MUTATION OF P53 IN HEAD AND NECK SQUAMOUS CELL CARCINOMA CORRELATES WITH BCL-2 EXPRESSION AND INCREASED SUSCEPTIBILITY TO CISPLATIN-INDUCED APOPTOSIS

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Abstract: Background. The p53 protein, a well-known tumor suppressor that functions primarily as a transcription factor, initiates cell cycle arrest and apoptosis after genotoxic stress. The antiapoptotic regulator Bcl-2 is a downstream modulator of p53-induced apoptosis. Loss of function of the p53 tumor suppressor through mutation is an important event that contributes to cellular transformation. Mutation of p53 is one of the most common genetic alterations in squamous cell carcinomas of the head and neck (SCCHN). We hypothesized that p53 mutation is associated with Bcl-2 expression and susceptibility to apoptosis in SCCHN.

Methods. Exons 5 to 8 of the p53 gene were sequenced in 22 SCCHN tumor samples and correlated with the Bcl-2 expression and apoptosis rates in these tumors. In addition, a Bcl-2–expressing SCCHN cell line, UMSCC74B, was stably transfected with a temperature-sensitive mutant p53 construct, and Bcl-2 expression levels were examined at the mutant and the wild-type temperatures.

Results. Bcl-2 expression was inversely correlated with wild-type p53 status in SCCHN tumors (p = .05). Furthermore, there was a modest increase (1.7-fold) in apoptosis in the wild-type p53 tumors compared with mutant p53 SCCHN. Immunoblotting of UMSCC74B cells stably transfected with the temperature-sensitive mutant p53 construct demonstrated that shifting these cells to the mutant p53 temperature (39.5°C) resulted in decreased expression of Bcl-2 compared with levels in cells grown at the wild-type p53 temperature (32.5°C). Further investigation showed that SCCHN cells expressing predominantly mutant p53 and decreased Bcl-2 were more susceptible to cisplatin-induced apoptosis than vector-transfected controls (p < .0001).

Conclusions. These results suggest that p53 mutation directly modulates Bcl-2 expression and therefore susceptibility to chemotherapy-induced apoptosis in SCCHN cells in vitro.

Keywords: p53; Bcl-2; apoptosis; head and neck cancer
The p53 protein, which functions primarily as a transcription factor, is a well-known tumor suppressor that has two major roles: (1) cell cycle arrest, and (2) initiation of apoptosis after genotoxic stress. Loss of function of the p53 tumor suppressor, through mutations in key regions of the p53 gene, is an important event that contributes to cellular transformation.\(^1\) Mutations of p53 have been shown, in vitro, to be quite devastating to the ability of the cell to regulate growth and cell cycle progression. For example, in a nasopharyngeal carcinoma cell line that has one wild-type p53 allele and a point mutation in the other allele, the mutant p53 allele acted dominantly to control transcriptional activity, govern cell growth, and induce preneoplastic progression.\(^2\) P53 mutation contributes to tumor formation in vivo as demonstrated in a nasopharyngeal carcinoma xenograft model, in which human nasopharyngeal cells transfected with wild-type p53 gene were growth inhibited compared with tumors transfected with mutant p53.\(^3\)

The significance of p53 mutational status in patients with cancer has been assessed by correlating p53 expression levels in tumors with pathologic features and clinical outcome. Overexpression of p53, as demonstrated immunohistochemically in patient tumor samples, is generally thought to represent mutant p53, because mutant p53 accumulates in the cell, whereas wild-type p53 is rapidly degraded. One study examined 149 squamous cell carcinoma tumors of the larynx and found that 50% of the tumors demonstrated p53 overexpression.\(^4\) Others have found that p53 overexpression was associated with reduced survival of patients with squamous cell carcinoma of the head and neck (SCCHN).\(^5,6\) The crucial role of p53 mutation in many epithelial cancers, including SCCHN, suggests that studying the downstream targets of p53 alteration will advance our understanding of tumorigenesis.

