

## The Expression of Phospho-AKT, Phospho-mTOR, and PTEN in Extrahepatic Cholangiocarcinoma

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**Abstract Purpose:** The protein kinase B (AKT) pathway plays a key role in the regulation of cellular survival, apoptosis, and protein translation, and has been shown to have prognostic significance in a number of cancers. We sought to define its role in extrahepatic cholangiocarcinoma.

**Experimental Design:** Two hundred twenty-one extrahepatic cholangiocarcinoma patients with clinicopathologic data, including survival, were arrayed into tissue microarrays. Phosphorylated AKT (p-AKT), phosphorylated mammalian target of rapamycin (p-mTOR), and total phosphatase and tensin homolog deleted on chromosome 10 (PTEN) protein expressions were studied with multiplex tissue immunoblotting assay.

**Results:** Expressions of p-AKT and p-mTOR were significantly increased in extrahepatic cholangiocarcinoma cases compared with normal and dysplastic bile duct epithelium ( $P < 0.05$  both). Decreased PTEN expression was observed in patients with increasing depth of invasion ( $P < 0.05$ ), T classification ( $P < 0.05$ ), and stage grouping ( $P < 0.05$ ), and the presence of invasion of the pancreas ( $P < 0.05$ ) and duodenum ( $P < 0.05$ ). Decreased PTEN expression ( $P = 0.004$ ) as well as decreased PTEN/p-AKT ( $P = 0.003$ ) and PTEN/p-mTOR ( $P = 0.009$ ) expression showed shorter survival by univariate but not by multivariate analysis.

**Conclusions:** The AKT pathway is activated in a subset of extrahepatic cholangiocarcinoma. Elevated PTEN expression correlates with longer survival. Quantitative data obtained by multiplex tissue immunoblotting may provide additional information than assessment of immunohistochemistry alone. Quantitative analysis of PTEN, PTEN/p-AKT and PTEN/p-mTOR shows differences in survival by univariate analysis.

Extrahepatic cholangiocarcinoma is a malignant neoplasm of biliary tract epithelia arising from the hepatic hilum to the distal bile duct, and constitutes approximately 70% to 90% of all cholangiocarcinomas (1). Although extrahepatic cholangiocarcinoma is a relatively uncommon neoplasm in the United States, it is more prevalent in Asia, including Korea (1–3). Currently, surgical resection is the mainstay of treatment, but it is curative only in a limited number of patients, primarily

those without advanced-stage disease (4). For patients who undergo surgical resection, the 5-year survival rate is approximately 20% (5). Several neoadjuvant therapies, including chemotherapy, radiation therapy, and photodynamic therapy have been studied, but have not shown significant survival benefit (1, 6). Therefore, the identification of new targets for early detection and/or the development of new therapeutic regimens based on a better understanding of the biological mechanisms are essential for reducing the mortality of extrahepatic cholangiocarcinoma patients.

The phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) signaling pathway plays an important role in regulating tumor cellular survival, apoptosis, and protein translation (7). PI3K is activated by receptor tyrosine kinases, and activation of receptor tyrosine kinases leads to allosteric joining to the cellular membrane and subsequent tyrosine phosphorylation of the regulatory subunit of PI3K. PI3K converts phosphatidylinositol 2 phosphate (PIP2) to phosphatidylinositol 3 phosphate (PIP3; refs. 7, 8). AKT is activated by phosphorylation at Thr308 by PIP3 and at Ser473 by mammalian target of rapamycin (mTOR) as a part of the mTOR complex (mTORC; ref. 8). The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a well-described negative regulator of the PI3K/AKT signaling pathway, which functions as a tumor suppressor gene by induction of G<sub>1</sub> phase cell cycle arrest through decreasing the levels of cyclin D1 (9). Rapamycin was initially considered as a promising modality for blocking

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### Translational Relevance

Extrahepatic cholangiocarcinoma is a malignant neoplasm of biliary tract epithelia, and constitutes approximately 70% to 90% of all cholangiocarcinomas. Surgical resection is the mainstay of treatment, but results in an approximate 5-year survival rate of only 20%. Neoadjuvant therapies, including chemotherapy, radiation therapy, and photodynamic therapy have failed to show significant survival benefit. Therefore, the identification of prognostic and potential predictive biomarkers of response to targeted agents may be useful in guiding therapy, especially if applicable to biopsy specimens. Utilizing multiplex tissue immunoblotting, we showed that PTEN expression provided predicted survival by univariate analysis. A combination biomarker of the ratio of PTEN/p-AKT stratifies patients better than PTEN alone. Similar results were seen with a PTEN/p-mTOR ratio. These findings may provide useful information, when applied to diagnostic samples, and potentially identify patients appropriate for mTOR analog-based chemotherapy or agents directed against AKT.

mTOR phosphorylation in several cancer types; however, cancer patients with high AKT activity are reported to minimally respond to mTORC1 inhibitors (10). Patients with high expression of activated (phosphorylated) AKT were also reported to be resistant to radiation therapy (11). Although a few reports described AKT, mTOR, or PTEN expression in cholangiocarcinoma (12–15), there is no comprehensive study of phosphorylated AKT (p-AKT), phosphorylated mTOR (p-mTOR), and PTEN expression in extrahepatic cholangiocarcinoma.

