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Mutation of the *p16/CDKN2* gene and loss of heterozygosity in malignant mucosal melanoma and adenoid cystic carcinoma of the head and neck

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Abstract. The purpose of this study was to investigate the molecular biological characteristics of malignant mucosal melanoma (MMM) and adenoid cystic carcinoma (ACC) of the head and neck. We analyzed the common genetic abnormalities that may help to identify the loci in the genes involved in the development of MMM and ACC of the head and neck by PCR-LOH on chromosomes 1p, 6q, 9p, 10q, 11q, 12q, 17p, and 19q. LOH was observed in 6 of 12 cases of MMM and in 12 of 15 cases of ACC informative for at least one of the loci analyzed. One distinct deleted region was identified at chromosome 9p21. In addition, to identify a possible involvement of *p16/CDKN2* PCR-SSCP and auto-DNA sequence analysis were also performed to detect any mutation of the *p16/CDKN2*. Particularly, 2 missense mutations were detected in codon 225 and 226, both in MMM and ACC. There were mutational hot spots in the *p16/CDKN2* gene. These results suggested that mutation of the *p16/CDKN2* gene was a common factor in the development of human MMMs and ACCs, while this gene may be correlated with development and/or progression of a subtype and play a role in the oncogenesis of these cancers.

Introduction

Malignant mucosal melanoma (MMM) and adenoid cystic carcinoma (ACC) are two of the malignant tumors with the poorest prognosis. According to a previous report, the effective treatment for these diseases is carbon ion radiotherapy using heavy charged particles (1).

Clinical trials of carbon ion radiotherapy for various cancers were initiated in June 1994 at the Hospital of the Research Center for Charged Particle Therapy, National Institute of Radiological Sciences (NIRS), using the world's first Heavy Ion Medical Accelerator in Chiba (HIMAC) dedicated to medical purposes (1). Preliminary results of phase II clinical trials have shown extremely favorable therapeutic results in the treatment of MMMs and ACCs that are otherwise intractable with conventional photon radiation (2,3). As stated above, radiotherapy with heavy charged particles is significantly effective in the therapy of MMMs and ACCs of the head and neck. Radiotherapy using the HIMAC has some characteristics in common with conventional photon radiation. Firstly, HIMAC irradiation inflicts fatal damage upon the DNA of cancer cells so that cancer cell restoration is made difficult. Secondly, HIMAC irradiation affects cells at any stage of the cell cycle. Thirdly, the HIMAC irradiation has a cytotoxic effect on cancer cells also in a hypoxic environment. Lastly, the distribution characteristics of HIMAC radiation are such as to minimize damage to normal tissues while limiting injury to the tumor only. However, no analysis method for the molecular biological assessment of the effectiveness of HIMAC radiation for MMMs and ACCs has yet been established. We therefore considered that the molecular biological characteristics such as loss of heterozygosity (LOH) and microsatellite instability (MSI) may become criteria for assessment of the effectiveness of HIMAC radiotherapy for MMM and ACC prior to therapy. In recent studies, LOH and MSI have been used for assessing allelic imbalances. For example, allelic imbalances including LOH and MSI in malignant cells have proven useful for mapping DNA regions potentially harboring candidate tumor suppressor genes (TSG). According to a previous study, a high incidence of LOH has been detected in head and neck squamous cell carcinomas (SCC) on several chromosome arms, including 2q, 3p, 4q, 5q, 7q, 8p, 9p, 10q, 11q, 13q, 18q, 20q, 21q, and 22q (4-7), where candidate TSGs may be present.

We thus made a literature search to look for evidence of prior gene analysis common to MMM and ACC, but the extant literature does not provide any information on gene

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seat ranking common to both diseases. There were few reports related to MMM, while most focused on cutaneous melanoma (8). However, there was a significant number of reports in the literature referring to gene analysis of ACC (9,10). The limited number of loci tested and the varietal incidence of genetic alterations in these studies make it difficult to assess the regions of chromosomes 1p, 6q, 9p, 10q, 11q, 12q, 17p, and 19q (11-14) involved in cutaneous melanoma and ACC of the head and neck. We paid particular attention to the 3 chromosomes, 6p, 17p, and 9p. First, while the chromosome region around 6p does not reveal a major presence of a TSG it does show a high level of expression of LOH in ACC and cutaneous melanoma. In contrast, the 17p and 9p regions included the TSG p53 and p16. In particular, *p16/CDKN2* loci have been reported in previous studies that have a high level of incidence of LOH in cutaneous melanoma and ACC (10,15).

