Loss of p53 Gene Mutation after Irradiation Is Associated with Increased Aggressiveness in Recurring Head and Neck Cancer

Oreste Gallo,1 Ilaria Chiarelli,1 Simonetta Bianchi, Anna Calzolari, Laura Simonetti, and Berardino Porfirie2

Institute of Otolaryngology Head and Neck Surgery [O. G. L. S.], Institute of Anatomic Pathology [S. B. A. C.], and Department of Clinical Physiopathology, Human Genetic Unit [I. C. B. P.], University of Florence, Viale Morgagni 85, I-50134 Florence, Italy

ABSTRACT

The p53 gene plays a pivotal role in the control of a checkpoint during G1 and in the apoptotic program. It has been postulated that alterations of p53 may influence radiosensitivity and prognosis in several malignancies. We studied the p53 gene status of 35 consecutive head and neck cancer patients who failed primary radiotherapy (RT) in preirradiated and postirradiated tumor samples using DNA single-strand conformational polymorphism analysis. Sixteen of 35 (46%) preirradiated samples presented with band shifts suggestive of point mutations in one or two exons. Eleven of these tumors (69%) showed the same shift even in the postirradiated samples. Exons 5 and 8 were prevalently affected in this group. Five tumors (31%) lost the mutation following RT. The missed mutations clustered in exon 7. All mutations were confirmed by sequencing. Actuarial analysis demonstrated increased survival in patients with tumors bearing a p53 gene mutation in both preirradiated and postirradiated samples (P = 0.05 and P = 0.01, respectively). We also found that loss of p53 gene mutation in postirradiated cancers is associated with a significantly shorter disease-free interval (P < 0.02) and a worse prognosis (P < 0.05). A possible explanation in such cases is clonal selection by RT of more aggressive and radioresistant cell subpopulations, which are wild-type for the p53 gene. Taken together, our data suggest that not only p53 gene status but also the pattern of mutations could modulate the response of tumor cell to RT in vivo.

INTRODUCTION

Radiation therapy continues to have a significant impact on the treatment of head and neck cancer and other malignancies. A major impediment to successful RT1 is the failure of some tumor types to respond to this form of treatment and the appearance of resistant populations upon relapse of an originally responsive malignancy. The persistence or recurrence of the disease after irradiation is usually a poor prognostic factor because it is frequently associated with unresponsiveness to other anticancer therapies and high risk of distant metastases (1, 2). Thus, it is evident that understanding the molecular basis of tumor cell resistance to irradiation is of great interest because of its biological, therapeutic, and prognostic implications.

In the past few years, an increasing accumulation of evidence provided new insights into the possible key role of the p53 gene in the regulation of the cellular mechanisms of resistance to DNA-damaging agents. Observations of the requirement of the wild-type p53 protein for radiation-induced G1-S-phase arrest (3) or radiation-induced apoptosis (4, 5) have contributed to this hypothesis, suggesting that the p53 gene is involved in the control of genomic integrity by regulating the G1-S transition following several genotoxic insults. In fact, after exposure to ionizing and nonionizing radiation, wild-type p53 protein rises dramatically in exposed normal cells inducing G1 arrest (6, 7). This p53 protein-induced arrest allows cell time to repair the damage before being fixed as mutations. Following a variety of genotoxic damage, the p53 gene may also trigger a programmed cell death or apoptosis with elimination of potentially oncogenic cells (reviewed in Ref. 8). The fine tuning of the balance between efficient DNA repair and apoptosis may represent the key to regulation of cellular radiosensitivity. p53 mutant cells, which are frequently observed in a large variety of malignancies, do not halt after DNA-damaging irradiation and continue to progress into the cell cycle with a decrease in DNA repair efficiency. Thus, p53 mutated cells might be expected to have increased sensitivity to radiation because of an accumulation of potentially lethal radiation-induced DNA lesions. However, several recent studies show that in some cells disruption of p53 confers resistance to radiation and other anticancer treatments through the inhibition of the p53-mediated apoptosis (9–11).

Taken together, these in vitro observations suggest that the p53 gene status may influence tumor response to irradiation, even if, to date, few clinical studies have verified the possible relation between p53 mutation and tumor radiosensitivity in vivo.

