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Watanabe T, Ueno H, Hiraoka N, Morizane C, Yamada T, Honda K, et al.	ACTN4 copy number increase as a predictive biomarker for chemoradiotherapy of locally advanced pancreatic cancer.	*Br. J. Cancer.*	112 (4)	704-13	2015
Isayama H, Ryozawa S, et al.	TOKYO criteria 2014 for transpapillary biliary stenting.	*Dig Endosc.*	27 (2)	259-64	2015
Aoki T, Morizane C, et al.	Streptozocin chemotherapy for advanced/metastatic well-differentiated neuroendocrine tumors: an analysis of a multi-center survey in Japan.	*J Gastroenterol.*	-	-	In press
Yamamoto Y, Hiraoka N, et al	A targeting ligand enhances infectivity and cytotoxicity of an oncolytic adenovirus in human pancreatic cancer tissues.	*J Control Release.*	192	284-93	2014

Yamaguchi T, Morizane C, et al	Multicenter retrospective analysis of systemic chemotherapy for advanced neuroendocrine carcinoma of the digestive system.	*Cancer Sci.*	105 (9)	1176-81	2014
Furukawa D, Hiraoka N, et al	Zinc finger protein 185 is a liver metastasis-associated factor in colon cancer patients.	*Mol Clin Oncol.*	2(5)	709-13	2014
Kishi Y, Hiraoka N, et al.	Basing treatment strategy for non-functional pancreatic neuroendocrine tumors on tumor size.	*Ann Surg Oncol.*	21 (9)	2882-8	2014
Qiu Y, Hiraoka N, et al.	Knowledge discovery for pancreatic cancer using inductive logic programming.	*IET Syst Biol.*	8 (4)	162-8	2014
Inagawa Y, Hiraoka N, et al.	A human cancer xenograft model utilizing normal pancreatic duct epithelial cells conditionally transformed with defined oncogenes.	*Carcinogenesis.*	35 (8)	1840-6	2014

Hayashi T, Hiraoka N, et al.	Potential diagnostic biomarkers: differential expression of LMP2/β1i and cyclin B1 in human uterine leiomyosarcoma.	*Tumori.*	100 (4)	99e-106e	2014
Yamazaki K, Hiraoka N, et al.	Upregulated SMAD3 promotes epithelial-mesenchymal transition and predicts poor prognosis in pancreatic ductal adenocarcinoma.	*Lab Invest.*	94 (6)	683-91	2014
Watabe Y,Honda K, et al.	Copy number increase of ACTN4 is a prognostic indicator in salivary gland carcinoma.	*Cancer Med.*	3 (3)	613-22	2014
Tanaka N, Honda K, Yamada T, et al.	Histological growth pattern and alpha-actinin-4 expression in thyroid cancer.	*Anticancer Res.*	34 (6)	3157-63	2014
Kondo S, Morizane C, Ueno H, et al.	A retrospective analysis of factors associated with selection of end-of-life care and actual place of death for patients with cancer.	*BMJ Open.*	4 (5)	e004352	2014

Shoji H, Morizane C, Ueno H, et al.	Twenty-six cases of advanced ampullary adenocarcinoma treated with systemic chemotherapy.	*Jpn J Clin Oncol.*	44 (4)	324-30	2014
Masuda M, Honda K, Yamada T, et al.	Alternative mammalian target of rapamycin (mTOR) signal activation in sorafenib-resistant hepatocellular carcinoma cells revealed by array-based pathway profiling.	*Mol Cell Proteomics.*	13 (6)	1429-38	2014
Hori M, Hiraoka N, et al.	Association of pancreatic Fatty infiltration with pancreatic ductal adenocarcinoma.	*Clin Transl Gastroenterol.*	5	e53	2014
Kobayashi E, Honda K, Yamada T, et al.	MicroRNA expression and functional profiles of osteosarcoma.	*Oncology.*	86 (2)	94-103	2014
Ohki R, Hiraoka N, et al.	PHLDA3 is a novel tumor suppressor of pancreatic neuroendocrine tumors.	*PNAS.*	111(23)	2402-13	2014
Matsubara A, Hiraoka N et al.	Intraductal dissemination of papillary adenocarcinoma of the amp	*Pathol Int.*	64 (1)	39-44	2014

	ulla of Vater in the pancreatic duct.				
Fujiwara Y, Azuma T, Yoshida M, et al.	Metabolomics evaluation of serum markers for cachexia and their intra-day variation in patients with advanced pancreatic cancer.	*Plos ONE*	9 (11)	e113259	2014

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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 (LPAC) 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 (Miyanaga *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 μm-thick sections.

