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# Inactivation patterns of *p16/INK4A* in oral squamous cell carcinomas

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Abbreviations: cdks, cyclin-dependent kinases; INK4, Inhibitor for cyclin-dependent kinase 4; SCC, Squamous cell carcinoma; pRb, retinoblastoma tumor suppressor protein

## Abstract

**The *p16/INK4A* is one of the major target genes in carcinogenesis and its inactivation has frequently been reported in other types of tumors. The purpose of this study is to evaluate inactivation patterns of *p16/INK4A* in oral squamous cell carcinoma. Six different oral cancer cell lines, SCC-4, SCC-9, SCC-15, SCC-25, KB, and SNUDH-379 were examined for inactivation of *p16/INK4A* genes. In the analysis of *p16/INK4A* gene inactivation, PCR amplification, direct sequencing, and methylation-specific PCR methods were adopted for evaluation of homozygous deletion, point mutation, and promoter hypermethylation, respectively. Homozygous deletion was detected in SCC-25 and SCC-9. SCC-15 showed hypermethylated promoter region within *p16/INK4A* gene. It is suggestive in the present study that inactivation patterns of *p16/INK4A* were mainly homozygous deletion, promoter methylation rather than point mutation in oral squamous cancer cell lines, so treatment modalities of oral squamous cell carcinoma should be focused on these types of inactivation.**

**Keywords:** homozygous deletion; oral squamous cell carcinoma; *p16/INK4A*; promoter methylation

## Introduction

Recent studies have revealed the molecular basis of cell cycle control and a critical role of collapsing this homeostatic mechanism in carcinogenesis. At the core of cell cycle control lies a late $G_1$ checkpoint in which critical biochemical activity is achieved by many kinds of regulating enzymes. Positioned in late $G_1$ phase, the regulatory mechanism centered around the retinoblastoma tumor suppressor protein (pRb) plays an important role by participating in decisions on cell cycle progression depending on the cellular environment (Bartek *et al.,* 1997). Many reports have focused on the critical role played by this regulatory mechanism known as the pRb pathway (Pardee, 1989; Stein *et al.,* 1990; Weinberg, 1995; Hertwig and Strauss, 1997; Sellers and Kaelin, 1997; Kim *et al.,* 2003).

The pRb-E2F connection is the heart of the pRb pathway in that phosphorylated pRb liberates E2F to commence DNA replication and helps cell cycle forward. But phosphorylation of pRb is tightly controlled by a variety of regulating enzymes including $p16^{INK4A}$ as an inhibitor of phosphorylation. Cell cycle driving force exerted by cdk 4 or 6/cyclin D is normally balanced with this protein and collapsing this homeostatic mechanism is one of the causative factors of carcinogenesis (Liggett and Sidransky, 1998). This protein is the product of *p16/INK4A* gene that is one of the main target tumor suppressor genes. The purpose of this study is to evaluate the role of *p16/INK4A* inactivation in the development of oral squamous cell carcinoma by analyzing its inactivation pattern of homozygous deletion, point mutation, and promoter methylation *via* PCR amplification, direct sequencing, and methylation-specific PCR methods, respectively.

## Materials and Methods

### Cell lines

Six oral squamous cancer cell lines were used in this study including SCC-4, SCC-9, SCC-15, SCC-25, KB, and SNUDH-379. HF-3 (human fibroblast cell line), Saos-2, and U-2 OS (Osteosarcoma cell lines) were used for positive controls. Of these, SCC-4, SCC-9, SCC-15, SCC-25, KB, Saos-2, and U-2 OS cell lines were obtained from ATCC (American Type Culture Collection, Rockville, MD). SNUDH-379 and HF-3

were kindly provided by Professor Hong (Seoul National University, Seoul, Korea). Tissue sources for SCC-4, SCC-9, SCC-15, SCC-25 cell lines were from the human tongue (Rheinwald and Beckett, 1981). SCC-15 was previously proven to be hypermethylated in the *p16/INK4A* promoter region (Cody *et al.,* 1999; Kim *et al.,* 2000), but was included in this experiment to be examined for other possible abnormalities including homozygous deletion and point mutation. The KB cell line, which had been used extensively in many studies (Eagle, 1955; Eagle and Foley, 1958; Salzman, 1961; Boshart *et al.,* 1984), was also included in this study. SNUDH-379 cell line was established from pathologically confirmed oral squamous cell carcinoma of a less well differentiated type involving maxilla of a 55-year-old female Korean. The author included this established cell line in this study to test possible alterations of *p16/INK4A* gene.

