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Molecules and Cells

Inactivation of p16^{INK4a} in Primary Tumors and Cell Lines of Head and Neck Squamous Cell Carcinoma

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Inactivation of the p16^{INK4a} gene by mutation and deletion is common in head and neck squamous cell carcinoma (HNSCC). The present study demonstrates that hypermethylation of the 5′ CpG islands can serve as an alternative mechanism for the inactivation of the p16^{INK4a} gene in this tumor. We studied 11 HNSCC cell lines and 17 oral squamous cell carcinoma (OSCC) primary tumors for p16^{INK4a} gene status by protein/mRNA and DNA genetic/epigenetic analyses to determine the incidence of its inactivation. Our study indicates that: (1) inactivation of p16 protein is frequent in HNSCC cell lines (6/11, 54.5%) and OSCC primary tumors (15/17, 88.2%), (2) inactivation of p16^{INK4a} protein is commonly associated with the presence of gene alteration such as mutation, homozygous deletion and especially aberrant methylation, and (3) genomic sequencing of bisulfite-modified DNA shows that the carcinoma develops a heterogeneous pattern of hypermethylation.

Keywords: DNA Methylation; Head and Neck Squamous Cell Carcinoma; p16^{INK4a}.

Introduction

Head and neck cancers are the sixth most common malignancy worldwide (Parkin *et al*., 1988). The molecular mechanisms involved in the development and progression of these carcinomas are not well known. Head and neck carcinogenesis is believed, as is the case with many cancers, to be a multistep process. The definition and sequence of the specific genetic events that lead to the development of invasive head and neck squamous cell carcinoma (HNSCC) has been the subject of intensive study by many different groups. Tumor suppressor gene inactivation is among the most common genetic mechanisms resulting in malignant transformation (Weinberg *et al*., 1994).

Deletions and mutations of the p16^{INK4a} gene have been studied in several oral cancers and cell lines (Lydiatt *et al*., 1995; Reed *et al*., 1996). Recently, the expression of the p16^{INK4a} gene has been shown to be silenced by aberrant methylation in several tumors such as lung, breast, colon, bladder, prostate, and head and neck organs (Gonzalez-Zulueta *et al*., 1995; Herman *et al*., 1995; Merlo *et al*., 1995).

The p16^{INK4a} gene encodes a cell cycle protein which inhibits cyclin-dependent kinase 4 and 6, preventing the phosphorylation of Rb protein and causing the inhibition of cell cycle progression from G1 to S phase (Lukas *et al*., 1995; Serrano *et al*., 1993). Genetic alterations of the p16^{INK4a} gene lead to its inactivation, resulting in the deregulation of cell proliferation and tumorigenesis.

In HNSCC, the inactivation of p16^{INK4a} by mutation was reported in 10% (Cairns *et al*., 1995a; Zhang *et al*., 1994), by homozygous deletion in 33% (Cairns *et al*., 1995b) and by hypermethylation in 20% (Merlo *et al*., 1995) of primary tumors. These studies illustrate that hypermethylation of the 5′ CpGs island within the promoter region of the p16^{INK4a} gene is one of the major mechanisms for the inactivation of this gene in human cancer.

To detect the hypermethylation of the p16^{INK4a} promoter region, we performed the chemical modification of cytosine to uracil by bisulfite treatment. In this reaction, cytosines are converted to uracil, but those that

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Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HNSCC, head and neck squamous cell carcinoma; LOH, loss of heterozygosity; MSP, methylation specific PCR; OSCC, oral squamous cell carcinoma.

are methylated (5-methylcytosine) are resistant to this modification and remain as cytosine (Wang *et al.*, 1980). This modified DNA can be amplified and sequenced, providing detailed information within the amplified region for the methylation status of all CpG sites.

To fully assess the $p16^{INK4a}$ gene status in HNSCC, we analyzed 11 cell lines and 17 OSCC primary tumors for the gene products and genetic/epigenetic alterations of DNA.

Materials and Methods

Primary tumors and margin samples Seventeen OSCC primary tumors were collected following surgical resection with prior consent from Kyungpook National University Hospital patients. Samples from tumor areas and non-neoplastic adjacent tissues were stored at −70°C until studied.

Immunohistochemical analysis of $p16^{INK4a}$ Immunohistochemical staining for $p16^{INK4a}$ was performed on formalin-fixed, paraffin-embedded tissue sections. After hydrogen peroxide treatment to block the endogenous peroxide activity, the slides were washed in distilled water and placed in 0.01 mol sodium citrate buffer (pH 6.0) for 10 min at 95°C, which was followed by rinsing with distilled water and phosphate-buffered saline. Pretreated slides were incubated in normal goat serum for 30 min to block nonspecific binding. Purified mouse anti-human p16 monoclonal antibody (Pharmingen, USA) at a dilution of 1:500 was incubated overnight at 4°C. After the phosphate-buffered saline wash, the primary antibody was visualized using biotinylated anti-mouse IgG (Vector ABC Kit; Vector laboratories, USA) with 0.05% 3, 3′-diaminobenzidine. After counter-staining with 0.05% methyl green, the slides were mounted and microscopic examination followed.

