

# Evaluation of phosphatidylinositol-3-kinase catalytic subunit (PIK3CA) and epidermal growth factor receptor (EGFR) gene mutations in pancreaticobiliary adenocarcinoma

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**Background:** Phosphatidylinositol-3-kinase (PI3K) activation involves the epidermal growth factor receptor (EGFR) and plays an important role in cell survival signaling in pancreaticobiliary cancer. EGFR gene mutations have been correlated with clinical response to EGFR inhibitors in patients with advanced non-small cell lung cancer. This study examined the prevalence of PIK3CA and EGFR mutations in pancreaticobiliary cancer where erlotinib, an EGFR inhibitor, is approved for therapy.

**Methods:** Thirty patients who underwent pancreatectomy for pancreaticobiliary carcinoma were identified. Genomic DNA was extracted from formalin fixed paraffin embedded tumor and adjacent normal tissue, and exons 9 and 20 (for the PIK3CA gene) and exons 18-21 (for the EGFR gene) were amplified by PCR and sequenced. Literature review on EGFR and/or PIK3CA mutations in pancreaticobiliary adenocarcinomas was conducted.

**Results:** No mutations in either PIK3CA or EGFR genes were identified. The study identified one synonymous single nucleotide polymorphism (SNP) (rs1050171) in the coding region of EGFR. A previously unreported change, suspected to be a SNP, was observed in intron 18 of EGFR (IVS18+15, C>T). Review of the literature showed EGFR mutation rate of 2% and 10.5% in pancreatic and biliary tract carcinomas, respectively. PIK3CA mutations were found in 3.6% and 11.7% of pancreatic and biliary tract carcinomas, respectively.

**Conclusions:** A low prevalence of EGFR or PIK3CA mutations exists in pancreatic cancer (<5%), indicating that mutation screening may not be as useful in determining prognosis or response to targeted inhibition.

**Key Words:** Phosphatidylinositol-3-kinase catalytic subunit (PIK3CA); epidermal growth factor receptor (EGFR); mutations; pancreas; biliary; cancer



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## Introduction

Epidermal growth factor receptor (EGFR)-mediated cell signaling, including the Ras/mitogen-activated protein kinase (MAPK) signaling pathway activation, plays an important role in angiogenesis, proliferation, and apoptosis (1-2). Tumors often exhibit alternations in receptor tyrosine kinases (TK) upstream of phosphatidylinositol-3-kinase (PI3K), the p110 catalytic subunit of PI3K (PIK3CA), the downstream kinase, Akt, and the negative regulator, PTEN (3). EGFR missense and deletion mutations were found in 13.4% of non-small cell lung cancer (NSCLC)

patients, within exons 18 through 21 of the kinase domain (4). Lynch *et al.* reported in-frame deletions and amino acid substitutions, clustered in the region of the ATP-binding pocket of the TK domain, in eight of nine patients with gefitinib-responsive NSCLC (5). While EGFR mutations are characteristic for NSCLC, PIK3CA mutations are also identified in glioblastomas, colorectal cancer, gastric cancer, and breast cancer (3,6). EGFR is expressed by many epithelial tumor cells, including biliary and pancreatic cancers (7-9).

Inhibition of activated protein kinases through the use of targeted small molecule drugs (i.e., gefitinib and erlotinib)

or antibody-based (i.e., cetuximab and panitumumab) strategies have emerged as an effective approach to cancer therapy (10-12). EGFR expression itself is not a definite predictor of response to EGFR TK inhibitors (13), however, EGFR mutations in NSCLC were found to predict sensitivity to gefitinib (4). Phase II studies have shown that TK inhibitors (TKI) induced response in over 70% of NSCLC patients harboring EGFR mutations (14).

Both pancreatic and biliary tract carcinoma are diagnosed at advanced stages when incurable, and outcomes even with surgery and chemotherapy, are poor (15-19). Combination of erlotinib and gemcitabine in advanced pancreatic cancer showed a modest increase in survival compared to gemcitabine alone, and resulted in the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approval for this regimen as first-line treatment of pancreatic cancer (20).

