p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis

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Summary

Intact p73 function is shown to be an important determinant of cellular sensitivity to anticancer agents. Inhibition of p73 function by dominant-negative proteins or by mutant p53 abrogates apoptosis and cytotoxicity induced by these agents. A polymorphism encoding either arginine (72R) or proline (72P) at codon 72 of p53 influences inhibition of p73 by a range of p53 mutants identified in squamous cancers. Clinical response following cisplatin-based chemo-radiotherapy for advanced head and neck cancer is influenced by this polymorphism, cancers expressing 72R mutants having lower response rates than those expressing 72P mutants. Polymorphism in p53 may influence individual responsiveness to cancer therapy.

Introduction

Apoptosis is a fundamental mechanism by which DNA-damaging anticancer agents cause cytotoxicity (Johnstone et al., 2002). p53 has a major function in transducing stress to the apoptotic machinery of the cell, consistent with the importance of p53 status as a determinant of cellular response to DNA-damaging drugs (Lowe et al., 1993, 1994). The presence of intact p53 function confers tumor sensitivity to DNA-damaging agents including cisplatin and hence potential curability of cancers such as germ-cell tumors (Riou et al., 1995). Despite the recognized role of p53 in determining cellular sensitivity to anticancer agents, cancers with intact p53 function are in a minority. Mutations in p53 are reported to occur in at least 50% of human cancers (Vogelstein et al., 2000). The resultant mutant proteins are frequently functionally compromised for apoptosis induction. Indeed, there is now evidence that apoptosis is the p53 function selected against in tumorigenesis (Schmitt et al., 2002). Wild-type p53 function is compromised in the majority of human tumors yet is intact in normal cells of the host (Vogelstein et al., 2000). As such, the selectivity of anticancer drugs cannot be accounted for by p53-dependent apoptosis alone. The p53 relative p73 also functions in transduction of specific types of DNA damage. For example, activation of the p73/c-abl pathway by some chemotherapeutic agents causes apoptosis via target genes such as *AIP*1 (Gong et al., 1999; Costanzo et al., 2002). p73 exists as several splice variants at both N and C termini. N-terminal variants lacking the transactivation domain may act as dominant-negative inhibitors of both p53 and p73 (Stiewe et al., 2002).

In squamous cell carcinomas (SCC), mutants occurring in the arginine allele (72R) are detected more commonly than those in the proline allele (72P) at the polymorphic codon 72 (Marin et al., 2000; Brooks et al., 2000). These findings were subsequently verified independently in other cancers (Tada et al., 2001; Furihata et al., 2002). The nonrandom retention and expression of

SIGNIFICANCE

Molecular genetic factors predictive of clinical outcome would be of great value in oncology. We show that p73 is a determinant of cellular response to a range of anticancer drugs and that polymorphism in p53 influences response in head and neck cancer, at least in part, via its influence on the inhibition of p73-dependent apoptosis by mutant p53. Patients whose cancers express p53 mutants with mutation in the 72arginine (72R) variant have a worse response to therapy than those expressing p53 mutants encoding proline at position 72 (72P). Expression of 72R mutants that efficiently inhibit p73 is associated with a particularly poor outcome to therapy. Our results thus establish a link between polymorphism in p53 and individual response to cancer therapy.

p53 mutants implies the existence of selective pressure(s) during tumorigenesis that confer a selective advantage to cells expressing 72R mutants, resulting in overrepresentation of 72R mutations in carcinomas. p73 and the related p63 are targets for transdominant inhibition by specific p53 mutants (Di Como et al., 1999; Marin et al., 2000; Strano et al., 2000, 2002; Gaiddon et al., 2001). A possible mechanism to account for the bias toward 72R mutants in carcinomas was afforded by the observation that inhibition of p73 is influenced by the polymorphism at codon 72. The 72R forms of mutants 143A and 175H more efficiently associate with p73 and inhibit p73-dependent target gene upregulation than equivalent 72P mutants (Marin et al., 2000), but other mutants associate with p73 in both 72R and 72P forms (Marin et al., 2000; Gaiddon et al., 2001). Because of its modulatory effects on p53 mutant function, it is an interesting possibility that treatment outcome in human cancer is influenced by the polymorphism (Soussi and Béroud, 2001). Such a model presupposes that p73 is an important determinant of cellular sensitivity to anticancer drugs, but definitive evidence of this is lacking. In the present work, we have investigated this hypothesis at a mechanistic and molecular pathological level.

Results

p73 is induced by anticancer agents and modulates cellular sensitivity

Steady-state levels of endogenous p73 were analyzed by Western blotting following cytotoxic drug treatment in a range of cancer cell lines, predominantly SCC of the head and neck but also including SCC of the cervix (C33a), vulva (A431), and immortalized skin keratinocytes (Hacat). Levels of endogenous p73 increased in each of the squamous carcinoma cell lines following exposure to cisplatin, doxorubicin, taxol, and etoposide (Figure 1A). There were variations in induction of p73 by different drugs in the individual cell lines. For example, doxorubicin caused an increase in p73 in all cell lines analyzed. Further, there was induction of p73 by each tested drug in HSC3 and BICR31, whereas doxorubicin, taxol, and cisplatin but not etoposide caused p73 induction in HN30. Quantitative PCR analysis revealed that expression of *AIP*1 mRNA was increased by drug treatment that caused upregulation of p73 (Figure 1B).

We next addressed the possibility that induction of p73 contributes to the cytotoxicity of these anticancer agents. Saos-2 cells were transiently transfected with full-length p73α together with the dominant-negative inhibitors p73Δ2 and p73DD or with the inactive point mutated p73DD (L371P). Saos-2 cells have been shown to activate an endogenous program of p73-dependent gene expression in response to doxorubicin, including upregulation of *AIP*1, and are thus a suitable cell line for such experiments (Costanzo et al., 2002). Both p73Δ2 and p73DD bind to p73 and inhibit its transactivating activity whereas p73DD (L371P) lacks this activity (Irwin et al., 2000; Fillippovich et al., 2001). Expression of p73α alone caused apoptosis, which was efficiently inhibited by p73Δ2 and p73DD but not by p73DD (L371P) (Figures 2A and 2B). To further establish a potential role for p73 in mediating cytotoxicity, we established Saos-2 cell lines expressing the dominant-negative inhibitors of p73 and tested their sensitivity to drug-induced apoptosis and cytotoxicity. Consistent with transient assays, cell lines expressing p73Δ2 and p73DD were less sensitive to apoptosis following exposure to cisplatin, doxorubicin, and taxol, but lines expressing the inactive p73DD (L371P) exhibited similar sensitivity to control Saos-2 cells (Figure 2C). In colony-forming assays, survival of lines expressing either dominant-negative form of p73 was higher than control cells and those expressing the inactive p73DD (L371P) following drug exposure (Figure 2D).

p53 mutants confer anticancer drug resistance: Effect of p53 polymorphism

These data imply a role for p73 in mediating the cytotoxicity of anticancer agents and show that cellular sensitivity can be modified by dominant-negative inhibition of p73. Specific p53 mutants are able to inhibit p73. For mutants 143A and 175H, this activity is enhanced if the p53 mutant expresses arginine (72R) rather than proline (72P) at codon 72 (Marin et al., 2000). We thus wished to determine whether expression of 72R and 72P variants of a wide range of p53 mutants influences cellular sensitivity to p73-dependent, drug-induced apoptosis in vitro. First, we tested in transient assays the ability of 72R and 72P polymorphic variants of mutants identified in head and neck squamous cell cancers (HNSCC) to inhibit apoptosis resulting from expression of p73 (Figure 3A). In total, 13/25 tested mutants produced at least a 50% inhibition of p73-dependent apoptosis (173L, 175H, 176Y, 179R, 179Y, 220C, 242Y, 245D, 245S, 248W, 249S, 273C, 282W). Although 72P variants of the positive mutants were clearly able to inhibit p73-induced apoptosis, the 72R variant more efficiently inhibited apoptosis than the corresponding 72P (Figures 3A and 3B). The remaining 12 mutants caused less than a 50% reduction in p73-induced apoptosis irrespective of the polymorphism (Table 1). Next, we wished to determine whether inhibition of p73 by the 72R and 72P variants of the p53 mutants correlates with the relative ability to form stable complexes. To achieve this, we tested a subset of the mutants in transfection/coimmunoprecipitation assays similar to those described previously to demonstrate association between mutant p53 and p73 (Di Como et al., 1999; Marin et al., 2000; Strano et al., 2000; Gaiddon et al., 2001). In these assays, 72R mutants more efficiently associated with p73, whereas the 72P variants of these mutants also bound to p73 but with lower efficiency than the 72R (Figure 3C). The superior ability of the 72R variant of mutants such as 173L, 175H, 179Y, and 245D to inhibit p73-dependent apoptosis was also reflected in their greater association with p73 relative to the 72P (Figure 3C). Consistent with its inability to inhibit p73-dependent apoptosis, mutant 142L did not detectably bind to p73 in either 72R or 72P forms (Figure 3C). p53-induced apoptosis was also inhibited by some of the p53 mutants, but in contrast to p73, this activity was not influenced by the polymorphism (Figure 3D).

To directly assess the effect of the p53 polymorphism on drug-induced apoptosis and cytotoxicity, we established isogenic Saos-2 cell lines expressing 72R and 72P forms of each p53 mutant. The steady-state levels of p53 protein in the cell lines analyzed were similar to those of endogenous p53 mutants such as seen in head and neck cancer cell lines (Figure 4). We initially analyzed Saos-2 cell lines expressing equal steady-state levels of the p53 mutants 173L, 175H, and 179Y (Figure 4A). These mutants were detected in individual patients in both 72R and 72P forms in HNSCC and, moreover, the polymorphic variants showed differences in their ability to inhibit p73 (Figure 3). Cells were exposed to various anticancer drugs and apoptosis assessed by flow cytometry. Expression of the three mutants reduced cellular sensitivity to drug-induced apoptosis com-

