High *EGFR* mRNA expression is a prognostic factor for reduced survival in pancreatic cancer after gemcitabine-based adjuvant chemotherapy

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) still presents a major therapeutic challenge and a phase III clinical trial has revealed that the combination of gemcitabine and a human epidermal growth factor receptor type I (HER1/EGFR) targeting agent presented a significant benefit compared to treatment with gemcitabine alone. The aim of this study was to investigate *EGFR* mRNA expression in resected PDAC tissues and its correlation with patient prognosis. We obtained formalin-fixed paraffin-embedded (FFPE) tissue samples from 88 patients with PDAC who underwent pancreatectomy, and measured *EGFR* mRNA levels by quantitative real-time reverse transcription-polymerase chain reaction. The high-level *EGFR* group had significantly shorter disease-free-survival (p=0.029) and overall-survival (p=0.014) as shown by univariate analyses, although these did not reach statistical significance, as shown by multivariate analyses. However, we found that high *EGFR* expression was an independent prognostic factor in patients receiving gemcitabine-based adjuvant chemotherapy (p=0.023). Furthermore, we measured *EGFR* mRNA levels in 20 endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) cytological specimens. Altered *EGFR* levels were distinguishable in microdissected neoplastic cells from EUS-FNA cytological specimens compared to those in whole cell pellets. In conclusion, quantitative analysis of *EGFR* mRNA expression using FFPE tissue samples and microdissected neoplastic cells from EUS-FNA cytological specimens could be useful in predicting prognosis and sensitivity to gemcitabine in PDAC patients.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal and aggressive human malignancies, and it is the fourth leading cause of tumor-related deaths in the industrialized world (1,2). The vast majority of patients with PDAC have poor outcomes due to the aggressive nature of the tumor and difficulties in early diagnosis due to the lack of early disease-specific signs and symptoms. Only 10-20% of patients with PDAC have a chance of curative resection (3) and, even if the curative resection is performed, the post-operative 5-year survival rate is only 15-25% due to a high recurrence rate (4,5). Two randomized clinical phase III trials of adjuvant chemotherapy (AC) for PDAC have shown significant increases in overall survival (OS) and disease-free survival (DFS). However, their efficacy was limited and insufficient (6,7). To improve the prognosis of patients with PDAC, individualized chemotherapy based on the gene expression profiles of the individual's own cancer tissues, could be a potent strategy.

Human epidermal growth factor receptor type 1 (HER1/EGFR) is a receptor tyrosine kinase. Binding of ligand growth factors, such as epidermal growth factor (EGF) and transforming growth factor (TGF)-α to EGFR leads to receptor phosphorylation and activation of downstream Ras/mitogen-activated protein kinase (MAPK) signaling, thereby enhancing the malignant behavior of cancer cells (8,9). There is increasing

evidence showing that the dysregulation of EGFR pathways by overexpression or constitutive activation can promote tumor growth and metastasis, and that this is associated with poor prognosis and tumor aggressiveness in many human malignancies, including pancreatic cancer (10-13). To improve the prognosis of PDAC patients, the blockade of the EGFR signaling pathway could be a potent strategy (9,14). The EGFR signaling blockade has been reported to decrease growth and metastasis in an orthotopic implantation murine model of pancreatic cancer cells (15) and to improve the efficacy of gemcitabine in human pancreatic tumor xenograft models (16).

At the time of diagnosis, >80% of PDAC patients present with either locally advanced or metastatic disease (3). Therefore, patients with unresectable advanced PDAC require cytopathological assessment using endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) or pancreatic juice specimens to predict their sensitivity to chemotherapeutic agents and prognosis. Quantitative mRNA analysis of genes associated with sensitivity to chemotherapeutic agents, or with patient prognosis could be suitable for clinical use as this method enables us to reproducibly detect gene expression, even with small samples (17). In the current study, we investigated the correlation between *EGFR* expression and the prognosis of patients with PDAC. To elucidate the role of *EGFR* expression in gemcitabine sensitivity, we also investigated the association between receptor expression levels and treatment outcomes in PDAC patients receiving gemcitabine-based AC. Furthermore, we quantified *EGFR* expression in cytological specimens obtained by EUS-FNA to examine the possible utility of such samples for quantifying the mRNA levels of these predictive factors.

Materials and methods

Patients and pancreatic tissues. Our study subjects comprised of 88 patients who underwent pancreatectomy for PDAC at the Department of Surgery and Oncology, Kyushu University Hospital (Fukuoka, Japan) from 1992 to 2007. The patients (54 male and 34 female) had a median age of 65 years (range, 36-86 years). Eighteen of the 88 patients received no AC (non-AC group). Thirty-six of the 88 patients received gemcitabine-based AC (GEM group), consisting of two or more cycles of 1,000 mg/m^2/d gemcitabine on days 1, 8 and 15 every 28 days, and three or more cycles of 1,000 mg/m^2/d gemcitabine on days 1 and 8 every 21 days. Nineteen of the 88 patients received other forms of AC (other AC group), including 5 patients orally administered S-1 (80-100 mg/body), 7 patients orally administered tegafur (400-800 mg/body), and 7 patients treated with a bolus of 5-fluorouracil (250-500 mg/body). The remaining 15 patients did not receive adequate AC due to their poor performance status. We recommended that patients had follow-up visits every 3 months for 2 years, then visits every 6 months for 3 years, and then annual visits. DFS was defined as the time from the date of pancreatic resection to the date of local or distant recurrence. The date of recurrence was defined as the date of the first subjective symptom heralding relapse, or the date of documentation of recurrent disease, independent of site, as assessed by diagnostic imaging techniques (whichever occurred first). Data for patients without recurrence were censored at the time of the last follow-up visit. OS was measured from the date of pancreatic resection to the date of death. Fifty-eight patients died during follow-up and the other patients were censored at the time of the last follow-up visit. Data were analyzed in December 2009 and follow-up data from all cases were available. The median observation time for DFS was 9 months (range, 0.5-114 months) and OS was 18 months (range, 0.5-114 months). The clinicopathological characteristics of the tumors collected from 88 patients are provided in Table I. Additionally,

Table I. Clinicopathological characteristics of the patients (n=88).

