Abrogation of the p16-retinoblastoma-cyclin D1 pathway in head and neck squamous cell carcinomas

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Abstract. In the present study, we analyzed p16, retinoblastoma (Rb), and cyclin D1 abnormalities in head and neck squamous cell carcinoma (HNSCC) tissues and cell lines from Korean patients. We found a 40% loss of heterozygosity at the D9S171 locus (9p21 region) these tissues. All eight of the HNSCC cell lines did not express the p16 protein, and in two of these cell lines (Amc-HN-6 and 8), this was due to a deletion of the p16 gene. Three of the cell lines (Amc-HN-3 to 5) that expressed the p16 mRNA had the same nonsense mutation at codon 50 (CGA-Arg to TGA-Ter). The Amc-HN-1 and Amc-HN-7 cell lines, which did not express the p16 mRNA, had a missense mutation at codon 9 (GCC-Ala to GTC-Val) and a silent mutation at codon 106 (CCC-Pro to CCA), respectively. The Amc-HN-2 cell line (p16 exon-positive/mRNA-negative) had a single base deletion at codon 38 (CGG-Arg to CG), which resulted in a frameshift and a consequent stop signal at codon 44. The Rb protein was detected in all of the eight cell lines, although it was inactive in five of these due to hyperphosphorylation. The inverse relationship between p16 and Rb was 62.5% (5/8). Cyclin D1 was overexpressed in all of the eight cell lines. Our results suggest that the abrogation of p16, the overexpression of cyclin D1, and the consequent inactivation of Rb could be important factors in the carcinogenesis of HNSCCs.

Introduction

Head and neck squamous cell carcinoma (HNSCC) remains a significant cause of morbidity and mortality, with ~540,000 new cases reported annually worldwide and 271,000 deaths reported annually resulting in a mortality rate of 50% (1). HNSCC is the most common cancer in head and neck malignancies and it is the sixth most common cancer worldwide (2). HNSCC ranks 5th in both the frequency and cause of cancer death in Western countries (3). HNSCC is very common, especially in India and South East Asia, and accounts for ~40% of all malignancies (4).

The retinoblastoma (Rb) protein is one of the most important cell cycle proteins of the G1 checkpoint. The activity of the Rb protein is regulated by its phosphorylation level, which is mediated by cell cycle regulators such as p16 and cyclin D1.

The tumor suppressor gene, p16 (MTS1/CDKN2A), located on human chromosome 9p21, encodes a protein that inhibits the binding of cyclin D1 to the cyclin-dependent kinases (CDK4 or CDK6), thus preventing the phosphorylation of the Rb protein (5,6). The underphosphorylated Rb inhibits the E2F-mediated transcriptional activation of the S phase genes, which are necessary for cell proliferation. The p16 protein is one of the central Rb-dependent regulators responsible for controlling cell proliferation at the G1-S checkpoint.

The cyclin D1 proto-oncogene is also a key regulator of the cell cycle at the restriction point in late G1. Cyclin D1 binds to CDK4/6 and subsequently induces cell proliferation by releasing the E2F transcription factor from the Rb protein via the phosphorylation of Rb (7-9). The amplification of the cyclin D1 gene and protein overexpression have been associated with aggressive behavior in HNSCC and other tumors including higher local recurrence, higher metastatic potential, and diminished survival (10-14).

The inactivation of p16, the overexpression of cyclin D1, and the subsequent inactivation of the Rb protein have been reported in a variety of human cancers (15-20). However, little is known about the p16-Rb-cyclin D1 status in HNSCC in Korean patients. Thus, this study sought to determine whether p16, Rb, and cyclin D1 alterations are important in the development of HNSCCs in a Korean population. We performed a loss of heterozygosity (LOH) analysis of eight Korean HNSCCs using nine microsatellite markers that cover chromosome 9p. We also examined eight HNSCC cell lines obtained from Korean patients, in order to reveal alterations in the p16 gene and protein expressions of p16, Rb, and cyclin D1. We discuss the correlation between p16, Rb, and cyclin D1 and the implication of such correlations in the carcinogenesis of HNSCC in Korean patients.