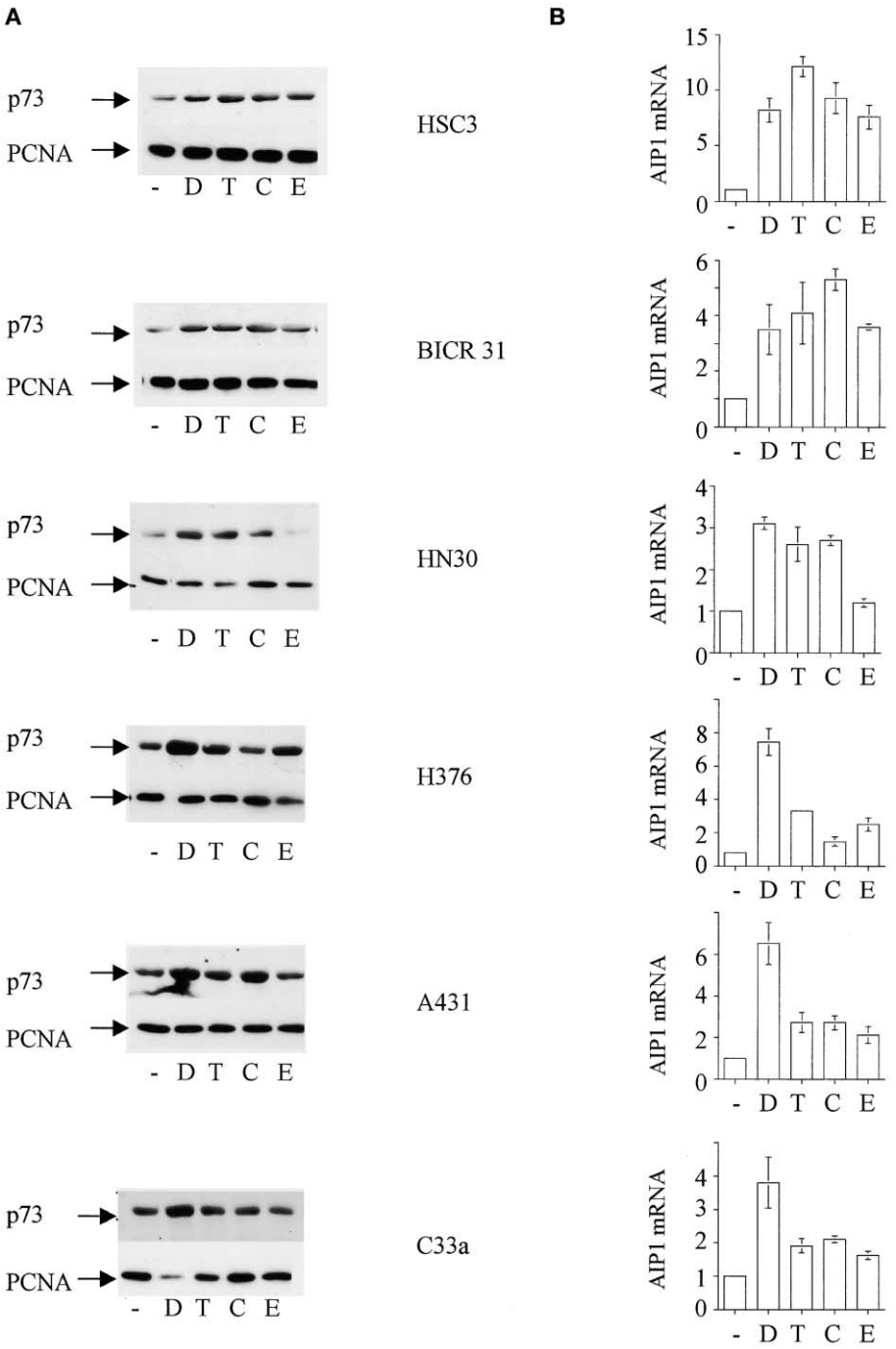

Figure 1. Anticancer drugs induce p73 function in squamous carcinoma cell lines

A: Cytotoxic drugs induce p73 in squamous carcinoma cell lines. The indicated cell lines were treated with chemotherapeutic drugs: — = no drug; D = doxorubicin; T = taxol; C = cisplatin; E = etoposide. The following concentrations were used: doxorubicin 1 μg/ml; taxol 500 ng/ml; cisplatin10 μg/ml; etoposide 10 μM. Twenty-four hours after drug exposure, cell lysates were prepared and steady-state levels of p73 determined by Western blotting. Equal protein loading in each lane was confirmed by reprobing the blot with PC-10, an antibody to PCNA.

B: Anticancer drugs activate *AIP*1. Squamous carcinoma cell lines were treated with the indicated drugs at the above concentrations. After 24 hr, total RNA was isolated and the level of *AIP*1 RNA determined using TaqMan PCR. Data shown are mean mRNA levels (± 1SD), normalized to GAPDH, relative to untreated cells from two independent experiments.

pared to control cells (Figures 4B and 4C). In cells expressing the 72R variant, apoptosis was reduced almost to levels seen in untreated control cells, whereas the reduction in apoptosis in cells expressing the 72P variant was less pronounced (Figure 4C). The mutant 142L, which did not detectably associate with p73, had only a minimal effect on drug-induced cytotoxicity in either the 72R or 72P form (Figure 4C). To extend these observations, we next analyzed cell lines expressing the 72R and 72P variants of the remaining 22 mutants identified in our patients. Mutants 176Y, 179R, 220C, 242Y, 245S, 245D, 282W, 249S, and 273C also showed higher efficiency of apoptosis inhibition when expressed as the 72R form relative to the 72P (Table 1). In contrast, expression of other mutants such as 142L, 152Q, 158G, and 161V had only small effects on cellular sensitivity to drug-induced apoptosis, irrespective of the polymorphism (Table 1). We next further analyzed a subset of these mutants to determine whether differences in apoptosis inhibition were related to the different abilities of the mutants to block induction of *AIP*1, a recognized target gene for p73 (Costanzo et al., 2002). Activation of the *AIP*1 promoter by cisplatin or doxorubicin treatment was inhibited by 173L, 175H, and 179Y, in each case the 72R variant more efficiently blocking activation of *AIP*1 than the

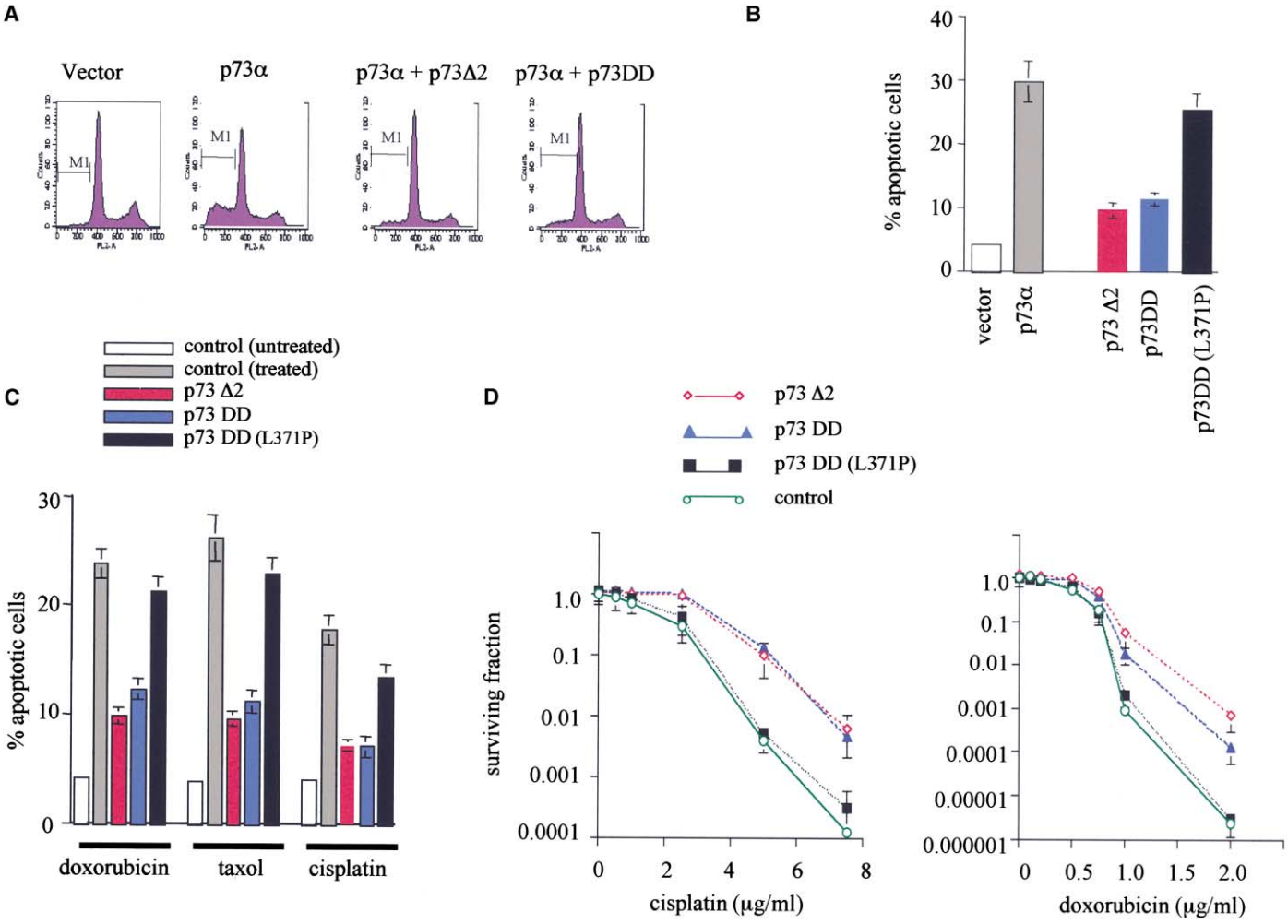

Figure 2. p73 is a determinant of cytotoxic drug action in human carcinoma cells

A: Inhibition of p73-dependent apoptosis by coexpression of dominant-negative p73 proteins. Saos-2 cells were transfected with the indicated expression plasmids (10 μg), and apoptosis was determined by flow cytometry after 24 hr as described in Experimental Procedures. In the plate designated as vector, cells received only empty expression vectors. p73Δ2 and p73DD are dominant-negative p73 proteins. The sub-G1 cell population is indicated as M1. The profiles shown are representative of four independent experiments.

B: Inhibition of p73-dependent apoptosis by dominant-negative p73 proteins p73Δ2 and p73DD, but not the inactive p73DD (L371P). The histogram summarizes the results of four independent transfection experiments. Data shown are mean apoptosis ± 1SD.

C: Abrogation of p73 function inhibits induction of apoptosis by DNA-damaging anticancer agents. Saos-2 cell lines stably expressing the indicated dominant-negative p73 proteins were treated with the indicated drugs (doxorubicin 1 μg/ml; taxol 500 ng/ml; cisplatin 10 μg/ml) and apoptosis determined by flow cytometry after 24 hr. Controls containing vector only DNA sequences were treated with drug vehicle only (untreated). Data shown are mean % sub-G1 cells ± 1 SD from at least three separate experiments.

D: Inhibition of p73 confers resistance to anticancer agents. Saos-2 cell lines stably expressing the indicated p73 proteins or containing empty vector only (control) were treated with varying concentrations of cisplatin or doxorubicin. Survival was determined by colony-forming assay. Data shown are means ± SD of at least three separate experiments. Similar results were obtained from several independent clones.

equivalent 72P mutant (Figure 4D). The mutant 142L, which failed to inhibit drug-induced apoptosis, also failed to inhibit *AIP*1 induction (Figure 4D). We next determined the effect of the polymorphism on the cytotoxicity of anticancer drugs in the cell lines used for apoptosis analysis. Cells were exposed to varying concentrations of each agent and colony survival determined. Mutants previously shown to most efficiently inhibit apoptosis also conferred the highest degree of resistance to cytotoxicity of cisplatin (Figure 4E). Thus, cell lines expressing the 72R variant of "hot spot" mutants 173L, 175H, 179 acquired the greatest resistance to cytotoxicity (Figure 4E and Table 1). Mutants, which did not inhibit drug-induced apoptosis, did not significantly increase resistance to the cytotoxic effects of anticancer drugs.

