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Membranous and cytoplasmic expression of epidermal growth factor receptor in metastatic pancreatic ductal adenocarcinoma

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Abstract. Recent studies indicate the clinical significance of the cellular localization of epidermal growth factor receptor (EGFR) in a variety of cancer types. Internalization of activated EGFR is reported to be closely associated with patient prognosis. This study investigated the clinical significance of the immunohistochemical localization of EGFR in patients with metastatic pancreatic cancers compared to those with surgically resected pancreatic cancers. Using 44 surgically resected primary pancreatic cancers and 40 primary or metastatic tumors from 20 autopsied patients with far advanced pancreatic cancers, the incidence of membranous and cytoplasmic EGFR overexpression was compared between primary tumors and far advanced tumors by immunohistochemistry using the Dako EGFR pharmDx™ kit, a global standard kit for EGFR assay. In the 44 surgically resected cancers, 13 (30%) exhibited membranous overexpression of EGFR, comprising 1 case (2%) with score 3+ and 12 cases (27%) with score 2+ and 10 (23%) exhibited cytoplasmic overexpression of EGFR. In the 40 tumors at a far advanced stage, the percentage of samples exhibiting positivity for membranous and cytoplasmic EGFR overexpression was 48% (19 of 40) comprising 7 (18%) with score 2+ and 12 (30%) with score 3+ and 33% (13 of 40), respectively. The far advanced tumors tended to show membranous and cytoplasmic EGFR overexpression more frequently than the surgically resected tumors, although the difference was not significant. These findings suggest that membranous and cytoplasmic overexpression of EGFR may be indicative of the potential aggressiveness of pancreatic cancers.

Introduction

Despite recent advances in diagnostic and therapeutic techniques, pancreatic carcinoma is one of the most lethal malignancies among cancers. The 5-year survival rate of patients having primary pancreatic cancer after complete resection does not reach 15% (1), while the overall 5-year survival rates in patients having inoperable pancreatic cancer are desperately low, ranging from 0.4 to 4% (2,3).

Currently, gemcitabine is a key drug not only for treating advanced pancreatic cancer (4) but also as an adjuvant chemotherapy regimen for resectable pancreatic cancer (5,6). Furthermore, molecular targeting of epidermal growth factor receptor (EGFR) or vascular endothelial growth factor (VEGF) has recently been developed to treat these lesions (7,8). Moore *et al* reported in a phase III trial of patients with advanced pancreatic cancer, that erlotinib, a tyrosine kinase inhibitor of EGFR, in combination with gemcitabine was superior to gemcitabine alone when progression-free and overall survival were compared between the two groups (8).

EGFR, one of the tyrosine kinase receptors of the ErbB family, is reported to be expressed immunohistochemically in 10-30% of patients with solid tumors including pancreatic carcinoma (9,10). Tyrosine phosphorylation in EGFR protein in cancer cells leads to activation of several downstream intracellular substrates and plays a pivotal role in tumor proliferation, invasion and metastasis (11). Recent studies have suggested that the EGFR gene copy number and expression obtained by fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC) predict the clinical response of a tumor to gefitinib, a tyrosine kinase inhibitor of EGFR, in patients with non-small cell lung cancer (12-14). Furthermore, recent studies have found that mutations of the EGFR gene at the restricted region, e.g., exon 19 and exon 21, were closely correlated with response to gefitinib therapy (15-20). However, the relevance of EGFR expression in pancreatic cancer with therapeutic response has remained to be verified (8).

Although immunohistochemical expression of EGFR can also be recognized as positive membranous staining,

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Table I. Clinicopathological characteristics of the patients and tumors.

Parameter	Surgically resected cancers (n=44)	Far advanced cancers (n=20)	
	n (%)	n (%)	
Age (mean \pm SD, years)	63.3 \pm 3.7	57.3 \pm 5.7	
<65	23 (52)	13 (65)	
\geq 65	21 (47)	7 (35)	
Gender			
Male	34 (77)	16 (80)	
Female	10 (23)	4 (20)	
Tumor site			
Head	36 (82)	12 (60)	
Body and/or tail	8 (18)	8 (40)	
Stage			
I	1 (2)		
II	32 (73)		
III	8 (18)		
IV	3 (7)		
Grade			
1	12 (27)	5 (25) ^a	2 (10) ^b
2	28 (64)	3 (15) ^a	9 (45) ^b
3	4 (9)	12 (60) ^a	9 (45) ^b
Median survival (mean \pm SD, month)	24.5 \pm 10.3		

^aPrimary cancers. ^bHepatic metastases. SD, standard deviation.

cytoplasmic expression of EGFR can frequently be observed in cancer cells of the pancreas. We previously reported that high cytoplasmic expression of EGFR in primary pancreatic cancer was significantly correlated with higher histological grade and poorer survival (10), suggesting that cytoplasmic EGFR expression could indicate a potentially aggressive or metastatic feature of pancreatic cancer. However, it is unclear whether localization of EGFR expression differs between primary and metastatic sites of pancreatic cancers at surgically resectable stages and those at inoperable far advanced stages.

