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Analysis of T Cell Receptor Variability in Fresh Tumor-infiltrating Lymphocytes from Human Head and Neck Cancer

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In this study, we analyzed T cell receptor (TCR) gene rearrangements in tumor-infiltrating lymphocytes (TIL) freshly obtained from 15 patients with head and neck cancer using the reversely transcribed polymerase chain reaction (RT-PCR) method. These TILs showed preferential expression of Vα10, Vα8 and Vα1, detected in 13 (87%), 11 (73%), and 9 cases (60%), respectively. The TCRVβ gene revealed diversity without preferential usage. The head and neck region is exposed to bacteria and viruses, so it is possible that the tumor site can become infected and accumulate T cells involved in infection and inflammation. Therefore, we also investigated TCR gene usage in T cells infiltrating in chronic sinusitis mucosa to address the question of whether the Vα1, Vα8, and Vα10 subfamilies are characteristic in TIL from squamous cell carcinoma of head and neck. TCR Vα10 gene usage was also the most common in Vα segment in T cells infiltrating the sinus mucosa, but Vα1 and Vα8 were not detected in the T cells in sinusitis. These results indicate that the Vα10 subfamily, the preferred T cell population in both TIL and T cells in inflammatory disease, might be involved mainly in inflammation or infection. On the other hand, Vα1 and Vα8 appear to be relatively specific populations for antitumor immunity in head and neck cancer.

Key words: T cell receptor — Tumor-infiltrating lymphocyte — Squamous cell carcinoma

TIL[1] are observed in malignant tumors and considered to play an important role in antitumor immunity. Human TIL are largely composed of CD3+α/β TCR+T cells and mostly activated *in situ*.[1] It is known that mature αβ T cells specifically recognize antigenic peptides presented by MHC molecules through their heterodimeric surface receptor, which associates the α and β polypeptides. The specific recognition is dependent upon interaction between MHC/peptide complex and the variable region of TCR molecules. Recently, it has become possible to analyze TCR gene usage of the variable region, and restricted TCR Vα gene usage in TIL has been revealed. Nitta *et al.* have documented preferential expression of Vα7 gene in melanoma TILs and glial tumor TILs.[2,3] Similarly, Vα7, Vα12, Vβ6 and Vβ20 were predominantly expressed among TIL derived from gastric signet ring cell carcinoma.[4] Vα7 seems to be preferentially expressed independently of the histological type of tumor.

Over 90% of head and neck cancer is squamous cell carcinoma. It is of interest to know whether TIL in the head and neck region also show preferential usage of the Vα7 gene, or whether they predominantly use other types of Vα and/or Vβ genes which may be relatively specific to squamous cell carcinoma. Here, we studied Vα and Vβ gene usage in TIL in head and neck cancer. Furthermore, to ascertain whether certain Vα are relatively specific in TIL compared to T cells in inflammation sites, we also analyzed TCR variability in T cells infiltrating mucosa of chronic sinusitis.

[1] Abbreviations: TIL, tumor-infiltrating lymphocytes; TCR, T cell receptor; cDNA, complementary DNA; RT-PCR, reversely transcribed polymerase chain reaction; MHC, major histocompatibility complex; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Patients Surgical specimens were obtained from 15 patients suffering from hypopharyngeal (6 cases), maxillary (4 cases), lingual (2 cases), laryngeal (1 case), gingival (1 case) and buccal (1 case) cancer, who had undergone surgery at Kumamoto University Hospital. The patients had received no anticancer drugs or radiation therapy before surgery. In each case, the malignancy was diagnosed by histological examination; all of the tumors were squamous cell carcinoma (7 well differentiated, 3 moderately differentiated, 5 poorly differentiated). The patients were 12 males and 3 females (mean age 63 years, ranging from 25 to 82). Control lymphocytes were obtained from patients suffering from chronic sinusitis. The patients had no symptoms of nasal allergy, there were no eosinophils in the nasal discharge, and non-specific IgE titer was less than 150 IU/ml (within the normal range).