We therefore analyzed in this study, the molecular biological characteristics of MMM and ACC and identified the *p16/CDKN2* sequence.

Materials and methods

Tissue samples. Tissue samples were taken from 27 patients, 12 with MMMs and 15 with ACCs of the head and neck, who attended the Hospital of the Research Center for Charged Particle Therapy, NIRS, during the 2 years from 2002 through 2003 (Table I). Informed consent was obtained from all patients and also from their families. In addition, this study was approved by the institutional ethics committee in NIRS and also by the Tokyo Dental College. Normal tissue specimens as control were prepared from each patient's peripheral blood samples at the time of biopsy. None of the tumors and normal samples had undergone chemotherapy and radiotherapy prior to biopsy. All tissues resected for biopsy were prepared using a 10% formalin solution for pathologic diagnosis. Histopathologic diagnosis was performed according to the International Classification of Tumors (16).

All normal samples were prepared by immediate DNA extraction by the method described below.

Tumor DNA extraction from paraffin sections and normal DNA extraction from blood. Multiple 10- μ m sections were cut and mounted on silanated slides and left overnight at 37°C. They were then dewaxed with xylene and water with different concentration levels of alcohol being successively added. The sections were lightly stained in Harris hematoxylin and compared with a hematoxylin and eosin-stained section on the same block. All samples had histologically confirmed MMM or ACC of the head and neck, and the tumor samples for DNA extraction were checked by observing in biopsy to ensure that they consisted of more than 80% tumor cells. Genomic DNA was extracted from several paraffin-embedded sections of all tumor samples using Takara DEXPAT® (Takara Bio Inc, Shiga, Japan). A standard protocol of DEXPAT® is shown below. Three 10- μ m thick paraffin-embedded tissue sections were prepared and placed in a microcentrifuge tube using sterilized tweezers. Of a gently stirred DEXPAT solution, 0.5 ml per bottle was added to the centrifuging tube and mixed with the sample to remove the resin. The microcentrifuge

Table I. Summary of clinical features in 12 MMMs and 15 ACCs.

Sample no.	Patient no.	Location	Age	Gender
MMM				
1	9	Palate	69	M
2	11	Nasal	77	M
3	17	Nasal	77	F
4	29	Nasal	78	M
5	48	Maxillary sinus	78	M
6	49	Nasal	77	F
7	52	Maxillary sinus	53	M
8	62	Maxillary sinus	52	M
9	66	Nasal	66	M
10	69	Palate	62	M
11	83	Ethmoidal sinuses	55	F
12	89	Orbit	55	M
ACC				
1	7	Sublingual gland	49	M
2	13	Parotid gland	58	F
3	27	Hard palate	44	M
4	30	Epipharynx	45	F
5	32	Maxillary sinus	49	M
6	54	Lacrimal gland	76	F
7	59	Maxillary sinus	64	M
8	64	Tongue	44	M
9	73	Epipharynx	26	F
10	75	Parotid gland	56	F
11	81	External acoustic meatus	50	F
12	86	Epipharynx	45	F
13	91	Parotid gland	52	F
14	96	Maxillary sinus	40	M
15	98	Maxillary sinus	37	F

MMM, malignant mucosal melanoma; ACC, adenoid cystic carcinoma; M, male; F, female.

tube was heated to and kept at 100°C for 10 min in a block heater and then centrifuged at 12,000 rpm for 10 min at 4°C. The thin paraffin layer was removed from the tube wall and the supernatant was collected with a micropipette. After the DNA samples had been extracted by phenol-chloroform extraction and refined, they were washed and precipitated with ethanol. The concentrations of extracted DNA were estimated by a spectrophotometric method and the DNA samples were kept at -80°C.

Normal cells were prepared from peripheral blood samples as control. The blood samples were used for analysis with the Dr.GentLE® (Takara Bio Inc) gene trapping kit. This kit is composed of three solutions; GentLE® solution I, II, and III. GentLE solution I lyses blood cells immediately after adding it to the whole blood, and a non-charged complex with nucleic acids is formed. The complex is collected by centrifugation, and the DNA precipitates are washed with GentLE solution II. Then GentLE solution III is added to isolate DNAs only, and DNAs are precipitated by adding isopropanol. The concentrations of the extracted DNA were estimated by a

Table II. Microsatellite markers used in our LOH study in MMMs and ACCs.

