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

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Abbreviations: OSCC, oral squamous cell carcinoma; CRK, CT10 regulator of kinase

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Abstract

CT10 regulator of kinase (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. In the present study, we evaluated the possibility of CRKII as an appropriate molecular target for cancer gene therapy. The expression of CRKII in 71 primary oral squamous cell carcinomas and 10 normal oral mucosal specimens was determined immunohistochemically, and the correlation of CRKII overexpression with clinicopathological factors was evaluated. Overexpression of CRKII was detected in 41 of 70 oral squamous cell carcinomas, the frequency being more significant than in normal oral mucosa. In addition, CRKII overexpression was more frequent in higher-grade cancers according to the T classification, N classification, and invasive pattern. Moreover, RNAi-mediated suppression of CRKII expression reduced the migration and invasion potential of an oral squamous cell carcinoma cell line, OSC20. Downregulation of CRKII expression also reduced the expression of Dock180, p130Cas, and Rac1, and the actin-associated scaffolding protein cortactin. These results indicate that the overexpression of CRKII is tightly associated with an aggressive phenotype of oral squamous cell carcinoma. Therefore, we propose that 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 causes inevitably

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].

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[9-11].

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[12,13]. Cellular homologues of v-CRK include the

c-CRK gene, which produces two alternatively spliced protein products: CRKI (28 kDa) and CRKII (40

kDa)[13,14]. The ubiquitous expression of CRK is observed in embryos and adults[13.] In addition, the

overexpression of CRK in malignant neoplasmas[15], including glioblastomas[16] and lung cancers[15,17],

has been reported. CRKI/II mRNA expression is enhanced in lung tumors at more advanced stages and

accompanies poor survival[17]. CRKI is composed of an SH2 and an SH3 domain, and CRKII has an

additional SH3 domain[13]. 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[18], growth factor receptor tyrosine

kinases, and a docking protein Gab1, which is involved in epithelial dispersal and morphogenesis[19-21].

The NH_2-terminal of the CRKII-SH3 domain constitutively interacts with proline-rich motifs present in

proteins, including C3G, a nucleotide exchange factor for Rap1[22], Dock180, an exchange factor for

Rac1[19,23], the Abl tyrosine kinase[24], tyrosine phosphatase[26], the p85 subunit of

phosphatidylinositol 3-kinase[26], and the c-Jun-NH_2-kinase[27]. 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[28] as well as the Rac exchange factor Dock180[29].

Based on these interactions, the proposed roles of CRK include the regulation of cell migration,

morphogenesis, invasion, phagocytosis, and survival[18]. However, the relationship between the

expression of CRKII and the invasiveness and metastatic potential of cells remains unknown for OSCC. In

this study, we examined CRKII expression in OSCC immunohistochemically, and then determined the

clinicopathological significance of CRKII expression in relation to various parameters, such as patient

characteristics and histopathological findings. We also performed siRNA analysis to assess whether CRKII

could be a potent molecular target for cancer gene therapy of OSCC.

2. Materials and Methods

2.1. Patients

Paraffin-embedded sections were obtained from biopsy specimens of 71 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[30]. As controls, samples of normal oral epithelium were obtained after informed consent from 10 patients undergoing routine surgical removal of their third molars.

2.2. Cell lines

Basically, we examined the expression of CRKII in the seven OSCC cell lines   (Ca9-22, SAS, SCC25, OSC20, HSC2, HSC3, and HSC4). Among them, the OSC 20 cell line expressed CRKII most strongly (date not shown). Therefore, we used the OSC20 cell line for our experiments. 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% 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 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. CRKII expression was defined as the

presence of specific staining in the nucleus and cytoplasm of tumor cells. The immunoreactivity of CRKII

was scored by staining intensity and immunoreactive cell percentage as follows[31-33]: 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 from 25% to 50% of tumor cells; 3 = tissue with strong staining in ≥50% of

tumor cells. Overexpression of CRKII was defined as staining index ≥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 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'-TCTCAGGCAGTGCAAATCAC-3' for CRKII (F);

5'-CGCTCCATACAATGAAAGCA-3' for CRKII (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%

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, 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 was expressed as a

percentage.

2.7. RNA interference (RNAi)

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)

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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 polyvinylidine difluoride membranes and immunoblotted with either anti-CRKII (H-53, Santa Cruz

Biotech), anti-p130 Cas (8G4-E8, Millipore, MA, USA), anti-Dock180 (H-4, Santa Cruz Biotech),

anti-Rac1 (102, BD Transduction Lab., CA, USA), or anti-cortactin (H-191, Santa Cruz Biotech, Inc., CA,

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 Fischer'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 CRKII expression and clinicopathological features

Immunohistochemistry with an anti-CRKII polyclonal antibody was performed on 71 patients with oral squamous cell carcinoma. Representative immunohistochemical stainings are shown in Figure 1. Overexpression of CRKII was undetectable in normal epithelium (date not shown). In the squamous cell carcinoma cells, strong CRKII staining was located at the invasive front and the diffuse invasive area (Fig.1D). Indeed, CRKII overexpression was recognized more frequently in OSCC (49 of 71, 68.1%) than in normal oral epithelium (0 of 10, 0%; $p<0.05$). Furthermore, overexpression of CRKII was more frequent in cancers with higher grades according to the T classification (T 3/4 vs. 1/2; $p<0.05$), 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 CRKII overexpression is a potent predictor of survival.