Cell lines Eleven HNSCC cell lines established at KCLB (Korean Cell Line Bank) and ATCC were used for analysis. Their primary sites and characteristics are indicated in Table 1.

DNA extraction Frozen tissues containing more than 70% neoplastic cells were ground and incubated at 50°C for 3 h in a lysis buffer containing 0.5% sodium dodecyl sulfate (SDS), 10 mM Tris·Cl (pH 8.0), 0.1 mM EDTA (pH 8.0), and proteinase K (20 mg/ml). DNA was then purified by phenol-chloroform extraction and ethanol precipitation.

RNA extraction Cultured cells were lysed in a guanidium isothiocyanate-phenol solution (RNAzol B, Biotex Lab, Houston, USA) and chloroform was added to sample lysates. Specimens were then centrifuged, and the aqueous DNA-containing phase was precipitated with isopropanol and resuspended with DEPC-H_2O.

Multiplex PCR amplification To analyze for the presence of homozygous deletion in HNSCC cell lines, we performed a multiplex-PCR on DNA extracted from cell lines. A fragment of $p16^{INK4a}$ exon 2 and p53 as a positive control were co-amplified in the same reaction. The primers for $p16^{INK4a}$ were 5′-CCATTCTGTTCTCTCTGGCA-3′ and 5′-GCATGGTTACTGCCTCTGGT-3′. PCR conditions were adjusted to one cycle at 95°C (7 min), 35 cycles of 95°C (30 s), 58°C (30 s), and 72°C (30 s). The PCR product was electrophoresed in a 2% agarose gel. This yielded 299-bp for $p16^{INK4a}$.

Analysis of $p16^{INK4a}$ expression by RT-PCR and direct sequencing The expression of $p16^{INK4a}$ was analyzed by RT-PCR. A 1 μg aliquot of RNA was reverse transcribed for the first-strand cDNA using the Reverse Transcription System (Promega, Madison, USA) and 2 μl of the final reaction product was used as a template for PCR. Amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed to evaluate the integrity of RNA. The PCR product was electrophoresed in a 2% agarose gel. This yielded 548-bp for $p16^{INK4a}$ and 239-bp for GAPDH. Direct sequencing analysis was performed on the RT-PCR products using an Omni Base™ DNA Sequencing Kit (Promega, Madison, USA). Primer sequences for this reaction were as follows; 5′-GGAGCACGATGGAGCCG (p16-S9), 5′-GGCCCTGTAGGACCTTCG (p16-S13). GAPDH primers were 5′-GTCATGAGTCCTTCCACTATGC-3′ and 5′-GAGTCCACTGGTTTACC-3′.

SSCP analysis The entire exon 1 and two fragments of exon 2 of $p16^{INK4a}$ were PCR amplified for SSCP analysis and 3 μl of the PCR products containing sequencing stop solution were

Table 1. Primary sites and tumor sources of cell lines.

Cell line	Primary site	Gen./age, race	ATCC/KCLB number
SCC 15	Tongue	M/55	ATCC CRL-1623
SCC 25	Tongue	M/70	ATCC CRL-1628
FaDu	Pharynx	M/56, Caucasian	ATCC HTB-43
CAL 27	Tongue	M/56, Caucasian	ATCC CRL-2095
RPMI 2650	Nasal septum	Unknown	KCLB-10030
SNU-46	Larynx, supraglottic	M/65, Mongoloid	KCLB 00046
SNU-585	Larynx, supraglottic	M/56, Mongoloid	KCLB 00585
SNU-899	Larynx, glottic	M/56, Mongoloid	KCLB 00899
SNU-1066	Larynx, glottic	M/62, Mongoloid	KCLB 01066
SNU-1076	Larynx, subglottic	M/60, Mongoloid	KCLB 01076
SNU-1214	Larynx, glottic	M/55, Mongoloid	KCLB 01214

KCLB: Korean Cell Line Bank.

applied to a 6% nondenaturing acrylamide gel. Electrophoresis was carried out at 8 W for 12 h in a cold chamber.