An anti-actinin-4 monoclonal antibody was established by our group (Miyanaga *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 μm thick) were hybridised with FISH probes at 37 °C for 48 h. The nuclei were counterstained with 4,6-duamidino-2-phenylindone. 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

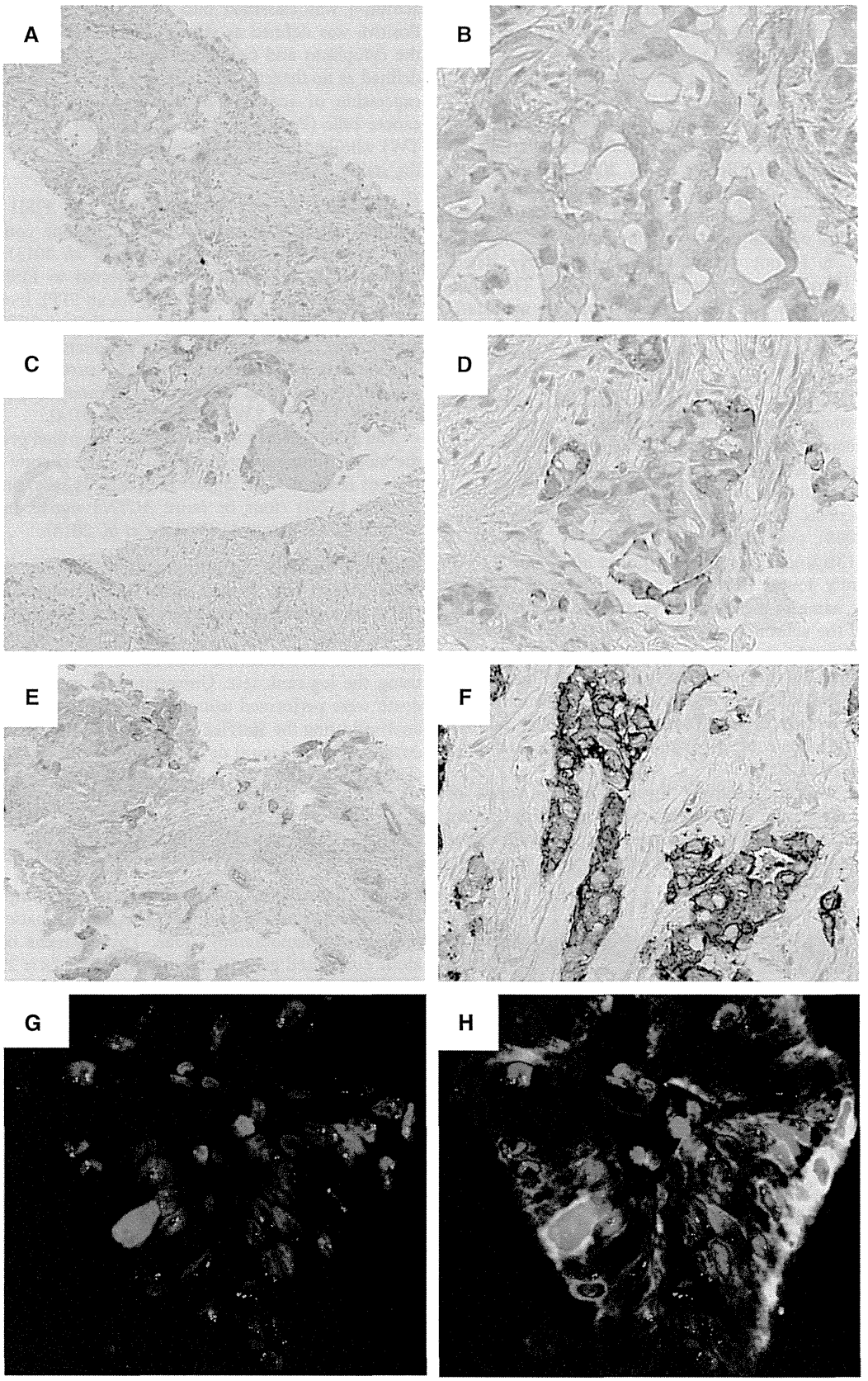

Figure 1. Immunohistochemical (IHC) and fluorescence *in situ* hybridisation (FISH) analyses of representative actinin-4 protein expression and *ACTN4* copy number, respectively, in LAPC biopsy specimens. (**A–F**) Immunohistochemical analysis of actinin-4 protein expression. Representative cases of 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

	Total		CRT		CT		
Characteristic	**Number**	**%**	**Number**	**%**	**Number**	**%**	***P*-value***
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).

