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Correlation between angiogenesis and reduction ratio measured using ^{201}Tl chloride single photon emission computed tomography in patients with oral cavity squamous cell carcinoma

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Objective: The aim of this study is to examine the correlation between tumor angiogenesis and response to preoperative radiotherapy evaluated using ^{201}Tl single photon emission computed tomography (Tl SPECT) in oral cavity squamous cell carcinoma (SCC). ***Methods:*** Tl SPECTs before and after preoperative radiotherapy were obtained from 11 patients diagnosed with SCC in oral cavity. Regions of interest were set around the tumor and scalp respectively, and the ratio of mean counts in the tumor to those in the scalp was calculated (T/N). Immunohistochemical staining for investigating microvessel density of pre-treatment biopsy specimen was performed using CD31 monoclonal antibody. We compared microvessel density with semi-quantitative parameters obtained using Tl SPECT (T/N at pre- an post-treatment, reduction ratio) and prognosis. ***Results:*** The subgroup with higher microvessel density showed a significantly higher reduction ratio than the one with lower microvessel density. Regarding prognosis, the subgroup with locoregional recurrent disease exhibited a significantly higher microvessel density than the one without recurrence. ***Conclusions:*** In SCC of the oral cavity, there was a significant correlation between microvessel density and response to preoperative radiotherapy. Namely, it was revealed that change of ^{201}Tl uptake after preoperative radiotherapy correlated with tumor angiogenesis of oral cavity SCC.

Key words: microvessel density, preoperative radiotherapy, oral cavity squamous cell carcinoma, ^{201}Tl chloride single photon emission tomography, CD31

INTRODUCTION

IN SQUAMOUS CELL CARCINOMA (SCC), some authors have reported that tumor angiogenesis is a prognostic factor in predicting the effect of treatment.[1,2] There are some studies using microvessel density (MVD) in evaluating angiogenesis and showing the usefulness of MVD in predicting radiosensitivity in esophageal cancer,[3] cervix carcinoma,[4] and head and neck carcinoma.[5–8] Furthermore, some authors used CD31 antibody and reported its usefulness in staining vascular endothelial cells.[3,5,7,9] On the other hand, in evaluating the effectiveness of radiotherapy, some authors have reported the usefulness of ^{201}Tl chloride single photon emission computed tomography (Tl SPECT) in brain tumor,[10–12] lung cancer,[13–16] bone and soft tissue sarcoma[17] and head and neck cancer,[18–22] because ^{201}Tl chloride (Tl) has a tendency to accumulate in lesions with tumor activity.[23] Nakahara et al.[24] examined the percent decrease in the quantitative value using Tl SPECT after preoperative chemoradiotherapy (%Dtl) for advanced esophageal SCC. They reported that in evaluation using %Dtl, there was a

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statistically significant difference among the subgroups with different histopathological grades. In our previous study, we also used a semi-quantitative parameter, percent decrease (reduction ratio: RR) calculated using Tl SPECT in evaluating preoperative radiotherapy for head and neck SCC, and obtained a significant difference in RR between the group responsive to radiotherapy and the non-responsive group.[25] Uptake of Tl on early SPECT (immediately after administration or 10 to 15 min postinjection) had relevance to blood supply.[26–29] If uptake of Tl is affected by blood supply, it is possible to predict the effectiveness of preoperative radiotherapy using Tl SPECT at pre-treatment. However, no study has investigated the relationship between MVD and semi-quantitative assessment using Tl SPECT. We immunochemically stained pre-radiotherapy biopsy specimen of SCC in oral cavity with anti-CD31 factor and compared MVD with RR. The aim of this study is to examine the correlation between tumor angiogenesis and RR in the assessment of the effectiveness of preoperative radiotherapy for patients with oral cavity SCC.