Bisulfite modification for DNA methylation analysis DNA methylation patterns in the CpG islands of the $p16^{INK4a}$ promoter region were determined by chemical modification of unmethylated, but not by the methylated, cytosine to uracil and subsequent PCR using primers specific for either methylated or the modified unmethylated DNA. The bisulfite-modification was performed essentially according to Olek *et al.* (1996). One hundred nanograms of *Eco*RI-digested genomic DNA were incubated in 0.3 M NaOH for 15 min at 50°C. Diluents of this material were mixed with 2 volumes of 2% LMP agarose. Agarose/DNA mixtures were directly pipetted into chilled mineral oil to form agarose beads (Herman *et al.*, 1996; Olek *et al.*, 1996). Aliquots of 100 µl of a 5 M bisulfite solution (2.5 M sodium metabisulfite, Sigma; 125 mM hydroquinone, Sigma; pH 5.0) were added to each reaction tube containing a single bead. The reaction mixtures were then incubated for 4 h at 50°C under exclusion of light. Modification was stopped by equilibrations against 1 ml of 1 × TE (6 × 15 min) followed by desulphonation in 500 µl of 0.2 M NaOH (2 × 15 min). The reaction was neutralized with a 1/5 vol of 1 M hydrochloric acid. The beads were used directly for PCRs.

PCR amplifications of bisulfite-modified DNA Primer sequences of p16-M1 were for the methylated reaction 5′-TTATTAGAG GGTGGGGCGGATCGC (sense) and 5′-GACCCCGAACC-GCGACCGTAA (antisense), and for the unmethylated reaction 5′-TTATTAGAGGGTGGGGTGGATTGT (sense) and 5′-CAACCCCAAACCACAACCATAA (antisense). Primer sequences of p16-M2 were for the methylated reaction 5′-TTATTAGAGGGTGGGGCGGATCGC (sense) and 5′-CC-ACCTAAATCGACCTCCGACCG (antisense), and for the unmethylated reaction 5′-TTATTAGAGGGTGGGGTG-GATTGT (sense) and 5′-CCACCTAAACAACCTCCAAC-CA (antisense). The genomic positions of the 5′ nucleotide of these two sense primers in relation to the major transcriptional positions start site are all +167, but the PCR products are 150-bp for p16-M1 and 234-bp for p16-M2.

Genomic sequencing of bisulfite modified DNA Genomic sequencing of bisulfite modified DNA (Frommer *et al.*, 1992) was accomplished using the solid-phase DNA sequencing approach. Bisulfite modified DNA (100 ng) was amplified with a $p16^{INK4a}$ outer primer (sense, 5′-TTTTTAGAGGATTTG-AG-GGATAGG; antisense, 5′-CTACCTAATTCCAATTC-CCCTACA). PCR conditions were as follows: 95°C for 7 min, then 35 cycles of 95°C (30 s), 56°C (30 s), 72°C (90 s), and finally 5 min at 72°C. To obtain products for sequencing, we performed a second round of PCR with a $p16^{INK4a}$ inner primer (sense, 5′-AGTATTGGAGGAAGAAAGAGGAG; antisense, 5′-TCCAATTCCCCTACAAACTTC). Primer pairs were purchased from Bioneer Science (Korea). Direct sequencing analysis was performed on the PCR products using an Omni Base™ DNA Sequencing Kit (Promega, Madison, USA).

Treatment with a demethylating agent The cell lines which displayed $p16^{INK4a}$ gene methylation were treated with a demethylating agent, 1.0 µm 5-aza-2′-deoxycytidine (Sigma Chemicals Co. St. Louis, USA) for 24 h. The concentration of cells was 1×10^5 per T-75 flask. After exposure, the cells were washed with medium in the absence of FBS and cultured for 24 h. Re-expression of the $p16^{INK4a}$ gene was examined by RT-PCR.

Results

Clinicopathological characteristics of patients The patients comprised 13 males (76.5%) and 4 females (23.5%), ranging in age from 41 to 77 with a mean age of 59. Tumor locations were as follows: soft palate, 4 (23.5%); gingiva, 3 (17.6%); tongue, 5 (29.4%); floor of mouth, 2 (11.8%); buccal mucosa, 3 (11.8%). Histological differentiation of tumors consisted of 10 (58.8%) well, 6 (35,3%) moderately, and 1 (5.9%) poorly differentiated carcinomas.

Table 2. $p16^{INK4a}$ methylation analysis in HNSCC cell lines and re-expression of $p16^{INK4a}$ mRNA by 5-aza-2′-deoxycytidine.

