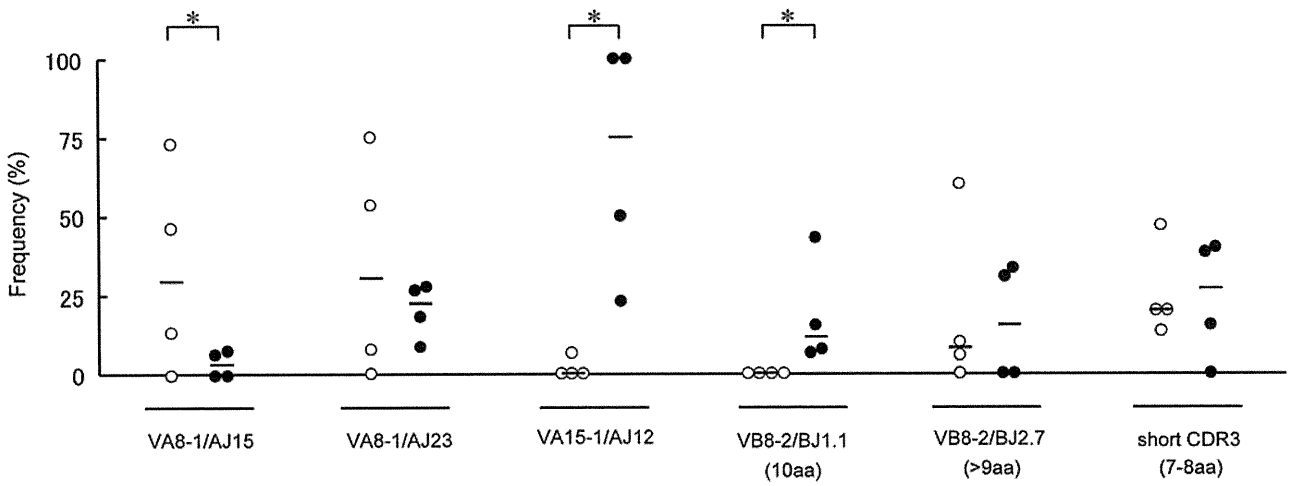

FIG. 5. Differential patterns of TCR gene usage between surviving and dying mouse brains. Frequencies for characteristic combinations of V and J gene usages were individually plotted. CDR3 length was taken into consideration for β chain: BJ2.7 (long) indicates CDR3 consisted of more than 9 aa, and short CDR3 indicates CDR3 consisted of 7 or 8 aa. Open circles indicate surviving mice, and closed circles indicate dying mice. Bars indicate the median for each group. Asterisks denote statistically significant ($p < 0.05$) differences between surviving and dying mice using the Mann-Whitney U test.

severity of TBEV infection. In our previous study (21), large numbers of $CD8^+$ T cells infiltrated the brains of TBEV-infected mice, yet no significant difference was observed between surviving and dying mice. We therefore investigated the individual characteristics of brain-infiltrating T cells in this study.

TCR repertoire analysis revealed that the frequencies of T cells bearing VA8-1, VA15-1, and VB8-2 were significantly increased in TBEV-infected mouse brains compared with those of spleens. However, there was no significant difference between surviving and dying mice. This indicated that once a certain amount of virus was inoculated, T cells with selected TCR V families accumulate in the brain regardless of disease severity. In contrast, clonality results and CDR3 sequencing analysis indicated a distinct difference between mouse groups, with frequencies of VA15-1/AJ12 and VB8-2/BJ1.1 gene usage higher in dying mice. One clone with an identical CDR3 sequence (CAAS GA GGYKVVF) was detected in all four dying mice, strongly suggesting that this clone was associated with severe encephalitis. High frequencies of VA8-1/AJ15 gene usage were characteristic of surviving mice, while high frequencies of VB8-2/BJ2.7 gene usage and clones with short CDR3 were observed in both surviving and dying mice. We therefore observed a unique increase of T-cell clones for each mouse group. Specific clones were found only in dying mice, while other clones were frequently found in surviving mice or were commonly found in every infected mouse. Thus, there are several T-cell clones that may be associated with the severity of TBEV infection, while the remaining clones may be different in

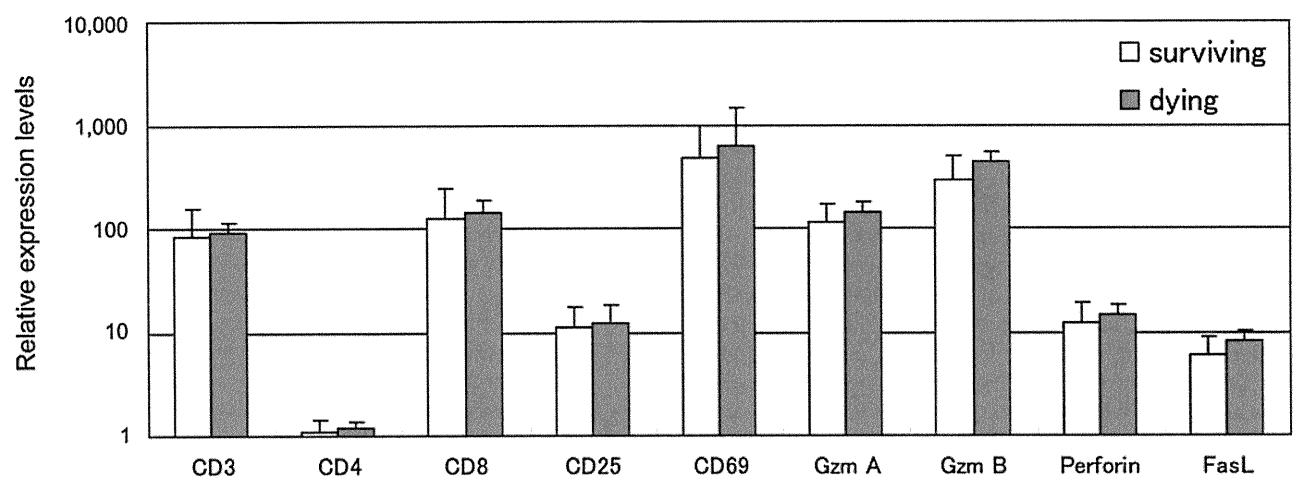

FIG. 6. Quantification of mRNA expression of T-cell-related antigens, activation markers, and apoptosis-related genes in brains using qPCR. Sample RNAs were extracted from brains of mock-infected and TBEV-infected surviving and dying mice at 13 dpi (n = 4). The mRNA expression levels in TBEV-infected brains were normalized by GAPDH expression and are shown as the relative expression levels compared with mock-infected brains. Vertical error bars indicate the standard deviation of three independent experiments.

each mouse. T cells associated with surviving mice may also be variable and were divided into two groups based on CDR3 sequence patterns for VA8-1 and VB8-2. Thus, specific mechanisms to overcome TBEV infection may exist. It is therefore further worthwhile to investigate the roles of the distinct clones identified in our study, especially clones with VA15-1/AJ12, VB8-2/BJ2.1 (10 aa), and VA8-1/AJ15, that exhibited different patterns between surviving and dying mice.

The large sequence variation observed in our data can be explained by multiple reasons. First, TCR-peptide MHC (pMHC) recognition is flexible (1), and different TCRs with similar binding capabilities can recognize identical antigen peptides. Such flexibility may therefore produce variations in induced T-cell clones in TBEV-infected brains. Another possible reason relates to the presence of quasispecies. Noncloned RNA viruses generally exist as a quasispecies (22,25), and it has been reported that the virus stock used in our study was a complex of quasispecies (19). Consequently, the different amino acids for the antigen peptides among the quasispecies might produce T-cell clone variations in TBEV-infected brains.

We must also consider why different characteristics between surviving and dying mice were observed despite of an absence of differences between these groups in TCR repertoire analysis. Common V gene usage means that the TCR could recognize identical or similar antigen peptides (12,33). However, as described above, TCR-pMHC recognition can allow for some cross-reactivity; for example, different TCRs can bind to the same pMHC, and a single TCR can bind to a different pMHC (29,46). Therefore, TBEV infection can induce multiple T-cell clones with certain V genes that recognize specific antigens, yet only a few may play a critical role.

Different roles may also exist for the distinct T-cell clonotypes found in surviving and dying mice. However, qPCR analysis failed to identify any difference in T-cell function-associated markers between these groups, and apoptosis-associated genes were almost equivalently expressed in both surviving and dying mice. As this was the result for total brain expression, further studies of each T-cell clonotype are needed. We also need to investigate if the difference in target antigens recognized by T cells has an impact on severity and fatality rather than activation level. Or perhaps the difference in T-cell clonotype resulted from encephalitis progression and requires further investigation.

We must exercise care, because virulent and host immune responses can be very different in experimental models, depending upon the subtype and strain of virus, the mouse strain, and so on. In addition, because our data are based on analysis at 13 dpi only, we may need to investigate immune responses at earlier time points post-infection. We previously reported increased levels of serum corticosterone in dying mice (21). Glucocorticoids are known to exert immunomodulatory effects by activating the hypothalamic-pituitary-adrenal axis and/or cytokine expression (2,31). Thus, the relationship between T-cell clone bias and the level of corticosterone is interesting and warrants further investigation.