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Abbreviations: HNSCC, head and neck squamous cell carcinoma; Rb, retinoblastoma; CDK, cyclin-dependent kinase; LOH, loss of heterozygosity; PBL, peripheral blood lymphocyte; RPA, ribonuclease protection assay

Key words: head and neck squamous cell carcinoma, p16, retinoblastoma, cyclin D1, alteration

Table I. Microsatellite markers on chromosome 9p for LOH analysis.

Marker	Primer	Sequence (5' to 3')	Location
D9S144	9CMP4R 9CMO4F	GGATAAATACACTGGAAAAGAGAT AAATATTATAGCAAGTTAATTACTGAA	9pter-p22
D9S156	AFM051xd6m AFM051xd6a	AGATGGTGGTGAATAGAGGG ATCACTTTTAACTGAGGCGG	9p23-p22
D9S162	AFM115yb4m AFM115yb4a	AATTCCCACAACAAATCTCC GCAATGACCAGTTAAGGTTC	9p23-p22
D9SIFNA	GT strand AC strand	TGCGCGTTAAGTTAATTGGTT GTAAGGTGGAAACCCCCACT	9p22
D9S171	AFM186xc3m AFM186xc3a	ACCCTAGCACTGATGGTATAGTCT AGCTAAGTGAACCTCATCTCTGTCT	9p21
D9S126	D9S126.PCR1.2 D9S126.PCR1.1	CAACTCCTCTTGGGAACTGC ATTGAAACTCTGCTGAATTTTCTG	9p21
D9S161	AFM087yd3m AFM087yd3a	CATGCCTAGACTCCTGATCC TGCTGCATAACAAATTACCAC	9p21
D9S200	C80R C80F	CCTCTCTGCATGCCCCAG GCATTTCACAGGAAATAATCTAAGG	9p21-p12
D9S166	AFM144zg7m AFM144zg7a	TCCTAATTCACTGGGAAAAC AAATCATGCAATTCATTTCA	9p12-q21

Materials and methods

Tissues, peripheral blood lymphocyte (PBL)s, and DNA extraction. We used samples of 8 surgically resected HNSCCs and PBLs of the corresponding patients. The tissues and PBLs were snap frozen using liquid nitrogen and stored immediately at -80˚C until analysis. The genomic DNA from the microdissected tumor tissues and PBLs were prepared using a QIAamp Tissue Kit (Qiagen, Chatwirth, CA, USA).

LOH. LOH analysis was performed using 9 microsatellite markers spanning chromosome 9p. The microsatellite markers used in this study are shown in Table I. The primers were purchased from Research Genetics (Huntsville, AL, USA). PCR was carried out in 10 μl reaction volumes that contained 100 ng genomic DNA, 1X PCR buffer (Gibco-BRL, Grand Island, NY, USA), 100 μM of each dATP, dGTP and dTTP, 5 μM dCTP, 1 μCi [α-^{32}P]dCTP, 2.5 pmol of each primer, and 1 unit Taq polymerase (Perkin-Elmer, Sunnyville, CA, USA). PCR was performed in a Thermocycler 9600 (Perkin-Elmer) under the following conditions: Initial denaturation at 94˚C for 2 min followed by 30 amplification cycles (94˚C for 30 sec, 58˚C for 30 sec, and 72˚C for 30 sec), and a final extension at 72˚C for 7 min. The PCR product was separated on a denaturing polyacrylamide gel and the bands were detected by autoradiography. LOH was scored when the intensity of one allele was absent or at least 70% reduced in the tumor alleles when compared to the corresponding wild-type alleles for the informative cases. Any additional bands on the tumor side were regarded as microsatellite instability.

