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-IREZEO 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/ mdrlb–/– 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 (A8935 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.
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\textsuperscript{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\textsuperscript{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\textsuperscript{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'-CTTACTTTTCAGTTCT-3' and 5'-GAGAATACAGCATTTTTAAGGA-3', respectively. The resulting PCR products were directly sequenced using the primer 5'-CAGTTCTACTTCTTACAACA-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 \( \beta \)-actin transcription start site, and a rabbit \( \beta \)-globin intron (18). The upstream MDR1 cDNA is translated in a cap-dependent manner, and the downstream zeocin resistance gene (ZE0) 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\textsubscript{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 \( \mu \)g/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 \( \mu \)g/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 \( \mu \)g/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')\textsubscript{2} fragment of MRK16 (100 \( \mu \)g/mL) followed by washing and incubation with R-phycocerythrin-conjugated streptavidin (400 \( \mu \)g/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 \( \mu \)g/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 DNA 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’-CACGTGGTTGGAACCTAACC-3’ and 5’-GAAGGCCAGAGCATAGATGC-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’-ATCACATTC-TTCCAGGAGCG-3’ and 5’-CCTCACCACCTTGT-GATGT-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 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’-GATATCAATGATA-TTCCAGGAGCGA-3’ and 5’-GCTTCACCACCTTGT-GATGT-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-32P]ATP, 200 μmol/L orthovanadate, 3 mmol/L MgCl2, 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], and 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/cm²) 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-32P]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 His1195 and Ile1196 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 His1195 and Ile1196 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 NIH3T3 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.
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 His1195 residue of P-gp, whereas the T3587G MDR1 results in a serine substitution for Ile1196. Importantly, both of these residues are located in the Walker B region of the second ATP-binding site of P-gp (Fig. 1A). The Ile1196 residue in the P-gp is highly conserved among the members of ATP-binding cassette transporter superfamilies, but His1195 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 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 B-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.
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 MDRI gene (24, 25).

We reported previously the construction of bicistronic vectors in which the MDRI gene is coexpressed with herpes simplex virus-thymidine kinase (26–28), α-galactosidase A (28, 29), O6-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 MDRI 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 BCRP on their cell surfaces. We also showed that the transfectants possess similar plasmid copy numbers (Fig. 2C) and similar levels of MDRI 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 MDRI retrovirus showed slightly lower P-gp expression levels than those transduced with WT MDRI 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.

Figure 5. ATP-binding activities in the mutant MDRI 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-azidoo-[α-32P]ATP and 200 μmol/L vanadate in the presence or absence 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-gp. 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-gp. D, relative ATP-binding activity of mutant P-gp. The trapped 8-azido-[α-32P]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.
Dubin-Johnson syndrome is an inherited disorder characterized by chronic conjugated hyperbilirubinemia due to the absence or dysfunction of the multidrug resistance-associate 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 Arg1392 and Met1393 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 Asp1286 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 Ile1286 results in the loss of ATP-binding activity but that the substitution of tyrosine for His1286 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 expect 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 subgroup 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 Gln141 residue of BCRP, is frequently observed in Japanese populations. Significantly, the Gln141 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 iflomotecan, 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.
Nonfunctional P-glycoprotein Mutant

References

Molecular Cancer Therapeutics

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

Kazuyoshi Mutoh, Junko Mitsuhashi, Yasuhisa Kimura, et al.

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