SCC-4, SCC-9, SCC-15, and SCC-25 cell lines were cultured in Ham's F12/DMEM (1:1) supplemented with 10% FBS, and 0.4 μg/ml hydrocortisone. KB and SNUDH-379 cell lines were cultured in DMEM supplemented with 10% FBS. Initial passages were performed when heavy tumor cell growth was observed and subsequent passages were performed every four or five day on all of these six cell lines. Twenty to twenty five passages were performed on SCC-4, SCC-9, SCC-15, SCC-25, and KB cell lines and between 15 and 20 on SNUDH-379. All cultured cells were in the late log phase growth at 75-80% confluence immediately before DNA isolation. Names, origins, sources, and culture media of the cell lines that were used are listed in Table 1.

## DNA isolation

Cell monolayers were washed in PBS and lysed in 2 ml of DNA lysis buffer (50 mL of STE (0.1 M NaCl, 10 mM Tris, pH 8.0) buffer, 1 ml of 0.5 M EDTA, 5 ml of 10% sodium dodecyl sulfate) and 100 μl of 20 mg/ml proteinase K was added to the samples. After the samples were incubated at 60°C for 2 h, DNA was isolated from the samples with phenol and chloroform, precipitated with ethanol, and re-dissolved in 50 μl of water.

## Determination of deletion and point mutation

### *Polymerase chain reaction (PCR) amplification*

Samples were examined for deletion of exon 1 and exon 2 of *p16/INK4A* gene by the PCR amplification method. Exon 1 was amplified as one fragment while exon 2 was split into two fragments. HF-3 cell line was used as an internal positive control for the *p16/INK4A* amplification. The primers used for PCR amplification were designed from the published *p16/INK4A* sequences (Zhang *et al.,* 1994) and are listed in Table 2.

Two hundred ng of purified DNA were amplified in a 20 μl solution containing 1×PCR buffer, 1.5 mM $MgCl_2$, 1 μM of each primer, 0.125 mM of each deoxynucleotide triphosphate, 5% dimethylsulphoxide (DMSO), and 1 U of Taq polymerase. Thirty-five cycles of PCR were completed using GeneAmp PCR system 2400 (PE applied biosystem, Foster City, CA) under the following conditions: denaturation at 95°C, 60 s; annealing at 60°C, 60 s; and extension at 72°C, 60 s. Amplified PCR products were electrophoresed on 1.8% agarose gels. The absence of visible bands amplified by PCR indicated homozygous deletion.

## Direct sequencing

PCR products were directly sequenced by a PCR-based sequencing method using the BigDye terminator cycle sequencing kit (PE applied biosystem, Foster City, CA) with Genetic Analyzer 310 (PE applied biosystem, Foster City, CA). Briefly, following the cleanup of PCR product using PCR purification kit (Qiagen, Valencia, CA) the 200 ng of purified PCR products and 4 pM of primers were mixed with

**Table 1.** Cell lines used.

| Name | Origin | Source | Culture Media |
|---|---|---|---|
| SCC-4 | Tongue squamous cell carcinoma | ATCC[a] | DMEM/F12(1:1), 10% FBS |
| SCC-9 | Tongue squamous cell carcinoma | ATCC | DMEM/F12(1:1), 10% FBS |
| SCC-15 | Tongue squamous cell carcinoma | ATCC | DMEM/F12(1:1), 10% FBS |
| SCC-25 | Tongue squamous cell carcinoma | ATCC | DMEM/F12(1:1), 10% FBS |
| KB | Mouth squamous cell carcinoma | ATCC | DMEM, 10% FBS |
| SNUDH-379 | Gingiva squamous cell carcinoma | SNUDH[b] | DMEM, 10% FBS |

a, American Type Culture Collection, Rockville, MD, USA; b, Seoul National University Dental Hospital, Seoul, Korea

BigDye reaction mixture to make a total volume of 20 µl. Twenty-five cycles of PCR reactions were done under the following conditions: denaturation at 96°C for 10 s; annealing at 50°C for 5 s; and extension at 60°C for 4 min. Reaction products were loaded into the Genetic Analyzer 310 (PE applied biosystem, Foster city, CA) for electrophoresis.