Median age	65 years (range, 36–86 years)
Gender (male/female)	54 (61.4%)/34 (38.6%)
Histological diagnosis	
Adenocarcinoma	86 (97.7%)
Adenosquamous carcinoma	2 (2.3%)
Adjuvant chemotherapy (AC)	
No	18 (20.5%)
Yes	55 (62.5%)
Gemcitabine-based AC	36 (40.9%)
Other AC	19 (21.6%)
Radiotherapy including IOR	
Yes	23 (26.1%)
No	53 (60.2%)
pT category	
pT1	5 (5.8%)
pT2	3 (3.4%)
pT3	78 (89.7%)
pT4	1 (1.1%)
pN category	
pN0	27 (31.0%)
pN1	60 (69.0%)
UICC stage	
I	6 (6.9%)
II	78 (89.7%)
III	1 (1.1%)
IV	2 (2.3%)
Histological grade	
G1	20 (23.3%)
G2	33 (38.4%)
G3	33 (38.4%)
Residual tumor category	
R0	55 (63.9%)
R1	31 (36.1%)
Vessel invasion	
Positive	55 (63.2%)
Negative	32 (36.8%)
Neural invasion	
Positive	72 (82.8%)
Negative	15 (17.2%)

in order to compare the *EGFR* expression levels in PDAC tissues to those in non-malignant pancreatic specimens, a total of 40 non-malignant pancreatic tissues, including 10 normal pancreatic tissues resected with bile duct carcinoma and 30 chronic pancreatitis tissues, were also obtained.

All resected specimens were fixed in formalin and embedded in paraffin, and all tissues adjacent to the specimens were evaluated histologically according to the criteria of the World Health Organization. Two pathologists were in agreement as regards the pathological features of all cases and the diagnoses were confirmed. The tumor stage was assessed according to the Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer guidelines (18). The study was approved by the Ethics Committee of Kyushu University and conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration.

Immunohistochemistry. A total of 25 sections (4-μm thick) from formalin-fixed paraffin-embedded (FFPE) specimens from 88 patients with PDAC and 15 sections from 40 non-malignant cases, including seven sections from normal pancreas resected with bile duct carcinoma, and 8 sections from chronic pancreatitis patients, were deparaffinized in xylene and hydrated in graded ethanol. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 30 min. Antigen retrieval was achieved by autoclaving the sections in citrate buffer at pH 6.0. The Histofine SAB-PO(R) kit (Nichirei, Tokyo, Japan) was used for immunohistochemical labeling. The sections were incubated with 1.5% normal goat serum/phosphate-buffered saline, followed by incubation with a rabbit polyclonal anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:50 dilution overnight at 4˚C. The sections were incubated with biotinylated anti-rabbit immunoglobulin solution for 20 min followed by peroxidase-labeled streptavidin for 20 min. Immunocomplexes were visualized using stable 3,3'-diaminobenzidine tetrahydrochloride (Dojin, Kumamoto, Japan). The sections were rinsed with distilled water and counterstained with hematoxylin for 10 sec. The amount of EGFR immunoreactivity was evaluated using the following scale according to the percentage of EGFR-positive cancer cells: 0, <5%; 1, 5–25%; 2, 26–50%; and 3, >51%. Staining intensity was scored semi-quantitatively as follows: 0, absent; 1, weak; 2, moderate; 3, strong. To perform the quantitative analysis of EGFR immunoreactivity, the following combined score was determined: Degree of staining = quantity x intensity. We also performed additional staining without primary antibodies as the negative control. All slides were evaluated independently by three investigators (H.F., A.H. and K.N.) without any knowledge of the background of each case.

Cytological specimens. Cytological specimens were obtained at the time of cytological examination and diagnosis from the pathological laboratory of Kyushu University Hospital. In brief, cytological specimens were divided into whole cell pellets (WCP) and into three or more smears as soon as possible after retrieval. Smears were processed in three different ways as described previously (17). Two smears were mounted on standard glass slides for Hemacolor staining (Merck KGaA, Darmstadt, Germany) and Papanicolaou staining, then used for rapid cytological diagnosis and strict cytological diagnosis, respectively. These two smears were examined histologically by cytopathologists and diagnosis was confirmed according to the Papanicolaou Classification. The third smear of each specimen was mounted on membrane slides (Leica Microsystems, Wetzlar, Germany) for laser capture microdissection (LCM). These smears were stained in 1% toluidine blue staining solution or by Hemacolor staining. Twenty cytological specimens were obtained from patients at the Kyushu University Hospital who underwent EUS-FNA cytology and who were cytopathologically diagnosed with PDAC.

Isolation of RNA. Total RNA was isolated from FFPE tissue samples using the RNeasy FFPE kit (Qiagen, Tokyo, Japan) with some modification to the manufacturer's instructions after macrodissection based on a review of representative hematoxylin and eosin-stained slides as described previously (19). Total RNA was extracted from cells isolated by micro-dissection according to the standard acid guanidinium thiocyanate-phenol-chloroform protocol (20), with or without glycogen (Funakoshi, Tokyo, Japan).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCR was performed using the Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and the LightCycler 480 II Real-Time PCR System (Roche Diagnostics) for 40 cycles of 15 sec at 95˚C and 1 min at 55˚C with the QuantiTect SYBR-Green Reverse Transcription-PCR kit (Qiagen) in accordance with the manufacturer's instructions (21). We designed specific primers for *EGFR* (forward primer, 5'-cctatgtgcagaggaa ttatgatcttt-3'; and reverse primer, 5'-ccactgtgttgagggcaatg-3') and *β-actin* (forward primer, 5'-tgagcgcggctacagctt-3'; and reverse primer, 5'-tccttaatgtcacgcacgattt-3'), and screened a database using BLASTN to confirm the primer specificities. The level of each mRNA was calculated from a standard curve constructed using total RNA from Capan-1, a human pancreatic cancer cell line. The level of *EGFR* mRNA was normalized to that of *β-actin*. The PCR product sizes of *EGFR* and *β-actin* primers were small [88 base pairs (bp) and 59 bp, respectively], which allowed for accurate and sensitive qRT-PCR analysis despite the fragmented RNA extracted from the FFPE tissue specimens (22,23).

