[CANCER RESEARCH 52, 6390–6393, November 15, 1992]

Advances in Brief

Mutations in the *p53* Gene in Radiation-sensitive and -resistant Human Squamous Carcinoma Cells[1]

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Abstract

Five of six human squamous cell carcinoma (SCC) cell lines characterized as radiation sensitive (SQ-38, SCC-9, SQ-9G) or radiation resistant (SQ-20B, SCC-35, JSQ-3) exhibited alterations of the *p53* gene. The point mutations and a deletion were detected by using single-stranded conformational polymorphism analysis and polymerase chain reaction-direct sequencing. Interestingly, three of three radiation-sensitive and two of three radiation-resistant cell lines revealed mutations in the *p53* gene. Point mutations were located in exons 4, 6, and 8 (at codons 72 and 298 in JSQ-3; 273 in SCC-35; 196 in SQ-38), and deletions consisted of 32 base pairs between codons 274 and 285 in SCC-9 and 1 base pair at codon 271 in SQ-9G. Three mutations resulted in substitutions for an arginine residue. Immunocytochemical analysis confirmed p53 protein overexpression in SCC-35 cells which contained a missense mutation at codon 273. In contrast to previous studies which linked alterations in *ras*, *myc*, and *raf* expression with radiation resistance, this study indicates that mutations in the tumor suppressor gene, *p53*, do not directly correlate with such resistance.

Introduction

SCC[3] of head and neck origin are frequently treated for cure using ionizing radiation therapy. The outcomes of such treatments are dependent on various tumor and host factors, including differing intrinsic radiation sensitivities (1, 2). The mechanisms by which oncogenes may modify the response of cells to ionizing radiation have not been established, although signal transduction and transcriptional regulation have been implicated (3, 4). Using a panel of three SCC cell lines classified as radiation resistant and three as radiation sensitive, analysis (5) of differential protein expression showed that 14 proteins were expressed more abundantly in the former group and 15 were expressed more abundantly in the latter group (6). It appears, therefore, that the phenotype for response to radiation killing may be associated with either gain or loss of certain protein expression.

We have previously reported the activation of the *raf* oncogene in a radiation-resistant SCC (7). Furthermore, reversion of resistance to sensitivity was accomplished by antisense modulation of c-*raf*-1 in these SCC cells (8). Other investigators have implicated the *ras* and *myc* oncogenes in radiation resistance (9, 10). *p53* has been shown to function as both an oncogene in its mutated form and a tumor suppressor gene in its wild-type form (11). Since *p53* has been shown to be frequently mutated in human cancer, including squamous cell carcinoma, we investigated whether mutations in this gene correlate with either the radiation-sensitive or -resistant cellular phenotype in these cell lines.

Materials and Methods

Head and Neck Cell Lines. Cells were a gift of R. R. Weichselbaum, M. D., University of Chicago. Establishment and characterization of these cell lines have been reported previously (5, 12). Cells were maintained in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.4 μg/ml hydrocortisone.

The details of radiobiological and clinical observations have been reported (6). Cells characterized as RR included SQ-20B, SCC-35, and JSQ-3. The D_0 values were 2.4, 2.3, and 2.5 Gy, respectively, and all three were originated from patients who suffered "in-field" radiation treatment failure. Cells characterized as RS included SQ-38, SCC-9, and SQ-9G. The D_0 values were 1.7, 1.3, and 1.7 Gy, respectively. Clinical data about SQ-38 are unclear, but SCC-9 and SQ-9G responded completely to radiation therapy.

Genomic DNA Amplification. Human genomic DNA (0.5 μg) from squamous cell lines was amplified by PCR through 25 cycles in a DNA Thermal Cycler (Perkin Elmer Cetus). The oligonucleotide primers were designed according to sequences flanking domains containing mutational hot spots in the *p53* gene (13, 14). The first temperature cycle consisted of an initial denaturation step for 5 min at 95°C followed by a 1-min annealing at 58°C and a 4-min extension at 70°C. Each of the additional 24 cycles was performed as follows: 1 min at 95°C; 1 min at 58°C; and 4 min at 70°C followed by 10 min for the last extension. The products were analyzed on a 2% Seakem GTG (FMC) agarose gel.