The objectives of this study were to determine the prevalence of EGFR and PI3K mutations in patients with pancreaticobiliary cancers. No studies had been reported at the time our research began of either EGFR or PIK3CA mutations in either disease. Several small reports have been published since, and this article will summarize the current literature in this field.

## Materials and methods

### Study population

This study was performed with approval of the Roswell Park Cancer Institute (RPCI) Institutional Review Board. The institutional pathology department reviewed all cases of pancreatic and biliary tract cancers following pancreatectomy diagnosed at RPCI over a period of five years between December 1, 1999, and November 30, 2004. All tumor blocks with adequate DNA for performing mutation analysis were selected for inclusion. Clinical data, including age, sex, ethnicity, and clinical stage, was obtained via chart review unblinded to mutation results. The samples were numbered consecutively to ensure patient confidentiality.

### Histopathological evaluation

Twenty micron curls from tumor samples were examined with hematoxylin and eosin stain of the same area to ensure that the DNA is being extracted from a slice with maximal tumor and not normal tissue. The study examined mutational "hotspots" within the PIK3CA and EGFR genes based on reports by Pao *et al.* and Broderick *et al.* (4,6,21-22). The most frequently reported alterations in the PIK3CA gene in adult neoplasms are missense mutations in exon 9, which encodes a portion of the helical domain

of the PIK3CA protein, and exon 20, which encodes the C-terminus of p110 $\alpha$  catalytic subunit. PIK3CA gene mutations are believed to be activating mutations, and NIH3T3 cells transfected with H1047R (exon 20) mutant p110 $\alpha$  constructs have increased lipid kinase activity as compared to cells transfected with wild-type p110 $\alpha$  (21).

Mutational analysis was also performed for exons 18-21 of the EGFR gene that encode the protein TK domain of the EGFR protein. In-frame deletions in a GXGXXG motif (exon 19) as well as the missense mutations G719S (exon 18) and L858R (exon 21) of the EGFR gene associated with response to gefitinib were studied (*Figure 1*).

### DNA extraction

A portion of snap frozen tumor biopsies from each patient were homogenized individually in TE buffer. Genomic DNA was extracted from each sample using a standard phenol/chloroform protocol, and the DNA quality was assessed by both spectrophotometry and visualization on an ethidium bromide stained agarose gel. Working dilutions of 50 ng/ $\mu$ L were prepared for each sample and DNA samples were stored at 4 °C.

### PCR and direct sequencing

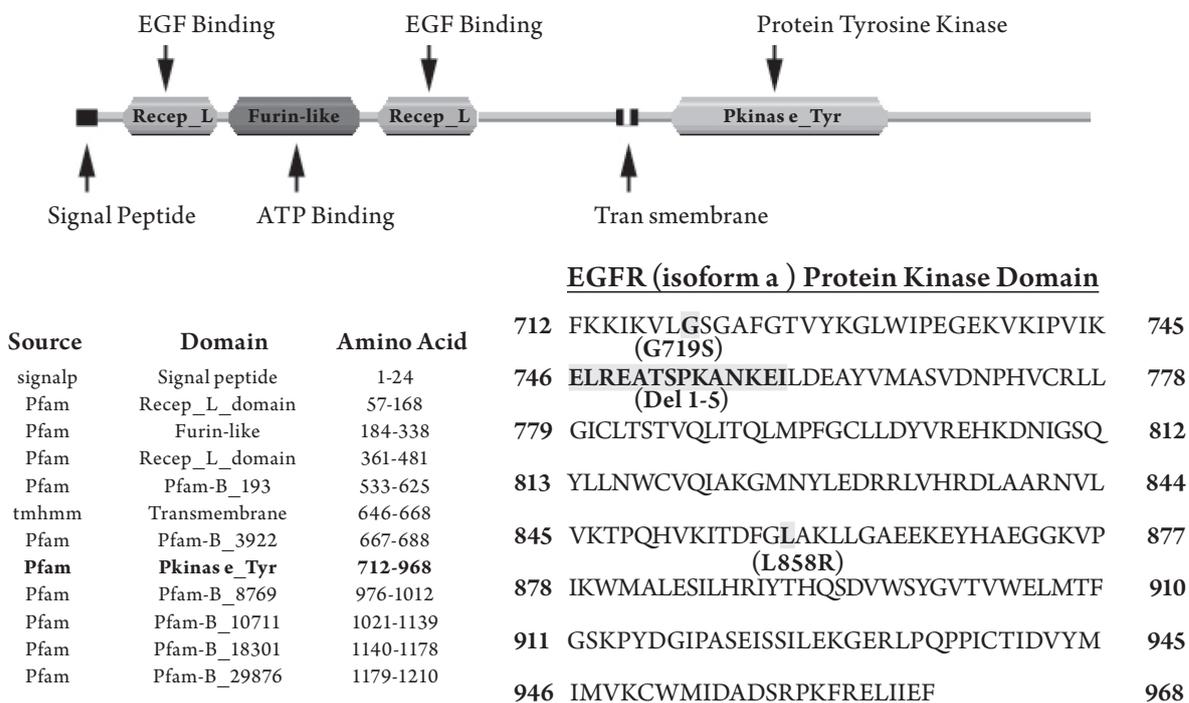
Primers sequences for exons 9 and 20 of the PIK3CA gene were designed using NCBI (<http://www.ncbi.nlm.nih.gov/>) published gene sequence information and the Primer 3 program (<http://frodo.wi.mit.edu/>). Bardelli *et al.* published primer sequences for exons 18-21 that were used for EGFR gene sequencing (23). PCR was performed using high-fidelity PCR reagents and individual exons were amplified for 35 cycles using standard reaction conditions. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA) and purified products were sequenced in both directions using an ABI Prism 3100. Sequence information for samples was compared directly to the human reference sequence (NCBI Build 36.1) and single nucleotide polymorphisms (SNPs) were identified using both the NCBI SNP and HapMap (<http://www.hapmap.org>) databases.

