Differential Expression of Cytokeratin after Orthotopic Implantation of Newly Established Human Tongue Cancer Cell Lines of Defined Metastatic Ability

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Two human tongue squamous cell carcinoma cell lines, SQUU-A and SQUU-B, were established from the same patient. Cervical lymph node metastasis was detected in the mice orthotopically implanted with SQUU-B (86.7%, 13/15), but not in those with SQUU-A (0/13). Histologically, SQUU-B showed invasive growth and intravasation in the tongue, whereas SQUU-A simply demonstrated expansive growth without intravasation. By Western blot analysis, nonmetastatic clone SQUU-A expressed cytokeratin (CK)13/4, 14, 16/6, 18/8, and 19, whereas a high metastatic clone SQUU-B expressed CK18/8 and 19. The reverse transcription-polymerase chain reaction technique showed that CK13/4 mRNA was expressed in both cell lines, but CK14 and 16 mRNA was expressed only in SQUU-A. CK13 was immunohistochemically expressed in both SQUU-A and SQUU-B transplanted into the tongues of nude mice; CK14 and 16 were detected in SQUU-A of the tongues, but not in SQUU-B. As seen in SQUU-B cell line, SQUU-B of the cervical lymph node metastasis did not exhibit CK13, 14, or 16. These results suggest that the loss or down-regulation of CK13, 14, or 16 is related to the invasive and metastatic ability of cancer. The cytoskeletal system is thus considered to be closely related to the malignant phenotype. (*Am J Pathol* 2000, 156:1317–1326)

Tongue cancer has a high metastatic ability. The survival of cancer patients often depends primarily on whether or not the tumor metastasizes; therefore, it is important to charac-

terize the phenotype of metastatic tumors and to elucidate both the responsible molecules and their functions.

Cytokeratins represent important structural components of the epithelial cytoskeleton and thus constitute major protein markers for cellular differentiation.¹ They have been widely used as molecular markers in the diagnosis of a variety of carcinomas as well as in the study of many nonmalignant lesions.² The CK19 fragment, referred to as CYFRA 21-1, has been reported to be useful as an independent prognostic marker of squamous cell carcinoma.^{3,4} Alterations in the expression patterns of cytokeratins have been reported in carcinoma or carcinoma cell lines using immunohistochemistry,^{5,6} Western blot analysis,^{5,7} two-dimensional gel electrophoresis,^{1,8} and *in situ* hybridization.⁹ However, few reports have analyzed them using metastatic models. It is necessary to analyze cancer cells that have differences in invasive and metastatic abilities to understand the cytokeratin gene regulation as well as the role of cytokeratin in the progression of carcinoma. An orthotopic implantation enabled us to establish the metastatic models.^{10,11} In the present study, we examined the relationship between the malignancy and the cytokeratin expression by using two established squamous cell carcinoma cell lines, SQUU-A and SQUU-B, derived from the same human tongue cancer, which demonstrated different invasive and metastatic potentials in the orthotopic implantation system.

Materials and Methods

Establishment of Cell Lines

SQUU-A and SQUU-B cell lines were established from local recurrences of the tongue cancer obtained from a 66-year-old Japanese woman. This tumor had been diagnosed to be a well differentiated squamous cell carcinoma. Surgical specimens were minced with a scalpel into small cubes measuring from 1 to 3 mm in size. These

Supported in part by grants-in-aid for Scientific Research (B) and (C) from the Ministry of Education, Science, Sports and Culture, Japan.

Accepted for publication December 9, 1999.

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tissue fragments were placed in tissue culture flasks in Eagle's MEM medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY), streptomycin (100 mg/ml), penicillin (100 IU/ml; GIBCO), and fungizone (1 mg/ml) and were then incubated at 37°C in a 5% CO₂ atmosphere. The cells were then cultured in Eagle's MEM medium supplemented with 10% fetal bovine serum and thereafter harvested by adding 0.05% trypsin and 0.53 mmol/L EDTA (GIBCO). Epithelioid cells were selected and cloned twice. We obtained two clones and designated them SQUU-A and SQUU-B.

