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Establishment and characterization of a spindle cell squamous carcinoma cell line

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BACKGROUND: Spindle cell squamous carcinoma (SCSC) is a rare and peculiar biphasic malignant neoplasm that occurs mainly in the upper aerodigestive tract. It consists of sarcomatoid proliferation of pleomorphic spindle-shaped cells and squamous cell carcinoma.

METHODS: Here, we established a SCSC cell line from a tumour arisen in gingiva. We characterized the feature of a SCSC cell line by immunohistochemistry. To know the biological feature, we examined the cell growth, invasiveness and epithelial-mesenchymal transition markers of a SCSC cell line in comparison with oral squamous cell carcinoma (OSCC) cell lines.

RESULTS: By immunohistochemical analyses, the primary tumour expressed cytokeratin and vimentin, indicating carcinosarcoma-like characters. This tumour also showed overexpression of p53 protein. Cultured SCSC cells resulted in bypass of crisis and maintenance over passage 100. The established SCSC cell line was spindle-shaped and showed identical immunohistochemical characters to those of primary tumour cells. Similar to the primary tumour, the cell line showed p53 overexpression and had p53 mutation at codon 132: AAG (lys) → AAT (asp). The SCSC cell line grew slower than two other OSCC cell lines (MSCC-1 and HSC-2), whereas SCSC cells had remarkable invasiveness in comparison with these cell lines. Moreover, SCSC cells expressed wnt-5a and vimentin mRNA at high levels, but did not express E-cadherin mRNA. This expression pattern of the markers was similar to that of mesenchymal cells, not of epithelial cells.

CONCLUSION: In the present study, we newly established a SCSC cell line with strong invasiveness. This is the first report on the establishment of SCSC cell line. The SCSC cell line can be a useful cell model for the study to know the cytodifferentiation and nature of SCSC.
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Keywords: cell line; epithelial-mesenchymal transition; establishment; invasion; spindle cell squamous cell carcinoma

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Introduction

Spindle cell squamous carcinoma (SCSC) is a rare and peculiar biphasic malignant neoplasm that occurs mainly in the upper aerodigestive tract. It consists of sarcomatoid proliferation of pleomorphic spindle-shaped cells and squamous cell carcinoma, either *in situ* or invasive. SCSC has been referred to by a variety of names, including pseudosarcoma (1), carcinosarcoma (2), pleomorphic carcinoma or spindle cell carcinoma (3–5), which reflect the divergent interpretation of the sarcomatoid component as reactive or neoplastic, mesenchymal or epithelial. It is generally accepted that the sarcomatoid cells are derived from squamous cells. We previously reported that the epithelial nature of the sarcomatoid component of SCSC was clearly revealed by a combination of immunohistochemical staining for keratins and electron microscopic demonstration of tonofilament-like filaments and/or desmosome-like structures (6). However, the biological significance, such as cytodifferentiation and nature remain unknown. Here, we newly established and characterized a SCSC cell line from SCSC in gingiva.

Materials and methods

All procedures of the present studies were performed in compliance with regulations administered by the Hiroshima University.

Table 1 Antibodies used for immunohistochemical analyses

Antibody	*Source*	*Dilution*
Cytokeratins (CAM5.2)	Beckton Dickinson	Pre-diluted
Vimentin	Dako	1:50
S-100 protein	Dako	1:400
αSMA	Dako	1:100
p53 protein	Dako	1:100
PCNA	Dako	1:100

αSMA, alpha-smooth muscle actin; PCNA, proliferating cell nuclear antigen.

Table 2 Summary of immunohistochemical findings of SCSC tissue and cell line

	Immunohistochemistry				
	CAM5.2	*Vimentin*	*αSMA*	*S-100*	*p53*
Primary tumour	+	+	−	−	+
Cell line	+	+	−	−	+

SCSC, spindle cell squamous carcinoma; αSMA, alpha-smooth muscle actin.

Cell culture

Tissue sample was obtained from a SCSC of a 73-year-old Japanese male tumour in gingiva. The tissue sample was cut into small pieces and placed on 90-mm Petri dishes (3003, Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) with RPMI-1640 (Nissui Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Boehringer Mannheim Corp., Indianapolis, IN, USA) and 100 U/ml penicillin-streptomycin (Gibco BRL, Grand Island, NY, USA) under conditions of 5% CO_2 in air at 37°C. When these outgrowth cultures formed confluent monolayer, the cells were subcultured after enzymatic removal with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) to passage 1 (P1). Then, we subcultured the cells with the same medium until over P100 and used for the following analyses. Oral squamous cell carcinoma (OSCC) cell line, HSC-2 was provided by the Japanese Cancer Research Resources Bank and was routinely maintained in RPMI-1640 (Nissui Co.) supplemented with 10% FBS (Gibco BRL) under conditions of 5% CO_2 in air at 37°C. Another OSCC cell line, MSCC-1 cell was previously established by us and was routinely maintained in Keratinocyte-SFM (Gibco BRL) supplemented with 10% FBS under conditions of 5% CO_2 in air at 37°C. Fibroblast was obtained from periodontal ligament and was routinely maintained in Dulbecco's Modified Eagle Medium (DMEM; Nissui Co.) supplemented with 10% FBS under conditions of 5% CO_2 in air at 37°C.

