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ORIGINAL ARTICLE

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Mutant *p53* expression enhances drug resistance in a hepatocellular carcinoma cell line

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Abstract Chemoresistance is a major problem in the treatment of hepatocellular carcinoma. Certain *p53* mutants may enhance drug resistance in cancer cells. To determine whether two frequently occurring *p53* mutants, R248Q and R273C, would increase the drug resistance of liver cancer cells, stable cell lines expressing these specific *p53* mutants were established by transfecting the *p53*-null Hep3B cells with mutant *p53* expression vectors, and then treating them with the anticancer drugs doxorubicin and paclitaxel. The cells expressing the *p53* mutant, R248Q, but not R273C, displayed cross-resistance to both drugs, in contrast to the control cells expressing the vector alone. Moreover, both the expression and the activity of the multiple drug resistance gene product, P-glycoprotein, were elevated in *p53* mutant R248Q-expressing cells. Reduced uptake of doxorubicin was also observed in the R248Q-expressing cells. These results suggest that expression of the *p53* mutant, R248Q, in liver cancer cells may enhance their drug resistance and that upregulation of P-glycoprotein activity may contribute to this protective effect.

Keywords *p53* mutation · P-glycoprotein · Doxorubicin · Drug resistance · Liver cancer

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide [1]. It is a common cancer in China, Southeast Asia, sub-Saharan Africa, and is occurring with increasing frequency in the United States [2]. Since most HCC patients are diagnosed at a late stage, they are usually not suitable for resection [3] and chemotherapy is an important therapeutic alternative. Unfortunately, response rates of HCC to most anticancer drugs are very low [4].

Doxorubicin (Dox) is one of the most commonly used anticancer drugs for HCC [4]. Suggested mechanisms for its cytotoxic effects include intercalation into DNA, inhibition of enzymes such as topoisomerase II, and the generation of free radicals [5, 6]. Paclitaxel (Taxol) is another anticancer agent used in HCC treatment and it has a different mode of action. It is a β-tubulin-stabilizing agent and its cytotoxic effect is attributed to its ability to prevent microtubule depolymerization and, thus, to inhibit formation of the mitotic spindle [7]. Reasons for the resistance of tumor cells to an anticancer drug vary from patient to patient and include decreases in intracellular drug concentrations due to activation of transporter proteins, reduced drug activation or increased detoxification of the drug, alterations of drug targets and increased repair of the damaged target, and abrogation of apoptosis [8].

P-glycoprotein (P-gp) is a membrane-linked transporter. In human cells, it is encoded by the multidrug resistance (*MDR1*) gene [9]. A key characteristic of P-gp is its ability to act on a group of structurally and functionally unrelated substrates, and substrates entering the cell are pumped out by P-gp through ATP hydrolysis as the energy source [8]. In normal human tissues, it is expressed in the kidney, liver, pancreas, intestine, and the blood-brain barrier for protection against xenobiotic and toxic substances. P-gp can efflux many anticancer drugs commonly used in chemotherapy, including Dox, vinblastine, and paclitaxel [8]. Overexpression of P-gp may enhance drug resistance in cancer cells by increasing the efficiency of drug efflux, and P-gp can be modulated by a number of compounds, including the calcium channel blocker, verapamil [8, 10].

p53 is one of the most important tumor suppressor genes in human cancer. The N-terminal acidic domain

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(amino acids 1–42) of this 393 amino acid, a 53-kDa nuclear phosphoprotein, is responsible for its transcriptional activity. The second domain (amino acids 64–92) is related to its negative regulation. The central core domain (amino acids 102–292) recognizes at least two repeats of the consensus *p53* binding sequence, 5′-PuPuPuC(A/T)-(T/A)GpyPyPy-3′, required for sequence-specific DNA binding. The oligomerization domain (amino acids 324–355) is responsible for tetramer formation that is required for protein-DNA interactions. The p53 C-terminus (amino acids 311–393) contains a nuclear localization sequence, exhibiting both non-specific DNA binding, as well as serving as a negative regulator for itself [11, 12, 13]. The p53 protein carries out its role primarily as a transcription factor to induce cell cycle arrest and to promote apoptosis in response to certain stress conditions or DNA damage [14, 15].