Modulation of cellular drug sensitivity by siRNA

To verify the hypothesis that mutant p53 increases resistance to anticancer agents via interaction with p73, we tested the ability of small inhibitory RNA (siRNA) oligonucleotides to modulate cellular drug sensitivity. To assess the effects on 72R and 72P p53 mutants, we initially analyzed the effect of siRNA against p53 in isogenic Saos-2 cells expressing 72R and 72P

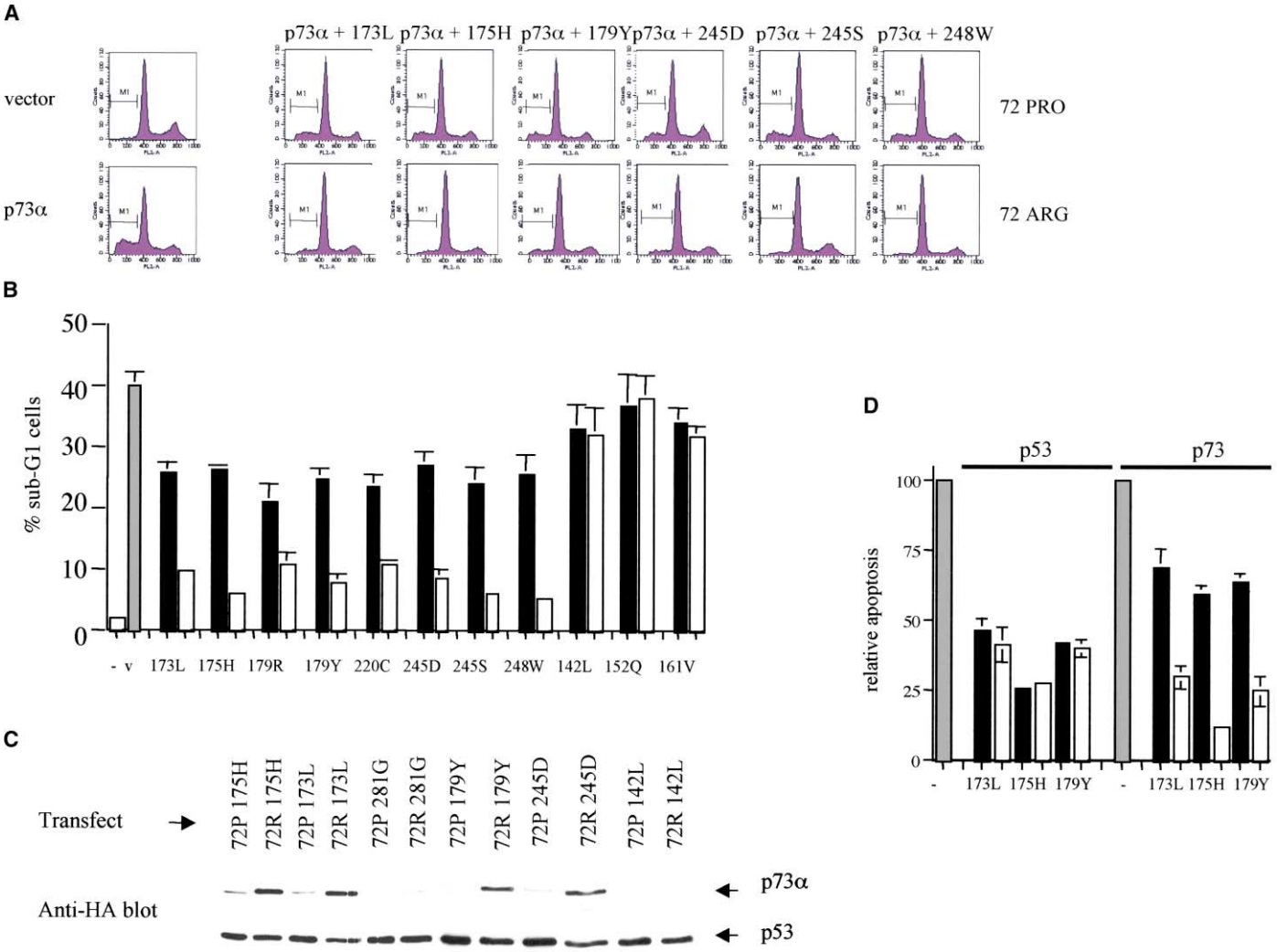

Figure 3. Polymorphism in p53 influences transdominant inhibition of p73 by a wide range of human tumor-associated mutants

A: Transient assays of p73-induced apoptosis in cells transfected with 72R and 72P p53 mutants. Saos-2 cells were transfected with plasmids expressing p73α (10 μg) and the indicated p53 mutants (20 μg), together with pCD20 (3 μg). Twenty-four hours following transfection, cell cycle distribution and DNA content were determined by flow cytometry as described in Experimental Procedures. Sub-G1 cells are designated M1. The flow cytometry profiles shown are representative of at least three experiments.

B: Summary of superior inhibition of p73-dependent apoptosis by 72R p53 mutants. Data shown are means (± 1SD) from at least three experiments in which Saos-2 cells were transfected with 10 μg of p73α and 20 μg of p53 expression plasmid.

C: Association of polymorphic p53 mutants with p73α correlates with inhibition of drug-induced apoptosis and cytotoxicity. Saos-2 cells were transfected with HA-tagged p73α (10 μg) and the indicated p53 mutants as the 72R or 72P variant (20 μg). Twenty-four hours later, cell lysates were prepared and subjected to immunoprecipitation with PAb1801. Immunoblotting was done with anti-HA polyclonal antiserum (Y11). Mutant 281G in our hands associates inefficiently with p73 and is used as a negative control. The remaining mutants are all from the HNSCC cases described in this study. 142L which does not affect sensitivity to drug-induced apoptosis associates very weakly with p73. The remaining mutants shown associate more efficiently with p73 when in the 72R form. Note that this correlates with more efficient inhibition of p73-dependent apoptosis.

D: Polymorphism in mutant p53 influences transdominant inhibition of p73 but not wild-type p53. Saos-2 cells were transfected with expression plasmids encoding either wild-type p53 or full-length p73α as indicated (gray panels), together with the indicated plasmids expressing either the 72P (black panel) or 72R (open panel) form of each p53 mutant. Sub-G1 cells were determined 24 hr after transfection by FACS assay. The graph shows percentage of sub-G1 cells relative to transfection of p53 or p73 alone (which are designated as 100%). Data are mean percentages of sub-G1 cells induced by p53 or p73 alone ± 1SD from at least four independent experiments.

forms of the mutant 173L, which had been demonstrated to efficiently inhibit p73 (Figure 3). There was a dose-dependent reduction in steady-state levels of both 72R and 72P forms of this mutant following treatment with p53-specific siRNA (Figures 5A and 5B). The control PGL3 siRNA did not affect p53 levels. We also tested the ability of siRNA to downregulate expression of the endogenous proteins in squamous carcinoma cell lines previously shown to upregulate p73 in response to cytotoxic drug exposure. Treatment with p53-specific siRNA reduced steady-state levels of p53 both in head and neck and other squamous carcinoma cell lines (Figure 5C and data not shown). Despite upregulating p73 levels (see Figure 1), cytotoxic drug treatment did not increase p53 levels, but treatment with siRNA caused a clear reduction in mutant p53 levels both in the pres-

Table 1. Inhibition of cisplatin-induced apoptosis by human tumor-associated p53 mutants

Mutant	% apoptosis 72P	% apoptosis 72R	% apoptosis 72P (siRNA)	% apoptosis 72R (siRNA)
Control (no drug)	2.67	3.1	4.9	4.9
Control	44.2 ± 5.9	44.2 ± 5.9	48.6 ± 5.2	48.6 ± 5.2
110H	41.2 ± 7.6	42.8 ± 6.0	ND	ND
133L	37.2 ± 4.2	35.5 ± 6.4	ND	ND
142L	40.6 ± 2.1	36.4 ± 6.9	46.5 ± 7.4	41.7 ± 8.3
152Q	39.7 ± 3.8	36.6 ± 2.2	ND	ND
158G	39.9 ± 5.1	37.8 ± 4.8	ND	ND
161V	40.8 ± 6.5	37.5 ± 8.2	ND	ND
168Y	36.1 ± 7.3	29.2 ± 1.9	ND	ND
172A	34.9 ± 4.7	27.3 ± 5.6	ND	ND
173L	21.3 ± 4.2	12.5 ± 1.7	35.8 ± 4.1	29.7 ± 1.5
175H	15.3 ± 0.9	6.9 ± 0.87	26.4 ± 1.3	19.4 ± 4.0
176Y	28.3 ± 4.9	20.8 ± 0.8	ND	ND
178Y	33.8 ± 2.2	26.2 ± 1.5	ND	ND
179R	20.4 ± 5.1	14.6 ± 2.7	29.9 ± 5.5	26.4 ± 3.9
179Y	20.0 ± 1.7	14.3 ± 3.1	32.3 ± 4.6	22.1 ± 3.8
206Y	35.2 ± 6.6	30.5 ± 1.8	ND	ND
220C	25.1 ± 4.4	16.4 ± 1.8	34.5 ± 7.2	27.3 ± 4.3
242Y	26.1 ± 1.3	20.4 ± 2.2	ND	ND
245D	24.2 ± 1.0	15.1 ± 0.9	29.6 ± 4.1	22.2 ± 1.8
245S	20.9 ± 5.8	10.6 ± 2.0	29.7 ± 1.5	17.9 ± 2.4
249S	21.3 ± 4.2	13.8 ± 2.2	31.2 ± 4.7	26.8 ± 3.8
248W	18.6 ± 2.3	11.5 ± 0.8	25.7 ± 3.9	18.2 ± 2.1
272A	31.5 ± 6.3	26.2 ± 3.1	ND	ND
273C	27.9 ± 5.1	18.8 ± 1.6	34.2 ± 1.2	25.8 ± 2.7
275Y	32.4 ± 2.6	26.5 ± 4.2	ND	ND
282W	29.8 ± 6.2	21.7 ± 4.6	37.1 ± 4.0	31.4 ± 0.8

All experiments were performed in Saos-2 cells stably expressing the indicated p53 mutants. Cells were treated with 10 μg/ml cisplatin and apoptosis determined after 24 hr by flow cytometry as described in Experimental Procedures. Where indicated, cells were exposed to 100 μM p53 siRNA 48 hr before cisplatin treatment. Data are mean apoptosis ± 1SD from two experiments.

ence and absence of drug exposure (Figure 5C). We also wished to determine whether siRNA to p53 caused reduced association of mutant p53 with p73. We therefore examined by immunoblotting endogenous complex formation with and without siRNA. The increase in p73 protein caused by drug exposure was unaffected by p53 siRNA (Figure 5D), but there was a clear reduction in the amount of bound mutant p53 immunoprecipitated with p73 in cells treated with p53 siRNA prior to exposure to cytotoxic drugs (Figure 5D). Thus, whereas total p73 protein levels are unaffected, p53 siRNA causes a significant reduction in p73 mutant p53 complex formation. In cells treated with p53 siRNA then exposed to anticancer drugs, there was a clear increase in apoptosis compared to cells either mock-transfected with siRNA or transfected with the PGL siRNA (Figure 5E). Although the magnitude of the enhancement of apoptosis caused by mutant p53 downregulation varied between cell lines, inhibition of mutant p53 expression caused a consistent increase in drug-induced apoptosis in all cell lines tested (Figure 5F).

In the Saos-2 cells expressing 72R and 72P mutants, we tested in colony survival assays the effect of p53 siRNA on drug-induced cytotoxicity (Figure 6). We initially analyzed clones expressing 173L, which we previously showed to inhibit p73 and increase drug resistance. When pretreated with p53 siRNA, such cells (expressing either 72R or 72P forms of the mutant) were substantially more sensitive to doxorubicin and cisplatin than untreated cells or cells treated with the PGL siRNA oligos (Figure 6A; Table 1). siRNA treatment had only a small effect on drug sensitivity in cells expressing the p53 mutant 142L, which does not detectably inhibit p73 in our assays (Table 1). We next analyzed Saos-2 cell lines expressing other p53 mutants to determine the utility of p53 siRNA to sensitize such cells to anticancer drugs. We tested mutants 175H, 179Y, 179R, 245S, 248W, 249S, 273C, and 282W. These hot spot mutants together comprise a substantial proportion of human tumor-associated mutants (Vousden and Lu, 2002). Each of the mutants had previously been shown to increase resistance to cisplatin, particularly when expressed in the 72R form (Table 1). Treatment with p53 siRNA increased the sensitivity of cell lines expressing each of these mutants (in both 72R and 72P forms) to cisplatin and doxorubicin (Table 1). Representative dose response curves are shown in Figure 6B.

