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A Novel Human Multidrug Resistance Gene *MDR1* Variant *G571A* (G191R) Modulates Cancer Drug Resistance and Efflux Transport

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Abstract

The human multidrug resistance gene *MDR1* encodes a membrane-bound transporter P-glycoprotein (Pgp) that confers the drug resistance of cancer cells by mediating an ATP-dependent drug efflux transport. We and others have reported a number of functionally significant *MDR1* variants, including *G1199A* and *G1199T*, that modulate cancer drug resistance and intracellular levels of antivirals. In this report, we describe a novel *G571A* variant of *MDR1* detected in 6.4% of leukemia patients. Because this nucleotide modification gives rise to an amino acid change from Gly to Arg at the 191 amino acid position of Pgp, we have developed and characterized the functional affect of the *G571A* variant in stable, recombinant cells. Using six chemotherapeutic drugs, doxorubicin HCl, daunorubicin HCl, vinblastine sulfate, vincristine sulfate, taxanes (paclitaxel), and epipodophyllotoxin (etoposide, VP-16), we found that the *MDR1*_{571A} variant selectively reduced the degree of Pgp-mediated resistance in drug-dependent manner. Although there was a minimal effect on doxorubicin and daunorubicin, the *MDR1*-dependent resistance on vinblastine, vincristine, paclitaxel, and etoposide was reduced by approximately 5-fold. The increased drug sensitivity in *MDR1*_{571A}, compared with *MDR1*_{wt} paralleled the intracellular drug levels. These data suggest that individuals with this novel *MDR1* variant, the *571A* genotype, may be more sensitive to the specific anticancer drugs that are Pgp substrates.

The human multidrug resistance gene (*MDR1* or *ABCB1*) encodes the P-glycoprotein (Pgp). As one of the ATP-binding cassette superfamily of transporters, Pgp is expressed broadly in epithelial tissues throughout the body, including intestine, liver, kidney, blood-brain barrier, and placenta (Schinkel et al., 1997). As a transmembrane protein, the active efflux drug transporter, Pgp, recognizes a large number of natural compounds and lipophilic xenobiotics (Lin and Yamazaki, 2003). Substrates of Pgp include anticancer, cardiovascular, anti-HIV, immunosuppressant, and β -adrenergic drugs. Because of the broad substrate recognition, it has been a challenge to predict effects of disrupting Pgp-mediated transport. Nevertheless, Pgp expression has been demonstrated to influence the treatment of cancers, infectious diseases (HIV, tuberculosis) (Camus et al., 2006), inflammatory bowel disease (Annese et al., 2006), and organ transplantation (Pauli-Magnus and Kroetz, 2004). Thus, variation in Pgp functions may lead to significant clinical consequence.

The human *MDR1* spans over 100 kb on human chromosome 7q21. The *MDR1* genome contain 29 exons that give rise to a 3843-bp sequence of transcripts encoding the 1280-amino acid Pgp protein with a molecular mass of 170 kDa. Pgp has 12 transmembrane

domains and two ATP binding sites (Gottesman et al., 1996; Ambudkar et al., 1999). Variations of *MDR1* sequences have been well studied, and a long list of single nucleotide polymorphisms (SNPs) has been reported; however, their functional affects are not yet fully understood (Woodahl and Ho, 2004). The high-frequency *MDR1* SNPs include C1236T (exon 12), G2677T or A (exon 21), and C3435T (exon 26). Some of these SNPs are genetically linked. Their effects on the protein function have been studied in vivo and in vitro (Kim et al., 2001a). In a recent study, *MDR1* SNPs that do not alter amino acid sequence also have been shown to result in functional variations. These sequence variations seemed to slow down Pgp translation, leading to a change in protein conformation and altered substrate specificity (Kimchi-Sarfaty et al., 2007).

Pgp is also expressed in leukocytes, hematopoietic stem cells, and leukemia cells. The high degree of *MDR1* expression contributes to multidrug resistance in patients with leukemia. In particular, genetic polymorphisms of *MDR1* have been found to affect therapeutic outcome in acute myeloid leukemia patients (Illmer et al., 2002). Our ongoing effort to identify functionally significant *MDR1* genetic variations in the coding sequences of *MDR1* in leukemia patients with either myelodysplasia (MDS) or acute myelogenous leukemia (AML) has led to the discovery of a number of novel variations that exhibit functional impacts. We found that the *MDR1 G1199A* variation, which results in a serine-to-asparagine transition at amino acid 400 in a cytoplasmic domain of Pgp, alters efflux and transepithelial transport and the drug sensitivity to chemotherapeutic agents (Woodahl et al., 2004). The *MDR1 G1199T*, on the other hand, exhibits some degree of reversal on cancer drug resistance compared with the wild-type *MDR1*.

In this report, we have characterized a new *MDR1* variation, *G571A*, which has given rise to G191R amino acid transition. This *MDR1* variation, located in exon 7 and mapped to within the third transmembrane domain of Pgp, exhibits 6.4% frequency in the MDS and AML patient population. Using stable recombinant HEK host cells expressing either *MDR1* wild-type or *MDR1 571 G→A* variant, we have systematically characterized the functional differences between the *MDR1_{wt}* and *MDR1_{571A}* cells. Our data indicate that the *MDR1_{571A}* variant selectively reduced the degree of Pgp-mediated resistance in a drug-dependent manner.

