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Differential sensitivities of the *MRP* gene family and γ-glutamylcysteine synthetase to prooxidants in human colorectal carcinoma cell lines with different p53 status

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Abstract

Recent molecular cloning studies have identified six members in the multidrug-resistance protein (*MRP*) gene family. However, the regulation of expression of these genes is largely unknown. We previously reported that expression of *MRP1*, encoding multidrug-resistance associated protein, and *γ-GCSh*, which encodes the heavy subunit of γ-glutamylcysteine synthetase (γ-GCS), could be up-regulated by prooxidants [Yamane *et al.*, J Biol Chem 1998;273:31075–85]. In the present study, we investigated whether different members of the *MRP* family exhibit different responses to induction by prooxidants, and whether p53 status influences the levels of induction. A panel of colorectal cancer cell lines with different p53 status, i.e. HCT116 containing wild-type p53, and HT29, SW480, and Caco2 containing mutant p53, was treated with *tert*-butylhydroquinone (t-BHQ) and pyrrolidinedithiocarbamate (PDTC). MRP1 and γ-GCSh mRNA levels were determined by the RNase protection assay, using gene-specific probes. We report here that induction of *MRP1* and *γ-GCSh* expression by these prooxidants varied among the different cell lines, and p53 mutations were not always associated with elevated levels of induction. These results suggest that the effects of p53 on the induced expression of *MRP1* and *γ-GCSh* depend on the environment of the cell and/or nature of p53 mutations. In an isogenic HCT116 cell line containing *p53*(-/-) alleles, we demonstrated that, as for *MRP1*, expression of *MRP2* and *MRP3* was induced by the prooxidants, whereas expression of *MRP4* and *MRP5* was not. MRP6 mRNA was not detectable. Induction of *MRP2* expression by prooxidants seemed to be independent of p53 status. Our results demonstrated the differential regulation of the *MRP* gene family by p53 mutation under oxidative stress.

Keywords: Multidrug-resistance protein; Drug transporter; Chemotherapy; Antioxidants; Gene regulation

1. Introduction

Cultured cells exposed to a single cytotoxic agent often develop cross-resistance to a wide range of structurally unrelated compounds. Two important mechanisms are responsible for the development of MDR: one is mediated by P-glycoproteins encoded by the *MDR* gene family (see the review in Ref. 1), and the other is mediated by the MRPs encoded by the *MRP* gene family (review in Ref. 2). Both P-glycoproteins and MRPs contain multiple transmembrane domains and two cytoplasmic ABCs. It is generally believed that these ABC transporters utilize ATP as an energy source to extrude cytotoxic compounds.

Mammalian *MRP1* was first isolated by molecular cloning from a doxorubicin-selected MDR cell line [3]. A transport assay using plasma membrane vesicles prepared from MRP1-overproducing cell lines demonstrated increased ATP-dependent, high-affinity transport activities of cytotoxic compounds conjugated with GSH or other organic anion moieties [4–6]. There are also reports suggesting that

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Abbreviations: MDR, multidrug resistance; MRP, multidrug-resistance protein; ABC, ATP-binding cassette; GSH, glutathione; γ-GCS, γ-glutamylcysteine synthetase; c-MOAT, canalicular multispecific organic anion transporter; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CRC, colorectal cancer cell; t-BHQ, *tert*-butylhydroquinone; PDTC, pyrrolidinedithiocarbamate; PCR, polymerase chain reaction; cDNA, complementary DNA; and ROS, reactive oxygen species.

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GSH may serve as a cofactor in MRP1-mediated transport [7].

It has been demonstrated that GSH levels play an important role in the regulation of MRP1 expression [8]. *De novo* synthesis of GSH is mainly controlled by the rate-limiting enzyme γ-GCS (EC 6.3.2.2). The mammalian γ-GCS holoenzyme is a heterodimer consisting of a 73-kDa heavy (catalytic) subunit (γ-GCSh) and a 29-kDa light (regulatory) subunit. In a number of biological systems, the expression patterns of *MRP1* and *γ-GCSh* are frequently coordinated [8–11].

