Preoperative radiotherapy contributes to induction of proliferative activity of CD8+ tumor-infiltrating T-cells in oral squamous cell carcinoma

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Abstract. In order to evaluate host immune response to cancer, many methods have been applied. However, in the field of oral squamous cell carcinoma, evaluation of host immune response on the basis of proliferative activity of tumor-infiltrating T-cells (TIL) has not been reported. Therefore, we applied double immunohistochemical staining of proliferation markers Ki-67 and CD8 to surgically resected and paraffin-embedded tissue sections for 35 cases of oral squamous cell carcinoma. With this method, there was a significant correlation between the percentage of Ki-67+CD8+TIL and the intra-tumor epithelium infiltration rate of CD8+TIL (P=0.0237). In the process of analysis, we found that the proliferative activity of CD8+TIL tended to correlate (P=0.0859) with clinical N factor (lymph node metastasis), which was previously reported to suppress host immune response. We therefore assumed there was another factor inducing host immune response. The proliferative activity of CD8+TIL was well correlated with preoperative radiotherapy (P=0.0200) while there was no significant correlation between the proliferative activity of CD8+TIL and other clinical factors; age, tumor size, clinical stage, pathological N factor (P=0.5410, 0.7769, 0.1041, and 0.1072, respectively). Our present results strongly imply that preoperative radiotherapy is a very important factor in oral squamous cell carcinoma inducing host immune response regardless of the clinical factors present.

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Introduction

CD8+ T-cells recognize tumor cells through tumor associated antigen (TAA) presented on the surface of major histocompatibility complex (MHC) class I molecules on the tumor cells via T-cell receptor (TCR) on CD8+ T-cells (1). However, the naïve CD8+ T-cell cannot be adequately activated by the tumor cell itself because activation of the naïve T-cell requires a co-stimulatory signal that is typically mediated by the interaction of a B7 family molecule expressed on the surface of the immune cell and CD28 on the T-cell (1). We had previously reported the importance of the introduction of B7 molecules into tumor cells in order to make the tumor cells play the role of antigen presenting cells (APC) (2,3). On the other hand, the already-activated effector CD8+ T-cells require TAA and MHC class I complex on the tumor surface but do not require a co-stimulatory signal in order to recognize tumor cells for tumor killing (4). In general, the effector CD8+ T-cell itself does not proliferate well at the target organ (5) because T-cell proliferation is induced through the recognition of both first and second signals (6) with the help of CD4+ T-cells in the lymph node (7,8).

A recent article revealed that tumor-infiltrating T-cells (TIL) show weak proliferative activity in hepatocellular carcinoma (9). In addition, in renal cell carcinoma (10), and colorectal carcinoma (11) patients, proliferative activity of CD8+TIL is well related with patient prognosis. For head and neck squamous cell carcinoma (HNSCC), no study has observed proliferative activity of TIL *in situ* to evaluate immune response to tumor cells.

Therefore, in the present study we evaluated CD8+TIL proliferative activity by Ki-67 and CD8 double staining on formalin-fixed paraffin-embedded tumor tissue samples.

Materials and methods

Oral squamous cell carcinoma samples. Surgically removed human oral tumors were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Sections (4 μm-thick) were prepared and stained with hematoxyline and eosin (H&E) for pathological diagnosis.

Table I. Clinical data for the 35 oral squamous cell carcinoma cases.

Age (year, mean ± SD)	68.1±11.4
Sex	
Female	16
Male	19
Tumor size	
T1	5 (14.3%)
T2	10 (28.6%)
T3	4 (11.4%)
T4	16 (45.7%)
Cervical nodal involvement	
N0	21 (60.0%)
N1	6 (17.1%)
N2	8 (22.9%)
N3	-
Clinical stage	
I	5 (14.3%)
II	5 (14.3%)
III	7 (20.0%)
IV	18 (51.4%)
Site	
Lips	1
Buccal mucosa	3
Floor of the mouth	7
Tongue	13
Gingiva	14
Differentiation grade	
Well differentiated	28 (80.0%)
Moderately differentiated	7 (20.0%)
Poorly differentiated	-
Preoperative chemotherapy	
Yes	10 (28.6%)
No	25 (71.4%)
Preoperative radiotherapy	
Yes	17 (48.6%)
No	18 (51.4%)

After the pathological diagnosis, paraffin blocks were utilized in accordance with the guidelines of the Ethics Committee of Gifu University School of Medicine. A total of 35 cases of squamous cell carcinoma including 28 cases of well differentiated squamous cell carcinoma and 7 cases of moderately differentiated squamous cell carcinoma were analyzed as described below.

