ROLE OF SQUAMOUS CELL CARCINOMA ANTIGEN 1 EXPRESSION IN THE INVASIVE POTENTIAL OF HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Abstract: Background. Serine proteases have important roles in tumor invasion and metastasis, and their inhibitors, serine protease inhibitors (serpins), are attractive targets for therapeutic strategies. On chromosome 18q21, there is a cluster of serpins: maspin, headpin, and squamous cell carcinoma antigen 1 (SCCA1)/SCCA2. Others and we have reported that the expression of these serpins is downregulated in head and neck squamous cell carcinoma (HNSCC) cells compared with normal squamous epithelial cells. In this study, we hypothesized that expression of SCCA1 is biologically disadvantageous to HNSCC cells.

Methods. HNSCC cell lines were transfected with a mammalian expression vector with SCCA1 cDNA. In vitro proliferation, migration, or invasive potential (matrigel assay) of the transfectants were assayed. In addition, the in vivo growth and invasion was analyzed using the floor-of-mouth model of nude mice.

Results. SCCA1 expression did not alter the in vitro growth rate of established HNSCC cells. However, SCCA1 expression significantly inhibited the in vitro invasion in matrigel assays. Furthermore, the in vivo growth and invasion in nude mice was also inhibited by SCCA1 expression.

Conclusions. Overexpression of SCCA1 in a HNSCC cell line inhibited its invasive potential. Loss of expression of the serpin SCCA1 may play a role in the malignant progression of HNSCC.

Keywords: serpin; SCCA1; head and neck cancer

Serine protease inhibitors (serpins) are known to play an important role in tumor invasion, metastasis, tumor suppression, and apoptosis. On chromosome 18q21, which is recognized as a region for frequent loss of heterozygosity (LOH) in head and neck squamous cell carcinoma (HNSCC), there is a cluster of serpins: maspin, headpin, and squamous cell carcinoma antigens 1 and 2 (SCCA1/SCCA2).1,2 Maspin is a serpin that was originally isolated from normal mammary epithelium by subtractive hybridization and differential display techniques.3 It has been demonstrated extensively that maspin inhibits breast cancer cell motility, invasion, and metastasis and functions as a tumor suppressor gene.1–6 We previously reported that maspin expression is
downregulated in HNSCC cells. Headpin is a serpin that is also downregulated in squamous cell carcinoma of the oral cavity and in squamous cell carcinoma cell lines of the head and neck. With their function as proteinase inhibitors, serpins may be involved in suppression of tumor growth and invasion.

The SCC antigen was first isolated biochemically from SCC tissue of the uterine cervix. Serum SCC antigen levels have been used as a tumor marker for patients with SCC of the gynecologic, esophageal, and head and neck regions. However, serum SCC antigen is also elevated in patients with nonmalignant lung or skin diseases. The SCC antigen is transcribed by two highly homologous genes, SCCA1 and SCCA2, and the SCC antigen detected in the serum is produced mainly by SCCA1. SCCA1 encodes a protein that has activity to specifically inhibit papain-like cysteine proteinases; papain; and cathepsins L, K, and S. We have previously reported that SCCA1 expression is downregulated in cultured HNSCC cells compared with normal oral keratinocytes and that SCCA1 is expressed not only in tumor cells but also in T lymphocytes peripheral to tumor cells. We also demonstrated that T lymphocytes peripheral to tumor cells are responsible for serum SCC antigen elevation in patients with HNSCC. The biologic role of SCC antigen expression in HNSCC cells remained unclear. Uemura et al reported that the intracellular location of SCCA1 is mainly in the cytosol and is not actively secreted, indicating that SCCA1 has an intracellular function as a serpin. In general, protease inhibitors are negative factors for cancer invasion and metastasis, and there is a discrepancy as to whether SCCA1 acts as a positive regulator of cancer progression. Because SCCA1 is a serine protease inhibitor, we hypothesized that SCCA1 expression is biologically disadvantageous for cancer cells. We overexpressed SCCA1 in HNSCC cells and analyzed the effect on cell growth and invasive potential in vitro and in vivo.