MATERIALS AND METHODS

Materials

Table 1 shows clinical characteristics of all patients. Eleven patients attending our institution from October, 1995 to July, 2000, who were diagnosed with SCC in oral cavity by pre-treatment biopsy, were enrolled in this study. Clinical tumor-node-metastasis classification of patients was based on the Union International Contre le Cancer tumor-node-metastasis clinical classification. They consisted of 9 males and 2 females, aged from 53 to 79 years (mean 66 years). Sites of tumor were mandible in 5 cases, floor of the mouth (FOM) in 4, retromolar trigone in 1, and buccal mucosa in 1. Averages of length and width of tumor were from 13 to 79 mm (mean 39 mm). After radiotherapy, all patients were performed operation comprising excision of primary tumor, mandibular resection, and neck dissection. This study followed the ethical standards of the committee on human experimentation of our institution, and all patients provided informed consent.

Radiotherapy

Preoperative external radiotherapy for the primary lesion and lymph node metastasis was performed in all 11 patients with a radiation dose from 32.0 to 51.2 Gy (mean 42.1 Gy). Of 11 patients, 10 were treated with accelerated hyperfractionated radiotherapy (AHF), and 4 were concurrent chemotherapy with carboplatin (CBDCA) (mean 332 mg).

Imaging

With respect to pre-treatment Tl SPECT, 10 of 11 patients were investigated from 1 to 30 days before the initiation of treatment (mean 9 days), and the remainder a day after the initiation. Post-treatment Tl SPECTs were performed from 18 to 51 days after the completion of radiotherapy (mean 30 days). SPECTs were taken at 15 min postinjection of Tl using a triple head rotating gamma camera (GCA9300, Toshiba Medical System Co., Tokyo, Japan) in 9 patients and dual head gamma camera (GCA7200, Toshiba Medical System Co., Tokyo, Japan) in 2, equipped with a low-energy parallel-hole collimator. The photopeak was set for 71 keV and a 40% symmetric window was used. Acquisition time was 30 min, and image data were aquired in a 128 × 128 matrix for GCA9300 and in a 64 × 64 for GCA7200, respectively. The reconstruction images were obtained with a Ramp filter following the ordered subsets expectation maximization (OSEM) algorithm using a Butterworth filter. Scatter and attenuation correlation was not performed. For semi-quantitative study, we set the region of interest (ROI) around the tumor. ROI of normal lesion was set in half scalp at the height of the cerebellum on the contralateral side, which was not included in the area of irradiation. We calculated the ratio of mean counts in ROI of the tumor (T) to those

Table 1 Clinical characteristics of patients

	Age (yrs)/Sex	TNM classification	Site of tumor	Size of tumor (mm)	Mandibular resection	Site of neck dissection
Case 1	79/M	T4N2bM0	Mandible	28 × 21	Segmental	Ipsilateral
Case 2	65/M	T4N2cMx	FOM	55 × 50	Marginal	Bilateral
Case 3	63/M	T4N2bM0	Mandible	101 × 56	Hemisection	Bilateral
Case 4	65/F	T4N2Mx	Mandible	58 × 22	Hemisection	Ipsilateral
Case 5	58/M	T4N2bMx	RMT	69 × 41	Hemisection	Ipsilateral
Case 6	76/F	T2N0Mx	Buccal mucosa	14 × 12	Marginal	Ipsilateral
Case 7	73/M	T4N1M0	Mandible	50 × 37	Segmental	Bilateral
Case 8	53/M	T4N0M0	FOM	49 × 28	Marginal	Bilateral
Case 9	67/M	T2N0M0	FOM	28 × 24	Marginal	Ipsilateral
Case 10	70/M	T3N0Mx	FOM	37 × 23	Marginal	Ipsilateral
Case 11	62/M	T2N0M0	Mandible	22 × 20	Marginal	Ipsilateral