Statistical analyses. Statistical analyses and graphical presentations were performed using JMP 7.01 software (SAS Institute, Cary, NC, USA). Values were expressed as the means ± SD. Data were analyzed using the Kruskal-Wallis test if comparisons involved three groups, and the Mann-Whitney U-test and Spearman's rank-correlation test if comparisons involved two groups as normal distributions were not obtained. *EGFR* expression was split into high- and low-level groups using recursive descent partition analysis, as described by Hoffmann *et al* (24). Categorical variables were compared using the χ^2 test (Fisher's exact probability test). Survival curves were constructed using the Kaplan-Meier product-limit method and were compared using the log-rank test. To evaluate independent prognostic factors associated with survival, multivariate Cox

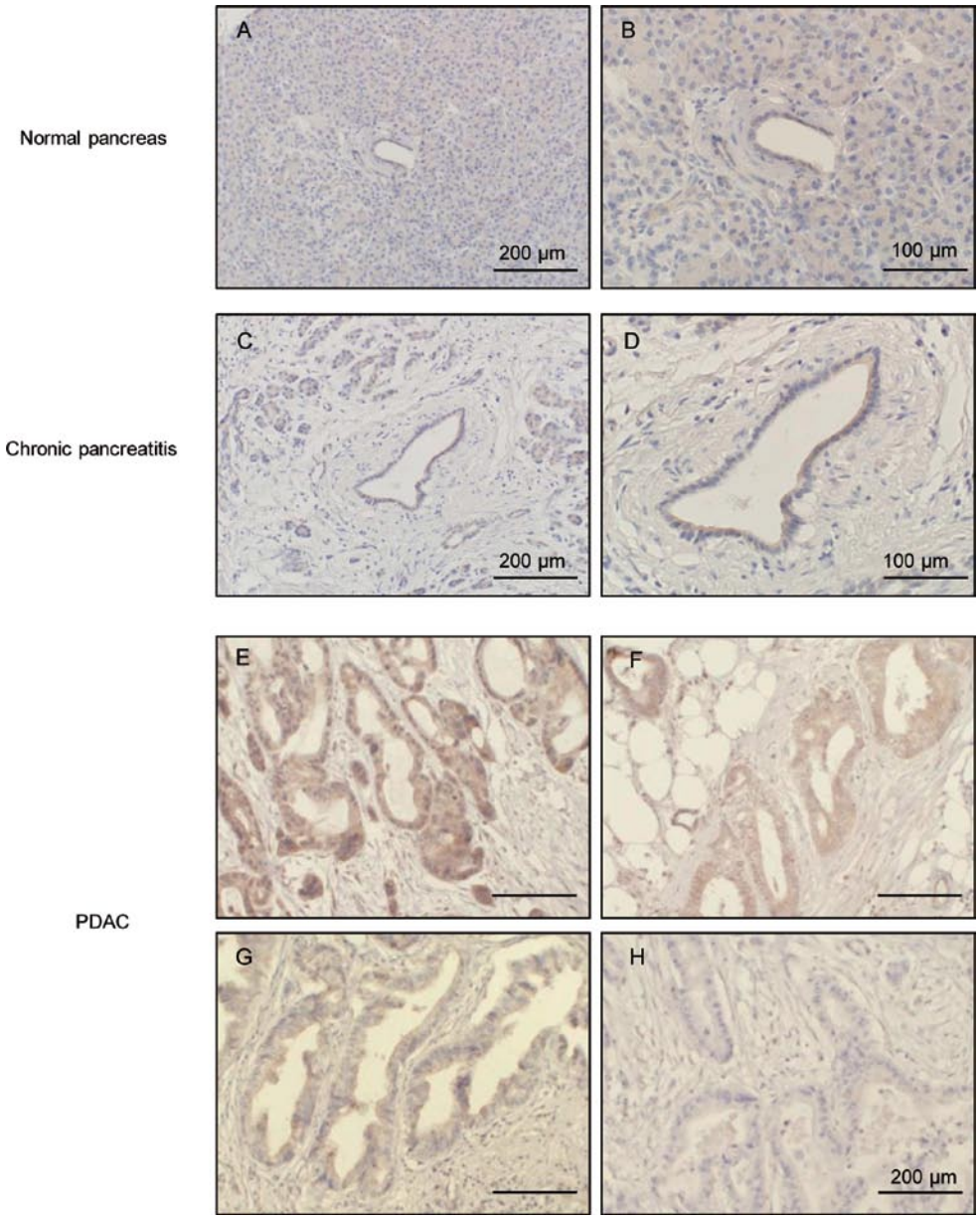

Figure 1. Immunohistochemical analysis of EGFR in normal pancreas, chronic pancreatitis and PDAC tissues. Weak to moderate immunoreactivity for EGFR was detected in some acinar cells and pancreatic ductal cells (A-D). In PDAC tissues, immunoreactivity for EGFR was observed on the surface and in the cytoplasm of cancer cells (E-G), with no immunoreactivity in the surrounding stroma (E-H). The immunoreactivity was different in respective cases (E, strong; F, moderate; G, weak expression; H, absent). Scale bars represent 200 μm (A, C, E-H) and 100 μm (B and D).

proportional hazards regression analysis was used. Statistical significance was defined as a p-value of <0.05.

Results

EGFR protein expression was correlated with EGFR mRNA expression. We performed immunohistochemical analyses on 15 sections of non-malignant pancreatic tissues, including 7 normal, 8 chronic pancreatitis tissues and 25 PDAC tissues. In agreement with the findings of previous studies (10,13), weak to moderate immunoreactivity for EGFR was detected in some acinar cells and pancreatic ductal cells (Fig. 1A-D). EGFR immunoreactivity was observed on the surface and in the cytoplasm of cancer cells within PDAC tissues, but none was observed in the surrounding stroma (Fig. 1E-G) (10,13). To investigate the correlation between EGFR immunoreactivity and *EGFR* mRNA expression levels within each FFPE tissue sample from resected PDAC tissue, we evaluated the degree of staining (quantity x intensity) for an anti-EGFR antibody, as the immunoreactivity was different in respective cases (Fig. 1E, strong; F, moderate; G, weak expression; and H, absent). We found a significant correlation between the degree of staining and *EGFR* mRNA expression levels [Fig. 2A; Spearman's rank-correlation coefficient (ϱ): 0.729, p<0.0001], and cases with a higher degree of immunoreactivity expressed significantly higher levels of *EGFR* mRNA compared with those with a lower degree of immunoreactivity (Fig. 2B; p=0.0005). These observations suggest that quantitative mRNA analysis of *EGFR* in macrodissected PDAC tissues may reflect *EGFR* protein expression levels in EGFR-

