Immunohistochemical Expression of Phospho-mTOR Is Associated With Poor Prognosis in Patients With Gallbladder Adenocarcinoma

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● ***Context.***—**Advanced gallbladder carcinoma (GBC) is a highly fatal disease with poor prognosis and few therapeutic alternatives. The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays a central role in cell growth and homeostasis. Its regulation is frequently altered in various tumors and is an attractive target for cancer therapy; however, its status in GBC remains unclear.**

Objective.—**To characterize immunohistochemical expression and prognostic significance of phospho-mTOR in advanced gallbladder carcinoma.**

Design.—**Phospho-mTOR expression was examined by immunohistochemistry in tissue microarrays containing 128 advanced GBCs and 99 cases of chronic cholecystitis, which were divided into 2 groups according to the presence or absence of metaplasia. To evaluate the association of the level of phospho-mTOR expression with clinical variables and patient survival, the advanced GBCs were classified as having low or high expression. Statistical analysis was performed by using a significance level of $P < .05$, and Kaplan-Meier curves were constructed for survival analysis.**

Results.—**Immunostaining for phospho-mTOR was positive in 82 of 128 tumors (64.1%) and in 24% of chronic cholecystitis cases (16% nonmetaplasia and 32% with metaplasia) ($P < .001$). Survival analysis indicated that a high phospho-mTOR immunohistochemical expression was associated with poorer prognosis in patients with advanced GBC ($P = .02$).**

Conclusions.—**Metaplasia is a common finding in chronic cholecystitis and is considered a precursor lesion of dysplasia. Our results suggest that the activation of mTOR occurs very early during the development of GBC, contributing to the carcinogenesis process. Phospho-mTOR expression is correlated with poor survival, supporting the potential of mTOR for targeted therapy.**

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Gallbladder cancer (GBC) is considered the most common malignant lesion of the biliary tract and the fifth most common among malignant neoplasms of the digestive tract.[1] It shows a marked geographic variation in its incidence,[2] with highest incidences seen in Native American and Latin American countries such as Mexico, Chile, and Bolivia.[3] In our region, it has an extremely high incidence (18.1/100 000)[4] and is the second leading cause of death by cancer among women.[5] This carcinoma is an aggressive tumor with a poor prognosis because most tumors are generally diagnosed in the advanced stages of the disease, with gallstones and chronic inflammation being the most commonly associated risk factors.[6,7]

The pathogenesis of most gallbladder cancers involves a progression from metaplasia to dysplasia to carcinoma in situ to invasive carcinoma during a period of about 15 years.[8] Two types of metaplasia, pseudopyloric and intestinal, are associated with an increased risk of gallbladder cancer; however, the risk is greater with intestinal metaplasia.[4] Severe dysplasia and carcinoma in situ have been found in more than 90% of patients with gallbladder carcinoma.[9] Adenoma has also been previously reported to be a precancerous lesion in the gallbladder, but the evidence indicates that the adenoma-carcinoma sequence is less significant in this organ and involves molecular alterations different from those observed in the metaplasia-dysplasia-carcinoma sequence.[10]

Despite the efforts of many investigators, GBC is a major challenge in oncology. Surgical resection remains as the only curative treatment for this disease[11]; the key prognostic factors for survival are the level of tumor wall infiltration, lymph node status, and tumor differentiation.[12–14] Only a minority of patients with advanced GBC are suitable candidates for lymphadenectomy and liver wedge resection. Median survival associated with unresectable or recurrent GBC is less than 6 months and fewer

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than 5% survive at 5 years,[15,16] thereby making adjuvant chemotherapy and/or radiotherapy very rational and attractive therapeutic options.[17] In this sense, various chemotherapy agents, including 5-fluorouracil, cisplatin, and gemcitabine, have been evaluated for the management of the disease in its advanced stages, but the results have been disappointing.[18–20] Several cytotoxic agents have been studied as single agents or in combination, with response rates in the 0% to 30% range; however, no chemotherapy regimen has been shown to prolong survival in patients with advanced GBC.[21] Therefore, a better understanding of pathologic molecular mechanisms of gallbladder carcinogenesis is essential for improving the diagnosis, for prognosis, and for developing novel targeted therapies for patients with advanced GBC.