Location	Sequence	Product size (bp)
MMM		
D1S160	F 5'-GCATCTAGCAAACAGCATGTG-3' R 5'-GCTGGAGGGCAGTGAAACT-3'	156
D1S243	F 5'-CACACAGGCTCACATGCC-3' R 5'-GCTCCAGCGTCATGGACT-3'	209
D9S104	F 5'-GATCTGGGTATGTCTTTCTG-3' R 5'-ACTGGGACTCTAACTAATGT-3'	181
D6S310	F 5'-GATCCAGATTGCAGAAGG-3' R 5'-GAAACAGGACCAGTAGGATAT G-3'	159
D9S736	F 5'-TTCTAGACCTCTCAGCAGAC-3' R 5'-GATAGTGTGGAGACACCAG-3'	157
D9S162	F 5'-GCAATGACCAGTTAAGGTTC-3' R 5'-AATTCCCACAACAAATCTCC-3'	181
D9S171	F 5'-AGCTAAGTGAACCTCATCTGTCT-3' R 5'-ACCCTAGCACTGATGGTATAGTCT-3'	159
D10S254	F 5'-ACTCCTCCCATGTAGGTACC-3' R 5'-TCCTGTAAAGATGTTACTGAG-3'	98
D11S29	F 5'-TCTAGTCCACCATCCTGTG-3' R 5'-ACAACACACTGCCACAAGAC-3'	143
TP53	F 5'-TTGCCTCTTCTTAGCACTG-3' R 5'-CCAAGACTTAGTACCTGAAG-3'	103
ACC		
D6S310	F 5'-GATCCAGATTGCAGAAGG-3' R 5'-GAAACAGGACCAGTAGGATATG-3'	159
D6S311	F 5'-ATGTCCTCATTGGTGTGTG-3' R 5'-GATTGACAGCCAGGAAGAT-3'	62
D6S441	F 5'-AACAAATTTGGTGACTGTTAAAGG-3' R 5'-TGGACAAATTGATTAGGAAGTAAAG-3'	103
D9S171	F 5'-AGCTAAGTGAACCTCATCTGTCT-3' R 5'-ACCCTAGCACTGATGGTATAGTCT-3'	159
D12S391	F 5'-AACAGGATCAATGGATGCAT-3' R 5'-TATTGGACAGTAAAGAAACCTGC-3'	135
D12S1301	F 5'-ACACAGATGTGCATCTATCTCG-3' R 5'-TATTGGACAGTAAAGAAACCTGC-3'	132
D12S1064	F 5'-AATATTGACTTCTCTTGCTACC-3' R 5'-ACTACTCCAAGGTCCAGCC-3'	80
TP53	F 5'-TTGCCTCTTCTTAGCACTG-3' R 5'-CCAAGACTTAGTACCTGAAG-3'	103
D19S210	F 5'-TCACACTCACTGGTCTCTC-3' R 5'-GGTGTGCCTGTGTGTAAG-3'	57
D19S246	F 5'-AGAGTGAGATTCCACCTTTC-3' R 5'-GAAACACATCATTTACCCAC-3'	64

spectrophotometric method and the DNA samples were kept at -80°C . From each DNA sample, 50 ng/ μl was used for the template of the PCR amplification procedure.

PCR and microsatellite analysis. We used 17 microsatellite markers from among those on 1p32, 6q23-25, 9p21, 10q23-25, 11q23.3, 12q12-13, 17p13.1, and 19q13.1 (Table II). All primers were obtained from Sigma-Aldrich Japan K.K. (Hokkaido, Japan). The DNA fragments of the above DNA

Table III. *p16/CDKN2* oligonucleotide primers.

