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F-18-FDG Positron Emission Tomography Findings Correlate Pathological Proliferative Activity of Oral Squamous Cell Carcinoma

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Background : It is still controversial whether FDG uptake is correlated with cellular proliferation and prognosis of oral squamous cell carcinoma (OSC). In this study, we performed PET study and immunohistochemical analysis to elucidate the relationship between FDG uptake and expression of cellular proliferative markers and pathological prognostic markers in patients with OSC. **Methods :** FDG PET and immunohistochemical staining have been carried out in sixteen patients with OSC. Tumor uptake of FDG was expressed with standardized uptake value (SUV). The expression of Ki-67, Topoisomerase IIα (Topo IIα), p53, and p63 in cancer cells was quantitatively assessed with positivity of the immunohistochemical staining. SUV was compared with the results of immunohistochemical analysis. **Results :** FDG PET study revealed that SUV ranged from 3.6 to 22.1 with average of 10.4. Average positive rate of Ki-67, Topo IIα, p53, and p63 was 68.9%, 58.9%, 72.0%, and 65.2%, respectively. Pearson product-moment correlation coefficient analysis revealed that SUV was significantly correlated with Ki-67 ($r=0.616$, $p=0.01$), Topo IIα ($r=0.677$, $p=0.004$), p53 ($r=0.613$, $p=0.01$), and p63 ($r=0.710$, $p=0.002$), respectively. **Conclusion :** The present preliminary study indicated that FDG uptake was closely correlated with pathological cellular proliferative and prognostic markers in patients with OSC.
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Key Words： Oral squamous cell carcinoma, FDG PET, Cellular proliferation, p53, Topoisomerase IIα, p63

Introduction

Oral squamous cell carcinoma (OSC) is an ordinary type of oral cancer. It is usually found by clinical examinations such as simple inspection and palpation ; however, it is difficult to evaluate the precise state of tumor progression. The accurate assessment of the tumor is important because the treatment of OSC is based on the accurate clinical TNM classification.[1] Recent advances in diagnostic imaging methods such as CT, MRI and PET have improved the diagnostic accuracy of OSC and the prognosis of patient with OSC.

In the field of head and neck tumors including OSC, 2-deoxy-2-[F-18] fluoro-D-glucose (FDG) PET and CT/MRI have shown increased diagnostic accuracy,[2] but FDG PET has been more advantageous than CT/MRI to detect tumor recurrence and lymph node metastasis,[3-6] secondary or occult malignancy, and distant metastasis.[7,8] FDG PET is a functional imaging which demonstrates increased glucose uptake as a metabolic marker for malignant tumor. FDG uptake in the tumor is influenced by the expression of glucose transporters and hexokinase, which play a key

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role in glucose uptake and glycolysis in the cell.[9] Malignant cells are known to have increased glucose consumption as their substrate for energy production or the maintenance of accelerated proliferative activity.

Although FDG uptake is correlated with cellular proliferation and prognosis in many types of cancer, it is still controversial whether FDG uptake is correlated with the histological markers of cellular proliferation and prognosis in OSC.[10] In this study, we aimed to elucidate the relationship between FDG PET and immunohistochemical markers for cellular proliferation and prognosis in patients with OSC.

Patients and methods

Patients

We studied 16 patients (10 females and 6 males, age from 52 to 82 year-old) with OSC at our institution from May 2003 to June 2006. Primary site of OSC in all patients was shown in Table 1. The pathological tumor staging was based on the TNM-classification by the International Union against Cancer.[11]

The origin of OSC was gingiva in eight, floor of mouth in four, tongue in two, and cheek and palate in one patient each. Ten patients showed marked keratinization and histologically classified into well differentiated carcinoma. All patients underwent resection of the tumor within one month after the FDG PET study, and histological examination was performed on the tumor specimen. Postoperative clinical course was assessed for more than 24 months in all patients. Existence or absence of recurrence was censored at 24 months after the surgery.

