RNAi-mediated down-regulation of α-actinin-4 decreases invasion potential in oral squamous cell carcinoma

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Abstract

The α-actinin-4, originally identified as an actin-binding protein associated with cell motility, invasion, and metastasis of cancer cells, appears to be overexpressed in various human epithelial carcinomas, including colorectal, breast, esophageal, ovarian, and non-small cell lung carcinomas. In the present study, we therefore evaluated whether α-actinin-4 might be appropriate as a molecular target for cancer gene therapy. In 64 primary oral squamous cell carcinomas and 10 normal oral mucosal specimens, and in seven human oral squamous cell carcinoma cell lines, α-actinin-4 expression was evaluated by immunological analyses, and correlations with clinicopathologic factors were examined. Overexpression of α-actinin-4 was detected in 38 of 64 oral squamous cell carcinomas (70.4%); significantly more frequently than in normal oral mucosa. The expression of α-actinin-4 was significantly associated with invasion potential defined by the Matrigel invasion assay. Cancer cell lines with higher α-actinin-4 expression had greater invasive potential. Moreover, RNAi-mediated decrease in α-actinin-4 expression reduced the invasion potential. These results indicated that the overexpression of α-actinin-4 was associated with an aggressive phenotype of oral squamous cell carcinoma. In conclusion, the present study indicated that α-actinin-4 could be a potential molecular target of gene therapy by RNAi targeting for oral squamous cell carcinoma.

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[20]. The primary therapeutic modality for OSCC is surgery. Although recent advancements in surgical techniques and anticancer agents have improved tumor regression and survival rate, 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[11,14,25]. Moreover, alterations in the expression of adhesion-related molecules are reportedly associated with poor prognosis among OSCC patients[2,3,29,30].

The α-actinin-4, an actin-binding and crosslinking protein, is believed to play important roles in forming stress fibers, promoting cell adhesion, and regulating cell shape and motility[10,18,23]. Of the 4 isoforms of α-actinin, the expression of α-actinin-2 and α-actinin-3 is limited to skeletal and cardiac muscle sarcomeres[4]. In contrast, α-actinin-1 and α-actinin-4, which are non-muscular α-actinins, are widely expressed. The latter 2 isoforms share a high degree of similarity (87% amino acid homology) but have different subcellular localizations[15]. The α-actinin-1 is localized within the end of actin stress fibers and adherens junctions, and associates them with the cell membranes[15]. In contrast, α-actinin-4 is present in the cytoplasm and nucleus, and it colocalizes with actin stress

fibers[15]. The expression of α-actinin-4 is highly concentrated at the leading edge of motile cells and in the cytoplasm of sharp cell extensions[15].

Recent reports have suggested that the increased expression of α-actinin-4 in the cytoplasm of various malignant neoplasms is correlated with poor prognosis, enhanced cell motility, advanced tumor stage, and lymph node metastasis[8,9,13,15-17,22,27]. However, the relationship between α-actinin-4 expression and invasiveness or metastatic ability in OSCC and the mechanisms underlying any such relationship remain unknown.

In this study, we therefore immunohistochemically examined α-actinin-4 expression in OSCC. We then determined the clinicopathological significance of α-actinin-4 expression in relation to various parameters such as patient characteristics and histopathological findings. Moreover, the siRNA analysis strongly suggested that α-actinin-4 would be a potent molecular target for cancer gene therapy in OSCC.

Materials and methods

Patients

Paraffin-embedded sections were obtained from biopsy specimens of 64 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[5]. 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

The following human OSCC cell lines were obtained from the Human Science Research Resource Bank (Osaka, Japan): SAS, SCC25, OSC20, HSC-2, HSC-3, HSC-4, Ca9-22, and the human keratinocyte cell line, HEKa, as a control. All cells were cultured under conditions recommended by their depositors.

Immunohistochemical staining and evaluation

Serial 4-μm thick specimens were taken from the 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 α-actinin-4 (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. α-actinin-4 expression was defined as the presence of specific staining in the cytoplasm and nuclei of tumor cells. The immunoreactivity of α-actinin-4 was scored by staining intensity and immunoreactive cell percentage as follows[9]: staining index 0 = tissue with no staining; 1 = tissue with faint staining or moderate staining in ≤25% of tumor cells; 2 = tissue with moderate staining or strong staining in ≤25% of tumor cells; 3 = tissue with strong staining in >25% of tumor cells. Overexpression of α-actinin-4 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 28 cycles (for α-actinin-4), 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: AGTATGAACGCAGCATCGTG for α-actinin-4(F); GGTGAGGATCTGGTTCTCCA for α-actinin-4(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

The BioCoat Matrigel invasion chamber (Becton Dickinson, Bedford, MA) was used. This contains an internal chamber with an 8-μm porous membrane bottom that was coated with Matrigel. Six-well cell culture inserts and a six-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 manufacture'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, 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 α-actinin-4 siRNA sequences were 5'-GGACAUGUUCAUCGUCCAUTT-3' and 5'-TTCCUGUACAAGUAGCAGGUA-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.

