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Overexpression of Cortactin Increases Invasion Potential in Oral Squamous Cell Carcinoma

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Abstract Cortactin, an F-actin binding protein, stabilizes F-actin networks and promotes actin polymerization by activating the Arp2/3 complex. Overexpression of cortactin has been reported in several human cancers. Cortactin stimulates cell migration, invasion, and experimental metastasis. However, the underlying mechanism is not still understood. In the present study, we therefore evaluated the possibility that cortactin could be appropriate as a molecular target for cancer gene therapy. In 70 primary oral squamous cell carcinomas and 10 normal oral mucosal specimens, cortactin expression was evaluated by immunological analyses, and the correlations of the overexpression of cortactin with clinicopathologic factors were evaluated. Overexpression of cortactin was detected in 32 of 70 oral squamous cell carcinomas; significantly more frequently than in normal oral mucosa. Cortactin overexpression was more frequent in higher grade cancers according to T classification, N classifications, and invasive pattern. Moreover, RNAi-mediated decrease in cortactin expression reduced invasion. Downregulation of cortactin expression increased the expression levels of E-cadherin, β-catenin, and EpCAM. The siRNA of cortactin also reduced PTHrP expression *via* EGF signaling. These results consistently indicate that the overexpression of cortactin is strongly associated with an aggressive phenotype of oral squamous cell carcinoma. In conclusion, we propose that cortactin could be a potential molecular target of gene therapy by RNAi targeting in oral squamous cell carcinoma.

Keywords Cortactin · Invasion · Metastasis · RNA interference · Oral squamous cell carcinoma

Abbreviations

OSCC	Oral squamous cell carcinoma
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
PTHrP	Parathyroid hormone-related protein

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the head and neck region and accounts for more than 90% of cancers of the oral cavity [1]. The primary therapeutic modality for OSCC is surgery. Although recent advances in surgical techniques and anticancer agents have improved tumor regression and survival for patients with OSCC, wide surgical resection of OSCC causes various oral dysfunctions. Therefore, new treatment strategies are urgently needed.

The presence of neck lymph node metastasis is strongly related to a poor prognosis in squamous cell carcinoma of the head and neck [2–4]. Moreover, alterations in the expression of adhesion-related molecules are reported to be associated with poor prognosis among OSCC patients [5–8].

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Gene amplification, leading to an increase of DNA copy numbers and overexpression of oncogenes in many tumors, is reported to contribute to the growth advantage of cells, subsequently changing their biological behaviors, and causing carcinogenesis [9–11] Chromosomal band 11q13 is a frequently amplified genomic segment in a large number of malignant neoplasms, and is thought of as a potential biomarker for diagnosis and prognosis [12, 13]. In head and neck squamous cell carcinoma, this amplification is one of the most frequently observed genetic alterations [14–23] and is reportedly correlated with aggressive tumor growth [12, 16, 22], the presence of lymph node metastases [20, 24–26], and poor prognosis [12, 22, 27]. The amplified 11q13 region is 3–5 megabases in size and includes four putative oncogenes: CCND1 (PRAD1), FGF3 (INT2), FGF4 (HST1), and EMS1. Because CCND1 and EMS1 were found to be overexpressed in all carcinomas carrying the 11q13 amplification, they are believed to be the more important candidate oncogenes [13].

Cortactin, which is encoded by the EMS1 gene, is amplified in 30% of head and neck squamous cell carcinomas and 13% of primary breast cancers [16, 28–31]. Cortactin is an actin-associated scaffolding protein that binds and activates the actin-related protein (Arp) 2/3 complex, and regulates branched actin networks in the formation of dynamic cortical actin-associated structures [32, 33] Amplification of the EMS1 gene and the overexpression of cortactin have been reported in breast cancer, bladder cancer, hepatocellular carcinoma, esophageal carcinoma, and head and neck squamous cell carcinoma [22, 23, 27, 34–38]. Cortactin overexpression has been postulated to mediate the increased invasive and metastatic behaviors of tumor cells because of its effects in the organization and the functioning of cytoskeleton and cell adhesion structures [37]. However, the relationship between cortactin expression and invasiveness and metastatic potential remain unknown for OSCC. In this study, we initially immunohistochemically examined cortactin expression in OSCC. We then determined the clinicopathological significance of cortactin expression in relation to various parameters such as patient characteristics and histopathological findings. Moreover, siRNA analysis was also performed to assess whether cortactin could be a potent molecular target for cancer gene therapy in OSCC.

