Analysis of a cancer stem cell marker EpCAM in an oral squamous cell carcinoma cell line as monolayers and multicellular aggregates

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The epithelial cell adhesion molecule (EpCAM) is a Ca²⁺-independent intracellular adhesion in epithelial tissues, and is highly expressed in several cancers. Despite much research on the function of EpCAM in cancers, complete understanding remains elusive. Recent studies have reported the possibility that EpCAM is a cancer stem cell marker. The expression level of cyclin D1 in monolayer cultures of a human oral squamous cell carcinoma cell line, HSC-4, was not affected by the depression of EpCAM expression using RNA interference (RNAi). On the other hand, suppression of EpCAM reduced the expression level of cyclin D1 in HSC-4 cells as multicellular aggregates (MCAs). Moreover, it was observed that localization of EpCAM protein in HSC-4 cells was altered between MCAs and monolayers. These results suggest that EpCAM regulates the cell growth of HSC-4 through the cyclin D1 expression under anchorage independent conditions. It is proposed that targeting EpCAM might lead to more useful therapies against oral squamous cell carcinoma. (J Osaka Dent Univ 2012; 46: 215-220)

Key words: Oral cancer cell line; EpCAM; Cancer stem cell; Cyclin D1; Oral squamous cell carcinoma; RNA interference; Anchorage independence

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is one of the most frequently occurring cancers of the head and neck region, and accounts for more than 90% of all malignant neoplasms of the oral cavity.¹,² Surgery, radiation and chemotherapy are standard treatments for oral cancer. Despite therapeutic advances, post surgical dysfunction is a serious problem. Many theories have been advanced for novel targets as markers for determining the prognosis of the disease. Nevertheless, additional functional analysis of molecular targets is needed for therapies of oral cancer.³⁵

Independent researchers have identified the epithelial cell adhesion molecule (EpCAM) in different carcinomas.⁶⁻⁹ EpCAM is a 34-42 kDa transmembrane glycoprotein that mediates Ca²⁺-independent homotypic cell-cell adhesion in epithelial cells,⁶ and is selectively expressed in the basolateral membrane of most epithelium tissues, with the exception of adult squamous epithelium and a number of specific epithelia.⁸,¹⁰ It has been reported that EpCAM is overexpressed in the tissues of several carcinomas and is a characteristic of cancer.¹¹⁻¹⁴ Moreover, some groups have reported that the expression level of EpCAM correlates with cell proliferation and the invasiveness of cancers including oral cancer, possibly making it an effective molecular target.¹¹,¹²,¹⁵,¹⁶ Recent studies have reported the possibility that EpCAM might be used as a marker of cancer stem cells found in malignancies of some tissues.¹⁷,¹⁸ Recently, treatments using the trifunctional monoclonal antibody catumaxomab have been applied in the clinical
research of non-small cell lung cancer and ovarian cancer.\textsuperscript{19–22} The antibody is characterized by a unique ability to bind three different cells involving tumor cells, T-cells and accessory cells, and its therapeutic potential has been indicated in EpCAM-positive carcinomas.\textsuperscript{21}

Cyclin D1 is known as a cell-cycle regulator, and is involved in formation of the cyclin dependent kinase (CDK) complex by binding to either Cdk 4 or Cdk 6.\textsuperscript{22} Phosphorylation and deactivation of RB are induced by the complex, and the E2F transcription factor activates some genes required for the progression from the G1 into the S phase.\textsuperscript{23} Unregulated cell proliferation is a profile of cancer, and cyclin D1 expression is involved in cell growth, malignancy, migration and oncogenic signal events of several cancers.\textsuperscript{24–27} In the present study, we found that EpCAM expression regulated the cell cycle regulator, cyclin D1, and could lead to active cell proliferation of an oral cancer cell line as multicellular aggregates (MCAs).