The mammalian target of rapamycin (mTOR) is a central signaling molecule downstream of the AKT signaling pathway.[22] mTOR controls protein synthesis, angiogenesis, and cell cycle progression and functions as a master switch between catabolic and anabolic processes.[23] Several lines of evidence suggest that mTOR is involved in the pathogenesis of several human cancers. Although mutations have not been reported in mTOR in human cancers, deregulation of upstream pathway effectors can lead to hyperactivation of the mTOR protein.[24–26] Recently, mTOR has been recognized as an important and attractive target for anticancer therapy. Several rapalogs with improved pharmacokinetic properties and reduced immunosuppressive effects, including temsirolimus (CCI779), everolimus (RAD001), and deforolimus (AP23573), have been developed.[27] In particular, everolimus (RAD001) has shown promising results in experimental studies, inhibiting tumor growth and having antiangiogenic effects.[28–30] Thus, mTOR pathway is a potential candidate whose analysis will enable new therapeutic targets and biomarkers to be identified and validated in this neoplasia. In this study, we investigated the association of phospho-mTOR expression in advanced gallbladder cancer tissue as well as clinicopathologic features and survival rates in patients with GBC.

MATERIALS AND METHODS

Patients and Tissue Samples

The present study was performed retrospectively on 227 gallbladder sample tissues from patients with neoplastic and nonneoplastic lesions who underwent surgery at the Hernán Henríquez Aravena Hospital (Temuco, Araucania Region, Chile) between 1987 and 2006. The samples analyzed consisted of 128 advanced GBCs and 99 cases of chronic cholecystitis (CC). None of the included patients with advanced GBC received any neoadjuvant or coadjuvant therapy, or radical surgery after the cholecystectomy.

The advanced GBCs were grouped according to pT stage (infiltration level) as subserous (pT2) and serous (pT3), using the TNM staging system for gallbladder cancer (American Joint Committee on Cancer, 7th edition).[31] Patients with diagnosis of CC were divided into 2 groups according to the presence or absence of metaplasia. Tissue samples were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. The clinicopathologic features for the patients with advanced GBC were obtained from medical records and are summarized in Table 1. Briefly, 97 cases were classified as pT2 and 31 cases as pT3, which were grouped as follows: stage II (70 cases), stage III A (19 cases), stage III B (20 cases), and stage IV B (19 cases). According to histologic differentiation, 34 were classified as well differentiated, 54 as moderately differentiated, and 32 as poorly differentiated. Complete postoperative follow-up was available for all 128 patients; of these, 114 (89.1%) had data that were used for the Kaplan-Meier survival analysis (14 patients who died less than 30 days postoperatively were excluded). This study was approved by the ethics committee of the Faculty of Medicine at the Universidad de La Frontera (Temuco, Araucania Region, Chile).

Table 1. Association of Phospho-mTOR Status (Low/High) With Clinicopathologic Characteristics of Advanced Gallbladder Carcinoma

Clinical and Pathologic Features	Patients, No. (%) (n = 128)	Phospho-mTOR Status		
		Low, No. (%) (n)	High, No. (%)	*P* Value[a]
Age, y				.85
<65	64 (50.0)	42 (65.6)	22 (34.4)	
≥65	64 (50.0)	41 (64.1)	23 (35.9)	
Sex				>.99[b]
Female	116 (90.6)	75 (64.7)	41 (35.3)	
Male	12 (9.4)	8 (66.7)	4 (33.3)	
Ethnicity				.49
Mapuche	21 (16.4)	15 (71.4)	6 (28.6)	
Hispanic	107 (83.6)	68 (63.6)	39 (36.4)	
Infiltration level				.21
Serosa	31 (24.2)	23 (74.2)	8 (25.8)	
Subserosa	97 (75.8)	60 (61.9)	37 (38.1)	
Histologic differentiation				.27
Well	34 (26.6)	23 (67.6)	11 (32.4)	
Moderate	54 (42.2)	36 (66.7)	18 (33.3)	
Poor	32 (25.0)	17 (53.1)	15 (46.9)	
Missing	8 (6.3)	7 (87.5)	1 (12.5)	
TNM stage				.71
II	70 (54.7)	43 (61.4)	27 (38.6)	
IIIa	19 (14.8)	13 (68.4)	6 (31.6)	
IIIb	20 (15.6)	15 (75.0)	5 (25.0)	
IVb	19 (14.8)	12 (63.2)	7 (36.8)	

Abbreviation: mTOR, mammalian target of rapamycin.

[a] Chi square exact probability test.
[b] Fisher exact test.