Direct Sequencing Analysis. The amplified DNA samples were purified and sequenced according to the previously published procedure (15). Briefly, one-third of purified PCR samples were mixed with 2 μl of the primer used for PCR amplification and 2 μl of the 5 × Sequenase reaction buffer in a final volume of 10 μl. The reaction mixture was then heat-denatured for 3 min and chilled. The labeling and termination steps were performed according to the protocol of the Sequenase kit (United States Biochemical). The reactions were then analyzed on a 6% polyacrylamide/8 M urea gel, dried, and exposed overnight to Kodak XAR-5 film.

Immunocytochemistry. Immunocytochemical analysis was performed with a modified version of the method described by the manufacturers (Oncogene Science). Cells were grown on glass slides and fixed for 30 min with cold methanol containing 0.3% H_2O_2. The slides were washed with phosphate-buffered saline containing 1% BRIJ-35 and incubated with a blocking solution containing normal goat serum at room temperature for 30 min. The cells were incubated with primary antibody (monoclonal PAb-2 from Oncogene Science) at 37°C for 1 h. Specifically bound antibody was then visualized by incubation with biotinylated secondary antibody, horse anti-mouse immunoglobulin, at room temperature for 1 h, followed by incubation with a streptavidin-horseradish peroxidase conjugate and substrate. The positive staining was observed by using diaminobenzidine as substrate.

Single-Strand Conformation Polymorphism Analysis. SSCP analysis was performed by using the method described by Gaidano *et al.* (16). Briefly, exons 4–9 of *p53* in genomic DNA of SSC were amplified as described elsewhere in the presence of the end-labeled primers with

Received 8/17/92; accepted 10/1/92.

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[1] This research was supported by Grants P01CA52066 and R01 CA45408 from the National Cancer Institute.

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[3] The abbreviations used are: SCC, squamous cell carcinomas; PCR, polymerase chain reaction; SSCP, single-stranded conformational polymorphism; RS, radiation sensitive; RR, radiation resistant.

[γ-^{32}P]ATP using T4 polynucleotide kinase. One μl of PCR products (100 μl) was diluted (1:100) in 0.1% sodium dodecyl sulfate-10 mM EDTA. Five μl of diluted samples were mixed (1:1) in 95% formamide, 1% xylene cyanol, 1% bromophenol blue, and 20 mM sodium hydroxide and denatured at 90°C. In the absence or presence of 10% glycerol, 1.5 μl of each sample were analyzed on a 6% polyacrylamide gel. The gel was dried and exposed to the Kodak XAR-5 film.

Results

To determine whether the genomic DNA from six human SCC of the head and neck origin had nucleotide alterations including point mutations in the *p53* tumor suppressor gene, a rapid and sensitive SSCP assay was used initially. One hundred- to 600-base pair-long fragments containing exons 4–9 were amplified and electrophoresed on nondenaturing acrylamide gels. The SSCP analysis of six SCC of head and neck origin revealed abnormal migration patterns in one of the examined exons of *p53* in five of six lines tested. As shown in Fig. 1, 5 of 6 SCC exhibited polymorphic bands in exons 4, 5–6, and 8–9, compared to a normal control, human placental DNA.

To investigate specific alterations in sequences of *p53* using the genomic DNA from the six SCC, amplified fragments (exons 4–9) were analyzed by direct sequencing (Fig. 2). Three of these were found to result in a missense mutation, and two in deletions involving exons 4, 6, and 8, in the regions of previously reported mutational hot spots (11). Interestingly, two RR-SCC, JSQ-3 and SCC-35, contain two point mutations (at codons 72 and 298) and one (at codon 273) known as one of