CD8 and Ki-67 double staining on the paraffin-embedded tissue sections. Sections (4 μm) were prepared and specimens were de-waxed and re-hydrated. Each specimen was autoclaved in 10 mM of citrate buffer, pH 7.0 at 121˚C for 10 min followed by washing with phosphate-buffered saline (PBS). The specimen was stained with 10 μg/ml of mouse anti-human CD8a (clone C8/144B, mouse IgG1, κ, Dako Cytomation Japan, Kyoto, Japan) for 1 h at room temperature (R/T) in a humidified chamber. After washing with PBS for 10 min x3, the specimen was stained with Histofine Simple Stain AP(M)® alkaline phosphatase-conjugated goat anti-mouse IgG (Nichirei biosciences, Tokyo, Japan) for 1 h at R/T. After washing with PBS for 10 min x3, the reaction was visualized with New Fuchsin® substrate (Dako Cytomation Japan) for 15 min. After fixing with 4% paraformaldehyde/PBS for 10 min, the specimen was washed with PBS for 10 min x3, and then autoclaved in 10 mM of citrate buffer, pH 7.0 at 121˚C for 10 min to degenerate reacted antibodies on the specimen followed by washing with phosphate-buffered saline (PBS). The specimen was treated with 0.3% H_2O_2, 0.1% sodium azide in PBS for 20 min followed by washing 3 times with PBS for 10 min each. Then the specimen was treated with 10 μg/ml of mouse anti-human Ki-67 antigen (clone MIB-1, mouse IgG1, κ, Dako Cytomation Japan) for 1 h at R/T. After washing with PBS for 10 min x3, the specimen was stained with Histofine Simple Stain MAX-PO(M) horseradish peroxidase-conjugated goat anti-mouse IgG (Nichirei Biosciences, Tokyo, Japan) for 1 h at R/T. After washing 3 times with PBS, the reaction was visualized with 0.035% H_2O_2 and 0.03% 3,3'-diaminobenzidine (Wako) in 50 mM Tris-HCl (pH 7.6) for 10 min. After 4% paraformaldehyde fixation for 10 min, the specimen was counterstained with hematoxylin and submitted for observation.

Analysis of stained sections. We observed each specimen at x400 magnification attached to a 10 mm square (1 mm divided) micrometer (Olympus, Tokyo, Japan). That is, we observed an actual area of 0.25 mm x 0.25 mm in each observation. We counted the numbers of 1) tumor-epithelium-infiltrated $CD8^+$ cells, 2) tumor-epithelium-infiltrated $CD8^+Ki\text{-}67^+$ cells, 3) tumor stroma-infiltrated $CD8^+$ cells, and 4) tumor stroma-infiltrated $CD8^+Ki\text{-}67^+$ cells separately. Only the tumor stroma within 0.25 mm from the tumor epithelium was observed because we could not tell how far the stroma was from the tumor epithelium if there was no tumor epithelium within the micrometer.

Statistical analysis. For statistical analysis, we used Mann-Whitney's U test for single parameter comparison. To analyze correlation of two parameters, we utilized regression analysis. Probability values <0.05 were considered significant.

Results

Proliferative activity of $CD8^+$ tumor-infiltrating T-cells was correlated with intra-tumor epithelial infiltration of $CD8^+$ T-cells. The clinicopathological data are summarized in Table I. The number of either Ki-67 and CD8 double positive cells or CD8 positive cells in tumor epithelium and tumor stroma is shown in Table II. Fig. 1 shows a representative result of the staining (case nos. 21 and 27). Because all of the specimens we stained showed a very good signal noise ratio we could easily distinguish intra-nuclear Ki-67 staining from

Table II. Summary of immunohistochemical staining for 35 patients.