Tissue Microarray

Before tissue microarray (TMA) construction, all tissue slides were histopathologically reevaluated by a pathologist. Two 2.0-mm tissue cores were taken from representative areas of GBC samples with a TMA Arrayer device (Pathology Devices TMArrayer, Westminster, Maryland) and mounted in a new recipient block. Four 4.0-μm-thick sections were consecutively cut from the recipient block and transferred to poly-L-lysine–coated glass slides. Hematoxylin-eosin staining was performed on TMA to confirm the presence of a tumor.

Immunohistochemistry for Phospho-mTOR

For the immunohistochemical procedure, 4-μm-thick sections were deparaffinized with xylene and rehydrated through an alcohol gradient. For antigen epitope retrieval, the specimens were heated (95°C) for 10 minutes in 10 mmol/L of citrate buffer (pH 6.0) in a pressure cooker. To reduce nonspecific background staining, the slides were incubated in 3% hydrogen peroxide (H_2O_2) for 10 minutes. After incubation with Ultra V Block (UltraVision LP detection system, Lab Vision Corporation, Fremont, California) for 7 minutes at room temperature, the tissues were incubated overnight at 4°C with a rabbit monoclonal antibody specific for phospho-mTOR (Phospho-mTOR, Ser2448, clone 49F9; Cell Signaling Technology, Beverly, Massachusetts) at a dilution of 1:25. This antibody detects mTOR only when it is phosphorylated at Ser2448. Labeling was detected with the DAB-Chromogen system (Dako North America Inc, Carpinteria, California) according to the manufacturer's protocol. After being counterstained with Harris hematoxylin, the section was dehydrated, cleared, and mounted. According to the manufacturer's recommendation, we used human colon carcinoma tissues as positive controls. Omission of the primary antibody and replacement with phosphate buffered saline served as negative controls.

Interpretation of Immunohistochemical Staining

The expression of phospho-mTOR was examined by 2 independent and specialized gastrointestinal pathologists (J.C.R., O.T.) without any information about clinicopathologic features or prognosis. Immunostaining for phosho-mTOR was evaluated by using a previously described scoring system.[32] The staining intensity and percentage of positive cells were scored semiquantitatively as negative (–), 0%–9%; weakly positive (+), 10%–25%; moderately positive (++), 26%–50%; and strongly positive (+++), 51%–100%. To compare the phospho-mTOR expression between chronic cholecystitis (with and without metaplasia) and advanced GBC, we considered that phospho-mTOR is overexpressed in tumors only when this protein is detected at high levels in neoplastic cells. Thus, the 227 samples were classified according to positive status into negative (–) and positive (+, ++, +++) expression. Nonetheless, to evaluate the association of phospho-mTOR expression level with clinical variables and patient survival, we arbitrarily grouped cases by their immunohistochemical intensity for phospho-mTOR, namely, as low (negative and weak staining intensity) or high (moderate and strong staining intensity).

Statistical Analysis

All statistical analyses were performed by using the statistical package SPSS version 17.0 (SPSS Inc, Chicago, Illinois). The associations between phospho-mTOR expression levels and clinicopathologic variables were examined by using the χ^2 test or Fisher exact probability test. For advanced GBC, Kaplan-Meier survival curves for patients with a high versus low expression of phospho-mTOR were plotted. Stratified log-rank tests were used to assess the difference between survival curves. The stratification factor was the infiltration level, since this covariate has been recognized as a strong predictor of survival in patients with advanced GBC. $P < .05$ was considered statistically significant.

RESULTS

We examined the phospho-mTOR expression by using standard immunohistochemistry protocols. Examples of staining intensity are illustrated in Figure 1, showing positive staining to phospho-mTOR in the cytoplasm of gallbladder cancer cells (Figure 1, D through F), whereas the chronic cholecystitis tissue shows a variable intensity (Figure 1, A through C). In most cases, phospho-mTOR showed a diffuse pattern of staining. The positive immunostaining of phospho-mTOR was detected in 64.1% (82 of 128) of all tumors. As summarized in Table 2, phospho-mTOR was highly expressed in advanced gallbladder cancer compared with chronic cholecystitis without or with metaplasia ($P < .001$), for which 16% and 32.4% of positivity, respectively, was observed.

To evaluate the association of phospho-mTOR expression levels with clinical variables and patient survival, the 128 advanced GBCs were classified as low or high according to intensity.

