Advances in Brief

High Frequency of *p16 (CDKN2/MTS-1/INK4A)* Inactivation in Head and Neck Squamous Cell Carcinoma[1,2]

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Abstract

The tumor suppressor gene *p16 (CDKN2/MTS-1/INK4A)* can be inactivated by multiple genetic mechanisms. We analyzed 29 invasive primary head and neck squamous cell carcinomas (HNSCC) for *p16* inactivation with immunohistochemistry utilizing a new monoclonal antibody (mAb), DCS-50. p16 staining of the primary lesions was correlated with genetic analysis including: (*a*) detailed microsatellite analysis of markers at the *p16* locus to detect homozygous deletion; (*b*) sequence analysis of *p16*; and (*c*) Southern blot analysis to determine the methylation status of the 5' CpG island of *p16*. Twenty-four of 29 (83%) head and neck squamous cell carcinoma tumors displayed an absence of p16 nuclear staining using immunohistochemistry. Of these 24 tumors, we found that 16 (67%) harbored homozygous deletions, 5 (21%) were methylated, 1 displayed a rearrangement at the *p16* locus, and 1 displayed a frameshift mutation in exon 1. These data suggest that: (*a*) inactivation of the *p16* tumor suppressor gene is a frequent event in squamous cell carcinomas of the head and neck; (*b*) p16 is inactivated by several distinct and exclusive events including homozygous deletion, point mutation, and promoter methylation; and (*c*) immunohistochemical analysis for expression of the *p16* gene product is an accurate and relatively simple method for evaluating *p16* gene inactivation.

Introduction

HNSCCs[4] comprise 5% of the 1,040,000 new cancers afflicting Americans annually; however, little is known about the molecular changes associated with oncogenesis (1, 2). Perturbations in cellular proliferation driven by the accumulation of genetic alterations in oncogenes and tumor suppressor genes give rise to neoplasms (3). Tumor suppressor gene inactivation is among the most common genetic mechanisms resulting in malignant transformation (4). For example, mutational inactivation of the *p53* tumor suppressor gene is one of the most common genetic changes in human cancer, and it is also found in approximately 45% of primary HNSCCs (5, 6).

Evidence for inactivation of additional tumor suppressor genes in

HNSCC is supported by frequent and early LOH at the 9p21 locus in these neoplasms (7). Located within this minimal region is the putative tumor suppressor gene *p16 (CDKN2/MTS-1/INK4A*; Refs. 8 and 9). The *p16* gene encodes a cell cycle protein which inhibits cyclin-dependent kinases 4 and 6, preventing phosphorylation of Rb protein and causing inhibition of cell cycle progression from G_1 to S-phase (10–12). Genetic alterations of the *p16* gene lead to its inactivation, resulting in deregulation of cell proliferation and tumorigenesis. Previous reports suggest that the incidence of *p16* gene mutations in HNSCC cell lines (44%) is much greater than that found in primary HNSCC tumors (10%; Refs. 13 and 14). This would suggest that the gene encoding *p16* may not be the primary target of LOH at 9p21 in HNSCC, implicating the existence of (an) additional tumor suppressor genes at this locus. However, alternative mechanisms of *p16* inactivation include homozygous deletion (8, 15) and methylation (16) of the 5' CpG island within the promotor region leading to *p16* inactivation. For most tumor types, these alternative mechanisms of *p16* inactivation are much more frequent than point mutation alone (14).

To delineate the role of *p16* as a tumor suppressor in the genesis of HNSCC, we examined 29 primary HNSCC tumors for *p16* gene inactivation using IHC and correlated these findings with genetic analysis of the *p16* locus. We found that *p16* is the most commonly inactivated tumor suppressor gene detected thus far in primary HNSCC and that immunohistochemistry is a sensitive method of detecting *p16* inactivation.

Materials and Methods

Primary Tumor and Margin Samples and DNA Extraction. Twenty-nine randomly selected primary HNSCC tumors were collected from July 1996 through August 1996 following surgical resection with prior consent from Johns Hopkins Hospital patients. These specimens were fresh frozen, then microdissected on a cryostat to select for greater than 70% neoplastic cells/ tumor. Blood was obtained by venipuncture from patients, and lymphocyte DNA was isolated as described for use as a normal control (7).