Preparation of TIL TIL were isolated from the surgical specimens. Each specimen was placed in sterile saline containing 1000 units/ml penicillin G (Meiji Pharmaceutical Co., Tokyo), 1000 μg/ml streptomycin (Meiji

Pharmaceutical Co.) and 500 μg/ml fungizone (Squibb Japan Inc., Tokyo), and allowed to stand for at least 30 min. Within 2 h of excision, necrotic and connective tissues were removed, and the tumor mass was minced with scissors into pieces 1–2 mm^3 in size. The pieces were then digested in RPMI-1640 medium (M.A. Bioproducts, Walkersville, MD) containing 800–1000 units/ml protease (Dispase: Godo Shusei Co., Tokyo: 10000 units protease/ampule containing 2 mg of enzyme protein and 2 mg of calcium acetate) and gently agitated with a magnetic stirrer. The resulting cell suspension was filtered through a 200 gauge stainless steel mesh and washed twice with RPMI-1640 medium. The cell suspension was layered onto Ficoll-Hypaque and centrifuged at 800*g* for 15 min to remove erythrocytes and dead cells. After centrifugation, the cell layer was collected, washed twice, and resuspended in RPMI-1640 medium. TIL containing tumor cells were resuspended in RPMI-1640

Table I. Sequences of T Cell Receptor α and β-Chain Primers

Primer	5′→3′ Sequence	Family members	Expected length
Vα1	TTGCCCTGAGAGATGCCAGAG	1.1, 1.2, 1.3	415
Vα2	GTGTTCCCAGAGGGAGCCATTGCC	2.1, 2.2	363
Vα3	GGTGAACAGTCAACAGGGAGA	3.1	405
Vα4	ACAAGCATTACTGTACTCCTA	4.1	430
Vα5	GGCCCTGAACATTCAGGA	5.1	371
Vα6	GTCACTTTCTAGCCTGCTGA	6.1	451
Vα7	AGGAGCCATTGTCCAGATAAA	7.1, 7.2	353
Vα8	GGAGAGAATGTGGAGCAGCATC	8.1, 8.2	395
Vα9	ATCTCAGTGCTTGTGATAATA	9.1	435
Vα10	ACCCAGCTGGTGGAGCAGAGCCCT	10.1	395
Vα11	AGAAAGCAAGGACCAAGTGTT	11.1	399
Vα12	CAGAAGGTAACTCAAGCGCAGACT	12.1	410
Vα13	GCTTATGAGAACACTGCGT	13.1	333
Vα14	GCAGCTTCCCTTCCAGCAAT	14.1	334
Vα15	AGAACCTGACTGCCCAGGAA	15.1	367
Vα16	CATCTCCATGGACTCATATGA	16.1	375
Vα17	GACTATACTAACAGCATGT	17.1	333
Vα18	TGTCAGGCAATGACAAGG	18.1	252
Cα	AATAGGTCGAGACACTTGTCACTGGAGA		
Cα	GAACCCTGACCCTGCCGTGTACC	Probe	
Vβ1	GCACAACAGTTCCCTGACTTGCAC	1.1, 1.2	199
Vβ2	TCATCAACCATGCAAGCCTGACCT	2.1, 2.2, 2.3	202
Vβ3	GTCTCTAGAGAGAAGAAGGAGCGC	3.1, 3.2	197
Vβ4	ACATATGAGAGTGGATTTGTCATT	4.1, 4.2, 4.3	228
Vβ5.1	ATACTTCAGTGAGACACAGAGAAAC	5.1	245
Vβ5.2	TTCCCTAACTATAGCTCTGAGCTG	5.2, 5.3	179
Vβ6	AGGCCTGAGGGATCCGTCTC	6.1, 6.2, 6.3	185
Vβ7	CCTGAATGCCCCAACAGCTCTC	7.1, 7.2	212
Vβ8	ATTTACTTTAACAACAACGTTCCG	8.1, 8.2, 8.3, 8.4	260
Vβ9	CCTAAATCTCCAGACAAAGCTCAC	9.1	207
Vβ10	CTCCAAAAACTCATCCTGTACCTT	10.1, 10.2	192
Vβ11	TCAACAGTCTCCAGAATAAGGACG	11.1, 11.2	200
Vβ12	AAAGGAGAAGTCTCAGAT	12.1, 12.2	224
Vβ13.1	CAAGGAGAAGTCCCCAAT	13.1	221
Vβ13.2	GGTGAGGGTACAACTGCC	13.2	243
Vβ14	GTCTCTCGAAAAGAGAAGAGGAAT	14.1	193
Vβ15	AGTGTCTCTCGACAGGCACAGGCT	15.1	202
Vβ16	AAAGAGTCTAAACAGGATGAGTCC	16.1	251
Vβ17	CAGATAGTAAATGACTTTCAG	17.1	245
Vβ18	GATGAGTCAGGAATGCCAAAGGAA	18.1	240
Vβ19	CAATGCCCCAAGAACGCACCCTGC	19.1	195
Vβ20	AGCTCTGAGGTGCCCCAGAATCTC	20.1	224
Cβ	TTCTGATGGCTCAAACAC		
Cβ	AGCGACCTCGGTTGGGAACAC	Probe	

