EGFR, but not COX-2, protein in resected pancreatic ductal adenocarcinoma is associated with poor survival

JOHAN BOURGHARDT FAGMAN[1,2], DAVID LJUNGMAN[1,2], PETER FALK[1,2], BRITT-MARIE IRESJÖ[1,2], CECILIA ENGSTRÖM[1,2], PETER NAREDI[1,2] and KENT LUNDHOLM[1,2]

[1]Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg; [2]Department of Surgery, Sahlgrenska University Hospital, SE-413 45 Gothenburg, Sweden

Received October 31, 2018; Accepted February 19, 2019

DOI: 10.3892/ol.2019.10224

Abstract. The effects of EGFR and COX-2 protein overexpression on clinical outcomes in pancreatic ductal adenocarcinoma (PDAC) patients remains unclear. Therefore, the aim of the present study was to evaluate the protein expression of epithelial growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) in tumor cells in surgically resected PDAC, in comparison with clinicopathological characteristics and clinical outcomes. Immunohistochemical staining of formalin-fixed paraffin-embedded tissue derived from surgically resected tumors was performed. Tissue slides were evaluated for membrane wild-type EGFR and cytoplasmic COX-2 staining using a histoscore system. Statistical associations between EGFR and COX-2 staining and clinicopathological characteristics were examined to predict survival. In a cohort of 32 resected PDAC patients, high EGFR protein expression in tumor cells was significantly associated with shorter median overall survival (7.9 vs. 39.2 months, P=0.0038). The corresponding hazard ratio (HR) for patients with high EGFR protein expression in tumor cells was 3.12 [95% confidence interval (CI): 1.39-7.00, P=0.006]. COX-2 protein expression was not associated with survival (22.6 vs. 24.5 months P=0.60; HR 1.22 95% CI: 0.59-2.51, P=0.60). Following multivariate Cox regression analysis, high EGFR protein expression in tumor cells (P=0.043) remained as significant independent prognostic factor for survival. In conclusion, high wild-type EGFR protein expression, but not COX-2 protein expression, in tumor cells is a prognostic factor for reduced overall survival following pancreatic tumor resection, supporting a role for EGFR in identifying resected patients that may benefit from EGFR-targeted therapy.

Correspondence to: Dr Johan Bourghardt Fagman, Department of Surgery, Sahlgrenska University Hospital, Vita Stråket 12, paviljong plan 2, SE-413 45 Gothenburg, Sweden
E-mail: johan.bourghardt@gu.se

Abbreviations: PDAC, pancreatic ductal adenocarcinoma; EGFR, epithelial growth factor receptor; COX-2, cyclooxygenase-2; HR, hazard ratio; immunohistochemistry, IHC; TBS, tris-buffered saline; DAB, 3,3'-diaminobenzidine; H-score, histoscore; CI, confidence interval; OS, overall survival

Key words: EGFR, COX-2, survival, pancreatic cancer

Introduction

For pancreatic ductal adenocarcinoma (PDAC), one of the deadliest malignant diseases (1,2), there has been poor progress in the development of new effective treatments. Surgical resection is still to date the only potentially curative treatment. However, only about 20% of patients present tumors eligible for resection at time of diagnosis (1). Despite surgery, many patients relapse or develop metastasizing disease (3,4). Hence, there is a need for therapy improvement and specific biomarkers that predict patient outcome or that can guide individualized treatment after surgery.

Epithelial growth factor receptor (EGFR) has been extensively studied in PDAC (5). EGFR protein overexpression has been reported in 40-70% of pancreatic cancers (6,7), with variable association with survival (8). Erlotinib, a small molecule EGFR inhibitor, is in clinical use in combination with gemcitabine for unresectable locally advanced or metastatic PDAC (9,10). The effectiveness of erlotinib in resected PDAC is less studied and remains unclear (11,12). Recent experimental studies by Ardito *et al* (13) and Navas *et al* (14) showing that knock-out of *Egfr* in KRAS-mutated mice completely blocked development of PDAC, suggest a critical role for EGFR activation in the pathogenesis of human KRAS-driven PDAC.

Cyclooxygenase-2 (COX-2), far less studied than EGFR in PDAC, has also been reported to be overexpressed in 60 to 70% of tumor patients (15,16). The association with survival is unclear (17-22). Further, no anti-COX-2 treatment has yet been proven effective in PDAC patients in combination with chemotherapy (23). Activation of COX-2 signaling has been reported to promote PDAC tumor growth in experimental models (24,25).