Cell Line	p16 expression	p16 gene alteration		Methylation status		Re-expression of p16 mRNA
	RT-PCR	Mutation	HD	M1/M2	*Bst*UI	
SCC-15	−	−	NoHD	M	M	+
SCC-25	−	−	HD	−	−	·
FaDu	307 bp-deleted	Deletion, MS	NoHD	−	−	·
CAL 27	+	NS	NoHD	−	−	·
RPMI 2650	+	−	NoHD	−	−	·
SNU-46	+	MS	NoHD	−	−	·
SNU-585	74 bp-deleted	Deletion, MS	NoHD	−	−	·
SNU-899	+	−	NoHD	−	−	·
SNU-1066	+	NS	NoHD	−	−	·
SNU-1076	−	MS	NoHD	M	M	+
SNU-1214	−	NS	NoHD	M	M	+

+, positive; −, negative; MS, missense mutation; NS, nonsense mutation; HD, homozygous deletion; NoHD, non homozygous deletion; M1 and M2, primers for methylation-specific PCR; M, methylated.

p16^{INK4a} expression and genetic alterations We analyzed 17 primary OSCCs and 11 cell lines for p16^{INK4a} expression using immunohistochemistry and RT-PCR, respectively. The comparison between p16-protein expression levels and gene alterations in HNSCC cell lines is summarized in Table 2. In our study, homozygous deletion was detected in one of the 11 cell lines (9%). We screened the expression of the p16^{INK4a} gene transcript in cell lines using RT-PCR. Normal sized RT-PCR products were detected in 5 cell lines, whereas no products were detected in 4 cell lines (36.4%) (Fig. 1A). Two cell lines (FaDu, SNU-585) displayed a shorter mRNA than the wild type and a direct sequence analysis of the RT-PCR products was then performed on these two cell lines. These were observed to have a point mutation at the 5′ splice-acceptor site of intron 2 in SNU-585, and at the 3′ splice-donor site of intron 1 in FaDu (Fig. 2). In two cell lines, SNU-1076 and SNU-1214, we detected missense mutation and nonsense mutation respectively, and these results were the same as those of Ku *et al.* (1999).

Fifteen (88.2%) of the OSCC tumors demonstrated a complete absence of p16^{INK4a} nuclear staining. Genetic analysis of the 17 primary OSCC tumors showed that 2 cases had gene mutations with concomitant 9p21 LOH and 2 tumors had only 9p21 LOH (Table 3). The results of 9p21 LOH for these 17 primary OSCC tumors were previously published (Choi *et al.*, 2000). One of the 2 mutated carcinomas had a missense mutation at codon 14 (Asp → Gly) and a nonsense mutation at codon 15 (Trp → STOP) of exon 1. The other cases also showed a nonsense mutation at codon 110 (Trp → STOP) of exon 2 (data not shown). Conversely, genetic mutation and LOH at 9p21 were not detected in any of the tumors which expressed p16^{INK4a} using immunohistochemical analysis.

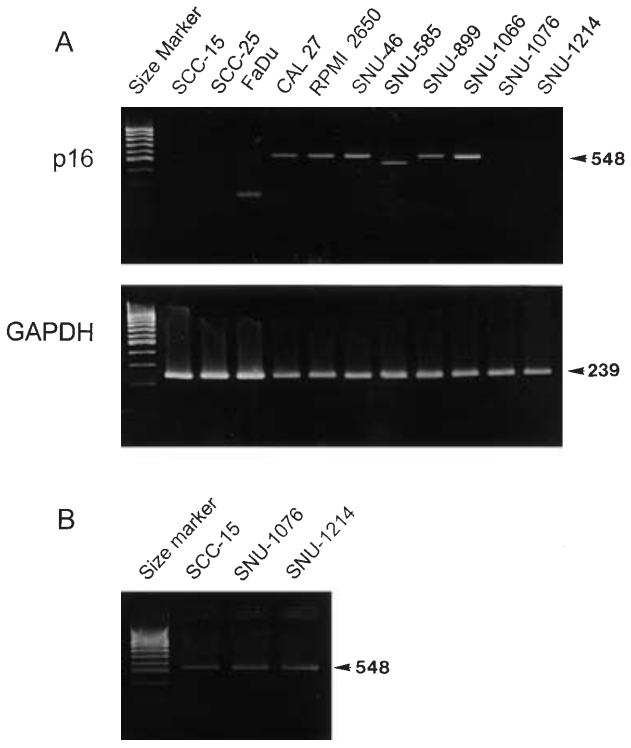

Fig. 1. RT-PCR analysis of the p16^{INK4a} gene in HNSCC cell lines. **A.** before and **B.** after treatment with demethylating agent. The GAPDH gene was used as a positive control. RT-PCR products of normal size (548 bp) derived from mRNA for the p16^{INK4a} exon 2 were observed in five cell lines (CAL 27, RPMI 2650, SNU-46, SNU-899, SNU-1066), and small-sized products were detected in two cell lines (FaDu, SNU-585).

