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Expression of Multiple Drug Resistance Conferring Proteins in Normal Chinese and Caucasian Small and Large Intestinal Tissue Samples

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Abstract: Multidrug resistance conferring proteins (MDRCP) are ATP-binding cassette (ABC) transporters known to significantly influence the absorption, distribution, metabolism, and elimination (ADME) and toxic behavior of many therapeutic agents. Research in the pharmacogenomics area has suggested that mutations and variable expression patterns of these MDCRPs may exist in tissue samples from different ethnic groups. The goal of this study was to examine the expression of P-glycoprotein (PGP), sister of PGP (S-PGP), multidrug resistance protein 3 (Mdr3), multidrug resistance like proteins 1−5 (MRP 1−5), and lung resistance associated protein (LRP) in tissue slides and protein lysates derived from normal adult small or large intestines of Caucasian or Chinese origin. Our results demonstrated ubiquitous expression of PGP, MRP 1, MRP 4, and LRP in the small and large intestinal epitheliums originating from both Caucasian and Chinese origin. S-PGP, Mdr3, MRP 2, and MRP 3 exhibited variable expression in the tissue slides and protein lysates derived from the Chinese and Caucasian small and large intestines. MRP 5 was not observed in any of the samples studied. The results suggest that MDCRPs may have distinct expression profiles in the small and large intestines that potentially vary with genetic background. These studies provide a foundation for further investigations to verify these findings across a wider number of patients of different ethnic backgrounds.

Keywords: Multidrug resistance; LRP; MRP; Mdr; P-glycoprotein

Introduction

Multidrug resistance is now considered a potentially major impedance to the effective therapeutic treatment of infectious and malignant diseases.[1,2] Several families of the multiple drug resistance conferring proteins (MDRCPs) have since been identified as participants in facilitating drug resistance.[3] MDRCPs are membrane-bound proteins that mediate the unidirectional transport of lipophilic compounds and/or their conjugates in an ATP-dependent transport process.[4−7] The presence of a shared homologous ATP-binding cassette (ABC) in each of these transporters has been utilized to

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identify a variety of related orthologues in different organisms including yeast, nematodes, plants, rodents, and humans.[8−11] Studies investigating the pharmacokinetic implications of human MDRCPs have been traditionally focused on two main ABC subfamilies: the Mdr (multidrug resistance protein, ABCB) subfamily and the MRP (multidrug resistance related protein, ABCC) subfamily.

The Mdr subfamily contains MDR-1/P-glycoprotein (PGP, ABCB1),[6,8,21] Mdr3 (ABCB4),[6,12] and the most recently discovered sister of P-glycoprotein (S-PGP, ABCB11).[6] There are more than 10 MRP subfamily members; however, most of the studies are focused on the characterization of MRP 1−5.[6,13−15] In addition to the multiple drug resistance proteins, a lung resistance related protein (LRP) is a major protein family member believed to be involved in nuclear-cytoplasmic transport.[16] LRP mediates drug resistance through a presumed transport process[17] and is widely distributed in normal tissues and overexpressed in multiple drug resistant cancer cells.[18,19]

The presence of a multiple drug resistance protein was first reported in 1976,[20] and the genes encoding *mdr1* in mouse and human were subsequently cloned.[8,21] Studies carried out in the *mdr1* gene knockout mouse strain indicated that the multiple drug resistance protein plays an important role in normal absorption and excretion of many commonly used pharmacological agents and xenobiotics.[22] It must be noted that the recognition of drug resistance has steadily increased with the increasing number of compounds being screened for absorption, distribution, metabolism, and elimination (ADME) properties. This is due in part to the large body of evidence showing that transfection of cells with individual MDRCP family members causes them to become resistant to many different classes of therapeutic agents.[3] In addition, the presence of endogenous orthologues of these multidrug resistant transporters in cell lines cannot be ignored.[23−25]

Despite the fact that both Mdr and MRP family members share the common ability to confer multiple drug resistance

