

Expression of Tumor Necrosis Factor- α and Interleukin-6 in Oral Squamous Cell Carcinoma

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To explore the role of cytokines in tumor development and clinical manifestations, we examined the expressions of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in tumor tissues obtained from 57 patients with oral squamous cell carcinoma (OSCC) and their relationships to pathological grade and staging. Enzyme-linked immunosorbent assay on the tumor tissues demonstrated elevated concentrations of TNF- α and IL-6 proteins and upregulated mRNA levels were detected by the reverse transcription-polymerase chain reaction method when compared to those in normal control tissues. These cytokines and their transcripts were localized in stromal macrophages and in the tumor cells in particular of the front area of tumor tissues, possibly indicating active synthesis of these cytokines by tumor cells. Larger-sized tumors (T3, 4) contained significantly greater levels of IL-6 proteins than small-sized tumors (T1, 2) ($P < 0.05$). The levels of these cytokines were significantly reduced in cases with effective pre-treatment with radiation or anti-cancer agents compared to those in the less effective group ($P < 0.05$, grade IIa vs. grade IV for both TNF- α and IL-6). The present study thus demonstrated enhanced expression of cytokines in OSCC tissues.

Key words: Oral cancer — Cytokine — *In situ* hybridization — Treatment effects

Cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) exert biological effects on tumor development. They are produced by a variety of cells including macrophages,¹ fibroblasts and endothelial cells, as well as neoplastic cells.^{1,2} Serum levels of such cytokines are increased in patients with ovarian cancer,³ malignant melanoma⁴ and renal cell carcinoma.⁵ Tissue homogenates from head and neck squamous cell carcinoma (HNSCC) also contain measurable levels of the cytokines.⁶ On the one hand, cytokines are cytotoxic to tumor cells, inhibiting the progression of the tumor or causing necrosis.^{7–9} On the other hand, they stimulate growth of tumor cells, through promotion of angiogenesis,¹⁰ modification of matrix proteins¹¹ or expression of adhesion molecules.^{12–14} Cytokines thus have not only a cytostatic action, but also a pathogenetic role in tumor development, being further implicated in invasion and metastasis.

The exact source of cytokines in tumor tissues is not, however, well established. While tumor tissues from colorectal carcinomas,¹⁵ renal cell carcinoma,⁵ gastric cancer¹⁶ and breast cancer¹⁷ contain cytokines, they are mainly produced by stromal macrophages, not by tumor cells. By contrast, it has been shown that neoplastic cells produce TNF- α ³ and IL-6 in ovarian cancers.^{3,18} Patients with HNSCC, including cases of oral squamous cell carcinoma (OSCC), showed increased serum levels of TNF- α

and IL-6^{19–21} that correlated with poor prognosis.²² It is yet to be determined, however, which cellular components produce these cytokines or whether the tissue levels of cytokines are associated with tumor size, lymph node metastasis or histological grade of malignancy. Since cytokine levels are useful in the determination of treatment modalities and may be predictors of prognosis, information on cytokine expression in OSCC is extremely important. We therefore performed a detailed analysis of TNF- α and IL-6 expressions in tumor tissues obtained from OSCC patients and here we discuss the significance of the observed changes of cytokine expressions.

MATERIALS AND METHODS

Materials Tissue samples were obtained from a total of 57 patients with OSCC operated in Hirosaki University Hospital, including 3 biopsy cases. Among them, 32 cases were untreated before the surgical resection of the cancer (cases with non-pretreatment: NT case) and 23 cases were preoperatively treated with radiation and/or anti-cancer agents (cases with preoperative treatment: PT case). Some cases were examined serially before and after pretreatment. Decision of the pretreatment regimens with radiotherapy and/or chemotherapy before the surgical resection of the cancer was made based on the tumor size, localization, and general condition of the patients. A hundred and twenty-eight samples were examined in total. Oral mucosal tissues without marked inflammatory changes, obtained from non-cancer patients, were used as normal

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controls (NC). For comparison, lymph nodes with metastatic foci (LN) were also examined. Tissue sampling followed the guideline of the ethical committee of the Hirosaki University, with the informed consent of patients.

