiol on P-gp-mediated transport and resistance. We examined the effects of either 1 or 100 pM E_2 treatment on the cellular accumulation of rhodamine 123 and vincristine in the MCF-7 and MCF-7/MDR cells. MCF-7/MDR cells treated with 100 pM E_2 showed higher rhodamine 123 uptake, compared with 1 pM E_2 treatment (Fig. 6a). In contrast, MCF-7 cells treated with either 1 or 100 pM E_2 showed similar levels of rhodamine 123 uptake (Fig. 6a). MCF-7 cells showed higher rhodamine 123 uptake than MCF-7/MDR cells for both E_2 treatments.

We additionally examined vincristine uptake in E_2-treated cells. MCF-7/MDR cells treated with 100 pM E_2 showed higher [^{3}H]vincristine uptake compared to the 1 pM E_2 treatment (Fig. 6b). In contrast, MCF-7 cells treated with either 1 or 100 pM E_2 showed similar levels of [^{3}H]vincristine uptake, which was at higher levels than MCF-7/MDR cells in both cases (Fig. 6b). These results therefore suggest that the down-regulation of P-gp by E_2 results in an increase of the cellular uptake of P-gp substrates.

We then examined the effects of E_2 on cellular drug resistance. As shown in Figure 7a, MCF-7/MDR cells treated with 100 pM E_2 show a higher sensitivity to vincristine than the same cells treated with 1 pM E_2. The IC_{50} values for vincristine in MCF-7 cells in the presence of 1 and 100 pM E_2 were determined to be 0.35 ± 0.04 and 0.34 ± 0.01 nM, respectively (Fig. 7a). In contrast, the IC_{50} values for vincristine in MCF-7/MDR cells in the presence of 1 and 100 pM E_2 were measured at 68.7 ± 2.9 and 8.4 ± 0.2 nM, respectively (Fig. 7a). Hence, MCF-7/MDR cells treated with 100 pM E_2 showed an eight-fold higher sensitivity to vincristine than the same cells treated with 1 pM E_2. The timecourse of the P-gp expression profile in the E_2-treated MCF-7/MDR cells further supports our hypothesis that the suppression of P-gp expression by E_2 leads to the sensitization of cells to anticancer agents (Fig. 7b).

Discussion

In our previous study, we reported for the first time that the physiological levels of estrogens (10–100 pM) down-regulate

