Pretreatment p53 Protein Expression Correlates with Decreased Survival in Patients with End-Stage Head and Neck Cancer[1]

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ABSTRACT

Relatively little is known about p53 changes in far-advanced head and neck cancer for several reasons: (*a*) most patients respond well to initial treatment; (*b*) most institutions do not encounter large numbers of these patients; (*c*) recurrent or metastatic disease is often within body cavities inaccessible for analysis; and (*d*) the variety of treatment regimens and disease sites makes meaningful conclusions difficult to draw in such a heterogeneous group. The purpose of this study was to evaluate the clinical significance of p53 mutations and overexpression in a homogeneous group of patients with end-stage squamous cell carcinoma of the head and neck.

Pretreatment tumor specimens from a homogeneous group of 16 patients with end-stage squamous cell carcinoma of the head and neck were obtained. All patients had recurred after surgery and radiation ± induction chemotherapy, and all met the criteria for enrollment in a Phase II chemotherapy trial consisting of 5-fluorouracil, *N*-phosphoacetyl-L-aspartate, and recombinant IFN-α. Each was analyzed for mutations in exons 5–8 of the *p53* gene and protein expression using the p53 polyclonal antibody CM-1.

No relationship was found between p53 immunostain or p53 mutations and age, gender, site of primary tumor, or site of disease recurrence. p53 alterations also did not correlate with response to Phase II chemotherapy. p53 immunostain (but not p53 mutations) correlated with a shorter survival ($P = 0.0124$) after diagnosis with end-stage disease. This suggests that mechanisms other than p53 mutations which alter the half-life of p53 protein may contribute to the outcome of these patients.

INTRODUCTION

While it is clear that p53 alterations occur early (1, 2) in the development of SCCHN,[3] there is controversy in the literature regarding the prognostic role of p53 in these tumors. Most studies have concluded that there is no correlation between p53 expression and disease progression (3) or survival (4) in patients with SCCHN, although at least one study did find a correlation between p53 mutations and disease progression (2). Exceptions to the general lack of correlation with survival are two studies which evaluated patients with advanced tongue base tumors (5) and patients with end-stage disease (6), with conflicting results.

Over half of adult cancers, including lung, breast, colon, esophagus, and skin cancers contain p53 mutations (7), and increased p53 expression is one of the most common genetic features in a variety of human malignancies. The *p53* gene product functions as a transcription factor (4), and has a central role in cell cycle regulation (8) and apoptosis (9).

p53 alterations may influence treatment response since wt p53 is required for G1 arrest following ionizing radiation (10), wt p53 protein has been shown to repress the activity of the human *MDR1* gene promoter *in vitro*, and mutant p53 protein has a stimulatory effect on MDR1 activity (11). Overexpression of the *MDR1* gene product, a M_r 170,000 transmembrane glycoprotein (P-glycoprotein), has been associated with drug resistance in a variety of malignancies (12). The purpose of this study was to evaluate the influence of p53 mutations and p53 protein expression on response to treatment and on survival in patients with incurable SCCHN who had already received standard therapy and were enrolled in a Phase II trial using combination chemotherapy and biological modifiers.

PATIENTS AND METHODS

Patient Characteristics. Formalin-fixed, paraffin-embedded tumor tissue was analyzed from 16 patients (9 males and 7 females) with distant metastases or uncontrolled local or regional tumor. These patients were enrolled between 1991 and 1994 in a Phase II trial of combination chemotherapy consisting of 5-FU (2600 mg/m^2/week by 24-h continuous infusion), PALA (250 mg/m^2/week by rapid i.v. infusion), and rIFN-α (10 million units × 3/week by s.c. injection) administered weekly until evidence of disease progression or intolerable toxicity ensued.

Information was available regarding gender, tumor stage, site of initial disease, and site of first recurrence (Table 1). All patients underwent surgery and radiation prior to enrollment in the trial. Pretreatment tissue from the primary disease site was available for analysis in all patients. Comparison tumor from a local or regional recurrence was available in five patients before and in five patients (four different, one overlap) after treatment with 5-FU/PALA/rIFN-α chemotherapy. To be considered

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[3] The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; wt, wild type; MDR1, multidrug resistance 1; P-gp, P-glycoprotein; 5-FU, 5-fluorouracil; PALA, *N*-phosphoacetyl-L-aspartate; SSCP, single-strand conformation polymorphism; HSP, heat shock protein.

evaluable for response to and toxicity of 5-FU/PALA/rIFN-α chemotherapy, a patient had to receive at least 6 weeks of therapy and satisfy the response criteria as outlined: complete remission, disappearance of all clinical evidence of tumor by scan and physical examination for a minimum of 4 weeks; partial remission, 50% or greater decrease in tumor size lasting a minimum of 6 weeks, with no new tumors appearing; stable disease, <50% decrease or <25% increase in tumor size for at least 8 weeks, with no new tumors; and progressive disease, increase in tumor size of at least 25%.