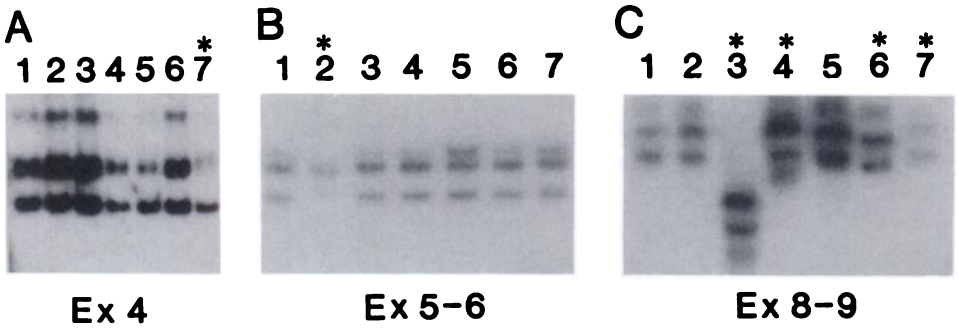

Fig. 1. SSCP analysis on a nondenaturing polyacrylamide gel. Each amplified fragment containing exons (*Ex*) 4–9 of *p53* was analyzed at 30 W for 6–8 h on a 6% polyacrylamide gel in the absence or presence of 10% glycerol. *A*, exon 4; *B*, exons 5–6; *C*, exons 8–9. *Lane 1*, human placenta; *Lane 2*, SQ-38; *Lane 3*, SCC-9; *Lane 4*, SQ-9G; *Lane 5*, SQ-20B; *Lane 6*, SCC-35; *Lane 7*, JSQ-3. *, samples showing different mobilities.

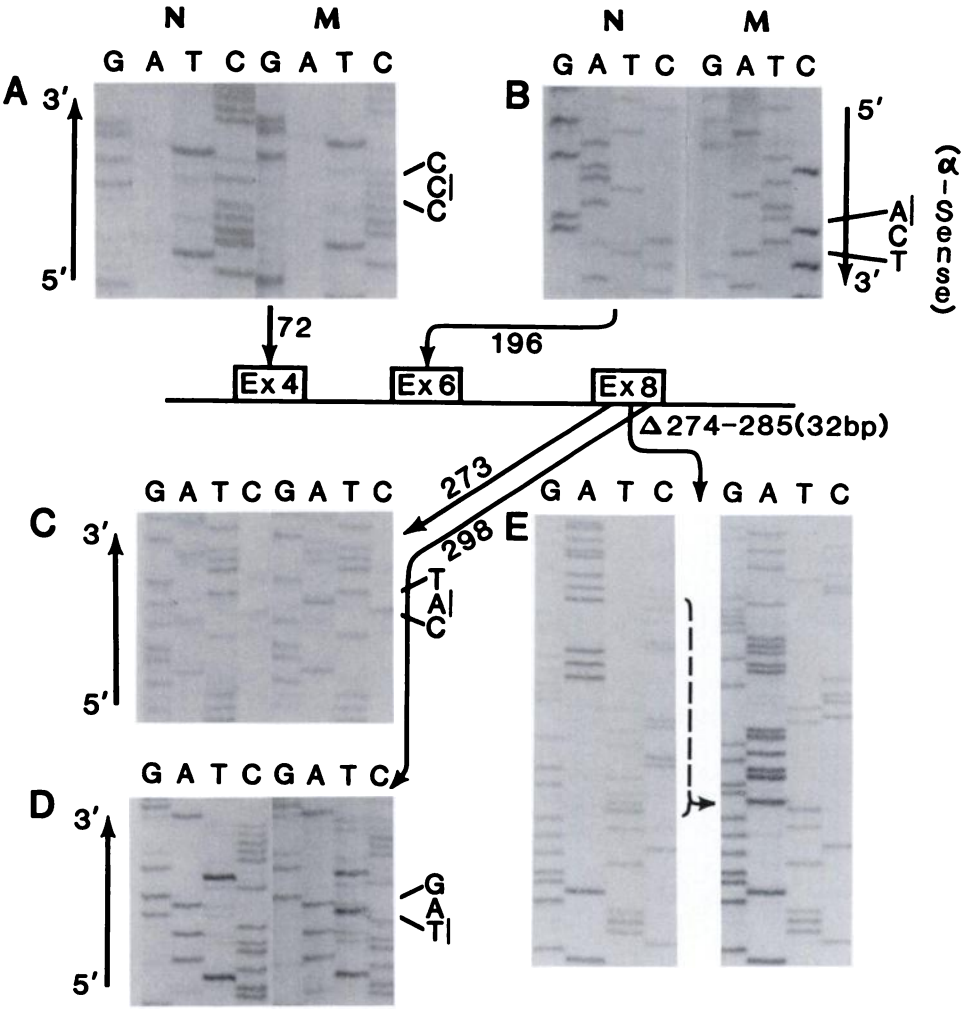

Fig. 2. Sequence analysis of *p53* genomic DNA. PCR-amplified fragments from the *p53* locus were directly sequenced without subcloning. Mutated sequences were compared to a control DNA containing normal sequences of *p53*. Each sequence is read according to indicated orientation 5′→3′. Mutations: *A*, at codon 72 (G→C) in JSQ-3; *B*, at codon 196 (G→A) in SQ-38; *C*, at codon 273 (G→A) in SCC-35; *D*, at codon 298 (G→T) in JSQ-3; *E*, 32-base pair (*bp*) deletion between codons 274 and 285 in SCC-9.