Materials and Methods

Chemicals and Drugs

All chemotherapeutic agents used for this study, doxorubicin HCl, daunorubicin HCl, vinblastine sulfate, vincristine (VCR), paclitaxel, and etoposide, were obtained through the drug service of the University of Washington Medical Center. Each drug was diluted with culture medium to a wide range of concentration. The multidrug resistance protein inhibitor GF120918 (Elacridar) was kindly provided by GlaxoSmithKline (Cambridge, Middlesex, UK) (Hyafil et al., 1993). Rhodamine 123 was from Sigma-Aldrich (St. Louis, MO). Bodipy FL-vinblastine and paclitaxel (Tubulin Tracker green reagent), tissue culture media DMEM, trypsin-EDTA, and antibiotic solutions were supplied by Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). *MDR1* antibody C219 was purchased from Calbiochem (San Diego, CA), and F4 antibody was purchased from Sigma-Aldrich. The monoclonal antibody MRK16 was from Kamiya Biomedical (Thousand Oaks, CA). The CellTiter-Glo luminescent cell viability assay kit was from Promega (Madison, WI). Other reagents were of analytical grades or higher.

Cell Culture

Recombinant HEK cells expressing *MDR1_{wt}* and *MDR1_{571A}* vectors were grown in complete media consisting of DMEM (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratories), 1% (v/v) antibiotic-antimycotic, and 1% (v/v) nonessential amino acid and grown at 37°C in the presence of 5% CO₂ in a humidified incubator. Cells were periodically treated with Geneticin (G-418; Calbiochem) at 200 µg/ml for routine maintenance.

Identification of *MDR1* 571 Variation

Samples were collected from patients 55 to 85 years old with confirmed MDS or AML by cytological or cytogenetic criteria recruited from the University of Washington Medical Center and by the Veterans Administration Puget Sound Health Care System according to approved protocols from the human subject review committee. Patients with an M3 subtype of AML were excluded from this study because of the variation of their therapy. Patients with recent exposure to a chemotherapeutic agent, low serum folate, or vitamin B₁₂ were also excluded. The blood or bone marrow samples were collected, and RNA was extracted from the isolated peripheral blood mononuclear cell as described before (Yang et al., 2002b). RNAs were subjected into RT-PCR using human *MDR1*-specific primer pairs, which cover the entire *MDR1* cDNA (GenBank accession no. M14758) regions. The amplified cDNA was separated in agarose gel, and the gel slices containing the cDNA fragment were isolated. The cDNAs were extracted using a QIAGEN gel extraction kit (QIAGEN, Valencia, CA) following the supplier's manual. The sequence of the extracted DNA was determined using the ABI automated DNA sequencer 370 (Applied Biosystems, Foster City, CA) in our DNA Sequencing and Gene Analysis center. Sequences were assembled using Sequencher software (Gene Codes Corporation, Ann Arbor, MI) or VectorNT software (Invitrogen) and compared with human *MDR1* reference (Gen-Bank accession no. M14758). These data were verified with a genomic sequence of the region of interest.

Generation of *MDR1_{wt}* and *MDR1_{571A}* Expression Vectors

The generation of *MDR1* expression plasmid had been reported in our laboratory as detailed previously (Yang et al., 2002a,b). The neomycin-resistant plasmid containing human *MDR1* wild-type cDNA (*MDR1_{wt}*) was employed as a template to generate the *MDR1_{571A}* variant. A site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used for this purpose. The primer sequences were as follows: forward, 5'-GTTATTGG TGACAAAATT AGAATGTTCT TTCAGTC-3' and reverse, 5'-GACTGAAAGAACATTCTAATTTTGTCCACCAATAAC-3'.

The generated clones were screened by size exclusion and restriction enzyme digestion. The full length of the cDNA containing mutation clones was verified by DNA sequencing using our DNA sequence center facility (Big-Dye 3.0 Chemistry and an ABI Prism 377 DNA Sequencer; Applied Biosystems).

Development and Characterization of Stable Recombinant HEK Cells Expressing Human *MDR1_{wt}* or *MDR1_{571A}* Variant

The pcDNA3.1 vector and *MDR_{wt}* and *MDR1_{571A}* plasmids were transfected into the mammalian HEK cells. In brief, HEK cells were seeded into a 24-well plate. Transfection was conducted when the cells reached approximately 90% confluence. The growth medium of the cells was replaced with serum-free medium (Opti-MEM; Invitrogen) for 30 min. The cells were then allowed to grow in a growth medium containing serum. At 24 h after transfection, cells from one or two wells were trypsinized and plated into a 100-mm Petri

dish at a dilution of approximately 1:20 in a DMEM growth medium. At 48 h post-transfection, G-418 at 500 µg/ml was added. The medium containing G-418 was refreshed every 3 to 4 days for 3 to 4 weeks, and cells that survived under G-418 selection pressure were used subsequently for clone selection. Single cell colony was picked into a 12-well plate. Cells grown from the single colony were seeded into new 12-well plates, and rhodamine 123 (R123) uptake and efflux were conducted to select the polyclonal cells with high Pgp activities. The cells with high Pgp activity were further expanded and tested. Cells with high Pgp activity stable for at least three times for R123 uptake were stored and used for functional evaluation.