In order to profile proteomic expression profiles of these target molecules, we used a recently developed multiplex tissue immunoblotting (MTI) method (16). Applying this method of transferring proteins from paraffin-embedded tissue sections to a stack of membranes that can then be applied conventional immunoblotting, multiple antigens can be assayed from a single tissue section (16–19). This approach permits simultaneously quantifying multiplex markers with preservation of the morphologic structure of the tissue and normalization to total protein content. In addition, we reconfirmed the expression profile of the MTI by immunohistochemistry. Utilizing this approach, we were able to show that quantitative analysis of PTEN expression provided survival information, as well as the capacity of PTEN/p-AKT and PTEN/p-mTOR to stratify patients better than PTEN alone by univariate analysis.

### Materials and Methods

**Patients and tumor samples.** The study subjects were composed of 221 patients with extrahepatic cholangiocarcinoma who were surgically resected at Asan Medical Center, University of Ulsan College of Medicine in Seoul, South Korea. Carcinomas with the epicenter in the extrahepatic bile duct were included, whereas carcinomas of the ampulla of Vater or pancreas, and those with obvious precancerous epithelial changes in the ampulla of Vater or pancreas were excluded. Carcinomas arising in the gallbladder or intrahepatic bile duct with extension to the extrahepatic bile duct were also excluded in this study. Medical records were reviewed to obtain data including age and gender

of patients, surgical procedure, survival time, and survival status. Data on tumor location, size, and growth pattern were obtained from reviewing pathology reports. Information on postoperative radiation and/or chemotherapy, and performance status of patients was not available for analysis. Material was obtained with appropriate human protection approvals from the Institutional Review Board of the Asan Medical Center and the Office of Human Subjects Research at the NIH.

**Tissue microarray construction.** Tissue microarrays were constructed from archival formalin-fixed, paraffin-embedded tissue blocks. For each tumor, a representative tumor area was carefully selected from a H&E-stained section of a donor block which was previously described (20, 21). In brief, 20 normal biliary epithelial, 67 biliary dysplasia, and 221 extrahepatic cholangiocarcinoma cases were included. Each case was represented with 2 cores of 1.5 mm diameter.

**Proteomic expression profiling by multiplex tissue immunoblotting.** The MTI was done as previously described (16, 17). In brief, tissue microarray slides were deparaffinized and treated with an enzyme cocktail solution (0.001% trypsin plus 0.002% proteinase-K, 10% glycerol, 50 mmol/L  $\text{NH}_4\text{HCO}_3$  pH 8.2; Fisher Scientific) for 30 min at 37°C. Slides were subsequently incubated with Probuffer complete protease inhibitor solution [0.5 mL phosphatase inhibitor I (Sigma), 0.5 mL phosphatase inhibitor II (Sigma), and 1 protease inhibitor tablet (Roche Diagnostics) in 50 mL PBS (pH 7.2)] for 20 min at room temperature. The proteins of treated slides were transferred to a 5-membrane stack of P-FILM (20/20 GeneSystems) using Tris-glycine transfer buffer (50 mmol/L Tris, 380 mmol/L Glycin) under serial conditions for 1 h at 55°C, for 0.5 h at 65°C, and for 2 h at 80°C. After the transfer the membranes were washed with TBS with Tween 20. Each membrane was incubated with anti-p-AKT, anti-p-mTOR, and total PTEN (1:100 dilution each; Cell Signaling) overnight and subsequently with FITC-conjugated antirabbit IgG (1:1,000; Molecular Probes) and streptavidin-linked Cy5 (1:1,000; Amersham Biosciences) for 30 min. Following immunodetection, total cellular proteins were measured by biotinylation of proteins followed by incubation of the membrane with streptavidin-Cy5. Following fluorescence-based detection (Microarray Scanner; PerkinElmer), signal intensity was quantified by correcting for background followed by determining the ratio of specific protein/total cellular protein for each sample. Interarray normalization was done by determining the ratio of specific protein/total cellular protein for each sample, and then intra-array normalization was done by adjusting the median expression level of normal biliary epithelia.

**Immunohistochemistry.** Tissue sections were deparaffinized and hydrated in xylene and serial alcohol solutions, respectively. Endogenous peroxidase was blocked by incubation in 3%  $\text{H}_2\text{O}_2$  for 10 min. The antigen retrieval was done in a steam pressure cooker with prewarmed antigen retrieval buffer pH 10 (Dako) at 95°C, for 10 min. To minimize nonspecific staining, sections were incubated with protein block (Dako) for 15 min. Primary antibodies were incubated overnight at 4°C. Antigen-antibody reactions were detected with DAKO LSAB+ peroxidase kit and DAB. Anti-p-AKT, anti-p-mTOR, and PTEN (Cell Signaling) were used at a dilution of 1:200. Immunostained sections were lightly counterstained with hematoxylin, dehydrated in ethanol, and cleared in xylene.

**Statistical analysis.** Statistical analyses were done using SAS (version 9.13) and R (<http://www.r-project.org>). Differential expression of p-AKT, p-mTOR, and total PTEN between and among normal biliary epithelia, dysplasia, and extrahepatic cholangiocarcinoma was compared by ANOVA and Duncan's tests after normalization of expression. Associations between categorical variables were examined using the Pearson's  $\chi^2$  and Fisher's exact tests. A recursive partitioning technique coupled with log-rank statistics was employed to identify the cutoff points that discriminate outcome of patients based on protein expression. Survival curves were calculated by the Kaplan-Meier method, and statistical significance was examined by the log-rank test and the Cox proportional hazards regression model. A *P* value < 0.05 was considered statistically significant.