Immunohistochemistry

Immunohistochemical analyses of both original tumour tissues and established culture cells were performed

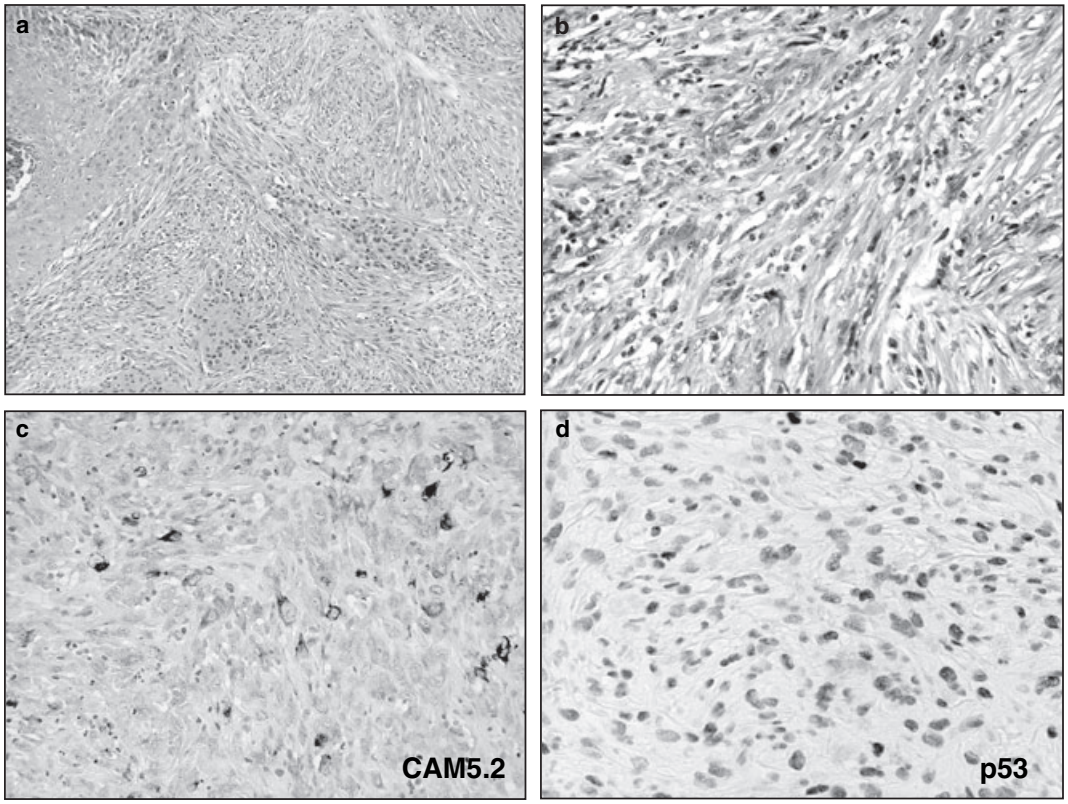

Figure 1 Histological and immunohistochemical findings of primary tumour of spindle cell squamous carcinoma (SCSC). (a) Haematoxylin and eosin (H & E) staining; squamous cell carcinoma and sarcomatoid area composed of spindle cells. Note the transition of both components (×100). (b) H & E staining; high magnification of pleomorphic spindle cells (×200). (c) Immunohistochemical staining; expression of cytokeratins in spindle cells (×200). (d) Immunohistochemical staining; expression of p53 protein in spindle cells (×200).

by ENVISION+ system (Dako, Glostrup, Denmark). Antibodies used are listed in Table 1. For immunohistochemical analyses of established culture cells, we used pleomorphic adenoma cells, fibroblast and oral cancer cell lines as positive and negative control for staining.

Cell growth

Cells were plated onto a 24-well multiwell plate (3047, Falcon, Becton Dickinson) and allowed to attach for 24 h. The culture medium was then replaced with fresh medium. After then, trypsinized cells were counted by Cell Counter (Coulter Z1, Coulter, Miami, FL, USA) at 0, 2 and 4 days.