**MATERIALS AND METHODS**

**Cell Culture.** YCU-N, a human head and neck squamous cell carcinoma (HNSCC) cell line (provided from Dr M. Tsukuda, Yokohama City University, Japan), was maintained in RPMI1640 medium, supplemented with 10% fetal bovine serum (FBS), trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA.4Na), and penicillin/streptomycin, 1000 IU/mL. Cells were maintained in a humidified incubator at 37°C under 5% CO₂.

**Transfection of YCU-N cells with Squamous Cell Carcinoma Antigen 1.** The preparation of the mammalian cell expression pEH/hygro/B resistance vector (provided by Stuart Orkin) containing the SCCA1-eGFP cDNA construct has been reported previously. Control cells were transfected with plasmid vector containing the SCCA1(p14 mutant)-eGFP cDNA construct.

YCU-N cells were inoculated into 100-mm dishes at 25% to 30% confluency. After 24 hours, the cells were rinsed with 10 mL of serum-free RPMI 1640 and transfected with 5 μg of plasmid DNA using lipofectin (Life Technologies, Japan), according to the conditions described by the supplier. Forty-eight hours after transfection, transfected cells were selected in complete medium containing 400 μg/mL hygromycin B for 2 to 3 weeks. After selection, single independent clones were randomly isolated using cloning rings, and each clone was plated separately.

**Immunoradiometric Assay for Squamous Cell Carcinoma Antigen.** Cells were plated in triplicate 6-cm dishes. After incubation for 24 hours, the cells were washed twice with phosphate-buffered saline (PBS), and the medium was changed to serum-free medium. After incubation for 24 hours, the conditioned medium was recovered. The concentration of SCC antigen in the conditioned medium was measured using an immunoradiometric assay (SCC RIABEAD, Kyoowa, Japan).

**Western Blot Analysis of Cultured Cells.** Western blot analyses were performed to detect the SCCA1 expression in the YCU-N cell line. Total cellular protein was extracted and quantified using the M-Per Mammalian Protein Extracted Reagent and “Coomassie” Protein Assay Reagent Kit (Pierce, Rockford, IL). Equal amounts (100 μg) of cell lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked for 1 hour with 5% non-fat dry milk in PBS, and then incubated with the purified monoclonal antibody against SCCA1 (clone 8H11, 1:1000) or an anti-β actin antibody (AC-15, 1:2000, Sigma, Saint Louis, MO) for loading control for 1 hour at 37°C. The membranes were then incubated with the horseradish peroxidase (HRP)—conjugated goat anti-mouse immunoglobulin G (IgG) secondary
antibody (1:5000 dilution) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hour at room temperature, followed by the detection with the enhanced chemiluminescence (ECL) system (Amersham International, Buckinghamshire, UK).

**In Vitro Proliferation Assays.** Transfected clone and control clone cells (5000/dish) were seeded onto 35-mm dishes in RPMI 1640 medium plus 10% FBS. The number of the cells was counted every 48 hours for 10 subsequent days, in triplicate assays, using a Coulter Counter (Beckman Coulter, Fullerton, CA). The mean values were used to generate growth curves.

**In Vitro Matrigel Invasion Assays.** Invasion of squamous carcinoma cells and stable transfectants in vitro was measured by the invasion of cells through matrigel-coated transwell inserts (Becton Dickinson, Bedford, MA). Transwell inserts of 8-μm pore size were coated with a final concentration of 1 μg/mL of matrigel in cold serum-free RPMI 1640. Cells were trypsinized, and 200 μL of cell suspension (3 × 10^5 cells/mL) was added in triplicate wells. After 24 hours of incubation at 37°C, media in the upper chamber were aspirated, and cells on the upper surface of the filter were removed with a cotton swab. Cells on the lower surface that passed through the filter were fixed and stained using Heme-3 and photographed. The number of the stained nuclei were counted (10 high-power fields per each chamber) and expressed as a percentage of the sum of the cells in the upper and lower wells.

**In Vivo Tumorigenicity and Invasion Assays.** All animal experiments were in accordance with the guidelines of Kyushu University. To assess the effect of SCCA1 on in vivo invasiveness and tumorigenicity, 4 × 10^6 cells of parental YCU-N cells, YCU-N cells expressing SCCA1, or SCCA1(p14 mutant) were injected in the oral floor of 6- to 8-week-old female BALB/c nude mice. Each group consisted of 11 mice. This animal model is an orthotopic model designed for evaluation of the invasiveness of head and neck cancer. The animals were monitored for tumor formation every week and put to death 7 to 12 weeks after injection. After postmortem necropsy, the tumor and surrounding structures of muscle and jawbone were formalin fixed and paraffin embedded for subsequent histopathologic examination. Serial 3-mm sections were cut and stained with hematoxylin-eosin. The histologic invasion morphology was evaluated following the classification of the mode of invasion reported by Simon et al. The sections were examined by two individuals simultaneously. Tumor invasion was evaluated and recorded for each animal.

