Impact of Methylation of the Gene p16^{INK4a} on Prognosis of Head and Neck Osteosarcoma

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Abstract

PURPOSE

Osteosarcoma occurring in the head and neck region is known as a malignant tumor that shows a relatively poor prognosis and, despite various treatments, clinicians have often been confounded by it. The existence or non-existence of the mutation of the gene p16^{INK4a} has been used in prognosis assessment. In this study, author have attempted to determine whether methylation of the gene p16^{INK4a} could be applied to forecast the progress of osteosarcomas in the head and neck region having been given poor prognoses in the diagnostic process and the early stage of treatment.

RESEARCH SUBJECT AND METHOD

Clinicopathologic investigations, immunohistochemical examinations, a methylation specific polymerase reaction (MSP) analysis, and a survival analysis were conducted on the tissues of 20 patients with mandibulofacial osteosarcoma.

RESULTS

Neither age, sex, size, smoking or non-smoking, nor region have showed a statistical significance with methylation or unmethylation of the gene p16^{INK4a} and expression rates demonstrated by immunohisto- chemical examinations. A chi-square test indicated that recurrence inclination has no relation with the expression rate of p16 protein (p=0.6615), but it showed a statistical significance with methylation of the gene p16^{INK4a} (p=0.0033). With respect to investigations of the survival rates, a Kaplan-Meier survival analysis found that the manifestation rate of p16 protein did not have an impact on survival (p=0.8864), but that the methylation of the gene p16^{INK4a} resulted in significant differences in survival rates (p=0.0105).

CONCLUSIONS

The above results show that methylation of the gene p16^{INK4a} could be one of the major factors that help determine the recurrence inclination and prognosis of osteosarcomas occurring in the head and neck region.

INTRODUCTION

Osteosarcoma occurring in the head and neck region is known as a malignant tumor that shows a relatively poor prognosis and, despite various treatments, clinicians have often been confounded by it. Even several-times repeated operations and chemotherapy often result in unexpected consequences, shortening the lives of patients. In addition to diagnostic means, the determination of prognoses of osteosarcoma is indispensable for patient treatment guidelines. General anatomical and pathological methods, immunohistochemical methods, and various gene analyzing methods have been used for the determination of prognoses. In particular, variations of the gene p16^{INK4a} are used for the assessment of prognoses of malignant tumors.

The gene p16^{INK4a} is located at 9p21, which is a short arm of chromosome #9. CDK4/6 intercepts the cell-cycle progress by effectively controling the kinase activity and encodes nuclear protein that can negatively control cell growth[1-3]. When there is no functional p16 protein, CDK4 adheres to cyclin D and stimulates the cell-cycle progress by changing pRb to phosphoric acid and isolating E2F. Studies have reported these activities in many malignant

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tumors and proposed that decreases in p16 manifestation are related both to the progress of tumors and survival rates in carcinoma patients[4-7]. Similarly, some studies reported that when p16 manifestation decreases, the survival rates of infant osteosarcoma patients also decrease[8]. Losses of function of the gene $p16^{INK4a}$ take place in many carcinomas and malignant tumors, and these losses seem to be caused by homozygous deletion[9-12], point mutation[13], and methylation of the promoter region[14-16]. That methylation of the promoter region, the CpG-rich region known as the CpG island of the gene $p16^{INK4a}$, is related to the loss of translation[17], has also been proved in some osteosarcomas[18-24].

Through a retrospective survey of the patients with head and neck osteocarcoma who received treatment in Pusan National University Hospital, the author tried to conduct the determination of prognoses of patients with head and neck osteosarcomas that are uncontrollable and aggressive. To identify the inactivity process of the gene $p16^{INK4a}$ and the immunohistochemical expression of p16 protein in head and neck osteosarcomas that have not spread to other regions, this study examined the course of methylation and compared the outcome with the clinical aspects of tumors, recurrence inclination, and survival rates. By doing so, the author attempted to determine whether methylation of the gene $p16^{INK4a}$ can be clinically applied to forecast the progress of osteosarcomas having been given poor prognoses in the diagnostic process and the early stage of treatment, thus helping assess the scope of treatment.

