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Frequent activation of mitogen-activated protein kinase relative to Akt in extrahepatic biliary tract cancer

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Background. Lack of effective adjuvant therapy against advanced extrahepatic biliary tract carcinoma (BTC) requires that new therapeutic methods, such as molecular targeted therapy, be developed. The mitogen-activated protein kinase (MAPK) and Akt signaling pathways, which activate cell proliferation and suppress apoptosis, respectively, may function as important targets for such therapies. The aim of this study was to examine the expression patterns of phosphorylated MAPK (p-MAPK) and phosphorylated Akt (p-Akt) proteins in BTC cell lines and clinical specimens. ***Methods.*** Expression of p-MAPK and p-Akt proteins in four human BTC cell lines and in frozen sections of 20 advanced extrahepatic BTC specimens was analyzed by Western blotting. Thirty formalin-fixed BTC specimens were immunohistochemically stained for p-MAPK and p-Akt using labeled streptavidin–biotin conjugates. ***Results.*** Expression of p-MAPK was observed in three of four (75%) BTC cell lines, whereas no expression of p-Akt was observed. Twenty-three of 30 formalin-fixed specimens stained positive for p-MAPK (77%), whereas only 47% stained positively for p-Akt. Expression of p-MAPK relative to that of p-Akt was also seen more frequently in the frozen specimens. ***Conclusions.*** The results of this study suggest that MAPK is activated more frequently than Akt in extrahepatic biliary tract carcinoma.

Key words: MAPK, Akt, phosphorylation, gallbladder cancer, bile duct cancer

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Introduction

In Japan, the age-adjusted cancer death rate related to biliary tract cancer (BTC) is high and continues to increase.[1] Despite advances in diagnosis and treatment, the prognosis of BTC patients is poor.[2–4] Detection of early-stage BTC is difficult; hence, most BTCs are unresectable at the time of diagnosis owing to invasion to the liver, metastases to lymph nodes and distant organs, or peritoneal dissemination.[5] Moreover, there is a high rate of recurrence of advanced BTC after surgery.[6,7] The benefit of chemoradiotherapy is marginal, and for only a limited number of patients with advanced biliary cancer.[8–11] Because of the lack of efficacious treatments that improve survival, novel treatment modalities for BTC are urgently required.

A balance between proliferative and apoptotic cell death signals controls epithelial cell multiplication. One of the most important types of receptors regulating cell proliferation and survival is the epidermal growth factor receptor (EGFR). EGFR activates phosphatidylinositol 3′-kinase (PI3K)/Akt, ras/mitogen-activated protein kinase (MAPK), and janus kinase-signal transducer and activator of transcription (JAK/STAT) signaling pathways. Activation of ras/MAPK results in mitogenesis or cellular proliferation, while the PI3K/Akt pathway controls cellular survival. Coordination of the two pathways within a single cell may depend on the cancer type or degree of cancer progression. In BTC, oncogenic mutations in K-*ras* that result in constitutive activation of MAPK[12,13] and PI3K/Akt signaling pathways[14] have been reported to contribute to carcinogenesis,[15–17] presumably by promoting uncontrolled growth of biliary tract epithelium. Although activated MAPK and Akt have been shown to be involved in the growth of human malignant tumors, reports of the activation (phosphorylation) status of MAPK and Akt in BTC are limited primarily to studies of patients with intrahepatic cholangiocarcinoma.[18,19] Thus, the phosphorylation status of

MAPK and Akt in extrahepatic BTCs, including gallbladder cancer, is not known.

The aim of this study was to examine the expression patterns of activated MAPK and Akt in BTC cells lines and surgically resected extrahepatic tumor specimens. In both types of BTC samples, MAPK activation was observed more frequently than Akt activation.

Materials and methods

Cells

Four cells lines were studied: bile duct cancer cell lines Sk-ChA-1 and Mz-1, and gallbladder cancer cell lines TGBC-2 and NOZ (all derived from humans). Sk-ChA-1, TGBC, and Mz-1 were gifts from Dr. T. Todoroki (Department of Surgery, University of Tsukuba, Tsukuba, Japan).[20] NOZ was a gift from Dr. S. Nagamori (Jikei University School of Medicine, Tokyo, Japan).[21] HSC-42 human gastric cancer cells, which overexpress phosphorylated Akt (p-Akt) continuously, served as a positive control in Western blots.[22] All cells were cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. NOZ, Sk-ChA-1, and TGBC-2 cells carry a mutation in codon 12 of K-*ras*, whereas Mz-1 cells encode wild-type K-*ras*.[23–25] MAPK is constitutively active (phosphorylated) in NOZ cells.[26]

Patients and samples

Thirty human BTC specimens resected from patients operated on at Kobe University Hospital between 1998 and 2004 were used in this study. Among the 15 male and 15 female patients (mean age ± SD, 71.3 ± 7.6 years; range, 52–86 years) were 15 with gallbladder cancer, 13 with bile duct cancer, and two with ampullary cancer. Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki and its later revision.