In conclusion, we have revealed an association between brain-infiltrating T-cell clones and severity in TBEV-infected mice, although the cause of this relationship is still unclear. Specifically, as the brain is originally free from adaptive immunity, it is unknown whether particular T-cell accumulation determines disease severity, or if a certain type of disorder induces particular T-cell accumulation by changing antigen presentation patterns. Further experiments are needed to elucidate which factor causes the difference in induced T-cell clones. Although our results are complicated, we believe our data are an initial step in better understanding of the mechanisms of viral encephalitis.

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Author Disclosure Statement

No competing financial interests exist.

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Up-regulation of EGF receptor and its ligands, AREG, EREG, and HB-EGF in oral lichen planus

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Objective. This study aimed to investigate the roles of the epidermal growth factor receptor (EGFR) family members and their ligands in oral lichen planus (OLP).
Study design. The expressions of 4 EGFR-like receptors and 6 EGF-like ligands were measured in OLP tissues from 10 patients and compared with the levels in normal oral mucosa (NOM) from 10 healthy donors.
Results. Of the receptors, only EGFR mRNA and protein were more highly expressed in OLP compared with NOM tissues. Regarding the ligands, the mRNAs of amphiregulin (AREG), epiregulin (EREG), and heparin-binding EGF-like growth factor (HB-EGF) were more highly expressed in OLP compared with NOM tissues. These ligands were strongly expressed by infiltrating lamina propria lymphocytes as well as epithelial keratinocytes in OLP lesions, as shown by immunohistochemistry.
Conclusions. The enhanced EGFR expression on the keratinocytes in OLP lesions and the up-regulation of EGF-like ligands in keratinocytes and infiltrating mononuclear cells could contribute to the carcinogenesis and pathogenesis of OLP. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;110:748-754)**

Oral lichen planus (OLP) is a chronic inflammatory mucosal disease that appears clinically as white papules, white plaques, or erythema with a reticulate or erosive pattern affecting predominantly the buccal mucosa, tongue, and gingiva.[1,2] Histological sections show hyperkeratosis and epithelial thinning with serrated rete

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Table I. Patient characteristics

Patient	Age	Sex	Allergy	Past history
1	37	F	Food (soymilk)	Hyperlipidemia, osteoporosis
2	64	M	None	None
3	65	M	None	Hypertension
4	59	M	None	Tinea
5	78	F	None	Emphysema
6	38	M	None	None
7	65	M	None	None
8	58	F	Pollinosis	Hypertension
9	70	F	None	Hypertension, hyperlipidemia
10	30	F	None	None

Five men, 5 women; mean age ± SD: 61.5 ± 15.9 years.

ridges as well as hydropic degeneration of basal epithelial cells with infiltrating bandlike lymphocytes, which are predominantly T cells.[3] Although the initial event in the development of OLP lesions and the factors determining OLP susceptibility remain unknown, previous studies have shown that immunological bias, bacterial or viral infections, or hypersensitivity reactions may be causal factors of OLP, and that the T-cell–mediated immune response against epithelial cells contributes to the pathogenesis.[4] It appears that OLP is a precancerous disorder,[5-7] and the World Health Organization has indeed categorized OLP as a precancerous condition, defined as "a generalized state associated with a significant increased risk of cancer."[8]

The proto-oncogene c-erb-B, referred to as epidermal growth factor (EGF) receptor (EGFR), is a well-known tyrosine kinase growth factor receptor. Four members of the EGFR family have been identified to date, namely c-erb-B/EGFR and its related products ErbB2, ErbB3, and ErbB4. The family members form homodimers or heterodimers in various combinations, and exhibit different ligand specificities for the 13 members of the EGF family.[9] Abnormal amplification of the EGFR gene has been observed widely in various human tumors, including lung carcinoma, and laryngeal and oral squamous cell carcinoma (SCC).[10-13] It is thus possible that EGFR family members and their ligands are involved in the development of oral SCC from OLP. In a previous study, the expression of ErbB2 was augmented in OLP.[14] However, detailed evaluations concerning the expressions of the EGFR family members and their ligands in OLP tissues have not been reported so far.

In the present study, we determined the expressions of 4 members of the EGFR-like receptors (c-erb-B/EGFR, ErbB2, ErbB3, and ErbB4) and 6 major EGF-like growth factors (EGF; amphiregulin [AREG], transforming growth factor α [TGFα], epiregulin [EREG], heparin-binding EGF-like growth factor [HB-EGF], and betacellulin [BTC]) in OLP and normal oral mucosa (NOM) tissues using quantitative real-time polymerase chain reaction (PCR) and immunohistochemistry.

MATERIALS AND METHODS

Patients and samples

OLP tissues were obtained from 10 patients with OLP (5 men, 5 women; mean age ± SD: 61.5 ± 15.9 years) (Table I). Patients with OLP were enrolled in this study if they presented with a histological diagnosis of OLP in the oral buccal mucosa, had no history of metal allergies or local or systemic allergic drug reactions, and had not received topical or systemic steroid therapy. All of the OLP samples were of the reticular type and taken as a spindle shape, before being divided into 2 samples. One was used for histopathological or immunohistochemical analysis and the other was used for RNA extraction. As control samples, biopsy specimens from the normal buccal mucosa of 10 healthy donors (5 men, 5 women; mean age ± SD: 26.5 ± 10.6 years) were taken in the same manner. The healthy donors had not experienced any diseases in the buccal mucosa.

The study was approved by the Research Ethical Committee of Tsurumi University, Yokohama Rosai Hospital, and National Hospital Organization, Sagamihara National Hospital, and informed consent for this study was obtained from all of the patients and healthy donors.

Preparation of tissue specimens

After biopsy, one half of each tissue specimen was immediately soaked in RNAlater RNA Stabilization Reagent (Qiagen, Tokyo, Japan) and stored at –20°C until RNA extraction.

RNA extraction and cDNA synthesis

OLP and NOM tissues were homogenized and subjected to RNA extraction using an RNeasy Mini Kit

(Qiagen). The total RNA was reverse-transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). One tenth of the cDNA was amplified with a SYBR Premix Ex Taq RT-PCR Kit (Takara Bio) according to the manufacturer's instructions.

Quantitative RT-PCR

Real-time PCR was performed using a LightCycler 2.0 (Roche Diagnostics, Tokyo, Japan), following the manufacturer's protocol. The PCR products were assessed by the fluorescence from the SYBR Green that intercalated with the DNA fragments, and melting curves were routinely recorded to verify the singularity of the products. The primer sequences for the genes tested in this study were: EREG, 5′-GTGATTCCATCATGTATCCCAGGAG-3′ (forward primer) and 5′-AGATGCACTGTCCATGCAAACAA-3′ (reverse primer); BTC, 5′-CTTCACTGTGTGGTGGCAGATG-3′ (forward primer) and 5′-ATGCAGTAATGCTTGTATTGCTTGG-3′ (reverse primer); EGF, 5′-CAACCAGTGGCTGGTGAGGA-3′ (forward primer) and 5′-GAGCCCTTATCACTGGATACTGGAA-3′ (reverse primer); AREG, 5′-GTGGTGCTGTCGCTCTTGATACTC-3′ (forward primer) and 5′-TCAAATCCATCAGCACTGTGGTC-3′ (reverse primer); HB-EGF, 5′-GGGCATGACTAATTCCCACTGA-3′ (forward primer) and 5′-GCCCAATCCTAGACGGCAAC-3′ (reverse primer); TGFA, 5′-AGATAGACAGCAGCCAACCCTGA-3′ (forward primer) and 5′-CTAGGGCCATTCTGCCCATC-3′ (reverse primer); EGFR, 5′-GGTGCGAATGACAGTAGCATTATGA-3′ (forward primer) and 5′-AAAGGTGGGCTCCTAACTAGCTGAA-3′ (reverse primer); ErbB2, 5′-CAGGCACCGCAGCTCATCTA-3′ (forward primer) and 5′-TCCCAGGTCACCATCAAATACATC-3′ (reverse primer); ErbB3, 5′-CCCAGCATCTGAGCAAGGGTA-3′ (forward primer) and 5′-TTTAGGCGGGCATAATGGACA-3′ (reverse primer); and ErbB4, 5′-TGATAGGCCGTTGGTTGTCTGA-3′ (forward primer) and 5′-CCAGGTAGACATACCCAATCCAGTG-3′ (reverse primer).

The amplified product using each primer pair was cloned into the pGEM-T vector (Promega, Tokyo, Japan) and the plasmid was linearized by enzymatic digestion for use as a quantification standard. A reference cDNA was used in every assay to assess the interassay precision. The cDNA levels among the samples were normalized by the expression level of the internal control gene GAPDH.

Histology and immunohistochemistry

For histopathology, 6-μm sections of paraffin-embedded OLP and NOM tissue specimens were stained with hematoxylin and eosin (HE). For immunohistochemical studies, the expressions of the EGFR-like receptors and EGF-like ligands were investigated using the ENVISION technique with 3,3-diaminobenzidine tetrahydrochloride as the indicator agent and commercially available monoclonal antibodies against EGFR (Abcam, Cambridge, UK), ErbB2 (Nichirei, Tokyo, Japan), ErbB3 (Abcam), HB-EGF (R&D Systems, Minneapolis, MN), AREG (R&D Systems), EREG (R&D Systems), and EGF (Abcam). To detect T cells, serial sections were stained with an anti-CD3 antibody (Nichirei).