MATERIALS AND METHODS

Tumor samples

Twenty formalin-fixed, paraffin wax-embedded tissue samples from 20 patients with osteosarcoma (OS) of head and neck were collected from the Department of Oral and Maxillofacial Surgery, Pusan National University Hospital, South Korea, and were used for the immunohistochemical analysis of p16. There were 9 females and 11 males with a mean age of 38.7 years (range 19-61 years). Tumor tissue was obtained by surgical biopsy at the site of the primary tumor prior to treatment. The diagnoses of all cases included in this study were based on histological examination with haematoxylin and eosin (H & E) staining. The patient underwent neoadjuvant chemotherapy, ablation surgery, and radiation therapy. The radiation therapy was optional treatment modality.

Clinicopathological analysis

The clinicopathological parameters investigated in this study were classified as follows: age ; size ($\geq$5 cm versus <5 cm); smoking history (positive versus negative); recurrence (- versus + versus ++); and survival period. Survival data were available for all cases, with a follow-up period ranging from 5 to 162 months. The recurrence inclination was classified according to three cases: when there is no recurrence (indicated as "-"); when the disease is controlled after a one-time occurrence (indicated as "+"); and when there are two or more recurrences despite several operations or treatments (indicated as "++"). The pathological stage was omitted. Only a size classification was conducted based on the assessment of MRI findings and an examination with the naked eye during the first medical examination. In addition, a survey was conducted on whether patients smoke or not and the relatedness between smoking and methylation of the gene $p16^{INK4a}$ was examined, as smoking is one of the most implicated carcinogenic factors in head and neck tumors.

Immunohistochemistry

Immunohistochemical analyses were performed using a mouse IgG monoclonal antibody against p16 (1:50 :Neo Markers Fremont, California, USA). Four-micrometre thick histological sections were cut, mounted on glass slides coated with 3-aminopropyltriethoxysilane, and air-dried overnight at room temperature. The sections were deparaffinized in xylene and dehydrated in ethanol. After dehydration, the endogenous peroxidase was blocked with methanol containing 3% H_2O_2 for 10 min. For staining with the above mentioned antibody, specimens were pretreated with citrate buffer (0.01 mol/l citric acid, pH 6.0) four times, each for 5 min at 100 ℃ in a microwave oven. Sections were incubated with the primary antibody at 4 ℃ overnight, followed by staining with a streptavidin-biotin-peroxidase kit (Zymed Laboratory Inc., South San Francisco, USA). The sections were then finally reacted in an aminomethan ethylcarbazole (AEC), peroxytrichloride substrate solution; counterstained with haematoxylin; and then mounted. Each case was scored criteria[4,25,26]. For example, if the neoplastic nuclei were stained throughout the tumor, the expression of p16 was considered to be normal. However, if the neoplastic nuclei failed to stain in all areas, although admixed non-neoplastic cells were stained, or if there was an absence of stained neoplastic nuclei in certain

areas, with positive staining of the admixed non-neoplastic nuclei, the expression of p16 in these neoplasm was judged to be decreased. Tonsil tissue was used as an external positive control.

DNA extraction from tumor tissue

Genomic DNA was extracted using standard proteinase K digestion and phenol/chloroform extraction methods. To avoid contamination of the DNA with normal tissue, manual microdissection was performed to extract tumor DNA from formalin-fixed, paraffin wax-embedded tissues. In each sample, a lesion with high cellularity was selected for the PCR assay.

