Expression and Gene Amplification of Actinin-4 in Invasive Ductal Carcinoma of the Pancreas

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Abstract

Purpose: An invasive growth pattern is one of the hallmarks of pancreatic ductal carcinoma. Actinin-4 is an actin-binding protein associated with enhanced cell motility, invasive growth, and lymph node metastasis. Actinin-4 might play an important role in the development and progression of pancreatic cancer.

Experimental Design: The expression of actinin-4 was examined immunohistochemically in 173 cases of invasive pancreatic ductal carcinoma. The copy number of the actinin-4 (ACTN4) gene was calculated by fluorescence in situ hybridization. The expression of actinin-4 was stably knocked down by short hairpin RNA, and tumorigenicity was evaluated by orthotopic implantation into mice with severe combined immunodeficiency.

Results: The expression level of actinin-4 was increased in 109 (63.0%) of 173 cases of pancreatic cancer. Kaplan-Meier survival curves revealed that patients with increased expression of actinin-4 had a significantly poorer outcome (P = 0.00001, log-rank test). Multivariate analysis by the Cox proportional hazard model showed that high expression of actinin-4 was the most significant independent negative predictor of survival (hazard ratio, 2.33; P = 0.000009). Amplification of the gene (defined as more than four copies per interphase nucleus) was detected in 11 (37.9%) of 29 cases showing increased expression of actinin-4. Knockdown of actinin-4 expression inhibited the destructive growth of cancer cells in the pancreatic parenchyma.

Conclusion: Recurrent amplification of chromosome 19q13.1-2 has been reported in pancreatic cancer, but the exact target gene has not been identified. Actinin-4 contributes to the invasive growth of pancreatic ductal carcinoma, and ACTN4 is one of the candidate oncogenes in this chromosome locus.

Invasive ductal carcinoma of the pancreas is one of the most aggressive forms of human malignancy, with a 5-year survival rate of <5% to 10% and a median survival of <6 months (1, 2). As a result, pancreatic cancer is the fourth leading cause of cancer death in the United States, and is the fifth in Japan (3). Massive local invasion to adjacent organs and/or metastasis to regional lymph nodes and distal organs are detected in the majority of patients at the time of diagnosis. To improve the prognosis of patients with pancreatic cancer, it will be necessary to elucidate the molecular mechanisms causing invasion and metastasis.

We have identified an actin-binding protein, actinin-4, as a biomarker of cancer invasion and metastasis (4). The expression of actinin-4 was closely associated with the invasive phenotype of breast cancer and was a prognostic indicator in patients with this disease (4). A microarray analysis revealed that actinin-4 was a significant prognostic indicator in patients with non–small cell lung cancer (5). The expression level of ACTN4 protein was increased in the majority of cases of colorectal cancer, and the increase in expression was most significant in dedifferentiated cancer cells infiltrating at the invasive front (6). In mouse models, colorectal cancer cells expressing actinin-4 showed infiltrative growth and metastasized into regional lymph nodes (6).

Oncogenic activation of the K-ras (KRAS) gene occurs in >90% of pancreatic ductal carcinomas and is detected even in premalignant intraepithelial lesions (7, 8). Transgenic mice with a K-rasG12D transgene develop hyperplasia of ductal epithelial cells (9), but the hyperplastic lesions infrequently
progress into invasive tumors, suggesting that additional genetic events must occur for the development of fully malignant pancreatic tumors (10). Recurrent amplification of the chromosome locus 19q13.1-2 has been reported in pancreatic cancer cell lines and primary pancreatic cancers (11–13). Earlier studies indicated that the \textit{AKT2} gene was the target of amplification, but \textit{AKT2} was not always overexpressed in pancreatic cancer cell lines with gene amplification (14, 15), and its down-regulation by small interfering RNA did not significantly affect cell viability (16), leaving the precise target gene(s) of the 19q13.1-2 amplicon undetermined.