In addition to *MRP1*, other *MRP* homologues, designated *MRP2–6*, have been identified by human expressed sequence tags or by using low stringency hybridization screening conditions (for a review, see Ref. 12). *MRP2*, expressed mainly in the canalicular membrane of hepatocytes, encodes cMOAT for hepatobiliary excretion of bilirubin glucuronides and other multivalent organic anions, including GSH *S*-conjugates [13–17]. Like *MRP1* [18, 19], transfection of *MRP2* into cultured cells confers elevated resistance to various antitumor agents [20, 21]. *MRP3*, which is the closest homologue of *MRP1* among the *MRP* family, shares 58% amino acid identity with *MRP1* [22, 23]. While *MRP3*-transfected cells confer resistance to antitumor agents (etoposide and methotrexate), unlike those overexpressing MRP1 and MRP2, these cells do not show increased GSH export [22]. An *in vitro* transport assay using membranes prepared from *MRP3*-transfected cells demonstrated that MRP3 has substrate specificity different from that of MRP1 and MRP2, that is, GSH conjugates, such as leukotriene C_4 and 2,4-dinitrophenyl-*S*-glutathione, are poor substrates of MRP3 [23]. While *MRP1, MRP2,* and *MRP3* are frequently overexpressed in many drug-resistant variants, overexpression of *MRP4* is infrequent in these cell lines [24]. A recent study demonstrated that elevated expression of MRP4, resulting from amplification of the *MRP4* gene, is correlated with resistance of these cells to the antiviral acyclic nucleoside analogues [25]. MRP5 and MRP6 share 36 and 45%, respectively, of their amino acid identities with MRP1. MRP5 is expressed in many human tissues with relatively high levels of expression in skeletal muscles and brain, whereas expression of MRP6 is relatively restricted, with elevated levels of expression in the liver and kidney [26]. Recent studies demonstrated that MRP5 functions as an ATP-dependent transporter of nucleotide analogues [27] and the natural cyclic nucleotides cyclic GMP and cyclic AMP [28], whereas the substrate specificities of MRP6 remain to be determined.

The mechanisms of expression of the *MRP* gene family in normal and drug-resistant tissues are not well understood. While gene amplification has been associated with the elevated expression of *MRP1* in some MDR cell lines [29–31], transcriptional up-regulation without DNA amplification has been implicated in some MRP-overexpressing cell lines [32]. We previously demonstrated that expression of *MRP1* and *γ-GCSh* is co-regulated by intracellular redox conditions that are ROS concentration dependent [8]. Expression of *MRP1* and *γ-GCSh* is up-regulated by prooxidants such as t-BHQ, 2,3-dimethoxy-1,4-sulfoximine, and menadione, which enhance the production of ROS. Likewise, suppression of ROS production by enhanced expression of GSH results in down-regulation of *MRP1* and *γ-GCSh*.

The present investigation was initiated to address two important issues relevant to regulation of the *MRP* gene family. First, in light of the diverse expression patterns among various members of the *MRP* family, we investigated whether expression of other members of this family would also respond to redox-regulation. This is particularly relevant considering that, as alluded to above, different members of MRP-encoding proteins exhibit overlapping but distinct substrate specificities. Co-induction of different MRP members would alter the drug resistance profile. Second, recent studies have revealed a strong correlation between the increased expression of p53 and MRP1 in clinical specimens [33]. Moreover, transfecting the temperature-sensitive p53 mutant into human LNCaP prostate cancer cells up-regulated *MRP1* expression in cells cultured under a nonpermissive temperature [34]. These findings are consistent with the notion that expression of *MRP1* is regulated by p53 status. Since it has been implicated that p53 plays an important role in regulating intracellular redox conditions [35–39], we investigated whether p53 mutations would affect the induced expression of *MRP1* and *γ-GCSh* in several human colorectal cancer cell lines containing mutant as well as wild-type p53. We chose human colorectal cancer cell lines because it has been suggested that redox conditions play an important role in the progression of colorectal carcinogenesis [40]. Moreover, we previously reported that expression of *MRP1* and *γ-GCSh* is frequently up-regulated in human colorectal cancers [10], a disease that frequently contains p53 mutations [41]. We report here that like *MRP1,* expression of *MRP2* and *MRP3* but not *MRP4* and *MRP5* could be induced by the prooxidant t-BHQ. We also found that not all the p53 mutations in the colorectal cancer cell lines could affect induced *MRP1* and *γ-GCSh* expression levels. In a pair of isogenic colorectal cancer cell lines, we found that null expression of p53 enhanced the induction of *MRP1, MRP3,* and *γ-GCSh* by t-BHQ as compared with its wild-type containing counterpart. These results demonstrated the differential response of the *MRP* gene family to prooxidant treatments and the effects produced by p53 in a gene-specific manner.

2. Materials and methods

2.1. Materials

Cell culture media (DMEM and McCoy's 5a) were purchased from GIBCO/Life Science. FBS, t-BHQ, PDTC, and oligonucleotides were obtained from the Sigma Chemical Co. ^{32}P-Labeled nucleoside triphosphates were purchased from DuPont NEN Research Products.

2.2. Cell lines

The human colorectal carcinoma cell lines HCT116, SW480, HT29, and Caco2 were obtained from Dr. Li-Kuo Su (M. D. Anderson Cancer Center). Cell lines 4016, 379.2, and 8054 were obtained from Dr. Bert Vogelstein (The Johns Hopkins University). All cell lines except for HT29 were grown in DMEM supplemented with 10% FBS. HT29 cells were grown in DMEM/McCoy's 5a (50% each) supplemented with 10% FBS and 20 mM glucose. Cells were maintained in a 5% CO_2 atmosphere at 37°. Cells in exponential growth were used for the induction experiments.

