p53 Mutations Do Not Predict Response to Paclitaxel/ Radiation for Nonsmall Cell Lung Carcinoma

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BACKGROUND. Mutations in the tumor suppressor gene p53 have been associated with resistance to ionizing radiation and chemotherapy. Paclitaxel and concurrent radiation (paclitaxel/RT) achieve high response rates with locally advanced nonsmall cell lung carcinoma (NSCLC). In vitro data and animal studies suggest that paclitaxel may have a unique ability to activate tumor cell apoptosis in the absence of wild-type p53 function. The authors sought to determine whether p53 mutations affect response to paclitaxel/RT in patients with locally advanced NSCLC.

METHODS. Thirty patients with Stage IIIA or IIIB NSCLC who participated in Brown University Oncology Group protocols utilizing paclitaxel/RT had tumor tissue that was adequate for analysis. Mutations were detected in tumor tissue by single-strand conformation polymorphism analysis of exons 5 through 8 of the p53 gene, and confirmed by direct sequencing.

RESULTS. Mutations in p53 were found in 12 of 30 patients (40%). The response rates (complete plus partial) of 75% for patients with tumors with p53 mutations, and 83% for patients with wild-type p53, did not differ significantly ($P = 0.70$).

CONCLUSIONS. p53 mutations do not predict response of patients with NSCLC to paclitaxel/RT. This finding is in striking contrast to results with other chemotherapeutic agents and ionizing radiation. These clinical data support in vitro data and animal studies regarding the unique mechanism of the action of paclitaxel. Further investigation is needed to determine the mechanism of lung tumor cell death after paclitaxel/RT. These results suggest that paclitaxel/RT may be an active regimen for patients with other locally advanced neoplasms with high rates of p53 gene mutations. ***Cancer* 1996; 78:1203–10.** © *1996 American Cancer Society.*

KEYWORDS: p53, paclitaxel, radiotherapy, combined modality therapy, lung neoplasms.

Treatment of locally advanced nonsmall cell lung cancer (NSCLC) with platinum-based chemotherapy and concurrent radiotherapy (RT) improves survival rates compared with RT alone, but the benefits are modest and more active therapies are needed.[1–5] Paclitaxel is a chemotherapeutic agent extracted from the bark of the Pacific yew (Taxus brevifolia), which has substantial activity against a range of tumor types, including ovarian, breast, and lung carcinomas.[6–12] In vitro studies and preliminary clinical trials suggest that paclitaxel is a useful radiosensitizing agent.[13–15]

The Brown University Oncology Group has accumulated extensive experience using paclitaxel/RT for locally advanced NSCLC. A Phase I study showed that the maximum tolerated dose of paclitaxel was 60 mg/m^2/week with 60-Gray (Gy) radiation.[16] This therapy was well tolerated and esophagitis was the dose-limiting toxicity.[17] The overall response rate of 84% in a subsequent Phase II Brown University study is comparable to that of the most active chemoradi-

ation combinations currently available, yet the toxicity is more tolerable.[18]

Paclitaxel interferes with mitotic spindle function by enhancing the rate and yield of microtubule assembly and preventing microtubule depolymerization.[19] This action can arrest a high percentage of cells at the G_2/M interface, the point in the cell cycle at which cells are most vulnerable to radiation damage.[20] In vitro studies have shown that although G_2/M arrest is necessary for paclitaxel-mediated radiosensitization, other cellular factors must be involved because some cell types develop G_2/M arrest without radiosensitization.[21]

Many genes have been recently identified whose products regulate the cell cycle.[22,23] Mutations in these genes are frequent in human carcinoma, and some of these alterations are associated with decreased response to chemotherapy and radiation.[24] The p53 gene product, which blocks cell entry into S-phase in response to DNA damage, is of particular interest in this regard.[25] Mutations in p53 are the most common known genetic alterations in human tumors, and they are found in approximately one-half of NSCLC.[26]

Apoptosis, or programmed cell death, is the major mechanism by which ionizing radiation and most chemotherapeutic agents cause tumor cell death.[27] Wild-type p53 function is required in vitro to induce apoptosis by a wide variety of chemotherapeutic agents and radiation.[28,29] Mutations in the p53 gene typically result in an abnormal protein that usually has a longer life than the wild-type protein.[29] Mutant p53 protein may also inactivate wild-type protein.[30-32] These features make p53 a potentially critical determinant of therapeutic response. Mutations in p53 are associated with resistance to chemotherapy in patients with NSCLC and other malignancies.[33-38]