### Determination of promoter methylation

Three types of methylation-specific PCRs were designed; methylation-specific PCRs with unmodified DNA samples and wild-type primer pairs, those with modified DNA samples and unmethylation-specific primer pairs, and those with modified DNA samples and methylation-specific primer pairs, respectively. Three primer pairs were used as wild-type, unmethylation-specific, and methylation-specific PCR primers, respectively. These primer pairs were designed after the published sequences (Herman *et al.,* 1996) and are listed in Table 2. Saos-2 and U-2 OS cell lines were used as positive control for unmethylation-specific, methylation-specific PCR, respectively. Reported methylation status of these two controls (Otterson *et al.,* 1995) was used to determine the internal positive controls. Sample DNAs were modified for unmethylation-specific and methylation specific PCRs. One µg of DNA in a volume of 50 µl was denatured by 0.2 M of NaOH for 10 min at 37°C. Thirty µl of 10 mM hydroquinone (Sigma, St. Louis, MO) and 520 µl of 3 M sodium bisulfite (Sigma, St. Louis, MO) of pH 5, both freshly prepared, were added and mixed, and the samples were incubated under mineral oil at 50°C for 16 h. Modified DNA was purified using the Qiagen DNA purification kit (Qiagen, Valencia, CA) according to the manufacturer and eluted into 50 µl of water. Modification was completed *via* treatment by 0.3 M of NaOH for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at -20°C for later use.

The PCR mixture contained 1×PCR buffer, 1.5 mM $MgCl_2$, 1 µM of each primer, 0.125 mM of each deoxynucleotide triphosphate, 5% dimethylsulphoxide (DMSO), 1 U of Taq polymerase, and 100 ng of unmodified or bisulfite-modified DNAs in a final volume of 50 µl. Reactions were hot-started at 95°C for 5 min before the addition of 1 U of Taq polymerase. Amplification was carried out for 35 cycles under the following conditions. Unmodified DNA was denatured for 30 s at 95°C, annealed for 30 s at 60°C, and extended for 30 s at 72°C with wild-type primer pairs. The condition for unmethylation-specific PCR was the same except that the annealing temperature was 56°C with unmethylation-specific primer pairs. Methylation-specific PCR was done under the same conditions as the unmethylation-specific PCR except that the annealing temperature was 58°C with methylation-specific primer pairs. Final extension was done

**Table 2.** Oligonucleotide primers used for polymerase chain reactions (PCR) and methylation-specific PCR (MSP).

| | Primer set | | Sequence | Size(bp) |
|---|---|---|---|---|
| PCR | p16E1[a] | Sense | 5'TCTGCGGAGAGGGGGAGAGCAGGCA3' | 279 |
| | | Antisense | 5'GCGCTACCTGATTCCAATTC3' | |
| | p16E2a | Sense | 5'ACAAGCTTCCTTTCCGTCATGCCG3' | 244 |
| | | Antisense | 5'CCAGGCATCGCGCACGTCCA3' | |
| | p16E2b | Sense | 5'TTCCTGGACACGCTGGTGGT3' | 242 |
| | | Antisense | 5'TCTGAGCTTTGGAAGCTCTCAG3' | |
| MSP | p16W[b] | Sense | 5'CAGAGGGTGGGGCGGACCGC3' | 140 |
| | | Antisense | 5'CGGGCCGCGGCCGTGG3' | |
| | p16U | Sense | 5'TTATTAGAGGGTGGGGTGGATTGT3' | 151 |
| | | Antisense | 5'CAACCCCAAACCACAACCATAA3' | |
| | p16M | Sense | 5'TTATTAGAGGGTGGGGCGGATCGC3' | 150 |
| | | Antisense | 5'GACCCCGAACCGCGACCGTAA3' | |

[a]E1: Exon 1, [b]W: wild-type primers, E2a: 5' fragment of Exon 2, U: unmethylation-specific primers, E2b: 3' fragment of Exon 2, M: methylation-specific primers.

for 5 min at 72°C in all three types of methylation-specific PCRs. Controls without DNA were performed for each set of PCRs. Each PCR product was directly loaded on 1.8% agarose gels, and directly visualized under UV illumination.