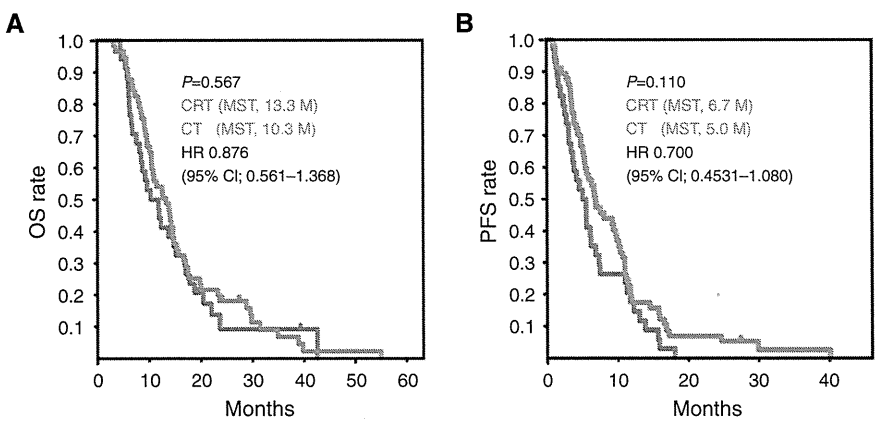

Figure 2. Kaplan–Meier analyses of overall survival (OS) and progression-free survival (PFS) in all locally advanced pancreatic cancer (LAPC) cases. The survival curves of all LAPC patients treated with chemotherapy alone (CT, blue lines) or with chemoradiotherapy (CRT, red lines) are shown. Statistically 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 acitinin-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

	Actinin-4 IHC					*ACTN4* FISH				
Characteristic	**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	
$\geqslant 1000\,U\,ml^{-1}$	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 statistically 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 ($\geqslant 1000\,U\,ml^{-1}$) 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

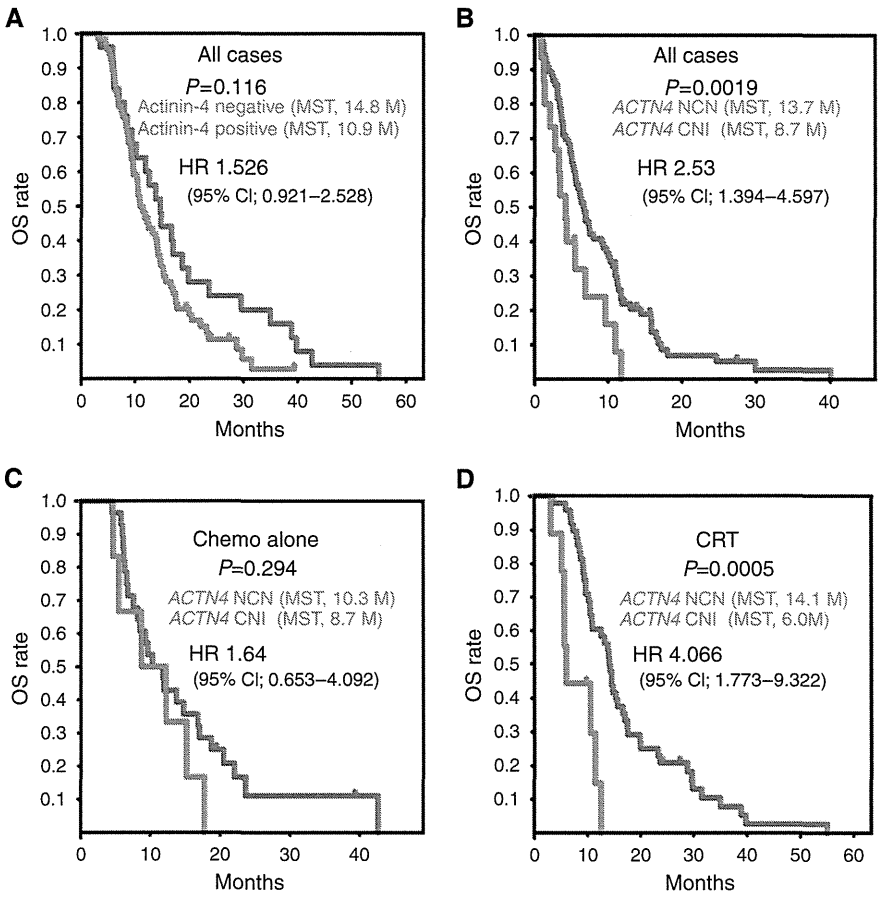

Figure 3. Kaplan–Meier analyses of survival relative to protein expression of actinin-4 and copy number of *ACTN4*. (**A**) Overall survival (OS) curves based on protein expression of actinin-4. The blue line represents patients with negative expression of actinin-4. The red line represents patients 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*

	Copy number status of *ACTN4*			
Status of actinin-4 with IHC	**NCN (%)**	**CNI (%)**	**Total**	***P*-value***
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 statistically significance.

