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The expression of several drug-resistance genes, including *MRP* and *p53*, increases with advancing stage of human prostate cancer. Altered transcription could account for the genotypic alterations associated with prostate cancer progression, and it was recently reported that the promoter of *MRP1* is activated in the presence of mutant p53. To determine whether there is a relationship between p53 status and the expression of MRP1, a human, temperature-sensitive p53 mutant (tsp Val<sup>138</sup>) was transfected into LNCaP human prostate cancer cells. In the transfected cell line (LVCaP), the wild-type p53 produced growth arrest at the G1/S interface of the cell cycle, inhibited colony formation, and induced p21<sup>waf1/cip1</sup>. Temperature shifting to 38°C (p53 mutant) produced a time-dependent increase in expression of MRP1. This change in MRP1 expression was also seen in isogenic cell lines in which p53 was inactivated by human papilloma virus (HPV)16E6 protein or by a dominant-negative mutant. Functional assays revealed a decrease in drug accumulation and drug sensitivity associated with mutant p53 and increased MRP1 expression. These results provide the first mechanistic link between expression of MRP1 and mutation of p53 in human prostate cancer and support recent clinical associations. Furthermore, these data suggest a mechanism tying accumulation of p53 mutations to the multidrug resistance phenotype seen in this disease.

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Regulation of expression of the multidrug resistance protein MRP1 by p53 in human prostate cancer cells

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The expression of several drug-resistance genes, including MRP and p53, increases with advancing stage of human prostate cancer. Altered transcription could account for the genotypic alterations associated with prostate cancer progression, and it was recently reported that the promoter of MRP1 is activated in the presence of mutant p53. To determine whether there is a relationship between p53 status and the expression of MRP1, a human, temperature-sensitive p53 mutant (tsp Val138) was transfected into LNCaP human prostate cancer cells. In the transfected cell line (LVCaP), the wild-type p53 produced growth arrest at the G1/S interface of the cell cycle, inhibited colony formation, and induced p21waf1/cip1. Temperature shifting to 38°C (p53 mutant) produced a time-dependent increase in expression of MRP1. This change in MRP1 expression was also seen in isogenic cell lines in which p53 was inactivated by human papilloma virus (HPV) 16 E6 protein or by a dominant-negative mutant. Functional assays revealed a decrease in drug accumulation and drug sensitivity associated with mutant p53 and increased MRP1 expression. These results provide the first mechanistic link between expression of MRP1 and mutation of p53 in human prostate cancer and support recent clinical associations. Furthermore, these data suggest a mechanism tying accumulation of p53 mutations to the multidrug resistance phenotype seen in this disease.

at 32°C these cells express a functionally wild-type protein (11, 12). We used the transfected cell line, LVCaP, to analyze the regulation of MRP1 by p53 in human prostate cancer.

**Methods**

**Cell Culture.** LNCaP.FGC 1740 cells frozen at passage 18 (American Type Culture Collection, Rockville, Maryland, USA) were routinely cultured in RPMI 1640 supplemented with 10% FBS, 15 mM HEPES buffer (pH 7.4), 2 mM L-glutamine and 100 μM nonessential amino acids (GIBCO BRL, Gaithersburg, Maryland, USA). LVCaP cells were routinely cultured in the LNCaP media supplemented with 50 μg/mL of Geneticin (GIBCO BRL).

RKO.mp53.13 was generated by transfecting parental RKO cells with a dominant-negative p53 mutant. Cells were maintained in a humidified atmosphere containing 5% CO₂/95% air and were free of contamination with mycoplasma or fungi.

**Transfections.** The pCMV p53 Val¹³⁸ vector was a generous gift from U. Moll (State University of New York at Stony Brook, New York, USA) and has been described previously (11). Amino acid 138 of the human p53 gene was mutated from alanine to valine (GCC → GTC). This mutation creates a temperature-dependent conformational change in the p53 protein so that it is functionally active at 32°C but not at 38°C. Transfections were carried out with 2 μg of the pCMV p53 Val¹³⁸ vector using Lipofectamine (GIBCO BRL) according to the manufacturer’s instructions. After transfection, cells were allowed to recover for 48 hours before selection with 500 μg/mL of Geneticin. Colonies were selected and expanded in LVCaP media containing 15% FBS.

