# E-cadherin increases squamous cell carcinoma antigen expression through phosphatidylinositol-3 kinase-Akt pathway in squamous cell carcinoma cell lines

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**Abstract.** Squamous cell carcinoma antigen (SCCA) has been used for the management of squamous cell carcinoma, especially for evaluating therapeutic effects and monitoring recurrence. It has been reported that SCCA has several biological activities and influences behavior of cancer cells. E-cadherin is a cell adhesion molecule and plays important roles in the process of cancer invasion and metastasis. Our previous studies have shown that blockage of E-cadherin action by anti-E-cadherin antibody treatment suppresses SCCA production in squamous cell carcinoma cells. This finding strongly suggests that E-cadherin regulates SCCA expression. The present study was, therefore, undertaken to investigate the correlation between E-cadherin and SCCA2. For this purpose, *E-cadherin* cDNA was transfected into squamous cell carcinoma cell lines, SiHa and SKG IIIa. Overexpression of E-cadherin increased SCCA2 expression together with cell aggregation. We also examined the involvement of phosphatidylinositol 3-kinase (PI3K)-Akt pathway, which is one of major signaling pathways from E-cadherin. E-cadherin transfection increased phosphorylated Akt expression concomitantly with the increase in SCCA2 expression, and the increased SCCA2 expression was inhibited by a PI3K inhibitor. In conclusion, SCCA2 is up-regulated by E-cadherin through PI3K-Akt pathway, suggesting that SCCA2, as well as E-cadherin, may be involved in the regulation of cancer behavior.

## Introduction

Squamous cell carcinoma antigen (SCCA) was first discovered in uterine cervical squamous cell carcinomas by Kato and Torigoe (1), and was widely used as a serological tumor marker for diagnosis and management of squamous cell carcinoma (2). Molecular studies have revealed that SCCA is encoded by two highly homologous genes, *SCCA1* and *SCCA2*, at 18q21.3 locus (3,4). Both SCCA1 and SCCA2 belong to the serine proteinase inhibitor (serpin) family (3-5). SCCA1 inhibits both serine and cysteine proteinases, while SCCA2 inhibits serine proteinases, chymase and cathepsin G (6-9). Accumulating data have shown that SCCA has several biological activities and influences behavior of cancer cells (10-14). In our previous studies, *SCCA2* mRNA expressed in squamous cell carcinoma tissues was higher than that in normal tissues, while *SCCA1* mRNA did not show significant differences between them (15). Therefore we speculate that SCCA2 has important biological functions in squamous cell carcinoma. In fact, our previous studies have revealed that SCCA2 suppresses apoptosis of human cancer cells, suggesting that SCCA2 serves as a survival factor (12).

E-cadherin is a most important cell adhesion molecule of epithelium that mediates calcium-dependent homophilic interactions at sites of cell-cell contact. It is well known that E-cadherin is involved in cancer behavior, especially in the process of invasion and metastasis (16-22). Cell-cell contact mediated by E-cadherin also activate the intracellular signal transduction system such as phosphatidylinositol 3-kinase (PI3K)-Akt pathway (23-26), suggesting that E-cadherin plays important roles in the regulation of cellular functions. We have found that blockage of E-cadherin action by anti-E-cadherin antibody treatment suppressed SCCA production in squamous cell carcinoma cell lines (27). This finding strongly suggests that E-cadherin regulates SCCA expression in squamous cell carcinoma cells. In the investigation of biological functions of SCCA, it is important to know how SCCA expression is regulated in squamous cell carcinoma. The present study was, therefore, undertaken to investigate whether E-cadherin affects SCCA expression in squamous cell carcinoma cells, if so, whether a PI3K-Akt pathway is involved in the effect of E-cadherin.

## Materials and methods

*Cell culture*. Human uterine cervical squamous cell carcinoma cell lines, SiHa and SKG IIIa, were used for E-cadherin

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transfection. SiHa was obtained from Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan) and SKG IIIa was kindly provided by Dr S. Nozawa (Keio University, Tokyo, Japan). SiHa was cultured in Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% heat-inactivated fetal calf serum (FCS), and SKG IIIa was cultured in Ham's F-12 medium (Sigma-Aldrich) with 10% FCS, and incubated at 37˚C under 5% $CO_2$ in a humidified atmosphere. Cell cultures were prepared by seeding $5x10^6$ cells.