FOM: floor of the mouth, RMT: retromolar trigone

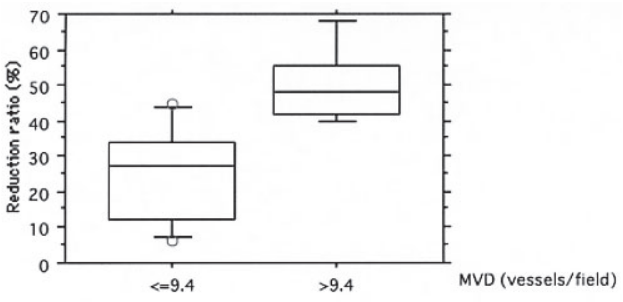

Fig. 1 Correlation between reduction ratio (RR) and microvessel density (MVD). RRs of subgroups with higher and lower MVD were 49.920 ± 11.119 and 25.250 ± 14.110%.

of the scalp (N) (T/N) and reduction ratio (RR) as follows: (pre T/N – post T/N)/(pre T/N) × 100%, where pre- and post-T/S stand for T/N of pre- and post-treatment Tl SPECT.

Immunochemical staining

In all patients, biopsy specimens before treatment were investigated using immunochemical staining. Four μm-thick sections of biopsy specimen were deparaffinized in xylene and dehydrated through graded concentrations of alcohol. Then, sections were heated to 95°C by microwave irradiation for 20 min in 10 MN citrate buffer solution. Endogenous peroxidase activity was blocked by treatment with 1% H_2O_2 in methanol for 20 min. Sections were subsequently washed in phosphate-buffered saline (PBS) and treated with blocking serum for 10 min. CD-31 staining was performed using biotin-avidin immunoperoxidase technique. Specimens were incubated for 60 min with the CD-31 mouse monoclonal antibody (NOVO, UK) at a dilution of 1:100. Then, they were incubated with biotin-labeled antimouse secondary antibodies (HISTOFINE, Nichirei, Japan) for 10 min at room temperature. Next, specimens were treated with avidin biotin complex reagent (HISTOFINE, Nichirei, Japan) for 10 min. The reaction products were visualized by immersing slides in 0.03 mol 3,3′-diaminobenzidine in 50 MN Tris buffer containing 0.6% hydrogen peroxide for 10 min. The slides were counterstained with Meyer's hematoxylin. Microvessels in the tumors were highlighted, and microvessel density was quantified. The stained slides were examined using Nikon ECLIPSE E800 (Nikon, Tokyo, Japan) at low-power magnification (×40 or 100 total magnification) to identify the areas of highest neovascularization of the tumor. A ×400 field (×40 objective and ×10 ocular), 0.196 mm^2, in each of these three areas was counted, and the average counts were recorded. A brown-staining endothelial cell clearly separated from adjacent microvessels, tumor cells and other connective-tissue elements was considered a single, evaluable microvessel. Counting of microvessels was performed by one pathologist (T.T.) without any knowledge of possible prognostic factors or other clinical data.

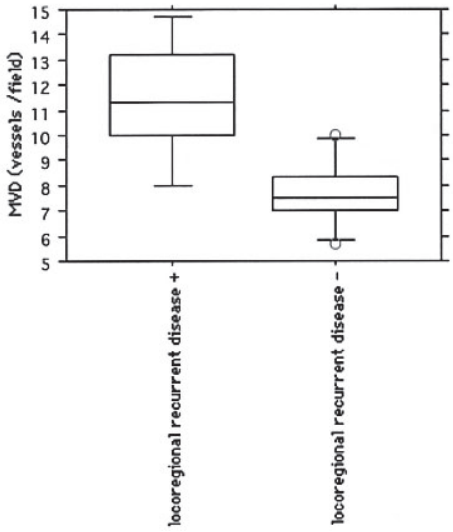

Fig. 2 Correlation between MVD and locoregional recurrent disease. MVDs of subgroup with and without recurrence were 11.480 ± 2.480 and 7.667 ± 1.435 vessels/field.