72R p53 mutants correlate with poor response in head and neck cancer

Taken together, the in vitro studies described above reveal roles for both p73 and p53 polymorphism in influencing anticancer drug sensitivity. We thus wished to determine whether treatment outcome in vivo is influenced by mutation and polymorphism in p53 in cancers arising in tissues expressing p73. We therefore analyzed expression of p73 and the sequence of p53 in a series of 70 inoperable head and neck cancers treated with chemo-radiotherapy. Each patient presented with locally advanced, unresectable TNM stage III/IV HNSCC and received multimodal treatment consisting of radiotherapy alternated with platinum-based chemotherapy. Clinico-pathological details of the cases are shown in Table 2.

The frequency of the codon 72 genotypes in the cases did not differ from that in normal tissue controls: cases RR = 54%; RP = 40%; PP = 6%. Controls: RR = 55%; RP = 50%; PP = 5% (χ^2 = 1.85; p= 0.40). Mutations were detected in 40/70

cases (57%). In cancers arising in individuals heterozygous R/P at codon 72 (n = 19), mutations were detected more commonly in the 72R allele (14 mutations in 72R and 5 mutations in 72P; p = 0.0394).

Twenty-five out of the twenty-seven patients with wild-type p53 (93%) gained complete response to treatment (CR), as opposed to only 23 out of the 40 patients with p53 mutations (58%) (p = 0.0044). At the time of analysis, 30/70 (43%) patients have died or progressed. Median time to death or progression of these 30 = 13.1 months (inter quartile range, IQR = 7.6 to 18.4). Median follow up for the 40 alive and progression-free patients = 33.9 months (IQR = 16.9 to 44.7).

Progression-free survival (PFS) was significantly longer for complete responders than for partial responders (13/48 progressed or died versus 14/19, respectively; % surviving progression free at 2 yr = 77% versus 28%; χ^2 = 25.7, log rank p < 0.0001). There was also significantly longer PFS for patients with wild-type p53 rather than those with mutant p53 (5/30 progressed or died versus 25/40 respectively; % surviving at 2 yr = 82% versus 46%; χ^2 = 11.4, log rank p = 0.0007). The significance of p53 mutation remained even in the presence of other known prognostic variables (age, performance status, stage, site of primary tumor, and nodal status) (p = 0.002). For the 40 cases with mutations, PFS was significantly longer for patients with mutant 72P rather than 72R (1/8 progressed or died versus 24/32, respectively; % surviving progression free at 2 yr = 83% versus 38%; χ^2 = 6.9, log rank p = 0.0087).

At the time of analysis, 26/70 patients had died (37%). The median time to death of these patients = 14.9 months (inter quartile range, IQR = 7.6 to 27.0). The median followup for the 44 living patients = 31.7 months (IQR = 17.2 to 44.7).

There was significantly longer survival for patients achieving CR than PR (11/48 dead versus 12/19 dead; % surviving at 2 yr = 85% versus 43%; χ^2 = 20.0, log rank p < 0.0001). Survival was also significantly longer for patients without mutations (5/30 dead versus 21/40, respectively; % surviving at 2 yr = 82% versus 62%; χ^2 = 7.6, log rank p = 0.0058). The significance of p53 status remained even in the presence of other known prognostic variables (p = 0.02).

For the 40 cases with mutations, there was significantly longer survival for patients with mutation in the 72P allele rather than 72R (0/8 dead versus 21/32, respectively; % surviving at 2 yr = 100% versus 53%; χ^2 = 8.1, log rank p = 0.0044).

p53-dependent inhibition of p73 correlates with clinical drug resistance

In this work, we have identified specific p53 mutants that inhibit p73-dependent apoptosis. We show that this activity is substantially enhanced when the mutants occur in the 72R form and we demonstrate partial reversal of drug resistance by inhibition of p53 expression. To assess the clinical effect of these mutants, we analyzed treatment outcomes in the patients in our series of HNSCC whose cancers contained these mutants.

Twenty-five cases contained p53 mutations (72R or 72P) that efficiently inhibited p73 (Table 3), and there were 15 cases with mutations that did not. Of the 25 patients, 11 (44%) achieved CR. Of the 15 cases, 12 (80%) achieved CR (p = 0.06). There was significantly longer PFS for the 15 patients with non p73-inactivating p53 mutations compared to the 25 patients (6/15 progressed or dead versus 19/25 progressed or dead, median PFS = 31.3 months (95% CI 17.3-not reached) versus 16.1 months (95% CI = 12.5–24.5)), χ^2 = 3.9, p = 0.05. There was no significant difference in survival between the 25 cases with p73-inactivating mutations and the remaining 15 patients (15/25 dead versus 6/15 dead, median survival = 29.3 months (95% CI = 16.8-not reached) versus 45.0 (95% CI = 21.0-not reached)), χ^2 = 0.9, p = 0.34.

Of the 25 cases expressing mutants which efficiently inactivate p73, 6 of the mutations were in the 72P allele and 19 in the 72R (Table 2). Of the 6 in the 72P allele, 5 (83%) achieved CR. Of the 19 in the R, 6 (32%) achieved CR (p = 0.06). At the time of analysis, 19 of the 25 patients had progressed or died (median PFS = 16.1 months, 95% CI = 12.5–24.5). PFS was significantly longer if mutation was in the 72P rather than 72R allele (1/6 progressed or died versus 18/19 progressed or died, median PFS not reached (95% CI 18.5–not reached) versus 13.5 (95% CI = 10.0 – 16.4)), χ^2 = 8.5, p = 0.0036). At the time of analysis, 15 out of the 25 patients had died (median survival = 29.3 months, 95% CI = 16.8–not yet reached). Survival was significantly longer for patients with mutations in the 72P rather than the 72R allele (0/6 dead versus 15/19 dead, median survival not reached (95% CI not reached–not reached) versus 19.4 (95% CI = 13.8–32), χ^2 = 9.1, p = 0.0026) (Figure 7A).

These data all imply that p73 may have an important function in mediating the activity of anticancer drugs in vivo and in vitro. It was therefore of interest to determine whether expression of p73 per se might be of prognostic value. We thus assessed p53 and p73 expression using immunocytochemistry in 50 cases for which sections were available (Figure 7B) and scored these cases for nuclear expression. p53 expression was detected in 31/50 cases and expression of p73 in 19/50 cases. After grouping patients into positive p73, wild-type p53 (n = 10), positive p73, mutant p53 (n = 9), negative p73, wild-type p53 (n = 11), and negative p73, mutant p53 (n = 20), survival was found to be similar between groups (log rank χ^2 = 2.7, log rank p = 0.45). Thus steady-state p73 expression does not appear in this small sample to be predictive of survival. However, analysis of response rates across the four groups revealed that p73 expression and p53 mutational status together may be predictive of response (χ^2 test for trend p value = 0.03). Thus, taking together p73 expression with p53 mutation status, p73 expression may have predictive value in terms of response.

Discussion

In this study, we present data implying an important role for p73 function in determining cellular sensitivity to chemotherapeutic agents. Several lines of in vitro experimental evidence from our studies support the hypothesis that p73 status influences responsiveness to such drugs. Firstly, we demonstrate induction of p73 in response to anticancer agents in squamous carcinoma cell lines from several tissues. Although p73 was initially reported not to be upregulated by DNA-damaging treatment (Kaghad et al., 1997), later work has demonstrated p73 induction by cisplatin and doxorubicin (Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999; Costanzo et al., 2002). We now extend these studies by showing p73 induction by a variety of cancer chemotherapeutic agents that act through distinct mechanisms. Upregulation was accompanied by increased expression of *AIP*1 in each of the cell lines examined. As such, our results suggest that the model of p73 action derived from studies of doxorubicin (Costanzo et al., 2002) may also be applicable to

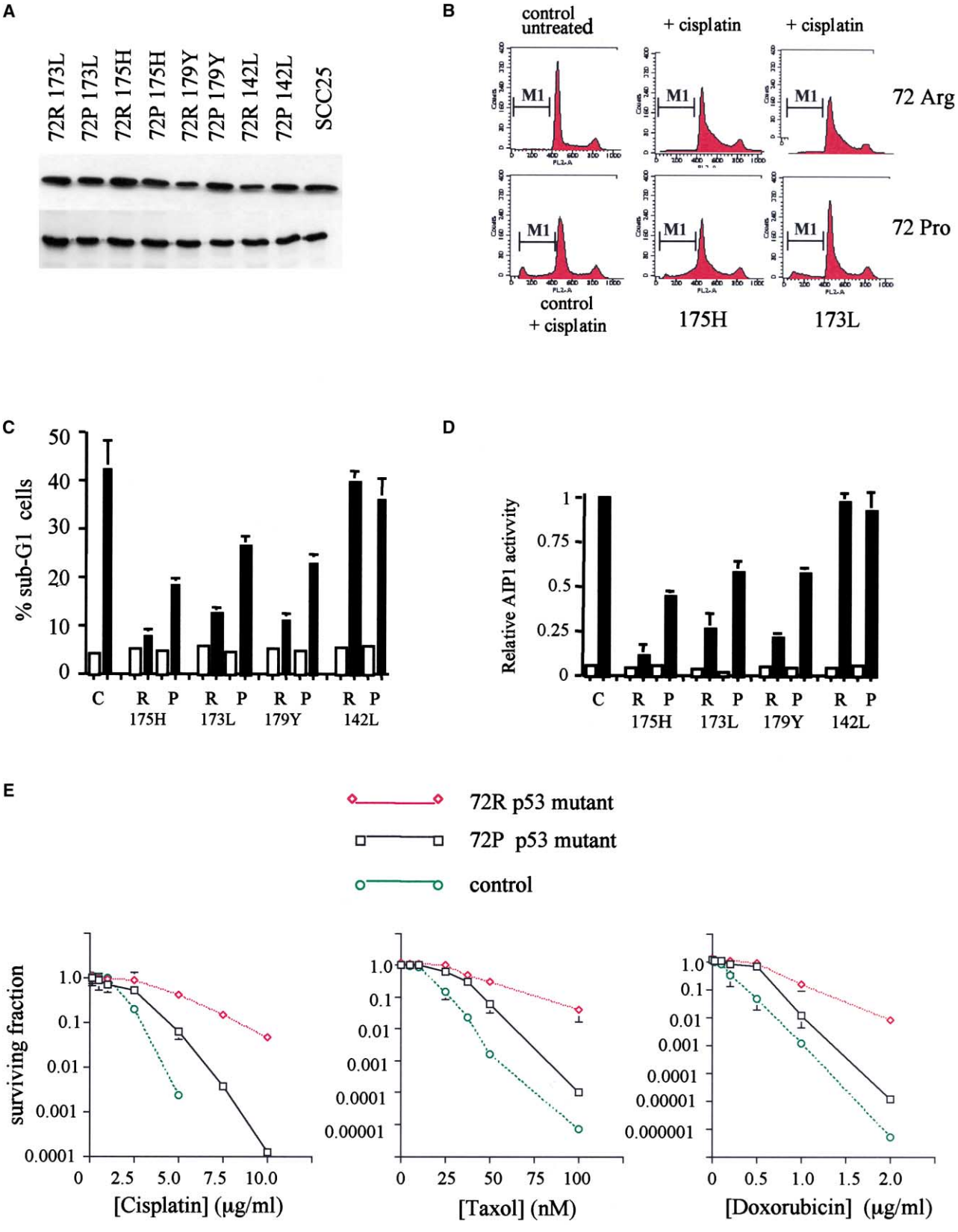

Figure 4. Polymorphism in p53 modulates sensitivity to anticancer drugs

A: Equal steady-state levels of mutant p53 proteins are expressed in the cell lines analyzed for apoptosis sensitivity. Saos-2 cells were engineered to stably express the indicated p53 mutants in either the arginine (72R) or proline (72P) polymorphic variant. Cell lysates were prepared from exponentially growing Saos-2 cells and subjected to Western blotting with antibody DO-1 (p53) and PC-10 (PCNA) to verify equal protein loading in each lane. SCC25 is a human

other anticancer agents. Furthermore, the diversity of the anticancer drugs that cause p73 induction and *AIP*1 upregulation supports the hypothesis that p73 may be an important cellular mediator of the cytotoxicity of multiple anticancer agents. Our data are complementary to those reported by Irwin and coworkers who also show induction of p73 by various anticancer agents (Irwin et al., 2003 [this issue of *Cancer Cell*]).

