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ACTN4 copy number increase as a predictive biomarker for chemoradiotherapy of locally advanced pancreatic cancer

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Background: Several clinical trials have compared chemotherapy alone and chemoradiotherapy (CRT) for locally advanced pancreatic cancer (LAPC) treatment. However, predictive biomarkers for optimal therapy of LAPC remain to be identified. We retrospectively estimated amplification of the *ACTN4* gene to determine its usefulness as a predictive biomarker for LAPC.

Methods: The copy number of *ACTN4* in 91 biopsy specimens of LAPC before treatment was evaluated using fluorescence *in situ* hybridisation (FISH).

Results: There were no statistically significant differences in overall survival (OS) or progression-free survival (PFS) of LAPC between patients treated with chemotherapy alone or with CRT. In a subgroup analysis of patients treated with CRT, patients with a copy number increase (CNI) of *ACTN4* had a worse prognosis of OS than those with a normal copy number (NCN) of *ACTN4* ($P=0.0005$, log-rank test). However, OS in the subgroup treated with chemotherapy alone was not significantly different between patients with a CNI and a NCN of *ACTN4*. In the patients with a NCN of *ACTN4*, the median survival time of PFS in CRT-treated patients was longer than that of patients treated with chemotherapy alone ($P=0.049$).

Conclusions: The copy number of *ACTN4* is a predictive biomarker for CRT of LAPC.

Despite progress in clinical cancer medicine in the fields of imaging technology, surgical management, therapeutic modalities and molecular-targeted therapy, the prognosis of pancreatic cancer has remained dismal. Every year in Japan, ~27 000 patients are diagnosed with pancreatic cancer, with almost the same number dying from this disease (Mayahara *et al*, 2012). Indeed, the 5-year overall survival (OS) rate of patients with pancreatic cancer is ≤5% (Johung *et al*, 2012).

Locally advanced pancreatic cancer (LAPC) is defined as a surgically unresectable disease without detectable metastasis. Effective therapy for patients with LAPC is not only crucial for

any hope of long-term survival, but also necessary for symptom management. Because survival rates for patients with LAPC are generally low, treatment recommendations often involve aggressive multimodal therapies (Savir *et al*, 2013). A multidisciplinary approach involving surgical oncologists, medical oncologists and radiation oncologists is strongly recommended for balanced discussion of management options (Pawlik *et al*, 2008; Katz *et al*, 2013; Mian *et al*, 2014).

At present, treatment options for LAPC include chemotherapy alone, induction chemotherapy followed by chemoradiotherapy (CRT) or definitive CRT. Numerous randomised trials have been

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performed to compare the survival benefit between chemotherapy alone and CRT for LAPC (Chauffert *et al*, 2008; Loehrer *et al*, 2011). Nevertheless, as there are some contradictory results, the most effective treatment has not yet been defined for patients with LAPC (Savir *et al*, 2013; Mian *et al*, 2014). Radiotherapy focussed on the primary site does not have a direct impact on distant metastatic lesions and radiotherapy should therefore be limited to patients without metastases (Berger *et al*, 2008). If pancreatic cancer oncologists can accurately evaluate the occult distant metastasis before deciding the therapeutic strategy, they should be able to choose the optimal therapy for individual patients with LAPC. However, it is not yet possible to accurately detect micrometastatic lesions using imaging technology. Therefore, elucidation of biomarkers that can accurately evaluate metastatic potential from biopsy samples obtained from patients with LAPC is very important for deciding the best personalised therapeutic strategy from multimodal therapies.

In 1998, we identified actinin-4 (gene name *ACTN4*) as an actin-binding protein that is closely associated with cancer invasion and metastasis (Honda *et al*, 1998; Hayashida *et al*, 2005). Immunohistochemical analysis (IHC) showed that overexpression of the actinin-4 protein was significantly correlated with a poor prognosis for breast (Honda *et al*, 1998), pancreas (Kikuchi *et al*, 2008), ovary (Yamamoto *et al*, 2007, 2009, 2012) and lung cancer (Miyayama *et al*, 2013; Noro *et al*, 2013).

We subsequently found that gene amplification of *ACTN4*, which is the gene name of the actinin-4 protein, is responsible for overexpression of the actinin-4 protein in a number of pancreatic cancer patients (Kikuchi *et al*, 2008). Using fluorescence *in situ* hybridisation (FISH), we then reported that gene amplification of *ACTN4* is a good biomarker for identification of patients with a poor prognosis for ovarian cancer (Yamamoto *et al*, 2009), salivary gland carcinoma (Watabe *et al*, 2014) and stage-I adenocarcinoma of the lung (Noro *et al*, 2013).

In this study, we retrospectively investigated the status of actinin-4 protein expression and *ACTN4* copy number in biopsy samples of LAPC patients. We confirmed the possibility that *ACTN4* copy number is useful as a predictive and prognostic biomarker of CRT for LAPC.

MATERIALS AND METHODS

Patients. A total of 91 patients who were diagnosed as having LAPC from May 2001 until December 2003 underwent chemotherapy alone or CRT at the National Cancer Center Central Hospital (Tokyo, Japan). All patients were diagnosed as adenocarcinoma of the pancreas by fine needle biopsy. This study was reviewed and approved by the institutional ethical committee and informed consent was obtained from the patients for this study.