The present study compared immunohistochemically the levels and localization of EGFR expression between surgically resected primary pancreatic cancers and far advanced cancers obtained at autopsy, in order to clarify the clinical impact of membranous and cytoplasmic EGFR overexpressions in far advanced pancreatic cancers.

Materials and methods

Patients and tumor specimens. This study was performed with approval by the Internal Review Board on Ethical Issues of the National Defense Medical College, Japan. The subjects of this study were 44 patients who underwent surgery with curative intent for primary pancreatic cancers between 1987 and 2000 at the National Defense Medical College Hospital, Tokorozawa, Japan. The clinicopathological characteristics of these cases are summarized in Table I.

The mean patient age was 63.3 years [\pm 3.7 standard deviation (SD)]. Thirty-four (77.3%) were men and 10 (22.7%) were women. More than 80% of tumors were located in the head of the pancreas. As for stage, approximately 90% of the patients were assigned to stage II or stage III (21). Histologically, all 44 patients had invasive ductal adenocarcinoma of the pancreas, and the majority of the patients had moderately differentiated tubular adenocarcinoma. The median survival time was 24.5 months (\pm 10.3 SD).

In addition, a total of 40 tumor specimens from primary sites and hepatic metastatic sites were obtained at autopsy from 20 patients who had died of inoperable far advanced pancreatic cancer between 1980 and 2001 at the same hospital (Table I).

Using these tumor specimens from a total of 64 patients, formalin-fixed paraffin-embedded tissue blocks were prepared, and sections were cut and stained with hematoxylin and eosin (H&E) for routine histopathological examination. Because surgically resected specimens had been cut routinely for pathology specimens once a week periodically, the duration of formalin fixation of the surgically resected specimens varied from 1 to 6 days. Likewise, the duration of formalin fixation of the autopsied tissues varied from 1 to 6 days. All specimens were diagnosed as ductal adenocarcinomas of the pancreas. After a histological review of the sections by three observers (T.E., H.T. and S.U.), a representative tissue block was selected from each surgically resected primary tumor, each primary tumor obtained by autopsy, and each metastatic tumor obtained

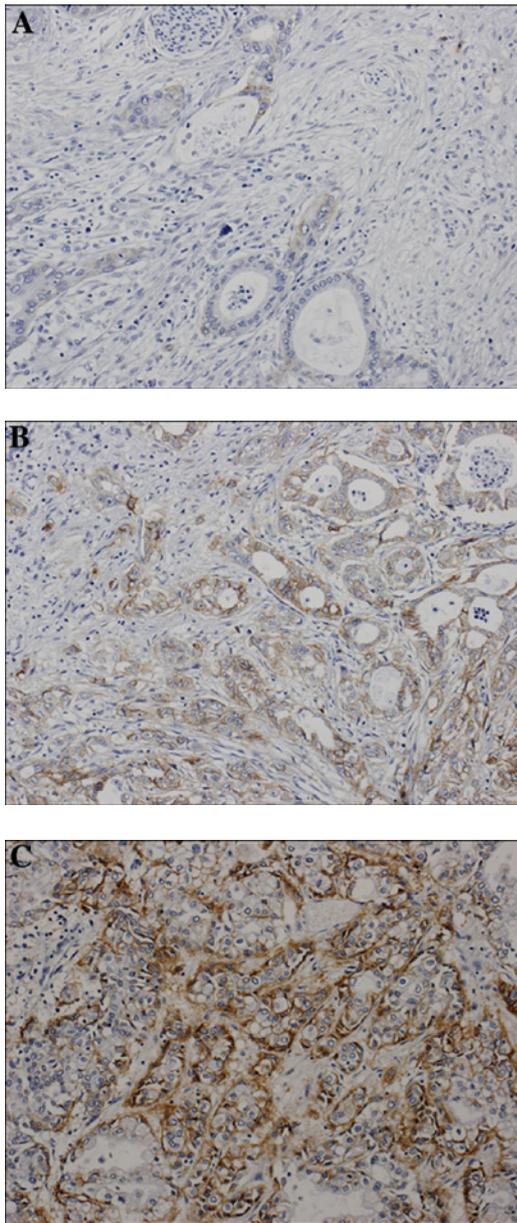


Figure 1. Representative cases of pancreatic ductal adenocarcinoma showing scores of 1+, 2+ and 3+ for membranous EGFR expression. (A) Score 1+, incomplete membrane staining is weakly visible. (B) Score 2+, the entire circumference of the cell membrane is weakly stained. (C) Score 3+, the entire circumference of the cell membrane is heavily stained. Immunoperoxidase stain, x200.