DNA Extraction from Fixed Tissue. DNA was extracted from formalin-fixed tissue blocks as previously described (13), with slight modifications. After confirmation of tumor location through hematoxylin and eosin tissue staining, tumor tissue was microdissected from surrounding stroma with a scalpel and placed in xylene followed by rehydration in graded alcohols to water. The tissue was then boiled for 10 min, and a suspension of Chelex 100 ion exchange resin was added (Bio-Rad Laboratories, Melville, NY) followed by proteinase K (0.4 μg/μl), boiling for 8 min, and centrifugation.

PCR-SSCP Analysis. Nested primers (14) were used to perform two sets of PCR reactions for exons 5 to 8 of the *p53* gene to obtain more efficient DNA amplification. In the first set, 4 μl extracted DNA, 2.8 μl deoxynucleotide triphosphates (1.25 mM of each deoxynucleotide triphosphate), 1 μl primer mix (each primer 20 μM), 0.25 μl (5 units/μl) AmpliTaq DNA polymerase (Perkin Elmer/Cetus, Norwalk, CT), 1 μl 10× PCR buffer, and 10.9 μl water were used to make a a 20-μl first reaction mixture. Samples were overlaid with 20 μl mineral oil. Eight PCR cycles of melting at 94°C for 1.6 min, annealing at 37°C for 1.6 min, and extension at 72°C for 1.6 min, followed by 20 cycles of 94°C for 1.3 min, 54°C for 1.3 min, and 72°C for 1.3 min. For the second set of cycles, 2 μl template DNA from the first set of cycles, 2.5 μl primer mix (each primer 4 μM), 0.20 μl AmpliTaq DNA polymerase, 0.25 μl (10 μCi/μl) [α-^{32}P]dCTP (DuPont New England Nuclear Products, Wilmington, DE), 1 μl 10× PCR buffer, and 2.55 μl water were used to make a 10-liter reaction mixture. Thirty-five cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min were then performed. The resulting DNA solution was diluted 1:12 in 0.1% SDS-10 mM EDTA and then mixed 1:1 with a solution containing 20 mM EDTA, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol. These samples were heated at 95°C for 5 min, chilled on ice, and 3 μl immediately loaded onto gels. For exons 5 to 7, 0.089 M Tris-0.089 M borate-0.002 M EDTA-10% glycerol-6% polyacrylamide gel was used and run at 3 W overnight. Autoradiography was performed at 24 h at −70°C with an intensifying screen (Fig. 1). Tumor DNA known to have a p53 mutation in the evaluated exon and a water blank substituting for DNA were used as positive and negative controls. Sequence analysis was not performed on positive SSCP specimens due to a lack of available tumor DNA.

Immunohistochemistry. Immunohistochemical analysis was performed (Fig. 2) using the avidin-biotin-peroxidase technique. Briefly, tissue sections were deparaffinized and rehydrated in graded alcohols. Endogenous peroxide activity was quenched with methanol in hydrogen peroxide. Incubation in blocking serum was carried out prior to the application of primary antibody. A rabbit polyclonal antibody to p53, CM-1 (Signet Laboratories, Dedham, MA), was used. The secondary antibody was goat antirabbit IgG (1:100 dilution; Vector Laboratories, Burlingame, CA). The reaction was developed using an avidin-biotin-peroxidase complex (1:25 dilution; Vector Laboratories). The chromagen 3′,3′-diaminobenzidine was used to identify sites of immunostain. For a lesion to be considered positive, it had to have intense immunostain in at least five tumor nuclei in a single high-power field. A head and neck carcinoma known to overexpress p53 and head and neck tumors without p53 primary antibody were used as positive and negative controls.

