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PRECLINICAL STUDIES

EGFR and HER2 inhibition in pancreatic cancer

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Summary The aim of this study was to investigate the effect of lapatinib, a selective inhibitor of EGFR/HER2 tyrosine kinases, on pancreatic cancer cell lines both alone and in combination with chemotherapy. Two cell lines, BxPc-3 and HPAC, displayed the greatest sensitivity to lapatinib (IC_{50}< 2 μM). Lapatinib also demonstrated some activity in three K-Ras mutated pancreatic cancer cell lines which displayed resistance to erlotinib. Drug effect/combination index (CI) isobologram analysis was used to study the interactions of lapatinib with gemcitabine, cisplatin and 5'deoxy-5'fluorouridine. Concentration-dependent anti-proliferative effects of lapatinib in combination with chemotherapy were observed. To evaluate the potential effect of lapatinib in pancreatic cancer tumours, and to identify a subset of patient most likely to benefit from lapatinib, expression of EGFR and HER2 were investigated in 72 pancreatic cancer tumour specimens by immunohistochemistry. HER2 membrane expression was observed in only 1 % of cases, whereas 44 % of pancreatic tumours expressed EGFR. Based on our in vitro results, lapatinib may provide clinical benefit in EGFR positive pancreatic ductal adenocarcinoma.

Keywords Lapatinib · Erlotinib · HER2 · EGFR · Immunohistochemistry · Pancreatic cancer

Introduction

Pancreatic cancer is a highly aggressive malignancy; it represents the fourth most frequent cause of cancer-related death in the world [1]. Pancreatic ductal adenocarcinoma (PDAC) comprises of 80-90 % of all pancreatic exocrine tumours [2]. Gemcitabine is the standard chemotherapy for advanced/metastatic disease [3]. However, overall response rates still remain low. Clinical trials combining gemcitabine with cytotoxic agents have been investigated. In advanced or metastatic pancreatic cancer, combinations of gemcitabine plus 5-fluorouracil (5-FU) [4], cisplatin [5], oxaliplatin [6] and irinotecan [7] have not shown significant benefit in overall survival compared to single-agent gemcitabine. Capecitabine, an orally administered fluoropyrimidine carbamate which converts to active 5-FU in the tumour site demonstrates efficacy in untreated advanced colorectal carcinoma [8] and the combination of gemcitabine and capecitabine in advanced pancreatic cancer improves overall survival, clinical benefit response and quality of life at a statistically significant level compared with standard gemcitabine treatment [9, 10]. Reports of expression of the ErbB receptor family, including the epidermal growth factor receptor (EGFR; ErbB1/HER1) and HER2; ErbB2/neu vary widely in PDAC. Studies have shown that immunohistochemical (IHC) expression of EGFR ranges from 41-70 % [11–13] in PDAC.

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EGFR over-expression is thought to confer a poor prognosis [14], correlating with poorly differentiated histology and a more advanced stage. Blocking EGFR tyrosine kinase signalling decreases the growth and metastasis of human pancreatic tumour xenografts [15]. The combination of erlotinib, an EGFR inhibitor plus gemcitabine is now considered to be the standard of care for advanced/metastatic pancreatic cancer [16].

Variable expression of HER2 has also been observed in PDAC. Previous studies have determined HER2 over-expression by IHC in 21-80 % [13, 17–19] of cases. Over-expression is more common in well and moderately differentiated tumours compared to poorly differentiated and anaplastic tumours [18]. Therefore, targeting EGFR and HER2 is a potential attractive therapeutic strategy for pancreatic cancer. In this study, we investigated the in vitro effect of lapatinib, a reversible, dual EGFR/HER2 tyrosine kinase inhibitor alone and in combination with chemotherapy in a panel of pancreatic cancer cell lines. We also determined the frequency of EGFR and HER2 expression in 72 pancreatic cancer specimens.