EGFR and COX-2 signaling pathways have been suggested to be linked. This has been reported in experimental studies of colorectal cancer and head and neck cancer, where EGFR is shown to upregulate COX-2 or vice versa (26,27). However, whether there is a link in human PDAC is currently unknown.

The effects of EGFR and COX-2 protein overexpression on clinical outcomes in PDAC patients remain unclear. Therefore, the aim of this study was to explore the prognostic and clinicopathological significance of both EGFR and COX-2 protein expression in tumor cells in patients with surgically resected PDAC aimed at cure.

Materials and methods

Patients and tumor tissue samples. Pancreatic tumor tissue sections were obtained from 32 patients with pancreatic ductal adenocarcinoma who underwent surgical tumor resection in 1998 to 2005 at Sahlgrenska University Hospital, Gothenburg, Sweden. All patients underwent surgery as primary treatment and none had received neoadjuvant chemotherapy. The group of patients consisted of 53% males and 47% females with a mean age of 64.1 years (range 50 to 80 years) at surgery. Median overall survival was 22.9 months (range 1.1 to 122.4 months) after surgery. All the tumors were histologically diagnosed as ductal adenocarcinoma and classified according to the TNM staging system by the Pathology department at Sahlgrenska University Hospital. The present study was approved by the Regional Ethical Review Board in Gothenburg, Sweden (reference number 002-06) and all participants gave written informed consent. Data was analyzed anonymously.

Histology and immunohistochemistry. Pancreatic tumor tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 4 μm were prepared. Immunohistochemistry (IHC) was performed as follows: After xylene deparaffinization, ethanol dehydration and antigen retrieval (water bath for 30 min at 98˚C in citrate buffer pH 6.0), sections were first blocked with 5% non-fat milk in 5 mM Tris-buffered saline (TBS), pH 7.8 for 30 min and then endogenous peroxidase activity was quenched in 0.3% H_2O_2 solution (Dual Endogenous Enzyme Block, Dako Envision K4065, Agilent Technologies, Santa Clara, CA, USA) for 15 min. Sections were incubated with primary antibodies diluted in 5% non-fat milk in TBS overnight at 4˚C, followed by incubation with polymer labelled secondary antibodies conjugated with HRP (Labelled polymer-HRP, Dako Envision K4065, Agilent Technologies), for 40 min at room temperature. Bound peroxidase was visualized by 15 min incubation in a 3,3'-diaminobenzidine (DAB) solution (Dako Envision K4065, Agilent Technologies). Sections were washed, counterstained with hematoxylin, dehydrated and mounted. The following antibodies were used: Monoclonal mouse anti-human wild-type EGFR (detects the membranous N-terminal part of the extracellular domain, but not the 2-7 truncated EGFR variant (EGFR-vIII), immunizing peptide aa 30-198), clone DAK H1 WT, M7298, Agilent Technologies, dilution 1:50, polyclonal rabbit anti-COX-2 (cytosolic detection), ab15191, Abcam, Cambridge, UK, dilution 1:200, negative control mouse IgG1 (X0931, Agilent Technologies) and rabbit IgG1 (X0903, Agilent Technologies) diluted in 5% non-fat milk in TBS. Negative control antibodies were diluted to the same protein concentration as the primary antibody. Methodology has been used and described previously (28,29).

Scoring of immunohistochemical stainings. Tumor tissue samples were evaluated for membranous EGFR and cytoplasmic COX-2 staining using a histoscore (H-score) system. Staining intensity of EGFR and COX-2 protein expression was scored from negative (score=0), low (score=1), intermediate (score=2) to high (score=3). The percentage of tumor cells showing positive staining was assessed separately. For both staining intensity and percentage of tumor cells, 10 high magnification (x200) fields per patient and staining were assessed and averaged. The final staining score is the product of the average intensity score and the average percentage of tumor cells showing positive staining and ranged from 0 to 270 (30). Scoring of tumor tissue samples was performed in blinded manner by JBF without knowledge of pathological and clinical data. For statistical analysis, EGFR and COX-2 scores were classified into two staining grades according to the median staining score (the high grade represents ≥ median; the low grade represents < median).