Follow-up

Follow-up data from 4 to 56 months after the first visit (mean 34 months) (from June, 1996 to June, 2003) were obtained. Clinical parameters were defined on the basis of the report of Spector et al.[30] They defined metastases as clinically established tumor deposits outside the primary organ site and confirmed histopathologically, and defined locoregional recurrent disease as tumor recurrence in the treated field within 2 years of the completion of treatment. Delayed regional metastases were defined as tumor metastases diagnosed clinically, histopathologically, or cytologically to the ipsilateral or the contralateral regional neck nodes 2 years or more after the completion of primary therapy. Distant metastases are defined as tumor spread to other organ systems and secondary primary malignancy is defined as malignant tumors in other organ systems that are not directly related to the laryngeal or hypopharyngeal cancers.

In 4 of 11 patients, disease control could be achieved. In contrast, 7 patients could not control diseases. Three of the 7 patients showed metastasis consisting of one had only metastasis, another metastasis and locoregional recurrent disease, and the other metastasis and locoregional recurrent disease, and distant metastasis. Another 3 of the 7 cases had locoregional recurrent disease, 2 showed only this finding, and 1 showed delayed regional metastasis. The remaining one patient exhibited only distant metastasis.

On the other hand, 5 of all 11 patients died and 2 are alive without disease. Three of the 11 cases moved to and attended other hospital, involving one verified alive without disease. The remaining one of 11 cases lives with distant metastasis and delayed regional metastasis.

