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Co-overexpression of cortactin and CRKII increases migration and invasive potential in oral squamous cell carcinoma

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Key Words: cortactin, CRKII, invasion, metastasis, oral squamous cell carcinoma

Running title: Co-overexpression of cortactin and CRKII in oral squamous cell carcinoma
Abbreviations: OSCC, oral squamous cell carcinoma; CRK, CT10 regulator of kinase; EMT, epithelial-mesenchymal transition

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Abstract

Cortactin stimulates cell migration, invasion, and experimental metastasis. Overexpression of cortactin has been reported in several human cancers. CRK was originally identified as an oncogene product of v-CRK in a CT10 chicken retrovirus system. Overexpression of CRKII has been reported in several human cancers. CRKII regulates cell migration, morphogenesis, invasion, phagocytosis, and survival; however, the underlying mechanisms are not well understood. We evaluated the possibility of the combination of cortactin and CRKII as an appropriate molecular target for cancer gene therapy. The expression of cortactin and CRKII in 70 primary oral squamous cell carcinomas and 10 normal oral mucosal specimens was determined immunohistochemically, and the correlation of cortactin and CRKII co-overexpression with clinicopathological factors was evaluated. Co-overexpression of cortactin and CRKII was detected in 31 of 70 oral squamous cell carcinomas, the frequency being significantly greater than in normal oral mucosa. In addition, cortactin and CRKII co-overexpression was more frequent in higher-grade cancers according to
the T classification, N classification, and invasive pattern. RNAi-mediated co-suppression of cortactin and
CRKII expression reduced the migration and invasion potential of an oral squamous cell carcinoma cell
line, OSC20. Downregulation of cortactin and CRKII expression also reduced the expression of vimentin,
fibronectin, and N-cadherin. These results indicate that the co-overexpression of cortactin and CRKII may
be tightly associated with an aggressive phenotype of oral squamous cell carcinoma. Therefore, we propose
that the combination of cortactin and CRKII could be a potential molecular target of gene therapy by
RNAi-targeting in oral squamous cell carcinoma.
1. 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 inevitably 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, it has been reported that an alteration in the expression of adhesion-related molecules is associated with poor prognosis in OSCC patients [5-8].

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 [9,10]. In head and neck squamous cell carcinoma, this amplification is one of the most frequently observed genetic alterations.
and is reportedly correlated with aggressive tumor growth [9,13,19], the presence of lymph node
metastases [17,21-23], and poor prognosis [9,19,24]. 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 [10]. 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 [13,25-28]. 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 [29,30]. 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 [19,20,24,31-35]. Cortactin
overexpression has been postulated to mediate the increased invasive and metastatic behaviors of tumor
cells because of its effects on the organization and functioning of cytoskeleton and cell adhesion structures [34].

CRK is an adaptor family member of proteins mostly composed of SH2 and SH3 domains known to mediate protein-protein interactions, and plays an important role in intracellular signal transduction [36-38].

CRK was originally isolated as the oncogene product of v-CRK in a CT10 chicken retrovirus. Its cellular homologues have been isolated from various species [39,40]. Cellular homologues of v-CRK include the c-CRK gene, which produces two alternatively spliced protein products: CRKI (28 kDa) and CRKII (40 kDa) [40,41]. The ubiquitous expression of CRK is observed in embryos and adults [40]. In addition, the overexpression of CRK in malignant neoplasms [42], including glioblastomas [43] and lung cancers [42,44], has been reported. CRKI/II mRNA expression is enhanced in lung tumors at more advanced stages and accompanies poor survival [44]. CRKI is composed of an SH2 and an SH3 domain, and CRKII has an additional SH3 domain [40]. The CRK-SH2 domain binds a specific phosphorylated tyrosine motif present
in proteins involved in cell spreading, actin reorganization, and cell migration. Such CRK-SH2 binding proteins include the focal adhesion components, p130Cas and paxillin [45], growth factor receptor tyrosine kinases, and a docking protein Gab1, which is involved in epithelial dispersal and morphogenesis [46-48].