The cells transfected with E-cadherin genes were cultured in adherent condition using 6-well plates (Falcon, NJ, USA) or in suspended condition using poly-HEMA-coated 6-well plates (Sigma-Aldrich) to observe morphological changes caused by E-cadherin transfection.

To study whether SCCA2 expression is regulated through PI3K-Akt pathway mediated by E-cadherin, a recommended concentration (20 $\mu$M) of a PI3K inhibitor (LY294002) (Carbiochem, San Diego, CA, USA) was added in the culture medium and the incubation was performed for 72 h. Three independent experiments were performed.

*Construction of the expression vector of E-cadherin.* The coding region of *E-cadherin* cDNA was amplified by reverse transcription (RT)-PCR. The first strand cDNA was generated by incubation of 3 $\mu$g of total RNA extracted from SKG IIIa using Isogen (Nippon Gene, Toyama, Japan) with Ready-To-Go First-Strand Beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's protocol. Following RT reaction, PCR was performed in 50 $\mu$l of mixture with final concentration of 1.0 mM of $MgSO_4$, 0.2 $\mu$M of dNTPs, 0.3 $\mu$M of each primer, 1X PCR buffer, and 1 U of KOD-plus-DNA polymerase (Toyobo, Osaka, Japan) and the following primers: sense primer, 5'-GCG TGGGAGGCTGTAT-3'; anti-sense primer, 5'-TCATCT CAAGGGAAGG-3'. The reaction was performed at 94˚C for 2 min followed by 35 cycles of 94˚C for 15 sec, 55˚C for 30 sec, and 68˚C for 1 min and final extension at 72˚C for 10 min. The PCR products were ligated with pCR-Blunt Vector (Invitrogen, Carlsbad, CA, USA) and nucleotide sequence of the ligated product was confirmed by the dideoxy nucleotide chain termination method using automated DNA sequencing device (LI-COR Inc., Lincoln, NE, USA). The sequenced products containing full length of *E-cadherin* cDNA coding region was ligated with the expression vector pRc/CMV (Invitrogen). This construct (pRc-E-cad) and the empty vector were used for gene transfection.

*Gene transfection.* Cultured SiHa and SKG IIIa cells were plated at density of ~$5x10^3$ cells per 6-well plate (Falcon), respectively. These cells were washed with Opti-MEM medium (Invitrogen) and transfected with 2.5 $\mu$g of pRc-E-cad or the empty vector alone by polyamine transfection method using the TransIT Reagents (Pan Vera, Madison, WI, USA), according to the manufacturer's protocol. The cells were cultured in the medium containing neomycin for 6 weeks to select independent clones.

*Western blot analyses.* Whole cell lysate was prepared using Red loading buffer reagents (New England BioLabs, Beverly,
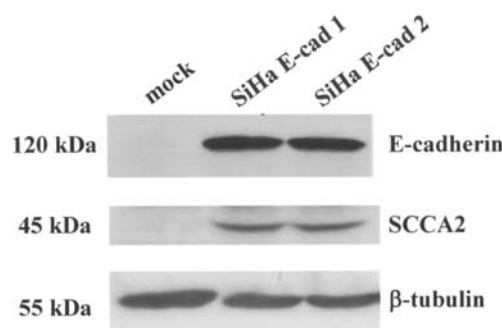