Orthotopic Implantation and Evaluation

Female BALB/c-*nu/nu* mice were obtained from SLC (Shizuoka, Japan). Six-week-old animals (28 mice) were used for the orthotopic transplantation experiment. This experiment was reviewed by the Committee of the Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University, and carried out under the control of the Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University, and the law (no. 105) and notification (no. 6) of the government of Japan. The tumor cells were harvested from subconfluent cultures by treating them with 0.05% trypsin and 0.53 mmol/L EDTA after washing twice with 0.02% EDTA in phosphate buffered saline (PBS). The dislodged cells were washed two times in medium with 10% fetal bovine serum. Viable cells were determined by the trypan blue dye (Wako, Osaka, Japan) exclusion test. The mice were anesthetized with diethyl-ether, and then viable tumor cells ($3.5 \times 10^5/0.035$ ml) were injected submucosally on the right side of the tongue. The mice transplanted with SQUU-B cells were sacrificed on day 35 after implantation. Mice implanted with SQUU-A cells were sacrificed on days 35 and 114. The tongues and cervical lymph nodes were excised and histologically examined.

Growth Curve of Cell Lines

SQUU-A cells of passage 80 and SQUU-B cells of passage 78 were used to estimate the population doubling time. The initial cell number was 1×10^4 cells per dish (Falcon 3001; 35×10 mm) containing 1 ml of culture medium. The number of cells was counted each day in three dishes by staining with crystal violet.

Morphology and Electron Microscopy of Cell Lines

All unstained cultured cells were photographed with the use of a phase contrast microscope. For the electron microscopic examination, the cultured cells in petri dishes were fixed with 3% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, for 2 hours at 4°C, washed with cold buffer, and fixed with 1% (v/v) osmic acid in the same buffer for 1 hour. The fixed cells were dehydrated by a graded ethanol series and then embed-

ded in Epon/Araldite (Nissin EM Co., Tokyo, Japan). Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and then examined by an electron microscope (JEOL 200CX, Tokyo, Japan).

One- or Two-Dimensional Gel Electrophoresis of Cell Lines

The preparation and fractionation of cellular proteins were performed as previously described.¹² The Triton X-100 insoluble cytoskeletal fractions were dissolved in sample buffer and used for polyacrylamide gel electrophoresis in sodium dodecyl sulfate. After electrophoresis, the gels were stained with 0.15% Coomassie blue, 50% methyl alcohol, and 10% acetic acid for 30 minutes and then destained with 7.5% methanol and 7.5% acetic acid for about 15 hours.

Western Blotting

After SDS or two-dimensional polyacrylamide gel electrophoresis, the portions of the gels containing cytokeratins were cut out and equilibrated with 25 mmol/L Tris-HCl, 192 mmol/L glycine, and 20% methanol for 30 minutes. The proteins of the polyacrylamide gels were transferred to nitrocellulose in the above buffer at 0.8 mA/cm². Protein-blotted nitrocellulose sheets were immediately immersed in PBS containing 2% bovine serum albumin (BSA) overnight at 4°C. After washing the prehybridized nitrocellulose sheets with two changes of 0.1% Tween/PBS at 5-minute intervals, they were then incubated for 2 hours at room temperature in a plastic bag containing a small volume (50 μ l/cm² of filter) of the first antibodies in 0.1% BSA/PBS. The characteristics of these antibodies are summarized in Table 1.^{1,7,13-21} After washing the sheets with three changes of 0.1% Tween/PBS at 5-minute intervals, they were immersed in 0.1% BSA/PBS with HRP-labeled IgG (1:500, Sigma, St Louis, MO) for 1 hour at room temperature. To remove the second antibody, the filters were washed in the same way as that described for washing the first antibodies. We used ECL detection reagents (Amersham Pharmacia Biotech, Tokyo, Japan).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cell lines by QuickPrep Total RNAs Extraction Kit (Amersham Pharmacia Biotech). Reverse transcription of 4 μ g total RNA was performed using *Not* I-d(T)¹⁸ (Amersham Pharmacia Biotech) and Ready-To-Go You-primer First-Strand Beads (Amersham Pharmacia Biotech) at 37°C for 1 hour. CK13/4, 14, and 16, and β -actin cDNAs were amplified with programs consisting of 94°C for 1 minute followed by each condition, and a final 10-minute extension at 72°C using the following primer pairs: CK13 sense 5'-GCAAGC-TTCTATCTGCACC-3', CK13 antisense 5'-ACCATC-AGGAGAGAGTCAGG-3', with cycling conditions of 1 minute at 96°C, 30 seconds at 58°C, and 30 seconds at