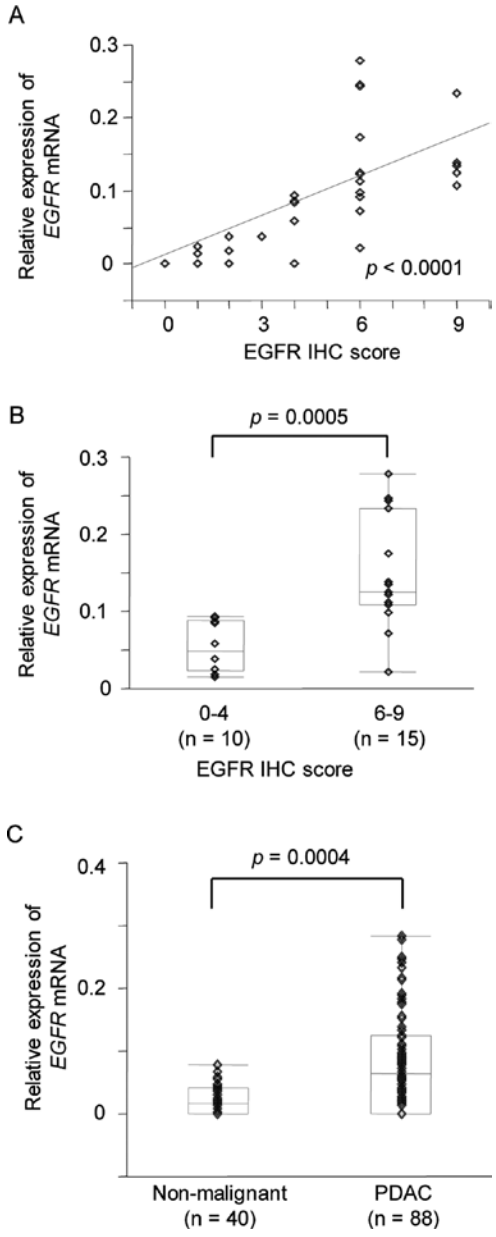

Figure 2. Correlation between EGFR immunoreactivity and *EGFR* mRNA expression levels in each FFPE tissue sample from resected PDAC tissues (A; n=25). We observed a significant correlation between the degree of staining (quantity x intensity) and *EGFR* mRNA expression levels [A; Spearman's rank-correlation coefficient (ϱ): 0.729, p<0.0001], and cases with a higher degree of staining (6-9; n=15) expressed significantly higher levels of *EGFR* mRNA compared to cases with a lower degree of staining (0-4; n=10) (B; p=0.0005). We found that *EGFR* expression levels in the PDAC samples (n=88) were significantly higher than those in the non-malignant samples (n=40) (C; p=0.0004).

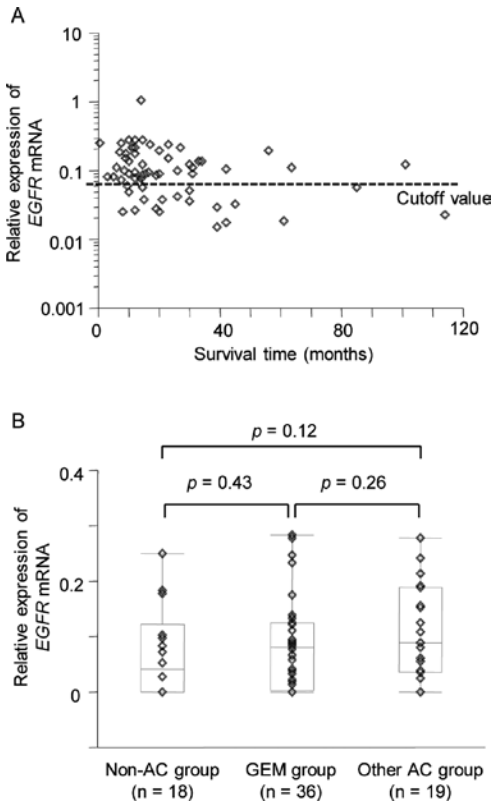

Figure 3. Quantitative analysis of *EGFR* mRNA expression levels in FFPE tissue samples from resected PDAC tissues (n=88) using qRT-PCR. After normalization to *β-actin* expression, we obtained 2 groups with high *EGFR* expression and low *EGFR* expression using a cut-off value (0.058) determined with recursive descent partition analysis, respectively (A). There was no significant difference in *EGFR* mRNA levels between the respective groups (non-AC group, n=18; GEM group, n=36; other AC group, n=19; p=0.26) (B).

expressing cancer cells. Additionally, although there was immunoreactivity for EGFR in some acinar and ductal cells in non-malignant cases, we found that *EGFR* expression levels in PDAC samples (n=88) were significantly higher than those in non-malignant samples (n=40) (Fig. 2C; p=0.0004).

Univariate and multivariate analyses of EGFR mRNA expression and survival time. We quantified *EGFR* mRNA expression levels in FFPE tissue samples from resected PDAC tissues using qRT-PCR. After normalization to *β-actin*, we obtained two groups (high *EGFR* expression and low *EGFR* expression) using a cut-off value (0.058) determined by recursive descent partition analysis of all patients (n=88) (Fig. 3A) (24). The high and low *EGFR* expression groups comprised of 46 and 42 cases, respectively. The relationship between *EGFR* mRNA expression and the clinicopathological factors seen in PDAC patients is shown in Table II. We found no significant correlation between *EGFR* mRNA expression and clinicopathological factors. In addition, there was no significant difference in *EGFR* mRNA levels between the non-AC group, the GEM group, and the other AC group (p=0.26; Fig. 3B).