Characterization of Pgp Expression by mRNA Quantitation

Total RNA from HEK cells transfected and *MDR1*_{wt} and *MDR1*_{571A} variant were extracted using the Ultraspec total RNA isolation kit according to the manufacturer's instruction (Biotecx Laboratories, Inc., Houston, TX). To compare the expression level, absolute quantitation of *MDR1* mRNA transcripts in transfected cellular samples was performed using the ABI Prism 7900HT Detection System (Applied Biosystems). An *MDR1* RNA standard was generated by in vitro transcription using the T7 promoter on the plasmid described previously (Yang et al., 2002b), and concentration was measured by absorbance at 260 nm and converted to the number of copies of *MDR1* RNA by the mol. wt. A dilution series of the *MDR1* RNA standard was used to generate a standard curve of the number of copies of *MDR1* mRNA versus threshold cycle value. After RNA isolation, 1 µg of total RNA of each sample was subjected to RT. Fifty nanograms of total RNA was analyzed in quadruplicate to obtain a threshold cycle value and an estimation of the number of copies of *MDR1* mRNA in recombinant HEK cells. To validate the assay, a housekeeping gene, *hGUS*, was detected at the same time to eliminate the sample variation.

Characterization of Pgp Expression at the Protein Level

Immunoblot analysis was used to detect protein expression. In brief, approximately 1×10^7 cells were pelleted and washed in PBS and lysed in a lysis buffer containing 1% Nonidet P40 and a protease inhibitor cocktail (Calbiochem). Protein concentration was measured by a bicinchoninic acid microplate assay protocol (Pierce Chemical, Rockford, IL), and all samples were diluted into the same concentration using the lysis buffer and mixed with a 5× loading buffer containing 0.1% SDS and β-mercaptoethanol. Electrophoresis and transfer of membrane protein were done according to instructions for Mini-PROTEAN II Electrophoresis (Bio-Rad, Hercules, CA). Proteins were transferred onto polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA) and blocked with 5% nonfat milk in Tris-buffered saline/Tween 20 buffer (20 mM Tris-HCl, 0.9% NaCl, 0.05% Tween 20, pH 7.6). Immunoblotting was performed with the anti-Pgp monoclonal antibody C219 or F4, followed by a secondary horseradish peroxidase-conjugated goat anti-mouse IgG. ECL plus reagents (GE Healthcare, Chalfont St. Giles, UK) were used as substrate, and blots were exposed to X-ray film for visualization of the protein bands.

Cellular Localization of Bodipy-Labeled Vinblastine, Paclitaxel, and Doxorubicin in *MDR1* Recombinant Cells

Control and recombinant *MDR1* HEK cells (wild type and 571 variant) were seeded onto eight-cell chamber slides for 2 days at 37°C. Cells were briefly washed with PBS. Doxorubicin HCl (7 µM), bodipy FL-vin-blastine (1 µM), and paclitaxel (Tubulin Tracker green reagent, 1 µM) were added to the cell in duplicate in a chamber containing serum-free DMEM and incubated for 30 min. The untreated control wells were filled with the medium only. The cells that were grown in the glass slides were washed three times with ice-cold PBS and dried. They were analyzed using a fluorescence microscope (Zeiss Axiovert 200; Carl Zeiss GmbH, Jena, Germany), and the presented images were recorded under a fixed

exposure and identical settings. For each treatment, at least 10 fields of the slides were viewed, and at least three photos were taken. The experiment was replicated twice.

Evaluation of Cancer Drug Resistance

The drug resistance of stable recombinant cells expressing *MDR1*_{wt} and *MDR1*_{571A} transfected was evaluated by measuring the cell viability after exposing them to a wide range of the selected substrates following our previous description (Woodahl et al., 2004; Yang et al., 2004). In brief, the cells (control, *MDR1*_{wt}, *MDR1*_{571A}) were seeded at 1×10^4 density/well in a 96-well plate format in a 100- μ l culture medium overnight. Media were replaced with 100 μ l of media containing a wide range of dilution of selected chemotherapeutic agents alone or with a Pgp inhibitor, GF120918. Each concentration of the drug was run in quadruplicate at least, or experiments were repeated at least twice. The culture was maintained at 37°C 5% CO₂ in a humidified incubator for 72 h. Cell viability was measured with a CellTiter-Glo cell viability assay kit (Promega). Data from each quadruplicate was converted to the percentage of their controls with 0% inhibition at zero concentration of the drug and maximal inhibition at the highest concentration. IC₅₀ values were obtained using SigmaPlot software. The relative resistance ratio was calculated comparing the IC₅₀ value of recombinant *MDR1* cells with that of control host cells. These data were replicated at least twice to validate the IC₅₀ values. The variations experiments were less than 10%.