Table 1 shows the clinicopathologic features of patients with GBC according to phospho-mTOR expression in a univariate analysis. The phospho-mTOR expression was not associated with any clinical or pathologic features, including age, sex, ethnicity, infiltration level, histologic differentiation, or TNM stage ($P \geq .05$).

Complete postoperative follow-up was available for all 128 patients with advanced GBC but only 114 (89.1%) were considered for analysis (14 patients died less than 30 days after surgery). The entire group (n = 114) had an estimated survival rate of 21.9% with a median survival of 15 months. Patients with a low phospho-mTOR expression (n = 73) had a survival rate of 24.7% with a median survival of 19.5 months, whereas patients with high phospho-mTOR expression (n = 41) had a survival rate of 17.1% with a median survival of 11.4 months. As shown in Figure 2, univariate analysis using the Kaplan-Meier method showed significant differences in survival rate for different phospho-mTOR expression levels. Patients whose tumors overexpressed phospho-mTOR had a poorer prognosis ($P = .02$).

COMMENT

The mammalian target of rapamycin is a serine/threonine protein kinase that supports cell growth, cell metabolism, cell proliferation, cell motility, cell survival, protein synthesis, and transcription such as angiogenesis and autophagy.[33] Current research indicates that mTOR integrates the input from multiple upstream pathways, including those of insulin, growth factors, and mitogens, and also functions as a sensor of cell nutrient and energy levels and redox status.[34] mTOR is activated by phosphorylation of Ser2448 through the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, activating the eukaryotic translation factor 4E (eIF4E) and the p70 ribosomal S6 kinase (p70S6 kinase) and participating in the inactivation of the eIF4E inhibitor 4E-BP1.[35] PI3K is a component of the AKT/mTOR signaling pathway that regulates various cellular processes related to tumorigenesis, such as cell proliferation, cell survival, adhesion, motility, and angiogenesis.[36] Gene amplifications, deletions, and somatic missense mutations of the *PIK3CA* gene have been reported in several cancers including colon,[37] breast,[38] ovary,[39] brain,[40] stomach,[41] lung,[42] and biliary tract cancer.[43] Therefore, mTOR plays a key role in cell growth and homeostasis, and its regulation is frequently altered in human tumors.[44] Recent studies[45]

