A novel function of EpCAM in oral squamous cell carcinoma cells under anchorage-independent conditions

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Received May 23, 2011; Accepted June 30, 2011

DOI: 10.3892/ijo.2011.1137

Abstract. Epithelial cell adhesion molecule (EpCAM), involved in Ca^{2+}-independent homotypic cell-cell adhesion in epithelial tissues, is overexpressed in several cancer types. Although studies investigating the function of EpCAM in cancers have shown that it plays a role in cell proliferation, invasion and metastasis, the overall function of EpCAM in cancer cells has remained elusive. Here, we report a novel function of EpCAM in multicellular aggregates (MCAs). EpCAM inhibition using RNA interference (RNAi) did not affect cell morphology, proliferation or expression of certain genes, including cyclin D1 in monolayer cultures of the human oral squamous cell carcinoma cell lines HSC-3 or HSC-4. However, in HSC-4 cells cultured as MCAs, suppression of EpCAM significantly reduced the expression levels of cyclin D1. Nuclear localization of the cyclin D1 protein was observed in MCAs of HSC-4 cells but not in MCAs of EpCAM knockdown HSC4 cells, suggesting that EpCAM regulates cyclin D1 expression and localization in HSC-4 cells under anchorage-independent conditions. We propose that targeting EpCAM might result in more efficient therapies under certain conditions of oral squamous cell carcinoma.

Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent cancer of the head and neck (1,2) with surgery, radiation, and chemotherapy being the standard treatments. Despite therapeutic advances, functional disorders after surgery often occur. Although many studies investigating molecular targets as markers to determine the prognosis of patients have been developed, further functional analyses are required to develop therapies for oral cancer (3-5).

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Key words: oral cancer cell line, EpCAM, cyclin D1, cell proliferation, squamous cell carcinoma, anchorage independence, RNA interference

In independent studies, epithelial cell adhesion molecule (EpCAM) has been observed to be expressed in several carcinomas (6-11). It is a 34-42-kDa transmembrane protein mediating Ca^{2+}-independent homotypic cell-cell adhesion in epithelial cells (12), and does not belong to the four major adhesion molecule families (cadherins, integrins, selectins, and the immunoglobulin superfamily) (13,14). EpCAM consists of an extracellular domain of 265 amino acids including two epidermal growth factor (EGF)-like repeats, a transmembrane domain of 23 amino acids and a short intracellular domain of 26 amino acids (15-17). Expression is restricted to the basolateral membrane of the majority of epithelium tissue, except in adult squamous epithelium and several specific epithelia (12,17,18). EpCAM has been shown to be overexpressed in several carcinomas including colon, lung, stomach, prostate, cervical, and breast cancer (19-22). Furthermore, the expression level of EpCAM reportedly correlates with cell proliferation and invasiveness of cancers including oral cancer, indicating EpCAM could be an efficient molecular target (19,21,23). Clinical studies investgating the efficacy of the monoclonal antibody, catumaxomab, in non-small cell lung cancer and ovarian cancer has indicated the efficacy of targeting EpCAM (24,25).

Cyclin D1 is an important regulator through the G1 phase of the cell cycle, and it forms a cyclin-dependent kinase (CDK) complex by binding Cdk4 and Cdk6 (26). Through phosphorylation and deactivation of retinoblastoma protein by the complex, the E2F transcription factor activates several genes required for advancement from the G1 into the S phase (27). Uncontrolled cell proliferation is a characteristic of cancer, and high levels of cyclin D1 expression promotes cell growth and malignancy of several cancer types (28-31).

Here we determined that EpCAM expression upregulated or maintained the cell cycle regulator, cyclin D1 in oral cancer cell lines as multicellular aggregates (MCAs).

Materials and methods

Cell culture. Human OSCC cell lines SAS, HSC-3, and HSC-4 (RIKEN BioResource Center, Ibaraki, Japan) were cultured in Dulbecco's modified Eagle's medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Cell monolayer cultures were prepared by plating on 10-cm cell culture dishes (Asahi Glass, Tokyo, Japan), 6-well plates (Multiwell Plates; Asahi Glass), and 96-well plates (Asahi Glass). To generate MCAs, cells were plated on 35-mm EZ BindShut® culture dishes (Asahi Glass).