Statistical analyses. Data are presented as median and range (continuous data), and as numbers and percentages (categorical data). Mann-Whitney U and Pearson's Chi-square tests were used to determine the association between EGFR and COX-2 protein expression in tumor cells and clinicopathological and molecular parameters. Overall survival (OS) was evaluated using Kaplan-Meier survival plots, and differences in survival were tested using log-rank (Mantel-Cox) tests. Cox proportional hazards regression analyses were used to estimate hazard ratios (HR) and 95% confidence intervals (95% CI). Multivariate Cox proportional hazards regression analysis was performed to assess independent prognostic factors using covariates found significant in univariate analysis (regional lymph node metastasis and EGFR score). All P-values corresponded to two-sided tests and $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were conducted using either SPSS version 22 (IBM Corp., Armonk, NY, USA) or GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Association between EGFR and COX-2 protein expression in tumor cells and clinicopathological characteristics. To assess EGFR and COX-2 protein expression in tumor cells in surgically resected pancreatic ductal adenocarcinoma (PDAC), protein expression level in PDAC samples was scored and divided into two subgroups: Low and high grade (Fig. 1) and was then compared to clinicopathological characteristics. In the study cohort, no significant associations of either EGFR or COX-2 protein expression in tumor cells were found with tumor location, tumor stage or regional lymph node metastasis (Tables I and II). However, we detected a weak positive correlation between EGFR and COX-2 protein expression in tumor cells (Spearman's rank correlation coefficient of 0.363, P=0.041, Fig. 2).

High EGFR protein expression in tumor cells associates with shorter overall survival in PDAC patients. In order to examine the prognostic impact of EGFR and COX-2 on survival outcome, we analyzed overall survival (OS) time

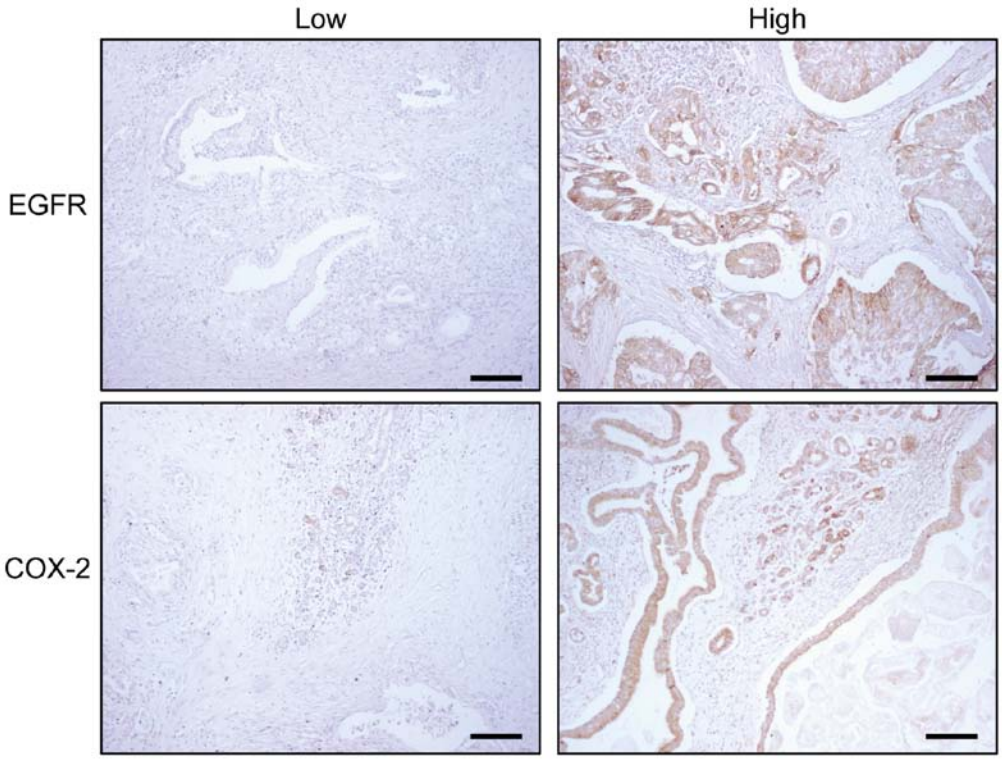

Figure 1. Immunohistochemistry staining of EGFR and COX-2 in pancreatic ductal adenocarcinoma tissue. Representative photomicrographs demonstrating low (left-hand panels) and high (right-hand panels) grade of membranous EGFR and cytoplasmic COX-2 expression in tumor cells. Scale bars, 100 μm (brown: Positive antibody staining, blue: Hematoxylin for nuclei staining). EGFR, epithelial growth factor receptor; COX-2, cyclooxygenase-2.