Histopathology. Fresh-frozen specimens were embedded in OCT (Tissue-Tek; Miles, Elkhart, IN). Tissue sections (5 mm) were cut on a cryostat, and the first two sections of each tumor were mounted on lysine-coated glass slides and stained with H&E. These slides were examined by a pathologist (W. H. W.), and microdissection was performed as needed to obtain greater than 70% neoplastic cells. Twenty-four sections were obtained from each tumor and mounted on 12 lysine-coated slides and stored at −20°C to be used for IHC staining. Fifty sections, 12-mm thick, were cut from each tumor and placed in SDS/proteinase K at 60°C for 4 h. This was followed by phenol-chloroform extraction of DNA and ethanol precipitation as described previously (17).

IHC. IHC was performed as described previously (18) with the use of a p16 monoclonal antibody, DCS-50 (12) (Lab Vision, Fremont, CA), which preferentially recognizes denaturation-resistant epitopes on the carboxy terminus of the p16 protein. Briefly, 5-mm thick frozen tumor sections were fixed on lysine-coated slides in a 1:1 cold acetone/methanol solution. After blocking, serum was applied for 15 min, and sections were reacted with primary p16

mAb in a 1:500 dilution overnight at 4°C. Immunolocalization was performed with the use of the Vectastain Elite ABC kit (Burlingame, CA), 3,3′-diaminobenzidine tetrahydrochloride (Vector, Burlingame, CA), chromagen, and nickel enhancement was used to localize p16 mAb binding in tissue sections. After 3,3′-diaminobenzidine tetrahydrochloride development, tissues were counterstained using Gill's hematoxylin and mounted in an aqueous medium. The slides were evaluated by three observers (W. H. W., J. C., A. L. R.) using standard light microscopy. A squamous cell carcinoma of the larynx cell line (JHU-020-SCC-L) with a homozygous deletion of p16 was used as a negative control. H82, a Rb−/− small cell lung cancer cell line was used as a positive control. For nonspecific staining, normal preimmune mouse serum (Vector) was used as a background control. Only nuclear staining was regarded as positive staining. Inflammatory cells and reactive stromal cells served as positive internal controls.

Allelic Loss. Nine polymorphic microsatellite markers at the *p16* locus on chromosome 9p21 were amplified using PCR analysis to detect dinucleotide repeat polymorphisms in primary HNSCC tumor DNA and corresponding blood lymphocyte DNA. Criteria for the detection of LOH and homozygous deletions have been described previously (15). Determination of homozygous deletion was based on the presence of one or more closely spaced microsatellite markers demonstrating apparent retention when flanked by markers showing clear LOH. This apparent retention is due to low level amplification of normal alleles from small amounts of nonneoplastic cells within the primary tumor without any contribution of the homozygously deleted region from the neoplastic cells (15). Primer sequences can be obtained from Research Genetics (Huntsville, AL; *D9S162, IFN-α, D9S171*, and *D9S736*) or from the Genome Data base (Johns Hopkins University, Baltimore, MD; *D9S1747-52*).

Southern Blot Analysis. Standard Southern blot analysis, including restriction digestion of genomic DNAs, blotting, and labeling of the probe fragment, was performed as described previously (16). Briefly, 10 μg of genomic DNA were digested with *Eco*RI alone or in combination with the methylation sensitive enzyme *Eag*I overnight. The cut DNA was separated on a 1% agarose gel and transferred to Zeta-Probe nylon membranes (Bio-Rad). Filters were hybridized with 25 ng of a random prime-labeled probe (PE1 probe) as described previously (17).

DNA Sequence Analysis of p16. Exons 1 and 2 of p16 were PCR amplified separately as described previously (8). The primers used in the PCR amplification contained dUMP residues at the 5′ end. These primers were used for rapid cloning into a modified CloneAmp vector (Life Technologies, Inc., Gaithersburg, MD). Competent DH5-α *Escherichia coli* were transformed with cloned products and pooled clones were sequenced by amplicycle sequencing (Perkin Elmer) as described (14).