3.2. Correlation between CRKII expression and survival analysis

The 5-year survival rates according to CRKII expression and other clinicopathological features were determined. There was a tendency of coupling between lower survival in patients and CRKII overexpression, as seen in the T classification, N classification, and invasive pattern. The association was significant by the log rank test (Fig. 2). These findings also strongly suggest that CRKII overexpression is a potent predictor of survival.

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

Cell migration and invasion are basic characteristics of tumor metastasis. To determine the effect of CRKII on the migration and invasion potential of cells, we transfected OSC20 cells with CRKII siRNA and performed wound healing and Matrigel invasion assays. Transfection with CRKII siRNA significantly decreased CRKII mRNA and protein levels, compared with that of non-transfected cells and cells transfected with scrambled siRNA (Fig. 3A, B). The induced downregulation of CRKII expression resulted in a 64.3% decrease in healing rate compared with the controls at 36 h after wounding (Fig. 3C). Concomitantly, the invasion index of OSC20 cells decreased significantly from 92.4% and 91.7% in cells treated with vehicle alone and scrambled siRNA, respectively, to 30.3% in those transfected with CRKII siRNA (Fig. 3D). Therefore, downregulation of CRKII expression by siRNA drastically suppresses the mobility of OSC20 cells in vitro.

3.4. Effect of decreasing CRKII expression on CRKII-p130Cas signaling

Dock180 is reported to positively regulate signaling of integrins with CRKII-p130Cas complexes at focal adhesions[29]. Phosphorylated p130Cas interacts with CRK, which in turn binds to Dock180, an unconventional Rac-guanine nucleotide exchange factor that activates Rac1[34-38]. The p130Cas-CRK complex is reported to trigger actin polymerization by a Rac-independent mechanism[29]. Cortactin is an actin-associated scaffolding protein that binds and activates the actin-related protein (Arp) 2/3 complex,

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and regulates branched actin networks in the formation of dynamic cortical actin-associated

structures[39,40]. Cortactin and CRK cooperate to trigger actin polymerization during *Shigella* invasion of

epithelial cells[41]. We therefore examined the effect of CRKII on the integrin-stimulated

Dock180-CRKII-p130Cas signaling pathway. CRKII-targeted siRNA transfection of OSC20 cells

significantly decreased p130Cas and Dock180, and Rac1 expression levels (Fig. 4). Moreover, the

CRKII-targeted siRNA transfection also decreased cortactin expression (Fig. 4). These results suggest that

downregulation of CRKII induces the suppression of integrin-stimulated signaling pathways that govern

the formation of focal adhesion and cortactin-mediated regulation of branched actin networks in OSCC.

4. Discussion

CRK has been reported to regulate cytoskeletal reorganization by integrin stimulation, and thereby modulate cell motility and adhesion[15]. The SH2 domain of CRK binds a phosphorylated tyrosine residue in the YXXXP motif of p130Cas and paxillin, the components of focal contacts[18]. The SH3 domain of CRK binds to DOCK180, which is reported to activate Rac regulation of cytoskeketal movement such as engulfment[19,23,29,34,42]. Increased CRKII activity suppresses apoptosis, induces lamellipodia formation and cell spreading in migratory cells[43], and encourages anchorage-independent growth[28]. It has been reported that increased expression of CRKI/II at the mRNA and protein level is observed in various cancers[15], including glioblastomas[16] and lung cancers[15,17]. In lung adenocarcinoma, CRKI/II mRNA expression is predominantly increased at more advanced stages and is associated with poor survival[17]. In the present study, we reveal that CRKII 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 CRKII expression with 5-year survival rates (log-rank, $p < 0.05$), similar to the T classification, N classification , and pattern of invasion, respectively. There were no significant differences in the correlation between CRKII overexpression clinical factors in the analysis using multivariate statistics. Because of uneven case distributions of CRKII overexpression negative and positive groups in T classification and N-classification, the correlation between CRKII overexpression and clinical factors may be affected. However, our findings are basically consistent with

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the results of a previous study that demonstrates the close relationship between elevated CRK levels and

poorer survival in lung adenocarcinoma patients[17]. Importantly, our study further suggests that CRKII

expression levels 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[44]. In this context, it seemed important to

demonstrate that CRKII expression could be associated with the migration and invasion capacity of the

human tongue squamous cell carcinoma cell line, OSC20. Our study shows that *de novo* expression of