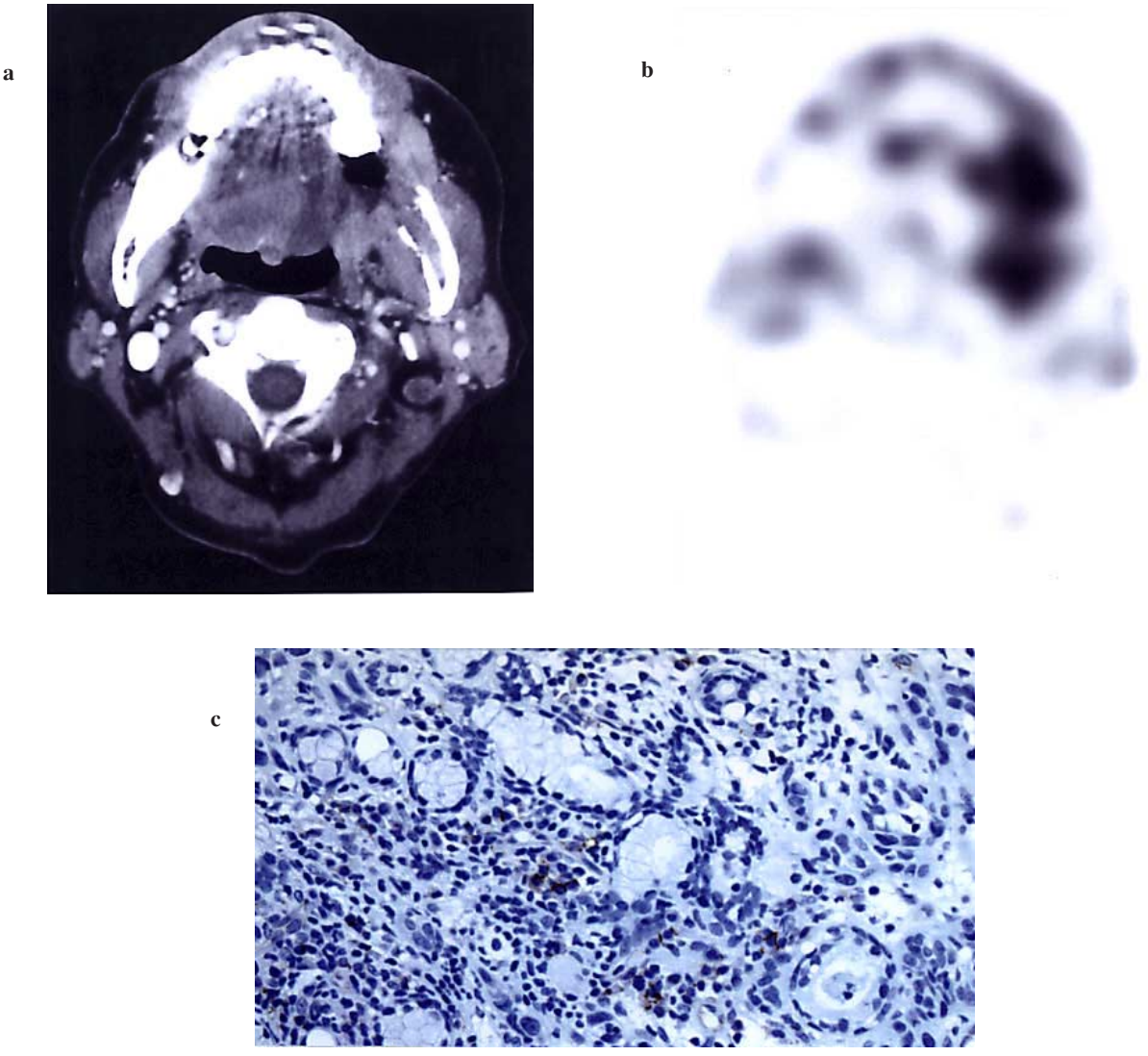

Fig. 3 a: Contrast-enhanced CT (CE-CT) showed tumor in the left side of the retromolar trigone. b: Pre-treatment thallium-201 single photon emission computed tomography (Tl SPECT) exhibited abnormal uptake by tumor. c: One of 3 hottest spots of the tumor showed extensive brown-stained area by CD31 monoclonal antibody (×40 objective). d: Post-treatment Tl SPECT revealed considerably diminished accumulation in tumor.

Statistics

The data analysis was done with Stat view software package (Version 5.0; Abacus Concepts, Inc., Berkeley, CA). Student's unpaired t-test was applied to test for independence between MVD, semi-quantitative values, and prognosis. When the p value was less than 0.05, the difference was defined as significant. For purpose of comparing tumor size with MVD and pre T/N, regression analysis was performed.