In the present study, we investigated the p53 gene status using SSCP analysis and sequencing in tumor biopsy specimens obtained before and after irradiation from 35 previously untreated patients with squamous cell carcinoma of the head and neck, who experienced persistence or recurrence of the disease after primary RT. The study was undertaken to verify whether p53 gene status and/or pattern of p53 mutations correlates with tumor response to RT and prognosis in such cases. Another aim was to assess whether the p53 gene itself is a possible target for radiation-induced mutation in neoplastic cells, according to recent observations on human radiation-induced tumors (12, 13).
PATIENTS AND METHODS

Patients. Formalin-fixed, paraffin-embedded tumor specimens were obtained from patients surgically treated during 1985–1989 for resectable recurrences from head and neck cancers after unsuccessful primary RT. Our series included only those patients for whom biopsy specimens of the original untreated tumor were also available. Thirty-five patients were selected for this study, and archival tumor materials from each case were identified retrospectively through a systematic search in the files of the Surgical Pathology Division, Institute of Anatomic Pathology. Thus, 70 tumor specimens were collected, including preirradiated and postirradiated tumors from each patient. All specimens and H&E-stained histological slides were reviewed by one pathologist (S. B.) to confirm the original diagnosis.

This study includes all but three (T2N2 oropharynx, T1N1 larynx, and T1N0 oral cavity squamous cell carcinomas) stage I-II head and neck cancers with clinically negative neck (23 larynx, 6 oral cavity, and 3 oropharynx) that received primary RT with curative intent. All patients received external beam therapy and were irradiated with 60Co or a 4/5 MeV linear accelerator. The daily dose ranged from 1.8 to 2.0 Gy. All patients received doses in the range of >60 to 72 Gy or greater, the median dose being 64 Gy. In all cases, treatments were initiated 3–10 weeks after initial diagnosis and completed by 5–7 weeks, with breaks not exceeding 1 week. All patients enrolled in the study after primary RT experienced a persistence (six cases) or a local recurrence of the disease during follow-up (overall disease-free interval, median, 9 months; range, 1–36), and in all cases a surgical salvage of radiation failure was attempted.

The criteria for distinguishing recurrence from second primary cancer were: (a) site of recurrence rigorously the same as the original malignancy and (b) time of relapse not exceeding 3 years from initial RT. All patients had a 5-year minimum follow-up starting from the date of the last surgical procedure.

All patients were followed up regularly by radiotherapists and otolaryngologists at 1–3-month intervals.

Sample Preparation. Formalin-fixed, paraffin-embedded tissue sections (7–8 μm) were placed onto standard microscope slides. Specimens were deparaffinized with xylene, rehydrated in serial graded water-ethanol solutions (100%, 90%, 70%, and 50%, respectively), and rinsed in deionized water. H&E stain was performed. The samples were once again dehydrated, but the slides were not fitted with the coverslip. The stained unmouted sections were examined with a microscope. The portion of normal tissue was identified and scraped with a sterilized blade, to obtain tumor cells exclusively, for DNA extraction. All of these procedures were repeated at least twice to detect possible gene mutations from different tumor areas.

DNA Isolation. DNA from paraffin-embedded tumor sections was extracted by overnight incubation at 55°C in an extraction buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2, 0.1 mg/ml gelatin, 0.45% NP40, 0.45% Tween 20, and 0.5 mg/ml proteinase K]. The sample was boiled for 8 min and centrifuged for 15 min at 14,000 rpm. The supernatant (1–5 μl) was used in the PCR mixture.

PCR and SSCP Analysis. Exons 5–8 of the p53 gene were amplified. Exon 5 was amplified with two pairs of primers giving rise to partially overlapped fragments. The primers used were: TP53 5US, 5'-TTATCTGGTCACTTTTGCCC (14); TP53 5UA, 5'-TCTATGCTGTGACTGCTTTG (14); TP53 5DS, 5'-TCCCACACCCCCGCCCCGCA (14); TP53 5DA, 5'-CCACCCCTGTGGTCCTTCC (15); TP53 6S, 5'-GCCCTGATTCCTCCTCAGT (15); TP53 6A, 5'-TAAACCCCTCTCCTCACAGAGA (14); TP53 7S, 5'-ACTGCGCTCATCTTGCGCT (15); TP53 7A, 5'-TGTGACGTTGCGAAGTGCC (15); TP53 8S, 5'-TAATAAGAGCAGGTAGGAC (15); and TP53 8A, 5'-TCCACCGTTCTTGGTCTCG (15).