**Cell growth.** Four milliliters of LVCaP and LNCaP cells were plated at 3.5 × 10⁴ cells/dish in 60 × 15 mm tissue culture dishes. After allowing the cells to attach to the plastic and grow for varying periods, cells were harvested by trypsinization and counted with an electronic counter. Three determinations of cell number were made for each time point.

**Clonogenic assay.** LVCaP and LNCaP cells were plated at 5 × 10² cells/mL in 60 × 15 mm tissue culture dishes. After 2–4 weeks, media was aspirated and plates were incubated for 3 minutes in methylene blue (2.5 g of methylene blue trihydrate, 250 mL water, and 250 mL 95% ethanol). The stain was removed and plates rinsed in tepid running water. When dry, colonies were counted using a colony counting pen.

**Cell-cycle analysis.** Cell-cycle analysis was performed by measuring uptake of bromodeoxyuridine (BrdU) and staining of DNA with propidium iodide. Briefly, LVCaP and LNCaP were incubated with 10 μM BrdU for 1 hour at 37°C and harvested by trypsinization. Cells were washed in ice-cold PBS (137 mM NaCl [pH 7.4], 2.3 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄), resuspended in 200 μL of PBS, and fixed by drop-wise addition of ice-cold 70% ethanol while vortexing. The cells were resuspended and incubated for 30 minutes in 2 N hydrochloric acid in PBS supplemented with 0.5% Triton-X100 and neutralized by rinsing once in 0.1 M sodium tetraborate (pH 8.5). Next, cells were resus-
pended with 50 μL of FITC-conjugated anti-BrdU antibody (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) diluted 1:5 in PBS supplemented with 1% BSA and 0.5% Tween-20 and incubated for 30 minutes. Cells were rinsed and resuspended in 1 mL of PBS containing 5 μg/mL of propidium iodide. Fluorescence intensities were determined by quantitative flow cytometry and profiles were generated on a Becton Dickinson FACScan analyzer.

Immunoblotting. Cell pellets were lysed on ice for 30 minutes with RIPA buffer (10 mM sodium phosphate [pH 7.2], 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 2 mM EDTA) supplemented with fresh 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 50 μg/mL leupeptin. After trituration through a 25-gauge needle, lysates were centrifuged at 14,000 g at 4°C for 10 minutes. Protein concentration was determined using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories Inc., Hercules, California, USA). Proteins (50–100 μg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes for detection of p53 and p21waf1/cip1 or polyvinylidene difluoride membranes for detection of MRP1. The blots were incubated in blocking solution consisting of 5% milk and 3% BSA in PBS-T for 2 hours at 25°C and were then immunoblotted with monoclonal anti-human p53 protein clone DO7 (DAKO Corp., Carpinteria, California, USA), or WAF1 (Ab-1) (Calbiochem-Novachem, San Diego, California, USA), or anti-MRP1 antibody (QCRL-1; Signet, Dedham, Massachusetts, USA), or anti–β-actin antibody (Sigma Chemical Co., St. Louis, Missouri, USA). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer’s protocol (Amersham Life Sciences Inc., Arlington Heights, Illinois, USA). Relative expression of proteins was determined by densitometric analysis of autoradiographs using Molecular Analyst Software on a Molecular Imager FX system (Bio-Rad Laboratories, Inc.).

Northern blot analysis. RNA was prepared using Trizol Reagent according to the manufacturer’s protocol (GIBCO BRL). Twenty micrograms of total RNA from each sample were electrophoresed, blotted onto nitrocellulose, and probed for MRP1. An α-32P-labeled β-actin probe was used to measure RNA loading. Relative expression of mRNA was determined by densitometric analysis of autoradiographs using Molecular Analyst Software on a Molecular Imager FX system.

Leukotriene C4 accumulation. Cells were seeded in 24-well plates and grown at 37°C (LNCaP) or 32°C (LVCaP). When cells were 80% confluent, LVCaP cells were shifted to 38°C and incubated for 60 hours. The growth medium was aspirated and replaced with 0.5 mL of RPMI 1640 containing 25 mM HEPES (pH 7.4) and 50 nM of [3H]leukotriene C4 (NEN Life Science Products Inc., Boston, Massachusetts, USA). Cells were incubated with [3H]leukotriene C4 for 2 hours and were then cooled on ice, washed three times with ice-cold PBS, and solubilized with 0.25 mL of 1% SDS. The radioactivity in each sample was determined by scintillation counting.