Cell lines and preparation of genomic DNA and total RNA. Eight HNSCC cell lines from Korean patients, Amc-HN-1, Amc-HN-2, Amc-HN-3, Amc-HN-4, Amc-HN-5, Amc-HN-6, Amc-HN-7, and Amc-HN-8, were obtained from the Asan Biological Sciences Research Center at Ulsan University (Ulsan, Korea). The genomic DNA was isolated using a standard SDS-proteinase K and phenol-chloroform extraction method. The total RNA was extracted using an RNeasy Total RNA Kit (Qiagen).

Detection of p16 deletion. The p16 deletion was detected by PCR of exons 1 and 2 of the p16 gene using the following primers (exon 1: Forward, 5'-GGAATTCGAAGAAAGA-3' and reverse, 5'-CGGATCCTCTACCCACCTGGATCGG-3'; exon 2: Forward, 5'-GCTTCCTTTCCGTCATGCCG-3' and reverse, 5'-CAAATTCTCAGATCATC-3') and Vent DNA polymerase (New England Biolabs, Beverly, MA, USA). The PCR products were resolved on a 10% polyacrylamide gel and stained with ethidium bromide.

Nucleotide sequence determination of the p16 gene. Exons 1 and 2 of the p16 gene in the Amc-HN-series HNSCC cell lines amplified by Vent polymerase (New England Biolabs), were cloned into the *HincII* site of pUC18. The nucleotide sequence was determined using an automated sequencer (ABI PRISM 377, Perkin-Elmer, Norwalk, CT, USA).

Ribonuclease protection assay (RPA). RPA was performed using the Multi-probe RNase Protection Assay System (PharMingen, San Diego, CA, USA). Ten micrograms of total

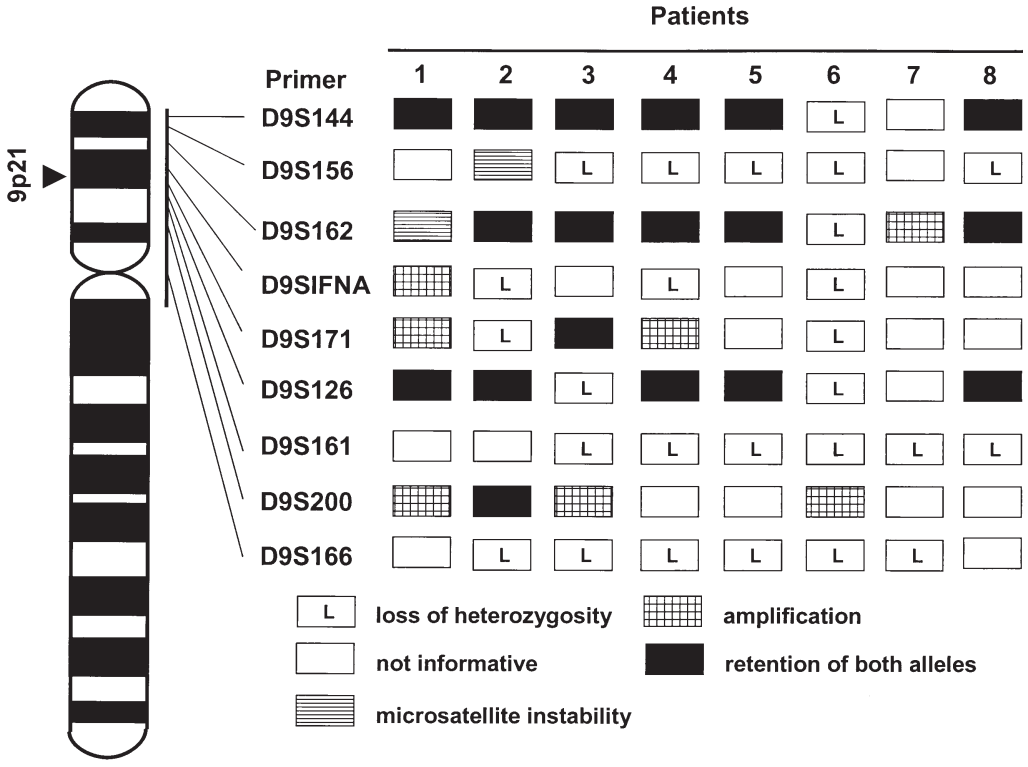

Figure 1. Deletion map of chromosome 9p. Microsatellite markers and map positions are illustrated. The arrow indicates the D9S171 (9p21) locus.

