Coexpression of EGFR and HER-2 in pancreatic ductal adenocarcinoma: A comparative study using immunohistochemistry correlated with gene amplification by fluorescencent *in situ* hybridization

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) is the major pancreatic tumor and carries an extremely poor prognosis. Coexpression of epidermal growth factor receptor (EGFR) and the HER-2 oncoprotein has been reported to be related to the invasion and an adverse clinical outcome of human pancreatic ductal adenocarcinomas. HER-2 amplification, as determined by fluorescent *in situ* hybridization (FISH) analysis, has been identified as a positive predictor of response to EGFR tyrosine kinase inhibitor treatment in some other cancers. The aim of this study was to investigate the coexpression rate and amplification status of HER-2 oncogene in EGFR positive pancreatic ductal adenocarcinoma (PDAC) by immunohistochemistry and FISH. Overexpression of EGFR ($\geq 2^+$) was seen in 65% (21/32) of the study cases. EGFR gene amplification was not detected in any of the 32 PDACs. Overexpression of HER-2 protein ($\geq 2^+$) was seen in 17% (5/28) of the study cases and in 24% (5/21) of EGFR positive cases. None of the EGFR negative tumors showed HER-2 overexpression or gene amplification. The HER-2 gene locus was amplified in 11% (3/28) of the study cases and in 14% (3/21) of EGFR positive PDACs. There was 60% concurrence between HER-2 gene amplification and HER-2 protein expression in this study. These results suggest that HER-2 is an important cooperating member of the EGFR signal pathway in a subset of PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) accounts for 2% of malignant tumors in both men and women, but was the fourth leading cause of cancer deaths after lung, colon and prostate for men, and lung, breast, and colon for women in the US in 2006 (1). Since most patients with PDAC present late in the course of their disease, and are detected in the late stages of the disease, their mortality rates are extremely high, and most patients die of the disease within a year (2).

Both epidermal growth factor receptor (EGFR) and HER-2 oncoprotein are members of the human EGFR family of transmembrane receptor tyrosine kinases and have crucial roles in the growth, repair, and differentiation of cells. In a normal pancreas, EGFR is rarely expressed only in the Langerhans islands and ductal cells, whereas EGFR overexpression has been reported to occur in >15-60% of both surgically resected pancreatic carcinoma and pancreatic carcinoma cell lines (3). The overexpression of EGFR has been shown to enhance the production of EGF and TGF-α by promoting autocrine and paracrine loops and cell proliferation.

EGFR inhibitors, including the monoclonal antibodies directing the receptors and the small molecule tyrosine kinase inhibitors (TKIs) have been implicated and demonstrated to have some effects in non-small cell lung cancers (NSCLCs) (4). The association with EGFR mutations, and sensitivity to the EGFR inhibitors in the NSCLC, have been described in retrospective studies (5,6). The associations with EGFR expression and EGFR copy numbers have also been reported. It has become increasingly clear that EGFR works in concurrence with other EGFR family members, especially HER-2 and ErbB3 in the NSCLCs (7). HER-2 has been identified as a potentially important component of the EGFR signaling network and particularly in the EGFR TKI-sensitive NSCLCs (8). HER-2 amplification, as determined by fluorescent *in situ* hybridization (FISH) analysis, was identified as a positive predictor of response to EGFR TKI treatment.

HER-2 overexpression has also been reported to be correlated with glandular differentiation and early oncogenesis in pancreatic cancer (9). Coexpression of EGFR and the HER-2 oncoprotein has been reported to be related to the invasion and an adverse clinical outcome in pancreatic adenocarcinomas. In this study, we performed

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immunohistochemical and FISH analyses of EGFR and HER-2 to determine the coexpression and amplification of EGFR and HER-2 in PDAC.

Materials and methods

Patients and tumor specimens. Representative tissue samples from 32 cases of nonselected PDAC were obtained from the surgical pathology PDAC patient database that underwent resection at Louisiana State University Health Science Center (LSUHSC) and from consultation files between January 1997 and March 2005. Hematoxylin-eosin stains were reviewed by two pathologists, and representative paraffin-embedded blocks were selected for further analysis. Clinicopathological characteristics of the 32 patients were evaluated including age, tumor size, and AJCC stage. There were 20 female and 12 male patients. The median age of the patients at the time of operation was 64 (range: 34-82) years.