Statistical analysis

Statistical analysis was carried out using StatView statistical analysis software (SAS, Cary, NC). Differences between the OLP and NOM tissues were determined to be significant for values of $P < .05$ using a Mann-Whitney U test.

RESULTS

Gene expression profiles of EGFR-like receptors in OLP and NOM

First, we determined the mRNA expression levels of EGFR-like receptors in OLP and NOM tissues by real-time PCR. Of the 4 EGFR-like receptors, c-erb-B/EGFR, ErbB2, ErbB3, and ErbB4, the expression of EGFR mRNA was significantly higher in OLP compared with NOM ($P < .05$) (Fig. 1, A). There were no significant differences in the ErbB2, ErbB3, and ErbB4 expressions between OLP and NOM.

Protein expression profiles of EGFR-like receptors in OLP and NOM

HE staining of OLP tissue specimens revealed lichenoid degeneration of the basal layer of the epithelium with bandlike infiltrates of lymphocytes in the superficial lamina propria (Fig. 1, B-e). We determined the protein expressions of the EGF/EGFR family members in OLP and NOM tissues by immunohistochemistry. Expression of EGFR was detected in both OLP and NOM tissue specimens, whereas expressions of ErbB2 and ErbB3 were not observed in OLP or NOM (Fig. 1, B-b, c, d, f, g, h). In NOM, the EGFR-positive cells were mainly keratinocytes in the basal and suprabasal layers (Fig. 1, B-b). In contrast, the EGFR-positive cells in OLP were mainly keratinocytes in the basal and upper spinous layers (Fig. 1, B-f). In addition to keratinocytes, mononuclear cells infiltrating the lamina propria were positive for EGFR in OLP.

Expression profiles of EGF-like growth factors in OLP and NOM

We next sought to determine which EGF-like growth factors were enhanced in the OLP lesions. We exam-

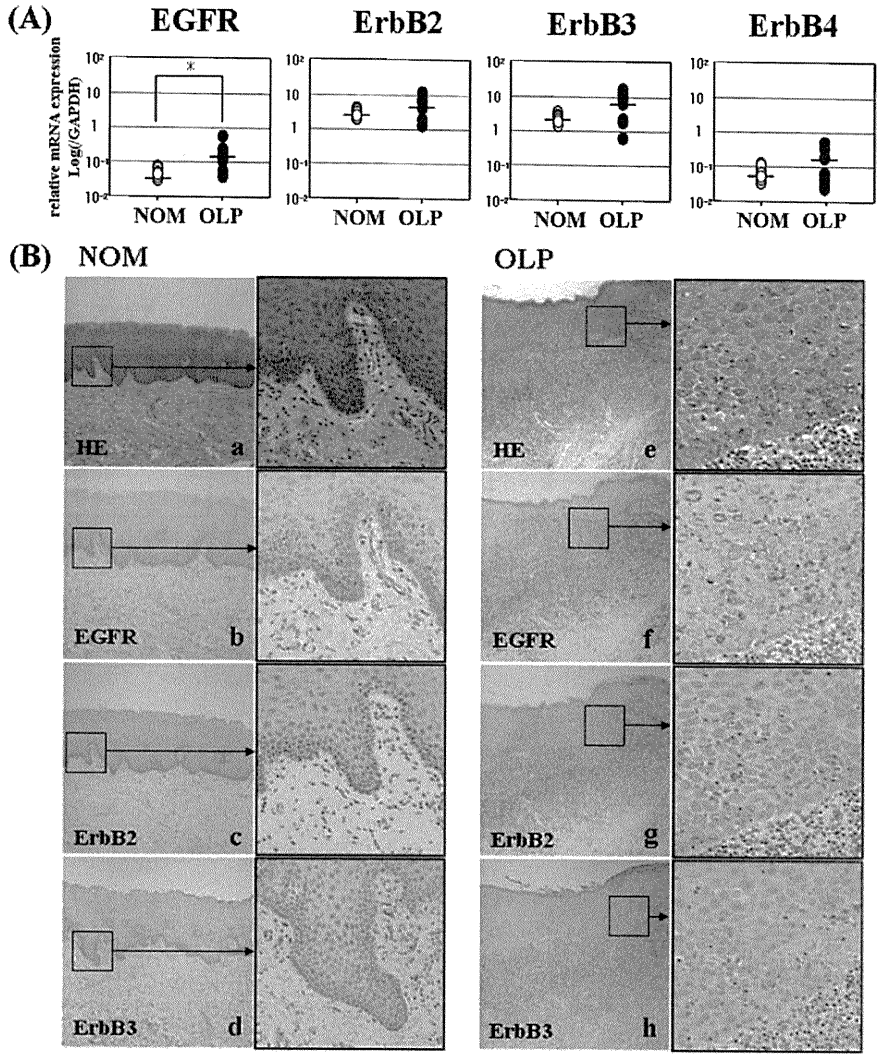

Fig. 1. Expression of EGFR-like receptors in OLP and NOM. **A,** The expression levels of mRNA of EGFR-like receptors were measured by real-time quantitative polymerase chain reaction (PCR). Each dot indicates a single sample. The GAPDH gene was used for the internal control. Out of 4 EGFR-like receptors, EGFR expression was significantly higher in OLP tissues compared with NOM tissues. $*P < .05$. **B,** Routine staining for HE and immunohistochemical analysis for the expression of EGFR, ErbB2, and ErbB3 in NOM (a to d) and OLP (e to h) tissues using commercially available monoclonal antibodies and the Envision technique using 3,3-di-aminobenzidine-tetrahydrochloride (DAB) as an indicator agent (magnifications: ×40 and ×200). Expression of EGFR was detected in both OLP and NOM tissue specimens. In NOM, EGFR-positive cells were detected in the basal layer (b), while they were also detected in the upper spinous layer, especially in the infiltrating mononuclear cells, in OLP (f).

ined the mRNA expression levels of the well-known EGF-like ligands EGF, AREG, TGFα, EREG, HB-EGF, and BTC in OLP and NOM by real-time PCR. There were significantly higher expression levels of AREG, EREG, and HB-EGF in OLP compared with NOM ($P < .01$) (Fig. 2, A). Interestingly, EGF expression was significantly lower in OLP than in NOM. TGFα and BTC showed no significant differences. These results were then confirmed by immunohistochemistry. AREG, EREG, and HB-EGF were predominantly detected in the basal layers of the epithelium in NOM, whereas they were focally found in the upper spinous layer in OLP (Fig. 2, B). Interestingly, a number of infiltrating mononuclear cells in OLP were positive for AREG, EREG, and HB-EGF (Fig. 2, B-n, o, p). Most of the infiltrating cells were CD3-positive lymphocytes in OLP lesions (Fig. 2, B-r). Otherwise, EGF was more highly expressed in NOM than OLP in keratinocytes of the basal and suprabasal layers (Fig. 2, B-q). Furthermore, mononuclear cells infiltrating the lamina propria were also positive for EGF in OLP.