Initially, we examined the independent markers that indicated poor prognosis in the 88 PDAC patients. Univariate analyses for DFS and OS (Table III) showed that conventional prognostic markers, such as pN status (p=0.0009 and p=0.0026, respectively), residual tumor category (R factor) (p<0.0001 and p<0.0001, respectively), and positive vessel invasion (p=0.0018 and p=0.0035, respectively) reached statistical significance, whereas the effect of AC did not (p=0.23 and p=0.066, respectively). High *EGFR* levels after normalization to *β-actin* were associated with a shorter DFS and OS (Table III and Fig. 4A-B; p=0.029 and p=0.014, respectively). Multivariate analysis based on the Cox proportional hazards model was performed on all parameters that were found to be significant by univariate analyses for DFS (Table IV) and OS

Table II. Relationship between *EGFR* mRNA expression and various clinicopathological factors.

Characteristics	*EGFR* mRNA expression[a]		P-value
	High-level group (n=46)	Low-level group (n=42)	
Age			0.808
≥65 years	24 (52.2%)	23 (54.8%)	
<65 years	22 (47.8%)	19 (45.2%)	
Adjuvant chemotherapy (AC)			0.484
No	8 (17.4%)	10 (23.8%)	
Yes	33 (71.7%)	22 (52.4%)	
Gemcitabine-based AC	21 (45.7%)	15 (35.7%)	
Other AC	12 (26.1%)	7 (16.7%)	
Radiotherapy			0.368
Yes	10 (21.7%)	13 (30.9%)	
No	29 (63.0%)	24 (57.1%)	
pT category			0.543
pT1/pT2	5 (10.9%)	3 (7.1%)	
pT3/pT4	41 (89.1%)	39 (92.9%)	
pN category			0.169
pN0	11 (23.9%)	16 (38.1%)	
pN1	34 (73.9%)	26 (61.9%)	
UICC stage			0.804
I	3 (6.5%)	3 (7.1%)	
II	41 (89.1%)	37 (88.1%)	
III/IV	1 (2.2%)	2 (4.8%)	
Histological grade			0.220
G1	10 (21.7%)	10 (23.8%)	
G2	14 (30.4%)	19 (45.2%)	
G3	21 (45.7%)	12 (28.6%)	
Residual tumor category			0.336
R0	26 (56.5%)	29 (69.0%)	
R1	18 (39.1%)	13 (30.9%)	
Vessel invasion			0.256
Positive	31 (67.4%)	24 (57.1%)	
Negative	14 (30.4%)	18 (42.9%)	
Neural invasion			0.891
Positive	37 (80.4%)	35 (83.3%)	
Negative	8 (17.4%)	7 (16.7%)	

[a]Cut-off value (0.058) was determined by recursive descent partition analysis of all patients (n=88).

(Table V). DFS was significantly dependent on the R factor (p<0.0001) and vessel invasion (p=0.038), whereas OS was significantly dependent on the R factor alone (p<0.0001). The effect of high *EGFR* levels did not reach statistical significance for either DFS or OS.

In order to determine which parameters were predictive for gemcitabine sensitivity, we then evaluated the correlation between each parameter and DFS in the GEM and non-AC groups. We found no significant correlation between the level of *EGFR* mRNA expression and clinicopathological factors in the GEM group (Table VI). Univariate survival analyses of the GEM group showed that pN status (p=0.0094), residual tumor (p=0.0004), and high *EGFR* level normalized to *β-actin* (Fig. 5A; p=0.068) reached statistical significance for DFS (Table VII). However, there was no significant correlation between the *EGFR* expression level and DFS in the non-AC group (p=0.30, Fig. 5C), although the number of patients who did not receive AC was limited. Multivariate analysis of the GEM group (Table VIII) showed that DFS was significantly dependent on both the R factor (p=0.0071) and high *EGFR* levels (p=0.010).

Similarly, we evaluated the correlation between each parameter and OS in the GEM and non-AC groups. Univariate survival analyses of the GEM group showed that the conventional prognostic markers, pN status (p=0.020), R factor (p=0.013), and high *EGFR* levels normalized to *β-actin* (Fig. 5B; p=0.054) reached statistical significance for OS (Table VII). However, the effect of *EGFR* expression levels

Table III. Univariate survival analysis of conventional prognostic factors and *EGFR* mRNA expression (n=88).

Characteristics	Number of cases	Median DFS (months)	P-value	Median OS (months)	P-value	5-year survival rate
EGFR mRNA expression[a]			0.029[b]		0.014[b]	
High	42	7.0		14.6		19.9%
Low	46	25.0		35.5		37.9%
Age			0.96		0.93	
≥65 years	47	12.0		26.0		24.6%
<65 years	41	8.0		19.0		35.4%
Adjuvant chemotherapy (AC)			0.23		0.066	
Yes	64	12.0		23.0		33.4%
No	20	7.0		12.1		14.3%
Radiotherapy			0.77		0.58	
Yes	23	12.0		23.0		29.5%
No	53	10.0		20.0		22.4%
pT category			0.34		0.54	
pT1/pT2	8	22.0		63.0		62.5%
pT3/pT4	80	8.0		23.0		25.5%
pN category			0.0009[b]		0.0026[b]	
pN0	27	36.0		45.0		49.3%
pN1	60	8.0		16.3		19.0%
UICC stage			0.14		0.28	
I	6	26.5		63.0		83.3%
II	78	8.0		20.9		25.3%
III/IV	3	16.0		19.8		0%
Histological grade			0.072		0.16	
G1/G2	54	14.0		30.0		31.9%
G3	33	8.0		14.6		27.4%
Residual tumor category			<0.001[b]		<0.001[b]	
R0	55	26.0		43.0		43.4%
R1	31	5.0		12.0		5.3%
Vessel invasion			0.0018[b]		0.0035[b]	
Positive	55	7.0		16.9		18.7%
Negative	32	31.0		45.0		45.0%
Neural invasion			0.95		0.76	
Positive	72	10.0		23.0		26.0%
Negative	15	14.0		19.0		24.3%

[a]Cut-off value (0.058) was determined by recursive descent partition analysis of all patients (n=88); [b]p<0.05.

Table IV. Multivariate DFS analysis (Cox regression model) of conventional prognostic factors and *EGFR* mRNA.