## Results

### Homozygous deletion rather than point mutation on the *p16/INK4A* loci

Six tumor DNA samples were subjected to PCR for the assessment of homozygous deletion. Exon 1 and exon 2 of *p16/INK4A* were independently amplified using HF-3 primers in the same reactions as an internal positive control. Because of the large size of exon 2 (307 bp), it was amplified as two separate fragments. Homozygous deletions of the *p16/INK4A* gene were detected in two of six oral cancer cell lines. Exon 1 and exon 2 of the *p16/INK4A* gene were entirely deleted homozygously in SCC-25, and only exon 1 was deleted in SCC-9 (Figure 1). On the other hand, no alterations were detected in SCC-4, SCC-15, KB, and SNUDH-379. We have directly se-

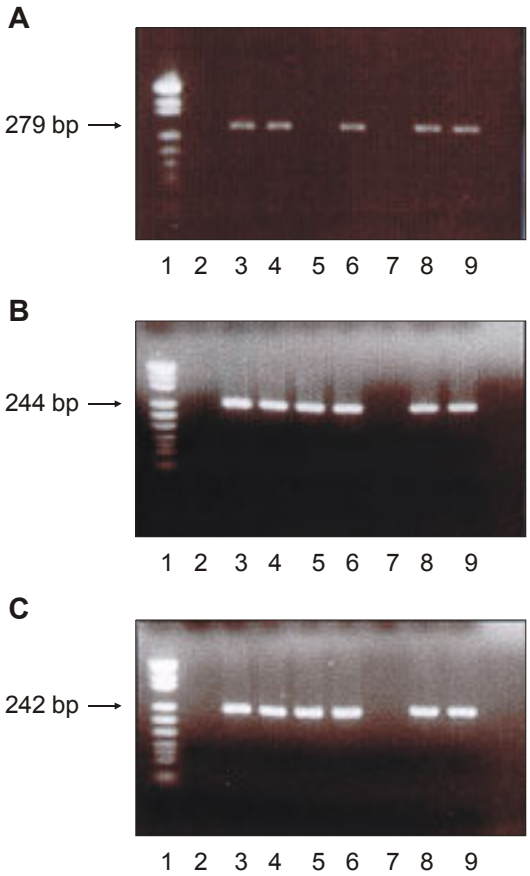


**Figure 1.** Analysis of *p16/INK4A* exon 1 (A), exon 2a (B), and exon 2b (C) by PCR. Each exon was amplified by PCR and 20 µl of PCR products were separated on a 1.8% agarose gel. The bands corresponding to the predicted molecular weights of *p16/INK4A* amplicons are indicated by arrows: A, 279 bp for exon 1; B, 244 bp for exon 2a; and C, 242 bp for exon 2b. Results showed the bands were lost at exon 1 in SCC-9 and SCC-25 (lane 5 and 7, respectively in A) and lost at exon 2 in SCC-25 (lane 7 in B and C). 1) DNA size marker; pUC 18 *Msp* I digest, 2) Absence of DNA (negative control), 3) HF-3 (positive control), 4) SCC-4, 5) SCC-9, 6) SCC-15, 7) SCC-25, 8) KB, 9) SNUDH-379.