All surgical specimens were cut into several pieces for the measurement of cytokine contents, immunohistochemical observation, estimation of transcript levels of cytokines by reverse transcription polymerase chain reaction (RT-PCR) and *in situ* hybridization (ISH). For the measurement of cytokine contents, enzyme-linked immunosorbent assay (ELISA) was used. The levels of cytokine contents were examined to address whether they were correlated with tumor size or lymph node metastasis. The determination of these parameters was based on the WHO's TNM clinical classification.²³⁾ Portions of tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.5) and embedded in paraffin, then processed for immunohistochemistry. For the evaluation of transcript levels, small pieces of tissues were frozen in liquid nitrogen and used for RT-PCR. ISH was conducted on the paraffin sections of the tissues using the same blocks as for immunohistochemistry. Conventional light microscopic examinations were made on hematoxylin-eosin (HE)-stained slides. Histological grade of malignancy in NT cases was determined on sections of maximal cut surface of the tumors based on Anneroth's criteria.²⁴⁾ Briefly, we divided the histological grade of malignancy for NT cases into two groups of high-grade malignancy (>13 points) and low-grade malignancy (<12 points). The effects of preoperative treatment were also determined histologically on the sections of maximal cut surface of the tumors in PT cases using Shimamoto's classification,²⁵⁾ in which the grade is defined as follows: I, no destruction of tumor cells; IIa, viable tumor cells occupy over 1/3 area; IIb, viable tumor cells below 1/3 area; III, only small clusters of non-viable cells or very few viable cells; IV, no tumor cells.

ELISA Tissue samples were stored at -80°C until assay. Then, they were thawed, and homogenized in PBS with a Polytron (Kinematica, Luzern, Switzerland). The homogenate was centrifuged at $1,000g$ twice for 10 min at 4°C ,

then the supernatant fraction was centrifuged again at 4°C at $105,000g$ for 40 min, and frozen at -80°C in aliquots until assay. Tissue protein levels were measured by the Bradford method (Bio-Rad, Richmond, CA).²⁶⁾ Immunoassays were performed using TNF- α and IL-6 ELISA kit (TFB Co., Tokyo). All samples were assayed in duplicate. **Immunohistochemical staining** The streptavidin-biotin (SAB) method (Histofine SAB kit, Nichirei, Tokyo) was applied to serial tumor sections using monoclonal anti-TNF- α antibody (Austral Biologicals Inc., San Ramon, CA) and monoclonal anti-IL-6 antibody (Genzyme Inc., Cambridge, MA). After deparaffinization and elimination of endogenous peroxidase activity with methanol containing 0.3% H_2O_2 , each primary antibody in 1:100 dilution was applied at 4°C overnight. The sections were then incubated with biotinylated rabbit anti-mouse antibody for 15 min at room temperature. Thereafter, the sections were reacted with streptavidin-peroxidase complex for 10 min. The reaction products were finally visualized with diaminobenzidine hydrochloride and lightly counterstained with hematoxylin. Specificity of immunoreactions was confirmed by pretreatment of the antibodies with excess antigens; each $100\ \mu\text{l}$ of antibodies was incubated with $4\ \mu\text{g}$ of recombinant human IL-6 (Boehringer Mannheim, Tokyo) and $5\ \mu\text{g}$ of recombinant human TNF- α (Boehringer Mannheim) for 1 h, respectively. Replacement of the first antibodies by un-immunized mouse sera or omission of the first antibodies during the process of immunostaining was also done to confirm the specificity.

For the quantitation of immunoreactions, positivities of TNF- α and IL-6 were graded as; +, positive cells less than 25%; 2+, 25–50%; 3+, over 50%.

ISH The synthesized DNA oligonucleotide probes are listed in Table I. Labeling of $4\ \mu\text{g}$ of oligonucleotide with a biotin moiety was performed in $50\ \mu\text{l}$ of reaction buffer at 85°C for 30 min using a Universal Linkage System (ULS) kit (Nichirei).²⁷⁾ After having been rinsed in diethylpyrocarbonate (DEPC)-treated PBS, deparaffinized sections were digested in $1.0\ \mu\text{g}/\text{ml}$ proteinase K (GIBCO BRL Inc., Gaithersburg, MD) for 20 min at 37°C , followed by postfixation in 4% paraformaldehyde and neu-

Table I. Primers for PCR and Oligonucleotide Probes for *in situ* Hybridization (ISH) Used in the Present Study

Primers for PCR	TNF- α	5' primer	5' TAG CCC ATG TTG TAG CAA ACC CTC AAG CT 3'
		3' primer	5' TCA CAG GGC AAT GAT CCC AAA GTA GAC CT 3'
	IL-6	5' primer	5' ATG AAC TCC TTC TCC ACA AGC GCC TTC 3'
		3' primer	5' GAT TCT TTG CCT TTT TCT GCA GGA ACT GG 3'
	β -Actin	5' primer	5' ATG GAT GAT GAT ATC GCC GCG CT 3'
		3' primer	5' GAC TCC ATG CCC AGG AAG GA 3'
Probes for ISH	TNF- α	5' CGG GGT TCG AGA AGA TGA TCT GAC TGC CTG 3'	
	IL-6	5' CAG GAA CTC CTT AAA GCT GCG CAG AAT GA 3'	