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-α-actinin-4 (4D10, Abnova, Taipei, Taiwan)[9] or anti-β-actin (9F2, Abcam, Cambridge, UK; 0.02 μg/ml). 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 α-actinin-4 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 α-actinin-4 mRNA expression and invasion index was determined using Person's correlation coefficient. P values less than 0.05 were considered significant.

Results

Correlation between α-actinin-4 overexpression and clinicopathologic features

Immunohistochemistry with an anti-α-actinin-4-specific monoclonal antibody was performed on a series of 64 patients with oral squamous cell carcinoma. Representative immunohistochemical stainings are shown in Figure 1. Overexpression of α-actinin-4 was undetectable in the normal epithelium (Fig. 1A). In the squamous cell carcinoma cells, strong α-actinin-4 staining was apparent at the invasive front and the diffuse invasive area. The α-actinin-4 overexpression was detected significantly more frequently in OSCC (38 of 64, 70.4%) than in normal oral epithelium (0 of 10, 0%; $p<0.001$, Table 1). Moreover, α-actinin-4 overexpression was significantly more frequent in higher grade cancers (grades 3/4 vs. grades 1/2; $p=0.004$, Table 1). Cox regression analysis was performed with the parameter of α-actinin-4 overexpression. There was no significant independent predictor of survival (Hazard ratio:1.50, 95%CI:0.769-2.94, P value:0.233).

The α-actinin-4 is associated with invasion pattern of OSCC

The α-actinin-4 protein expression of OSCC cell lines SAS, SCC25, OSC20, HSC-2, HSC-3, HSC-4, and Ca9-22 was analyzed by western blot analysis.

The α-actinin-4 protein was detectable in all OSCC cell lines. The α-actinin-4/β-actin intensity ratio varied from 0.151 to 0.289 (Fig. 2A). To characterize the invasion potential of OSCC cells *in vitro*,

we used Matrigel invasion chambers. The highest invasion index was noted in HSC-3, followed by SAS and OSC20 with similar indices, and the index was lowest in HSC-2 (Fig. 2B). The α-actinin-4 protein expression level was significantly correlated with the invasion index of the OSCC cells (Fig. 2C).

Effect of decreasing α-actinin-4 expression on the invasion potential of OSC20 cells

To determine the effect of decreasing α-actinin-4 expression on invasion potential, we transfected OSC20 cells with α-actinin-4 siRNA (Fig. 3A,B) and performed the Matrigel invasion assay. Transfection with α-actinin-4 siRNA significantly decreased the mRNA and protein levels of α-actinin-4, compared to those in non-transfected cells and cells transfected with scrambled siRNA (Fig.3A, B). Concomitantly, the invasion index of the OSC20 cells decreased significantly from 77.4% (in cells treated with vehicle alone) and 84.0% (in cells transfected with scrambled siRNA) to 39.3% in cells transfected with α-actinin-4 siRNA (Fig. 3C).

Discussion

The α-actinins, which are members of the spectrin gene superfamily, are rod-shaped proteins that bind and crosslink actin[21]. There are two isoforms of α-actinin, the muscular type and the non-muscular type[4]. In non-muscle cells, in addition to being involved in actin stress fiber formation, the α-actinins play an important role in promoting cell adhesion and in regulating cell shape and motility[10,18,23]. In particular, α-actinin-4 has been proposed as a new marker of motility in non-neoplastic cells and tumor cells, and it has also been associated with the metastatic potential of malignant cells[1,15]. In this study, we examined the overexpression of α-actinin-4 in biopsy specimens of human OSCC patients using immunohistochemistry. Overexpression of α-actinin-4 is recognized in various cancers. In breast cancer and non-small cell lung cancer, the overexpression and subcellular localization of α-actinin-4 is significantly correlated with poorer prognosis[8,15]. Increased expression of α-actinin-4 can enhance cell motility and promote lymph node metastasis in colorectal cancer[17] and is significantly correlated with advanced tumor stage as well as lymph node metastasis in esophageal cancer[9]. In ovarian carcinoma, high expression of α-actinin-4, resulting from its cytoplasmic accumulation, is strongly associated with higher histological grade, advanced clinical stage, greater residual tumor, and poor prognosis[27]. Moreover, the down-regulation of α-actinin-4 can decrease the invasion potential in pancreatic cancer[13]. In contrast, decreased expression of α-actinin-4 is reported in neuroblastoma and prostate carcinoma[12,22]. Hence, the clinicopathological

significance of α-actinin-4 overexpression is controversial. In agreement with previous reports, the present data showed that, from the clinicopathological standpoint, increased expression of α-actinin-4 is significantly correlated with invasion pattern in OSCC. These findings support the conclusion that α-actinin-4 affects the cell motility and invasion of OSCC. As reported in studies finding similar clinicopathological impacts of α-actinin-4 in other carcinomas, α-actinin-4 may be ubiquitously expressed in various malignant tumors and involved in invasion and metastasis. In the previous reports, overexpression of α-actinin-4 is correlated with poor prognosis[8,15,27]. In this study, since there was no correlation between survival date and overexpression of α-actinin-4, α-actinin-4 expression was not a prognostic factor in OSCC patients.

