Overexpression of OATP1B3 Confers Apoptotic Resistance in Colon Cancer

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Abstract
Organic anion transporting polypeptide 1B3 (OATP1B3, SLCO1B3) is normally expressed in hepatocytes. In this study, we showed frequent overexpression of OATP1B3 in colorectal adenocarcinomas. Quantitative reverse transcription-PCR analysis of 17 colon tumors indicated tumoral overexpression of OATP1B3 by ~100-fold, compared with 20 normal colon samples (P < 0.0001). Using immunohistochemistry on a tissue microarray containing 93 evaluable colon tumor specimens, we detected immunostaining of OATP1B3 in 75% of colon adenocarcinomas (81%) and no immunostaining in normal samples. To determine the functional effects of OATP1B3 expression on drug-induced apoptosis, we used camptothecin and oxaliplatin on a panel of colorectal cancer cell lines stably overexpressing OATP1B3. The results indicated that OATP1B3 overexpression enhanced cell survival in RKO, HCT-8, and HCT116(p53−/−) cells that harbor wild-type p53 but not in Caco-2 and HCT116(p53−/−) cells that lack p53, compared with the respective empty vector controls (P < 0.01). The terminal deoxynucleotidyl transferase-mediated nick-end labeling assay confirmed that HCT116(p53−/−) cells overexpressing OATP1B3 had significantly lower apoptotic levels compared with empty vector control (P < 0.001). The overexpression of OATP1B3 reduced the transcriptional activity of p53, with subsequent reductions in transcript and protein levels of its downstream transcription targets (P21WAF1 and PUMA). Overexpression of a point mutation (G583E) variant of OATP1B3 lacking transport activity did not confer an antipapoptotic effect or affect p53 transcriptional activity, suggesting that the antiapoptotic effect of OATP1B3 may be associated with its transport activity. Taken together, our results suggest that OATP1B3 overexpression in colorectal cancer cells may provide a survival advantage by altering p53-dependent pathways. [Cancer Res 2008;68(24):10315–23]

Introduction
Colorectal cancer (CRC) represents a major public health problem accounting for over 1 million cases of new cancer and about half a million deaths worldwide annually (1, 2). The lifetime risk of developing CRC is 1 in 17, affecting men and women alike, with 90% of cases occurring after the age of 50 years (3). CRC is thought to develop from the progressive accumulation of genetic mutations, many of which affect the control of apoptosis (4–6). Abnormalities in apoptosis mechanisms may promote the selection of cells that are resistant to apoptosis and consequently have increased rates of mutations (7).

Organic anion transporting polypeptide 1B3 (OATP1B3; gene name, SLCO1B3) belongs to the organic anion transporting polypeptide (OATP/SLC0) superfamily and is expressed on the basolateral membrane of hepatocytes around the central veins (8). When expressed in the normal liver, OATP1B3 acts as an uptake transporter for a variety of endogenous compounds (e.g., bile acids, cholecystokinin, conjugated steroids, and thyroid hormones) as well as xenobiotic compounds (e.g., pravastatin and paclitaxel; refs. 9–12). OATP1B3 has been shown to be overexpressed in various human cancer tissues as well as in cancer cell lines derived from colon, pancreas, gall bladder, lung, and breast cancers (9, 13, 14). Recent studies have reported that certain members of the OATP family are overexpressed in breast and brain tumors and may play a role as regulators of cellular processes such as proliferation and apoptosis (15–20). However, it is not known whether tumoral expression of OATP1B3 has any pathobiological significance. In this study, we investigated the expression of OATP1B3 in established colorectal adenocarcinomas and its functional effect on cancer cell survival after chemotherapy treatment using in vitro CRC cell line models.