p16/CDKN2	Sequence	Product size (bp)
Exon 1	5'-GAAGAAAGAGGAGGGGTG-3' 5'-GCGCTACCTGATTCCAATTC-3'	340
Exon 2	5'-ACACAAGCTTCCTTTCCGTCAT-3' 5'-TCTGAGCTTTGGAAGCTCT-3'	426
Exon 3	5'-TTGATTATTTGTTTTTTTTGGTAGGTT-3' 5'-CAAATTCTCAAATCATCAATCCTCACCT-3'	161
Exon 4	5'-AGGAATTCGGTAGGGACGGCAAGAGAGG-3' 5'-GAAGCTTGGGGGAAGGCATATATCTACG-3'	215

sample were amplified with PCR in a 10- μl final volume containing 0.25 units of HotStarTaq[®] DNA polymerase (Qiagen GmbH, Hilden, Germany) in 10X PCR buffer (50 mM KCL, 10 mM Tris-Cl, pH 8.0, 1.5 mM MgCl₂), 0.2 mM deoxynucleotide triphosphate, 0.2 μM of each primer, 2.5 mM of MgCl₂ and 2.5 ng genomic DNA. The amplification protocol specifies initial denaturation for 15 min at 94°C , 40 cycles of 20 sec each at 94°C , 30 sec at 62°C , 30 sec at 72°C , followed by a final extension step of 72°C for 6 min according to the protocol of Nakamoto *et al* (17). After dilution with an adequate volume of formamide-dye mixture (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), the PCR products were heat-denatured (98°C , 5 min), chilled on ice, and electrophoresed on 6% urea-formamide-polyacrylamide gel at 3 W for 2 h, depending on the fragment size. Silver staining of the gels was performed using the DNA silver staining kit (GE Healthcare UK Ltd, Buckinghamshire, UK). To ensure reproducibility in each sample with LOH, all tests were performed under the same conditions.

We carried out the previously described process twice to confirm the reproducibility of the results.

Assessment of LOH and microsatellite instability (MSI). LOH in the tumor DNA samples was assessed by scanning densitometry and analyzed with NIH Image software (version 1.62, <http://rsb.info.nih.gov/nih-image/>). The intensities of the signals from the tumor DNA were compared with those of normal DNA as a control. The criterion for the presence of LOH was a reduction in signal intensity of $>50\%$. Commonly deleted regions were defined by considering the loci most frequently showing LOH, together with multiple interstitial deletions. MSI in the DNA samples was assessed as positive in cases with additional bands in the tumor sample that were not observable in the normal samples and in cases showing a band shift in the tumor sample that contrasted with the pattern of the normal bands.

Detection of *p16/CDKN2* gene alteration by PCR-SSCP and auto-DNA sequence analysis. To screen the sequence variations of the *p16/CDKN2* gene, PCR-SSCP analysis was performed as described previously (18,19). Four sets of oligonucleotide primers as summarized in Table III were used to amplify the entire coding region (exons 1 to 4) of the *p16/CDKN2* gene. Sequences for all annotated exons and adjacent intronic

Table IV. Frequency of LOH at each microsatellite marker in MMMs and ACCs.

MMM			ACC		
Locus symbol	LOH/informative cases	Frequency of LOH (%)	Locus symbol	LOH/informative cases	Frequency of LOH (%)
D6S310	1/12	8.3	D6S310	1/15	6.7
D9S171	4/12	33.3	D9S171	8/15	53.3
TP53	0/10	0.0	TP53	0/8	0.0
D1S160	0/8	0.0	D6S441	0/15	0.0
D1S243	0/12	0.0	D6S311	0/13	0.0
D9S104	0/11	0.0	D12S391	6/15	40.0
D9S736	1/12	8.3	D12S1301	1/15	6.7
D9S162	1/12	8.3	D12S1064	0/14	0.0
D10S254	1/12	8.3	D19S210	1/15	6.7
D11S29	1/10	10.0	D19S246	1/14	7.1

The former 3 markers are common to MMM and ACC; the remaining 7 markers are specific to each.