### Statistical analysis

A description of the presence/absence of these known mutations in each of the two malignancies was tabulated and reported as a percentage of the total number of samples screened.

### Review of literature

For the literature review, the electronic databases PubMed



**Figure 1** Mutations of exons 18-21 of the EGFR gene, including deletions in exon 19, and missense mutations G719S (exon 18) and L858R (exon 21)

and MEDLINE, as well as the Cochrane library and the American Society of Clinical Oncology (ASCO) abstracts were searched using the key words pancreatic, pancreas, biliary, cholangiocarcinoma, cancer, EGFR, PIK3CA, and mutation, in all possible combinations, limited to humans and English-language studies. All articles published by the year 2011 were included. The authors adhered to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.

**Results**

**Demographics and clinicopathological features of study population**

Table 1 summarizes the clinicopathological findings for the 30 patients included in this study. The number of males was equal to the number of females. The median age was 63.8 (range, 46 to 84) years. Most of the patients were Caucasians, and over 86% (26 patients) were diagnosed with pancreatic adenocarcinoma.

Twenty patients (67%) had a TNM stage II disease, 5 patients (17%) had a stage I disease, and 5 patients (17%) had advanced disease (stage III or IV). The patients who were found to have advanced disease had a clinically

resectable tumor, yet final pathological report showed a T4 tumor and/or micrometastatic disease.

One patient was treated with neoadjuvant chemoradiotherapy with doxorubicin, 5-fluorouracil (5-FU), and cisplatin. Eighteen (60%) patients were treated with adjuvant therapy, two-third of them with a combination of chemoradiotherapy and the remaining third with chemotherapy alone. Ten patients were treated with 5-FU or capecitabine, four patients received gemcitabine, and two patients received a combination of both. One patient was treated with 5-FU in combination with streptozotocin, mitomycin, and leucovorin.

Subsequent therapies at the time of metastases detection were gemcitabine, oxaliplatin, or taxane based. Only one patient received gemcitabine in combination with erlotinib (Tarceva®, OSI Pharmaceuticals, LLC).