**Results**

**Clinicopathologic characteristics of the patients.** Clinicopathologic characteristics of cases are summarized in Table 1. The ages of the patients ranged from 30 to 84 years (mean, 61 years). One hundred thirty-four patients were men and 87 were women. The tumor sizes ranged from 0.4 to 6 cm (mean, 2.6 cm). Thirty-four cases were T<sub>1</sub> tumors, 80 T<sub>2</sub>, 84 T<sub>3</sub>, and 23 T<sub>4</sub>. The length of the patients' follow-up time ranged from 1 to 128 months, and median survival at last follow-up was 34 months.

**Table 1.** Clinicopathologic characteristics of patients with extrahepatic cholangiocarcinoma who were examined in this study

Variables	No. of patients (N = 221)
Mean age	61 y
Gender	
Male	134
Female	87
Mean tumor size	2.6 cm
Histologic subtype	
Adenocarcinoma, NOS	188
Papillary carcinoma	15
Intestinal type adenocarcinoma	5
Mucinous carcinoma	4
Adenosquamous carcinoma	5
Clear cell carcinoma	1
Signet ring cell carcinoma	1
Sarcomatoid carcinoma	2
pT classification	
pT <sub>1</sub>	34
pT <sub>2</sub>	80
pT <sub>3</sub>	84
pT <sub>4</sub>	23
Lymph node metastasis	
Present	74
Absent	147
Hepatic invasion	
Present	7
Absent	214
Pancreatic invasion	
Present	100
Absent	121
Duodenal invasion	
Present	23
Absent	198
Perineural invasion	
Present	150
Absent	71
Vascular invasion	
Present	64
Absent	157
Type of surgery	
Pylorus preserving pancreaticoduodenectomy	93
Whipple's operation	59
Bile duct resection	46
Hepatic lobectomy with bile duct resection	18
Pancreaticoduodenectomy with extended hepatic lobectomy	3
Pylorus preserving pancreaticoduodenectomy with bile duct resection	1
Whipple's operation with bile duct resection	1

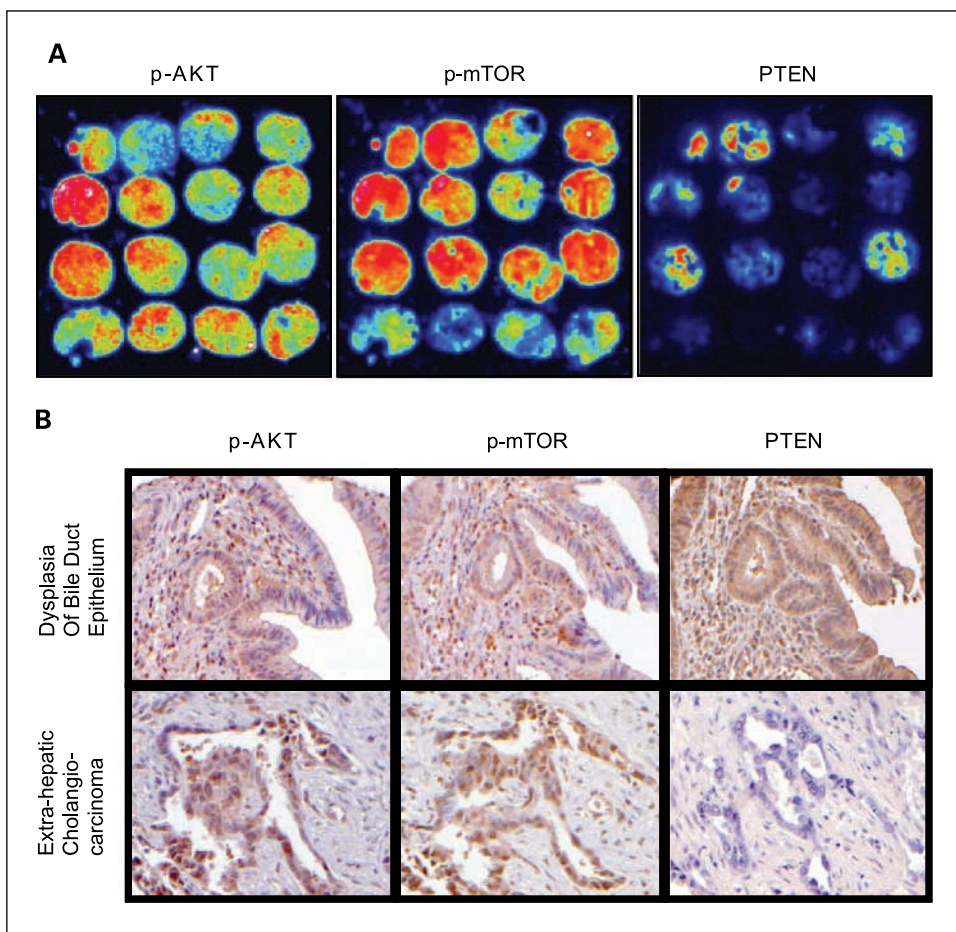
**Expression of p-AKT and p-mTOR.** We analyzed the expression patterns of p-AKT and p-mTOR proteins using MTI assay from 221 patients with extrahepatic cholangiocarcinoma. A representative expression signal to p-AKT, p-mTOR, and total PTEN of 16 cases by the MTI assay is shown in Fig. 1A. The expression pattern was confirmed by immunohistochemistry (Fig. 1B). As shown in previous studies (16–18), there is strong correlation between MTI and immunohistochemistry, with the added benefit of normalization for total protein, and quantification. After normalization, we calculated the relative expression of p-AKT and p-mTOR among normal biliary epithelia, dysplasia, and cancer cases (Fig. 2). No significant difference of p-AKT and p-mTOR expression was observed between normal bile duct epithelium and dysplastic epithelium. However, patient specimens with extrahepatic cholangiocarcinoma showed significantly higher p-AKT ( $P < 0.05$ , post hoc Duncan test) and p-mTOR ( $P < 0.05$ , post hoc Duncan test) expression than those with normal and dysplastic biliary epithelia (Fig. 2A). Overall, p-AKT expression was elevated in 26.3% (15 of 57 cases) of dysplasia and 84.2% (186 of 221 cases) of extrahepatic cholangiocarcinoma after normalization. Expression of p-mTOR was similar to that of p-AKT, with increased mTOR expression detected in 28.1% (16 of 57 cases) of dysplasia and 83.7% (185 of 221 cases) of extrahepatic cholangiocarcinoma, respectively (Fig. 2B). We also observed a statistically significant positive correlation between p-AKT and p-mTOR ( $r = 0.45$ ;  $P < 0.001$ ; Fig. 2C), supporting that the two proteins are involved in the same signaling pathway as previously reported in cancers from other organs (22).