To evaluate the effect of α-actinin-4 siRNA on invasive potential, we used chemically synthesized α-actinin-4 siRNA to achieve direct homology-dependent posttranscriptional gene silencing. When the time course experiment of α-actinin-4 siRNA was assessed by RT-PCR, the potential of α-actinin-4 mRNA inhibition peaked at 72h post transfection, and the obvious effect of α-actinin-4 inhibition was maintained for 5 days at least (*data not shown*). Since this anti-α-actinin-4 mAb specifity reacts α-actinin-4 on immunoblotting analysis(Abcam, Cambridge), the slightly lower inhibition of α-actinin-4 protein expression compared with mRNA expression might be due to the longevity of the protein than cross-reactivity to α-actinin-1[7]. Our results revealed that the α-actinin-4 siRNA significantly inhibited the invasiveness of OSC20 cells. The invasion of

malignant tumors is mediated by the interactions between epithelial cancer cells and the extracellular matrix. Hence, the behavior of malignant tumor cells infiltrating the stroma may be affected by interactions with the extracellular matrix[24]. Dynamic regulation of the actin cytoskeleton by the various classes of actin-binding proteins plays a crucial role in cell movement[6]. In colorectal cancer, immunofluorescence microscopy revealed that α-actinin-4 and β-catenin were colocalized in bleb-like membrane protrusions on the free surface of tumor cells[13]. E-cadherin is reported to regulate this association between α-actinin-4 and β-catenin, and the dynamic shift of β-catenin from the cell adhesion complex into a complex containing α-actinin-4 may evoke cell movement and mediate cancer invasion and metastasis[13]. In the present study, the down-regulation of α-actinin-4 significantly reduced the invasion potential of OSC20 cells in vitro. Although we did not directly demonstrate the association between α-actinin-4 and the extracellular matrix proteins, such as E-cadherin and β-catenin, the disruption of such interactions may be involved in enhancing the invasion potential of OSC20 cells.

RNAi technology is a specific and powerful tool to turn off the expression of oncogenic target genes[26]. In oral cancer, the possibility of RNA-mediated gene therapy has been reported[19,28]. We successfully applied RNA silencing to inhibit the expression of α-actinin-4, thereby decreasing the invasion potential of OSCC. Therefore, we propose that RNAi-mediated gene silencing of α-actinin-4 might be a useful modality for OSCC treatment in the future.

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Ethical approval: Not required

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Figure Legends

Figure 1

Representative immunohistochemical staining for α-actinin-4. (A) Negative staining without α-actinin-4 overexpression is shown in normal oral epithelium (40×). (B) Well-differentiated squamous cell carcinoma demonstrating strong α-actinin-4 expression (staining index of 3) and diffuse invasion (40×). (C) Immunohistochemical staining for α-actinin-4 demonstrates strong cytoplasmic expression in the cancer nests (200×). (D) α-actinin-4 overexpression (intense staining) is shown in squamous cell carcinoma cells at the invasive front of the tumor (200×).

Figure 2

(A) Representative western blot analysis for α-actinin-4 in SAS, SCC25, OSC20, HSC-2, HSC-3, HSC-4, Ca9-22, and HEKa cells. The α-actinin-4/β-actin intensities are the mean ± SD of triplicate experiments. (B) Invasion index (%) was expressed as the ratio of mean number of cells that passed through the Matrigel chamber to mean number of cells that passed through the control chamber, according to the manufacture's recommendations. The data are presented as means of three separate experiments, each performed in triplicate; bars, SD. (C) Correlation between α-actinin-4 protein

expression and invasion index (%). α-actinin-4 protein expression was highly correlated with invasion index (Pearson's correlation, r=0.755, P<0.05). ●, SAS cells; ▲, SCC25 cells; ○, OSC20 cells; ■, HSC-2 cells; △, HSC-3 cells; ◆, HSC-4 cells; □, Ca9-22 cells.

Figure 3

(A) Representative RT-PCR for the suppression of α-actinin-4 in OSC20 cells. Cells were transfected with scrambled siRNA (control) or α-actinin-4 siRNA. After 72h, isolated total RNA was analyzed using RT-PCR. (B) Western blot analysis for the suppression of α-actinin-4 in OSC20 cells. Western blot analyses show α-actinin-4 and β-actin expression in the whole cell lysate proteins. (C) The graph shows significant decrease of the invasion index in OSC20 cells treated with α-actinin-4 siRNA (P<0.01).