No.	Age	Sex	Primary site	T	N	M	Differentiation	Location and Ki67+ % of CD8 T-cells: Epithelial Ki67+CD8+/ CD8+		Stromal Ki67+CD8+/ CD8+	
1	55	M	Tongue	1	0	0	mod	7/120	(5.83%)	8/300	(2.67%)
2	65	F	Tongue	2	1	0	well	37/192	(19.27%)	91/201	(45.27%)
3	64	M	Floor	4	2	0	mod	4/125	(3.20%)	30/665	(4.51%)
4	82	F	Gingiva	2	2	0	well	11/70	(15.71%)	28/338	(8.28%)
5	70	M	Buccal mucosa	4	2	0	well	0/62	(0.00%)	17/321	(5.30%)
6	51	F	Tongue - Floor	4	2	0	well	7/38	(18.42%)	16/101	(15.84%)
7	79	M	Tongue	2	0	0	well	15/235	(6.38%)	36/309	(11.65%)
8	64	F	Gingiva	4	2	0	well	5/100	(5.00%)	13/305	(4.26%)
9	94	F	Buccal mucosa	4	0	0	well	4/105	(3.81%)	29/301	(9.63%)
10	78	M	Tongue	1	0	0	well	8/131	(6.11%)	13/346	(3.76%)
11	75	M	Gingiva	4	2	0	well	4/105	(21.05%)	39/301	(12.96%)
12	54	M	Tongue	4	0	0	mod	4/19	(7.55%)	16/336	(4.76%)
13	70	M	Tongue	2	0	0	well	7/117	(5.98%)	17/313	(5.43%)
14	78	M	Gingiva	1	0	0	well	4/153	(2.61%)	5/325	(1.54%)
15	63	M	Gingiva	4	0	0	mod	10/127	(7.87%)	14/303	(4.62%)
16	45	F	Tongue	2	0	0	well	21/100	(21.00%)	35/448	(7.81%)
17	73	F	Tongue	2	0	0	well	11/104	(10.58%)	20/368	(5.43%)
18	46	F	Gingiva	4	0	0	mod	5/38	(13.16%)	45/241	(18.67%)
19	79	F	Gingiva	4	1	0	well	8/66	(12.12%)	2/310	(0.65%)
20	70	M	Gingiva - Floor	3	0	0	well	5/106	(4.72%)	12/384	(3.13%)
21	73	F	Lip	1	0	0	well	4/73	(5.48%)	21/356	(5.90%)
22	74	M	Floor	4	0	0	well	8/81	(9.88%)	29/321	(9.03%)
23	81	F	Tongue	1	0	0	well	2/50	(4.00%)	15/326	(4.60%)
24	66	M	Gingiva	2	1	0	well	4/137	(2.92%)	37/174	(21.26%)
25	59	M	Gingiva - Floor	3	1	0	well	64/139	(46.04%)	59/302	(19.54%)
26	53	F	Gingiva	4	2	0	well	0/1	(0.00%)	10/96	(10.42%)
27	61	M	Floor	4	0	0	well	42/112	(37.50%)	69/319	(21.63%)
28	50	M	Tongue	2	1	0	well	5/63	(7.94%)	22/236	(9.32%)
29	70	M	Gingiva	4	1	0	mod	23/58	(39.66%)	11/104	(10.58%)
30	62	M	Buccal mucosa	4	0	0	well	0/127	(0.00%)	8/302	(2.65%)
31	48	M	Tongue	2	0	0	well	18/100	(18.00%)	7/403	(1.74%)
32	69	F	Floor	2	2	0	mod	5/66	(7.58%)	20/294	(6.80%)
33	85	F	Gingiva	4	0	0	well	63/120	(52.50%)	40/227	(17.62%)
34	73	F	Gingiva	3	0	0	well	2/101	(1.98%)	4/300	(1.33%)
35	70	M	Tongue	3	0	0	well	0/19	(0.00%)	25/350	(7.14%)