**PTEN expression.** There was no difference of PTEN expression among cases with normal, dysplastic, and malignant biliary epithelia (data not shown). Cases with T<sub>1</sub> TNM stage had a significantly higher relative PTEN expression (mean, 16.08; relative expression intensity) than those with other classifications (T<sub>2</sub>, 8.92; T<sub>3</sub>, 7.18; T<sub>4</sub>, 3.98;  $P < 0.05$ , post hoc Duncan test; Fig. 3A). Patients with invasion of the pancreas had significantly less PTEN expression (mean, 5.94) than those without pancreas invasion (mean, 9.95;  $P < 0.05$ , post hoc Duncan test). Cases with duodenal invasion had statistically less PTEN expression (mean, 3.98) than those without duodenal invasion (mean, 9.04;  $P < 0.05$ , post hoc Duncan test; Fig. 3B). Patients with higher stage (IIB and III) had significantly less PTEN expression (6.45 and 3.98, respectively) than those with lower stages IA and IIA, (17.23 and 8.14, respectively;  $P < 0.05$ , post hoc Duncan test; Fig. 3C).

We have recently shown that the measurement of the depth of tumor invasion from the basement of membrane to the portion of deepest tumor is a better indicator of patient survival than the current tumor pT classification used in the American Joint Committee on Cancer tumor staging system (23). Therefore, the depth of tumor invasion was also compared (Fig. 3D). Patients with less tumor cell invasion (<0.5 cm of depth of invasion) had a higher PTEN expression (mean PTEN, 3.41) than >1.2 cm invasion tumor cell invasion (mean PTEN 1.61;  $P < 0.05$ , post hoc Duncan test). Intermediate tumor cell invasion (0.5–1.2 cm invasion) had a mean PTEN 2.94, which does not reach statistical significance.

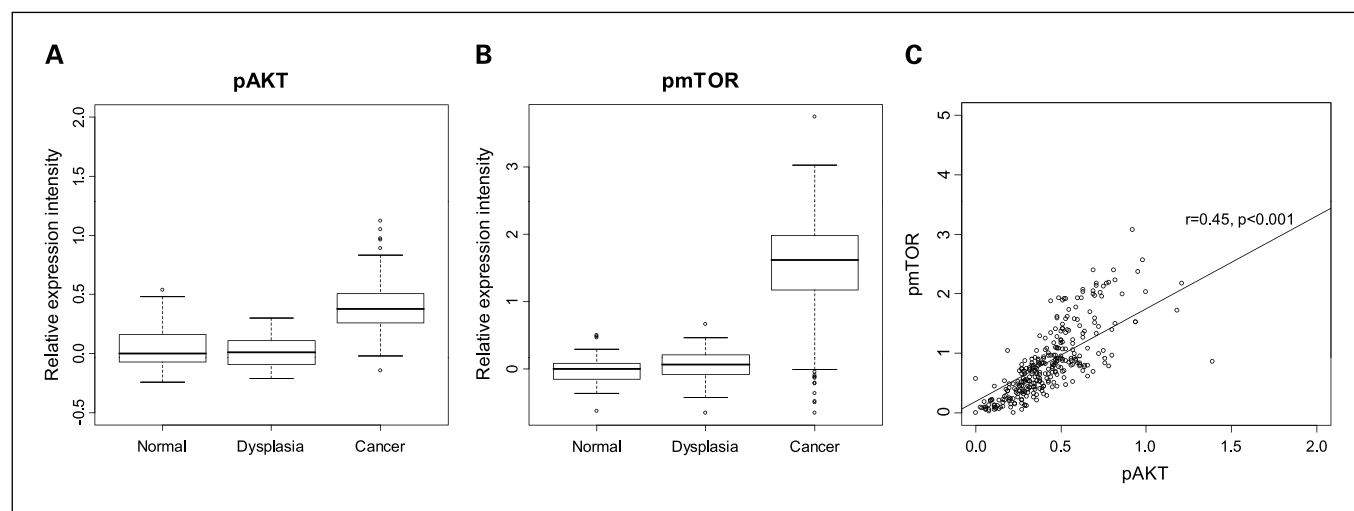
**Survival analysis.** For survival analysis, patients were categorized as high or low expressers based on the median expression of the marker of interest. Although patients with high