Bisulphite modification and methlyation-specific PCR(MSP)

Bisulphite modification was performed using a DNA modification kit (Intergen, Purchase, NY, USA) according to the manufacturer's protocol. The modified DNA was used for MSP. The sequences of the primers were: methylated-specific primers, 5' TTATTAGAGGGTGGGGCGGATCGC 3' (sense) and 5' GACCCCGAACCGCGACCGTAA 3' (antisense); unmethylated-specific primer, 5' TTATTAGAGGGTGGGGTGGATGT 3' (sense) and 5' CAACCCCAAACCACAACCATAA 3' (antisense). PCR products identified by methylation primers and unmethylation primers were 145 bp and 154 bp respectively. PCR was carried out for 35 cycles after the first denaturation at 95 ℃ (95℃ for 45 seconds, the annealing temperature 60 ℃ for 45 seconds, and 72 ℃ for 1 minute), followed by a final 4 minutes extension at 72 ℃. DNA of the colo-205 cell line, which is known to show hypermethylation of the gene $p16^{INK4a}$ promoter by the MSP method, was used as a positive control[27]. In addition, DNA from normal skeletal muscle was used as a negative control. Each PCR product (10 μl) was loaded directly onto 10% polyacrylamide gels, stained with ethidium bromide, and directly visualized under UV illumination.

Statistical analysis

Fisher's Exact test was conducted on the relatedness between clinical pathological variables, methylation of p16 and the immunohistochemical manifestation rates of the gene $p16^{INK4a}$. A chi-square test was conducted on the matters related to recurrences. SAS version 8.3 (SAS Institute Inc., North Carolina, USA) was employed for the analysis of survival rates, and the Kaplan-Meier survival curve[28] was used to compare survival rates in terms of p16 methylation and the immunohistochemical expression rates. The difference in the survival rates of the two groups was verified with a long-rank test. When a p-value was less than 0.05, it was determined to have statistical significance.

RESULTS

Clinicopathologic findings (Table 1)

The patients were aged between 19 and 61 (average age: 35.75). Twenty head and neck osteosarcoma patients consisted of 9 females and 11 males. Five cases occurred in the maxilla and 15 cases in the mandible. Among the

Table 1. Clinicopathologic parameters in 20 cases head and neck osteosarcoma.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
Gender	F	M	F	M	F	F	F	F	M	M	M	M	M	F	M	M	F	M	M	F
Age	35	40	61	35	57	25	29	21	33	33	60	39	42	60	42	49	31	40	19	24
Size (cm)	≥5	<5	≥5	≥5	≥5	<5	<5	<5	<5	≥5	≥5	≥5	≥5	<5	<5	≥5	<5	<5	<5	<5
Location	Lt. Mx.	Lt. Mn.	Rt. Mn.	Rt. Mn.	Rt. Mx.	ant. Mx.	Rt. Mn.	Rt. Mn.	Lt. Mn.	Lt.. Mn.	Rt. Mn.	Lt. Mn.	Rt. Mx.	Rt. Mn.	Lt. Mn.	Lt. Mn.	ant. Mx.	Lt. Mn.	Lt. Mn.	Rt. Mn.
Recurrence	++	+	-	++	-	+	-	-	-	++	+	++	-	+	-	-	++	-	-	-
Methylation	+	+	-	+	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-
Immunohisto-chemistry	-	-	-	+	-	+	-	-	-	+	+	+	+	-	+	+	-	+	-	-

S1-20:samples; M:male; F:female; Rt.:right; Lt.:left; Mx.:maxilla; Mn.:mandible; recurrence inclination:when there is no recurrence (indicated as "-"); when the disease is controlled after a one-time occurrence (indicated as "+"); and when there are two or more recurrence despite several operations or treatments (indicated as "++")

five cases occurred in the maxilla, two cases occurred in the anterior region of the maxilla and the rest occurred in the side regions. The 15 cases of the mandible occurred in the lateral region of the mandible. The clinical and CT or MRI findings at the first medical examination indicated that the sizes of 9 cases were larger than 5cm and those of 11 cases smaller than 5 cm. Of the 20 patients, 9 patients (45%) had a smoking habit for a long period of time. With respect to recurrence inclination, 11 cases (55%) showed no recurrence, 4 cases (20%) one-time recurrence, and 5 cases (25%) several times of local recurrences. A survival rate analysis conducted on all the 20 patients and the survival period ranged from 5 to 162 months.