Table II. Summary of 9p allelic abnormalities in HNSCCs in Korean patients.

Primer	Site	LOH/IC (%)	AM/IC (%)	MI/IC (%)
D9S144	9pter-p22	1/7 (14.3)	0/7 (0)	0/7 (0)
D9S156	9p23-p22	5/6 (83.3)	0/6 (0)	2/6 (33.3)
D9S162	9p23-p22	1/8 (12.5)	1/8 (12.5)	1/8 (12.5)
D9SIFNA	9p22	3/4 (75)	1/4 (25)	0/4 (0)
D9S171	9p21	2/5 (40)	2/5 (40)	0/5 (0)
D9S126	9p21	2/7 (28.6)	0/7 (0)	0/7 (0)
D9S161	9p21	6/6 (100)	0/6 (0)	0/6 (0)
D9S200	9p21-p12	0/4 (0)	3/4 (75)	0/4 (0)
D9S166	9p21-q21	6/6 (100)	0/6 (0)	0/6 (0)

IC, informative cases; AM, amplification; MI, microsatellite instability.

RNA were hybridized with p16 and Rb probes which were transcribed *in vitro* in the presence of the GACU nucleotide pool, RPA template set, RNasin, and [α-^{32}P]UTP (DuPont NEN, Boston, MA, USA) using the T7 polymerase according to the manufacturer's manual. The RNA duplexes protected from RNase treatment were isolated and electrophoresed in a standard 6% acrylamide gel containing 7 M urea. The gel was dried and placed on an XAR film (Kodak, Rochester, NY, USA) with intensifying screens and exposed at -80˚C.

Preparation of nuclear extracts and Western blotting. The cells were harvested with trypsin-EDTA and washed with PBS. The cell pellets were resuspended in 400 μl cold buffer A [10 mM Hepes-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% NP-40, and 0.2 mM PMSF] and allowed to swell on ice for 30 min. The cells were then vortexed for 30 sec and centrifuged for 10 sec. The pellet was resuspended in 1 to 3 vol cold buffer C [20 mM Hepes (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF] and incubated on ice for 30 min. The cellular debris was removed by centrifugation for 2 min at 4˚C. Forty micrograms of the nuclear extracts were separated on a 4-20% gradient SDS-polyacrylamide gel (Novex, San Diego, CA, USA). The protein transferred onto the nitrocellulose membrane was probed with p16, Rb or cyclin D1-specific antibodies (PharMingen).

Results and Discussion

LOH at region 9p in HNSCC tissues. LOH at D9S171 (region 9p21) was found in 2 of 5 cases (40%; Fig. 1 and Table II). Although only 5 cases were informative, this result suggests that the genetic alteration(s) near or including the p16 tumor suppressor gene occurred during the development of HNSCCs. This frequency of LOH was higher than the LOH frequencies of 15% (21) and 19% (22) reported in previous studies. Other reports have shown a similar (41%) (23) or increased (75%) (24) LOH at 9p21 in HNSCC. Other markers exhibiting a high frequency of LOH were D9S161 (100%, 6 of 6), D9S166 (100%, 6 of 6), D9S156 (83%, 5 of 6), and D9SIFNA (75%, 3 of 4). This suggests that an extensive genetic alteration occurred in many loci on chromo-

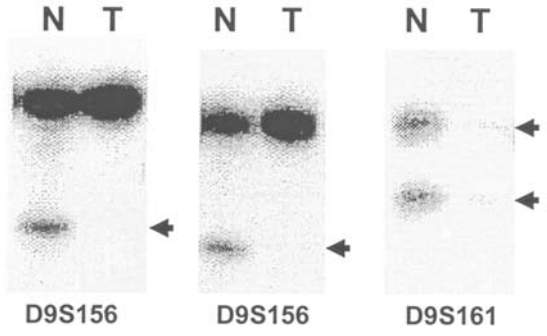

Figure 2. Representative LOH patterns. T, HNSCC tissue; N, corresponding PBL of the same patient. The arrows indicate the absence or a decreased band intensity of over 70% in the HNSCC tissues.