The NH₂-terminal of the CRKII-SH3 domain constitutively interacts with proline-rich motifs present in proteins, including C3G, a nucleotide exchange factor for Rap1 [49], Dock180, an exchange factor for Rac1 [46,50], the Abl tyrosine kinase [51], tyrosine phosphatase [52], the p85 subunit of phosphatidylinositol 3-kinase [53], and the c-Jun-NH₂-kinase [54]. The binding proteins of the COOH-terminal of the SH3 domain are still poorly understood. CRKII has been identified as a mediator of cell migration associated with p130Cas and paxillin [55] as well as the Rac exchange factor Dock180 [56].

On the basis of these interactions, the proposed roles of CRK include the regulation of cell migration, morphogenesis, invasion, phagocytosis, and survival [45].

In previous studies, we found that the overexpressions of cortactin and CRKII are each tightly
associated with an aggressive phenotype of oral squamous cell carcinoma [57,58]. Moreover, the CRK-cortactin complex has been reported to play a major role in actin polymerization downstream of tyrosine kinase signaling [59]. In our previous report, we showed that the downregulation of CRKII decreased the expression levels of cortactin in the OSC20 cell line, which indicates that the CRK-cortactin complex may also play a major role in actin polymerization in OSCC [58].

In this study, we examined cortactin and CRKII expression in OSCC immunohistochemically, and then determined the clinicopathological significance of cortactin and CRKII co-expression in relation to various parameters, such as patient characteristics and histopathological findings. We also performed double siRNA analysis to assess whether cortactin and CRKII could be potent molecular targets for cancer gene therapy of OSCC.
2. Materials and Methods

2.1 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 [60]. As controls, samples of normal oral epithelium were obtained after informed consent was provided by 10 patients undergoing routine surgical removal of their third molars.

2.2 Cell lines

Basically, we examined the expression of each of cortactin and CRKII in the seven OSCC cell lines (Ca9-22, SAS, SCC25, OSC20, HSC2, HSC3, and HSC4). Among them, the OSC20 cell line expressed
cortactin and CRKII most strongly (data not shown). Then, we performed the co-suppression of CRK II and cortactin by RNAi with the OSC20 cell line. A human OSCC cell line (OSC20) was obtained from the Human Science Research Resource Bank (Osaka, Japan). The cells were cultured under conditions recommended by their depositors.

2.3 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 then placed in an autoclave at 121°C for 5 min for antigen retrieval. Endogenous peroxidase was blocked by incubation with 0.3% H2O2 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) or CRKII (H-53, Santa Cruz Biotech, Inc., CA, USA). The sections were
incubated with the primary 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 prepared by replacement of the primary antibody with phosphate-buffered saline. Cortactin and CRKII expressions were defined as the presence of specific staining in the nucleus and cytoplasm of tumor cells. The immunoreactivity of cortactin and CRKII was scored by staining intensity and immunoreactive cell percentage as follows \[57,58,61,62\]: staining index 0 = tissue with no staining; 1 = tissue with faint or moderate staining in \(\leq 25\%\) of tumor cells; 2 = tissue with moderate or strong staining from 25\% to 50\% of tumor cells; and 3 = tissue with strong staining in \(\geq 50\%\) of tumor cells. Overexpression of each of cortactin and CRKII was defined as staining index \(\geq 2\).