2.3. RNA isolation and RNase protection assay

The procedures used for the isolation of RNA, the preparation of human *MRP1* and *γ-GCSh* probes, and RNase protection assays have been described previously [8]. Quantitative analyses of mRNA levels were performed by densitometric scanning of the autoradiographs using an SI personal densitometer (Molecular Dynamics, Inc.). The autoradiographic signals corresponding to each mRNA species were converted into digitized images using computer software provided by the vendor.

RNase protection assays were also used to determine MRP2–MRP6 mRNA levels. The respective antisense probes were synthesized from recombinant plasmid templates containing the PCR products generated using the following primer sets (numbers refer to the translation start cite; forward/reverse primers):

MRP2. 5′ ^{818}CCTGGCTTGAACAAGAATCA/5′ ^{1058}CCAATCCACAAATATGTGTC;
MRP3. 5′ ^{3173}GATACGCTCGCCACAGTC/5′ ^{3420}CAGTTGCCGTGATGTGGCT;
MRP4. 5′ ^{1765}TAGTGACTCATCAGTTGCAG/5′ ^{2284}AGTACCAGTTAAGATCTAGC;
MRP5. 5′ ^{216}GATAACTTCTCAGTGG/5′ ^{593}ATGGCAATGCTCTAAAG; and
MRP6. 5′ ^{433}CTTGCCAGCTACCAACGCT/5′ ^{818}TTGCCTTGTTGTGCCT.

The PCR products were cloned into a pCRII-TOPO vector (Invitrogen) and sequenced. For synthesizing antisense probes, the respective plasmid DNAs were linearized by restriction endonucleases *Ava*II, *Eco*RV, *Dra*I, *Fok*I, and *Xho*I, respectively, and probes were synthesized using either T7 (for *MRP2*, *MRP4*, and *MRP5*) or SP6 (*MRP3* and *MRP6*) polymerase to generate the expected protection fragments.

2.4. Other procedures

Measurements of total cellular glutathione (GSH + 2xGSSG) followed the methods described previously [8].

3. Results

3.1. Induction of MRP1 and γ-GCSh expression by prooxidants in colorectal carcinoma cell lines with different p53 status

We previously demonstrated that expression of *MRP1* and *γ-GCSh* could be induced by prooxidants [8]. To investigate the effects of p53 status on the induction of these genes, we used a panel of colorectal cancer cell lines containing either wild-type p53 (HCT116) or mutant p53 (SW480, HT29, and Caco2). SW480 and HT29 contain mutations at codon 273 (CGT → CAT) [42], and Caco2 contains a deletion and a termination signal at codon 204 [43]. These cell lines were treated with 100 μM t-BHQ for various lengths of time. Total cellular RNA was prepared, levels of MRP1 and γ-GCSh RNA were determined by the RNase protection assay (Fig. 1A), and the corresponding transcripts were quantified by densitometry. Results from three independent assays were statistically analyzed (Table 1). In most cases, increased γ-GCSh mRNA levels could be seen 2 hr after the treatment, and levels continued to increase throughout the entire 24-hr testing period. The maximal levels of γ-GCSh induction varied among these cells lines, in descending order: HT29, Caco2, HCT116, and SW480. These results showed that induction of *γ-GCSh* expression by t-BHQ in these cell lines was independent of p53 status, perhaps because of the nature of p53 mutations and/or cell environment. Induction of MRP1 mRNA expression by t-BHQ was also seen in HCT116, HT29, and Caco2 cells. The levels of induction were only modest (1.5- to 2.5-fold), but were statistically significant. These levels of induction were low compared with those in other cell lines treated with the same amount of t-BHQ [8] and other cytotoxic agents [9,11]. Time–course dependent induction of *γ-GCSh* with another prooxidant, PDTC (100 μM), was also seen in these cell lines (Fig. 1B). However, the levels of induction were generally lower than those induced by t-BHQ. No statistically significant induction of MRP1 by PDTC was evident in these cells (Table 1), suggesting that PDTC is, comparatively, a weaker inducer.

To further analyze whether p53 plays a role in the modulation of *γ-GCSh* induction by prooxidants, we used a set of isogenic HCT116 cell lines constructed by a somatic cell knockout technique. In the 4016 cell line, one *p53* allele was replaced by a drug-resistance marker (neomycin), whereas in 379.2 cells, both alleles were replaced [39]. In the 8054 cell line, *p53* alleles remain wild-type, but both *p21* alleles have been knocked out. [40]. These cells were similarly treated with t-BHQ, and induction of *MRP1* and *γ-GCSh* expression was analyzed by the RNase protection assay. Figure 2A shows that the induced expression levels of γ-GCSh mRNA in 4016 and 8054 cells were comparable to those of HCT116 cells, whereas the induced expression levels in 379.2 cells were much higher than those in the other two cell lines (Table 1). These results indicated that