Materials and methods

Cell lines, cell culture and reagents The human pancreatic cell lines BxPc-3, MiaPaCa-2 and Panc-1 were obtained from the European Collection of Cell Cultures (ECACC, UK). HPAC was obtained from the German Tissue Repository DSMZ and AsPc-1 from American Type Culture Collection (ATCC, USA). AsPc-1, BxPc-3 and HPAC were maintained in RPMI medium 1640 supplemented with 5 % FBS, MiaPaCa-2 and Panc-1 were maintained in DMEM supplemented with 5 % FBS (Sigma-Aldrich). All cells were maintained in a humidified atmosphere containing 5 % CO_2 at 37 °C. All cell lines were free from Mycoplasma as tested with the indirect Hoechst staining method.

Lapatinib was kindly provided by GlaxoSmithKline and was prepared as a 10 mM stock in DMSO. Erlotinib was purchased from Sequoia Research Products (Pangbourne, UK) and prepared to a 10 mM stock in DMSO. Chemotherapeutic agents, cisplatin (Platinol™, Bristol-Myers Squibb) and gemcitabine (Gemzar™, Eli Lilly) were obtained from St. Vincent's Hospital, Dublin and 5'deoxy-5'fluorouridine (5'dFUrd) from Sigma.

EGFR and HER2 enzyme-linked immunosorbent assay EGFR (R&D Systems, Minneapolis, MN) and HER2 (Calbiochem) were measured by enzyme-linked immunosorbent assay (ELISA). EGFR and HER2 protein concentrations were estimated from a standard curve and expressed as pg/μg of total protein.

Proliferation assays

1×10^3 cells/well of BxPc-3 and HPAC were plated in 96-well plates. After 24 h, media containing lapatinib was added to appropriate wells. For combination assays, fixed ratio assays were used with the concentration ratio of lapatinib to chemotherapeutic drugs, cisplatin (1:0.3), gemcitabine (1:0.01) and 5dFUrd (1:8), added after 24 h at clinically relevant concentrations and incubated for a further 5 days until the control wells had reached approximately 80-90 % confluency. Cell survival was determined by the acid phosphatase assay. Briefly, media was removed from the plates and each well was washed with 100 μl PBS. 100 μl of freshly prepared phosphatase substrate (10 mM *p*-nitrophenol phosphate (Sigma) in 0.1 M sodium acetate (Sigma), 0.1 % triton X-100 (BDH), (pH 5.5)) was added to each well. The plates incubated in the dark at 37 °C for 1.5 h. The enzymatic reaction was stopped by the addition of 50 μl of 1 M NaOH and plates were read at 405 nm with a reference wavelength of 620 nm. Synergy was assessed using the CalcuSyn software (Biosoft version 2), using the Chou-Talalay method [20].

Immunohistochemistry analysis

Patients

The patient group consisted of 72 patients diagnosed with primary tumours of the pancreas. All patients were treated at St. Vincent's University Hospital, Dublin. Pathological material was examined on each case by SK. Representative 4-μm sections of formalin-fixed paraffin-embedded pancreatic tumour tissue were cut using a microtome, mounted onto poly-l-lysine coated slides and dried overnight at 37 °C. Slides were stored at room temperature until required.

Immunohistochemistry

Staining was performed using an automated staining apparatus for IHC (Autostainer, DakoCytomation) according to the manufacturer's guidelines. Optimum primary antibody dilutions were predetermined using known positive control tissues. Negative and known positive control sections were included in each run. Deparaffinisation and antigen retrieval consisted of 40-minute incubation in pH 6.0 buffer (HER2) or 20-minute incubation in pH 9.0 buffer (EGFR) (TARGET Retrieval, DakoCytomation) in a 95 °C water bath followed by cooling to room temperature. In the Dako Autostainer, sections were treated with 3 % H_2O_2 for 10 min to quench endogenous peroxidase and then rinsed. Quenched sections were incubated with antibodies, EGFR (NovaCastra,UK)(dilution 1/200) and HER2

(DakoCytomation)(dilution 1/300) for 30 min, followed by incubation with Dako EnVision+™ dual linked reagent for 30 min. The antigen-antibody complex was visualised using diaminobenzidine (DAB) substrate as a chromogen which was applied with fresh reagent twice for 5 min each time. Sections were then counterstained with haematoxylin, dehydrated in a graded series of ethanols and xylene, and mounted. Slides were reviewed by light microscopy.

