The Incidence of p53 Mutations Increases with Progression of Head and Neck Cancer

Jay O. Boyle, John Hakim, Wayne Koch, Peter van der Riet, Ralph H. Hruban, R. Arturo Roa, Russell Correa, Yolanda J. Eby, J. Michael Ruppert, and David Sidransky


Abstract

To establish a genetic model of the progression of head and neck squamous carcinoma we have defined the incidence and timing of p53 mutations in this type of cancer. We sequenced the conserved regions of the p53 gene in 102 head and neck squamous carcinoma lesions. These included 65 primary invasive carcinomas and 37 noninvasive archival specimens consisting of 13 severe dysplasias and 24 carcinoma in situ lesions. The incidence of p53 mutations in noninvasive lesions was 19% (7/37) and increased to 43% (28/65) in invasive carcinomas. These data suggest that p53 mutations can precede invasion in primary head and neck cancer. Furthermore, the spectrum of codon hotspots is similar to that seen in squamous carcinoma of the lung and 64% of mutations are at G nucleotides, implicating carcinogens from tobacco smoke in the etiology of head and neck squamous carcinoma.

Introduction

Cancers arise through a series of well defined genetic changes that accumulate during histopathological progression (1-3). This process has been best delineated through an analysis of colorectal cancer in which the progression from adenoma to carcinoma was correlated with specific genetic changes (4). Alterations of p53 are the most common mutation in human cancers (5) and often occur in the transition from early to invasive lesions in colorectal cancer (6) and other epithelial tumors (7). However, recent reports have suggested that alterations of p53 may occur even earlier in squamous epithelium during progression to cancer (8, 9).

HNSC\(^3\) kills more than 11,000 Americans each year (10), yet little is known of the genetic events involved in its progression (11). We examined the genetic progression for HNSC by determining the timing and incidence of p53 mutations in noninvasive lesions as well as in primary invasive carcinomas. We found that p53 mutations occur in noninvasive lesions and continue to increase in frequency in invasive carcinomas. We have also catalogued the spectrum of p53 mutations in HNSC for comparison with p53 mutations in other types of carcinomas. As expected, HNSC shares a p53 mutation spectrum similar to that found in squamous carcinoma of the lung. Moreover, the types of p53 mutations in HNSC also implicate tobacco-derived carcinogens in the etiology of this disfiguring and often fatal disease.

Materials and Methods

Surgically resected specimens of invasive HNSCs were collected with consent from patients at Johns Hopkins Hospital. Tumors were fresh frozen and later carefully cryostat-micrdissected to enrich for neoplastic cells. Cases with less than 50% neoplastic cells were not included in the analysis. More than 50 12-\(\mu\)m sections were cut and placed in sodium dodecyl sulfate/Proteinase K followed by extraction with phenol/chloroform and ethanol precipitation as described (6). Archival lesions consisting of dysplasia or CIS were identified retrospectively (1991-1993) through a systematic search in the files of the surgical pathology division of the Department of Pathology. These formalin-fixed, paraffin-embedded lesions were microdissected to enrich for neoplastic cells and then deparaffinized in xylene. DNA from tissue was then digested, extracted, and precipitated with ethanol as described above.

p53 Gene Mutations. From primary fresh frozen tumor DNA a 1.8-kilo-base segment of the p53 gene encompassing exons 5-8 was amplified by the PCR as described (12). A UDP cloning site was added to the 5' end of the primers (a = 5'-CAU CAU CAU CAU TIC ACT TGT GCC CTG ACT T-3' and d = 5'-CUA CUA CUA CTG GAA ACT TTC CAC TAC AT-3') to allow cloning into a Cloneamp (BRL) plasmid vector (pSPORT) (13). From archival samples the p53 gene was amplified in two segments. One segment included exons 5 and 6 utilizing primers a and b (b = 5'-CAU CUA CUA CUA CCA CTG ACA ACC ACC CCTT-3') and the other segment included exons 7 and 8 utilizing primers c (c = 5'-CAU CAU CAU CCA AGG GCG ACT GGC CTC-3') and d. Following amplification with uracil-containing primers, the PCR products were extracted with phenol/chloroform and run on a 1% agarose gel. The product was extracted from the gel and treated with 1 unit of uracil DNA glycosolase, and one-half of the total product was annealed to the plasmid vector according to the manufacturer's instructions (13). Competent DH5-\(\alpha\) cells were transfected with plasmid by heat shock, plated on ampicillin plates, and incubated overnight at 37°C. More than 100 colonies were pooled, and plasmid DNA was isolated by alkaline lysis.