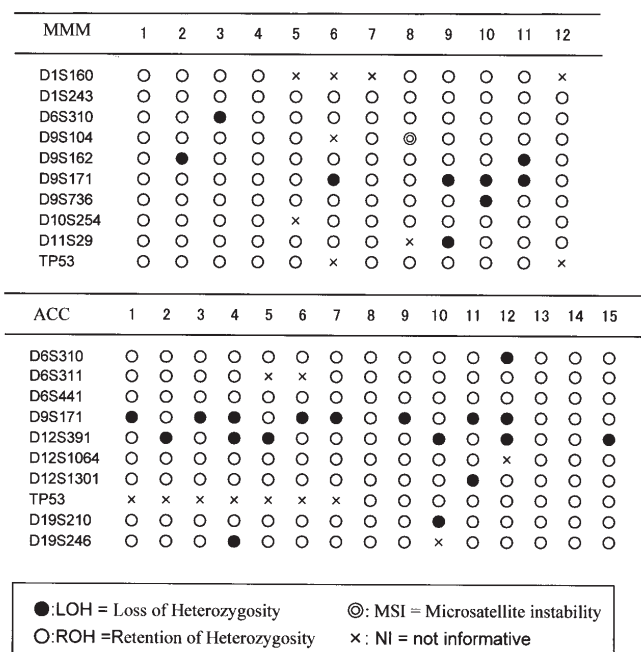


Figure 1. Deletion mapping of chromosomes 1p, 6q, 9p, 10q, 11q, and 17p in 12 MMMs, and chromosomes 6q, 9p, 12q, 17p, and 19q in 15 ACCs. Case numbers are shown at the top and locus symbols on the left.

sequences were extracted using the sequences made public on the NCBI database (<http://www.ncbi.nlm.nih.gov/>). Primers for PCR amplification and sequencing were designed using the Primer 3 program (version 0.2, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR was carried out in a final volume of 25 μ l of a reaction mixture containing 1X LA-PCR buffer (Takara Bio Inc), 2 μ M of each primer, 200 μ M of each dNTP, 100 ng of template DNA, and 0.01 unit of LA-Taq DNA polymerase (Takara Bio Inc). Thirty-five cycles of denaturation were performed at 94°C for 10 sec each, followed by annealing at 54°C for 10 sec, and extension at 72°C for 30 sec. After amplification, the PCR products were electrophoresed on 10% polyacrylamide gels under different conditions at 4°C, 15°C, and room temperature.

After electrophoresis, the gel was silver-stained using a DNA silver staining kit (GE Healthcare UK Ltd). Suspected mutations were identified by re-amplification with the same primer used in PCR-SSCP analysis, ligated into pCR2.1 vector (Invitrogen Japan K.K., Tokyo, Japan), and sequenced on an Applied Biosystems 3730xl DNA analyzer (Applied Biosystems Ltd, CA, USA).

Results

Analysis of LOH and MSI in MMM. We analyzed tumors from 12 patients with MMM using PCR-LOH assay with 10 microsatellite markers. LOH was detected in six of 12 samples (50.0%) in at least one region. The incidence of LOH for 10 microsatellite markers is shown in Table IV. The results of the LOH analysis in tumor samples are shown in the deletion map in Fig. 1. The highest incidence of LOH was found at the marker D9S171 at 9p21 (33.3%). This locus has already been reported as a candidate TSG locus for cutaneous melanoma (20). The marker D9S171 has been mapped around the *p16/CDKN2* gene locus on 9p21 (21).

Another frequently occurring LOH location was the marker D6S310 at 6q23-25 (8.3%), D9S736 at 9p21 (8.3%), D9S162 at 9p21 (8.3%), and D11S29 at 11q23.3 (10.0%). In addition, incidence of MSI was found at the microsatellite marker D9S104 in sample no.8. For all of them, it was not possible to assume a common deletion domain because expression was \leq 20%.

Typical examples of LOH, informative (INF) and not informative (NI) cases are shown in Fig. 2.

Analysis of LOH and MSI in ACC. The results of PCR-LOH analysis of the tumor samples with LOH are shown in Fig. 1, and Table IV. Fifteen tumor samples and normal tissues were studied for LOH using 10 microsatellite markers. We detected LOH in 80.0% (12/15) of the ACCs, the most frequently deleted locus being D9S171 at 9p21. Of the 15 informative cases, 8 (53.3%) showed LOH at D9S171. In the location of D12S391, a frequency of LOH of 40% (6/15) was detected. In addition, frequently detected loci were D6S310 at 6q23-25