Apoptosis, the process of programmed cell death, is mediated either by mitochondria or cell surface receptors.\(^7\) The mitochondrial pathway is mediated by the Bcl-2 family of proteins. Bax, a proapoptotic regulator in the Bcl-2 family, is one of several genes that undergoes initiation of transcription as a result of p53 activation and DNA binding.\(^8,9\) The expression levels of the antiapoptotic regulator Bcl-2 can be modulated by the binding of activated p53 to a negative response element in the 5'-untranslated region of the Bcl-2 gene.\(^10\) The ratio of Bcl-2 and Bax proteins is thought to contribute to the relative susceptibility of cells to stimuli that induce apoptotic cell death.\(^11\) Specifically, p53-induced apoptosis can be blocked by forced overexpression of Bcl-2 protein.\(^12-14\) Therefore, it is possible that Bcl-2 and Bax may be important components of apoptosis downstream of p53 mutaion.

This study was undertaken to test the hypothesis that mutation of the p53 gene in SCCHN is associated with modulation of Bcl-2 expression chemotherapy-induced apoptosis. Exons 5 to 8 of the p53 gene were sequenced in 22 SCCHN tumors, where the expression status of Bcl-2 and the rate of apoptosis had been previously determined. To further explore the mechanism of p53 and Bcl-2 interaction, we stably transfected an SCCHN cell line (UMSCC74B) containing wild-type p53 with a temperature-sensitive p53 mutant construct designed to express mutant p53 at 39.5°C and wild-type p53 at 32.5°C.\(^15,16\) Stably transfected cells grown at the wild-type and mutant p53 temperatures were analyzed for apoptotic regulatory protein expression and susceptibility to cisplatin-induced apoptosis.

**MATERIALS AND METHODS**

**Tissue Specimens and Cells.** Tumor tissue was obtained from 22 patients with SCCHN undergoing primary surgical resection for head and neck cancer at the University of Pittsburgh Medical Center from 1994 to 1996, following approval by the Institutional Review Board. The SCCHN cell line UMSCC74B was obtained from Dr. Thomas Carey (University of Michigan) and was used for the temperature-sensitive mutant p53 experiments.

**Sequence Analysis.** The 22 formalin-fixed, paraffin-embedded SCCHN tumors were deparaffinized in xylene. DNA was then digested, extracted, and precipitated with ethanol as described.\(^17\) From the DNA harvested, a 1.8-kilobase segment of the p53 gene containing exons 5 to 8 was amplified by polymerase chain reaction (PCR) as described.\(^18\) Sequences of exons 5 to 8 of the p53 genes were determined as described.\(^16\) The sequences corresponding to the wild-type p53 were differentiated from mutant p53, and the location and type of mutation (missense mutations or deletions) were determined. All mutations were confirmed by a second PCR reaction followed by recloning and resequencing.
Plasmid Construct and Transfection. The murine temperature-sensitive p53 construct, LTRp53 cG(val), was generously provided by Dr. A. Levine. Cells transfected with this plasmid have been shown to predominantly express the mutant form of the protein at 39.5°C and the wild-type protein at 32.5°C. The construct was co-transfected with pSV2-neo into the SCCHN cell line, UMSCC74B, in a ratio of 50:1 with the GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA). Cells stably transfected with pSV2-neo alone were used as a negative control; two different vector-transfected control clones were isolated (Neo 1 and 2). Transfected cells were grown in media supplemented with G418 (0.2 μg/mL, Gibco, Invitrogen, Carlsbad, CA). Thirteen G418-resistant temperature-sensitive clones (TS 1–13) were isolated, expanded, and maintained under G418 selection pressure.