Table 2
Sensitivity of LNCaP and LVCaP cells to vincristine and doxorubicin at 38°C

<table>
<thead>
<tr>
<th>Drug</th>
<th>LNCaP</th>
<th>LVCaP</th>
</tr>
</thead>
<tbody>
<tr>
<td>vincristine</td>
<td>8.3 ± 0.9</td>
<td>240 ± 43a</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>180 ± 0.5</td>
<td>360 ± 28a</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD of three separate experiments. aP < 0.05 versus LNCaP cells.
Drug sensitivity. Exponential growing cells were plated into 96-well tissue culture plates at a density of 1 \( \times 10^4 \) cells per well. Various concentrations of drugs were added to each well, and the plates were incubated at 38.5°C for 72 hours. Cell viability was measured by the MTT assay (4).

Results

Growth characteristics of cells expressing temperature-sensitive p53. To determine whether or not p53 status affected expression of MRP, we generated a stable clone expressing a temperature-sensitive p53 mutant (tsp Val138). Figure 1a demonstrates that at the restrictive temperature (38°C; mutant p53), the LVCaP cells proliferated with a log-phase doubling time of 28 hours. In contrast, proliferation was inhibited at the permissive temperature (32°C; wild-type p53) (Figure 1a). The parental cell line, LNCaP, expresses wild-type p53 (13) and proliferated at both the permissive and the restrictive temperatures; log-phase doubling times were 48 and 32 hours, respectively (Figure 1b).

Temperature shift also affected clonogenicity. As shown in Table 1, at the restrictive temperature (38°C; mutant p53) LVCaP cells had a 48% cloning efficiency. In contrast, shifting to the permissive temperature (32°C; wild-type p53) led to a complete loss of clonogenic capacity. Temperature shifting had no significant effect on the clonogenicity of LNCaP cells (Table 1).

Effect of p53 on cell-cycle distribution and related gene expression. We determined the effect of temperature shifting on cell-cycle distribution by BrdU uptake and propidium iodide staining. As seen in Figure 2, incubation of LVCaP cells at the permissive temperature (32°C; wild-type p53) produced G1/S arrest; the cells returned to a normal cell-cycle distribution when shifted to the restrictive temperature (38°C; mutant p53). In contrast, temperature shifting produced no significant differences in cell-cycle distribution in the parental LNCaP cell line (data not shown).

To confirm that the G1/S arrest seen in LVCaP cells was p53-mediated, we studied the effect of temperature shifting on p21wafl/cip1. As seen in Figure 3, the expression of p21wafl/cip1 was markedly increased when LVCaP cells were shifted from the restrictive temperature (38°C; mutant p53) to the permissive temperature (32°C; wild-type p53). p21wafl/cip1 was barely detectable at 38°C in LVCaP cells, increased greatly at 8 hours, and increased further after 16 hours of culture at 32°C (Figure 3). Temperature shifting had no effect on the expression of p21wafl/cip1 in the parental cell line (Figure 3).

Wild-type p53 represses the expression of genes such as Bcl-2 (14) and MAP4 (15). As shown in Figure 4, the expression of Bcl-2 protein was low in LVCaP cells cultured at the permissive temperature (32°C; wild-type p53) and increased after shift to the restrictive temperature (38°C; mutant p53). Effect of functional status of p53 on expression of MRP1. LVCaP cells cultured at the permissive temperature (32°C; wild-type p53) expressed low levels of MRP1 protein (Figure 5). Shifting LVCaP cells to the restrictive temperature (38°C; mutant p53) increased the expression of MRP1 9-fold over a 60-hour period (Figure 5). In contrast, the parental cells (LNCaP) did not express MRP1 at either the restrictive or the permissive temperatures (Figure 5). To assess whether p53 affected MRP1 expression at the RNA level, we isolated total cellular RNA from LVCaP and LNCaP cells incubated at 38°C or 32°C for varying periods and measured MRP1 mRNA by Northern analysis. Figure 6 shows that growth of LVCaP cells at 38°C increased the expression of MRP1 mRNA; from 12 hours to 48 hours, there was a 2.4-fold increase in the expression of MRP1 mRNA. MRP1 mRNA was low in LNCaP cells and did not change with temperature shift.