CRKII raises the migration and invasion potential of tongue squamous cell carcinoma cells. Moreover,

immunohistochemical analysis of CRKII revealed strong positivity at the invasive front of the diffuse

invasion pattern. However, the mechanism by which CRKII increases the invasive potential remains

unclear. The molecular complex composed of paxillin-CRKII-Dock180 is reported to govern one major

aspect of cell migration, which is the activation of Rac1[45]. Subsequently, Rac1 controls actin

cytoskeletal dynamics and integrin adhesions that are essential for lamellipodium extension during cell

migration[46,47]. The expression of farnesylated Dock180 induced the spreading of spindle-shaped

NIH3T3 cells, which suggests that Dock180 may be involved in cytoskeletal reorganization[42]. In lung

cancer cell lines, the expression of Dock180 is reported to correlate with growth rate[15]. As p130Cas

contains multiple binding motifs for SH2 and SH3 domains, it localizes at focal adhesions and becomes

tyrosine-phosphorylated upon integrin stimulation[48-50]. Tyrosine-phosphorylation of p130Cas recruits

CRKII to focal adhesions[51,52]. The formation of CRKII-p130Cas complexes and their

hyperphosphorylation is upregulated by Dock180[29]. Dock180 overexpression induces accumulation of

p130Cas, Dock180, and CRKII at focal adhesions[29]. Our results suggest that the suppression of CRKII

may cause the inhibition of the formation of CRKII-p130Cas complexes, which result in the effects on the

binding of DOCK180 to SH3-domain of CRKII and additional Rac1 binding to those complex in the

integrin-stimulated signaling pathways that govern the formation of focal adhesion and cortactin-mediated

regulation of branched actin networks in OSCC.

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[53].

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[53]. Therefore, our data demonstrating the 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. The interaction of p130Cas and paxillin with CRK

mediates signal transmission from extracellular stimulation to the reorganization of the actin

cytoskeleton[28,54]. 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[55]. CRK was

shown to directly interact with tyrosine-phosphorylated cortactin and to mediate cortactin-dependent actin

polymerization required for *Shigella* uptake[41]. The CRK-cortactin complex has been reported to play a

major role in actin polymerization downstream of tyrosine kinase signaling[41]. In this report,

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.

In summary, we demonstrate the significance of CRKII overexpression and its potential as a

prognostic factor for OSCC as well as the possibility of an epithelial-mesenchymal-like transition in OSCC.

RNAi technology is a specific and powerful tool to turn off the expression of oncogenic target genes[56].

In oral cancers, the possibility of RNA-mediated gene therapy has been reported[57,58]. We successfully

applied RNA silencing to inhibit the expression of CRKII, and thereby decreased the invasive potential of

OSCC. Thus, we propose that RNAi-mediated gene silencing of CRKII could be a useful modality for

OSCC treatment in the future.

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Figure Legends

Figure 1

Representative immunohistochemical staining of CRKII.

(A and C) H-E staining, and (B and D) immunohistochemical staining of CRKII(A and B: 100×

magnification, C and D: 40× magnification. Well-differentiated squamous cell carcinoma (A) demonstrates

strong CRKII expression (staining index of 3) and diffuse invasion (C). Well-differentiated squamous cell

carcinoma (B) demonstrates strong cytoplasmic expression in cancer nests, and intense staining is shown

in squamous cell carcinoma cells at the invasive front of the tumor (D).

Figure 2

Kaplan-Meier curves for 5-year survival analysis.

Kaplan-Meier curves for 5-year survival were examined about CRKII overexpression. The

associations was significant using the log rank test (CRKII overexpression: overexpression (+)/(-);

$p<0.05$).

Figure 3

RNAi of CRKII in OSC20 cells.

OSC20 cells were transfected with either scrambled or CRKII siRNA. (A) After 72 h, isolated total RNA was analyzed by RT-PCR for CRKII or GAPDH and (B) protein extracts were used for western blotting of 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 (closed circle), scrambled (open circle), and RNAi (square) (right). The graph shows a significant decrease in the wound healing rate in OSC20 cells treated with CRKII siRNA ($p<0.001$). (D) 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 CRKII siRNA ($p<0.001$).