Amplification consisting of 32 cycles was carried out in 25 μl of 1.5 mM MgCl2 Perkin Elmer Cetus buffer with 1 μM of exon-flanking primer set, 50 μM each deoxynucleotide triphosphate, and 0.5 units Perkin Elmer Cetus AmpliTaq. Temperature and time for the reaction cycles were 95°C for 1 min, 62°C for 1 min, and 72°C for 30 s.

PCR products were heat denatured and subjected to SSCP analysis using electrophoresis on 6% polyacrylamide gels with 5–10% glycerol (16). Electrophoresis was carried out at 45 W for 4 h at 4°C; the gels were silver stained and dried on filter paper.

All samples were subjected to PCR and SSCP analysis at least twice to confirm the results obtained. To exclude the silent CGA/CGG dimorphism in codon 213, PCR products from samples showing SSCP abnormalities in exon 6 were subjected to restriction analysis with TaqI.

LOH was evaluated in those samples missing mutation in relapsed tumors after RT. The polymorphism revealed by MspI digestion of a 107-bp fragment obtained from amplification of p53 intron 6 was used (18). Another intragenic short tandem repeat polymorphism (AAAAA), was investigated (19).

Nucleotide Sequence Analysis. Abnormal bands detected by SSCP analysis were eluted from acrylamide gels, and amplified by PCR using the same primers as utilized for SSCP analysis, with a modified 5′ end to contain the M13(−20) sequence. These PCR products were purified on 2% NuSieve gels, phenol extracted, and subjected to automated sequencing with the Taq Dye Primer Cycle Sequencing kit (Applied Biosystems). Both strands were sequenced for confirmation of the mutations. p53 wild-type samples were directly sequenced from the original PCR products.

Statistical Analysis. The statistical analysis for actuarial survival was performed using the Kaplan-Meier method (20). The period started at the time of diagnosis. Survival analysis was performed using the log rank test.

The Mann-Whitney U test was performed to compare the disease-free intervals between groups of patients (e.g., those maintaining p53 mutation after RT and those missing it). A χ2 test with small numbers (21) was used to determine heterogeneity between the mutational frequencies of the groups of patients. P values <0.05 were considered to be statistically significant. Statistical analysis was performed using the Stata (Stata Corporation, College Station, TX) and StatXact (Cambridge, MA) programs.
Table 1  Disease-free interval in 35 recurring head and neck squamous cell carcinomas according to p53 status before and after RT

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Disease-free interval median (mo)</th>
<th>p53 Status</th>
<th>Exon affected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before RT</td>
<td>After RT</td>
</tr>
<tr>
<td>11</td>
<td>22 (1-35)*</td>
<td>Mutated</td>
<td>Mutated</td>
</tr>
<tr>
<td>5</td>
<td>4 (1-12)</td>
<td>Mutated</td>
<td>Wild-type</td>
</tr>
<tr>
<td>19</td>
<td>9 (2-36)</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
| *One sample was mutated in both exons 5 and 8.
* Range.

Table 2  Sequence analysis of abnormal p53 SSCP bands found in 16 primary head and neck squamous cell carcinomas and their recurrences after RT