**Statistical Analysis.** Statistical analyses were performed using the Mann–Whitney U test or Fisher exact probability test. Differences with a p value < .05 were considered to be significant.

**RESULTS**

**Transfection of Squamous Cell Carcinoma Antigen 1 in YCU-N Human HNSCC Cell Line.** Fifteen independent clones were selected after 2 to 3 weeks of growth in medium supplemented with hygromycin B. Two representative clones, which were expressing the exogenous SCCA1 protein intensively, YCU-NSCCA1-6 and YCU-NSCCA1-7, were selected for use in subsequent experiments. Western blot analysis indicating the levels of SCCA1 expression of the selected clones and the parental cell line, YCU-N cells, is illustrated in Figure 1. The concentration of SCC antigen in the media of the transfected clones was measured using an immunoradiometric assay. The SCC concentration was significantly high in the medium conditioned by the SCCA1 transfected cells (12.3 ± 1.1 ng/mL/10^6 cells) compared with the vector-transfected control (2.1 ± 0.9 ng/mL/10^6 cells).

**In Vitro Invasive Potential among Transfected Clones.** Matrigel invasion assay was performed to examine the in vitro invasion of the SCCA1 transfected clones compared with parental YCU-N cells and the control vector transfecant. Staining of transwell inserts of SCCA1 transfected clone cells that invaded through the matrigel were significantly less than parental and control vector, SCCA1p14m transfected, clones.
We found no marked difference in the invasion between the parental and control vector clones. However, a significant reduction in the invasive potential was noted with SCCA1 transfected clones (Figure 3B).

(Figure 3A). In Vivo Invasive Potential among Transfected Clones. An orthotopic floor-of-mouth model was used to evaluate the effect of SCCA1 transfection to HNSCC cells. From 7 weeks after cancer cell injection, some mice in the control vector group began to have anorexia because of obstruction to feeding and were put to death successively. As shown in Figure 4, the transplanted tumor of the SCCA1p14m group was significantly larger than the SCCA1 transfected SCCA1-7. The average time span from cell injection to animal sacrifice (because of anorexia) was shorter in the SCCA1p14m group (8 weeks, 3 days) than the SCCA1-7 group (11 weeks). On grading the tumor invasion following Simon classification, the number of mice classified in stages 1, 2, 3, and 4 were four (36%), three (27%), four (36%), and 0 in the NSCCA1-7 group and one (10%), three (27%), four (36%), and three (27%) in NSCCA1p14m group. An apparently significant correlation was the higher proportion of stage I and II in NSCCA1-7 group (63%) than in the NSCCA1p14m group (37%) (p < .05) (Table 1).

DISCUSSION
HNSCC is known to be highly invasive with early metastasis. Proteases that are involved in extracellular proteolysis and their inhibitors play an important role in cancer invasion and metastasis. An imbalance between proteases and protease inhibitors in cancer cells may result in progression of the disease. Unlike the metalloprotease family, there are only few data on serine proteases and their inhibitors (serpins) regarding their role in the invasive behavior of HNSCC.

FIGURE 1. Western blot analysis revealed high levels of squamous cell carcinoma antigen 1 (SCCA1) expression in clones NSCCA1-4, 5, 6, 7, and 8 compared with the nontransfected control (YCU-N). NSCCA1-6 and NSCCA1-7 were chosen for further examination.