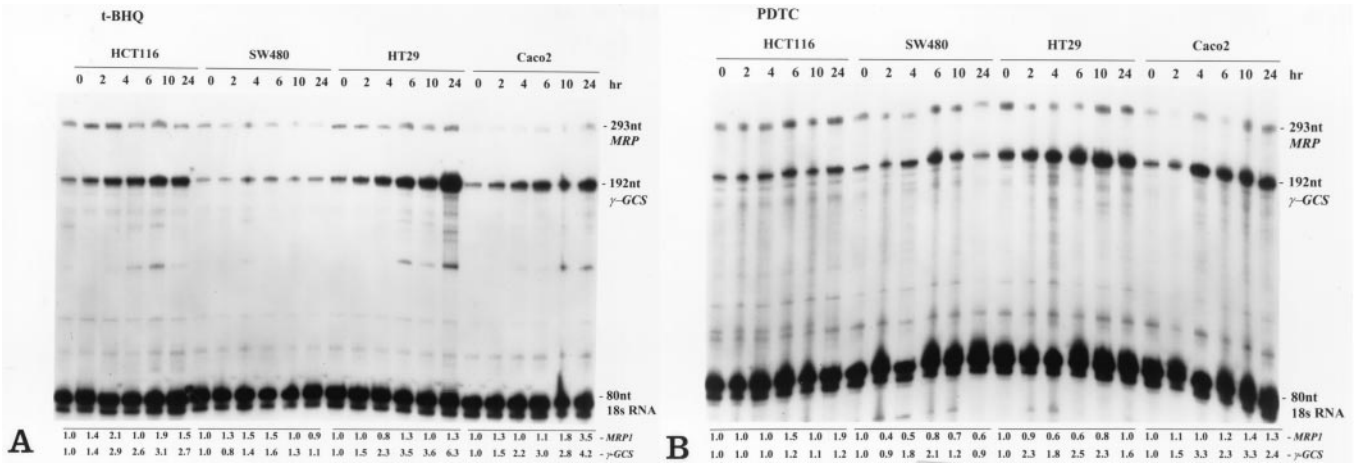

Fig. 1. Induction of *MRP1* and γ-*GCSh* expression in four human colorectal cancer cells treated with prooxidants, as determined by RNase protection assays. (A) t-BHQ (100 μM), and (B) PDTC (100 μM). Signals at 293, 192, and 80 nucleotides (nt) correspond to *MRP1*, γ-*GCSh*, and 18S rRNA protection fragment sizes, respectively. The autoradiographs were densitometrically scanned. Fold increases or decreases in levels of expression with 0 hr as the reference point were calculated for each cell line and are indicated underneath each lane. Numbers are averages of two independent experiments.

homozygous deletion of p53 is associated with elevated levels of γ-GCSh mRNA induced by t-BHQ. Moreover, induction of MRP1 mRNA by t-BHQ in 379.2 cells was higher than in 4016 cells, suggesting that homozygous deletion of *p53* also affects the induced expression of *MRP1*. Deletion of *p21* did not increase the induction of *MRP1* and γ-*GCSh* expression by t-BHQ (compare the 8054 and HCT116 lines in Table 1). When the 379.2 cells were treated with PDTC, the expression levels of γ-GCSh and MRP1 mRNA were enhanced when compared with the other cell lines (Fig. 2B, Table 1). These results suggested that null expression of p53 influences the inducibility of γ-GCSh and, to a much lesser extent, that of MRP1 mRNA by the prooxidants. Moreover, Table 1 shows that the prooxidant-induced expression level of γ-GCSh mRNA was correspondingly higher that that of MRP1 mRNA in all the cell lines, indicating that γ-GCSh is more sensitive than MRP1 to induction by these prooxidants.

Attempts to determine MRP1 protein by western blot using polyclonal antibody prepared in this laboratory [11] were not successful due to the low level of MRP1 expression, although this antibody could faithfully detect the expression of MRP1 in other cell lines. We note that treating these cell lines with t-BHQ and PDTC under the current conditions dramatically sensitized the cells to MRP1-related antitumor drugs, e.g. doxorubicin and VP-16 (data not

Table 1
Relative levels of MRP1 and γ-GCSh mRNA in colon cancer cells treated with t-BHQ or PDTC