trahization in 2 mg/ml glycine buffer for 10 min at room temperature. After immersion in 0.2 N HCl at room temperature for 20 min, the sections were incubated with specific oligonucleotide probe labeled with biotin in hybridization buffer containing 20 µg/ml yeast-tRNA (Ambion Inc., Austin, TX), 0.02 M Tris-HCl (pH 8.0), 0.0025 M EDTA, 1% Denhart's medium, 0.3 M NaCl, 50% formamide, and DEPC-H₂O overnight at 42°C. They were then rinsed with 2× standard saline citrate (SSC) and 50% formamide solution at 42°C. The hybridized sections were digested with 1 µg/ml RNase dissolved in TNE solution (0.01 M Tris-HCl, 0.5 M NaCl, 0.001 M EDTA) for 30 min at 37°C. They were washed again in 0.2× SSC followed by 2× SSC at 42°C for 20 min. Sections were transferred in buffer 1 (0.1 M Tris-HCl, 0.15 M NaCl) and treated with 1.5% blocking reagent (DIG Nucleic Acid Detection kit, Boehringer Mannheim) in buffer 1. The sections were then incubated with streptavidin (1:200 dilution, Vector Inc., Camarillo, CA) for 30 min at room temperature. The hybridized complex was reacted with biotinylated alkaline phosphatase (1:500 dilution, Vector Inc.) for 30 min and finally visualized using New Fuchsin Substrate System (Dako, Carpinteria, CA).

The specificity of reactions was confirmed by blocking tests involving pretreatment of sections with 10 µg/ml RNase for 2 h at 37°C before hybridization or hybridization of the sections with biotin-labeled sense probes.

RT-PCR Fifteen samples of both NT and PT cases rich in tumor parenchyma (confirmed by examination of frozen sections) and 7 lymph node specimens in addition to 7 controls were used for the quantitation of mRNA levels of TNF-α and IL-6. Total RNA was extracted from each sample with Isogen (Nippon Gene, Osaka). Complementary DNA (cDNA) was synthesized from 5 µg of total RNA in 25 µl of reactive solution using Moloney murine leukemia virus RT (Stratagene, Stratascript (TM) RT-PCR kit, Cambridge, CA). After heat inactivation of RT, 2.5 units of DNA polymerase (Takara Shuzo Co., Kyoto) and specific primers (Table I) were added to the above solution, and cDNA was amplified by PCR using a DNA thermal cycler (RTC-100, Funakoshi Inc., Tokyo). PCR assays for both cytokines and β-actin were performed in 20 µl of solution using 28 cycles with steps of 94°C for 1 min for denaturation, 72°C for 2 min for primer extension, 64°C for 1 min of TNF-α and 63°C for 1 min of IL-6 for annealing, respectively. The PCR products (10 µl) were electrophoresed on 2% agarose gel containing 0.5 µl ethidium bromide and visualized with ultraviolet light. The products of message bands were densitometrically quantified using the NIH image software (National Institutes of Health, Bethesda, MD) and values were normalized by the value of each β-actin level.

Statistical analysis Statistical analysis was performed with Mann-Whitney's *U*-test to compare the mean values

between two groups. If necessary, the Kruskal-Wallis test was added. The criterion of statistical significance was a *P* value of less than 0.05.

RESULTS

ELISA TNF-α protein was undetectable in NC cases except for one case (14.9 pg/mg). By contrast, OSCC tissues contained high levels of TNF-α and the mean value was significantly greater in the OSCC (NT) group (27.0±41.3 pg/mg, mean±SD) than that in NC (2.1±5.7 pg/mg) (*P*<0.05) (Fig. 1). TNF-α protein was detected in 6 of 13 LN tissues (46%), ranging from 10.1 to 48.3 pg/mg (17.1±21.7 pg/mg). Levels of IL-6 protein were also elevated in OSCC tissues compared to NC. The mean value of IL-6 protein was significantly greater in OSCC (NT) (94.2±112.5 pg/mg) than that in NC (7.8±18.2 pg/mg) (*P*<0.05). The mean value of IL-6 was greater in LN (154.6±220.0 pg/mg) than in NC, but there was no statistically significant difference in the levels of TNF-α between these two groups.

Levels of IL-6 protein were significantly greater in larger tumors (124.5±101.1 pg/mg in T3 and T4) compared to those in small-sized tumors (69.9±118.5 pg/mg in T1 and T2) (*P*<0.05) (Fig. 2). There was a similar trend toward a greater value of TNF-α protein in tumors of larger size, although it was not statistically significant. The presence of lymph node metastasis did not affect the levels of cytokine contents in the primary OSCC tissues of NT group (data not shown). Comparison of TNF-α and IL-6 levels in tissues of the NT group with histological grade of malignancy did not reveal significant differences between groups of low and high grades (data not shown).

