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Association of epidermal growth factor receptor and mitogen-activated protein kinase with cystic neoplasms of the pancreas

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The molecular pathobiology of pancreatic cystic neoplasms is poorly understood. The aim of this study was to know the involvement of epidermal growth factor receptor (EGFR) and its downstream targets in the serous cystic neoplasms and the mucinous cystic neoplasms of the pancreas. In a total of 72 pancreatic cystic neoplasms, including 39 serous cystic neoplasms and 33 mucinous cystic neoplasms, we examined the expression of native and phosphorylated EGFR, mitogen-activated protein kinase (MAPK), and AKT by immunohistochemistry and somatic mutations in *EGFR*, *KRAS*, *BRAF*, and *PIK3CA*, by direct sequencing. We also assessed the copy numbers of *EGFR* transcripts and the amplification of the *EGFR* gene in some of the samples. We found that EGFR, phosphorylated EGFR, MAPK, and phosphorylated MAPK were evidently expressed in 100, 54, 100, and 69% of the serous cystic neoplasms, and in 12%, none, 33, and 27% of the mucinous cystic neoplasms, respectively; the expression was significantly higher and more prevalent in the serous cystic neoplasms than in the mucinous cystic neoplasms. The expression of AKT and phosphorylated AKT was low in both the types of neoplasms. On average, *EGFR* transcripts in the serous cystic neoplasms and the mucinous cystic neoplasms increased 53.5- and 2.5-fold, respectively, as compared with that in normal tissues, with the increase in the former being significantly greater than that in the latter. Amplification of the *EGFR* gene was not detected in any of the examined serous cystic neoplasms. None of the tumors had mutations in any of the examined portions of the genes, except two mucinous cystic neoplasms with mutations in codon-12 of *KRAS*. These results indicate that EGFR and MAPK are actively involved in the pathobiology of serous cystic neoplasms and may therefore be potential diagnostic markers and therapeutic targets in patients with the above mentioned types of neoplasms.
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Cystic neoplasms of the pancreas besides intraductal papillary mucinous neoplasms are classified into two types: serous cystic neoplasms and mucinous cystic neoplasms. Serous cystic neoplasms are usually solitary neoplasms that mostly occur in middle-aged and elderly women.[1–3] Occasionally, they are enlarged and/or multi-centric, and obstruct the pancreatic duct, thereby causing symptoms.[2,4] Furthermore, although serous cystic neoplasms are almost entirely benign, some malignant cases that involve the adjacent organs and/or metastasize to the liver have been reported.[5–7] Patients who have symptoms associated with serous cystic neoplasms usually require surgical treatment.[1,2] Mucinous cystic neoplasms develop almost exclusively in middle-aged and elderly women, and are associated with a significant risk of malignant change from adenoma to carcinoma.[8] Therefore, it is generally accepted that mucinous cystic neoplasms need to be

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resected completely.[9] Patients with non-invasive mucinous cystic neoplasms have a fair prognosis; however, those with invasive mucinous cystic neoplasms have a poor prognosis, with a 5-year survival rate of 16–30%.[10,11]

Although surgical resection is the treatment of choice for these cystic neoplasms, some patients may not be able to undergo surgery because of their medical condition. Medical treatment is preferred in such cases; however, no effective medicine is available for these cystic neoplasms at present. One of the reasons for this is the lack of knowledge regarding the mechanisms underlying the development and progression of these neoplasms.

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein with ligand-inducible tyrosine kinase activity and is one of the four members of the ERBB family.[12] The ligand-binding EGFR dimerizes and undergoes autophophorylation, which results in the transmission of stimulatory signals to the rat sarcoma viral oncogene homolog (RAS) or phosphatidylinositol-3-kinase (PI3K).[12] RAS is a GTP-binding molecule that activates signaling cascades, including those related to mitogen-activated protein kinases (MAPKs).[13,14] PI3K is a membrane-bound lipid kinase that activates the v-akt murine thymoma viral oncogene homolog (AKT).[15] The RAS-MAPK and PI3K-AKT signaling pathways have essential roles in cellular proliferation and differentiation. Aberrant signaling involving the EGFR and its downstream molecules is implicated in the development of cancers of the head and neck, breast, lung, colon, and pancreas.[16–21] Recently, EGFRs have come to be regarded as clinically important because of the development of a number of anti-EGFR drugs, including extracellular domain-targeted antibody-based drugs such as cetuximab, panitumumab, and zalutumumab, and intracellular kinase domain-targeted small-molecule-based drugs such as erlotinib and gefitinib. These drugs enable the medical treatments of some cancers.[22]

In this study, we studied the involvement of EGFR and its related signaling molecules, including *KRAS*, *BRAF*, *PIK3CA*, MAPK, and AKT, in the serous and mucinous cystic neoplasms of the pancreas.