Immunohistochemical stain. EGFR and HER-2 expressions were evaluated with an immunohistochemical technique on 5-μm tissue sections obtained from paraffin-embedded specimens fixed in 10% neutral-buffered formalin. Immunohistochemical staining for HER-2 was performed in 28 cases with the HercepTest kit (Dako, Carpinteria, CA). Immunohistochemical staining for EGFR was performed in 32 cases using the EGFR pharm Dx kit (Dako). Positive staining for EGFR and HER-2 were evaluated by both manual microscopic evaluation and automated cellular image system (ACIS) (Chromavision Inc., CA, USA) to validate the manual analysis. Manual microscopic scoring was evaluated according to the manufacturer's instructions using the following scale: 0 and 1^+, normal HER-2 /EGFR expression and 2 and 3^+, HER-2/EGFR overexpression. The high color staining covered at least 10% of the specimen for a positive evaluation of overexpression. For ACIS evaluation in each slide, a minimum of 6 areas were chosen in which the intensity of color was evaluated and then quantified to give HER-2 and EGFR expression scores that were dependent on median staining intensity of the 6 areas.

Fluorescent in situ hybridization (FISH). The PathVysion kit (Vysis, Downers Grove, IL) was used for FISH studies. FISH for HER-2 was performed in 28 cases with dual color HER-2 Spectrum Orange/CEP17 spectrum green-labeled probe. FISH for EGFR was performed in 32 cases with the dual color EGFR Spectrum Orange/CEP7 spectrum green-labeled probe. The CEP rapid assay protocol (Vysis) was used. Slides containing 3-μm sections of the tumor were dehydrated by serial treatment with increasing concentrations of ethanol. Prior to hybridization, the slides were treated with the Paraffin pretreatment reagent kit (Vysis). DNA denaturation was carried out at 75˚C for 10 min (probe mixture) or 5 min (slides). For interpretations of EGFR, CEP7 (chromosome 7, green signal) and EGFR (red signal) were counted in a minimum of 30 cells using a fluorescence microscope. For interpretations of HER-2, CEP17 (chromosome 17, green signal) and HER-2 (red signals) were counted using a fluorescence microscope in a minimum of 30 cells. A ratio of red to green signals of 2.0 or >2.0 was interpreted as cases with gene amplification, and cases with non-amplification were defined as a ratio of <2.0.

Table I. Summary data of pancreatic ductal adenocarcinoma cases.

Case No.	Age (yr)/sex	Stage	IHC-HER2	HER-2 Gene Ratio	IHC-EGFR
1	75/F	I	1	1.1	3
2	50/F	I	1	1.0	2
3	82/F	I	1	1.0	1
4	54/F	I	2	1.2	1
5	34/F	I	1	1.19	1
6	77/F	I	-	-	1
7	59/F	I	1	1.02	3
8	76/F	I	1	1.05	2
9	62/F	I	2	2.02	1
10	64/F	II	1	1.07	2
11	64/M	II	3	2.3	3
12	58/M	II	1	1.0	2
13	60/M	II	1	1.12	1
14	80/F	II	3	6.3	3
15	73/F	II	1	1.0	3
16	57/M	II	1	1.1	2
17	57/M	II	1	1.03	3
18	70/F	II	1	1.22	3
19	79/M	II	1	0.94	1
20	59/M	II	1	1.07	2
21	47/F	II	1	0.98	2
22	62/M	II	0	0.98	3
23	50/M	II	1	1.1	2
24	60/M	II	-	-	1
25	60/F	II	1	1.08	0
26	53/M	II	-	-	1
27	59/F	II	1	1.05	3
28	70/F	II	1	1.05	3
29	66/M	II	1	1.43	3
30	71/F	II	2	3.4	3
31	64/F	III	-	-	1
32	77/F	III	1	1.09	3

Results

The clinicopathological characteristics, including the patient population, and tumor staging used in this study, are summarized in Table I. The EGFR IHC scores and gene amplification results for 32 PDACs are in Table II. HER-2 gene amplification and IHC scores for 28 PDACs are listed in Table III.