containing 10% heat-inactivated FCS (Kyoto Biken Laboratories, Inc., Kyoto), cryopreserved and stored in liquid nitrogen until use, as described before.[5]

Preparation of lymphocytes infiltrating maxillary sinus mucosa in chronic sinusitis Lymphocytes infiltrating maxillary sinus mucosa in chronic sinusitis were isolated by a technique similar to that described above. Control lymphocytes were also cryopreserved and stored in liquid nitrogen until use.

Preparation of RNA and cDNA synthesis Total cellular RNA was prepared from TIL and lymphocytes infiltrating the maxillary sinus mucosa by the guanidinum thiocyanate method using Isogen (Nippon Gene, Tokyo). Total RNA was detected by electrophoresis in a 1% regular agarose gel. Four micrograms of total RNA was used for the synthesis of single-strand cDNA using reverse transcriptase. The RNA was incubated for 10 min at 70°C with random hexamer (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD), and then with 0.5 m*M* dNTP, 0.01 *M* DTT and 200 units of Superscript reverse transcriptase (GIBCO BRL Life Technologies, Inc.) in a final volume of 20 μl 1×Synthesis buffer (50 m*M* KCl, 20 m*M* Tris-HCl pH 8.4, 2.5 m*M* $MgCl_2$), for 50 min at 42°C and 5 min at 95°C. The reaction mixture was incubated with 2 units of *E. coli* RNase H (GIBCO BRL Life Technologies, Inc.) for 20 min at 37°C.

Amplification of cDNA by PCR A 1 μl sample of cDNA was mixed with a PCR mixture containing dNTP (50 μM each), a primer (5′Vα, 3′Cα, 5′Vβ, 3′Cβ) and 1×PCR buffer (50 m*M* KCl, 10 m*M* Tris-HCl pH 8.8, 2.5 m*M* $MgCl_2$ and 0.01% gelatin) in a final volume of 48 μl. Sequences of the individual primers are shown in Table I. The mixture was denatured for 10 min at 98°C, and then 1 U of *Taq* polymerase (Perkin Elmer, Cetus, CA) was added. The PCR profile used was as follows: denaturation at 95°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s for 35 cycles, followed by final extension at 72°C for 10 min on a DNA thermal cycler (Perkin Elmer). PCR products were analyzed by 2% agarose gel electrophoresis.