Secondly, we show that abrogation of p73 function by expression of dominant-negative inhibitors reduces apoptosis and cytotoxicity produced by the same range of anticancer drugs. Previous work has shown that dominant-negative inhibition by p73Δ2 or p73ΔN abrogates cytotoxicity of cisplatin (Fillippovich et al., 2001; Zaika et al., 2002). Dominant-negative inhibitors such as p73Δ2 and p73DD may also inhibit the function of the p73 paralog p63 (Irwin et al., 2000). The TA form of p63 can activate apoptosis in response to DNA damage, whereas the N-terminally truncated ΔN forms lack this activity. Western blotting and RT-PCR revealed that expression of p63 was almost exclusively of the ΔN form in the cell lines used in our studies (data not shown), consistent with the hypothesis that the effect of dominant-negative inhibition is mediated through p73. This is also supported by studies using siRNA against p73 reported in the accompanying paper (Irwin et al., 2003).

p73 expression is deregulated via E2F1 by expression of oncogenes (Irwin et al., 2000; Stiewe and Putzer, 2000; Brooks et al., 2002; Zaika et al., 2001). As a result, full-length p73 is overexpressed in some cancers relative to normal tissue (Zaika et al., 1999; Cai et al., 2000; Brooks et al., 2002) and we now show by immunocytochemistry that p73 is overexpressed in advanced HNSCC. Taken together with these studies, our results and those of Irwin and coworkers suggest that the relatively greater steady-state levels of full-length p73, and/or its enhanced inducibility, in neoplastic versus normal tissue may contribute to the oncotoxic selectivity of DNA-damaging agents.

Previous work has shown that expression of specific p53 mutants can inhibit apoptosis induced by some anticancer agents (Li et al., 1998; Blandino et al., 1999). Furthermore, inhibition of p73 by p53 mutants has been demonstrated by several investigators (Di Como et al., 1999; Marin et al., 2000; Strano et al., 2000; Gaiddon et al., 2001). Because our data implied an important role for p73 in mediating drug cytotoxicity, we wished to explore the possibility that these phenomena might be mechanistically linked. We show that expression of a subset of p53 mutants able to inhibit p73 confers resistance to apoptosis and cytotoxicity induced by chemotherapeutic drugs. The close correlation between inhibition of p73 and increased drug resistance supports such an association. Consistent with these observations, downregulation of mutant p53 using siRNA increased the sensitivity of multiple cancer cell lines to drug-induced apoptosis. These results suggest a model in which expression of mutant p53 alters cellular sensitivity to anticancer drugs by inhibition of p73. Because we had also observed that this inhibition is affected by the polymorphism at codon 72 of p53, this raised the possibility that the in vivo efficacy of chemotherapy is influenced by the polymorphism. The effect of p53 status on response and survival in head and neck cancer has been investigated previously. In one such study, mutations in p53 were associated with loco-regional failure, but not overall survival following radiotherapy (Koch et al., 1996). In other studies, overall survival following radiotherapy was significantly worse in cases with p53 mutations (Gallo et al., 1999; Alsner et al., 2001). Mutation in p53 is also associated with lower response rates to chemotherapy in head and neck cancer (Cabelguenne et al., 2000; Fouret et al., 2002). However, no study has examined the influence of mutation and polymorphism in p53 on outcome. Furthermore, few studies have sought to correlate outcome with the properties of the p53 mutants, although specific mutations may be associated with shorter survival in oral cancer (Yamazaki et al., 2003). In the present work, we present evidence that clinical response to combined modality chemo- radiotherapy in advanced head and neck cancer is influenced both by the properties of the p53 mutants and the allelic variant at codon 72. Specifically, we have observed that p53 mutants associated with less favorable response to chemo-radiotherapy are those which most efficiently inactivate p73 in vitro. Consistent with in vitro studies of the effect of the polymorphism on inhibition of p73, response was less favorable when the mutant occurred in the 72R rather than 72P form. Further, we present evidence that p53 mutation and polymorphism may also influence both progression-free and overall survival. Verification of our results will require study of larger numbers of patients and of other tumor types. Despite these caveats, our results suggest that the ability of p53 mutants to abrogate p73 function may be important in vivo as well as in vitro and support the assertion that future studies of the effect p53 on outcome in oncology will need to incorporate analysis

tongue squamous carcinoma cell line and is included to show that levels of p53 protein in the Saos-2 cells are comparable to endogenous mutant p53 in head and neck cancer cell lines.

B: Inhibition of cisplatin-induced toxicity in cells stably expressing 72R and 72P variants of p53 mutants 173L and 175H. Exponentially growing cells were treated with cisplatin (10 μg/ml) for 2 hr and then grown in drug-free medium for a further 24 hr after which apoptosis was determined by flow cytometry. The figure shows representative FACS profiles, and the sub-G1 cells are designated as M1.

C: Increased cellular resistance to anticancer agents by mutant p53 correlates with inhibition of p73. The data shown are mean % sub-G1 cells (± 1 SD) in the indicated cell lines following exposure for 2 hr to cisplatin (10 μg/ml). The data are from three experiments. Similar results were obtained from other independent clones.

D: Abrogation of drug-induced apoptosis is associated with inhibition of p73-dependent activation of *AIP*1. Saos-2 cell lines expressing the indicated p53 mutants were transfected with *AIP*1 luciferase and pSV2-βgal. Eighteen hours later, the cells were treated with cisplatin (10 μg/ml). Luciferase activity was determined after 12 hr. Data shown are mean luciferase activity, corrected for β-galactosidase, from three experiments. Equivalent results were obtained from at least two independent clones expressing each mutant.

E: Polymorphism in p53 modulates the cytotoxicity of anticancer drugs which act through p73. Saos-2 cell lines stably expressing p53 mutants were treated with varying concentrations of the indicated drugs for 2 hr, then grown in drug-free medium until visible colonies appeared. Data shown are means ± 1 SD of at least three independent experiments. Symbols are as follows: open circles = vector only cells; open square = 72P form of the mutant; open diamonds = 72R form of the mutant. In the left-hand figure, the mutant = 175H; In the middle figure, the mutant = 173L; In the right-hand figure, the mutant = 179Y.

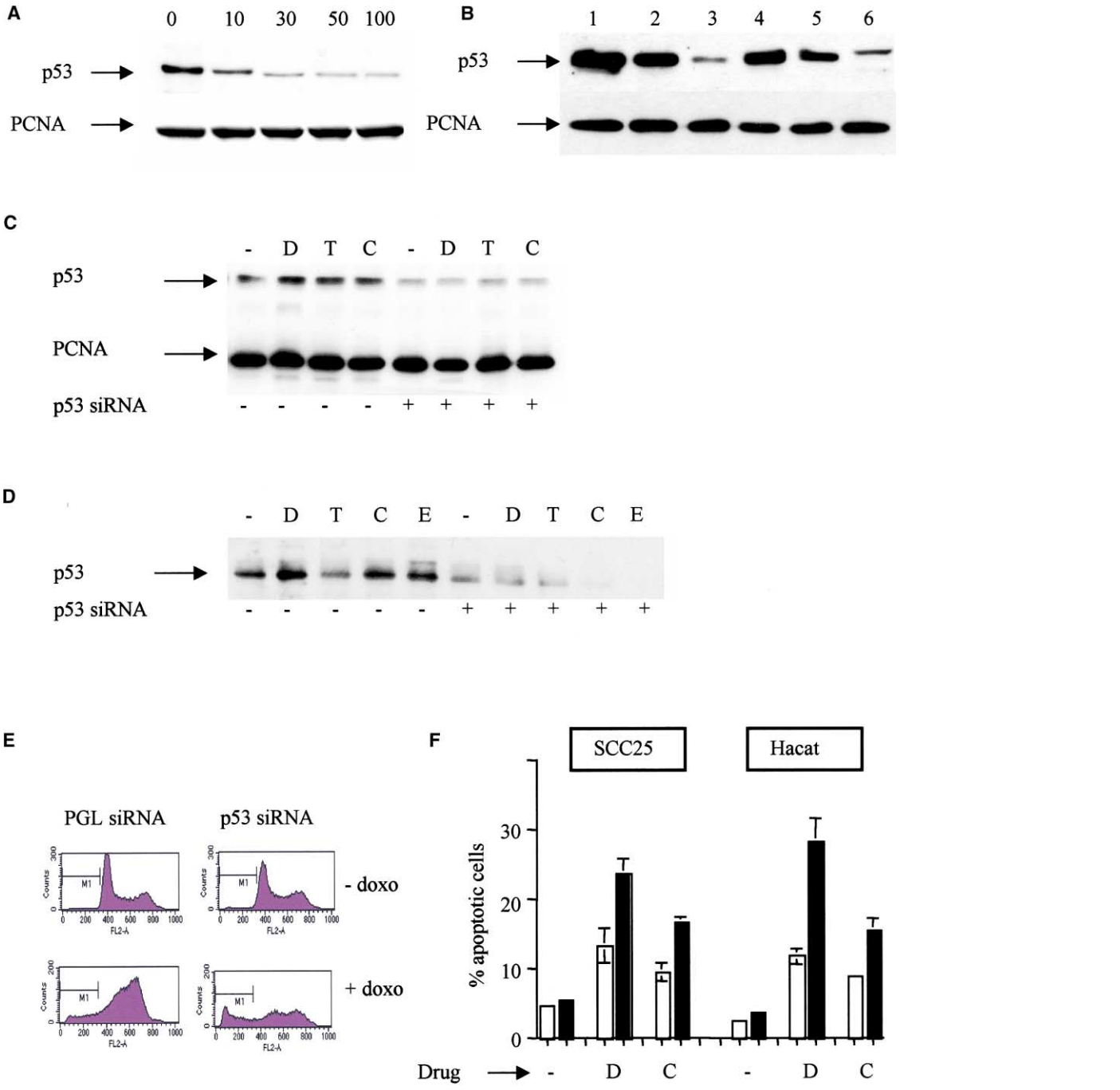

Figure 5. Inhibition of mutant p53 expression sensitizes cells to anticancer agents

A: Dose-response of reduction in p53 expression with increasing p53 siRNA. Saos-2 cells expressing 72R 173L p53 were treated with the indicated concentrations (nM) of p53 siRNA. p53 expression was determined by Western blotting after 48 hr of treatment.