Materials and Methods
Cells, plasmids, and reagents. The human CRC lines Caco-2, HCA-7, HCT-8, and RKO cells were obtained from the American Type Culture Collection. The isogenic HCT116(p53−/−) (p53-wild-type) and HCT116(p53−/−) (p53-null) were gifts from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD; ref. 21). All cells were maintained in DMEM supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. The expression plasmid for OATP1B3 (wild-type) was prepared by inserting the open reading frame of the OATP1B3 cDNA sequence (NM 019844) into the pcDNA3.1-Zeo+ vector (Invitrogen). The expression plasmid for OATP1B3 G583E mutant was prepared using the QuikChange site-directed mutagenesis kit (Stratagene) and the following primers: 5′CTCAAGGAATTCTAGCTCCAATATATTTTG and 5′TAAGAACACTAGGGATGCAACCATGAG. The plasmids used for the reporter assay, pcDNA3-Fp53, PG13-luc (containing the OATP1B3 promoter and PUMA-Luc reporter plasmid s have been described elsewhere (22). The polyclonal antibody against the COOH-terminal peptide sequence of OATP1B3 has been previously used and shown to be specific for OATP1B3.

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optimal concentrations of zeocin (800 µg/ml) and P2IWA.F1 (WA-1) were from Abcam. The antibodies against caspase 3, PUMA, poly(ADP-ribose) polymerase (PARP), and β-actin were from Cell Signaling. The antibody against NOXA was from Imageon. The antibody against p53 (BP53.12) was obtained from Chemicon. The reagents for immunohistochemistry were from Biogenex. Fluorescently labeled deoxycholic acid (FITC-DCA) was a gift from Dr. Jesse Martinez (University of Arizona, Tucson, AZ) and previously reported to induce signaling changes in a similar manner to unlabeled deoxycholic acid (24).

Quantitative reverse transcription-PCR and immunoblotting analyses on colon tumors. Total RNA was isolated from 20 normal colonic mucosa samples and 17 primary colon tumors (7 samples were from matched donors). Single-stranded cDNA was synthesized from a total RNA amount of 1 µg using the iScript cDNA synthesis kit (Bio-Rad). Quantitative reverse transcription-PCR (RT-PCR) was performed using an iCycler with the iQ SYBR-green Supermix (Bio-Rad) and the following gene-specific primers reported previously (25, 26); OATP1B3.fw, 5'-GTCCAGT-CATTGGCCTTGCA-3'; OATP1B3.rv, 5'-CAACCAACCGAGTCTCCTTAGG-3'; OATP1B1.fw, 5'-TGAAACCGGTTGAGATTCG-3'; OATP1B1.rv, 5'-TCTCTATGAGATGTCAGTGTG-3'; OATP1A2.fw, 5'-AGAACGAACCGAGACGATCAT-3'; OATP1A2.rv, 5'-GAGTTTTCCCCATCCTAGTACA-3'; β-actin.fw, 5'-GCATCCCTACCCGTAGATC-3'; and β-actin.rv, 5'-GATAGACCACGCTT-GATGAC-3'. Reactions were performed in duplicate and mRNA copy numbers were quantified using purified PCR products as calibration samples. The results were normalized to 10^6 copies of β-actin mRNA.

The overexpression of OATP1B3 protein in colon tumors was confirmed by immunoblotting using protein lysates prepared from normal and cancerous colonic tissues from the same donors (n = 5 pairs). Tissue homogenate containing 25 µg of total protein was subjected to immunoblotting and probed for OATP1B3 and β-actin. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia).

Immunohistochemistry. A tissue microarray contained 93 deidentified, archival cases of colon adenocarcinomas of all American Joint Committee on Cancer criteria stages and 12 normal colonic mucosa sections. An avidin-biotin immunoperoxidase assay was performed after the antigen retrieval procedure using citrate buffer, and a polyclonal antibody against OATP1B3 (1:200 dilution) was used (23). The immune reaction was visualized using 3,3′-diaminobenzidine (DAB), and nuclei were counterstained with hematoxylin. The specificity of immunoreactive signals was verified by omitting either primary or secondary antibody as well as by incubating with polyclonal OATP1B3 antisera that has been neutralized by preincubation with the antigenic peptide.

OATP1B3 expression was evaluated by an experienced pathologist (E.H.). The intensity of immunostaining was assigned as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3). In normal colonic mucosa, the staining for OATP1B3 was negative. We therefore designated staining of intensity of ≥1 as "positive" staining for OATP1B3. Similarly, the immunohistochemical staining for p53 was performed on the same colorectal tumor microarray using a monoclonal p53 antibody (BP53.12, 1:100 dilution). The accumulation of p53 protein was evaluated by an experienced pathologist (E.M.B.). The staining for p53 was designated to be positive if ≥10% of tumor cells exhibit p53 immunostaining (27). In normal colonic mucosa, there was no accumulation of p53 protein.