Mutations of the *p53* tumor suppressor gene have been estimated to occur in close to 50% of human tumors and *p53* mutation is not a random process. According to the *p53* mutation database (www.iarc.fr/p53), the majority of DNA mutations are located in the core domain responsible for DNA binding, and include hot spots at codons 175, 245, 248, 249, 273, and 282. Unlike other tumor suppressor genes (e.g. *RB* and *p16*), in which the most common mutations found are deletions or nonsense mutations, in *p53* most mutations within the core domain are often single amino acid substitutions, resulting in a missense protein with a new amino acid sequence [16]. The hot spot mutations at codons 248 and 273 occur with highest frequency in human cancers. In liver cancer, the most frequent amino acid substitutions occurring at these two codons are arginine to glutamine at codon 248 (R248Q) and arginine to cysteine at codon 273 (R273C). Generally, cancers with *p53* mutations are more aggressive and consequently are more difficult to treat [17]. A simple consequence of *p53* mutation is losing the sequence-specific DNA binding activity and results in a loss-of-function phenotype. If a *p53* mutant has an intact oligomerization domain, it may be able to inhibit the wild-type function by forming a dysfunctional tetramer with the wild-type p53 through a dominant-negative mechanism. In addition to losing the wild-type *p53* activity, some frequently occurring *p53* mutants may have a gain-of-function property associated with novel oncogenic functions [13, 18, 19].

Chemotherapy is the primary treatment approach for most HCC patients, although several lines of evidence suggest that certain *p53* mutations may be associated with the drug resistance phenotype [18, 19]. The relationship between common *p53* mutations and an increase in drug resistance is still unclear in liver cancer cells. In the present study, the two *p53* hot spot mutants, R248Q and R273C, were expressed in a *p53*-null human liver cancer cell line, Hep3B, which has a 7-kb *p53* gene deletion [20], to examine their oncogenic function for resistance to Dox and paclitaxel. An increase in resistance to both anticancer agents was observed in the cells expressing the *p53* mutant, R248Q, together with an elevated P-gp expression that may be associated with its protective effect against the anticancer drugs.

Materials and methods

Construction of the mutant *p53* expression vector

The mutant *p53* fragments at codons 248 (R;C**G**G to Q;C**A**G) and 273 (R;**C**GT to C;**T**GT) were generated through oligonucleotide-directed mutagenesis. Overlapping primers (CGGCATGAACC**A** GAGGCCCATCCT and AGGATGGG CCTC**T**GGT TCATGCCG for codon 248; AGCTTTGAGGT G**T**GTGT**T**TGT GCCT and AGGCACAAACAC**A**CACCTCAA A**G**CT for codon 273) were used to introduce the single base pair changes at the desired positions. The fragments were cloned into a pcDNA3 vector (Invitrogen, Carlsbad, Calif.) to construct the mutant *p53* expression vectors. The mutant sequences were verified by DNA sequencing using an ABI automated sequencer (Perkin-Elmer, Boston, Mass.).

Cell culture and transfection

The Hep3B cell line (HB-8064; American Type Culture Collection, Rockville, Md.) was grown in Dulbecco's modified Eagle's minimal essential medium high glucose supplemented with 10% fetal bovine serum, non-essential amino acids, and antibiotics in an atmosphere containing 5% CO_2 at 37°C. All culture reagents were supplied by Gibco-BRL (Grand Island, N.Y.). To generate cell lines expressing the exogenous *p53* mutants, the expression vectors were stably transfected into the cells using the lipofectin reagent (Gibco-BRL). Two days after transfection, the cells were grown under 400 μg/ml G418 selection for 2 weeks. Resistant clones were expanded and positive clones expressing the p53 protein were confirmed through Western blot analysis.

Western blot analysis

Cells were lysed with 50 m *M* Tris (pH 7.5), 100 m *M* NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and protein inhibitor mixture (Roche, Nutley, N.J.). Protein concentrations were measured by the Bradford assay (Bio-Rad Laboratories, Munich, Germany). Samples of 15 μg cellular protein were separated on SDS-polyacrylamide gels (6% for P-gp and 8% for p53) and transferred to nitrocellulose membranes (Immobilon-P; Millipore, Billerica, Mass.). The membranes were blocked with 5% non-fat milk and primary antibody incubation was performed with 1:2500 DO-1 (Santa Cruz, Santa Cruz, Calif.) for the p53 protein, 1:500 G-1 (Santa Cruz) for P-gp, and 1:5000 Ab-1 (Oncogene, Germany) for α-tubulin. The signals were visualized by an enhanced chemiluminescence (Amersham, Uppsala, Sweden) method according to the manufacturer's instructions.

