Evaluation of the Combined Effect of p53 Codon 72 Polymorphism and Hotspot Mutations in Response to Anticancer Drugs

Faina Vikhanskaya,1 Mobin M. Siddique,1 Ming Kei Lee,1 Massimo Broggini,3 and Kanaga Sabapathy1,2

Abstract Mutations in p53 are common events during carcinogenesis and have been suggested to affect sensitivity to chemotherapy. Recently, the common polymorphism at codon 72, resulting in either an arginine (72R) or a proline (72P) residue, was shown to differentially affect the response to anticancer drugs. Here, we have generated isogenic lung cancer cell lines to evaluate the effect of six p53 hotspot mutations (R175H, G245S, R248W, R249S, R273H, and R282W) in conjunction with the codon 72 polymorphism, for their response to a variety of anticancer drugs, either alone or in combination. The data indicate that 72R mutations do not confer general resistance to cisplatin, etoposide, gemcitabine, vinblastine, and taxol. For doxorubicin, cells expressing 249-72R were more resistant than the 249-72P cells. Combined treatment with cisplatin + etoposide resulted in an additive effect in cells expressing most 72R and 72P mutations, except for the 175-72R cells which were refractory to combined treatment. However, combined treatment with cisplatin + gemcitabine resulted in the absence of an additive effect in cells expressing the 273-72R and 282-72R mutants, unlike their 72P counterparts. Nonetheless, all p53 mutants (72R or 72P) equally inhibited p73-mediated transcriptional activity in lung cancer cells, suggesting that the selective resistance conferred by some 72R mutants to certain drugs is probably due to other p73-independent effects of these mutants. Together, the data show that the status of codon 72 polymorphism and p53 mutations can be used as a means for prediction of treatment response, although variables for each cancer type require detailed evaluation.

P53 is arguably the most critical tumor suppressor gene and mutations in the p53 gene are considered to represent the most common genetic alterations in human cancer (1–3). About 90% of the p53 mutations are found in the central DNA-binding domain of p53 (1, 3). These mutations therefore affect the DNA-binding activity of p53, leading to the loss of p53-mediated transcriptional activation and hence, p53 functions such as apoptosis, and cell cycle arrest and repair (1, 2). Besides the gene mutations, several reports have focused on p53 polymorphisms as risk factors for malignant disease. A polymorphic site at codon 72 in exon 4 encodes either an arginine amino acid (Arg72R) or a proline residue (Pro72P; refs. 4–6). This polymorphism is located in a proline-rich region of the p53 protein that is required for the growth suppression and apoptosis mediated by p53 but not for cell cycle arrest (7–9). The two polymorphic variants of wild-type p53 have been shown to have some differences in biochemical properties such as differential binding to components of the transcriptional machinery (10). Moreover, it has also been suggested that the 72R variant is much more susceptible to degradation by the human papillomavirus E6 protein (11) and recent analysis has shown that the 72R variant is more efficient in inducing cell death than the 72P variant in some cell types (12, 13). In addition, head and neck cancer cells expressing the wild-type 72R variant were shown to be more sensitive to a variety of anticancer drugs and head and neck cancer patients had a longer survival compared with their 72P-expressing counterparts (13), indicating that the status of the p53 codon 72 polymorphism could influence the outcome of cancer therapy.