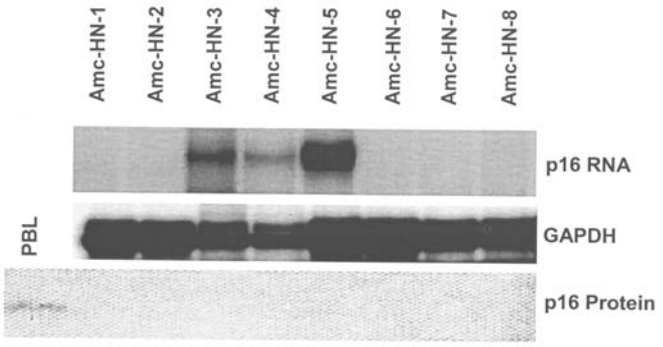

Figure 4. Alterations of the p16 mRNA and protein detected by RPA and Western blotting in the HNSCC cell lines. GAPDH, glyceraldehyde-3-phosphate dehydrogenase mRNA.

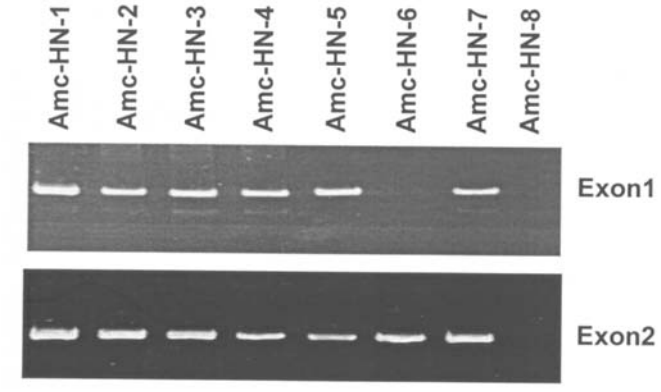

Figure 3. The p16 gene deletion in the HNSCC cell lines. The PCR analyses amplifying p16 exons 1 and 2 were performed with genomic DNAs from the cell lines.

some 9p during the carcinogenesis of HNSCC. For example, patient No. 6 showed LOH in 8 of the 9 microsatellite markers. Representative LOH patterns are shown in Fig. 2. We further characterized the alterations of p16 in the 8 HNSCC cell lines from Korean patients.

P16 alterations. Two cell lines (Amc-HN-6 and Amc-HN-8) had a deletion in exon 1 of the p16 gene. The Amc-HN-8 cell line contained deletions in both exons 1 and 2 (Fig. 3). We could not detect p16 transcripts in these 2 cell lines by RPA analysis. Amc-HN-1, Amc-HN-2, and Amc-HN-7, which were shown to contain p16 exons 1 and 2, did not express the p16 transcripts (Fig. 4). Amc-HN-3, Amc-HN-4, and Amc-HN-5, which did show the p16 transcripts, did not express the p16 protein. These results suggest that these 6 cell lines contained mutations in or around the p16 gene. All of the 8 HNSCC cell lines examined did not express the p16 protein (Fig. 4).

Zhang *et al* (25) and Lydiatt *et al* (22) reported that the frequency of p16 deletions and mutations in HNSCC cell lines was 44 and 56.2%, respectively. In this study, we found that genetic alterations in p16 are less frequent in primary HNSCC tissues (40%) than in the HNSCC cell line (100%). This could be due to the fact that p16 deletions result from adaptation to the tissue culture, as suggested by other investigators who found relatively low frequencies of p16 alterations in primary tumors in comparison to cell lines (26). Alternatively, the lower frequency of the p16 alteration could result from the high sensitivity of the PCR-based assay, which allows for a positive reaction even in the presence of an extremely small amount of normal tissue contamination.