Figure 1. Effects of E-cadherin transfection on SCCA2 expression. *E-cadherin* cDNA was transfected into a human uterine cervical cancer cell line, SiHa. Two independent clones (SiHa E-cad 1 and SiHa E-cad 2) were obtained, in which E-cadherin was highly expressed. Expression of E-cadherin and SCCA2 was examined by Western blotting. Data are representative of three independent experiments. ß-tubulin was used as an internal control.
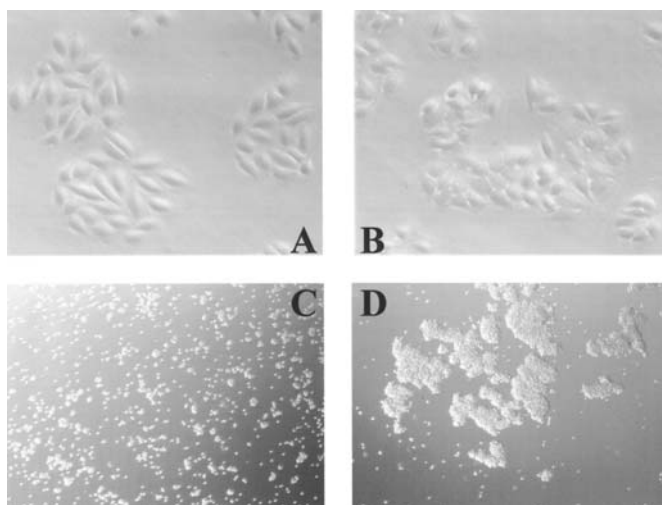


Figure 2. Cell morphology of the E-cadherin transfectant (SiHa E-cad 1). The mock cells (A) and the E-cadherin transfectant cells (B) were cultured in the adherent condition. The mock cells (C) and the E-cadherin transfectant cells (D) were cultured in the suspended condition using poly-HEMA-coated 6-well plates. Magnification: x200 in A and B; x40 in C and D.

MA, USA) without trypsin treatment. Total protein (40 $\mu$g) in each lane was electrophoresed on 10% SDS-polyacrylamide gel. After SDS-PAGE was completed, proteins were transferred to the polyvinylidene difluoride membrane (ATTO, Tokyo, Japan) with semi-dry type blotting. The transferred membrane was stained by an immunochemical technique consisting of the following procedure: after blocking the membrane with the blocking solution (5% skimmed milk with 0.1% Tween-20 dissolved in Tris-buffered saline, pH 7.5), the blotted membrane was incubated with the first antibody, which was diluted in the blocking solution. As the first antibody, the monoclonal antibody against SCCA2 was used (kind gift of Dr Gary A. Silverman), and as monoclonal antibodies against E-cadherin (Santa Cruiz Biotechnology, Delaware Avenue, USA), phosphorylated-Akt (Ser473), Akt (Cell Signaling Technology, Beverly, MA, USA) and ß-tubulin (Sigma) were used. Then, the membrane was incubated with the peroxidase conjugated second antibody diluted in the blocking solution. Finally, ECL-Western blot detection
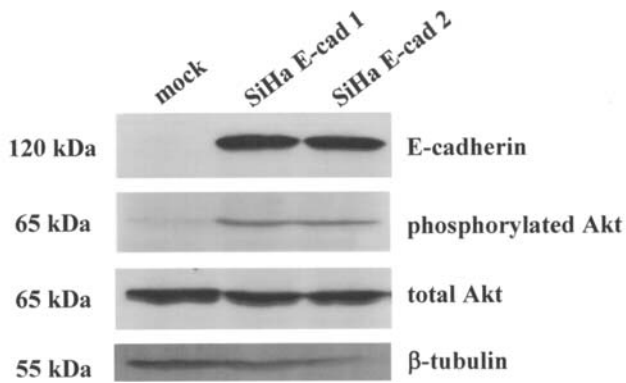
Figure 3. Phosphorylated Akt expression in the E-cadherin transfectants. Phosphorylated Akt expression was analyzed by Western blotting in two clones (SiHa E-cad 1 and SiHa E-cad 2), in which E-cadherin was highly expressed. Data are representative of three independent experiments. ß-tubulin was used as an internal control.