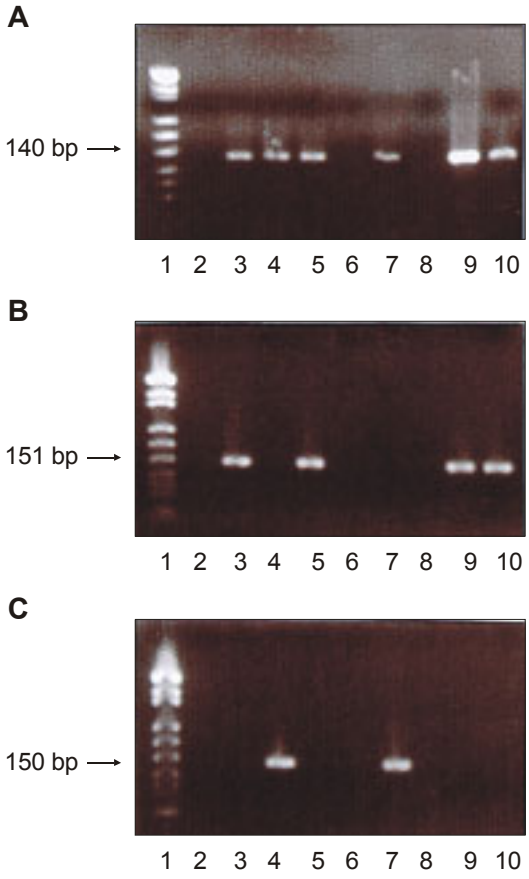


**Figure 2.** Analysis of methylation status of *p16/INK4A* promoter regions by methylation-specific PCR. (A) Methylation-specific PCR product of unmodified DNA with wild-type primer pairs. All of the experimental cell lines showed visible bands at predicted molecular weights (140 bp) except the SCC-9 and SCC-25 (lane 6 and 8, respectively) which in previous experiment were proven to have deletions of *p16/INK4A* genes (Figure 1A). (B) Methylation-specific PCR product of modified DNA with unmethylation-specific primer pairs. The bands were seen at predicted molecular weights (151 bp) in SCC-4, KB, SNUDH-379 (lane 5, 9, 10, respectively). (C) Methylation-specific PCR product of modified DNA with methylation-specific primer pairs. The band was seen at predicted molecular weights (150 bp) only in SCC-15 (lane 7). 1) DNA size marker; pUC 18 *Msp* I digest, 2) Absence of DNA (negative control), 3) Saos-2 (positive control for unmethylation-specific PCR), 4) U-2 OS (positive control for methylation-specific PCR), 5) SCC-4, 6) SCC-9, 7) SCC-15, 8) SCC-25, 9) KB, 10) SNUDH-379

**Table 3.** Results of Methylation-specific PCR.

| Primer set | SCC-4 | SCC-9 | SCC-15 | SCC-25 | KB | SNUDH-379 |
|---|---|---|---|---|---|---|
| p16-W[a] | + | - | + | - | + | + |
| p16-U[b] | + | - | - | - | + | + |
| p16-M[c] | - | - | + | - | - | - |

[a], W: unmodified or wild-type primers; [b], U: unmethylation-specific primers; [c], M: methylation-specific primers.

quenced all six tumor DNA samples for detection of point mutations but none of them showed any point mutations.

### Methylation status in the promoter region of the *p16/INK4A* gene

Primer sequences were chosen for regions containing frequent cytosines to distinguish wild-type from modified DNA. Besides, CpG pairs near the 3' end of the primers were selected in determining the primer sequences to provide maximal discrimination in the PCR between unmethylated and methylated DNA. All the samples were subjected to methylation-specific PCR for the detection of methylation in the promoter region of the *p16/INK4A* gene. In PCR using wild-type primer pairs with unmodified samples, PCR products were detected in SCC-4, SCC-15, KB, and SNUDH-379 but not in SCC-9 and SCC-25 (Figure 2A). In PCR using unmethylation-specific primer pairs with bisulfite-modified samples, PCR products were detected in SCC-4, KB, and SNUDH-379 but not in SCC-9, SCC-15 and SCC-25 (Figure 2B). In PCR using methylation-specific primer pairs with bisulfite-modified samples, only SCC-15 yielded PCR product (Figure 2C). The results of methylation-specific PCRs are summarized in Table 3.

## Discussion

In the amplification study for exon 1, no bands were found in SCC-9 and SCC-25 as shown at lane 5 and 7, respectively in Figure 1A. In the case with exon 2, the band was lost in SCC-25 only at lane 7 in Figure 1B and C. From the fact that *p16/INK4A* was deleted only at exon 1 in SCC-9 and deleted at both exon 1 and 2 in SCC-25, we have concluded that each of the exons is independently deleted and any deletion of these exons can lead to inactivation of *p16/INK4A* gene. In this study, the author adopted PCR amplification method, not RT-PCR method, for assessment of the deletion because the reaction product would be complementary DNA and not genomic DNA in RT-PCR. *p16/INK4A* gene is made up of three exons, which were spliced by two introns: a 5' region of 126 bp (exon 1), a middle region of 307 bp (exon 2), and 3' region of 11 bp (exon 3). In the deletion analysis, we only included exon 1 and exon 2 because the 3' region (exon 3) contains only 11 bp of genes and this was considered negligible.