The p16 mutations in HNSCC cell lines. We then determined the nucleotide sequence of p16 exons 1 and 2 in the HNSCC cell lines (Amc-HN-1, Amc-HN-2, Amc-HN-3, Amc-HN-4, Amc-HN-5, and Amc-HN-7). Amc-HN-1, Amc-HN-2, and Amc-HN-7 were p16 DNA-positive/p16 RNA-negative, while Amc-HN-3, Amc-HN-4, and Amc-HN-5 were p16 DNA-positive/p16 RNA-positive/p16 protein-negative. Amc-HN-1 had a missense mutation at codon 9 (GCC-Ala to GTC-Val) of exon 1. Amc-HN-2 had a single base deletion at codon 38 (CGG-Arg to CG) of exon 1, which resulted in a frameshift and consequent stop signal at codon 44. Amc-HN-7 had a silent mutation at codon 106 (CCC-Pro to CCA) of exon 2. Amc-HN-3, Amc-HN-4, and Amc-HN-5 all contained a nonsense mutation at the same site (codon 50, CGA-Arg to TGA-Ter) of exon 2. This mutation could result in the truncation of the p16 protein, which may not be detected by Western blotting. The same mutation was reported by Poi *et al* (27) and by Tripathi *et al* (28) in HNSCC and cervical carcinoma, respectively. The missense mutation at codon 9 (Amc-HN-1), the single base deletion at codon 38 (Amc-HN-2), and the silent mutation at codon 106 (Amc-HN-7), have not been previously reported.

Since the mutations in Amc-HN-1 and Amc-HN-7 did not affect the protein function, the absence of p16 mRNA in these cell lines could be due to unknown mechanisms including p16 promoter methylation.

Rb alterations. Our next step was to analyze Rb expression since p16 controls cell proliferation via the Rb pathway. Rb was detected in all the 8 cell lines examined at both the mRNA and protein levels. We examined the phosphorylation status of Rb using an Rb-specific antibody that can detect both hyperphosphorylated and hypophosphorylated Rb. Five cell lines (Amc-HN-1, Amc-HN-4, Amc-HN-5, Amc-HN-7, and Amc-HN-8) expressed hyperphosphorylated (inactive) Rb (Fig. 5). This suggests that the abrogation of p16 and the consequent inactivation of Rb could be important in the

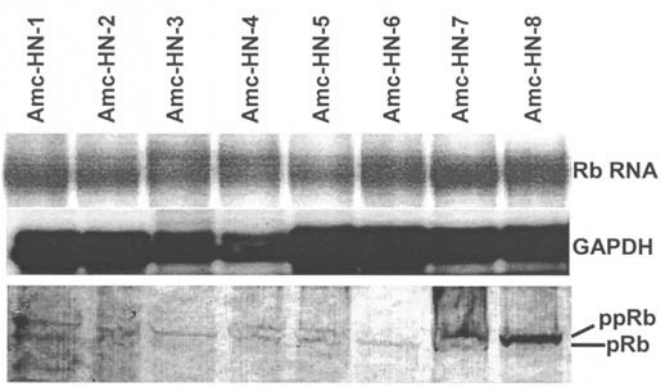

Figure 5. mRNA and protein alterations of Rb detected by RPA and Western blotting in the HNSCC cell lines. Western blotting was performed with a monoclonal antibody that detects both hyperphosphorylated (ppRb) and hypophosphorylated (pRb) Rb proteins.