Materials and Methods

Patients

Paraffin-embedded sections were obtained from biopsy specimens of 70 patients with OSCC who underwent radical surgery in our department. Tumor stage was classified according to the TNM classification of the International Union Against Cancer, histological differentiation was defined according to the WHO classification, and invasion pattern was determined according to Bryne's classification [22]. As controls, samples of normal oral epithelium were obtained after informed consent from ten patients undergoing routine surgical removal of their third molars.

Cell Lines

SAS, a human OSCC cell line, was obtained from the Human Science Research Resource Bank (Osaka, Japan). The cells were cultured under conditions recommended by their depositors.

Immunohistochemical Staining and Evaluation

Serial 4-μm thick specimens were taken from tissue blocks. Sections were deparaffinized in xylene, soaked in target retrieval solution buffer (Dako, Glostrup, Denmark) and placed in an autoclave at 121°C for 5 min for antigen retrieval. Endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol for 30 min. Immunohistochemical staining was performed using the Envision system (Envision+, Dako, Carpinteria, CA). The primary antibody used was directed against cortactin (4D10, Abnova, Taipei, Taiwan). The sections were incubated with the monoclonal antibody overnight at 4°C. Reaction products were visualized by immersing the sections in diaminobenzidine (DAB) solution, and the samples were counterstained with Meyer's hematoxylin and mounted. Negative controls were performed by replacing the primary antibody with phosphate-buffered saline. Cortactin expression was defined as the presence of specific staining in the cytoplasm and cytoplasm membrane of tumor cells. The immunoreactivity of cortactin was scored by staining intensity and immunoreactive cell percentage as follows [37]: staining index 0 = tissue with no staining; 1 = tissue with faint or moderate staining in ≦25% of tumor cells; 2 = tissue with moderate or strong staining in 25% to 50% of tumor cells; 3 = tissue with strong staining in ≧50% of tumor cells. Overexpression of cortactin was defined as staining index ≧2.

RNA Isolation and Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA) and first-strand cDNA was synthesized from 1 μg total RNA using Oligo d (T) primer (Invitrogen) and ReverTra Ace (Toyobo, Osaka, Japan). For PCR analysis, cDNA was amplified by Taq DNA polymerase (Takara,

Otsu, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous expression standard. Each PCR program involved a 3-min initial denaturation step at 94°C, followed by 23 cycles (for cortactin), or 18 cycles (for GAPDH) at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, on a PCR Thermal Cycler MP (Takara). Primer sequences were as follows: TGGGGAGGGGAA TATACACA for cortactin (F); CTCTAGAGGAAGCC CCTCGT for cortactin (R); GCCCCATTCGTTCAAG TAGTCA for E-cadherin (F); TTCCGAAGCTG CTAGTCTGAGC for E-cadherin (R), TGGCCTGG TTTGATACTGACCT for β-catenin (F); CTCTACAGGC CAATCACAATGC for β-catenin (R); CCAGAACAAT GATGGGCTTT for EpCAM (F); ACGCGTTGTGA TCTCCTTCT for EpCAM (R); GGTGGCACCA AAGCTGTATT for FGFR (F); GGTGCAGGAGAGGA GAACTG for EGFR (R); ACAGTTGGAGTAGCC GGTTG for PTHrP (F); TCAGCTGTGTGGATTTCTGC for PTHrP (R); ATGTCGTGGAGTCTACTGGC for GAPDH (F); and TGACCTTGCCCACAGCCTTG for GAPDH (R). The amplified products were separated by electrophoresis on ethidium bromide-stained 2% agarose gels. Band intensity was quantified by Image J software.

Invasion Assay

A BioCoat Matrigel invasion chamber (Becton Dickinson, Bedford, MA) was used for the invasion assay. This contains an internal chamber with an 8-μm porous membrane bottom that was coated with Matrigel. Six-well cell culture inserts and a 6-well multiwell companion plate were used for the experiment. The membranes were rehydrated with warm serum-free medium for 2 h. The internal chamber was filled with 1.25×10^5 cells in medium containing 10% FBS as a chemoattractant. Cells were incubated for 72 h at 37°C in a 5% CO_2 atmosphere. After the incubation, noninvading cells were removed from the top of the wells with a cotton swab, and cells that transferred to the inverse surface of the membrane were subjected to Diff-Quick staining. Cells were counted under a microscope at 100× magnification. For the control cell count, cells that passed through a control chamber without Matrigel were counted. All experiments were performed in triplicate, and cell numbers at least in 4 fields/well were counted. The ratio of the cell count that passed through the Matrigel chamber to the control cell count was defined as the invasion index, expressed as a percentage.