FDG PET study

Fluorine-18 was produced in an in-house cyclotron (BC1710, Japan Steel Works, Muroran, Japan), and FDG was synthesized by the method that has been reported.[12] PET images were obtained with a whole-body PET scanner (SET 2400W, Shimadzu Corporation, Kyoto, Japan) with a 59.5 cm transaxial field of view, 20 cm axial field of view, which produced 63 image planes, spaced 3.125 mm apart. Transaxial spatial resolution was 4.2 mm full width at half maximum (FWHM) at the center of the field of view and axial resolution was 5.0 mm FWHM. A whole-body imaging by the simultaneous emission-transmission method with a rotating external source (Ge-68/Ga-68) was started at 60 min after the injection of 5 MBq (185 mCi)/kg (body weight) FDG. Four to five bed positions from the head to the thigh were imaged for 8 min per position. Patients fasted for at least 6 hours before FDG injection. The study protocols of FDG PET were approved by the Institutional Review Board, and all the patients gave informed consent to be included in the study and undergo the examination.

Attenuation-corrected transaxial images were reconstructed by the ordered subsets expectation maximization (OS-EM) algorithm into 128×128 matrix with pixel dimensions of 4.0 mm in a plane and 3.125 mm axially. Finally, 3 consecutive slices were added to generate transaxial images with slice thickness of 9.8 mm for visual interpretation and quantitative analysis by using standardized uptake value (SUV). Coronal images with slice thickness of 9.8 mm were also reconstructed from transaxial images.

PET data were visually interpreted by two nuclear physicians independently, and statistical analysis was performed using SUV.

Table 1 Characterictics of Patients with Squamous Cell Carcinoma of the Oral Cavity

No. of Patients	Sex	Age	Localization	TNM Classification (pStage)	Histological Differentiation	Prognosis
1	M	82	Tongue	T2N0M0 (II)	Well	No recurrence
2	F	81	Gingiva, Mandible	T4N0M0 (IV)	Well	Dead
3	M	73	Gingiva, Mandible	T2N0M0 (II)	Well	No recurrence
4	M	52	Floor of mouth	T1N0M0 (I)	Well	No recurrence
5	M	64	Floor of mouth	T2N0M0 (II)	Well	No recurrence
6	F	61	Gingiva, Maxilla	T3N0M0 (III)	Well	Recurrent
7	M	75	Tongue	T2N0M0 (II)	Well	No recurrence
8	F	68	Gingiva, Mandible	T2N0M0 (II)	Well	Recurrent
9	F	66	Floor of mouth	T1N0M0 (I)	Well	No recurrence
10	F	65	Cheek	T1N1M0 (III)	Well	No recurrence
11	M	76	Gingiva, Maxilla	T4N0M0 (IV)	Moderate	Dead
12	F	72	Gingiva, Mandible	T2N1M0 (III)	Moderate	No recurrence
13	M	64	Gingiva, Mandible	T2N2bM0 (IV)	Moderate	Dead
14	F	71	Gingiva, Mandible	T2N0M0 (II)	Moderate	Dead
15	F	70	Floor of mouth	T2N0M0 (II)	Moderate	No recurrence
16	F	81	Palate	T2N2bM0 (IV)	Poor	Dead

No recurrence : Alive without recurrence at 24 months after operation, Dead : Died of OSC within 24 months, Recurrence : Recurrence of OSC within 24 months.

Table 2 Primary Antibodies and Antigen Retrieval Method for Immunohistochemical Staining

Antibodies	Clone	Dilution	Antigen Retrieval*	Source
Anti-Ki-67	MIB-1	×100	B	DakoCytomation , Glostrup, Denmark
Anti-Topo IIα**	3F6	×50	B	Novocastra Lab., Newcastle upon Tyne, UK
Anti-p53	DO7	×25	A	Novocastra Lab., Newcastle upon Tyne, UK
Anti-p63	Ab-4	×100	B	NeoMarkers, Fremont, CA, USA

*A : Antigen retrieval with 20% $ZnSO_4$ solution for 20 min at 98°C
B : Antigen retrieval with 0.1M citric acid buffer, pH 6.0, for 20 min at 98°C
** Topo IIα : Topoisomerase IIα

Immunohistochemical study

After histopathological diagnosis was established, immunohistochemical staining of Ki-67, Topoisomerase IIα (Topo IIα), products of p53 and p64 (p53, and p63, respectively) was carried out using 4 μm-thick paraffin sections from the resection samples in all OSC cases. The paraffin sections were dewaxed with xylene and endogenous peroxidase activity was blocked using 3 % hydrogen peroxide in methanol solution for 30 min at room temperature. Then the sections were rehydrated in a degraded ethanol series and followed by the treatment of antigen retrieval with microwave, if necessary, which was detailed in Table 2. After the treatment with normal horse serum to prevent non-specific staining, the sections were incubated with each primary antibody at 4°C overnight. Details of primary antibodies used in this study were shown in Table 2. Then, the sections were incubated with biotinylated secondary antibody for 30 min at room temperature, followed by avidin-biotin-peroxidase complex solution according to the manufacturer's instructions (VECTASTAIN, Vector Laboratories, Burlingame, CA, USA). The peroxidase was visualized with 0.02% 3-3'-diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 in 0.01M tris-phosphate buffer, pH7.4. Finally, the sections were counterstained lightly with hematoxylin.