At first diagnosis, multidetector computed tomography (CT) involving the chest and abdomen was performed for assessment of the local extension of the primary tumour, and for exclusion of distant metastasis. The CT-based criteria regarding tumour unresectability included enhancement or occlusion of the coeliac trunk, common hepatic artery, superior mesenteric artery or aorta (Ikeda *et al*, 2007; Mayahara *et al*, 2012).

Immunohistochemistry. Formalin-fixed, paraffin-embedded (FFPE) pathology blocks, which were made to diagnose the biopsy specimens, were cut into 4 μ m-thick sections.

An anti-actinin-4 monoclonal antibody was established by our group (Miyayama *et al*, 2013; Noro *et al*, 2013) (Abnova, Taipei, Taiwan). Immunostaining of actinin-4 was performed using the Ventana DABMap detection kit and an automated slide stainer (Discovery XT; Ventana Medical System, Tucson, AZ, USA) (Watabe *et al*, 2014). The immunohistochemical staining of

actinin-4 was classified into two groups: positive and negative. Positive was defined as strong protein expression of actinin-4 in the cytoplasm and cell membranes of cancer cells. Negative was defined as no detection of actinin-4 protein in cancer cells or weak expression of actinin-4 in the cytoplasm or cell membrane of cancer cells (Figure 1). Two independent investigators (TW and YW) who had no clinical information about these cases evaluated the staining pattern.

Fluorescence *in situ* hybridisation. The FISH probes of the bacterial artificial chromosome (BAC) clone containing *ACTN4* were prepared by our group (Noro *et al*, 2013) (Abnova). The labelled BAC clone DNA was subjected to FISH as previously described. Sections that were cut from an FFPE biopsy block (4 μ m thick) were hybridised with FISH probes at 37 °C for 48 h. The nuclei were counterstained with 4,6-diamidino-2-phenylindole. The number of fluorescence signals corresponding to the copy number of *ACTN4* in the nuclei of 20 interphase tumour cells was counted (TW and YW) (Watabe *et al*, 2014).

The FISH patterns were defined as described previously. Briefly, the biopsy samples were grouped as normal copy number (NCN) (two or fewer *ACTN4* signals in >90% of cells) and copy number increase (CNI) (four or more *ACTN4* signals in >10% of the tumour cells) (Figure 1) (Watabe *et al*, 2014).

Statistical analysis. Significant correlations were detected by using Fisher's exact test. Overall survival and progression-free survival (PFS) were measured as the period from first diagnosis to the event or last follow-up and were estimated by Kaplan–Meier analysis. Significant differences between curves of OS or PFS were assessed using the log-rank test. Univariate and multivariate analyses for death were performed using the Cox regression model. Data were analysed using the StatFlex statistical software package (version 6.0; Artiteck, Osaka, Japan) or the R-project (<http://www.r-project.org/>) (Honda *et al*, 2005, 2012; Noro *et al*, 2013).

RESULTS

Patient characteristics and survival benefit comparison between chemotherapy alone and CRT. In all, 34 patients with LAPC underwent chemotherapy alone. The regimens of chemotherapy alone comprised gemcitabine (GEM) alone ($n=29$), combination of GEM and erlotinib ($n=1$), combination of GEM and S-1 ($n=3$) or S-1 alone ($n=1$). A total of 57 patients with LAPC underwent CRT. The regimens of CRT comprised radiotherapy (RT) and 5-fluorouracil (5-FU) ($n=39$), RT and GEM ($n=10$) and RT and S-1 ($n=8$). The median age of patients and tumour size for all of the cases was 63.0 years and 37.4 mm, respectively. Statistical significances of patient characteristics with respect to age, gender, Eastern Cooperative Oncology Group Performance Status (PS), tumour size, lymph node metastasis and location of tumours were calculated. No statistically significant differences were observed between any of these factors and chemotherapy alone or CRT (Table 1).

The statistical significance of differences between the benefit of chemotherapy alone and that of CRT for OS and PFS was also calculated. In the absence of biomarker selection, no statistically significant differences in survival benefits in terms of OS and PFS were found between patients treated with chemotherapy alone and those treated with CRT (Figure 2).

Prognostic impact of protein expression of actinin-4 in patients with LAPC. We previously showed that protein overexpression of actinin-4 is a prognostic biomarker for resectable invasive ductal adenocarcinoma of the pancreas (Kikuchi *et al*, 2007). We investigated the protein expression level of actinin-4 in LAPC by using IHC. The 91 patients with LAPC were classified into one