2.4 RNA isolation and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA
was synthesized from 1 μg of total RNA using Oligo d(T) primer (Invitrogen) and ReverTra Ace (Toyobo, Osaka, Japan). For PCR analysis, Taq DNA polymerase was used to amplify cDNA (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), 25 cycles (for CRKII), 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:

5’-TGGGGAGGGGAATATACACA-3’ for cortactin (F); 5’-CTCTAGAGGAAGCCCCTCGT-3’ for cortactin (R); 5’-TCTCAGGCAGTGCAAATCAC-3’ for CRKII (F); 5’-CGCTCCATACAATGAAAGCA-3’ for β-catenin (F); 5’-CTCTACAGGCCAATCACAATGC-3’ for β-catenin (R); 5’-GGCTCAGATTCAGGAACAGC-3’ for vimentin (F); 5’-TGGCCTGGTTTGATACTGACCT-3’ for β-catenin (R); 5’-TTCCGAAGCTGCTAGTCTGAGC-3’ for E-cadherin (R); 5’-TTCCGAAGCTGCTAGTCTGAGC-3’ for E-cadherin (F); 5’-TGGCCTGGTTTGATACTGACCT-3’ for β-catenin (F); 5’-CTCTACAGGCCAATCACAATGC-3’ for vimentin (F); 5’-TGGCCTGGTTTGATACTGACCT-3’ for β-catenin (R); 5’-GGCTCAGATTCAGGAACAGC-3’ for vimentin (F);
5'-GCTTCAACGGCAAGTTCTC-3’ for vimentin (R); 5'-TCGAGGAGAAATTCCAATG-3’ for fibronectin (F); 5'-ACACACGTGCACCTCATCAT-3’ for fibronectin (R);

5’-GGACAGTTCTGAGGGATCA-3’ for N-cadherin (F); 5’-GGATTGCCTCCATGTCTGT-3’ for N-cadherin (R); 5’-ATGTCGTGGAGTCTACTGGC-3’ for GAPDH (F); and

5’-TGACCTTGCCCACAGCCTTG-3’ 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.

2.5 Wound healing assay

Cell migration was evaluated by a scratched wound-healing assay on plastic plate wells. In brief, cells were grown to confluence and then wounded using a pipette tip. Three wounds were made for each sample, and all were photographed at 0 h and subsequent time points. Cell migration was evaluated by measuring
the width of the wound at the identical position.

2.6 Invasion assay

A BioCoat Matrigel invasion chamber containing an internal chamber with an 8-μm porous membrane bottom-coated with Matrigel (Becton Dickinson, Bedford, MA) was used for the invasion assay. 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% CO₂ 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, cells that passed through a control chamber without Matrigel were counted. All experiments were performed in triplicate, and cell numbers were counted in at least 4 fields/well. The ratio of the cell count that passed through the
Matrigel chamber to the control cell count was defined as the invasion index, and is expressed as a percentage.

2.7 RNA interference (RNAi)

The cortactin siRNA sequences were 5′-CAAGACCGAUGGAUAAGUTT-3′ and 5′-ACUUAUCCAUUCGGUCUUGTT-3′. The CRKII siRNA sequences were 5′-GUAUCAGAAGGGAUAGGUATT-3′ and 5′-UACCUAUCCCUUCUGAUACTT-3′. The scrambled control siRNA sequences were 5′-CGUAUGCGCGUACUCUAAUTT-3′ and 5′-TTGCAUACGCGCAUGAGAUUA-3′. All sequences were submitted to the National Institutes of Health Blast program to ensure gene specificity.

All siRNAs were purchased from Takara Bio Inc. (Otsu, Japan). Cells were transfected with double-stranded RNA using TransIT-siQUEST® transfection reagent (Mirus, Madison, WI, USA) according
to the manufacturer’s protocol. The OSC20 tongue cancer cell line was used for this experiment. Briefly, 1.0 × 10^5 OSC20 cells were plated in each well of six-well plates and allowed to grow for 24 h, until they reached 50% confluence. Cells were then transfected with siRNA at a concentration of 200 nM by using the transfection reagent in serum-free medium. Following 24 h of incubation, the medium was replaced with serum-enriched medium and the cells were cultured for an additional 24 h.