Immunohistochemical analysis of p16 protein and statistical correlation (Table 2)

Expression of p16 protein was reduced in 11 tumors. Expression of p16 protein in nuclei and cytoplasm was confirmed in the remaining 9 tumors (Fig. 1). Of the tumors larger than 5 cm, three cases showed a lowered expression of p16 protein. Fisher's Exact test indicated no statistical significance between the rates of expression and the sizes of tumors (p=0.1748). Neither was there any statistical significance between the average age and the expression rate of p16 protein (p=0.3698), being no association between the patient's age at the outbreak of the disease and immunohistochemical manifestation. Regarding the association between recurrence inclination and immunohistochemical analysis, a chi-square test showed no statistical significance (p=0.6615) (Table 3).

Methylation of the gene $p16^{INK4a}$ promoter and statistical correlation (Table 4)

As the reduced expression rate of p16 protein can be caused by various factors, we investigated the course of methylation to examine the inactivity process of the gene $p16^{INK4a}$. Of the 20 tumors, methylation of the gene $p16^{INK4a}$

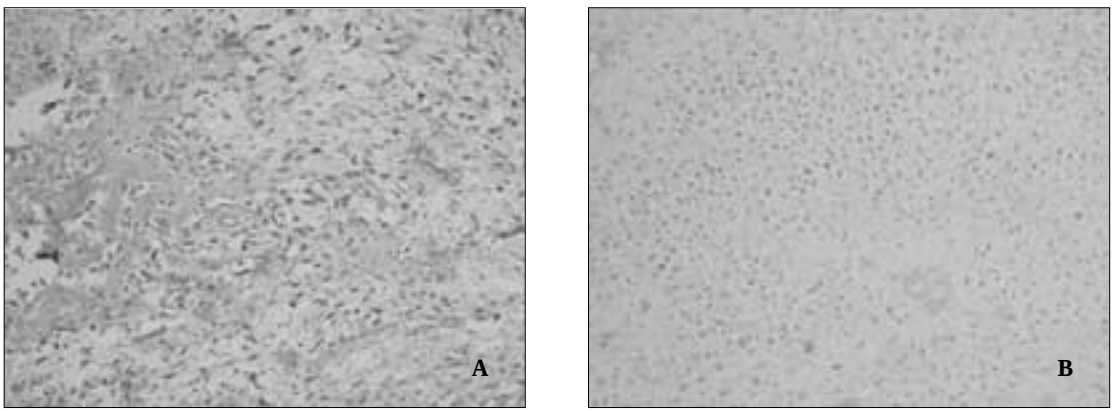

Fig. 1. Immunohistochemical staining for p16 (A) Immunohistochemistry showing nuclear / cytoplasmic staining in the majority of tumor cells (×200). (B) Loss of p16 protein expression was observed in the tumor cells (×200).

Table 2. Correlations between the Immunohistochemical Expression of p16 Protein and the Clinicopathologic Findings.

	p16 Protein expression Positive	p16 Protein expression Negative	p-value
age	> avg 3 (15%)	> avg 7 (35%)	0.3698
	< avg 6 (30%)	< avg 4 (20%)	
size	< 5 3 (15%)	< 5 8 (40%)	0.1748
	≥ 5 6 (30%)	≥ 5 3 (15%)	
smoke	+ 7 (35%)	+ 2 (10%)	0.2160
	- 2 (10%)	- 9 (45%)	

avg:average age(35.75 years); size:larger or smaller than 5 cm; smoke:smokers (indicated as "+") or nonsmokers (indicated as "-")

Table 3. Correlations between the Immunohistochemical Expression of p16 Protein and the Recurrence Inclination.