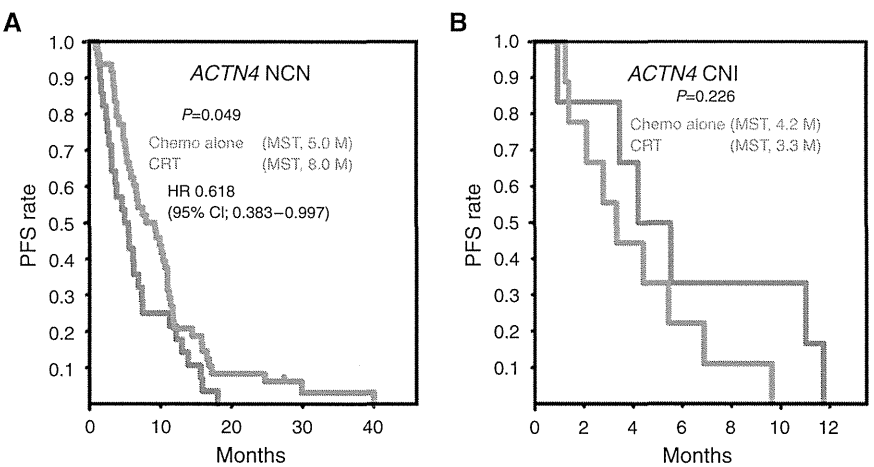

Figure 4. Kaplan–Meier analyses of progression-free survival (PFS) in CNI and NCN subgroups of *ACTN4*. The PFS curves of patients with a NCN of *ACTN4* (**A**) or a CNI of *ACTN4* (**B**), treated with chemotherapy alone (chemo alone, blue line) or with chemoradiotherapy (CRT, red line). The y axis 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.

REFERENCES

Berger AC, Garcia Jr. M, Hoffman JP, Regine WF, Abrams RA, Safran H, Konski A, Benson 3rd AB, MacDonald J, Willett CG (2008) Postresection CA 19-9 predicts overall survival in patients with pancreatic cancer treated with adjuvant chemoradiation: a prospective validation by RTOG 9704. *J Clin Oncol* **26**(36): 5918–5922.

Chauffert B, Mornex F, Bonnetain F, Rougier P, Mariette C, Bouche O, Bosset JF, Aparicio T, Mineur L, Azzedine A, Hammel P, Butel J, Stremsdoerfer N, Maingon P, Bedenne L (2008) Phase III trial comparing intensive induction chemoradiotherapy (60Gy, infusional 5-FU and intermittent cisplatin) followed by maintenance gemcitabine with gemcitabine alone for locally advanced unresectable pancreatic cancer. Definitive results of the 2000-01 FFCD/SFRO study. *Ann Oncol* **19**(9): 1592–1599.

Colucci G, Giuliani F, Gebbia V, Biglietto M, Rabitti P, Uomo G, Cigolari S, Testa A, Maiello E, Lopez M (2002) Gemcitabine alone or with cisplatin for the treatment of patients with locally advanced and/or metastatic pancreatic carcinoma: a prospective, randomized phase III study of the Gruppo Oncologia dell'Italia Meridionale. *Cancer* **94**(4): 902–910.

Crane CH, Varadhachary GR, Yordy JS, Staerkel GA, Javle MM, Safran H, Haque W, Hobbs BD, Krishnan S, Fleming JB, Das P, Lee JE, Abbruzzese JL, Wolff RA (2011) Phase II trial of cetuximab, gemcitabine, and oxaliplatin followed by chemoradiation with cetuximab for locally advanced (T4) pancreatic adenocarcinoma: correlation of Smad4(Dpc4) immunostaining with pattern of disease progression. *J Clin Oncol* **29**(22): 3037–3043.

Hayashida Y, Honda K, Idogawa M, Ino Y, Ono M, Tsuchida A, Aoki T, Hirohashi S, Yamada T (2005) E-cadherin regulates the association between beta-catenin and actinin-4. *Cancer Res* **65**(19): 8836–8845.