**Fig. 1.** Phosphorylated AKT (p-AKT), phosphorylated mTOR (p-mTOR), and PTEN expression by multiplex tissue immunoblotting and immunohistochemical staining. *A*, signal intensity from maximum to minimum is white-yellow-red-green-blue-black in order. Cases with higher intensity to p-AKT and p-mTOR showed lower intensity to total PTEN. *B*, immunohistochemical staining of p-AKT, p-mTOR, and PTEN protein and dysplasia and extrahepatic cholangiocarcinoma.



p-AKT expression group had shorter 1-, 3-, and 5-year survival rates (79.7%, 46.1%, and 36.3%, respectively) than those with low p-AKT expression (83.3%, 83.3%, and 83.3%, respectively), the difference was not statistically significant ( $P = 0.06$ ). Cases with high p-mTOR expression showed shorter 1-, 3-, and 5-year

survival rates (70.6%, 22.1%, and 22.1%, respectively) than those with lower p-mTOR expression (82.2%, 51.5%, and 41.0%, respectively), but there was no statistically significant difference between the two groups ( $P = 0.06$ ). Patients with low PTEN expression (median survival, 18 months) had a



**Fig. 2.** Box plot of relative expression rate of p-AKT and p-mTOR protein expressions among normal biliary epithelia, dysplasia, and cancer cases. *A*, cases with extrahepatic cholangiocarcinoma had significantly higher expression to p-AKT than those with normal and dysplastic epithelia ( $P < 0.05$ , post hoc Duncan test). *B*, cases with extrahepatic cholangiocarcinoma had significantly higher expression to p-mTOR than those with normal and dysplastic epithelia ( $P < 0.05$ , post hoc Duncan test). *C*, correlation between p-AKT and p-mTOR expression ( $R = 0.45$ ;  $P < 0.001$ ).

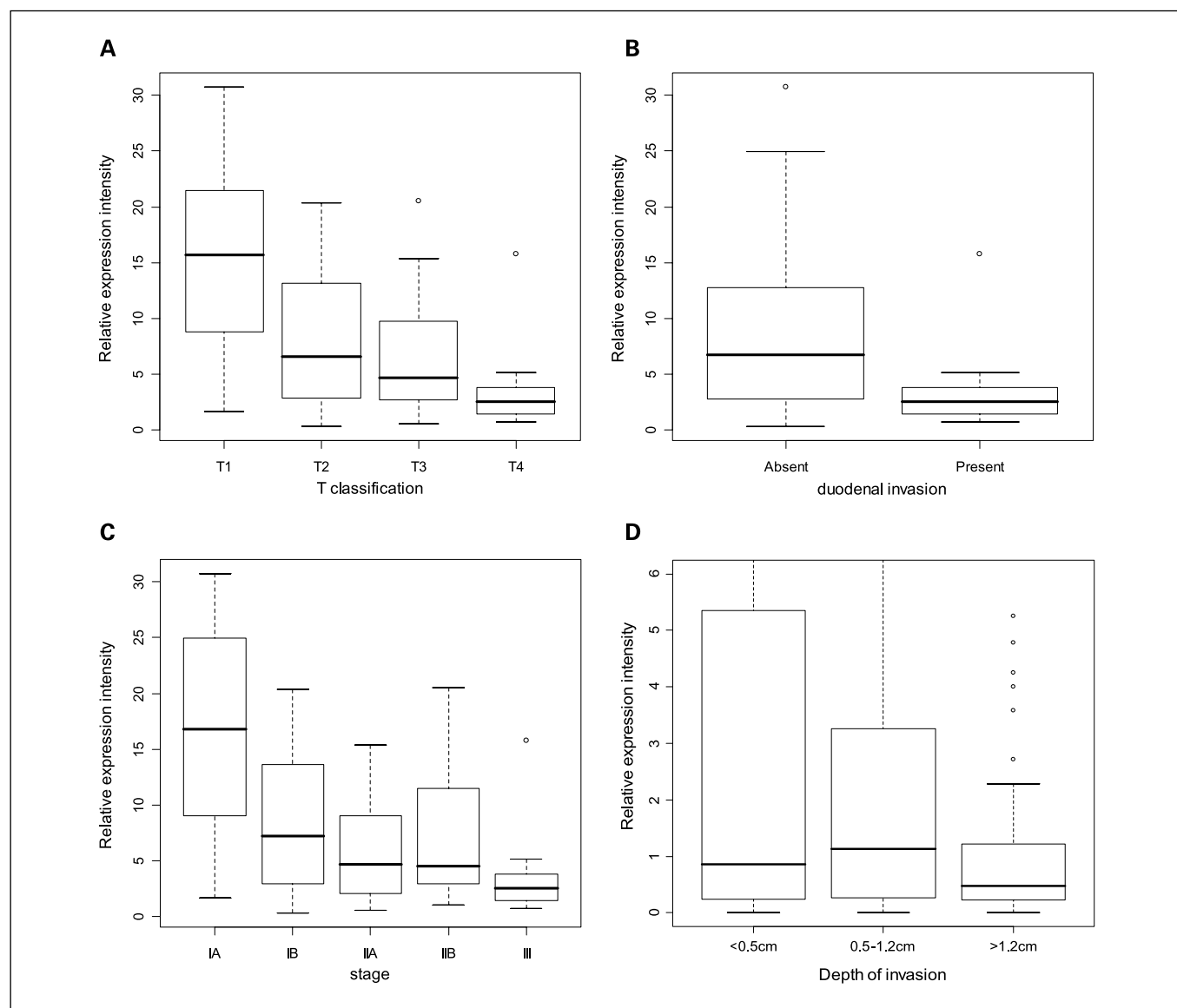
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significantly worse patients' survival time than those with high PTEN expression (median survival, 39 months; log-rank test,  $P = 0.004$ ). Patients with low PTEN had 1-, 3-, and 5-year survival rates of 80.0%, 13.3%, and 13.3%, respectively, whereas those in the high-expression group had 1-, 3-, and 5-year survival rates of 80.7%, 52.3%, and 42.2%, respectively (Fig. 4).