	-	+	++	chi-square	p-value
p16 Protein expression Positive	4	2	3	0.8264	0.6615
p16 Protein expression Negative	7	2	2		

recurrence inclination:when there is no recurrence (indicated as "-"); when the disease is controlled after a one-time occurrence (indicated as "+"); and when there are two or more recurrence despite several operations or treatments (indicated as "++")

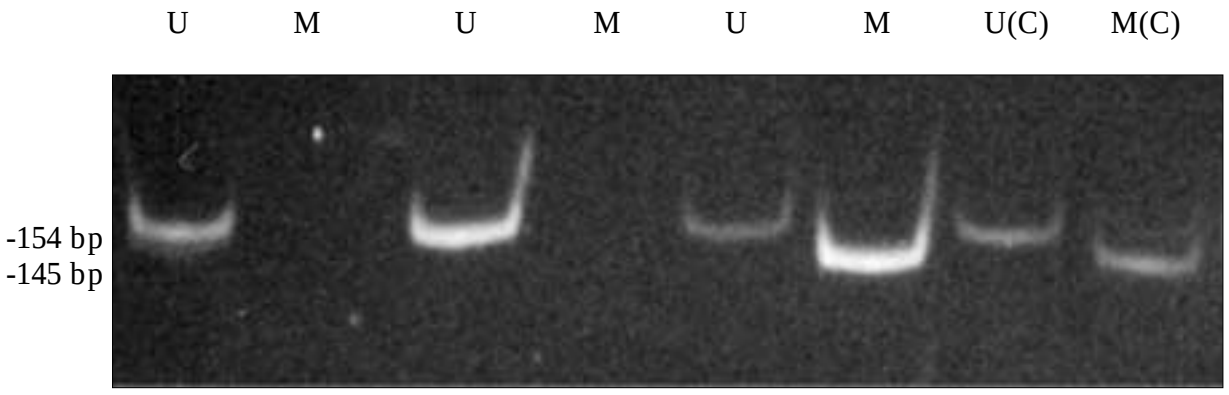

Fig. 2. Methylation-specific PCR analysis of $p16^{INK4a}$ PCR products amplified using unmethylated (U) and methylated (M) specific primers. O1,O2,O3 = Samples, C = Control

Table 4. Correlations between the gene $p16^{INK4a}$ Methylation index and the Clinicopathologic Findings/ the Immunohistochemical Expression of p16 protein.

	Unmethylation	Methylation	p-value
age	> avg 5 (25%)	> avg 5 (25%)	0.141
	< avg 9 (45%)	< avg 1 (5%)	
size	< 5 8 (40%)	< 5 3 (15%)	1.000
	≥ 5 6 (30%)	≥ 5 3 (15%)	
smoke	+ 6 (30%)	+ 3 (15%)	1.000
	- 8 (40%)	- 3 (15%)	
p16 Protein expression	+ 6 (30%)	+ 3 (15%)	1.000
	- 8 (40%)	- 3 (15%)	

avg:average age(35.75 years); size:larger or smaller than 5cm; smoke:smokers (indicated as "+") or nonsmokers (indicated as "-")

was confirmed in 6 tumors (30%). Unmethylation was confirmed in the remaining 14 tumors (Fig. 2). Of the tumors larger than 5 cm, methylation of the gene $p16^{INK4a}$ was observed in three cases. Fisher's Exact test showed that no statistical significance could be confirmed between size and methylation (p=1.000). Neither was there any statistical significance between average age and methylation of the gene $p16^{INK4a}$ (p=0.141), being no correlation between them. Neither was there any statistical significance between smoking or non-smoking and

Table 5. Correlations between the gene p16^{INK4}a Methylation index and the Recurrence Inclination.

	-	+	++	chi-square	p-value
Unmethylation	11	2	1	11.4286	0.0033
Methylation	0	2	4		

recurrence inclination:when there is no recurrence (indicated as "-"); when the disease is controlled after a one-time occurrence (indicated as "+"); and when there are two or more recurrence despite several operations or treatments (indicated as "++")

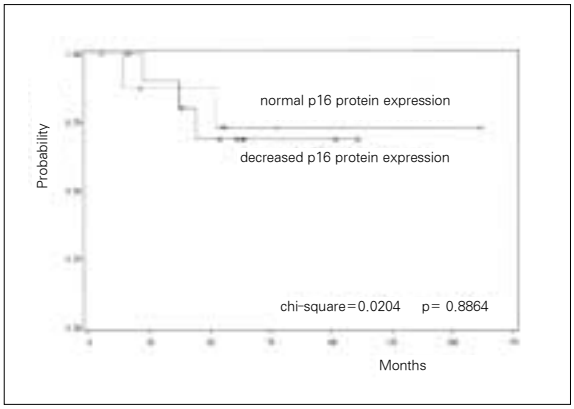

Fig. 3. Effects on survival of p16 protein expression. Decreased p16 protein expression was not associated with a significant reduction in overall survival (p=0.8864).