RNA Interference (RNAi)

All siRNAs were purchased from Takara Bio Inc. (Otsu, Japan). Cells were transfected with double-strand RNA using TransIT-siQUEST® transfection reagent (Mirus, Madison, USA) according to the manufacturer's protocol. The SAS tongue cancer cell line was used for this experiment. Briefly, 1.0×10^5 SAS cells were plated in each well of six-well plates and allowed to grow for 24 h, till they reached 50% confluence. Cells were then transfected with siRNA at a concentration of 200 nM using the transfection reagent and serum-free medium. Following 24 h of incubation, serum-rich medium was added. The EMS1 siRNA sequences were 5′-CAAGACCGAAUG GAUAAGUTT-3′ and 5′-ACUUAUCCAUUCGGUC UUGTT-3′. The scrambled control siRNA sequences were 5′-CGUAUGCGCGUACUCUAAUTT-3′ and 5′-TT GCAUACGCGCAUGAGAUUA-3′. All sequences were submitted to the National Institutes of Health Blast program to ensure gene specificity.

Western Blot Analysis

Cells were harvested by trypsinization, washed, and precipitated by centrifugation. The Mammalian Cell Extraction Kit (BioVision Research Products, Mountain View, CA) was used for the extraction of proteins. All subsequent manipulations were performed on ice. The cells were incubated in the Extraction Buffer Mix. The lysed cells were centrifuged at 15,000 rpm for 3 min and the supernatant was collected as the cytoplasmic fraction. Protein concentration of each sample was measured with micro-BCA protein assay reagent (Pierce Chemical Co.). Samples were denatured in SDS sample buffer and loaded onto 12.5% polyacrylamide gels. After electrophoresis, the proteins were transferred onto a polyvinylidine difluoride membrane and immunoblotted with anti-cortactin (H-191, Sanra Cruz, California, USA), anti-E-cadherin (Cell Signaling, MA, USA), anti-β-actin (Cell Signaling, MA, USA), anti-EpCAM(HEA-125, Gene Tax, San Antonio, TX), or anti-EGFR(H11,Thermo, Cheshire, UK). Signals were detected using a horseradish peroxidase-conjugated secondary antibody (ECL antimouse IgG, Amersham Biosciences, Piscataway, NJ; 0.01 μg/ml), and then visualized using an ECL Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Statistical Analysis

Statistical analysis was performed using StatMate® (ATMS Co., Tokyo, Japan). The associations between cortactin expression and clinicopathologic features were assessed by Fischer's exact test. To determine significant prognostic factors related to survival, multivariate analysis was performed using the Cox proportional hazards regression model. Continuous data are given as mean ± standard deviation. Data sets were examined by one-way analysis of

variance (ANOVA) followed by Scheffe's post-hoc test. The correlation between cortactin mRNA expression and invasion index was determined using Person's correlation coefficient. *P* values less than 0.05 were considered significant.

Results

Correlation Between Cortactin Overexpression and Clinicopathologic Features

Immunohistochemistry with an anti-cortactin-specific monoclonal antibody was performed on a series of 70 patients with oral squamous cell carcinoma. Representative immunohistochemical stainings are shown in Fig. 1. Overexpression of cortactin was undetectable in the normal epithelium (Fig. 1a). In the squamous cell carcinoma cells, strong cortactin staining was apparent at the invasive front and the diffuse invasive area. Cortactin overexpression was detected significantly more frequently in OSCC (32 of 70, 45.7%) than in normal oral epithelium (0 of 10, 0%; $p<0.01$). Moreover, cortactin overexpression was significantly more frequent in cancers with higher grade according to T classification (T 3/4 vs. 1/2; $p<0.001$), N classification (N 3/4 vs. 1/2; $p<0.05$), or invasive pattern (grade 3/4 vs. 1/2; $p<0.001$, Table 1).

