Activation of $p53$ Gene Expression in Premalignant Lesions during Head and Neck Tumorigenesis

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Abstract

With the goal of identifying a potential intermediate biomarker in the multistep process of head and neck cancer development, we conducted immunohistochemical analyses for $p53$ expression in 33 patients with head and neck squamous cell carcinomas whose tissue sections contained adjacent normal epithelium, hyperplastic, and/or dysplastic lesions. Fifteen of 33 (45%) squamous cell carcinomas of the head and neck expressed $p53$, but none of four normal control patients (cancer-free nonsmokers) expressed detectable $p53$ in oral mucosa specimens. To determine when $p53$ expression is initiated during head and neck tumorigenesis, we examined the normal and premalignant lesions adjacent to the tumors. Five of 24 (21%) samples of normal epithelium adjacent to tumors, 7 of 24 (29%) samples of hyperplasia, and 9 of 20 (45%) samples of dysplasia expressed $p53$. Quantitative image analysis demonstrated not only a gradual increase in the amount of $p53$ expression as tissue abnormalities progressed but also a topological change in expression. Whereas $p53$ expression, when present, was limited to the basal layer in normal epithelium adjacent to tumor, the expression of $p53$ expanded into the parabasal and superficial layers in hyperplasia and dysplasia. We conclude that $p53$ expression can be altered in very early phases of head and neck tumorigenesis. Thus, it may be an excellent candidate for risk assessment and may serve as an intermediate biomarker in chemoprevention trials.

Introduction

Head and neck cancer provides a unique model system for the study of tumorigenesis and development of biomarkers for two reasons: (a) tumorigenesis in the head and neck has been hypothesized to be a manifestation of “field cancerization,” the predisposition of an entire field of tissue to the development of multiple cancers through repeated carcinogenic insult to that field (1); (b) this malignant process is thought to require multiple steps (2-4), such that premalignant lesions often precede frank malignancy (5-7). An important component and one of the driving forces of the multistep tumorigenesis process may be the accumulation of genetic damage, the rate of which reflects the degree of staining from slide to slide, we attached a cell block section of progress toward malignancy (12). These important genetic events in the development of head and neck cancer are not yet well understood.

Alteration of the $p53$ gene is the most commonly implicated genetic event in a number of human solid tumors (2, 13-16). Mutation or increased expression of the $p53$ protein has also been reported in head and neck squamous cell carcinomas (17-19). However, few data are available on the frequencies of $p53$ expression in “normal” tissue adjacent to tumors and premalignant lesions during head and neck tumorigenesis (18). The $p53$ gene may be activated in certain stages of head and neck tumorigenesis, and as such it may serve as a regulatory marker in the process of tumorigenesis.

There is an important need to identify potential biomarkers of tumorigenesis that can be utilized on premalignant lesions to determine the level of risk for tumor development and/or to determine the effect of preventive intervention on the tissue at risk. Such biomarkers might reflect the effect of specific genetic events important for tumor development or they might be a detection of the genetic event itself. With the goal of possibly identifying a biomarker for the process of head and neck cancer development, we have studied $p53$ expression in head and neck squamous cell carcinomas and their adjacent premalignant lesions and normal epithelium. In particular, we wished to determine whether $p53$ expression increases prior to tumor development and, if so, how early during tumorigenesis this abnormality could be detected. This information could then be applied to assess tumor risk and response to preventive intervention in tissues at increased risk (e.g., leukoplakia) for the development of head and neck cancer.

Materials and Methods

Formalin-fixed, paraffin-embedded tumor specimens were obtained from patients with head and neck cancer whose tumors were surgically resected at the University of Texas M. D. Anderson Cancer Center in 1990 or 1991. Thirty-three specimens containing not only carcinomas but also adjacent normal tissue and premalignant lesions (i.e., hyperplasia or dysplasia) were selected for this study. Four biopsy specimens of oral mucous epithelium obtained from normal individuals (i.e., cancer-free nonsmokers) were used as normal controls. All specimens and hematoxylin-eosin-stained histological slides were reviewed by one pathologist (J. Y. R.) to identify normal, hyperplastic, dysplastic, and tumor areas according to criteria described previously (20). Sections 4 µm thick were mounted on aminoalkylsilane-coated slides (Histology Control Systems, Glen Head, NY) for immunohistochemical analysis, and sections 8 µm thick were mounted on glass slides for microdissection for further studies.