Membrane expression of EGFR and HER2 proteins was based on the intensity of the immunohistochemical staining of the tumour area. For EGFR and HER2, the intensity of the immunostaining (negative, 1+, 2+, or 3+) and the percentage of tumour cells with positive staining were assessed according to the Herceptest® guidelines (Dako): negative = no membrane staining or <10 % of cells stained; 1+ = incomplete membrane staining in >10 % of cells; 2+ = >10 % of cells with weak to moderate complete membrane staining; and 3+ = strong and complete membrane staining in >10 % of cells. Cytoplasmic and nuclear only staining was considered negative.

Statistical analysis

The concentration of drug which caused 50 % inhibition of cell growth (IC_{50} of the drug) was determined by using CalcuSyn software (Biosoft, version 2.0). Multiple drug effect analysis was used to determine the interaction between lapatinib and chemotherapeutic drugs (synergy, addition, or antagonism). Combination indices (CI) at the ED_{50} (effective dose of concentration that inhibits 50 % of growth) were calculated using the model of Chou and Talalay [20] for fixed concentration drug combinations. In this analysis, synergy is defined as CI values statistically significantly<1.0, antagonism as CI values statistically significantly>1.0 and additivity as CI values not statistically significantly different from 1.0. The chi-square test was used to evaluate the relationships between the IHC expression of EGFR and the following clinicopathologic parameters: age, gender, tumour size, tumour grade and nodal status. A *p*-value of≤0.05 was considered significant.

Results

Expression of EGFR and HER2 in a pancreatic cancer cell lines

The relative levels of EGFR and HER2 (pg/μl total protein) were investigated in a panel of five pancreatic cancer cell lines by ELISA. EGFR was highly expressed in Panc-1, BxPc-3 and AsPc-1, moderately expressed in HPAC and expressed at a low level in MiaPaCa-2. HER2 expression was highest in HPAC, moderately expressed in BxPc-3 and MiaPaCa-2, and at a low level in AsPc-1 and Panc-1 (Table 1, supplementary data 1).

Sensitivity of pancreatic cancer cell lines to lapatinib

The sensitivity of five pancreatic cancer cell lines, AsPc-1, BxPc-2, HPAC, MiaPaCa-2 and Panc-1 to lapatinib was tested. The cell lines showed varying degrees of sensitivity to lapatinib; however, BxPc-3 (high EGFR, moderate HER2) and HPAC (moderate EGFR, high HER2) displayed the greatest sensitivity (Fig. 1, Table 1). Panc-1 (high EGFR, low HER2) was the most resistant cell line to lapatinib (IC_{50} 4.1 μM).

We compared the efficacy of lapatinib to erlotinib, an EGFR inhibitor approved for metastatic pancreatic cancer, in our panel of pancreatic cancer cell lines. Only BxPc-3 and HPAC showed some sensitivity to erlotinib (IC_{50} <2 μM), whereas growth of

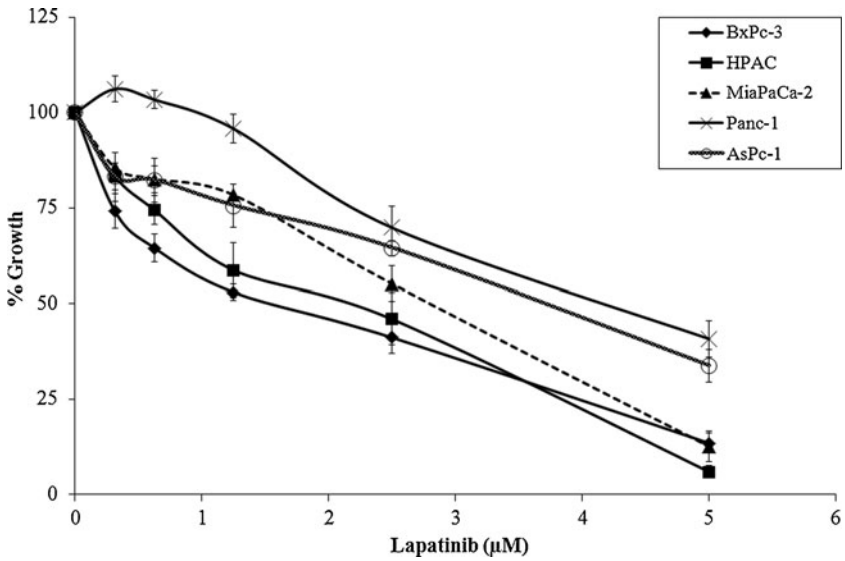

Fig. 1 Proliferation assay of pancreatic cancer cells treated with increasing concentrations of lapatinib. Graph expressed as percentage growth (relative to control) versus concentration of lapatinib