Immunoblotting and Densitometry. Cells were maintained at the wild-type (32.5°C) or mutant (39.5°C) temperatures for at least 24 hours before analysis and then lysed in a detergent solution containing protease inhibitors. This cell lysis solution was prepared by combining 10 mL of detergent solution, prepared as previously described, with one pulverized protease inhibitor tablet (Roche Diagnostics, Mannheim, Germany). Protein levels were determined by the Bio-Rad Protein Assay method (BIORAD Laboratories, Hercules, CA). Forty micrograms of total protein was then resolved on a 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by semi-wet blotting. Filters were blocked with phosphate-buffered saline (PBS) containing 5% dry milk powder and 0.2% Tween-20 overnight at 4°C or for 1 hour at room temperature, rinsed with 50 mM TRIS (pH 7.4), with 1% dry milk powder, 0.9% NaCl, and 0.5% Tween 20) three times for

Table 1. p53 sequencing results for 22 tumors squamous cell carcinoma of the head and neck.

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Abbreviations: SCCHN, squamous cell carcinoma of the head and neck; M, mutant p53; W, wild-type p53; +, positive Bcl-2 staining; –, negative Bcl-2 staining.
15 minutes, and incubated for 1 hour with a mouse anti-human bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-human bcl-2 antibody (Santa Cruz Biotechnology). Membranes were then incubated with a horseradish peroxide–conjugated secondary antibody, developed with a Luminol Reagent (Santa Cruz Biotechnology), and exposed to film. Quantitation of the signal was performed by use of a Molecular Dynamics Personal Densitometer SI and ImageQuant software (Image Product International, Chantilly, VA).

Annexin V Binding Assays. After treatment with cisplatin for 24 hours, the p53-transfected cells were detached by trypsinization, counted and pelleted (1000 revolutions per minute [rpm] for 5 minutes). Cell pellets were washed once with PBS (pH 7.4) and resuspended in 100 μL annexin V binding buffer (10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic (HEPES) acid, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂); 5 × 10⁵ cells were transferred to a 12-× 75-mm tube and 5 μL of Annexin V-Cy3 (BioVision Research Products, Mountain View, CA) was added per tube and allowed to incubate at room temperature for 15 minutes in the dark. Then the stained cell suspension was placed on the slides and covered with coverslips. The membrane of apoptotic cells is stained bright orange with this method when analyzed with a fluorescence microscope. The ratio (percentage) of apoptotic to total cells (apoptotic plus nonapoptotic cells) was calculated for each high-power field. For each treatment condition, 5 to 10 high-power fields were quantitated on each section.

Statistics. The association between p53 mutational status and the presence or absence of the expression of Bcl-2 in SCCHN tumors was determined with the Fisher’s exact test. The difference in rates of apoptosis by p53 status was analyzed with the Wilcoxon test. The proportion of apoptotic cells affected by cisplatin and/or transfection with the temperature-sensitive vector was analyzed by two-way and three-way factorial analysis of variance. The raw data were first transformed by
taking the square root of the arcsine of the proportion of observed apoptotic cells.

RESULTS

p53 Mutational Status in SCCHN Tumors. Mutant p53 status has been extensively examined by immunohistochemistry in SCCHN on the basis of the assumption that overexpression of p53 represents mutated p53 because of the longer half-life of the mutant protein.4 To more specifically determine the p53 status in primary SCCHN tumors, DNA was isolated from 22 SCCHN tumors, and exons 5 to 8 of the p53 gene were directly sequenced. The type, frequency, and localization of the p53 mutations in these tumors are summarized in Figure 1. Heterogeneity of the p53 mutational status was detected in a single SCCHN tumor, so the tumor was designated as wild type only if no mutant sequences could be identified. As shown in Table 1, 13 (59%) of 22 SCCHN tumors demonstrated mutant p53 sequences, whereas nine (41%) of 22 tumors were wild-type p53. This relatively high incidence of p53 mutation in SCCHN tumors is consistent with prior reports that used immunohistochemistry.4