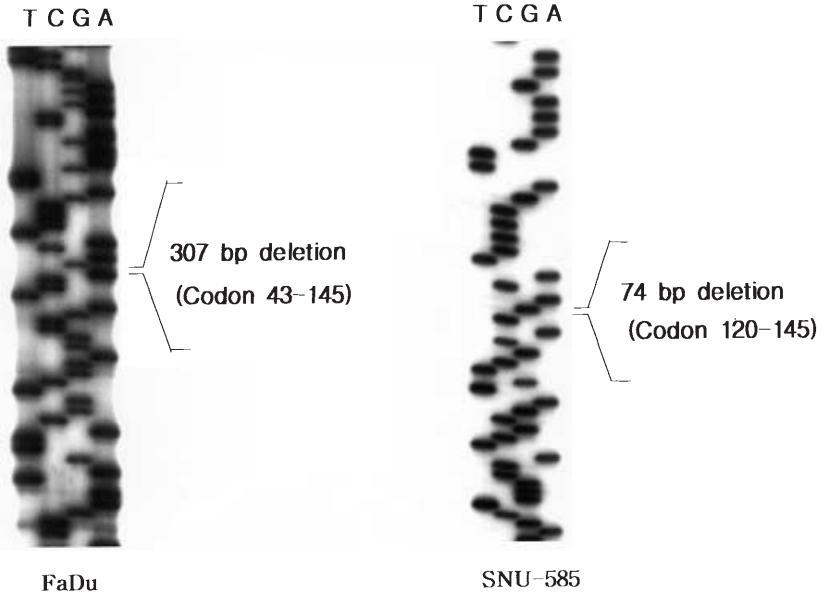

Fig. 2. Direct sequence analyses in two cell lines that displayed a deletion of exon 2 in their RT-PCR products. These cell lines were observed to have point mutations at the 3′ splice-donor site of intron 1 in FaDu, and at the 5′ splice-acceptor site of intron 2 in SNU-585.

Table 3. $p16^{INK4a}$ expression and gene alteration in 17 primary OSCCS.

Case	$p16^{INK4a}$ expression by IHC	$p16^{INK4a}$ gene alteration		N/T	$p16^{INK4a}$ methylation status		
		Mutation	9p21 LOH		M1	M2	*Bst*UI
Mutated							
K-3	+		–	N	–	–	–
				T		M	
K-4	+ +		–	N	–	–	–
				T	M	M	
Non-Mutated							
K-5	–		+	N	–	–	–
				T	M	M	
K-6	–		ND	N	–	–	–
				T	M	M	M
K-7	–		ND	N	–	–	–
				T	M	M	M
K-8	–	Exon 1 codon 14, 15 Asp, Trp → Gly, STOP	+	N	–	–	–
				T	–	–	–
K-9	–		–	N	–	M	–
				T	M	M	
K-10	–		–	N	–	–	–
				T	M	M	
K-11	–		–	N	–	–	–
				T			
K-12	–	Exon 2 codon 110 Trp → STOP	+	N	–	–	–
				T	M	M	–
K-13	–		+	N	M	M	–
				T	M	M	M
K-14	–		–	N	M	M	M
				T	M	M	M
K-15	–		–	N	–	–	–
				T			
K-16	–		–	N	M	–	–
				T	M	M	M
K-17	–		–	N	M	M	–
				T	M	M	
K-18	–		–	N	M	M	–
				T	M	M	
K-19	–		–	N	–	–	–
				T	M	M	M

+, positive; –, negative; ND, not determined; M, methylated.

MSP analysis of HNSCC cell lines Because $p16^{INK4a}$ promoter expression is frequently inactivated by *de novo* methylation, we screened the methylation status of the $p16^{INK4a}$ promoter in the primary carcinomas and cell lines. After the bisulfite modification of DNA, methylation specific-PCR was employed for the detection of methylated and non-methylated DNA sequences of $p16^{INK4a}$ (Herman *et al.*, 1996).

All the samples were subjected to amplification of the first exon of $p16^{INK4a}$ with methylation-modified (p16-M) and -unmodified (p16-U) primers. To verify further the methylation status of the $p16^{INK4a}$ promoter, we treated the PCR products amplified with $p16^{INK4a}$ inner primers with *Bst*UI, because these products are longer than those amplified with M1 or M2. The *Bst*UI recognition site, CGCG, will remain intact after bisulfite treatment if both Cs are methylated, but will become TGTG if unmethylated. Thus, products amplified with unmethylated products failed to be cleaved, but in a completely methylated sample one would expect to see the 166-, 115-, and 57-bp fragments.

Methylated DNA was detected in the cell lines SCC-15, SNU-1076 and SNU-1214, and analyses of these promoter sequences revealed exclusive methylation of this site of the $p16^{INK4a}$ promoter (Fig. 3). The amplified $p16^{INK4a}$ products of SCC-15, SNU-1076 and SNU-1214 were fragmented with *Bst*UI and others were not cleaved (data not shown).