Table 1 IC_{50} values of lapatinib and erlotinib in pancreatic cancer cell lines (results are expressed as IC_{50} ± SD, n=3), K-ras mutational status, EGFR and HER2 expression levels

Cell line	Lapatinib (μM)	Erlotinib (μM)	K-Ras status	EGFR expression[a]	HER2 expression[a]
BxPc-3	1.2±0.3	2.0±0.6	Wild-type	High	Moderate
HPAC	1.9±0.6	2.0±0.2	Mutated	Moderate	High
MiaPaCa-2	2.3±0.3	> 10	Mutated	Low	Moderate
AsPc-1	3.5±0.2	> 10	Mutated	High	Low
Panc-1	4.1±1.0	> 10	Mutated	High	Low

[a] EGFR and HER2 expression levels were calculated from ELISA pg/μl of total protein (supplementary data 1)

MiaPaCa-2, Panc-1 and AsPc-1 was not significantly inhibited with erlotinib concentrations up to 10 μM.

Combination assays with lapatinib and chemotherapeutic drugs

Combinations of lapatinib with chemotherapy agents were tested in the two cell lines which displayed the greatest sensitivity to lapatinib, BxPc-3 and HPAC. The IC_{50}s for cisplatin, gemcitabine and 5'dFUrd (intermediate metabolite of capecitabine) were determined for BxPc-3 and HPAC using an in vitro proliferation assay (Supplementary data 2). We then analysed the combination of lapatinib with cisplatin, gemcitabine or 5'dFUrd (Table 2). Pre-treatment with lapatinib was performed 24 h prior to the addition of chemotherapy drug. CalcuSyn analysis of the combination of lapatinib and cisplatin revealed a synergistic interaction (CI=0.75±0.27) in HPAC cells and an additive-synergistic interaction (CI=0.93±0.05) in BxPc-3 cells (Fig. 2a & b). The combinations of lapatinib and 5'dFUrd were additive in both cell lines, whereas the interaction of lapatinib and gemcitabine was additive to slightly antagonistic in both HPAC (CI=1.03±0.04) and BxPc-3 (CI=1.11±0.2) (Table 2).

Patient clinicopathological characteristics

Expression of EGFR and HER2 was evaluated in 72 pancreatic tumours. This study included 36 male and 34 female patients with a median age of 62 years (Table 3). Adenocarcinoma accounted for 83 % of all tumour types. Tumour grade was diagnosed as 19 % well differentiated 51 % moderately and 19 % poorly differentiated. Thirty-seven patients (51 %) had positive lymph node status, whereas twenty-one (29 %) were negative. Detailed characteristics are shown in Table 3.

Table 2 Mean combination index values at the ED_{50} for lapatinib plus chemotherapy drug combinations

Chemotherapeutic agent	Combination index values (ED_{50})	
	BxPc-3	HPAC
Cisplatin	0.93±0.05	0.75±0.27
Gemcitabine	1.11±0.20	1.03±0.04
5'dFUrd	0.96±0.12	0.99±0.10

EGFR expression in pancreatic cancer Seventy-two cases were evaluated for EGFR staining. Membrane EGFR-positivity was observed in 44 % (32/72) of tumours (Fig. 3a-d and Table 4). Of the 72 cases examined, 15 % (11/72) scored 1+, 10 % (7/72) scored 2+, 19 % (14/72) scored 3+ and no immunostaining was observed in 56 % (40/72) samples. In addition to membrane EGFR immunostaining, both cytoplasmic and nuclear staining were also observed, however these were scored as