Colony survival assay

The cells were seeded at 5×10^4 cells or 2×10^4 cells, respectively, in culture dishes 24 h before treatment with 0.125 μg/ml Dox for 4 h or 32 ng/ml paclitaxel for 2 days. These treatment conditions produced a countable colony number, as determined in preliminary experiments. After a 2-week recovery period, the colonies were fixed with 3.7% formaldehyde and stained with Giemsa solution (1:50). Surviving colonies were quantitated using the Eagle Eye Gel II documentation system (Stratagene, La Jolla, Calif.).

Flow cytometry and TUNEL analysis of apoptotic cells

To analyze the apoptotic response of the cells to Dox through flow cytometry, both floating cells and attached cells were harvested following exposure to Dox (0.5 μg/ml) for various times. The cells were fixed in 70% ethanol, incubated with RNase A (100 μg/ml), and stained for DNA with propidium iodide (PI) at 50 μg/ml. The PI fluorescence (DNA content) of individual cells was measured using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, N.J.). The data obtained were processed with the software WinMDI version 2.1. The apoptotic response of the cells to Dox treatment was also evaluated by the TUNEL (TdT-mediated dUTP nick end labeling) assay using an "In situ cell death detection kit, AP" (Roche) according to the manufacturer's instructions. In this method, terminal deoxynucleotidyl transferase (TdT), was used to label DNA strand breaks in apoptotic cells after treatment with the Dox at 0.5 μg/ml or with drug-free medium for 20 h. Finally, the labeled DNA strand breaks were detected by incubation with the BCIP/NBT reagent (Zymed, South San Francisco, Calif.), and then the cells were counterstained with hematoxylin (Zymed) and analyzed by light microscopy.

Rhodamine-123 efflux assay

Flow cytometric analysis of rhodamine-123 (Rho-123; Sigma, St. Louis, Mo.) efflux was performed as described with some modification [21]. Cells were first incubated with 0.38 μg/ml Rho-123 for 1 h and then incubated in dye-free medium with or without the P-gp inhibitor verapamil at 10 μg/ml for 1 h for dye efflux. The cells were collected through trypsinization and kept on ice before analysis. The cellular levels of Rho-123 were measured in terms of fluorescence intensity with laser excitation at 488 nm using a 530/30 nm bandpass filter on a FACScan flow cytometer. Background fluorescence was determined with untreated cells. Data acquired were analyzed by WinMDI version 2.1 software and the mean fluorescence value of Rho-123 was recorded. The percentage of dye efflux was calculated as (R0−Rt)/R0 as described previously [22], where R0 represents the mean fluorescence value immediately after dye uptake and Rt represents the remaining mean fluorescence value after dye efflux. Three separate experiments were carried out for each treatment.

Analysis of Dox accumulation

Intracellular Dox accumulation was determined by flow cytometry following incubation of the cells with various concentrations of Dox. The cellular level of Dox red fluorescence was evaluated using a 570/26 nm bandpass filter. The amount of Dox uptake at 1 μg/ml in the vector-alone-transfected culture was designated as 100% for comparison. To analyze the relative amount of Dox uptake through fluorescence microscopy, an equal number of the vector-alone-expressing cells and the *p53* mutant R248Q-expressing cells (248-15), were mixed and grown on coverslips 24 h before treatment with Dox at 1 μg/ml for 8 h. To identify the mutant *p53*-expressing cells in the mixed culture, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with the anti-p53 monoclonal antibody, DO-1 (Santa Cruz). The immune complexes were detected with 1:250 Alexa Fluor 488-labelled goat anti-mouse IgG antibody (Molecular Probes, Eugene, Ore.). The red fluorescence of Dox and the green fluorescence of the *p53* staining were visualized by laser excitation at 488 nm and image capture by fluorescence microscopy (Olympus, Tokyo, Japan).

Statistical analysis

Statistical analyses for significance differences in growth inhibition, colony numbers, sub-G_1 cells, Rho-123 efflux, and Dox uptake, between control and treated cells were assessed using the *t*-test with Sigma plot 4.0 software, and were considered significant for P values < 0.05.