After identification of premalignant and carcinoma areas, immunohistochemical staining was applied on the adjacent sections. A monoclonal anti-$p53$ antibody (clone D07; BioGenex, Inc., San Ramon, CA), which has been shown to react with both wild-type and mutant forms of the $p53$ protein, was applied to paraffin-embedded sections (21). Immunohistochemical analysis involved a modification of the avidin-biotin-immunoperoxidase method described previously (22).

To overcome the quantitative complications associated with variations in degree of staining from slide to slide, we attached a cell block section of

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Fig. 1. Immunohistochemical demonstration of p53 expression in head and neck tumorigenesis. A, a few cells in normal epithelium adjacent to tumor expressing p53; B, transitional areas within normal epithelium where the level of p53 expression increased dramatically from one area to another (arrow); C, increased level of p53 expression in hyperplasia; D, even higher level of expression of p53 in dysplasia, where p53 expression expanded into parabasal and superficial layers; E, F, a squamous cell carcinoma in which the level of p53 expression is low in one area (E) and increased in another area (F).

Paraffin-embedded A431 cells to each slide; these cells are known to express a mutated p53 gene (CGT to CAT at codon 273). Thus, the A431 cells could act as an internal control for each head and neck tumor section and different sections could be quantitatively compared.

To assess the amount and distribution of p53 expression on the immunostained slides, we quantitated the expression of the p53 gene product on digitized images using the Magiscan Image Analysis System (Joyce-Loebl, Ltd., Dukesway, England) attached to a Nikon light microscope with a controller-driven stage. The quantitative evaluation involved visual identification of premalignant lesions of the epithelial layer and tumors and manual circling of each nucleus with a light pen on particular stained areas. Each circled region was characterized by a measurement of total integrated density over the nucleus, the area of the measured nuclear region, and its relative coordinates. The specific intensity of each region was calculated as the integrated intensity over each nucleus divided by the area of that nucleus and was normalized to that measured in the internal control, A431 cells. Since the location of each object measured was recorded in relative coordinates, the pattern of p53 expression could be displayed as a two-dimensional array. This allowed the visualization of the topological distribution of p53 expression in each tissue section.

To determine whether a high degree of p53 expression was associated with p53 mutation, we identified the selected area by direct comparison with p53-immunostained slides in the unstained tissue under the microscope. After paraffin sections were dewaxed for 10 min in two changes of xylene and air dried, selected areas of tissue were rehydrated with sterile water and dissected from the glass slides using a 23-gauge needle and syringe under the dissecting microscope. The dissected tissue fragments were placed into 100 μl of deionized water, and DNA was released by boiling in a water bath for 10 min. The residual tissue was pelleted, and an aliquot of supernatant was taken for PCR using primers described previously (14). To ensure consistency and reproducibility and to eliminate PCR artifact, all assays were performed a minimum of three times using separate PCR.

A human non-small cell lung cancer cell line, WTH460, has been known to be a wild type p53 expression and used as a control for SSCP. Both tumor and normal areas of the same section were examined for exons 5–8 of p53. Analysis and direct sequencing of PCR products were performed essentially as described previously (14, 23).

**Fig. 2.** Demonstration of frequency (%) of p53 expression in head and neck tumorigenesis. The frequency of detectable p53 expression increased as tissues progressed from normal epithelium adjacent to tumor (ANL), to hyperplasia (HYP), to dysplasia (DYP), and to squamous cell carcinomas (SCC).

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3 The abbreviations used are: PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.
Results

To identify alterations in p53 expression in premalignant lesions and squamous cell carcinomas in the head and neck area, we performed immunohistochemical analysis using an anti-p53 monoclonal antibody on paraffin-embedded sections. In the normal oral mucosal epithelium controls, cells expressing p53 were infrequent and restricted to the basal layer (Fig. 1A). In contrast, the histologically normal epithelium adjacent to p53-positive tumors showed more cells expressing significant p53 levels (Fig. 1B). Interestingly, as shown in Fig. 1C, one area in the adjacent normal epithelium showed an abrupt increase of p53 expression without histological change. As the tissue progressed to hyperplasia (Fig. 1D), dysplasia (Fig. 1E), and squamous cell carcinoma, the frequencies of p53-positive cells increased continuously. In squamous cell carcinomas, p53 expression could be very heterogeneous; whereas one area expressed no detectable p53, another area of the tumor had a marked increase of p53 expression (Fig. 1F).