**MATERIALS AND METHODS**

**Cell culture**

The human OSCC cell line HSC-4 used in the present experiments was obtained from RIKEN BioResource Center (BRC; Ibaraki, Japan). It was maintained at 37°C in a humidified atmosphere of 95% air and 5% CO\textsubscript{2} in Dulbecco's Modified Eagle's Medium (DMEM; Mediatech, Manassas, VA, USA) to which had been added 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA). Cell monolayer cultures were prepared by plating on 10 cm cell culture dishes (Asahi Glass, Tokyo, Japan) and 6-well plates (Multiwell Plates; Asahi Glass). To generate MCAs, the cells were plated on 35 mm EZ Bind Shut\textsuperscript{+} culture dishes (Asahi Glass), and maintained.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated using TRIzol (Invitrogen) from culture cells. cDNA was synthesized using Random Primer 6 (New England BioLabs, Ipswich, MA, USA) and SuperScript\textsuperscript{TM} III reverse transcriptase (Invitrogen). For PCR analysis, cDNA was amplified by TaKaRa Ex Taq\textsuperscript{\textregistered} Hot Start Version (Takara Biotechno-

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Size (bp)</th>
<th>Primer set</th>
<th>Primer sequence</th>
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<td>5'-GAATGGGCTCAAAAACCTGGGA-3'</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5'-ACGCGTTTGTACCTCCTTC-3'</td>
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<tr>
<td>Cyclin D1</td>
<td>409</td>
<td>Forward</td>
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<tr>
<td></td>
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<tr>
<td>GAPDH</td>
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<td>5'-ACAGTCACGGCAGCTTTCTT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5'-TGGAAGATGTTGATGGGATT-3'</td>
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**Immunofluorescence staining**

Monolayer cultures of HSC-4 cells on 8-chamber slides (Lab-Tek\textsuperscript{®} Chamber Slide\textsuperscript{TM}; Electron Microscopy Sciences, Hatfield, PA, USA) were fixed with 3.5% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with Image-iT\textsuperscript{TM} FX Signal Enhancer (Invitrogen). Ber-EP 4 (Santa Cruz Biotechnology, Santa Cruz, CA) and A-20 (Santa Cruz Biotechnology) directed against EGF-like domain I and the C-terminus of EpCAM, respectively, were used as primary antibodies. Next, Alexa 488 (Invitrogen) and Alexa 594 (Invitrogen) conjugated IgGs were used as secondary antibodies. After incubation with antibodies, Slow Fade\textsuperscript{®} gold antifade reagent with DAPI (Invitrogen) was added, and coverslips were mounted. The reaction of the cells as MCA cul-

**Table 1** PCR Primer sequences used in RT-PCR

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ture was performed in siliconized 1.7 mL tubes with procedures similar those for to monolayers, and the cells were transferred onto slides and mounted. The specimens were observed using an immunofluorescence microscope (Leica Microsystems Japan, Tokyo, Japan).

RESULTS

Effect of reduced EpCAM expression on cyclin D1 expression in HSC-4 cells as monolayers

To determine the effect of EpCAM expression on cyclin D1 expression in an oral cancer cell line as monolayers, EpCAM knockdown was carried out in HSC-4 cells by transfection with EpCAM siRNA. In the control cells transfected with or without scrambled RNA, the EpCAM mRNA level was not affected (Fig. 1 A). However, the expression level of EpCAM was considerably reduced in the EpCAM siRNA transfected cells (Fig. 1 B). Cyclin D1 expression of HSC-4 cells was not obviously influenced by downregulation of EpCAM mRNA (Fig. 1 B). Furthermore, it was likely that the cyclin D1 expression level of HSC-4 cells was almost the same, in a concentration-dependent manner, as that of EpCAM siRNA (Fig. 1 B and C). These results indicate that the EpCAM expression did not influence the cyclin D1 expression level in HSC-4 cells as monolayers.

Effect of reduced EpCAM mRNA on cyclin D1 expression in HSC-4 cells as MCAs

To examine the function of EpCAM in an oral cancer cell line under anchorage independent conditions, transfection of EpCAM siRNA was carried out in HSC-4 cells as MCAs. RT-PCR showed a marked reduction in the EpCAM mRNA level of EpCAM siRNA transfected HSC-4 cells as MCAs (Fig. 2). Furthermore, the cyclin D1 level was also reduced in EpCAM siRNA transfected HSC-4 cells as MCAs. These data indicate that the EpCAM expression might be associated with the cyclin D1 expression in MCAs of HSC-4.