Characteristic		Number of samples	α-actinin-4 overexpression (−)	α-actinin-4 overexpression (+)	*p* value
Normal epithelium		10	10	0	0.005
Squamous cell carcinoma		64	26	38	
Gender	Male	35	12	23	0.389
	Female	29	13	16	
Age	60≦	19	7	12	0.688
	60＞	45	19	26	
T classification	T1 ＋ T2	41	18	23	0.458
	T3 ＋ T4	23	8	15	
N classification	N0	50	19	31	0.419
	N1 ＋ N2	14	7	7	
Differentiation	Well	56	22	34	0.564
	Moderate/Poor	8	4	4	
Pattern of invasion	Grades 1/2	39	22	17	0.004
	Grades 3/4	25	5	20	

Table 1

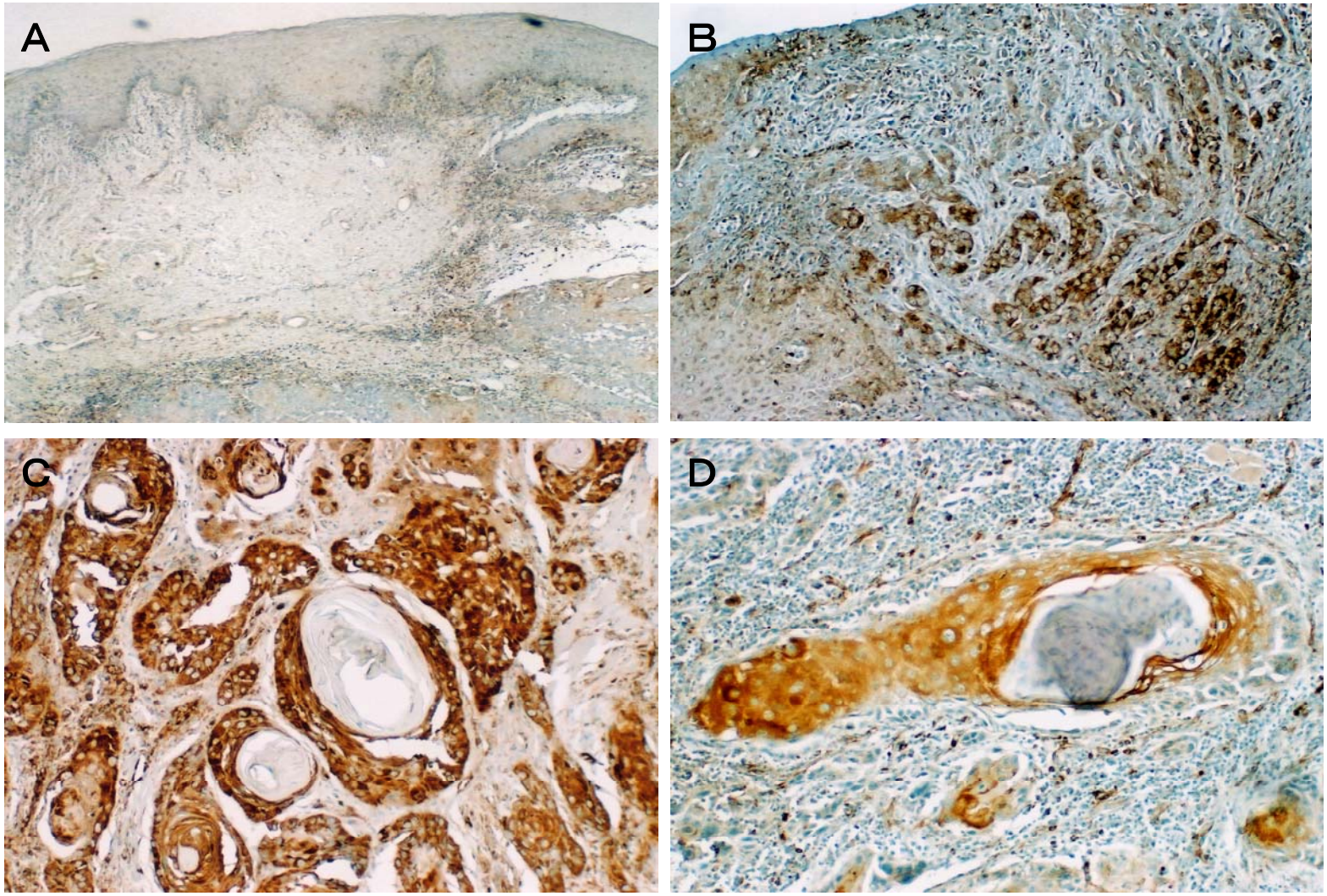

Fig.1

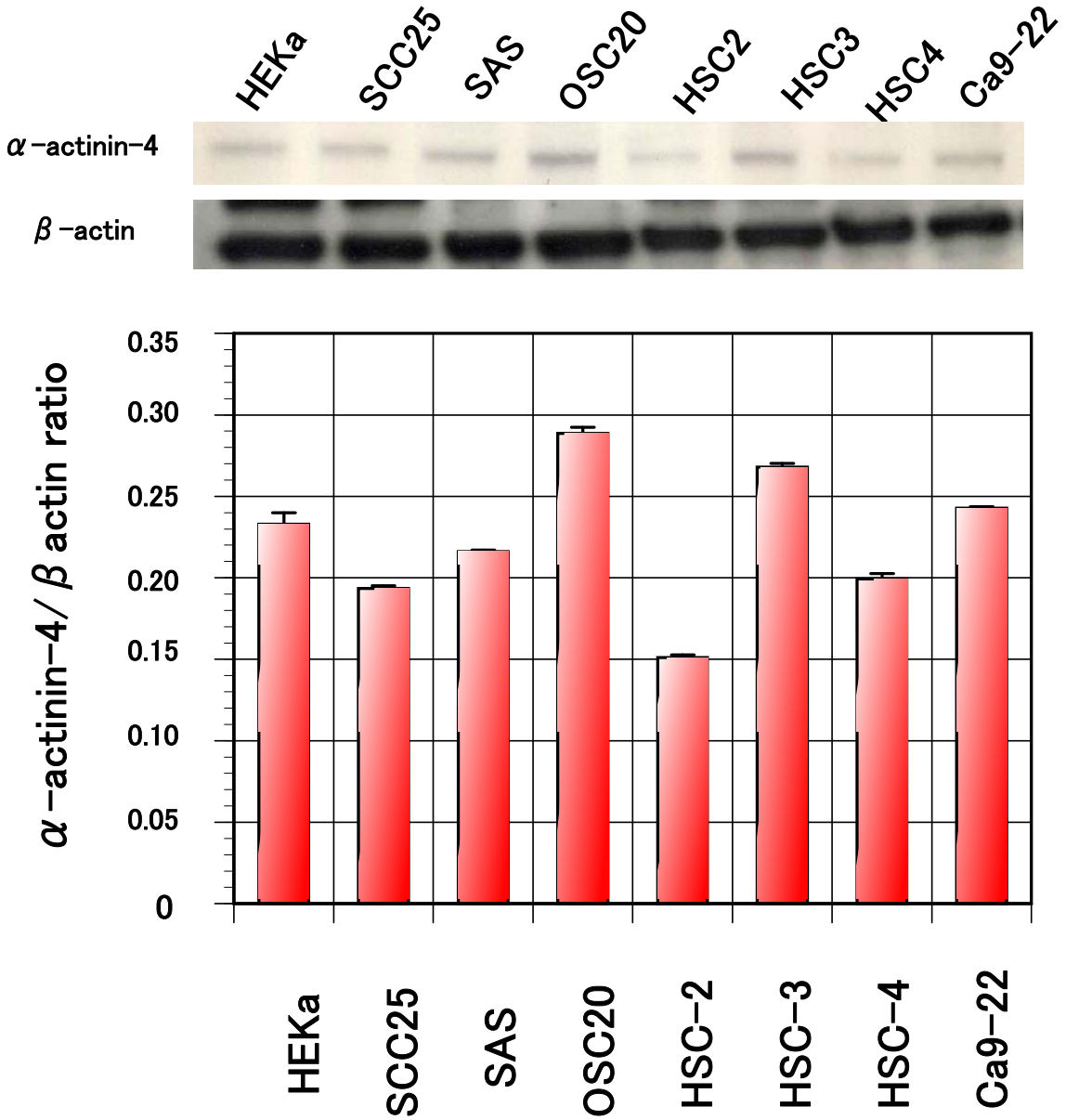
HEKa
SCC25
SAS
OSC20
HSC2
HSC3
HSC4
Ca9-22
α-actinin-4
β-actin
α-actinin-4/β actin ratio
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
HEKa
SCC25
SAS
OSC20
HSC-2
HSC-3
HSC-4
Ca9-22

Fig.2A

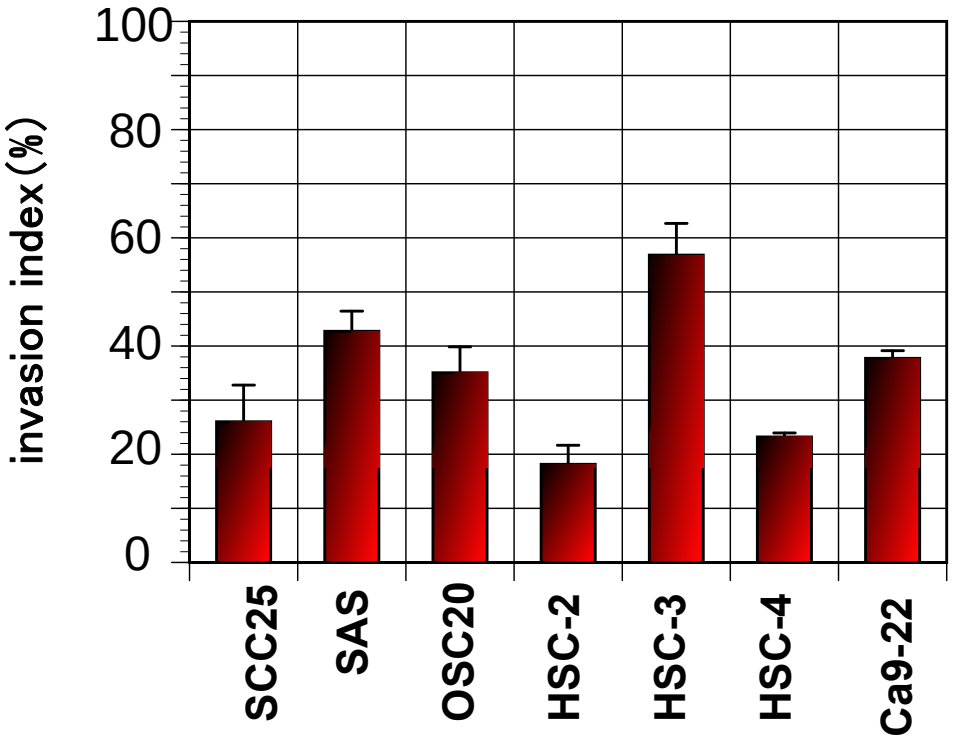
invasion index (%)
100
80
60
40
20
0
SCC25
SAS
OSC20
HSC-2
HSC-3
HSC-4
Ca9-22