Results

Loss of p16 Expression Using IHC Analysis. We obtained 29 primary invasive HNSCCs and analyzed them for *p16* inactivation using IHC. Following DNA isolation, fresh-frozen tumor sections were stained using the p16 monoclonal antibody DCS-50 (12). In each experiment, a positive control cell line (H82) showed strong staining of the nuclei with scant cytoplasmic staining, whereas the negative control cell line (JHU-020-SCC-L) was consistently devoid of nuclear staining but exhibited slight, diffuse cytoplasmic staining. Twenty-four (83%) of the HNSCC tumors demonstrated a complete absence of p16 nuclear staining. In all cases, strong nuclear staining was observed in reactive stromal fibroblasts and lymphocytes (Fig. 1). Positive staining was not observed when the sections were reacted with nonspecific mouse serum at a 1:1000 dilution.

Genetic Analysis. Fine mapping of the 29 HNSCC tumors using closely spaced microsatellite markers at the *p16* locus detected the presence of a homozygous deletion in 16 of the 24 (67%) tumors with absent staining using IHC. Conversely, homozygous deletions were not detected in any of the tumors which expressed p16 using IHC. Fig. 2 shows LOH in two primary HNSCC tumors, in which the microsatellite markers at p16 display apparent retention of heterozygosity. This apparent retention occurs from amplification of residual normal cells within the primary tumor since the homozygously deleted region
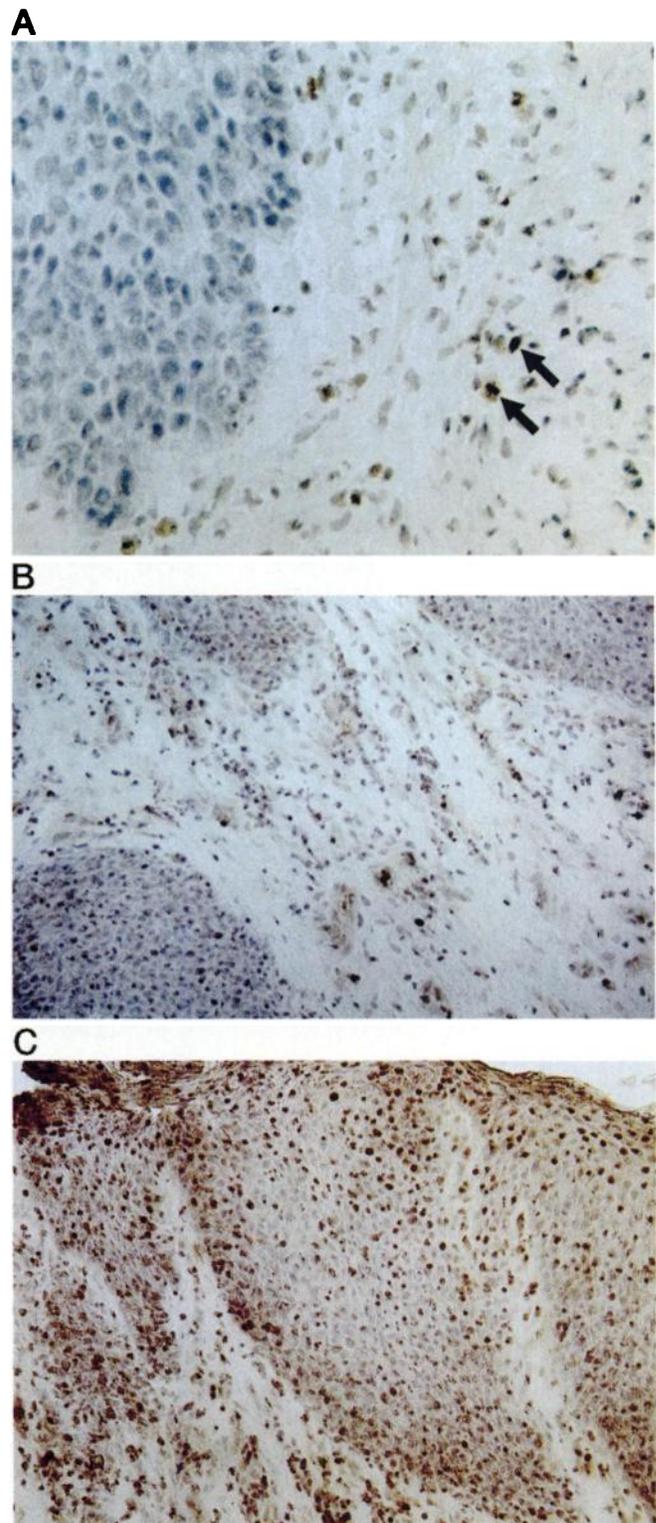
Fig. 1. IHC analysis of *p16* in primary HNSCC. Primary HNSCC stained with p16 mAb demonstrating the absence of nuclear staining in tumor with positive staining of stromal elements (*A*). *Arrows* also identify positively staining lymphocytes. *B*, nuclear and cytoplasmic staining in tumor nests and adjacent stromal elements. Tumor sections are counterstained with Gill's hematoxylin. *C*, nonmalignant epithelium with positively staining nuclei and stroma.