Immunohistochemical analysis

Specimens were sliced into 4-μm-thick sections, placed on glass slides, and deparaffinized. Following activation of antigens with microwave radiation (400 W, 5 min), the slides were immersed in 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. The specimens were then incubated with primary antibodies overnight in a humidified chamber at 4°C. Primary antibodies included rabbit polyclonal phospho-p44/42 MAPK (Thr202/Tyr204) antibody (Cell Signaling Technology, Beverly, MA, USA) at a 1:50 dilution and rabbit polyclonal p-Akt (Ser473) antibody (Cell Signaling Technology) at a 1:100 dilution. After secondary incubation with biotinylated anti-mouse IgG secondary antibody and treatment with peroxidase-conjugated streptavidin, the sections were washed in cold phosphate-buffered saline (PBS; 0.01 M, pH 7.2). Peroxidase activity was visualized with 3,3′-diaminobenzidine tetrahydrochloride and 0.03% hydrogen peroxidase in 0.01 M PBS. Slides were counterstained with hematoxylin, dehydrated, and mounted in a routine fashion. For each primary antibody, normal bile duct epithelium was stained and used as an internal control for normal cell staining. Negative controls were duplicate sections stained simultaneously in which the primary antibody was replaced by nonspecific rabbit serum.

Assessment of immunostaining

Results of phosphorylated MAPK (p-MAPK) and p-Akt immunostaining were classified by staining intensity: "positive" was defined as increased staining intensity relative to corresponding normal biliary tract epithelium, and "negative" was defined by no significant increase in staining over normal tissues. Similar to previous reports, p-MAPK immunostaining was scored as positive when immunoreactivity was seen in more than 30% of tumor cells,[27] and p-Akt immunostaining was considered positive when immunoreactivity was seen in more than 50% of tumor cells.[22] For each sample, immunoreactivity scores were derived from tumor cell counts in three randomly selected fields of view.

Western blotting analysis

Cells were trypsinized and scraped, followed by two washes with cold PBS (10 mM, pH 7.4). Ice-cold RIPA buffer [1 × PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin] was added, and each suspension was homogenized for 15 s. Cell homogenates were then centrifuged at 5700 g for 10 min at 4°C, and the lysate supernatants were collected and stored at –80°C until use. For Western blot analyses, SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer was added to an aliquot of each lysate (at a final concentration of 65 mM Tris, 5% 2-mercaptoethanol, 3% SDS, and 10% glycerol), and the sample was heated at 95°C for 5 min prior to SDS-PAGE. Concentrations of lysate proteins were measured with the DC Bio-Rad assay according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA).

Of the 30 resected tumors, we were able to obtain 20 fresh-frozen samples, which were used for the Western blot analysis. Each snap-frozen sample was suspended

in RIPA buffer and sonicated using an ultrasonicator (Tomy Seiko, Tokyo, Japan). The cell suspension was then centrifuged at 5700 g for 10 min at 4°C, and the lysate supernatants were collected. After addition of SDS-PAGE loading buffer (to a final concentration of 65 mM Tris, 5% 2-mercaptoethanol, 3% SDS, and 10% glycerol), each lysate was heated at 95°C for 5 min. Concentrations of lysate proteins were determined by using the DC Bio-Rad assay according to the manufacturer's protocol (Bio-Rad Laboratories).