B: 72R and 72P p53 mutant expression is specifically downregulated by siRNA treatment. Saos-2 cells were treated with 100 nM siRNA and analyzed by Western blotting with antibody DO-1 48 hr later. To confirm equal loading, the blots were also probed with antibody to the control protein PCNA as shown. Lanes 1–3: Saos-2 expressing 72P 173L p53, lanes 4–6: Saos-2 cells expressing 72R 173L p53. Lanes 1 and 4: untreated (no si RNA); Lanes 2 and 5: 30 μM siRNA; Lanes 3 and 6: 100 μM siRNA.

C: Inhibition of endogenous mutant p53 expression by siRNA. HN5 cells were treated with 100 nM p53 siRNA (+) or PGL3 siRNA (−). Forty-eight hours later, cells were either untreated (—) or exposed to doxorubicin (D; 1 μg/ml), taxol (T; 500 ng/ml), or cisplatin (C; 10 μg/ml). After 24 hr, cell lysates were prepared and analyzed for p53 and PCNA by Western blotting. p53 levels are unaffected by cytotoxic drugs but downregulated by p53 siRNA.

D: p53 siRNA inhibits endogenous complex formation between mutant p53 and p73. Hacat cells were treated with 100 nM p53 siRNA (+) or PGL3 siRNA (−). Forty-eight hours later, cells were either untreated (—) or exposed to doxorubicin (D; 1 μg/ml), taxol (T; 500 ng/ml), cisplatin (C; 10 μg/ml), or etoposide (E; 10 μM). After 24 hr, cell lysates were prepared and p53-p73 complexes analyzed as described in Experimental Procedures. Immunoprecipitation was performed with ER-15 and blotting with DO-1. Following treatment with p53 siRNA (designated as +), there is a marked reduction in p53 complexed with p73 compared to cells pretreated with PGL3 siRNA.

of the polymorphism (Soussi and Béroud, 2001). Additional prognostic data may be afforded by analysis of p73 expression. The results of initial immunocytochemical studies that we present suggest that p73 expression, when taken together with p53 mutation status, may have predictive value in terms of response but not survival. However, the steady-state expression of p73 revealed by immunocytochemistry may not be representative of levels induced in tumors by chemotherapy. Moreover, p73 is expressed in multiple splice variants at both C and N termini, further complicating interpretation of immunocytochemical analysis. Nevertheless, additional studies of the prognostic utility of p73 expression are clearly warranted.

It should be noted from our data that mutants such as 142L, 152L, 158G, and 161V lacking detectable ability to abrogate p73 function also appear to be preferentially selected in the R allele. As such, the greater effect of 72R p53 mutants on p73 is unlikely to be the only mechanism underlying both the bias for selection of 72R mutants in neoplastic development and also the less favorable outcome in cancers with mutations in the 72R allele. This implies either that 72R mutants possess other enhanced functions relative to the 72P allele or that selection during tumorigenesis favors clones in which the wild-type activity of the 72R protein has been inactivated. The latter hypothesis would require that the 72R wild-type protein possesses functions which more efficiently mediate tumor suppression and which are selected against in tumorigenesis. Additional studies are required to address these possibilities, but the frequent preferential retention of 72R mutants clearly implicates gain of function for a subset of mutants specifically selected for in cancers. This in turn correlates with less favorable response to treatment as evidenced by the particularly poor outcome of the patients in our series where cancers contained 72R mutants that efficiently abrogate p73.

Combined modality treatments for advanced head and neck cancer are associated with significant toxicity. It is, therefore, crucial to make rational treatment decisions based on distinctive features of each cancer and to spare unnecessary toxicity to patients with a low probability of response. Although resistance to cancer chemotherapy is likely to be regulated by multiple factors, the data we present herein suggest that the p53-p73 interaction and its regulation by p53 polymorphism is an important determinant of response and survival in head and neck cancer. Our results suggest that patients with specific p53 mutations in the 72R form are those least likely to gain complete response to chemotherapy.

Experimental procedures

Cell lines

Squamous carcinoma cell lines and Saos-2 $p53^{-/-}$ cells were maintained in DMEM with 10% fetal bovine serum. To generate cell lines stably expressing dominant-negative inhibitors, Saos-2 cell lines were transfected with expression plasmids, then grown in the presence of 400 μg/ml G418. Isolated colonies were ring cloned and expression of transfected sequences confirmed by Western blotting and/or RT-PCR.

Drug treatment

Cells were exposed to anticancer drugs at concentrations determined in colony-forming assays to be cytotoxic. For analysis of p73 induction, the following concentrations were used: etoposide 10 μM; cisplatin 1 μg/ml and 10 μg/ml; taxol 500 ng/ml; doxorubicin 1 μg/ml.

Immunoblotting

Cells were harvested in lysis buffer (50 mM Tris, 250 mM NaCl, 0.1% Nonidet NP-40, 5 mM EDTA, 50 mM NaF, 1 mM PMSF with protease inhibitor cocktail (Roche)). Antibodies were as follows: p73: ER-15 (Neomarkers) affinity purified, 1:100; p53: DO-1 ascites, 1:1000; PCNA: PC-10 ascites, 1:1000). All lanes contained equal amounts of protein as determined by the Bradford method. Association between p53 mutants and p73 was analyzed by immunoblotting essentially as described by Marin et al. (2000). Briefly, lysates were prepared from subconfluent 3 cm dishes of cells in RIPA buffer and divided into equal portions. Lysates were precleared with protein G sepharose then subjected to immunoprecipitation with ER-15 (p73), DO-1 (p53), or Pab419 (control antibody). Immunoprecipitated proteins were resolved on 10% polyacrylamide gels and immunoblotted with either DO-1, ER-15, or Pab419. Proteins were detected using a secondary anti-mouse light chain antibody (HRP-conjugated rat anti-mouse light chain, Pharmingen No: 559751) and visualized with the enhanced chemiluminescent system from Amersham. To compare complex formation with p73 between the two polymorphic variants of p53, Saos-2 cells were transfected with 15 μg pcDNA3 p73α and 15 μg $pCB6^{+}$ p53 (in either 72R or 72P forms). Twenty-four hours later, lysates were prepared and processed for immunoblotting exactly as described above.

Plasmids

$pCB6^{+}$72R wt p53 and $pCB6^{+}$72P wt p53 (Crook et al., 1994) were the plasmids for p53 expression and for in vitro mutagenesis using the Stratagene Quikchange system. pcDNA3 HAp73α and pcDNA3 HAp73β (De Laurenzi et al., 1998) were generous gifts of Dr. Gerry Melino and were used for expression of p73. pcDNA3 p73Δ2 was generously given to us by Dr. K.K. Khana. pcDNA3 p73DD and p73DD (L371P) were kindly provided by Drs. Meredith Irwin and William Kaelin. pcDNA3 p73DD encodes a dominant-negative form of p73 that binds to full-length p73 and abrogates activation of p73-inducible promoters (Irwin et al., 2000). The point mutant p73DD (L371P) does not bind to full-length p73 and lacks transdominant inhibitory activity (Irwin et al., 2000). p53*AIP*1 luciferase was generously provided by Dr Silvia Soddu.

Analysis of apoptosis and cytotoxicity

p73-induced apoptosis was assessed in Saos-2 cells by transient transfection and flow cytometry. Exponentially growing cells received 10 μg of p73 expression plasmid and 3 μg of pcDNA3 CD20, together where appropriate with p53 mutants or dominant-negative inhibitors at a ratio of 1:1 (10 μg: 10 μg) and 1:2 (10 μg: 20 μg). Twenty-four hours after transfection, cells were harvested, stained with propidium iodide, and apoptosis was determined as the % of sub-G1 cells. Drug-induced apoptosis was determined 24 hr after treatment with chemotherapeutic drugs by flow cytometry. p53-dependent apoptosis was assessed using essentially similar experiments. Briefly, cells received 10 μg of either $pCB6^{+}$72R wt p53 or $pCB6^{+}$72P wt p53 together with 72R or 72P p53 mutants at a ratio of 1:1 and 1:2. Apoptotic cells were quantified by flow cytometry as described above.

Cytotoxicity was determined in standard colony-forming assays. Briefly, 10^{2}–10^{6} cells were seeded in quadruplicate onto 9 cm tissue culture dishes. 24 hr later, they were treated for 2 hr with cytotoxic drugs at a range of

E: Downregulation of mutant p53 results in increased drug-induced apoptosis. Hacat cells were pretreated with either p53 siRNA or PGL3 siRNA as indicated, then exposed to doxorubicin (1 μg/ml). Twenty-four hours later, cells were analyzed by flow cytometry. Sub-G1 cells are designated M1.
F: Increased apoptosis in squamous carcinoma cell lines following p53 siRNA treatment. SCC25 and Hacat cells were pretreated with p53 siRNA (black panels) or mock pretreated (open panels). Forty-eight hours later, cells were exposed to doxorubicin (D) or cisplatin (C). After 24 hr, apoptosis was determined by flow cytometry. Data shown are means, ± 1SD, of two experiments.