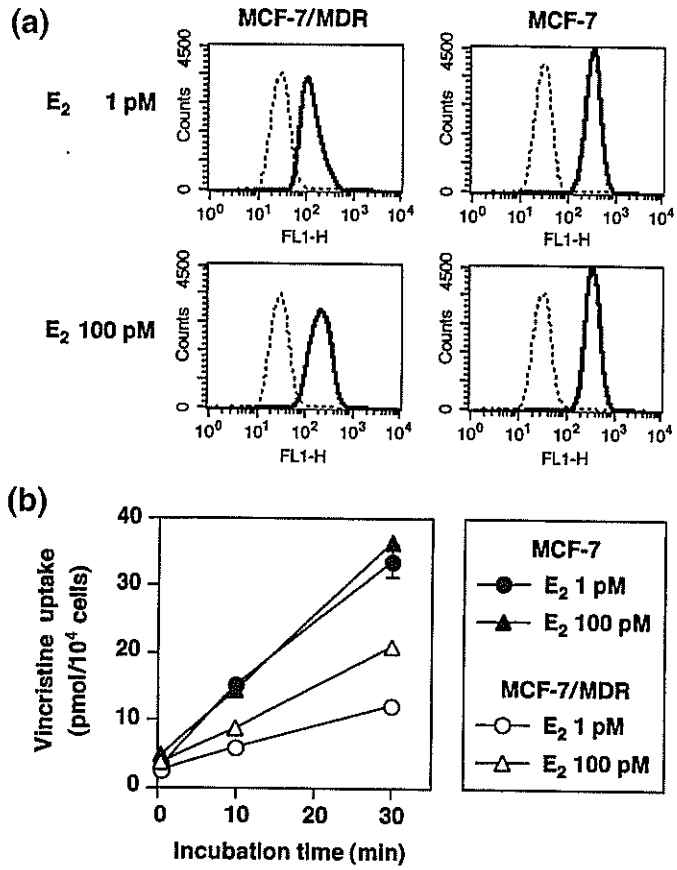

Fig. 6. Analysis of rhodamine 123 and [^{3}H]vincristine uptake in E_2-treated MCF-7/MDR cells. (a) Rhodamine 123 uptake. MCF-7 and MCF-7/MDR cells were cultured in basal medium in the presence of either 1 or 100 pM E_2 for 4 days. After trypsinization, the cells were incubated with (—) or without (---) 100 nM rhodamine 123 in basal medium supplemented with the same concentrations of E_2 for 20 min. After washing of the cells, the cellular uptake of rhodamine 123 was measured by FACSCalibur. (b) [^{3}H]Vincristine uptake. MCF-7 (▲ ●) and MCF-7/MDR (△ ○) cells were cultured in basal medium in the presence of either 1 pM (● ○) or 100 pM (▲ △) E_2 for 4 days. The cells were then washed and incubated with 50 nM [^{3}H]vincristine for 0.5, 10, and 30 min in basal medium supplemented with the same concentration of E_2. After washing of the cells, the cells were lyzed, and [^{3}H]vincristine uptake was measured by liquid scintillation. Data are the mean ± SD values from triplicate determinations. Where a horizontal bar is not shown, the SD is low and contained within the symbol.

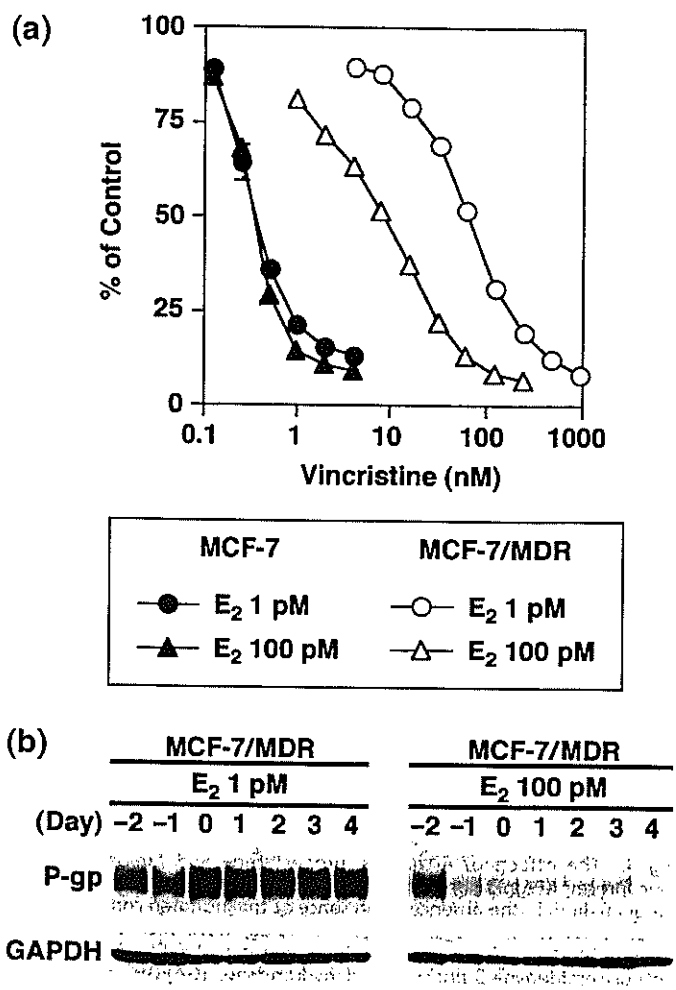

Fig. 7. Drug sensitivity levels of E_2-treated MCF-7/MDR cells. (a) Drug sensitivity to vincristine. MCF-7 cells (● ▲) and MCF-7/MDR cells (○ △) were cultured in basal medium in the presence of either 1 pM (● ○) or 100 pM (▲ △) E_2 for 2 days. Vincristine was then added to the cultures and the cells were incubated for an additional 4 days. Cell numbers were determined using a cell counter. Data are the mean ± SD values from triplicate determinations. Where a horizontal bar is not shown, the SD is low and contained within the symbol. (b) Timecourse analysis of the P-gp expression levels in E_2-treated MCF-7/MDR cells. MCF-7/MDR cells were cultured in basal medium in the presence of either 1 or 100 pM E_2 for up to 6 days. Cell pellets were obtained each day and cell lysates were subjected to western blot analysis with the anti-P-glycoprotein (P-gp) monoclonal antibody, C219.