Southern blot analysis The amplified products were confirmed by Southern blot analysis using a Cα (5′-GAACCCTGACCCTGCCGTGTACC-3′) or Cβ (5′-AGCGACCTCGGTTGGGAACAC-3′) specific oligonucleotide probe. The amplified products were electrophoresed in 2% agarose gel, and transferred to nylon membranes (Hybond-N, Amersham International plc, UK) according to the standard procedure. Nylon membranes were prehybridized for 30 min at 45°C in 0.1× standard sodium citrate and 0.5% SDS, and hybridized for 2 h at 40°C with ^{32}P-kinased Cα probe or Cβ probe. The nylon membranes were washed twice for 10 min in 2×standard sodium citrate and 0.1% SDS at room temperature, and then washed twice for 10 min in 6× standard sodium citrate and 0.1% SDS. After washing, the nylon membranes were exposed to X-ray film for 1 h, 12 h and 24 h at −70°C.

RESULTS

Analysis of TCR V gene segment expression in TIL from head and neck cancer TIL obtained from 15 patients with head and neck cancer were studied for TCR V gene expression. In all cases where Vα-Cα gene rearrangement was visualized on agarose gel electrophoresis with ethidium bromide staining, positive hybridization was obtained on Southern blot hybridization with a Cα-specific probe. Fig. 1 shows the positive hybridization by Southern blot hybridization in case S.Y. (hypopharyngeal cancer). The PCR products of TCR Vα1, 2, 6, 8, 10, 14 and 15 were clearly detected. Analysis of Vα expression in TIL from 15 tumor specimens is shown in Table II. A limited number of 1 to 10 TCR Vα gene segments was observed in all TIL. TCR Vα1, Vα8 and Vα10 genes were preferentially expressed in 9 (60%), 11 (73%) and 13 (87%) out of 15 cases, respectively. When the relationship between the predominant usage of V-region genes and the degree of histological differentiation or the progression of the tumor was studied, no correlation was observed.

Analysis of Vβ expression is shown in Table III. Positive TCR Vβ rearrangements were visualized on agarose gels with ethidium bromide staining, and positive hybridization was similarly obtained on Southern blot hybridization with Cβ-specific probe. Fig. 2 shows the positive hybridization by Southern blot hybridization in case S.Y. (hypopharyngeal cancer). The PCR products of all TCR Vβ except for Vβ2 were clearly detected. The usage of

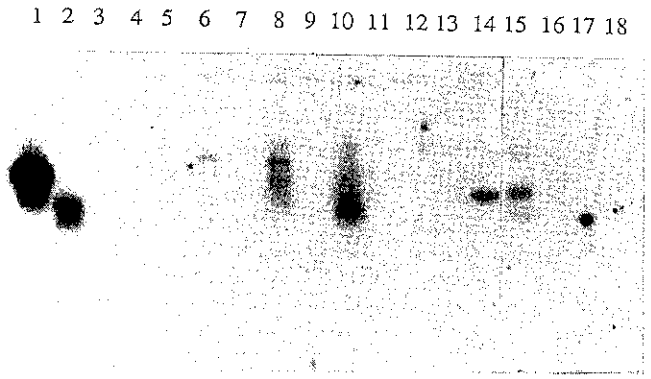

Fig. 1. Southern blot analysis of reverse-transcribed and PCR-amplified TCR Vα gene segment in case S.Y. (hypopharyngeal cancer). The PCR products were hybridized with a Cα-specific oligonucleotide probe. TCR Vα1, α2, α6, α8, α10, α14 and α15 genes were detected.