Characteristics	Relative risk	95% Confidence interval	P-value
High *EGFR* levels[a]	1.208	0.680-2.192	0.523
pN category (pN1)	1.939	0.966-4.166	0.063
Residual tumor category (pR1)	4.957	2.647-9.281	<0.0001[b]
Positive vessel invasion	1.942	1.036-3.825	0.038[b]

[a]Cut-off value (0.058) was determined by recursive descent partition analysis of all patients (n=88); [b]p<0.05.

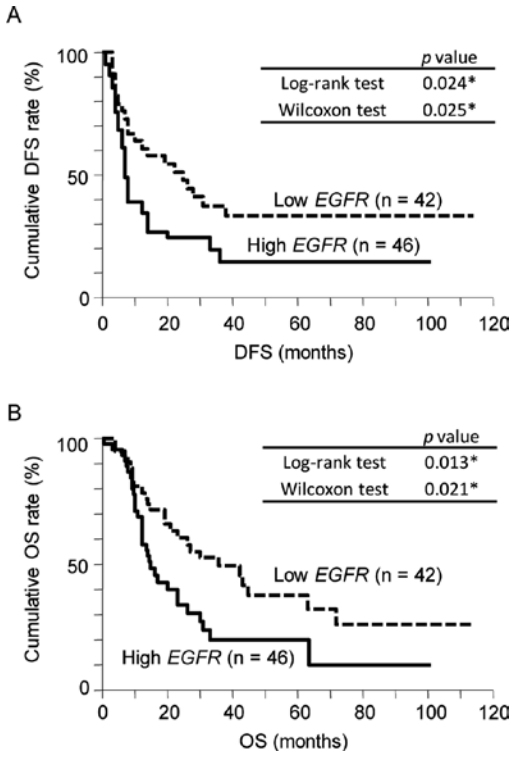

Figure 4. DFS and OS after resection of PDAC with high versus low *EGFR* expression. High *EGFR* mRNA levels were associated with a shorter DFS (A, p=0.029) and a shorter OS (B, p=0.014). *p<0.05.

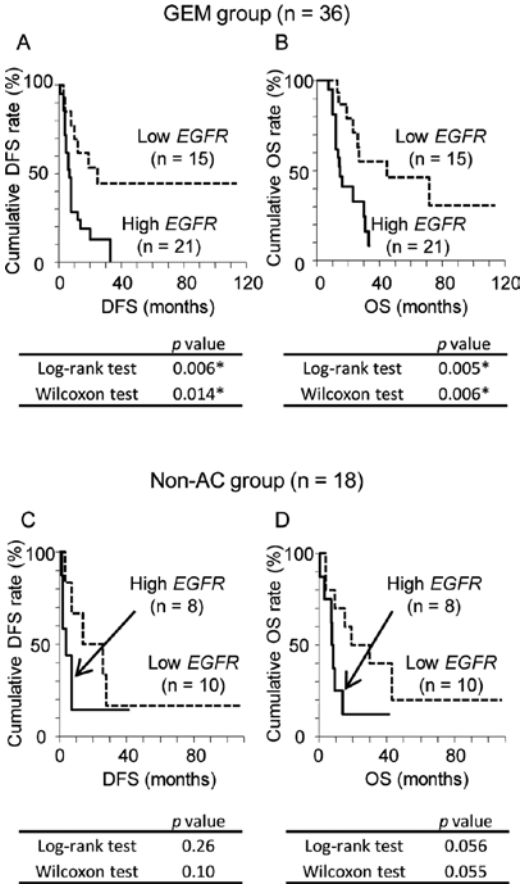

Figure 5. DFS and OS after resection of PDAC with high versus low *EGFR* expression in the GEM (A and B; n=36) and non-AC (C and D; n=18) groups. High *EGFR* mRNA levels were associated with a shorter DFS (A, p=0.068) and a shorter OS (B, p=0.054) in the GEM group. In contrast, there was no significant correlation between *EGFR* expression levels and DFS (C, p=0.30) or OS (D, p=0.07) in the non-AC group. *p<0.05.

did not reach significance in the non-AC group (p=0.07, Fig. 5D). Multivariate analysis of the GEM group (Table IX) showed that OS was significantly dependent on pN status (p=0.024), R factor (p=0.045), and high *EGFR* levels (p=0.023). These data suggest that high *EGFR* expression is a significant predictor for reduced DFS and a significant prognostic indicator for reduced OS, especially in those patients receiving gemcitabine-based AC.

Quantitative analysis of EGFR expression in cells micro-dissected from cytological specimens. In order to apply this prediction of outcome for PDAC patients receiving gemcitabine-based chemotherapy based on *EGFR* expression levels to a clinical setting, we quantified the *EGFR* mRNA levels in cytological specimens obtained from 20 patients with PDAC who underwent EUS-FNA cytological examination at our institute. Although some samples contained abundant neoplastic cells, most samples contained a large amount of blood and inflammatory cells and scarce clusters of neoplastic cells (Fig. 6A-B). Therefore, we quantified the *EGFR* mRNA levels in the WCP and LCM samples, and then compared the expression levels between the two. We were unable to detect clear differences in *EGFR* mRNA levels in the WCP samples. However, we distinguished higher and lower expression levels of the mRNA in the LCM samples (Fig. 6C). These data suggest that the quantification of *EGFR* expression levels in microdissected neoplastic cells could be a potent tool for

Table V. Multivariate OS analysis (Cox regression model) of conventional prognostic factors and *EGFR* mRNA.

Characteristics	Relative risk	95% Confidence interval	P-value
High *EGFR* levels (>0.058)[a]	1.649	0.958-2.873	0.071
pN category (pN1)	1.671	0.888-3.350	0.114
Residual tumor category (pR1)	3.059	1.762-5.324	<0.0001[b]
Positive vessel invasion	1.784	0.979-3.416	0.059

[a]Cut-off value (0.058) was determined by recursive descent partition analysis of all patients (n=88); [b]p<0.05.

Table VI. Relationship between *EGFR* mRNA expression and various clinicopathological factors in the GEM group (n=36).

