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Methylation, a Major Mechanism of p16/CDKN2 Gene Inactivation in Head and Neck Squamous Carcinoma

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We studied 11 head and neck squamous carcinoma (HNSC) cell lines and 46 primary tumors for p16 gene status by protein, mRNA, and DNA genetic/epigenetic analyses to determine the incidence, the mechanism(s), and the potential biological significance of its inactivation. Of the 11 cell lines, only 1 showed intact p16 and 10 lacked its protein and mRNA; DNA analysis of these 10 cell lines showed 2 homozygous deletions, 6 methylations at exon 1 and 2, and 2 with no detectable abnormalities. In primary tumors, 16 (34.7%) of the 46 showed detectable p16 protein and mRNA; of these, 12 had no DNA abnormalities and 4 had only exon 2 methylation. Loss of p16 expression was found in three tumors with concurrent mutation at exon 2 and methylation at exon 2 (two) and both 1 and 2 (one). Of the 30 tumors that lacked p16 protein, 27 also lacked mRNA, 1 had detectable p16 mRNA, and 2 failed RT-PCR amplification. Twenty-two of the thirty tumors showed DNA alterations and eight manifested no abnormalities; DNA alterations comprised 6 homozygous deletions, 2 concurrent mutations and methylation of exon 2, and 13 with methylation at exon 1 and exons 1 and 2 (12 with methylation only and 1 with mutation) at exon 1. Except for patients' gender ($P = 0.02$), no significant correlation between p16 and clinicopathological factors was observed. We conclude that in HNSC 1) intragenic p16 alterations are infrequent events, 2) methylation of exon 1 constitutes a common mechanism in silencing the p16 gene, 3) p16 inactivation may play an important role in the early development and progression of HNSC, and 4) no association between p16 alterations and conventional clinicopathological factors was noted in this cohort. ***(Am J Pathol 1997, 151:1767–1774)***

The identification of a common tumor suppressor gene associated with the pathogenesis of diverse tumor entities is central to understanding their evolution and the development of both molecular diagnostic markers and novel therapeutic strategies for future management of cancer patients. The p16 tumor suppressor gene is located on chromosome 9p21 and encodes a 16-kd cyclin-kinase inhibitor with ankyrin motifs that bind with CDK4 and CDK6.[1,2] This interaction blocks the passage of cells from G1 to S by inhibiting cyclin-D-dependent phosphorylation of the Rb protein and maintains its binding to the E2F transcriptional factor.[3]

Although evidence for a high incidence of p16 alterations in a variety of cell lines and primary tumors has been reported,[4–9] this has been contested by others.[10–12] Studies of head and neck squamous carcinoma (HNSC) have also shown widely variable results.[11,13–16] Underlying these discrepancies are the varied methodologies, analytical strategies, and patient populations between these studies.[17] To fully assess the p16 gene status in HNSC, 11 cell lines and 46 primary tumors were analyzed for the gene product and mRNA and DNA genetic and epigenetic alterations, and the results were correlated with conventional clinicopathological factors and DNA content analysis.

Materials and Methods

Tumor

Matched pairs of fresh normal mucosa and tumor specimens from 46 resected primary HNSCs received at the frozen section of the Department of Pathology, The University of Texas, M.D. Anderson Cancer Center, from November 1992 to December 1995 formed the materials for this study. Normal mucosa was obtained from the farthest margin of resection after frozen section verification. Invasive carcinoma specimens were carefully dissected from grossly viable tumor and were subjected to frozen section for evaluation of tumor content. Specimens with less than 80% neoplastic tissue were excluded from this study. All specimens were obtained by one pathologist (A. K. El-Naggar) and promptly snap-frozen and kept

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Table 1. Primers Used for the PCR of Genomic and cDNA