<table>
<thead>
<tr>
<th>Identification</th>
<th>Exon</th>
<th>Codon</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Mutation in the recurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5460</td>
<td>5</td>
<td>144</td>
<td>C-*T</td>
<td>Gln-*stop</td>
<td>Yes</td>
</tr>
<tr>
<td>3048†</td>
<td>5</td>
<td>155</td>
<td>C-*T</td>
<td>Thr-*Asn</td>
<td>Yes</td>
</tr>
<tr>
<td>1219</td>
<td>5</td>
<td>157</td>
<td>G-*T</td>
<td>Val-*Phe</td>
<td>Yes</td>
</tr>
<tr>
<td>8590</td>
<td>5</td>
<td>176</td>
<td>G-*T</td>
<td>Cys-*Phe</td>
<td>Yes</td>
</tr>
<tr>
<td>7883</td>
<td>5</td>
<td>179</td>
<td>C-*T</td>
<td>His-*Tyr</td>
<td>Yes</td>
</tr>
<tr>
<td>8788</td>
<td>6</td>
<td>205</td>
<td>A-*G</td>
<td>Tyr-*Cys</td>
<td>Yes</td>
</tr>
<tr>
<td>1531</td>
<td>7</td>
<td>236</td>
<td>A-*G</td>
<td>Tyr-*Cys</td>
<td>No</td>
</tr>
<tr>
<td>3245</td>
<td>7</td>
<td>242</td>
<td>G-*T</td>
<td>Cys-*Phe</td>
<td>Yes</td>
</tr>
<tr>
<td>2133</td>
<td>7</td>
<td>245</td>
<td>G-*A</td>
<td>Gly-*Asp</td>
<td>No</td>
</tr>
<tr>
<td>7175</td>
<td>7</td>
<td>255</td>
<td>Ins T</td>
<td>Frameshift</td>
<td>No</td>
</tr>
<tr>
<td>5357</td>
<td>7</td>
<td>255</td>
<td>Del ATC</td>
<td>In-frame deletion</td>
<td>No</td>
</tr>
<tr>
<td>3048†</td>
<td>8</td>
<td>264</td>
<td>Del CTA</td>
<td>In-frame deletion</td>
<td>Yes</td>
</tr>
<tr>
<td>0610</td>
<td>8</td>
<td>273</td>
<td>G-*A</td>
<td>Arg-*His</td>
<td>Yes</td>
</tr>
<tr>
<td>8084</td>
<td>8</td>
<td>290</td>
<td>A-*G</td>
<td>Arg-*Gly</td>
<td>No</td>
</tr>
<tr>
<td>0716</td>
<td>8</td>
<td>283</td>
<td>Del G</td>
<td>Frameshift</td>
<td>Yes</td>
</tr>
<tr>
<td>0205</td>
<td>8</td>
<td>285</td>
<td>G-*T</td>
<td>Glu-*stop</td>
<td>Yes</td>
</tr>
<tr>
<td>7982</td>
<td>8</td>
<td>298</td>
<td>G-*T</td>
<td>Glu-*stop</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* Two mutations found in case 3048.

RESULTS

In our study, we demonstrate, using SSCP analysis, in 16 of 35 (46%) preirradiated tumor samples, band shifts suggestive of p53 gene point mutations that were all confirmed by sequencing. p53 gene mutations occurred most commonly in tumors of the oral cavity (3/7, 43%) and larynx (12/24, 50%), whereas 1 of 4 oropharyngeal cancers had p53 sequence alterations at SSCP analysis. The distribution of p53 SSCP alterations detected in preirradiated tumor samples was: four cases in exon 5 (all were laryngeal cancers), one case in exon 6 (a laryngeal cancer), five cases in exon 7 (three were laryngeal cancers), and, finally, five cases in exon 8 (three were laryngeal cancers). Moreover, one laryngeal carcinoma showed band shifts in both exons 5 and 8.

The comparative analysis of p53 mutational status in the original and relapsed tumors is shown in Table 1. The same p53 mutation was detected both before and after RT in 11 of 35 patients (31%). Exons 5 and 8 were prevalently affected in this group.

Five recurring cancers (14%) lost the mutation in the postirradiated biopsy specimens. The missed mutations clustered in exon 7 (four cases), whereas one case involved exon 8.

Although the numbers are small, a heterogeneity test showed that the distribution of p53 mutations in the samples mutated both before and after RT was statistically different from that observed in the group missing the mutation (P < 0.01). Sequence analysis of all mutated tumor samples is summarized in Table 2.

To assess the nature of the lack of p53 mutations in the samples from relapsed tumors that were originally mutated, preirradiated tumor samples were typed at two intragenic p53 polymorphisms (18, 19). All five cases were informative at least for one system and were assayed for LOH in the post-RT samples: no allelic losses were found.