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in different cells, evidence demonstrates that there is a dramatic difference in their structure, tissue localization, and substrate specificity. Even within the MRP subfamily, the tissue distribution and subcellular localization of MRP 1 and MRP 2 are strikingly different. The level of MRP 1 is high in lung, testis, and muscle and very low in the liver,[26,27] whereas MRP 2 expression is high in liver and relatively low in other tissues. Furthermore, MRP 2 is predominantly expressed in the apical membrane of human liver cells, while a small amount of MRP 1 is expressed in the basolateral membrane of these tissues.[28] It is not fully understood whether structural differences in MDRCPs as well as their different tissue distribution may imply that each protein may play a specific role in regulating different physiological functions. However, some evidence points to a possibility that the Mdr and MRP members may play synergistic functions to "pump" out toxic compounds to the extracellular environment in several tissues[29,30] and thus protect the cells from the cytotoxic agents. There is also strong evidence suggesting that several of the MDRCPs transporters work in conjunction with metabolizing enzymes as to eliminate foreign compounds from the body as effectively as possible.[31,32]

The main area of our interest is the role of these MDRCP isoforms in influencing the absorption of therapeutic agents in the human gastrointestinal (GI) tract. In order to better understand the significance of the individual MDRCP isoform in the GI tract, their physiological expression patterns must first be elucidated. Several laboratories have already begun to assess the expression of these transporters in human intestinal tissues; however, more work is still needed to fully elucidate the significance and frequency of expression differences. In the present study, we report our observations on the expression patterns of MDRCP isoforms in the small and large intestines of a Caucasian and a Chinese male, using immunocytochemical analysis. In addition, individual protein lysates ($n = 3$ for each sample) were used to investigate the presence of the MDRCPs with immunoblotting. Our goal is to provide a physiological basis for the further functional and pharmacogenetic investigations of the role of these MDRCPs.

Material and Methods

Materials. Formalin-fixed, paraffin-embedded human small and large intestine tissue slides (Chinese-derived) were purchased from Maxim Biotech (South San Francisco, CA). Caucasian-derived tissue sections were collected at St. Peter's Hospital in New Brunswick, NJ. Samples were collected from patients undergoing resection or biopsy from different regions of the small and large intestine. After the pathologist had processed the tissue for clinical diagnosis, a sample of the discarded tissue was collected, fixed in formalin, and processed for immunohistochemical evaluation. Samples were used only if determined to have no pathologic conditions.The Caucasian and Chinese small and large intestinal tissue samples were from two separate male patients of similar age. All comparisons within a region were performed with tissue from the same patient. Three protein lysate samples of the small and large intestines from normal adults of Chinese and Caucasian origin were obtained from Biochain (Hayward, CA). Antibodies were purchased from Kamiya Antibodies, Inc. (Seattle, WA), except those against PGP and MRP 1, which were purchased from Santa Cruz (Santa Cruz, CA). Secondary antibody conjugates and DAB staining agents were purchased from Zymed (South San Francisco, CA).

Immunohistochemistry. The Maxim Biotech staining protocol was followed for all of the paraffin-embedded tissue samples. Briefly, the slides were deparaffinized in xylene and rehydrated in gradient ethanol (from 100% down to 85%). Then the slides were immersed in 3% hydrogen peroxide dissolved in methanol and blocked with nonimmune serum. After blocking, the slides were incubated with primary antibody dissolved in PBS at optimal titration (ranging from 1:500 to 1:1000) for 2−3 h, and with HRP-conjugated secondary antibody for a half-hour. Next the slides were

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stained with hematoxylin and rinsed with PBS. The images were recorded with a Leica microscope equipped with Image software SPOT 32 at a magnification of 400×.