Biochemical analysis has shown that 72R mutants were capable of binding to p73, the structural and functional homologue of p53, and inactivating it (14). This property of mutant p53 to bind and neutralize p73-mediated apoptosis was enhanced when the p53 was in the 72R form compared with the 72P form, especially when tested using the human SAOS2 osteosarcoma cells (14). In addition, this negative inhibitory effect was shown to be specific for mutant forms of p53, because wild-type p53 was shown not to bind p73 (15, 16). Accordingly, clinical response following cisplatin-based chemotherapy for advanced head and neck cancer was shown to be influenced by this polymorphism, as cancers expressing 72R mutants were found to have lower response rates than those expressing the 72P mutants (17). Thus, polymorphism at codon 72 of p53 can have opposing effects on response to therapy according to the status of p53 (13, 17).
Hitherto, it is not clear if the expression of mutant 72R forms would contribute to chemotherapeutic resistance and poor prognosis in all cancer types. We have now done a systematic analysis using the p53 null H1299 human lung cancer cell line, to evaluate the sensitivity of isogenic cell lines expressing the six common hotspot p53 mutations (i.e., R175H, G245S, R248W, R249S, R273H, and R282W), in conjunction with the codon p72 polymorphism, to several anticancer drugs. These cells have been evaluated for their response to individual drugs and to combination of drugs to mimic the clinical therapeutic regimens. Our results indicate that the expression of mutant 72R forms do not always confer resistance to anticancer drugs, suggesting that there may be cell type and mutation specific factors that influence the outcome of drug response. Detailed results are discussed.

Materials and Methods

DNA constructs, generation of isogenic cell lines, drug treatment, and cell survival assays. Human p53 cDNA was cloned in pCDNA3 (Invitrogen, Carlsbad, CA) eukaryotic expression vector that was used as a template for site-directed mutagenesis. Template (50 ng) was used for each PCR reaction with 5 units of Pfu I and 1× Pfu Buffer (Promega, Madison, WI), 1 μL of 10 mM deoxynucleotide triphosphate, and one pair of the six hotspot-specific primers. All the PCR products were digested with Dpn I to remove any parental DNA and these were transformed in Escherichia coli strain XL1-Blue. Single colony was picked and plasmids were extracted and sequenced to confirm the induction of the mutation. p53 mutants that were generated are as follows: p53aa175 (arginine-histidine), p53aa245 (glycine-serine), p53aa248 (arginine-tryptophan), p53aa249 (arginine-serine), p53aa273 (arginine-histidine), and p53aa282 (arginine-tryptophan). These were sequenced to confirm the mutations (Fig. 1A and B).

H1299 cells were transfected with the p53 mutant constructs; 5 μg of plasmid were used to transfect cells in 10-cm culture dish by lipofection (Invitrogen). After transfection, cells were selected in presence of Geneticin (800 μg/mL) to obtain stable cell lines. All drugs used were obtained from Sigma (St. Louis, MO) and were dissolved according to manufacturer’s instructions, except for Gemcitabine that was obtained from the National Cancer Centre outpatient clinic.
Cell survival was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) metabolic viability assay. Exponentially growing cells were (5 × 10^3) transferred into 96-well tissue culture plates (Nunc, Roskilde, Denmark) in 100 µL complete medium. After 72 hours of incubation, the cells were exposed to increasing concentration of drugs. After 24 hours of treatment, cells were replaced with fresh medium and incubated for another 48 hours. Cell viability in wells was determined by the ability of live and early apoptotic cells with intact mitochondria to convert the soluble salt of MTT into insoluble formazan precipitate. Aliquots of 20 µL MTT (6 mg/mL) were added to each well for 4 hours, the color formed was quantified using a spectrophotometric microplate reader MRX 11 at 570 nm after solubilization in propanol-2/HCl.

For combination treatment, cisplatin was used in concentrations ranging from 5 to 50 µmol/L and cells were exposed for 2 hours. This drug was then washed out and wells were replaced with constant concentration of gemcitabine (4 µg/mL) or etoposide (10 µmol/L), concentrations at which both drugs gave a single dose survival of ~60%. Cells were treated with cisplatin alone at all times and in all the combination experiments.

All experiments were done in triplicates, and at three independent times. The SDs are indicated in the graphs.