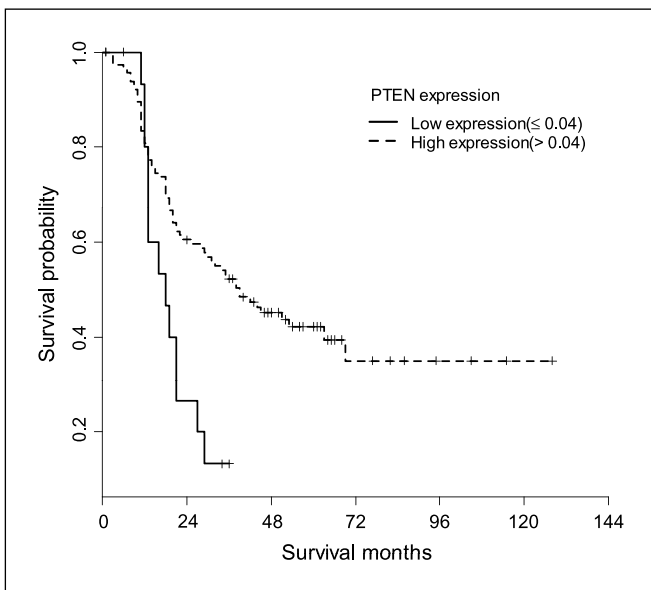
**Expression profile by PTEN/p-AKT or p-mTOR ratio.** To determine if the combination of PTEN and p-AKT together has a better predictive potential for determining the survival probability of patients with extrahepatic cholangiocarcinoma, we analyzed the ratio of PTEN/p-AKT or PTEN/p-mTOR in patients with extrahepatic cholangiocarcinoma. By recursive partitioning coupled with log-rank test, the best cutoff point

to discriminate patients' survival based on PTEN/p-AKT ratio most was 0.77. Patients with low PTEN/p-AKT expression ( $\leq 0.77$  of PTEN/p-AKT ratio; median survival, 18 months) had a significantly worse patients' survival time than those with high PTEN/p-AKT expression ( $>0.77$ ; median survival, 45 months, log-rank test,  $P = 0.003$ ). The 1-, 3-, and 5-year survival rates for patients with low PTEN/p-AKT expression were 72.6%, 30.0%, and 23.4%, respectively, whereas the 1-, 3-, and 5-year survival rates for those with high expression were 84.1%, 56.4%, and 46.2%, respectively (Fig. 5A).

The best cutoff point to discriminate patients' survival based on PTEN/p-mTOR ratio most by recursive partitioning technique was 0.33. Patients with low PTEN/p-mTOR expression ( $<0.33$  of PTEN/p-mTOR; median survival, 18 months) had a



**Fig. 3.** Box plot of relative expression rate of PTEN and its association with other clinicopathologic factors. *A*, cases with  $T_1$  classification (mean, 16.1) had a significantly higher relative PTEN expression than those with other classifications;  $T_2$ , 8.9;  $T_3$ , 7.2;  $T_4$ , 4.0 ( $P < 0.05$ , post hoc Duncan test). *B*, patients with duodenal invasion (mean, 4.0) had significantly less PTEN expression than those without duodenal invasion (mean, 9.0;  $P < 0.05$ , post hoc Duncan test). *C*, patients with higher stage grouping (advanced disease; mean PTEN stage III, 4.0; IIB, 6.5) had significantly less PTEN expression than those with lower stage (IIA, 8.1; IB, 9.6; IA, 17.2;  $P < 0.05$ , post hoc Duncan test). *D*, patients with less tumor cell invasion ( $<0.5$  cm of depth of invasion) had a statistically greater PTEN expression (mean PTEN, 3.41) than cases with deeper tumor cell invasion ( $>1.2$  cm invasion, mean PTEN 1.61;  $P < 0.05$ , post hoc Duncan test), but no statistical difference with those with depth of invasion between 0.5 and 1.2 cm (PTEN mean, 2.94).



**Fig. 4. A.** Kaplan-Meier survival analysis of extrahepatic cholangiocarcinoma according to PTEN expression. Patients with low PTEN expression (median survival, 18 mo;  $n = 17$ ) had a significantly worse patients' survival time than those with high PTEN expression (median survival, 39 mo;  $n = 117$ ; log-rank test,  $P = 0.004$ ).

significantly worse patients' survival time than those with high PTEN/p-mTOR expression ( $>0.33$ ; median survival, 39 months; log-rank test,  $P = 0.009$ ). The 1-, 3-, and 5-year survival rates for patients in the low PTEN/p-mTOR expression group were 76.2%, 22.9%, and 11.4%, respectively, whereas the 1-, 3-, and 5-year survival rates for those in the high expression group were 81.3%, 52.9%, and 43.3%, respectively (Fig. 5B).