Cox regression analysis was performed with the parameters of histologic differentiation (T classification, N classification, and pattern of invasion) and cortactin overexpression. Cox regression analysis revealed a correlation between N classification (hazard ratio: 3.71, 95% CI: 0.420–2.20, P: 0.004), pattern of invasion (hazard ratio: 2.65, 95% CI: 0.055–1.89, P: 0.038), and cortactin overexpression (hazard ratio: 2.80, 95% CI: 0.091–1.97, P: 0.032, Table 2). These findings strongly suggested that cortactin overexpression would be a significant independent predictor of survival.

Effect of Decreasing Cortactin Expression on the Invasion Potential of SAS Cells

To determine the effect of decreasing cortactin expression on invasion potential, we transfected SAS cells with cortactin siRNA (Fig. 2a, b) and performed the Matrigel invasion assay. Transfection with cortactin siRNA significantly decreased the mRNA and protein levels of cortactin, compared with those in non-transfected cells and cells transfected with scrambled siRNA (Fig. 2a, b). Concomitantly, the invasion index of the SAS cells decreased significantly from 13.2% (in cells treated with vehicle alone) and 12.4% (in cells transfected with scrambled siRNA) to 0.02% in cells transfected with cortactin siRNA (Fig. 2c). Furthermore, the mRNA expressions of E-cadherin, β-catenin and EpCAM were significantly decreased in cortactin-targeted siRNA transfected SAS cells (Fig. 3a). A very similar tendency was seen in the protein levels, with the exception of β-catenin (Fig. 3b). Therefore, downregulation of cortactin expression by siRNA drastically suppressed the mobility of SAS cells in vitro.

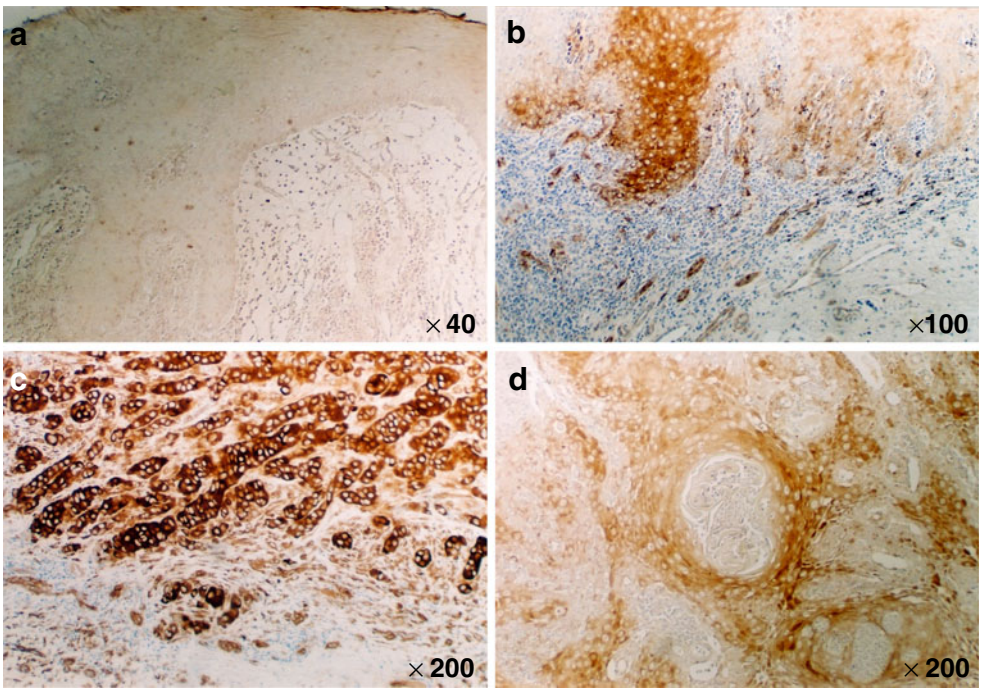

Fig. 1 Representative immunohistochemical staining for cortactin. **a** Negative staining without cortactin overexpression is shown in normal oral epithelium (40×). **b** Well-differentiated squamous cell carcinoma demonstrating strong cortactin expression (staining index of 3) and diffuse invasion (40×). **c** Immunohistochemical staining for cortactin demonstrates strong cytoplasmic expression in the cancer nests (200×). **d** Cortactin overexpression (intense staining) is shown in squamous cell carcinoma cells at the invasive front of the tumor (200×)