Honda K, Okusaka T, Felix K, Nakamori S, Sata N, Nagai H, Ioka T, Tsuchida A, Shimahara T, Shimahara M, Yasunami Y, Kuwabara H, Sakuma T, Otsuka Y, Ota N, Shitashige M, Kosuge T, Buchler MW, Yamada T (2012) Altered plasma apolipoprotein modifications in patients with pancreatic cancer: protein characterization and multi-institutional validation. *PLoS One* **7**(10): e46908.

Honda K, Yamada T, Endo R, Ino Y, Gotoh M, Tsuda H, Yamada Y, Chiba H, Hirohashi S (1998) Actinin-4, a novel actin-bundling protein associated with cell motility and cancer invasion. *J Cell Biol* **140**(6): 1383–1393.

Honda K, Yamada T, Hayashida Y, Idogawa M, Sato S, Hasegawa F, Ino Y, Ono M, Hirohashi S (2005) Actinin-4 increases cell motility and promotes lymph node metastasis of colorectal cancer. *Gastroenterology* **128**(1): 51–62.

Huguet F, Andre T, Hammel P, Artru P, Balosso J, Selle F, Deniaud-Alexandre E, Ruszniewski P, Touboul E, Labianca R, de Gramont A, Louvet C (2007) Impact of chemoradiotherapy after disease control with chemotherapy in locally advanced pancreatic adenocarcinoma in GERCOR phase II and III studies. *J Clin Oncol* **25**(3): 326–331.

Ikeda M, Okusaka T, Ito Y, Ueno H, Morizane C, Furuse J, Ishii H, Kawashima M, Kagami Y, Ikeda H (2007) A phase I trial of S-1 with concurrent radiotherapy for locally advanced pancreatic cancer. *Br J Cancer* **96**(11): 1650–1655.

Johung K, Saif MW, Chang BW (2012) Treatment of locally advanced pancreatic cancer: the role of radiation therapy. *Int J Radiat Oncol Biol Phys* **82**(2): 508–518.

Katz MH, Marsh R, Herman JM, Shi Q, Collison E, Venook AP, Kindler HL, Alberts SR, Philip P, Lowy AM, Pisters PW, Posner MC, Berlin JD, Ahmad SA (2013) Borderline resectable pancreatic cancer: need for standardization and methods for optimal clinical trial design. *Ann Surg Oncol* **20**(8): 2787–2795.

Kikuchi S, Honda K, Handa Y, Kato H, Yamashita K, Umaki T, Shitashige M, Ono M, Tsuchida A, Aoki T, Hirohashi S, Yamada T (2007) Serum albumin-associated peptides of patients with uterine endometrial cancer. *Cancer Sci* **98**(6): 822–829.

Kikuchi S, Honda K, Tsuda H, Hiraoka N, Imoto I, Kosuge T, Umaki T, Onozato K, Shitashige M, Yamaguchi U, Ono M, Tsuchida A, Aoki T, Inazawa J, Hirohashi S, Yamada T (2008) Expression and gene amplification of actinin-4 in invasive ductal carcinoma of the pancreas. *Clin Cancer Res* **14**(17): 5348–5356.

Loehrer Sr. PJ, Feng Y, Cardenes H, Wagner L, Brell JM, Cella D, Flynn P, Ramanathan RK, Crane CH, Alberts SR, Benson 3rd AB (2011) Gemcitabine alone versus gemcitabine plus radiotherapy in patients with locally advanced pancreatic cancer: an Eastern Cooperative Oncology Group trial. *J Clin Oncol* **29**(31): 4105–4112.

Mayahara H, Ito Y, Morizane C, Ueno H, Okusaka T, Kondo S, Murakami N, Morota M, Sumi M, Itami J (2012) Salvage chemoradiotherapy after primary chemotherapy for locally advanced pancreatic cancer: a single-institution retrospective analysis. *BMC Cancer* **12**: 609.

Mian OY, Ram AN, Tuli R, Herman JM (2014) Management options in locally advanced pancreatic cancer. *Curr Oncol Rep* **16**(6): 388.

Miyanaga A, Honda K, Tsuta K, Masuda M, Yamaguchi U, Fujii G, Miyamoto A, Shinagawa S, Miura N, Tsuda H, Sakuma T, Asamura H, Gemma A, Yamada T (2013) Diagnostic and prognostic significance of the alternatively spliced ACTN4 variant in high-grade neuroendocrine pulmonary tumours. *Ann Oncol* **24**(1): 84–90.

Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W. National Cancer Institute of Canada Clinical Trials G (2007) Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* **25**(15): 1960–1966.