Effects of preoperative treatment on the cytokine levels in OSCC tissues of the PT group were then analyzed. The mean value of TNF-α was significantly smaller (*P*<0.05) in groups with a higher grade of pretreatment effects (grade IV, 2.7±4.5 pg/mg, grade IIb, 5.5±6.2 pg/mg) than that in a group with a lower grade of pretreatment effects (grade IIa, 127.9±216.0 pg/mg) (Fig. 3). There was also significant reduction of IL-6 in a group with a higher grade of pretreatment effects (grade IV, 41.3±36.1 pg/mg) compared to a group with lower grade of pretreatment effects (grade IIa, 121.7±77.3 pg/mg) (*P*<0.05).

Immunohistochemistry Immunoreactive TNF-α was positive in the perinuclear cytoplasm of OSCC cells in most cases. Infrequently, strong positive reactions appeared diffusely in the cytoplasm (Fig. 4A). Strong positivity (3+) was detected in 3 of 9 cases (33.3%) of a group with high-grade histological malignancy, but in none of 6 cases of a group with low-grade histological malignancy. TNF-α was also positive in tumor cells in all LN specimens. Vascular endothelial cells adjacent to the tumor cells were strongly positive for TNF-α, but the reaction of stromal cells and normal squamous cells was

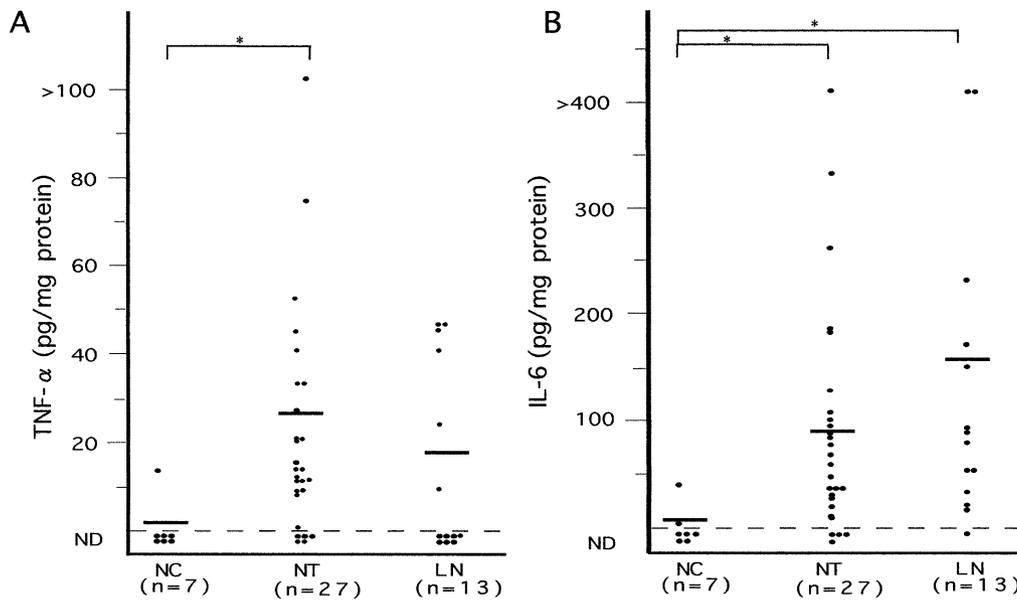


Fig. 1. Tissue levels of TNF- α (A) and IL-6 (B) in cases of oral squamous cell carcinoma (OSCC) measured by enzyme-linked immunosorbent assay. Levels of TNF- α and IL-6 were significantly elevated in the OSCC group without preoperative treatment (NT) compared to normal controls (NC). Metastatic lymph node (LN) also showed greater levels of IL-6 but not TNF- α compared to NC. ND, not detectable; * $P < 0.05$.