Comparison of EGFR overexpression with amplification in PDAC. EGFR overexpression (≥2) was seen in 21 of 32 (65%) cases. Of the 32 PDACs, 13 (41%) showed 3^+ labeling,

Table II. Clinical characteristrics of cases with EGFR overexpression and amplification.

		IHC			FISH	
	n	3+	2+	Negative	Gene amplification	Negative
PDAC	32	13	8	11	0	32
		41%	25%	34%	0%	100%
Stage						
I	9	2	2	5	0	9
II	21	10	6	5	0	21
III	2	1	0	1	0	2

Table III. Clinical characteristrics of cases with HER-2 overexpression and amplification.

		IHC			FISH	
	n	3+	2+	Negative	Gene amplification	Negative
PDAC	28	2	3	23	3	25
		7%	10%	83%	11%	89%
Stage						
I	8	0	2	6	0	8
II	19	2	2	15	3	16
III	1	0	0	1	0	1

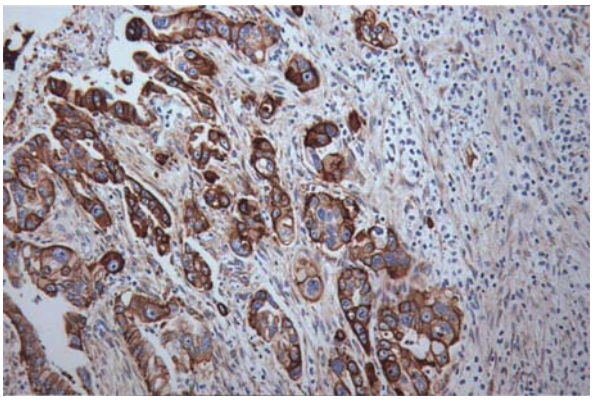

Figure 1. Overexpression of EGFR in PDAC by immunohistochemical stain.

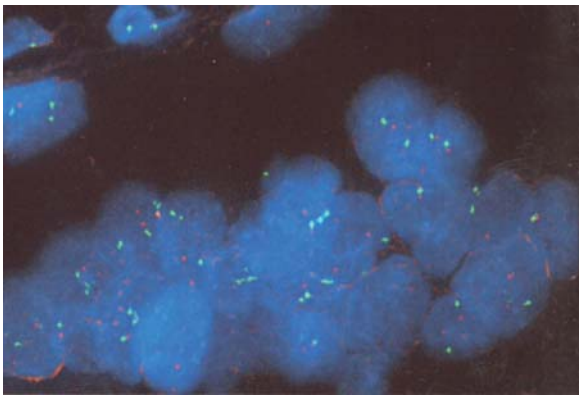

Figure 2. FISH of EGFR in PDAC. No amplification of the EGFR gene was observed.

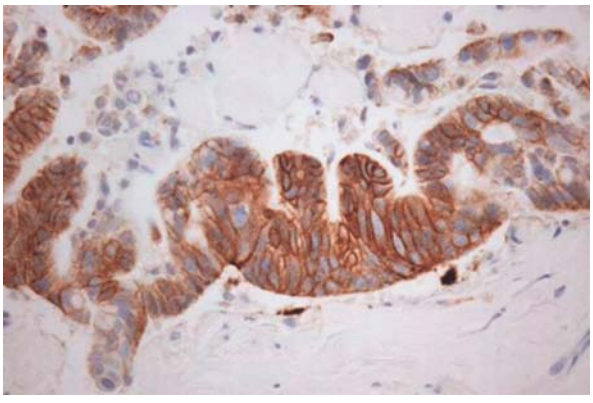

Figure 3. Overexpression of Her-2 in PDAC by immunohistochemical stain.