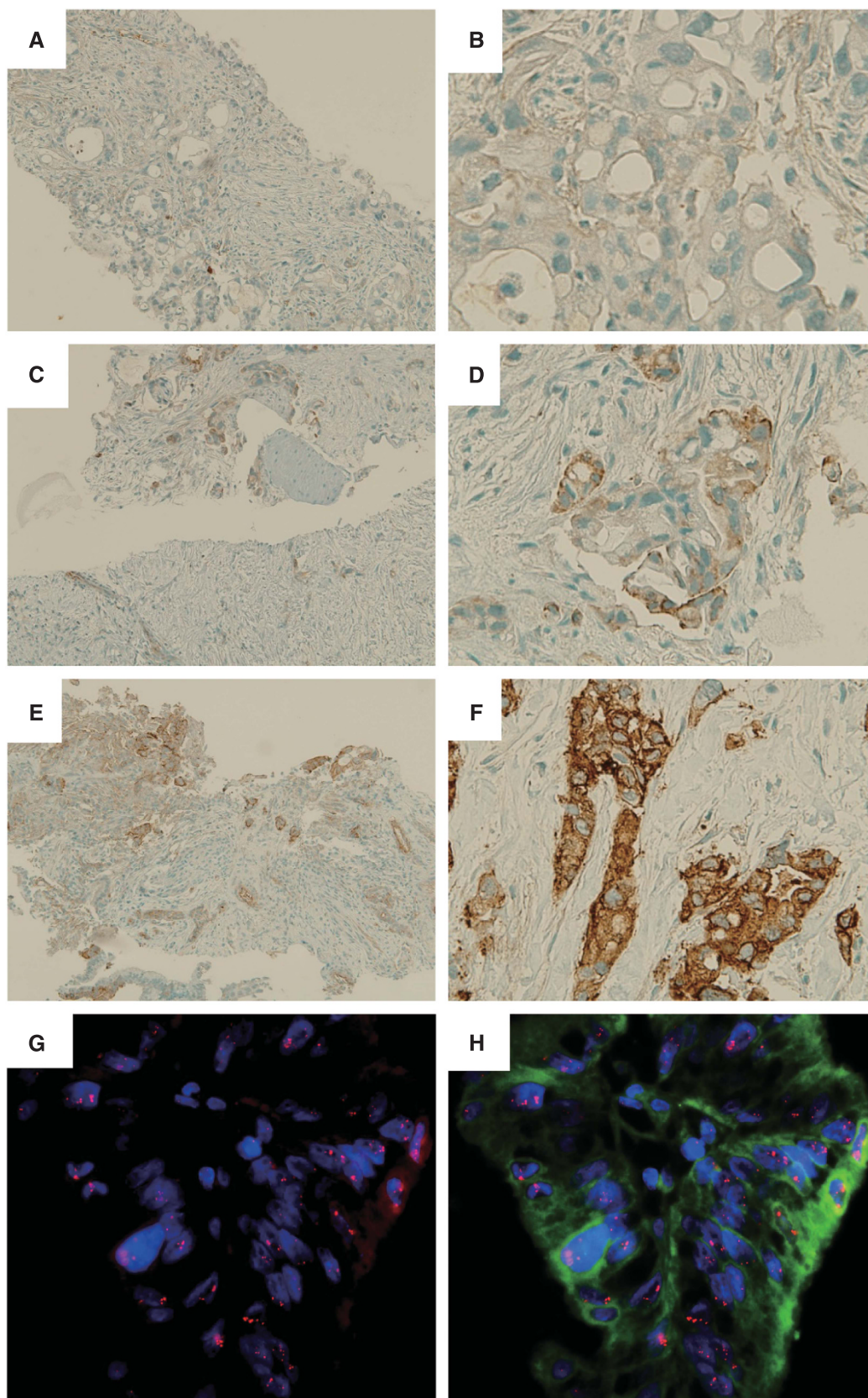


Figure 1. Immunohistochemical (IHC) and fluorescence *in situ* hybridisation (FISH) analyses of representative actinin-4 protein expression and *ACTN4* copy number, respectively, in LAPC biopsy specimens. (A–F) Immunohistochemical analysis of actinin-4 protein expression. Representative cases of no expression (A, B), weak expression (C, D) and strong expression (E, F) of actinin-4 protein in LAPC cells. (A), (C) and (E) are low-magnitude images. (B), (D) and (F) are high-magnitude images of regions of (A), (C) and (E), respectively. (G, H) Fluorescence *in situ* hybridisation analysis of representative cases with a copy number increase (CNI) in *ACTN4*.

Table 1. Baseline patient characteristics

Characteristic	Total		CRT		CT		P-value*
	Number	%	Number	%	Number	%	
Median age, years (63.0)							0.0501
< 63.0	45	49.5	32	56.1	13	38.2	
≥ 63.0	46	50.5	25	43.9	21	61.8	
Gender							1
Male	53	58.2	33	57.9	20	58.8	
Female	38	41.8	24	42.1	14	41.2	
PS							0.2681
0	26	28.6	17	29.8	9	26.5	
1	63	69.2	40	70.2	23	67.6	
2	2	2.2	0	0.0	2	5.9	
Median tumour size, mm (37.4)							0.3862
< 37.4 mm	44	48.4	14	41.2	30	52.6	
≥ 37.4 mm	47	51.6	20	58.8	27	47.4	
Lymph node metastasis							1
Negative	64	70.3	40	70.2	24	70.6	
Positive	27	29.7	17	29.8	10	29.4	
Location of the tumour							0.0501
Head of pancreas	43	47.3	22	38.6	21	61.8	
Body or tail of pancreas	48	52.7	35	61.4	13	38.2	
CA19-9							
< 1000 U ml ⁻¹	62	68.1	39	68.4	23	67.6	1
≥ 1000 U ml ⁻¹	29	31.9	18	31.6	11	32.4	

Abbreviations: CRT = chemoradiotherapy; CT = chemotherapy; PS = Eastern Cooperative Oncology Group Performance Status. *P-value: Fisher's exact test (two sided).