Cell lines	p53 Status	t-BHQ		PDTC	
		Maximal induction (fold)		Maximal induction (fold)	
		MRP1	γ-GCSh	MRP1	γ-GCSh
HCT116	Wild type	1.52 ± 0.33* (10)[a]	2.78 ± 0.63* (10)	1.48 ± 0.35 (24)	1.31 ± 0.30 (24)
SW480	p53 Mutant	1.25 ± 0.25 (6)	1.77 ± 0.34 (6)	1.00 ± 0.15 (6)	2.29 ± 0.34*** (6)
HT29	p53 Mutant	1.61 ± 0.28* (6)	7.07 ± 1.14*** (24)	0.94 ± 0.10 (24)	2.05 ± 0.50*** (10)
Caco2	p53 Mutant	2.49 ± 1.11* (24)	4.51 ± 0.60*** (24)	1.36 ± 0.27 (10)	3.57 ± 1.21*** (10)
4016	p53 (+/−)	1.14 ± 0.11 (10)	2.76 ± 0.30* (10)	1.40 ± 0.29 (4)	4.02 ± 0.30*** (4)
379.2	p53 (−/−)	2.32 ± 0.42*** (10)	5.01 ± 1.63*** (10)	4.42 ± 1.09*** (24)	4.89 ± 1.60*** (24)
8054	p21 (−/−)	1.44 ± 0.38 (6)	2.14 ± 0.38 (6)	1.04 ± 0.10 (6)	2.00 ± 0.49 (6)

Values are means ± SD ($N = 3$).
[a] Time (in hr) at which maximal levels of induction were observed.
* Statistically significant ($P \geq 0.05$, Student's *t*-test), compared with the untreated control.
** Statistically significant ($P \geq 0.05$, Student's *t*-test), compared with HCT116 p53 (+/+).

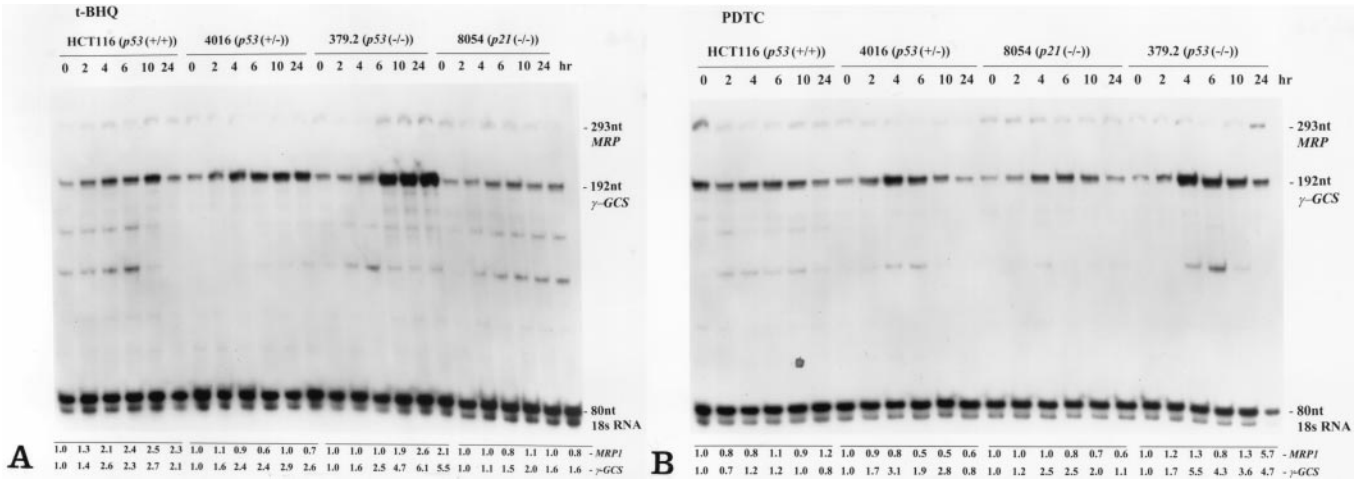

Fig. 2. Induction of *MRP1* and γ-*GCSh* expression by prooxidants in four isogenic cell lines with different genotypes. HCT116 contains wild-type *p53* and *p21*, the 4016 line contains a heterozygous *p53* deletion, the 379.2 line contains homozygous deletions, and the 8054 line contains homozygous deletion of *p21*. For details, see the legend to Fig. 1. Cells were treated with 100 μM t-BHQ (A) or with 100 μM PDTC (B).

shown). These findings preclude the investigation into whether the induced expression of MRP1 and γ-GCSh by these prooxidants would confer elevated drug resistance (data not shown).

3.2. Induction of GSH by prooxidants in colorectal cancer cells

The observation that elevated γ-GCSh mRNA levels could be induced transiently by prooxidants suggests that the intracellular GSH pool may also be altered in these cells. γ-GCS is the rate-limiting enzyme in the biosynthesis of GSH. Its regulation involves many signaling pathways [44–47] including the feedback suppression of γ-GCSh expression [8] and of its enzymatic activities [48]. It is important to determine the homeostatic levels of GSH in these cells upon exposure to the prooxidants. Figure 3A shows that, among the six colorectal cancer cell lines tested, significantly increased GSH levels were seen in HCT116, 8054, and HT29 cells treated with t-BHQ, whereas in cells treated with PDTC, a significant increase was seen in only the HCT116 cells. No significantly increased GSH levels were seen in the other cell lines. Moreover, the levels of GSH induction apparently did not correlate with the levels of γ-GCSh mRNA induction. These results suggest that there is post-transcriptional regulation of GSH levels in the colorectal cancer cells exposed to prooxidants.