Following resolution of lysates on e-PAGEL (E-R7.5L, Atto, Tokyo, Japan), proteins were transferred to polyvinylidene difluoride nitrocellulose membranes (Millipore, Bedford, MA, USA). Blots were blocked at room temperature for 1 h in 5% skim milk (Blockace, Yukijirushi Nyugyo, Osaka, Japan), and then incubated in 1% Tris-buffered saline (TBS)-Tween overnight at 4°C with primary antibodies against rabbit polyclonal p44/42 MAPK (dilution 1:1000), phospho-p44/42 MAPK (Thr202/Tyr204) (dilution 1:1000), or p-Akt (Ser473) (dilution 1:1000). Membranes were washed three times in TBS-Tween and then incubated for 1 h in 1% TBS-Tween with the required secondary antibody–horseradish peroxidase (HRP) conjugate (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive proteins were detected by chemiluminescence with SuperSignal West Dura (Pierce, Rockford, IL, USA) and visualized by using a Lumino Imaging Analyzer FAS-1000 (Toyobo, Osaka, Japan). Immunoblots with anti-β-actin antibody (Sigma, Saint Louis, MO, USA) served as internal loading controls.

Statistics

The Mann-Whitney *U* test, χ-squared test, or Fisher's exact test was used to assess correlations between clinicopathological features and the expression of p-MAPK and p-Akt. P values < 0.05 were considered statistically significant.

Results

Expression of p-MAPK (Thr202/Tyr204) and p-Akt (Ser 473) in BTC cell lines

Because MAPK and Akt activity are both regulated by phosphorylation, a direct correlation can be made between kinase activity and signal intensity in anti-p-MAPK and anti-p-Akt immunoblots. Western analysis revealed expression of p-MAPK in three (Sk-chA-1, TGBC-2, NOZ) of the four cell lines examined (75%), and all three of these cell lines had K-ras abnormalities. In contrast, no expression of p-Akt was observed in the four biliary tract cell lines (Table 1, Fig. 1A).

Table 1. Expression of phosphorylated mitogen-activated protein kinase (p-MAPK) and phosphorylated Akt (p-Akt) in cell lines and surgical specimens of biliary tract cancer

	Overexpression % (positive samples/samples tested)		
	p-MAPK	p-Akt	*P*
Cell lines	75 (3/4)	0 (0/4)	0.03
Surgical specimens	77 (23/30)	47 (14/30)	0.02

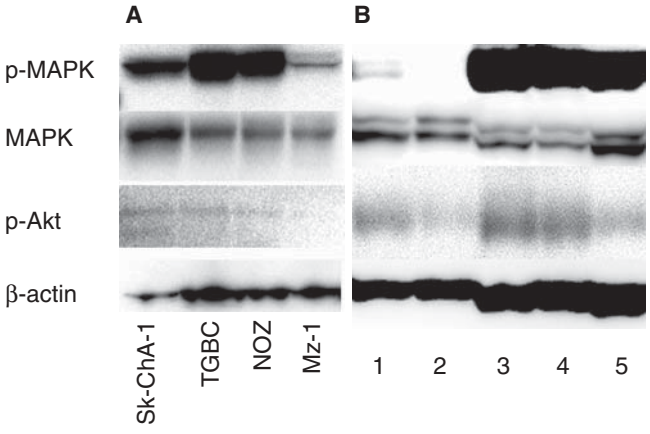

Fig. 1A,B. Protein levels of mitogen-activated protein kinase (*MAPK*), activated (phosphorylated) MAPK (*p-MAPK*), and activated (phosphorylated) Akt (*p-Akt*) in cancer cell lines (**A**) and biliary tract cancers (**B**). Data are from biliary tract cancer specimens resected from case numbers 1–5

Expression of p-MAPK (Thr202/Tyr204) and p-Akt (Ser 473) in paraffin-embedded and frozen BTC specimens

Figure 2 shows representative immunohistochemical stainings of normal bile duct epithelium and tumor cells. As reported previously,[18] weak p-Akt immunoreactivity was detected in the nuclei and cytoplasm of nontumor epithelial cells. In normal epithelial cells, immunoreactivity to p-MAPK was detected mostly in the nuclei, with a faint signal in the cytoplasm. Relative to normal epithelium, 77% of surgical specimens scored positive for an increased signal with anti-p-MAPK, whereas only 47% scored positive for enhanced p-Akt detection. These values indicate a significantly higher percentage of cells that overexpress p-MAPK compared with p-Akt (Table 1). The positive rates for detection of each phosphoprotein did not differ significantly with regard to clinicopathological variables, including tumor location (Table 2). There was a significant positive correlation between expression of p-MAPK and that of p-Akt (Table 3).