Materials and methods

Materials

Seventy-two cystic neoplasms of the pancreas (39 serous cystic neoplasms, 33 mucinous cystic neoplasms) that had been surgically resected at Tokyo Women's Medical University Hospital between 1983 and 2008 were retrospectively analyzed. The clinicopathological features, including the patients' sex and age, and the histological types of the tumors, are shown in Table 1. This study was approved by the ethics committee of Tokyo Women's Medical University.

Immunohistochemistry

Immunohistochemical staining for the native and phosphorylated forms of EGFR, MAPK, and AKT was performed using formalin-fixed and paraffin-embedded tissue sections. The antibodies used were anti-EGFR (DAK-H1-WT; Dako, Glostrup, Denmark), anti-phosphorylated EGFR (DAK-H1–1197; Dako), anti-MAPK (137F5; Cell Signaling Technology Inc., Danvers, MA, USA), anti-phosphorylated MAPK (20G11; Cell Signaling Technology Inc.), anti-AKT (C67E7; Cell Signaling Technology Inc.), and anti-phosphorylated AKT (736E11; Cell Signaling Technology Inc.). EGFR and phosphorylated EGFR were manually stained by using CSAII, a biotin-free tyramide signal amplification system (Dako), according to the manufacturer's instructions. MAPK, phosphorylated MAPK, AKT, and phosphorylated AKT were manually stained using a Histofine SAB-PO kit (Nichirei Corp., Tokyo, Japan), according to the manufacturer's instructions. The primary antibodies against EGFR, phosphorylated EGFR, MAPK, phosphorylated MAPK, AKT, and phosphorylated AKT were diluted to 1:200, 1:100, 1:250, 1:100, 1:300, and 1:50, respectively. Staining for EGFR and phosphorylated EGFR was evaluated by using the Zymed evaluation guidelines: score 0, no staining;

Table 1 Characteristics of the examined cases of cystic neoplasms of the pancreas

	Sex (M:F)	*Mean age*	*Histological type*	*No. of cases*
SCNs	7:32	56.4	Microcystic serous cystadenoma	25
			Macrocystic serous cystadenoma	12
			Solid serous adenoma	2
			Total	39
MCNs	0:33	46.3	Mucinous cystic neoplasm with low- or moderate-grade dysplasia	29
			Mucinous cystic neoplasm with high-grade dysplasia	2
			Mucinous cystic neoplasm with an associated invasive carcinoma	2
			Total	33

Abbreviations: F, female; M, male; MCN, mucinous cystic neoplasm; SCN, serous cystic neoplasm.

1+, faint or barely perceptible membrane staining; 2+, moderate membrane staining; and 3+, strong membrane staining. In this study, scores greater than 1+ (ie, 2+ and 3+) were considered to indicate evident expression as described elsewhere.[23] Staining for MAPK, phosphorylated MAPK, AKT, and phosphorylated AKT was evaluated by comparing the intensities of neoplastic cells with that of islet cells because islet cells show consistent staining in the examined tissues: score 0, no staining; 1+, lower-intensity staining; 2+, equal-intensity staining; and 3+, greater-intensity staining. The staining characteristics of 500 cells were evaluated and the final score was the sum total of the product of the staining intensity and the corresponding tumor percentage. Total scores that exceeded 100 were considered to indicate evident expression as described elsewhere.[24]

RNA Extraction and Quantification

Frozen tissues from seven serous cystic neoplasms and eight mucinous cystic neoplasms were used for RNA studies. The frozen sections were prepared and tumor and normal tissue were separately dissected manually from the sections. Because these studied cystic neoplasms were well separated from normal parenchymal tissues, the dissected tissues were composed of neoplastic epithelial cells and supporting stromal cells, the former exceeding far more volume than the latter. Normal parenchymal tissues were composed of acini, ducts, and islets. Total RNA was extracted from the dissected tissues by using the mirVana RNA Isolation kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Next, 2 μg of the extracted total RNA was converted to cDNA by using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). We determined the copy number of the transcripts by quantitative real-time PCR assays performed using TaqMan Gene Expression Assays and the 7500 Real-Time PCR system (Applied Biosystems), according to the manufacturer's instructions. *GAPDH* was used as an endogenous reference gene for normalization of expression among the samples. Plasmid clones harboring the TaqMan PCR products were generated using a PCR cloning kit (Stratagene, Cedar Creek, TX, USA) and used as standards for quantitation.