Results

Increased resistance of the *p53* mutant-expressing cells to Dox

To analyze the effect of mutant *p53* expression on anticancer drug treatment, stable clones expressing the *p53* mutants, derived from Hep3B, were established after transfection and confirmatory *p53* expression identification by Western blotting. Cells transfected with the pcDNA3 vector alone were used to generate the control cells without *p53* expression. Representative clones expressing the highest level of the mutant *p53* were selected, including 248-13 and 248-15 (for the *p53* mutant R248Q), and 273-5 and 273-13 (for the *p53* mutant R273C) (Fig. 1). The cells' survival response to drug treatment was examined using a colony survival assay. A significant increase in cell survival was observed in the *p53* mutant R248Q-expressing cells. The R248Q-expressing clone, 248-13, exhibited a 5.9-fold increase in colony numbers (235 ± 19.2, $P < 0.001$), and a further increase to 13.4-fold was found in the clone 248-15 (534 ± 66.8, $P < 0.001$), as compared to cells expressing the vector alone (40.0 ± 4.4). No significant difference in colony numbers was found between the vector expressing cells and cells expressing the *p53* mutant, R273C (Fig. 2).

Analysis of apoptotic cells following Dox treatment

The apoptotic responses of the cells to Dox were evaluated by flow cytometric analysis. This treatment removes low molecular weight DNA fragments from apoptotic cells and results in the appearance of a sub-G_1 peak as an apoptosis indicator [18]. In the vector-alone-expressing cells the proportion of sub-G_1 cells increased from $0.93 \pm 0.11\%$ at 0 h (no treatment) to $23.9 \pm 6.45\%$ at 36 h after Dox treatment. No protective effect against Dox-induced increase in sub-G_1 cells was found in the clones expressing the *p53* mutant, R273C, as compared to the vector-alone-expressing cells (Fig. 3). In the

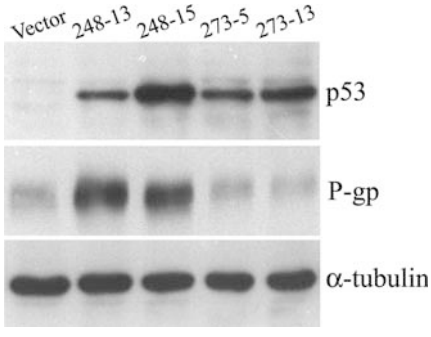

Fig. 1 Expression of p53 protein and P-gp. Cell lysates from Hep3B-derived clones were subjected to Western blot analysis for p53 protein and P-gp. α-Tubulin served as a protein loading control

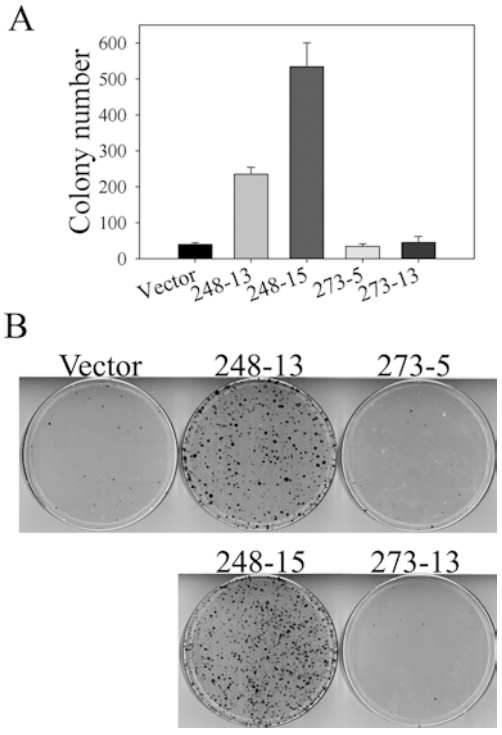

Fig. 2A, B Colony formation assay of the *p53* mutants following Dox treatment. The cells were treated with Dox at 0.125 μg/ml for 4 h. Surviving colonies after 2 weeks of culture were quantitated. **A** The results presented are the means of three independent experiments and are plotted as the total number of colonies formed; error bars indicate SD. **B** Representative images of the colony formation plates. The survival advantage of the R248Q mutant was also observed with Dox treatment at 0.0625 μg/ml (data not shown)