Immunoblot Analysis. Protein aliquots (40 μg) were loaded on nitrocellulose membrane (Millipore, Bedford, MA) using the Bio-Dot SF microfiltration apparatus (BioRad, Hercules, CA) utilizing the manufacturer's recommended protocol. Western blot analysis using each of these antibodies was determined in our laboratory previously with separate tissue samples (unpublished data) to confirm their specificity. The membrane was blocked with 5% fat-free milk powder in TBS and then incubated with the appropriate primary antibody in TBS containing 1% fat-free milk powder. The membranes were washed 3× for 7 min with TBS/T and then incubated for 1 h with an appropriate HRP-labeled secondary antibody. The membranes were washed 3× for 10 min with TBS/T and incubated with SuperSignal substrate (Pierce, Rockville, IL) for detection. Chemiluminescence images were captured using a Nucleotech 920 CCD camera (Nucleotech, San Mateo, CA), and the densitometry of each band was analyzed with the GelExpert program.

Results

Immunohistochemical Staining. Figure 1 presents the results of the MDRCP isoform immunohistochemical staining in the small intestine from the Chinese donor. When contrasted with the control slide (A), it appears that PGP (B) and MRP 2 (G) exhibited faint expression, and Mdr3 (C) exhibiting a stronger localization in the small intestine villous region. LRP (E) appeared to have high expression in the small intestine, and MRP 1 (F), MRP 3 (H) and MRP 4 (I) also appeared to be expressed along the villi. S-PGP (D) and MRP 5 (J) were not significantly observed in the small intestine slides derived from the Chinese donor.

Staining results of the human large intestinal tissue slides of the Chinese donor are illustrated in Figure 2. These results showed that all of the Mdr-related family members (B−D) were expressed in the large intestinal epithelium. LRP (E) was also observed in the large intestinal tissue slides derived from this Chinese donor. These results indicated that all of the MRP family members (F−I), with the exception of MRP 5 (J), were expressed in the large intestine of the Chinese donor.

The results of small intestine MDRCP staining from the Caucasian donor are illustrated in Figure 3. For the Mdr subfamily, S-PGP (D) was not detected in the small intestine, whereas both PGP (B) and Mdr3 (C) were observed in this tissue. The staining with LRP (E) antibody revealed expression in the Caucasian donor. The MRP family members MRP 1 (F), MRP 2 (G), and MRP 4 (I) were expressed, whereas MRP 3 (H) and MRP 5 (J) were not present in the small intestine. Figure 4 illustrates the results of the Caucasian large intestinal donor, where the immunohistochemistry results of the MDRCP isoforms indicated a pattern similar to that shown in Figure 3 for the small intestine.

Immunoblot Analysis. Immunoblot analysis was performed using commercially available normal human adult