Development of in vitro model cell lines overexpressing OATP1B3. CRC cell lines Caco-2, HCT-8, RKO and isogenic HCT-116 cells with wild-type or null p53 status were used to generate stable cell line models overexpressing OATP1B3 or the G583E variant of OATP1B3. Cells were transfected with the pcDNA3-based mammalian expression plasmid containing the open reading frame of OATP1B3 or the empty vector (control) using Lipofectamine 2000 (Invitrogen) and selected with the optimal concentrations of zeocin (800 µg/ml or 520 µmol/L for HCT-8 and Caco-2; 400 µg/ml or 260 µmol/L for RKO and isogenic HCT-116) for 3 wk. OATP1B3 protein expression levels in the resulting resistant clones were examined by immunoblotting.

Cell viability assay. The functional effect of OATP1B3 overexpression on tumor cell survival after the incubation with chemotherapeutic drugs, camptothecin (CPT) or oxaliplatin was examined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay or ATP-based assay (Cell Titer Glo; Promega). Cells were plated at a density of 5,000 to 20,000 cells per well in a 96-well plate and treated with CPT (10 µmol/L), oxaliplatin (10 µmol/L), or vehicle (DMSO), and cell viability was assessed. The relative cell viability in the drug-treated group was calculated compared with the vehicle-treated group.

Apoptosis assay: terminal deoxynucleotidyl transferase-mediated nick-end labeling assay. HCT-116p53+/− cells stably expressing OATP1B3 or empty vector were seeded into 8-well chamber slides. Cells were treated with CPT (10 µmol/L) or vehicle (DMSO) for 24 h and stained by terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL; In situ cell death detection kit; Roche Diagnostics). Twenty random fields at ×200 magnification, ×1,000 cells were examined and photographed using fluorescence microscopy. The percentage of apoptosis was calculated by counting TUNEL-positive (red fluorescence) and healthy nonapoptotic cells.

Luciferase assay. To investigate whether the antiapoptotic effects of OATP1B3 involve the suppression of exogenous or endogenous p53 transcriptional activity, luciferase activity assays were performed in HCT-116p53+/− and HCT-116p53−/− cells using the PG13-luc, P2IWA.F1-luc, or PUMA-luc reporter plasmid, as previously described (22). Briefly, PG13-luc is a reporter plasmid that has a firefly luciferase under the control of 13 tandem repeats of the p53 response elements, and P2IWA.F1-luc and PUMA-luc reporter plasmids contain a firefly luciferase under the P2IWA.F1 and PUMA gene promoter sequences with p53 response elements (28, 29). HCT-116p53−/− cells were transiently transfected with the expression plasmids for OATP1B3, p53, or empty vector as well as PG13-luc or P2IWA.F1-luc and pRL-TK using Fugene 6 (Roche Diagnostics). After 48 h, luciferase activity normalized for renilla luciferase activity was obtained using a Dual Luciferase assay kit (Promega). In separate experiments, HCT-116p53−/− cells stably overexpressing OATP1B3 or empty vector were transfected with the PG13-luc or PUMA-luc and pRL-TK. After 24 h, cells were treated with CPT (10 µmol/L) or vehicle (DMSO) for 4 h and further incubated in fresh medium for 24 h. Luciferase activity was measured as previously described.

Chemotherapy treatment and immunoblotting analyses. HCT-116p53−/− cells stably expressing OATP1B3 or empty vector were treated with CPT (10 µmol/L), oxaliplatin (10 µmol/L), or vehicle (DMSO). After incubation, cells were harvested in radioimmunoprecipitation assay buffer and subjected to immunoblotting. After the incubation with respective primary and secondary antibodies, immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia). β-actin was used as a gel loading control.