BCRP expression by post-transcriptional processes without affecting the *BCRP* transcript levels.[18] Because P-gp also belongs to the ABC transporter superfamily, in addition to BCRP, we examined the effects of estrogens on P-gp expression in our current study and demonstrate that E_2-mediated down-regulation of P-gp expression occurs in ER-α-positive human breast cancer cells.

It is significant that E_2 down-regulates P-gp expression only in the ER-α-positive cells, MCF-7/MDR and T-47D/MDR, but not in the ER-α-negative cells, MDA-MB-231/MDR and NCI/ADR-RES (Fig. 2). Estrone and diethylstilbestrol were also found to down-regulate P-gp expression in MCF-7/MDR cells (Fig. 4a), but progesterone did not cause such effects (Fig. 4b). Moreover, the antiestrogen drug, tamoxifen, strongly reverses the E_2-mediated down-regulation of P-gp in MCF-7/MDR cells (Fig. 4c). These results suggest that a functional ER-α and its downstream pathways are important for estrogen-mediated P-gp down-regulation.

In a previous study we demonstrated that estrogens down-regulate BCRP expression without affecting its gene promoter activity.[18] In this study we also screened the 60 cell line panel of the NCI for ER-α- and P-gp-positive cells. However, the ER-α-positive MCF-7 and T-47D cells that we used in our present experiments do not express endogenous P-gp. Moreover, NCI/ADR-RES, a cell line in the NCI panel that does overexpress P-gp, is likely to be of ovarian origin, and thus lacks any expression of ER-α.[22] We therefore established *MDR1*-transduced cells, MCF-7/MDR and T-47D/MDR, to enable us to examine the effects of estrogens on P-gp expression in ER-α-positive cells. We show herein that estrogens down-regulate P-gp expression in *MDR1*-transduced cells, but there remains a possibility that estrogens may not exert such an effect in ER-α-positive human breast cancer cells, if they were to activate the *MDR1* promoter. To clarify this point, the effects of estrogen on P-gp expression in ER-α-positive human breast cancer cells that express endogenous P-gp will need to be assessed in the near future, when such cell lines become available.

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It has been reported that murine *mdr* mRNA is expressed at high levels in the gravid uterus and that these levels dramatically increase during pregnancy, compared with the relatively low levels of expression observed in the non-gravid uterus.[28] In addition, it was previously shown that *mdr* mRNA and murine P-gp are induced at high levels in the uterine secretory epithelium by a combination of estrogen and progesterone treatments.[29] In humans, the high expression levels of P-gp in the syncytiotrophoblast of the placenta has been reported during the early phase of pregnancy.[30] However, although these reports suggest a possible role for estrogen/progesterone in the control of P-gp expression, most studies have focused only on the regulation of *MDR1/mdr* mRNA expression. In our present study, we have shown that estrogens down-regulate P-gp expression in ER-α-positive human breast cancer cells, without affecting *MDR1* transcription levels. It is not clear at present whether our observations are in any way linked with the findings of previous studies of *MDR1* regulation in the uterus, but our present data do suggest the possibility of a role for estrogens in the control of P-gp expression in breast cancer cells and possibly also in normal breast tissues.

Clinical trials of P-gp inhibitors have now been conducted against various malignancies, including advanced breast cancers.[31–34] Tidefelt *et al.* examined the effects of the cyclosporinee derivative, PSC 833, on the concentration of daunorubicin in leukemic blast cells *in vivo* during treatment.[35] They have shown that PSC 833 treatment results in a higher increase of cellular daunorubicin concentrations in P-gp-positive leukemic cells than in P-gp-negative leukemic cells. These results suggest that PSC 833, by interacting with P-gp, can increase the cellular uptake of daunorubicin in leukemic blasts *in vivo*.[35] However, the results of clinical trials using verapamil or other P-gp inhibitors, in combination with either doxorubicin, vincristine, paclitaxel or epirubicin, against breast cancers have resulted in only minor responses, with PR rates of 10–20%. The evidence provided so far is therefore not entirely sufficient to evaluate the possible effectiveness of P-gp inhibition during chemotherapies against breast cancer.