according to EGFR and COX-2 protein expression in tumor cells. The median OS for the study cohort was 22.9 months. EGFR protein expression and regional lymph node metastasis were significantly associated with survival (Fig. 3). High EGFR protein expression in tumor cells was significantly associated with shorter OS (median OS: 7.9 months vs. 39.2 months P=0.004, Fig. 3A). In contrast, we did not detect any significant difference in median OS between patients with high COX-2 and low COX-2 protein expression in tumor cells (median OS: 22.6 months vs. 24.5 months P=0.596, Fig. 3B). To further evaluate the association between EGFR and COX-2 protein expression, patients were divided into four groups: Low EGFR/Low COX-2; Low EGFR/High COX-2; High EGFR/Low COX-2; High EGFR/High COX-2. Analysis of OS in these groups showed that patients with high EGFR score have shorter median OS in both the Low COX-2 and High COX-2 subgroups (median OS: Low EGFR/Low COX-2=31.7 months vs. High EGFR/Low COX-2=19.9 months and Low EGFR/High COX-2=53.4 months vs. High EGFR/High COX-2=7.5 months, P=0.038, Fig. 3C). Furthermore, median OS in patients with regional lymph node metastasis was also significantly shorter than OS in patients without regional lymph node metastasis (median OS: 21.0 months vs. 36.7 months, P=0.025, Fig. 3D). However, the established prognostic factor tumor stage (31) was not significantly associated with survival (data not shown).

The hazard ratio (HR) for death in patients with high EGFR protein expression in tumor cells (when compared with low EGFR protein expression in tumor cells) was 3.12 (95% CI: 1.39-7.00, P=0.006, Table III). The corresponding HR for regional lymph node metastasis was 2.65 (95% CI: 1.10-6.39, P=0.030). In multivariate analysis, where regional lymph node metastasis and EGFR score were included, only high EGFR protein expression score remained as significant independent prognostic factor for overall survival (P=0.043, Table III).

To conclude, these results indicate that high EGFR, but not COX-2, protein expression in tumor cells is an independent prognostic factor for poor survival in resected PDAC patients.

Discussion

In this study, we demonstrated that EGFR protein expression is a prognostic factor for survival in resected PDAC patients. Patients with high EGFR protein expression in tumor cells had significantly shorter overall survival. In contrast, COX-2 protein expression was not significantly associated with clinical outcomes. Thus, EGFR seems to be a more significant predictor than COX-2 for survival in resected PDAC patients.

In agreement with previous studies, we showed that high EGFR protein expression is associated with poor survival in PDAC patients (8,30,32,33). The study with the largest sample size, by Valsecchi and colleagues (30) demonstrated similarly to our study that high membrane EGFR protein expression was significantly associated with shorter overall survival. Yet, other studies have not detected any significant association (7,34,35). A reason for these differences may be the use of different antibodies. We used an antibody that only detects wild-type EGFR, whereas many of the studies reporting no association used antibodies that detect both wild-type and a 2-7 truncated EGFR variant (EGFR-vIII). This truncated EGFR variant is the most common form of mutant EGFR and does not require ligand binding to activate downstream signaling. However, the

Table I. Clinicopathological characteristics and EGFR status.

Characteristic	EGFR High, n (%)	EGFR Low, n (%)	P-value[a]
All	15 (47)	17 (53)	
Age, years			
<65	11 (73)	8 (47)	0.165[b]
≥65	4 (27)	9 (53)	
Sex			
Female	7 (47)	8 (47)	0.982
Male	8 (53)	9 (53)	
Tumor location			
Head	13 (88)	16 (94)	0.471
Others	2 (12)	1 (6)	
Tumor stage			
T1	3 (20)	0 (0)	0.103
T2	6 (40)	6 (35)	
T3	6 (40)	8 (47)	
T4	0 (0)	3 (18)	
Regional lymph node metastasis			
N0	10 (67)	6 (35)	0.077
N1	5 (33)	11 (65)	
COX-2			
Low	9 (60)	7 (41)	0.288
High	6 (40)	10 (59)	

[a]Chi-square test, except [b]Mann-Whitney U test. Distribution of EGFR staining categorization according to clinicopathological characteristics. Values are presented as the number of patients and percentages (in parentheses). EGFR, epidermal growth factor receptor; EGFR Low, EGFR staining grade low; EGFR High, EGFR staining grade high; N0/N1, no presence/presence of regional lymph node metastasis; COX-2, cyclooxygenase-2.