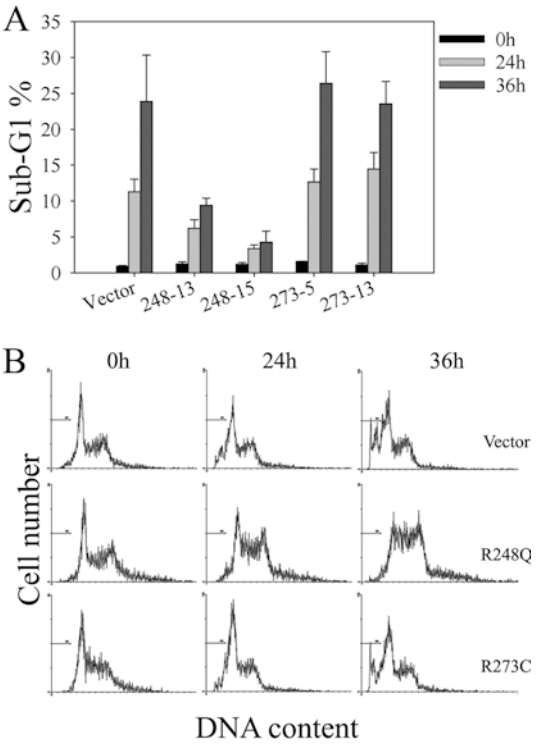

Fig. 3A, B Analysis of apoptotic cells by flow cytometry following Dox treatment. The cells were treated with Dox at 0.5 μg/ml and analyzed by flow cytometry for sub-G_1 cells. **A** The results presented are the means of three independent experiments; error bars indicate SD. **B** Representative flow cytometry profiles of DNA content (PI fluorescence) of vector-expressing cells, R248Q-expressing cells (248-15), and R273C expressing cells (273-5). The horizontal bars indicate sub-G_1 cells

R248Q-expressing clones, however, significantly fewer sub-G_1 cells were detected during the entire time course and these clones exhibited a reduction in sub-G_1 cells of 60.6% (248-13, $P = 0.006$) and 78.2% (248-15, $P = 0.002$) after 36 h exposure to Dox, as compared to the vector-alone-expressing cells. The results suggest the cells expressing the R248Q mutant may have a survival advantage against Dox-induced cytotoxicity. The protective effect of the R248Q mutant against Dox-induced apoptosis was further confirmed in a TUNEL assay, in which a smaller proportion of positive-staining cells was observed in the R248Q-expressing clones following exposure to Dox for 20 h (Fig. 4).

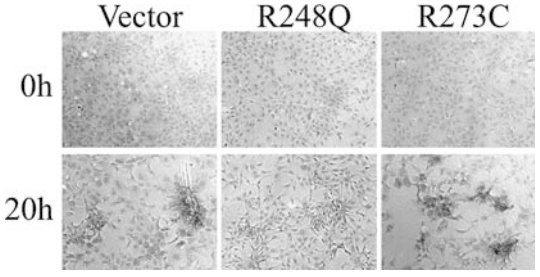

Fig. 4 Detection of apoptotic cells by TUNEL analysis following Dox treatment. The expressing the vector alone, the *p53* mutant, R248Q, and the p53 mutant, R273C, were treated with Dox at 0.5 μg/ml for 20 h and analyzed for apoptosis with the TUNEL assay. The cells were counterstained with hematoxylin and representative microscopic images of the cells are shown (×100)

Increased resistance of *p53* mutant-expressing cells to paclitaxel

It is apparent that cells expressing the *p53* mutant, R248Q, had a survival advantage against Dox treatment. The resistance of the *p53* mutant expressing cells to another anticancer agent, paclitaxel, whose mode of action is different from that of Dox, was also examined. As shown in Fig. 5, the R248Q-expressing clones, 248-13 and 248-15, exhibited a 5.2-fold (444 ± 53.7, $P < 0.001$) and 6.5-fold (550 ± 6.94, $P < 0.001$) increase in surviving colonies, respectively, after paclitaxel treatment, as compared with the vector-alone-expressing clone (84.8 ± 24.8). For the clones expressing another *p53* mutant, R273C, no obvious difference in colony number was observed. These results are consistent with both a growth advantage against Dox and paclitaxel in the cells expressing the *p53* mutant, R248Q.