Exon	Primer	Sequence (5′ to 3′)	Position	Enzyme
p16/cDNA	M4U	GCAGC ATGGA GCCTT CGGCT GAC	13–30 (L272211)	
	M4D	CTGGT TCTTT CAATC GGGGA TGT	452–474 (L272211)	
P16/exon1	M4U	GCAGC ATGGA GCCTT CGGCT GAC	13–30 (L272211)	*Cfo*I, *Sma*I
	1108R	GCGCT ACCTG ATTCC AATTC	321–340 (U12818)	*Sac*II
P16/exon2	M1	TTCCT TTCCG TCATG CCG	93–110 (U12819)	
	M2	ACCAG CGTGT CCAGG AAG	267–284 (U12819)	
P16/exon2	M3	ACACG CTGGT GGTGC TGCA	275–293 (U12819)	
	42R	TTCTC AGATC ATCAG TCCTC	460–479 (U12819)	
P16/exon2	M1	TTCCT TTCCG TCATG CCG	93–110 (U121819)	*Cfo*I, *Sma*I
	42R	TTCTC AGATC ATCAG TCCTC	460–479 (U121819)	*Sac*II
Interleukin-1	DM151	GTCTC TGAAT CAGAA ATCCT TCTATC		
	DM152	CATGT CAAAT TTCAC CGCTT CATCC		
P53/exon 7	7US	ACTGG CCTCA TCTTG GGC		
	7DS	GGCAC AGCAG GCCAG TGT		

at −80°C until used. Ficoll-Hypaque-isolated peripheral blood lymphocytes were also obtained from every patient and snap-frozen until used.

Cell Lines

Eleven HNSC cell lines established at M.D. Anderson Cancer Center (10 primary and 1 metastatic) were exponentially grown, harvested, and snap-frozen for analysis. An aliquot of each cell line with 1×10^6 cells/ml was used for cytospin preparations and keratin immunostaining. All cell lines were immunoreactive to a cocktail of low and high molecular weight keratin antibodies, confirming their epithelial nature.

DNA Extraction

Frozen tissues were ground and incubated for 3 hours in a lysis buffer containing 1% sodium dodecyl sulfate (SDS), 0.1 mol/L NaCl, 50 mmol/L EDTA (pH 8.0), and 200 μg/μl proteinase K. DNA was then purified using standard procedures with phenol/chloroform and precipitated with ethanol.

RNA Extraction

Tumor tissues were homogenized by a tissue teaser (Biospec Products, Bartlesville, OK). Cells were lysed in a guanidinium isothiocyanate-phenol solution (RNAzol B, Biotecx Lab, Houston, TX), and chloroform was added to sample lysates. Specimens were then centrifuged, and the aqueous RNA-containing phase was precipitated with isopropanol, resuspended with DEPC-H_2O, and treated with RNAse-free DNAse I at a concentration of 0.1 μ/μl. RNA concentration was determined spectrophotometrically (Pharmacia, Alameda, CA). RNA analysis was electrophoretically performed under denaturing conditions on 2% agarose and 6% formaldehyde gel.

Protein Extraction

Frozen tissues were homogenized in 500 μl of lysis buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.1 mg/ml phenylmethylsulfonyl fluoride), incubated on ice for 30 minutes, and then centrifuged; the supernatant was collected and protein concentration was determined spectrophotometrically using a DC protein assay kit (Bio-Rad Laboratory, Hercules, CA).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

A 1-μg aliquot of RNA was reverse transcribed for the first-strand cDNA using the Gene Amp RT-PCR system (Perkin Elmer Cetus, Branchburg, NJ), and 20 μl of the final reaction product was used as a template for PCR. This reaction mixture was added to 80 μl of 100 μmol/L dNTP and 2 mmol/L $MgCl_2$ in 10% glycerol and 2.5 U of *Taq* polymerase in 1X PCR buffer. Amplification was carried out with 0.4 μmol/L primer M4U-M4D for P16/CDKN2 cDNA and DM151-DM152 (Perkin Elmer Cetus) for the human interleukin (IL) gene as an internal control. PCR was conducted at 94°C (5 minutes), followed by 35 cycles of 94°C (1 minute), 55°C (1 minute), and 72°C (2 minutes). Reaction product was separated on a 2% agarose gel and stained with ethidium bromide.