In many types of malignant tumors, homozygous deletion of *p16/INK4A* gene and hypermethylation of the promoter CpG island are main causes of tumorigenesis (Mead *et al.,* 1994; Brenner *et al.,* 1996; Marchini *et al.,* 1997; Drexler, 1998; Tsutsumi *et al.,* 1998; Wei *et al.,* 1999). In primary tumors, the rate of *p16/INK4A* point mutation approximates 10% or so (Sartor *et al.,* 1999; Wu *et al.,* 1999). In an experimental study using eight oral squamous cancer cell lines, point mutation was found in five of six cell lines as the causative factor for *p16/INK4A* gene inactivation (Sartor *et al.,* 1999). In the present study, authors could not find any point mutations in direct sequencing procedures of all six cell lines. This difference was partially due to the difference in cell lines. There was not a single matched cell line in these two studies. In addition to the complete mismatch of the cell lines, the cause of this difference may partly have been the sample size. The sample numbers of both studies were inadequate for statistical evaluation. It is discreetly considered that for a comparative study using data, the sample size should be increased.

There have been some differences in the past between the alteration rate of *p16/INK4A* gene and the expression rate of its gene product. Expression loss of $p16^{INK4A}$ protein has approached to 70% in non-small cell lung carcinomas (Otterson *et al.,* 1994). But the rate of *p16/INK4A* gene alteration in the same diagnosis was much lower in other experiments (Bonetta, 1994; Mead *et al.,* 1994). Some mechanism other than point mutation and deletion of genes was speculated and it is now known that hypermethylation of the promoter region causes transcriptional silencing and thus blocks translational procedures. In normal conditions, cytosines in CpG-poor region are methylated in contrast to the cytosines in the CpG islands,

which are hardly methylated. This normal type of reading frame is recognized by the unmodified primers, which are wild-type primers. All forms of cytosines, dispersed in CpG-poor region or clustered as in CpG island, will be replaced by uracil when it is treated by bisulfite if they are unmethylated. But if methylated, cytosines remain the same in spite of its treatment by bisulfite. In this experiment, three primer pairs were used as unmodified, unmethylation-specific, and methylation-specific primers. Unmethylation-specific and methylation-specific primer pairs detected these uracils and 5-methylcytosines in reading frames, respectively. Methylation-specific PCR is sensitive and specific for methylation of virtually any block of CpG sites in CpG islands requiring only a small sample and being able to rule out pseudopositive reaction caused by partial digestion of methylation-sensitive enzymes used in other methylation studies (Herman *et al.,* 1996). In methylation-specific PCR with wild-type primer pairs, the bands were intact except in SCC-9 and SCC-25 (lane 6 and lane 8, respectively in Figure 2A) meaning that the promoter regions of the cell lines were intact except in SCC-9 and SCC-25 regardless of the methylation status. In methylation-specific PCR with unmethylation-specific primer pairs, the bands were lost in SCC-9, SCC-15, and SCC-25 (lane 6, 7, and 8 in Figure 2B). Considering the deletion of *p16/INK4A* gene was previously identified in SCC-9 and SCC-25, the only abnormality found in this result was SCC-15 and this was confirmed by further experiments with methylation-specific primer pairs. As was expected, the result was positive only in SCC-15 in methylation-specific PCR with methylation-specific primer pairs (lane 7 in Figure 2C). The result shows that CpG islands in promoter region of *p16/INK4A* gene are hypermethylated only in SCC-15 of six oral squamous cancer cell lines selected. Promoter hypermethylation plays a role in the inactivation of *p16/INK4A* gene in oral squamous cell carcinomas.

Authors analyzed inactivation patterns of *p16/INK4A* genes to evaluate the role of *p16/INK4A* inactivation in the development of oral squamous cell carcinoma. Three kinds of inactivation patterns were examined; homozygous deletion by PCR amplification, point mutation by direct sequencing, and promoter methylation by methylation-specific PCR methods, respectively. *p16/INK4A* gene was deleted at exon 1 and exon 2 in SCC-25, and partially deleted at exon 1 in SCC-9. Promoter region of *p16/INK4A* gene was hypermethylated in SCC-15. But we could not find any evidence of point mutations in all the six cell lines. In this study, inactivation patterns of *p16/INK4A* were mainly homozygous deletion, promoter methylation rather than point mutation in oral squamous cancer cell lines, and it is suggested that treatment modalities of oral squamous cell carcinoma should be focused on these types of inactivation.

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