Apoptosis assay. Annexin V and propidium iodide costaining were used to determine apoptotic cells. Briefly, cells were harvested, washed once in PBS and were incubated with FITC-conjugated Annexin V (BD Biosciences, San Diego, CA) and 50 µg/mL PI in binding buffer [10 mmol/L HEPES (pH 7.4), 0.14 mol/L NaCl, and 2.5 mmol/L CaCl₂] for 15 minutes in the dark at room temperature.
analyzed by flow cytometry immediately after incubation. All experiments were done at least twice.

**Luciferase assay.** Transient transfections of H1299 cells were done with the 10 μg of the indicated expression plasmids and the 2 μg each of the luciferase reporter plasmid (i.e., p21, AIP-1, bax, or gadd45 promoter luciferase plasmid), and the plasmid encoding the β-galactosidase gene for evaluating the transfection efficiency. Cells were collected 48 hours post-transfection. Luciferase assays were done in triplicates, as described (19). P73-mediated transactivation was determined using SAOS2 cells with the tetracycline-inducible p73β which were transfected with the mutant p53 constructs and the reporter constructs as described. All reporter assays were done at least three times independently, and representative results are shown.

**Western blot analysis.** Proteins were analyzed by immunoblotting using anti-p53 (DO-1) and anti-actin antibodies, as described (20).

**Statistical analysis.** Normal test was used to test for statistical significance of the differences in the arg and pro allele status, and only those with P < 0.05 were considered significant. To quantify deviations from additive effects induced by sequential treatment of two drugs, a statistical Student’s test was employed, as described (21). For a given drug dose, we determined survival fraction (Sf) of cells: SfA for the first drug used in sequential schemes and SfB for the second. Following combined treatment, we determined SfAB. The results obtained were defined according to the following criteria: SfAB = SfA × SfB indicated an additive effect, SfAB < (1)SfA × SfB, a synergistic effect, and SfAB > SfA × SfB, an antagonistic effect, as described (21).

**Results**

**Generation of mutant p53 expressing isogenic H1299 cell lines.** We generated the six p53 hotspot mutations (i.e., R175H, G245S, R248W, R249S, R273H, and R282W), either in the 72R or 72P forms by site-directed mutagenesis, in the pCDNA vector. All constructs were sequenced for verification (Fig. 1A and B) and tested for their ability to transactivate the following gene promoter-luciferase constructs: p21, AIP-1, and bax. Compared with wild-type p53 (both 72P and 72R) that activated the p21 promoter efficiently, none of the 12 mutants constructs were able to activate from the p21 promoter (Fig. 1C). Moreover, none of the 12 mutants were able to activate from the apoptotic AIP-1 and bax gene promoters (Fig. 1D and E), indicating that these mutations were indeed inactivating mutations. H1299
human lung cancer cell lines were transfected with these constructs and stable cells were generated. None of the p53 mutants inhibited cell growth (data not shown). Pooled cultures of cells were collected and used for each construct to exclude any variations due to clonal variability. Cells expressing the various p53 mutants were tested for expression of the mutant p53 by reverse transcription-PCR analysis and immunoblotting and were found to express equal amounts of p53 (data not shown; Fig. 2) and hence were used for evaluation of their response to various chemotherapeutic drugs.

Effect of p53 mutational and polymorphic status on sensitivity of H1299 cells to several anticancer drugs. All the H1299-derived cells were tested for their ability to respond to a wide range of concentrations of the following anticancer drugs acting with different mechanisms of action: cisplatin, etoposide, gemcitabine, vinblastine, taxol, and doxorubicin, to evaluate if the polymorphic status in conjunction with the p53 mutations would affect cellular response. Cellular response to drugs was determined by the standard MTT assay, which determines the metabolic viability, due either to the cytostatic and cytotoxic effects of the drugs (22). Cells expressing the 175, 245, 248, and 282 mutations showed varying levels of resistance to cisplatin compared with vector-expressing cells (Fig. 3). Although there was a tendency for 72P-expressing 175 and 282 cells to be more resistant than their 72R counterparts, there were no statistically significant differences between these polymorphic variants for all the mutations analyzed (Fig. 3). Similarly, taxol treatment resulted in marginal resistance of cells expressing the 249 mutant, but there were no significant differences between the 72R and 72P forms (Fig. 3). However, sensitivity to gemcitabine, vinblastine, and etoposide was neither affected by the presence of any mutations nor affected by the p53 polymorphic status (Fig. 3).