Multiplex PCR Amplification and Deletion Analysis

Genomic DNA was PCR amplified by primers M4U-1108R for p16 exon 1 and M1-M2 and M3-42 R for exon 2 (Table 1). The PCR reaction was conducted in 1X PCR buffer (Promega, Madison, WI), 1 μmol/L primers, 200 μmol/L dNTPs, 2.5 U of *Taq* polymerase, 5% dimethylsulfoxide (DMSO), and 200 ng of template DNA. Conditions were adjusted to one cycle at 95°C (5 minutes), 20 cycles at 94°C (1 minute), 56°C (1 minute), and 72°C (1 minute), and 15 cycles at 94°C (1 minute), 52°C (1 minute), and 72°C (1 minute). The PCR product was electrophoresed in 2% agarose gel. This yielded 217-, 192-, and 205-bp bands, respectively. Samples that failed amplification were re-amplified in a separate PCR mixture simultaneously with interferon (IFN) primers for assessing intragenic deletion in exons 1 and 2.

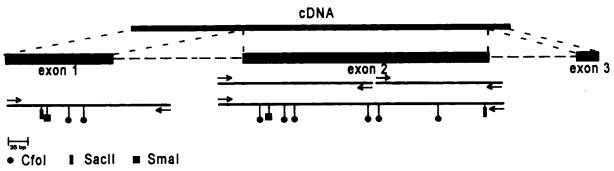

Figure 1. Restriction map outline and PCR strategy for p16 RT-PCR, methylation, deletion, and SSCP analysis. The **top solid bar** represents the p16 462-bp cDNA fragment. Exons 1 and 2 were amplified as 217-bp and 387-bp fragments for methylation and deletion (**lower thin bar** with enzyme sites). The *Cfo*I, *Sac*II, and *Sma*I methylation-sensitive enzymes were used. Exon 2 was amplified as two fragments (192 and 205 bp) for SSCP analysis (middle thin two bar). The primer sets used are indicated by **arrows** (see Table 1).

Single-Strand Conformation Polymorphism (SSCP) Analysis and Direct Sequencing

The entire exon 1 and two fragments of exon 2 were PCR amplified for SSCP analysis. Five microliters of PCR products were denatured by heating at 95°C (5 minutes) in 5 μl of sequencing stop solution (USB, Cleveland, OH) and applied to 8% nondenaturing acrylamide gel. The gel was subjected to silver staining by a color silver stain kit (Pierce, Rockford, IL). For each SSCP-positive specimen, a 20-μl PCR product was electrophoresed in 1% Nusieve/GTG agarose gel (FMC, Rockland, ME), and the bands were excised. The gel slide was melted by heating to 68°C (10 minutes) and purified by Magic DNA purification systems (Promega), and the PCR product was mixed with one of the primers, boiled for 5 minutes, and cooled to 37°C for 5 minutes. Sequencing was then performed using the version 2.0 Sequence DNA sequencing kit (USB).

Western Blot Analysis

One hundred micrograms of total protein was size fractionated on 12% acrylamide gel. The proteins were blotted onto a nitrocellulose membrane with a transblot cell (Bio-Rad). A $p16^{ink4}$ polyclonal antibody (PharMingen, San Diego, CA) was used for the reaction. Proteins were detected by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

DNA Methylation Assay

Figure 1 depicts the restriction sites for different enzymes and the strategy used for the analysis. In this assay, DNA methylation will only be detected if all sites of a restriction enzyme in a given DNA fragment tested are methylated, and incomplete methylation is interpreted as a lack of this process.[18,19] One microgram of genomic DNA was digested for 2 hours in 20 μl of 5 U/μl by the following methylation-sensitive restriction enzymes: *Cfo*I, *Sma*I, and *Sac*II (Boehringer Mannheim, Indianapolis, IN).[19] To ensure complete digestion of genomic DNA and the presence of excess enzyme, a 5-μl aliquot of the supernatant was added to 5 μl of a previously amplified fragment of exon 1 for further digestion.[20] In every instance, exon 1 digestion was accomplished (data not present), confirming the presence of excess enzyme. Enzymes were inactivated by 0.2 mmol/L EDTA at 65°C for 5 minutes, and multiplex PCR was conducted with 100 ng of digested genomic DNA. Primer sets M4U-1108R for exon 1, M1-42R for exon 2, and 7US-7DS of the p53 gene as a control (Table 1) were used. Under this setting, intact DNA serves only as a template for amplification. A 15-μl aliquot of PCR products was used for electrophoresis in 2% agarose gel. To confirm accuracy of p16 exons 1 and 2 band size, bands were excised and sequenced.