Pgp-Mediated Efflux Transport Studies Using Rhodamine123 as a Substrate

Cells were seeded into 24-well plates precoated with poly-D-lysine at 5×10^5 /well and incubated for 3 days at 37°C until 95% confluence. Cells were washed briefly with Dulbecco's phosphate-buffered saline plus glucose containing medium (DPBSG), pH 7.2, and loaded with 0.5 ml of DPBSG or inhibitor for 10 min. The DPBSG or inhibitor was then replaced with 1 μ M rhodamine 123 or rhodamine 123/inhibitor in DPBSG buffer for 30 min at 4°C. After moving the rhodamine123, 1 ml of DPBSG or DPBSG with inhibitor at 37°C was added into each well, and efflux was started in a water bath at 37°C. At the indicated time point, the cells were washed twice with ice-cold DPBSG. One percent Triton X-100 (0.5 ml) was added into each well. The amount of intracellular rhodamine123 was measured using a fluorometer (PerkinElmer Victor3V; PerkinElmer Life and Analytical Sciences, Waltham, MA) at $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{ex}} = 530$ nm.

Results

A Novel MDR1 Variant, 571 G→A Variant in Leukemia Patients

As an ongoing project to explore the effects of *MDR1* sequence variation in functional impact of Pgp, we collected mononuclear cells from leukemic patients undergoing bone marrow evaluation. We then sequenced the entire coding sequence of the cDNA derived from the RNA samples. These sequences were validated with direct sequence analysis of the genomic DNA for the respective regions. Of 78 samples, we found a novel variant in five subjects exhibiting the heterozygous genotype, *G571G/A*. The genotypic frequency was recorded at 6.4%, and the allelic frequency for the *571A* was estimated to be 3.2% (Table 1). A representative of the sequence chromatograph from patients showing the A/G overlapping is presented in Fig. 1. The G→A transition at position 571 translates into an amino acid change from Gly to Arg at position 191 of the human *MDR1* protein Pgp. Based on the predicted Pgp structure, it is mapped to exon 5 and predicted to be located within the third transmembrane domain. Such variation is likely to modulate the Pgp efflux transporter function. Hence, we developed stable, recombinant cells to evaluate functional significance of this novel *MDR1* variation at cellular and functional levels.

Development and Characterization of an *MDR1* Recombinant Cell Expressing *G571A*

To evaluate the functional impact of the novel *MDR1* variant, *571 G→A* (191 Gly→Arg amino acid), we expressed it in a mammalian host, HEK cells. The stable recombinant HEK cells were selected with G-418 before full characterization with respect to the *MDR1* sequence and protein expression. The function of the stable recombinant cells expressing *G571A* was compared with previously established recombinant HEK cells expressing *MDR1* wild type (517G at the 191 amino acid position).

By direct sequencing confirmation of the *MDR1* DNA isolated from the stable recombinant HEK cells, we found that these cells faithfully retained the *G571A* variant with no other sequence modifications. Using fluorescence R123 efflux assay, we identified three to five clones that exhibited consistent Pgp functions for at least 2 months. The Pgp protein expression levels were evaluated by immunoblot analysis. As shown in Fig. 2, the selected HEK recombinant cells expressing *G571A* exhibited comparable levels of Pgp protein and RNA transcript (based on mRNA quantitation) with the cells expressing wild-type *MDR1*. Furthermore, immunofluorescent staining of the cells using the *MDR1* monoclonal antibody F4 exhibited similar profile of Pgp expression for both in *MDR1* wild type and *G571A*, and expression levels are significantly higher than those of the control cells (data not shown). Both *MDR1* wild type and *G571A* exhibited similar molecular mass (~160 kDa) using Western immunoblot analysis (Fig. 2). These cells were used for all subsequent functional analyses.

Effects of *G571A* Variant on Pgp Efflux Function and Pgp-Dependent Drug Resistance

To determine the effects of *MDR1 571 G→A* transition on Pgp transporter function, we first evaluated the intracellular concentration of HEK recombinant cells expressing either *MDR1_{wt}* or *MDR1_{571A}*. Expression of *MDR1* in HEK host cells reduced the intracellular levels of a fluorescence substrate R123 by 37 and 33% in *MDR1_{wt}* and *MDR1_{571A}* variant, respectively (Table 2). However, the differences in intracellular concentration of R123 between the recombinant HEK cells expressing *MDR1_{wt}* and *MDR1_{571A}* were not statistically significant. The reduction in intracellular R123 levels was due to Pgp expression because this function can be reversed with an established Pgp inhibitor, GF120918. In the presence of 1 μ M GF120918, both Pgp-expressing cells retained R123 levels similar to that of Pgp-negative control cells (Table 2).