FIGURE 2. In vitro proliferation of the squamous cell carcinoma antigen 1 (SCCA1) transfected clones. Triplicate cultures of YCU-N (parental cell line), NSCCA1p14m (vector control), and SCCA1 transfected NSCCA1-6 and NSCCA1-7 were cultured under 10% serum.
Serpins play a role in extracellular matrix remodeling and cell migration. The SCC antigen that is coded by two tandemly repeated genes SCCA1 and SCCA2 belongs to the ovalbumin serpin (ov-serpin) family. The SCCA1/SCCA2 gene locates at chromosome 18q21.3, which is a cluster region of the ov-serpin family such as maspin and headpin. Although the SCC antigen has been recognized as a tumor marker for squamous cell carcinomas including HNSCC, we have previously reported that the expression of SCCA1 is downregulated in HNSCC cells compared with normal keratinocytes. The expression of the other members in the serpin family, such as headpin and maspin, is also suppressed in HNSCC cells. In this study, we demonstrated that overexpression of SCCA1 suppresses the invasive potential of HNSCC cells in vivo and in vitro.

In this study, we have used the floor-of-mouth model to analyze the in vivo effect of SCCA1 transfection. Because tumors growing subcutaneously in the flank rarely invade the surrounding structures, further assessment of invasive potential will only be possible with the floor-of-mouth model. We stably transfected SCCA1 cDNA in a human HNSCC cell line YCU-N cells that have low endogenous SCCA1 expression and high invasive potential. The SCCA1 transformant cell lines, SCCA1-6 and SCCA1-7 cells, revealed no change in the in vitro growth. However, the invasive potential of YCU-N cells was suppressed by SCCA1 cDNA transfection.

Maspin, a member of the serpin family, is downregulated in breast cancer. Transfection of maspin in a mammary carcinoma cell line resulted in inhibition of in vivo cancer cell growth and invasion. Sheng et al reported that maspin exhibits its inhibitory activity toward plasminogen activators such as urokinase-type plasminogen activators.
ogen activator (uPA) and tissue plasminogen activator (tPA). However, Bass et al\textsuperscript{18} have shown that maspin inhibits cell migration in the absence of protease inhibitory activity, and controversy still exists concerning the protease-inhibitory activity and tumor-suppressing activity of maspin.

In contrast to maspin, only a few reports exist of SCCA1 regarding the direct biologic effect on cancer cells. SCCA1 inhibits the papain-like cysteine proteinases cathepsins S, L, and K.\textsuperscript{11} Strojan et al\textsuperscript{19} reported that cathepsin L is expressed in HNSCC cells and is involved in their invasive behavior. Kos et al\textsuperscript{20} reported that cathepsin L protein expression in HNSCC tissues is significantly higher than normal tissue using quantitative immunoreactive assays. Inhibition of cathepsins that are involved in the invasive potential of HNSCC may have a role in the inhibitory activity of SCCA1 against HNSCC cells.

It has been demonstrated that high serum levels of the SCC antigen correlate with poor clinical outcome of patients with squamous cell carcinomas of the cervix, esophagus, and head and neck. However, the biologic mechanism of this serum SCC antigen elevation has been unclear. If the cancer cells themselves are expressing and secreting the SCC antigen, it conflicts with our data that SCCA1 is disadvantageous for cancer cells to invade. We previously reported that T lymphocytes surrounding the cancer tissue instead of the cancer cells are responsible for serum SCC antigen elevation in patients with HNSCC.\textsuperscript{12} Iwasaki et al\textsuperscript{21} reported that transcriptional activation of SCC antigen through the E1AF pathway inhibits the invasiveness of SiHa cervical cancer cells. They also indicated that antisense SCCA transfection enhanced the in vitro invasive potential of cervical cancer cells, which is consistent with our finding that SCCA1 inhibits cancer cell invasion.

Suminami et al\textsuperscript{22} reported that SCCA1 overexpression in a murine cell line enhanced in vivo growth of the tumor cells by mediating protection from apoptosis. The discrepancy of our results could be explained by the difference of the cell line used in the experiments or the difference of the exogenous expression level of SCCA1. Several reports indicate that an elevated SCCA2/SCCA1 mRNA expression ratio reflects the aggressiveness of squamous cell cancer, suggesting SCCA2 to be a positive regulator and SCCA1 to be a negative regulator for cancer cells.\textsuperscript{23,24} These data agree with our findings that SCCA1 expression inhibits the malignant potential of HNSCC cells.

In conclusion, we have shown that SCCA1 expression inhibits invasion of HNSCC cells in vitro and in vivo. Loss of expression of the serpin SCCA1 may play a role in the malignant progression of HNSCC and have a potential to be a molecular target in the treatment of HNSCC.

REFERENCES