Tamoxifen has also been reported to overcome P-gp-mediated drug resistance[36] and clinical trials of high oral doses of tamoxifen have been conducted to examine for possible MDR-reversing effects.[37–41] The earliest studies were conducted using tamoxifen alone, and subsequent studies used tamoxifen in combination with other MDR modulators. Unfortunately, these trials proved to be only moderately successful. The patients enrolled in these studies had presented with either colorectal or renal carcinomas, and it may thus be difficult to discuss the results of these studies in the context of our present analyzes. Human breast cancer cells grow in the presence of estrogens, which are supplied from the ovary and placenta. In the presence of estrogens, tamoxifen may increase the P-gp expression of ER-α-positive cells. Hence, the possible effects of tamoxifen on the P-gp expression levels in human breast cancer cells *in vivo* is an important area for future studies.

In conclusion, we find that estrogen decreases P-gp expression in *MDR1*-transduced, ER-α-positive human breast cancer cells *in vitro* via post-transcriptional processes. Furthermore, E_2-mediated P-gp down-regulation induces a reduction in the cellular resistance to anticancer agents. These results may therefore serve to provide a better understanding of the expression control of ABC transporters. In addition, the modulation of P-gp expression in breast cancer cells by small molecules may be a useful strategy for sensitizing such cells to anticancer agents.

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PRECLINICAL STUDY

Coexistence of the loss of heterozygosity at the PTEN locus and HER2 overexpression enhances the Akt activity thus leading to a negative progesterone receptor expression in breast carcinoma

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Abstract Serine/threonine kinase Akt/PKB is known to regulate divergent cellular processes, including apoptosis, proliferation, differentiation, and metabolism. Akt is activated by a variety of stimuli, through such growth factor receptors as HER2, in phosphoinositide-3-OH kinase (PI3K)-dependent manner. A loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) function also activates Akt. It has recently been shown that Akt activation is associated with a worse outcome among endocrine treated breast cancer patients and that it also inhibits the progesterone receptor (PR) expression via the PI3K/Akt pathway in breast cancer cells. Therefore, the PI3K/Akt signaling pathway has recently attracted considerable attention as a new target for effective therapeutic strategies. In the present study, we investigated the relationship between Akt activation and either HER2 overexpression or PTEN gene alteration, as well as the PR expression. We analyzed the incidence of LOH at the PTEN locus in 138 breast cancer patients, using our new system for microsatellite analysis, called high-resolution fluorescent microsatellite analysis (HRFMA). We showed Akt activation to significantly correlate with HER2 overexpression or LOH at the PTEN gene locus while inversely correlating with the PR expression. In addition, when LOH at the PTEN gene locus and HER2 overexpression occurred simultaneously, the incidence of Akt activation and reduced PR expression was significant. The association between Akt activation and PR negative expression was observed even in the ER-positive cases. Our results suggest that simultaneous PTEN LOH and HER2 overexpression enhances Akt activation and may thus lead to a negative PR expression.

Keywords PTEN · LOH · HER2 · Akt · Hormone receptors · Progesterone receptor · Breast cancer

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Introduction

Akt, also known as protein kinase B, is a serine/threonine protein kinase which has recently emerged as a crucial regulator of widely divergent cellular processes, including apoptosis, proliferation, differentiation, and metabolism [1]. A disruption of normal Akt/PKB signaling frequently occurs in several human cancers, and this enzyme appears to play an important role in cancer progression and cell survival [1]. Akt is activated by a variety of stimuli, through growth factor receptors such as HER2, in phosphoinositide-3-OH kinase (PI3K)-dependent manner. Another major mechanism of Akt activation is a loss of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) function [1, 2].