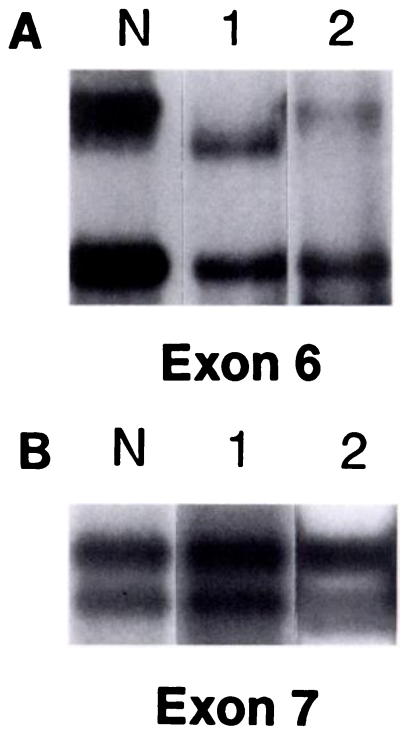

Fig. 1. Representative SSCP band shifts in *Lane 1* (*A*, patient 2) and *Lane 2* (*B*, patient 8). *N*, normal tissue.

The proportion of p53-positive samples was compared between groups using Fisher's exact test and survival curves generated using log rank analysis. Phase II chemotherapy did not significantly alter patient survival. Thus, although a minority of patients did not fit the protocol criteria for evaluation of chemotherapeutic response and toxicity, all 16 were considered evaluable for survival.

RESULTS

Eleven of 16 patients completed the chemotherapeutic regimen. Five patients received less than full treatment either due to toxicity or early death. There were no complete responses and two partial responses to treatment. Neither the ability to evaluate response nor evidence of response significantly altered patient survival. There were no treatment-related deaths. All patient mortality was due to progressive head and neck cancer or its sequelae.