Flow Cytometry

Single-cell suspension from solid tissues was prepared by mechanically mincing fresh tissue in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA). DNA/RNA analysis was performed with the EPICS Profile cytometer (EPICS Division of Coulter Corp., Hialeah, FL). Histograms were analyzed using the histogram analysis menu option of the profile software.

Results

The patient population comprised 33 males (71.7%) and 13 females (28.3%), ranging in age from 33 to 92 years with a mean age of 58.2 years. Tumor locations were as follows: oral cavity, 8 (7.4%); tongue, 18 (39.1%); larynx, 13 (28.3%); and oropharynx, 7 (15.2%). Histological differentiation of tumors consisted of 4 (8.7%) well, 28 (60.9%) moderately, and 14 (30.4%) poorly differentiated carcinomas. Tumor stage included 8 each (17.4%) stage I and II, 7 (15.2%) stage III, and 22 (47.8%) stage IV. Tumor DNA content analysis revealed 28 (60.9%) diploid and 19 (41.3%) aneuploid histograms with proliferative indices ranging from 2 to 44.4% with a mean of 13.2%. The DNA index of the DNA aneuploid tumors ranged from 1.12 to 2.2 with a mean of 1.59.

Tables 2 and 3 present the results of the DNA alterations in HNSCs lacking p16 protein and mRNA. P16 protein was detected in 1 of the cell lines and was absent in 10 (Figure 2A). mRNA analysis by RT-PCR matched the p16 protein results in all cell lines; only one cell line (1483) had detectable mRNA (Figure 3A, upper panel). DNA analysis of the cell lines showed two (18.1%) homozygous deletions (182T and 1386T), six (54.5%) methylations at exon 1 and 2, and two (18.1%) with no alterations (177T and 188 T). Of the 46 primary tumors, 16 (34.7%) had detectable protein by Western blotting (Figure 2B) and 30 (65.2%) lacked p16 protein. Of these, mRNA was absent in 27, there was failed amplification in two, and mRNA was present in one (Figure 3B, upper panel, tumor 27). The latter case had concurrent DNA mutation and exon 2 methylation.

DNA analysis of tumor specimens showed three (6.5%) with mutations (Figure 4) that consisted of C to A and T to A at codons 68 and 70 in cases 27, 26 (Figure 5), and 42, resulting in the following amino acid changes: Arg → Leu, Glu → Val, and Glu → Val, respectively. Multiplex PCR of exons 1 and 2 and IFN, as a control, revealed six (13.0%) homozygous deletions (Figure 6).

Table 2. DNA Genetic and Methylation Alteration in HNSC Cell Lines Lacking p16 Protein and mRNA

						Methylation				
				SSCP		Exon 1			Exon 2	
Number	Cell line	p16	RT-PCR	Exon 1	Exon 2	*Cfo*I	*Sac*II	*Sma*I	*Cfo*I	*Sma*I
1	138T	−	−	−	−−	−	−	+	−	+
2	159T	−	−	−	−−	−	−	+	−	+
3	167T	−	−	−	−−	−	−	+	−	+
4	177T	−	−	−	−	−−	−		−	−
5	182T	−	−	del	del		del		del	
6	183T	−	−	−	−−	−	+	+	−	+
7	188T	−	−	−	−−	−	−	−	−	−
8	212T	−	−	−	−−	+	−	+	−	+
9	212L	−	−	−	−−	+	−	+	−	+
10	1386T	−	−	del	del		del		del	

−, negative; +, positive; del, deletion.

Further analysis of these six tumors with PCR for microsatellite markers near the p16 locations showed retention of heterozygosity at either a proximal or a distal marker in five and loss of heterozygosity at these loci in one (data not shown).