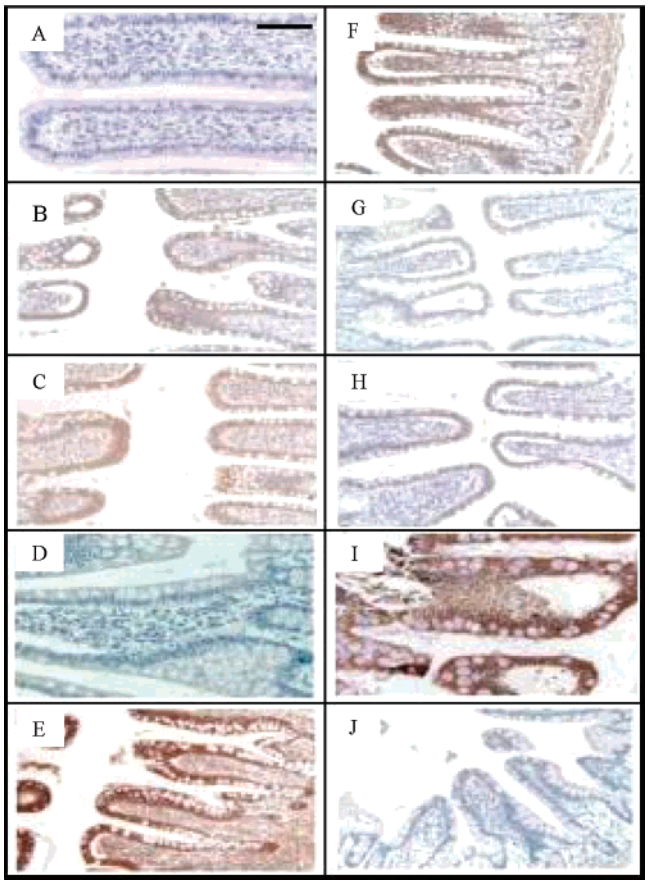

Figure 1. Immunohistochemical staining results of (A) control, (B) PGP, (C) Mdr3, (D) S-PGP, (E) LRP, (F) MRP 1, (G) MRP 2, (H) MRP 3, (I) MRP 4, and (J) MRP 5 isoforms in the normal small intestine of a Chinese donor. The slides were pretreated with citric buffer, incubated with polyclonal anti-rabbit primary antibody followed by the secondary antibody, and stained with DAB staining kit from Zymed. The control slide was incubated with nonimmune rabbit IgG instead of primary antibody. The pictures were taken using a Leica microscope (magnification bar, 5 μm).

GI protein lysates samples from Asian Chinese and Caucasian. All samples were normalized to the expression of human β-actin to provide semiquantitative comparisons between the ethnic samples for each antibody. In Figure 5A, PGP, Mdr3, S-PGP, and LRP appeared to be expressed in all the Chinese and Caucasian samples. These transporter proteins appeared to be more highly expressed in the Chinese small intestine when contrasted to the Caucasian small intestine, with PGP, Mdr3, and LRP reaching statistical significance ($p < 0.05$). However, no statistically significant changes were observed in the expression of these proteins in the large intestines derived from Chinese and Caucasian sources.

Immunoblot analyses of the same tissue protein lysates utilizing MRP family member specific antibodies are illustrated in Figure 5B. MRP 1 demonstrated significantly higher ($p < 0.05$) expression levels in the Chinese small intestinal lysates when contrasted to the Caucasian samples. In general, MRP 1, MRP 2, and MRP 4 appeared to