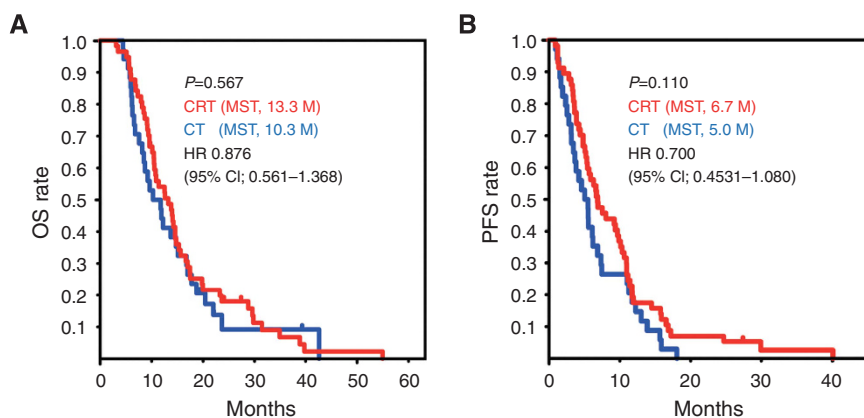


Figure 2. Kaplan-Meier analyses of overall survival (OS) and progression-free survival (PFS) in all locally advanced pancreatic cancer (LAPC) cases. The survival curves of all LAPC patients treated with chemotherapy alone (CT, blue lines) or with chemoradiotherapy (CRT, red lines) are shown. Statistically significant differences in OS (**A**) and PFS (**B**) were calculated using a log-rank test. Median survival time (MST) is shown in months (M). The clinical benefit of CT vs CRT was calculated by univariate Cox regression analysis (hazard ratio (HR) and 95% confidence interval (95% CI)). The y axis is the rate of OS or PFS, and the x axis is the time after first diagnosis (months).

of two groups based on actinin-4 protein expression; positive (66 patients, 72.5%) and negative (25 patients, 27.5%). We investigated correlations between protein expression of actinin-4 and the following patient characteristics: age, gender, PS, size of tumour, lymph node metastasis and treatment strategy (chemotherapy alone or CRT). Protein expression of actinin-4 was statistically correlated with tumour location ($P = 0.0379$; Table 2).

We determined whether protein expression of actinin-4 provided benefit for OS to patients with LAPC by comparing the OS of cases of LAPC with and without actinin-4 expression (total, $n = 91$). No statistically significant difference in OS between

actinin-4 protein-positive and -negative cases was found ($P = 0.116$, log-rank test; Figure 3A). However, although a statistical significance was not found by Kaplan-Meier analysis, the median survival time (MST) of OS of cases positive for actinin-4 protein was 10.9 months, which was shorter than the MST of the negative cases (14.8 months) by 3.9 months (Figure 3A).

Determination of the copy number of ACTN4 by FISH, and prognostic impact of copy number of ACTN4 for LAPC. It is known that gene amplification of ACTN4 is responsible for

Table 2. Association of protein expression of actinin-4 and copy number increase in *ACTN4* with clinicopathological characteristics of locally advanced pancreatic cancer

Characteristic	Actinin-4 IHC					ACTN4 FISH				
	Positive	%	Negative	%	P-value*	Positive	%	Negative	%	P-value*
Median age, years (63.0)					0.159					0.41
<63.0	36	54.5	9	36.0		9	60.0	36	47.4	
≥63.0	30	45.5	16	64.0		6	40.0	40	52.6	
Gender					0.6348					0.02
Male	37	56.1	16	64.0		13	86.7	40	52.6	
Female	29	43.9	9	36.0		2	13.3	36	47.4	
PS					0.3506					0.679
0	21	31.8	5	20.0		3	20.0	23	30.3	
1	44	66.7	19	76.0		12	80.0	51	67.1	
2	1	1.5	1	4.0		0	0.0	2	2.6	
Tumour size					0.8647					1
<37.4 mm	33	50.0	12	48.0		7	46.7	38	50.0	
≥37.4 mm	33	50.0	13	52.0		8	53.3	38	50.0	
Lymph node metastasis					0.4478					0.059
Negative	48	72.7	16	64.0		7	46.7	57	75.0	
Positive	18	27.3	9	36.0		8	53.3	19	25.0	
Location of the tumour					0.0379					0.156
Head of pancreas	31	47.0	18	72.0		10	66.7	33	43.4	
Body or tail of pancreas	35	53.0	7	28.0		5	33.3	43	56.6	
CA19-9					0.451					0.227
<1000 U ml ⁻¹	43	65.2	19	76.0		8	53.3	54	71.1	
≥1000 U ml ⁻¹	23	34.8	6	24.0		7	46.7	22	28.9	
Therapy					1					1
CT	25	37.9	9	36.0		6	40.0	28	36.8	
CRT	41	62.1	16	64.0		9	60.0	48	63.2	

Abbreviations: *ACTN4* = actinin-4; CRT = chemoradiotherapy; CT = chemotherapy; FISH = fluorescence in situ hybridisation; IHC = immunohistochemistry; PS = Eastern Cooperative Oncology Group Performance Status. *P-value: Fisher's exact test (two sided). Bold entries indicate statistical significance.

overexpression of actinin-4 protein in a number of patients with pancreatic cancer. In addition, gene amplification of *ACTN4* predicts a poorer prognosis than protein overexpression of actinin-4 in ovarian (Yamamoto *et al*, 2009), lung (Noro *et al*, 2013) and salivary gland cancer (Watabe *et al*, 2014). To evaluate the significance of *ACTN4* as a prognostic factor for LAPC, we determined the copy number of *ACTN4* in patients with LAPC by FISH. Of the 91 LAPC patients whom we examined, 76 patients were classified as NCN (83.5%) and 15 patients were classified as CNI (16.5%). Although only 1 of the 25 cases who were negative for actinin-4 protein (4.0%) had a CNI of *ACTN4*, 14 of the 66 cases who were actinin-4 protein positive (21.2%) had a CNI of *ACTN4* (Table 3). We also analysed association of the *ACTN4* copy number, as assessed by FISH analysis, with clinicopathological characteristics. There were statistically significant differences between gender and copy number of *ACTN4* ($P=0.02$; Table 2).