Using three methylation-sensitive enzymes for exons 1 and 2 of p16, 6 of the 11 (54.5%) cell lines and 20 (43.4%) of the 46 primary tumors manifested methylation at either exon 1 or 2 or both (Figure 3, A and B, lower panels). Methylation varied between different enzymes in some cell lines and few primary tumors. Similar observations have previously been reported.[18] Of the 20 tumors, 4 had methylation at exon 1, 7 at exon 2, and 9 at both exons (Table 3). All cases with methylation were repeated at least twice with identical results. All 13 tumors with methylation at exon 1 or both 1 and 2 lacked p16 transcription, comprising 43.3% of all 30 tumors lacking translation and/or transcription and 54.1% of the 24 tumors without homozygous deletion. In one tumor (42), concurrent mutation and methylation at exons 1 and 2 were observed. The functional status of such mutation and its role in the abrogation of p16 is unknown. Of the seven tumors with

Table 3. DNA Genetic and Methylation Analysis in Primary HNSC Lacking p16 Protein

				Methylation				
	SSCP			Exon 1			Exon 2	
Number	Exon 1	Exon 2	Sequence	*Cfo*I	*Sac*II	*Sma*I	*Cfo*I	*Sma*I
2	−	−−	−	−	+	+	−	−
3	del	−−	−		del		−	−
6	−	−−	−	−	−	−	−	−
7	−	−−	−	+	+	+	−	+
8	−	−−	−	−	−	−	−	−
9	−	−−	−	−	−	−	−	−
10	−	−−	−	−	−	+	−	+
12	−	−−	−	+	+	−	+	−
17	−	−−	−	+	−	+	+	−
20	del	−−	−		del		−	−
21	−	−−	−	−	−	+	−	+
22	−	−−	−	−	−	+	−	+
23	−	−−	−	−	−	+	−	+
24	−	−−	−	−	−	−	−	+
25	del	−−	−		del		−	−
26	−	+−	CTC-CAC	−	−	−	−	+
27	−	+−	GCC-GAC	−	−	−	−	+
28	−	−−	−	+	+	+	−	+
29	−	−	−	−	−	−	−	−
31	del	−−	−		del		−	−
33	−	−−	−	−	−	−	−	−
34	del	−−	−		del		−	−
36	−	−−	−	−	+	−	−	−
37	−	−−	−	−	−	−	−	−
38	−	−−	−	+	+	−	−	−
42	−	+−	CTC-CAC	−	+	+	−	+
43	−	−−	−	−	−	−	−	−
44	−	−−	−	−	+	−	−	−
45	−	−−	−	−	−	−	−	−
46	del	−−	−		del		−	−

−, negative; +, positive; del, deletion.

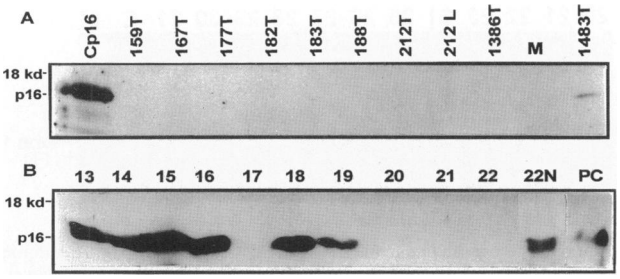

Figure 2. Western blot analysis of p16 protein in HNSC cell lines (**A**) and carcinoma specimens (**B**). Cp16 and PC, p16 positive control protein (Santa Cruz); N, protein from normal tissue of case 22.

exon 2 methylation, four had p16 protein and mRNA and three lacked protein expression (two had concurrent mutation and one had no DNA alteration). Eight (26.6%) of the thirty tumors that lacked p16 protein and mRNA had no DNA alteration and only one showed abnormal methylation at exon 2 (tumor 24).

Table 4 presents the correlation between clinicopathological factors and DNA content analysis as well as p16 status in this cohort. Significant statistical association between p16 alterations and gender was noted (P = 0.02); 92.3% of tumors from females had p16 alterations whereas 54.5% of tumors in males show alterations. There were apparent differences between males and females in the incidence of intragenic DNA or methylation abnormalities. Our data show a trend for a higher incidence of p16 abnormalities in the larynx than in tongue and/or other sites combined. No significant association between histological differentiation, DNA ploidy, and tumor stage and p16 status was found.