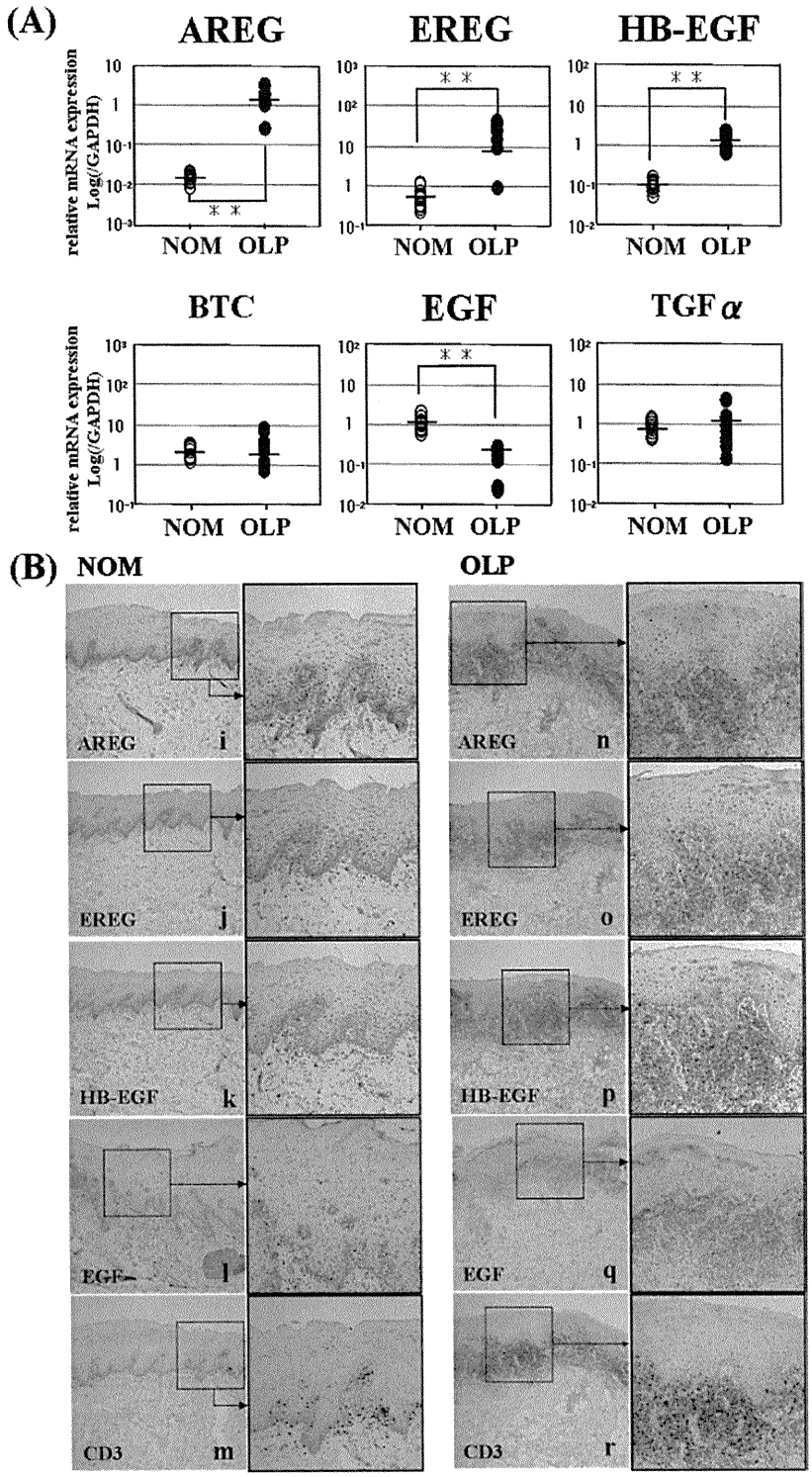

Fig. 2. Expression of EGFR-related growth factors in OLP and NOM. **A,** The mRNA expression levels of EGF-related growth factors were measured by real-time quantitative PCR. Out of 6 EGF-like ligands, AREG, EREG, and HB-EGF were expressed significantly higher in OLP tissues compared with NOM tissues. $^{**}P < .01$. **B,** Immunohistochemical analysis for the expression of AREG, EREG, HB-EGF, EGF, and CD3 in NOM (i to m) and OLP (n to r) tissues (magnifications: ×40 and ×100). AREG, EREG, and HB-EGF were expressed in both keratinocytes and infiltrating mononuclear cells at the lamina propria in OLP lesions (n, o, p). EGF was more highly expressed in NOM than in OLP in the keratinocytes of the basal and suprabasal layers (q). Furthermore, mononuclear cells infiltrating the lamina propria were also positive for EGF in OLP.

DISCUSSION

In the present study, we showed that there was a significantly higher level of EGFR expression in OLP compared with NOM. This suggests that the keratinocytes in OLP may be more sensitive to stimulation with ligands for EGFR than those in NOM. Similar to the present observation, it has been shown that the expression of EGFR is localized to the basal layer in NOM and is widely distributed in the spinous layer in leukoplakia.[15] Thus, in the normal state, EGFR is expressed in the basal layer where cells proliferate constantly, whereas it is expressed in the upper spinous layer in abnormal conditions, such as OLP or leukoplakia. The histopathology of OLP shows saw-tooth–shaped elongation of the rete ridges. Because EGFR is essential for keratinocyte proliferation, it is possible that the up-regulated EGFR expression is involved in the mechanisms of the rete ridge elongation. We further showed that the EGFR ligands AREG, EREG, and HB-EGF were more highly expressed in OLP than in NOM, whereas EGF was down-regulated in OLP. EGF can elicit a variety of biological actions, including the proliferation and differentiation of epithelial and mesenchymal cells.[16] In addition, EGF plays a central role in not only maintaining the homeostasis of epithelial cells, but also inflammation and immunological responses.[17] OLP lesions show a shift of expression from EGF to AREG, EREG, and HB-EGF. Furthermore, these ligands were secreted mainly by infiltrating mononuclear cells at the lamina propria in OLP lesions (Fig. 2, B). Therefore, it is possible that the control of T cells in OLP patients may provide a new effective treatment strategy for OLP. Regarding AREG and HB-EGF, it has been shown that AREG and HB-EGF in combination with TNFα stimulate keratinocyte proliferation.[18] We and others have shown that TNFα is up-regulated in OLP,[19,20] suggesting that TNFα may combine with AREG and HB-EGF to act as powerful mitogens for keratinocytes in OLP.

It is well established that SCC develops from OLP lesions, but the mechanism of the carcinogenesis in OLP is still unclear.[5-7] EGFR has been shown to be involved in the carcinogenesis of various cancers, including lung carcinoma, and laryngeal and oral SCC.[10-13] It has also been shown that AREG, EREG, and HB-EGF are secreted by oral SCC cells or cigarette extract–stimulated cells.[21-25] Recently, it was reported that the interaction of AREG and EGFR is essential for tobacco smoke–induced DNA synthesis in a human oral epithelial cell line, suggesting that tobacco-related carcinogenesis in the oral mucosa may involve EGFR signaling via AREG.[26] Taken together with our observations that both EGFR and its ligands are up-regulated in OLP, we suggest that the amplification of EGFR signaling in OLP may cause changes in the activation of other cell-surface proto-oncogene proteins and/or the transcription of other proto-oncogenes, thereby distorting the control of epithelial cell growth, and eventually leading to a malignancy.

Alternatively, some investigators have suggested that the atrophic epithelium of OLP may be more susceptible to the action of carcinogens.[27] Another study has suggested that the chronic inflammatory host response to the atypical cells of epithelial dysplasia can appear virtually indistinguishable histopathologically from OLP, particularly in milder cases of epithelial dysplasia.[28] Such ambiguity may contribute to the controversy related to the malignant transformation potential of OLP, but further studies based on analyses of signaling pathways will promote our understanding of the mechanisms of OLP and aid diagnosis and treatment.

It has also been suggested that, in OLP lesions, the close interaction between infiltrating T cells and keratinocytes as well as intrinsically released substances, such as cytokines and growth factors, may change the nature of epithelial cells.[4] We observed that AREG, EREG, and HB-EGF were immunohistochemically detectable not only in keratinocytes but also in the infiltrating mononuclear cells (Fig. 2, B). In this study, EGF-like ligands released from infiltrating mononuclear cells adjacent to the epithelium bound to EGFR on epithelial keratinocytes, thus stimulating epithelial cell division. Recent studies have shown that AREG and EREG play roles in immune-related responses.[29,30] EREG seems to be essential for the down-regulation of interleukin (IL)-18, whereas AREG acts as a Th2 cytokine and enhances resistance to nematodes. It is possible that AREG and EREG also regulate the inflammatory process via immune modulation in OLP lesions, although the precise mechanisms require further investigation.

In conclusion, the expression levels of the EGF/EGFR family members, especially EGFR and its ligands, may play a role in the pathogenesis of OLP. Further analyses of the EGFR signaling pathway in OLP would clarify the premalignant nature of OLP, and EGFR and its ligands may be possible new targets for the treatment of OLP.

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Evidence for the changes of antitumor immune response during lymph node metastasis in head and neck squamous cell carcinoma

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Objective. This study aimed to elucidate the differences in antitumor immune responses between primary tumors and metastatic regional lymph nodes in head and neck squamous cell carcinoma (HNSCC).
Study design. The clonality of tumor-infiltrating lymphocytes in tissue specimens from 17 HNSCC patients was examined regarding their T-cell receptor (TCR) repertoires and their complementary determining region 3 (CDR3) size spectratyping. Cytokine expression profiles and T-cell phenotypes also were measured by using real-time quantitative polymerase chain reaction.
Results. The host immune responses to HNSCC cells, reflected by the TCR repertoire, differed between primary tumors and metastatic lymph nodes. $CD8^+$-T cells and T helper type 1 (T_H1)/T cytotoxic 1 (T_C1) cell cytokine production in metastatic and nonmetastatic lymph nodes were similar.
Conclusions. The antitumor immune response to HNSCC cells changes during lymph node metastasis, and HNSCC cells can escape the cytotoxic immune responses mediated by $CD8^+$-T cells and T_H1/T_C1 cells. These results suggest that lymph node metastasis might be associated with changes in the nature of the primary tumor antigens. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;110:341-350)**

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant tumor worldwide, characterized by locoregional disease with a propensity for metastasis to the cervical lymph nodes.[1] The presence of lymph node metastasis is the single most important prognostic factor for survival.[2] Despite advances in surgical and adjuvant chemoradiotherapy, the incidence of and mortality from HNSCC are increasing in several countries, and the 5-year survival rate of 50% has failed to improve over the past few decades.[1,3]

There has been an ongoing debate regarding the clinical, pathologic, and biologic prognostic factors for

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HNSCC. Cervical lymph node metastasis cannot always be predicted from the size or extent of invasion of primary tumors. Furthermore, little is known about the molecular mechanisms leading to lymph node metastasis, and more evidence is needed to clarify the metastatic mechanisms of HNSCC.