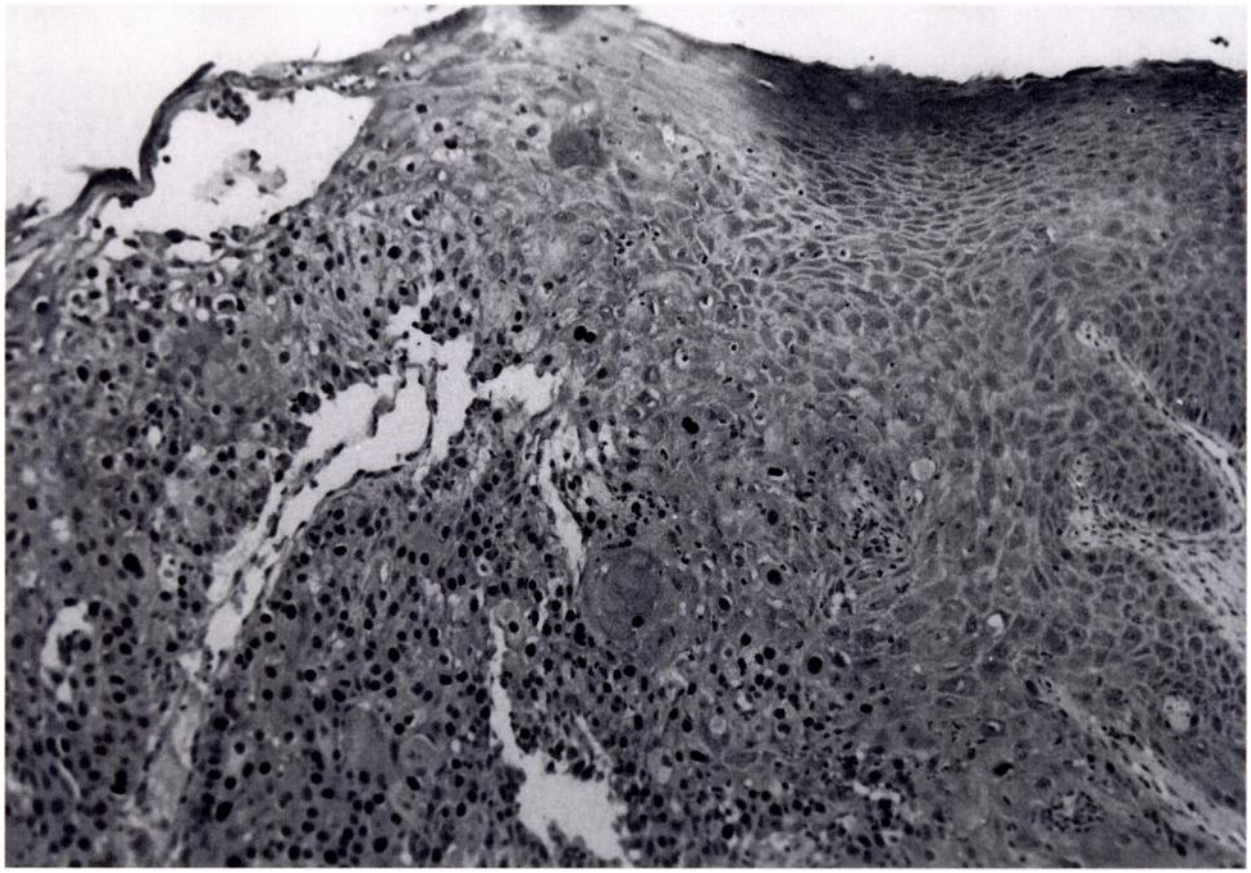

Fig. 2 p53 nuclear stain in invasive head and neck squamous cell carcinoma from patient 8.

In pretreatment tissue, 7 (44%) of 16 specimens had increased p53 protein expression, and p53 mutations were identified in 8 (50%) of 16 samples (Table 1). Mutations were identified in each of the four exons analyzed. No correlation was found between either p53 expression or p53 mutations and gender, site of primary tumor, tumor stage, site of first disease recurrence (Table 1), or age (data not shown). There was concordance between p53 mutations and protein expression in 11 (69%) of 16 cases. In five patients with tissue available before and after standard radiation/chemotherapy but prior to Phase II chemotherapy, protein/mutation analysis was the same in three patients and different in two patients. In one patient, a p53 mutation and increased p53 overexpression were identified after but not before treatment; in the second patient, the reverse occurred, with decreased expression/absence of a mutation post-treatment.

In five patients with specimens available both before standard radiation/chemotherapy and after receiving 5-FU/PALA/rIFN-α chemotherapy, p53 protein/mutation analysis was the same in two patients and different in three patients: in two of these patients, a mutation found before treatment was not identified in the postchemotherapy specimen, while in the third patient a p53 mutation and increased p53 expression were identified after Phase II chemotherapy but not before treatment.

There was no correlation between either pretreatment p53 mutations or increased protein expression and survival calculated from the time of initial diagnosis (Table 2). On the other hand, when survival was calculated from the time of diagnosis with end-stage disease (Table 2 and Fig. 3), increased p53 protein expression (but not p53 mutations) correlated with a poor outcome ($P = 0.0124$).

DISCUSSION

Patients with end-stage head and neck cancer have few options. They have failed standard therapy, and further treatment using radiation, surgery, or standard chemotherapy is either inadvisable or inevitably futile. Such patients may be offered investigational chemotherapy. To date, no studies have demonstrated a survival benefit with this approach. All of the patients enrolled in this study meet these criteria.

Studying the tumors of patients with far-advanced head and neck cancer is more difficult than investigating those with early or newly diagnosed disease for a variety of reasons. By the time most patients with far-advanced disease are evaluated, their primary tumors have been treated and residual or recurrent tumor is either intertwined with scar or otherwise not readily accessible to biopsy. Tissue is generally obtained only if confirmation of disease recurrence or persistence is essential, and is

Table 1 Clinical and tumor characteristics in 16 patients with end-stage head and neck cancer

Patient	Sex	Primary	Initial stage	Recurrence	Prior treatment[a]	Response[b]	Survival[c] (mo)	IHC[d]	SSCP[e]
1	M[f]	Larynx	III	Local only	S + XRT	NE	1, DOD	−	
2	F	Larynx	III	Locoregional	S + XRT	SD	10, DOD	−	Exon 6
3	M	Pharynx	III	dis	S + XRT	PD	5, DOD	−	
4	M	Larynx	III	Regional + dis	S + XRT + CMT	NE	9, DOD	+	
5	F	Pharynx	III	Locoregional	S + XRT	PD	11, DOD	−	
6	F	Larynx	III	Local + dis	S + XRT	NE	7, DOD	−	
7	M	Larynx	IV	Local only	S + XRT + CMT	NE	1, DOD	+	Exon 7
8	M	Pharynx	III	Local only	S + XRT	SD	7, DOD	+	Exon 7
9	M	Oral	IV	Local only	S + XRT	PD	7, DOD	+	Exon 8
10	M	Larynx	IV	Locoregional	S + XRT + CMT	PR	22, AWD	−	
11	F	Oral	I	Local only	S + XRT + CMT	PD	21, DOD	−	
12	M	Oral	IV	Locoregional	S + XRT	PD	5, DOD	+	Exon 7
13	F	Pharynx	III	Local only	S + XRT	PD	2, DOD	+	Exon 5
14	F	Larynx	IV	Regional	S + XRT + CMT	NE	11, AWD	−	Exon 8
15	F	Oral	II	Rreginal + dis	S + XRT	PR	14, AWD	−	Exon 8
16	M	Oral	III	Locoregional	S + XRT + CMT	SD	6, DOD	+	