Characteristics	*EGFR* mRNA expression[a]		P-value
	High-level group (n=46)	Low-level group (n=42)	
Age			0.176
≥65 years	11 (52.4%)	4 (26.7%)	
<65 years	10 (47.6%)	11 (73.3%)	
Gender			0.736
Male	14 (66.7%)	9 (60.0%)	
Female	7 (33.3%)	6 (40.0%)	
Radiotherapy			0.084
Yes	2 (9.5%)	5 (33.3%)	
No	18 (90.5%)	8 (66.7%)	
pT category			1.000
pT1/pT2	1 (4.8%)	0 (0%)	
pT3/pT4	20 (95.2%)	15 (100%)	
pN category			0.694
pN0	4 (19.0%)	4 (26.7%)	
pN1	17 (81.0%)	11 (73.3%)	
UICC stage			1.000
I	-	-	
II	20 (95.2%)	14 (93.3%)	
III/IV	1 (4.8%)	1 (6.7%)	
Histological grade			0.297
G1	4 (19.0%)	3 (20.0%)	
G2	5 (23.8%)	7 (46.7%)	
G3	12 (57.2%)	5 (33.3%)	
Residual tumor category			0.282
R0	12 (57.2%)	12 (80.0%)	
R1	9 (4.8%)	3 (20.0%)	
Vessel invasion			0.499
Positive	15 (71.4%)	9 (60.0%)	
Negative	6 (28.6%)	6 (40.0%)	
Neural invasion			1.000
Positive	17 (81.0%)	13 (86.7%)	
Negative	4 (19.0%)	2 (13.3%)	

[a]Cut-off value (0.058) was determined by recursive descent partition analysis of all patients (n=88) and the GEM group (n=36).

predicting the outcome of PDAC patients, even when specimens contain abundant contaminated cells.

Discussion

There is increasing evidence showing the usefulness of immunohistochemical analysis of molecular markers, including EGFR, for predicting the clinical outcome of PDAC patients (10,11,13,25,26). Immunohistochemical analysis is a valid method as it shows protein expression. However, the clinical introduction of immunohistochemical assessment for predicting sensitivity to chemotherapeutic agents is still problematic due to difficulties in quantitative measurement (inter-observer variations in interpretation) and the lack of calibrated quantification techniques (27-29). In addition, only 10-20% of patients with PDAC are candidates for curative resection (3). Therefore, the remaining 80–90% of patients with advanced PDAC need cytopathological assessment with EUS-FNA, or pancreatic juice, to predict their sensitivity to chemotherapeutic agents for individualized chemotherapy. The present analysis of *EGFR* mRNA is quantitative (even considering the small amount of specimens available, including cytological specimens). For these reasons, quantitative mRNA analysis of genes associated with tumor sensitivity, or with resistance to anti-tumor agents, could be preferred to immunohistochemical analysis in a clinical setting. Furthermore, we introduced the use of LCM to obtain target cells from EUS-FNA cytological specimens (17). As a result, we found that *EGFR* mRNA levels in microdissected neoplastic cells were easier to distinguish than those in WCP, suggesting that quantification of the expression levels of individual genes in microdissected neoplastic cells could be a potent tool for

Table VII. Univariate survival analysis of conventional prognostic factors and *EGFR* mRNA expression in the GEM group (n=36).

Characteristics	Number of cases	Median DFS (months)	P-value	Median OS (months)	P-value	5-year survival rate
EGFR mRNA expression[a]			0.0068[b]		0.0054[b]	
High	42	7		14.6		8.2%
Low	46	25		45.0		46.0%
Age			0.57		0.92	
≥65 years	17	8		27.0		24.8%
<65 years	23	10		23.0		26.8%
Gender			0.45		0.71	
Male	26	14		27.0		20.9%
Female	14	6		13.7		27.7%
Radiotherapy			0.32		0.24	
Yes	9	12		27.0		34.3%
No	27	8		19.0		12.1%
pT category			0.25		0.09	
pT1/pT2	1	4		10.0		0.0%
pT3/pT4	39	10		26.0		26.1%
pN category			0.0094[b]		0.020[b]	
pN0	9			45.0		46.7%
pN1	31	8		19.0		19.0%
UICC stage			0.62		0.35	
I	0	-		-		-
II	37	8		23.0		27.4%
III/IV	3	2		19.0		0.0%
Histological grade			0.086		0.071	
G1/G2	23	14		31.0		33.3%
G3	17	8		19.0		13.9%
Residual tumor category			0.0004[b]		0.013[b]	
R0	26	19		30.2		39.6%
R1	14	5		13.7		0.0%
Vessel invasion			0.079		0.26	
Positive	26	8		23.0		23.6%
Negative	14	25		31.0		29.2%
Neural invasion			0.56		0.84	
Positive	33	9		26.0		23.5%
Negative	7	8		15.6		50.0%

[a]Cut-off value (0.058) was determined by recursive descent partition analysis of all patients (n=88) and the GEM group (n=36); [b]p<0.05.

Table VIII. Multivariate DFS analysis (Cox regression model) of conventional prognostic factors and *EGFR* mRNA expression levels in the GEM group (n=36).

Characteristics	Relative risk	95% Confidence interval	P-value
pN status (pN1)	2.654	0.892-11.41	0.083
Residual tumor category (pR1)	3.197	1.383-7.365	0.0071[b]
High *EGFR* levels (>0.058)[a]	3.016	1.292-7.742	0.010[b]

[a]Cut-off value (0.058) was determined by recursive descent partition analysis of all patients (n=88) and the GEM group (n=36); [b]p<0.05.

Table IX. Multivariate OS analysis (Cox regression model) of conventional prognostic factors and *EGFR* mRNA expression levels in the GEM group (n=36).

Characteristics	Relative risk	95% Confidence interval	P-value
pN status (pN1)	3.451	1.157-14.89	0.024[b]
Residual tumor category (pR1)	2.442	1.021-5.858	0.045[b]
High *EGFR* levels (>0.058)[a]	2.882	1.154-8.194	0.023[b]

[a]Cut-off value (0.058) was determined by recursive descent partition analysis of all patients (n=88) and the GEM group (n=36); [b]p<0.05.