DNA Extraction and Southern Blotting

Genomic DNA was isolated from the separately dissected tumor and normal tissues obtained from six frozen serous cystic neoplasm specimens as described above by digestion with proteinase-K and extraction with phenol, chloroform, and isoamyl alcohol as described elsewhere.[25] Southern blotting was performed as follows: 5 μg of the genomic DNA was digested with *Eco*RI or *Pvu*II, electrophoresed on a 0.8% agarose gel, and transferred onto a nylon membrane as described elsewhere.[25] Hybridization was performed using the ExpressHyb solution (Takara Bio Inc., Otsu, Japan) and radioactive probes were prepared using a random priming kit (Roche Diagnostics, Basel, Switzerland) containing [α-^{32}P]dCTP (Applied Biosystems), according to the manufacturers' instructions. The templates used for generating the radioactive probes were PCR products harboring exon-19 of the *EGFR* gene and a unique genomic portion of chromosome-15 amplified from normal human genomic DNA by using paired primers (shown in Supplementary Table 1S).

Mutation Analyses

Tumor and normal tissues were manually dissected from the formalin-fixed and paraffin-embedded tissues obtained from 39 serous cystic neoplasms and 33 mucinous cystic neoplasms, and used for genomic DNA extraction as described elsewhere.[26] Somatic mutations in exons 18–21 of *EGFR*, exon-1 of *KRAS*, exon-15 of *BRAF*, and exons 10 and 21 of *PIK3CA* were analyzed as follows: The extracted genomic DNA was PCR-amplified (primers shown in Supplementary Table 1) according to protocols described elsewhere.[27] The PCR products were treated with ExoSAP-IT (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. Finally, direct sequencing was performed using a BigDye Terminator (Applied Biosystems) and a 3130x Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions.

Statistical Analyses

Statistical analyses were performed using Microsoft Office Excel 2007 (Microsoft Corp., Seattle, WA, USA) or Dr SPSS II (SPSS Inc., Chicago, IL, USA). *P*-values <0.05 were considered statistically significant.

Results

EGFR, MAPK, and AKT Expression in the Cystic Neoplasms of the Pancreas

To know the involvement of EGFR and its downstream targets in serous cystic neoplasms and mucinous cystic neoplasms, the expression of native and phosphorylated EGFR, MAPK, and AKT was immunohistochemically evaluated in 39 serous cystic neoplasms and 33 mucinous cystic neoplasms. Evident expression of EGFR, phosphorylated EGFR, MAPK, and phosphorylated MAPK was seen in 100% (39/39), 54% (21/39), 100% (39/39), and 69% (27/39) of the serous cystic neoplasms, respectively (Figures 1 and 2, and Tables 2 and 3). Interestingly, EGFR expression was quite distinct,