Discussion

The incidence and the biological significance of p16 alterations in different neoplastic categories, including HNSC remains uncertain.[4,15,17,21–24] At issue is the apparent discrepancy between findings in cell lines and primary tumors and also among different studies of similar neoplastic entities.[1,4,10] The majority of these investigations have employed a variety of molecular techniques and study designs. These, together with evidence that an alternative transcript of the p16 gene[5,25–27] and CpG islands methylation may contribute to its inactivation, further complicate the evaluation of this gene.[28–30]

Our study shows that DNA mutation and homozygous deletion of p16 occur in only a small number of cell lines and primary tumors of HNSCs. Only two cell lines had homozygous deletions (182T and 1386T), and nine primary tumors had intragenic DNA alterations (six with homozygous deletion and three with missense mutations).

Although our primary tumor results are in agreement with most studies,[10–12,14,16,31,32] they differ from those reported by others.[15] In the latter study, the analysis of closely spaced microsatellite markers rather than targeting the p16 gene itself may have led to the high reported incidence of homozygous deletion.[15] Supporting such a

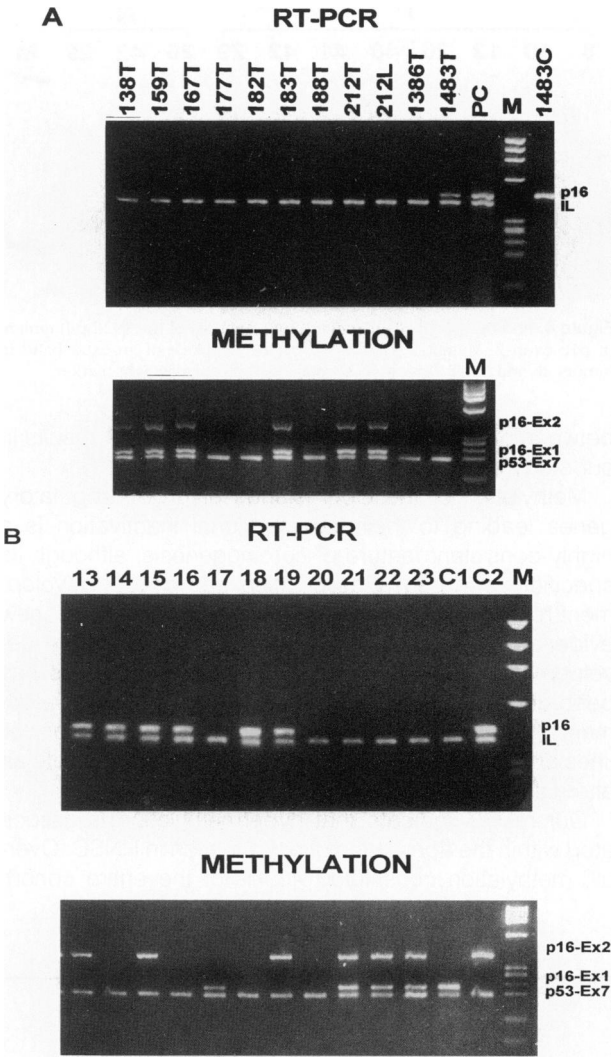

Figure 3. **A:** Photograph of RT-PCR and methylation analysis of HNSC cell lines. **Upper panel:** p16 cDNA (462 bp) co-amplified with interleukin (IL) cDNA (421 bp); lack of p16 mRNA is noted in all cell lines except in 1483T. **Lower panel:** Methylation analysis of *SmaI*-digested genomic DNA of p16 exons 1 and 2 co-amplified with p53 exon 7 as control. The presence of exon 1 (217 bp) or exon 2 (387 bp) bands in cell lines 138T, 159T, 167T, 183T, 212T, and 212L indicate methylation. M, molecular weight marker; PC, positive control HL100 cell line; 1483C, RT-PCR from 1483T RNA with p16 primers only. **B:** RT-PCR and methylation photomicrograph of primary HNSC. **Upper panel:** Note absence of p16 mRNA in cases 17 and 20 to 23. **Lower panel:** Methylation of exons 1 and 2 is noted in cases 21 to 23, exon 2 only in cases 13, 15, and 19 and exon 1 only in case 17. C1 and C2 are exons 1 and 2 PCR products of undigested genomic DNA from normal tissue (sample 23) as controls.