Figure 4

Western blot analysis of proteins modulated by the suppression of CRKII expression. OSC20 cells were transfected with either scrambled siRNA or CRKII siRNA. After 72 h, proteins of whole cell lysates were analyzed by western blot analysis. Western blot analysis shows the decreased expression of Dock180, p130Cas, Rac1, and cortactin.

| | CRK Ⅱ overexpression | | p *value* |
	(−)	(＋)	
Normal epithelium	10	0	p＜0.05
Squamous cell carcinoma	22	49	
Gender　　Male	13	27	p=0.753
Female	9	22	
Age　　　≦60	2	19	p＜0.05
＞60	20	30	
T classification　T1 ＋ T2	18	28	p＜0.05
T3 ＋ T4	4	21	
N classification　N0	21	35	p＜0.05
N1 ＋ N2	1	14	
Differentiation　Well	17	44	p=0.161
Moderate/Poor	5	5	
Pattern of invasion　Grades1/2	20	23	p＜0.001
Grades3/4	2	26	

Table 1. Correlation of CRKII overexpression with clinicopathological features

(A)

(C)

(B)

(D)
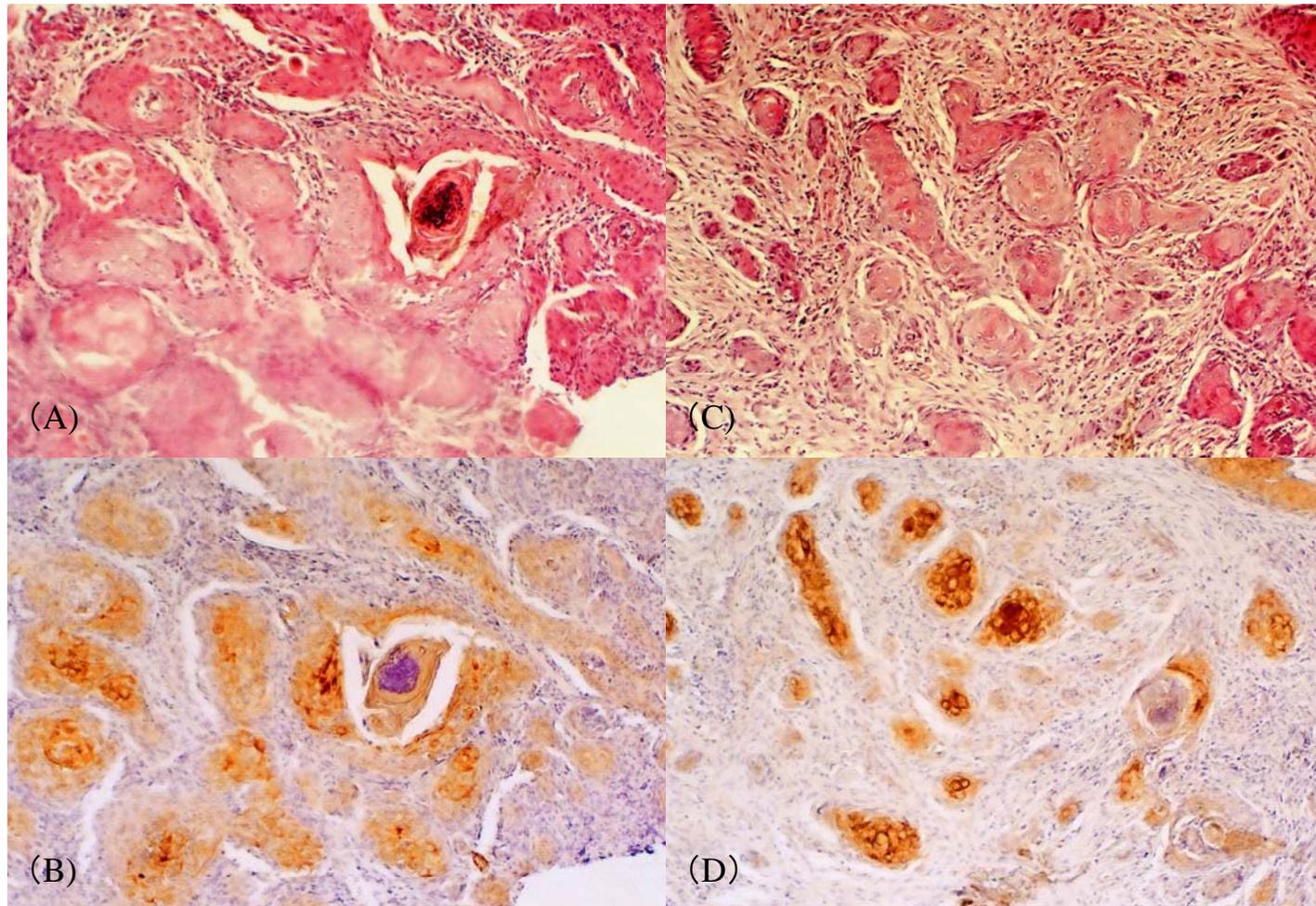
Fig. 1

Fig. 2A

Fig. 2B

Fig. 2C

Fig. 2D

Mock   RNAi   scrambled

CRK Ⅱ

GAPDH

Fig. 3A

Fig. 3B

Fig. 3C

Fig. 3D

Fig. 4