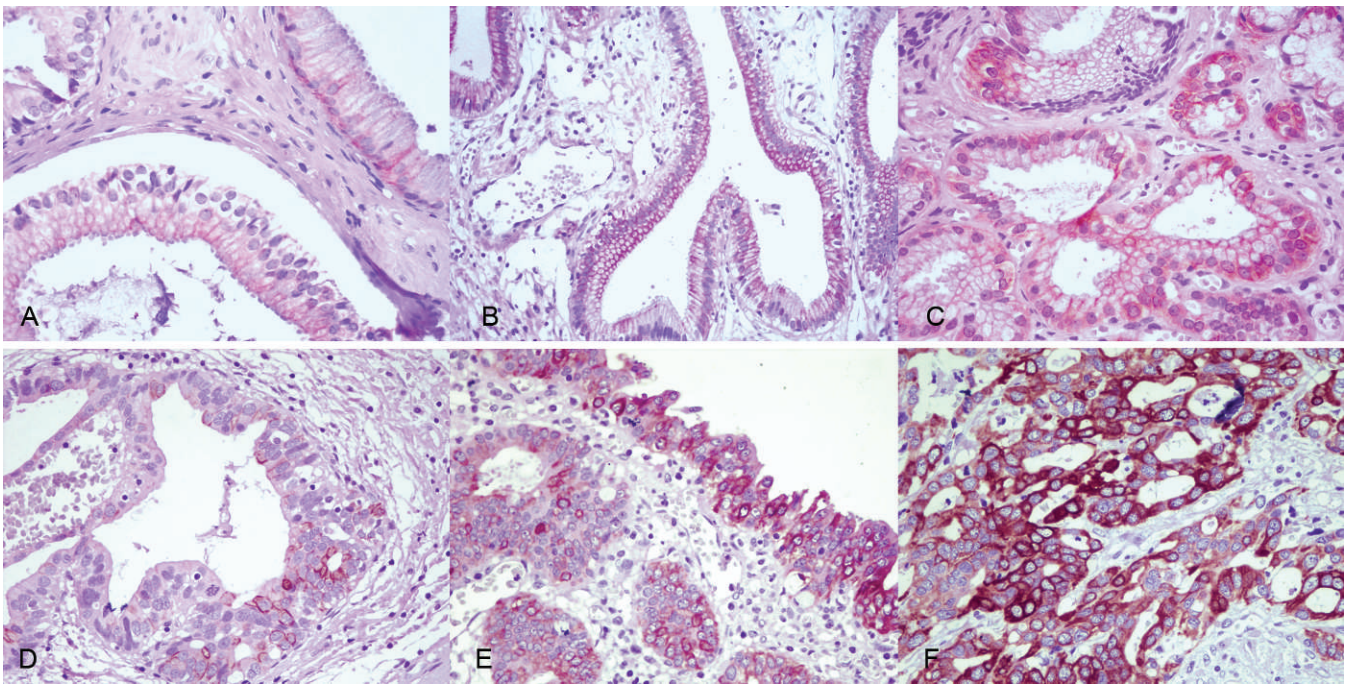

Figure 1. *Immunohistochemical phospho-mTOR expression in gallbladder tissues. Chronic cholecystitis without metaplasia, weak intensity (A) and moderate intensity (B). Chronic cholecystitis with pseudopyloric metaplasia, moderate intensity (C). Advanced gallbladder carcinoma with weak intensity (D), moderate intensity (E), and strong intensity (F) (hematoxylin-eosin, original magnifications ×100 [A through F]).*

suggest that the mTOR pathway is crucial for regulating the translation of specific proteins associated with cancer progression. Moreover, studies on several types of cancers have shown that the aberrant activation of mTOR is associated with poor prognosis.[46,47] However, mTOR activation status in gallbladder cancer has not been thoroughly investigated.

In the present study, we examined the phospho-mTOR expression by immunohistochemistry in 128 advanced gallbladder carcinomas and 99 cases of chronic cholecystitis. Cases of CC were divided into 2 groups according to the presence or absence of metaplasia. Our results showed a strong positive staining for phospho-mTOR in 64.1% of gallbladder tumors, indicating that this might be a potential biomarker of prognosis in advanced gallbladder cancer. The positive staining for phospho-mTOR was significantly higher in advanced cancers than in metaplastic and nonmetaplastic groups of CC whether compared separately or compared together. In addition, we found a nonsignificant trend toward an increased phospho-mTOR expression from CC without metaplasia (positivity rate, 16%) compared to CC with metaplasia (positivity rate, 32.4%). Metaplasia is a common finding in chronic cholecystitis and is considered a precursor lesion of dysplasia. Different studies have demonstrated the presence of some form of metaplasia in gallbladders removed for chronic cholecystitis or cholelithiasis. Pseudopyloric or gastric metaplasia is the most frequent, being identified in greater than 50% of cases, and its presence is associated with intestinal metaplasia.[48] In turn, intestinal metaplasia is frequently associated with dysplasia.[49,50] These studies support the notion that the sequence for gallbladder carcinogenesis is a progression from pseudopyloric metaplasia to dysplasia via intestinal metaplasia, with the subsequent development of invasive carcinoma.[48] Our results suggest that the activation of mTOR occurs very early during the development of GBC, and it might be involved in the onset of reactive epithelial changes in the gallbladder mucosa. Studies on other cancers have also shown that mTOR activation can occur frequently in normal tissue and preinvasive lesions, resulting in an abnormal initiation of synthesis protein and increased cell proliferation and survival. Feng et al[51] (2008) reported a positive mTOR expression in 94.1% of nonneoplastic gastric epithelia; the most intense expression appeared to be

Table 2. Phospho-mTOR Expression in Chronic Cholecystitis and Advanced Gallbladder Carcinoma According to Positive Status

Groups	N	Negative, No. (%) –	Weak, No. (%) +	Moderate, No, (%) ++	Strong, No. (%) +++	PR, %	*P* Value[a]	
Nonmetaplastic cholecystitis (a)	25	21 (84.0)	1 (4.0)	2 (8.0)	1 (4.0)	16.0	(ac)	<.001
Metaplastic cholecystitis (b)	74	50 (67.6)	6 (8.1)	13 (17.6)	5 (6.8)	32.4	(ab)	.13
Advanced carcinoma (c)	128	46 (35.9)	37 (28.9)	28 (21.9)	17 (13.3)	64.1	(bc)	<.001

Abbreviations: mTOR, mammalian target of rapamycin; PR, positivity rate.