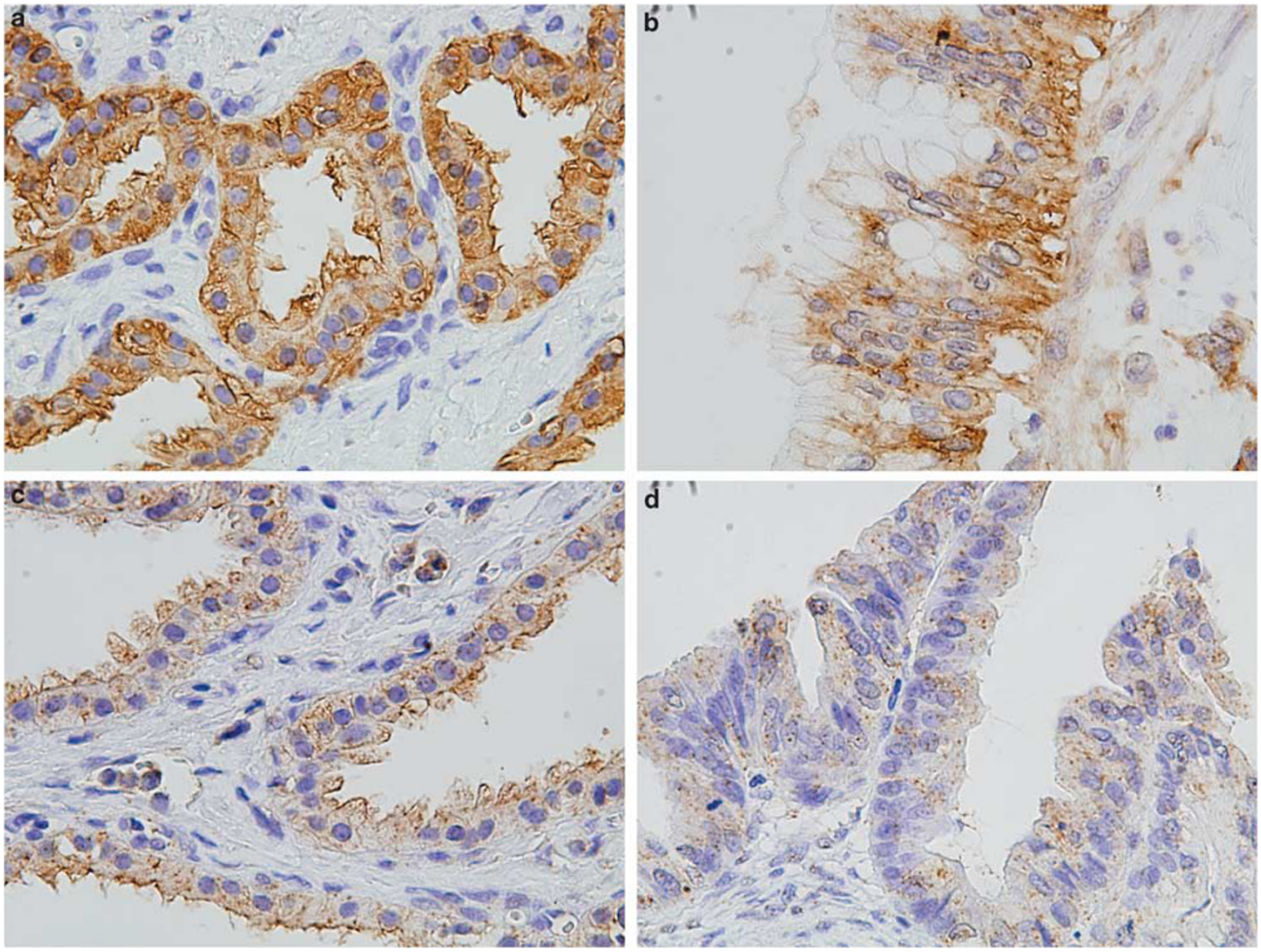

Figure 1 Expression of the native and phosphorylated forms of EGFR in cystic neoplasms, as evaluated by immunohistochemistry. All images have been taken through a ×40 objective lens. Expression of EGFR (**a**, **b**) and phosphorylated EGFR (**c**, **d**) in serous cystic neoplasms (**a**, **c**) and mucinous cystic neoplasms (**b**, **d**), respectively.

and fairly strong membrane staining was observed in most of the serous cystic neoplasms (38/39, 97%). Expressions of phosphorylated EGFR and phosphorylated MAPK were not associated with each other. In addition, the expressions were not significantly associated with sex, age, and histological type, that is, microcystic, macrocystic, and solid type. Expression of AKT and phosphorylated AKT was not evident in any of the serous cystic neoplasms (Figure 3 and Table 3). Expression of EGFR, phosphorylated EGFR, MAPK, and phosphorylated MAPK was evident in 12% (4/33), none (0/33), 33% (11/33), and 27% (9/33) of the mucinous cystic neoplasms (Figures 1 and 2, and Tables 2 and 3). The expression was not associated with the histological types of the mucinous cystic neoplasms. The invasive and non-invasive components in the cases of mucinous cystic neoplasms with an associated invasive carcinoma were studied and no difference was found between them. Expression of AKT and phosphorylated AKT was not detected in any of the mucinous cystic neoplasms (Figure 3 and Table 3). Expression of EGFR, phosphorylated EGFR, MAPK, and phosphorylated MAPK was found to be significantly more frequent and more intense in the serous cystic neoplasms than in the mucinous cystic neoplasms (EGFR, $P<0.0000001$; phosphorylated EGFR, $P<0.0000001$; MAPK, $P<0.0000001$; phosphorylated MAPK, $P=0.0009$; determined using χ^2-test with Yates' correction (Tables 2 and 3)). These results indicate that EGFR and MAPK are actively involved in the development and/or maintenance of serous cystic neoplasms.

Mechanisms of EGFR Overexpression

To elucidate the mechanism of EGFR overexpression in cystic neoplasms, we determined the copy numbers of *EGFR* transcripts by using quantitative real-time reverse transcription (RT)-PCR analysis of frozen tissues obtained from seven serous cystic neoplasms and eight mucinous cystic neoplasms. The copy number of *EGFR* transcripts was much higher in all seven serous cystic neoplasms than that in the matched normal tissues (53.5 times higher on