within the neoplastic cells does not provide a template for amplification. This analysis has been previously compared with the presence of homozygous deletion using standard Southern blot analysis and fluorescence *in situ* hybridization in primary tumors (15).

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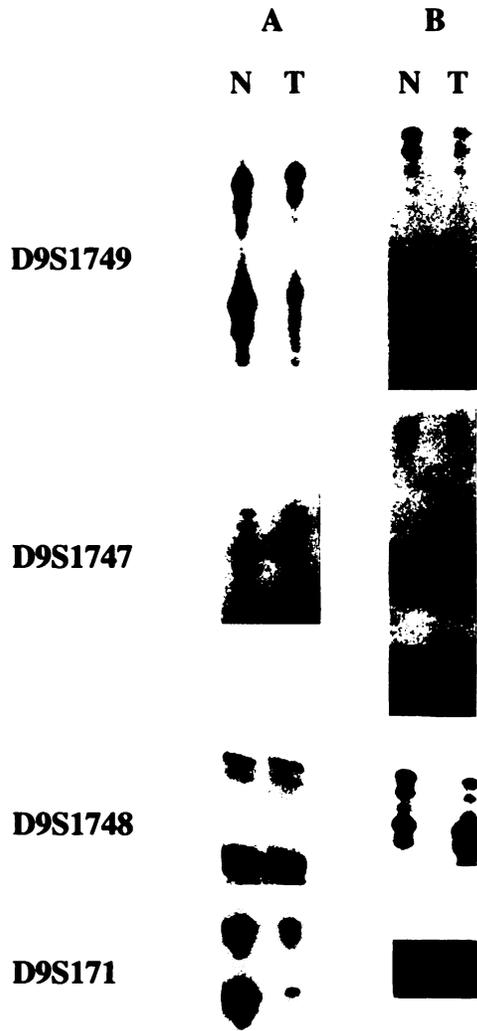Fig. 2. Demonstration of homozygous deletions by microsatellite analysis. *A*, primary head and neck tumor showing apparent retention of heterozygosity, indicating homozygous deletion at *D9S1748* flanked by LOH indicated by loss of the lower allele at *D9S171*, *D9S1747*, and *D9S1749*. *B*, primary head and neck tumor showing apparent retention of heterozygosity, indicating homozygous deletion at *D9S1747* flanked by LOH indicated by loss of the upper allele in *D9S1748*, *D9S1749*, and at *D9S171*. p16 is located between *D9S1747* and *D9S1748*, and the distance between these two markers is approximately 100 kb. *N*, normal tissue; *T*, tumor tissue.