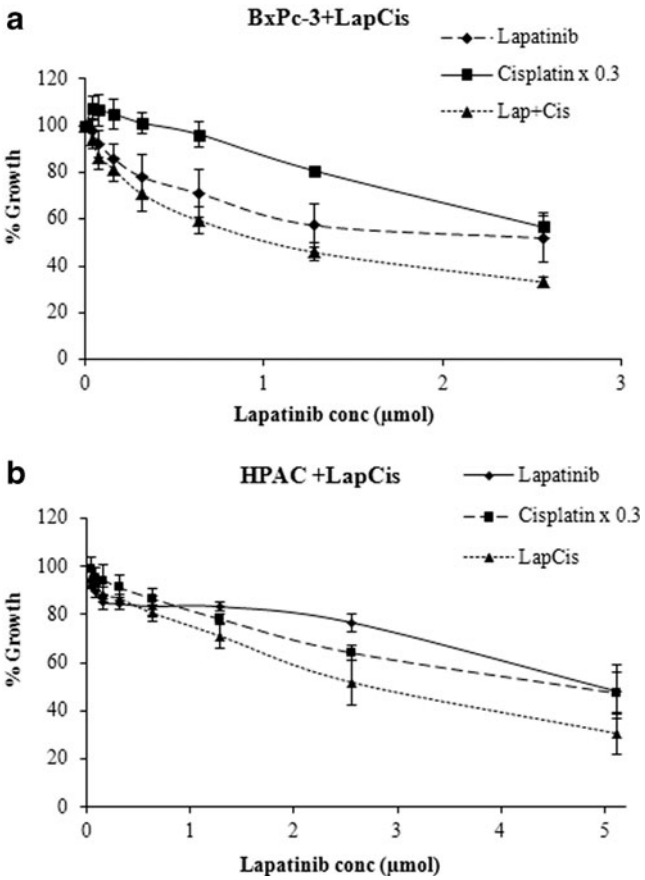

Fig. 2 Fixed combination proliferation assays of pancreatic cancer cell lines, **a** HPAC and **b** BxPc-3 treated with lapatinib in combination with cisplatin. Fixed ratio of lapatinib to cisplatin (1:0.3)

Table 3 Patient characteristics

	Number of patients (%)
Age (Unknown=2)	Median 62 yrs (range 36-91)
≤ 60	25 (35 %)
>60	45 (63 %)
Sex (Unknown=2)	
Male	36 (50 %)
Female	34 (47 %)
Tumour type (Unknown=2)	
Adenocarcinoma	60 (83 %)
Other	10 (14 %)
Tumour size (Unknown *n*=13)	
≤3 cm	23 (32 %)
>3 cm	36 (50 %)
Tumour Grade (Unknown=7)	
Well differentiated	13 (18 %)
Moderately differentiated	37 (51 %)
Poorly differentiated	15 (21 %)
Lymph node status (Unknown=14)	
Positive	37 (51 %)
Negative	21 (29 %)

negative. Clinicopathologically, EGFR over expression was associated with adenocarcinoma tumour type (p=0.04). No correlation was observed between EGFR status and tumour size, grade or lymph node status. A higher percentage of poorly differentiated tumours were positive for EGFR (60 % versus 38 % of well differentiated and 43 % of moderately differentiated), although this difference did not reach statistical significance.

HER2 expression in pancreatic cancer

Using the Herceptest® scoring system for grading HER2 staining, HER2 2+ immunostaining was only observed in the membrane of 1 out of the 72 pancreatic cancer cases (Fig. 4a-d).

Discussion

In this study, we show concentration-dependent antiproliferative effects of lapatinib in five pancreatic cancer cell lines tested. Lower lapatinib IC_{50} values were associated with higher levels of HER2 expression. However, cell lines with low HER2 and high EGFR expression also responded at concentrations of lapatinib<5 μM. The plasma concentration of lapatinib, achieved with a daily dose of 1000 mg, ranges from 1.1-1.6 μM [21]. Konecny et al. [22] found that increased EGFR expression was associated with improved in vitro response to lapatinib in endometrial cancer cells. However, other studies showed that lapatinib is more a potent cell growth inhibitor in HER2 overexpressing breast cancer cell lines [23] and is independent of EGFR expression [24]. Three pancreatic cancer cell lines AsPc-1, MiaPaCa-2 and Panc-1 showed significant resistance to erlotinib (IC_{50}>10 μM), but showed sensitivity to lapatinib.