When all cases of LAPC were considered, the difference in OS between cases with a CNI and those with NCN of *ACTN4* was statistically significant ($P=0.0019$, log-rank test). The MST of OS in the cases with a CNI of *ACTN4* (8.7 months) was also significantly shorter than the MST of NCN cases (13.7 months) by 5 months ($P=0.0019$; Figure 3B).

Prognostic impact of the serum level of CA19-9 in patients with LAPC. The serum level of CA19-9 has been reported to be a prognostic factor for patients with LAPC (Berger *et al*, 2008; Mayahara *et al*, 2012; Yang *et al*, 2013). We confirmed the usefulness of the serum level of CA19-9 as a prognostic factor for patients with LAPC in our study. The LAPC patients were

classified into one of two groups: CA19-9 high expression (≥ 1000 U ml⁻¹) and CA19-9 low-intermediate expression (< 1000 U ml⁻¹), as previously described (Mayahara *et al*, 2012). There was a statistically significant difference in OS between the CA19-9 high-expression group and the CA19-9 low-intermediate-expression group ($P=0.0003$, log-rank test; Supplementary Figure 1). The MST of the CA19-9 high-expression group was 9.3 months, which was shorter than the MST of the CA19-9 low-intermediate-expression group (14.6 months) by 5.3 months.

Univariate analysis indicated that the risk factors for death of LAPC patients were: lymph node metastasis, serum level of CA19-9 (cutoff value; 1000 U ml⁻¹) and copy number status of *ACTN4*. The hazard ratios (HRs) for the death of patients with LAPC of lymph node metastasis, CA19-9 and a CNI of *ACTN4* were: 1.606 (95% confidence interval (CI); 1.008–2.560, $P=0.0463$), 2.354 (95% CI; 1.479–3.761, $P=0.0003$) and 2.531 (95% CI; 1.394–4.597, $P=0.0023$), respectively. By multivariate analysis, the serum level of CA19-9 (HR; 2.325, 95% CI; 1.416–3.818, $P=0.0009$) and a CNI of *ACTN4* (HR; 2.645, 95% CI; 1.439–4.861, $P=0.0017$) were independent risk factors for the death of patients with LAPC. The HR of CNI of *ACTN4* (HR; 2.531) was slightly higher than that of the serum level of CA19-9 (HR; 2.354; Table 4).

Evaluation of OS in subgroup analyses of treatment strategy with copy number of *ACTN4*. A biomarker that can evaluate the potential for metastatic activity in tumour cells has the possibility of use as a predictive biomarker of CRT. It is known that *ACTN4* is an oncogene that is associated with cancer metastasis and cell invasion. In order to evaluate the benefit for OS based on the copy