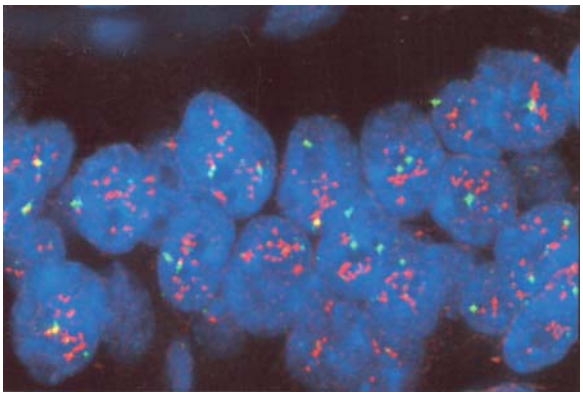

Figure 4. FISH analysis of HER-2 in PDAC demonstrating amplification of HER-2.

and 8 of 32 (25%) showed 2+ labeling for EGFR by IHC. EGFR overexpression is shown in Fig. 1. Patient age, sex, and tumor stage did not have a significant correlation with EGFR overexpression. EGFR gene amplification was not detected in any of the 32 PDACs by FISH (Fig. 2).

Comparison of HER-2 overexpression with amplification in PDAC. Of the 28 PDAC cases, 2 (7%) showed 3+ labeling, and 3 (10%) showed 2+ labeling for HER-2 by IHC. HER-2 overexpression is shown in Fig. 3. HER-2 overexpression also failed to show any correlation with patient age, sex, and tumor stage in our study. HER-2 gene locus was amplified in 3 of 28 PDACs (11%). Two of three (67%) HER-2 gene amplified cases showed 2+ labeling, and one (33%) showed 3+ labeling by HER-2 IHC. HER-2 amplification by FISH is shown in Fig. 4. HER-2 amplification did not have a significant correlation with patient age, sex, and tumor stage.

Coexpression of EGFR and HER-2 in PDAC. Of the 21 EGFR positive PDAC cases, 5 (24%) showed positive HER-2 stain by IHC. Of the 21 EGFR positive PDAC cases, 3 (14%) showed HER-2 gene amplified in FISH studies.

Discussion

EGFR belongs to a receptor family that has three additional proteins including ErbB-2(HER-2), ErbB-3 and ErbB-4 (11). The EGFR family has a central role in the pathogenesis and progression of variable carcinomas (12,13). The proteins and the growth factors of the EGFR family form a tightly integrated system in which a signal that is received by an individual receptor type is often transmitted to other receptors of the same family (14). This family of receptors is implicated in cell-cell and cell-stromal communication primarily through signal transduction. The epidermal growth factors and their ligands affect the transcription of various genes by phosphorylating or dephosphorylating a series of transmembrane proteins and intracellular signaling intermediates (15). They ultimately affect cell proliferation, survival, motility, and adhesion in various carcinomas including PDAC (16).

Gene amplification, overexpression and activating mutations of EGFR have been reported in various human cancers. In our study, 13 of 32 (41%) cases scored 3^+, and 8 of 32 (25%) cases scored 2^+ for EGFR overexpression by IHC. Previous studies have shown that overexpression of EGFR was correlated with shorter postoperative survival in PDAC (17). Gene amplification of EGFR has been demonstrated to occur in different tumor types and is usually associated with EGFR protein overexpression, but overexpression of EGFR in the absence of gene amplification has also been described in various cancers (18). PDAC cases with EGFR overexpression ($\geq 2^+$) labeled by IHC in our study failed to demonstrate gene amplification by FISH. EGFR gene amplification has been reportedly demonstrated in 22-32% of primary NSCLC, and in 6% of primary breast carcinomas (5,19). However, there are no previous data for EGFR gene amplification in PDAC.