In vitro invasion assay

Invasion was measured by an invasion assay device using of a 24-well cell culture insert with 8 mm pores (3097, Falcon, Becton Dickinson). *In vitro* invasion assay was performed by the method of Kalebic et al. (7) with minor modification (7, 8). The filter was coated with 20 μg of EHS extract (Iwaki Garasu, Tokyo, Japan), which was a reconstituted basement membrane substance. The lower compartment contained 0.5 ml of serum-free medium. After trypsinization, 1.5×10^5 cells were resuspended in 100 μl of serum-free medium and placed in the upper compartment of the cell culture insert for 12 h at 37°C in a humidified 95% air/5% CO_2 atmosphere. At the end of incubation, cells were fixed with methanol and stained with haematoxylin and eosin. Cells on the upper surface of the filter were wiped off with a cotton swab, and the invasiveness of the cells was determined by counting of the penetrating cells onto the lower side of the filter through the pores under a microscope at ×100 magnification. We assayed three times and randomly selected three fields were counted for each assay.

RNA preparation and reverse transcription polymerase chain reaction analysis

Total RNA was isolated from cultures of confluent cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Preparations were quantified and their purity was determined by standard spectrophometric methods. cDNA was synthesized from 1 μg total RNA by using the ReverTra Dash kit (Toyobo Biochemicals, Tokyo, Japan). The oligonucleotide reverse transcription polymerase chain reaction (RT-PCR) primers were listed in Table 2. Aliquots of total cDNA were amplified with 1.25 U of rTaq-DNA polymerase (Qiagen), and amplifications were performed in a PC701 thermal cycler (Astec, Fukuoka, Japan) for 30 cycles after an initial 30 s denaturation at 94°C, annealed for 30 s at 60°C and extended for 1 min at 72°C in all primers. The amplification reaction products were resolved on 1.5% agarose/TAE gels (Nacalai Tesque, Inc., Kyoto, Japan), electrophoresed at 100 mV and visualized by ethidium bromide staining.