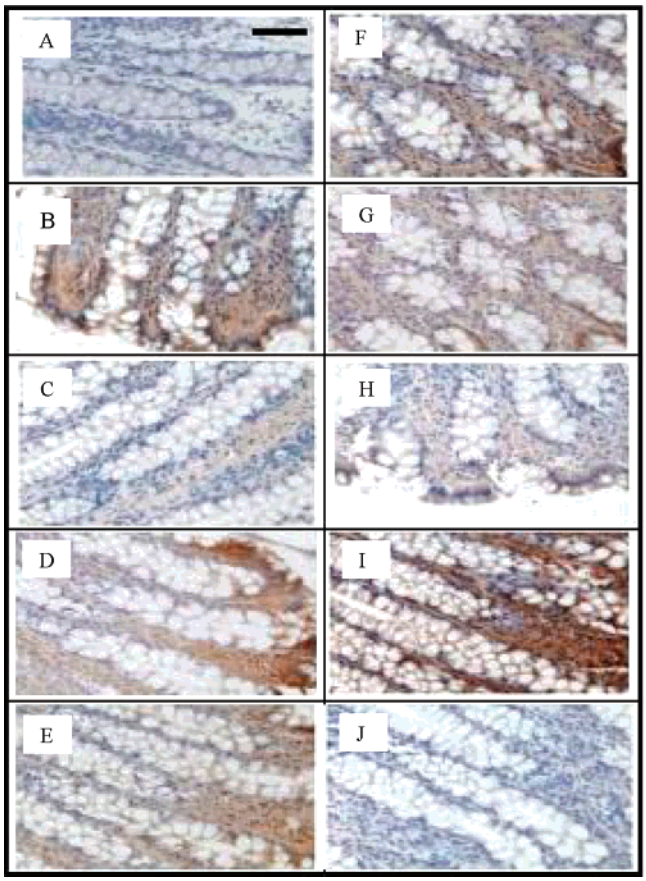

Figure 2. Immunohistochemistry staining results of (A) control, (B) PGP, (C) Mdr3, (D) S-PGP, (E) LRP, (F) MRP 1, (G) MRP 2, (H) MRP 3, (I) MRP 4, and (J) MRP 5 isoforms in the normal large intestine of a Chinese donor. The slides were pretreated with citric buffer, incubated with polyclonal anti-rabbit primary antibody followed by secondary antibody, and stained with DAB staining kit from Zymed. The control slide was incubated with nonimmune rabbit IgG instead of primary antibody. The pictures were taken using a Leica microscope (magnification bar, 5 μm).

demonstrate higher expression in both the small and large intestines samples of Chinese and Caucasian origin. MRP 3 demonstrated faint, variable expression levels in the small and large intestine samples from both sources, whereas MRP 5 was not detected.

Discussion

Oral drug administration is the most convenient route for dosage form delivery; however, it requires effective absorption of drug across the GI epithelium. Besides the traditional factors that have to be considered for oral absorption, such as solubility, lipophilicity, and molecular size, the discovery of MDRCPs along the GI tract also presents a great challenge for the development of orally bioavailable drug candidates.[20,22−25] While these proteins are viewed to hinder therapeutic intervention, it is important to understand that MDRCPs are an important part of the body's detoxification mechanism and they play a critical role in maintaining normal physiological processes.[22,33,34]

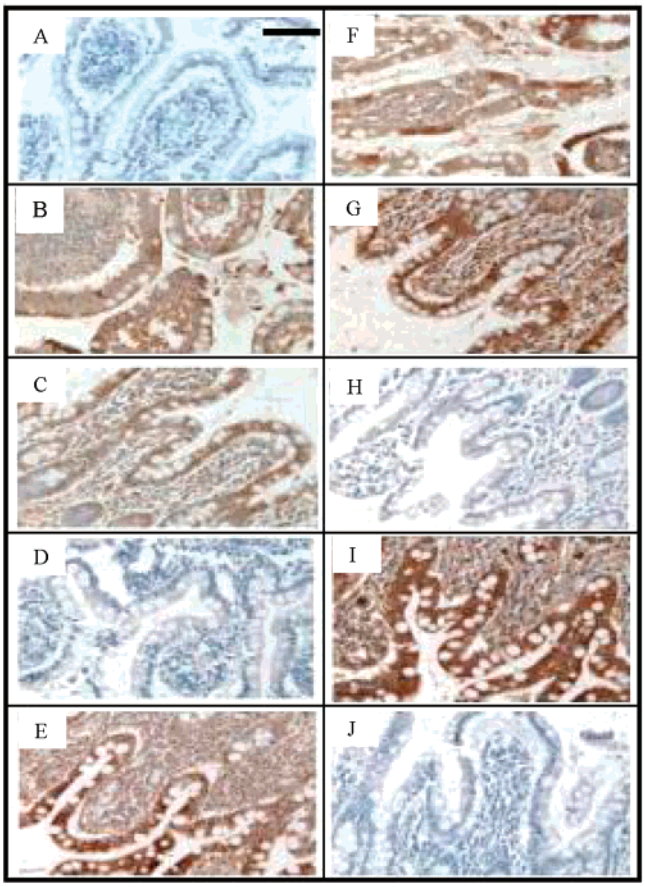

Figure 3. Immunohistochemistry staining results of (A) control, (B) PGP, (C) Mdr3, (D) S-PGP, (E) LRP, (F) MRP 1, (G) MRP 2, (H) MRP 3, (I) MRP 4, and (J) MRP 5 isoforms in the normal small intestine of a Caucasian donor. The slides were pretreated with citric buffer, incubated with polyclonal anti-rabbit primary antibody followed by secondary antibody, and stained with DAB staining kit from Zymed. The control slide was incubated with nonimmune rabbit IgG instead of primary antibody. The pictures were taken using a Leica microscope (magnification bar, 5 μm).