To substantiate the relationship between p53 function and MRP1 expression, we measured the expression of MRP1 in additional paired human cancer cell lines in

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**Figure 3**

Effect of temperature on the expression of p53 and p21wafl/cip1 in LVCaP and LNCaP cells. Identical amounts (50 µg) of total protein were resolved using a 15% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with monoclonal anti-human p53, monoclonal anti-human β-actin, and monoclonal anti-human p21wafl/cip1 antibodies as described in Methods. Results are representative of three similar experiments.

**Figure 4**

Effect of temperature on the expression of Bcl-2 in LVCaP cells. Identical amounts (100 µg) of total protein were resolved using a 15% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with monoclonal anti-human Bcl-2 and monoclonal anti-human β-actin antibodies as described in Methods. Results are representative of three similar experiments.
which one member of the pair harbors a transcriptionally inactive p53. In the A875/E6 melanoma cell line, p53 is degraded by HPV16E6. In the RKO.mp.53.13 colon cancer cell line, p53 is inactivated through transfection with a dominant-negative mutant (16). Figure 7 demonstrates that inactivation of p53 resulted in a doubling of MRP1 protein level in A875/E6 cells and a 14-fold increase of the protein in RKO.mp.53.13 cells.

**Effect of MRP1 expression on drug accumulation and sensitivity.** To determine whether the increased expression of MRP1 seen with mutant p53 had functional consequences, we studied the accumulation of an MRP1-specific substrate (leukotriene C4) using the LVCaP model. As shown in Figure 8, the cellular accumulation of leukotriene C4 was significantly reduced in LVCaP cells grown at 38°C, compared with that in LNCaP cells (P < 0.01; Figure 8a) or compared with that in LVCaP cells growing at 32°C (P < 0.01; Figure 8b). Temperature shifting had no effect on drug accumulation in LNCaP cells (data not shown). Similar results were obtained with doxorubicin, another MRP1 substrate (data not shown).

Increased expression of MRP1 also changed the sensitivity to chemotherapeutic drugs that are transported by this protein. At 38°C (restrictive temperature; mutant p53), LVCaP cells were 29-fold more resistant to vincristine compared with LNCaP cells (IC50 240 ± 43 vs. 8.3 ± 0.9 nM) and were twofold more resistant to doxorubicin (IC50 360 ± 28 vs. 180 ± 0.5 nM) (Table 2). LVCaP cells did not proliferate at 32°C (permissive temperature, wild-type p53), making comparisons of drug sensitivity inaccurate.

**Discussion**

The hypothesis that mutation of p53 might contribute to drug resistance in prostate cancer was suggested by our analysis of drug-resistance proteins during the progression of human prostate cancer (1). To test this hypothesis, we created the LVCaP cell line by stable transfection of LNCaP cells with a vector containing a temperature-sensitive p53 mutant. The Val138 mutation is in the DNA binding region of p53 and abrogates DNA binding at the restrictive temperature by altering the conformation of the tertiary structure of the protein (11).

LVCaP cells display the anticipated phenotype after temperature shifting. At the permissive temperature, p53 adopts a wild-type conformation and is functionally active (11, 12). This was confirmed in the LVCaP cell line as measured by induction of p21waf1/cip1 (Figure 3). p21waf1/cip1 is part of the G1/S cell-cycle checkpoint and overexpression of this protein in LVCaP cells produces G1/S arrest (Figure 2) and inhibition of cell proliferation (Figure 1). Induction of p21waf1/cip1 also markedly inhibited the ability of LVCaP cells to form colonies (Table 1), but did not produce apoptosis (Figure 2). This pattern of growth arrest, failure to form colonies, yet lack of initiation of apoptosis, can be explained by the expression of the antiapoptotic protein, Bcl-2, in this cell line (Figure 4). In addition, these properties make the LVCaP model a particularly useful one for the study of both biologic and pharmacologic consequences of p53 alterations.