Effects of the OATP1B3 G583E variant. A point mutation variant (G583E) of OATP1B3 lacking transport activity was used to assess the relationship between OATP1B3 transport activity and its antiapoptotic effect. Luciferase activity assays were performed using the PG13-luc plasmid after transient transfection of OATP1B3 (wild-type), OATP1B3 G583E variant, or empty vector in HCT-116p53−/− cells. HCT-116p53−/− cells stably overexpressing OATP1B3 (wild-type), OATP1B3 G583E variant, or empty vector were developed and used to assess the cellular uptake of a fluorescently labeled substrate (FITC-DCA) by measuring the green fluorescent signal associated with cell lysates after incubation with FITC-DCA (10 µmol/L, 10 min) and subsequent PBS washes. In addition, HCT-116p53−/− cells stably overexpressing OATP1B3 (wild-type), OATP1B3 G583E variant, or empty vector were treated with CPT (10 µmol/L, 24 h) to measure cell survival and protein levels of p53 and its target genes, P2IWA.F1 and NOXA.

Statistical analysis. The results were expressed as the mean with SD. Statistical significance between groups was determined using the Mann-Whitney test, the unpaired Student’s t test, or ANOVA. P values of <0.05 were considered to indicate statistical significance.

**Results**

OATP1B3 mRNA and protein are frequently overexpressed in colorectal adenocarcinomas. The results from the quantitative
RT-PCR analysis indicated that OATP1B3 mRNA is markedly overexpressed (96-fold differences in the median values) in the colorectal adenocarcinoma samples tested compared with normal colonic mucosa \((P < 0.0001\) by Mann-Whitney test; Fig. 1A). In seven cases, the tumoral and normal colon tissue samples analyzed were from the same donors and OATP1B3 mRNA was up-regulated in all seven tumors compared with the matched normal colon tissue samples (average fold differences in seven matching pairs was 76, ranging from 3–176). The expression levels of closely related members of the OATP family (namely OATP1B1 and OATP1A2) in these samples were also measured using quantitative RT-PCR. The results indicated minimal expression of OATP1B1 and OATP1A2 mRNA in both tumor and normal colonic mucosa, suggesting that marked overexpression is specific to OATP1B3, not accompanied by other tested OATP family members (data not shown). The results from OATP1B3 immunoblotting analysis confirm that OATP1B3 protein is overexpressed in colon tumors \((n = 3\) of 5) but not in the adjacent normal colonic tissues from the same donor (Fig. 1B). OATP1B3 expression in colon tissue seems to be tumor specific and not related to the characteristics of an individual patient.

The OATP1B3 protein expression was assessed by immunohistochemistry. Because of the known expression of OATP1B3 in human hepatocytes (10), liver sections served as positive controls and were used to confirm the specificity of OATP1B3 antiserum by antigenic peptide blocking (Fig. 1C). In normal colon tissue, OATP1B3 protein expression was not detectable, consistent with its minimal mRNA expression in normal colon (Fig. 1C). In contrast, colon tumor sections showed intense cytoplasmic OATP1B3 immunostaining, whereas normal colon tissue showed no detectable immunostaining.

### Table 1. Summary of OATP1B3 immunostaining from colon tumor tissue microarray

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<th>OATP1B3 positive staining (intensity of $\geq 1$)</th>
<th>Normal colon</th>
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The OATP1B3 immunostaining was assessed by using the avidin-biotin complex method. The positive signal was visualized by DAB staining (dark brown color). Normal human liver was used as a positive control to confirm the specificity of polyclonal OATP1B3 antiserum and showed membranous immunostaining for OATP1B3. Colon tumor tissue showed intense cytoplasmic OATP1B3 immunostaining, whereas normal colon tissue showed no detectable immunostaining.
colon tumor tissue array containing 93 evaluable tumor and 12 normal colon tissue specimens. Normal colonic mucosa did not show OATP1B3 staining (intensity, 0); however, tumor tissue sections showed OATP1B3 staining primarily in the cytoplasm. We therefore designated staining of intensity of ≥1 as “positive” staining for OATP1B3. The results indicate that 81% of colon tumor sections evaluated (n = 75 of 93 specimens evaluated) were positive for OATP1B3 staining (Table 1). OATP1B3 expression was prevalent in all clinical stages with no statistically significant association with tumor stage (Fisher’s exact test, P > 0.05). In addition, there was no statistically significant association between OATP1B3 immunostaining and any known clinicopathologic factors (e.g., gender, age, tumor location, and tumor size; Fisher’s exact test, P > 0.05).