Southern Blot Analysis of p16 Methylation Status. Exon 1 of *p16* was used as a probe for Southern blot analysis on genomic DNA from 11 of 13 tumors, without a homozygous deletion of the region (by microsatellite analysis), to assess p16 inactivation due to promotor methylation. Five tumors exhibited methylation and one tumor demonstrated a rearrangement event at the *p16* locus. We did not detect additional homozygous deletions with this analysis. Fig. 3 is a Southern blot showing *p16* methylation in several samples and the rearrangement in tumor 18. This methylation has been previously associated with the transcriptional block of *p16* in cell lines (16, 19). All five tumors with methylation (and one with rearrangement) displayed a complete absence of p16 staining using IHC. Furthermore, the methylation status of these tumors was independently confirmed in a blinded manner using a novel PCR-based methylation assay (20).

DNA Sequencing Results of Exons 1 and 2. Sequencing of exons 1 and 2 in the same tumors noted above revealed one tumor with a polymorphism in exon 2 and one tumor with a frameshift mutation in exon 1. The somatic mutation would be expected to make aberrant or truncated protein and was negative for p16 staining. All molecular

analysis was performed blinded with respect to the IHC staining. Table 1 compiles the results in all 29 tumors correlated with IHC staining. Absence of IHC staining of p16 in our hands demonstrated a near perfect correlation with *p16* gene inactivation by homozygous deletion, methylation, or point mutation. In one tumor with absent p16 staining (tumor 28), we could not identify a genetic alteration.

Discussion

We have demonstrated that the *p16* tumor suppressor gene is frequently targeted in primary HNSCC. This is the first comprehensive analysis of the mechanisms and frequency of *p16* gene inactivation in primary HNSCC. Analysis of *p16* is fraught with difficulties in primary tumors for several reasons. First, multiple mechanisms of *p16* inactivation warrant extensive genetic studies, including a search for homozygous deletions, methylation, and point mutations. Second, standard methods to assess homozygous deletions and methylation status require large amounts of tumor DNA of excellent quality. Even with large amounts of tumor DNA, homozygous deletions can easily escape detection due to the masking effect of nonneoplastic cells.

We have shown that IHC analysis is a straightforward method to detect *p16* inactivation regardless of the precise genetic mechanisms involved. Our results demonstrate an excellent correlation between IHC and genetic analysis. Thus, IHC analysis appears to be a reliable and practical alternative to a comprehensive genetic analysis of *p16* status. Others have described the use of IHC analysis in some primary tumors with a polyclonal antibody (21, 22). In one preliminary study of
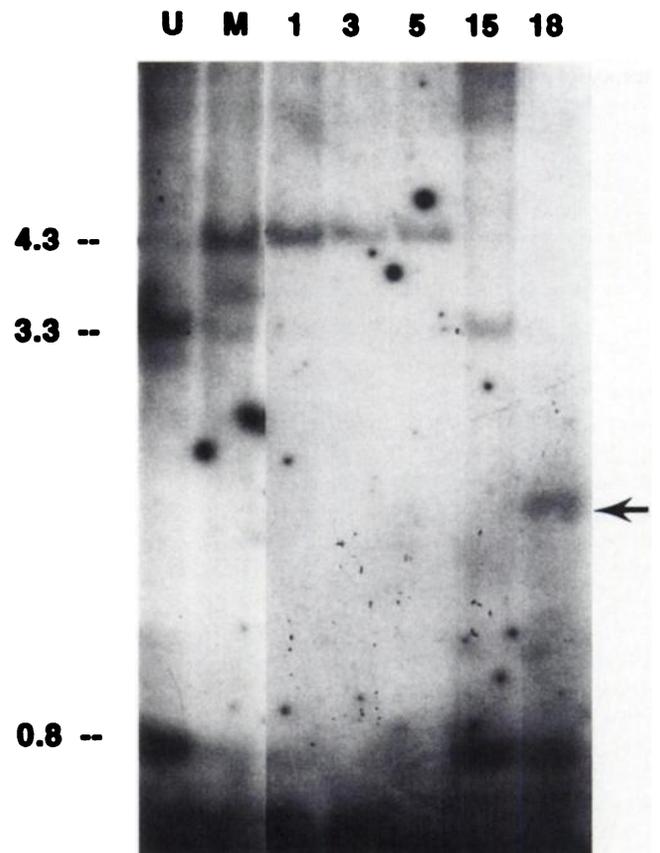
Fig. 3. Methylation of the 5' CpG island in p16. Southern blot analysis with exon 1 probe from p16 demonstrates methylation of tumors 1, 3, and 5 by protection of the 4.3-kb band. Tumor 15 is unmethylated, and tumor 18 demonstrates a genomic rearrangement (*arrow*), probably due to a small deletion. The methylated (*M*) control cell line demonstrates only the 4.3-kb flanking cut after digestion with *Eco*RI and *Eag*I, whereas the unmethylated (*U*) control demonstrates further digestion resulting in 3.3-, 0.8-, and 0.5-kb (not shown) fragments. For further details, see Ref. 15.