Recent evidence suggests that paclitaxel may have a unique ability to activate apoptosis in the absence of wild-type p53 function.[39,40] Given the high response rate of NSCLC to paclitaxel/RT and the frequency of p53 gene mutations in this disease, it seemed likely that a significant fraction of tumors with p53 mutations responded to this regimen. We sought to test this hypothesis by determining whether p53 gene mutations predicted response to paclitaxel/RT in patients treated in our studies for locally advanced NSCLC.

METHODS

Eligibility

All patients who participated in Brown University Oncology Group studies of paclitaxel/RT for NSCLC and who had adequate tissue available for molecular studies were included in this study. Subjects included 19 patients who received weekly paclitaxel, 60 mg/m^2, for 6 weeks by 3-hour intravenous infusion with concurrent 60-Gy radiation in a Phase II study; 5 patients who received lower weekly paclitaxel dosages (30–50 mg/m^2 for 6 weeks) with 60-Gy radiation as part of a Phase I study; and 2 patients who received neoadjuvant weekly paclitaxel, 60 mg/m^2, for 5 weeks with 45-Gy radiation. Also included are 4 patients who received weekly carboplatin (area under the curve = 2), with paclitaxel 50 mg/m^2 intravenously over 1 hour, for 7 weeks with 66-Gy radiation. Eligibility criteria and details of paclitaxel administration, RT, and dose modifications have been described previously.[15-17]

Analysis of p53 Gene Mutations

Samples were selected based on pathologic review of tissue sections to assure the presence of tumor tissue and minimize the amount of normal tissue present. Ten to 20 10-μm sections were collected from paraffin blocks using a standard microtome. This tissue was deparaffinized using multiple rinses with xylene followed by absolute ethanol in a microcentrifuge tube with alternate vortexing and brief centrifugations. After the final centrifugation, all ethanol was removed and the tissue pellet briefly dried in vacuo. Proteinase K was then added in buffer containing sodium dodecyl sulfate, Tris buffer, and ethylenediamine tetraacetic acid. Samples were vortexed and then incubated at 50 °C with gentle agitation for 1–2 days. Samples were extracted with 50% phenol/50% chloroform followed by chloroform extraction and ethanol precipitation. The resulting DNA pellet was dried in vacuo and resuspended in water. The concentration of the resulting DNA was measured spectrophotometrically and adjusted to 20–100 μg/mL with water and stored at −20 °C.

Polymerase chain reaction (PCR) reactions included paired probes specific for p53 exons 5, 6, 7, or 8. The following primers were employed: p53 exon 5,5′-TCCTCTTCCTGCAGTACTC-3′ and 5′-AGCTGCTCACCATCGCTA-3′; p53 exon 6,5′-TGATTGCTCTTAGGTCTGGC-3′ and 5′-AAACCAGACCTCAGGCGGCT-3′; p53 exon 7,5′-TCCTAGGTTGGCTCTGACT-3′ and 5′-CAAGTGGCTCCTGACCTG-3′; and p53 exon 8,5′-TGGTAATCTACTGGGACGG-3′ and 5′-TCCTGCTTGCTTACCTCG-3′. Fifty-μL PCR reactions included 200–1000 ng of purified sample DNA, paired p53 primers, 60 μM dATP, dGTP, and dTTP; 10 μM dCTP; 2–10 μCi α^{32}P dCTP; PCR buffer II (Perkin Elmer, Norwalk CT), 1.5 mM $MgCl_2$; and 1.25 units of AmpliTaq™ polymerase (Perkin Elmer). PCR was performed using a "hot start" technique with a Perkin Elmer Thermal Cycler 480 with denaturation at 94 °C for 3 minutes, then 35 cycles of 94 °C for 1 minute, 59 °C for 1 minute, and 72 °C for 1 minute, followed by 72 °C for 10 minutes.