3.3. Differential inducibility of the MRP family by prooxidants

To investigate the inducibility of other *MRP* members by the prooxidants and the role of p53 in the induction, we treated the two isogenic cell lines, HCT116 and 379.2, with either t-BHQ or PDTC for various lengths of time ranging from 2 to 24 hr. RNase protection assays were carried out using gene-specific probes to determine the levels of different MRP mRNAs in the treated cells. Figure 4 shows an example of autoradiographs using probes specific for the *MRP2–MRP6* sequences. Densitometric analyses of signals in the autoradiographs are presented in Table 2. Several important conclusions may be drawn. First, like *MRP1*, expression of *MRP2* and *MRP3* can be induced by t-BHQ and PDTC, whereas expression of *MRP4* and *MRP5* was induced only marginally. Expression of *MRP6* was not detectable in these cell lines. The inability to detect *MRP6* expression in these cells was not due to the RNase protection assay, because a predicted protection signal of 360 nt was detected in RNA prepared from human liver, a tissue source that expresses MRP6 mRNA [26]. Similar results were obtained in cells treated with PDTC (Fig. 4B). Second, it appears that expression of *MRP3*, like *MRP1*, was modulated by p53 knockout, whereas *MRP2* expression seemed to be independent of p53 status. Third, the kinetics of induction of *MRP* expression varied. Induced expression of *MRP1* and *MRP2* occurred at earlier time points than did induced expression of *MRP3* (Figs. 1, 2, and 4). These results demonstrated the differential inducibilities of the *MRP* gene family to the treatment of prooxidants in colorectal cancer cells and the influence of p53 status on the levels of induction.

4. Discussion

A previous study from this laboratory demonstrated that expression of *MRP1* and γ-*GCSh* is frequently up-regulated in human colorectal carcinomas [10]. The increase in expression levels ranged from several-fold to more than 100-fold. We also have demonstrated that expression of *MRP1* and γ-*GCSh* is regulated by redox conditions [8]. Because p53 plays an important role in regulating intracellular redox