Growing pharmacogenomic evidence points to the fact that the regional expression and genetic characteristics of these transporters might differ across populations, which could potentially influence a drug's pharmacokinetic profile. Genetics-based differences may be explained by the close inspection of single nucleotide polymorphisms (SNP) and larger mutations in several MDRCP genes that are also known to be the underlying cause of a variety of human genetic disorders.[6,33] Expression-based differences may arise from ethnicity, sex, and age differences between subjects and be confounded by many environmental factors. To provide insight into the potential phenotypic differences in

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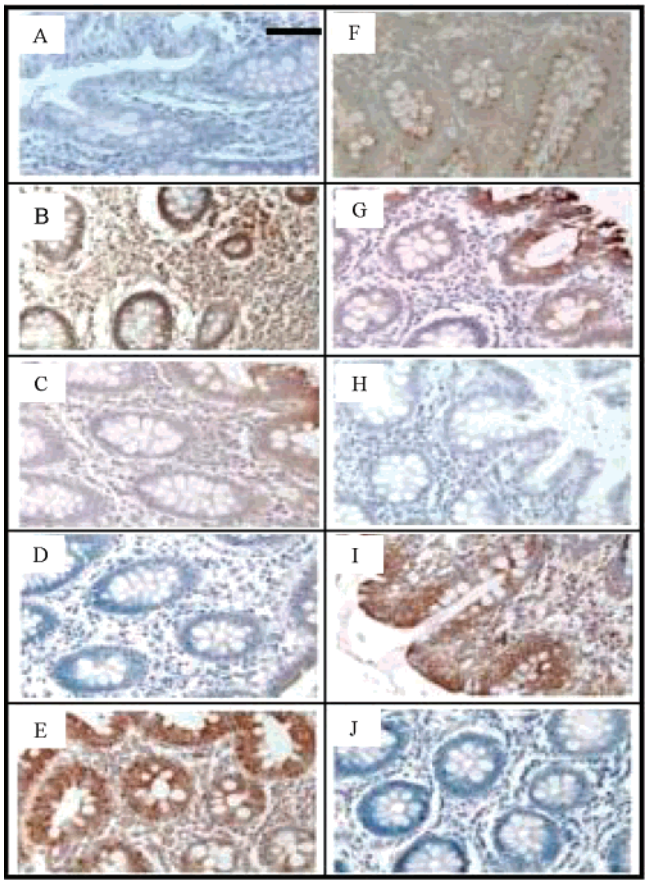

Figure 4. Immunohistochemistry staining results of (A) control, (B) PGP, (C) Mdr3, (D) S-PGP, (E) LRP, (F) MRP 1, (G) MRP 2, (H) MRP 3, (I) MRP 4, and (J) MRP 5 isoforms in the normal large intestine of a Caucasian donor. The slides were pretreated with citric buffer, incubated with polyclonal anti-rabbit primary antibody followed by secondary antibody, and stained with DAB staining kit from Zymed. The control slide was incubated with nonimmune rabbit IgG instead of primary antibody. The pictures were taken using a Leica microscope (magnification bar, 5 μm).