Association of Mutant p53 with Decreased Apoptosis and Bcl-2 Expression in SCCHN Tumors. p53 is an important mediator of apoptosis after genomic damage. Activated p53 has been found to negatively regulate expression of the antiapoptotic protein Bcl-2 and positively regulate expression of the proapoptotic protein Bax.9,10 Bcl-2 and Bax proteins are thought to work cooperatively to control apoptosis11 and may be instrumental in p53-mediated apoptotic cell death. To test this hypothesis, the p53 mutational status was correlated with Bcl-2 expression as determined by immunohistochemistry in the same 22 SCCHN tumors. As shown in Table 1 and Figure 2, eight (88.9%) of nine wild-type p53 tumors were negative for Bcl-2, whereas only six (46.2%) of 13 mutant p53 tumors were negative for Bcl-2 (p = .05). The p53 status of the SCCHN tumors was also correlated with the percentage of apoptotic cells in these same tumors as determined by staining for DNA fragmentation.21 As shown in Figure 3, the wild-type p53 SCCHN tumors were associated with a 1.73-fold increase in the percentage of apoptotic cells compared with the mutant p53 tumors. In addition, Bax expression was examined by immunostaining in the tumor samples and found to be expressed ubiquitously in the tumor specimens (data not shown). These results suggest an association of increased Bcl-2 expression and decreased apoptosis with mutant p53 in SCCHN tumors.

Bcl-2 Expression Is Modulated by p53 Mutation in a SCCHN Cell Line. To more directly test the hypothesis that Bcl-2 expression is regulated by p53 mutation, an SCCHN cell line, UMSCC74B, was...
stably transfected with a murine temperature-sensitive p53 construct, LTRp53 cG(val). This construct contains a missense mutation in the region of the p53 gene that encodes the DNA-binding domain. The protein expression product of this p53 construct alters its conformation during temperature shift. At 32.5°C, the p53 protein can enter the nucleus, where it can initiate transcription, similar to wild-type p53. In contrast, at 39.5°C, the p53 protein is restricted to the cytoplasm. The UMSCC74B cell line was selected for transfection because it expresses Bcl-2 and contains wild-type p53 (T. Carey, personal communication, March 2002). Clones were screened for p53 expression at 37°C, and those cells expressing total levels of p53 at least 30% greater than pSV2-neo transfected control cells were considered to be stably transfected with the LTRp53cG(val) construct (data not shown). As shown in Figure 4, there was a decrease in the level of Bcl-2 protein expression in representative clones (TS 4 and TS 13) with induction of mutant p53. However, there was no change in the levels of Bax expressed in the cells maintained at the mutant temperature compared with the wild-type temperature beyond that observed with temperature shift alone.

**p53 Mutation Increases Susceptibility to Cisplatin-induced Apoptosis.** Platinum-containing agents, including cisplatin, are among the most commonly used chemotherapy agents in the treatment of patients with SCCHN. Cisplatin has been shown to exert its antitumor effects, at least in part, by inducing apoptosis. To determine whether modulation of p53 mutational status was correlated with susceptibility to apoptosis, cells transfected with the p53 temperature-sensitive vector were grown at the wild-type and mutant p53 temperatures, treated with cisplatin (20 μM), and assessed for apoptosis by annexin 5 binding 24 hours later. As shown in Figure 5, cells expressing predominantly mutant p53 demonstrated a higher rate of cisplatin-induced apoptosis than vector-transfected control cells (p < .0001). The increased apoptosis detected at the mutant temperature is consistent with the decreased expression of the antiapoptotic regulatory protein Bcl-2 in the same cells.

**DISCUSSION**

The results of this study suggest that Bcl-2 expression is correlated with mutant p53 status in SCCHN tumors. Furthermore, there was a modest increase in apoptosis in the wild-type p53 tumors compared with mutant p53 tumors. In contrast, induction of mutant p53 in SCCHN cells in vitro resulted in decreased expression of Bcl-2 compared with cells grown at the wild-type p53 temperature and increased susceptibility to cisplatin-induced apoptosis. This divergence between the data obtained in the transfected SCCHN cell line likely reflects the complex biology of p53 in the regulation of apoptosis in this cancer. Hence, modulation of Bcl-2 expression may be one of the mechanisms by which mutation of p53 contributes to tumor formation.