We have previously found that both the expression of MRP1 and p53 increased with advancing stage in human prostate cancer specimens (1). However, our previous measurements of p53 in human prostate cancer specimens were done by immunohistochemistry, which does not definitively discriminate between the mutant and wild-type protein. Rather, the detection of p53 by immunohistochemistry correlates well with the presence of the mutant protein because of its increased half-life. Using the LVCaP model, we find that MRP1 expression is regulated by the transcriptional activity of p53. Thus, when p53 is wild-type, the expression of MRP1 is repressed (Figure 5), and when p53 is mutant, the expression of MRP1 increases (Figure 5). Similar observations were made in paired, isogenic, human melanoma and colon cancer cells in which p53 was inactivated either by HPV6E-induced degradation or by transfection with a dominant-negative p53 mutant (Figure 7). The level
of control of MRP1 by p53 appears to occur at least in part at the mRNA level as a shift from transcriptionally active to inactive p53 produced increases of MRP1 mRNA (Figure 6). These data are consistent with the work by Wang and Beck, who demonstrated the ability of wild-type p53 to suppress the promoter activity of MRP1 (2). Finally, p53 mutation and overexpression of MRP1 protein leads to increased function of the transporter as measured by the accumulation of leukotriene C4 (Figure 8), an MRP1-specific substrate and the decreased sensitivity to vincristine and doxorubicin (Table 2). The decreased sensitivity to drugs was not due to changes in MDR1 gene expression, as P-gp was not detectable in either cell line (G.F. Sullivan and W.N. Hait, unpublished observations).

The significance of these results is strengthened by several factors. First, the relationship between p53 and MRP1 expression was originally suggested by studies done in human cancer specimens including prostate (1), colorectal (17) and non-small cell lung cancer (18). Second, unlike previous reports on the regulation of MRP1 and MDR1 that focused on activation of promoter/reporter constructs (2, 6), our results demonstrate that p53 can regulate the expression of the endogenous MRP1 gene, producing changes in both mRNA and protein (Figures 5, 6, and 7). The promoter regions of ABC transporters are complex, and no obvious p53-binding motif has been reported. However, repression of transcription by wild-type p53 may occur through indirect means rather than direct binding to promoter or enhancer elements (19). For example, Murphy et al. demonstrated that a microtubule-associated protein, MAP4, was transcriptionally repressed by wild-type p53, yet MAP4 does not contain an obvious p53 binding motif (15). They went on to show that a complex of wild-type p53, mSin3a, and histone deacetylase (20) mediated repression of MAP4 transcription. Third, inactivation of p53 by HPVE6 protein or by expression of a dominant-negative mutant in iso-

genic melanoma and colon carcinoma cell lines produced similar effects on MRP1 (Figure 7).

Because p53 mutations are found in approximately 50% of human cancers, this suggests that upregulation of MRP1 might be part of a cellular response to conditions of oxidative stress in which the likelihood of fixing mutations into the genome is increased. The fact that MRP1 participates in the efflux of glutathione-, glucuronate-, and sulfate-conjugated lipophilic xenobiotics and endotoxins has implicated it in a redox-controlled detoxification pathway (21–23). We also observed that the expression of GSTπ is rapidly lost as prostate epithelium undergoes malignant transformation (1). This is believed to be a consequence of hypermethylation of cytidine residues in the regulatory
sequences of the GST\(\text{p}\) promoter (24, 25). The loss of GST\(\text{p}\) can create a pro-oxidant cellular environment by shutting down a major phase II detoxification enzyme in the cancer cells (26). Because of this, many xenotoxic and endotoxins may remain in a mutagenic and electrophilic state ready to interact with nucleophilic substrates, such as DNA and protein (27). In this biotransformed state, these toxins cannot be transported out of the cell by MRPI and pose an increased threat to the stability of the genome. Therefore, the upregulation of MRPI might represent a required compensation by cancer cells.

In summary, our results indicate that the functional status of p53 can regulate the expression of MRPI in human prostate cancer cells. Because p53 mutations become increasingly frequent as prostate cancer advances in stage, this observation may explain one component of the drug-resistance phenotype of this common malignancy.

Acknowledgments

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