Follow-up data available on all 35 patients studied showed a statistically significant difference in survival among patients with p53 mutated and nonmutated RT-relapsed cancers. In fact, in our series we found that patients with a p53-mutated original tumor had an increased survival rate than those with wild-type cancer (P = 0.05; Fig. 1a). Moreover, this difference was more significant considering the p53 gene status in postirradiated tumor biopsies. In fact, patients with p53 mutations in relapsed cancers had a higher survival rate than those of patients with no p53 gene mutations in recurring tumor (P = 0.01; Fig. 1b). Detailed analysis of disease-free and follow-up data according to the pattern of p53 mutations showed that patients with missed p53 mutations following RT had a shorter disease-free interval and a worse prognosis (P < 0.05) than those observed in relapsed patients with no tumor change at the p53 gene level after RT. Kaplan-Meier survival curves according to p53 status before and after RT are shown in Fig. 2. Although differences
DISCUSSION

In this study, we have shown that in about 50% of the 35 consecutive head and neck cancers relapsed after primary RT, an alteration of the p53 tumor suppressor gene was identified by SSCP and confirmed by sequencing.

Studies suggest that the p53 gene is an essential component of the apoptotic program in tumor cells, induced by anticancer agents, including irradiation (10, 22). However, the exact role of the p53 gene in tumor resistance to anticancer therapies is still unclear. In fact, recent observations postulated that the biological consequences of loss of p53 function could be cell-type specific (23), being associated with increased radioresistance in several tumor cell lines (24) or with no survival effects on other ones (25, 26). Moreover, in an experimental tumor model with rat epithelial cells bearing a mutation of the p53 gene following exposure to benzo(a)pyrene, thus closely resembling the head and neck carcinogenesis model, Biard et al. (27) found enhanced sensitivity to ionizing radiation of p53 mutated epithelial cells.

Since the rate of the p53 alterations in our series of RT-treated recurring cancers is similar to that reported in several studies on overall head and neck tumors (8, 28, 29), we argue that the p53 gene status could not directly affect the response of head and neck carcinomas to irradiation. These data are in agreement with the recently reported absence of correlation between p53 gene mutations and radiosensitivity in head and neck cancer cell lines by Jung et al. (25) and Brachman et al. (26).

What was unexpected was the change of the p53 gene status in relapsed tumors following irradiation in some cancers examined and its prognostic implications. In fact, we found that the detection of a p53 gene mutation in tumor cells can provide prognostic information in patients with head and neck cancer, who experienced tumor relapse after primary RT. In our patients, the p53 gene mutations correlated with increased survival because of a low risk of distant metastases and locoregional recurrence after surgical salvage in relapsed cancers. This was true considering p53 gene status in tumor samples obtained either before or after RT. However, the link between p53 mutations and prognosis was stronger in postirradiated tumors than in preirradiated ones (Fig. 1, P = 0.01 versus P = 0.05, respectively). In fact, the data reported here show that ionizing radiation could be accompanied with a change in the p53 gene status in recurring tumors. More precisely, we found the loss of the original p53 mutations in five tumors (14%). The missed mutations clustered in exon 7. All of them affected the IV evolutionarily conserved region (residues 234–258) of the p53 (8). This region includes the L3 loop domain (residues 236–251) that participates in DNA contact and protein stabilization (30). The missed mutation in exon 8 involved codon 280, and Arg280 also belongs to the DNA-binding domain (30).

Disease-free survival analysis showed that those patients who lost p53 mutations in postirradiated tumor samples had a worse prognosis than those without a change in the p53 gene status in postirradiated tumors (P = 0.01 and P = 0.05, respectively). Thus, the loss of p53 mutations was associated with increased aggressiveness in relapsed cancers.

Loss of p53 mutation after irradiation has been recently reported by Kemp et al. (31) in mice lacking one p53 allele.
(p53+/−) as consequence of a p53 gene deletion with LOH in irradiated murine cells and an increased appearance of p53 null clones. In this study, the authors suggested that in p53-mutated cells the p53 gene itself is a genetic target for radiation-induced mutagenesis, being deleted or additionally altered in cells surviving irradiation.