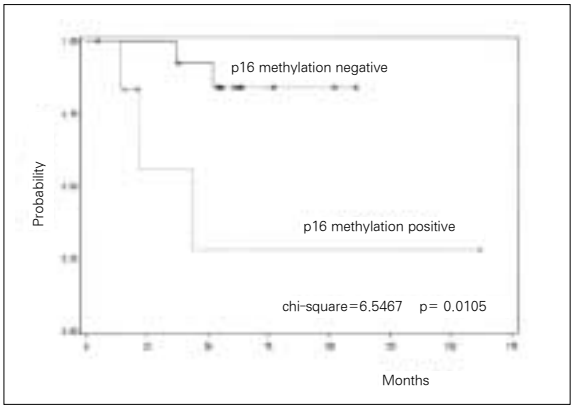

Fig. 4. Effect of p16^{INK4a} promoter hypermethylation. Patients with methylation had a significant poorer prognosis (p=0.0105).

methylation of the gene p16^{INK4a} (p=1.000).

However, a chi-square test showed statistical significance between recurrence inclination and methylation of the gene p16^{INK4a} (p=0.0033), and this suggests that methylation of the gene p16^{INK4a} can have influences on patients with osteosarcomas that show several-times aggressive recurrences (Table 5). In addition, Fisher's Exact test indicated that there was no statistical significance between methylation of the gene p16^{INK4a} and the expression rate of p16 protein determined by an immunohistochemical analysis (p=1.000), thus suggesting no correlation between the degeneration of the gene level and the mutation of the protein level. This implies that immunohistochemical analysis of proteins does not reflect the exact nature of genes.

Survival analysis

A Kaplan-Meier survival analysis was conducted for the existence or non-existence of p16 protein expression. A log-rank test on the two groups found that the chi-square value was 0.0204 and the p-value 0.8884 (>0.05), showing no statistical significance (Fig. 3). Assessment of p16 protein by an immunohistochemical analysis showed no difference in the survival rate, making it difficult to judge the expression of p16 protein as a prognostic factor. This is a different result from those of other studies. However, a survival analysis according to the existence or non-existence of the gene p16^{INK4a} methylation found that the chi-square value was 6.5467 and the p-value 0.0105 (<0.05), showing a significant difference (Fig. 4). That is, in the case of osteosarcomas occurring in the head and neck region, the existence of the gene p16^{INK4a} methylation has an impact on the survival rate, thus making it possible to consider methylation as a prognostic factor.

DISCUSSION

One of the important phenomena from the perspective of general genetics is a mutation in which a base changes but in epigenetics, the methylation process in which a methyl adheres to a base is important. Methylation of the

CpG island may serve as a defense mechanism that incapacitates rambling genes like transposon that flow in from the outside. Promotor CpG islands of genes that flow in from the outside are methylated to block gene manifestation. As time passes, cytosine with a methyl group appears to be replaced by thymine, ultimately causing rambling genes to lose their functions gradually. Research shows the functions of the tumor suppressor genes come from methylation of the CpG islands of the genes and that the loss of functions of the tumor suppressor genes is caused by mutations, deletion, and methylation of the promoter area.

The key proteins controlling cellular proliferation at the G1/S checkpoint include pRB, p16^{INK4a} and cyclin D1[29]. There is mounting evidence to suggest that alteration of any 1 component of this intricate cell-cycle control system is sufficient to shift the balance in favor of proliferation. Structural abnormalities involving the RB, ^{INK4a} and cyclin D1 genes have been previously demonstrated in a variety of human tumors, including osteosarcomas[30,31]. Greater than 60% of osteosarcomas show loss of heterozygosity at chromosome 13p14[32-34], which harbors the RB gene. RB mutations have been demonstrated in nearly one-third of sporadic osteosarcomas[35-37], and patients with germline RB mutations have an increased incidence of this tumor[38]. A smaller number of osteosarcomas show abnormalities of the gene p16^{INK4a}, with an observed frequency of mutations varying from 7% to 23%[10,12,21,39].