HER2/erbB2/neu is a member of the type I subclass of receptor tyrosine kinases, which has been associated

with several types of human cancers. Numerous studies have demonstrated HER2/erbB2 to be amplified and/or overexpressed in 20%–30% of all primary breast cancers and it is also generally associated with a poor prognosis [3–5]. One of the major signaling pathways utilized by the erbB families is the PI3K/Akt pathway. The ligand of HER2 has not yet been identified, however, HER2-containing heterodimers are potent activators of the multiple signaling pathways involved in proliferation, invasion, and survival [6]. Studies in breast cancer cells, primary breast tumors, and transgenic mice all indicate that when HER2 is overexpressed, it is constitutively associated with HER3 [7] and HER2–HER3dimers strongly activate the PI3K/Akt pathway. This is supported by the findings of a previous report which showed tumor cells overexpressing HER2 to exhibit a constitutive Akt activity [8]. In addition, Akt activation has been shown to be positively associated with HER2 overexpression in breast carcinoma obtained from human materials [9–11].

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a novel tumor suppressor gene that was initially identified through its mutation in a variety of tumor types [12, 13]. Loss of PTEN function, due to PTEN mutation, PTEN haploinsufficiency from the loss of heterozygosity (LOH) at the PTEN locus, and epigenetic downregulation of PTEN, has been reported in nearly 50% of all breast cancers and also in many other cancer types [12, 14, 15]. The gene product of PTEN reverses the activities of PI3K by dephosphorylating the D3 position of its lipid products, phosphatidylinositol-3, 4 bis-phosphate (PtdIns(3,4)P2), phosphatidylinositol-3,5 bis-phosphate (PtdIns(3,5)P2), and 3,4,5-tri-phosphate (PtdIns(3,4,5)P3) [16], thereby inhibiting the activity of PI3K. The fundamental in vivo role of PTEN appears to inhibit the PI3K-dependent activation of Akt. In fact, the loss of the PTEN function has been revealed to lead to the activation of the PI3K/Akt function [2].

Mutation of the PTEN gene is rare in breast carcinomas, however, LOH at 10q23 (PTEN locus) is observed in approximately 30%–40% of sporadic breast cancers [17–19]. Regarding the PTEN expression, a reduced or absent PTEN protein expression has been recognized in 8%–50% of breast cancer cases [20–24]. A reduced expression of the PTEN protein has been shown to be associated with Akt activation [21, 23].

Recently, the activation of Akt has been shown to be associated with a worse outcome among endocrine treated breast cancer patients [9, 10, 25]. We also reported that Akt activation is associated with resistance to endocrine therapy for metastatic breast cancer [26]. Another study revealed that progesterone receptor (PR) expression is inhibited in breast cancer cells via the PI3K/Akt pathway, and it is not mediated via a reduction of estrogen receptor (ER) levels or activity, based on an in vitro study [27]. In addition, cancer cell lines with activated Akt have been shown to be especially sensitive to the antagonism of mammalian target of rapamycin (mTOR), which is understream of Akt [28]. Therefore, the PI3K/Akt signaling pathway currently attracts considerable attention as a new target for effective therapeutic strategies, especially for endocrine therapy.

In the present study, we investigated the relationship between Akt activation and HER2 overexpression or PTEN gene alteration, as well as the PR expression. We analyzed the incidence of LOH at the PTEN locus in 138 breast cancer patients, using our new system for microsatellite analysis, called high-resolution fluorescent microsatellite analysis (HRFMA). We showed Akt activation to be significantly correlated with the HER2 overexpression or LOH at the PTEN gene locus and inversely correlated with the PR expression. In addition, when LOH at the PTEN gene locus and HER2 overexpression occurred simultaneously, both the incidence of Akt activation and a reduced PR expression were significant. The association between Akt activation and PR negative expression was observed even in the ER-positive cases.

Materials and methods

Patient population and breast cancer and normal tissue specimens

Primary human breast carcinoma specimens and corresponding normal tissues or peripheral blood were obtained from 138 patients who underwent surgery at the Department of Surgery and Science, Kyushu University Hospital, from 1994 to 2003. Informed consent was obtained from all the patients prior to tissue acquisition. Clinical data were obtained from the medical records. The specimens, taken immediately after resection, were placed in liquid nitrogen and then were used for an analysis of genomic DNA. The remaining tissues were routinely subjected to histopathological analyses by histopathological specialists at our hospital. The histopathological diagnosis was determined according to the criteria of the Japanese Breast Cancer Society [29]. Genomic DNA was extracted as previously described [30].