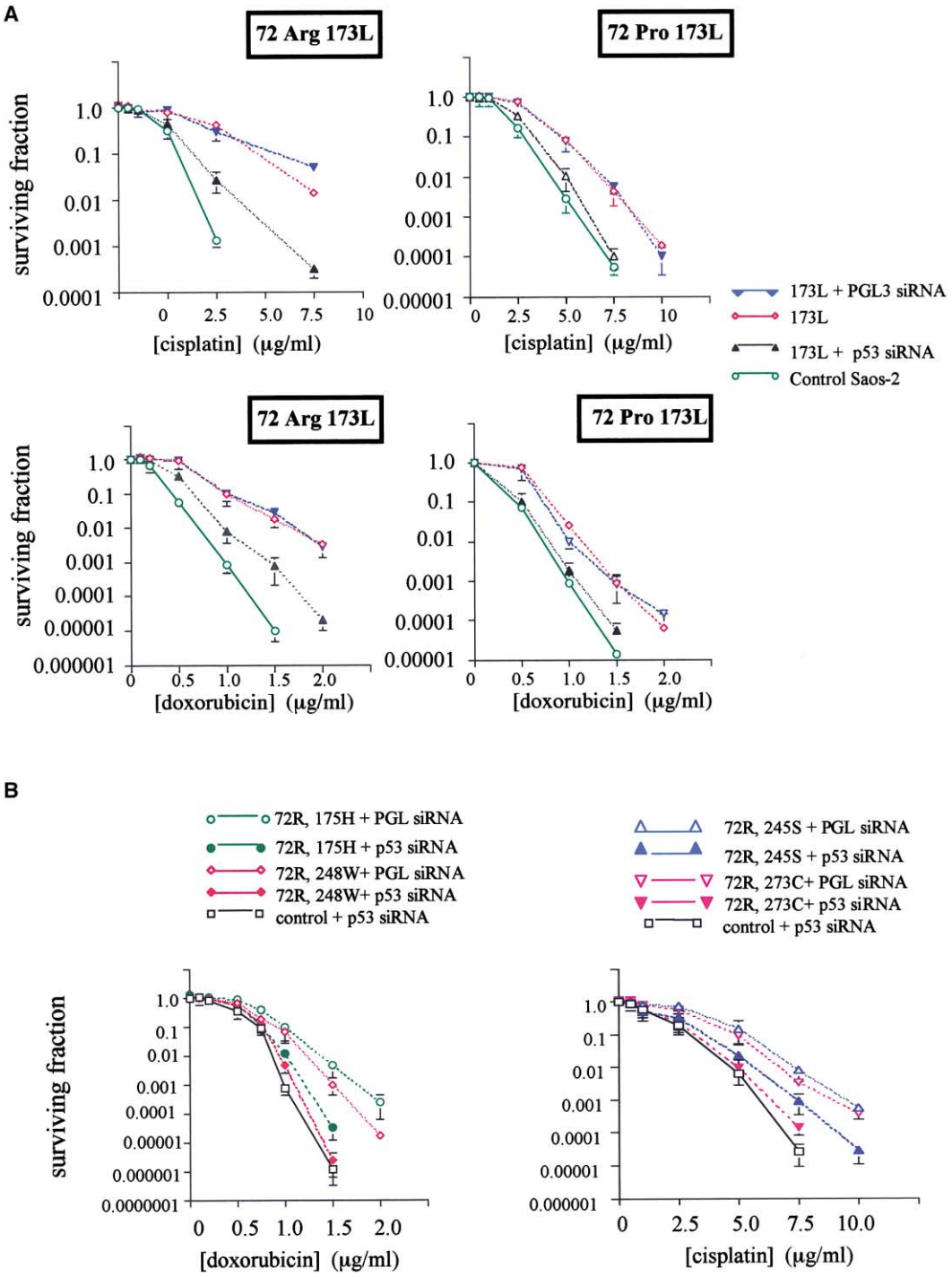

Figure 6. siRNA modulates chemosensitivity in cells engineered to express 72R and 72P variants of hot spot p53 mutants

A: Saos-2 cells expressing 72R 173L p53 were treated with siRNA (100 nM) to p53 or with PGL siRNA as indicated. Forty-eight hours later, cells were exposed to various concentrations of cisplatin or doxorubicin. Survival was determined by colony-forming ability. Data are mean surviving fraction (± 1SD) of three experiments. Control Saos-2 cells do not express p53.

B: Inhibition of expression of hot spot p53 mutants increases sensitivity to doxorubicin and cisplatin. Saos-2 cell lines engineered to express the indicated hot spot p53 mutants were treated with 100 nM siRNA as shown. Forty-eight hours later, they were exposed to varying concentrations of doxorubicin or cisplatin and colony survival was determined as described in Experimental Procedures. Data shown are mean surviving fraction (± 1SD) of two independent experiments.

Table 2. Clinico-pathological characteristics of head and neck cancers

Sex	Age	Primary site	Subsite	T	N	M Stage	72 Germline	p53 gene	Mt allele	Resp.	PFS	OS	A/D	
M	37	Rhinopharynx	—	4	2c		IV	Arg/Arg	173 Val>Leu	Arg	CR	23.3	32.0	D
M	55	Hypopharynx	Pyriform sinus	2	3		IV	Pro/Pro	220 Tyr>Cys	Pro	CR	41.4	41.4	A
M	40	Hypopharynx	Pyriform sinus	4	0	3	IV	Arg/Arg	220 Tyr>Cys	Arg	PR	4.7	7.3	D
M	49	Hypopharynx	—	4	2c		IV	Arg/Arg	Wild-type	None	CR	43.3	43.3	A
M	67	Hypopharynx	—	3	0		III	Arg/Pro	Wild-type	None	CR	36.7	36.7	A
M	50	Hypopharynx	—	4	3		IV	Arg/Pro	Wild-type	None	CR	49.9	49.9	A
M	58	Hypopharynx	—	4	0		IV	Arg/Arg	175 Arg>His	Arg	PR	12.7	29.3	D
									Δ 11bp exon4	Arg				
M	66	Larynx	—	3	2b	2	IV	Arg/Pro	172 Val>Ala	Arg	CR	31.2	44.9	D
M	41	Hypopharynx	Pyriform sinus	2	2c		IV	Pro/Pro	175 Arg>His	Pro	PR	23.2	23.2	A
M	59	Oropharynx	—	4	0		IV	Arg/Pro	248 Arg>Gln	Pro	CR	18.5	58.7	A
M	65	Oral cavity	Oral floor	4	0	2	IV	Arg/Arg	Wild-type	None	CR	20.3	20.3	A
M	45	Oropharynx	Soft-palate	1	2b	3	IV	Arg/Pro	Wild-type	None	TD	1.5	1.5	D
M	59	Larynx	Vocal cord	4	0		IV	Arg/Arg	175 Arg>His	Arg	PR	13.5	13.9	D
M	65	Larynx	Vocal cord	3	1	2	III	Arg/Pro	176 Cys>Tyr	Arg	CR	15.1	17.6	D
M	49	Hypopharynx		1	3		IV	Arg/Arg	Wild-type	None	CR	42.8	42.8	A
M	65	Cervical esophagus		3	2c		IV	Arg/Pro	179 His>Arg	Arg	PR	12.0	19.4	D
F	62	Rhinopharynx		4	2a		IV	Arg/Arg	Wild-type	None	CR	52.7	52.7	A
M	58	Hypopharynx	Pyriform sinus	4	2c		IV	Arg/Pro	276 Ala>Thr	Arg	CR	16.7	16.7	A
M	76	Hypopharynx	Pyriform sinus	2	2c	3	IV	Arg/Arg	Wild-type	None	CR	22.4	22.4	A
M	56	Hypopharynx		2	3		IV	Arg/Arg	245 Gly>Ser	Arg	PR	16.4	26.9	D
M	68	Oropharynx		2	2c		IV	Arg/Arg	161 Ala>Val	Arg	CR	44.7	44.7	A
M	59	Larynx		2	2c		IV	Arg/Arg	Wild-type	None	CR	26.8	26.8	A
M	54	Oropharynx		4	0		IV	Arg/Arg	Wild-type	None	CR	49.0	49.0	A
M	75	Larynx	Epiglottis	2	3	3	IV	Arg/Arg	175 Arg>His	Arg	PD	1.4	1.4	D
									249 Arg>Ser, 250	Arg				
M	41	Hypopharynx	Pyriform sinus	3	2b	3	IV	Arg/Arg	Wild-type	None	CR	43.5	43.5	A
M	36	Oropharynx	Soft-palate	3	2c	1	IV	Arg/Arg	133 Met>Leu	Arg	CR	45.8	45.8	A
M	67	Hypopharynx	Pyriform sinus	3	2b		IV	Arg/Arg	Wild-type	None	CR	29.0	29.0	A
F	76	Oropharynx	Tonsil	4	2c	2	IV	Arg/Pro	Wild-type	None	CR	44.6	44.6	A
M	53	Larynx		4	2	2	IV	Arg/Arg	Wild-type	None	CR	15.4	15.4	A
M	62	Oropharynx	Base of tongue	3	2c	2	IV	Arg/Pro	248 Arg>Trp	Arg	CR	12.5	12.5	D
M	54	Larynx		0	3	3	IV	Arg/Pro	173 Val>Leu	Pro	CR	14.3	14.3	A
M	57	Hypopharynx	Pyriform sinus	4	2a		IV	Arg/Pro	280 Arg>Lys	Arg	PR	10.8	14.5	D
M	47	Hypopharynx	Pyriform sinus	3	1	2	III	Arg/Pro	Wild-type	None	PR	20.6	20.6	A
M	66	Oral cavity	Soft-palate	3	2c		IV	Arg/Pro	242 Cys>Tyr	Arg	CR	16.1	21.1	A
M	59	Oropharynx	Tonsil	4	2b	3	IV	Arg/Pro	206 Leu>Tyr	Arg	CR	17.0	17.0	A
M	67	Larynx		4	0	3	IV	Arg/Arg	Wild-type	None	TD	1.5	1.5	D
M	54	Oropharynx	Tonsil	4	1	3	IV	Arg/Pro	245 Gly>Ser	Arg	PR	10.0	13.8	D
M	47	Oral cavity	Oral floor	4	2b		IV	Arg/Pro	241 Ser>Phe	Pro	CR	42.4	42.4	A
M	68	Larynx	Glottis	3	1	2	III	Arg/Arg	152 Pro>Leu	Arg	CR	27.9	27.9	D
M	56	Oropharynx	Tonsil	4	1	2	IV	Arg/Arg	Wild-type	None	CR	18.1	18.1	A
M	70	Oropharynx	Tonsil	4	2b	1	IV	Arg/Arg	Wild-type	None	CR	53.4	53.4	A
F	55	Oral cavity	Tongue	4	0		IV	Arg/Pro	175 Arg>His	Arg	PR	4.4	7.7	D
M	40	Rhinopharynx		2	3		IV	Arg/Arg	273 Arg>Cys	Arg	CR	24.5	36.5	D
M	51	Rhinopharynx		2	2b		IV	Pro/Pro	Wild-type	None	CR	59.8	59.8	A
F	68	Oropharynx	Tonsil	4	0		IV	Arg/Pro	179 His>Tyr	Pro	CR	36.5	36.5	A
M	45	Oropharynx	Soft-palate	1	2b	3	IV	Arg/Arg	Wild-type	None	TD	1.5	1.5	D
M	66	Oropharynx	Base of tongue	2	2c		IV	Arg/Arg	Wild-type	None	CR	20.4	20.4	D
F	30	Rhinopharynx		4	0	3	IV	Arg/Arg	273 Arg>His	Arg	PR	39.0	39.0	A
M	55	Oropharynx	Base of tongue	4	2c		IV	Arg/Arg	245 Gly>Asp	Arg	PR	30.9	31.9	A
M	67	Oropharynx	Base of tongue	4	2b	3	IV	Arg/Pro	Wild-type	None	CR	15.2	15.2	A
F	72	Hypopharynx	Pyriform sinus	4	0		IV	Arg/Pro	Wild-type	None	PR	15.4	15.4	A
M	70	Oral cavity	Tongue	2	2c	3	IV	Arg/Pro	Wild-type	None	TD	1.9	1.9	D
F	59	Larynx	Vocal cord	4	0		IV	Arg/Arg	142 Pro>Leu	Arg	CR	18.2	18.2	A
M	57	Hypopharynx		4	3		IV	Arg/Arg	179 His>Tyr	Arg	PR	9.8	16.8	D
M	50	Hypopharynx	Pyriform sinus	4	2b		IV	Arg/Pro	181 Arg>His	Arg	CR	31.3	31.3	A
M	46	Larynx	Glottis	4	2c		IV	Arg/Pro	245 Gly>Asp	Pro	CR	37.4	37.4	A
M	59	Hypopharynx	Pyriform sinus	3	1	2	III	Arg/Pro	158 Arg>Gly	Arg	CR	15.3	15.3	D
M	54	Larynx	Vocal cord	3	0	2	III	Arg/Arg	176 Cys>Phe	Arg	PR	15.0	17.3	A
F	61	Larynx	Epiglottis	3	0	2	III	Arg/Pro	266 Gly>Val	Arg	NE	9.9	9.9	D
M	60	Oral cavity	Oral floor	4	2a		IV	Arg/Pro	273 Arg>Cys	Arg	CR	25.1	35.6	D
M	60	Hypopharynx	Pyriform sinus	4	2b		IV	Arg/Arg	Wild-type	None	CR	45.9	45.9	A
M	59	Hypopharynx	Pyriform sinus	4	2b	2	IV	Arg/Arg	F'shift; ter cod 169	Arg	PR	13.2	13.2	A
M	57	Oropharynx	Tonsil	4	2b	2	IV	Arg/Pro	163 Tyr>His	Pro	CR	5.1	5.1	A
M	44	Oropharynx	Base of tongue	4	2a	2	IV	Arg/Arg	Wild-type	None	CR	6.2	6.2	A
M	62	Hypopharynx	Pyriform sinus	4	2b	3	IV	Arg/Arg	175 Arg>His	Arg	PR	7.7	7.7	D
M	56	Oropharynx	Base of tongue	4	3	2	IV	Pro/Pro	Wild-type	None	CR	7.2	7.2	D
M	55	Hypopharynx	Pyriform sinus	3	0	1	III	Arg/Arg	Wild-type	None	CR	51.3	51.3	A
M	54	Rhinopharynx		4	3	3	IV	Arg/Arg	175 Arg>His	Arg	CR	17.3	21.0	D
M	74	Hypopharynx	Pyriform sinus	3	0	2	III	Arg/Pro	Wild-type	None	CR	45.6	45.6	A
F	66	Trachea		4	1		IV	Arg/Pro	Wild-type	None	CR	8.5	8.5	A

CR = complete response; PR = partial response; TD = death due to toxicity; PD = progressive disease; NE = not evaluable.