**Sequence information**

No mutations were identified in either exons 9 and 20 of the PIK3CA gene or in exons 18-21 of the EGFR gene in the 30 pancreaticobiliary tumors that were analyzed. One single nucleotide polymorphism (SNP), rs45455192, located in intron 8 of the PIK3CA gene was identified in a single tumor sample. This particular SNP has no reported

**Table 1** Patients' demographics and clinicopathological features (N=30)

Characteristic	Frequency	
	No.	%
Age at diagnosis, years		
Median	63.8	
Range	46-84	
Male sex	15	50%
Race/ethnicity		
Caucasians	27	90%
African-American	2	7%*
Not reported	1	3%*
Type of cancer		
Pancreatic adenocarcinoma	26	87%*
Cholangiocarcinoma	3	10%
Ampulla of Vater	1	3%*
Stage (TNM)		
I	5	17%*
II	20	67%*
III	3	10%
IV	2	7%*
Adjuvant treatment	18	60%
Chemoradiotherapy	12	40%
Chemotherapy alone	6	20%
Untreated	9	30%
Unknown	3	10%

\*Percentages were rounded off

frequency data. No unusual clinical findings were seen in the patient who provided this sample (*Figure 2A*).

An unreported variant in intron 18 of the EGFR gene was identified in one tumor sample (*Figure 2B*). This variant (C>T) occurred sixteen base pairs immediately following exon 18 (IVS18+16) and was near a reported SNP (rs17337107). This variant was queried as a possible splice site mutation, but no evidence for this change adversely affecting splicing was identified using splice site prediction software.

### Review of literature

Review of the literature yielded thirteen relevant articles and abstracts relating to EGFR and/or PIK3CA mutations in human pancreatic adenocarcinoma. This is summarized in *Table 2*, including the results of this current study. Overall only 7 EGFR mutations were found among 347 pancreatic cancers (2%). Kwak *et al.* demonstrated disease stabilization with EGFR inhibition (erlotinib with

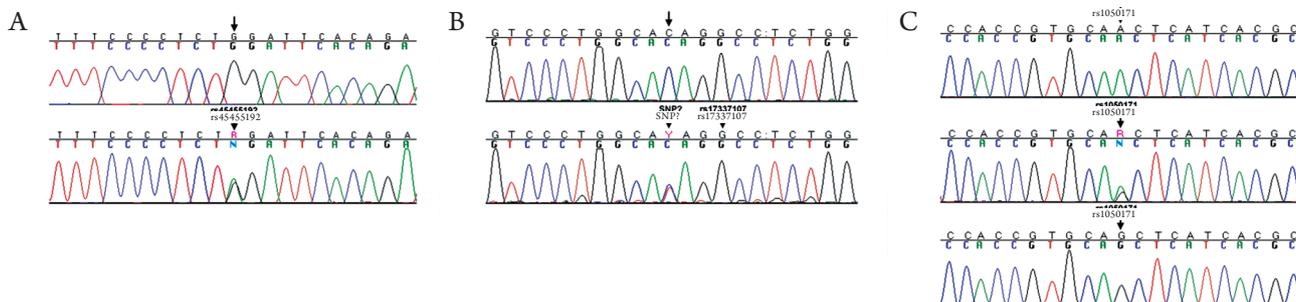
capecitabine) in 5 out of 55 cases, including both (2/2) pancreatic cancers with EGFR mutations (24). PIK3CA mutations were identified in 2 out of 56 cases (3.6%) of pancreatic adenocarcinoma. Jimeno *et al.* found that 2 of 10 human tumors were sensitive to EGFR inhibition, including the single (1/1) pancreatic cancer with PIK3CA mutation (30).

Similar search yielded eight articles and abstracts that investigated biliary tract carcinomas. The summary of these publications is presented in *Table 3*, including the results of this current study. A total of 19 EGFR mutations (10.5%) and 18 PIK3CA mutations (11.7%) were found in 180 and 154 biliary tumors, respectively. The latter percentage was influenced by the presence of PIK3CA mutations in one third of Chinese study population (8).