Adult mice. Adult mice (3-6 weeks old; ICR male and female; Japan SLC, Inc., Shizuoka, Japan) were maintained according to the institutional guidelines of the Research Institute for Microbial Diseases, Osaka University. The mice were used for RNA isolation from testis, kidney, liver, brain, intestine, stomach, heart, lung, tongue, and ovary.

RT-PCR. Total RNA was isolated with TRIzol (Invitrogen) from cultured cells and tissues of the adult mice. cDNA was synthesized using Random Primer 6 (New England BioLabs, Ipswich, MA) and SuperScript™ III Reverse Transcriptase (Invitrogen). cDNA was amplified by Takara Ex Taq® Hot Start Version (Takara Biotechnology, Shiga, Japan) for PCR analysis. Primer sequences for PCR were EpCAM (Fw, 5'-GA ATGGCTCAAAACTTGGGA; Rv, 5'-ACGCGTTGTGATC TCCTTCT), Cyclin D1 (Fw, 5'-CCCTCGGTGTCCTACTTCA; Rv, 5'-GTTTGTTCTCCTCCGCCTCT), GAPDH (Fw, 5'-ACA GTCAGCCGCATCTTCTT; Rv, 5'-TGGAAGATGGTGATGG GATT), mEpCAM (Fw, 5'-ATGGACCTGAGAGT GAACGG; Rv, 5'-CACGGCTAGGCATTAAGCTC), mGapdh (Fw, 5'-CC CACTAACATCAAATGGGG; Rv, 5'-CCTTCCACAATGCCA AAGTT). Quantification of band intensities were performed with LAS4000 mini (Fujifilm, Osaka, Japan). Expression levels of each sample were normalized against GAPDH mRNA expression.

RNA interference (RNAi). Short interfering RNA (siRNA) treatment was performed using Oligofectamine (Invitrogen) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The sequences of siRNAs synthesized by Thermo Fisher Scientific (Waltham, MA) were si-EpCAM, 5'-GUUUA CGGCCAGCUUGUAGdTdT and 5'-CUACAAGCUGGCCG UAAACdTdT; si-scrambled RNA, 5'-UUCUCCGAACGUGU CACGUdTdT and 5'-ACGUGACACGUUCGGAGAAdTdT.

To perform the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, siRNAs were transfected into HSC-4 cells as monolayers in 96-well plates using Oligofectamine reagent. To perform RT-PCR, siRNAs were transfected into HSC-3 and HSC-4 cells as monolayers in 6-well plates using Lipofectamine 2000 reagent and for HSC-4 cells as MCAs; monolayer cells were transfected using Lipofectamine 2000 and plated on 35-mm EZ BindShut culture dishes.

Cell proliferation assay. Proliferation of siRNA transfected cells after incubation for 0, 24, 48, or 96 h in 96-well plates was assessed using the MTT assay (CellTiter 96® Non-Radioactive Cell Proliferation Assay; Promega, Tokyo, Japan) according to the manufacturer's instructions. The absorbance at 570 nm was measured using a 96-well plate reader (Labsystems Multiskan MS-UV; Labsystems, Helsinki, Finland).

Immunofluorescence staining. MCA cultures of HSC-4 cells were fixed with 3.5% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with Image-iT™ FX Signal Enhancer (Invitrogen). Cyclin D1 antibody was used as primary antibody. Next, Alexa488 conjugated IgG (Invitrogen) was used as secondary antibodies. After incubation with the antibodies, SlowFade® Gold antifade reagent with DAPI (Invitrogen) was added and coverslips mounted. The specimens were observed using an immunofluorescence microscope (Leica Microsystems Japan, Tokyo, Japan).