OATP1B3 overexpression provides a survival advantage to colon cancer cells. We assessed the expression of OATP1B3 in a number of established CRC cell lines, RKO, HCT-8, HCA-7, and Caco-2 cells. The quantitative RT-PCR results indicated that

Figure 2. The overexpression of OATP1B3 conferred a cell survival advantage after CPT or oxaliplatin treatment to cells harboring wild-type p53 but not to cells lacking p53. Cell viability after CPT (10 μmol/L, 24 h) or oxaliplatin (10 μmol/L, 72 h) treatment was determined using the MTT assay and expressed as the relative percentage compared with the vehicle (DMSO)-treated groups. A, Caco-2, RKO, and HCT-8 cells. B and C, isogenic HCT-116 cells with wild-type or null p53 status. Increased OATP1B3 protein expression in cells stably transfected with OATP1B3 was confirmed by Western blotting (WB) using a polyclonal OATP1B3 antiserum (bottom; **, P < 0.01; ***, P < 0.001, ANOVA with post hoc Tukey test).

Figure 3. The overexpression of OATP1B3 in HCT-116p53+/+ cells provided apoptotic resistance after CPT treatment. A, HCT-116p53+/+ cells stably transfected with OATP1B3 or the empty vector were treated with CPT (10 μmol/L, 24 h). Apoptotic cells were visualized by TUNEL staining (red fluorescence, arrows). Representative images and the results from quantitative analyses indicate that OATP1B3 overexpression is associated with a significantly decreased rate in apoptosis (P < 0.001, unpaired t test). B, after treatment with CPT (10 μmol/L, 24 h), the cleavage/activation of caspase-3 and PARP was substantially less in HCT-116p53+/+ cells stably overexpressing OATP1B3 compared with their empty vector controls.
OATP1B3 mRNA is expressed in all the cell lines tested except Caco-2 cells. The OATP1B3 mRNA levels per 10^6 copies of β-actin were as follows: 22707 (HCA-7), 1473 (RKO), 250 (HCT-8), and not detectable (Caco-2). Considering a possibility that the functional effect of OATP1B3 overexpression may be affected by cell line–dependent alterations, we developed cell line models stably overexpressing OATP1B3 or empty vector using Caco-2, RKO, and HCT-8 and examined whether OATP1B3 expression alters cell viability upon CPT (10 μmol/L, 24 hours) treatment. The results indicated that OATP1B3 overexpression led to a significant cell survival advantage after CPT treatment in RKO and HCT-8 cells but not in Caco-2 cells (Fig. 2A). Noting that the cellular p53 status differs between RKO and HCT-8 (p53 wild-type) and Caco-2 (harboring a p53 mutation causing a premature stop codon; ref. 30), we examined whether cellular p53 status is an important factor determining the effect of OATP1B3 on cancer cell survival using isogenic HCT-116 cells with wild-type and null p53 status. OATP1B3 overexpression enhanced cell survival upon CPT (10 μmol/L, 24 hours) or oxaliplatin treatment (10 μmol/L, 72 hours) in HCT-116(p53+)/+ cells but not in HCT-116(p53+)/− cells sharing the same genetic background (Fig. 2B and C, top). These findings indicate that p53-dependent pathways may be potentially important for the OATP1B3 effect to enhance cell survival after chemotherapy treatment.

To examine whether OATP1B3 confers a survival advantage through apoptotic pathways, we measured the apoptosis outcome in HCT-116(p53+)/+ cells stably overexpressing OATP1B3 using the TUNEL assay after CPT treatment (10 μmol/L, 24 hours). A representative image shown in Fig. 3A shows that HCT-116(p53+)/+ cells overexpressing OATP1B3 are protected against apoptosis induced by CPT (10 μmol/L) compared with the empty vector control cells. The quantitative analyses of apoptotic (TUNEL positive) and healthy nonapoptotic cells indicated that stable overexpression of OATP1B3 in HCT-116(p53+)/+ cells leads to a significant decrease (by 2.7-fold) in apoptosis (P < 0.001; Fig. 3A). Consistent with these results, the activation/cleavage of caspase-3 and PARP was substantially reduced in CPT-treated HCT-116(p53+)/+ cells overexpressing OATP1B3 compared with CPT-treated empty vector controls (Fig. 3B, lanes 2 versus 4).