Sequencing. Double-stranded DNA obtained from plasmid was sequenced by the dyeoxyrib method utilizing Sequenase (USB) and \[^{35}P\]dATP or \[^{32}P\]dATP (12). Prior to termination, a 30-min incubation with 0.5 units of Klenow fragment (USB) was added to eliminate "stop" bands. Sequencing reaction products were then separated on a 6% urea/polyacrylamide gel and exposed to film. All mutations were confirmed by a second PCR reaction followed by resequencing and resequencing.

Clinical Data. All information was obtained from patient records and assessed as follows. Tobacco exposure was classified as: light, nondrinker, quit >20 years ago; moderate, <1 pack/day or quit 5-20 years ago; and mild, nonsmoker or quit >20 years ago. Alcohol exposure was classified as: light, nondrinker, quit >20 years ago, or special occasions only; moderate, <12 ounces/week or quit 5-20 years ago; and heavy, >12 ounces/week.

Results

To determine the relative timing of p53 gene mutations in HNSC, we sequenced 65 fresh primary invasive tumor samples and 37 archival preinvasive lesions. Nineteen % (7 of 37) of the early preinvasive lesions contained p53 mutations, compared to 43% (28 of 65) of the primary invasive HNSCs (Table 1). Only 7 p53 mutations were found in preinvasive lesions: 5 of 24 in CIS lesions (21%); and 2 of 13 in dysplastic lesions (15%). Both of the latter mutations were in lesions of severe dysplasia; no lesions of mild or moderate dysplasia contained p53 mutations. The difference in incidence of p53 mutations between noninvasive and invasive lesions was found to be statistically significant (\(P < 0.02\) by \(\chi^2\) analysis).

Closer analysis of the specific p53 mutations found in all lesions reveals that 72% (26 of 36) were missense mutations and 28% (10 of
occurred near small repeating sequences, perhaps secondary to replicative errors as described by others (14). Additionally, one tumor (H15) contained an unusual pyrimidine dimer 2-base pair mutation at codon 245. Although common in UV-induced skin tumors (15, 16), 36) would produce truncated proteins. This represents a high percentage of mutations resulting in truncations, similar to that seen in esophageal cancer (5). Of these, four were nonsense mutations, four may also be induced by reactive oxygen free radicals or severe exposure to carcinogens (17). Eighty-two % of the invasive tumors (23) occurring on a different allele. In contrast, 5 of 7 this allele may represent some residual contamination from surrounding nonneoplastic tissue, or it may mean that the second allele had not been lost in these early lesions. Mutations were spread over a wide range of codons yet occurred in the most highly conserved regions of p53 (Fig. 1) as previously reported (5). The mutations also occupied a spectrum similar to that seen in squamous cell carcinoma of the lung (18), but they differed from mutations seen in other epithelial tumors (5).

Clinical data were available for 26 of the 27 patients with primary invasive HNSC tumors containing mutations in the p53 gene (Table 1). Of these, 16 of 25 point mutations (64%) were at guanine nucleotides, including 8 G→A, 5 G→T, or 3 G→C; these changes often associated with benzoapyrenes, nitrosamines, and possibly oxygen radicals from cigarette smoke (18–21). Thirteen of these 16 patients were heavy smokers, and five of them also had histories of heavy drinking. Only one patient with a G→A transition was a nonsmoker and nondrinker. Overall, 25 of 26 patients with mutations had histories of moderate to heavy smoking. In contrast, 11 of 38 patients without mutations did not smoke. Although the exact amount was quite variable and difficult to quantify accurately, carcinogen exposure was significant in all but one of the patients with specific mutations previously found to be associated with known carcinogens in cigarette smoke. The presence or absence of p53 mutations did not correlate with age or sex of the patient nor...
p53 mutations occur outside the conserved regions, we did not sequence all the exons within p53 (5). Therefore, the total number of p53 mutations in these tumors is probably slightly higher. The validity of our approach is supported by recent articles suggesting similar p53 mutations in a smaller number of HNSC tumors (29, 30). Our findings in turn support recent case reports demonstrating p53 staining and/or mutations in selected lesions with severe dysplasia in squamous epithelium (8, 9). The significant frequency of p53 mutations in early lesions suggests that direct damage of DNA may be important in HNSC progression because of the role of wild type p53 in inducing a G1-S arrest following DNA damage (31). It appears that ongoing DNA damage exerts selective pressure on p53 to mutate early, allowing clonal outgrowth and progression. The pattern of p53 mutations with regard to affected codons and specific nucleotide changes implicates cigarette smoke and is reminiscent of changes seen in lung cancer (5, 18). Additionally, codons 220, 245–248, and 278–281 appear to be particular “hot spots” for p53 mutations in HNSC, based on previous reports (22, 29, 30) and our own data. Our work further supports growing evidence that cigarette smoke is a prime mutagenic agent in cancers of the aerodigestive tract.