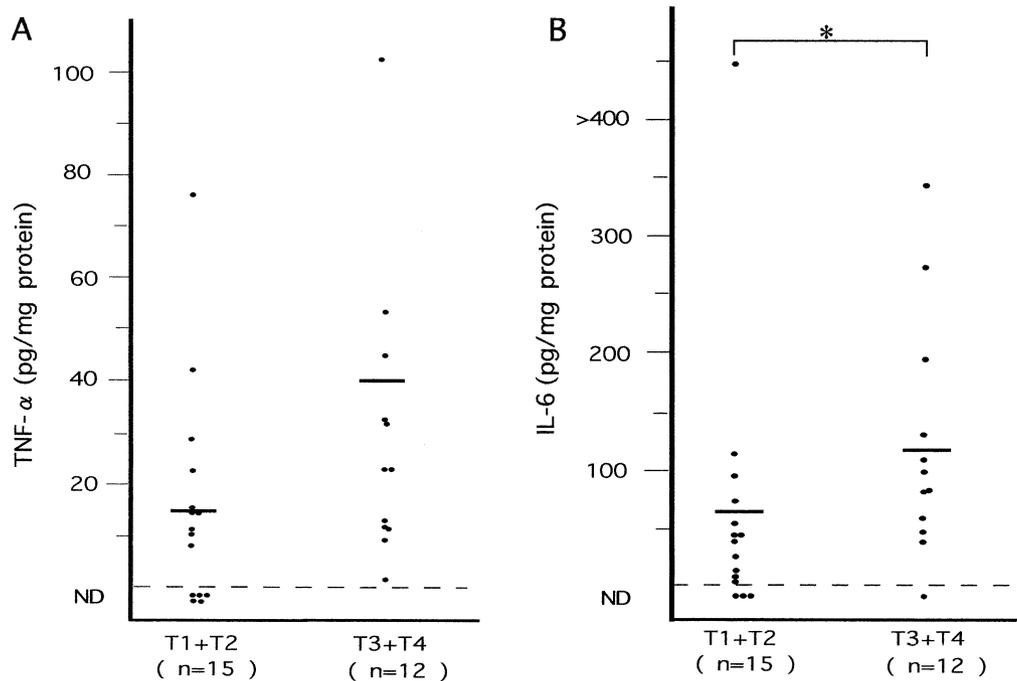


Fig. 2. Relation of tumor size to tissue levels of TNF- α (A) and IL-6 (B) in cases of preoperatively non-treated oral squamous cell carcinoma. Tumor size was graded based on WHO's TNM classification (1992). Levels of IL-6 were significantly greater in a group of T3 and T4 than those in a group of T1 and T2. There was a similar trend in the levels of TNF- α , although it was not statistically significant. ND, not detectable; * $P < 0.05$.

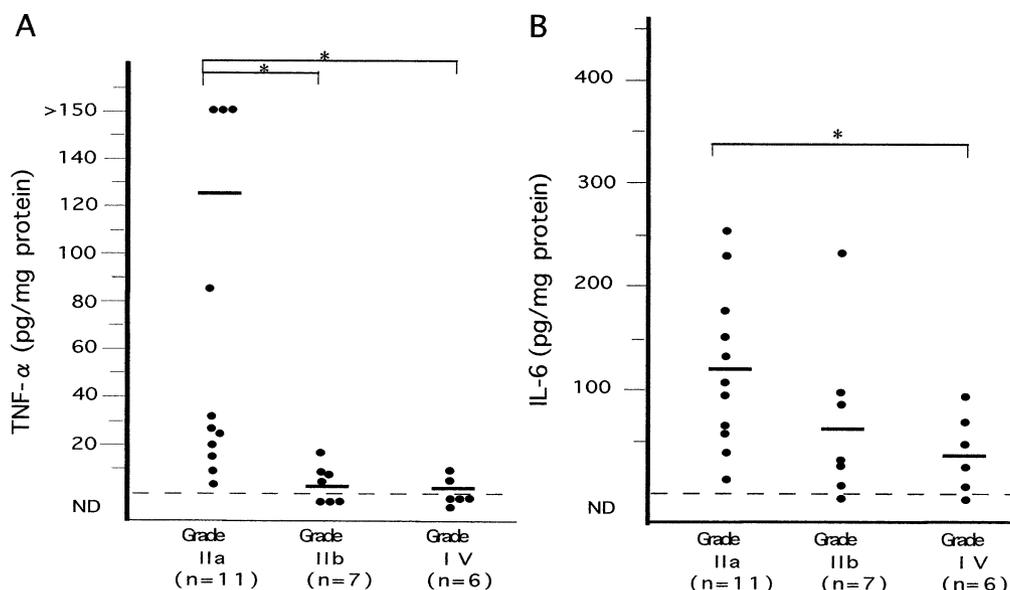


Fig. 3. Tissue levels of TNF- α (A) and IL-6 (B) and effects of preoperative treatment in oral squamous cell carcinoma. Effects of treatment were histologically graded based on Shimamoto's criteria (1971) (see text), in which grade IV was assigned to a group in whom pretreatment was effective. The levels of both TNF- α and IL-6 were significantly greater in grade IIa (less effective pretreatment) than those in grade IV.

equivocal (Table II). The endothelial reaction appeared to be weakened in the area remote from the tumor.