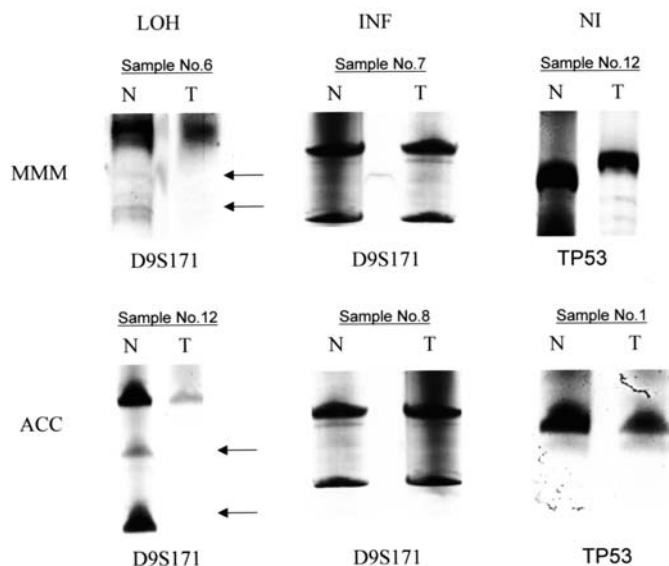


Figure 2. Illustration of microsatellite polymorphism analysis in MMM and ACC specimens. Case numbers are shown at the top, and locus symbols on the left. Paired normal (N) and tumor (T) samples for patients 1, 6, 7, 8, and 12 demonstrating loss of the upper allele (LOH), retained heterozygosity (INF) and not informative (NI), respectively.

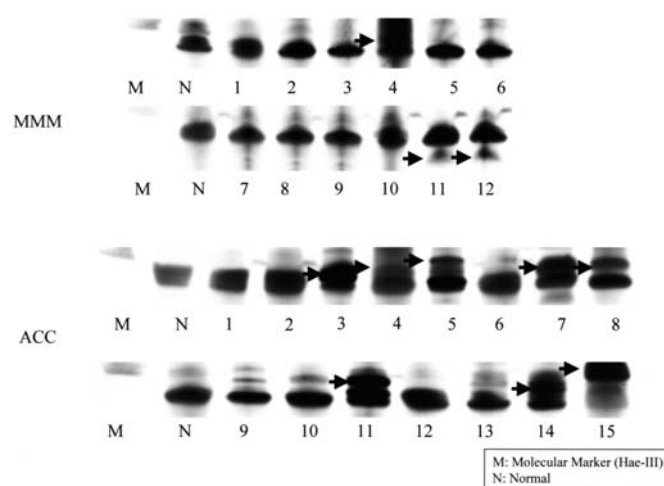


Figure 3. PCR-SSCP analysis for exon 4 of the *p16/CDKN2A* gene in MMMs and ACCs. Case numbers are shown below each column. N, corresponding normal tissue; T, tumor tissue.

(6.7%), D12S1301 at 12q12-13 (6.7%), D19S210 at 19q13.1 (6.7%), and D19S246 at 19q13.1 (7.1%). MSI was not found in ACCs. Interestingly, a locus of D9S171 overlapped with the proximal, commonly deleted region in MMM.

Typical examples of LOH, informative (INF) and not informative (NI) cases are shown in Fig. 2.

SSCP and auto-sequencing of the *p16/CDKN2* gene in MMM.

We investigated exons 1 to 4 of the *p16/CDKN2* gene by PCR-SSCP analysis in 12 primary tumors. We found mobility shifts in 3 cases, sample nos. 4, 11, and 12, of the 8 cases with LOH detected at D9S171 (30.0%) in exon 4 (Fig. 3), and these bands were analyzed by automatic sequencer. In tissue sample no. 4, 3 different nucleotide substitutions were

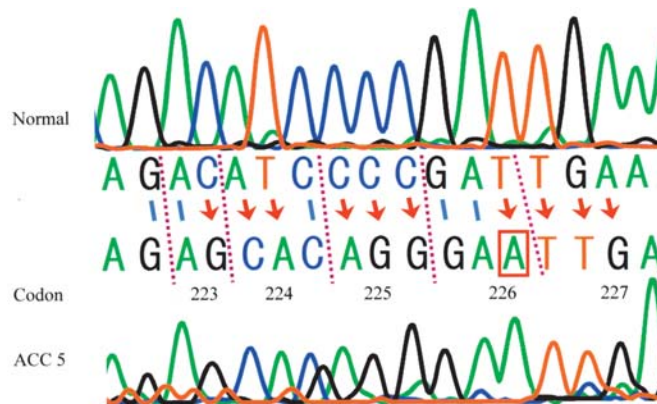


Figure 4. Sequencing analysis of the *p16/CDKN2* gene. The 223rd codon was changed from AC (Asp) to AG (Glu), the 224th codon was changed from ATC (Ile) to CAC (His), the 225th codon was changed from CCC (Pro) to AGG (Arg), and the 226th codon was changed from GAT (Asp) to GAAT (1 bp insertion, Glu) in ACC sample no. 5.