Table 1 Correlation of cortactin overexpression and clinicopathologic features

		cortactin overexpressiom		*p* value
		(−)	(+)	
Normal epithelium		10	0	$p<0.01$
Squamous cell carcinoma		38	32	
Gender	Male	22	17	$p=0.689$
	Female	16	15	
Age	60≦	10	11	$p=0.464$
	60>	28	21	
T classification	T1 + T2	33	13	$p<0.001$
	T3 + T4	5	19	
N classification	N0	34	21	$p<0.05$
	N1 + N2	4	11	
Differentiation	Well	32	28	$p=0.695$
	Moderate/Poor	6	4	
Pattern of invasion	Grades1/2	32	10	$p<0.001$
	Grades3/4	6	22	

Effect of Decreasing Cortactin Expression on PTHrP Expression Via the EGFR Signaling Pathway

PTHrP was firstly discovered as a causative protein for hypercalcemia, which is frequently encountered during the terminal phase of malignant tumors [39, 40]. PTHrP contributes to the malignancy of oral cancers downstream of EGFR signaling [41]. We therefore examined the effect of decreasing cortactin expression on PTHrP expression *via* the EGFR signaling pathway. Cortactin-targeted siRNA transfection of SAS cells significantly decreased EGFR and PTHrP gene expressions (Fig. 3a). In terms of protein level, EGFR expression was decreased by the cortactin-targeted siRNA transfection (Fig. 3b). These results suggest that cortactin also may act as a mediator of EGFR signaling, as well as PTHrP, in OSCC.

Discussion

Cortactin has been described as an actin associated scaffolding protein. It binds and activates the Arp 2/3 complex and regulates the branched actin networks in the formation of dynamic cortical actin-associated structures [32, 33]. Cortactin is also thought to relate to functions involving membrane dynamics and cortical actin assembly, including cell migration, morphogenesis, adhesion, receptor-mediated endocytosis, and pathogen invasion to improve the connection with the list of functions [42]. The amplification of cortactin has been reported in 30% of head and neck squamous cell carcinomas and 13% of primary breast cancers [16, 28–31]. In head and neck squamous cell carcinoma, the amplification of cortactin correlates with poor prognosis [21]. In nude mice with esophageal squamous cell carcinoma, tail vein injection of cortactin siRNA-transfected cells decreased lung metastasis and prolonged survival time compared with controls [37]. In addition, in the same animal model, amplification and overexpression of cortactin contribute to metastasis, anoikis resisitance [37], and carcinogesis [38]. In NIH3T3 fibroblasts, overexpression of EMS1/cortactin increases cell motility and invasion in vitro [43]. Enhancement of migration ability facilitates tumor invasion, which is the principal mechanism reported to account for the role of cortactin in tumor metastasis [33]. The ectopic expression of cortactin potentiates bone metastasis of breast cancer by increasing the adhesive affinity of tumor cells for bone marrow endothelial cells [44]. Therefore, the overexpression of cortactin endows cancer cells with various capabilities for metastasis.

In previous studies, cortactin overexpression has reported to be correlated with carcinogenesis [38], lymph

Table 2 Multivariate analysis (Cox regression) of different prognostic parameters 95% CI, 95% confidence interval

Parameter	Hazard ratio	95% CI	*P* value
T classification (T1 + T2 versus T3 + T4)	0.987	−0.932–0.906	0.987
N classification (N0 versus N1 + N2)	3.71	0.420–2.20	0.004
Differentiation (Well versus Moderate/Poor	0.346	−2.371–0.249	0.346
Pattern of invasion (Grades 1/2 versus Grades 3/4)	2.65	0.055–1.89	0.038
Cortactin overexpression (− versus +)	2.80	0.091–1.97	0.032