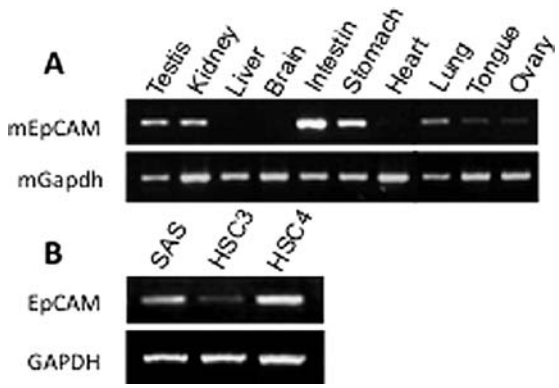

Figure 1. EpCAM expression in mouse tissues and human oral cancer cell lines. (A) EpCAM expression in the tissues of adult mice; and (B) EpCAM expression in SAS, HSC-3, and HSC-4 cells were analyzed by RT-PCR.

Statistical analysis. A Mann-Whitney U test was performed using StatView 5.0 (Abacus Concepts Inc., Berkeley, CA) to assess the statistically significance difference between samples. Data are presented as the mean ± SD. P-values < 0.05 were considered statistically significant.

Results

EpCAM expression associates with oncogenesis in the tongue. To confirm the association of EpCAM expression with oncogenesis in the tongue, we analyzed EpCAM mRNA levels in mouse tissues and human oral cancer cell lines using RT-PCR. EpCAM mRNA was clearly detected in testis, kidney, intestine, and lung of mice, and faintly in the tongue and ovary (Fig. 1A). In oral cancer cell lines derived from tongue, EpCAM mRNA was strongly detectable in SAS and HSC-4 cells and weakly in HSC-3 (Fig. 1B), indicating that the expression levels of EpCAM were different depending on the locus of derived tissues and differentiation of cells. These data suggest that EpCAM expression associates with carciogenesis in the tongue.

Effects of downregulation of EpCAM in oral cancer cell lines as monolayers. Previous studies suggest that EpCAM is related to the prolifration and invasion of cancer cells (19,21,23). To confirm the function of EpCAM on proliferation in oral cancer cell lines as monolayers, EpCAM knockdown in HSC-3 and HSC-4 cells by transient transfection with EpCAM siRNA was performed. EpCAM mRNA levels decreased drastically in the siRNA transfected cells (Fig. 2B and C). In the control cells transfected with or without scrambled RNA, the expression level of EpCAM was not affected (Fig. 2B and C). Downregulation of EpCAM did not significantly influence HSC-4 cell proliferation (Fig. 2A) or cyclin D1 expression (Fig. 2B). Downregulation of EpCAM in HSC-3 cells did not affect the expression level of cyclin D1