OSCC showed positive immunoreactions for IL-6 in the cytoplasm of tumor cells and stromal cells. Strong reactions were found in particular in the tumor cells around the keratin pearls (Fig. 4B). In non-neoplastic tissues, weakly positive reactions were found in the prickle cell layer of the squamous epithelia and submucosal inflammatory cells. There was no obvious positive reaction in the cornified layer. Control sections showed no reactions to TNF- α or IL-6 (Fig. 4C).

ISH TNF- α mRNA was expressed as dot-like or diffuse signals in the cytoplasm of tumor cells (Fig. 4D). Some tumor cells with marked nuclear polymorphism were strongly positive for TNF- α mRNA. Stromal cells including infiltrating macrophages and fibroblasts were also positive for TNF- α mRNA, whereas there was no apparent positive reaction in endothelial cells of vessels in the tumor area. IL-6 mRNA was localized diffusely in the cytoplasm of tumor cells (Fig. 4E). Macrophages, fibroblasts and endothelial cells in stroma were weakly positive. Control sections were completely negative (Fig. 4F).

RT-PCR Strong signals of both TNF- α and IL-6 were detected in most of the examined tumor samples (Fig. 5). TNF- α mRNA levels were elevated nearly 4–7 fold in OSCC tissues of NT and PT groups compared to the levels of NC ($P < 0.05$ vs. NC for both) (Fig. 6). By contrast, the

expression levels were not high in most LN cases. IL-6 mRNA levels were similarly elevated 3–6 fold in OSCC tissues of the NT and PT groups ($P < 0.05$ vs. NC for both). There was no significant increase in IL-6 mRNA levels in LN compared to NC.

DISCUSSION

In the present study, TNF- α and IL-6 levels in the homogenates from OSCC tissues detected by ELISA were significantly higher than the levels in normal mucosa. Immunohistochemical staining revealed the localization of these cytokines in the tumor cells, some stromal macrophages and fibroblasts in the sections of OSCC. RT-PCR demonstrated augmented transcript (mRNA) levels of both TNF- α and IL-6 in the tumor tissues and ISH also demonstrated the presence of enhanced transcript signals in tumor cells. These findings are in keeping with the previous observation that tumor cells in HNSCC were positive for immunoreactive TNF- α ^{6,28–30} and further suggest for the first time the possibility that tumor cells in OSCC themselves produce TNF- α and IL-6 *in vivo*.

It is not known why some tumor cells actively produce cytokines, as in HNSCC or OSCC detected in this study, whereas others such as breast cancer,¹⁷ colorectal cancer¹⁵ or renal cell carcinoma⁵ do not express them. Ohno *et al.*¹⁶ found increased TNF levels in the serum and portal