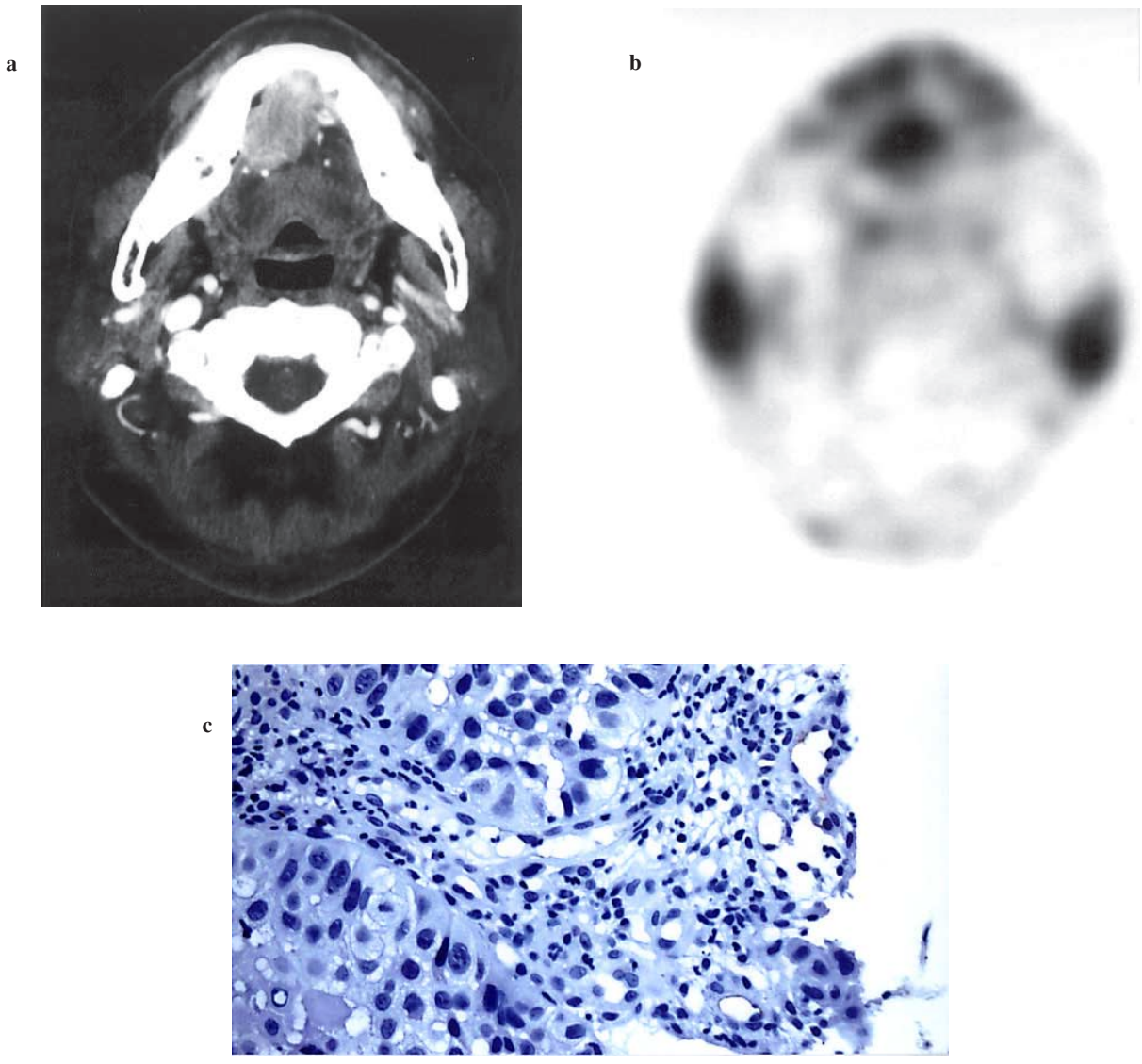

Fig. 4 a: CE-CT showed tumor in the floor of the mouth. b: Pre-treatment Tl SPECT exhibited abnormal uptake by tumor. c: One of 3 hottest spots of the tumor showed a few brown-stained areas by CD31 monoclonal antibody (×40 objective). d: Post-treatment Tl SPECT revealed slightly diminished uptake by tumor.

RESULTS

Size of tumor had a neither marked correlation with MVD (r = 0.263) nor with pre T/N (r = 0.077). The subgroup with higher MVD than average (>9.4 vessels/field) showed higher RR (49.920 ± 11.119) than the subgroup with lower MVD (≦9.4) (25.250 ± 14.110) (p = 0.0114) (Fig. 1). Pre T/N (p = 0.4350) had no significant difference between subgroups with higher (5.205 ± 2.121%) and