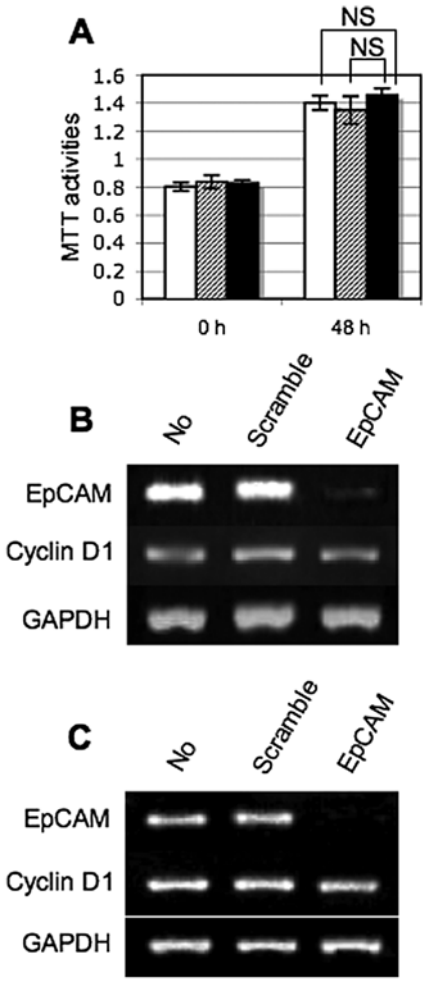

Figure 2. Effects of EpCAM siRNA on HSC-3 and HSC-4 monolayers. (A) HSC-4 cells plated on 96-well plates of $5x10^3$ cells per well. After incubation for 48 h, activity of HSC-4 cells transfected with (shaded column) or without (white column) scrambled siRNA (200 nM) or transfected with EpCAM siRNA (200 nM) (black column) were determined using MTT assay. Data are presented as the mean ± SD from four independent experiments (Mann-Whitney U test, no-siRNA vs scramble siRNA and no-siRNA vs EpCAM siRNA; NS, not significant). (B) HSC-4 cells were plated on 6-well plates of $1x10^5$ cells per well, and transfected with scrambled siRNA (200 nM) or EpCAM siRNA (200 nM). After 24 h, cells were analyzed for EpCAM and cyclin D1 mRNA by RT-PCR. Representative results are shown from two independent experiments. (C) HSC-3 cells plated on 6-well plates of $1x10^5$ cells per well and transfected with siRNAs. EpCAM and cyclin D1 mRNA were analyzed by RT-PCR. Representative results are shown from two independent experiments.

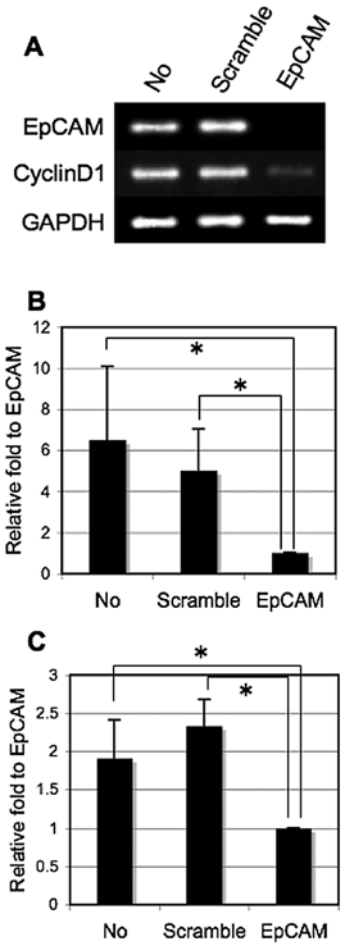

Figure 3. Effects of EpCAM siRNA on HSC-4 cells as MCAs. (A) siRNA transfected HSC-4 cells as MCAs were plated on 35 mm EZ BindShut culture dishes of $2x10^5$ cells. After 24 h, cells were analyzed for EpCAM and cyclin D1 level by RT-PCR. Representative results are shown from three independent experiments. (B) EpCAM/GAPDH intensities are the mean ± SD from three independent experiments (Mann-Whitney U test; $^*P<0.05$). (C) The cyclin D1/GAPDH intensities are the mean values with SD from three independent experiments (Mann-Whitney U test; $^*P<0.05$).

(Fig. 2C). These results indicate that EpCAM expression did not impact cell proliferation and cyclin D1 expression level in HSC-3 and HSC-4 cells as monolayers.

Effects of downregulation of EpCAM on cyclin D1 level in oral cancer cell line as MCAs. To investigate the role of EpCAM in oral cancer cell lines under anchorage-independent conditions, transfection of siRNA was carried out using Lipofectamine 2000 in HSC-4 cells as MCAs. Suspension culture of HSC-4 cells transfected with siRNA in non-adhesive culture dishes (EZ BindShut) formed MCAs indistinguishable from the control MCAs (data not shown). Dramatic reduction of the EpCAM mRNA level in EpCAM siRNA transfected MCAs was detected by RT-PCR (Fig. 3A and B). Data suggest that