Table II. Usage of TCR Vα Genes in Head and Neck Cancer

Case	Age	Sex[a]	Diagnosis	Histology[b]	TCR Vα families
M.M.	75	M	Maxillary cancer	SCC(well)	1, 2, 3, 6, 8, 10, 13, 15
F.N.	51	F	Hypopharyngeal cancer	SCC(well)	1, 7, 10, 15
S.H.	59	M	Hypopharyngeal cancer	SCC(well)	5, 10
K.K.	67	M	Hypopharyngeal cancer	SCC(well)	1, 3, 8, 10
H.K.	25	M	Lingual cancer	SCC(well)	8, 15
N.N.	76	F	Gingival cancer	SCC(well)	1, 3, 7, 8, 10, 13, 14
S.T.	58	M	Buccal cancer	SCC(well)	1, 8, 10, 14
C.I.	61	M	Maxillary cancer	SCC(mod)	1, 3, 6, 8, 10, 14, 15
T.O.	64	M	Maxillary cancer	SCC(mod)	3
S.Y.	61	M	Hypopharyngeal cancer	SCC(mod)	1, 2, 6, 8, 10, 14, 15
K.I.	82	F	Maxillary cancer	SCC(poor)	8, 10
M.I.	67	M	Laryngeal cancer	SCC(poor)	2, 3, 8, 10
M.I.	69	M	Hypopharyngeal cancer	SCC(poor)	1, 2, 3, 7, 8, 10, 12, 14, 15, 18
T.S.	67	M	Hypopharyngeal cancer	SCC(poor)	1, 10
T.M.	67	M	Lingual cancer	SCC(poor)	8, 10, 13

a) M, male; F, female.
b) SCC(well), SCC(mod), SCC(poor), well, moderately and poorly differentiated squamous cell carcinoma.

Table III. Usage of TCR Vβ Genes in Head and Neck Cancer

Case	Age	Sex[a]	Diagnosis	TCR Vβ families
M.M.	75	M	Maxillary cancer	1, 2, 4, 5.1, 6, 7, 8, 9, 10, 11, 12, 13.1, 13.2, 14, 15, 18, 19, 20
F.N.	51	F	Hypopharyngeal cancer	1, 2, 3, 4, 5.1, 5.2, 6, 7, 8, 9, 10, 11, 12, 13.1, 13.2, 14, 15, 16, 17, 18, 19, 20
S.H.	59	M	Hypopharyngeal cancer	2, 3, 4, 5.1, 6, 8, 9, 10, 11, 12, 13.1, 13.2, 14, 15, 16, 17, 18, 19, 20
K.K.	67	M	Hypopharyngeal cancer	1, 2, 3, 4, 5.1, 5.2, 6, 7, 8, 9, 10, 11, 12, 13.1, 13.2, 14, 15, 16, 17, 18, 20
H.K.	25	M	Lingual cancer	2, 3, 4, 5.1, 6, 13.1
N.N	76	F	Gingival cancer	1, 2, 3, 4, 5.1, 5.2, 6, 7, 8, 9, 10, 11, 12, 13.1, 13.2, 14, 15, 17
S.T.	58	M	Buccal cancer	13.2, 15, 18, 19, 20
C.I.	61	M	Maxillary cancer	1, 2, 3, 4, 5.1, 5.2, 6, 7, 8, 9, 10, 11, 12, 13.1, 13.2, 14, 16, 17, 18, 20
T.O.	64	M	Maxillary cancer	1, 2, 3, 4, 5.1, 5.2, 6, 7, 8, 9, 10, 11, 12, 13.1, 13.2, 14, 15, 16, 17, 18, 20
S.Y.	61	M	Hypopharyngeal cancer	1, 3, 4, 5.1, 5.2, 6, 7, 8, 9, 10, 11, 12, 13.1, 13.2, 14, 15, 16, 17, 18, 19, 20
K.I.	82	F	Maxillary cancer	1, 2, 3, 4, 5.1, 5.2, 6, 7, 8, 9, 12, 13.1, 13.2, 14, 16, 17
M.I.	67	M	Laryngeal cancer	1, 2, 3, 4, 5.1, 5.2, 6, 7, 8, 9, 12, 13.1, 13.2, 14, 15, 16, 17, 18, 20
M.I.	69	M	Hypopharyngeal cancer	1, 2, 3, 4, 5.1, 6, 7, 12, 13.1, 13.2, 14, 15, 17, 18, 19, 20
T.S.	67	M	Hypopharyngeal cancer	1, 2, 3, 4, 5.1, 5.2, 6, 13.1, 13.2, 14, 17, 20
T.M.	67	M	Lingual cancer	1, 2, 3, 4, 5.1, 5.2, 6, 7, 12, 13.1, 13.2, 16, 17, 18

a) M, male; F, female.