Table 1. Range of Monoclonal Antibodies Used for Western Blotting

Antibody	Dilution	Reactivity	Ref. no.	Source
K8.12	1:100	CK 13, 16	13	BioMakor
AE-1	1:100	CK 14, 16, 19 (most type I CK)	7	PROGEN
6B10	1:100	CK 4	14	SIGMA
Ks6.KA12	1:100	CK 6	15	PROGEN
RCK105	1:5	CK 7	16	PROGEN
M20	1:10	CK 8	17	PROGEN
KS-1A3	1:100	CK 13	18	SIGMA
LL002	1:100	CK 14	19	CYMBUS BIOTEC
Ks18.04	1:20	CK 18	1	PROGEN
K4.62	1:100	CK 19	20	BioMakor
CCI610	1:100	vimentin	21	YLEM

72°C for a varying number of cycles of amplification (18–20,22,24,25,35). CK4 sense 5'-CCCCTC-CACGTGGAGATTCA-3', CK4 antisense 5'-CCTGCA-GATGGATAAGAGGG-3', with cycling conditions of 1 minute at 96°C, 1 minute at 58°C, and 1 minute at 72°C for 35 cycles; CK14 sense 5'-ACTACCTGCAGCCGC-CAGTT-3', CK14 antisense 5'-CAGTTCTTGGTGCGA-AGGAC-3'; CK16 sense 5'-TGCGCACGCCCTTTTGCA-GA-3', CK16 antisense 5'-GCTGTTCTCCAGGGATGCTT-3'; β -actin sense 5'-GAAAATCTGGCACCACACCTT-3', β -actin antisense 5'-TTGAAGGTAGTTTCGTGGAT-3'.²² The amplification of CK14, 16, and β -actin cDNA were performed with cycling conditions of 1 minute at 96°C, 30 seconds at 60°C, and 30 seconds at 72°C for 35 cycles. β -actin was used as internal control of the reaction. Aliquots from amplified samples were subjected to electrophoresis through 1% agarose gel and DNA fragments were visualized by ethidium bromide staining. The PCR products of CK13 were cloned in pT7Blue T-vector (Novagen, Madison, WI). Sequence analysis of amplified fragments was performed in an automated sequencer (Applied Biosystem, Chiba, Japan). The nucleotide sequence was determined by the dideoxynucleotide chain termination method using fluorescent labels with dye terminator kits (Applied Biosystem).

Immunohistochemistry

The specimens were fixed in 10% formalin solution and then embedded in paraffin to prepare serial sections. Immunohistochemical staining was performed by the labeled streptavidin biotin method (LSAB, Nichirei, Tokyo, Japan) after the fixed sections were deparaffinized, rehydrated, and microwaved with 0.01 mol/L sodium citrate

buffer, pH 6.0. Anti-CK13 (KS-1A3, Sigma),¹⁸ anti-CK14 (LL002, Cymbub Biotec)¹⁹ and anti-CK16 (LL025, Novocastra Laboratories, Newcastle, UK)²³ antibodies were used. The working dilutions were 1:100, 1:4, and 1:10, respectively. The final color reaction was developed in a diaminobenzidine substrate.