The histopathology of pancreatic cancer invariably reveals massive infiltration of small cancer nests lacking a glandular structure. This invasive growth pattern seems to be an intrinsic feature of pancreatic carcinogenesis and might reflect a specific underlying genetic alteration. The \textit{ACTN4} gene that encodes actinin-4 has been mapped to chromosome 19q13.7 in the vicinity of the amplification described above (13). On the basis of these histopathologic and genetic observations, we hypothesized that the \textit{ACTN4} gene might be a target of the 19q13.1-2 amplification and may play a significant role in the invasive growth of pancreatic ductal carcinoma. Because gene amplification is known to activate several oncogenes by increasing their expression levels (17), we first investigated the expression of actinin-4 in clinical samples of pancreatic cancer in order to assess the clinical relevance of any expression changes.

### Patients and Methods

**Immunohistochemistry.** Immunohistochemical analysis was done on tissue specimens from 173 patients with pancreatic ductal carcinoma who had undergone surgical resection between 1990 and 2003 at the National Cancer Center Hospital (Tokyo, Japan) without any prior therapy. The tumors were staged according to the International Union Against Cancer (UICC) tumor-node-metastasis classification (18). Other pathologic variables (macroscopic type, lymphatic invasion, intrapancreatic nerve invasion, and cancer-stroma relationship; Table 1) were categorized according to the Japan Pancreas Society's classification of pancreatic carcinoma (2nd English edition).

### Table 1. Prognostic significance of actinin-4 expression in 173 cases of invasive ductal carcinoma of the pancreas

<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis*</th>
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<tr>
<td></td>
<td>Hazard ratio</td>
<td>95% Confidence interval</td>
<td>( P ) (^a)</td>
<td>Hazard ratio</td>
<td>95% Confidence interval</td>
<td>( P ) (^a)</td>
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<tr>
<td>Age (y)</td>
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<tr>
<td>(&lt;\text{65}/\geq\text{65})</td>
<td>1.18</td>
<td>0.85-1.65</td>
<td>0.321584</td>
<td>1.35</td>
<td>0.59-3.08</td>
<td>0.471917</td>
<td></td>
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<tr>
<td>Gender</td>
<td></td>
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<tr>
<td>Male/female</td>
<td>1.12</td>
<td>0.80-1.57</td>
<td>0.515322</td>
<td>0.86</td>
<td>0.52-1.44</td>
<td>0.572695</td>
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<tr>
<td>UICC stage (^b)</td>
<td></td>
<td></td>
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<tr>
<td>I-I/II-III/IV</td>
<td>2.59</td>
<td>1.31-5.12</td>
<td>0.006191</td>
<td>1.35</td>
<td>0.59-3.08</td>
<td>0.471917</td>
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<tr>
<td>Extent of primary tumor (^c)</td>
<td>1.81</td>
<td>1.16-2.82</td>
<td>0.008528</td>
<td>0.86</td>
<td>0.52-1.44</td>
<td>0.572695</td>
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<td>Lymph node metastasis (^c)</td>
<td>2.73</td>
<td>1.82-4.08</td>
<td>0.000001</td>
<td>1.96</td>
<td>1.20-3.19</td>
<td>0.007271</td>
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<td>Distant metastasis (^c)</td>
<td>2.40</td>
<td>1.68-3.44</td>
<td>0.000002</td>
<td>1.73</td>
<td>1.18-2.54</td>
<td>0.005023</td>
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<tr>
<td>N(0)/N(1) (^b)</td>
<td>2.06</td>
<td>1.44-2.95</td>
<td>0.000076</td>
<td>1.54</td>
<td>1.03-2.28</td>
<td>0.033537</td>
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<tr>
<td>Lymphatic invasion (^c)</td>
<td>2.12</td>
<td>0.89-1.77</td>
<td>0.189521</td>
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<tr>
<td>Intrapancreatic nerve invasion (^c)</td>
<td>1.28</td>
<td>0.91-1.80</td>
<td>0.162522</td>
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<td>Macroscopic type (^c)</td>
<td>1.14</td>
<td>0.81-1.60</td>
<td>0.458187</td>
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<td>Nodular/infiltrative Cancer-stroma relationship (^c)</td>
<td>2.27</td>
<td>1.57-3.27</td>
<td>0.000012</td>
<td>2.33</td>
<td>1.61-3.39</td>
<td>0.000009</td>
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\(^a\) Univariate and multivariate analyses with Cox proportional hazards model.

\(^b\) Based on the International Union Against Cancer tumor-node-metastasis classification (6th edition).