Many experimental and clinical studies have suggested that the immune system may exert some control over the development of certain tumors. An earlier study demonstrated that the immunologic surveillance system is continually sensitized against transformed cells and provides the first line of defense against the development of certain tumors.[4] Solid malignant tumors are often infiltrated by tumor-infiltrating lymphocytes (TILs), and extensive lymphocyte infiltration has been associated with a more favorable prognosis in patients with various cancers.[5,6] TILs can be propagated in vitro in the presence of cytokines and can acquire strong, and sometimes specific, antitumor activities.[7] Cytokine-activated TILs have been used for immunotherapy in patients with metastatic melanoma and other tumors.[8,9] HNSCC often contain large mononuclear cell infiltrates, composed mainly of T cells, which could reflect an in situ immune reaction against the malignant HNSCC cells.[10] Extensive lymphocyte infiltrates have been associated with a more favorable prognosis in patients with HNSCC.[11] These observations suggest that the antitumor response mediated by T cells may contribute to the control of HNSCC growth. However, other studies have shown that TILs isolated from metastatic tumors are functionally deficient, and this deficiency at the tumor site could be associated with the tumor metastasis.[12,13]

During an immune response to tumor antigens, antigen-specific T cells undergo clonal expansion. T cells bearing T-cell receptors (TCRs) recognize antigens in the form of peptide fragments in association with major histocompatibility complex (MHC) class I and class II molecules on antigen-presenting cells.[14] The fine specificity of T cells is determined by the TCRs displayed on the cell surface, which comprise heterodimers of an α- and a β-chain or of a γ- and a δ-chain. The variable regions of these chains are responsible for antigen recognition and are encoded by variable (V), joining (J), and diversity (D) gene segments. We previously developed an adaptor ligation–mediated polymerase chain reaction (AL-PCR) method that allows TCR repertoires to be defined based on the expression levels of transcripts, even when only small numbers of cells are available. This method enables us to amplify all of the variable regions of the rearranged TCR genes through PCR cycles without skewing. Applying this method to a microplate hybridization assay (MHA) is simple and reproducible and enables the rapid analysis of TCR repertoires.[15] Random insertions of nongerminal element (N) nucleotides or deletions of nucleotides have been observed in the VN (D) NJ junction region, designated the complementary determining region 3 (CDR3), and are thought to be responsible for the antigenic peptide content.[16,17] Thus, any specific recognition of antigens by CDR3 can lead to the clonal expansion of T cells. Because CDR3 has different sequences and lengths, it is possible to analyze the diversity of TCRs using a CDR3-size spectratyping method that provides a rapid scan of all TCR V-region transcripts grouped according to the utilized V-region gene and the chain length.[18] In HNSCC, TCR diversity is largely due to the $\alpha\beta$-chain.[19] We previously reported that TCR repertoires were similar among multiple tissue specimens from different sites in the same tumors, and that a limited number of TILs locally expanded in response to tumor antigens.[20]

Cytokines are also key molecules modulating the function of T cells. T helper type 1 (T_H1), T_H2, T cytotoxic type 1 (T_C1), and T_C2 cells play immunoregulatory roles.[21] Alterations in T_C1/T_C2 subsets may cause host antitumor immune responses.[22] The phenotype of TILs in HNSCC is composed mainly of $CD8^+$-T lymphocytes; however, changes in TIL phenotype and cytokine production associated with metastasis are a controversial issue.

In the present study, we hypothesized that restricted TCR usage may reflect prolonged exposure of the host immune system to putative tumor-associated antigens, and that primary HNSCCs can escape these specific T-cell immune responses, allowing them to be transported to and proliferate in the cervical lymph nodes where they then form metastatic lesions. To elucidate the differences in antitumor immune responses between primary tumors and regional lymph nodes, we examined the characteristics of the TILs within HNSCC tissue specimens regarding TCR repertoires and CDR3-size spectratyping. In addition, we established the phenotype of T cells and the cytokine environment using real-time quantitative PCR.

MATERIALS AND METHODS

Patients and samples

HNSCC tissue specimens were obtained from 17 patients with HNSCC who underwent surgical treatment including unilateral or bilateral neck dissection in Tsurumi University Hospital or Toshiba Rinkan Hospital (Table I). The patient group comprised 10 women and 7 men with an age range of 49-90 years (mean 67.1 y). Out of 17 patients, 3 patients were recurrent in cervical lymph nodes within 1 year. The diameter of the primary tumor (T) and the number of lymph nodes involved with the metastatic tumor (N) were classified according to the Union Interna-

Table I. Characteristics of patients with head and neck squamous cell carcinoma

Patient	*Age, y*	*Gender*	*Primary site*	*TN*	*Clinical stage*	*Lymph node metastasis*	*Recurrence*	*Past History*
1	74	F	Lower gingiva	T4N0	IV	−	−	None
2	80	F	Lower gingiva	T1N0	I	−	−	Stomach cancer
3	90	M	Lower gingiva	T3N0	III	−	−	Pulmonary tuberculosis, malaria, cataract
4	70	F	Buccal mucosa	T2N0	II	−	−	Colon polyp
5	78	M	Tongue	T3N0	III	−	−	None
6	67	F	Tongue	T2N0	II	−	−	Hypertension
7	72	F	Lower gingiva	T2N0	II	−	−	None
8	57	F	Tongue	T2N0	II	−	−	None
9	73	M	Buccal mucosa	T2N0	II	−	−	Hypertension, stomach cancer
10	72	M	Mouth floor	T2N1	III	+	−	Hypertension
11	77	F	Tongue	T3N2c	IV	+	+	Uterine fibroid, cataract
12	49	F	Mouth floor	T4N2b	IV	+	−	None
13	70	F	Upper gingiva	T2N1	III	+	−	None
14	72	F	Tongue	T4N2c	IV	+	+	Tongue leukoplakia
15	55	M	Tongue	T4N2b	IV	+	+	Rhinosinusitis
16	55	M	Tongue	T3N1	III	+	−	None
17	58	M	Tongue	T4N2c	IV	+	−	Stomach ulcer

tionale Contre le Cancer (UICC). Clinical stage was determined by the UICC TN staging system. Informed consent was obtained from each of the patients. The study was approved by the local Research Ethical Committees at Tsurumi University, Toshiba Rinkan Hospital, and National Sagamihara Hospital. Specimens were taken from HNSCC primary tumors and regional lymph nodes. Control peripheral blood samples were obtained from 10 healthy age-matched volunteers. The tissue specimens were obtained immediately after surgery and were soaked in RNAlater (Qiagen, Hilden, Germany). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by using lymphocyte separation medium (H-SMF; Jimro Co., Gunma, Japan) gradient centrifugation. The specimens were cryopreserved until RNA extraction.

TCR repertoire analysis

Crude cellular RNAs from HNSCC specimens were extracted using an RNeasy Lipid Tissue Kit (Qiagen) according to the manufacturer's instructions. The AL-PCR and MHA methods were carried out as described previously.[15] Briefly, 1 mg total RNA was reverse-transcribed to double-stranded complementary DNA (cDNA) by using a SuperScript II cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, except for priming with a BSL-18B primer adaptor containing a Not I site. The P20EA/P10EA universal adaptors were ligated at the 5′ ends of the BSL-18B primed cDNA. Three rounds of Ca- and Cb-specific PCR were performed using Ca and Cb sequence–specific oligonucleotide probes (SSOPs), respectively, to prepare amplified and biotinylated TCR cDNA pools. Hybridization was carried out between biotinylated PCR products and AV or BV SSOPs immobilized on carboxylate-modified enzyme-linked immunosorbent assay plates (Sumitomo Bakelite, Tokyo, Japan). Positive hybridization was detected using p-nitrophenylphosphate (Nacalai Tesque, Osaka, Japan), and the visualized signals were estimated at 405 nm using a Multiskan JX Microplate Reader (Thermo Labsystems, Helsinki, Finland). Relative expansion of the TCRAV or TCRBV repertoires were calculated using the following formula: frequency (%) = 100 × (corresponding SSOP signal)/(ΣTCRV SSOP signals). In the TCR repertoire analysis, PBMCs from 10 healthy donors were used as control samples.

T-Cell clonality analysis with CDR3-size spectratyping

The second PCR products described above were labeled by 20 cycles of PCR amplification with fluorescent dye–labeled C-region SSOPs. After mixing with size markers (CEQ DNA Size Standard Kit 600; Beckman Coulter, Fullerton, CA), the labeled PCR products were loaded onto a polyacrylamide sequencing gel (CEQ Separation Gel LPA I; Beckman Coulter), and their sizes and fluorescence intensities were determined by using an automated capillary DNA sequencer (CEQ 8000; Beckman Coulter). Data were analyzed by using CEQ 8000 Genetic Analysis System software (Beckman Coulter).