2.8 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 Extraction Buffer Mix. The lysed cells were centrifuged at 15,000 rpm for 3 min, and the resultant supernatant was used as the cytoplasmic fraction. Protein concentration of each sample was measured with the micro-BCA
protein assay reagent (Pierce Chemical Co., Rockford, USA). Samples were denatured in SDS sample buffer and loaded onto 12.5% polyacrylamide gels. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes and immunoblotted with anti-cortactin (H-191, Santa Cruz, California, USA), anti-CRKII (H-53, Santa Cruz Biotech), or anti-β-actin (Cell Signaling, MA, USA). Incubation with a horseradish peroxidase-conjugated secondary antibody (ECL antimouse IgG, Amersham Biosciences, Piscataway, NJ; 0.01 μg/ml) was performed, and signals were visualized with an ECL Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.9 Statistical analysis

Statistical analysis was performed using StatMate® (ATMS Co., Tokyo, Japan). The correlation between CRKII expression and the clinicopathological features was assessed by Fisher’s exact test.

Continuous data are given as the means ± standard deviation. Data sets were examined by one-way analysis of variance (ANOVA) followed by Scheffe’s post hoc test. Survival analysis was carried out with
Kaplan-Meier curves and the related log-rank tests. P values less than 0.05 were considered significant.
3. Results

3.1 Correlation between cortactin and CRKII co-expression and clinicopathological features

Immunohistochemistry with an anti-CRKII polyclonal antibody or an anti-cortactin-specific monoclonal antibody was performed on 70 patients with oral squamous cell carcinoma. Representative immunohistochemical stainings are shown in Figure 1. Overexpression of cortactin and CRKII was undetectable in normal epithelium (data not shown). In the squamous cell carcinoma cells, strong cortactin or CRKII staining was located at the invasive front and the diffuse invasive area (Fig. 1B and D). Indeed, cortactin and CRKII co-overexpression was recognized more frequently in OSCC (31 of 70, 44.3%) than in normal oral epithelium (0 of 10, 0%; p<0.01). Furthermore, co-overexpression of cortactin and CRKII was more frequent in cancers with higher grades according to the T classification (T 3/4 vs. 1/2; p<0.001), N classification (N 3/4 vs. 1/2; p<0.05), and invasive pattern (grade 3/4 vs. 1/2; p<0.001, Table 1).

These findings strongly suggest that cortactin and CRKII co-overexpression is a potent predictor of
3.2 Correlation between cortactin and CRKII co-expression and survival analysis

The 5-year survival rates according to cortactin and CRKII co-expression and other clinicopathological features were determined. There was a tendency for an association between lower survival in patients and cortactin and CRKII co-overexpression, as seen in the T classification, N classification, invasive pattern, and separate cortactin or CRKII overexpression (data not shown). The association was significant by the log-rank test (Fig. 2). These findings also strongly suggest that cortactin and CRKII co-overexpression is a potent predictor of survival.

3.3 Effect of cortactin and CRKII on the migration and invasion of OSC20 cells

Cell migration and invasion are basic characteristics of tumor metastasis. To determine the effect of cortactin and CRKII co-expression on the migration and invasion potential of cells, we transfected OSC20 cells with cortactin siRNA and CRKII siRNA, and performed wound healing and Matrigel invasion
assays. In the previous study, we reported the invasion potential of OSCC cell lines (Ca9-22, SAS, SCC25, OSC20, HSC2, HSC3, and HSC4) [62] and found that SAS and HSC3 showed higher invasion potential than OSC20. However, the efficiency of RNAi in SAS and HSC3 cells was limited and, accordingly, although the cells revealed the tendency of reduction of cell migration and invasion potential, but the differences were not significant ($p = 0.31$ in SAS and $p = 0.37$ in HSC3). Hence, we used OSC cell in this study, because the RNAi co-suppression of cortactin and CRK II in OSC was most efficient among the cells tested. Transfection with cortactin siRNA and CRKII siRNA significantly decreased cortactin and CRKII mRNA and protein levels, respectively, compared with those of non-transfected cells and cells transfected with scrambled siRNA (Fig. 3A, B). The induced downregulation of cortactin and CRKII co-expression resulted in a 38.7% decrease in healing rate compared with that of the controls at 36 h after wounding (Fig. 3C, D). Concomitantly, the invasion index of OSC20 cells decreased significantly from 86.6% and 86.1% in cells treated with vehicle alone and scrambled siRNA, respectively, to 15.8% in those transfected with...
CRKII siRNA (Fig. 3E). Therefore, downregulation of cortactin and CRKII co-expression by siRNA drastically suppresses the mobility of OSC20 cells in vitro.