Noro R, Honda K, Tsuta K, Ishii G, Maeshima AM, Miura N, Furuta K, Shibata T, Tsuda H, Ochiai A, Sakuma T, Nishijima N, Gemma A, Asamura H, Nagai K, Yamada T (2013) Distinct outcome of stage I lung adenocarcinoma with ACTN4 cell motility gene amplification. *Ann Oncol* **24**(10): 2594–2600.

Otey CA, Carpen O (2004) Alpha-actinin revisited: a fresh look at an old player. *Cell Motil Cytoskeleton* **58**(2): 104–111.

Pawlik TM, Laheru D, Hruban RH, Coleman J, Wolfgang CL, Campbell K, Ali S, Fishman EK, Schulick RD, Herman JM. Johns Hopkins Multidisciplinary Pancreas Clinic T (2008) Evaluating the impact of a single-day multidisciplinary clinic on the management of pancreatic cancer. *Ann Surg Oncol* **15**(8): 2081–2088.

Savir G, Huber KE, Saif MW (2013) Locally advanced pancreatic cancer. Looking beyond traditional chemotherapy and radiation. *JOP* **14**(4): 337–339.

Watabe Y, Mori T, Yoshimoto S, Nomura T, Shibahara T, Yamada T, Honda K (2014) Copy number increase of ACTN4 is a prognostic indicator in salivary gland carcinoma. *Cancer Med* **3**(3): 613–622.

Welsch T, Keleg S, Bergmann F, Bauer S, Hinz U, Schmidt J (2009) Actinin-4 expression in primary and metastasized pancreatic ductal adenocarcinoma. *Pancreas* **38**(8): 968–976.

Yamamoto S, Tsuda H, Honda K, Kita T, Takano M, Tamai S, Inazawa J, Yamada T, Matsubara O (2007) Actinin-4 expression in ovarian cancer: a novel prognostic indicator independent of clinical stage and histological type. *Mod Pathol* **20**(12): 1278–1285.

Yamamoto S, Tsuda H, Honda K, Onozato K, Takano M, Tamai S, Imoto I, Inazawa J, Yamada T, Matsubara O (2009) Actinin-4 gene amplification in ovarian cancer: a candidate oncogene associated with poor patient prognosis and tumor chemoresistance. *Mod Pathol* **22**(4): 499–507.

Yamamoto S, Tsuda H, Honda K, Takano M, Tamai S, Imoto I, Inazawa J, Yamada T, Matsubara O (2012) ACTN4 gene amplification and actinin-4 protein overexpression drive tumour development and histological progression in a high-grade subset of ovarian clear-cell adenocarcinomas. *Histopathology* **60**(7): 1073–1083.

Yang GY, Malik NK, Chandrasekhar R, Ma WW, Flaherty L, Iyer R, Kuvshinoff B, Gibbs J, Wilding G, Warren G, May KS (2013) Change in CA 19-9 levels after chemoradiotherapy predicts survival in patients with locally advanced unresectable pancreatic cancer. *J Gastrointest Oncol* **4**(4): 361–369.

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Review

TOKYO criteria 2014 for transpapillary biliary stenting

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It is difficult to carry out meta-analyses or to compare the results of different studies of biliary stents because there is no uniform evaluation method. Therefore, a standardized reporting system is required. We propose a new standardized system for reporting on biliary stents, the 'TOKYO criteria 2014', based on a consensus among Japanese pancreatobiliary endoscopists. Instead of stent occlusion, we use recurrent biliary obstruction, which includes occlusion and migration. The time to recurrent biliary obstruction was estimated using Kaplan–Meier analysis with the log–rank test. We can evaluate both plastic and self-expandable metallic stents (uncovered and covered). We also propose specification of the cause of recurrent biliary obstruction, identification of complications other than recurrent biliary obstruction, indication of severity, measures of technical and clinical success, and a standard for clinical care. Most importantly, the TOKYO criteria 2014 allow comparison of biliary stent quality across studies. Because blocked stents can be drained not only using transpapillary techniques but also by an endoscopic ultrasonography-guided transmural procedure, we should devise an evaluation method that includes transmural stenting in the near future.

Key words: biliary drainage, biliary stent, biliary stricture, metallic stent, obstructive jaundice

INTRODUCTION

IN MOST STUDIES of biliary stents used to treat malignant biliary obstruction, the primary outcome is stent patency, which is typically the time between stent placement and occlusion regardless of the kind of prosthesis.[1–7] However, the definition of stent patency differs considerably across studies, including randomized controlled trials of covered versus uncovered self-expandable metal stents (SEMS) for distal malignant biliary obstruction (Table 1), [7–12] and this heterogeneity has hindered the comparison of studies.[13,14]

Dysfunction in uncovered SEMS is caused mainly by SEMS occlusion as a result of tumor ingrowth, overgrowth, or sludge accumulation. Consequently, we have used the term 'stent patency'. Subsequently, the covered SEMS was developed to prevent tumor ingrowth and has become a treatment option for distal malignant biliary obstruction.[6,7,15–17] After the introduction of covered SEMS, however, other stent-related events emerged, such as stent migration, which does not cause stent occlusion or dysfunction, but causes recurrent biliary obstruction. Such emergent problems complicate the analysis of stent patency.