Table II. Clinicopathological characteristics and COX-2 status.

Characteristic	COX-2 Low, n (%)	COX-2 High, n (%)	P-value[a]
All	16 (50)	16 (50)	
Age, years			
<65	10 (63)	9 (56)	0.468[b]
≥65	6 (37)	7 (44)	
Sex			
Female	7 (44)	8 (50)	0.723
Male	9 (56)	8 (50)	
Tumor location			
Head	14 (88)	15 (94)	0.544
Others	2 (12)	1 (6)	
Tumor stage			
T1	1 (6)	2 (13)	0.813
T2	6 (38)	6 (38)	
T3	8 (50)	6 (38)	
T4	1 (6)	2 (13)	
Regional lymph node metastasis			
N0	8 (50)	8 (50)	1.000
N1	8 (50)	8 (50)	
EGFR			
Low	9 (56)	6 (37)	0.288
High	7 (44)	10 (63)	

[a]Chi-square test, except [b]Mann-Whitney U test. Distribution of COX-2 staining categorization according to clinicopathological characteristics. Values are presented as the number of patients and percentages (in parentheses). COX-2, cyclooxygenase-2; COX-2 Low, COX-2 staining grade low; COX-2 High, COX-2 staining grade high; N0/N1, no presence/presence of regional lymph node metastasis; EGFR, epidermal growth factor receptor.

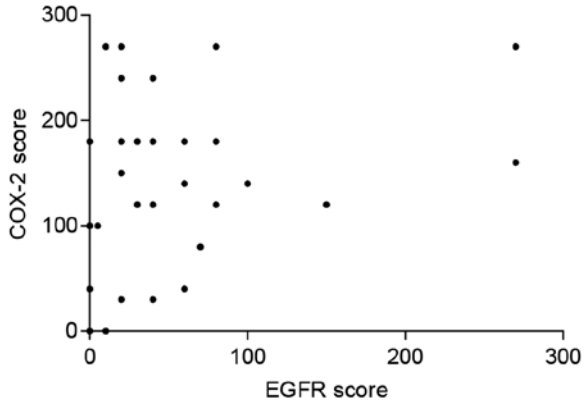

Figure 2. Correlation between EGFR and COX-2 scores. Correlation analysis revealed a positive correlation for EGFR and COX-2 protein expression in tumor cells (Spearman's rank correlation coefficient of 0.363, P=0.041. EGFR, epithelial growth factor receptor; COX-2, cyclooxygenase-2.

importance of EGFRvIII expression in PDAC is unclear (36). Therefore, conclusions about the prognostic value of EGFR should be drawn with caution. However, recent studies have given a better insight into the biology of EGFR-mediated signaling in PDAC and suggest a critical role for EGFR in development of PDAC. Ardito *et al* (13) and Navas *et al* (14) knocked out *Egfr* in KRAS-mutated mice and found that loss of EGFR completely blocked pre-malignant lesion development. Together, these studies support that EGFR is important for KRAS-driven pancreatic tumorigenesis. Since most PDAC carry KRAS mutations (37), EGFR activation is likely to be crucial also in the pathogenesis of human PDAC as it increases aggressiveness of the tumors (32,38) and causes shorter survival as shown in this and previous studies.

Our results on COX-2, showing no significant association between high protein expression and overall survival, confirmed those obtained by others (16,19-22). However, two more recent studies by Juuti *et al* (17) and Matsubayashi *et al* (18) demonstrated significant effects of COX-2 overexpression and clinical outcomes in PDAC patients. These studies included more patients than our study (128 and 299 patients), which may be one reason explaining the differing results. Another