Results

Tumorigenicity and Metastasis Assay

By histopathological examination on day 35, metastasis of the cervical lymph nodes was detected in 86.7% of the mice transplanted with SQUU-B (13 of 15 mice), whereas no metastasis was observed in the mice transplanted with SQUU-A (0 of 10) (Table 2). SQUU-A-implanted mice developed grossly evident tumors in the tongues (100%). SQUU-A cells formed well or moderately differentiated tumor tissue and showed expansive growth with clear parenchymal-stromal border (Figure 1A). No intravascular invasion was detected. There was no metastasis in the mice transplanted with SQUU-A (0 of 3) on day 114. The tumors grew large until they reached the edge of the tongue on day 114. The histological views on days 35 (Figure 1, A and C) and 114 after implantation of SQUU-A were similar, except that the necrotic tissue and the remarkable infiltration of lymphocyte were observed on day 114 (data not shown). SQUU-B cells growing orthotopically also exhibited moderate differentiation (Figure 1B). However, they showed invasive growth into the muscular tissue (Figure 1D), whereas the invasion into both blood and lymphatic vessels was also evident (Figure 1, E and F).

Table 2. Tumorigenicity, Metastasis and Histological Views

Clones	Autopsy day	Tumorigenicity of local growth	Cervical lymph node metastasis	Histological views of local growth
SQUU-A	35	10/10*	0/10	Expansive growth
	114	3/3	0/3	No intravasation
SQUU-B	35	15/15 (100%)	13/15 (86.7%)	Invasive growth
				Intravasation

After the orthotopic implantation of these clones of human tongue cancer into tongues of nude mice, the tumorigenic and metastatic capability was determined while the histological views were examined. Please see Materials and Methods for the procedures.

*Data are shown as the number of mice in which local tumor growth or metastasis were observed/number of evaluable mice.

Figure 1. Microscopic views of local tumor growth in nude mice after orthotopic implantation. Nonmetastatic clone SQUU-A shows expansive growth (**A** and **C**), while a high metastatic clone SQUU-B shows invasive growth (**B**) into the muscular tissue (**D**), blood and lymphatic vessels (**E** and **F**). Original magnifications, $\times 18$ (**A** and **B**), $\times 140$ (**C** and **D**), $\times 200$ (**E**), $\times 300$ (**F**). **Arrows** show invasive tumors.

Characteristics of Cell Lines

In the exponential growth phase, doubling time of the population was found to be 27.5 hours for SQUU-A cells and 23 hours for SQUU-B, respectively. SQUU-A (Figure 2A) and SQUU-B (Figure 2B) were polygonal in shape and grew in a typical cobblestone pattern containing oval or round nuclei with 1 or 2 evident nucleoli. SQUU-A cells

were slightly larger than SQUU-B cells. Piling up of SQUU-A cells was partially seen, whereas SQUU-B cells developed multiple layers throughout. Ultrastructural appearances of these clones were basically similar. The cells of both clones contained a large central nucleus with 1 or 2 nucleoli, and desmosomes were observed between the cells; the cytoplasm was large, with many polysomes and mitochondria. There were some differ-

Figure 2. Phase contrast micrographs of nonmetastatic clone SQUU-A (A) and a high metastatic clone SQUU-B (B). Electron microscopy of SQUU-A (C) and SQUU-B (D). The intermediate filaments are more prominent in SQUU-A (C) than in SQUU-B (D). Original magnifications, $\times 263$ (A and B), $\times 8000$ (C), $\times 22,700$ (D). Arrows show desmosomes.

ences in the formation of intermediate filaments among these clones; the intermediate filaments were more prominent in SQUU-A (Figure 2C) than in SQUU-B (Figure 2D).