Figure 5. Effects of E-cadherin transfection on SCCA2 expression in SKG IIIa cells. *E-cadherin* cDNA was transfected into SKG IIIa cells. Two independent clones (SKG IIIa E-cad 1 and SKG IIIa E-cad 2) were obtained, in which E-cadherin was highly expressed. Expression of E-cadherin, SCCA2, and phosphorylated Akt were examined by Western blotting. Data are representative of three independent experiments. ß-tubulin was used as an internal control.
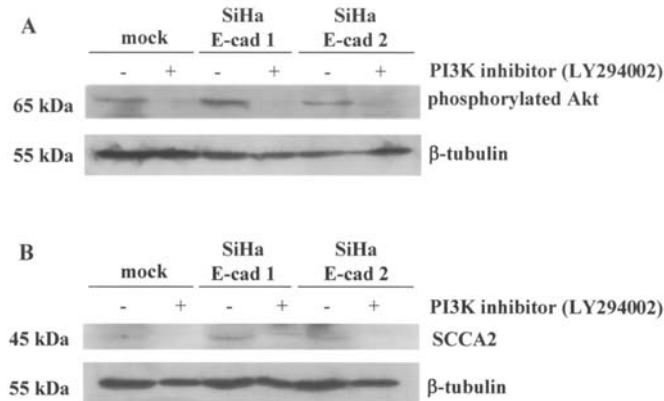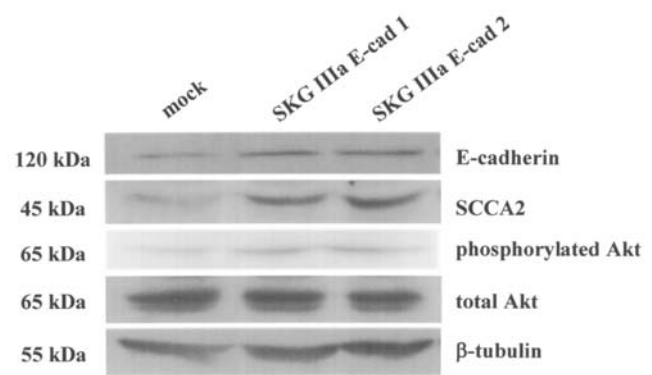


Figure 4. Effects of PI3K inhibitor on expression of phosphorylated Akt and SCCA2 in the E-cadherin transfectants (SiHa E-cad 1 and SiHa E-cad 2). The E-cadherin transfectant cells were cultured with the PI3K inhibitor (LY294002, 20 $\mu$M) for 72 h. Expression of phosphorelated Akt and SCCA2 was examined by Western blotting. (A) Phosphorylated Akt expression 20 min after PI3K inhibitor treatment. (B) SCCA2 expression 72 h after PI3K inhibitor treatment. ß-tubulin was used as an internal control. Data are representative of three independent experiments.
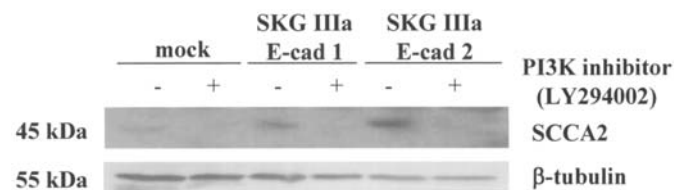


Figure 6. Effects of PI3K inhibitor on E-cadherin-stimulated SCCA2 expression in SKG IIIa cells. The E-cadherin transfectant cells (SKG IIIa E-cad 1 and SKG IIIa E-cad 2) were cultured with the PI3K inhibitor (LY294002, 20 $\mu$M) for 72 h. SCCA2 expression was examined by Western blotting. Data are representative of three independent experiments. ß-tubulin was used as an internal control.

system (Amersham Pharmacia Biotech, Buckinghamshire, UK) was applied and then the membrane was exposed to hyperfilm-ECL (Amersham).

**Results**

*Establishment of clones with overexpression of E-cadherin and effects of E-cadherin transfection on SCCA2 expression in SiHa cells.* To examine the effect of E-cadherin on SCCA2 expression, *E-cadherin* cDNA was transfected into SiHa cells. E-cadherin and SCCA2 expression were confirmed by Western blotting. As shown in Fig. 1, two independent clones (SiHa E-cad 1 and SiHa E-cad 2) were obtained, in which E-cadherin was highly expressed. In these clones, SCCA2 expression was also highly expressed compared to the mock cells (control) (Fig. 1).

*Cell morphology of E-cadherin transfectants.* The morphological changes of the mock cells (control) and the E-cadherin

transfectant cells (SiHa E-cad 1) are shown in Fig. 2. The mock cells showed a spindle shape and looked similar to thin-layer cobblestones in the adherent condition (Fig. 2A). The E-cadherin transfectant cells showed a round shape with a three-dimensional effect and were aggregated in the adherent condition (Fig. 2B). In the suspended condition using poly-HEMA-coated culture plates, the mock cells were disconnected (Fig. 2C), whereas the E-cadherin transfectant cells were clearly aggregated (Fig. 2D). These morphological changes by E-cadherin transfection indicate that E-cadherin actually functions in the transfectant cells.