Fig.2B

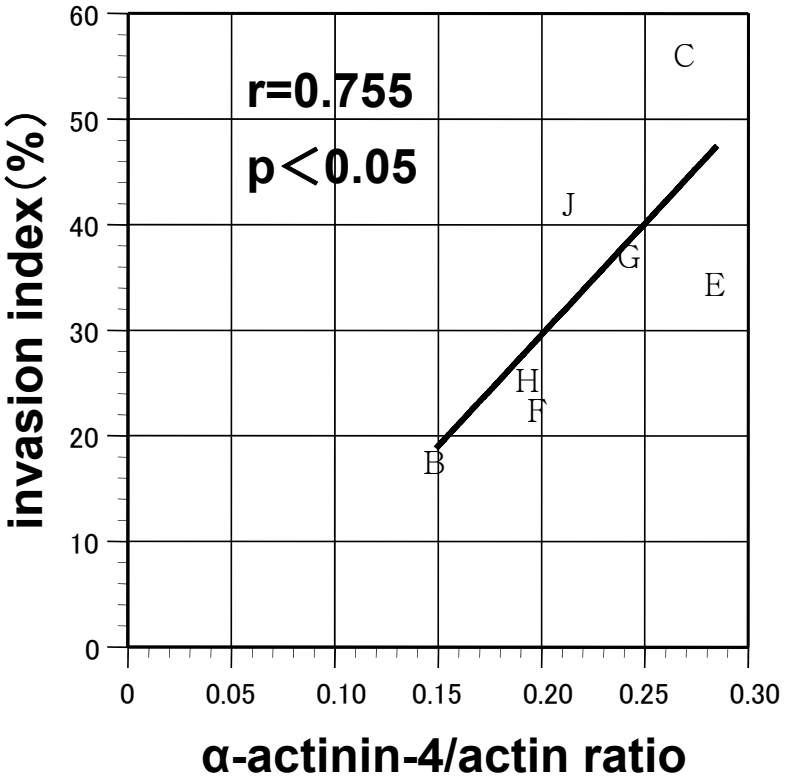
r=0.755
p＜0.05
C
J
G
E
H
F
B
invasion index(%)
α-actinin-4/actin ratio
60
50
40
30
20
10
0
0
0.05
0.10
0.15
0.20
0.25
0.30

Fig.2C

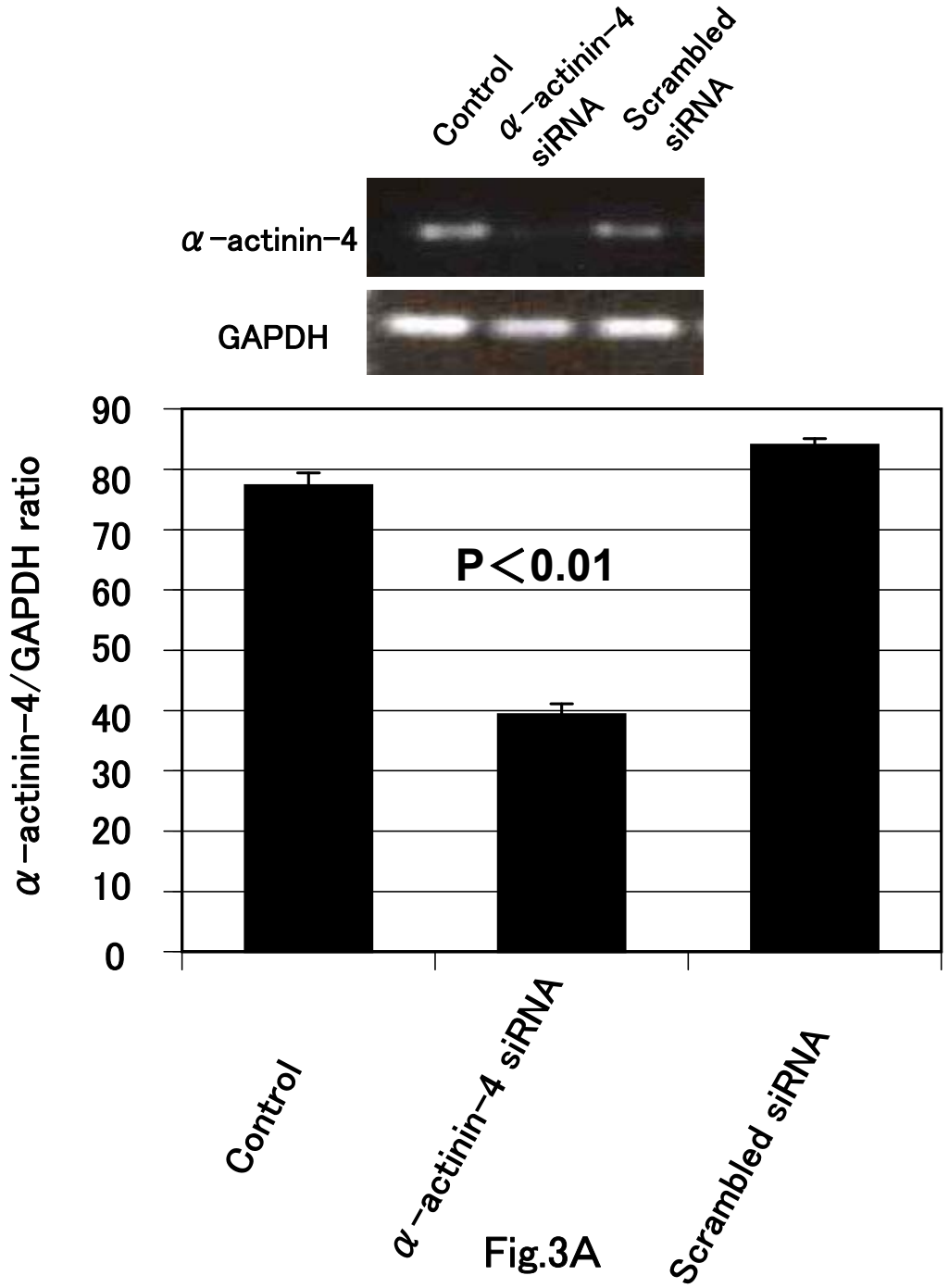
Control
α-actinin-4 siRNA
Scrambled siRNA
α-actinin-4
GAPDH
α-actinin-4/GAPDH ratio
90
80
70
60
50
40
30
20
10
0
P＜0.01
Control
α-actinin-4 siRNA
Scrambled siRNA