Table 1 *Alterations in p53 in radiation-sensitive and radiation-resistant squamous carcinoma cell lines*

Cell line	RS/RR[a]	Immuno-staining	PCR/sequencing		Amino acid substitution
			Codon	Mutation	
SQ-20B	RR	±			
SCC-35	RR	+	E8, 273	G→A	Arg→His
JSQ-3	RR	−	E4, 72	G→C	Arg→Pro
		−	E8, 298	G→T	Glu→stop
SQ-38	RS	−	E6, 196	C→T	Arg→stop
SCC-9	RS	−	E8, 32 base pairs	Deletion	Frame-shift
SCC-9G	RS	−	E8, 271 base pairs	Deletion	Frame-shift

[a] RS, radiation sensitive; RR, radiation resistant; +, positive; −, negative; ±, heterogeneous staining.

hot spot codons (about 30% of missense mutations), whereas three RS-SCC, SQ-38, SCC-9, and SQ-9G, had one point mutation at codon 196, with the deletion between codons 274 and 285 and a deletion at codon 271, respectively. Table 1 summarizes the observed *p53* mutations in these cell lines. No specific pattern of mutations was revealed, but three resulted in the substitution for an arginine residue.

Next, an immunocytochemical analysis was performed to examine the expression and the location of the mutated p53 protein in these cells. Interestingly, the three cell lines characterized as RR in Fig. 3, *C–E*, showed either a positive immunostaining or one with a heterogeneous pattern, whereas none of RS cell lines in Fig 3, *F–H*, showed positive staining. Only SCC-35 cells showed a strong nuclear staining with the p53 antibody (pAb-2) as intense as a positive control, MDA 231, a breast cancer cell line known to contain a point mutation in the *p53* gene at codon 280.

Discussion

Mutations in *p53* are among the most common genetic alterations in human cancers (11). Both inherited and sporadic forms of malignancies have been found with *p53* mutations (14). Elevated *p53* expression in squamous cell carcinoma of the head and neck (associated with a history of heavy smoking) have been observed in 67% of 73 patients, using two independent antibodies (17). There was no apparent correlation in the expression of *p53* to prior treatment or clinicopathological