We next determined the effect of *571 G→A* transition on cancer drug resistance and we compared cytotoxicity using six commonly used drugs that fall within four classes of chemotherapeutic agents that are known substrates of Pgp. HEK recombinant cells expressing *MDR1* variants were exposed to these compounds at varying concentrations for 3 days. Cell survival was recorded and shown in Table 3. We found that the expression of *MDR1_{wt}* or *MDR1_{571A}* conferred varying drug resistance to the HEK cells. The record range was from 10- to 150-fold for the drugs tested. For the two anthracyclines, doxorubicin and daunorubicin, insignificant differences were found between *MDR1_{wt}* and *MDR1_{571A}*-expressing cells. However, effects of *571 G→A* transition significantly altered the drug resistance profiles for vinblastine, vincristine, paclitaxel, and etoposide. The *G→A* transition slightly reduced the *MDR1* resistance to vincristine and vinblastine. In contrast, recombinant *MDR1_{571A}*-expressing cells exhibited approximately 4-fold reductions in resistance to paclitaxel (IC_{50} = 382 versus 91 nM) and etoposide (2518 versus 568 nM) compared with *MDR1_{wt}* (Table 3). None-theless, both the wild type and *571A* variant of *MDR1* conferred significant resistance to all tested drugs, compared with that of the Pgp-negative control (Table 3). These data are reproducible and were replicated at least twice. Taken together, these data indicate that the *G→A* transition at the 571 nucleotide position of *MDR1* with predicted Gly→Arg at 191 amino acid change seemed to reduce cancer drug

resistance from nominal to approximately 5-fold for a selective subset of chemotherapeutic drugs commonly used for leukemia therapy.

Effect of a Pgp Inhibitor on Reversing *MDR1*-Dependent Efflux Transport and Drug Resistance

To further characterize the time course of Pgp efflux transport variations for the *MDR1*_{571A} variant and to verify the effect of Pgp, we employed GF120918 as a Pgp inhibitor (Hyafil et al., 1993). GF120918 has been shown to be a potent inhibitor of Pgp but also inhibits breast cancer resistance protein. As the HEK host cell did not express a significant endogenous level of breast cancer resistance protein, GF120918 is used presently as a specific Pgp inhibitor. We proceeded to determine the time course of transport efflux in recombinant HEK cells expressing either *MDR1*_{wt} or *MDR1*_{571A}. The recombinant cells expressing *MDR1*_{wt} exhibited a faster decline to lower intracellular R123 concentrations compared with that of *MDR1*_{571A}, suggesting that Pgp derived from *MDR1*_{571A} is less efficient in efflux transport of this substrate (Fig. 3). The time and the *MDR1* dependent decline in R123 activity was Pgp dependent because the efflux activity was reversed by the presence of 1 μ M GF120918. The GF120918 did not influence the time course of intracellular R123 in control host cells.

We next determined whether the Pgp inhibitor GF120918 could reverse drug resistance in HEK cells expressing *MDR1*_{wt} or *MDR1*_{571A}. To do so, varying concentration of doxorubicin, vincristine, and paclitaxel were incubated with these cells in the presence or absence of 1 μ M GF120918. Fifty percent effective inhibitory concentrations (IC₅₀ values) were derived from a respective dose-response curve. As shown in Fig. 4, the IC₅₀ values for the three drugs were reduced significantly in the presence of GF120918 irrespective of whether the recombinant cell expressed *MDR1*_{wt} (Fig. 4A) or *MDR1*_{571A} (Fig. 4B). Taken together, these data suggest that both R123 efflux transports and higher IC₅₀ values observed for cancer drugs in the recombinant cells expressing *MDR1*_{wt} or *MDR1*_{571A} are Pgp dependent.

Differential Accumulation of Doxorubicin, Vinblastine, and Paclitaxel in the Recombinant *MDR1* Cells

To evaluate whether the reduction in intracellular drug concentration is a potential mechanism of differences in IC₅₀ values recorded for the wild type and 571A variant of *MDR1* recombinant cells, we surveyed the intracellular localization and distribution of three fluorescence anticancer drugs. Doxorubicin (a fluorescent compound), bodipy fluorescent-labeled vinblastine, and paclitaxel (labeled with tubulin tracker green) were used for this purpose. All three compounds produce green fluorescence for evaluation of drug localization and distribution. After exposing the cells to each of the three compounds for 30 min at 37°C, they were recorded with a fluorescence microscope and photographed at fixed exposure parameters. As shown in Fig. 5, doxorubicin, a DNA-interacting drug widely used in chemotherapy, exhibited a high degree of drug localization in the nucleus but did not seem to produce a differential staining pattern between the two *MDR1* variants. Bodipy FL-vinblastine is a fluorescent analog of the vinblastine that inhibits cell proliferation by capping microtubule ends, whereas Paclitaxel bis-acetate provides green fluorescent staining of polymerized tubulin in living cells, specifically, paclitaxel binds to the β -subunit of tubulin. Therefore, both compounds are good candidates for our staining purpose to visualize the drug uptake and distribution in the cells. For vinblastine and paclitaxel, fluorescence intensity of these two drugs in control cells was much higher than the two *MDR1* recombinant cells. Paralleled with the differences in IC₅₀ values, the green fluorescence for vinblastine and paclitaxel in 571A variant cells was some-what brighter than the *MDR1* wild-type cells under identical imaging conditions, suggesting a more

efficient efflux transport function in cells expressing *MDR1*_{wt}. Together, these results collected with the three fluorescent anticancer compounds in *MDR1* recombinant cells suggest that variations in intracellular drug levels probably lead to reduced drug resistance in *MDR1*_{571A}, compared with *MDR1*_{wt}.