Fig.3A

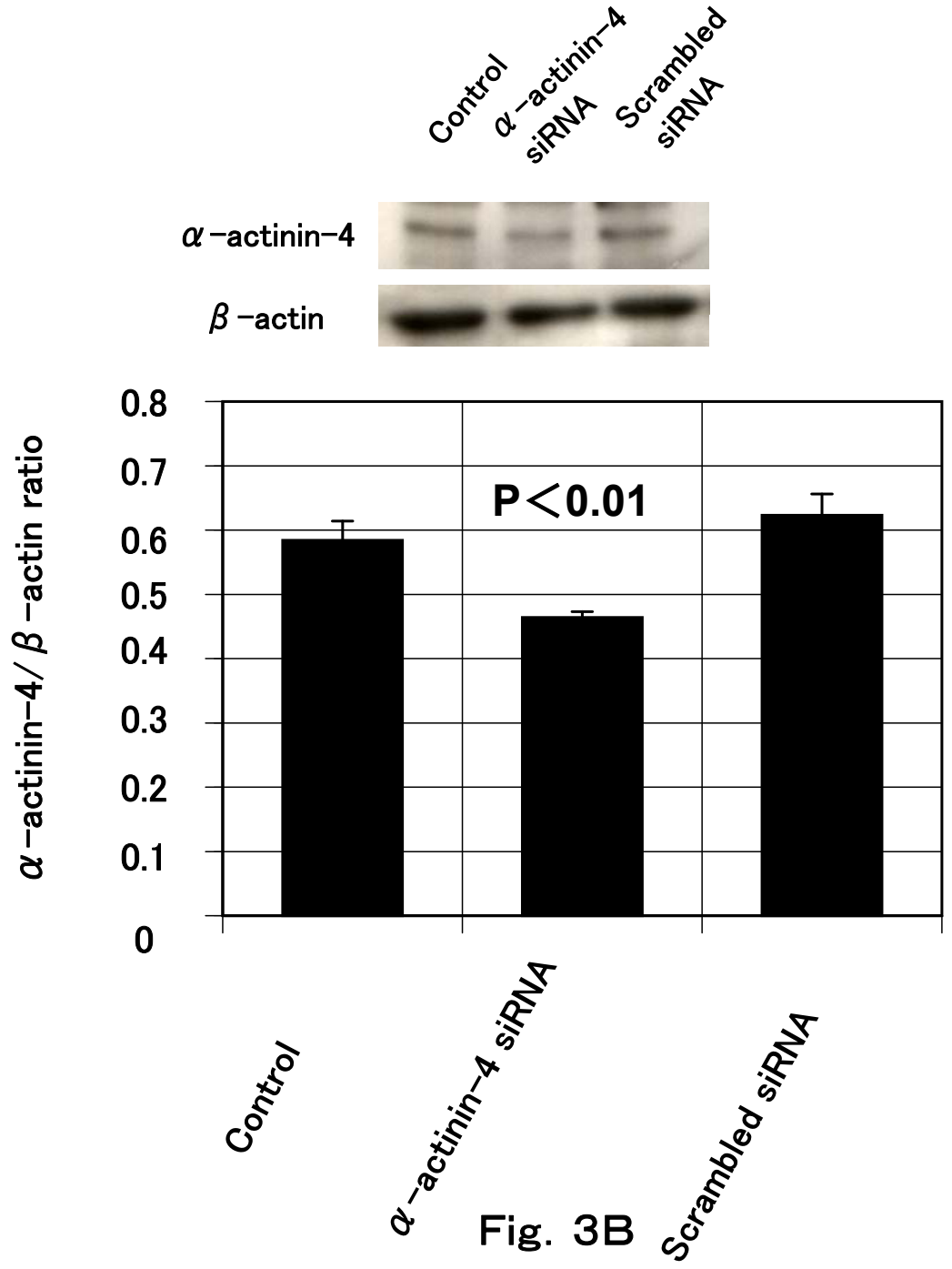
Control
α-actinin-4 siRNA
Scrambled siRNA
α-actinin-4
β-actin
α-actinin-4/ β-actin ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
P＜0.01
Control
α-actinin-4 siRNA
Scrambled siRNA