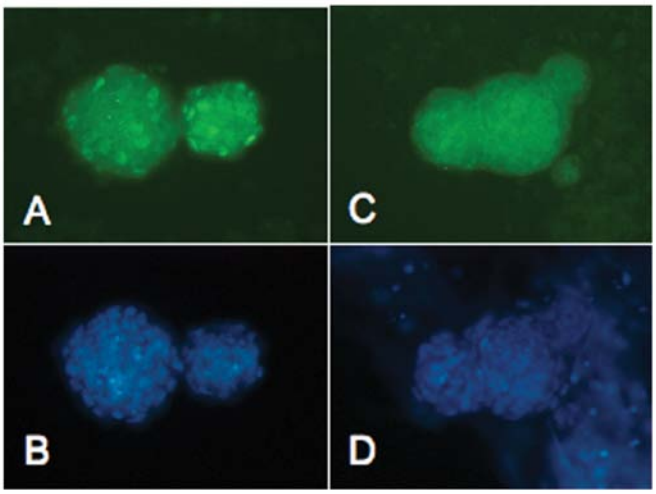

Figure 4. Localization of Cyclin D1 protein in HSC-4 cells as MCAs culture. Control (A and B) or siRNA transfected (C and D) HSC-4 cells as MCAs were plated on 35 mm EZ-BindShut culture dishes. After 48 h, localization of Cyclin D1 was assessed by immunofluorescent staining (A and C). Nuclei were counterstained with DAPI (B and D). Results are representative of at least three independent experiments.

cell surface EpCAM molecules are not involved with cell aggregation. Cyclin D1 expression in EpCAM downregulated MCAs was significantly repressed (Fig. 3A and C), indicating that the EpCAM expression could be associated with the cyclin D1 expression in MCAs of HSC-4 cells.

To examine the localization of cyclin D1 in oral cancer cell lines as MCAs, immunofluorescence staining was performed using cyclin D1-specific antibody. Specific staining was distinctly localized to the nucleus in some cells in control MCAs (Fig. 4A). On the other hand, nuclear signals of cyclin D1 staining could not be detected in MCAs from HSC4 cell-transfected siRNA for EpCAM (Fig. 4C). These results show that nuclear restricted cyclin D1 protein localization disappeared in EpCAM eliminated MCAs of HSC4 cells.

Discussion

The expression patterns of EpCAM in normal tissues of mice and in human OSCC cell lines suggest that EpCAM levels increase during carcinogenesis from the tongue and their over-expression was maintained in parts of tumor tissues because its expression is highly conserved between humans and mice (14,32). This is consistent with previous studies reporting that EpCAM expression is related to cell proliferation in several cancers through the regulation of specific genes including cell cycle regulators (19,21,23,33). Although EpCAM did not contribute to cell proliferation in HSC-4 and HSC-3 cells as monolayers, RNAi-mediated downregulation of EpCAM expression reduced the mRNA expression level of cyclin D1 in MCAs and eliminated cyclin D1 protein in nuclei of MCAs. These data indicate that cell proliferation through cyclin D1 expression in HSC-4 cells is regulated by EpCAM under anchorage-independent cell aggregation conditions. Thus, EpCAM plays various roles depending on the environment of the surrounding cells as well as cell types.

EpCAM is known to be a marker of poor prognosis including metastasis in several carcinomas (34-38). Metastasis is a multi-step process, during which primary tumor cells invade adjacent tissues, intravasate, translocate through the vasculature, arrest in distant capillaries, extravasate into the surrounding tissue, and finally proliferate into second tumors (39). Metastatic cancer cells need anoikis resistancy and growth ability in single cells and small cell clusters that are anchorage-independent. MCAs represent a culture model of anchorage-independence (40) and HSC-4 cells with or without siRNA treatment for EpCAM exhibited survival in MCAs culture, indicating that HSC-4 cells have the ability of anoikis resistance independent of EpCAM. Thus, EpCAM may assist cancer cells to initiate growth in a small clusters losing anchoradge-dependency in delivery through the lymphatic and circulatory systems or arrest around the target tissue.

EpCAM, one of the most highly and frequently expressed tumor-associated antigens, is found on a wide range of epithelial cancers (22). Furthermore, EpCAM was identified as a cancer stem cell antigen (41-43). It has also been highlighted as a target antigen for cancer immunotherapy, and many antibody-based therapeutic approaches targeting EpCAM are currently in development (44-49). Alternatively, the possibility of RNAi-based therapeutics has been reported (50). We successfully applied RNA silencing to inhibit the expression of EpCAM, thereby decreasing cyclin D1 levels in cancer cells. Therefore, we propose the possibility that RNAi-mediated gene silencing of EpCAM may be a useful strategy for treatment of tongue cancer metastasis.

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