\(^c\) Based on the Japan Pancreas Society’s classification of pancreatic carcinoma (2nd English edition).

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antibody was purchased from Cell Signaling Technology. Anti-AKT2 mouse monoclonal antibody (F-7) was purchased from Santa Cruz Biotechnology. Anti-β-actin mouse monoclonal antibody (AC-15) was purchased from Abcam. Anti-Ki67 antigen mouse monoclonal antibody (MiB-1) was purchased from Dako.

**Western blot analysis.** Cells were extracted with lysis buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% NP40, and 1 mg/ml Na3VO4] containing a protease inhibitor cocktail (Sigma-Aldrich) on ice for 30 min. Cell lysates were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). After incubation with primary antibodies at 4°C overnight and relevant secondary antibodies at room temperature for 1 h, the reaction was detected with enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences; ref. 22). Blot

![Western blot analysis](image).
A pathologist (N. Hiraoka) and cut into 5-formalin-fixed paraffin-embedded tissue block was selected by a kit (Abbott Molecular) as described previously (24). A representative hybridization was done using the methods for PathVysion DNA probe j than expression-negative cases (\( P = 0.00001 \), log-rank test). B. Kaplan-Meier analysis of overall survival for patients with clinical stage I to III pancreatic ductal carcinoma (\( n = 87 \)). Actinin-4 expression – positive cases (\( n = 50 \)) had significantly poorer prognosis than expression-negative cases (\( n = 37; P = 0.00192, \) log-rank test).

Intensity was quantified with a LAS-3000 image analyzer and Multi-Gauze software (Fuji Film; ref. 23). Intensity was quantified with a LAS-3000 image analyzer and Multi-Gauze software (Fuji Film; ref. 23).

**Fluorescence in situ hybridization analysis.** Fluorescence in situ hybridization was done using the methods for PathVysion DNA probe kit (Abbott Molecular) as described previously (24). A representative formalin-fixed paraffin-embedded tissue block was selected by a pathologist (N. Hiraoka) and cut into 5-\( \mu \)m-thick sections. Hybridization was done at 37°C for 14 to 18 h with the denatured ACTN4 (RP11-118P21) or AKT2 (CTB-166E20) locus-containing bacterial artificial chromosome probe labeled with SpectrImOrange (Abbott Molecular). The specimen was counterstained with 4,6-dianidino-2-phenylindone. The number of fluorescence signals in 20 interphase tumor cell nuclei were counted independently by at least two investigators (S. Kikuchi and H. Tsuda or K. Onozato) and averaged.

**Establishment of actinin-4 knockdown clones.** ACTN4 knockdown clones were established by the stable transfection of short hairpin RNA into BxPC-3 cells. A synthesized double-stranded oligonucleotide (5-gatgtctggctagctca-t3') targeting ACTN4 mRNA was cloned into the pBASI-hU6 Neo plasmid (Takara Bio), and the cells were transfected with LipofectAMINE 2000 reagent (Invitrogen). Twenty-four hours later, the transfection medium was replaced with RPMI 1640 containing 0.4 mg/mL of G418 (Geneticin, Invitrogen) to select clones with neomycin resistance.

**Fluorescence cytochemistry.** Cells grown on collagen-coated cover glasses (Asahi Technoglass) were fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were incubated with anti-E-cadherin mouse monoclonal antibody and then with anti-mouse IgG Alexa Fluor 488 (Invitrogen). Filamentous actin fibers were visualized with Alexa Fluor 488 phalloidin (Invitrogen; ref. 25).

**Results**

**Expression of actinin-4 in invasive ductal carcinoma of the pancreas.** The expression of actinin-4 protein was examined immunohistochemically in surgical specimens from 173 patients with pancreatic cancer (Fig. 1). The actinin-4 expression level in pancreatic cancer cells was increased compared with nonneoplastic duct epithelial cells (Fig. 1A and B). The expression of actinin-4 was limited to the apical and lateral membranes of cancer cells showing intraepithelial spreading (Fig. 1C), but this polarized distribution seemed to be lost in cancer cells that were dissociated from the glandular structure (Fig. 1D). Intense actinin-4 staining was observed in the periphery of cancer nests and in the membrane of solitary cells infiltrating the stroma (Fig. 1E and F).