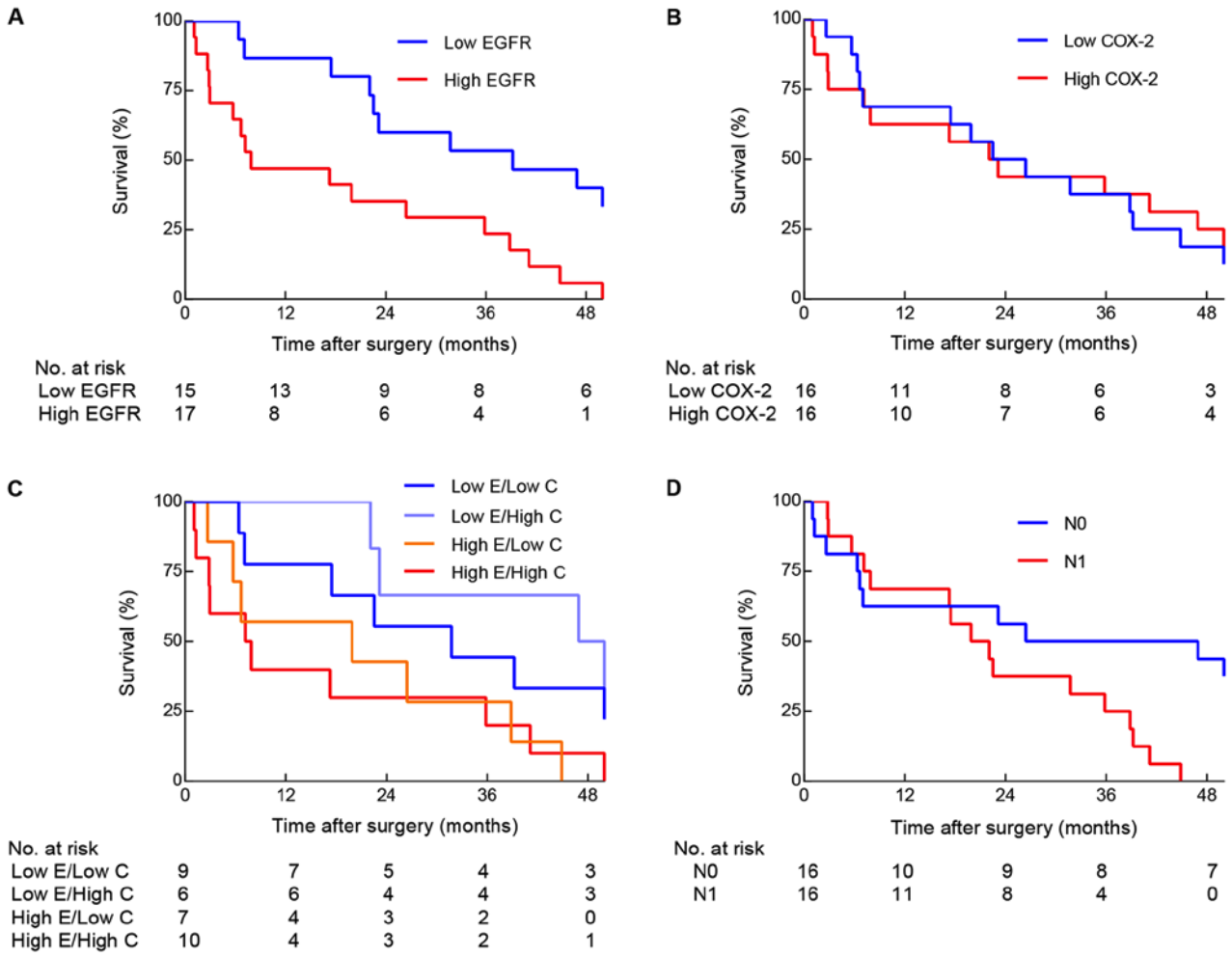

Figure 3. EGFR and regional lymph node metastasis are significant prognostic factors for survival. Kaplan-Meier analysis of overall survival following resection with curative intent for PDAC according to (A) EGFR score (P=0.004), (B) COX-2 score (P=0.596), (C) subgroup analysis of EGFR score (termed E) status in COX-2 score (termed C) Low and High groups (P=0.038), and (D) regional lymph node metastasis. N0/N1, no presence/presence of regional lymph node metastasis (P=0.025; log rank test). EGFR, epithelial growth factor receptor; COX-2, cyclooxygenase-2.

reason could be the use of different antibodies (39). Yet another reason may be differences in the evaluation of the IHC staining. We used a histoscore system, where staining intensity and percentage of tumor cells showing positive staining were assessed separately before combined into a staining score, in which relatively more weight is given to higher-intensity staining in a given tumor sample.