Discussion

As a continued effort to elucidate the pharmacogenetic impact of *MDR1* sequence variations on the drug disposition and pharmacokinetics, we discovered a novel genetic variant *G*→*A* transition at 571 nucleotide position of *MDR1* in humans. This genetic variation translates into a Gly→Arg transition at the 191 amino acid position of Pgp. This variant was identified as heterozygous in five of 78 leukemia patients, representing a 3.2% allelic frequency (Table 1). Whether the same frequency exists in healthy subjects or patients with other types of diseases remains to be determined. With the aid of well characterized stable recombinant cells expressing *MDR1* (191Gly) and the variant (191Arg), we found that both express a similar degree of functional Pgp on cell surfaces. Depending on the cancer drug tested, the variant seemed to exhibit efflux transport and related drug resistance at equal or lower potency.

Although the exact mechanism leading to substrate dependent variations is not clear for the Pgp transition at 191 Gly→Arg, it is possible that the net positive charge may contribute to the altered efflux of Pgp function. Amino acid 191 is mapped to the third transmembrane domain, where the added positive net charge may regulate the substrate affinity and transport. Although this is plausible, Arg in the same position may also alter overall conformation of Pgp leading to 4-to 5-fold IC₅₀ reduction of vinblastine, vincristine, paclitaxel, and etoposide. However, in the absence of Pgp protein crystal, the conformational details at atomic levels remain elusive.

Previous studies using site-directed mutagenesis to probe Pgp functions also found selective alterations of transport function for specific drug substrates (Loo and Clarke, 1993a,b, 1994a,b). It is generally believed that multiple binding domains in the drug-binding pocket exist for Pgp, which may account for the drug-specific alteration in Pgp efflux because of amino acid sequence variations (Martin et al., 2000; Loo et al., 2003). The observed differential sensitivity to cytotoxic agents because of 571 *G*→*A* may be important in modulating the efficacy of chemotherapy. The cancer patients with *MDR1* 571A or 571A/G genotype will probably be more sensitive to vinblastine, vincristine, and paclitaxel and equally sensitive to doxorubicin or daunorubicin. However, subjects exhibiting *MDR1* 571A or 571A/G genotypes will also be more susceptible to dose-limiting toxicities because *MDR1* is expressed widely in healthy cells and tissues, including those in gut mucosa, liver, and kidney, in drug clearance or elimination. This and other functional results could be used in the future to provide more effective drug selection and pharmacologic optimization in leukemia treatment and to overcome chemoresistance in other diseases. Our results imply that the patients with 571A may be more responsive to paclitaxel, etoposide, vincristine, or vinblastine than those with the wild-type 571G genotype.

Reports in the literature are contradictory with regard to the influence of *MDR1* polymorphisms (primarily *G2677T* and *C3435T*) on Pgp expression and function at the cellular level. As described earlier, multiple binding domains may exist in Pgp, and variations in the amino acid sequence of the protein may alter binding and efflux of some drugs but not others. Thus, the selection of substrates may be important in assessing changes in Pgp activity and could explain some observed differences. In addition, some vectors carry unique membrane components and host cells that may influence the activity of Pgp. For example, retroviral vectors containing *MDR1* variants at 2677, designed to express Ala893

or Ser893 in NIH-3T3 mouse embryonic cells, suggested that Ser893-expressing cells exhibited decreased digoxin accumulation than Ala893 cells, indicating enhanced Pgp efflux (Kim et al., 2001b). On the other hand, a vaccinia virus-based transient expression system of several *MDR1* polymorphisms (*A61G*, *T307C*, *G1199A*, *G2677T*, and *G2995A*) in HeLa cells showed no difference in Pgp expression and efflux of various drugs (Kimchi-Sarfaty et al., 2002). Some of these differences may be because of the transient protein expression system. The cells used to express the variant *MDR1* genes can also have variable intrinsic transporters, many of which contribute to the efflux of Pgp substrates. The recombinant stable expression system we have developed to generate cell lines that continuously express *MDR1* variants will be useful for study in detailing the complex interactions of *MDR1* polymorphisms.

We have now validated an expression system consisting of *MDR1* mammalian expression vectors and host cells capable of reproducibly expressing Pgp variants. This strategy can be readily adapted to investigate other SNPs and haplotypes of *MDR1* and provide insight into the functional significance on the more than 30 SNPs reported in the literature. For instance, the influence of C3435T may be evaluated to determine whether or not this synonymous polymorphism changes expression or activity of Pgp, with which it has been reported to be associated. In addition, the effect of *MDR1* haplotypes can also be evaluated in this system because multiple SNPs can be expressed at once in the same plasmid. Multiple SNPs found on the same chromosome are assigned to a specific haplotype, and some attempts have been made to determine the role of *MDR1* haplotypes in Pgp variability, but the data are still inconclusive (Kim et al., 2001b; Johne et al., 2002; Kroetz et al., 2003). Our expression system may be useful in sorting out the role of *MDR1* haplotypes. By design, this continuous system focuses on genetic modifications in the coding region of *MDR1* that alter the structure of Pgp. Other strategies specifically designed to evaluate the effects of promoter and intronic SNPs may be needed to address regulation of *MDR1* expression.