In order to extend these findings, we confirmed the methylation status of all the intervening CpG dinucleotides using bisulfite genomic sequencing (Fig. 4). These results demonstrate almost complete methylation of CpG dinucleotides of the $p16^{INK4a}$ promoter in SCC-15, SNU-1076 and SNU-1214. To confirm whether silencing of the $p16^{INK4a}$ gene in these three cell lines is due to methylation, we checked to

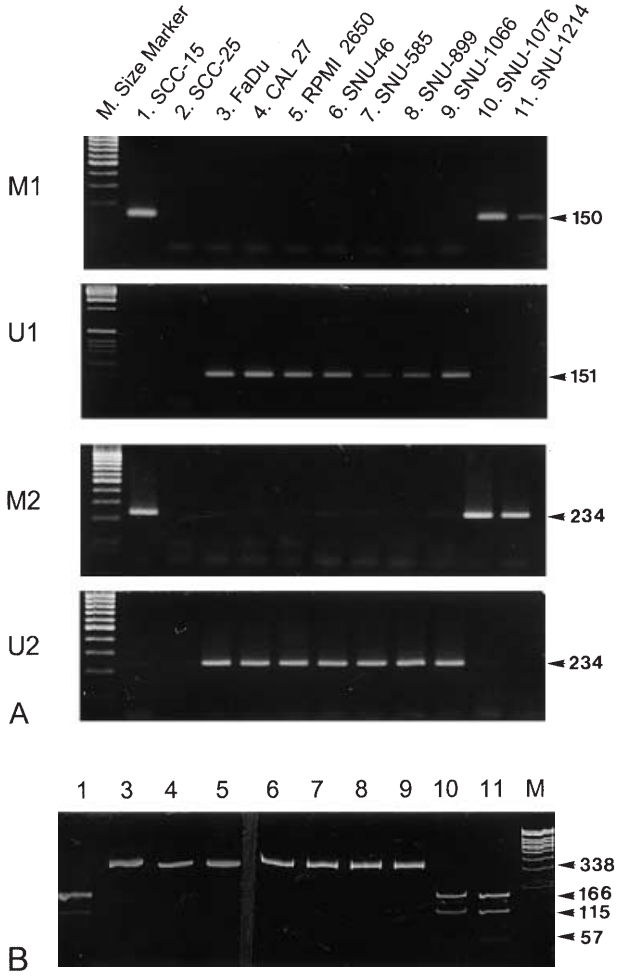

Fig. 3. Methylation-specific PCR of p16^{INK4a}. Primer sets for amplification were designated as unmethylated (U1, U2) or methylated (M1, M2). **A.** Amplification of bisulfite-treated DNA from HNSCC cell lines with p16-M1 (M2) and p16 U1 (U2). **B.** The amplified products with the p16^{INK4a} inner primer were restricted with *Bst*UI.

see if p16^{INK4a} mRNA is re-expressed after treatment with the demethylating agent 5-aza-2′-deoxycytidine for 24 h. As expected, RT-PCR products were detected in all three cell lines after the treatment (Fig. 1B). p16^{INK4a} promoter hypermethylation of the SCC-15 cell line was previously identified by Cody *et al.* (1999). They also showed the re-expression of p16^{INK4a} mRNA by 5-aza-2′-deoxycytidine treatment.

MSP analyses of primary OSCC tumors In clinical samples, two of the seventeen tumors that showed p16^{INK4a} protein expression had no CpG islands methylation of this gene. Twelve of fifteen cases of the primary OSCC tumors without mutation showed bands amplified with the methylation-modified primer (Table 3). Also there were some methylation bands in normal samples (K-9, K-13, K-14, K-16, K-17, K-18). There were no relationships between the methylation of these normal samples and clinicopathological characteristics such as age, site, differentiation and lymph node metastasis. The methylation status of the p16^{INK4a} promoter was verified further by *Bst*UI treatment. As shown in Fig. 5, the *Bst*UI site in the p16^{INK4a} gene of 9 cases (K-5, K-8, K-9, K-10, K-11, K-12, K-15, K-17, K-18) was unmethylated and only the 234-bp fragment was seen. The six cases (K-6, K-7, K-13, K-14, K-16, K-19) which are known to have no p16^{INK4a} gene mutation, showed partial methylation at this site. Normal and tumor tissues of K-14 showed both methylated and unmethylated bands, but the signal of tumor samples was more strong than in normal tissues (Fig. 5). It has been suggested that methylation of a normal sample is an age-related phenomenon (Case K-14 77 yrs vs average 59 yrs). In one tumor (K-13), concurrent LOH and methylation were observed. Genomic sequencing of bisulfite modified DNA of the primary tumor showed the coincidence of C and T bands, suggesting the heterogeneous state of p16^{INK4a} gene promoter methylation.