Table V. Somatic mutations of the *p16/CDKN2* gene in MMMs and ACCs.

Case	Base substitution	Codon	Amino-acid substitution
Sample no. 4 in MMMs	CCC→TCC	225	Pro→Ser
	GAT→ATG	226	Asp→Met
	TGA→GTGA	227	Stop→Val (1-bp insertion)
Sample no. 5 in ACCs	GAC→GAG	223	Asp→Glu
	ATC→CAC	224	Ile→His
	CCC→AGG	225	Pro→Arg
	GAT→GAAT	226	Asp→Glu (1-bp insertion)
Sample no. 14 in ACCs	CCC→deletion	225	3-bp deletion

identified. These substitutions were from CCC to TCC at codon 225, GAT to ATG at codon 226, and TGA to GTGA (1-bp insertion) at codon 227, resulting in an amino acid substitution, Pro to Ser, Asp to Met, and Stop to Val (Table V). However, nucleotide substitutions were not detected in samples no. 11 and 12.

SSCP and auto-sequencing of the *p16/CDKN2* gene in ACC.

By PCR-SSCP analysis of exons 1 to 4 of the *p16/CDKN2* gene, we identified mobility shifts in 8 of 15 cases (53.3%) in exon 4 (Fig. 3). Auto-sequencer analysis was performed, on sample no. 5, and 4 novel nucleotide substitutions at exon 4 were identified. These substitutions were from GAC to GAG at codon 223, ATC to CAC at codon 224, CCC to AGG at codon 225, and GAT to GAAT (1-bp insertion) at codon 226, and resulted in an amino acid replacement from Asp to Glu, Ile to His, Pro to Arg, and Asp to Glu (Fig. 4, Table V). Sample no. 14 showed a frameshift mutation at codon 225 (CCC, 3-bp deletion) (Table V). While it was possible to detect a band shift for sample nos. 3, 4, 7, 8, 11, and 15 (in SSCP), investigation of the gene sequence using a sequencer showed that no sequence abnormality or shift had occurred.

Discussion

MMM of the head and neck constitute approximately half of all malignant melanoma cases (22). These melanomas occur mainly in the upper respiratory tract in 56% and oral cavity in 44% (23). Within the oral cavity, the commonest site is the hard palate in up to 80%, followed, in decreasing order of frequency, by the upper and lower gingiva and mucosa of buccal. According to the report of Shah *et al.*, MMM of the head and neck exhibit a far more aggressive behavior than on the skin (24). They are more prone to metastasis into and recurrence in regional and distant sites.

ACC generally developed in the major and minor salivary glands. Seifert *et al.* reported in the WHO International histological classification of tumors that ACC is considered to have aggressive biological characteristics in terms of local recurrence, perineural spread and late distant metastasis (25). Among all salivary gland tumors, approximately 40% are malignant ones, and when the ACC are combined with mucoepidermoid carcinoma the total amounts to 80% or more. ACCs occur mainly in the palatine gland in approximately 35% and in the sublingual gland in approximately 30% (26). ACC has characteristics of a tendency towards permeation and intensive recurrence, and prognosis is generally poor.

In this study, we have analyzed and examined the molecular biological characteristics of MMM and ACC, by analyzing LOH and MSI for allelic imbalance. We carried out investigations to achieve a better understanding of the extent of alterations affecting chromosomes 1p32, 6q23-25, 9p21, 10q23-25, 11q23.3, 12q12-13, 17p13.1, and 19q13.1. From the results, LOH at one or more loci was found in 6 of the 12 informative MMM at 50% and 8 of the 15 informative ACC at 80%. The most frequently deleted region was D9S171 in the location of the *p16/CDKN2* gene on 9p21. The frequency rate was 33.3% in MMM and 53.3% in ACC. On the other hand, a 40% level of LOH incidence was detected in the D12S391 region located in chromosome 12q12-13. Yet, we focused our attention on microsatellite deletion in this region in MMM and ACC because the literature does not evidence any significant presence of TSG in this region. Therefore the hitherto unreported presence of some as-yet unknown TSGs cannot be ruled out. Further study is needed to shed light on this. LOH at a low frequency was detected on D6S310, D9S162, D9S736, D11S29, D12S1301, D19S210, and D19S246. However the incidence of LOH in all of these locations fell short of 20% and it was therefore not justified to consider them as common deletion domains. In this context, we are particularly interested in two regions, excluding *p16/CDKN2* which is a microsatellite domain of D9S171. The two regions are 6q23/25, which is a domain of D6S310, and p53 which is located at 17p13.1. The reason why these two regions arouse our particular interest lies in the fact that they do not exhibit LOH expression. Our research thrust will therefore concentrate on an analysis of the particular molecular biological characteristics of the 9p21 and *p16/CDKN2* regions.