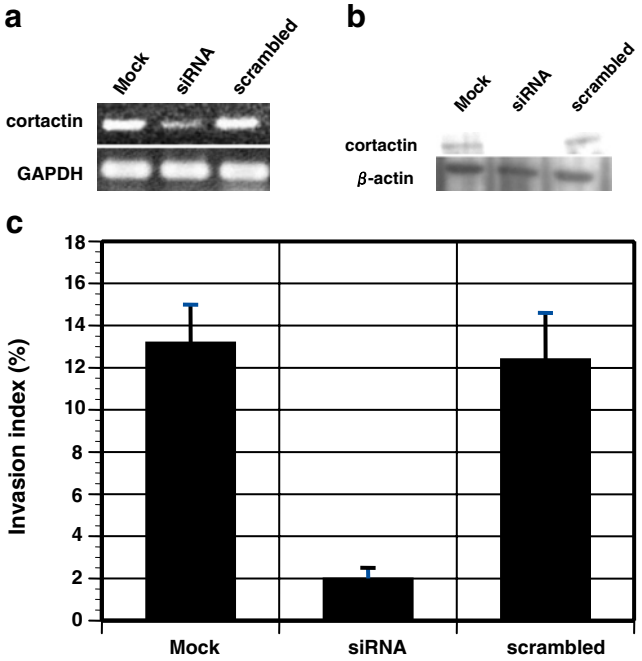

Fig. 2 **a** Representative RT-PCR for the suppression of cortactin in SAS cells. Cells were transfected with scrambled siRNA (control) or cortactin siRNA. After 72 h, isolated total RNA was analyzed using RT-PCR. **b** Western blot analysis for the suppression of cortactin in SA cells. Western blot analyses show cortactin and β-actin expression in the whole cell lysate proteins. **c** The graph shows a significant decrease of the invasion index in SAS cells treated with cortactin siRNA ($P<0.01$)

node metastasis [37], and poor prognosis [21]. In this study, cortactin overexpression was strongly correlated with cancers of higher grade according to T classification, N classification, and invasive pattern. Additionally, Cox regression analysis revealed a correlation between N classification, pattern of invasion, and cortactin overexpression, respectively. We demonstrated that cortactin overexpression in OSCC could reflect a large tumor size, regional lymph node metastasis, and diffuse invasion. Moreover, it was suggested that cortactin expression could be a prognostic factor in OSCC patients.

The present study also demonstrated that cortactin expression could be associated with invasiveness in the human tongue squamous cell carcinoma cell line, SAS. Our study indicated that the de novo overexpression of cortactin increased the invasion potential of tongue squamous cell carcinoma cells. Moreover, the immunohistochemical staining of cortactin revealed strong positivity in the invasive front of the diffuse invasion pattern. However, the mechanism by which cortactin increases the invasive potential remains unclear.

There is a correlation between the ability of cells to locally degrade the matrix at invadopodia, which are actin-containing protrusions extending into the matrix and participating in matrix degradation, and their invasive potential as measured in other in vivo and in vitro assays for motility and invasion [45]. Cortactin binds to F-actin in vitro, colocalizing with cortical actin at ruffling membranes, and possesses actin-bundling activity that is modulated by c-Src, suggesting a role in membrane motility [46, 47]. Cortactin is reportedly recruited to cell-cell adhesive

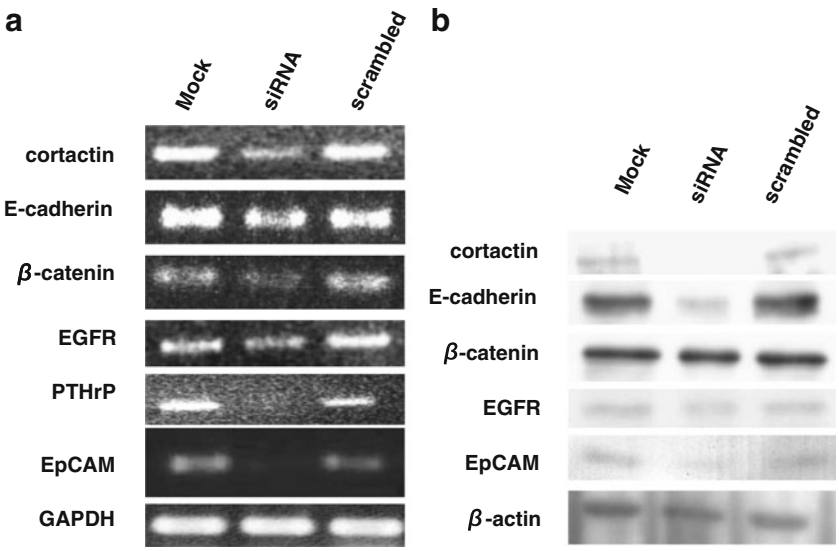

Fig. 3 **a** Representative RT-PCR for the suppression of cortactin in SAS cells. Cells were transfected with scrambled siRNA (control) or cortactin siRNA. After 72 h, isolated total RNA was analyzed using RT-PCR. The siRNA of cortactin reduced the mRNA expression levels of E-cadherin, β-catenin, EpCAM, FGFR, and PTHrP. **b** Western blot analysis for the suppression of cortactin in SAS cells. Western blot analysis show the decreased expression of E-cadherin, EpCAM, and EGFR