Discussion

Many authors have identified tumors with an absence of p16^{INK4a} gene mutation (Castellano *et al.*, 1997; El-Naggar *et al.*, 1997; Reed *et al.*, 1996), suggesting that other mechanisms of inactivation of the gene may also exist. Recent attention has been drawn to epigenetic events including cytosine methylation and its role in tumorigenesis. Most methylation changes that have been identified occur in clustered CpG dinucleotides of approximately 1 kb called CpG islands. CpG islands within genes and *de novo* methylation of CpG islands within gene promoters are associated with gene silencing of X-linked and imprinted genes (Park and Lee, 1997b). DNA methylation is necessary for normal mammalian development and may be involved in gene repression during embryonic development and differentiation (Park and Chapman, 1997a).

In this study we have analyzed the status of the p16^{INK4a} gene in HNSCC cell lines and primary OSCC tumor samples.

There were 4 cell lines that showed no RT-PCR product of the p16^{INK4a} gene. Analysis of a portion of the p16^{INK4a} gene containing a CpG island revealed that three of these four cell lines had aberrant methylation at this site. Treatment with the demethylating chemical 5-aza-2′-deoxycytidine caused re-expression of p16^{INK4a} in these three cell lines, consistent with the inhibition of p16^{INK4a} expression by aberrant DNA methylation. The absence of the RT-PCR product of the other cell line is due to homozygous deletion.

The sequence analyses of SNU-585 and FaDu are the same as those performed by Akanuma *et al.* (1999) with different cell lines, HSC-2 (from mouth floor, 74 bp deletion) and HSC-6 (from tongue, 307 bp deletion). Why the deleted sizes of the p16^{INK4a} gene are the same in different cell lines is uncertain, but the deletion due to a splicing mistake is supposed to be a common event in HNSCC cell lines.

DNA mutation and LOH of p16^{INK4a} occur only in a small number of primary tumors of HNSCC. Only two

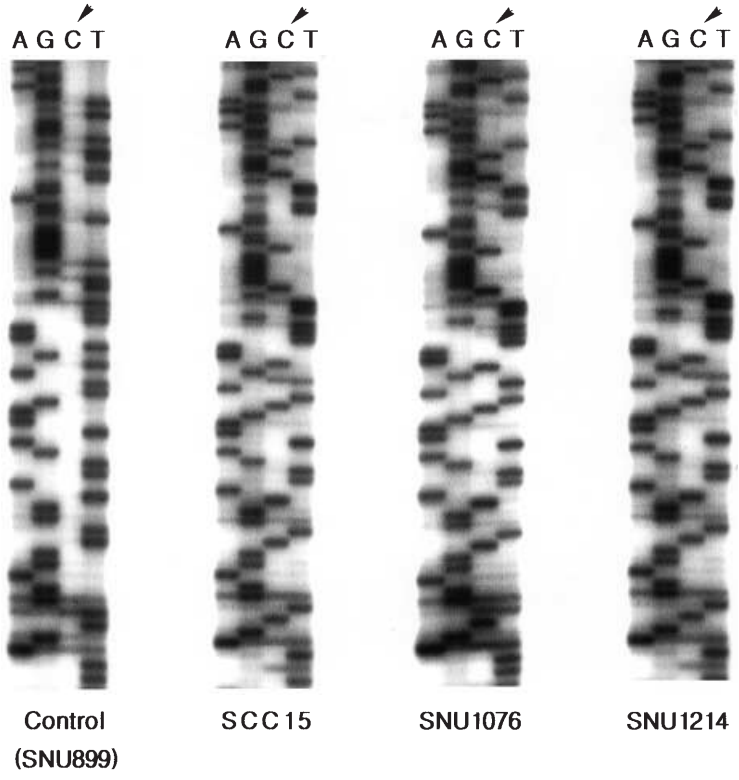

Fig. 4. Genomic sequencing of bisulfite-modified $p16^{INK4a}$ DNA. All cytosines in the unmethylated cell line SNU-899 have been converted to thymine, while all cytosines in CpG dinucleotides in the methylated cell lines SCC-15, SNU-1076 and SNU-1214 remain as C, indicating methylation.

cases (11.8%) had nonsense and/or missense mutations, and 4 cases (23.5%) had LOH. The inactivation of the $p16^{INK4a}$ gene by hypermethylation was 35.3%. We found 6 primary tumors which displayed the absence of immunohistochemical staining for $p16^{INK4a}$ without an obvious genetic alteration or aberrant methylation. These may have a mutation or methylation outside of the region we sequenced. All six tumors with methylation displayed the complete absence of $p16^{INK4a}$ expression through immunohistochemical analysis.

That there was no significant association between $p16^{INK4a}$ alteration and the histological differentiation stage suggests that the imbalance of DNA methylation begins at the earliest stages of the neoplastic process (Baylin *et al.*, 1998).