Western blot analysis detected the expression of actinin-4 protein in 9 of out of 11 (81.8%) pancreatic cancer cell lines examined, whereas AKT2 protein was detected only in Panc-1 cells (Supplementary Fig. S1).

**Clinical significance of actinin-4 expression in pancreatic cancer.** The staining intensity of actinin-4 was classified as “positive” when the actinin-4 expression level was equal to or higher than that of vascular endothelial cells, and “negative” when it was less than that of vascular endothelial cells (Fig. 1G). Of the 173 cases, there were 109 (63.0%) actinin-4 expression–positive cases and 64 (37.0%) expression-negative cases. The overall survival of positive cases was significantly worse than that of negative cases (\( P = 0.00001, \) log-rank test; Fig. 2A). Even in the 87 patients with clinical stages I to III, actinin-4 expression–positive cases had significantly poorer outcome than did expression-negative cases (\( P = 0.00192, \) log-rank test; Fig. 2B). Thirty-one (28.4%) out of the 109 actinin-4 expression–positive cases and 24 (37.5%) of the 64 actinin-4 expression–negative cases received postoperative chemotherapy (gemcitabine and others). There was no statistically significant difference in this respect between the groups (\( P = 0.2167, \) \( \chi^2 \) test).

Univariate analysis with the Cox proportional hazards model (Table 1) revealed that clinical stage (\( P = 0.0062 \), extent of

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**Gene Amplification of ACTN4**

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**Scanning electron microscopy, cell migration assay, and cell growth assay.** These assays and procedures are available online in the “Supplementary Methods.”

**Animal experiments.** Female severe combined immunodeficiency mice (C.B-17/ICrGr- scid) were purchased from Clea Japan and maintained in a specific pathogen-free environment. A laparotomy was done under general anesthesia, and 1 \( \times \) 10^6 cells were injected orthotopically into the parenchyma of the pancreas with fine tubulin needles, as described previously (26). The mice were sacrificed 5 weeks later, and serial sections of the entire pancreas were stained using H&E. The maximum diameter of the tumors was measured under a dissecting microscope (Nikon Instruments). All animal experimental procedures were reviewed and approved by the ethics committee of the National Cancer Center Research Institute (Tokyo, Japan).

**Statistical analyses.** Statistical analyses, including Kaplan-Meier analysis with log-rank test, \( \chi^2 \) test, and the Cox proportional hazards regression model, were done with the StatFlex statistics package (version 5.0; Aritech). The Wilcoxon rank sum test was done using a tool in the R project software package.8

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8 http://www.r-project.org
primary tumor \( (P = 0.0085) \), lymph node metastasis \( (P = 0.000001) \), distant metastasis \( (P = 0.000002) \), lymphatic invasion \( (P = 0.000076) \), and immunoreactivity of actinin-4 \( (P = 0.000012) \) were significantly correlated with the prognosis of the 173 patients with pancreatic cancer. Multivariate analysis indicated that actinin-4 expression was the most significant independent predictor of unfavorable prognosis \( (P = 0.000009; \) hazard ratio, 2.33; 95% confidence interval, 1.61-3.39),

![Fig. 3. Amplification of the ACTN4 and AKT2 genes in pancreatic cancer. A-F, fluorescence in situ hybridization analysis of copy numbers of the ACTN4 (A, C, and D) and AKT2 (B, E, and F) genes in a pancreatic cancer cell line (Panc-1; A and B) and clinical samples of pancreatic cancer (C-F). Increased copy numbers of the ACTN4 (C) and AKT2 (E) genes: two copies of the ACTN4 (D) and AKT2 (F) genes. G, correlation of actinin-4 protein expression and gene amplification. Average copy number of the ACTN4 gene determined by fluorescence in situ hybridization in actinin-4 expression – positive cases (gray columns, \( n = 29 \)) and expression-negative cases (unshaded columns, \( n = 17 \)). Average signal number of \(<4\) (per interphase nucleus) was defined as gene amplification (*). There is a significantly higher frequency of ACTN4 amplification in actinin-4 expression – positive cases than in expression-negative cases \( (P = 0.017, \chi^2 \) test).](#)
followed by the presence of lymph node metastasis ($P = 0.0073$; hazard ratio, 1.96; 95% confidence interval, 1.20-3.19) and distant organ metastasis ($P = 0.0050$; hazard ratio, 1.73; 95% confidence interval, 1.18-2.54).