Overall, 15 of 33 (45%) samples of squamous cell carcinomas of the head and neck expressed detectable p53. Interestingly, in many cases, p53 expression could be detected early in the multistep tumorigenesis process. In particular, 5 of 24 (21%) samples of normal epithelium adjacent to tumors, 7 of 24 (29%) samples of hyperplasia, and 9 of 20 (45%) samples of dysplasia expressed p53. (Fig. 2.) Increased p53 expression in premalignant lesions observed only in those specimens that exhibited detectable p53 expression on the tumor except two premalignant lesions, where positive p53 expression was observed in one hyperplasia and one dysplasia; but there were no p53 expression in their squamous cell carcinomas.

We also assessed the amount and distribution of p53 expression on immunostained slides using an image analyzer attached to a computer-assisted microscopic image system. The location of each nucleus scored was represented on a two dimensional display as a dot, the color of which was indicative of its normalized specific intensity. Since all samples contained identical internal controls, different
samples could be quantitatively compared one to another. A pseudo-color range from deep blue at lowest intensity to white at highest intensity (as indicated in the color bar at the bottom of Fig. 3) was used to indicate the level of p53 expression. Only a few cells of the normal epithelium adjacent to the tumor expressed p53, and these were again restricted to the basal layer (Fig. 3A). Of particular note, in certain cases, some areas of normal epithelium adjacent to the tumor showed a dramatic increase in p53 expression (Fig. 3B). As the tissue progressed toward carcinoma, not only the number of cells expressing p53 increased but also the level of expression in each cell. Furthermore, p53 expression expanded into the parabasal and superficial layers, particularly in hyperplastic (Fig. 3C) and dysplastic lesions (Fig. 3D). Interestingly, in early lesions, p53 expression appeared to be somewhat controlled since there appeared to be a down-regulation of expression away from the basal and parabasal layers. This level of regulation seemed to be lost in tumors. Nevertheless, by image analysis, we also demonstrated a topologically heterogeneous display of expression of this gene in some squamous cell carcinomas where one area expressed no p53 (Fig. 3E), whereas another area expressed p53 very strongly (Fig. 3F). This phenomenon occurred in 8 of the 15 (53%) p53-positive tumors. The normalized specific intensities of p53 expression in positive areas in positive cases (arbitrary units, mean ± SD) were: normal epithelium adjacent to tumor, 0.028 ± 0.025; hyperplasia, 0.053 ± 0.012 (P = 0.038); dysplasia, 0.054 ± 0.035 (P = 0.032); and squamous cell carcinoma, 0.207 ± 0.100 (P < 0.001) (Fig. 4). During tumorigenesis process the highest increment of p53 expression was from dysplasia to squamous cell carcinoma.

To determine whether tissue expression of p53 was associated with a gene mutation, we performed a combination of PCR-SSCP and direct genomic sequencing on one representative case that had a high level of p53 expression by immunohistochemical staining of the tumor but not in its adjacent normal epithelium. We screened exons 5 through 8 of the p53 gene in both adjacent normal epithelium and squamous cell carcinoma obtained by microdissection. An electrophoretic mobility shift between the control and the target samples was pathognomonic of a mutation and was detected by SSCP analysis. No mobility shift was found by SSCP in its adjacent normal epithelium or in control cell line that did not express p53 (Fig. 5A). Direct sequencing of both control H460 cells and the adjacent normal epithelium showed a wild-type p53 which had 10 intact base pairs (GCGTCCGCGC) at codon 174 (Fig. 5B, arrows). In C, the 10 base pairs have been deleted in the tumor sample (T) (arrows). This finding confirmed that there was a mutation where we observed a large mobility shift by SSCP and this resulted in increased levels of detectable p53 in the tumor.

Discussion

The purpose of this study was to identify a potential intermediate biomarker in the multistep process of head and neck carcinogenesis. Our finding that 15 of 33 (45%) samples of squamous cell carcinomas of head and neck over expressed p53 is similar to those of others (17–19). One important observation of this study was that p53 expression could be very heterogeneous within the same tumor sample. This finding strongly suggests the tumor heterogeneity even in the same tumors and raises the question of tumor clonality. To address this question in detail, we are currently analyzing p53 gene sequence using tissue specimens microdissected from the areas in which p53 is expressed differentially within the tumor specimen.