Fig. 1 Effect of EpCAM siRNA on HSC-4 monolayers. Monolayer HSC-4 cells were plated on 6-well plates at concentrations of $1 \times 10^5$ cells per well, and transfected with scrambled siRNA 200 nM (A) and, with EpCAM siRNA 100, 200, 400, 600, and 800 nM (B). After 24 hours, the cells were analyzed for the mRNA level of EpCAM and cyclin D1 by RT-PCR (A and B). The relative intensities of the cyclin D1 expression bands were then assayed (C).
Differences in the localization of EpCAM in HSC-4 cells as monolayers and MCAs

To examine localization of EpCAM in oral cancer cell lines as monolayers and MCAs, immunofluorescence staining of both cultures was performed using EpCAM specific antibodies. In HSC-4 cells as monolayers, specific staining by Ber-EP 4 was distinctly localized to the cellular membrane of the cell adhesion surface (Fig. 3 A). In addition, specific staining by Ber-EP 4 in HSC-4 cells as MCAs was localized in similar areas to those in the monolayers (Fig. 3 C). On the other hand, specific staining by A-20 was distinctly localized near the nucleus in the cytoplasm in HSC-4 cells as monolayers (Fig. 3 B). In contrast, in HSC-4 cells as MCAs, specific staining by A-20 was sparsely localized in the cytoplasm and nucleus (Fig. 3 D). In short, specific staining by Ber-EP 4 in HSC-4 as monolayers did not show any difference between that in HSC-4 as MCAs, whereas specific staining by A-20 in HSC-4 as monolayers was different from that in HSC-4 as MCAs.

DISCUSSION

It has been reported that the EpCAM expression is involved in cell growth in several cancers through regulation of certain genes including cell cycle regulators.\textsuperscript{11, 12, 15} Our data indicated that although an RNAi-mediated decrease in EpCAM expression reduced the expression level of cyclin D1 mRNA in MCAs, EpCAM did not contribute to cyclin D1 expression in HSC-4 cells as monolayers. By these results, it is suggested that cell proliferation through cyclin D1 expression in HSC-4 cells is regulated by EpCAM under conditions of anchorage independent cell aggregation. Therefore, EpCAM has different functions depending on the condition around the cells.

EpCAM expression has been reported to indicate a poor prognosis, such as metastasis, in several carcinomas.\textsuperscript{28-30} The development of metastasis requires multisteps including anoikis resistance.\textsuperscript{31}
MCAs have been used as a culture model of anchorage independence and HSC-4 cells, whether treated with EpCAM siRNA or not, have exhibited survival in MCAs culture, indicating that HSC-4 cells are independent of anoikis resistance. Therefore, EpCAM may be involved in the proliferation of cancer cells in anchorage independency during delivery through the circulatory and lymphatic vessels.

Previous reports have shown that cleavage of EpCAM into extracellular (EpEX) and intracellular (EpiCD) domains is involved in the proliferation of cancer cells. This is activated by the shedding of EpEX and the nuclear translocation of EpiCD. A nuclear complex with EpiCD induces gene transcription and proliferation. In our data, intracellular localization of EpiCD in aggregated HSC-4 cells is clearly different from that in monolayer HCS-4 cells. Although the details of localization of EpiCD need further examination, we postulate that cell aggregation stimulates cleavage of EpCAM and translocation of EpiCD from the membrane to the cytoplasm and nucleus, and nuclear EpiCD may upregulate the cyclin D1 level, and as a result, maintain cell growth in MCAs of HSC-4 cells.

Recently, EpCAM has been noted as a cancer stem cell antigen in the pancreatic and liver region. Therefore, EpCAM should be investigated as an effective target antigen for cancer immunotherapy. The therapeutic potential in EpCAM-positive carcinoma has been indicated. The systemic delivery of RNAi-based therapeutics has been demonstrated, and gene silencing by siRNA may lead to cancer therapies. Reduced EpCAM expression using RNA silencing has induced the reduction of the cyclin D1 level of cancer cells as MCAs. Therefore, RNAi-mediated gene silencing of EpCAM should be an effective treatment for oral cancer metastasis in the future.

REFERENCES