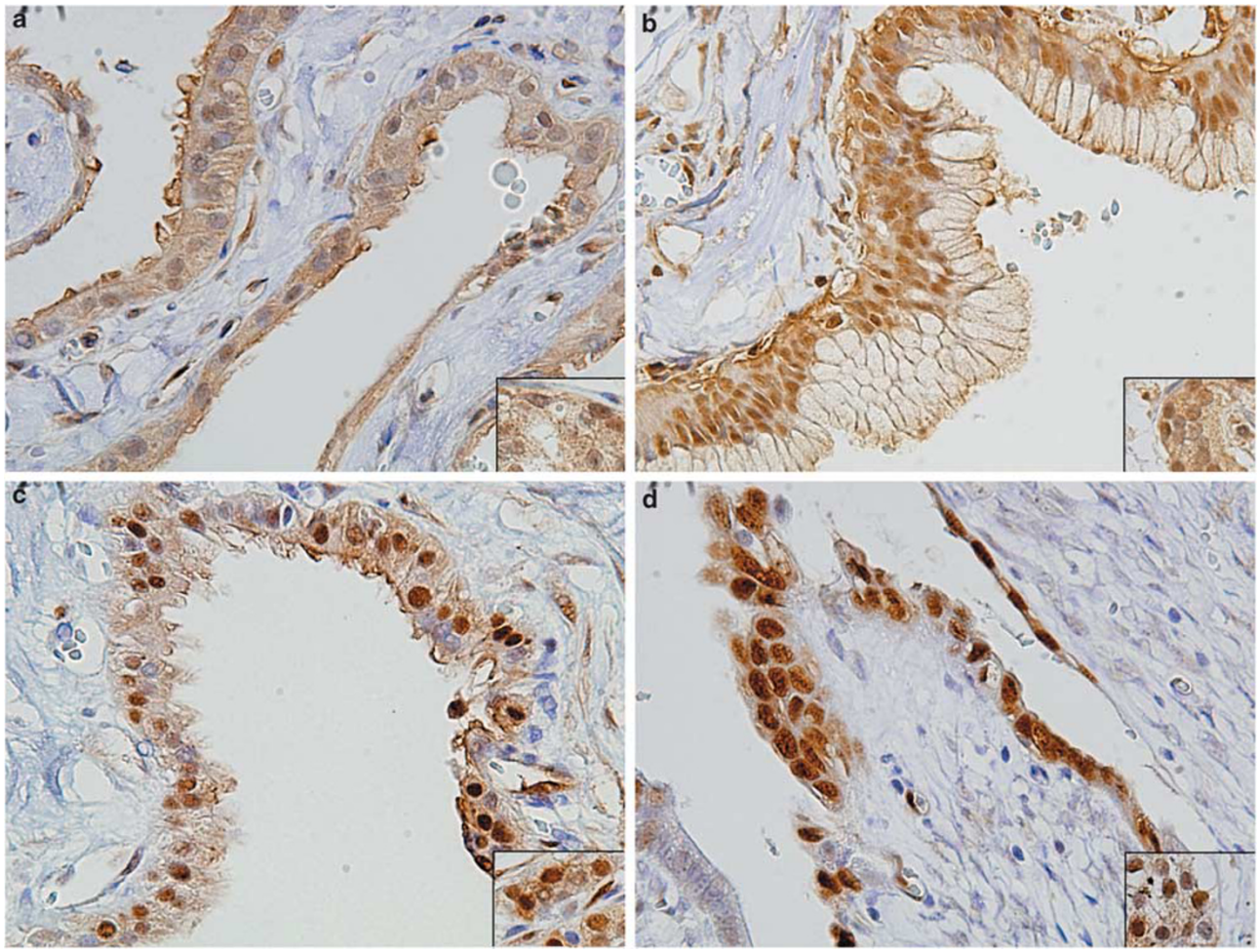

Figure 2 Expression of the native and phosphorylated forms of MAPK in cystic neoplasms, as evaluated by immunohistochemistry. All images have been taken through a ×40 objective lens. Expression of MAPK (**a**, **b**) and phosphorylated MAPK (**c**, **d**) in serous cystic neoplasms (**a**, **c**) and in mucinous cystic neoplasms (**b**, **d**), respectively. The insets show internal control staining in islets, for each stain.

Table 2 Expression of EGFR and phosphorylated EGFR in the cystic neoplasms of the pancreas, as evaluated by immunohistochemistry

	Evaluation score[a]				
	3+, strong (%)	*2+, moderate (%)*	*1+, faint (%)*	*0, none (%)*	P
EGFR					
SCNs	38 (97)	1 (3)	0 (0)	0 (0)	<0.0000001
MCNs	1 (3)	3 (9)	2 (6)	27 (82)	
Phosphorylated EGFR					
SCNs	0 (0)	21 (54)	18 (46)	0 (0)	<0.0000001
MCNs	0 (0)	0 (0)	1 (3)	32 (97)	

Abbreviations: EGFR, epidermal growth factor receptor; MCN, mucinous cystic neoplasm; SCN, serous cystic neoplasm.
[a]Staining intensity was evaluated according to Zymed's evaluation guideline.

average; Figure 4). By contrast, in the mucinous cystic neoplasms, the copy number of *EGFR* transcripts was only slightly increased than that in the matched normal tissues (2.5 times higher on average; Figure 4). The difference between the increase in the copy number of *EGFR* transcripts in the case of the serous cystic neoplasm and that in the case of the mucinous cystic neoplasm was significant ($P<0.0000001$; Student's *t*-test). Next, we used Southern hybridization to determine whether the *EGFR* gene was amplified in the serous cystic neoplasms. *EGFR* gene amplification was not detected in any of the six serous cystic neoplasms examined (Figure 5). These results indicate that