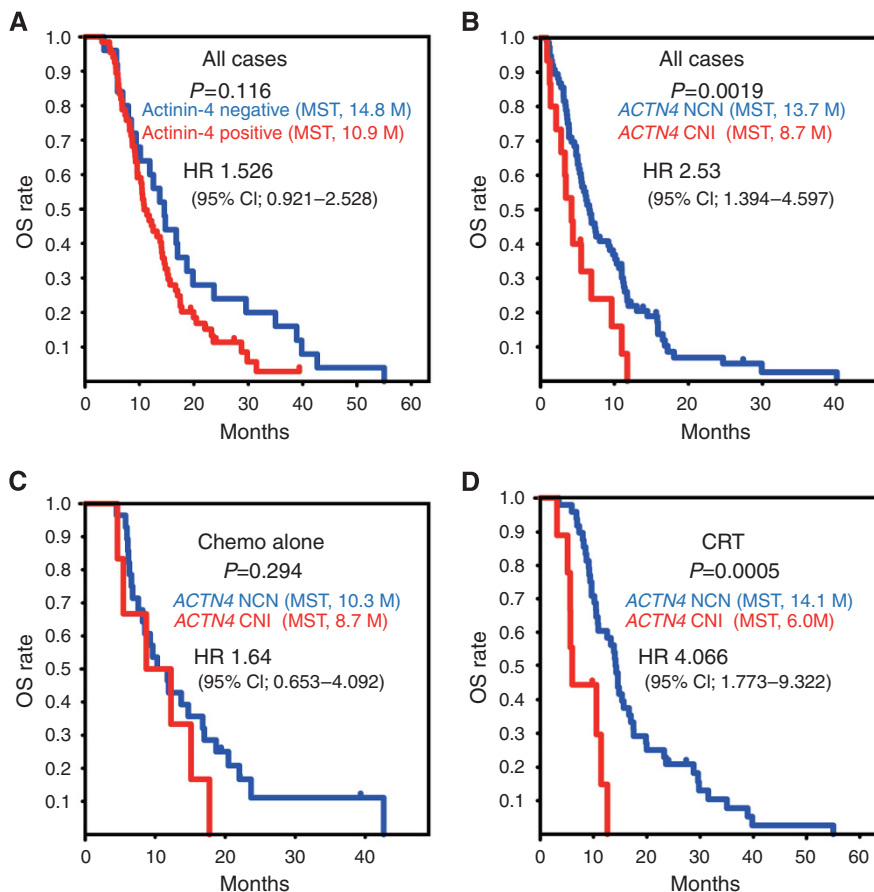


Figure 3. Kaplan–Meier analyses of survival relative to protein expression of actinin-4 and copy number of ACTN4. (A) Overall survival (OS) curves based on protein expression of actinin-4. The blue line represents patients with negative expression of actinin-4. The red line represents patients with positive expression of actinin-4. (B–D) The OS curves based on ACTN4 copy number status in all cases (n = 91) (B), in the subgroup treated with chemotherapy alone (Chemo alone, n = 34) (C) and in the chemoradiotherapy (CRT)-treated subgroup (n = 57) (D). The blue lines represent patients who were evaluated as normal copy number (NCN) of ACTN4. The red lines represent patients who were evaluated as copy number increase (CNI) of ACTN4. Statistical parameters were calculated as described for Figure 2. The y axis is the rate of OS, and the x axis is the time after first diagnosis (months).

Table 3. Statistical analysis of the association between the status of protein expression of actinin-4 and the copy number of ACTN4

Status of actinin-4 with IHC	Copy number status of ACTN4			P-value*
	NCN (%)	CNI (%)	Total	
Negative	24 (96.0)	1 (4.0)	25	0.042
Positive	52 (78.8)	14 (21.2)	66	
Total	76 (83.5)	15 (16.5)	91	

Abbreviations: ACTN4 = actinin-4; CNI = copy number increase; IHC = immunohistochemistry; NCN = normal copy number. *P-value: Fisher's exact test (one sided). Bold entry indicates statistically significance.

number status of ACTN4 for each treatment strategy, the patients with LAPC were classified into one of two subgroups on the basis of treatment strategies: a chemotherapy-alone group and a CRT group. We then analysed the impact of the copy number status of ACTN4 on OS of each subgroup. No statistical significance was observed between OS of patients with a NCN and with a CNI of ACTN4 in the chemotherapy-alone subgroup (P = 0.294, log-rank test). The MST of CNI and NCN of ACTN4 patients was almost the same at 8.7 and 10.3 months, respectively (Figure 3C).

Univariate Cox regression analysis indicated that the HR for death of CNI patients compared with NCN patients was 1.64 (95% CI; 0.653–4.092) in the chemotherapy-alone subgroup, and no statistically significant difference was found between CNI and NCN patients in the chemotherapy-alone subgroup (P = 0.291).

In contrast, in the subgroup who underwent CRT, the OS of CNI of ACTN4 patients was significantly worse than that of patients with a NCN (P = 0.0005, log-rank test), and the MST of CNI of ACTN4 patients (6.0 months) was definitely shorter than that of NCN of ACTN4 patients (14.1 months; Figure 3D). Univariate Cox regression analysis of the CRT groups indicated that the HR for death of CNI patients compared with that for NCN patients was 4.066 (95% CI; 1.773–9.322), and the difference between CNI and NCN groups was statistically significant (P = 0.0009). The HR for death in the comparison between CNI and NCN of ACTN4 (4.066) patients in the CRT subgroup was higher than that of the HR in the comparison between the CNI and NCN of ACTN4 patients in all 91 cases (HR; 2.531; Table 4).

We also calculated the prognostic impact of the serum level of CA19-9 in each subgroup of therapeutic strategy on OS. The OS of patients with high expression of CA19-9 was significantly worse than that of patients with low–intermediate expression of CA19-9 in both subgroups of the chemotherapy-alone group (P = 0.00218, log-rank test; Supplementary Figure 2) and the CRT group (P = 0.0095; Supplementary Figure 3).

Table 4. Univariate and multivariate Cox proportional hazard models to predict survival of patients with locally advanced pancreatic cancer receiving chemotherapy or chemoradiotherapy

	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Median age, years (63.0)						
<63.0/≥63.0	0.959	0.624–1.474	0.8498			
Gender						
Male/female	0.802	0.519–1.249	0.334			
PS						
0/1 and 2	1.126	0.697–1.819	0.6270			
Median tumour size, mm (37.4)						
<37.4 mm/≥37.4 mm	1.066	0.665–1.709	0.7902			
Lymph node metastasis						
Negative/positive	1.606	1.008–2.560	0.0463	1.199	0.7654–1.978	0.0740
Location of the tumour						
Head/body or tail of pancreas	0.764	0.492–1.185	0.2294			
CA19-9						
<1000/≥1000 U ml ⁻¹	2.354	1.479–3.761	0.0003	2.325	1.416–3.818	0.0009
Actinin-4 IHC						
Negative/positive	1.526	0.922–2.528	0.1004			
ACTN4 FISH						
NCN/CNI	2.531	1.394–4.597	0.0023	2.645	1.439–4.861	0.0017

Abbreviations: ACTN4 = actinin-4; 95% CI = 95% confidence interval; CNI = copy number increase; FISH = fluorescence in situ hybridisation; HR = hazard ratio; IHC = immunohistochemistry; NCN = normal copy number; PS = Eastern Cooperative Oncology Group Performance Status. Bold entries indicate statistical significance.