F, female; M, male; Floor, floor of the mouth; well, well differentiated squamous cell carcinoma; mod, moderately differentiated squamous cell carcinoma.

membrane CD8 staining. In the process of the analysis, we found several cases that had many Ki-67 positive CD8+TIL infiltrations in accordance with multiple CD8 T-cell infiltration into tumor epithelium (Fig. 1B). Thus, we analyzed whether there was any correlation between intra-epithelial infiltration of CD8+TIL and proliferative activity of CD8+TIL. As shown in Fig. 2, there was a significant correlation between the intra-epithelial/tumor stroma CD8+TIL ratio and the percentage of Ki-67+CD8+TIL in the tumor tissue (P=0.0237).

Ki-67 positive CD8+ T-cell percentage was correlated with preoperative radiotherapy. We analyzed the relationships between the percentage of Ki-67+CD8+TIL and clinicopathological parameters. As shown in Fig. 3, there was no statistical significance in age, tumor size, and clinical stage (P=0.5410, P=0.7769, P=0.1041, respectively), while there was a tendency towards correlation with clinical N factor (P=0.0859). In terms of evaluation of the preoperative therapy, there was a significant correlation between the preoperative therapy and percentage of Ki-67+CD8+TIL. As

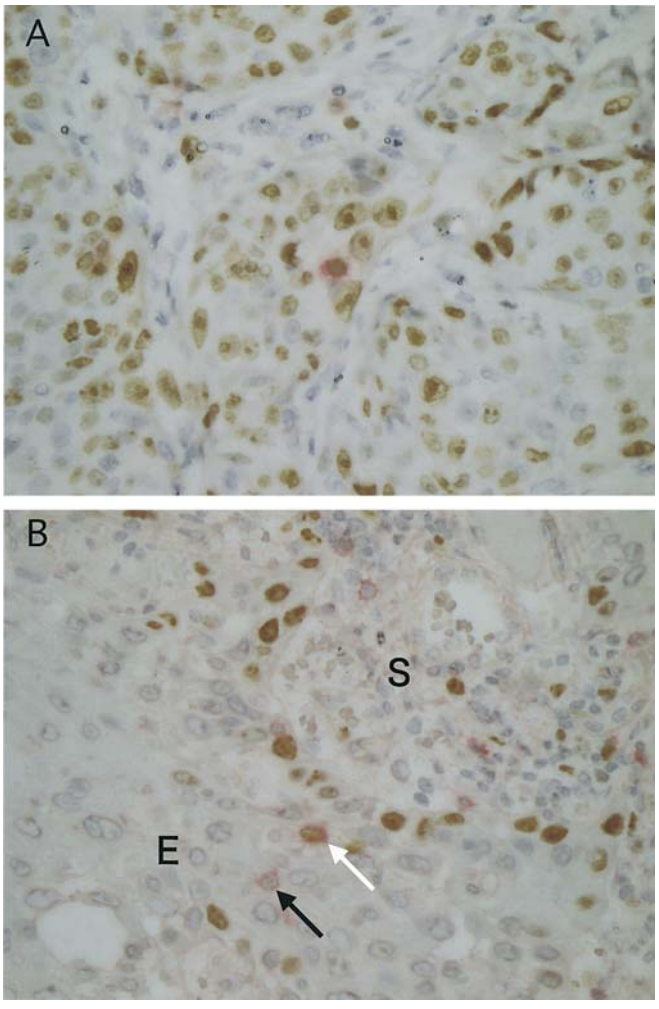

Figure 1. Representative double staining of Ki-67 and CD8 on oral squamous cell carcinoma tissue sections. (A) Proliferative activity of CD8+TIL low representative case (No. 21, x400). (B) Proliferative activity of CD8+TIL high representative case (No. 27, x200). In both cases, although many Ki-67+ tumor cells (brown nuclear staining) were observed, we could clearly distinguish Ki-67+CD8+TIL (brown nuclear staining with red membrane staining) from Ki-67+ tumor cells. White arrow indicates Ki-67+CD8+TIL, and black arrow indicates Ki-67-CD8+TIL. E, tumor epithelium; S, tumor stroma.