Table 3 Expressions of MAPK, phosphorylated MAPK, AKT, and phosphorylated AKT in the cystic neoplasms of the pancreas, as evaluated by immunohistochemistry

	Staining score[a]		
	>100 (%)	*≤100 (%)*	P
MAPK			
SCNs	39 (100)	0 (0)	<0.0000001
MCNs	11 (33)	22 (67)	
Phosphorylated MAPK			
SCNs	27 (69)	12 (31)	0.0009
MCNs	9 (27)	24 (73)	
AKT			
SCNs	0 (0)	39 (100)	—
MCNs	0 (0)	33 (100)	
Phosphorylated AKT			
SCNs	0 (0)	39 (100)	—
MCNs	0 (0)	33 (100)	

Abbreviations: MAPK, mitogen-activated protein kinase; MCN, mucinous cystic neoplasm; SCN, serous cystic neoplasm.
[a]Staining was evaluated by comparing the intensities of neoplastic cells with that of islet cells: score 0, no staining; 1+, lower-intensity staining; 2+, equal-intensity staining; and 3+, higher-intensity staining. The staining characteristics of 500 cells were evaluated and the final score was the sum total of the product of the staining intensity and the corresponding tumor percentage.

EGFR overexpression in the serous cystic neoplasms is associated with an increase in the number of transcripts without amplification of the *EGFR* gene.

Mutations in EGFR, KRAS, BRAF, and PIK3CA

We checked for the presence of somatic mutations in the hot spots of *EGFR*, *KRAS*, *BRAF*, and *PIK3CA* in 38 serous cystic neoplasms (one serous cystic neoplasm sample was excluded because the DNA obtained was of poor quality) and 33 mucinous cystic neoplasms by using direct sequencing. None of the serous cystic neoplasms or mucinous cystic neoplasms had mutations in the examined regions of the genes, except two mucinous cystic neoplasms harboring somatic mutations in codon-12 of *KRAS* (G12D mutation (GGT to GAT)). Of these, one was a mucinous cystic neoplasm with high-grade dysplasia (cystadenocarcinoma *in situ*) and this case showed expressions of MAPK and phosphorylated MAPK but no evident expression of EGFR or AKT. The other case was a mucinous cystic neoplasm with low-grade dysplasia (cystadenoma) and this case showed no evident expression of EGFR, MAPK, or AKT. We also found synonymous single-nucleotide polymorphisms in *EGFR* as follows: ACA in 99 alleles and ACT in 43 alleles at codon-629; CAA in 30 alleles and CAG in 112 alleles at codon-787; and ACA in 1 allele and ACG in 141 alleles at codon-725. All of these polymorphisms were found to be registered in the database of single-nucleotide polymorphisms of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov:80/).

Discussion

In this study, we found that serous cystic neoplasms showed high levels of EGFR expression and evident expression of MAPK, whereas mucinous cystic neoplasms showed only a modest level of expression. Overexpression of EGFR in the serous cystic neoplasms was associated with an increase in the number of transcripts without amplification of the *EGFR* gene. In the case of serous cystic neoplasms, no somatic mutations were detected in any of the examined hot spots of *KRAS*, *BRAF*, and *PIK3CA*. By contrast, somatic mutations were found in codon-12 of *KRAS* in a fraction of the mucinous cystic neoplasms. These results indicate that EGFR and its downstream target, MAPK, are actively involved in the development and/or maintenance of serous cystic neoplasms.

EGFR is overexpressed in a wide variety of epithelial neoplasms, including pancreatic cancer.[16,17] With regard to cystic neoplasms of the pancreas, Yeh *et al*[28] and Zhao *et al*[29] independently found that EGFR is infrequently expressed in the mucinous cystic neoplasms. However, there have been no published reports on EGFR expression in the serous cystic neoplasms. Therefore, to the best of our knowledge, our study is the first to show that serous cystic neoplasms frequently overexpress EGFR. EGFR overexpression in cancer is usually associated with amplification of the *EGFR* gene. In colorectal and breast cancers, amplification of the *EGFR* gene is invariably associated with overexpression of EGFR.[18,19] However, cancers that overexpress EGFR do not always show amplification of the *EGFR* gene,[19] indicating that amplification of the *EGFR* gene causes EGFR overexpression, but EGFR overexpression is not always caused by *EGFR* gene amplification. Our study indicated that EGFR overexpression in the serous cystic neoplasms is associated with an increase in the copy number of transcripts without amplification of the *EGFR* gene. The increase in *EGFR* transcripts may be due to the activation of the transcriptional machinery involving complex control systems.[30–32] The mechanism of the increase in the transcript copy numbers in the serous cystic neoplasms is an important area for further study. Furthermore, it would have been of major interest to test EGFR expressions and gene amplifications in malignant serous neoplasms; however, such malignant cases were not available in this study.