Immunohistochemical staining for Ki-67, Topo IIα, p53, and p63 was quantitatively evaluated by counting 200 to 500 tumor cells in the area where positive nuclei were gathered. Labeling index (LI) was defined as the percentage of immunoreactive tumor cells in totally counted tumor cells. Immunoreactive tumor cells were defined as nuclear staining for each antibody.

Statistical Analysis

The relationship between LI of Ki-67, Topo IIα, p53, p63 and SUV was statistically assessed using Pearson product-moment correlation coefficient analysis. $P<0.05$ was considered significant.

Results

All patients showed FDG accumulation in the primary tumor of the oral cavity. A representative case is shown in Figure 1. As shown in Table 3, SUV in 10 patients with well differentiated squamous cell carcinoma showed SUV ranged from 3.6 to 22.1 (10.7±6.2). SUV in five patients with moderately differentiated squamous cell carcinoma ranged from 6.1 to 21.2 (10.2±6.2). SUV in a patient with poorly differentiated squamous cell carcinoma was 8.9. SUV showed no correlation with tumor size, lymph node metastasis, nor pathological stage.

Immunoreactivity for Ki-67, Topo IIα, p53, and p63 was detected only in the nuclei, not in the cytoplasm (Fig. 2). In well differentiated squamous cell carcinoma, these markers were usually seen in the periphery of the alveolus. LI of Ki-67 ranged from 36.1% to 90.0% with the average of 68.9%. In 10 patients with well differentiated squamous cell carcinoma, LI of Ki-67 ranged from 36.1% to 87.3% (63.1%±15.7%). Five patients with moderately differentiated squamous cell carcinoma showed LI of Ki-67 ranged from 70.5% to 90.0% (78.1%±7.3%). Positive rate of Ki-67 in a patient with poorly differentiated squamous cell carcinoma was 81.0%. No significant difference in the LI was noted between well differentiated squamous cell carcinoma and moderately differentiated squamous cell carcinoma.

LI of Topo IIα, p53, and p63 in all patients were from 20% to 86.2% with the average of 58.9%, from 58.6% to 89.8% with the average of 72%, and from 16.2% to 93.5% with the average of 65.2%, respectively (Table 3). Comparison of the results of immunohistochemical staining revealed that no significant difference in the expression of Topo IIα, p53, and p63 was noted between well differentiated squamous cell carcinoma and moderately differentiated squamous cell carcinoma.

The correlation between SUV and LI of Ki-67 was statistically analyzed. There is a significant correlation with the correlation coefficient (r) of 0.616 and p value of 0.01 (Fig. 3A). SUV was also significantly