Table 3. Clinical outcome of patients expressing p53 mutations which efficiently inhibit p73 function

Mutation	Mutant allele	Response[a]	PFS[2]	OS[b]	Alive/dead
173L	Arg	CR	23.3	32.0	Dead
173L	Pro	CR	14.3	14.3	Alive
175H	Arg	PR	13.5	13.9	Dead
175H	Arg	PR	4.4	7.7	Dead
175H	Pro	PR	23.2	23.2	Alive
175H	Arg	PD	1.4	1.4	Dead
175H	Arg	PR	7.7	7.7	Dead
175H	Arg	PR	12.7	29.3	Dead
176Y	Arg	CR	15.1	17.6	Dead
176F	Arg	PR	15.0	17.3	Alive
179R	Arg	PR	12.0	19.4	Dead
179Y	Pro	CR	36.5	36.5	Alive
179Y	Arg	PR	9.8	16.8	Dead
220C	Arg	PR	4.7	7.3	Dead
220C	Pro	CR	41.4	41.4	Alive
242Y	Arg	CR	16.1	21.1	Alive
245D	Pro	CR	37.4	37.4	Alive
245D	Arg	PR	30.9	31.9	Alive
245S	Arg	PR	16.4	26.9	Dead
245S	Arg	PR	10.0	13.8	Dead
248W	Arg	CR	12.5	12.5	Dead
248Q	Pro	CR	18.5	58.7	Alive
273C	Arg	SD	39.0	39.0	Alive
273C	Arg	CR	25.1	35.6	Dead
273C	Arg	CR	24.5	36.5	Dead

[a] CR: complete response; PR: partial response; PD: progressive disease; SD: stable disease.
[b] PFS: progression-free survival in months; OS: overall survival in months.

concentrations. After drug exposure, cells were grown until visible colonies appeared at which point they were fixed in methanol and stained with Giemsa. Survival was calculated relative to cells receiving drug vehicle alone. Each such assay was repeated a minimum of three times.

Analysis of *AIP*1 activation

Expression of *AIP1* mRNA was analyzed using TaqMan quantitative PCR as described (Kaeser and Iggo, 2002). Total RNA was prepared using the Qiagen Rneasy miniprep kit according to the manufacturer's instructions. Activity of the p53*AIP*1 promoter was determined in transient assays by transfection of p53*AIP*1 luciferase. Cells were transfected with 1 μg of *AIP*1 luciferase and 2 μg of pSV2 β-galactosidase using lipofectamine (Qiagen). Twenty-four hours later, cells were exposed to cytotoxic drugs for 2 hr as described above, then grown in drug-free medium for a further 24 hr at which time luciferase and β-galactosidase activity was determined using the Promega system.

siRNA studies

Sequences of oligonucleotides for inhibition of p53 and the PGL3 control siRNA (Elbashir et al., 2001) were kindly provided by Dr. M. Irwin and Professor W. Kaelin. The sequence of the p53 oligonucleotide is: 5′ r(CUACUUC CUGAAAACAACG)d(TT) 3′ 5′ r(CGUUGUUUUCAGGAAGUAG)d(TT)3′.

siRNA oligos were obtained from Xeragon and transfected using the Transmessenger transfection reagent (Qiagen). In preliminary studies, cells were treated with 10–100 μM siRNA according to the manufacturer's instructions in order to determine the optimum concentration to downregulate the target genes. In later studies, cells were transfected with 100 μM oligos for 3 hr, after which the cells were grown for a further 48 hr in normal medium, then treated with anticancer drugs for 2 hr. The medium was replaced with drug-free medium and the cells grown until visible colonies appeared. The effect of siRNA treatment on sensitivity to apoptosis was assessed by flow cytometry 48 hr after drug treatment.

Patients and tumors

The patient population comprised all cases of locally advanced, inoperable TNM stage III/IV HNSCC presenting to the Medical Oncology Department of Cuneo General Hospital. Tissue was obtained as paraffin sections at the time of diagnosis or fresh-frozen tissue from patients with locally advanced, unresectable TNM stage III/IV head and neck cancers at the time of diagnosis. In each case, the diagnosis and presence of an adequate proportion of tumor tissue was confirmed by histopathological review. Patients underwent combined treatment consisting of radiotherapy and one of the following cisplatin-based chemotherapy regimens: (1) cisplatin/5-fluorouracil; (2) cisplatin/5-fluorouracil/paclitaxel; (3) cisplatin/gemcitabine. For some patients, normal tissue was obtained from blood or uninvolved tissue (confirmed by histopathological analysis). Genomic DNA was isolated from blood using the Qiagen kit and from frozen normal tissue with proteinase K digestion. In cases where blood or uninvolved tissue was not available, normal tissue was obtained by microdissection using sections stained with haematoxylin and eosin to identify areas of normal tissue.

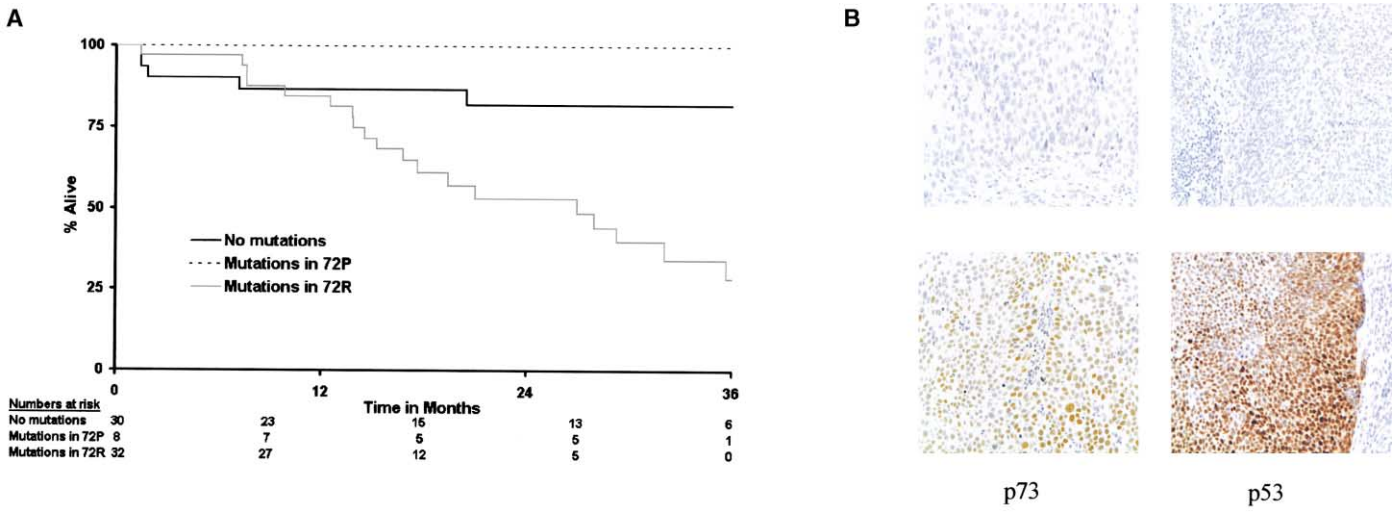

Figure 7. Clinical outcome in head and neck cancer is influenced by p53 polymorphism

A: p53 mutations predict worse survival. If mutations exist, those occurring in the 72R form predict worse survival. Survival curves for all 70 patients, split by presence or absence of mutations (log rank p = 0.0058), and whether the mutation occurred in the 72R or 72P form (log rank p = 0.0044).

B: Immunocytochemical analysis of p53 and p73 expression in head and neck cancers The figure shows examples of positive (lower panels) and negative (upper panels) cases for each protein.

Analysis of p53

Germline codon 72 genotypes were determined using genomic DNA isolated from patient-matched normal tissue. Amplification was performed with the following primers: Forward primer: 5′CAATGGTTCACTGAAGACC 3′; Reverse primer: 5′ CTGTCCCAGAATGCAAGA 3′. Following PCR, amplified products were extracted using the Qiagen QIAquick PCR purification kit and then bidirectionally sequenced using the ABI PRISM Big Dye Cycle Sequencing Kit and appropriate primers. To identify p53 mutations, each coding exon of the gene was amplified using exon-specific primers and high-fidelity *Pfx* polymerase (Gibco, BRL). Amplification products were gel-purified and ligated into pCRblunt (Invitrogen). Individual plasmid clones of each exon were then sequenced. A minimum of 12 clones for each exon were sequenced. When mutations occurred in 72 R/P germline heterozygotes, we further identified the polymorphic allele in which the mutation occurred. Tumor tissue was isolated by microdissection as described above and the presence of the mutation confirmed by sequencing. A fragment containing codon 72 was then amplified and sequenced using the primers and conditions given above.

Immunocytochemistry

Expression of p53 and p73 was analyzed by immuncytochemistry using the following antibodies: p73: ER-15 (Neomarkers used at 1:150 dilution); p53: DO-1 (Calbiochem used at 1:500). Sections were subject to antigen retrieval prior to application of antibodies. Stained sections were scored for nuclear expression by a head and neck pathologist (BAG) who was unaware of the clinical status of each patient.

Statistical analysis

Chi-squared tests were used to test the frequencies of the codon 72 genotypes between cases and controls. Fisher's exact tests were used to analyze any differences in complete and partial response rates between the presence or absence of mutations and mutant allele types. A test for proportions was used to analyze the frequency of mutations in R and P and a χ^2 test for trend used to analyze the proportions of complete and partial responses across different grouping levels of p53 and p73. Survival times were calculated as the date of diagnosis to date of death, or date of censor if alive. Progression-free survival times were calculated as the date of diagnosis to date of progression, or date of censor if not progressed. Survival curves were constructed using the method of Kaplan and Meier (Kaplan and Meier, 1958), and the log rank test (Peto et al., 1977) was used to assess the independent prognostic ability of both the presence of mutations and the allelic variant of mutation. Cox Proportional Hazards analysis (Cox, 1972) was then undertaken to test the prognostic ability of the presence of mutations in a multivariate setting alongside the prognostic factors of sex, age, performance status, stage, site of primary tumor, and nodal status. Statistical analysis was carried out independently using SAS statistical software (SAS Institute, Cary, North Carolina).

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