contacts in response to homophilic cadherin ligation [48]. Moreover, one of the molecular mechanisms that links cadherins and actin assembly is likely to involve the interaction between E-cadherin and the Arp2/3 actin nucleator complex [48]. Cadherin adhesive ligation can recruit the Arp2/3 complex to the cell surface [49], and cortactin can interact with Arp2/3 (*via* an NH_2-terminal acidic [NTA] domain) and F-actin (*via* the fourth of six tandem repeats located in the NH_2-terminal half of the molecule) [50]. In contrast, cortactin inhibits the disassembly of Arp2/3-generated actin filaments, and potentially stabilizes the cortical actin network [51]. Cortactin activity is necessary for the Arp2/3-dependent actin assembly that occurs in response to E-cadherin homophilic ligation [48].

Colocalization and association of cortactin with E-cadherin have been reported in epithelial cells [48]. In previous reports, reduction of cortactin expression levels had no effect on E-cadherin or β-catenin levels [48, 52]. However, RNAi-mediated downregulation of cortactin resulted in significant reduction of intercellular adhesion [52]. Additionally, cortactin downregulation delayed the formation of early nascent E-cadherin-based-cell-cell contacts [48] Catenin links cadherin with the actin cytoskeleton, and can also form a complex with EGFR [53]. It was reported that EpCAM expression could be associated with invasiveness in human tongue cancer cell lines [8]. The EpCAM overexpression decreased adhesion mediated by the cadherin-catenin complex [54, 55]. In this study, siRNA of cortactin resulted in the downregulation of adhesion molecules such as E-cadherin, β-batenin, and EpCAM levels, in contrast to previous reports [48, 52]. It is suggested that cortactin expression might also affect expression levels of these molecules and thereby contribute to invasive ability in OSCC cells.

Epidermal growth factor (EGF) is enriched in the oral region, and most OSCC cells express abundant EGF receptor(EGFR) [56]. The increased expressions of EGFR or its ligand are associated with reduced disease-free survival [57]. The overexpression of cortactin in head and neck squamous cell carcinoma cells attenuates ligand-induced downregulation of the EGFR, which leads to sustained receptor signaling to the mitogenic extracellular signal-regulated kinase (ERK)/ mitogen-activated protein kinase pathway (MAPK) [58]. PTHrP was first reported as a major factor responsible for hypercalcemia in malignancies [59], and acts classically as a stimulator of osteoclastic bone resorption [60]. EGF signaling up-regulates PTHrP gene expression through the MAPK cascades, leading to malignant conversion of OSCC by enhanced cell proliferation, migration, and invasion [41]. EGF activated ERK, p38 MAPK, and JNK in OSCC, and in particular, ERK and p38 MAPK were involved in PTHrP expression [41]. In this report, we examined the effect of cortactin expression on the PTHrP expression *via* EGF signaling by the siRNA downregulation of cortactin. Cortactin down-regulation reduced EGFR and PTHrP mRNA expression levels. It is suggested that cortactin expression might contribute to PTHrP expression *via* EGF signaling and accordingly enhance cell proliferation and invasiveness of OSCC.

In summary, we showed the significance of cortactin expression as a potential prognostic factor of OSCC and the possibility of an association between cortactin and PTHrP expression *via* EGF signaling. RNAi technology is a specific and powerful tool to turn off the expression of oncogenic target genes [61]. In oral cancer, the possibility of RNA-mediated gene therapy has been reported [62, 63]. We successfully applied RNA silencing to inhibit the expression of cortactin, thereby decreasing the invasion potential of OSCC. Therefore, we propose that RNAi-mediated gene silencing of cortactin might be a useful modality for OSCC treatment in the future.

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Ethical Approval Not require

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