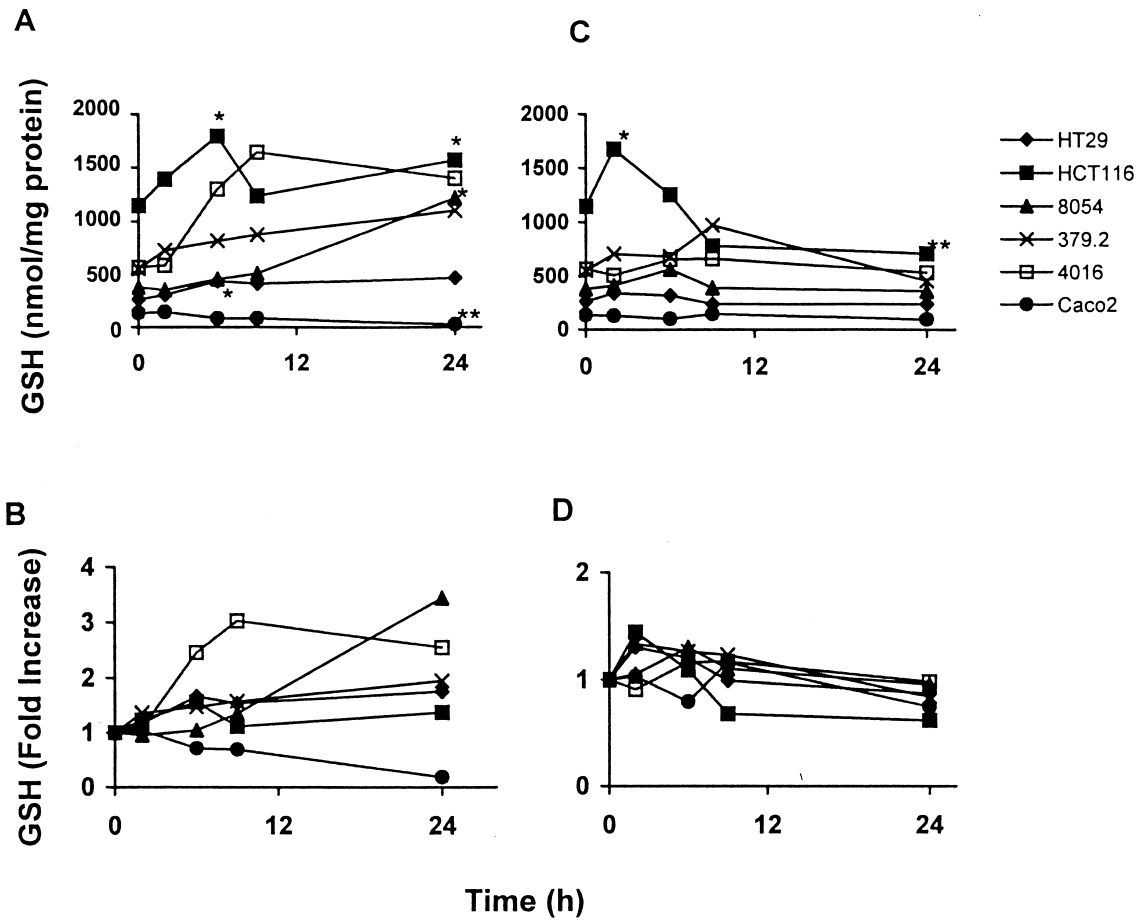

Fig. 3. Induction of GSH in various human colorectal cancer cell lines by prooxidants. Panels A and B show results of treatment with 100 μM t-BHQ; panel C and D, 100 μM PDTC. Panels A and C show GSH contents. Panel B and D show fold increases (decrease) in reference to the 0-hr treatment. Data are averages of at least three independent determinations. For simplicity, no standard error bars are indicated, but those values statistically significant ($P \leq 0.05$, Student's *t*-test) for increase (*) or decrease (**) compared with 0-time values are marked.

conditions, and because p53 mutations are frequently found in colorectal carcinomas [41], as an initial step to elucidating the mechanism by which *MRP1* and γ-*GCSh* expression is up-regulated during colorectal carcinogenesis, we investigated the expression of *MRP1* and γ-*GCSh* in a panel of colorectal cancer cell lines with different p53 mutations. Our results showed that prooxidants such as t-BHQ could only induce low levels (1.5- to 5-fold) of γ-*GCSh* expression. The expression levels of induced *MPR1* were greatly reduced (1.3- to 3-fold). Furthermore, not all the mutant p53-containing cell lines exhibited elevated MRP1 or γ-GCSh mRNA levels as compared with cells containing wild-type p53. Nonetheless, in the isogenic cell lines carrying null *p53* alleles, we observed that homozygous deletion of p53 increased the induction of *MRP1* and γ-*GCSh* expression by prooxidants. These results collectively suggest that the effects depend upon cell environment.

Our observation that p53 mutation is associated with elevated expression of *MRP1* in some human colorectal cancer cells may have clinical implications. Because p53 expression in normal colorectal epithelium is very low, accumulation of p53 in malignant cells is frequently associated with p53 mutations. A recent study showed that *MRP1* expression correlates with aberrant p53 accumulation [46]. Using immunohistochemistry, we observed a significant correlation between MRP1 expression and p53 accumulation ($P < 0.03$) in 68 human colorectal carcinomas. We also observed that *MRP1* expression was less frequent in adenomas (56%) than in carcinomas (88%), correlating with the p53 mutation frequency during colorectal cancer progression. Similar results have been noted by another group [49]. These findings, together with those from a human prostate cancer study [33], suggest that p53 mutations play a role in the up-regulation of *MRP1* in human cancers.

The mechanisms by which p53 regulates *MRP1* expression have not been elucidated clearly. A previous study demonstrated that co-transfection of wild-type p53 markedly suppresses *MRP* promoter activity in a transient transfection assay using reporter constructs [50]. Moreover, restoration of wild-type p53 in a p53 null cell line suppresses endogenous *MRP1* expression [50]. Our present study