OATP1B3 overexpression interferes with p53 transcriptional activity. Using p53-responsive reporter assays, we examined whether OATP1B3 expression interferes with the transcriptional activity of p53. We first examined the effect of OATP1B3 expression on the transcriptional activity of exogenous p53 by transiently transfecting the expression plasmids for p53 and OATP1B3 as well as p53-responsive reporter plasmids (PG13-luc and P21WAF1-luc). Relative luciferase activity after cotransfection of p53 and OATP1B3 in HCT-116(p53+)/− cells was substantially lower than that after

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**Figure 4.** Coexpression of OATP1B3 affects p53 transcriptional activity. A, the results from the p53-responsive PG13-luc and P21WAF1-luc reporter assays in HCT-116(p53+)/− cells show that OATP1B3 expression causes a decrease in the transcriptional activity of exogenous p53. B, the results from immunoblotting analysis indicate that cotransfection of OATP1B3 with p53 results in decreases in the protein level of P21WAF1, a p53 downstream target in HCT-116(p53+)/− and HCT-116(p53+)/+ cells. C, the results from the p53-responsive PG13-luc or PUMA-luc reporter assay in HCT-116(p53+)/− cells stably overexpressing OATP1B3 or empty vector show that CPT treatment increased reporter activity in the empty vector controls but not in cells overexpressing OATP1B3. D, HCT-116(p53+)/− cells stably overexpressing OATP1B3 or the empty vector showed induction of p53 protein upon chemotherapy treatment. However, the levels of the p53 downstream targets, P21WAF1 and PUMA, were substantially lower or undetectable in chemotherapy-treated OATP1B3 overexpressing cells compared with chemotherapy-treated empty vector control cells (lanes 2 vs. 4 for CPT treatment; lanes 6 vs. 8 for oxaliplatin treatment). *, P < 0.05; **, P < 0.01; ***, P < 0.001, different from the rest, ANOVA followed by post hoc Tukey test.
transfection of p53 alone (Fig. 4A). To ascertain the inhibitory effect of OATP1B3 on p53 transcriptional activity, we examined the protein levels of p53 and its downstream targets using HCT-116p53/−/− or HCT-116p53+/+ cells after transient expression of p53 and/or OATP1B3. The results indicate that cotransfection of OATP1B3 and p53 results in a substantial decrease in the protein levels of a p53 downstream target, P21WAF1, compared with transfection of p53 alone (Fig. 4B).

In addition, we investigated the effect of OATP1B3 overexpression on the transcriptional activity of endogenous p53 by comparing the effects of CPT treatment in HCT-116p53/−/− cells stably overexpressing OATP1B3 or empty vector. The results indicated that CPT treatment increased PG13-luc or PUMA-luc reporter activity in the empty vector controls but not in cells overexpressing OATP1B3 (Fig. 4C). These results suggested that OATP1B3 expression causes a decrease in p53 transcriptional activity. The inhibitory effect of OATP1B3 on endogenous p53 transcriptional activity was further verified by examining the protein levels of p53 and its downstream targets, P21WAF1 and PUMA. HCT-116p53/−/− cells stably overexpressing OATP1B3 or the empty vector showed induction of p53 protein upon CPT (10 μmol/L, 24 hours) or oxaliplatin (10 μmol/L, 72 hours) treatment. However, the levels of the p53 downstream targets, P21WAF1 and PUMA, were substantially lower or undetectable in chemotherapy-treated OATP1B3-overexpressing cells compared with chemotherapy-treated empty vector control cells (Fig. 4D, lanes 2 versus 4 for CPT treatment and lanes 6 versus 8 for oxaliplatin treatment). These results from the p53-responsive reporter assays and immunoblotting analysis suggest that OATP1B3 overexpression interferes with the transcriptional activity of p53 and its downstream signaling.