### Discussion

EGFR activation influences different intertwining signaling pathways, including Ras/MAPK, phospholipase C, PI3K/Akt, signal transducer and activator of transcription, and Src/FAK pathways (43). EGFR is expressed by pancreatic tumor cells (7), and has been associated with lymph node involvement, metastasis and disease recurrence (44,45), and overall worse prognosis (46). High EGFR expression has been reported also in biliary cancer (8,9,47,48). Tan *et al.* demonstrated that activation of EGFR is closely involved in cell dissociation in pancreatic cancer through activating MEK/ERK signaling pathway (49). Cytoplasmic overexpression of EGFR plays a significant role in the progression of pancreatic ductal adenocarcinoma, especially in the invasion and acquisition of aggressive clinical behavior (46). EGFR also contributes greatly to cholangiocarcinoma progression, associated with lymph node metastasis, aberrant p53 expression, proliferating activity, and carcinoma differentiation (50). EGFR is activated by bile acids and functions to induce COX-2 expression by an MAPK cascade that may contribute to progression of cholangiocarcinomas (51). Paez *et al.* searched for somatic genetic alterations in NSCLC specimens from Japan and the US by examining exons 2 through 25 of EGFR. They found missense and deletion mutations of EGFR in 13.4% of tumors, all within exons 18 through 21 of the kinase domain. The EGFR mutations were more frequent in adenocarcinomas, females, and Japanese patients (25% mutation prevalence *vs.* 1.6% in Americans) (4). The common EGFR mutations in NSCLC are exon 19 deletions and the L858R point mutation in exon 21 (52).

PI3K activation has been shown to play an important role in cell survival signaling in a number of cell types (53).



**Figure 2** Variation identified in the PIK3CA and EGFR genes in pancreaticobiliary tumor samples. A. A reported SNP (rs45455192) located 56 base pairs prior to the start of exon 9 in the PIK3CA gene was identified in one tumor sample. The nucleotide location of the SNP is indicated by the arrows (↓) in the reverse sequence of both the apparently more common “C” allele and the heterozygous C/T condition; B. A previously unreported variant (SNP?) in the EGFR gene was identified in a single tumor. This variant is located in intron 18 (IVS18+16) and is near a reported SNP (rs17337107); C. As a point of reference, a common SNP (rs1050171) in the EGFR gene is shown for three tumor samples. Consistent with Hardy-Weinberg Equilibrium, all three genotypes were identified in the tumors samples analyzed proportionate to the expected frequencies (heterozygosity =0.480) for this dbSNP

**Table 2** Summary of publications investigating EGFR and PIK3CA mutations in pancreatic adenocarcinoma

#	Author	Year	No. of patients	EGFR mutations	EGFR mutation type	PIK3CA mutations	Response to inhibitor	Origin
1	Samuels <i>et al.</i> (21)	2004	11	ND	ND	0/11 (0%)	ND	U.S.
2	Kwak <i>et al.</i> (24)	2006	55	2/55 (3.6%)	Exon 19 Deletion	ND	2/2 disease stabilization	U.S.
3	Immervoll <i>et al.</i> (25)	2006	43	0/43 (0%)		ND	ND	Norway
4	Tzeng <i>et al.</i> (26)	2007	31	0/31 (0%)*		ND	ND	U.S.
5	Lee <i>et al.</i> (27)	2007	66	1/66 (1.5%)	Exon 20 Substitution (2453G>A, C818Y)	ND	ND	Korea
6	Willmore-Payne <i>et al.</i> (28)	2007	12	0/12 (0%)**		ND	ND	U.S.
7	Morinaga <i>et al.</i> (29)	2008	42	0/42 (0%)		ND	ND	Japan
8	Jimeno <i>et al.</i> (30)	2008	10	0/10 (0%)		1/10 (10%)	1/1 Sensitive	U.S./ Europe
9	Iyer <i>et al.</i> (31)	2008	27	0/27 (0%)		0/27 (0%)	ND	U.S.
10	Ueno <i>et al.</i> (32)	2009	31	0/31 (0%)		ND	ND	Japan
11	Dewald <i>et al.</i> (33)	2009	5	1/5 (20%)	Gain ~4n	ND	ND	U.S.
12	Janku <i>et al.</i> (34)	2010	8	ND	ND	1/8 (12.5%)	NA	U.S.
13	Lozano-Leon <i>et al.</i> (35)	2011	34	3/34 (8.8%)	R841R, T571T, R831C	ND	As wild-type	Spain
TOTAL				7/347 (2%)		2/56 (3.6%)		

ND, not done; NA, not available. \*Tzeng *et al.* found EGFR mutations in 26/31 (83.8%) samples, yet all were silent mutations. 25 patients had 2361G>A in exon 20, and one patient had 2508C>T in exon 21. \*\*Willmore-Payne *et al.* found no activation mutations. our samples had polymorphism in exon 20