Determination of nucleotide sequence of CDR3 regions

Among the recurrent patients, patient 11 showed similar CDR3 signal patterns within the primary tumor and the metastatic lymph node in the skewed TCR subfamilies; therefore, we determined the nucleotide sequences of the CDR3 region of patient 11. PCR was performed using

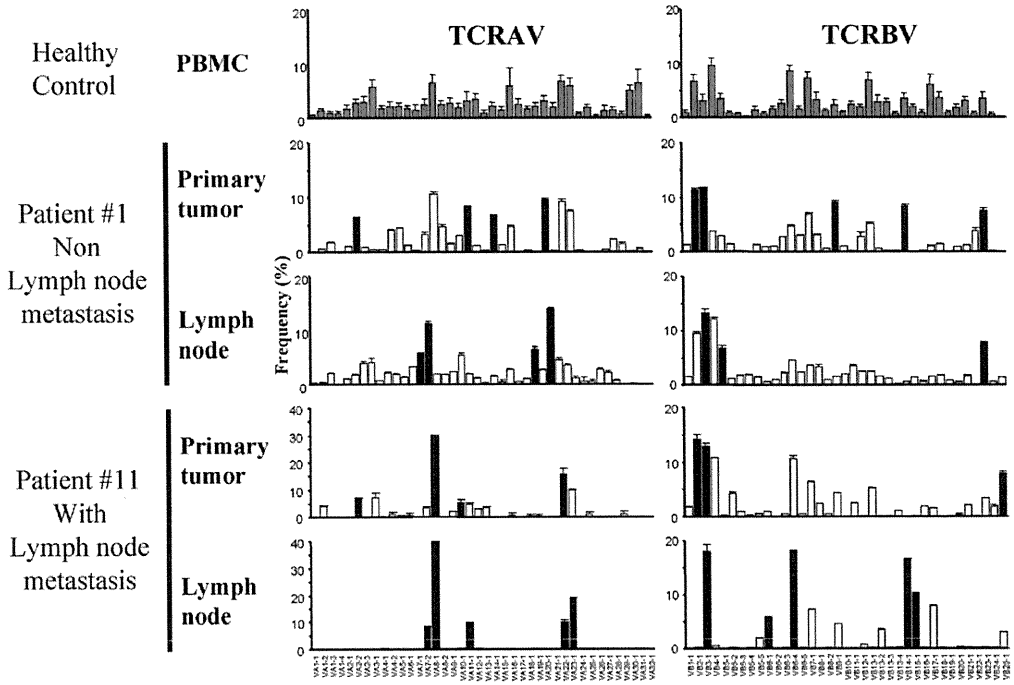

Fig. 1. Representative results of T-cell receptor variable region α-chain (TCRAV) and TCR β-chain (TCRBV) repertoires in patients with head and neck squamous cell carcinoma (HNSCC). TCRAV (**left**) and TCRBV (**right**) repertoires were analyzed in primary tumors and regional lymph nodes. Percentage frequencies of expression levels in each segment are shown. Segments showing significantly elevated expression levels relative to the control are shown by solid bars. *PBMC,* Peripheral blood mononuclear cells.

the cDNA clones from both tissues, with a forward primer specific for the variable region (VA8-1 or VA22-1 or VB3-1) and a reverse primer specific for the constant region (CA4 or CB4), under the conditions described above. The primers used in this study were: VA8-1: TGGGCGAAAAGAAAGACCAAC; VA22-1: GACAAGGGAAGCAACAAAGGT; VB3-1: ACTTCAGTGAGACACAGAGAAA; CA4: ATAGGCAGACAGACTTGTCACTG; and CB4: ACACCAGTGTGGCCTTTTGGGTG.

After elution from the agarose gel, the PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI). DH5-competent cells were transformed with the recombinant plasmid DNA. Sequence reactions were performed using the GenomeLab DTCS Quick Start Kit (Beckman Coulter) and analyzed by using CEQ 8000 Genetic Analysis System software (Beckman Coulter).

Real-time PCR

The mRNA expression levels of CD markers and cytokines, including CD4, CD8, interleukin (IL) 2, IL-4, IL-5, and interferon (IFN) γ, were examined in tissue specimens by real-time quantitative PCR using a LightCycler (Roche Diagnostics, Tokyo, Japan). Total RNA was reverse-transcribed into cDNAs using a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). One-tenth of each cDNA sample was amplified using a Sybr Premix Ex Taq RT-PCR Kit (Takara Bio) according to the manufacturer's instructions. Primer pairs were obtained from Takara Bio. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an internal control. The results were expressed as the ratio of the amount of the respective cytokine-specific cDNA to the amount of GAPDH-specific cDNA.

Statistical analyses

Statistical analyses were carried out by using StatView version 5·0 for Windows (SAS Institute, Cary, NC). The nonparametric Mann-Whitney rank test was applied for comparison of the results from the real-time PCR assays. The results of the comparisons are given as median values. P values of $<.05$ were considered to indicate statistical significance.

RESULTS

TCR repertoire analysis

TCR repertoires were analyzed for primary tumors and lymph nodes from 17 patients (Table I). To inves-

Table II. T-Cell receptor variable region T-cell receptor α-chain (TCRAV) and β-chain (TCRBV) segments with high levels of expression*

Patient	Lymph node metastasis	TCRAV		TCRBV	
		Primary tumor	Lymph node	Primary tumor	Lymph node
1	−	2-2,11-1,14-1,20-1	7-2,8-1,20-1,22-1	2-1,3-1,9-1,14-1.23-1	3-1.5-1.23-1
2	−	7-2,12-1,17-1,21-1,23-1	8-1,22-1	2-1,6-4	3-1,5-1
3	−	12-1,23-1	8-1	1-1,2-1,3-1	3-1,5-1
4	−	—	22-1	2-1	5-1
5	−	4-1	11-1,22-1	3-1,4-1,5-1	3-1,5-1
6	−	1-4,15-1,16-1,21-1,25-1,29-1	—	17-1,23-1,25-1	5-1
7	−	12-1,13-1,22-1,24-1	—	2-1,6-4,6-5,14-1,22-1	2-1,6-3,6-5
8	−	—	—	2-1,3-1,5-1,13-3,20-1	2-1,3-1
9	−	8-1,11-1,22-1,23-1	8-1,11-1,22-1	2-1,6-3,6-4,6-5	3-1,5-1
10	+	3-1,11-1,12-1,22-1,23-1	4-1,8-1,8-2,22-1	1-1,2-1,6-3,13-3,21-1	3-1,5-1
11	+	2-2,8-1,10-1,22-1	7-2,8-1,11-1,22-1,23-1	2-1,3-1,25-1	3-1,6-1,6-4,14-1,15-1
12	+	12-1,22-1	10-1	3-1,5-1	2-1,3-1,5-1
13	+	—	11-1,22-1,24-1	3-1,5-1,5-5	2-1,3-1,6-4
14	+	12-1,22-1,24-1	13-1,21-1,24-1,29-1,32-1	2-1,5-1,14-1,23-1	2-1,3-1,5-1,5-3,5-5
15	+	4-1,8-2,22-1	9-1,23-1	1-1,2-1,5-1,21-1	2-1,5-1
16	+	4-1,20-1	22-1	3-1,6-4	3-1,5-1
17	+	8-1,9-1,13-1,24-1	22-1	2-1,12-1,14-1	2-1,3-1,5-1

*Significant increase in frequency was defined as >3 SDs above the mean percentage; based on peripheral blood mononuclear cells.

tigate the pattern of antitumor response to HNSCC, we initially examined the TCR repertoire within HNSCC tissue specimens. The representative results of TCR repertoire analysis are shown in Fig. 1. We defined the increase as significant when: 1) the percentage increase was >3 SDs greater than the mean percentage based on PBMCs from 10 healthy controls; and 2) the absolute percentage increase was >5%. The expression level was defined as unchanged if these criteria were not met (Table II).

The high frequency of TCR variable segments reflected the presence of antigen-specific T cells driven by HNSCC cells. We found that the skewed TCR subfamilies varied markedly in both the primary tumors and the lymph nodes within individuals (Fig. 1; Table II). There was no difference in the mean numbers of variable segments between nonmetastatic and metastatic patients (Table II). Significant increases above the control were detected in limited AV and BV segments, such as VB2-1, VB3-1, and VB5-1, in both the primary tumors and the lymph nodes. However, significant increases were observed within primary tumor alone or lymph node alone in most variable segments other than these segments. This indicates a difference in the TCRAV and TCRBV repertoires between primary tumors and lymph nodes in individual patients.

T-Cell clonality in increased TCR repertoires

We used CDR3-size spectratyping to study if T-cell clonality was present in the increased TCR repertoires. T-Cell clonality was confirmed by CDR3-size spectratyping, and T cells bearing skewed subfamilies were therefore subjected to CDR3-size spectratyping of the TCR repertoires. Almost all of these TCR repertoires demonstrated monoclonal expansions in primary tumors and in metastatic lymph nodes. The TCR repertoires in nonmetastatic lymph nodes often showed polyclonal patterns, which reflected the variety of TCRs [Fig. 2 (a)]. Interestingly, some subfamilies showed monoclonal patterns in pathologically nonmetastatic lymph node specimens [Fig. 2 (b)]. In patients with lymph node metastasis, monoclonal spectratyping patterns were often observed in different TCR families. These monoclonal signals may be caused by the tumor antigens (Fig. 2d). In patient 11, the CDR3 signal patterns were similar in specimens from the primary tumor and metastatic lymph node [Fig. 2 (c)].