Control reactions utilized human placental DNA as well as samples with known p53 mutations. Ten μL of PCR product was added to 45 μL of deionized formamide containing 10mM NaOH and 0.01% xylene cyanol and 0.01% bromophenol blue (BioRad, Melville, NY). Single-strand conformation polymorphism (SSCP) analysis was performed by heat denaturing PCR products at 94 °C for 2 minutes and then quick chilling them on ice. Approximately 5–20 μL of samples were electrophoresed in a 0.4-mm gel comprised of specialized electrophoresis media (MDEE, AT Biochem, Malvern, PA) with 0.7 × TBE at 4–12 watts using a BioRad sequencing gel electrophoresis system (BioRad, Melville, NY) at 25 °C for 8–30 hours. Autoradiography of dried gels was performed at −70 °C for 4–150 hours with XAR or XRP-1 X-ray film (Kodak, Rochester, NY) with or without intensifying screens. Sample lanes were compared with PCR products prepared from wild-type and mutant p53 loci for mobility shifts. All mutations were confirmed in an independent PCR/SSCP assay.

For direct sequencing, the PCR/SSCP product of interest was excised from the gel, purified by Qiagen spin column (Qiagen, Chatsworth, CA), and reamplified by PCR. The resulting DNA was subjected to chain termination sequencing using a ^{32}P end-labeled p53 specific primer and a Sequenase sequencing kit (USB, Cleveland, OH), and analyzed on a 6–8% polyacrylamide sequencing gel containing 7 M urea.

STATISTICAL ANALYSIS

Patient characteristics including age, sex, disease stage, and response were compared between groups with and without a p53 mutation by Fisher's exact test (two-sided). Ninety-five percent confidence intervals for the response rates were determined based on the binomial distribution. The effect of p53 mutation on response rate as well as the statistical power of the analysis were assessed using Z test for proportions.

RESULTS

From May 1993 to May 1995, 64 patients with (American Joint Committee on Cancer) Stage III NSCLC were treated in Brown University studies with regimens that utilized paclitaxel/RT. Thirty of these patients had tumor tissue that was adequate for analysis by PCR/SSCP. Twelve of these 30 patients (40%) had mutations in p53 exons 5, 6, 7, or 8, as determined by PCR/SSCP (Fig 1). There were five mutations observed in exon 5, one in exon 6, two in exon 7, and four in exon 8. Bands corresponding to wild-type p53 were present in all cases. Each mutation was confirmed in an independent SSCP assay. The specific mutation was identified in 10 of the 12 samples by direct DNA sequencing of