In summary, we report here a novel polymorphism of *MDR1 G571A* found in the leukemia patient. This *MDR1* polymorphism confers moderate drug resistance to recombinant HEK cells but is less efficient than that of *MDR1* wild type for selected drugs. Further studies to correlate the in vitro data with in vivo finding may help us to understand its clinical significance.

Acknowledgments

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ABBREVIATIONS

<i>MDR1</i> or <i>ABCB1</i>	the human multidrug resistance gene
Pgp	a membrane-bound transporter P-glycoprotein that confers the drug resistance of cancer cells
SNP	single nucleotide polymorphism
MDS	myelodysplasia
AML	acute myelogenous leukemia
HEK	human embryonic kidney

GF120918, Elacridar	[<i>N</i> -(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide]
DMEM	Dulbecco's modified Eagle's medium
RT	reverse transcriptase
PCR	polymerase chain reaction
R123	rhodamine 123
PBS	phosphate-buffered saline
DPBSG	Dulbecco's phosphate-buffered saline plus glucose containing medium

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Fig. 1.

A representative nucleotide sequence alignment of human *MDR1* from wild type and a subject with the 571 A/G genotype. RNA from patients was amplified using RT-PCR. The RT-PCR fragment was sequenced and aligned with the human *MDR1* database (GenBank accession no. M14758) using Vector NTI (Invitrogen). One of the subjects exhibiting G/A at 571 was presented. The upper part of the chromatograph comes from a patient exhibiting *MDR1* wild type at the 571 position.

Fig. 2.

Evaluation of *MDR1* protein and mRNA expression in recombinant *MDR1* HEK cells by Western blot and real-time RT-PCR. For Western blot, transfected and mock cell lysates (1 μ g/lane) were separated on SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The blot was blocked with 5% milk and probed with the anti-human *MDR1* monoclonal antibody F4. After the incubation with secondary goat anti-mouse IgG, the signal was detected by adding ECL-Plus chemiluminescent horseradish peroxidase reagent and exposed to X-ray film. Lane 1, molecular mass marker; lane 2, *MDR1* wild type; lane 3, *MDR1* 571 variant; lane 4, mock cells. For quantitative RT-PCR, total RNA was extracted from each cell type. *MDR1* RNA copy number was estimated by real-time RT-PCR with an ABI Prism 7900HT Detection System using an ABI *MDR1* probe and an in vitro transcribed *MDR1* mRNA as standard. Each sample was run in quadruplicate, and data were shown as mean $\pm$ S.D.

Fig. 3.

Time-dependent efflux of rhodamine 123 in recombinant *MDR1* HEK cells. Rhodamine 123 at 1 μ M (○) or R123 with 1 μ M *MDR1*-specific inhibitor GF120918 (□) was added to the control (C) and *MDR wt* (A) and *571A* (B) variant expressed in HEK host cells for 45 min at 4°C as described under *Materials and Methods*. After removing the R123 and a quick wash with PBS, efflux was started and terminated at each time point. The intracellular remaining of R123 was measured. The data showed that the efflux activity in *MDR1* expressed cells was approximately 2 times compared with control cells. Efflux activity in *MDR1 wt* was slightly higher than the *571A* variant, and this activity could be inhibited with a Pgp inhibitor, GF120918.

Fig. 4.

Effect of a Pgp-specific inhibitor GF120918 on reversing *MDR1*-dependent drug resistance in recombinant *MDR1* HEK cells. HEK control cells and *MDR1* expressed cells were seeded at 1×10^4 /well in a 96-well plate. Respective chemotherapeutic drugs (doxorubicin, vincristine, or paclitaxel) at indicated concentrations (in quadruplicate) were added and exposed for 3 days with or without GF120918 inhibitor (at 1 μ M concentration). Cell viability was measured by adding the chemiluminescent reagent following the manufacturer's instruction. These data were analyzed as the percentage of the control, and IC₅₀ values were obtained from the SigmaPlot program. The IC₅₀ data (open column) shown is mean $\pm$ S.E. Resistance conferred to HEK cells because of *MDR1* expression

(open column) can be abolished with a Pgp inhibitor, GF120918 (hatched column). These data were replicated at least twice. A, *MDR1 wt* recombinant cells; B, *MDR1 571A* recombinant cells; C, HEK host control cells (lacking MDR1 expression).

Fig. 5.