lower MVD (6.387 ± 2.604%) (p = 0.4274). Post T/N showed no significant difference between subgroups with higher (4.115 ± 1.925%) and lower MVD (3.241 ± 1.731%) (p = 0.4350), either. Although the subgroup with locoregional recurrent disease showed significantly higher MVD than the one without disease (p = 0.0109) (Fig. 2), there were no significant differences in MVD between the subgroups with or without metastasis (p = 0.7306) or distant metastasis (p = 0.9579). In RR, no significant difference was noted between the subgroups with or without metastasis (p = 0.2473), locoregional recurrent disease (p = 0.3117), or distant metastasis (p = 0.2853). Figure 3a showed pre-treatment contrast-enhanced CT (CE-CT) of a 58-year-old male diagnosed with SCC in the left side of the retromolar trigone (RMT) (Case 5). On his pre-treatment Tl SPECT, pre T/S was 4.180 (Fig. 3b) and MVD of biopsy tissue was 12.7 (Fig. 3c). He undertook preoperative radiotherapy with 41.6 Gy (AHF). Post-treatment Tl SPECT of this patient was obtained (Fig. 3d). Post T/N was 2.034 and RR was 51.3%. He developed recurrent disease in the ipsilateral neck 6 months after the first visit. Figure 4a shows CE-CT of a 70-year-old male with SCC in the floor of the mouth (Case 10). Figure 4b exhibits his pre-treatment Tl SPECT, and pre T/N was calculated as 4.622. MVD was counted as 8.3 (Fig. 4c). His irradiated dose by AHF was 41.6 Gy. In his post-treatment Tl SPECT, post T/N and RR were 3.264 and 29.4%, respectively (Fig. 4d). He was verified to be alive without disease in November, 2001.

DISCUSSION

Regarding radiosensitivity, the contention that hypoxia causes failure against treatment is generalized. The degree of oxygenation depends on the arrangement, blood flow rate, blood oxgen content of microvessels, and the tissue's oxgen consumption rate.[31] In squamous cell carcinoma (SCC), some authors reported that tumor angiogenesis was a prognostic factor in predicting the effect of radiotherapy.[1,2] Some studies have used microvessel density (MVD) in evaluating angiogenesis and showing the usefulness of MVD in predicting radiosensitivity in esophageal cancer,[3] cervix carcinoma,[4] and head and neck carcinoma.[5–9]

In considering the relation between MVD and radiosensitivity, studies performed by Cooper et al.[4] and Brun et al.[9] showed no significant difference. In contrast, Kamijo and colleagues[5] reported that the subgroup with higher MVD exhibited significantly better radiosensitivity in biopsy specimens from laryngeal SCC patients, showing agreement with our result. However, their result with respect to prognosis was different from ours. They reported that the locoregional control rate of patients with higher MVD was significantly better than that of those with lower MVD. It was revealed that tumors with a high MVD had a significantly better neck control rate from a study on head and neck SCC in cervical lymph nodes.[7] Hironaka and colleagues[3] noted that patients of esophageal cancer whose tumors showed high MVD had significantly better survival. Only one study disclosed that the 5-year survival rate for patients with low vascularity tumors was significantly higher than that for those with high vascularity tumors in cervix carcinoma,[4] supporting our study. On the other hand, one report investigating MVD of oral SCC patients disclosed no significant correlation between MVD and overall survival.[9]

Although the number of the patients enrolled in this study was small, we think that tumor angiogenesis and the effectiveness of radiotherapy are independent prognostic factors, based on the study by Cooper et al.[4] Regarding MVD and the response to radiotherapy or prognosis, Brun et al.[9] suggested that possible explanations for the discordant results were differences in technique and in the distribution of tumor subsites. In comparing MVD and locoregional recurrent disease, which was defined as tumor recurrence in the treated field within 2 years of completion of treatment, the subgroup with locoregional recurrent disease obtained significantly higher MVD, while the subgroup with higher MVD showed a significantly better response. As a possible explanation for this result, the response to radiotherapy is better in well oxygenated status but in treated areas with better angiogenesis better nutrition is likely to occur and promote regrowth of any remaining micrometastasis.