We have recently described an assay capable of detecting a small percentage of mutant-containing cancer cells among an excess percentage of normal cells (12, 32). Establishing the genetic steps in the progression of HNSC may allow us to target early genetic events for novel screening techniques in saliva from these HNSC patients. Our results suggest that p53 mutations can be detected in a significant minority of early lesions including severe dysplasia and CIS. However, preliminary observations suggest that second primary tumors may have different p53 mutations as reported by Roth et al. (22) and as seen in patient H27. Furthermore, our information suggests that p53 mutations peak somewhat later in the progression to invasive lesions, which would seem to negate the use of this assay for initial screening. Rather, testing for reemergence of the initial p53 mutation might be indicative of recurrence with need for immediate intervention. The availability of additional molecular markers for screening will depend on the development of a reasonable model that identifies early steps in the genetic progression of HNSC.

Discussion

It is now known that cancer is caused by a series of genetic changes, each potentially leading to a clonal outgrowth of cells through a selective growth advantage (3, 4). Determining the nature and timing of these changes in HNSC is critical to both a clinical and biological understanding of the disease. It appears that p53 gene mutations do occur in some early lesions and increase with invasion in squamous cell carcinoma of the head and neck. Because little is known about specific histopathological progression in the upper aerodigestive tract, we chose to begin with severe dysplasia and carcinoma in situ since these lesions are known to progress to invasive cancer in 20–40% of cases (24, 25). Our findings clearly indicate that early lesions in squamous epithelium can have p53 mutations and establish mutation of the p53 gene in the general progression from noninvasive to invasive disease. As demonstrated by the genetic model of colorectal carcinoma (4), it is the accumulation and not necessarily the order of genetic missides that determines progression. The finding of a significant percentage of noninvasive lesions with p53 mutations comports with this model.

Our analysis of p53 mutation is rather rigorous, involving amplification, cloning, and sequencing of DNA derived from carefully microdissected lesions. We found this to be the most reliable method since other screening modalities, such as RNase protection (26), single-strand conformation analysis (27), or denaturing gel electrophoresis (28), can miss certain mutations. Because only a small percentage of mutations occur outside the conserved regions, we did not sequence all the exons within p53 (5). Therefore, the total number of p53 mutations in these tumors is probably slightly higher. The validity of our approach is supported by recent articles suggesting similar p53 mutations in a smaller number of HNSC tumors (29, 30). Our findings in turn support recent case reports demonstrating p53 staining and/or mutations in selected lesions with severe dysplasia in squamous epithelium (8, 9). The significant frequency of p53 mutations in early lesions suggests that direct damage of DNA may be important in HNSC progression because of the role of wild type p53 in inducing a G1-S arrest following DNA damage (31). It appears that ongoing DNA damage exerts selective pressure on p53 to mutate early, allowing clonal outgrowth and progression. The pattern of p53 mutations with regard to affected codons and specific nucleotide changes implicates cigarette smoke and is reminiscent of changes seen in lung cancer (5, 18). Additionally, codons 220, 245–248, and 278–281 appear to be particular “hot spots” for p53 mutations in HNSC, based on previous reports (22, 29, 30) and our own data. Our work further supports growing evidence that cigarette smoke is a prime mutagenic agent in cancers of the aerodigestive tract.

We have recently described an assay capable of detecting a small percentage of mutant-containing cancer cells among an excess percentage of normal cells (12, 32). Establishing the genetic steps in the progression of HNSC may allow us to target early genetic events for novel screening techniques in saliva from these HNSC patients. Our results suggest that p53 mutations can be detected in a significant minority of early lesions including severe dysplasia and CIS. However, preliminary observations suggest that second primary tumors may have different p53 mutations as reported by Roth et al. (22) and as seen in patient H27. Furthermore, our information suggests that p53 mutations peak somewhat later in the progression to invasive lesions, which would seem to negate the use of this assay for initial screening. Rather, testing for reemergence of the initial p53 mutation might be indicative of recurrence with need for immediate intervention. The availability of additional molecular markers for screening will depend on the development of a reasonable model that identifies early steps in the genetic progression of HNSC.