[a] Surgery (S), radiation (XRT), and/or neoadjuvant chemotherapy (CMT) prior to phase II chemotherapy.
[b] Response (PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable) to Phase II chemotherapy.
[c] DOD, dead of disease; AWD, alive with disease.
[d] IHC, immunochemistry; presence (+) or absence (−) of p53 overexpression in pretreatment tumor tissue.
[e] SSCP analysis of exons 5–8 of the *p53* gene in pretreatment tumor tissue.
[f] M, male; F, female; dis, distant.

Table 2 Overall survival (months) in patients with and without p53 alterations in pretreatment tumor

	No. of patients	From diagnosis			After Phase II chemotherapy		
		Mean	Med[a]	Range	Mean	Med	Range
IHC							
Normal p53	9	35.6	32	10–100	14.6	11	1–22
Altered p53	7	33	22	8–89	5.3	6	1–9
SSCP							
Normal p53	9	35.9	35	10–100	10.7	9	1–211
Altered p53	7	32.1	22	8–89	6.1	7	2–11

[a] Med, median; IHC, immunohistochemistry.

usually accomplished by the least invasive means, yielding small samples for analysis. Thus, it is difficult to obtain specimens for research. In addition, these patients have been treated with radiation and or chemotherapy, either of which may alter the target gene to be analyzed, obscuring spontaneous genetic events that might otherwise have led to tumor progression.

As a result, most p53 analysis in SCCHN has been performed at the time of initial diagnosis. To avoid the problems outlined above, we chose to focus on tumor tissue obtained at diagnosis of the primary tumor, before treatment with radiation or chemotherapy commenced. To analyze a relatively homogeneous population, we chose patients with end-stage disease who were eligible for a Phase II chemotherapy trial.

Field *et al.* (6) studied the role of p53 abnormalities in 24 patients with end-stage SCCHN who met the criteria for enrollment into a chemotherapy trial. Their evaluation using immunohistochemistry alone demonstrated that, while p53 immunostain did not correlate with survival from the time of initial diagnosis, there was a significant correlation between increased p53 protein expression and progression to death after the patient was believed to have "end-stage disease." They found no correlation between p53 staining and age, sex, site of primary tumor, or site of recurrence.

Our findings in a similar patient population expand on their results. As is characteristic of patients with far-advanced head and neck tumors, the individuals whose tumors we evaluated were heterogeneous in (*a*) their disease stage at presentation; (*b*) the regimen of treatment administered; (*c*) the response to therapy; and (*d*) the time course to end-stage SCCHN. Thus, the most consistent element in the cancer of these individuals is that they all developed end-stage SCCHN. That p53 immunostain, but not p53 mutations, correlated with a poor prognosis suggests either that mutations occurred outside the exons analyzed and therefore were not detected, or that factors other than gene mutations are important to the behavior of recurrent SCCHN. The former proposition is unlikely, since 98% of p53 mutations are found in exons 5–8 (7). In addition to inactivation by mutant p53 protein, wt p53 can be inactivated (3) by binding to viral proteins (SV40 T antigen, E1b protein of adenovirus, HPV E6) and host proteins (mdm 2). Accumulation of wt p53 in cells has been reported in tumors expressing a mutant *ras* oncogene (15). Unlike wt p53, some mutant p53 proteins are able to form