by autopsy. These tumor tissue blocks were subjected to immunohistochemical studies.

Histological classification. The three observers graded the degree of tumor differentiation. Tumor differentiation was classified into Grade 1 (well-differentiated type), Grade 2 (moderately differentiated type) and Grade 3 (poorly differentiated type), according to the degree of tubular formation (21). The grade of each primary cancer was defined according to the findings in the widest area of the representative section of the cancer.

Immunohistochemistry. Immunohistochemical staining for EGFR was performed using the EGFR pharmDx™ kit (Dako,

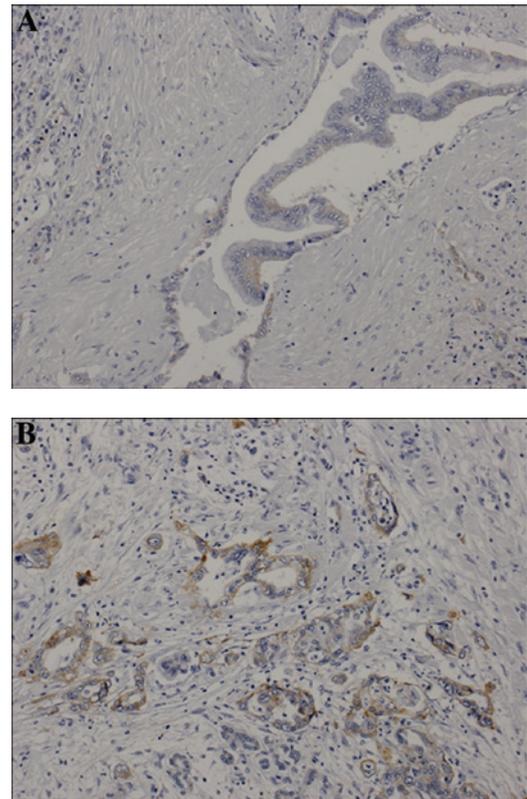


Figure 2. Representative cases of pancreatic ductal adenocarcinoma showing scores of 1+ and 2+ for EGFR cytoplasmic expression. (A) Score 1+, faint diffuse cytoplasmic staining is detected. (B) Score 2+, moderate to strong cytoplasmic staining and strong granular staining is observed. Immunoperoxidase stain, x200.

Carpinteria, CA, USA), a global standard kit for EGFR assay approved by the US Food and Drug Administration (US FDA). Sections were deparaffinized in two sequential xylene baths (5 min), 100% ethanol (3 min) and 95% ethanol (3 min), followed by a 5-min single wash in wash-buffer solution (Dako). Subsequently, at room temperature, the section was rinsed in wash-buffer for 5 min, incubated in proteinase K solution (Dako) for 5 min, rinsed again in the wash-buffer for 5 min, incubated in peroxidase blocking agent for 5 min, rinsed, incubated with the primary EGFR antibody or negative control reagent for 30 min, rinsed, incubated with visualization reagent for 30 min, rinsed twice with the buffer, incubated with substrate chromogen solution for 5 min and finally rinsed again with the buffer. Slides were counterstained with hematoxylin and rinsed gently in reagent quality water. The positive and negative controls used were formalin-fixed, paraffin-embedded pellets of HT-29 and CAMA-1 cell lines, which expressed and did not express EGFR, respectively (Dako).

Immunohistochemical evaluation. Immunohistochemical evaluation was performed for both the cell membrane and cytoplasm, separately, for the primary or metastatic carcinoma samples. The level of membranous EGFR expression was stratified into 4 groups (scores 0, 1+, 2+ and 3+) according to the criteria for the HER2 test (HercepTest) (22). In detail, when membranous staining was observed in <10% of the tumor

Table II. EGFR immunostaining in the surgically resected cancers and the far advanced cancers obtained at autopsy.