$p16^{INK4a}$ was inactivated by hypermethylation in about 20% of the cases studied (Gonzalez *et al.*, 1997; Jares *et al.*, 1999). In our results, $p16^{INK4a}$ promoter methylation by MSP is abundant when compared with other studies. This is because we amplified the bisulfite-modified $p16^{INK4a}$ promoter DNA by a second round of PCR, and MSP is not quantitative method. Six out of 12 cases which showed methylation-modified bands failed *Bst*UI treatment. These methylated bands may result from methylation of only the primer- binding region of $p16^{INK4a}$ DNA within the tumor.

Methylation of the CpG islands of tumor suppressor genes leading to their transcriptional inactivation is a highly consistent feature of tumorigenesis. Studies of primary tumors and OSCC cell lines indicate that methylation may constitute an alternative mechanism in silencing of the $p16^{INK4a}$ gene.

A recently described bisulfite-modified PCR-based method called MSP is an excellent alternative, but it tends to be a qualitative, rather than a quantitatively accurate method. Southern hybridization approaches provide an overall methylation status of CpG islands, but can only provide information about those CpG sites found within sequences recognized by methylation-sensitive restriction enzymes (Reed *et al.*, 1996). A more sensitive method of methylation-sensitive restriction enzyme digestion followed by PCR is prone to false-positive results since any uncleaved DNA will be amplified by PCR yielding a positive result for methylation. The chemical modification of cytosine to uracil by bisulfite treatment and a direct sequencing method is technically rather difficult and labor-intensive and less sensitive than the Southern analysis. Compared with these methods, MSP is more sensitive, facilitating the detection of low numbers of methylated alleles and the study of DNA from small samples. But MSP is supposed to give positive results even if a very small

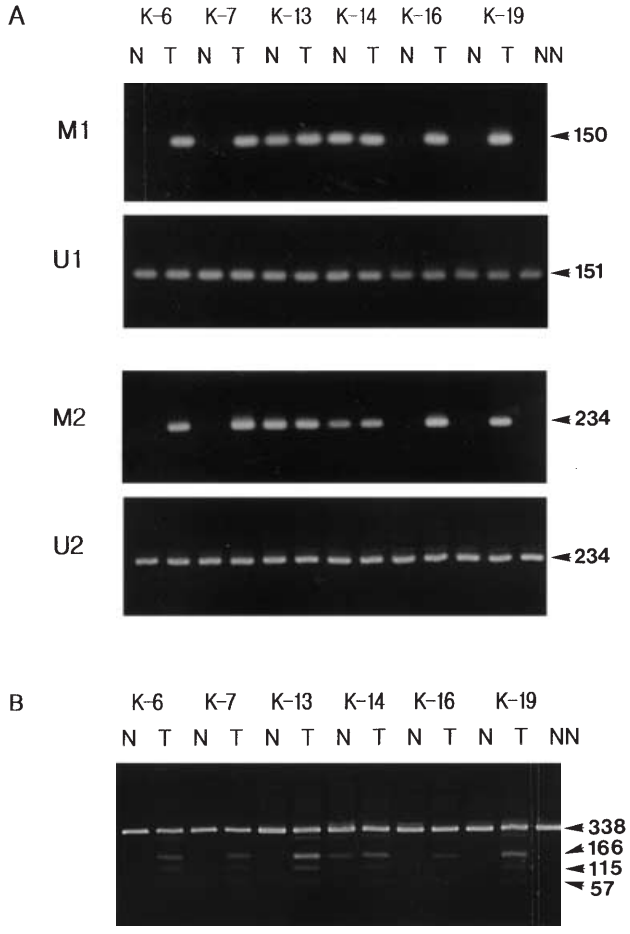

Fig. 5. MSP of p16^{INK4a}. Primer sets for amplification were designated as unmethylated (U) or methylated (M). **A.** amplification of bisulfite-treated DNA from OSCC patients with the p16-M1 (M2) and p16-U1 (U2). **B.** The amplified products with the p16^{INK4a} inner primer were restricted with *Bst*UI. NN designates neck lymph node.

portion of alleles is methylated. However, products amplified with methylation-modified primers failed to be cleaved or else the fragmented bands were very weak as compared with those of PCR products. So, MSP should be verified by *Bst*UI treatment.

In the case of cell lines, the methylation status of the p16^{INK4a} promoter region determined by MSP confirmed that *Bst*UI restriction site analysis validated the PCR product specificity. But primary tumor samples showed very different results in MSP and *Bst*UI treatment. This is because of the heterogeneous methylation status of primary tumor samples.

In conclusion, our study indicates that inactivation of the p16^{INK4a} protein is frequent in HNSCC cell lines (6/11, 54.5%) and OSCC primary tumors (15/17, 88.2%) and is commonly connected with the presence of gene alterations such as mutation, homozygous deletion and especially aberrant methylation. Genomic sequencing of bisulfite-modified DNA also shows that carcinomas develop a heterogeneous pattern for hypermethylation.

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