A plastic stent (PS) is still often placed to relieve the symptoms of biliary obstruction, and it has been evaluated in a similar way to the covered SEMS. Additionally, the evaluation of endoscopic ultrasonography (EUS)-guided transmural stenting requires other methods because of the presence of different causes of stent dysfunction and other types of complication. The proposed criteria do not include differences in biliary drainage procedures.

Here, we attempt to resolve these difficulties and propose a standardized reporting system for biliary stents, the 'TOKYO criteria 2014', for malignant biliary obstruction. These criteria were based on a consensus among expert Japanese pancreatobiliary endoscopists.

TECHNICAL AND FUNCTIONAL SUCCESS

TECHNICAL AND FUNCTIONAL success rates should be documented. We recommend that technical success was defined as successful deployment of a SEMS/PS in the intended location with sufficient coverage of the stricture.

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Table 1 Definitions of stent patency in each fully published RCT on SEMS for distal malignant biliary obstruction

Isayama *et al.* 2004[7]	The stent patency period was calculated as the interval between stent insertion and its obstruction, or death, with a patent stent.
Krokidis *et al.* 2011[8]	Primary patency of the endoprosthesis was defined as the time interval between initial placement and recurrence of obstruction. If there was no evidence of obstruction during the patient's life, the patency period was considered to be equal to the survival period, but censored.
Krokidis *et al.* 2010[9]	Primary patency was defined as the interval between initial placement and recurrence of obstruction. If there was no evidence of obstruction during the patient's life, the patency period was considered equal to the survival period but was censored.
Kullman *et al.* 2010[10]	Uneventful follow up for 12 months, death with a patent stent, and confirmed stent failure (details were not described).
Telford *et al.* 2010[11]	Patients not experiencing recurrent biliary obstruction were censored at the date of last follow up or date of death.
Ung *et al.* 2013[12]	Stent dysfunction was defined as a more than a twofold increase in the serum bilirubin level compared with the reference value, increasing alkaline phosphatase, dark urine combined with pale stools or occurrence of cholangitis or other clinical and radiological signs of stent dysfunction.

RCT, randomized controlled trial; SEMS, self-expandable metal stent.

We recommend that functional success was defined as a 50% decrease in or normalization of the bilirubin level within 14 days of stent placement, although this definition has differed considerably across studies.[3,4]

TIME TO RECURRENT BILIARY OBSTRUCTION

THE RECENT DEVELOPMENT of the covered SEMS made the analysis of successful stenting more difficult. Using only uncovered SEMS, the focus was on stent patency because the main cause of symptoms was 'stent occlusion' as a result of tumor ingrowth or sludge accumulation. However, the migration of a covered SEMS/PS is not 'stent occlusion', although it can cause symptoms of biliary obstruction. Therefore, we should not use the term 'stent patency' but should devise an adequate term for biliary luminal patency. The term 'recurrent biliary obstruction' may be suitable for such events instead of 'stent occlusion or dysfunction', and 'time to recurrent biliary obstruction' may be better than 'stent patency'.

We recommend that recurrent biliary obstruction was defined as a composite endpoint of either occlusion or migration, and time to recurrent biliary obstruction refers to the time from SEMS/PS placement to the recurrence of biliary obstruction. We recommend that the time points of occlusion and migration be defined as the points at which symptoms associated with occlusion or migration are observed. Time to recurrent biliary obstruction was estimated using the Kaplan–Meier method[18] and compared between groups using the log–rank test.[19] In the estimation of time to recurrent biliary obstruction, patient death and complications other than recurrent biliary obstruction requiring SEMS removal are treated as censored cases at the time of death or SEMS removal, respectively. Non-obstruction rates at 3, 6, and 12 months estimated using the Kaplan–Meier method should also be given, where the non-obstruction rate is the rate of patients not subject to recurrent biliary obstruction at a given time-point. The median time to recurrent biliary obstruction estimated using the Kaplan–Meier method should also be given.