A second important observation in this study was that 21% of the samples were shown to express p53 in histologically normal epithelium adjacent to the tumor. This represented one-half of the cases that eventually showed p53 expression in the tumor. Thus histologically normal epithelium which had already been exposed to tobacco and/or alcohol had a significant degree of p53 expression, whereas normal control epithelium taken from cancer-free, nonsmoking individuals did not express p53. These results suggest that alterations of p53 expression can occur in the very early stages of head and neck tumorigenesis, particularly in carcinogen-exposed epithelium. This observation also supports the concept of field cancerization, whereby
the whole epithelium accumulates genetic damage over time and is at increased risk for developing multiple independent lesions that may become malignant. It should be kept in mind that all samples studied were at 100% risk of tumor development. Finding of p53 expression in normal epithelium which is not associated with tumors but has been exposed to tobacco and/or alcohol would be significant, because it would indicate that this epithelium is at increased risk for transformation to carcinoma. The determination of p53 expression, therefore, may be a useful biomarker for assessing the potential for development of tumors in a high-risk population.

In premalignant lesions adjacent to the tumors, p53 expression increased continuously, not only in the incidence but also in the amount of p53 expressed, as the tissue progressed from normal to hyperplasia to dysplasia to squamous cell carcinoma. This finding supports the concept of the multistep process of tumorigenesis in the head and neck region. There is evidence that in several other human tumors, [e.g., colon (2), ovarian (24), and thyroid cancer (25)] activation of p53 is a relatively late event associated with tumor progression. The timing of p53 alteration may vary among tumors; in tumors of the aerodigestive tract, such as those of the esophagus (14, 26) or lung (27), p53 alteration may occur in the early stages of tumorigenesis. Our current study confirmed the most recent study in which expression of mutated p53 occurred in distant epithelia of head and neck cancer patients (28). More importantly, however, we addressed the timing of p53 expression in this multistep tumorigenesis where p53 protein can accumulate not only in dysplastic lesions but also in histologically normal and hyperplastic lesions adjacent to the tumor during the process of head and neck tumor development. Whether this increased p53 expression in premalignant lesions is a result of p53 mutation or is a cellular checkpoint reaction to toxic exposure remains to be determined.

The consequence of p53 alteration in head and neck tumorigenesis has not yet been explored. One possibility is that p53 alteration can enhance genomic instability and thus augment the accumulation of subsequent genetic events necessary for tumor development (29). To visualize the accumulation of genetic alterations during head and neck tumorigenesis, we have recently studied chromosomes 7 and 17 by in situ hybridization using chromosome-specific centromeric DNA probes. We found that histologically normal epithelium adjacent to tumors showed polymorphisms for both chromosomes (30). Moreover, the frequency of cells with polyploidy increased as the tissue changed from histologically normal epithelium to hyperplasia to dysplasia to cancer (30). A similar observation was made for proliferative dysregulation in head and neck tumorigenesis where the expression of proliferating cell nuclear antigen increased gradually as tissue progressed through the tumorigenic stages (31). To determine whether p53 alterations enhance genetic change in head and neck cancer development, we are currently studying the relationship between p53 mutation and chromosomal changes in this same multistep model.

The presence of altered p53 in histologically normal epithelium adjacent to tumor and the increased frequency of p53 expression as the tissue progressed toward malignancy suggest that p53 alteration might be a useful biomarker in assessing risk of tumor development in carcinogen-exposed normal or premalignant lesions. In this study, we analyzed p53 protein by immunohistochemistry. It still remains to be seen whether p53 protein expression is associated with gene mutation as was shown in a representative case. The tumor sample that expressed p53 demonstrated a deletion mutation at codon 174, and we expect that thorough analysis will delineate the relationship between p53 expression and mutation. Finally we hope to determine whether the detection of p53 alteration in aerodigestive tract tissue is useful for identifying individuals with the greatest risk of developing tumors in the future as well as an intermediate biomarker of response during chemoprevention trials.

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References


23. Holtstein, M., Meteaf, R. A., Wehrl, J. A., Montasano, R., and Harris, C. C. Frequent...


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