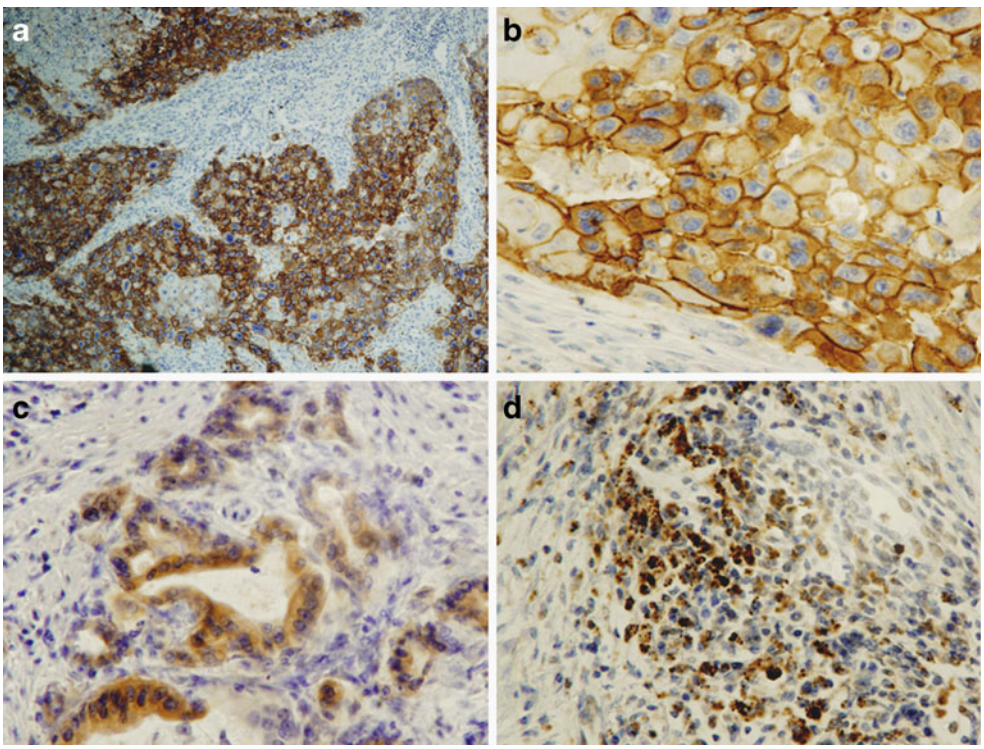

Fig. 3 Representative cases of EGFR expression in PDAC by IHC; **a** strong membrane score 3+, magnification 40× **b** strong membrane staining, score 3+, magnification 400× **c** strong cytoplasmic staining, magnification 200× **d** nuclear EGFR staining, magnification 100×

Table 4 EGFR protein expression intensity scores by IHC

	EGFR+ve expression	EGFR intensity score		
		1+	2+	3+
EGFR membrane positivity	32/72 (44 %)	11 (15 %)	7 (10 %)	14 (19 %)
Age (Un=2)				
≤60 (*n*=25)	10/25 (40 %)	4 (16 %)	4 (16 %)	2 (8 %)
>60 (*n*=45)	21/45 (47 %)	7 (16 %)	3 (7 %)	11 (24 %)
Sex (Un=2)				
Male (*n*=36)	15/36 (42 %)	2 (6 %)	4 (11 %)	9 (25 %)
Female (=34)	16/34 (47 %)	9 (27 %)	3 (9 %)	4 (12 %)
Tumour size (Un=13)				
≤3 cm (*n*=23)	12(52 %)	7 (30 %)	2 (9 %)	3 (13 %)
>3 cm (*n*=36)	15 (42 %)	3 (8 %)	5 (14 %)	7 (19 %)
Tumour type (Un=2)				
Adenocarcinoma (*n*=60)	30/60 (50 %)*	11 (18 %)	7 (12 %)	12 (20 %)
Non-Adenocarcinoma (*n*=10)	1/10 (10 %)	0	0	1 (10 %)
Tumour Grade (Un=7)				
Well (*n*=13)	5/13 (38 %)	2 (15 %)	2 (15 %)	1 (8 %)
Moderately (*n*=37)	16/37 (43 %)	5 (14 %)	4 (11 %)	7 (19 %)
Poorly (*n*=15)	9/15 (60 %)	4 (27 %)	1 (7 %)	4 (27 %)
Lymph node status (Un=14)				
Positive (*n*=37)	19/37 (51 %)	9 (24 %)	4 (11 %)	6 (16 %)
Negative (*n*=21)	6/21 (29 %)	1 (5 %)	2 (10 %)	3 (14 %)