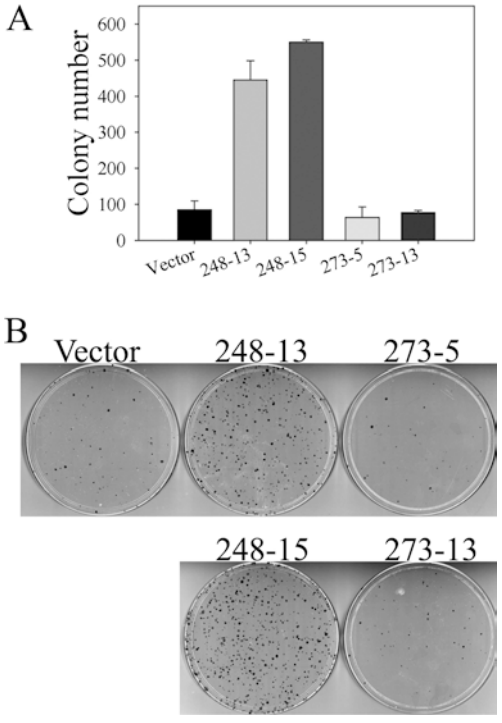

Fig. 5A, B Colony formation assay of the *p53* mutants following paclitaxel treatment. The cells were treated with paclitaxel at 32 ng/ml for 48 h. Surviving colonies after 2 weeks of culture were quantitated. **A** The results presented are the means of three independent experiments and are plotted as the total number of colonies formed; error bars indicate SD. **B** Representative images of the colony formation plates. The survival advantage of the R248Q mutant was also observed with paclitaxel treatment at 8 ng/ml and 16 ng/ml (data not shown)

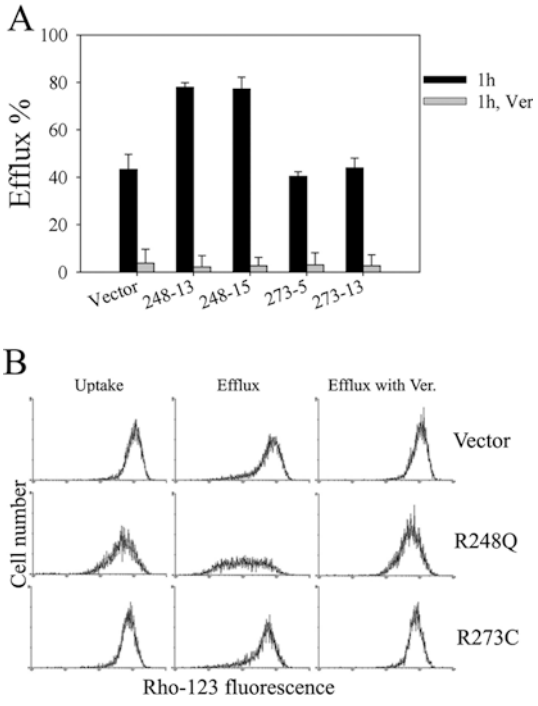

Fig. 6A, B Rho-123 efflux assay. The cells were incubated with Rho-123 at 0.38 μg/ml for 1 h followed by 1 h of dye efflux. The experiment was done in parallel with the P-gp inhibitor, verapamil (*Ver*) at 10 μg/ml. The fluorescence intensity was measured by flow cytometry and the percentage dye efflux was calculated as described in Materials and methods. **A** The results are presented as the means of three independent experiments; error bars indicate SD. **B** Representative flow cytometry profiles of Rho-123 fluorescence on the log scale versus cell numbers of the 1 h uptake, 1 h uptake followed by 1 h efflux with or without verapamil

Expression and functional analysis of P-gp in R248Q-expressing cells

Both Dox and paclitaxel are substrates for P-gp [8], and the possibility that P-gp may be associated with the protective effect of the *p53* mutant, R248Q, against drug treatment was investigated. Western blot analysis revealed that both clones expressing the *p53* mutant, R248Q, exhibited an increase in P-gp expression as compared to the cells expressing the vector alone, which expressed only the basal level of P-gp (Fig. 1). To assess whether the efflux activity of overexpressed P-gp was functionally active, the cells were incubated with the fluorescent dye, Rho-123, which is a P-gp substrate, and its intracellular level was measured flow cytometrically before and after 1 h of dye efflux. As shown in Fig. 6, a significantly higher percentage of dye efflux was observed in both R248Q-expressing clones, with $77.9 \pm 2.01\%$ (248-13, $P = 0.0036$) and $77.3 \pm 4.95\%$ (248-15, $P = 0.0059$), as compared to the vector-alone-expressing cells with $46.9 \pm 8.52\%$. However, when the same experiment was performed in the presence of the P-gp inhibitor, verapamil, the percentage of dye efflux was reduced substantially. The results suggest that the cells expressing the R248Q mutant have an elevated P-gp activity, and the results are in agreement with the increased P-gp expression detected by Western blotting (Fig. 1).