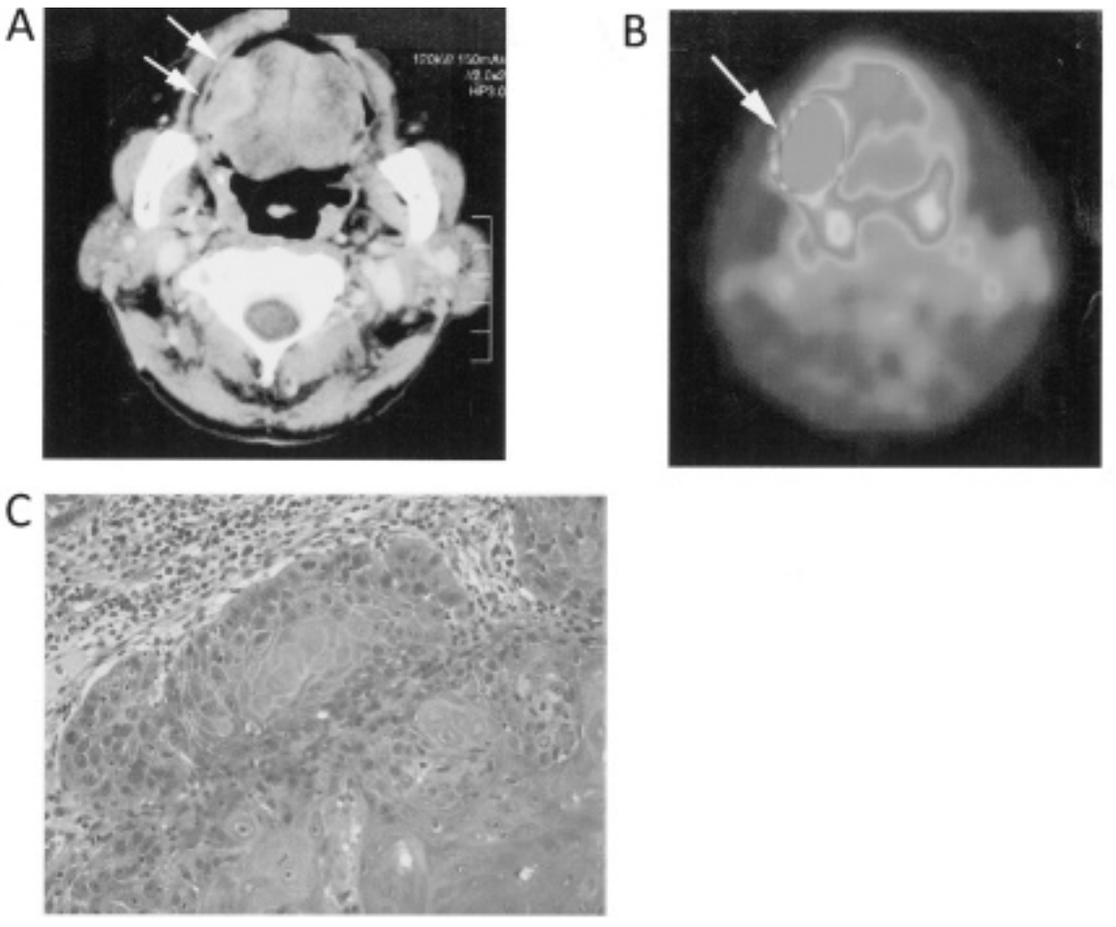

Fig. 1 A representative case (Patient no. 1) with oral squamous cell carcinoma
(A) CT shows an enhanced mass in the tongue on the right side (arrow).
(B) An axial section of FDG PET image shows an increased accumulation of FDG with maximum SUV value of 22.1 (arrow).
(C) Hematoxylin and eosin staining of the specimen obtained by the surgical removal of the tumor shows histological diagnosis of squamous cell carcinoma.

Table 3 Results of ^{18}F-FDG PET (maximal SUV) and Immunohistochemical Staining (% positivity of stained cells)

No. of Patients	^{18}F-FDG maxSUV	Ki-67 (%)	Topo-IIα (%)	p53 (%)	p63 (%)
1	22.1	90.0	72.5	89.8	85.3
2	18.5	77.8	80.4	80.0	83.0
3	14.1	54.2	52.7	83.0	97.7
4	11.8	68.6	75.2	64.5	74.6
5	10.7	48.2	86.2	91.7	75.1
6	10.4	74.2	76.2	88.3	93.5
7	7.4	60.0	66.3	80.0	46.2
8	4.7	68.2	52.3	65.0	50.9
9	3.8	36.1	32.0	61.3	16.2
10	3.6	60.0	20.0	58.6	54.0
11	21.2	90.0	78.5	86.6	79.8
12	9.0	75.7	68.2	60.6	78.9
13	7.8	75.0	41.9	74.7	59.2
14	6.9	70.5	40.8	81.5	51.3
15	6.1	60.0	54.5	68.0	57.4
16	8.9	81.0	44.8	85.7	52.2