[a] χ^2 exact probability test.

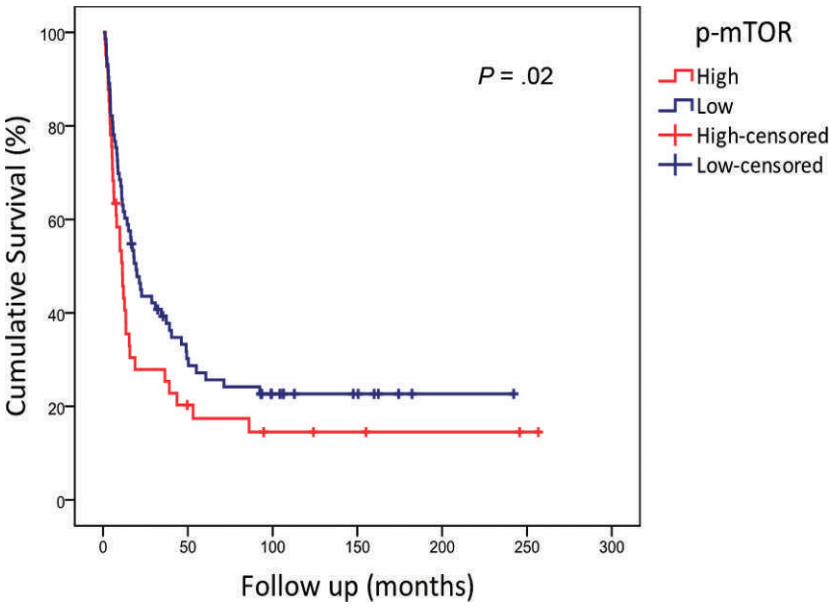

Figure 2. *Kaplan-Meier curves for cumulative survival rate of patients with advanced gallbladder carcinoma according to phospho-mTOR expression in advanced gallbladder cancer. The red line indicates patients whose tumors express high levels of phospho-mTOR and the blue line indicates patients with low phospho-mTOR expression (P = .02; stratified log-rank test). Abbreviation: p-mTOR, phospho-mTOR.*

present predominately within the deep foveolar pits, the isthmus, and the areas of intestinal metaplasia. In lung adenocarcinoma, the expression of phospho-mTOR has been shown to be high in invasive adenocarcinoma (84.1%), but also in adenocarcinoma in situ (90.2%) and atypical adenomatous hyperplasia of the lung (81.8%). Normal parenchyma , including normal appearing pneumocytes and bronchiolar metaplasia surrounding a tumor showed positivity for phospho-mTOR expression in only 25.9% of cases, mainly in pneumocytes and bronchiolar metaplasia.[52]

A univariate analysis using the Kaplan-Meier method showed that aberrant expression of phospho-mTOR was significantly associated with poorer prognosis in patients with advanced GBC. Studies on other human cancers, such as gastric cancer, extrahepatic cholangiocarcinoma, esophageal squamous cell carcinoma, and ovarian cancer, have shown that phospho-mTOR expression is closely related to poor prognosis.[53]

Remembering that the main prognostic factors for GBC are grade, depth of wall infiltration, and lymph node metastasis, our findings suggest that phospho-mTOR immunohistochemical overexpression might be an independent prognostic factor in patients with advanced GBC. The survival rate for patients with advanced GBC is poor, around 10% at 5 years in cases with serosal involvement with or without lymph node metastasis.[21,54] An important requirement for improving clinical outcomes is the identification of predictive biomarkers that can define the tumor subtypes and patient populations that are most likely to respond to the use of mTOR inhibitors or to combined therapies, chemotherapy or radiotherapy.[27] mTOR is inhibited by rapamycin, an immunosuppressive agent that arrests cells in the G1 phase of the cell cycle and induces apoptosis.[55] Rapamycin has very poor water solubility and chemical stability, which severely limit its bioavailability.[56] Thus, several rapalogs with improved pharmacokinetic properties and reduced immunosuppressive effects, including temsirolimus (CCI779), everolimus (RAD001), and deforolimus (AP23573), have been developed.[57,58]

Our data suggest that the AKT/mTOR pathway is a potential candidate whose analysis represents a great opportunity for identifying and validating new therapeutic targets in gallbladder cancer. Future studies are needed to confirm our findings as well as to elucidate the exact mechanism of the mTOR activation and its potential pathogenic role in GBC.

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