EGFR undergoes autophosphorylation and transmits an active signal to the RAS-MAPK pathway or the PI3K-AKT pathway.[12] Our study showed that phosphorylated EGFR and phosphorylated MAPK were expressed in a majority of the serous cystic neoplasms, whereas the level of expression of

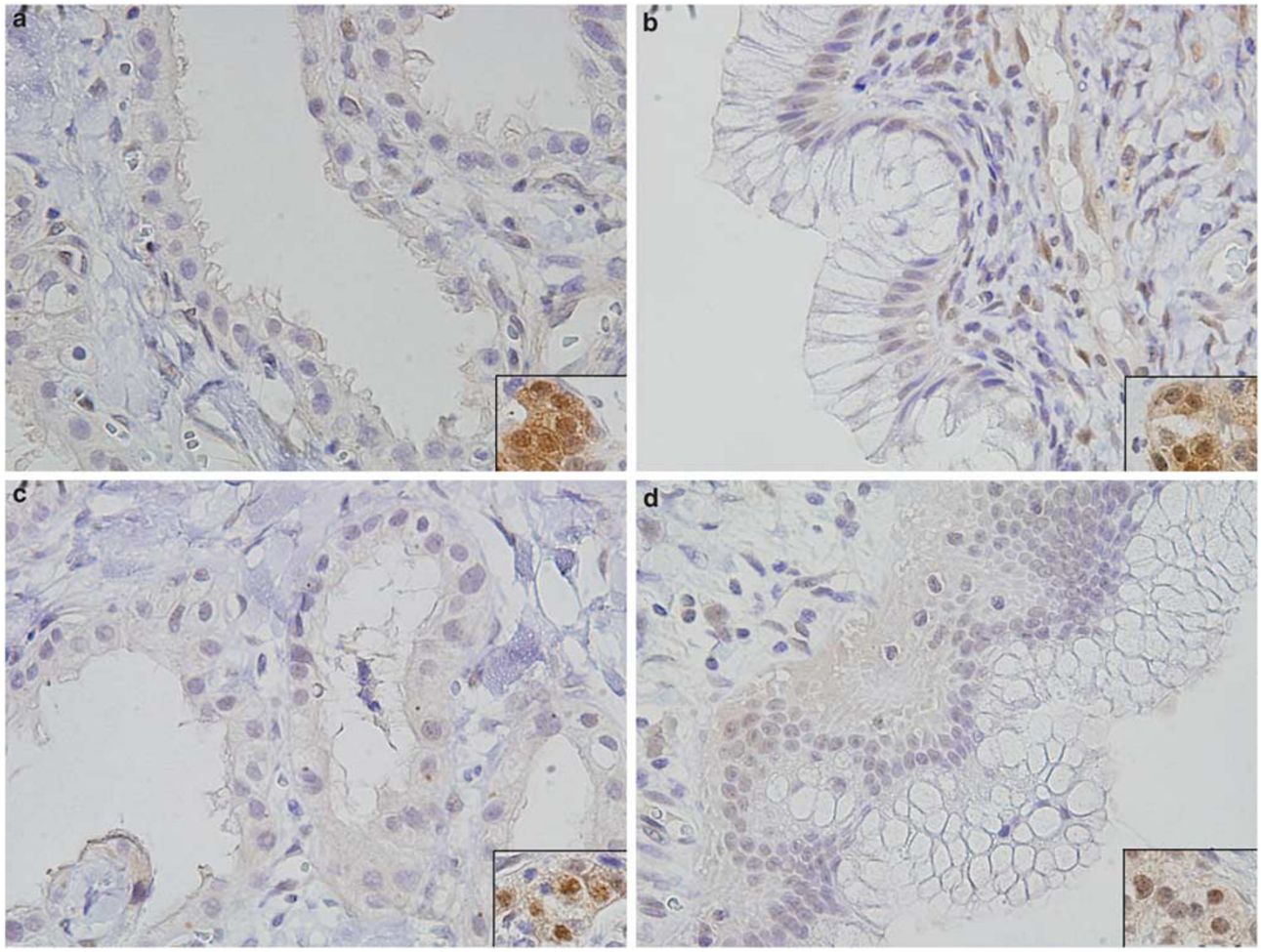

Figure 3 Expression of the native and phosphorylated forms of AKT in cystic neoplasms, as evaluated by immunohistochemistry. All images have been taken through a ×40 objective lens. Expression of AKT (**a**, **b**) and phosphorylated AKT (**c**, **d**) expression in serous cystic neoplasms (**a**, **c**) and in mucinous cystic neoplasms (**b**, **d**), respectively. The insets show internal control staining in islets, for each stain.