**Gene amplification of ACTN4 in pancreatic cancer.** Recurrent amplification of the chromosome 19q13 locus containing the AKT2 gene has been reported in the pancreatic cancer cell line Panc-1. We investigated whether the ACTN4 gene was included within the amplicon. Fluorescence *in situ* hybridization revealed an average of 13.7 ACTN4 (Fig. 3A) and 27.0 AKT2 (Fig. 3B) fluorescence signals per interphase Panc-1 cell. Consistently, real-time PCR showed that the copy numbers of the ACTN4 and AKT2 genes were increased 6-fold and 20-fold, respectively, in comparison with the immortalized near-diploid pancreatic ductal cell line H6C7 (Supplementary Fig. S2).

Fluorescence *in situ* hybridization was then done in surgical specimens of 46 randomly selected pancreatic cancers with positive expression of actinin-4 ($n = 29$) and negative expression of actinin-4 ($n = 17$; Fig. 3C-F). There was a significant difference in the frequency of ACTN4 gene amplification between actinin-4 expression–positive and expression-negative cases ($P = 0.017$, $\chi^2$ test), when gene amplification was defined as average fluorescence signals of $>4$ in 20 interphase tumor cell nuclei: 11 of 29 actinin-4 expression–positive cases (37.9%; gray columns; Fig. 3G) but only 1 of 17 expression-negative cases (5.9%; unshaded columns; Fig. 3G) showed gene amplification of ACTN4.
Knockdown of ACTN4 expression. In order to examine the involvement of actinin-4 in the invasive growth of pancreatic cancer cells in vivo, we established, from the pancreatic cancer cell line BxPC3, two stable clones whose expression of actinin-4 had been knocked down by short hairpin RNA transfection (KD-ACTN4, lanes 1 and 2) and two control (nonsilencing) clones (Control, lanes A and B). We have previously reported that transient knockdown of actinin-4 expression by small interfering RNA significantly reduces the motility of BxPC3 cells in an in vitro migration assay (27). Western blot analysis confirmed the down-regulation of actinin-4 protein expression in the two knockdown clones (lanes 1 and 2; Fig. 4A), but not in the control clones (lanes A and B).

Knockdown of actinin-4 expression resulted in the alteration of cell shape and distribution of the actin cytoskeleton (Fig. 4B-G). Control cells were poorly connected, and filamentous actin was concentrated at the cell periphery (lamellipodia; Fig. 4C). Conversely, KD-ACTN4 cells showed tight intercellular connections, and marked extension of actin stress fibers along cellular long axes was observed (Fig. 4B). Actinin-4 competes with E-cadherin for binding to β-catenin (27). Knockdown of ACTN4 may have restored the cell adhesion function of E-cadherin (Fig. 4D).

Scanning electron microscopy revealed the development of numerous microvilli on the dorsal surface of control cells (Control, Supplementary Fig. S3), whereas microvilli were...
poorly developed in KD-ACTN4 cells (KD-ACTN4, Supplementary Fig. S3). Knockdown of actinin-4 expression reduced cell migratory activity (Supplementary Fig. S4).

**Suppression of invasive growth by knockdown of actinin-4.**

Supplementary Fig. S5 illustrates the growth kinetics of KD-ACTN4 and control cells. KD-ACTN4 cells showed modest reduction of cell proliferation in vitro. However, the growth of KD-ACTN4 cells transplanted orthotopically into the pancreas of severe combined immunodeficiency mice was markedly suppressed (Fig. 5). Control cells formed masses at the sites of injection, destroying the parenchyma of the pancreas (Control; Fig. 5A) and spreading along the pancreatic ducts (KD-ACTN4; Fig. 5B and C). There were significant differences between the diameters of tumors generated by KD-ACTN4 clones and by control clones (Fig. 5F). However, Ki67 labeling revealed no apparent difference in cell proliferation activity between tumors generated by KD-ACTN4 and by control cells (Fig. 5D and E), consistent with the in vitro cell growth kinetics (Supplementary Fig. S5).