*Chi-squared p=0.04

Yamasaki et al. [25] reported that sensitivity to erlotinib in breast cancer cell lines is independent of EGFR levels and also unrelated to HER2, p-Akt, p-ERK, 1/ERK2 (p42/p44), and p27 levels. Recent evidence suggests that mutated K-Ras predicts for lack of sensitivity to EGFR inhibitors [26]. Approximately 80 % of pancreatic tumours contain point

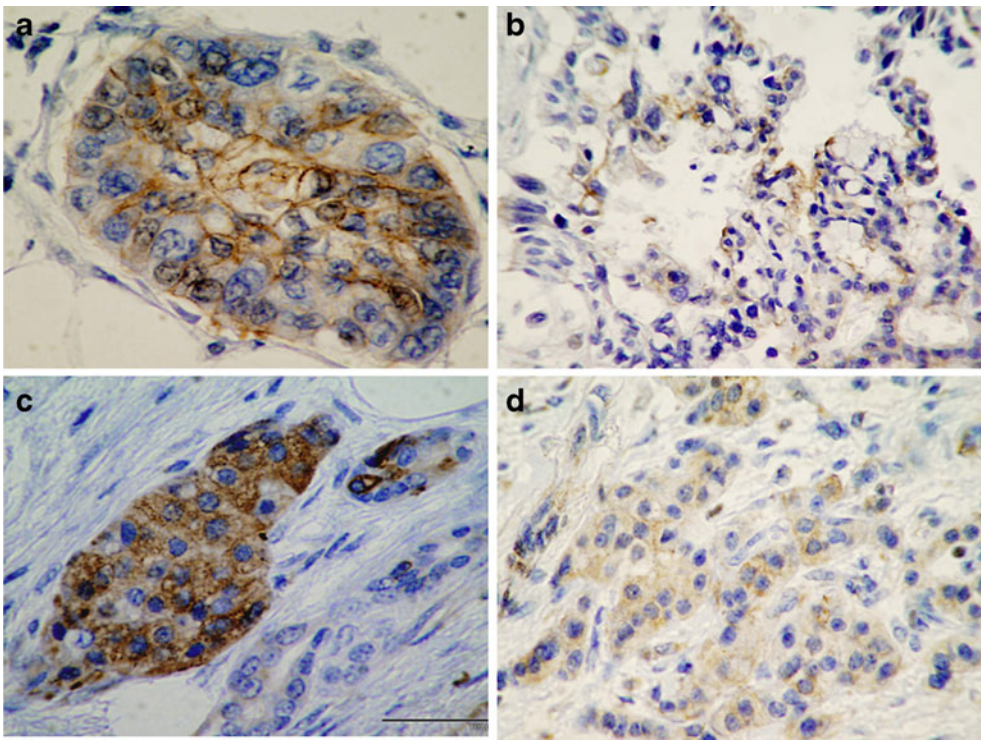

Fig. 4 Representative cases of HER2 expression in PDAC by IHC; **a** moderate membrane score 2+, magnification 400× **b** weak membrane staining, score 0, <10 % of tumour, magnification 200× **c** strong cytoplasmic staining, score 0, magnification 400× **d** weak cytoplasmic staining, score 0, magnification 200×