Cytokeratin Expression in Cell Lines

The type of cytokeratin isoforms was determined by immunoblotting using the monoclonal mouse antibody against each cytokeratin per catalog of Moll et al¹ after sodium dodecyl sulfate-polyacrylamide gel electrophoresis or two-dimensional gel electrophoresis (Figures 3 and 4). CK13/4, 14, and 16/6 were expressed only in the nonmetastatic clone SQUU-A, but not in the metastatic clone SQUU-B. Candidate spots for CK18/8 and 19 were detected in both SQUU-A and SQUU-B. The expression of CK18 was greater in SQUU-B than in SQUU-A. The expression of CK19 was similar for both clones (Figures 3 and 4). These results are summarized in Table 3. Vimentin was not expressed in SQUU-A or SQUU-B (data not shown). The presence of CK13/4, 14, and 16 mRNA in SQUU-A and SQUU-B was studied by RT-PCR technique (Figures 5 and 6). CK14 and 16 mRNA were detectable in SQUU-A, but not in SQUU-B (Figure 5). CK13/4 mRNA was detected in both SQUU-A and SQUU-B (Figures 5 and 6), though a substantial difference was observed in the amounts of CK13 mRNA between SQUU-A and

Figure 3. Coomassie blue-stained two-dimensional gel analyses of the cytoskeletal extracts from culture cells (A and B) and immunoblotting using monoclonal anti-cytokeratin antibodies in two-dimensional gel analyses of cytoskeletal extracts (C–F). CK13, 14, and 16 are detectable in nonmetastatic clone SQUU-A (A, C, and D), but not in a high metastatic clone SQUU-B (B). SQUU-A is stained with anti-CK13 and 16 (K8.12; C). SQUU-A is stained with anti-CK14 and 16 (AE1; D). Both SQUU-A (E) and SQUU-B (F) are stained with anti-CK 8 (M20).

Figure 4. Coomassie blue-stained sodium dodecyl gradient gel electrophoresis of cytoskeletal extracts from culture cells (A) and immunoblotting using monoclonal anti-cytokeratin antibodies after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (B–G). Nonmetastatic clone SQUU-A is positive reaction with anti-CK4 (6B 10; B), anti-CK6 (Ks6. KA12; C), anti-CK13 (KS1A3; D), anti-CK 14 (LL002; E), but a high metastatic clone SQUU-B is negative with them (B–E). Both clones are positive with anti-CK18 (K18.04; F) and anti-CK19 (K4.62; G). Lane 1, SQUU-A; lane 2, SQUU-B.

SQUU-B. The signal of CK13 in SQUU-A was detectable after 18 cycles, exponentially increased up to 22 cycles, and was followed by plateau phase (data not shown), whereas the signal in SQUU-B was detected only after 25 cycles (Figure 6), thus indicating that the level of CK13 mRNA in the former is 1000 times higher than that in the latter. The CK13 sequence of amplification products (1567 bp) obtained from SQUU-A was identified with that from SQUU-B.

Cytokeratin Expression in Primary and Metastatic Tumor Tissue

The expression pattern of cytokeratin in the primary and metastatic tumor tissue was examined by immunohistochemistry. CK13 was detectable in both SQUU-A and SQUU-B transplanted into the tongues of nude mice (Figure 7, A and D). However, a small number of SQUU-B

Table 3. The Major Cytokeratin (CK) Polypeptides Detected in Human Tongue Epithelia and SQUU Cell Lines

CK no.	Tongue epithelium*	SQUU-A†	SQUU-B‡
4	+	+	–
5	+	±	±
6	+	+	–
7	–	–	–
8	–	+	+
13	+	+	–
14	+	+	–
15	(+)	±	±
16	+	+	–
17	(+)	±	±
18	–	+	+
19	(+)	+	+

*Characteristics for tongue epithelia cytokeratins are in general agreement with the relative molecular weight values estimated from SDS-polyacrylamide gel electrophoresis by Franke et al.²⁴ +, component always observed in substantial amounts; (+), component present in minor or variable amounts; –, component not observed.

†Cytokeratins of both clones were estimated from immunoblotting.

‡CK5, 15, and 17 were not examined using immunoblotting.