A link between EGFR and COX-2 signaling pathways has been suggested in some cancers, mainly in experimental studies of colorectal cancer and head and neck cancer, where EGFR upregulates COX-2 or vice versa (26,27). For PDAC, a recent study by Hu *et al* (40) using PDAC cell lines reported that EGFR and COX-2 are linked and that overactive EGFR signaling leads to overexpression of COX-2 and subsequent secretion of VEGF, promoting angiogenesis which contribute to PDAC tumor cell growth. However, there is no support in the literature for such an association in human PDAC. Although we found a weak positive correlation between EGFR and COX-2 score, this potential link between the signaling pathways will require further mechanistic studies in appropriate experimental models.

The results presented in this study, suggest that EGFR is a superior predictor of survival than COX-2 since only EGFR score in tumor cells was shown to be a prognostic factor. To our knowledge, there is only one previous study that has examined the relationship between both EGFR and COX-2 tumor expression and survival among pancreatic cancer patients in the same study. Lozano-Leon and colleagues (41) reported no significant associations between EGFR or COX-2 expression and survival. In contrast to our study, where we used whole tissue sections, they performed IHC on tissue microarrays, which may have its limitations in survival analysis in small patient materials and when the number of tumor cores is limited (42).

A limitation of our study is the small number of patients. This may explain why we did not find a significant association between an established prognostic factor such as tumor stage and survival (31). However, we could still demonstrate an association between another established prognostic factor, regional lymph node metastasis, and overall survival.

As there are no effective treatments, to date, for pancreatic cancer, it is important to develop molecular biomarkers that predict clinical outcomes. This may help direct more effective targeted therapy in high-risk patients such as patients with EGFR protein overexpression in their tumors. Since the EGFR inhibitor, erlotinib, is already in clinical use in combination with gemcitabine for unresectable locally advanced or metastatic PDAC (9,10), there should be no restrictions as to

Table III. Prognostic factors of overall survival in 32 patients with pancreatic ductal adenocarcinoma following resection.

	Univariate analysis			Multivariate analysis[a]		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Age, years						
<65	1	0.62-2.71	0.487			
≥65	1.30					
Sex						
Female	1	0.43-1.78	0.701			
Male	0.87					
Tumor location						
Head	1	0.46-5.19	0.480			
Others	1.55					
Tumor stage						
T1-2	1	0.38-1.60	0.491			
T3-4	0.78					
Regional lymph node metastasis						
N0	1	1.10-6.39	0.030	1	0.66-4.48	0.267
N1	2.65			1.72		
EGFR						
Low	1	1.39-7.00	0.006	1	1.03-6.15	0.043
High	3.12			2.52		
COX-2						
Low	1	0.59-2.51	0.596			
High	1.22					

[a]Significant factors found via univariate analysis, regional lymph node metastasis status and EGFR score, were included in the Cox multivariate model. Univariate and multivariate Cox proportional hazards regression analysis. HR, hazard ratio; CI, confidence interval; N0/N1, no presence/presence of regional lymph node metastasis; EGFR, Epidermal growth factor receptor; COX-2, cyclooxygenase-2.

consider evaluations of EGFR-targeted therapy for resected PDAC patients. Our work underscores the importance of biomarkers, such as wild-type EGFR, for identifying patients for inclusion in large randomized studies.

In conclusion, our results show that high wild-type EGFR protein expression, but not COX-2 protein expression, in tumor cells is a prognostic factor for reduced survival following pancreatic tumor resection. This supports a role for wild-type EGFR as a biomarker in identifying resected PDAC patients that may benefit the most from EGFR-targeted therapy.

Acknowledgements

Not applicable.

Funding

The present research was supported by grants from the Swedish Cancer Society (grant nos. CAN 2015/400 and 2017/401), Sahlgrenska Academy (grant no. V 2012/294), Assar Gabrielsson's and Lundgren's Foundations (grant nos. 2017-1691 and 2018-2314).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DL and PF performed the experiments. DL, CE and JBF analyzed the data and performed the statistical analyses. DL, PF, BMI, KL and JBF conceived the study and designed the experiments. PN, KL and JBF interpreted data and revised the manuscript critically for important intellectual content. All authors read, reviewed and approved the final manuscript.

Ethics approval and consent to participate

Human pancreatic tumor tissue sections were obtained from the Pathology Department at Sahlgrenska University Hospital (Gothenburg, Sweden). The present study was approved by the Regional Ethical Review Board in Gothenburg, Sweden (reference no. 002-06) and all participants gave written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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