mutations of the K-Ras protooncogene [27, 28] and K-Ras mutation is associated with unfavourable outcomes [29]. Lung adenocarcinoma tumours containing K-Ras mutations are resistant to EGFR inhibitors, gefitinib and erlotinib [30]. In colorectal cancer, mutations of K-Ras are also associated with resistance to cetuximab [31]. In a study of gemcitabine with or without erlotinib in patients with advanced pancreatic cancer (PA.3 trial), Moore et al. [32] subsequently examined the mutational status of K-Ras, where tumour was available. Greater benefit for overall survival was observed for patients treated with erlotinib plus gemcitabine who had wild-type K-Ras, however, this finding was not significant ($p=0.34$). All of the pancreatic cancer cell lines used in this study carry mutated K-Ras, except BxPc-3 [33] (www.sanger.ac.uk/genetics/CGP/cosmic/), which is also the cell line that is most sensitive to lapatinib. Three of the four K-Ras mutated cell lines are resistant to erlotinib at concentrations up to 10 μM but display sensitivity to lapatinib with IC_{50}s ranging from 2.3-4.1 μM. We also examined in vitro interactions between lapatinib and chemotherapeutic agents: cisplatin, gemcitabine and 5'dFUrd in two pancreatic cancer cell lines. The combination of lapatinib with cisplatin is synergistic-additive, while the interaction of lapatinib and 5'dFUrd is additive; however, additive to slightly antagonistic interactions were observed for the combination of lapatinib and gemcitabine in both cell lines. A phase II trial of lapatinib and gemcitabine revealed that this combination may not be effective in pancreatic cancer [34]. Phase II clinical trials investigating the combination of lapatinib and capecitabine for second-line treatment of metastatic pancreatic cancer are currently on-going (ClinicalTrials.gov, NCT00881621) [35]; however, it has been found that this combination does not improve the overall survival in the first-line treatment of advanced pancreatic cancer patients [36].

Many studies show that EGFR is over-expressed in pancreatic cancer [12], our IHC results are in agreement, whereby EGFR was over-expressed in 44 % of pancreatic tumours with significant correlation to adenocarcinoma tumour type ($p=0.04$). We found that membrane HER2 is not frequently over-expressed in pancreatic cancer (1/72). Conflicting results exist on the over-expression of HER2 in pancreatic cancer, some of these differences, in older studies, may be partly due to the use of different HER2 antibodies, and scoring criteria employed. Our study used the antibody used in the Herceptest kit (A0485, Dako), which has been shown to be a reliable, cost-effective alternative to Herceptest [37], and the Dako score for HER2 staining criteria which was optimised for breast cancer. Amplification of the HER2/neu gene has been show to occur in 20-30 % of breast cancers [38]; however, very low [17] HER2/neu gene amplification have been observed in PDAC, which may support the lack of HER2 over-expression in our study.

While most studies agree that over-expression of membrane EGFR and HER2 correlates with clinicopathological outcomes, we observed cytoplasmic and nuclear staining for EGFR and HER2. A study by Ueda et al. [39] showed that cytoplasmic EGFR over-expression in the invasive component of PDAC, correlated with higher grade and worse patient prognosis, whereas membrane EGFR and HER2 over-expression were more frequently localised in the intraductal component. Cytoplasmic HER2 has also been detected in colon cancers and associated with tumour differentiation [40]. The role of nuclear EGFR in PDAC tumours observed in this study has not been elucidated. However, there is evidence to suggest that EGFR may enter the nucleus and directly function as a transcriptional factor [41]. Nuclear EGFR has been associated with poor clinical outcome in breast cancer [42] and oropharyngeal squamous cell carcinomas [43].

Conclusion

Our study suggests that pancreatic cancer cells are sensitive to lapatinib, including cells that are resistant to erlotinib, and that lapatinib combined with chemotherapeutic drugs such as cisplatin and capecitabine warrant further investigation in pancreatic cancer. IHC analysis of EGFR and HER2 suggest that while HER2 is not frequently over-expressed in pancreatic cancer, EGFR is expressed in 44 % of the pancreatic tumours tested. However, as EGFR expression may not a good predictor of response to EGFR inhibition, additional predictive biomarkers will be required to identify the pancreatic cancer patients that may benefit from lapatinib treatment.

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Disclosure/Conflict of Interest The authors have no conflict of interest to declare.

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