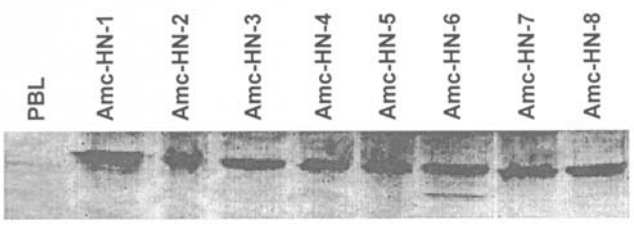

Figure 6. The overexpression of cyclin D1 detected by immunoblotting in the HNSCC cell lines. The leftmost lane shows the cyclin D1 expression level of normal PBLs with the same protein loading.

carcinogenesis of HNSCCs. The inverse relationship between p16 and Rb was 62.5% (5/8).

Cyclin D1 overexpression. Cyclin D1 is deregulated in many types of human tumor cells. The overexpression of cyclin D1 was especially known to contribute to the oncogenic transformation of cells (29-31). The expression level of cyclin D1 affects cell proliferation through binding to CDK4 and CDK6. Thus, we analyzed cyclin D1 protein expression using Western blotting. Cyclin D1 was overexpressed in all the HNSCC cell lines examined in comparison to the expression levels of normal PBLs (Fig. 6). The elevated cyclin D1 level induced the hyperphosphorylation of the Rb proteins in the HNSCC cell lines in the absence of the p16 protein. All of the eight HNSCC cell lines that did not express the p16 protein expressed cyclin D1 at a high level. This was consistent with the report that the loss of p16 in tumor cells is related to constitutive cyclin D1 expression (32).

Cyclin D1 overexpression has been reported to supersede Rb-mediated growth inhibition in esophageal tumors and in small cell lung carcinoma (13,33). This could be related to the phosphorylation and inactivation of the Rb gene product or to the growth-promoting activity by cyclin D1, independent of Rb (33). These reports could explain our finding that the active proliferative properties of the 3 HNSCC cell lines (Amc-HN-2, Amc-HN-3, and Amc-HN-6) expressing active (hypophosphorylated) Rb, were due to the cyclin D1 overexpression. The alterations of p16, Rb, and cyclin D1 in the HNSCC cell lines studied are summarized in Table III. All of the 8 (100%) HNSCC cell lines did not express the p16 proteins, and in 2 (25%) and 4 (50%) of these cell lines, this was due to p16 homozygous deletions and mutations,

Table III. Summary of p16, Rb, and cyclin D1 alterations in HNSCC cell lines in Korean patients.

	p16				Rb			
Cell lines	DNA	RNA	Protein	Mutation	RNA	ppRb	pRb	CycD1
Amc-HN-1	+	-	-	9th GCC_{Ala} to GTC_{Val}	+	+	+	+
Amc-HN-2	+	-	-	38th CGG_{Arg} to CG (G deletion) stop at 44th codon	+	-	+	+
Amc-HN-3	+	+	-	50th CGA_{Arg} to TGA_{Ter}	+	-	+	+
Amc-HN-4	+	+	-	50th CGA_{Arg} to TGA_{Ter}	+	+	+	+
Amc-HN-5	+	+	-	50th CGA_{Arg} to TGA_{Ter}	+	+	+	+
Amc-HN-6	-	-	-	ND	+	-	+	+
Amc-HN-7	+	-	-	106th CCC_{Pro} to CCA_{Pro}	+	+	+	+
Amc-HN-8	-	-	-	ND	+	+	+	+

ppRb, Hyperphosphorylated Rb; pRb, hypophosphorylated Rb; CycD1, cyclin D1 overexpression; ND, not determined.

respectively. The absence of the p16 protein (100%) and the overexpression of cyclin D1 (100%) were related to the hyperphosphorylation (inactivation, 63%) of the Rb proteins.

In this study, we showed the alterations in the p16-Rb-cyclin D1 tumor-suppressive pathway in HNSCCs in Korean patients. Our results suggest that the loss of the p16 protein, cyclin D1 overexpression, and the consequent inactivation of the Rb proteins, are important factors in the development of HNSCCs.

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