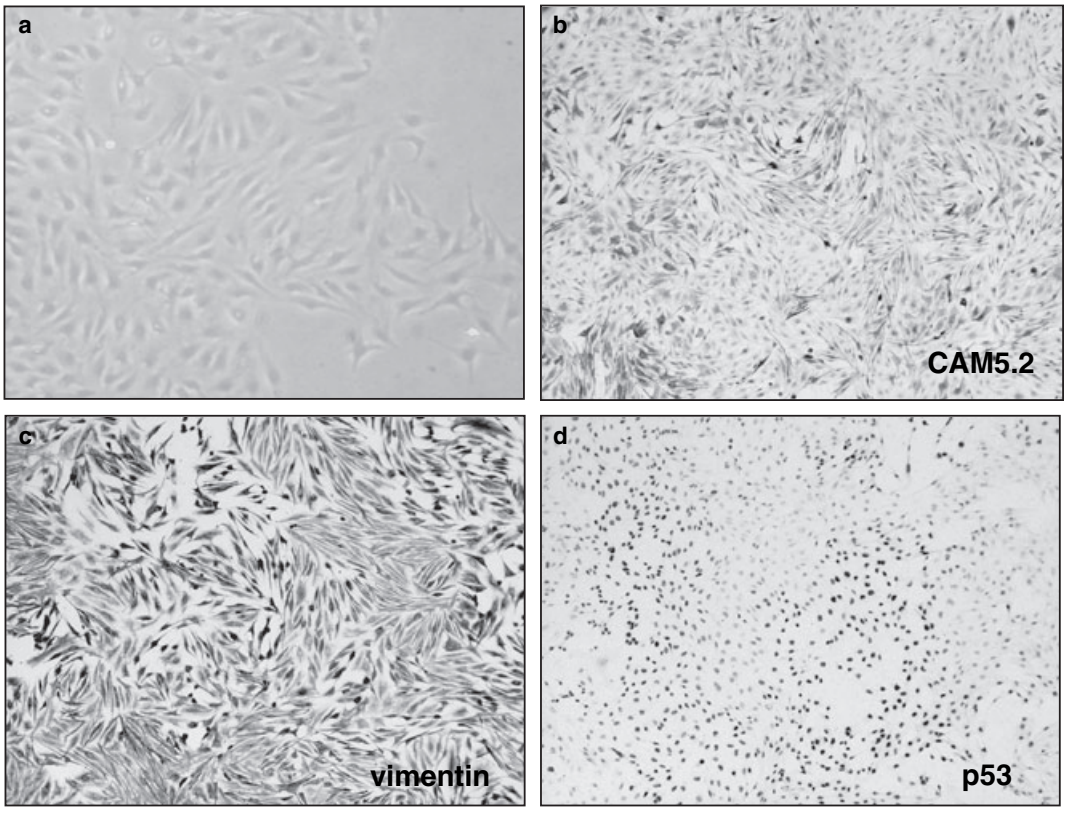

Figure 2 Histological and immunohistochemical findings of established spindle cell squamous carcinoma (SCSC) cell line. (a) Spindle shape under phase contrast microscopy (×100). (b) Immunohistochemical staining; expression of cytokeratins in SCSC cells (×100). (c) Immunohistochemical staining; expression of p53 protein in SCSC cells (×100).

Results

We established a SCSC cell line from a SCSC arisen in gingiva. The patient did not have a history of pre-existing squamous cell carcinoma and was treated by radical surgical excision without irradiation and chemotherapy. Histologically, the tumour widely infiltrated into connective tissues, muscles and mandible with ulcer formation. The tumour was mainly composed of pleomorphic spindle cells accompanying small areas of squamous cell carcinoma (Fig. 1a,b). Transition between both areas was observed and, immunohistochemically, the spindle cells as well as the squamous cell carcinoma cells were positive for cytokeratins (Fig. 1c). The former were also positive for vimentin. The tumour cells showed frequent positivity for PCNA and strong expression of p53 protein regardless of their shapes (Fig. 1d). S-100 and α-smooth muscle actin were negative. Immunohistochemical results are summarized in Table 2. The diagnosis of SCSC was made on the basis of these histological and immunohistochemical findings.

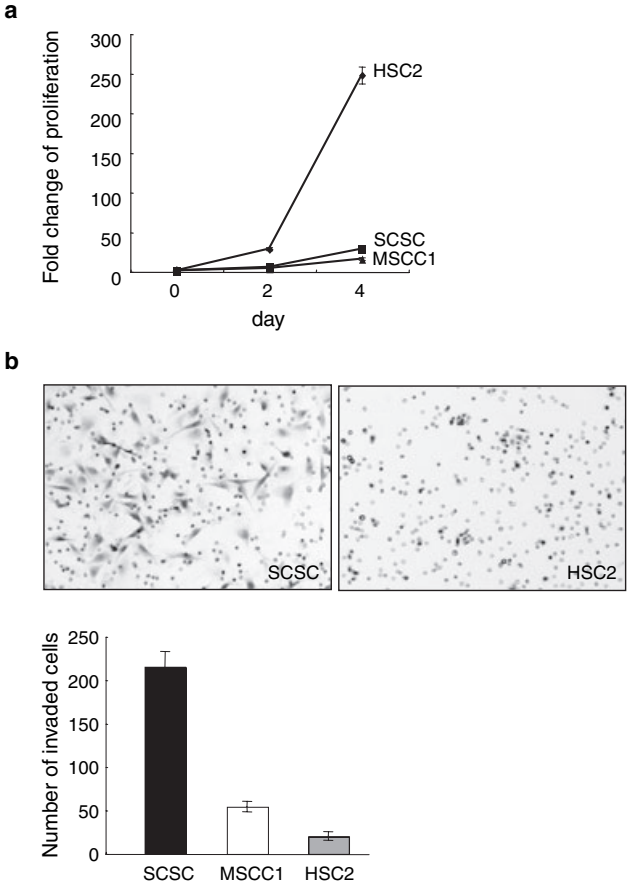

Figure 3 Characterization of spindle cell squamous carcinoma (SCSC) cells. (a) Cell proliferation of SCSC cells, compared with oral squamous cell carcinoma (OSCC) cell lines, HSC-2 and MSCC-1 cells. (b) Invasiveness of SCSC cells. The number of cells penetrated onto the lower side of the filter through pores is counted in SCSC, MSCC-1 and HSC-2 cells under a microscope (×100 magnification). We assayed three times and randomly selected three fields were counted for each assay. The number of SCSC, HSC-2 and MSCC-1 cells that penetrated through the filter was 214.4 ± 18.1, 20.2 ± 5.0 and 54.4 ± 6.1 at 12 h respectively.