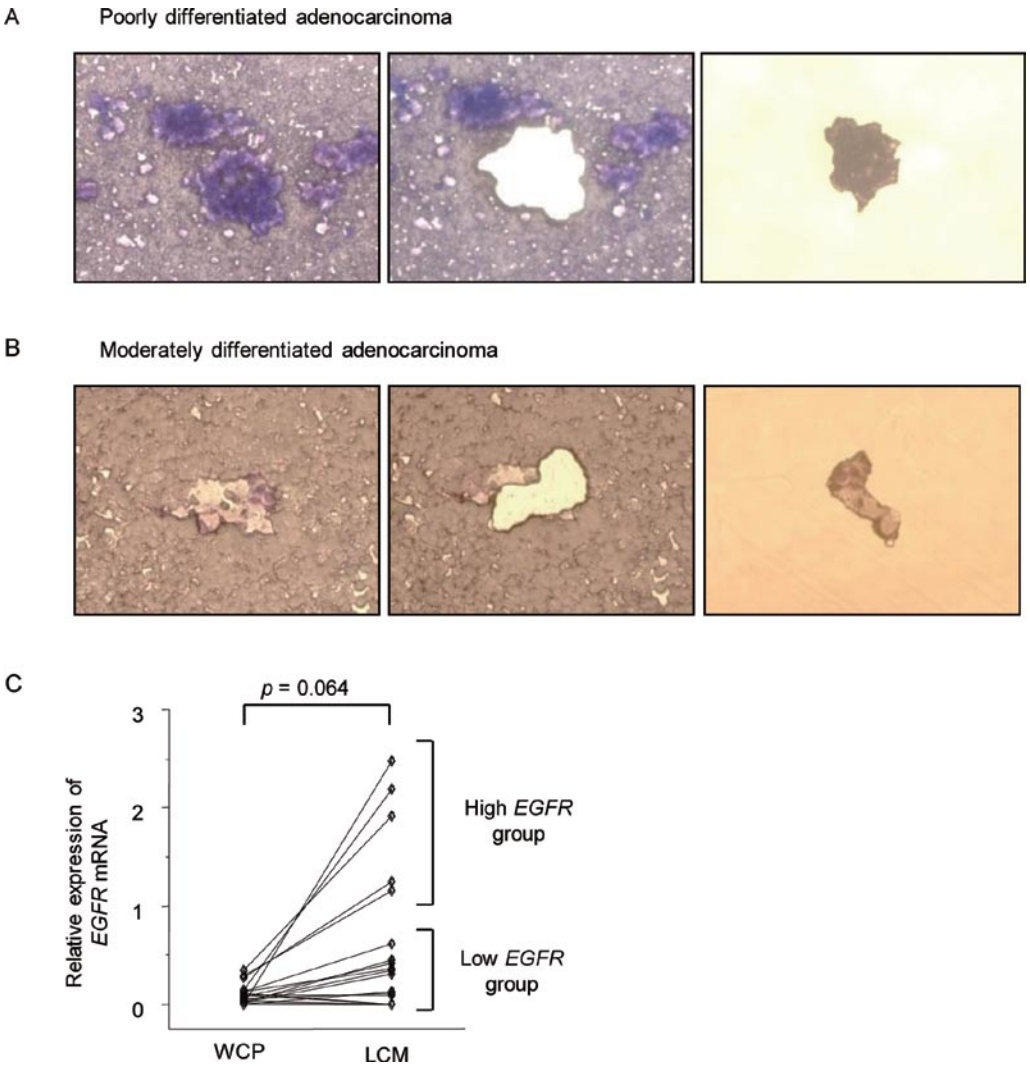

Figure 6. Quantitative analyses of *EGFR* mRNA in EUS-FNA cytological specimens. Representative micrographs of cytological specimens obtained from patients with PDAC who underwent EUS-FNA cytological examination (A and B). Most samples contained a large amount of blood and inflammatory cells with scarce clusters of neoplastic cells. (C) Quantitative analysis of *EGFR* in EUS-FNA cytological specimens (n=20). Although we did not detect clear changes in expression levels in the WCP samples, we distinguished samples having higher and lower *EGFR* expression levels in the microdissected neoplastic cells (C).

predicting sensitivity to anti-tumor agents, even when specimens contain contaminated cells. However, further investigations, including prospective studies, are required before this approach can be introduced into the clinical setting.

As EGFR plays a crucial role in controlling the activity of the Ras/MAPK signaling pathway (8,9), great efforts have been made to develop strategies targeting EGFR (30). In xenograft models of pancreatic cancer, the combination of gemcitabine and EGFR-targeted therapy significantly inhibited lymph node and liver metastases and improved OS (16). A randomized, placebo-controlled phase III trial comparing erlotinib, an EGFR tyrosine kinase inhibitor (TKI), plus gemcitabine to gemcitabine alone in patients with locally advanced or metastatic pancreatic cancer, demonstrated that

the combination of erlotinib and gemcitabine provided a small, yet statistically significant survival benefit (31). However, the efficacy of EGFR TKI in pancreatic cancer trials has not met expectations, not as it has in a proportion of patients with non-small cell lung cancer (NSCLC) (30). This is likely due to differences in the presence of activating mutations within EGFR, which are associated with prolonged responses in NSCLC (32). Many studies have identified activating EGFR gene mutations in only a small number of cases in PDAC patients (33,34). However, Tan *et al* demonstrated that higher genetic amplification of the *EGFR* region of chromosome 7 is associated with better clinical responses to erlotinib treatment in advanced NSCLC patients (35). Therefore, there is a possibility that quantitative analysis of *EGFR* mRNA expression levels could be helpful in predicting sensitivity to erlotinib in PDAC patients. However, further investigations incorporating larger patient numbers are required to evaluate the usefulness of this approach.

In conclusion, we demonstrate that quantitative analysis of *EGFR* mRNA expression using FFPE tissue samples is useful for predicting the prognosis of PDAC patients receiving gemcitabine-based AC. In addition, quantitative analysis of *EGFR* mRNA in neoplastic cells microdissected from EUS-FNA specimens is useful for determining treatment for patients with PDAC, even when the tumor is unresectable. Thus, quantitative analysis of genes associated with sensitivity to cytotoxic agents could be a potent tool for individualized chemotherapy.

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