Intranuclear or intracellular accumulation of doxorubicin, vinblastine, or paclitaxel analogs in recombinant *MDR1* HEK cells as shown by fluorescence intensity and distribution. Control and *MDR1* recombinant HEK cells were incubated with doxorubicin (A, D, and G), bodipy-FL-vinblastine (G, E, and H), or paclitaxel (C, F, and I) for 30 min at 37°C in a chamber slide. Cells were washed with PBS three times and viewed under fluorescence microscopy and photographed at constant exposure for all slides (1000× magnification for doxorubicin and 400× for vinblastine and paclitaxel). Less accumulation of doxorubicin, vinblastine sulfate, or paclitaxel by exhibiting weak fluorescence was observed in both wild-type and 571 variants compared with control cells. The result was consistent with the drug cytotoxicity data and R123 efflux data. A to C, HEK host cells; D to F, *MDR1 wt* recombinant cells; G to I, *MDR1 571A* recombinant cells. Intracellular drug concentrations, estimated based on mean $\pm$ S.D. fluorescence intensity (arbitrary units for equivalent cell surface area) for *MDR1 wt* and *MDR1 571A* recombinant cells are as follows: doxorubicin = 93.1 ± 3.3 versus 88.4 ± 0.8 ; vinblastine = 44.4 ± 0.5 versus 72.7 ± 0.51 ($p < 0.005$); and paclitaxel = 37.7 ± 1.8 versus 101.2 ± 2.15 ($p < 0.05$).

TABLE 1
Frequency of human *MDR1* 571 genotypes in leukemia patients

Total RNA from peripheral blood mononuclear cell was used two times, or bone marrow of patients was extracted and amplified by RT-PCR. The PCR product was purified and sequenced. Data were analyzed and compared with the human *MDR1* sequence (GenBank accession no. M14758)

Number of Subjects	<u><i>MDR1</i> G571 Genotype</u>		
	G/G	G/A	A/A
78	73	5	0
Genotype frequency (%)	93.6	6.4	0

TABLE 2
Intracellular level of Pgp substrate rhodamine 123 in recombinant cells expressing human
***MDR1*_{wt} and *MDR1*_{571A} variant**

Rhodamine 123 at 1 μ M in buffer was added into 24-well plates containing control, recombinant HEK cells expressing *MDR1*_{wt} or *MDR1*_{571A} for 45 min at 37°C. Plates were quickly washed with precooled DPBSG buffers twice. One percent Triton X-100 (0.5 ml) was added into each well. Intracellular fluorescence was measured with a Victor 3V plate reader operating at excitation of 485 nm and emission of 530 nm. Protein concentration was determined by bicinchoninic acid microplate assay. The raw FL count was normalized to fluorescence units per microgram of protein. For the GF inhibition experiment, 1 μ M GF120918 (GF) was exposed to the cells for 5 min prior to the addition of R123. The data expressed was the mean $\pm$ S.D. of quadruplicate samples.

Recombinant Cells	Intracellular R123 Concentration		
	Without Inhibitor	With GF Inhibitor	Less than Control %
	<i>arbitrary units/μg protein</i>		
Control	61.1 $\pm$ 6.84	74.3 $\pm$ 1.18	0
<i>MDR1</i> _{wt}	38.5 $\pm$ 2.40	68.5 $\pm$ 8.29	37
<i>MDR1</i> _{571A}	41.0 $\pm$ 0.63	66.8 $\pm$ 1.94	33

Effects of *MDR1* 571 G→A (Pgp 191 Gly→Arg amino acid) variation on cytotoxicity of recombinant HEK cells to chemotherapeutic agents^a

TABLE 3

Drug	Control: IC ₅₀ ^b	Recombinant HEK Cells			
		MDR1 _{wt} (191Gly)		MDR1 _{571A} (191Arg)	
		IC ₅₀	R Value ^c	IC ₅₀	R Value
<i>nM</i>					
Doxorubicin	25.1 ± 2.55	250.2 ± 24.5	9.971	244.6 ± 18.6	9.75
Daunorubicin	22.3 ± 1.40	241.1 ± 13.8	10.81	184.6 ± 8.90 *	8.28
Vinblastine	3.2 ± 2.5	142.6 ± 13.5	44.56	32.3 ± 1.90 **	10.10
Vincristine	4.1 ± 0.80	630.0 ± 72.0	153.66	142.1 ± 8.6 **	34.66
Paclitaxel	8.5 ± 1.38	382.0 ± 21.6	44.94	91.9 ± 14.77 **	10.81
Etoposide	99.6 ± 9.95	2518.3 ± 288.15	25.28	568.3 ± 50.88 **	5.71

* $P < 0.05$.
** $P < 0.01$.

^aCells were seeded at 1×10^4 /well in a 96-well plate. Chemotherapeutic drugs were serially diluted in culture medium, put into each well in quadruplicate, and exposed for 3 days. Cell viability was measured by chemiluminescent assay as described. Student's *t* test analysis was used to compare *MDR1*_{wt} and *MDR1*_{571A} variants.

^bThe IC₅₀ values were estimated by using a curve fitting program. Data shown was mean ± S.D.

^cRelative resistance (*R* value) was obtained by dividing the IC₅₀ value with the value of the corresponding control from each drug tested.