We could subculture cancer cells up to 100 passages. Established SCSC cells showed spindle shape (Fig. 2a) and identical immunohistochemical reaction to that of pleomorphic spindle cells in primary tumour (Fig. 2b,c). SCSC cells showed p53 overexpression similarly to primary tumour and had a p53 point mutation at codon 132, AAG (lys) → AAT (asp). To characterize SCSC cells, we examined the cell proliferation and invasiveness compared with OSCC cell lines, HSC-2 and MSCC-1 cells. Cell growth of SCSC cells was slower than HSC-2 (Fig. 3a). Interestingly, SCSC cells had significantly higher invasiveness than HSC-2 and MSCC-1 cells by *in vitro* invasion assay (Fig. 3b,c). The number of SCSC, HSC-2 and MSCC-1 cells that penetrated through the filter was 214.4 ± 18.1, 20.2 ± 5.0 and 54.4 ± 6.1 at 12 h respectively.

Taki et al. have previously reported that loss of E-cadherin and wnt-4 and upregulation of wnt-5a were epithelial-mesenchymal transition markers (9). Further, we examined the expression of epithelial-mesenchymal transition markers, wnt-4, wnt-5a, E-cadherin and vimentin by RT-PCR and compared their expressions with those of other OSCC and fibroblast cell lines. SCSC cells expressed wnt-5a and vimentin mRNA at high levels, but did not express E-cadherin mRNA. This expression pattern was similar to that of fibroblast, but not to that of OSCC cells.

Discussion

Spindle cell squamous carcinoma has been reported as a mostly polypoid tumour with a predilection for occurrence in elder males (10). The present tumour showed ulcerative, but not polypoid. Moreover, the present tumour arisen in gingiva widely infiltrated into mandibular bone. SCSC occurs mainly in the upper aerodigestive tract, especially in the vocal cord, oesophagus and oral cavity. In oral cavity, lower lip is described as the most frequently involved site (10). Although the causative factors for SCSC are essentially unknown, a role for radiation or trauma has been emphasized by some authors (11). The patient of present case did not have a history of pre-existing squamous cell carcinoma or radiation therapy. Histologically, this tumour was dominantly consisted of pleomorphic spindle cells. SCSC cells established in the present study showed spindle-shaped and showed similar immunohistochemical expression pattern to pleomorphic spindle cells in primary tumour.

We and Zarbo et al. (4) demonstrated that the epithelial nature of pleomorphic spindle cells was revealed by a combination of the immunohistochemical expression of keratins and the ultrastructural characteristics of epithelial cells (4, 6). In fact, we demonstrated that SCSC cells expressed cytokeratins. Previous report showed loss of E-cadherin and wnt-4 and upregulation of wnt-5a were epithelial-mesenchymal transition markers (9). We found that SCSC cells expressed wnt-5a and vimentin mRNA at high levels, but did not express E-cadherin mRNA (Fig. 4). This expression pattern was similar to that of fibroblast, not of OSCC cells. These

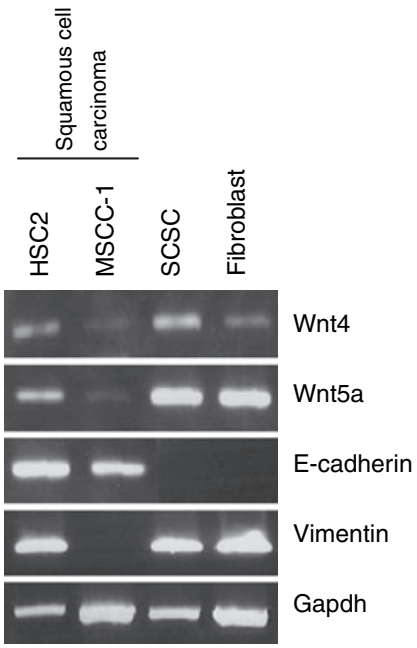

Figure 4 Expression of epithelial-mesenchymal transition markers, wnt-4, wnt-5a, E-cadherin and vimentin in spindle cell squamous carcinoma (SCSC) cells, oral squamous cell carcinoma (OSCC) cells and fibroblasts by reverse transcription polymerase chain reaction (RT-PCR).

findings suggest that the nature of SCSC cells may be similar to mesenchymal cells. However, the positivity for cytokeratins showed the epithelial nature of SCSC cells. Overall, these findings support the theory of epithelial origin and mesenchymal transition of SCSC. Recent molecular studies of these tumours in different organ sites have shown evidence for the sarcomatoid transformation from the epithelial component, thereby supporting a monoclonal hypothesis (12–14). As the pathogenesis and the biological implications of SCSC as mentioned above remain unknown, we believe that SCSC cell line established in this study can be a useful model.

So far, there have been a few studies for molecular analysis of SCSC (15–17). Moreover, there is no study on establishment of SCSC cell line. By using this cell line, we found that SCSC cell line had strong invasiveness by *in vitro* invasion assay in spite of the low proliferative activity (Fig. 3). In the future, we will examine the molecular analysis of SCSC to know the pathogenesis and the biological implications.

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