A link between p53 mutation and the antiapoptotic regulatory protein Bcl-2 has been demonstrated previously in several types of human cancer. For example, in a leukemia cell line stably transfected with the same temperature-sensitive construct used in this study, RNA expression levels of Bcl-2 decreased on temperature shifting. Also, in transitional cell carcinoma of the bladder, a Bcl-2/Bax ratio greater than 1 and p53 gene mutation were significantly associated with each other and closely correlated with the early relapse after treatment. Bax expression was found to be ubiquitous in both the SCCHN tissues and the transfected cells. In addition, modulation of p53 mutational status did not influence Bax expression levels. In one study examining SCCHN
tumors, most of the Bcl-2–positive cancers (70%) were also p53 mutants. A potential mechanistic explanation is supported by the identification of a negative response element in the 5'-untranslated region of the Bcl-2 gene that is modulated by p53. Therefore, p53 mutation and subsequent loss of function would lead to increased Bcl-2. This is, indeed, what we observed in the human SCCHN tumor specimens. However, tumors are often heterogeneous with respect to p53 mutational status, and the immunohistochemical evaluation of tumors for Bcl-2 expression is not quantitative. The in vitro experiments were designed to provide more quantitative and mechanistic information regarding the expression of Bcl-2 after mutation of p53. In tissue culture, the expression of Bcl-2 was decreased when the cells were maintained at the mutant p53 temperature compared with the cells at the wild-type temperature. The biologic significance of this down-regulation of Bcl-2 expression was confirmed by the increased susceptibility to cisplatin-induced apoptosis in these cells. Several previous studies have reported a decrease in Bcl-2 expression with p53 mutation. For example, in invasive breast carcinoma, the presence of p53 mutation was associated with low expression levels of Bcl-2 and increased apoptosis. Also, in non-small-cell lung carcinoma, an inverse relationship was observed between p53 mutation and Bcl-2 overexpression. One possible explanation for the variable effects of p53 mutation on Bcl-2 expression could be that, depending on the type or location of the mutation, the mutated p53 protein may still retain some ability to negatively regulate Bcl-2 expression. In addition, the mutation in the temperature-sensitive construct had a missense mutation that produced a substitution of valine for alanine in position 135 of mouse p53. It is likely that murine p53 mutations are not equivalent to human p53 mutations. It is also possible that p53 regulation of Bcl-2 in cancer cells may be tumor type specific (ie, leukocyte vs SCCHN).

In response to DNA damage, p53 induces cell cycle arrest and apoptosis. The significance of p53 mutation in the abrogation of apoptosis, with subsequent contribution to tumor formation, is incompletely understood. The modest increase in apoptosis in tumors with wild-type p53 compared with tumors with mutant p53 is supported by findings in previous studies. For example, in oral cancer, the presence of mutant p53 protein showed a significant inverse correlation to the extent of apoptosis. Also, in studies of p53-deficient transgenic mice, loss of p53 was associated with decreased rates of cell death rather than increased rates of cell proliferation.

The primary implication of this study is that p53 mutation contributes to SCCHN progression possibly through modulation of Bcl-2. The clinical significance of the role of mutant p53 in tumor formation for patients with SCCHN has been supported by the early success of the experimental antitumor agent ONYX-015. ONYX-015 is an attenuated adenovirus that was designed to selectively target mutant p53. Antitumor results have been observed in phase I/III clinical trials in which patients with SCCHN were treated with ONYX-015. The combination of ONYX-015 treatment with chemotherapy has been shown to have an even stronger antitumor effect than either treatment alone in patients with SCCHN. The signaling pathways that result in this antitumor activity after mutant p53 degradation are not completely understood. This study was undertaken to explain the mechanism by which mutation of p53 contributes to tumor cell survival.

The sequencing and immunohistochemical data in this study implicate Bcl-2 in the dysregulation of p53-induced apoptosis because of mutation of p53. Further studies to explain the mechanism of p53-induced apoptosis will facilitate the design of specific therapeutics that target the molecules downstream of p53 activation and perhaps synergize with treatments that target mutant p53, such as ONYX-015, in patients with SCCHN.

REFERENCES