Fig. 3B

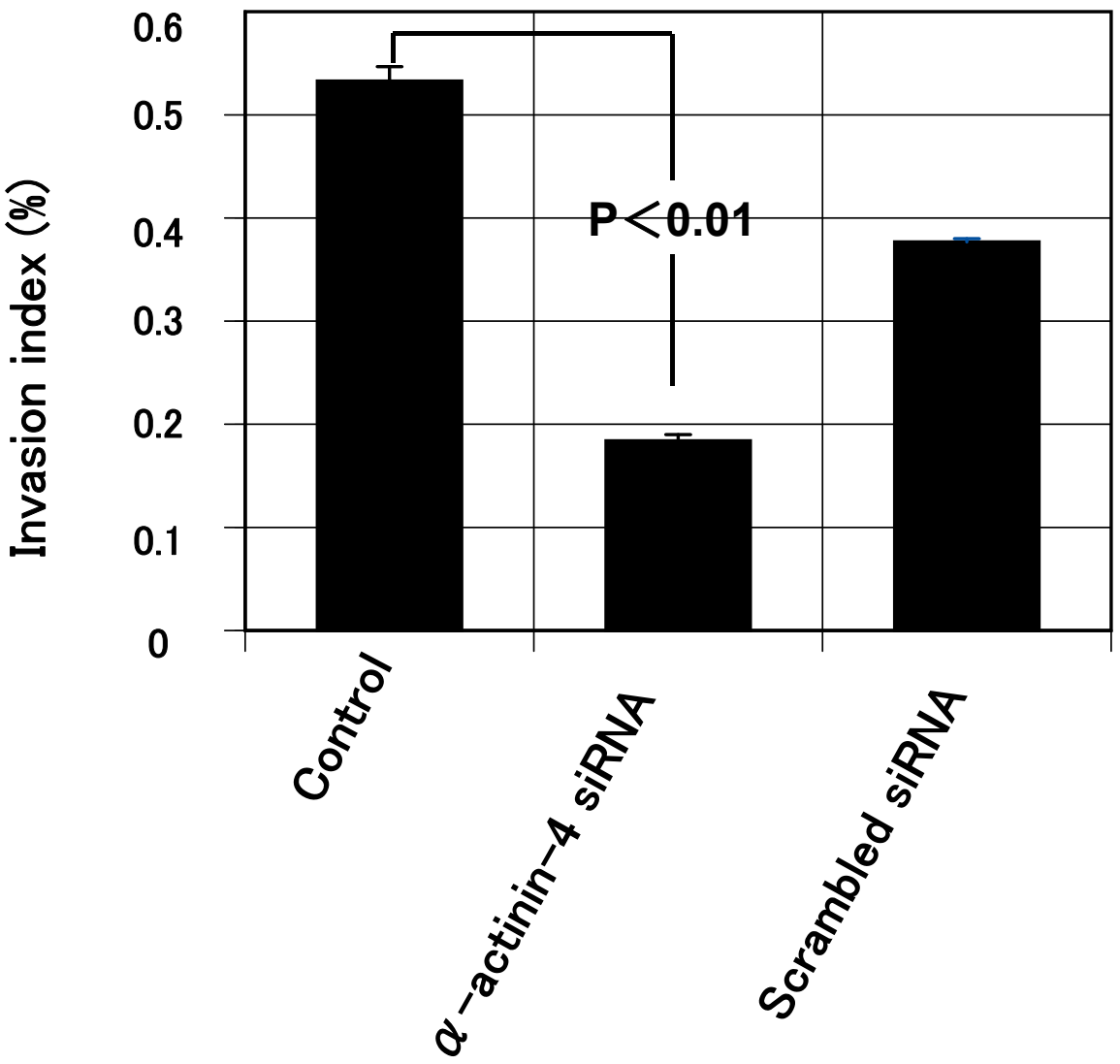
Invasion index (%)
0.6
0.5
0.4
0.3
0.2
0.1
0
P＜0.01
Control
α-actinin-4 siRNA
Scrambled siRNA

Fig.3C