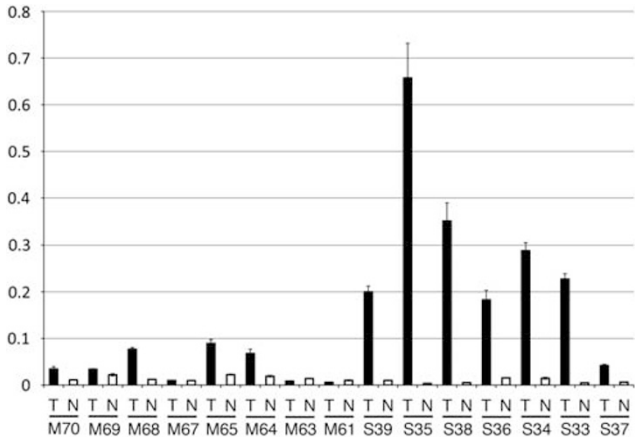

Figure 4 Copy numbers of *EGFR* transcripts as detected by quantitative real-time PCR. The relative expression of the *EGFR* transcripts is plotted against the number of *GAPDH* transcripts in tumor tissues (T) and in matched normal (N) tissues. The sample numbers labeled M represent the mucinous cystic neoplasms and those labeled S represent the serous cystic neoplasms.

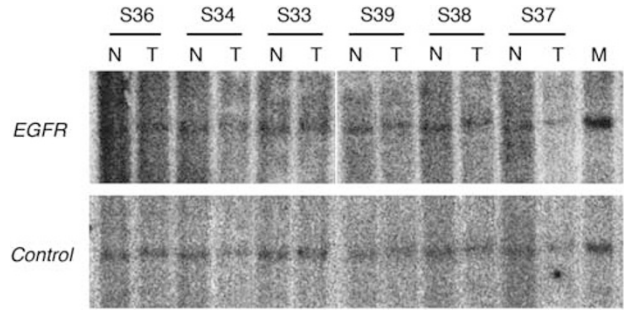

Figure 5 Southern blot analysis of the *EGFR* gene and a unique genomic portion on chromosome-15 (control) in tumor (T) and matched normal (N) tissues obtained from serous cystic neoplasms. M denotes MIA PaCa2, the pancreatic cancer cell line.

phosphorylated AKT was low; this suggests that activating signals are transmitted from EGFR to the RAS-MAPK pathway rather than to the PI3K-AKT pathway. Furthermore, no gain-of-function mutations were found in any of the hot spots of *KRAS*,

BRAF, or *PIK3CA* that were studied, suggesting that EGFR has a primary role in the activation of MAPK in the serous cystic neoplasms. The lack of mutations in *KRAS* in the serous cystic neoplasms in our study is consistent with the results of a previous study.[33]

Current knowledge indicates that responsiveness to anti-EGFR therapy depends on several molecular features, including amplification of the *EGFR* gene, overexpression of EGFR, mutation of the *EGFR* gene, and the absence of self-activating mutations in the signaling molecules downstream from the *EGFR* gene. In colorectal cancers, *EGFR* gene amplification is associated with responsiveness to and the survival benefits of anti-EGFR therapy.[34] In non-small-cell lung cancer, *EGFR* gene amplification, EGFR expression, and somatic mutations in a kinase domain of *EGFR* are associated with responsiveness to and the survival benefits of anti-EGFR therapy.[20,35–37] Mutations in the *EGFR* kinase domain are frequently found in female Asian patients with lung adenocarcinoma who have never smoked,[35] but are rarely found in patients with other types of cancers, including pancreatic cancer.[38–40] Mutations in *KRAS* or *PIK3CA* are associated with non-responsiveness to anti-EGFR therapy.[41,42] Our study indicated that in the case of serous cystic neoplasms, overexpression of the EGFR protein with increased copy number of *EGFR* transcripts occurs without amplification of the *EGFR* gene. The serous cystic neoplasms did not show any mutations in exons 18–23 of *EGFR*, which encode the kinase domain, nor in any of the examined hot spots in *KRAS*, *BRAF*, or *PIK3CA*. These results indicate that further studies are warranted to analyze the potential of EGFR inhibitors as therapeutic agents for serous cystic neoplasms, especially in symptomatic patients in whom it is difficult to perform surgical resection because of complications. In conclusion, EGFR and MAPK are actively involved in the pathobiology of serous cystic neoplasms and may therefore be potential diagnostic markers and therapeutic targets in the case of serous cystic neoplasms.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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