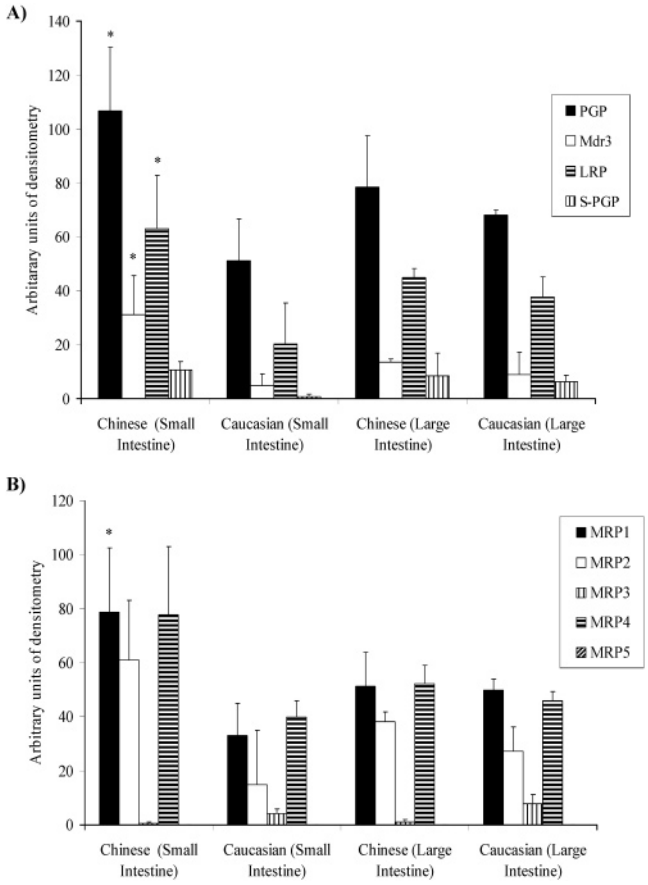

Figure 5. (A) Quantitative immunoblot analysis of PGP, Mdr3, S-PGP, and LRP 1 in human protein lysates derived from the small and large intestines of Chinese and Caucasian male donors. (B) Quantitative immunoblot analysis of MRP 1−5 in the human protein lysates derived from the small and large intestines of Chinese and Caucasian male donors. The values are indicated as mean ± SD and came from three separate experiments. *Y*-axis values are given in densitometry absorption units. Student's *t* test was performed to contrast the Chinese vs the respective Caucasian samples, with an asterisk (*) signifying $p < 0.05$.

expression of MDRCPs as a function of ethnicity, we have used immunohistochemical staining and immunoblot analysis to detect the presence of different isoforms in the normal human small and large intestine tissue slides and protein lysates from Chinese and Caucasian donor, respectively.

The results of the immunohistochemical analysis revealed similar expression patterns in both donors for MRP 4, MRP 5, and LRP, whereas MRP 3 demonstrated distinctively different ethnicity-based expression. The remainder of the MDRCPs demonstrated variable expression patterns in these samples. For example, PGP (Figure 1B) and MRP 2 (Figure 1G) were faintly observed in the small intestinal slide from Chinese donor with immunohistochemical staining, but they were detected in the Chinese large intestinal slides (Figure 2). In contrast, both PGP and MRP 2 exhibited apparently stronger immunoreactivity on the slides derived from the Caucasian donor. Interestingly, the immunoblot analyses suggested that higher expression of PGP and MRP 2 occurred in Chinese small intestine as compared to the Caucasian (Figure 5). The large inter- and intraindividual variability in the expression pattern of these proteins in different ethnic groups might result in differences.

MRP 3 has been assumed to act as a conjugate export pump[35,36] and postulated to play a role in the transport of biliary and intestinal excretion of organic anions.[37] Konig

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et al. have demonstrated that MRP 3 is mainly expressed in the basolateral side of the small intestine; thus MRP 3 may enhance the drug absorption and secretory efflux through the enterocytes.[35] In the present study, MRP 3 demonstrated immunoreactivity in the Chinese tissue slides (Figures 1H and 2H), with little expression observed in the Caucasian tissue samples. Immunoblot analysis of the protein lysates supported the observation of lower expression levels of MRP 3 in the small and large intestines of Chinese and Caucasian males (Figure 5B).