The classic mode of PI3K activation involves its binding to phosphorylated tyrosine residues of receptor tyrosine kinases, such as EGF and the platelet-derived growth factor receptor. PI3K/Akt signaling promotes small-cell lung carcinoma (SCLC) growth, survival, and chemotherapy resistance (54). The PI3K pathway is activated in multiple advanced cancers through inactivation of the PTEN

tumor suppressor gene (6). Systematic analysis of kinase genes has identified mutations in PI3K p110 catalytic subunit gene PIK3CA in human cancers (3,21,23). These missense mutations, H1047R, E545K and E542K, cluster in two conserved gene locations, and are mutations that confer constitutive kinase activity (21,55). PIK3CA gene is also amplified at high frequencies in squamous cell lung

**Table 3** Summary of publications investigating EGFR and PIK3CA Mutations in Biliary Tract Carcinoma

#	Author	Year	No. of patients	EGFR mutations	EGFR mutation type	PIK3CA mutations	PIK3CA mutation type	Response to inhibitor	Origin
1	Gwak <i>et al.</i> (36)	2005	22	3/22 (13.6%)	Del Exon 19	ND		ND	Korea
2	Bekaii-saab <i>et al.</i> (37)	2005	40 <sup>^</sup>	0/40 (0%)		ND		ND	US
3	Leone <i>et al.</i> (38)	2006	40	*6/40 (15%)	**	ND		ND	Italy
4	Aglietta <i>et al.</i> (39)	2007	49	7/49 (14.3%)	***	5/49 (10.2%)	***	ND	Italy
5	Riener <i>et al.</i> (40)	2008	68	ND		2/68 (2.9%)	E542K, E545K	ND	Switzerland
6	Iyer <i>et al.</i> (31)	2008	3	0/3 (0%)		0/3 (0%)		ND	US
7	Lubner <i>et al.</i> (41)	2010	26	3/26 (11.5%)	EGFR vIII <sup>†</sup>	ND		1/3 CR (NS)	US
8	Xu <i>et al.</i> (42)	2010	34	ND		11/34 (32.4%)	E545K/D, E542K	ND	China
TOTAL				19/180 (10.5%)		18/154 (11.7%)			

ND, not done; NA, not available; CR, Confirmed Response; NS, not significant. <sup>^</sup>Total number of hepatocellular and biliary carcinomas. \*Leone *et al.* found two substitution mutations (C775Y, T790M) in exon 20, two missense point mutations (A864T, E872K) in exon 21, and one patient with two substitution mutations (G882S, V843I) and a silent one (L858R), all in exon 21. \*\*Leone *et al.* also found silent mutation 787 (Gln; CAG-to-CAA) in exon 20 in 36 of 40 samples. \*\*\*Aglietta *et al.* found five hotspot mutations of PI3K (codon 545, 546, 1048 and 1059) in four cases (10.2%); Mutations of EGFR have been detected in 7 out of 49 samples (14.3%). One of them was a new stop-codon mutation. <sup>†</sup>Lubner *et al.* found EGFR intron 1 polymorphism and Q787 G > G SNP in 15 and 6 of 26 samples, respectively

carcinoma, head and neck, gastric, and cervical cancers (56).

Carcinoma of the pancreas is the fourth leading cause of cancer mortality in the U.S. Unfortunately its survival has not improved substantially over the past thirty years, with median survival in the metastatic stage of six months (16,17).

TK inhibitors have been shown to improve the outcome in patients with lung and pancreatic cancers (43). EGFR overexpression by immunohistochemistry is significantly higher in pancreatic tumor cells when compared to normal pancreatic cells (7). Erlotinib is a human EGFR type 1 (HER1)/EGFR TK inhibitor. As a single first or second line agent pancreatic disease control for more than eight weeks was achieved in 20% of patients (57). The drug was approved by the FDA initially for advanced NSCLC, and in 2005 for advanced pancreatic cancer combined with gemcitabine (58). So far only erlotinib has been shown to improve survival in pancreatic adenocarcinoma, with one-year survival of 23% in the erlotinib group compared to 17% with gemcitabine monotherapy (20).