Figure 5. Detection of CK4, 14 and 16 mRNA expression by RT-PCR. Total RNA was extracted from nonmetastatic clone SQUU-A and a high metastatic clone SQUU-B. CK4 mRNA is expressed in both clones (A). CK14 and 16 mRNA are detectable in SQUU-A, but not in SQUU-B (B and C).

cells in the primary tissue were negative for CK13, thus showing a heterogeneous staining pattern. Both CK14 and 16 were expressed in SQUU-A of the tongue (Figure 7, B and C), whereas neither CK14 nor 16 was expressed in SQUU-B of the tongue (Figure 7, E and F). The cornified sites of SQUU-A in the tongue did not express CK14 (data not shown). SQUU-B in the cervical lymph nodes of the nude mice did not express CK 13, 14, or 16 (Figure 7, G–I). As positive control, the expression of cytokeratin in the tongue epithelia of nude mice was estimated. CK13 was labeled in the suprabasal cell layers (Figure 7A). CK14 reacted in the basal cell layers (Figure 7B). CK16 was stained in the suprabasal cell layers with a partial reaction in the basal cell layers (Figure 7C).

Discussion

We established two cell lines, SQUU-A and SQUU-B, derived from human oral squamous cell carcinoma and orthotopically transplanted them into the tongues of the nude mice. SQUU-A exhibited an expansive growth, without either intravasation or cervical lymph node metastasis. SQUU-B invaded the muscle with intravasation and cervical lymph node metastasis. SQUU-A and SQUU-B

Figure 6. Detection of CK13 mRNA expression by RT-PCR. Total RNA was extracted from nonmetastatic clone SQUU-A and a high metastatic clone SQUU-B. Eighteen cycles of amplification of CK13 (lanes 1 and 2), 25 cycles of amplification (lanes 3 and 4) and 35 cycles of amplification (lanes 5 and 6). A CK13 signal in SQUU-A is detectable after 18 cycles, while the signal is detected in SQUU-B only after 25 cycles.

Figure 7. Immunohistochemical staining of nonmetastatic clone SQUU-A and a high metastatic clone SQUU-B transplanted into the node mice. SQUU-A in the tongues of nude mice is positive with anti-CK13 (KS-1A3; **A**), anti-CK14 (LL002; **B**) and anti-CK16 (LL025; **C**). SQUU-B in the tongues of nude mice shows positive reaction with anti-CK13 (* shows negative staining area; **D**). SQUU-B is negative with anti-CK14 (**E**) and -16 (**F**). SQUU-B of the cervical lymph node metastasis is negative with anti-CK13 (**G**), anti-CK14 (**H**), and anti-CK16 (**I**). **Arrows** show tumors. Original magnifications, $\times 200$.

thus showed different cellular properties regarding the tumor invasive potentials and the frequency of cervical lymph node metastasis when transplanted orthotopically into nude mice.

The invasive and metastatic potentials in carcinoma have been considered to be regulated by the alterations of cell motility and morphology.²⁵ The cytoskeleton is considered to play a role in maintaining and controlling cell motility and morphology.²⁶ Cytokeratins are epithelial intermediate filaments. The cytokeratin expression pattern has been reported to contribute to both the migratory and invasive ability.²⁷ In general, each epithelial cell expresses specific pairs of acidic type I and basic type II (CK13/4, CK14/5, CK16/6, CK18/8).²⁸ The normal lateral tongue epithelium expresses CK13/4, 14/5, 15, 16/6, 17, and 19, but does not express CK18/8. CK13 is expressed in the suprabasal cells of the lateral tongue. CK14/5 and 19 are expressed in their basal cells.²⁹ The nonmetastatic clone SQUU-A expressed CK13/4, 14, 16/6, 18/8, and 19, and thus resembled the expression patterns of human tongue tissue. However, the invasive and metastatic clone SQUU-B expressed only CK18/8 and 19. In addition, CK13, 14, and 16 were not expressed in SQUU-B of the cervical lymph node metastasis of the nude mice. These results lead us to hypothesize that the alteration of intermediate filaments caused by the loss or down-regulation of CK13, 14, and 16 may be involved in the acquisition of invasive and metastatic potentials.