LOH analysis

LOH at the PTEN locus was analyzed in this study using our new system for microsatellite analysis, called HRFMA [30–32]. We used two microsatellite markers, D10S1765 and D10S1173 [32]. The oligonucleotide primers were synthesized and then purified by HPLC. The sequences of the primers for PCR analysis of D10S1765 were as follows: forward, 5′-CAATGGAA CCAAATGTGGTC, and reverse, 5′-AGTCCGAT AATGCCAGGATG. The sequences of the primers for PCR analysis of D10S1173 were as follows: forward, 5′-CATGCCAAGACTGAAACTCC, and reverse, 5′-AAACCCCAATGCCATAATGG. PCR reactions using genomic DNA were performed using a TAKARA GeneAmp PCR Reagent Kit and then were run in the Perkin-Elmer GeneAmp PCR system 9700 (Norwalk, CT, USA). The PCR reactions were performed as previously described [32]. The data were processed by the ABI software GeneScan. The cases showing heterozygosity were informative. These results were analyzed by a comparison of the peak value of the tumor DNA with that of normal control DNA. A reduction of more than 30% in the peak value in tumor DNA in comparison to normal control DNA was judged to indicate LOH [32].

Antibodies and immunohistochemistry

Monoclonal antibodies 6F11 and 1A6 (Ventana Medical Instruments, Tucson, AR, USA) were used for ER and PR staining. For HER2 evaluation, the monoclonal antibody CB11 (Ventana Medical Instruments) was used. Phosphorylated-Akt (pAkt), which is considered as the activated Akt, was detected using polyclonal antibodies against phosphorylated Ser 473 (Cell Signaling Technology, Beverly, MA, USA).

Tissue samples were fixed by immersion in buffered formalin and then were embedded in paraffin. The immunostaining of these paraffin sections was performed using the Ventana Discovery™ automated staining instrument (Ventana Medical Instruments) as previously described [10]. A negative control reaction with no primary antibody was always performed alongside the reaction-containing sample.

Immunostaining was evaluated without knowledge of the clinical and pathological parameters. ER and PR were recorded as positive if 10% or more of the nuclei in the invasive component of the tumor were stained [33]. HER2 was scored into four groups, score 0, 1+, 2+ and 3+, by the widely accepted criteria that assessed the intensity and completeness of membrane staining [34, 35]. The cases with HER2 2+ and 3+ were regarded as positive for HER2 overexpression. A specimen was considered positive for pAkt if 10% or more of the cytoplasm in the invasive component of the tumor was positively stained [10].

Statistical analysis

The *t*-test and the Fisher exact test were used to analyze correlations between variables such as pAkt, PTEN, HER2 and PR.

Multivariate logistic regression analysis was performed in order to elucidate association between pAkt, having the binary response +/–, and the coexistence of PTEN LOH and HER2 overexpression. The method enables us to quantify associations and interactions between pAkt and each combination of PTEN LOH and HER2 overexpression via estimations of odds ratios. Here odds ratios bigger than 1 (less than 1) correspond to probabilities of going into the pAkt+ (–) group, and thus large odds ratio values indicate that positive expression of pAkt, which means Akt activation, occurs in many cases. To cope with relatively small frequencies, the exact method was utilized for estimating parameters of the logistic regression model. In contrast to the standard one assuming sufficiently large sample size, the exact logistic regression method is a conservative approach valid for small samples [36]. Associations among PR, PTEN and HER2 were also studied by the exact logistic regression.

All statistical tests were carried out with use of the SAS statistical software (version 9.1) at the two-sided 0.05 significance level.

Results

LOH at the PTEN gene locus in breast carcinoma

The 138 breast tumors were analyzed for LOH at the PTEN gene locus, and the results from 131 cases (94.9%) were considered informative. We investigated these 131 informative cases in this study. An allelic loss at at least one marker was observed in 31 (23.7%) of these 131 informative cases and these cases were diagnosed to have LOH. The representative results of LOH analyses, not-informative case, retention of heterozygosity (ROH) and LOH, were shown in Fig. 1. The clinico-pathological features of these cases are shown in Table 1.