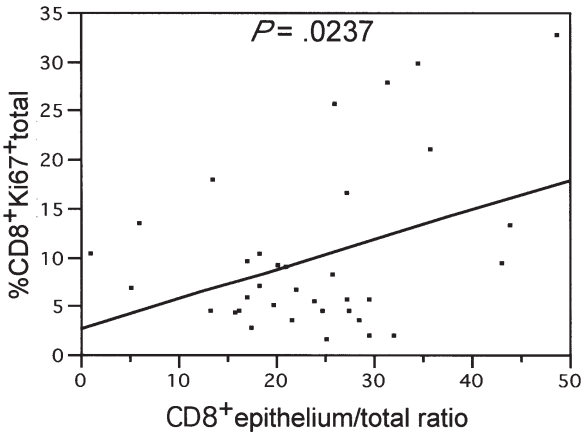

Figure 2. Relationship between proliferative activity of CD8+TIL and location of CD8+TIL. Scattered plot demonstrates the percentage of Ki-67+ CD8+TIL and intra-epithelial CD8+TIL ratio. Regression analysis was performed for statistical analysis.

shown in Fig. 4, preoperative radiotherapy showed a significant correlation (P=0.0200) although preoperative chemotherapy did not show any correlation (P=0.1825).

Discussion

Monoclonal antibody, clone MIB-1 was developed by Key *et al* and the antibody reacts with nuclear antigen Ki-67 that is expressed in cells which are not in G0 (12). The antibody has been utilized in many experiments especially in immunohistochemistry because it is able to detect Ki-67 antigen in paraffin-embedded tissue sections (12). In the present study, we utilized this antibody for evaluation of CD8+ T-cell proliferative activity by CD8 and Ki-67 double staining. As shown in Fig. 1, our staining method gave a good signal/noise ratio threfore we could easily distinguish Ki-67 positive CD8+ T-cells from other Ki-67 positive cells. A recent study revealed that only activated dendritic cells (DCs) that migrated from the periphery to draining lymph nodes, but not resting steady-state DCs residing within lymph nodes, induced T-cell activation and proliferation through the interaction of chemokine CCL19 and its receptor CCR7 (13). In addition, in order to induce target cell apoptosis, receptor mediated endocytosis of granzyme B after contact with the target cell is important under the assistance of perforin (14,15). The studies described above suggest that CD8+ T-cells that contact with a tumor target (Fig. 1B) specifically respond to target cells, and the proliferating CD8+TIL (Fig. 1) was supplied from the tumor-draining lymph node after activation. Fig. 2 demonstrates the significant tumor-specific CD8+TIL response with combined evaluation of histological location of CD8+TIL and its proliferative activity on the same paraffin-embedded tissue section (P=0.0237). There was no significant correlation between proliferative activity of CD8+TIL and either age, tumor size (T), or clinical stage (P=0.5410, P=0.7769, P=0.1041, respectively) (Fig. 3A-C). However, there was a tendency toward correlation between proliferative activity of CD8+TIL and clinical N factor (P=0.0859) (Fig. 3D) although there was no statistical significance to the correlation of proliferative activity of CD8+TIL and pathological N factor (P=0.1072) (Fig. 3E). In a recent study, DCs in sentinel lymph nodes that had no tumor metastasis showed expression of activation marker although DCs in lymph nodes with tumor metastasis lost expression of activation marker but not S-100 (16). In addition, the sentinel lymph node without tumor micro-metastasis gave higher interferon gamma (IFN-γ), interleukin-2 (IL-2), granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-10 production while the lymph node with tumor micrometastasis gave no higher cytokine production (17). This means that the results shown in Figs. 2 and 3D were contradictory. CD8+TIL proliferative activity as an indicator of host immune response appeared to correlate with tumor metastasis, which induced immuno-suppression according to the above studies. Therefore, we considered why tumor metastasis seemed to induce immune response but not immuno-suppression. We assumed that there was another factor that would induce immune response even in clinical N