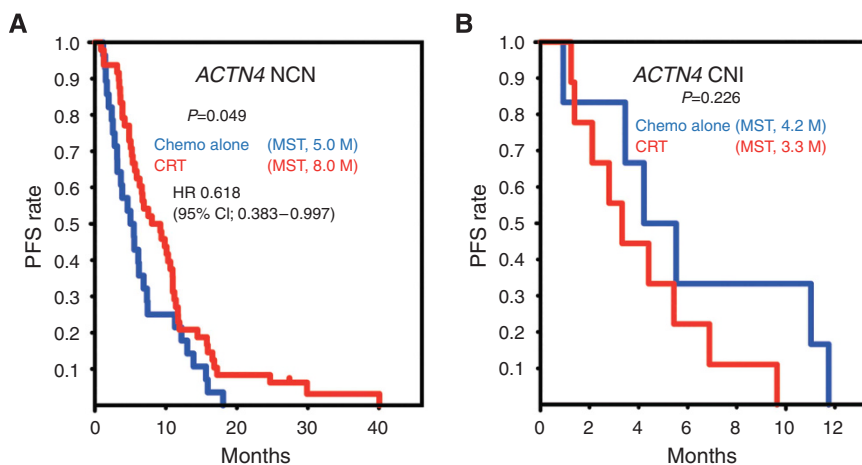


Figure 4. Kaplan–Meier analyses of progression-free survival (PFS) in CNI and NCN subgroups of ACTN4. The PFS curves of patients with a NCN of ACTN4 (A) or a CNI of ACTN4 (B), treated with chemotherapy alone (chemo alone, blue line) or with chemoradiotherapy (CRT, red line). The y axis is the rate of PFS and the x axis is the time after first diagnosis (months). Statistical parameters were calculated as described for Figure 2.

The benefit for PFS of CRT-treated patients who were selected by copy number status of ACTN4. We further examined the ability of ACTN4 copy number to function as a predictive biomarker for CRT using subgroup analysis of the copy number status of ACTN4. We classified the patients into CNI and NCI subgroups of ACTN4 and compared PFS in these CNI and NCI subgroups of ACTN4 patients between the two arms of chemotherapy alone and CRT. Kaplan–Meier analysis indicated a statistically significant difference in the PFS of NCN patients in the chemotherapy-alone group compared with that of the CRT subgroup ($P = 0.049$; Figure 4A). The median PFS of the patients who were evaluated as NCN of ACTN4 in the CRT subgroup was 8.0 months, whereas that for NCN of ACTN4 patients in the chemotherapy-alone subgroup was 5.0 months. Thus, the median

PFS of patients with NCN of ACTN4 in the CRT subgroup was longer than that of patients with NCN of ACTN4 in the chemotherapy-alone subgroup by 3 months. The HR for tumour progression of patients with NCN of ACTN4 in the CRT subgroup compared with the chemotherapy-alone subgroup was 0.618 (95% CI; 0.383–0.997). No statistically significant difference in the PFS of patients with a CNI of ACTN4 was noted between the chemotherapy-alone and the CRT subgroups ($P = 0.226$; Figure 4B). However, the MST of PFS in patients with a CNI of ACTN4 in the chemotherapy-alone subgroup (4.2 months) was slightly longer (0.9 month longer) than that of patients with a CNI of ACTN4 in the CRT subgroup (3.3 months). For both cohorts, there were no statistically significant differences in OS between the chemotherapy-alone and the CRT subgroups (data not shown).

DISCUSSION

In this study, we demonstrated that CNI of *ACTN4* is a predictive biomarker for the therapeutic strategy of LAPC. Although there have been a large number of studies and trials regarding the best chemotherapeutic strategy for extension of survival of patients with LAPC (Colucci *et al*, 2002; Huguet *et al*, 2007; Moore *et al*, 2007; Chauffert *et al*, 2008; Loehrer *et al*, 2011), the optimal therapy for patients with LAPC has not yet been decided upon. Clinical trials have reported contradictory results. Thus, the ECOG E4201, FFCD/SFRO and LAP07 phase III trials reported that the MST of OS in patients who received CRT was improved (Loehrer *et al*, 2011), decreased (Chauffert *et al*, 2008) or showed no statistically significant survival benefit compared with patients who received chemotherapy alone. The results of these studies suggest that there is a potential benefit to selecting appropriate patients for intensified treatment.

In order to select either chemotherapy or CRT as a treatment strategy, the metastatic potential of the tumour itself needs to be accurately evaluated. This is because radiotherapy can only exert a direct physicochemical effect on the tumour at the primary tumour site that is exposed to radiation, whereas chemotherapy can access both the primary tumour and distant metastasis. Therefore, patients with latent metastatic lesions, including lesions that cannot be detected using modern technology, should receive only strong chemotherapy, whereas patients who definitely have no distant metastatic lesions before initial treatment should receive CRT in order to exert sufficient physicochemical impact on the primary tumour site. Our finding that *ACTN4* copy number is a predictive marker for selection of therapy for LAPC should therefore prove valuable for optimisation of treatment strategy and help to provide the maximum personalised medicine for individual patients. Other predictive markers for treatment selection strategy have been suggested. Smad4 (*Dpc4*) is a tumour-suppressor gene involved in cell motility that is inactivated in 53% of pancreatic cancers. Prospective validation of smad4 expression in cytological specimens suggested that smad4 may be a predictive biomarker, and that analysis of smad4 levels may lead to personalised treatment strategies for patients with LAPC (Crane *et al*, 2011).

In the present paper we could not find any statistically significant difference in OS or PFS between LAPC patients who were treated with either chemotherapy alone or with CRT (Figure 2), again suggesting the need for a predictive marker for selection of patients for specific treatment. The potential predictive marker we considered was gene amplification of *ACTN4*.