TCR Vβ is more diverse than that of TCR Vα, except in cases H.K. and S.T.

Analysis of TCR gene segment expression in lymphocytes infiltrating maxillary sinus mucosa in chronic sinusitis

We analyzed the lymphocytes infiltrating maxillary sinus mucosa in chronic sinusitis. Lymphocytes obtained from 7 patients with chronic sinusitis were studied. The results of analysis of Vα and Vβ expression are shown in Table IV. Interestingly, the usage of Vα and Vβ genes was more restricted in chronic sinusitis. Vα10 gene was preferentially expressed in 5 (71%) out of 7 cases. On the other hand, preferential expression of Vα1 and Vβ8 in TIL was not detected in chronic sinusitis. No other Vα gene was preferentially expressed in chronic sinusitis. The usage of Vβ was more restricted, except in case 3, but no Vβ gene was preferentially expressed.

DISCUSSION

TIL are considered to be involved in host immune response against cancer. Indeed, the intensity of lymphocyte infiltration in breast cancer tissue was found to be correlated with the clinical prognosis of breast cancer patients.[6] In this study, T cell infiltration was ample in infiltrating papillotubular carcinoma, which had a better prognosis, while it was minimal in scirrhous carcinoma.

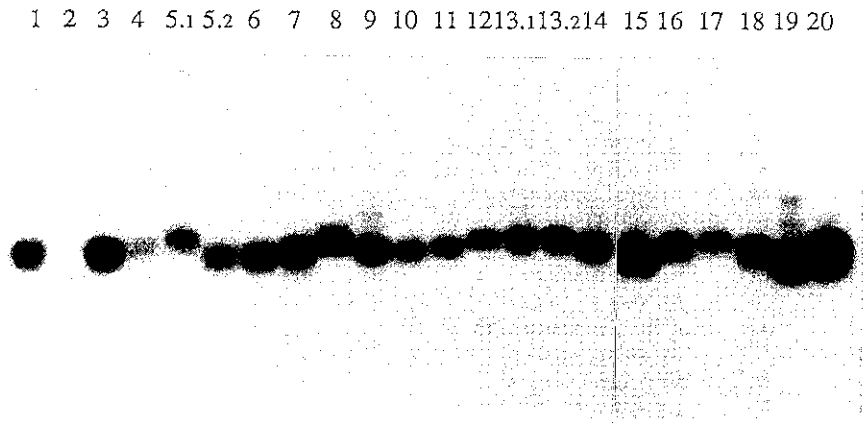

Fig. 2. Southern blot analysis of reverse-transcribed and PCR-amplified TCR Vβ gene segment in case S.Y. (hypopharyngeal cancer). The PCR products were hybridized with a Cβ-specific oligonucleotide probe. All TCR Vβ genes except for Vβ2 were detected.

Table IV. Usage of TCR V Genes in Chronic Sinusitis

Case	Age	Sex[a]	TCR Vα families	TCR Vβ families
K.M.	59	F	10	2, 3, 4, 5.1, 5.2, 12, 13.2, 14
T.K.	39	M	14	1, 2, 3, 4, 5.1, 13.1
S.N.	65	F	3, 10, 15	1, 2, 3, 4, 5.1, 5.2, 6, 7, 8, 9, 10, 11, 12, 13.1, 13.2, 14, 15, 17, 18
Y.M.	25	M	10	1, 2, 4, 5.1, 6, 7, 8, 13.1, 13.2
D.N.	49	M	10	1, 2, 3, 4, 5.1, 5.2
K.H.	27	M	3,10	1, 2, 3, 4, 5.1, 6, 8, 13.1, 13.2
S.H.	47	M	Not detected	2, 3, 12, 13.1, 13.2, 20

a) M, male; F, female.