	No. of cases (%)							
	Total	Membranous EGFR reactivity				Cytoplasmic EGFR reactivity		
		0	1+	2+	3+	0	1+	2+
Surgically resected cancers	44	24 (55)	7 (16)	12 (27)	1 (2)	22 (50)	12 (27)	10 (23)
Far advanced cancers	40	6 (15)	15 (38)	7 (18)	12 (30)	4 (10)	23 (58)	13 (33)
Primary cancers ^a	20	3 (15)	9 (45)	3 (15)	5 (25)	2 (10)	13 (65)	5 (25)
Hepatic metastases ^a	20	3 (15)	6 (30)	4 (20)	7 (35)	2 (10)	10 (50)	8 (40)

^aNo significant difference between membranous and cytoplasmic EGFR reactivity.

Table III. Expression of EGFR stratified according to histological grading between the surgically resected cancers and the far advanced cancers obtained at autopsy.

Histological grade	No. of tumors (%)				
	Total	Membranous	P-value ^a	Cytoplasmic	P-value ^a
		EGFR overexpression		EGFR overexpression	
Surgically resected cancers	44	13 (30) ^b		10 (23) ^c	
Grade 1	12	1 (8)	0.07	1 (8)	0.2
Grade 2/3	32	12 (38)		9 (28)	
Far advanced cancers	40	19 (48) ^b		13 (33) ^c	
Grade 1	7	3 (43)	0.8	1 (14)	0.3
Grade 2/3	33	16 (48)		12 (36)	

^aP-value indicates comparisons between Grade 1 and Grade 2/3 tumors. No significant difference was noted between EGFR expression and histological grade (Grade 1 vs. 2/3). ^bP=0.09, statistically significant difference between surgically resected and advanced cancers. ^cP=0.3, statistically significant difference between surgically resected and advanced cancers.

cells, a score of 0 was assigned, regardless of the intensity of the staining. If faint or barely perceptible membranous staining was detected in >10% of the tumor cells, a score of 1+ was assigned. Scores of 2+ and 3+ were assigned when weak to moderate staining and strong staining, respectively, were observed on the entire membrane in >10% of the tumor cells (Fig. 1). Cases showing a score of 2+ or 3+ were defined as showing overexpression.

Cytoplasmic staining was divided into 3 grades (0, 1+ and 2+), as grading of the intensity of the immunoreaction was difficult for the cytoplasm. The level of cytoplasmic staining was categorized as follows: when cytoplasmic staining was observed in <10% of the tumor cells, a score of 0 was assigned. If faint or barely perceptible cytoplasmic staining was detected in >10% of tumor cells, a score of 1+ was assigned. A score of 2+ was assigned when moderate or strong staining, respectively, was observed in >10% of the tumor cells. Cytoplasmic granular staining was also scored as 2+. Cases showing a score of 2+ were judged as showing overexpression (Fig. 2).

Statistical analysis. We used the χ^2 test or Fisher's exact test to determine the correlation between EGFR expression and histological grade. Differences were considered to indicate

statistical significance at a P-value <0.05. All statistical analyses were performed using Statview 5.0 software (SAS Institute Inc., Cary, NC, USA).

Results

The expression profiles of membranous and cytoplasmic EGFR in both surgically resected cancers and far advanced cancers obtained at autopsy are shown in Table II. In the 44 surgically resected cancers, 13 (30%) exhibited membranous overexpression of EGFR, comprising 1 case (2%) of score 3+ and 12 cases (27%) of score 2+ and 10 (23%) exhibited cytoplasmic overexpression of EGFR.

In the primary tumors in the 20 far advanced cancers, the percentage of samples with positivity for membranous EGFR overexpression was 40%, (8 of 20), comprising 3 cases (15%) of score 2+ and 5 cases (25%) of score 3+, and the percentage of samples showing positivity for cytoplasmic EGFR overexpression was 25% (5 of 20). In the hepatic metastases in the 20 far advanced cancers, the positivity of membrane EGFR overexpression was 55%, (11 of 20), comprising 4 cases (20%) of score 2+ and 7 cases (35%) of score 3+, and the positivity of cytoplasmic EGFR overexpression was 40% (8 of 20).

In a total of 40 tumors at a far advanced stage, the percentage of samples showing positivity for membranous EGFR overexpression was 48% (19 of 40) comprising 7 cases (18%) of score 2+ and 12 cases (30%) of score 3+, and the percentage of samples showing positivity for cytoplasmic EGFR overexpression was 33% (13 of 40). Therefore, the far advanced tumors tended to show membranous and cytoplasmic EGFR overexpression more frequently than the surgically resected tumors, although the difference was not significant.