The *ACTN4* gene encodes the actinin-4 protein, an actin-bundling protein that was isolated by our group in 1998 (Honda *et al*, 1998). Its protein overexpression is closely associated with cancer invasion and cell motility. Actinin-4 has one actin-binding domain at the N-terminus, and actinin-4 monomers can form a homodimer by binding in the opposite direction to form a dumbbell-shaped structure (Otey and Carpen, 2004). The actinin-4 homodimer can strongly bind F-actin and subsequently form bundling F-actin. Moreover, the bundling F-actin formed by actinin-4 makes strong contact with the cell membrane, following which cellular protrusions that are associated with cell motility are formed on the cell membrane (Welsch *et al*, 2009). The protein overexpression of actinin-4 in cancer cells stimulates dynamic remodelling of the actin cytoskeleton, and it is for this reason that actinin-4-overexpressing cancer cells have metastatic potential (Hayashida *et al*, 2005). Indeed, there are some reports that patients with cancers showing protein overexpression of actinin-4 have significantly worse OS than patients with cancers who are negative for actinin-4 (Honda *et al*, 1998; Yamamoto *et al*, 2007; Noro *et al*, 2013). Moreover, Kikuchi *et al* (2008) reported that protein overexpression of actinin-4 was a poor prognostic factor

for invasive ductal adenocarcinoma of the pancreas. However, in the present study we could not find a statistically significant positive correlation between actinin-4 protein overexpression and poor prognosis. One difference between our present study and the previous study of Kikuchi *et al* (2008) was that in the latter study protein expression of actinin-4 was immunohistochemically evaluated using whole pathological sections that were obtained from surgical samples, whereas in the present study protein expression of actinin-4 was immunohistochemically evaluated using biopsy specimens of LAPC. In the study of Kikuchi *et al* (2008), the staining pattern of endothelial cells as an internal control was used to accurately evaluate the protein expression level of actinin-4 in tumour cells. However, accurate evaluation of the protein expression level of actinin-4 from biopsy specimens was more difficult than from whole pathological sections because the biopsy specimens did not always include endothelial cells. These technical problems may therefore explain the difference in the results of the two studies. One cause of protein overexpression of actinin-4 in cancer cells is amplification of the *ACTN4* gene (Kikuchi *et al*, 2008) and it has been reported that the CNI of *ACTN4* is a better prognostic predictor than protein expression of actinin-4 (Yamamoto *et al*, 2009; Noro *et al*, 2013; Watabe *et al*, 2014). We found a statistically significant difference in OS between patients with a CNI and those with a NCN, and patients with a CNI had a worse prognosis in terms of OS than NCN patients (Figure 3B). Furthermore, multivariate Cox regression analysis indicated that a CNI of *ACTN4* and high serum CA19-9 levels were independent prognostic factors for the death of patients, and that the HR of CNI of *ACTN4* was higher than that of high CA19-9 levels (Table 4). These data confirmed the usefulness of CA19-9 as a prognostic factor for LAPC and further suggested that *ACTN4* might be a prognostic factor for LAPC.

Subgroup analyses of CNI and NCN patients who were treated with chemotherapy alone or with CRT using FISH to calculate *ACTN4* copy number showed that whereas the copy number of *ACTN4* may be a predictive biomarker for CRT of LAPC, CA19-9 was not a predictive biomarker for either chemotherapy alone or CRT. Thus, there was no statistically significant difference in OS between CNI and NCN patients in the subgroup who were treated with chemotherapy alone (Figure 3C). However, in the subgroup of patients who were treated with CRT, the CNI patients with an MST of 14.1 months had a significantly longer survival time than NCN patients who had an MST of 6.0 months (Figure 3D). In contrast, serum CA19-9 levels showed statistically significant differences in terms of OS for both subgroups (Supplementary Figures 1–3).

Our data further confirmed the usefulness of *ACTN4* as a predictive biomarker for CRT in the study of the PFS of patients with LAPC who were classified into CNI and NCN of *ACTN4* groups and were then further classified into subgroups based on therapeutic strategies. We found a statistically significant difference in good prognosis of PFS between the NCN group treated with CRT (MST of PFS of 8.0 months) compared with the NCN group treated with chemotherapy alone (5.0 months; Figure 4A). Interestingly, although no statistically significant difference in PFS was found between the subgroups of CNI of *ACTN4* who were treated with chemotherapy alone or with CRT, the MST of PFS was the reverse of that seen in the NCN group, with the MST of chemotherapy alone being 4.2 months and that of CRT being shorter at 3.3 months. These data suggest that, when considering therapy for LAPC patients, patients with a NCN of *ACTN4* should at least undergo CRT (Figure 4B). However, no statistically significant difference in benefit in OS was noted in subgroup analysis of CNI and NCN of *ACTN4* groups. It was considered that the number of patients in the subgroup of *ACTN4* was too small to statistically prove the clinical benefit of chemotherapy alone in the subgroup with CNI of *ACTN4*.

In conclusion, we showed that the copy number of *ACTN4* is not only a prognostic biomarker, but also a candidate predictive biomarker for the decision regarding effective treatment strategy. Although this was a retrospective study, it suggested that patients without gene amplification of *ACTN4* should undergo CRT. Although it was concluded that *ACTN4* is a biomarker of potential metastasis, this does not necessarily contraindicate a potential function for *ACTN4* copy number as a predictive biomarker for CRT of LAPC. More detailed analyses, including a prospective study, should be carried out to prove this possibility.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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