The T cell infiltration was significantly higher in patients without lymph node metastasis. Furthermore, Rosenberg *et al.* have shown that TILs expanded with interleukin 2 are therapeutically effective.[7-9]

By examining the expression of the gene for the variable region of α- and β-chains of the T cell receptor of TIL, Nitta *et al.* showed that freshly isolated TIL from intraocular melanoma exhibited preferential usage of the TCR Vα7 gene, and they have reported the same preferential usage in TIL within glial tumor.[2,3] Ikeda *et al.* have reported that cytotoxic TIL in gastric signet ring cell carcinoma predominantly expressed Vα7, Vα12, Vβ6, and Vβ20.[4] These results indicate preferential gene usage of TIL, as demonstrated in chronically inflamed tissues such as multiple sclerosis,[10,11] rheumatoid arthritis,[12] primary biliary cirrhosis,[13] and auto-immune thyroiditis.[14] In contrast, Ferradini *et al.* have claimed that TIL are extremely diverse, with no preferential TCR gene segment usage.[15] Hence, to explore whether TIL from squamous cell carcinoma of the head and neck have preferential gene usage, we analyzed the TCR gene usage by employing RT-PCR technology. The head and neck region is exposed to bacteria and viruses, so the growth of malignant tumor can involve infection and inflammation where T cells also participate in immune response. Therefore, we obtained T cells in chronic sinusitis and analyzed the TCR gene usage to compare it to that of TIL.

Analysis of the TCR Vα gene expressed on the lymphocytes infiltrating sinus mucosa revealed more restricted usage than that of TIL. Only Vα3,Vα10, Vα14, and Vα15 subfamilies were detected in T cells infiltrating in sinus mucosa. Among them, Vα10 was the most frequent, being observed in 5 of 7 cases. In contrast, Vα1 and Vα8 subfamilies were not found in the lymphocytes in sinus mucosa. Thus, the repertoire analysis indicated that Vα10 subfamily had common Vα segment usage in both TIL and the lymphocytes infiltrating in sinus mucosa, whereas the usage of Vα1 and Vα8 subfamilies found in TIL was not seen in the lymphocytes in inflamed sinus mucosa. Ikeda *et al.* have shown that T cells with Vα7, which was most frequently observed in TIL of

signet ring cell carcinoma, also comprised the most frequent population in the T cells of benign ulcerative lesions in 2 patients with duodenal or gastric ulcer. [4] To explain this phenomenon, they proposed that T cells with the TCR V products may be recognized by their TCR glycoprotein expressed amply in the stomach tissue, so that the T cell population could preferentially appear in the gastric mucosa. In the head and neck region, T cells with Vα10 might be a similar type of T cell population. Alternatively, T cells with Vα10 in TIL may be mainly involved in infection occurring at the tumor site. The Vα1 and Vα8 subfamilies were observed in the tumor site but not in chronic sinusitis, suggesting that Vα1 and Vα8 subfamilies in TIL are major populations related to antitumor immunity against head and neck squamous cell carcinoma. To pursue this hypothesis, further study of cytotoxic T cell lines and clones established from TIL is in progress.

As for TCR Vβ gene usage, there was no preferential usage at tumor sites. On the other hand, T cells of inflamed sinus tissues revealed preferential usage of Vβ1, Vβ2, Vβ3, Vβ4, Vβ5, Vβ13.1 and Vβ13.2, all of which were expressed in at least 5 out of 7 cases. It is uncertain whether preferential utilization of certain Vβ TCRs exists in TIL. Preferential Vβ TCR gene usage has been reported in TIL,[4, 16–19] but several groups have found no preference.[15, 20]

In conclusion, analysis of TIL freshly isolated from squamous cell carcinoma of the head and neck suggested that oligoclonal T cell populations are amplified at the tumor site and that these T cell populations, especially T cells with Vα1 or Vα8, might be involved in tumor-specific immune response against squamous cell carcinoma in the head and neck.

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