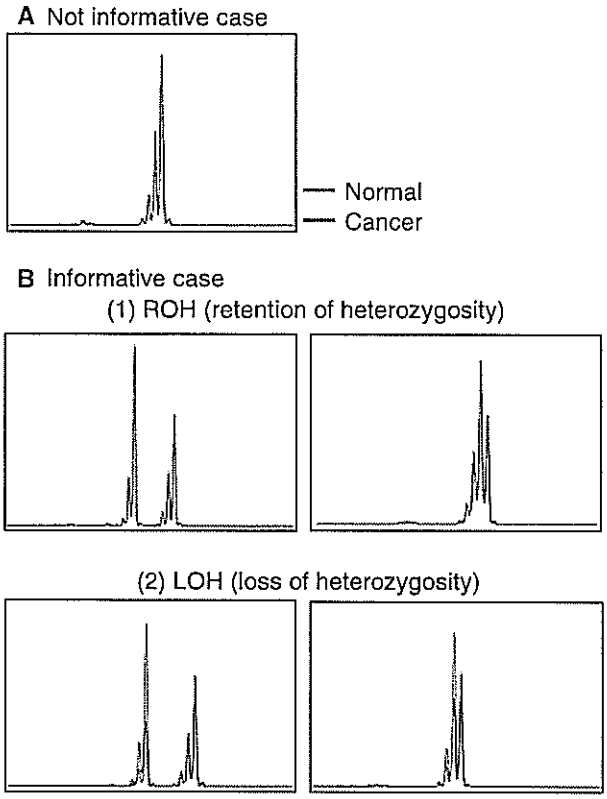

Fig. 1 Microsatellite analysis for the detection of PTEN LOH. Using genomic DNA samples derived from cancer and corresponding normal control cells, the microsatellite sequences were independently amplified by PCR, using two fluorescently labeled primers. The PCR products were then mixed and electrophoresed in the same lane in an automated DNA sequencer. The cases showing heterozygosity were informative. These results were analyzed by comparison of the peak value of the tumor DNA with that of normal control DNA. A reduction of more than 30% in the peak value in tumor DNA in comparison to normal control DNA was judged to indicate LOH. The representative results for each category are shown. (**A**) Not-informative case. (**B**) (1) ROH (retention of heterozygosity) (2) LOH (loss of heterozygosity). Red lines: cancer; green lines: normal

Association between pAkt expression and HER2 expression, PTEN LOH and PR expression

We analyzed the pAkt expression to evaluate the Akt activation. Of these 131 cases, 48 cases (36.6%) were diagnosed to be positive for pAkt expression. In HER2-positive cases, pAkt was significantly more expressed, namely Akt was more activated ($P = 0.001$). PTEN LOH was also significantly associated with the pAkt expression, namely Akt was more activated in cases with PTEN LOH ($P = 0.002$). On the other hand, the PR expression was inversely correlated with the pAkt expression significantly ($P = 0.011$) (Table 2).

Table 1 Clinico-pathological features

Variables	Number (%)
Age (year)	55.6 ± 12.4
Axillary lymph node metastases	
Negative	72 (55.0)
Positive	50 (38.2)
N.D.	9 (6.8)
Clinical stage	
I	25 (19.1)
II	70 (53.4)
III	29 (22.1)
IV	7 (5.3)
ER	
Negative	48 (36.6)
Positive	83 (63.4)
PR	
Negative	73 (55.7)
Positive	58 (44.3)
HER2	
0, 1+	94 (71.8)
2+, 3+	37 (28.2)
pAkt	
Negative	83 (63.4)
Positive	48 (36.6)
PTEN	
ROH	100 (76.3)
LOH	31 (23.7)

Table 2 Association between pAkt expression and HER2 expression, PTEN LOH and PR expression

Factors	*n*	pAkt		*P*-value
		Negative ($n = 83$)	Positive ($n = 48$)	
HER2 overexpression				
Negative	94	68 (72.3)	26 (27.7)	=0.001
Positive	37	15 (40.5)	22 (59.5)	
PTEN				
ROH	100	71 (71.0)	29 (29.0)	=0.002
LOH	31	12 (38.7)	19 (61.3)	
PR				
Negative	73	39 (53.4)	34 (46.6)	=0.011
Positive	58	44 (75.9)	14 (24.1)	

Association of PTEN LOH and HER2 overexpression

We next examined the correlation between LOH at PTEN locus and HER2 expression. Of these 131 cases, HER2 overexpression was recognized in 37 (28.2%) cases. Interestingly, PTEN LOH also significantly correlated with HER2 overexpression ($P < 0.001$) (Table 3).