Benassi *et al.*[19] have conducted a study on the ^{INK4a} gene in osteosarcoma and found that pRB and p53 cell-growth control pathways are deregulated by methylation of the gene p16^{INK4a} rather than mutation or deletion. The present study also put stress on CpG-island methylation and immunohistochemical analysis rather than mutation or deletion. Smoking is considered to be one of the risk factors having an influence on the malignant tumors in the head and neck region, and studies have also been conducted on it. Chang et al.[40] compared normal persons with patients with squamous cell carcinoma in the head and neck region and reported that methylation of p15 could be caused by smoking and drinking. The present study found that the smoking history of patients with head and neck osteosarcomas had no specific relation with methylation of the gene p16^{INK4a} or immunohistochemical manifestation rates. This result may be attributed to the fact that, unlike squamous cell carcinoma, the aspects of tumors in the case of osteosarcomas are not caused on the surface. Rodriguez-Galindo *et al.*[41] reported that prognoses were not good following local recurrences of osteosarcomas. In studies on osteosarcomas occurring in various regions, Maitra et al.8 reported that a decrease in pRB expression did not have an impact on the survival rate but that expression of p16 protein decreased the survival rate, thereby asserting that the gene p16^{INK4a} can be a diagnostic factor. The present study also found that local recurrences are correlated to methylation and that this has an impact on the survival rate. However, this study could not find statistically that p16 protein expression itself is related to local recurrences. In fact, the immunohistochemical expression rate at the protein level does not correspond to promoter methylation, and it is difficult to determine their correlation. Three tumors with promoter methylation showed normal expression of p16 protein in our series. If one considers Knudson's two-hit hypothesis, in which tumor suppressor gene function is lost by independent inactivation events involving both parental alleles, the present results might indicate that p16 methylation had occurred in only one allele in these cases. Therefore, the author acknowledges that, in the case of head and neck osteosarcomas, methylation of the gene p16^{INK4a} is related to local recurrences but that it is difficult to regard the immunohistochemical expression rate as a correlation factor for local recurrences.

In their study on leiomyosarcoma of soft tissue, Kawaguchi *et al.*[42] reported that, when expression of p16 protein decreased, methylation of the gene p16^{INK4a} increased depending on the immunohistochemical expression amount, and that prognoses became worse. They also reported that when the tumor sizes were big, p16 expression decreased, leading to worse prognoses, and that when there were methylated genes, the expression rates decreased and prognoses became worse. The present study found that methylation of the gene p16^{INK4a} had not direct relation to tumors' sizes, location and patients' ages and sexes. The immunohistochemical analysis also showed no statistical significance between methylation and the manifestation rates of p16. Unlike the findings of Kawaguchi's study[42] on leiomyosarcoma, the present study found that there was no statistical significance between the survival rate and the existence of the immunohistochemical course of p16 protein, and that the existence of methylation of the gene p16^{INK4a} had an impact on the survival rate. In actual clinical practices, as a prediction of a prognosis following the diagnosis of osteosarcoma in the head and neck region, the examina-

tion of gene methylation will be more reliable than the assessment of the p16 expression rate.

The immunohistochemical expression rates are important to the diagnosis of malignant tumors. But the assessment of the prognoses of patients in clinics is related to the survival of patients. Therefore, when the expression rates or methylation of specific genes are found to have an impact on prognoses, it will help present various treatment options, in addition to the increased number of specific cures. Based on the findings of the present study, the examination of the course of the gene $p16^{INK4a}$ methylation can be used as a prognostic factor in determining the survival rate and recurrence tendency of patients with head and neck osteocarcoma. Further studies by various cancer research groups will be necessary in order to include more cases and conduct more extensive scans.

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