3.4 Effect of decreasing cortactin and CRKII co-expression on EMT markers

Cortactin and CRK cooperate to trigger actin polymerization during Shigella invasion of epithelial cells [59]. We therefore examined the effect of cortactin and CRKII on EMT in OSC20 cells.

Cortactin- and CRKII-targeted siRNA transfection of OSC20 cells significantly decreased fibronectin, vimentin, and N-cadherin mRNA expression levels (Fig. 4). However, the mRNA expression levels of E-cadherin and β-catenin were not affected by the cortactin- and CRKII-targeted siRNA transfection (Fig. 4). These results suggest that downregulation of cortactin and CRKII induces the suppression of EMT in OSCC.
4. Discussion

Cortactin is also thought to be related 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 [39]. The amplification of cortactin has been reported in 30% of head and neck squamous cell carcinomas and 13% of primary breast cancers [13,25-28].

In head and neck squamous cell carcinoma, the amplification of cortactin correlates with poor prognosis [18]. 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 those of controls [34]. In addition, in the same animal model, amplification and overexpression of cortactin contributed to metastasis, anoikis resistance [34], and carcinogenesis [35]. In NIH3T3 fibroblasts, overexpression of EMS1/cortactin increases cell motility and invasion in vitro [63]. Enhancement of migration ability facilitates tumor invasion, which is the principal mechanism reported to account for the
role of cortactin in tumor metastasis [30]. The ectopic expression of cortactin potentiates bone metastasis of breast cancer by increasing the adhesive affinity of tumor cells for bone marrow endothelial cells [64]. Therefore, the overexpression of cortactin endows cancer cells with various capabilities for metastasis. In previous studies, cortactin overexpression was reported to correlate with carcinogenesis [35], lymph node metastasis [34], and poor prognosis [18].