In squamous cell carcinoma, the expression of CK13 is usually down-regulated.^{8,30} Moll et al stated that a decrease in the CK13 expression was associated with an increase in the grade of malignancy in a transitional cell carcinoma.³¹ Schaafsma also observed a decrease in the CK13 expression in higher-grade transitional cell carcinomas, particularly in the areas of muscle invasion.³²

Heyden et al suggested that in lesions with moderate or severe dysplasia, and in carcinoma *in situ*, all cell layers expressed CK14, but were negative for CK13. CK14 expression in carcinoma was heterogeneous, with some regions containing CK14-negative basal cells, and both CK14 and 13 disappeared in most invasive carcinoma cells.^{33,34} Most cases of breast invasive carcinoma were negative for the antibody to CK14, and a positive correlation was observed between the presence of CK 14 and the expression of type VII collagen, one of the basement components.³⁵ In prostatic invasive carcinoma, the loss of expression of CK14/5 has been reported, though the loss is probably due to the nature of the tumor origin.⁹

CK16 has been an indicator of hyperproliferation in epidermis.³⁶ There have so far been no reports on the relationship between the expression of CK16 and the ability of invasion and metastasis.

CK19 fragment, CYFRA 21-1, has been reported to be useful as a marker for follow-up in the treatment of lung cancer^{3,4} or head and neck carcinoma.³⁷ CK19 mRNA is used to detect the cancer cells of micrometastasis in the lymph nodes³⁸ or circulating cancer cells in the peripheral blood by using RT-PCR.³⁹ We observed the expression of CK19 independent of the metastatic ability in our models.

CK14 and 16 mRNA were expressed in the nonmetastatic clone SQUU-A, but not in the high metastatic clone SQUU-B. SQUU-A cells expressed CK13 protein in both cultured and orthotopically implanted conditions. On the other hand, SQUU-B cells, which expressed little CK13 mRNA and protein in culture, appeared to express a high level of CK13 protein after implantation into the tongues of nude mice, and those cells in the cervical lymph node metastatic region also expressed little CK13 protein, based on immunohistochemical detection methods. The expression of the CK13 gene in SQUU-B cells may be up-regulated by its transcriptional activation or by the stabilization of its transcripts or protein after their implantation into the tongues of nude mice, because of the orthotopic environment.

The loss of CK13 expression in metastasized SQUU-B cells suggests three possibilities concerning the mechanism for metastasis. (i) In most SQUU-B cells, which express little CK13 protein prepared from the culture, CK13 gene expression may be induced after the implantation of SQUU-B cells into the tongue due to the orthotopic environment. However, a portion of the cells may lose the ability to express CK13 gene in association with the invasion of these cells, because such a process changes their microenvironment. The heterogeneous expression of the CK13 protein in SQUU-B cell-transplanted tumors may reflect such a change in their microenvironment. (ii) Some SQUU-B cells that lack CK13 protein may migrate to the cervical lymph nodes as soon as they are transplanted, and then start growing without adapting to the orthotopic environment, thus resulting in CK13 gene expression. (iii) SQUU-B cells that express CK13 in primary tissue may express no CK13 in the metastatic regions due to changes in the environment. To elucidate these possibilities we need to identify the existence of metastatic tumors much sooner after transplantation. CK13 is the pair to CK4. The loss or reduction of CK13 protein in SQUU-B cell line may result in the inconsistency with the expression of the CK4 protein and CK4 mRNA, though it is necessary to think about other factors.

Based on our previous findings, we think that the loss of CK14 expression correlated to the acquisition of the invasive and metastatic ability, though it is possible that the loss of CK13 and 16 may be related to this ability. We thus plan to introduce the complementary DNA of CK14 into a high metastatic clone SQUU-B, and examine the changes in the invasive and metastatic potentials by orthotopic implantation.