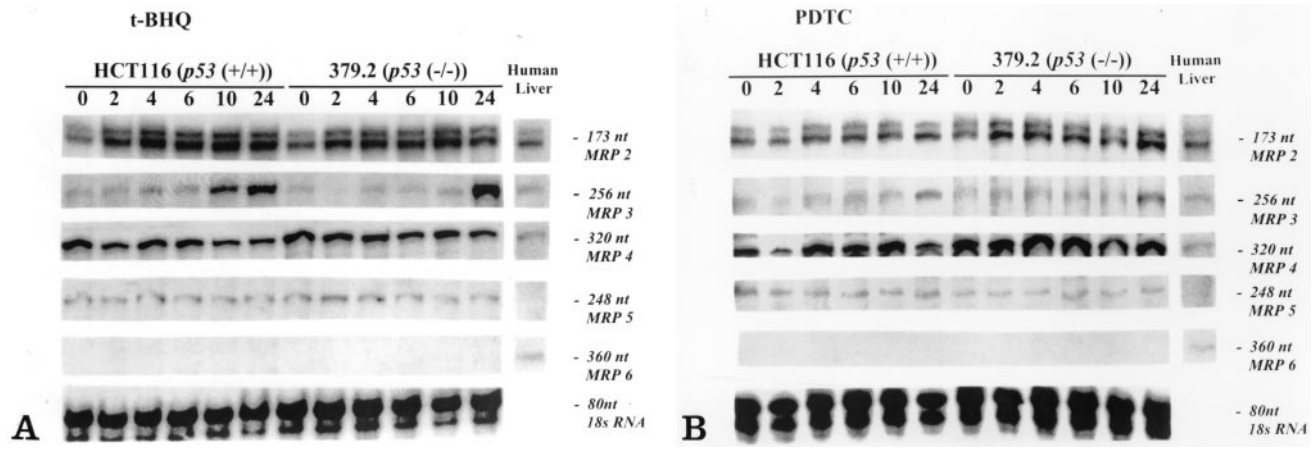

Fig. 4. Induction of different members of the *MRP* gene family in HCT116 and 379.2 cells treated with 100 μM t-BHQ (A) or 100 μM PDTC (B). Only signals corresponding to the expected protected sizes are shown. Total RNA from human liver was used as the control for the *MRP6* probe because the expression of MRP6 mRNA in the treated cells was too low to detect. Note that different members of the *MRP* family responded differently to the treatment with these prooxidants.

showed higher levels of induced expression of *MRP1* and *γ-GCSh* in a null-p53 cell line (379.2) than in a wild-type isogenic counterpart. These results are consistent with the idea that p53 plays a suppressive role in the regulation of *MRP1* expression. No p53 consensus sequence was found in the *MRP1* reporter constructs [50]. These results rule out a direct binding to a *cis*-acting element in the *MRP1* promoter region and suggest that the suppressive effects may be mediated by p53 downstream signals [51]. One of the important downstream *p53* genes is $p21^{CIP\text{-}1/WAF1,}$ which codes for a cdk inhibitor involved in p53-mediated cell cycle regulation [44] and G_2 arrest [40]. It is unlikely that the suppressive role of p53 in the regulation of *MRP1* and *γ-GCSh* expression is mediated by p21, however, because homologous deletion of *p21* failed to increase the induced expression of *MRP1* and *γ-GCSh*. Further research on the molecular basis underlying p53 modulation of *MRP1* and *γ-GCSh* expression in colorectal carcinogenesis is needed.

The successful molecular cloning of additional *MRP* genes has increased the complexity of the MDR system. While the expression of this gene family in normal tissues and in drug-resistant variants has been largely determined [22, 27], regulation of these genes by extracellular influences has not. Our present results demonstrating the differential inducibilities of the *MRP* gene family by prooxidants and by p53 mutations strongly suggest that diverse regulation mechanisms are associated with the expression of this gene family. We have demonstrated that among the six *MRP* genes investigated, *MRP3* is the most sensitive to induction by prooxidants (Table 2). It is interesting to note

Table 2
Maximal induction of MRP1, MR2, MRP3, MRP4, MRP5, and MRP6 mRNA in two isogenic colorectal cancer cell lines treated with t-BHQ or PDTC

MRP	HCT116 p53 (+/+)		379.2 p53 (−/−)	
	Maximal induction (fold)		Maximal induction (fold)	
	t-BHQ	PDTC	t-BHC	PDTC
MRP1	1.52 ± 0.343* (10)[a]	1.48 ± 0.35 (10)	2.32 ± 0.42*** (1)	4.42 ± 1.09*** (24)
MRP2	3.24 ± 1.45* (24)	1.34 ± 0.41* (24)	2.75 ± 0.91* (10)	2.16 ± 0.64* (24)
MRP3	8.74 ± 5.52* (24)	3.45 ± 0.77* (24)	19.67 ± 1.00*** (24)	3.77 ± 1.33* (24)
MRP4	0.97 ± 0.98 (10)	1.94 ± 0.56 (10)	1.03 ± 0.05 (10)	1.19 ± 0.06 (24)
MRP5	1.23 ± 0.25 (10)	1.30 ± 0.12 (24)	1.36 ± 0.47 (10)	1.23 ± 0.12 (10)
MRP6	N[b]	N	N	N

Values are means ± SD ($N = 3$).
[a] Time (in hr) at which maximal levels of induction were observed.
[b] N = not detectable.
* Statistically significant ($P \leq 0.05$, Student's *t*-test), compared with the untreated control.
** statistically significnt ($P \leq 0.05$, Student's *t*-test), compared with HCT116 p53 (+/+).

that, in the Eisai hyperbilirubinemic rat (EHBR), which contains mutated cMORT/MRP2 and exhibits defective transport of bile organic anions and glutathione and glucuronide conjugates [16], expression of *MRP3* in the liver is increased. These results suggest a compensatory relationship between *MRP2* and *MRP3* expression. Such compensatory expression was also observed in *mdr1a*(-/-) mice in which expression of *mdr1b* is elevated [52]. Expression of *MRP3* in rats could be induced by phenobarbital treatment [53]. These results, together with those presented here, are consistent with the notion that *MRP3* expression is sensitive to many extracellular stimuli. Further studies are required to elucidate the regulation of each of the *MRP* genes under various environmental influences. These studies should provide a better understanding of the evolution of multidrug resistance and enable us to design strategies to combat it.

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