Dox accumulation

The elevated activity of the P-gp in the R248Q-expressing cells suggests that drug resistance may be attributed to reduced drug accumulation. The relative amount of Dox accumulation between cells was analyzed by both flow cytometry and fluorescence microscopy. Flow cytometry showed that intracellular Dox accumulation increased with concentration and a significant reduction in drug accumulation was found in both R248Q-expressing clones, as compared to the cells expressing the vector alone (Fig. 7A, $P < 0.029$ at 0.5 μg/ml, and $P < 0.002$ at 1 μg/ml). The difference in drug accumulation was further demonstrated by fluorescence microscopy observations of the mixed culture treated with Dox (Fig. 7B). There was a strong partitioning of the Dox red fluorescence in the nucleus and there was a lower amount of drug accumulation in the *p53* mutant R248Q-expressing cells (stained with FITC). This is in agreement with the flow cytometry results, and

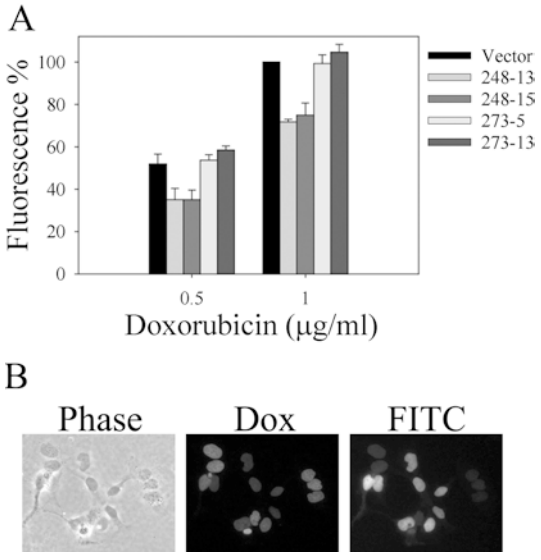

Fig. 7A, B Analysis of Dox accumulation. **A** The cells were treated with 0.5 and 1 μg/ml Dox for 8 h. The relative levels of Dox accumulation were estimated by flow cytometry and the fluorescence intensity of vector-expressing cells at 1 μg/ml was set as 100% for comparison. The results are presented as the means of three independent experiments; error bars indicate SD. **B** Mixed population of cells expressing the vector alone and cells expressing the *p53* mutant, R248Q (248-15), were grown on coverslips and treated with 1 μg/ml Dox for 8 h followed by immunostaining to identify the *p53* mutant expressing cells. The red fluorescence of Dox and the green (FITC) fluorescence of *p53* staining was visualized with 488 nm excitation and observed by fluorescence microscopy

suggests a reduction of Dox accumulation in cells expressing the *p53* mutant, R248Q.

Discussion

Mutation of the *p53* tumor suppressor gene is the most common genetic alteration occurring in human cancers and the presence of *p53* mutations may predict reduced responses in chemotherapy [18, 19]. Some *p53* mutants may express their protective effects through a gain-of-function mechanism [18, 19, 23]. Chemoresistance is a major obstacle in HCC [4]. In the present study the gain-of-function property for drug resistance of the two common *p53* mutants, R248Q and R273C, was examined in human liver cancer cells. Since most *p53* mutants have a dominant negative effect in overwhelming the wild-type *p53* activity [24], the evidence for a gain-of-function property needs to be demonstrated in a *p53*-null background to rule out the possibility that the observed oncogenic function is due to its dominant negative effect [18, 19, 20, 22]. In the present study a *p53*-null liver cancer cell line, Hep3B, was employed.

Dox is one of the most commonly used anticancer drugs for the treatment of HCC [4]. A previous study has shown that Hep3B cells expressing an exogenous wild-type *p53* are more sensitive to Dox [25]. In the present study, an increase in drug sensitivity or in drug resistance was not observed in cells expressing the *p53* mutant, R273C. In contrast, a significant protective effect was observed in cells expressing the *p53* mutant, R248Q. Dox is an apoptosis-inducing agent [6, 26] and the apoptotic response found in cells expressing the R248Q mutant was significantly lower than in the control cells expressing the vector alone. These results are consistent with a protective effect conferred by the R248Q mutant. This protective effect was observed in the absence of the wild-type *p53* and therefore represents a gain-of-function property for drug resistance. The protective effect of the R248Q mutant occurred with paclitaxel as well as with Dox, suggesting a cross-resistance phenotype that may relate to a protective mechanism against various agents with different modes of action.

In the present study, expression of the multiple drug transporter, P-gp, was elevated in cells expressing the *p53* mutant, R248Q. The increase in P-gp caused a more effective dye efflux as demonstrated in the Rho-123 functional assay. P-gp can transport a great variety of structurally and functionally distinct molecules, including many chemotherapeutic agents [8]. Since both Dox and paclitaxel are substrates for P-gp [8], it is hypothesized that the elevation of P-gp activity may contribute to the protective effect by reducing drug accumulation. Flow cytometric analysis and microscopy observations for the reduced Dox red fluorescence in cells expressing the *p53* mutant, R248Q, support this hypothesis. A relationship between P-gp over-expression and drug resistance has been shown in several clinical studies. In human breast cancer and in HCC, the response to chemotherapy response is inversely related to P-gp expression [26, 27].