Next, to examine if the clonal peaks with similar sizes were due to clonal expansion of identical T cells, we determined the nucleotide sequences of the CDR3 region of cDNA clones from patient 11. TCRAV and BV gene segments were described according to the nomenclature reported by Arden et al.[23] and Wei et al.[24] TCRAJ and BJ gene segments were also described according to the nomenclature reported by Koop et al.[25] and Toyonaga et al.[26] cDNA clones with identical nucleotide sequences were obtained from the primary tumor and the metastatic lymph node in this patient. There were some common amino acid sequences in the CDR3 regions of the cDNA clones of the VA8-1 and VA22-1 subfamilies. However, we observed identical nucleotide sequences in the CDR3 cDNA clones from

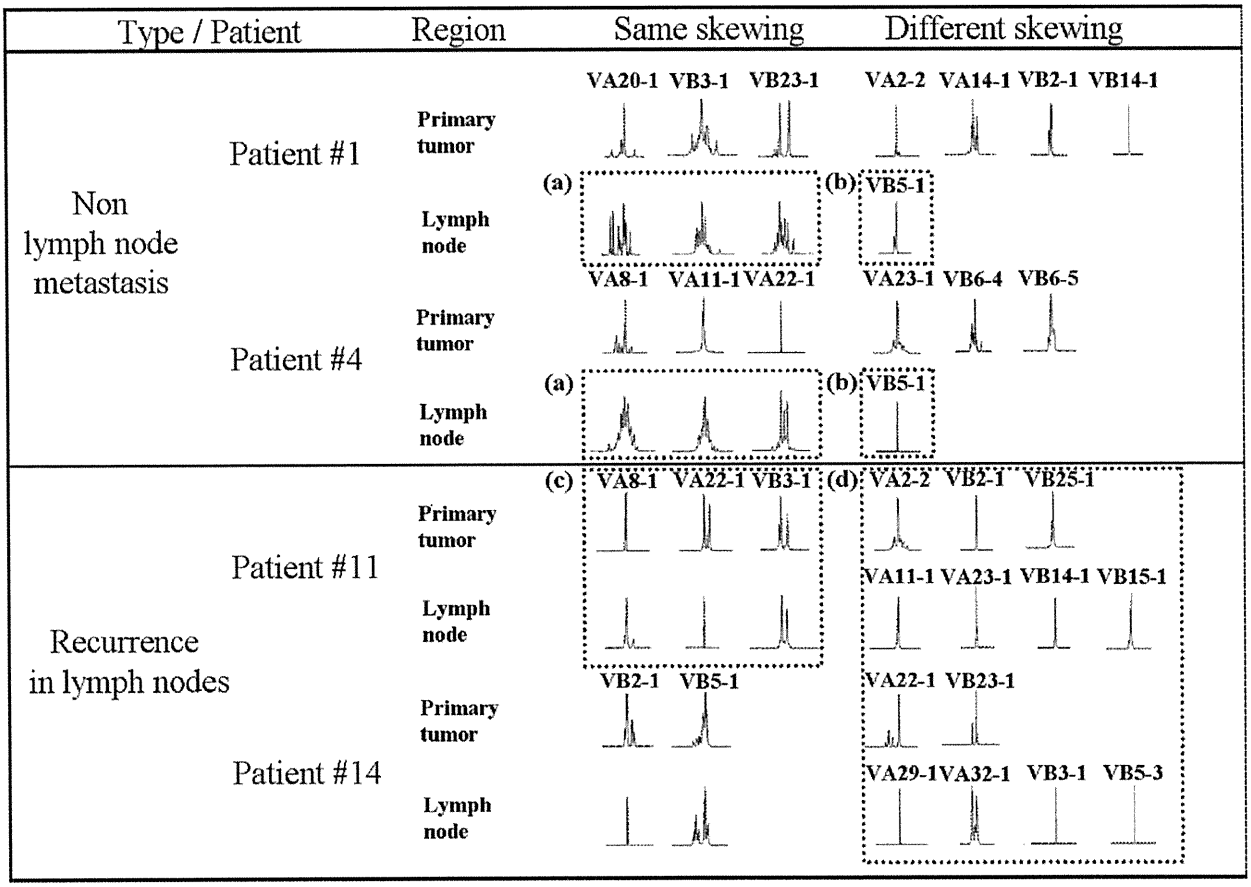

Fig. 2. Complementary determining region 3 (CDR3)–size spectratyping profiles of primary tumors and regional lymph nodes. In patients with nonmetastatic lymph nodes, the lymph nodes often showed polyclonal spectratyping patterns, representing maintenance of the variety of T-cell receptors (TCRs) (a). In differently skewed TCR families, monoclonal spectratyping patterns were also observed in the nonmetastatic lymph nodes (b). In patient 11, the CDR3 signal patterns were similar in the primary tumor and the metastatic lymph node (c). In patients with lymph node metastasis, monoclonal spectratyping patterns were often observed in different TCR families (d).

the primary tumor and the metastatic lymph nodes for the VB3-1 subfamily (Table III). Thus, the nucleotide sequences of the CDR3 regions in patient 11 partially differed between the primary tumor and the metastatic lymph node, indicating that the antitumor immune response had changed during lymph node metastasis.

Quantification of T-cell phenotype markers and cytokine expression

mRNA expression was examined to identify the $CD4^+$- and $CD8^+$-T–cell phenotype of the T cells infiltrating the HNSCC specimens. We detected no differences in tissue-associated $CD4^+$- and $CD8^+$-T–cell mRNA expression between the metastatic and nonmetastatic lymph nodes, indicating that cytotoxic T cells did not infiltrate the metastatic lymph nodes (Fig. 3). We then examined IL-2, IL-4, IL-5, and IFN-γ mRNA expression levels to determine the cytokine environment in the HNSCC specimens (Fig. 3). The expression levels of these cytokines were not significantly different between metastatic and nonmetastatic lymph nodes (Fig. 3). Thus, no significant increases in $CD8^+$-T–cell or T_H1/T_C1 cytokine production could be identified in the metastatic lymph nodes.

DISCUSSION

Using sensitive and reliable AL-PCR and MHA methods,[15] we investigated the clonality of TILs infiltrating HNSCC specimens regarding their TCR repertoires. Clonality analysis, coupled with quantitative analysis of the TCR repertoire, was able to provide important information about TILs infiltrating HNSCC. When tissue samples containing a small number of T cells are used for the clonality analysis, the clonality might be increased through PCR cycles. However, an increase in clonality, accompanied by an elevated fre-

Table III. Alignment of amino acid sequences of complementary determining region 3 regions from complementary DNA clones from patient 11

	Sample	*Frequency*	*AV/BV segment*	*AV/BV sequence*	*N/N-D-N*	*AJ/BJ sequence*	*AJ/BJ segment*
VA8-1	Primary tumor	8/11	AV13-1	CAA	LP	FSGGYNKLIFGAGTRLAVHP	AJ04
		3/11	AV13-1	CAA	L	TGGGNKLTFGTGTQLKVEL	AJ10
	Lymph node	9/9	AV13-1	CAA	L	TGGGNKLTFGTGTQLKVEL	AJ10
VA22-1	Primary tumor	6/10	AV9-2	CAL	SGA	NTGNQFYFGTGTSLTVIP	AJ49
		4/10	AV9-2	CAL	PNSGY	STLTFGKGTMLLVSP	AJ17
	Lymph node	7/9	AV9-2	CAL	SGA	NTGNQFYFGTGTSLTVIP	AJ49
		2/9	AV9-2	CAL	PNSGY	STLTFGKGTMLLVSP	AJ17
VB3-1	Primary tumor	8/17	BV28	CAS	SFG	NQPQHFGDGTRLSIL	BJ1-5
		5/17	BV28	CAS	GTGTSGSR	TQYFGPGTRLLVL	BJ2-5
		2/17	BV28	CAS	SPQMGQGA	YNEQFFGPGTRLTVL	BJ2-1
		1/17	BV28	CAS	GTGTSGSR	ETQYFGPGTRLLVL	BJ2-5
		1/17	BV28	CAS	SYPFSLADNEQF	FGPGTRLTVL	BJ2-3
	Lymph node	13/15	BV28	CAS	SLLETANTGELFFGE	GSRLTVL	BJ2-6
		2/15	BV28	CAS	SPTPL	TQYFGPGTRLLVL	BJ2-5

quency of expression provides evidence for the existence of a large population of monoclonal T cells in the lesions.

We found increased T-cell clonality in the majority of TCR variable segments, with increased frequencies in primary tumors and metastatic lymph nodes. TILs, like most mature T cells, probably recognize tumor antigens through the TCR, illustrating the relevance of our results. Previous studies have shed light on the specific subsets of lymphocytes expressing TCRs that are important in tumor surveillance. The relative contributions of αβ and γδ T cells in blocking primary tumor formation were explored in αβ T-cell −/− (lacking the TCR β-chain) or γδ T-cell −/− (lacking the TCR δ-chain) mice.[27] In humans, it has been predicted from cancer immunosurveillance finding that transplant patients who are immunosuppressed, or individuals with primary immunodeficiencies, have significantly higher relative risks of cancer development.[28] These studies provide strong support for the existence and physiologic relevance of cancer immunosurveillance. However, metastasis is associated with failure of the cancer immunosurveillance system, which would have allowed cancer cells to be transported to and proliferate within metastatic lesions.