Frozen resected tumors were obtained for 20 of the 30 cases in this study. Although overall expression

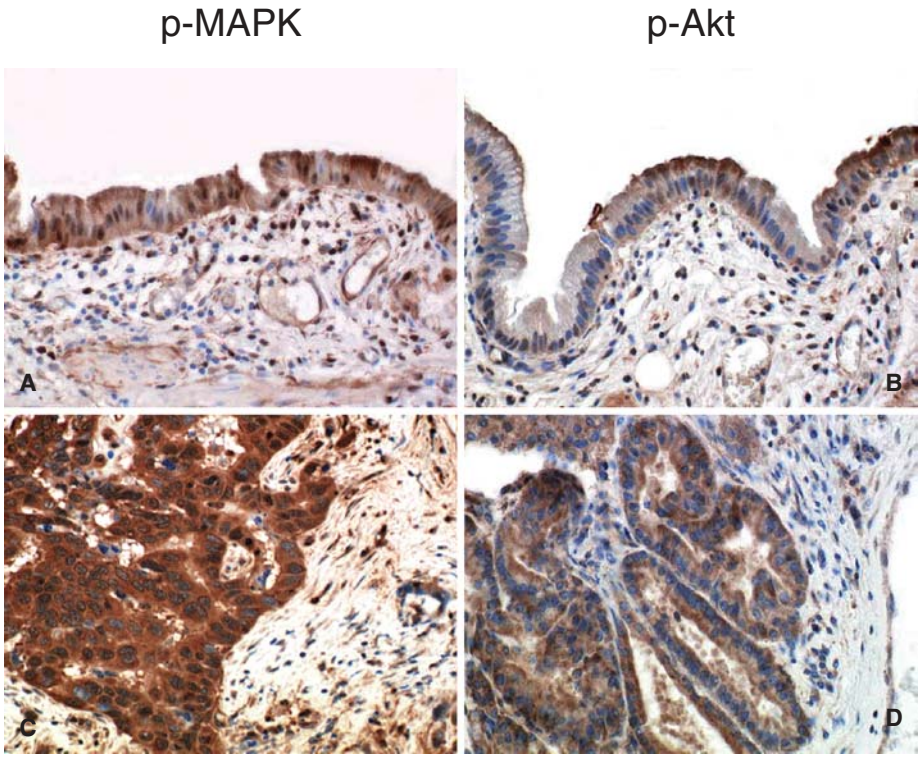

Fig. 2A–D. Immunohistochemical analysis of p-MAPK and p-Akt expression in gallbladder normal epithelium and cancer. Representative immunohistochemical staining of normal gallbladder epithelium for p-MAPK (**A**) and p-Akt (**B**), and of cancer for p-MAPK (**C**) and p-Akt (**D**)

Table 2. Expression of p-MAPK and p-Akt according to clinicopathological findings

		Overexpression (%)			
Factor	*n*	p-MAPK	*P*	p-Akt	*P*
Age					
≤70 years	12	92		42	
>70 years	18	67	0.11	50	0.65
Sex					
Male	15	67		54	
Female	15	87	0.20	40	0.46
Histological type					
Differentiated	27	78		48	
Undifferentiated	3	67	0.67	33	0.63
Location					
Gallbladder	15	87		47	
Bile duct	13	62		54	
Ampulla of Vater	2	100	0.21	0	0.36
pT category					
pT1	7	86		43	
pT2	6	67		67	
pT3	8	75		63	
pT4	9	78	0.67	22	0.18
pTNM stage					
I	6	83		33	
II	7	71		71	
III	5	80		80	
IV	12	75	0.96	25	0.08

Table 3. Association between expression of p-MAPK and p-Akt in surgical specimens of biliary tract cancer

	p-Akt expression		
	Positive	Negative	*P*
p-MAPK expression			
Positive	13	10	
Negative	1	6	0.049

levels of MAPK were similar among the samples, the relative expression of p-MAPK (an indicator of MAPK activity) was significantly elevated in 13 (65%) of the biliary cancer samples (Fig. 1B). Overexpression of p-Akt was detected in only six cases (30%), significantly fewer than for p-MAPK ($P = 0.03$). There was agreement between the expression results by Western blot analyses and those by immunohistochemistry in 85% of p-MAPK analyses and 80% of p-Akt analyses. Reproducibility of the assessment was good or moderate (Cohen's $\kappa = 0.68$ and 0.60 after categorization into positive and negative expression of p-MAPK and p-Akt, respectively).