*Involvement of PI3K-Akt pathway in E-cadherin stimulated SCCA2 expression in SiHa cells.* We examined whether E-cadherin-stimulated SCCA2 expression is mediated through a PI3K-Akt pathway because PI3K is a well-known mediator of E-cadherin-mediated biological events (23-26). For this purpose, the expression of phosphorylated Akt, downstream of PI3K, was first examined. The E-cadherin transfectant cells (SiHa E-cad 1 and SiHa E-cad 2) showed apparent increases in phosphorylated Akt compared with the mock cells (control) while there was no difference in total Akt expression between the mock cells and E-cadherin transfectant cells (Fig. 3). Furthermore, we examined the effect of PI3K inhibitor on E-cadherin-stimulated SCCA2 expression. Phosphorylated Akt expression was suppressed by the PI3K inhibitor (Fig. 4A),

and SCCA2 expression stimulated by E-cadherin was also suppressed by the PI3K inhibitor (Fig. 4B).

*Effects of E-cadherin transfection on SCCA2 expression in SKG IIIa cells.* We examined the effect of E-cadherin transfection on SCCA2 expression in another squamous cell carcinoma cell line, SKG IIIa. *E-cadherin* cDNA was transfected into SKGIIIa cells in a manner similar to SiHa cells. As shown in Fig. 5, two independent clones (SKG IIIa E-cad 1 and SKG IIIa E-cad 2) were obtained, in which E-cadherin was highly expressed, compared with the mock cells (control). In these clones, SCCA2 expression increased concomitant with the increase in phosphorylated Akt expression (Fig. 5). SCCA2 expression stimulated by E-cadherin transfection was clearly suppressed by the PI3K inhibitor in SKG IIIa cells (Fig. 6).

## Discussion

A number of studies have shown that altered expression of E-cadherin is involved in cancer behavior (16-22). Loss of E-cadherin plays important roles in the process of cancer invasion and metastasis because detachment of cancer cells from the primary tumor is the first step of metastasis (16,18-20). On the other hand, it is well known that E-cadherin-mediated cell aggregation is closely associated with cancer cell survival (28-32). The present study showed that increased expression of E-cadherin induces not only cell aggregation but also the increase in SCCA2 expression in squamous cell carcinoma cells. SCCA has several biological activities and influences behavior of cancer cells (10-14). Especially, SCCA2 has been reported to serve as a survival factor because SCCA2 suppresses apoptosis of human cancer cells (12). It has been reported that human skin squamous cell carcinoma cells transfected with SCCA2 genes showed significantly increased tumor growth as compared with controls when injected in nude mice (33). Therefore, it is likely that the activated E-cadherin-SCCA2 system contributes to cancer cell survival and the subsequent tumor growth in squamous cell carcinoma.

The present study also showed that E-cadherin-stimulated SCCA expression is mediated through the PI3K-Akt pathway. PI3K is a well-known mediator of E-cadherin-mediated biological events (23-26). This result is consistent with our previous data that E-cadherin-mediated SCCA production is inhibited by the PI3K inhibitor in squamous cell carcinoma cell lines (27). It has been reported in keratinocytes that E-cadherin-mediated cell adhesion regulates the expression of proteinases and their inhibitors (26), and that calcium-induced enhancement of cell adhesion increases SCCA production by keratinocytes (34). These regulations by E-cadherin in keratinocytes are mediated by activation of the PI3K-dependent pathway (25). However, the molecular mechanism by which E-cadherin-dependent PI3K-Akt pathway stimulates SCCA expression is unclear, although the promoter region of SCCA has been analyzed (35-37).

We have a hypothesis on the mechanism of cancer metastasis; the decrease in E-cadherin expression causes the detachment of cancer cells from the primary tumor, and

thereafter the cancer cells that have acquired the activated E-cadherin-SCCA system may aggregate, survive, and grow, resulting in the formation of metastatic tumors. This study may provide new information on the mechanism of cancer metastasis.

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