SURVIVAL TIME

THE SURVIVAL TIME should be reported for each study arm in comparative studies. Note that time to recurrent biliary obstruction is underestimated when premature patient death without recurrent biliary obstruction is treated as censored in the Kaplan–Meier method. Consequently, data on survival time in each study arm are required for an appropriate evaluation of time to recurrent biliary obstruction. Recently, competing risk analysis to solve this problem has been increasingly reported. Competing risk analysis treats premature death without SEMS/PS occlusion or recurrent biliary obstruction as informative censoring instead of non-informative censoring, as is done in the conventional Kaplan–Meier method. The application of this new method to all clinical trials of biliary stents should be discussed.

CAUSES OF RECURRENT BILIARY OBSTRUCTION

THE DETAILS OF recurrent biliary obstruction should be clarified in the evaluation of biliary stent, rate of each cause and median time from the placement.

Occlusion

We recommend that stent occlusion is defined as present when there is biochemical evidence of cholestasis; i.e. elevated liver enzymes compared with baseline values, accompanied by biliary dilation on imaging studies or

Table 2 Severity grading of complications other than recurrent biliary obstruction after stent placement

	Mild	Moderate	Severe
Pancreatitis	Requirement of admission or prolongation of hospitalization for 3 days	Hospitalization of 4–10 days or at least one of the following: 1. Requirement for stent removal. 2. Organ failure that resolves within 48 h. 3. Local or systemic complications without persistent organ failure.	Hospitalization for >10 days
Cholangitis	Antibiotics only	Febrile or septic illness requiring >3 days of hospitalization or endoscopic or percutaneous intervention	Hospitalization for >10 days, septic shock or organ failure
Cholecystitis	Conservative treatment only (antibiotics and/or no oral intake)	Hospitalization >3 days or requiring any intervention; percutaneous, endoscopic drainage, stent removal and surgery	Hospitalization >10 days, septic shock or organ failure
Bleeding	No requirement for transfusion	Transfusion of ≤4 units without angiographic intervention and surgery	Requirement for transfusion of ≥5 units or intervention (angiographic or surgical)
Perforation	Possible or only very slight leak of fluid or contrast, medically for ≤3 days	Any definite perforation treated medically for 4–10 days or endoscopic/percutaneous intervention	Hospitalization >10 days or surgery
Other and complications associated with stent placement procedure	Conservative treatment only	Prolonged hospitalization >3 days	Requirement for intervention or surgery

endoscopic findings suggesting it. The causes of SEMS/PS occlusion can be categorized as follows: tumor ingrowth/mucosal hyperplasia; tumor overgrowth; sludge with/without stones; hemobilia; food impaction; bile duct kinking; and others.

Tumor ingrowth is diagnosed when radiological findings show a persistent biliary stricture inside an uncovered or covered SEMS after the bile duct has been cleaned. Sometimes, we can observe the tissue growing into the SEMS cavity on endoscopic examination. Similarly, tumor overgrowth is diagnosed as a persistent stricture outside the SEMS. Occasionally, mucosal hyperplasia cannot be distinguished from tumor ingrowth as a cause of SEMS occlusion despite biopsies or cholangioscopy.[20] Consequently, tumor ingrowth or mucosal hyperplasia should be documented only if radiological and endoscopic findings can clearly determine a primary cause of SEMS occlusion. Sometimes, uncovered SEMS occluded within a short period of time as a result of embedding into tumor tissue. This phenomenon may occur in the case with soft tissue, but it is difficult to distinguish from tumor ingrowth as a result of rapid tumor growth. Thus, we did not define it.

Sludge, hemobilia, and food impaction as causes of SEMS/PS occlusion are diagnosed when reintervention reveals a large amount of sludge, clots, or food residue, respectively, in an occluded SEMS/PS. Kinking of the bile duct is caused by a straight stent in a tortuous bile duct, and it tends to occur in cases requiring high axial force for SEMS placement.[17,21,22] The proximal or distal end of the stent causes kinking of the bile duct and obstructs the bile flow. These complications can occur with a covered or an uncovered SEMS, or with a plastic stent. Other causes include SEMS collapse; other causes should be documented when considered a cause of stent occlusion.

Migration

Stent migration is diagnosed when a reintervention reveals a completely or partially migrated SEMS/PS as a cause of recurrent biliary obstruction. We recommend that the time point of symptomatic migration be defined as the point when symptoms associated with stent migration are observed. The time of asymptomatic migration detected incidentally should also be recorded but not defined as recurrent biliary obstruction. In asymptomatic migration, subsequent bile duct obstruction should be defined as recurrent biliary obstruction. The types of migration; i.e. proximal or distal, are also documented. Although migration in some cases is likely to be caused by enhanced inner pressure of the bile duct as a