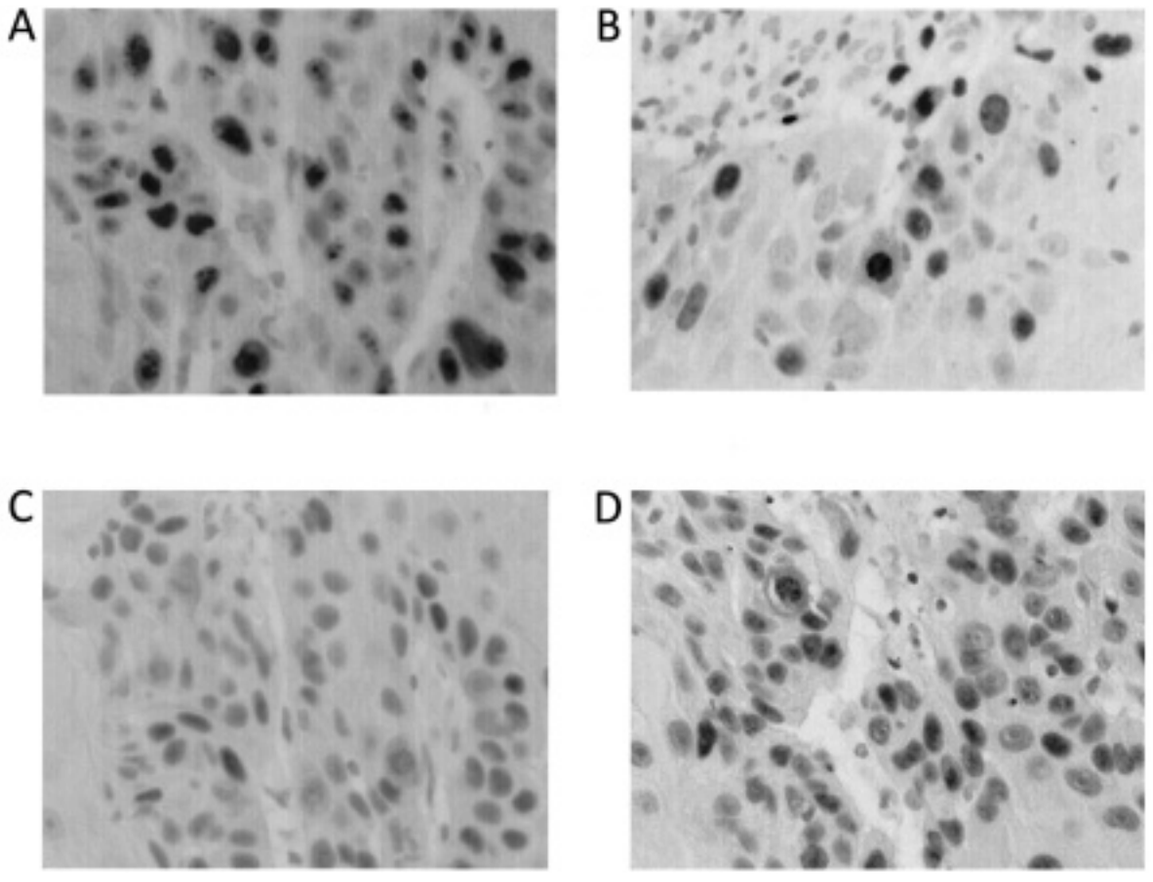

Fig. 2 Results of the immunohistochemical staining with Ki-67 (A), Topo IIα (B), p53 (C), and p63 (D) in a representative case (Patient no. 1) with oral squamous cell carcinoma.

correlated with Topo IIα ($r=0.677$, $p=0.004$), p53 ($r=0.613$, $p=0.01$), and p63 ($r=0.710$, $p=0.002$) (Fig. 3B, C, D).

Clinical follow-up disclosed that 5 patients (No. 2, 11, 13, 14, and 16) died of OSC and 2 patients (No. 6 and 8) recurred within 24 months after operation. Although SUV of died patients (12.7 ± 6.7) was higher than that of alive patients (9.4 ± 5.4), the difference was not statistically significant.

Discussion

The results of the present study revealed that the uptake of FDG was correlated with the proliferative activity as determined by the expression of Ki-67, Topo IIα , and also with p53 and p63 as pathological prognostic markers in OSC.