More recently Lowe et al. (11), analyzing the therapeutic responsiveness of genetically defined tumors expressing or devoid of the p53 gene in immunocompromised mice, postulated a key role of the p53 status in modulating tumor response to RT. They showed that tumors expressing the p53 gene (p53+/+) contained a high proportion of apoptotic cells and typically regressed after treatment with γ-radiation. In contrast, p53-deficient tumors (p53−/−) treated with the same regimens continued to enlarge and contained few apoptotic cells.

Although in head and neck cancers the more frequent p53 gene aberrations detected are single-point mutations and less frequently deletions of one or both p53 alleles (8), in our study LOH analysis of the informative cases showed the absence of p53 null cells in relapsed malignancies, suggesting that the loss of p53 mutation following RT was not due to a deletion of the p53 gene (data not shown). Conversely, assuming a clonal heterogeneity of tumor cell populations, particularly at the p53 gene level (11, 32), it is possible that tumor cells carrying p53 mutation(s) in some exon(s) could be more sensitive to radiation. Therefore, only p53 wild-type cell clones may survive and be enriched in resistant or recurring malignancy with loss of p53 mutated cell populations. This model is also suggested by the different proportion of p53-immunoreactive tumor cells detected in these cases (data not shown). This clonal selection could also have a prognostic impact since more aggressive tumor cell populations would ensue, leading to worse prognosis. According to this hypothesis and assuming that radiation failure is frequently due to radioreistant tumor cells (33), comparative analysis of the site of p53 mutations in tumors with and without a change in the p53 gene status after RT in our series suggests that cells harboring mutation in exon 7 should be those responding better to RT, whereas cells mutated in exons 5 and 8 could be more radioreistant. In fact, all but one recurring cancer originally mutated in exon 5 or 8 did not change the p53 gene status in relapsed malignancies, whereas all but one cancer missing p53 mutations after RT had exon 7 alterations in the original tumor. Moreover, all cancers missing p53 mutations following irradiation were unresponsive to RT or had the shortest disease-free interval with a higher risk of unsuccessful surgical salvage and poor prognosis. Interestingly, Brachman et al. (26, 28) found mutations at exon 7 only in tumor cell lines obtained from original radiosensitive head and neck squamous cell carcinomas, whereas tumor cell lines from RT-relapsed malignancies did not show a mutation(s) of exon 7 and were often p53 wild type.

According to Nowell’s clonal evolution model of tumor progression (34), a cell acquiring a genetic change might have a selective growth advantage. Clonal expansion of these cells following irradiation driven by its radioreistance would lead to recurrence and tumor progression (32). The data presented here suggested that different p53 mutations, because of their different radiosensitivity, may influence tumor progression and prognosis of head and neck irradiated cancers. This possible involvement of different p53 functional domains in the modulation of the tumor cell response to anticancer therapies is further supported by the ability of different mutations to alter different properties of the p53 protein, such as its DNA-binding or -transactivating activities (35–37), and, more recently, by variable prognostic significance of different p53 mutations in breast cancer patients (38).

Our study suggests that alterations of the p53 tumor suppressor gene function is common in head and neck cancers recurring after RT and may have a prognostic relevance. The site of p53 mutations we reported in relapsed tumors may influence in vivo tumor responsiveness to RT and salvage therapies. In fact, the loss or the acquisition of a p53 gene mutation in relapsed tumor cells following irradiation seem to involve specific p53 gene exons and have a prognostic relevance in our patients. Moreover, in our series p53 tumor mutations seem to have a favorable prognostic impact in head and neck cancer patients who failed their primary RT. Finally, our data demonstrate that after RT the relapsed malignancy may be genetically different from the original cancer, at least at the p53 gene level, possibly as result of an in vivo clonal expansion of radioreistant cell clones.

ACKNOWLEDGMENTS

We are grateful to P. L. Mattiuz for discussion and to V. Boddi for help with statistical analysis.

REFERENCES


Loss of p53 gene mutation after irradiation is associated with increased aggressiveness in recurring head and neck cancer.

O Gallo, I Chiarelli, S Bianchi, et al.


Access the most recent version of this article at: [http://clincancerres.aacrjournals.org/content/2/9/1577](http://clincancerres.aacrjournals.org/content/2/9/1577)