**Antiapoptotic effect of OATP1B3 seems to be associated with its transport function.** To investigate a potential association between the antiproliferative effect of OATP1B3 and its transport function, we used a point mutation (G583E) variant of OATP1B3 that lacks transport activity (31). In contrast to OATP1B3 (wild-type), the OATP1B3 variant (G583E) did not affect the p53 transcriptional activity after transient transfection with the PG13-luc plasmid in HCT-116p53/−/− cells (Fig. 5A). The cellular uptake of the fluorescently labeled bile acid (FITC-DCA; 10 μmol/L, 10 min) was substantially lower in HCT-116p53+/+ cells stably overexpressing OATP1B3 (wild-type) compared with cells stably overexpressing the OATP1B3 G583E variant or empty vector controls. Overexpression of the OATP1B3 G583E variant does not confer a cell survival advantage after CPT treatment to HCT-116p53/−/− cells, in contrast to OATP1B3 (wild-type). The levels of the p53 downstream targets, P21WAF1 and NOXA, are increased after CPT treatment in HCT-116p53/−/− cells stably overexpressing OATP1B3 (wild-type) but not in those stably overexpressing the OATP1B3 G583E variant.

**Discussion**

CRC remains one of the leading causes of cancer-related death worldwide and resistance to chemotherapy is a major issue in the management of recurrent and metastatic CRC. In this...
study, we show for the first time that OATP1B3 is aberrantly overexpressed at both mRNA and protein levels in the majority of colorectal adenocarcinomas and that OATP1B3 overexpression confers an antiapoptotic effect against chemotherapy treatment in colon cancer cells by altering the p53-dependent pathways. These findings may explain one of the potential mechanisms contributing to chemotherapeutic resistance in tumors harboring wild-type p53.

The expression of OATP1B3 in colon tumors and in the colon tumor cell lines (data not shown) was mainly cytoplasmic and clearly different from the membranous expression pattern in the normal liver. Cytoplasmic localization of OATP1B3 in tumor cells has also been found in primary breast cancer tissues (14). The aberrant expression of OATP1B3 in the cytoplasm of colon tumors was the impetus to investigate whether OATP1B3 functions differently in the setting of malignancy. The results from the current study suggest that OATP1B3 overexpression in the cytoplasm of colon tumor cells confers apoptotic resistance. Previous studies have reported cytoplasmic localization of other membrane proteins in cancer cell line models due to a defect in plasma membrane protein recycling or tumoral changes in signaling (e.g., PI3K activation; refs. 32, 33). However, the mechanism responsible for the cytoplasmic expression of OATP1B3 in colon tumors remains to be determined. In further studies, we have found that OATP1B3 expression is increased in premalignant adenomatous polyps (data not shown). However, in polyps with low malignant potential (hyperplastic), OATP1B3 was not detected, comparable with our observations in normal colonic mucosa. These results suggest that OATP1B3 up-regulation may be an early event associated with colorectal tumorigenesis and maintained throughout colon cancer progression and not just a marker of cellular proliferation. We aim to further investigate the pathobiological significance of OATP1B3 overexpression in colon epithelium by assessing the levels and variability of OATP1B3 expression in larger groups of colon polyps with differing malignant potential and histology.

In the present study, the prosurvival/antiapoptotic effect of OATP1B3 seems to involve the interference with the p53-dependent apoptosis pathways. OATP1B3 expression substantially decreased the levels of p53 downstream targets despite elevated p53 protein levels after chemotherapy treatment. The p53 protein, often called 'a guardian of the genome,' is the master regulator of apoptosis after exposure to DNA damage, hypoxia, and cytotoxic drugs, and it is the most commonly mutated gene in human cancers (34). Although p53 mutations have been associated with resistance to chemotherapy (35, 36), the predictive value of p53 mutations in determining clinical outcomes in CRC patients has not been clearly established (37–40). This may be in part due to methodologic problems assessing p53 mutation status in clinical samples (41, 42). Resistance to CRC treatment is observed in patients whose tumors harbor wild-type p53. Therefore other mechanisms may interfere with p53 function, altering response to chemotherapy. Our results suggest that OATP1B3 overexpression may be one of the mechanisms underlying chemotherapy resistance in tumors harboring wild-type p53. These findings may provide additional insights into understanding the complexity in determining chemotherapy response. In an attempt to identify the p53 wild-type/mutant status in colon tumors overexpressing OATP1B3 and to discover possible correlations between these two proteins in clinical samples, we performed immunohistochemical staining for p53 using a monoclonal p53 antibody (BP53.12) on the same tumor microarray that was used for OATP1B3 staining. On analysis, approximately one third of the samples showed an accumulation of p53 protein (26 of the total 89 tumor samples evaluable for p53 staining and 20 of 75 OATP1B3 overexpressing tumors). The distribution of p53-positive tumors was comparable among the OATP1B3 staining designations indicating no apparent correlation between p53 detection and OATP1B3 expression in these clinical samples (P = 0.325, Fisher's exact test). Further evaluation of a possible relationship between p53 accumulation and OATP1B3 expression is warranted given our in vitro results and the inherent limitations of immunohistochemistry as a means of assessing p53 mutational status. It is possible that the apparent lack of association between p53 staining and OATP1B3 overexpression may be due to other regulatory factors that affect the complex p53 pathway and mask the molecular relationships between p53 and OATP1B3 (43).