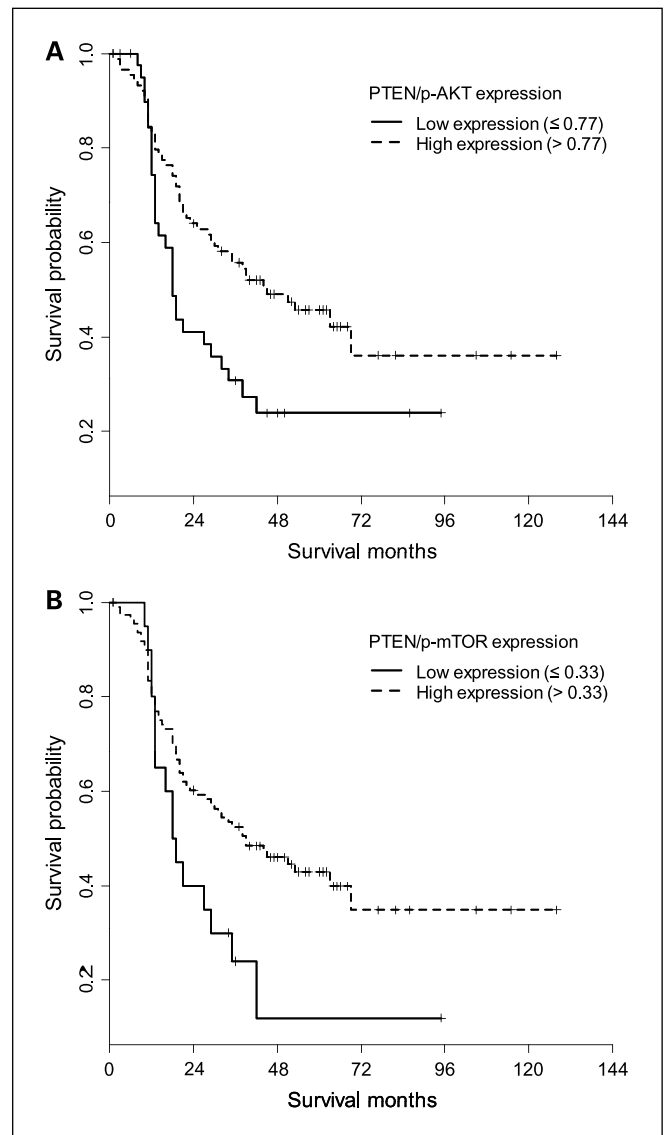
**Association between survival and other clinicopathologic factors.** Of the other clinicopathologic variables, T classification ( $P = 0.0002$ ), lymph node metastasis ( $P = 0.0001$ ), differentiation ( $P < 0.0001$ ), pancreatic invasion ( $P = 0.01$ ), duodenal invasion ( $P = 0.003$ ), liver invasion ( $P = 0.005$ ), and vascular invasion ( $P = 0.04$ ), were also significantly associated with survival. In contrast, survival was not associated with perineural invasion and resection marginal status.

**Multivariate analysis of clinicopathologic factors.** The independent prognostic significance of PTEN/p-AKT, as well as other clinicopathologic parameters, was determined using the Cox proportional hazards model (Table 2). Using this multivariate analysis, only lymph node metastasis ( $P = 0.008$ ) and differentiation ( $P = 0.0002$ ) remained significant (Table 2). PTEN/p-AKT did not obtain statistical significance in this analysis ( $P = 0.09$ ).

## Discussion

In a murine model of intrahepatic cholangiocarcinoma, the disruption of the PTEN gene was associated with increased level of p-AKT, GSK-3 $\beta$ , mTOR, and ERK, suggesting that PTEN may involve in cholangiocarcinogenesis by affecting its downstream genes (24). We sought to examine the expression status of pathway protein profiles rather than a single protein in one pathway. Our current study is the largest to show that PI3K/AKT pathway activation is a negative factor in the survival of extrahepatic cholangiocarcinoma patients, and is the first report

to show that decreased PTEN/p-AKT and/or decreased PTEN/p-mTOR protein ratio may provide the overall survival information of extrahepatic cholangiocarcinoma patients. Although there was no survival difference based on single protein expression status of p-AKT and p-mTOR, when combined expression status of those proteins were compared, different survival time in patients with extrahepatic cholangiocarcinoma was observed. Although PTEN expression alone could stratify survival, the ratio-based measurements with p-AKT and p-mTOR improved this discrimination. The combination of biomarkers may be more informative than a single marker when analyzing pathways and signaling. A previous study showed that AKT and p-AKT expression was associated



**Fig. 5. A.** Kaplan-Meier survival analysis of extrahepatic cholangiocarcinoma according to PTEN/p-AKT expression. Patients with low PTEN/p-AKT expression (median survival, 18 mo;  $n = 42$ ) had a significantly worse patients' survival time than those with high PTEN/p-AKT expression (median survival, 45 mo;  $n = 91$ ; log-rank test,  $P = 0.003$ ). **B.** Kaplan-Meier survival analysis of EHCC according to PTEN/p-mTOR expression. Patients with low PTEN/p-mTOR expression (median survival, 18 mo;  $n = 21$ ) had a significantly worse patients' survival time than those with high PTEN/p-mTOR expression (median survival, 39 mo;  $n = 112$ ;  $P = 0.009$ ).