p53 polymorphism affects the response of H1299 cells expressing 249p53 to doxorubicin. Although the polymorphic status of p53 did not influence drug sensitivity to several drugs, it affected the response to doxorubicin. The 249-72R-expressing cells were found to be more resistant than the 249-72P and vector-expressing cells over a range of drug concentrations (Fig. 4A). By contrast, the polymorphism-specific effect was not noted in cells expressing all the other p53 mutations to doxorubicin (Fig. 4A). We thus analyzed if the resistance conferred by 249-72R expression was due to reduced apoptosis, by staining with Annexin V and propidium iodide. 249-72R-expressing cells were found to undergo less apoptosis compared with vector expressing cells or 249-72P-expressing cells at various concentrations of doxorubicin treatment (at 0.25 μmol/L: 249-72R, 24.5%; V, 66.8%; 249-72P, 80.0%; at 0.5 μmol/L: 249-72R, 27.4%; V, 97.2%; 249-72P, 93.9%;
suggestion that the resistance conferred by 249-72R expression could be due to reduction in cell death.

Some 72R mutants are refractory to combination drug treatment. Clinical protocols for lung cancer chemotherapy generally use two or more drugs rather than single agents. As such, we evaluated the effects of p53 polymorphism and mutations in response to sequential treatment with various concentrations of cisplatin followed 24 hours later with a single dose of either gemcitabine or etoposide. Treatment with cisplatin followed by gemcitabine resulted in an additive effect resulting in enhanced cell death compared with treatment with cisplatin alone, in cells expressing most of the mutants, especially at lower doses (Fig. 5A). However, there were no significant differences between cells expressing either the 72P or 72P forms, except for the 273 and 282 mutants. Cells expressing the 273-72P or 282-72P mutants showed a significant antagonistic effect to the combination of drugs (P < 0.05; Fig. 5A). By contrast, there was a clear additive effect in cells expressing the 72P forms of these mutations (P < 0.05; Fig. 5A). However, treatment with cisplatin + etoposide resulted in a different pattern of response. 273 and 282 expressing cells, regardless of their polymorphic status, were equally sensitive and displayed an additive response to the combined treatment (Fig. 5B). Similar results were obtained with 245, 248, and 249 expressing cells, except for the 175-expressing cells (Fig. 5B). Cells expressing 175-72R were refractory to combined treatment, displaying a significant antagonistic effect compared with the additive effect found with the 175-72P-expressing cells (P < 0.05; Fig. 5B).

To ascertain if the differences observed by MTT assay were due to decreased cell death of the various 72R-mutant expressing cells, we analyzed the extent of apoptosis by staining with Annexin V and propidium iodide. Combined treatment with cisplatin and gemcitabine resulted in additive increase in apoptotic rate in vector, 273-72P- or 282-72P-expressing cells (Fig. 5C). By contrast, 273-72R- or 282-72R-expressing cells were refractory to additional gemcitabine treatment (cisplatin versus cisplatin + gemcitabine: 39.4% versus 66.4%; 273-72P: 60.4% versus 88.6%; 282-72P: 31.7% versus 74.4%; 273-72R: 40.5% versus 43.5%; 282-72R: 58.4% versus 59.5%; Fig. 5C). Similarly, combined treatment with cisplatin and etoposide resulted in an additive apoptotic effect in vector and 175-72P-expressing cells, whereas 175-72R-expressing cells were refractory to additional etoposide treatment (cisplatin versus cisplatin + etoposide → vector: 33.1% versus 50.1%; 175-72P: 55.3% versus 75.7%; 175-72R: 54.2% versus 59.1%; Fig. 5D). Treatment with gemcitabine or etoposide alone did not result in any difference in cell death rates among