CRK has been reported to regulate cytoskeletal reorganization by integrin stimulation, and thereby modulate cell motility and adhesion [42]. Increased CRKII activity suppresses apoptosis, induces lamellipodia formation and cell spreading in migratory cells [65], and encourages anchorage-independent growth [55]. It has been reported that increased expression of CRKI/II at the mRNA and protein levels is observed in various cancers [42], including glioblastomas [43] and lung cancers [42,44]. In lung adenocarcinoma, CRKI/II mRNA expression is predominantly increased at more advanced stages and is associated with poor survival [44].
In the present study, we reveal that cortactin and CRKII co-overexpression strongly correlates with cancers of higher grades in T classification, N classification, and invasive pattern. Additionally, Kaplan-Meier analysis revealed a significant association of cortactin and CRKII co-expression with 5-year survival rates (log-rank, p<0.05), similar to the T classification, N classification, and pattern of invasion (data not shown). However, there were no significant differences in the correlation between cortactin and CRKII co-overexpression with clinical factors in the analysis using multivariate statistics. Uneven case distributions of cortactin and CRKII co-overexpression-negative and -positive groups in T classification and N classification may have affected the correlation between cortactin and CRKII co-overexpression and clinical factors. However, including our previous reports, our findings are basically consistent with the results of a previous study that demonstrated the close relationship between elevated CRK levels and poorer survival in lung adenocarcinoma patients [14]. Importantly, our study further suggests that cortactin and CRKII co-expression level could be a prognostic factor in OSCC patients.
Cell motility is a complex event dependent on the coordinated remodeling of the actin cytoskeleton, on regulated assembly, and on turnover of focal adhesion [66]. In this context, it seemed important to demonstrate that cortactin and CRKII co-expression could be associated with the migration and invasion capacity of the human tongue squamous cell carcinoma cell line OSC20. Our data show that de novo co-expression of cortactin and CRKII raises the migration and invasion potential of tongue squamous cell carcinoma cells. Moreover, immunohistochemical analysis of cortactin and CRKII revealed strong positivity at the invasive front of the diffuse invasion pattern. However, the mechanism by which cortactin and CRKII increase the invasive potential remains unclear. We showed that the suppression of CRKII may cause inhibition of the formation of CRKII-p130Cas complexes, which affects the binding of DOCK180 to SH3-domain of CRKII and additional Rac1 binding to those complexes in the integrin-stimulated signaling pathways that govern the formation of focal adhesion and cortactin-mediated regulation of branched actin networks in OSCC[58].
In a previous report, cortactin is described as being required for adhesive contact formation through interaction with E-cadherin and promoting F-actin accumulation in adhesive complex; inhibition of cortactin activity reduced cadherin adhesive contact zone extension significantly [67]. With regard to the main component of adhesive junction, the downregulation of E-cadherin is generally accepted as a hallmark of EMT. It has been reported that many key transcription factors such as snail family proteins and zinc finger E-box binding family proteins activated directly or indirectly by Smad 2/3 were identified to inhibit the expression of E-cadherin at the transcriptional level [68]. The ectodomain of E-cadherin interacts with other E-cadherin in neighboring cells in a homotypic manner. The cytoplasmic domain of E-cadherin binds to β-catenin, which interacts with α-catenin and cortactin and anchors to the actin cytoskeleton [67,69]. Taking into consideration the important role of cortactin in the assembly of adhesion junction complex, regulation of cortactin may be involved in the disruption of adhesion junction during EMT [70]. Additionally, it has been reported that CRKII performs a critical role in promoting the
epithelial-mesenchymal-like transition in epithelial cells, and that stable overexpression of CRKII activates the downstream effectors, Rac1 and Rap, and promotes the spreading of MDCK (normal kidney) cells [71].

CRKII stimulates the breakdown of epithelial adherens junctions by inhibiting membrane accumulation of E-cadherin and β-catenin, and promotes cell dispersal in moderately differentiated breast cancer cell lines [71]. We previously reported that inhibition of the formation of CRKII-Dock180-p130Cas complexes by the reduction of CRKII strongly suggests that CRKII is involved in promoting the epithelial-mesenchymal-like transition in OSCC [58]. The interaction of p130Cas and paxillin with CRK mediates signal transmission from extracellular stimulation to the reorganization of the actin cytoskeleton [72]. CRK integrates multiple signals that could selectively lead to interactions between CRK and paxillin, CRK and p130Cas, or Rac activation. In turn, Rac can mediate Arp2/3-dependent actin polymerization through its interaction with IRSp53 and the WASP-family protein, WAVE [73]. CRK was shown to interact directly with tyrosine-phosphorylated cortactin and to mediate cortactin-dependent actin polymerization
required for \textit{Shigella} uptake \cite{59}. The CRK-cortactin complex has been reported to play a major role in actin polymerization downstream of tyrosine kinase signaling \cite{59}. In our previous study, downregulation of CRKII also decreased the expression levels of Rac1 and cortactin in the OSC20 cell line, which indicates that the CRK-cortactin complex may also play a major role in actin polymerization in OSCC \cite{58}. In our data, the downregulation of cortactin and CRKII induces the downregulation of vimentin, fibronectin, and N-cadherin expression levels as mesenchymal markers, but not E-cadherin and $\beta$-catenin expression levels as epithelial markers. Considering these findings, our data suggest that the downregulation of cortactin and CRKII may inhibit the decrease of the adhesion complexes by the downregulation of E-cadherin and $\beta$-catenin, and inhibit the reorganization of the actin cytoskeleton during EMT. The co-overexpression of cortactin and CRK II may enhance the epithelial-mesenchymal-like transition in OSCC, especially located at the invasive front and the diffuse invasive area, as seen in Fig. 1B and 1D. As a result, co-overexpression of cortactin and CRK II may increase migration and invasive potential in OSCC and correlate with T.
classification, N classification, invasive pattern, and prognosis significantly in this study.