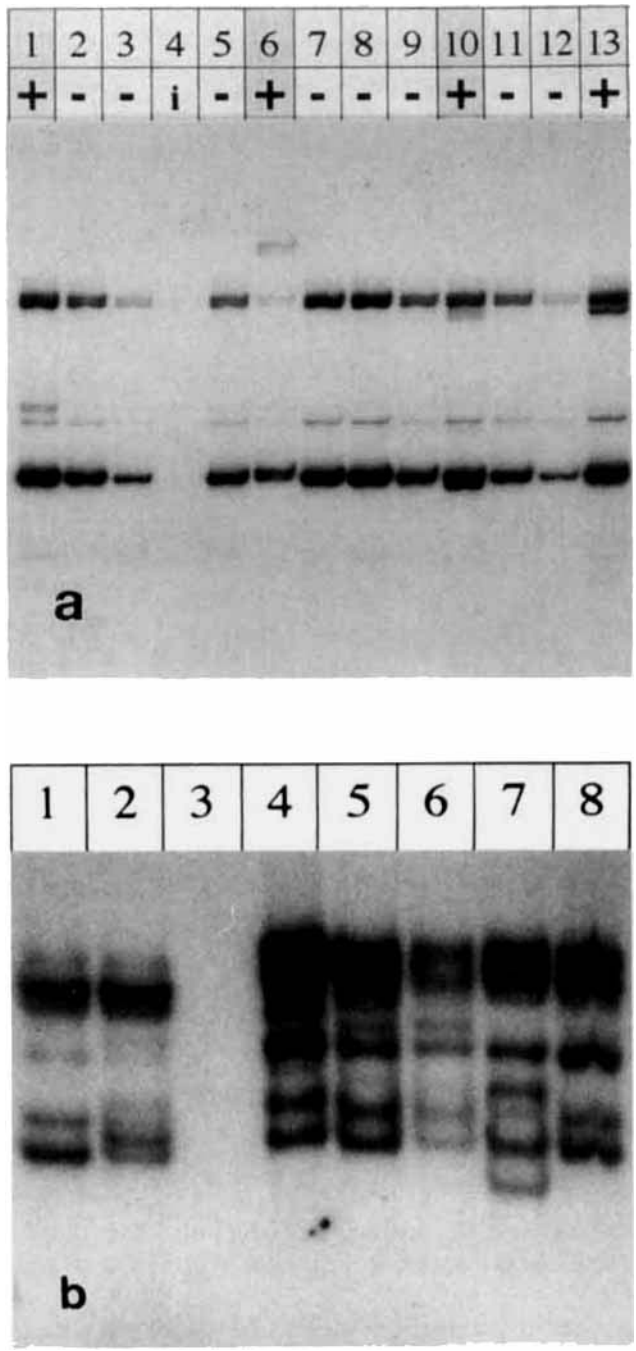

FIGURE 1. Polymerase chain reaction (PCR)/single-strand conformation polymorphism (SSCP) analysis of nonsmall cell lung cancer (NSCLC) tumor biopsies for p53 exons 5 and 8. In each panel, note the altered mobility band(s) in tumor samples with mutations relative to germline controls. Panel a shows an autoradiograph for p53 exon 5. Lane 1: positive control; lane 2: germline control, lanes 3, 5, 7, 8, 9, 11, and 12: NSCLC samples with germline exon 5 sequences; lane 4: insufficient DNA sample; lanes 6, 10, and 13: NSCLC samples with exon 5 mutations. Panel b shows an autoradiograph for p53 exon 8. Lanes 1 and 8: germline control; lane 2: positive control; lane 3: no added DNA control, lanes 4–7: NSCLC samples with exon 8 mutations.

TABLE 1
p53 Mutations by DNA Sequencing

Exon	Codon	Nucleotide	Amino acid
5	148	GAT → TAT	Asp → Tyr
5	157	GTC → CTC	Val → Leu
5	158	CGC → CTC	Arg → Leu
5	163	TAC → AAC	Tyr → Asn
5	168	CAC → TAC	His → Tyr
7	252	CTC → CCC	Leu → Pro
8	273	CGT → TGT	Arg → Cys
8	273	CGT → CAT	Arg → His
8	273	CGT → CTT	Arg → Leu
8	277	TGT → TTT	Cys → Phe

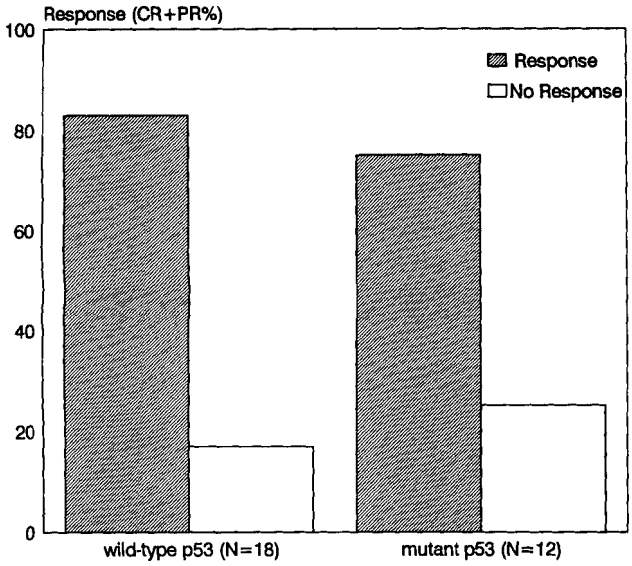

FIGURE 2. Response rates according to the presence or absence of p53 mutations.

the PCR product. In each case, the nucleotide change predicted an alteration in amino acid sequence (Table 1).