Fig. 5 continued. C, the indicated cell lines were treated with cisplatin alone (25 μmol/L) or in combination with gemcitabine (1 μg/mL) as indicated above and the percentage of apoptotic cells were determined 48 hours later by Annexin V and propidium iodide staining. D, the cell lines were treated with cisplatin alone (50 μmol/L) or in combination with etoposide (80 μmol/L) and the percentage of apoptotic cells were determined 48 hours later as described above. All apoptotic assays were done at least twice.
All cell lines (data not shown; Fig. 3). These data are consistent with the results obtained with MTT assay (Fig. 5A and B) and confirm that expression of 72R mutants result in reduced apoptosis. Together, the data indicate that the presence of mutations in the 72R form can result in resistance to some forms of drug treatment, and this seems to be drug and mutation specific.

Both 72R and 72P mutants are equally capable of inhibiting p73-mediated transactivation in H1299 cells. It has been suggested that the 72R mutants are able to specifically inhibit p73-mediated cellular transformation and were also shown to down regulate cisplatin-mediated AIP-1 promoter activity, compared with their 72P counterparts, in SAOS2 cells (14, 17). We therefore analyzed if the 72R mutants would preferentially inhibit p73-mediated transactivation, thereby providing an explanation for the observed differences in the response to various drugs. The effect of various p53 mutants on p73-mediated transactivation of the gadd45 promoter was tested in all the H1299 cell lines, and all the p53 mutants were found to inhibit p73-mediated transactivation (Fig. 6A). Importantly, there were no significant differences between the 72R or the 72P forms in their ability to inhibit p73-mediated transactivation (Fig. 6A). Because our results were contradictory to the earlier findings in SAOS2 cells, we tested if the 72R mutants used in our model had the ability to specifically inhibit p73-mediated transactivation using the SAOS2 cells inducibly expressing p73 (18). Tetracycline-induced expression of p73 resulted in activation of the gadd45 promoter activity, and all the 72R mutants tested were able to inhibit p73-mediated activity much more than the 72P mutants (Fig. 6B), consistent with earlier reports. Thus, the data show that 72R mutants have the ability to preferentially inhibit p73-mediated transactivation in SAOS2 osteosarcoma cells but not in H1299 lung cancer cells.

Discussion

We have investigated the possible role of codon 72 p53 polymorphism in response to a wide range of anticancer drugs used for lung cancer therapy, either alone or in combination, using isogenic cell lines expressing various p53 mutants to evaluate the prognostic value of the p53 mutants. Three essential findings have emerged from this study: (i) the individual p53 mutants have specific properties against different chemotherapeutic drugs, as the expression of a specific p53 mutant results in resistance to one drug but does not affect the sensitivity against another drug; (ii) although the 72R mutants tend to confer resistance to anticancer drugs, it is not an universal phenomenon and depends on the mutation and drug used; (iii) 72R mutants do not preferentially inhibit p73-mediated transcriptional activity compared with the 72P mutants in H1299 cells (differently from SAOS2 cells), highlighting the cell type–specific effects of the 72R mutants.