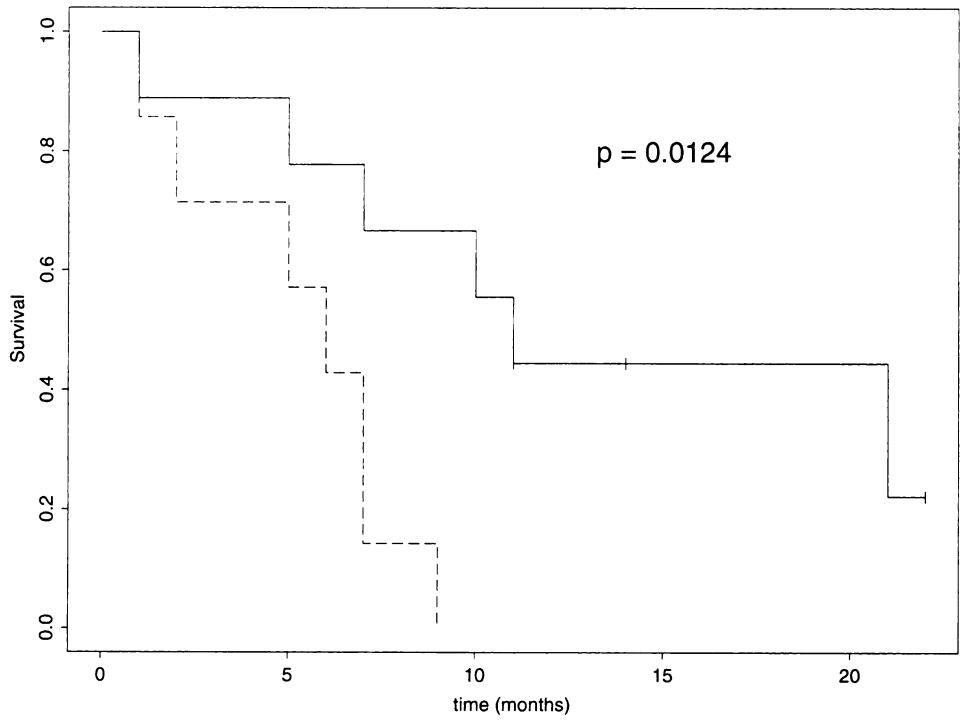

Fig. 3 Overall survival following diagnosis with end-stage disease in patients with (---) and without (—) p53 immunostain.

complexes (7) with a M_r 70,000 HSP70. Mutants that form a complex with HSP70 have a longer half-life than normal p53. Although mutant p53 proteins that do not complex with HSP70 may still have an extended half-life, they are more weakly transforming *in vitro* than those proteins that associate with HSP70 (16).

Disparity between p53 mutations and immunostain in 31% of tumors is consistent with the findings of others (15, 17). p53 immunostain is influenced both by p53 mutations and posttranslational events. A p53 mutation may result in a mutant protein with a prolonged half-life, a single base change which does not alter the protein, or the absence of detectable p53 protein due to a truncated protein or a p53 deletion. Posttranslational effects, including the binding of viral, host, or HSPs, can influence the half-life of both wt and mutant p53 proteins.

A lesser incidence of p53 immunostain but not p53 mutations in women was found which approached statistical significance ($P = 0.06$). Although the explanation for this is not clear, it is interesting that all three of the subjects with p53 mutations but negative p53 immunostain were women.

The fact that 5 of the 10 samples retested after either standard treatment or Phase II chemotherapy had different molecular and immunohistochemical results is noteworthy. Although most metastatic and recurrent tumors contain the same wt or altered codon in the *p53* gene as is found in the primary tumor, *p53* gene differences between the primary and the metastatic or recurrent tumor from the same patient have been seen both in tobacco-related (18) and other (19) tumors. Thus, at least two plausible reasons for the differences in our p53 results before and after treatment can be proposed. One possibility is that during tumor progression, an aggressive clone, differing in p53 sequence or protein expression from the predominant primary tumor clone, survived treatment and became the predominant clone in the tumor recurrence. An alternative possibility is that the perceived tumor recurrence was instead a second primary tumor.

In pretreatment tumor specimens, p53 protein expression but not p53 mutations correlated with a worse prognosis in patients with end-stage SCCHN after enrollment in a Phase II chemotherapy trial. This suggests that some posttranslational event altering the half-life of p53 is important in tumor behavior. This small series is the second to correlate increased p53 immunostain in end-stage SCCHN with poor survival and should prompt confirmation in a larger patient population, preferably in a prospective Phase II or Phase III trial.

REFERENCES

1. Sauter, E. R., Cleveland, D., Trock, B., Ridge, J. A., and Klein-Szanto, A. J. P. p53 is overexpressed in fifty percent of pre-invasive lesions of head and neck epithelium. Carcinogenesis, *15:* 2269–2274, 1994.