contention is the complete absence of residual normal contaminating bands at some proximal or distal markers with loss of heterozygosity and the presence of these bands in the same specimen at the marker interpreted as homozygous deletion in the authors' illustrations.[15] We observed retention of heterozygosity in at least one of the flanking loci of p16 in five of the six cases of homozygous deletion. We contend that our combined analysis of the p16 products and their genetic and epigenetic alterations allows for a more comprehensive and accurate evaluation of this gene. This is supported by the agreement

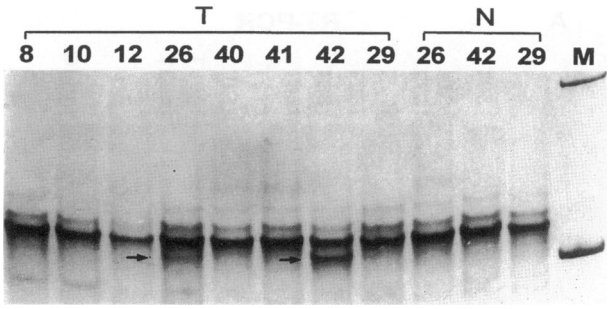

Figure 4. Photograph of silver-stained SSCP analysis of the 192-bp fragment of p16 exon 2. Mutation is indicated by the presence of an extra band in tumors 26 and 42. T, tumor; N, normal; M, molecular weight marker.

between protein and mRNA analyses and DNA results in our study and those of others.[3,12,33,34]

Methylation of the CpG islands of growth-regulatory genes leading to their transcriptional inactivation is a highly consistent feature of carcinogenesis, although its specificity and causal association with cancer development remain undetermined.[35,36] Recently, however, new evidence for a distinct pattern of methylation in the development of neuroblastoma and lung carcinomas has been demonstrated, suggesting a role in tumor development.[37,38] In addition, studies of primary and tumor cell lines of HNSC indicate that methylation may constitute an alternative mechanism in silencing the p16 gene.[32,39]

Our results indicate that hypermethylation is associated within the abrogation of p16 function in HNSC. Overall, methylation constituted 43.4% of the entire cohort.

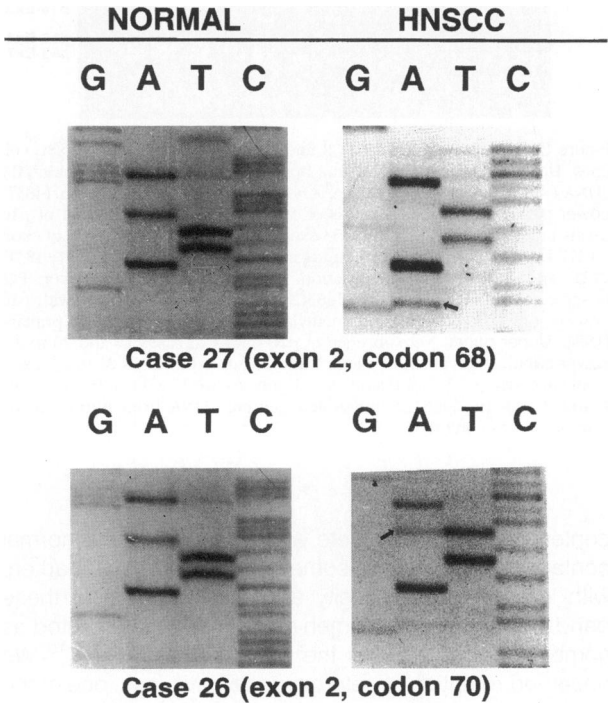

Figure 5. Sequencing analysis of p16 exon 2 in two mutated HNSCs. Note transversion mutation of codon 70 (CTC to CAC) and 68 (GCC-GAC) of cases 26 and 27, respectively (**arrows**). The relatively high level of wild-type signal could be attributed to either clonal heterogeneity and/or contamination by host nontumor elements or alternatively due to a mutation restricted to one allele.