We have used a total of six hotspot mutations in this study, of which 248 and 273 are DNA-contact mutants and 175, 245, 249, and 282 are conformational mutants (23). Previous studies have indicated that expression of the 175 conformational mutant resulted in increased resistance to etoposide in H1299 cells, whereas expression the 248 DNA-contact mutant did not alter etoposide sensitivity (24). Our results extend these findings and indicate that expression of the conformational mutants, 175, 249, and 282 result in resistance to cisplatin + etoposide, doxorubicin, and cisplatin + gemcitabine, respectively. On the contrary, one of the DNA-contact mutants (i.e., 248) did not significantly affect cellular survival, consistent with previous studies (24), indicating that the resistance to drugs is mutation and drug specific. Moreover, many of the mutants used in this study, in either the 72R or 72P forms, did not confer survival advantage compared with vector-expressing cells in response to many drugs, indicating that these mutations do not confer a gain of function to the mutant p53. These data, seemingly paradoxical, are consistent with many previous reports that have shown a lack of survival advantage in H1299 and other cancer cell lines to several chemotherapeutic drugs (25–29). However, differences in sensitivity to drugs, when observed, were only generally seen when the mutations were in the 72R form, but not in the 72P form, which were not investigated in the earlier studies. These findings suggest that although the mutations can confer resistance when in the 72R form, this is not a common characteristic of the 72R mutants.

These data raise the possibility that there may be several mechanisms through which resistance is conferred by the various 72R mutants, which seem dependent on mutant type...
and the drug employed. Although earlier studies have suggested that the 72R mutants were able to inhibit p73-mediated cellular transformation and transactivation of target gene promoters (14, 17), this seems cell type specific. We also noticed that the 72R mutants inhibited p73 activity in SAOS2 cells but not in H1299 cells. Moreover, all the mutants used in this study were transcriptionally inactive and were unable to activate the apoptotic box and AIP-1 gene promoters by themselves, further suggesting that the resistance conferred by the mutants might be due to transcriptional-independent mechanisms. In this respect, it is noteworthy that mutant p53, especially in the 72R forms was shown to preferentially bind to p73 and inhibit its activity in SAOS2 cells, and this was suggested as one mechanism for resistance conferred by the 72R mutants (17). However, in that cellular system, although most 72R mutants were able to inactivate p73 activity, some of the mutants which were unable to inactivate p73 function were also able to confer resistance to drug treatment, further indicating that 72R mutants may have other mechanisms that compromise their sensitivity to drugs (17). Moreover, recent data suggest that mutant p53 does not effectively bind to p73 in H1299 cells (30), supporting our observations. It has been recently proposed that the enhanced binding of 72R wild-type p53 compared with 72P to the nuclear export protein CRM1 leads to nuclear export and efficient localization to the mitochondria, thereby leading to enhanced apoptosis (12). Thus, it is possible that some of the 72R mutants are preferentially complexed with other proteins that would inhibit their mitochondrial localization and hence confer resistance compared with their 72P mutant counterparts. This hypothesis requires further investigation. Furthermore, it remains to be investigated if some of the 72R mutants have selectively acquired gain of functions, such as activation of growth promoting gene products such as epidermal growth factor-receptor and human telomerase reverse transcriptase (30, 31), leading to drug resistance.

This report also shows the intrinsic differences in cell types that may affect the property of the p53 mutants. Many previous studies have highlighted the association between p53 mutations and poor response to cancer therapy in several cancer types (32–37). However, mutations in p53 did not affect response to gemcitabine in transitional cell carcinoma patients (38). In addition, patients with mutant p53 ovarian tumors were found more responsive to paclitaxel-based chemotherapy (39), indicating that p53 status might differentially affect drug response in different cell types. Although many studies have tried to correlate the genotypic and allelic frequencies of 72P and 72R with various cancer predisposition (40–42), very few studies have rigorously investigated the role of p53 polymorphisms and mutations in influencing drug sensitivity, besides the recent studies by Crook et al. (17). Our data thus highlights the significance of evaluating the role of p53 mutations in conjunction with the polymorphic status before the therapeutic regimen is decided upon. Such evaluation using not only single treatment, but rather combining drugs, as reported in the present work, would serve as a useful platform against which clinical data can be systematically compared and hence used for eventual mutation-specific treatment of cancer.

Acknowledgments

We thank Dr. Karen Vousden (Beaston Institute for Cancer Research, Glasgow, United Kingdom) for the tetracycline-inducible p73-SAOS2 cells.

References