The results from our current study indicated that OATP1B3 overexpression in colon tumors is associated with the lower transcriptional activation of well-known p53 downstream target genes, P21WAF1, NOXA, and PUMA. These results are consistent with previously published reports documenting that P21WAF1, NOXA, and PUMA are transcriptionally regulated by p53 and play important roles in apoptosis (44–46). Our results also suggest that OATP1B3 overexpression in colon cancer cells harboring wild-type p53 reduces the activation of the DNA damage response protein PARP, an important marker of apoptosis (47). These findings are consistent with decreased apoptosis levels and attenuation of caspase activation in the presence of OATP1B3 overexpression.

OATP1B3 is known to mediate the transport of various endogenous and exogenous substrates including steroid and thyroid hormones, prostaglandins, and glutathione (8). Our results indicate that OATP1B3 overexpression in p53 wild-type HCT-116 cells confers a prosurvival/antiapoptotic effect and also maintains its transporter function in this setting. The overexpression of the OATP1B3 variant (G583E) lacking the transport activity resulted in no prosurvival/antiapoptotic effect, suggesting that the antiapoptotic effect of OATP1B3 may be associated with its transport activity. However, it is not a certainty that the antiapoptotic effect of OATP1B3 is due to alterations in the cellular transport of chemotherapy drugs because the antiapoptotic effect of OATP1B3 was observed with two drugs (CPT and oxaliplatin) that have differing OATP1B3 transport properties. There are differing reports regarding the transport of CPT and its structurally related analogue, CPT-11, by OATP1B3 (48–50). Oxaliplatin and other platinum drugs have been shown not to interact with organic anion transporters including OATP1B3 (50). Although our current results with OATP1B3 G583E variant suggest an association between the antiapoptotic effect of OATP1B3 and its transport activity, we cannot rule out the possibility that a point mutation of G583E in OATP1B3 affects both the antiapoptotic function and the transport activity of OATP1B3 simultaneously, and that the two functions are not necessarily linked. The results from our current study suggest potential complexity in the molecular mechanisms underlying the antiapoptotic effect of OATP1B3 overexpression. Further investigation using additional OATP1B3 variants or specific inhibitors of the transporter activity would be necessary to confirm the association between the antiapoptotic function and transport activity of OATP1B3 and to determine the involvement of...
additional molecular mechanisms. Certain transporters such as MDR1 have been shown to be localized in cytoplasmic organelles/vesicles such as mitochondria and golgi apparatus and to be functionally active as transporters (51, 52). Interestingly, MDR1 has been also reported to provide protection against apoptosis independently of its transport function (53, 54). Additional studies are required to clarify the molecular mechanisms and/or transport substrates contributing to the functional changes associated with OATP1B3 overexpression.

In conclusion, OATP1B3 is frequently overexpressed in a majority of colon tumor samples but not in normal colonic mucosa. Overexpression of OATP1B3 in colon cancer cells conferred an antiapoptotic advantage against chemotherapy treatment, by interfering with p53 transcriptional activity. These findings provide further justifications to investigate the molecular interactions between OATP1B3 and p53 as well as the clinical significance of OATP1B3 overexpression as a potential factor in determining chemotherapy resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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