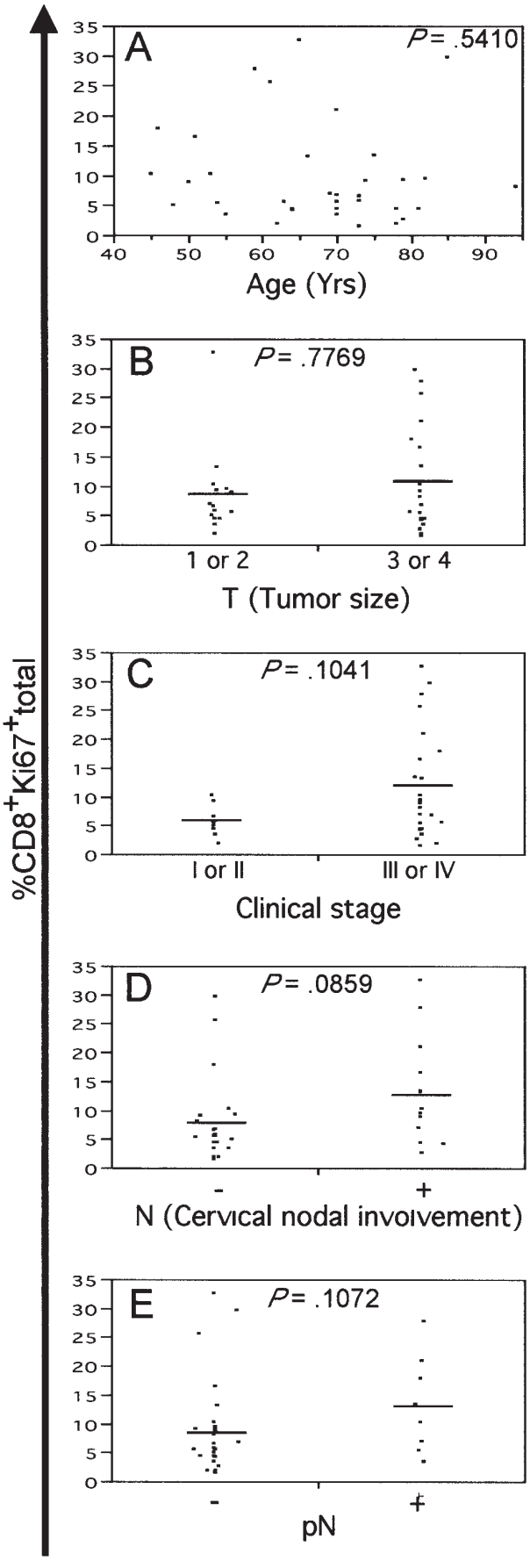

Figure 3. Relationship between proliferative activity of CD8+TIL and clinical factors. Double and single scatter plots are shown. Regression analysis or Mann-Whitney's U test was performed for each statistical analysis.