MRP 4 had similar, apparently high expression levels in both the Chinese and Caucasian tissue slides. These results were supported by immunoblot analysis revealing the presence of MRP 4 in Chinese and Caucasian small and large intestinal protein lysates (Figure 5B). MRP 5 was not observed in any of the samples tested. MRP 4 and MRP 5 are assumed to possess the glutathione-conjugate transport activity.[6] In addition, both MRP 4 and MRP 5 have been implicated in the efflux of chemotherapeutic agents,[38] with a particular affinity for nucleoside-based drugs.[39] The tissue distribution of MRP 4 has been controversial. For instance, Kool et al. argued that MRP 4 is expressed only at very low levels in few tissues, and it is not overexpressed in the tumor cell lines they tested.[15] On the other hand, Sampath et al. showed that MRP 4 had strong expression in liver and in a number of cancer cell lines.[40] The expression of MRP 4 in this present study suggests that further analyses are required to assess its impact on the ADME and toxicity of therapeutic agents.

The lack of MRP 5 expression was consistent with the findings by Borst and co-workers,[41] who claim that the commercially available MRP 5 antibodies do not detect the protein in tissue sections. On the other hand, when cells become cancerous, a subsequent increase in the expression of MRP 5 is observed in the cells making them capable of effluxing MRP 5 specific chemotherapeutics.[39,40] Therefore, under normal physiological conditions, intrinsic MRP 5 may not be appreciably expressed.

The results of the present study revealed that LRP was expressed in both the small and large intestines of Chinese and Caucasian origin (Figure 1−4E), which was confirmed in the immunoblot analyses of the corresponding protein lysates (Figure 5A). LRP was first identified in several non-PGP multidrug resistance cell lines of different histogenetic origin.[42] Immunohistochemical staining analyses have shown that LRP has a high expression level in normal epithelial cells and tissues chronically exposed to xenobiotics and potentially toxic agents, such as bronchial, intestinal, and kidney tubule cells.[43]

Mdr3, a splice variant of PGP, is mainly expressed in the liver and the kidney[44] and has been implicated in cholestasis;[45,46] Mdr3 was expressed in all of the samples tested with immunohistochemical staining (Figure 1−4C). The results of the immunoblot studies suggest that Mdr3 is significantly higher expressed in Chinese small intestine as compared to the Chinese large intestine and Caucasian samples. Currently the physiological study of Mdr3 is focused on its role in trafficking phospholipid and bile salt derivatives.[45]

S-PGP (bile salt export protein; BSEP) is a novel member of the ATP-binding cassette superfamily that is highly homologous to PGP, but has some substrate specificity differences.[6,47] S-PGP has been illustrated to transport calcein-acetoxymethyl ester (AM), while S-PGP was incapable of effluxing rhodamine 123, a known substrate of PGP.[48] In addition, despite the high similarity in structure between S-PGP and PGP, the overexpression of S-PGP in cells had no effect on uptake of vincristine, daunomycin, paclitaxel, and digoxin.[48] In the present study, S-PGP demonstrated relatively weak expression in the Chinese-derived large intestine, but was faintly expressed in the Chinese small intestine and in the Caucasian-derived samples

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(Figure 1−4D). Immunoblot studies revealed similar results with relatively weak expression in the small and large intestines of Chinese and Caucasian samples (Figure 5A). These results suggest that S-PGP may not play a prominent role in drug efflux in the Chinese or Caucasian GI tracts.

Overall, these results illustrate the expression patterns of several MDRCPs in both Chinese and Caucasian tissue samples. The present studies were performed on a limited sample size and, thus, serve as a preliminary platform for future work to establish the significance of the expression differences. Further work is also needed to elucidate the function of these proteins to properly assess their relevancy in influencing the ADME and toxicity behavior of therapeutic agents. Combined, these results provide a strong justification for further investigation into the physiological, molecular, and functional characterization of the studied MDRCPs. Potential elucidated genomic differences in the expression of these MDRCPs could have a significant impact in the therapeutic efficacy of drugs among different ethnic populations.

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