We observed nearly identical response rates (complete plus partial responses) to paclitaxel/RT in patients with and without p53 mutations (Fig. 2). A 75% response rate (9 of 12; 95% confidence interval [CI], 43–95%) was observed for tumors with p53 mutations as compared with an 83% response rate for tumors without evidence of p53 mutations (15 of 18; 95% CI, 59–96%); $P = 0.7$. The clinical characteristics of the patients with and without p53 gene mutations are shown in Table 2. p53 status was not associated with significant differences in patient sex or clinical stage (IIIA or IIIB). There was a trend toward p53 mutations occurring in younger patients that did not reach statistical significance ($P = 0.08$). p53 mutations appeared to be less common in adenocarcinomas (1 of 8) compared with other histologic tumor types (7 of 18), but this difference also did not reach statistical significance ($P = 0.10$). Response rate to paclitaxel/RT was not predicted by tumor histology or stage (IIIA or IIIB).

TABLE 2
Patient Characteristics According to the Presence of p53 Mutations

Characteristic	p53 mutation	Wild-type p53	*P* value
No. of patients	12	18	
Mean age (yrs)	59	67	0.08
Sex			
Male	6	11	0.70
Female	6	7	0.70
Disease stage			
IIIA	5	7	1
IIIB	7	11	1
Histology			
Adenocarcinoma	1	7	0.10
Squamous	4	6	1
Large	4	3	0.40
Poorly differentiated	3	2	0.40
Treatment			
Paclitaxel/RT	9	15	0.70
Paclitaxel/carboplatin/RT	3	3	0.70

RT: radiotherapy.

In patients who responded, treatment results were frequently dramatic. Figure 3a shows a pretreatment computed tomography (CT) scan from a patient with a Stage IIIB squamous cell lung carcinoma with extension into the mediastinum. The tumor had a p53 exon 5 mutation but there was marked tumor regression (partial response) to paclitaxel/RT (Fig. 3b).

DISCUSSION

Paclitaxel with concurrent RT is a promising treatment modality for locally advanced NSCLC. Phase I and Phase II studies by the Brown University Oncology Group have documented high response rates and a favorable toxicity profile for this regimen.[16–18] Our results indicate that response to this combined modality therapy is not affected by mutations in the p53 tumor suppressor gene.

The lack of dependence of paclitaxel/RT treatment outcome on p53 status is in contrast to results reported with other chemotherapeutic agents. In vitro data in multiple histologic cell types indicate that wild-type p53 function is required for the efficient activation of apoptosis in response to most chemotherapeutic agents and ionizing radiation.[41] Lowe et al. showed that ionizing radiation, 5-fluorouracil, etoposide, and doxorubicin induced apoptosis in E1A *ras*-trans-

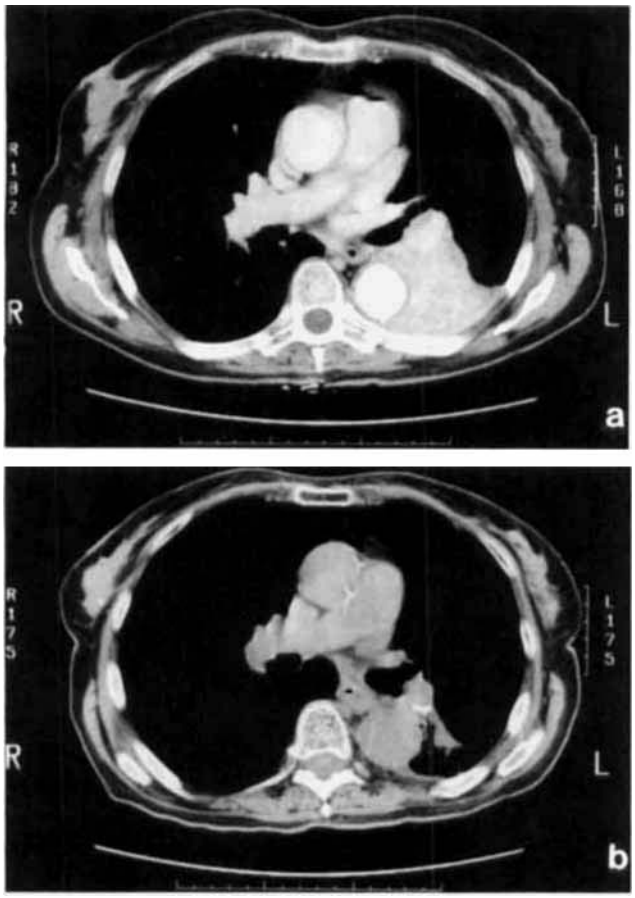

FIGURE 3. (a) Response of a patient with a p53 exon 5 mutation to paclitaxel/radiotherapy. Pretreatment computed tomography (CT) scan showing a Stage IIIB squamous cell lung carcinoma invading the mediastinum. (b) Posttreatment CT scan. There is substantial reduction in the lung mass after paclitaxel/RT.