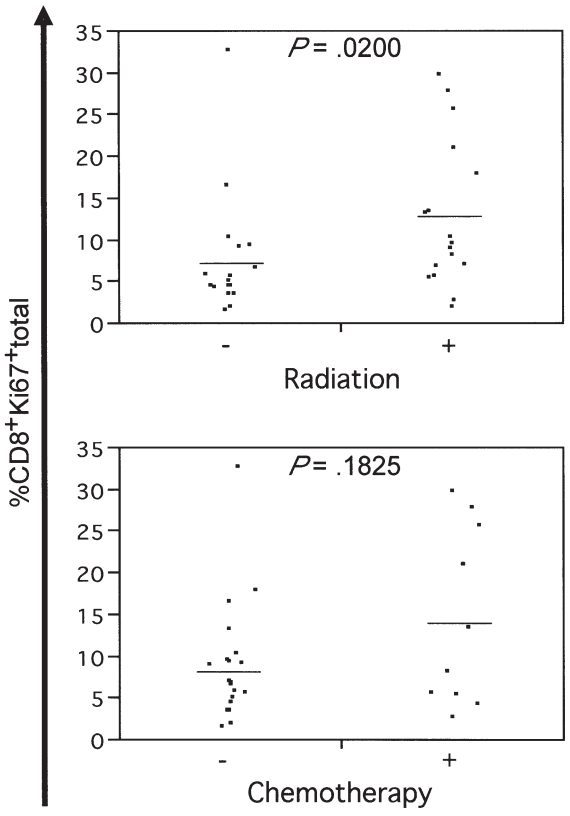

Figure 4. Relationship between proliferative activity of CD8+TIL and preoperative therapy. Single scatter plots are shown. Mann-Whitney's U test was performed for statistical analysis.

factor positive patients, and found that many patients had undergone regimens of preoperative chemotherapy and/or preoperative radiotherapy (Table I). Therefore, we performed further statistical analysis on the basis of the preoperative therapy. As shown in Fig. 4, preoperative radiotherapy was significantly correlated with CD8+TIL proliferative activity. Radiotherapy is well-known for inducing tumor apoptosis (18,19). Apoptotic or necrotic cells are engulfed by DCs and adequate protein is processed to the peptide for presentation onto MHC molecules (20). These findings support our results.

In oral squamous cell carcinoma, cytokine immunotherapy has been evaluated in clinical trials (21,22). However, there has been no report of proliferative activity analysis for evaluation of host immune response to tumor cells in oral cancer. There are reports on proliferation activity analysis for evaluation of host immune response in colorectal and renal cell carcinomas, mainly emphasizing the importance of this evaluation method for patient prognosis (10,11). The present study found that preoperative radiotherapy induces host immune response (Fig. 4), therebydemonstrating another significant utility of this evaluation method.

Preoperative radiochemotherapy is an important prognostic factor in oral cancer treatment (23). However, the complications of radiotherapy including osteo-necrosis (24) and inadequate coverage or treatment volume results in tumor recurrence should be considered (25). On the other hand, there are studies showing that dissection of draining lymph nodes

prior to antitumor vaccination significantly diminishes antitumor activity in a murine model (26) as well as human cases (27). Our results and the above findings suggest that preoperative immunotherapy would complement the weak points of radiotherapy while preoperative radiotherapy would complement immunotherapy by introducing an anti-tumor immune response of the host. Thus, these therapies are complimentary to each other. A reduced adverse effect is an other important benefit of immunotherapy because, in oral cancer, systemic chemotherapy leads to Grade IV severe toxic effects in some patients (28). For example, in recent clinical trials, dendritic cell-based immunotherapy was reported to be safe (29,30), and tumor antigen vaccination in combination with IL-12 showed mild adverse effects (Grade I-II) even though the patients in the study had end-stage cancer (31). Radioimmunotherapy has been employed in the field of head and neck squamous cell carcinoma treatment (32-34) as well as in other cancers (35). However, in these studies, the host immune response evoked via radio-labeled monoclonal antibody was not clearly evaluated. Based on our results, preoperative radioimmunotherapy can be used to induce cytotoxic T-cell response to cancer cells in oral squamous cell carcinoma cases. Finally, we would like to emphasize the importance of preoperative adjuvant therapy not only for the induction of immune response but also to maintaining the immune response after surgery. In general, surgical resection of primary tumor and regional lymph nodes strongly induce immunesuppression (36). However, a recent article demonstrated that central memory T-cells were maintained in the stroma of bone marrow (37). This means that preoperative adjuvant therapy inducing and enhancing the host immune response contributes to the maintenance of central memory T-cells even after surgical resection of primary tumor and regional lymph nodes.

In conclusion, in the present study we demonstrated a significant correlation between preoperative radiotherapy and proliferative activity of CD8+TIL. This is the first study to clearly demonstrate the significance of preoperative radiotherapy for induction of a tumor-specific CD8+ T-cell response at the site of tumor tissue.

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