Mutated forms of EGFR have been studied in NSCLC patients and reported in ≤17% of NSCLC (20). However, only 1 exon 18-21 mutation was seen among 34 lung cancers that could be successfully sequenced. It is still not clear which clinicopathological parameters in NSCLC might be the best predictors of response to EGFR inhibitors and overall survival (21,22). EGFR exon 18-21 mutations have been reported to be ethnically different, being rare in certain populations such as the Middle East or Western country patients with NSCLC (23). The high rate of EGFR gene amplifications could potentially facilitate studies on the predictive role of gene copy number changes for response to anti-EGFR therapies in these patients (23). EGFR exon 19 and 21 mutations have been sought in 40 pancreatic tumors (classification not specified) (7). One recent study reported that no alterations in EGFR exons 18-21 were detected in KRAS-positive or KRAS-negative cases of pancreatic ductal adenocarcinoma (24). Several designed anticancer therapies targeting EGFR receptors are being tested, and available research data suggest some activity of treatment with EGFR-TKIs in PDAC, but the experience is limited (25,26).

HER-2/neu, often referred to as C-erbB-2 proto-oncogene mapped to chromosome 17q21, encodes a 185 kDa transmembrane glycoprotein, designated as p185HER2 (27,28). In our study, the positive rate of membranous overexpression of HER-2 protein ($>2^+$) was seen in 17% of cases by IHC. The reported rates of HER-2 overexpression in PDAC vary between 20 and 80% (29,30). HER-2 overexpression was found to be a poor prognostic factor in breast carcinoma and PDACs (16). The positive rate of HER-2 gene amplification in our study was seen in 11% of cases by FISH. Correlation between HER-2 gene amplification by FISH and HER-2 protein overexpression by IHC of the PDAC in our study was 3 of 5 (60%) in IHC positive cases. Overexpression of HER-2 is frequently associated with gene amplification (31). Our results have shown better correlation between HER-2 overexpression by IHC and HER-2 gene amplification by FISH in PDACs than previously reported (29). HER2/neu gene amplification has been reported as a very important prognostic factor for patients with breast cancer since it correlates to the chemotherapy response. However, in PDAC cases, there is controversial data regarding its biological significance.

Tumors that co-express different ErbB receptors are often associated with a more aggressive phenotype and a worse prognosis. Dimerization with other members of the EGFR family is responsible for the formation of active receptors being able to bind a growth factor neuregulin or to be active in signal transduction (32). In our study, five of the 21 (24%) EGFR positive PDAC cases showed positive HER-2 stain by IHC. Of the 21 EGFR positive PDAC cases, three (14%) were amplified HER-2 in FISH studies. None of the EGFR negative cases showed HER-2 overexpression or gene amplification. There are reported data establishing a link between expression of EGFR and activation of HER-2 in breast cancer patients (17). Adverse prognostic value of HER-2 overexpression has been observed, especially when HER-2 is in the phosphorylated state or coexpressed with EGFR in invasive breast cancers (17). Some data suggest that HER-2 likely augments EGFR-mediated oncogenicity and addiction to EGFR signaling. One possible reason is the effect that HER-2 has on EGFR cycling. EGFR/HER-2 heterodimers are preferentially recycled back to the plasma membrane compared with EGFR homodimers that are more often targeted for degradation (33). The growth and survival of carcinoma cells appear to be sustained by a network of receptors/ligands of the ErbB family.

This phenomenon has been reported as an important point for therapeutic approaches since the response to anti-EGFR agents might depend on the total level of expression of EGFR receptors and ligands in tumor cells (13). HER-2 has been identified as a potentially important component of the ErbB signaling network in the EGFR TKI-sensitive lung cancers. Substantially, HER-2 amplification, as determined by fluorescence *in situ* hybridization analysis, was identified as a positive predictor of response to EGFR TKIs (10).

In conclusion, our results show 60% concurrence between HER-2 protein expression by IHC and HER-2 gene amplification in PDAC. In contrast, no concurrence was demonstrated between EGFR protein overexpression by IHC and EGFR gene amplification. Twenty-one percent of EGFR overexpression cases showed HER-2 co-expression. These

results suggest that HER-2 appears to be one of the cooperating EGFR family members of the EGFR signal pathway in PDAC. Further studies might be needed for evaluation of co-expression of HER-2 and mutant EGFR in PDAC.

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