**Discussion**

Although the molecular mechanisms causing cancer invasion and metastasis are highly complicated, the acquisition of enhanced motility by cancer cells is a prerequisite. During the process of cell movement, actinin-4 protein levels are increased and highly concentrated at the leading edge of motile cells (4). We recently showed that increased expression of actinin-4 significantly enhances cell motility and mediates invasive growth and lymph node metastasis by colorectal cancer (6). In this study, actinin-4 was found to be overexpressed in the majority of invasive ductal carcinomas of the pancreas (Fig. 1), and increased expression of actinin-4 protein was significantly correlated with poor prognosis of patients with pancreatic cancer (Fig. 2). Knockdown of actinin-4 expression enhanced intercellular connections (Fig. 4) and significantly reduced the motility of a highly motile pancreatic cancer cell line (Supplementary Fig. S4; ref. 27). These observations lead us to conclude that actinin-4 plays a biologically significant role in pancreatic carcinogenesis. We showed that gene amplification may underlie the increased expression of actinin-4 protein. Gene amplification of ACTN4 was significantly more frequent in cases with increased expression of actinin-4 (Fig. 3G). However, several cases with increased expression of actinin-4 had a normal copy number of the ACTN4 gene, and other molecular mechanisms could not be excluded. An invasive growth pattern seems to be an intrinsic feature of pancreatic cancer, and it is reasonable to assume that ACTN4 is the target of the 19q13 amplification. Nevertheless, because several other candidate genes have been isolated from the chromosome region (16, 28, 29), the ACTN4 gene may not be the sole target.

Recently, a familial pancreatic cancer gene on 4q32-34 was identified as PALLD, which encodes the protein palladin (30), another component of the actin-containing microfilaments that control cell shape, adhesion, and movement. Palladin binds to actinin and functions as a scaffold of the actin cytoskeleton (30, 31). The missense mutation of PALLD in the affected family was mapped to the actinin-binding domain of palladin (31). Transfection of the mutant palladin cDNA impaired the organization of the actin cytoskeleton and increased cell motility. Palladin mRNA was overexpressed in precancerous ductal dysplasia and carcinoma of the pancreas. Thus, PALLD mutation and palladin overexpression may have something to do with the functions of actinin-4.

Thus far, two non–muscle actinin isoforms, actinin-1 and actinin-4, have been identified. Enhanced actin stress fiber formation in KD-ACTN4 cells (Fig. 4B) may reflect a shift of filamentous actin from actinin-4 to actinin-1. A germ line mutation in the actinin4 gene is responsible for familial focal segmental glomerulosclerosis (32). Mice deficient in the Actn4 gene manifest severe glomerular dysfunction (33), and failure of foot process extension by glomerular podocytes is thought to be the major cause of focal segmental glomerulosclerosis. Knockdown of ACTN4 in pancreatic cancer cells inhibited the formation of microvilli (Supplementary Fig. S3), probably through the same mechanism. Actinin-4 seems to be essential for the invasive growth of pancreatic cancer and may represent a candidate drug target. However, because of the lack of redundancy with actinin-1 in glomerular function, renal side effects may be a concern for therapeutics targeting actinin-4.

Actinin-4 is a multifunctional protein whose functional role is determined by partner proteins that form complexes with it. As well as the cell adhesion and cytoskeleton proteins, actinin-4 (or an unspecified non–muscle actinin) has been reported to interact with molecules of various functions, including BERP (34), Na+/H+ exchanger 3 (35), DNaseY (36), ATK1 (37), plasminogen activator inhibitor type-1 (38), histone deacetylase 7 (39), androgen receptor (40), and HER2/Neu/ErbB2 (41). For example, actinin-4 physically interacts with AKT1, and knockdown of ACTN4 has been reported to inhibit the phosphorylation and nuclear translocation of AKT1. The AKT signaling pathway is known to be involved in regulating a variety of biological processes such as cell survival, proliferation, and motility (42).

In summary, we have identified increased expression and gene amplification of actinin-4 in pancreatic ductal carcinoma and clarified its clinical and biological significance. We believe that the findings reported here provide novel insights into diagnostic and therapeutic approaches to this devastating disease.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


Clinical Cancer Research

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