**Table 2.** Multivariate analysis for the prognosis

Variable	P	Relative risk (95% confidence interval)
PTEN/p-AKT	0.09	1.05 (0.99-1.11)
pT classification	0.10	1.95 (0.88-4.36)
Lymph node metastasis	0.008*	1.96 (1.19-3.21)
Duodenal invasion	0.96	0.97 (0.29-3.20)
Liver invasion	0.66	1.39 (0.32-6.13)
Pancreatic invasion	0.31	0.55 (0.18-1.74)
Vascular invasion	0.08	1.59 (0.95-2.65)
Differentiation	0.0002*	1.96 (1.38-2.78)

\*Significant at the level of  $P < 0.05$ .

with improved prognosis in cholangiocarcinoma (12). Using immunohistochemistry, Javle et al. analyzed a small number of cholangiocarcinoma cases ( $n = 24$ ) without providing the specific location of the tumor in the bile duct. However, AKT activation is a well-known poor prognosis marker in many other malignant neoplasms, including prostate cancer (25), malignant melanoma (26), pancreatic cancer (27), and non-small cell lung cancer (28). Our data support that AKT activation was associated with poor prognosis in extrahepatic cholangiocarcinoma. This discrepancy in the prognostic significance of AKT activation could be related to small sample sizes, differences in the preparation or selection of specimens (cholangiocarcinoma versus extrahepatic cholangiocarcinoma), or technical differences in staining or scoring. Activated (phosphorylated) AKT signaling was also reported to be associated with radioresistance (13). Similar to another previous study (14), no positive associations were identified between p-AKT expression and other clinicopathologic variables in the present study.

The PI3K/AKT pathway is one of the commonly activated pathways in many other malignant neoplasms, but only a few previous studies with a small number of cases were done on cholangiocarcinoma (12–15). Previous studies reported various rates of p-AKT expression in extrahepatic cholangiocarcinoma ranging from 54% to 96% (12–14), whereas the reported p-mTOR expression rate in a single study was 64% (15), lower than that observed in the present study. The differences in p-AKT expression rates in these studies are from several potential sources: different antibodies, scoring methodologies, or differences in patient cohorts. Immunohistochemical studies with manual scoring are not a quantitative measurement but a qualitative assessment. Although immunohistochemistry is considered a gold standard for evaluation of protein expression in histopathology, several difficult issues remain in evaluation of immunohistochemistry, including a subjective of determination of intensity (29). Image analysis overcomes some of these issues, but it lacks normalization to account for differences in tissue handling and processing (30). In order to overcome this limitation of immunohistochemistry, we examined protein expression with MTI, which could measure protein expression, incorporating quantitation and normalization. Normalization methods for immunohistochemistry have been suggested (31), but remain technically challenging (30). The MTI method permits rigorous comparisons of protein expression across histogeographic zones of histology as well as interrogation of multiple antigens from a

single section of tissue (16–19). In the present study, we observed p-AKT and p-mTOR expressions were significantly increased from normal biliary epithelial cells to infiltrating malignant neoplastic epithelial cells. In concordance with the results of previous studies (12–14), activated AKT expression was observed in 84.2% of extrahepatic cholangiocarcinoma cases after normalization. Furthermore in the present study we showed that the survival of extrahepatic cholangiocarcinoma patients could be clearly discriminated by specific relative expression level of active AKT and mTOR proteins in relation with loss of expression of total PTEN protein by univariate analysis. As a potential biomarker, assays such as the MTI measurement of PTEN/p-AKT may have utility in diagnostic biopsies as a means of selecting patients for neoadjuvant therapy, or when applied to resection specimens, selection of chemotherapeutic regimens. Application of this approach would require an appropriate clinical trial to show utility.

Silencing of PTEN expression in cholangiocarcinoma cell lines has been described to occur by several different mechanisms, including homozygous deletion (32) and interference by microRNA (miR-21; ref. 33). The clinical implication of loss of PTEN protein expression in extrahepatic cholangiocarcinoma has not been previously investigated. Several studies showed that loss of PTEN protein expression could be used as a prognostic factor in other malignancies, including breast (34), endometrial (35), hepatocellular (36), and gastric carcinomas (37). In this study, we showed that loss of PTEN expression was associated with worse survival outcome in patients with extrahepatic cholangiocarcinoma by univariate analyses. We also observed that patients with low PTEN expression were associated with increased T classification, duodenal invasion, and disease stages. In addition, we showed low expression of combined PTEN/p-AKT and PTEN/p-mTOR. Uegaki et al., using immunohistochemistry, showed in endometrial carcinoma that PTEN positive/p-AKT negative tumors were associated with better prognosis than the other three combinations of PTEN and p-AKT (38). Qualitative assessment of immunohistochemistry prevents a meaningful ratio-based assessment of survival with multiple biomarkers. In this study, we observed that the patients with low PTEN/p-AKT expression measured by quantitative means had worse prognosis than those with a higher pTEN/p-AKT ratio. The survival time in patients with extrahepatic cholangiocarcinoma were compared with log-rank statistics based on their relative protein expression status, which was previously described elsewhere (23). This approach may show survival of the patients more precisely with difference in

protein expression than simply dichotomized patients by interpretation as positive or negative based on immunohistochemistry.

It is postulated that rapamycin may inhibit proliferation of tumor cell growth in these patients population with extrahepatic cholangiocarcinoma. Recently this insight was concreted by Sawada et. al. (39) who observed that rapamycin treatment inhibited the growth of cholangiocarcinoma cell lines. Our paper suggests the PI3K/AKT pathway as a potential target for intervention in extrahepatic cholangiocarcinoma with rapamycin analogs.

In summary, we evaluated p-AKT, p-mTOR, and total PTEN expression by MTI assay, and observed that the PI3K/AKT path-

way is activated in a subset of extrahepatic cholangiocarcinoma. Our data show that PTEN, PTEN/p-AKT, and PTEN/p-mTOR can all function as biomarkers of survival by univariate analysis. Quantitative assessment of biomarkers, alone and by ratios or other approaches may offer new tools to discriminate survival.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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