To assess proliferative activity of OSC, we used two different immunohistochemical markers such as Ki-67 and Topo IIα. Proliferative markers can be classified into three main categories: (i) growth fraction markers; (ii) markers of specific phases of the cell cycle; and (iii) cell cycle time markers. Ki-67 antibodies identify an antigen expressed in late G1, S, G2, and M phases of the cell cycle. They are growth fraction markers and now widely used to evaluate proliferative activity by immunohistochemical analysis.[13,14] Topo IIα is a cell cycle related protein and expressed in normal and neoplastic cells in the S, G2, and M phases.[15] Topo IIα is an enzyme that exerts an important role in DNA topology, repair, and replication by breaking and rejoining the DNA double helix. Previous studies have shown that the expression of Topo IIα is closely related with that of Ki-67 in many tumors.[16] In the present study, the expression of Topo II α and Ki-67 was weakly correlated but no statistical significance was observed in OSC ($r=0.348$)

As markers of cellular proliferation relating the cell cycle, tritiated thymidine and bromodeoxyuridine are used for the indices for the S-phase. Duration of the cell cycle can be evaluated by the potential doubling time and argyrophilic nucleolar organizer regions (AgNORs) are known to be a histochemical marker of the cell cycle. AgNORs count has been known as a significant prognostic indicator in OSC.[17] Cellular proliferation is regarded not only as one of the most important biological markers in oncogenesis, but also as a prognostic marker of patients.

In the present study, we analyzed the expression of p53 and p63. The expression of both p53 and p63 does not usually reflect proliferative activity which is

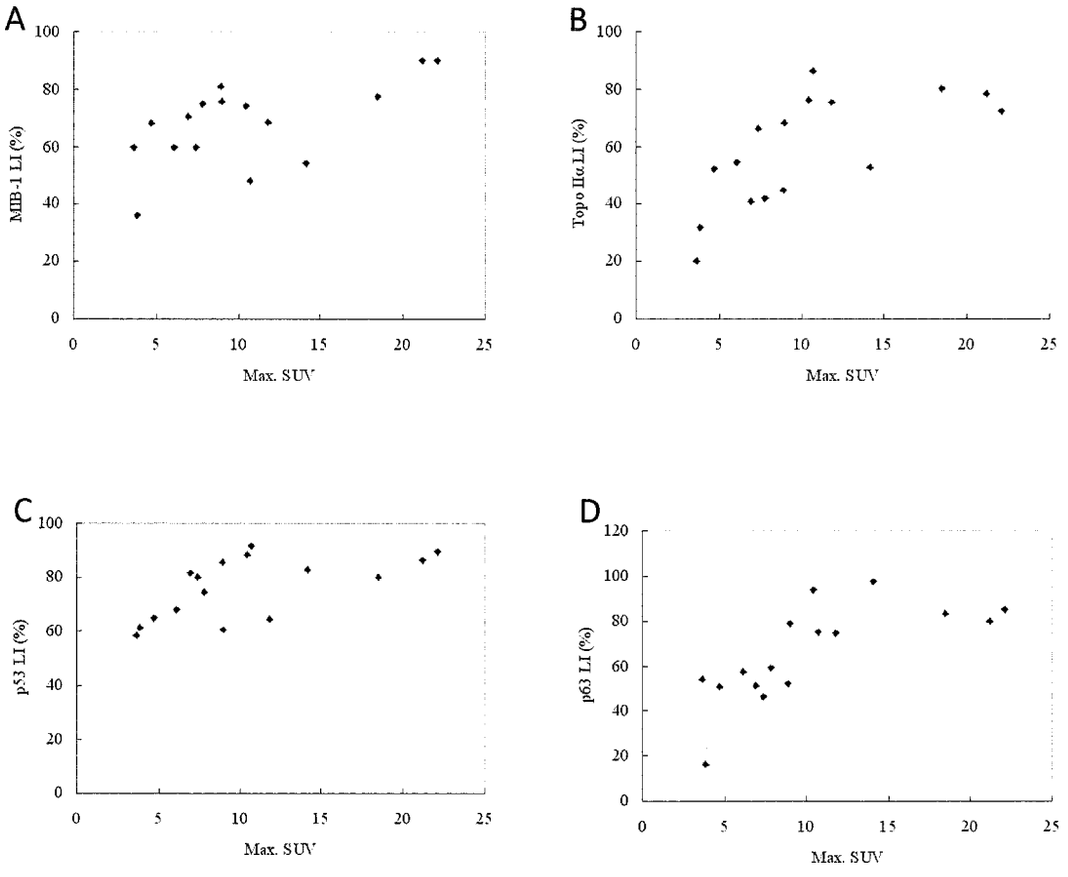

Fig. 3 Correlation between FDG accumulation with markers of cellular proliferation and p53/p63 of oral squamous cell carcinoma as determined by immunhistochemical staining.
(A) A significant correlation is noted between maximum SUV and labeling index of Ki-67 ($r=0.616$, $p=0.01$).
(B) A significant correlation is noted between maximum SUV and labeling index of Topo IIα ($r=0.677$, $p=0.004$).
(C) A significant correlation is noted between maximum SUV and labeling index of p53 ($r=0.613$, $p=0.01$).
(D) A significant correlation is noted between maximum SUV and labeling index of p63 ($r=0.710$, $p=0.002$).