2. Boyle, J. O., Hakim, J., Koch, W., van der Riet, P., Hruban, R. H., Roa, R. A., Correo, R., Eby, Y. J., Ruppert, J. M., and Sidransky, D. The incidence of p53 mutations increases with progression of head and neck cancer. Cancer Res., *53:* 4477–4480, 1993.

3. Sauter, E. R., Ridge, J. A., Trock, B., Cleveland, D., Whitley, K. V., Mohr, R. M., and Klein-Szanto, A. p53 overexpression in primary and metastatic head and neck carcinomas. Laryngoscope, *105:* 653–656, 1995.

4. Field, J. K., Pavelic, Z. P., Spandidos, D. A., Stambrook, P. J., Jones, A. S., and Gluckman, J. L. The role of the p53 tumor suppressor gene

in squamous cell carcinoma of the head and neck. Arch. Otolaryngol. Head Neck Surg., *119:* 1118–1122, 1993.

5. Sauter, E. R., Ridge, J. A., Gordon, J., and Eisenberg, B. L. p53 overexpression correlated with increased survival in patients with squamous carcinoma of the tongue base. Am. J. Surg., *164:* 651–653, 1992.

6. Field, J. K., Malliri, A., Butt, S. A., Gosney, J. R., Phillips, D., Spandidos, D. A., and Jones, A. S. p53 expression in end stage squamous cell carcinoma of the head and neck prior to chemotherapy treatment: expression correlates with a very poor clinical outcome. Int. J. Oncol., *3:* 431–435, 1993.

7. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancer. Science (Washington DC), *253:* 49–53, 1991.

8. Yin, Y., Tainsky, M. A., Bischoff, F. Z., Strong, L. C., and Wahl, G. M. Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell, *70:* 937–948, 1992.

9. Lane, D. P. p53, guardian of the genome. Nature (Lond.), *358:* 15–16, 1992.

10. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. Participation of p53 protein in the cellular response to DNA damage. Cancer Res., *51:* 6304–6311, 1991.

11. Chin, K-V., Ueda, K., Pastan, I., and Gottesman, M. M. Modulation of activity of the promoter of the human *MDR1* gene by ras and p53. Science (Washington DC), *255:* 459–462, 1992.

12. El Rouby, S., Thomas, A., Costin, D., Rosenberg, C. R., Potmesil, M., Silber, R., and Newcomb, E. W. *p53* gene mutation in B-cell chronic lymphocytic leukemia is associated with drug resistance and is independent of *MDR1/MDR3* gene expression. Blood, *82:* 3452–3459, 1993.

13. Goodrow, T. L., Prahalada, S. R., Storer, R. D., Manam, S. V., Leander, K. R., Kraynak, A. R., van Zwieten, M. J., Nichols, W. W., and Bradley, M. O. Polymerase chain reaction/sequencing analysis of ras mutations in paraffin-embedded as compared with 3T3 transfection and polymerase chain reaction/sequencing of frozen tumor deoxyribonucleic acids. Lab. Invest., *66:* 504–511, 1992.

14. Navone, N. M., Troncoso, P., Pisters, L. L., Goodrow, T. L., Palmer, J. L., Nichols, W. W., von Eschenbach, A. C., and Conti, C. J. p53 protein accumulation and gene mutation in the progression of human prostate carcinoma. J. Natl. Cancer Inst., *85:* 1657–1669, 1993.

15. Wynford-Thomas, D. p53 in tumor pathology: can we trust immunocytochemistry? J. Pathol., *166:* 329–330, 1992.

16. Hinds, R. W., Finaly, C. A., Quartin, R. S., Baker, S. J., Fearon, E. R., Vogelstein, B., and Levine, A. J. Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spots" mutant phenotypes. Cell Growth & Differ., *1:* 571–580, 1990.

17. Battifora, H. p53 immunohistochemistry: a word of caution. Hum. Pathol., *25:* 435–437, 1994.

18. Lohmann, D., Putz, B., Reich, U., Bohm J., Prauer, H., and Hofler, H. Mutational spectrum of the *p53* gene in human small-cell lung cancer and relationship to clinicopathological data. Am. J. Pathol., *142:* 907–915, 1993.

19. Hayashi, Y., Yamashita, J., and Yamaguchi, K. Timing and role of p53 gene mutation in the recurrence of glioma. Biochem. Biophys. Res. Commun., *180:* 1145–1150, 1991.