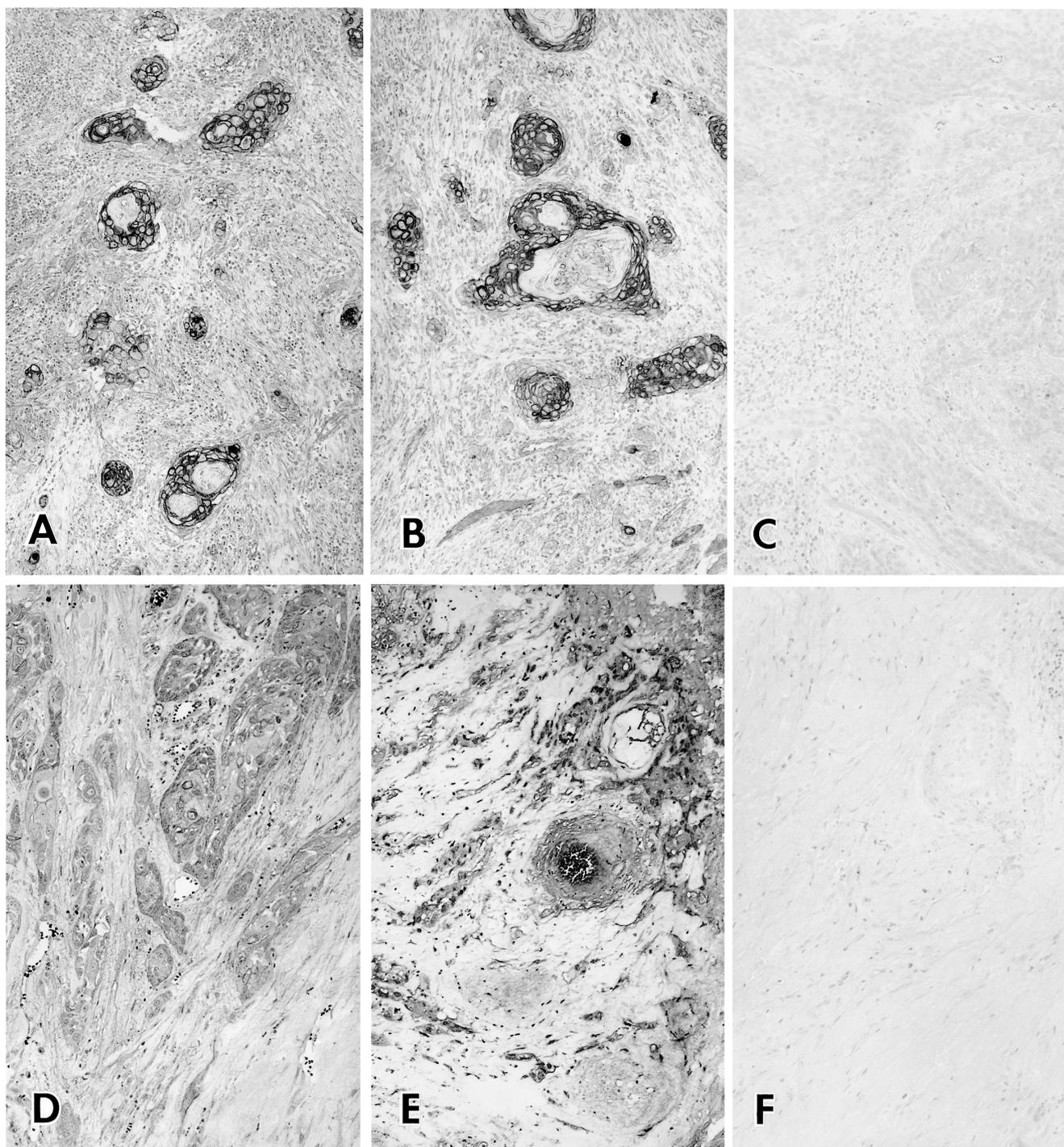


Fig. 4. Expression of cytokines and their mRNAs detected by immunohistochemistry and *in situ* hybridization. Both immunoreactive TNF- α (A, $\times 200$) and IL-6 (B, $\times 200$) were localized in tumor cells as well as in some stromal macrophages. Endothelial cells were also strongly positive for TNF- α . Pretreatment of antibodies with excessive antigens diminished the reactions to TNF- α (C) and IL-6 (not shown). *In situ* hybridization gave positive signals of TNF- α mRNA (D, $\times 200$) and IL-6 mRNA (E, $\times 200$). Endothelial cells were negative for TNF- α mRNA. Control section pretreated with anti-sense probe showed negative reactions to TNF- α mRNA (not shown) and IL-6 mRNA (F, $\times 100$).

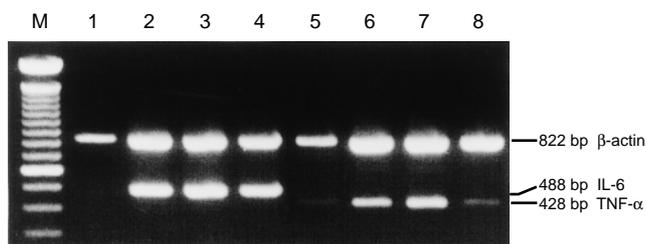


Fig. 5. Detection of mRNAs of TNF- α and IL-6 by RT-PCR. There was amplified expression of mRNA levels of TNF- α (428 bp) and IL-6 (488 bp) in tissues from oral squamous cell carcinoma of the preoperatively non-treated group (NT; lanes 2 and 6) and treated group (PT; lanes 3 and 7), as well as metastatic lymph node (LN; lanes 4 and 8), compared to those in normal mucosa (NC; lanes 1 and 5). Upper bands are of β -actin mRNA. M stands for marker. Lanes 1-4, IL-6 mRNA; lanes 5-8, TNF- α .

blood in patients with gastric cancer, but they could not detect production of TNF in the tumor tissues. They concluded that circulating macrophages were responsible for the TNF production. Naylor *et al.*³⁾ reported in almost 75% of cases of ovarian cancer the expression of TNF genes and a close correlation between TNF protein levels and histological grade of malignancy. In their studies, TNF- α mRNA was found in macrophages and some epithelial tumor cells, but TNF receptors were not uniformly distributed in tumor cells. It is known that TNF- α synthesis is upregulated in cultured tumor cells when they lose their receptors for TNF on the cell membrane, resulting in resistance to the cytotoxicity.²⁾ The resistant tumor cells with augmented TNF production activate stromal macrophages to release metalloproteases, thus perhaps promoting tumor cell invasion.³¹⁾

Table II. Results of Immunohistochemical Reactions of Tumor Necrosis Factor- α (TNF- α) and Interleukin-6 (IL-6) in Oral Squamous Cell Carcinoma (OSCC)