determined by the expression of Ki-67 or Topo IIα. The present study indicated that the expression of p53 and p63 showed weak correlation with the expression of Ki-67, but no statistical significance was observed ($r=0.354$, 0.476, respectively). Both p53 and p63 are multifunctional proteins that control cell cycle, apoptosis, DNA repair, and cellular proliferation and differentiation. In squamous cell carcinoma of the head and neck, strong nuclear expression of p53 and p63 are closely related with proliferative activity of the tumor as well as prognosis of the patient.[18,19] Both p53 and p63 are highly expressed in cancer cells and act in close cooperation with each other to stop cell division. Products of p53 act in G1/S check point of the cell cycle, respectively. The results of the present study revealed that the expression of p53 and p63 was weakly correlated but no statistical significance was observed ($r=0.488$). In approximately 50% of squamous cell carcinomas of the head and neck, function of wild type p53 is abrogated by the mutation. Consequently, nonfunctioning and more stable p53 proteins are generated and accumulated in the nucleus of the tumor cells.[20] On the other hand, p63 expressed in at least six isoforms and supposed to play diverse roles in the differentiation and proliferation of the keratinocyte.[21]

Based on these knowledge and the results obtained in the present study, cellular proliferation as determined by the FDG uptake might reflect tumor progression and may have prognostic value in OSC.

The association of FDG uptake and cellular proliferation has been examined in many types of cancers such as lung cancer and brain tumor, but there are a few reports of the associations in head and neck cancer, especially in OSC.[22] Minn et al. showed a clear correlation between the proliferative activity and the uptake of FDG in thirteen patients with malignant head and neck tumors.[23] In addition, another report

indicated that tumors with higher SUV and higher AgNORs score had significantly higher incidence of residual viable tumor cells after chemoradiotherapy.[17] The study suggests a close association between SUV and proliferative activity in OSC, because previous studies demonstrated significant association between AgNORs score and proliferative activity. A recent study by Linecker et al. disclosed that FDG uptake was significantly correlated with the prognosis of patients with OSC.[24] Histological marker would possibly evaluate noninvasively the clinical course of patients. However, no study has been proved the correlation of FDG uptake with histological markers including Ki-67, Topo IIα, p53, and p63 in patients with OSC.

In this study with small number of patients, neither the uptake of FDG nor the immunohistochemical expression of proliferative and prognostic markers showed correlation with the actual prognosis of patients with OSC.

The limitations of the present study were that the number of patients included in the study was small. Although FDG uptake in the tumor was correlated with the proliferative markers and pathological prognostic markers such as p53 and p63 in the present study, further study with increasing number of patients would be preferable to confirm the results. Another limitation is that neither the uptake of FDG nor the expression of pathological proliferative and prognostic markers showed significant correlation with the actual prognosis of patients. The prognosis of patients should be correlated with the results of FDG PET and pathological markers in a large number of patients in the further studies. Moreover, metastasis of cervical lymph nodes affects the prognosis of patients with OSC. In the present study, lymph node metastasis was noted in four patients (No.10, 12, 13, 16). However, SUV of the primary tumor of patient No. 10 was as low as 3.6, and the other two patients (No.12, 13) with moderately differentiated OSC and a patient (No.16) with poorly differentiated OSC had SUV of 9.0, 7.8, and 8.9, respectively. These values in patients with well and moderately differentiated OSC were not relatively higher than average SUV of each histological type. We should confirm prognostic factors for OSC by the multivariate analysis with FDG uptake in the primary tumor, pathological lymph node metastasis, and expression of immunohistochemical markers.

In conclusion, significant correlation was indicated between FDG accumulation and cellular proliferation determined by the immunohistochemical expression of Ki-67, Topo IIα, and pathological prognostic markers such as p53 and p63 in OSC. FDG PET may be useful for evaluating clinical status and for considering clinical management of patients with OSC.

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