When these cases were stratified according to histological grade, higher grade (Grades 2 and 3) cancer tissues tended to show membranous EGFR overexpression more frequently (12 of 32, 38%) than the lower grade (Grade 1) cancer tissues (1 of 12, 8%) in the surgically resected pancreatic cancers, although the difference was statistically marginal ($P=0.07$). The percentage of cytoplasmic EGFR overexpression did not differ statistically between the low grade (Grade 1) tumors (1 of 12, 8%) and higher grade (Grades 2 and 3) tumors (9 of 32, 28%) in the surgically resected cases.

The tissues of the far advanced cancers showed similar rates of membranous and cytoplasmic overexpressions, regardless of histological grade. In the 40 far advanced tumors, membranous EGFR overexpression was detected in 3 (43%) of 7 Grade 1 cases and in 16 (48%) of Grade 2 or 3 cases. In these far advanced tumors, cytoplasmic overexpression of EGFR was detected in 14% (1 of 7) of Grade 1 tumors and 36% (12 of 33) of Grade 2 or 3 tumors (Table III).

Discussion

In the present study, we demonstrated that EGFR overexpression in the cell membrane and cytoplasm was common in both surgically resected and far advanced pancreatic carcinomas. The occurrences of membranous and cytoplasmic EGFR overexpression tended to be higher in the tumors at far advanced stages than in the tumors that were at surgically resectable stages as determined using a global standard kit for EGFR assay.

Cytoplasmic EGFR expression in the far advanced cancers may be explained by the hypothesis of epithelial-to-mesenchymal transition which is thought to be an important mechanism for promoting cancer invasion and metastasis (23). Persistently activated EGFR can decrease intercellular adhesion between tumor cells and enhance cancer cell migration. Willmarth *et al* showed that EGF-activated EGFR in MCF10A cells enhanced signal transduction predominantly from the endosomes rather than from the membrane (24). Barr *et al* (25) suggested that continuously EGF-treated EGFR induced endocytosis of E-cadherin, a cell-to-cell adhesion protein, which enhanced invasiveness in several human cancer cell lines. Ueda *et al* previously reported that EGFR overexpression in the cytoplasm of pancreatic cancers was associated with poorer clinical outcome of patients (10,26). The present study corroborated that not only membranous overexpression but also cytoplasmic overexpression of EGFR is important for the acquisition of highly aggressive and metastatic properties of pancreatic carcinomas.

In the present study, the rate of EGFR overexpression in surgically resected cancers tended to be higher in higher grade (Grades 2 and 3) tumors than in low grade (Grade 1) tumors. It

is not surprising that poorly differentiated pancreatic cancers exhibited a higher incidence of EGFR overexpression as the patients with pancreatic carcinoma with altered EGFR activity tend to show a more aggressive clinical course and a poorer clinical outcome (27). Aggressive tumors appear to require the activation of an EGFR-mediated autocrine signaling in order to maintain proliferation. Therefore, we suppose the possibility that cytoplasmic EGFR protein, which is newly synthesized within the endoplasmic reticulum, would be processed at the cellular surface. Some investigators reported that binding of EGF to EGFR activates its receptor tyrosine kinases and accelerates its internalization through clathrin-coated pits followed by the efficient lysosomal targeting of internalized receptors, which results in receptor downregulation and degradation. Thus, the ligand-induced internalization of EGFR, so-called endocytosis trafficking, is characterized as activated EGFR (28-30). If the EGFR ligands dissociated EGFR localized in endosomes, EGFR would be deactivated and recycled to the plasma membrane.

We should consider the possibility that EGFR localization and its activity in advanced or metastatic pancreatic cancers may be modulated by chemotherapy or radiation therapy which those patients had received. It is known that ionizing radiation, hypoxia and oxidative stress can also phosphorylate EGFR with ligand independence, which is sequentially internalized and shuttled into the nucleus (31). Li *et al* (32) reported that the non-small cell lung cancer H226 cells which acquire resistance to cetuximab, an anti-EGFR antibody, showed decreased membranous EGFR accompanied by EGFR expressed with nuclear localization. These findings imply that EGFR localization of cancer cells may be an important determinant of responsiveness to specific therapies.

In conclusion, we demonstrated using immunohistochemistry that membranous and cytoplasmic EGFR overexpression was frequently noted in surgically resected and far advanced pancreatic cancers. These findings suggest that membranous and cytoplasmic overexpression of EGFR may be indicative of the potential aggressiveness of pancreatic cancers.

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