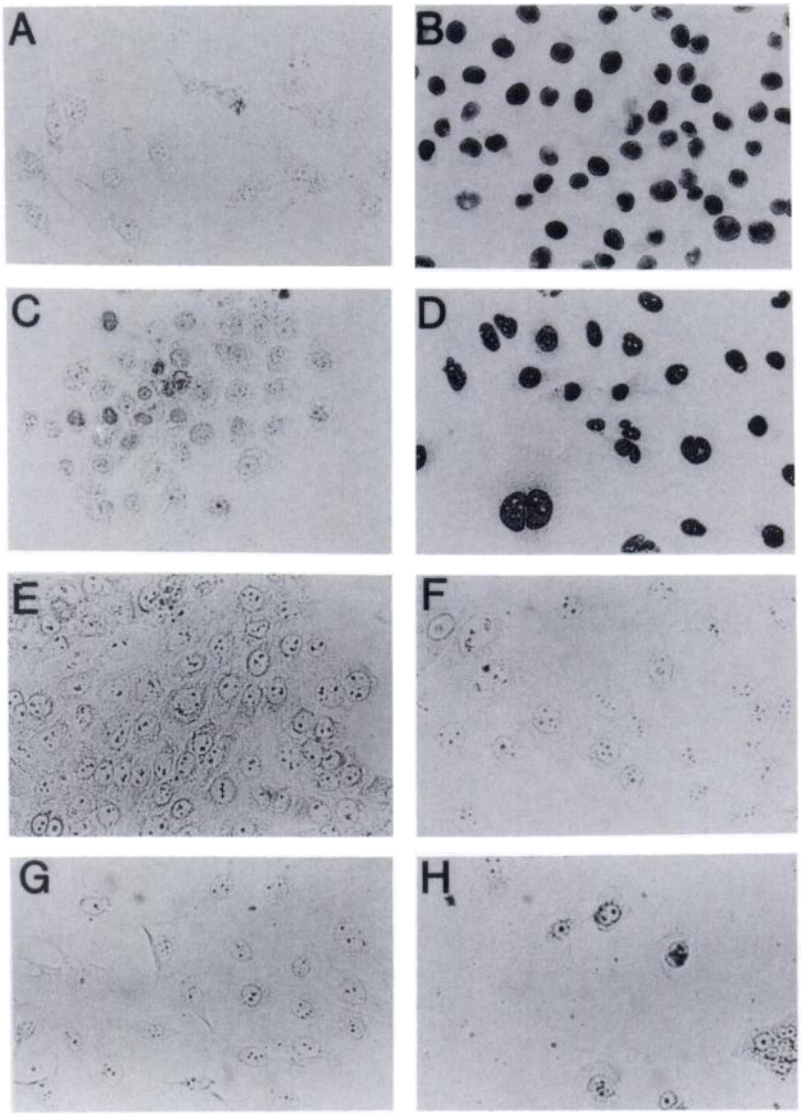

Fig. 3. Immunocytochemistry of *p53*. Cells were fixed in cold methanol for 10 min. *p53* was detected with monoclonal anti-*p53* antibody (PAb-2 from Oncogene Science) and visualized with biotinylated goat anti-mouse immunoglobulin followed by immunoperoxidase staining. *A*, NIH/3T3 (a negative control); *B*, MDA 231 (a positive control); *C*, *E*, and *G* (RR), SQ-20B, SCC-35, and JSQ-3, respectively; *D*, *F*, and *H* (RS), SQ-38, SCC-9, and SQ-9G, respectively.

parameters. *p53* has been implicated in cell cycle regulation (11), interaction with other regulatory proteins (18), and oncogenes (19).

Recently, Kastan *et al.* (20) have reported that *p53* may play a role in cellular response to γ-radiation damage. Cells that either lack *p53* gene expression or overexpress a mutant *p53* do not exhibit a G_1 arrest, but G_2 arrest is unaffected. This suggests that wild-type *p53* may be involved in DNA synthesis inhibition following radiation damage of DNA and provide a cell cycle "check point" (21). The fidelity of DNA repair during cell cycle arrest may play a role in the capacity of cells to tolerate radiation injury and therefore have an impact on radiation sensitivity. In this study, we investigated alterations of the *p53* gene and correlated these to the response of cells to ionizing radiation by analyzing the *p53* gene in six human SCC cell lines characterized as RS or RR.

Our results are consistent with previously reported observations indicating that SCC of head and neck origin frequently contain mutated *p53*. Four point mutations and two deletions were found, resulting in three substitutions for the arginine residue. Gusterson *et al.* (22) have studied expression of *p53* in 47 SCC of head and neck origin, observing 16 (34%) staining positive for the protein. They observed mutations in exons 5 and 7. In this study mutations were found in exons 4, 6, and 8.

The mutations observed in this sampling of head and neck SCC cell lines are compatible with previous reports showing a high frequency of the mutations of missense types. Two cell lines, SCC-9 and SQ-9G, contained deletions: (*a*) 32 base pairs from the third nucleotide of codon 274 to the first nucleotide of codon 285 in SCC9; and (*b*) 1 base pair at codon 271 in SQ-9G, resulting in a frame shift. These particular cell lines were sensitive to ionizing radiation.

The genetic alterations that may lead to modification of responses of mammalian cells to killing by ionizing radiation have not yet been identified, although the activation of several oncogenes has been implicated to be associated with resistance to ionizing radiation (7, 9, 10). Our data show that five (three RS and two RR) of six SCC cell lines contain the altered *p53* gene, suggesting that *p53* is not directly correlated to modification of the response of cells to ionizing radiation.

Acknowledgments

We would like to thank Dr. S. Paik for advice on immunostaining assay and Dr. Richard Schlegel for critical review of the manuscript.

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