The domain of the *p16/CDKN2* gene is one of the *p16* family, and the *p16* gene has a length of 25 kb and is situated on chromosome 9p21. It has been reported in the earlier literature to be involved in various cancers (27). One role played by p16 as a TSG is that the growth suppressing activity

of the retinoblastoma susceptibility gene product, *pRb*, is down-regulated by cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) whose activity is negatively regulated by CDK inhibitors of the p16 family (28,29). The *p16* gene may be a critical target in human carcinomas. Caldas *et al.* reported that frequent allelic losses were detected in location of the *p16/CDKN2* gene in many types of tumors including breast cancer, bladder cancer, head and neck cancer, esophageal SCCs, pancreatic adenocarcinomas and non-small cell lung carcinomas (30). In particular, Nakanishi *et al.* reported that the *p16/CDKN2* gene is commonly deleted in head and neck SCCs (53%) (5). These results suggest that a putative suppressor associated with many cancers is located at this region, but there have been no reports of *p16/CDKN2* gene LOH in both MMM and ACC of the head and neck. Our attention therefore focuses on mutation in *p16/CDKN2* as a TSG.

In this study, we have examined somatic mutations of the *p16/CDKN2* gene to determine whether these are associated with MMM and ACC of the head and neck. Eight somatic mutations of the *p16/CDKN2* gene were detected in 3 patients (1 MMM and 2 ACCs). These mutations have not been identified in any other type of tumor (Table V).

Previous studies have reported somatic mutation in exon 2 of *p16/CDKN2* (10,31). Mutations have also been reported at codon 80 in salivary duct carcinomas (10). This finding aroused our interest. All mutations detected were located within exon 4 of the *p16/CDKN2* gene. Interestingly, we found 2 somatic mutations in the *p16/CDKN2* gene in all samples of both MMM and ACC showing abnormal SSCP patterns, both mutations were present at codon 225 and codon 226, which created a truncated *p16/CDKN2* gene product. Thus, codon 225 and codon 226 may be mutational hot spots of the *p16/CDKN2* gene in MMM and ACC of the head and neck. These findings suggest that the *p16/CDKN2* gene has another potential tumor-suppression function, seeing that the mutation was detected in another domain not previously reported. However, the functional inactivation of *p16/CDKN2* by this mutation still remains to be clarified. For example, in ACC sample no. 5, a 1-bp insertion was detected in codon 226, resulting in a sequence shift after stop codon 227, disrupting the stop sequence. In order to determine the implications of this, the sequence following *p16/CDKN2* will need to be further clarified and the functionality of the proteins expressed confirmed in order to determine the influence of mutation at exon 4. On the other hand, a number of studies have found a relation between *p16/CDKN2* and the hypermethylation status of its promoter gene in ACC (32). Therefore, in further study, we need to identify the protein functionality of *p16/CDKN2*. Apart from the three patients mentioned above, none of the patients showed evidence of mutation, but did reveal mobility shifts. We concluded that this might be a case of polymorphism. It is also possible that these mobility shifts resulted from an artifact occurring during the PCR-SSCP. We used 12-cm long 10% polyacrylamide gel strips during the process, and such short strips of gel can not be divided into bands with PCR-SSCP. This may be a weakness of the study design, and clarification of this point will require further study using longer gels.

We will cautiously approach the issue of whether there is a relation between the effectiveness of HIMAC radiotherapy

and the biological characteristics of the *p16/CDKN2* gene. As mentioned above, on the one hand, p16/CDKN2 has an influence on the down-regulation of the cell cycle and, on the other, HIMAC is characterized by the unique feature that its radiation is effective on cells regardless of cell status and of the cell cycle. No analytical procedure for assessing the molecular biological effects of HIMAC radiation has been established yet. However, the discovery of *p16/CDKN2* mutations different from those of earlier literature publications might suggest the possibility of some relation between the effect of the HIMAC and these mutations.

Further studies are needed to clarify the role of the *p16/CDKN2* gene in the development and progression of MMMs and ACCs of the head and neck.

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