formed fibroblasts with wild-type p53, but had little effect on cells that lacked p53.[28] Wild-type p53 was also required for chemotherapy-mediated killing of fibrosarcoma cells transplanted into athymic nude mice.[29] Although tumors with wild-type p53 regressed after treatment with gamma radiation or doxorubicin and contained a high proportion of apoptotic cells, similarly treated p53 deficient tumors continued to enlarge and contained few apoptotic cells.

Recent clinical trials have also documented the importance of wild-type p53 for response to chemotherapeutic agents (Table 3). Dohner et al. reported that p53 gene alterations predict failure to respond to purine analogs in chronic B-cell leukemias.[35] Wattel et al. reported that p53 mutations are associated with resistance to chemotherapy in acute myeloid leukemia, myelodysplastic syndromes, and chronic lymphocytic leukemia.[34] Less information regarding the influence of p53 mutations on solid tumors is available. Lenz et al. showed that p53 mutations predicted resistance to 5-fluorouracil and leucovorin in patients with metastatic colon carcinoma.[36] Kawasaki et al. found that patients with Stage IIIB or IV NSCLC containing p53 mutations were significantly more likely to be resistant to cisplatin-based chemotherapy.[33] The only notable example of p53 mutations failing to predict response the chemotherapy is the evaluation by Preudhomme et al. in Burkitt's lymphoma and L_3 acute lymphatic leukemia (ALL).[43]

Our findings could be explained if tumor cell killing was achieved by p53-independent pathways. Both p53-dependent and p53-independent apoptotic pathways have been described in other systems.[43] Clarke et al. showed that although apoptosis in mouse thymocytes resulting from DNA damaging agents (radiation or etoposide) was dependent on functional p53, apoptosis mediated by glucocorticoids was not blocked by homozygous p53 gene deletions.[44] These findings may explain the lack of dependence on p53 status in Burkitt's lymphoma and (French American British classification) L_3 ALL for all such treatment protocols included corticosteroids. This suggests that alternate cellular pathways to apoptosis in NSCLC might be exploited by paclitaxel/RT.

Our results with NSCLC provide clinical support for in vitro evidence suggesting that paclitaxel can function in the absence of wild-type p53. Fisher et al. examined the induction of apoptosis by chemotherapy and ionizing radiation in E1A *ras*-transformed fibroblasts derived from mice with homozygous deletions of the p53 gene and in their wild-type counterparts.[39] Virtually all treatments tested, including radiation, DNA intercalating agents, antimetabolites, antibiotics, topoisomerase inhibitors, and alkylating agents, efficiently induced apoptosis in cells derived from wild-type p53 animals, but failed to do so in cells derived from p53 deficient animals. In striking contrast, paclitaxel induced apoptosis and killed tumor cells independent of p53 status. These investigators, as well as Blagosklonny et al.,[45] further showed that the cyclin-dependent kinase inhibitor p21 (WAF1), whose transcription is regulated by p53, could be induced by paclitaxel treatment in p53 deficient tumor cells. These findings suggest that paclitaxel-mediated blockade at G_2/M can activate cell cycle control pathways to induce apoptosis independent of p53. Further clinical studies are needed to determine if the p53 independent cell death observed in NSCLC was due to paclitaxel alone, RT alone, or the combination of paclitaxel/RT.