In summary, we demonstrate the significance of cortactin and CRKII co-overexpression and its potential as a prognostic factor for OSCC as well as the possibility of epithelial-mesenchymal-like transition in OSCC. RNAi technology is a specific and powerful tool to turn off the expression of oncogenic target genes [74]. In oral cancers, the possibility of RNA-mediated gene therapy has been reported 75,76. We successfully applied double RNA silencing to inhibit the expression of cortactin and CRKII, and thereby decreased the invasive potential of OSCC. Thus, we propose that RNAi-mediated gene silencing of cortactin and CRKII could be a useful modality for OSCC treatment in the future.

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Overexpression of CRKII increases migration and invasive potential in oral squamous cell carcinoma.


63. Patel AS, Schechter GL, Wasilenko WJ, Somers KD. Overexpression of EMS1/cortactin in NIH3T3


**Figure Legends**

**Figure 1**

Representative immunohistochemical staining of cortactin and CRKII.

Immunohistochemical staining of cortactin (A and B) and CRKII (C and D) (A and C: 40× magnification, B and D: 100× magnification). Well-differentiated squamous cell carcinoma demonstrates strong cortactin and CRKII expression (staining index of 3) and diffuse invasion (A and C).

Well-differentiated squamous cell carcinoma demonstrates strong cytoplasmic expression in cancer nests, and intense staining is shown in squamous cell carcinoma cells at the invasive front of the tumor (B and D).

**Figure 2**

Kaplan-Meier curves for 5-year survival analysis.

Kaplan-Meier curves for 5-year survival were examined for cortactin and CRKII co-overexpression.
The associations were significant using the log-rank test (cortactin and CRKII co-overexpression:

overexpression (+)/(-); p<0.05).

Figure 3

Double RNAi of cortactin and CRKII in OSC20 cells.

OSC20 cells were transfected with either scrambled or cortactin and CRKII siRNA. (A) After 72 h, isolated
total RNA was analyzed by RT-PCR for cortactin, CRKII, or GAPDH, and (B) protein extracts were used
for western blotting of cortactin, CRKII, or β-actin. (C) The wound healing process was photographed at 0,
12, 24, and 36 h after wounding (left), and healing rates were determined as described in Materials and
Methods; mock (blue), scrambled (green), and RNAi (red) (right). (D) The graph shows a significant
decrease in the wound healing rate in OSC20 cells treated with cortactin and CRKII siRNA (p<0.001). (E)

Invasion of OSC20 cells (left) and the percentage of invaded cells (right) were determined as described in
Materials and Methods. The graph shows a significant decrease in the invasion index of OSC20 cells treated with cortactin and CRKII siRNA (p<0.001).

Figure 4

RT-PCR analysis of mRNA modulated by the suppression of cortactin and CRKII co-expression.

OSC20 cells were transfected with either scrambled siRNA or cortactin and CRKII siRNA. After 72 h, mRNA was analyzed by RT-PCR. RT-PCR analysis shows the decreased expression of fibronectin, vimentin, and N-cadherin. However, the expression of E-cadherin and β-catenin showed no change.
Fig. 1
CRK II overexpression (+)
cortactin overexpression (+)
cortactin/CRK II overexpression (+)
cortactin/CRK II overexpression (−)

P < 0.05

Fig. 2

Survival rate (%)

Follow up (Months)
siRNA of cortactin/CRK II

Fig. 3A

Fig. 3B
Fig. 3C
Wound Healing Assay of siRNA of cortactin/CRK II

Fig. 3C
Invasion Assay of siRNA of cortactin/CRK II

Mock scrambled RNAi

Invasion index (%)

Fig. 3D

P < 0.001
The reduction of the EMT markers by the siRNA of cortactin/CRK II

Fig. 4
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Table 1