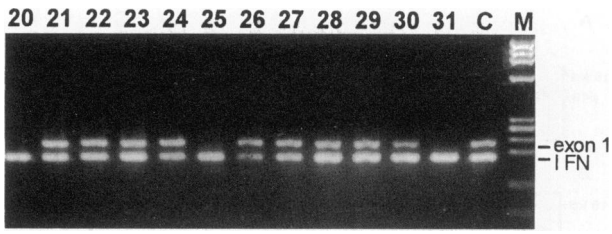

Figure 6. Photograph of multiplex PCR analysis of p16 exon 1 and interferon (IFN) in HNSC. C, sample 31 normal DNA; M, molecular weight marker. Homozygous deletion is noted in specimens 20, 25, and 31.

Exon 1 methylation, however, comprised 54.0% of the 24 tumors lacking transcription, excluding those with homozygous deletion. In contrast, four of the seven tumors with methylation restricted to exon 2 had detectable protein, and the two with concurrent mutations lacked p16 protein; that one of the latter tumors retained mRNA (tumor 27) suggests that the mutation may have led to truncated protein. These data suggest, as previously indicated, that exon 2 methylation plays a minor role in the inactivation of p16.[19]

Previous studies[19,32,35,36,39] of HNSC have shown a slightly lower incidence of methylation than ours, although the relatively small number of tumors and enzymes tested and the lack of information on p16 protein and mRNA status in these investigations are most likely the contributing factors for this difference.[19,39] In that context, the lack of p16 protein in one of our tumors with exon 2 methylation (tumor 24) and the absence of DNA and methylation abnormalities in eight (26.7%) neoplasms and two cell lines suggest that different mechanisms, including post-transcriptional and/or a minor alteration beyond the sensitivity of our techniques, may play a role.[28,32,39–41]

Table 4. Correlation between p16 Alterations and Clinicopathological Factors in Primary HNSC

Characteristic	p16 alteration		*P* value
	No	Yes	
Site			
Tongue	9 (50%)	9 (50.0%)	
Larynx	2 (14.3%)	11 (84.6%)	0.16
Others	5 (35.7%)	10 (66.7%)	
Sex			
Males	15 (46.8%)	18 (56.2%)	
Females	1 (7.7%)	12 (92.3%)	0.02
Differentiation			
WD	1 (25.0%)	3 (75.0%)	
MD	8 (28.5%)	20 (71.4%)	0.39
PD	7 (50.0%)	7 (50.0%)	
DNA ploidy			
Diploid	11 (40.7%)	17 (60.7%)	
Aneuploid	5 (27.7%)	13 (72.2%)	0.53
Proliferative index			
≥10%	11 (33.3%)	22 (66.6%)	
<10%	6 (46.1%)	7 (53.9%)	0.51
Stage			
I and II	6 (40.0%)	10 (62.5%)	
III and IV	10 (33.3%)	20 (66.6%)	0.70

WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

The fact that p16 inactivation is noted in the majority of tumors tested suggests that alterations of this gene constitute an early event in HNSC tumorigenesis. This is further supported by the lack of significant statistical association between p16 alteration and histological differentiation stage or DNA content. Tumors from female patients showed 92.3% alterations whereas only 54.5% of tumors from male patients had such alterations. The reason for these findings is unclear, although an association with an upstream X chromosome regulator may play a role. We, however, found no difference between the methylation status of tumors in male and female patients. It is also interesting that a trend for site predilection of p16 alteration was noted; laryngeal tumors had preponderantly more p16 abnormalities than those of the tongue and other nonlaryngeal sites. This could either be related to site specificity or to local regional etiological factors. A recent study of bladder carcinomas has shown high p16 alterations in bilharzial than histologically comparable nonbilharzial tumors,[42] suggesting an etiological association between this parasitic infection and tumorigenesis.

As methylation appears to play a major role in p16 inactivation of HNSC, modulation of this phenomenon may have an impact on their biology. Several lines of *in vivo* and *in vitro* evidence indicate that treatment with 5-deoxyazacytidine leads to demethylation of p16 and other methylated genes. There may be a potential role of methylation-modifying agents in the future biological therapy of some of these patients.[36,39–41,43,44]

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