The 5' upstream sequences of CK4,⁴⁰ 5,⁴¹ 6,⁴² 14,⁴¹⁻⁴³ and 16⁴⁴ genes are known to harbor AP-2 binding motifs. In particular, AP-2 has been shown to regulate CK14/5 expression.⁴¹⁻⁴³ But recently AP-2 has been found to be necessary for full transcription of CK4.⁴⁰ AP-2 alone does not seem to be sufficient for CK14 in SQUU-A, because CK4 mRNA is expressed in SQUU-A and SQUU-B. The 5' upstream sequences of CK14 and 5 gene have SP-1 binding motifs.⁴¹ In rabbit corneal epithelial cells, such a surge in the SP-1/AP-2 ratio decreases the expression of CK14 gene, and the switch of the SP-1/AP-2 ratio may play a role in the reciprocal expression of the CK3 and 14 genes.⁴⁵ Recently NF- κ B

proteins, especially p65, have been found to suppress CK14/5 promoters on HeLa cells or keratinocytes.⁴⁶ Transcription factor AP-2, SP-1, and NF- κ B may have caused the difference in the expression of CK, especially CK14/5, between SQUU-A and SQUU-B. The regulatory regions in desmocollin (type II),⁴⁷ desmoglein (type I and III),⁴⁸ laminin (B1 chain),⁴⁹ matrix metalloproteinase-2 (MMP-2),⁵⁰ tissue inhibitor of metalloproteinase-2 (Timp-2),⁵¹ and VEGF⁵² genes all contain AP-2 binding motifs, and the laminin (B2 chain) genes also contain binding sites for AP-2-like nuclear protein.⁵³ Type IV collagen,⁵⁴ type VII collagen,⁵⁵ desmocollin (type II),⁴⁷ MMP-9,⁵⁰ Timp-2,⁵¹ and VEGF⁵² have also SP-1 binding motifs. NF- κ B binding motifs have been observed in MMP-9.⁵⁰ A reduction or loss of desmocollin (type II), desmoglein,⁵⁶ or basement membrane (laminin, type IV collagen, type VII collagen) has been reported to be related to the invasion and metastasis of oral cancer.^{57,58} An interesting finding is that the transcriptional factors of CK14/5 are common to those of desmocollin, desmoglein, the components of basement membrane, MMP, and Timp.

Gene encoding CK type I has been mapped to human chromosome 17q12-q23.⁵⁹ Mapping studies of functional keratin genes have localized CK13 and CK19 to chromosome 17q21-q23, and CK14 and CK16 to chromosome 17q12-q21. The genes are organized from 5' to 3' in the following order: 5'-CK19-CK15-CK17-CK16-CK14.⁶⁰ Both CK14 and CK16 mRNA disappeared in the high metastatic clone SQUU-B, whereas both keratin and CK13 mRNA were expressed in the nonmetastatic clone SQUU-A. A loss of chromosome 17q12-q21, which mapped CK16-CK14 genes in the highly metastatic clone SQUU-B, may thus have occurred. However, this may not be valid for chromosome 17q12-q21. The loss of 17q has not been reported in head and neck cancer using the comparative genomic hybridization methods.⁶¹

Point mutations in the keratin intermediate filament genes for CK5 or 14 have been reported as causes for hereditary skin blistering disorders such as epidermolysis bullosa simplex.^{62,63} In addition, the keratin mutation has been reported to influence the cytoskeletal architecture.⁶⁴ As a result, such point mutations may alter intracellular level of cytokeratin proteins of CK13/4, 14, and 16. However, there was no difference in the sequence of the CK13 cDNA of 1567 bp between SQUU-A and SQUU-B cells. Mutations of the promoter regions of these genes may cause a difference in the expression of these proteins between SQUU-A and SQUU-B. We therefore plan to investigate the mutation of CK14 genes using a PCR analysis of genomic DNA in the near future.

We established two cell lines, which have differences in invasive and metastatic ability in an orthotopic implantation system, and suggested that the loss or down-regulation of CK13, 14, or 16 is related to the invasive and metastatic ability of cancer. We expect that further molecular analyses of SQUU-A and SQUU-B will provide us with important information related to both invasion and metastasis.

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