Discussion

Recent studies have implicated the MAPK as well as the PI3K/Akt pathways in the growth of various

cancers.[17,28] In this study of extrahepatic BTC, MAPK was found to be activated more frequently than Akt. For both proteins, increased activation did not correlate with any particular site of biliary tract tumor.

Activation of K-*ras* results in the constitutive activation of both MAPK and PI3K/Akt pathways, ultimately promoting carcinogenesis and cancer progression. Previous studies of BTC suggest an important relationship between mutations in K-*ras* and activation of MAPK and PI3K/Akt,[27,29] and we and others have reported the importance of *ras* gene mutations in carcinogenesis and cancer progression in the gallbladder.[15,30] On the basis of these findings, we established a novel orthotopic inoculation model using NOZ cells that express mutant K-*ras* and constitutively activated MAPK to induce gallbladder cancer in nude mice.[26,31] Against this established model, we observed that a MAPK inhibitor could inhibit tumor progression and survival, suggesting that selective inhibitors targeting MAPK signaling pathway may have important implications in the treatment of patients with BTC.

Changes in ras/MAPK signaling pathways have been reported to occur frequently in cholangiocellular carcinoma cells.[27,28] However, the effect of changes in this pathway in extrahepatic bile duct or gallbladder cancer is unclear. In this study, MAPK activation (expression of p-MAPK) was seen in all cell lines encoding a K-*ras* mutant, whereas MZ-1 cells (which do not encode the K-*ras* mutation) did not show p-MAPK expression. The results in surgical specimens, which were similar to those of Javle et al.[19] with cholangiocarcinoma, show that MAPK is frequently activated in extrahepatic biliary tract tumors.

In this study, we examined p-MAPK expression by using both Western blot analysis and immunohistochemistry. Regarding immunohistochemical methods, Tannapfel et al.[27] reported that expression of p-MAPK was consistently detected in cholangiocellular carcinomas, but that the percentage of positive cells varied considerably among samples (12%–89%, median 31%). Using these findings as a guide, we classified cases with 30% or more immunohistochemically p-MAPK-positive cells as "positive" in this study. Some cases showed different expression results by Western blot and by immunohistochemistry, perhaps because of differences between Western blot and immunohistochemistry assessment criteria. Nonetheless, Western blot analysis confirmed high levels of activated MAPK in extrahepatic BTC as well.

Activation of PI3K-Akt signaling has been reported to suppress apoptosis.[32,33] However, several reports also state that Akt is often overexpressed and hyperactivated in a variety of human tumors, including ovary,[34] pancreas,[35] thyroid,[36] prostate,[37] breast,[38] and myeloma cells.[39] Two similar reports on BTC demonstrate that p-Akt is frequently detected by immunohistochemistry in bile duct cancer specimens: Tanno et al.[18] suggest that pharmacological or genetic modulation of Akt activity might have important therapeutic implications for bile duct cancer patients treated with radiation, and Javle et al.[19] suggest that a positive relationship exists between Akt expression and prognosis in cholangiocarcinoma. Relative to these reports, the low frequency of Akt activation in our study suggests that differences in antibody or patient background may influence test results.

In this study, no positive associations were observed between p-MAPK/p-Akt expression and clinicopathological factors, including tumor stage and pT category. This result may indicate that activation of MAPK and Akt are early events in biliary carcinogenesis. Kiguchi et al.[40] reported increased MAPK activity in gallbladder carcinogenesis in the erbB2 transgenic mouse model, and Dai et al.[41] reported Akt activation as an early event in melanoma tumorigenesis.

The association between alterations of MAPK and Akt pathways is unclear. Although these two signaling molecules are thought to regulate independent survival pathways, cross-signaling between them has been hypothesized to occur. In cholangiocarcinoma, a noteworthy correlation was observed between overexpression of p-Akt and MAPK in tumors.[19] Furthermore, the activation rate of MAPK was high relative to that of Akt or other related factors, which also suggests cross-regulation of these pathways.[19] In the present study (like in the previous report), the rate of activation of MAPK was higher than that of Akt, and there was a significant correlation between abnormalities of activated MAPK and Akt. Although our data may support the existence of cross-signaling in biliary cancers, further study will be required to confirm the relationship between these two factors, because the number of cases in the present study was small.

Our findings lead us to conclude that the MAPK pathway is more frequently activated than the Akt pathway in extrahepatic BTC. Therefore, inhibition of MAPK signaling pathways may be useful in the treatment of extrahepatic biliary tract cancers.

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