In solid tumors the wild-type p53 allele is generally lost in patients with mutations of one p53 allele.[32,46,47] Due to the limited amount of tissue available after bronchoscopy or CT-guided needle biopsy and the

TABLE 3
Response Rates According to the Presence of p53 Mutations

Tumor type	No. of patients	Chemotherapy	Response rate %		P values
			Mutant p53	Wild-type p53	
Chronic B-cell leukemias[35]	48	Purine analogs	0[a]	56[a]	< 0.001
Chronic lymphocytic leukemia[34]	46	Chlorambucil, fludarabine, CHOP	12.5[a]	80[a]	0.020
Acute myelogenous leukemia[34]	90	Multiagent	33[b]	81[b]	0.005
Myelodysplastic syndromes[34]	51	Multiagent intensive or low dose AraC	8[a]	60[a]	0.004
Burkitt's lymphoma and L_3 acute lymphoblastic leukemia[42]	48	Multiagent	78[b]	76[b]	NS
Colon carcinoma[36]	37	5-FU/LV	18[a]	40[a]	0.038
Nonsmall cell lung cancer (NSCLC)[33]	95	Platinum based	16[a]	65[a]	0.005
NSCLC (current study)	30	paclitaxel/RT	75[a]	83[a]	NS

AraC: cytosine arabinoside; CHOP: cyclophosphamide, doxorubicin, vincristine, and prednisone; NS: not significant; 5-FU/LV: 5-fluorouracil and leucovorin; RT: radiation.
[a] Complete plus partial response.
[b] Complete response.

lack of normal tissue from the same individual, we were unable to formally assess loss of heterozygosity (LOH). Wild-type p53 bands were present in all of the samples with p53 mutations. Because LOH typically occurs in NSCLC with p53 mutations,[47] the wild-type bands may represent infiltrating leukocytes or other normal tissue that was present within all biopsy samples. Because p53 functions as a dimer, mutant p53 protein may sequester and inactivate wild-type protein in nonfunctional heterodimers,[30,31,32] thus, the function of even a retained wild-type allele may be lost due to this dominant negative effect. However, it is possible that resistance to chemotherapy occurs only when both wild-type p53 genes are lost, and by retaining one wild-type allele, these tumors were still able to undergo apoptosis. In-vitro tumors with a single wild-type p53 allele can still undergo apoptosis, but this ability is diminished.[28] Thus, tumors that retain a single wild-type p53 gene may still be capable of undergoing apoptosis.

Because the majority (> 90%) of p53 mutations occur in exons 5–8, we examined this region by SSCP. The frequency of p53 mutations identified in this study is similar to that reported by other groups for NSCLC (40–50%).[48–52] All mutations were confirmed in independent PCR/SSCP assays. The specific mutation was identified in ten of these samples and in each case predicted a missense mutation of p53.

The response rate to paclitaxel/RT in this subset of patients with evaluable tissue was comparable to those of the entire group of patients treated in Brown University protocols, including patients not evaluable for p53 mutations. The calculated statistical power of this study to detect a 50% decrease in response rate in tumors with p53 mutations was 90%. Although our relatively small sample size precludes detection of minor differences in response to paclitaxel/RT according to p53 status, it is unlikely that statistical limitation prevented us from detecting the substantial dependence observed in other studies (Table 3). The screening technique of PCR/SSCP detects greater than 90% of p53 mutations. We statistically investigated whether our conclusions were sensitive to failure to detect all p53 mutations. Even if two responding tumors that were originally classified as having wild-type p53 actually had p53 mutations, the Fisher's exact test comparing the response rates yielded the same P value ($P = 0.7$).

Only a few of the genes involved in mediating apoptosis in mammalian cells have been identified and characterized. Multiple genes have been shown to be involved in cell cycle control in yeast, but mammalian homologues have not been identified for most of these genes. Some of these gene products may be responsible for the initiation of apoptosis in tumors with p53 gene mutations in response to paclitaxel/RT. The identification of these genes in mammalian cells may permit a more detailed analysis of the mechanism of action of this combination therapy.

In conclusion, p53 gene mutations do not predict response to paclitaxel/RT in NSCLC. This finding is in marked contrast to the dependence of other chemotherapeutic agents and radiotherapy on wild-type p53 and suggests a unique mechanism of action for this

therapy. Our results provide clinical support for in vitro observations that paclitaxel can bypass mutant p53 and lead to tumor cell death by alternate pathway(s). These findings further suggest that paclitaxel/RT may be an active regimen for other neoplasms with frequent p53 gene mutations. To this end, Brown University is currently evaluating paclitaxel/RT for patients with locally advanced pancreatic and gastric tumors.[53]

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