Antibody	Grade of malignancy	Tumor cell ^{a)}		
		+	2+	3+
TNF- α	Low (n=6)	4.6 (66.7)	2.6 (33.3)	0/6 (0)
	High (n=9)	5.9 (55.6)	1.9 (11.1)	3.9 (33.3)
	LN ^{b)} (n=10)	4.10 (40)	5.10 (50)	1.10 (10)
IL-6	Low (n=6)	0/6 (0)	0.6 (0)	6.6 (100)
	High (n=9)	2.9 (22.2)	1.9 (11.1)	7.9 (66.7)
	LN ^{b)} (n=10)	1.10 (10)	0.10 (0)	9.10 (90)

() Percentage of positive cases.

a) Results of TNF- α and IL-6 staining were graded into +, 2+, 3+ as -25%, 25-50%, 50-100% of positive cells.

b) Lymph node with metastasis.

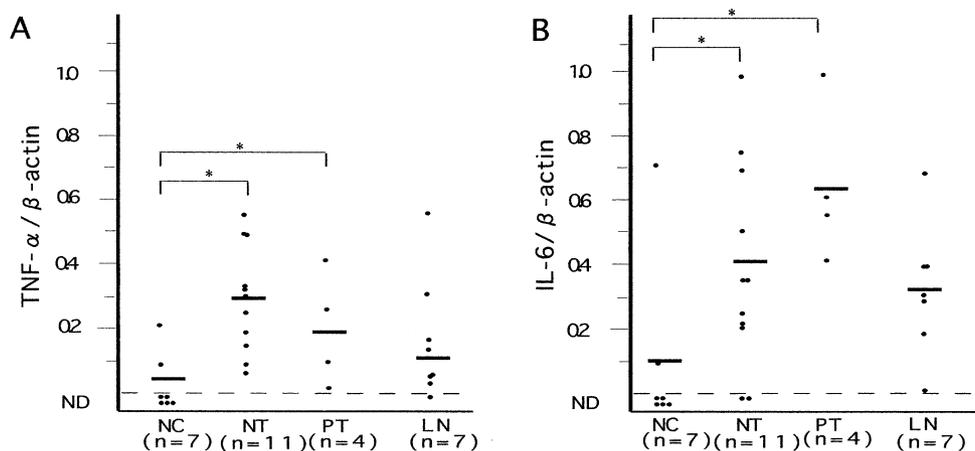


Fig. 6. Densitometric analysis of mRNA levels of TNF- α (A) and IL-6 (B) by RT-PCR. The scale shows the values of cytokine mRNA normalized with respect to β -actin levels. The levels of TNF- α and IL-6 were significantly greater in oral squamous cell carcinoma of the non-treated (NT) and preoperatively treated (PT) groups compared to those in normal mucosa (NC). By contrast, the levels in metastatic lymph node (LN) were not elevated. ND, not detectable; * $P < 0.05$.

We found strong signals of IL-6 mRNA in tumor cells, but weak signals in macrophages and stromal fibroblasts. Since squamous cells are activated to produce cytokines *in vitro* under stimulation with inflammatory cells,³²⁾ or when they are obtained from patients with lichen planus,^{33,34)} radicular cyst³⁵⁾ or psoriasis,³⁶⁾ it may be reasonable to consider that tumor cells in OSCC are active for IL-6 synthesis. Cellular interaction between tumor cells and stromal components may therefore be a possible determinant of cytokine production in tumor cells. The intensified expression of immunoreactive TNF- α and IL-6 in the front area of OSCC may be ascribed to strong cellular interactions between tumor cells and stromal cells.

In the present study, the elevated levels of tissue cytokines were associated with increased tumor size, but not with lymph node metastasis. These results suggest that cytokine production in OSCC is not regulated by circulating macrophages or immune reactions in response to lymph node metastasis, but is largely dependent on the local tumor growth of the primary lesion. This contention is consistent with the findings that TNF- α and IL-6 levels were significantly decreased in cases with effective preoperative treatment (grade IV).

Cytokines have an affinity for endothelial cells, which contain abundant receptors of these cytokines.²⁾ TNF- α induces expression of cell adhesion molecules to recruit

tumor cells to vessels, promoting metastatic processes.¹⁴⁾ In the present study, TNF- α protein was strongly expressed in endothelial cells in microvessels within the tumor, whereas the mRNA was not detected on the vessels, indicating attachment or uptake of TNF- α by endothelial cells, and not production of this cytokine. By contrast, transcripts of IL-6 were weakly detected on the vessel walls. The difference of transcript expression between IL-6 and TNF- α may suggest the tissue-specific production of these cytokines in OSCC tissues. In addition, dissociation between the location of cytokines and their transcripts may reflect topographic differences of cytokine production, binding and degradation. Further studies are required to elucidate the role of cytokines in the biological behavior of OSCC.

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