Tl has a tendency to accumulate in lesions with tumor activity. According to a prior *in vivo* study,[32] it was realized that sodium potassium pump activity was more important than blood flow in the mechanism of Tl uptake by tumor. Furthermore, Takekawa et al.[33] showed that uptake of Tl on the delayed scan (at 120 min postinjection) might be regulated by Na-K ATPase in adenocarcinoma of the lung. In another report,[34] it was revealed that there was a significant correlation between the Tl index on delayed image and the cell proliferation ratio in patients with small cell lung carcinoma. In this way, many investigators have assessed the effectiveness for determination of the malignant viability of tumors of Tl SPECT.[35–37]

With respect to early image, in brain tumors, some authors have compared the uptake of Tl with other radiopharmaceuticals, CE-CT, CE-MRI.[26–29] Nakagawara et al.[26] indicated that Tl uptake tended to correlate with early index of HSA-D SPECT, which was affected by tumor vascularity. In a report of Ohnishi et al.,[27] Tl uptake on early image correlated with the intensity of the lesion on CE-CT, and enhancement on CT was attributed to destruction of the blood brain barrier (BBB) and vascularity. Taki and colleagues[28] reported that the Tl uptake of early SPECT coincided with the intensity of the lesion on CE-MRI. Ueda et al.[29] compared Tl uptake on early image with CT, MRI, cerebral angiography and histopathological investigation. They revealed that early uptake of thallium in tumors was related to tumor vascu-

larity and the destruction of BBB. Two more reports indicated that Tl uptake was related to blood supply affected by tumor vascularity and the disruption of BBB.[38,39]

In evaluating Tl uptake, from the histopathological point of view, Yoshii et al. suggested that increased necrosis and vascularization in non-glial brain tumor specimens were closely related to high uptake using preoperative Tl SPECT.[39] But, in our study using early Tl SPECT, uptake of Tl at neither pre- nor post-treatment showed a significant correlation with tumor vascularity. Shintani et al.[1] reported that radiation therapy decreased MVD in oral SCC specimens after preoperative radiotherapy compared to before treatment, but found no relation between the change of MVD and the pathological response, that is the anti-tumoral effect of radiotherapy was not equivalent to the anti-vascular effect of irradiation. Our investigation revealed that in comparison with pre T/N, radiotherapy decreased post T/N and changes between them (RR) had a significant association with MVD in pre-treatment specimens. Our study indicated that RR obtained using early Tl SPECT could reflect changes in tumor angiogenesis.

In this study, there were some limitations in evaluating MVD. First, the number of patients enrolled in our investigation was small. Tumor progression cannot occur without angiogenesis. Therefore, it was generally predicted that larger tumor had higher MVD, but there was no significant correlation between tumor size and MVD. Referring to the reports of Gleich et al.,[40,41] MVD in the T1 oral cavity cancers was higher than the T2–T4 tumors. However, they obtained a significant correlation between tumor size and MVD counted using factor VIII-related antigen (FVIIIAg) in T2–T4 oral SCC. Furthermore, they proposed that since the oral cavity is extremely vascular by nature, early oral tumor development might be independent of angiogenesis. We examined 11 T2–T4 oral cavity SCC and found that tumor size did not affect MVD, which may be due to the use of CD-31 staining.

Second, in a report using surgical specimens in which MVD was counted by immunohistochemical staining, the authors detected the highest density of microvessels in the periphery of tumor.[42] We used pre-treatment biopsy specimens, and there is a possibility that we did not examine the specimens showing the highest microvessel count. Third, the method of counting microvessels used in this study did not sufficiently consider the geometry of MVD. There is a report actually describing irregular microvessel networks in cancer.[39] Secomb et al. suggested that a heterogeneous network structure leads to dispersion in hemodynamic variables.[43] Heterogeneous structure of tumor microcirculation can exert a substantial effect on the occurrence of hypoxic micro-regions. A new method of analyzing microvessel density advocated by Kamijo et al. takes vessel shape and size into consideration. They indicated that tumors with high TP:TA (the ratio of the total microvessel perimeter to tumor area) had significantly greater radiosensitivity than those with low TP:TA.[44] In this study, we counted MVD only in the three hottest areas.

CONCLUSION

There was a significant difference between tumor angiogenesis and RR obtained using Tl SPECT in evaluating the effectiveness of preoperative radiotherapy for patients with oral SCC.

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