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Table 1 *Genetic analysis versus IHC staining for p16 in HNSCC*

HNSCC tumor	HD[a]	Methylation status	Sequence analysis	p16 IHC staining
1		M		−
2	HD	−		−
3		M		−
4		M		−
5		M		−
6	HD	−		−
7	HD	−		−
8	HD	−		−
9	HD	−		−
10	HD	−		−
11	HD	−		−
12	HD	−		−
13	HD	−		−
14		−		+
15	HD	−		−
16		−		+
17	HD	−		−
18		R		−
19	HD	−		−
20		−		+
21		M		−
22		−	FS	−
23	HD	−		−
24		−		+
25	HD	−		−
26	HD	−		−
27	HD	−		−
28		−		−
29		−		+

[a] HD, homozygous deletion; R, rearrangement by Southern blot; −, negative; +, positive; M, methylated promoter; FS, frameshift mutation in exon 1.

non-small cell lung carcinomas, the investigators generated similar results [38/61 (63%) tumors displayed the absence of p16 staining], with a high frequency of *p16* inactivation (23). Although these data support our observations, it was not combined with detailed genetic analysis. Moreover, our use of a mAb that does not cross-react with other proteins appears to simplify the interpretation of our analysis. This approach should be of value for assessing other primary tumors and small lesions for which sufficient DNA is not available for detailed genetic analysis.

Although we tested all known mechanisms of genetic inactivation of *p16*, it is important to note that some missense mutations of *p16* have been detected by other investigators (8, 13, 23). Tumors with these missense mutations may well stain using IHC analysis and might be misinterpreted as indicating wild-type *p16* status. However, most tumor types (exception pancreatic carcinoma) display a low rate of point mutations and even fewer missense mutations (24). In many ways our results parallel those reported in several studies on IHC analysis of Rb protein (21, 22, 24). Most point mutations in this suppressor gene demonstrate complete inactivation of *Rb* with the absence of protein staining in primary tumors. IHC analysis has proven reliable for the assessment of *Rb* genetic status despite multiple mechanisms of inactivation (22, 25).

We found 1 tumor (tumor 28) which displayed the absence of IHC staining for p16 without an obvious genetic alteration. This tumor may have a mutation outside of the region we sequenced or may harbor a small homozygous deletion. As noted previously, small nested deletions which target *p16* may not always be detected if certain markers are noninformative or because of their wide spacing (15). We also did not test for homozygous deletion of exons 2 or 3 using Southern blot analysis. There have also been reports of poor staining due to artifact induced by tissue fixation in paraffin-embedded archival specimens, resulting in ambiguous staining results (20–22). We considered this possibility remote because we used only fresh-frozen tissue, yielding reproducible staining results.

Allelic loss at 9p21 occurs frequently and early in HNSCC (7). It is not yet known whether *p16* inactivation is the target of this early loss

event in primary HNSCC. Although further investigation is required to determine the temporal occurrence of *p16* loss in HNSCC, this study suggests that the inactivation of *p16* may be the most common genetic change delineated in primary invasive HNSCC.

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