Cholangiocarcinoma is a rare and aggressive tumor that is similar to pancreatic adenocarcinoma, both in histological features and in clinical outcome (18,59,60). Philip *et al.* reported EGFR expression rate of 81% in patients with unresectable or metastatic biliary disease. Following anti-EGFR therapy, 17 percent of patients were

progression free at six months; however, EGFR expression in baseline tumor specimens did not correlate with treatment benefit (48,61).

Gefitinib (Iressa), another EGFR inhibitor, inhibits pancreatic cancer cell growth through EGFR-dependent pathways and delays anchorage-independent growth and invasiveness (62). It was approved in Japan and the US for the treatment of NSCLC. The original rationale for its use was the observation that EGFR is abundantly expressed in lung carcinoma tissue in comparison to adjacent normal lung (63). However, EGFR expression as detected by immunohistochemistry is not an effective predictor of response to gefitinib (13). Presence of specific mutations in the EGFR gene has been shown to correlate with clinical response to the EGFR inhibitor gefitinib in patients with advanced non small cell lung cancer (4).

Erlotinib has been shown to have efficacy in pancreatic and biliary cancers, yet there was no published data on predictive value or prevalence of the abovementioned mutations in these tumor types, therefore this study was undertaken. The study failed to identify mutations in either PIK3CA or EGFR genes for any of the thirty pancreaticobiliary tumor samples that were analyzed. It did identify one synonymous SNP (rs1050171) in the coding region of EGFR, and a previously unreported change, suspected to be a SNP, in intron 18 of EGFR

(IVS18+15, C>T). The main limitation of our study is the small population size for pancreatic cancer and biliary tract cancer. Therefore, we conducted a review of the literature to explore the total number of patients and mutation detection. The review showed an EGFR mutation rate of 2% and 10.5% in pancreatic and biliary tract carcinomas, respectively. PIK3CA mutations were demonstrated in 3.6% and 11.7% of pancreatic and biliary tract carcinomas, respectively. This pooled data from the literature is in concordance with our study, showing similar rates in pancreatic adenocarcinoma. The prevalence of EGFR and PIK3CA mutations reported in the literature for pancreatic cancer was less than 5%. This finding may explain in part why erlotinib provides only a modest improvement in survival, as many other factors might play a role in the prognosis. Another limitation results from the inclusion of a single patient who received neoadjuvant therapy, thus the desmoplastic component of the tumor might have interfered with sequencing.

Genome-wide analysis is being utilized to identify mutations that might have an importance in diagnosis, prognosis, and treatment of pancreatic cancer. Harada *et al.* found frequent dysregulation of SKAP2/SCAP2 gene (7p15.2) in pancreatic cancer (64). Vincent *et al.* found numerous target genes that were hypermethylated and silenced or hypomethylated and overexpressed (65), while Jones *et al.* reported that pancreatic cancers have approximately 63 genetic alterations, mainly point mutations, which affect cellular signaling pathways (66).

In contrast to pancreatic cancer, biliary tract cancer had a higher prevalence of both EGFR and PIK3CA mutations, slightly over 10%, a value similar to that of EGFR mutation in NSCLC (4). Xu *et al.* reported that one third of Chinese patients with cholangiocarcinoma had PIK3CA mutations (42). This relatively high prevalence rate in Asian population might explain the varied response to treatment in different populations. Despite the fact that biliary tract cancer and pancreatic cancer share similar clinicopathological characteristics, the variation in EGFR and PIK3CA mutation rates might indicate that they have different pathophysiology. This research provides the background for designing future correlative prospective trials with EGFR inhibitors. It highlights the importance of studying the biology of each tumor due to their noted variability. It is conceivable that these mutational studies will improve our understanding of tumor biology, and may refine targeted approaches in these dismal diseases. Surgical attention must be given to the creation of fresh frozen specimen banks, as sensitivity of mutation detection may be higher in fresh frozen rather than paraffin embedded specimens. The role of other

mutations, such as K-RAS, predictive of response to EGFR inhibition with monoclonal antibodies in colon cancer, needs further investigation in these diseases. Future targeted therapy should take into account treatment regimens- as monotherapy or in combination with current chemotherapy.

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