To gain an insight into the clonality of TILs regarding increased TCR variable segments, we analyzed CDR3. We detected some differences in monoclonal patterns in the different TCR subfamilies between primary tumors and metastatic lymph nodes by CDR3 spectratyping. Furthermore, to investigate the changes of CDR3 regions during lymph nodes metastasis, we determined the nucleotide sequences of the monoclonal patterns in the same TCR subfamilies between primary tumors and metastatic lymph nodes, especially in patients with recurrences. The CDR3 signal patterns of patient 11 were similar for the VA8-1, VA22-1, and VB3-1 subfamilies; however, in the VB3-1 subfamily, the T-cell clonality in the primary tumor lesion partly changed during lymph node metastasis. This observation indicates that immunologic responses to tumor antigens differ between primary tumors and metastatic lymph nodes within individuals. This suggests that the development of lymph node metastases may be associated with changes in the nature of the primary tumor antigen. TILs have been considered to be a manifestation of the host immune reaction to the cancer cells, although their pathophysiologic significance in human cancer tissue remains controversial. One of the major reasons for this was the premise that human cancer arises through evasion of the host immune surveillance, either as a result of weak immunogenicity of the tumor cells or as a result of certain immunosuppressive effects of the tumor cells. Accordingly, the characteristics of TILs should be investigated.

To examine the characteristics of TILs, we examined the T-cell phenotype and cytokine production within HNSCC tissue specimens using real-time quantitative PCR. T cells are functionally characterized by their pattern of cytokine production, and the local production of cytokines probably affects the function of TILs. We detected no significant differences in tissue-associated $CD4^+$- and $CD8^+$-T–cell mRNA expression between metastatic and nonmetastatic lymph nodes. Accumulating evidence indicates that the presence of $CD8^+$-T cells in TILs contributes to improved survival of patients.[29] This suggests that the immune reaction mediated by $CD8^+$-T cells with a cytotoxic phenotype can reduce the aggressiveness of cancer cells, despite presumptive immunosuppressive environments. The pres-

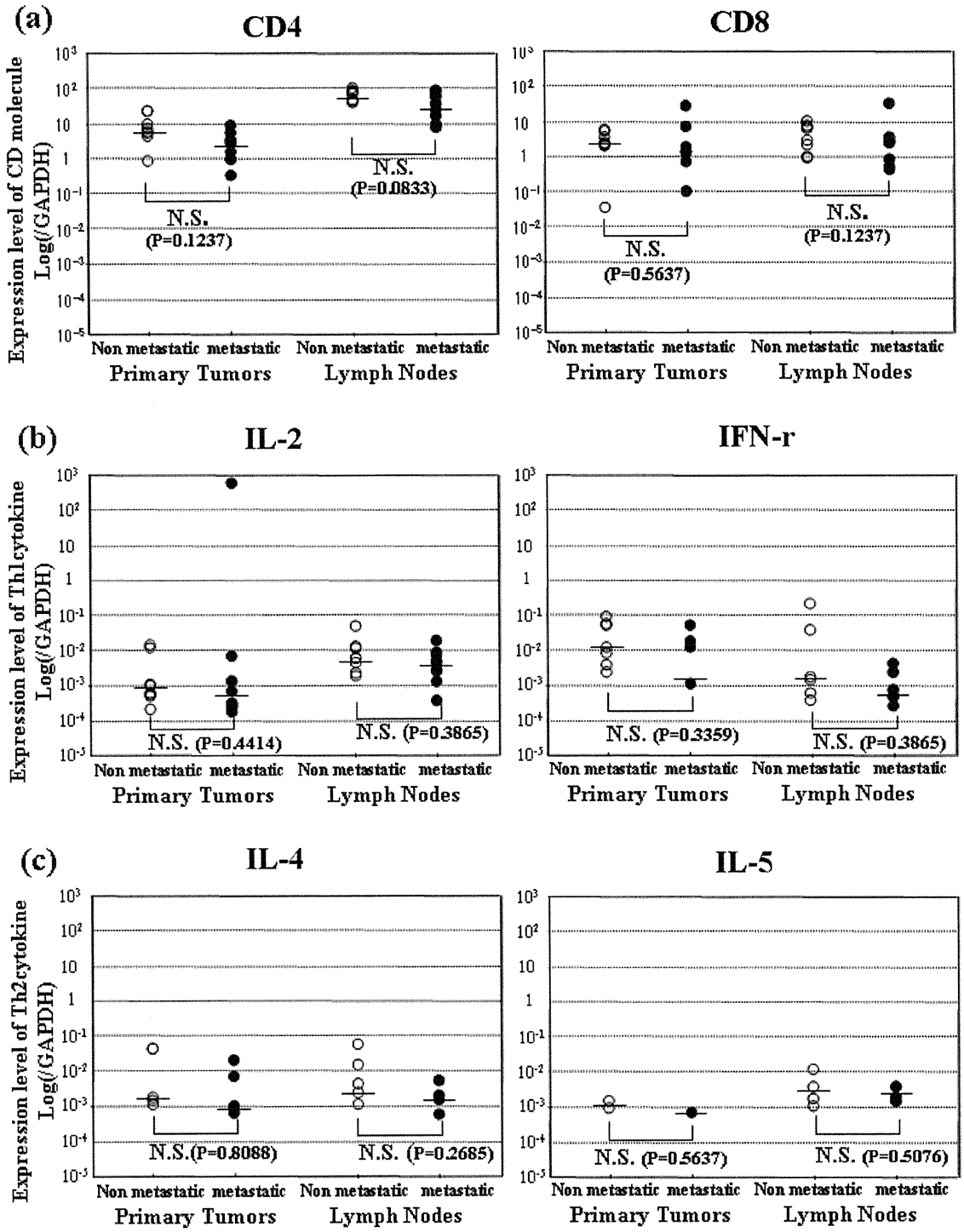

Fig. 3. Comparison of T-cell phenotypes and expression levels of several cytokines between primary tumors and regional lymph nodes. Expression levels were compared between nonmetastatic lesions (n = 9) and metastatic lesions (n = 8). The mRNA expression levels of CD molecules (**a**), the T_H1 cytokines interleukin (IL) 2 and interferon (IFN) γ (**b**), and the T_H2 cytokines IL-4 and IL-5 (**c**) were measured by real-time quantitative polymerase chain reaction (PCR). Each dot indicates a single sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an internal control. There were no significant increases in CD molecules or T_H1 and T_H2 cytokines between metastatic and nonmetastatic lesions. The expression levels of $CD4^+$- and $CD8^+$-T cells are not increased in metastatic lymph nodes compared with nonmetastatic lymph nodes (**a**). No T_H1 or T_H2 cytokines were significantly increased in metastatic lesions compared with nonmetastatic lesions (**b** and **c**). *N.S.*, Not significant.

ence of activated T helper cells correlated with the frequencies of cytotoxic T lymphocytes that exerted cytotoxic activity in metastatic tumors. In the present study, the cytotoxic activity mediated by $CD8^+$-T cells and T_H1/T_C1 cytokine production was not increased in metastatic lymph nodes, and tumor antigens must therefore have a means of escaping the host immune response.

T-Cell–based immunotherapies have been used, especially in patients with metastatic melanoma, although this type of therapy may not be useful for other solid malignant tumors.[13] Considering the immunosurveillance environment regarding TCR repertoire, TIL phenotype, and cytokine production should improve our understanding of the potential role played by tumor immune evasion in the failure of immunotherapeutic treatments in cancer patients.

In clinical settings, once HNSCC has been diagnosed and treatment is considered, the next step is to identify any lymph node metastasis. If lymph node metastasis is suspected after radiographic imaging, surgical approaches, such as neck dissection, are attempted to remove the metastatic lymph nodes. Metastasis is not an early event in HNSCC; however, >50% of newly diagnosed HNSCC patients are not cured and will suffer local or distant relapse; indeed, 10% of newly diagnosed HNSCC patients present with distant metastases. Recurrent and/or metastatic HNSCC patients have a poor prognosis, with a median survival of ≤1 year.[30] Given the importance of nodal status, the accurate staging of cervical lymph nodes is critical. However, routine analyses for the detection of lymph node metastases from neck dissection specimens is typically limited to histologic examination of 1 or only a few sections of each node stained with hematoxylin-eosin. It has been suggested that micrometastases might occur in histopathologically nonmetastatic lymph nodes in HNSCC. Furthermore, micrometastases often increase the risk of recurrence.[31] Another study shows that micrometastases occur in 3%-7% of nodes and 9%-22% of patients with clinically N0 neck disease.[32] In the present study, we detected monoclonal T cells in nonmetastatic lymph nodes. This monoclonal T-cell expansion may be to the result of micrometastasis to the lymph nodes. However, the independent prognostic and clinical relevance of micrometastases in the cervical lymph nodes remains unclear. Analyses of the TCR repertoire and the functions of TILs would also be useful for improving our understanding of tumor malignancy and micrometastasis.

In the present study, we demonstrated that the host immune response, as represented by the TCR repertoire, partly differs between primary tumors and metastatic lymph nodes within individuals. Furthermore, we found no increases in $CD8^+$-T cells or T_H1/T_C1 cytokine production in metastatic lymph nodes compared with nonmetastatic ones. These findings indicate that lymph node metastasis may be associated with changes in the nature of primary tumor antigens, and that there is a possibility that primary HNSCC can escape T-cell immune responses, be transported to and proliferate in the cervical lymph nodes, and then form metastatic lesions. Further studies of TIL functions and tumor antigens will be required to determine the mechanisms of tumor immune evasion. The results of such studies will not only promote our understanding of the mechanisms of pathogenesis and aid diagnosis of lymph node metastasis in HNSCC, but should also provide important information necessary for the development of an effective strategy for T-cell–based immunotherapies.

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