The cause of increased P-gp expression in cancer cells is still unclear. It has been hypothesized that certain *p53* mutants may contribute to the upregulation of P-gp in cancer cells. In multiple cell lines, including Caco-2 and MCF-7, the *MDR1* promoter is induced by the *p53* mutants, R175H and D281G, whereas wild-type *p53* has either no effect or an inhibitory effect. In the Saos-2 cell, the 3′ region of the *MDR1* promoter is activated by the *p53* mutants, R175H and R248Q [8, 28, 29, 30]. In a transfection study using the *p53*-null colon carcinoma cell line, Caco-2, the expression of endogenous P-gp was shown to be increased by stably transfecting the cells with the *p53* mutant, D281G [29]. However, conflicting results have been reported. The mutant *p53* R175H inhibits a 322-bp segment (−189 to +33) of the *MDR1* promoter in the Saos-2 cells and certain *p53* mutants, including L252P and R273H, induce slight inhibition of the *MDR1* promoter activity in the lung carcinoma cell line, H358 [30, 31]. These contradictory results may be because of the cell types, the *MDR1* promoter region, and the nature of the *p53* mutants used. In addition to these in vitro studies, only approximately half of the clinical specimen studies show a positive association between *p53* mutation and P-gp overexpression [8]. Detection of the p53 mutant with antibodies targeting most, but not a specific form of, mutant p53 in clinical

specimen studies and overexpression of the *p53* mutant in in vitro studies may also explain the differences observed.

Numerous studies suggest that the *MDR1* promoter is suppressed by wild-type *p53* through a sequence-specific binding [28, 29, 32, 33]; however, contradictory results have been reported [31, 34]. Although it is still unclear how wild-type *p53* regulates the *MDR1* gene, previous studies with a yeast *p53* functional assay [35, 36] have indicated that both core domain *p53* mutants, R248Q and R273C, fail to transactivate a reporter gene regulated by a *p53* responsive element and therefore lose the wild-type *p53* transactivating activity [37]. Thus, the observed elevation of the P-gp in the *p53* mutant expressing cell is unlikely to result from the wild type *p53* transactivating activity. The present results suggest an upregulation of P-gp in Hep3B cells expressing the *p53* mutant, R248Q, and provide additional information about a conditional association between specific *p53* gain-of-function mutant expression and its effect on the activity of P-gp. However, further studies are required to assess the in vivo relevance of these findings.

In the present study, although both R248Q-expressing clones displayed similar elevated levels and activity of P-gp, in the colony formation assay, there were more surviving colonies observed after Dox treatment in the clone (248-15) expressing a higher level of the *p53* mutant, than in the clone (248-13) expressing a lower level. This observation agrees with the appearance of fewer apoptotic cells found in the high expresser. Similar differences in the drug resistance responses between the high and low *p53* mutant-expressing cells were also found in *p53* mutant studies in the *p53*-null human lung adenocarcinoma cell line, H1299, to the anticancer drug, etoposide, and in the *p53*-null murine myeloid cell line, M1/2, to the anticancer drugs, cisplatin and α-amanitin. These studies showed that the degree of protection from drug-induced apoptosis correlates directly with level of p53 mutant protein expression [18, 23]. Besides the increased ability of P-gp, which can mediate drug resistance by reducing intracellular drug accumulation below its effective cytotoxic level, a direct abrogation of the apoptotic response may also contribute to the drug resistance phenotype. Overexpression of the antiapoptotic factor, *Bcl-2*, may render liver cancer cells more resistant to Dox and paclitaxel [38], and c-*myc* expression may be associated with drug resistance conferred by certain *p53* mutants [23]. The apoptosis-related genes may also mediate a protective effect against drug treatment, and their involvement in drug resistance cannot be ruled out, and needs further investigations.

In HCC, most patients are diagnosed at an inoperably advanced stage and chemotherapy is the primary treatment option [3]. The present study suggests that expression of certain *p53* mutants in liver cancer cells may enhance drug resistance. Extensive clinical data are required to evaluate the in vivo relevance of this observation in order to provide a rational basis for optimizing therapeutic options.

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