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The ERK/MAPK pathway is overexpressed and activated in gallbladder cancer

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ABSTRACT

Gallbladder cancer (GBC) is a highly fatal disease with poor prognosis and few therapeutic alternatives. Molecular profiling has revealed that the deregulation in the ERK/MAPK signaling pathway plays a crucial role in many disease and malignancies, including GBC. The aim of this study was to measure the expression of ERK1/2 and p-ERK1/2 in a population with high GBC-related mortality, such as the Chilean population, and characterize the protein expression of this ERK/MAPK pathway in seven GBC cell lines. Immunohistochemistry (IHC) for ERK1/2 and p-ERK1/2 was performed in 123 GBC tissues and 37 chronic cholecystitis (CC) tissues. In addition, protein expression analysis by western blot for ERK1/2, p-ERK1/2, EGFR, ERBB2 and ERBB3 were performed in seven GBC cell lines (GB-d1, G415, NOZ, OCUG-1, TGBC-1, TGBC-2 and TGBC-24). A higher ERK1/2 and p-ERK1/2 expression was found in GBC tissues compared to chronic cholecystitis (CC) tissues ($P < 0.001$). However, neither significant differences in overall survival nor significant associations with any of the clinicopathological features were found by comparing low and high expression of both ERK1/2 and p-ERK1/2. Western blot analysis of seven GBC cell lines showed that, in general, GB-d1, G415 and NOZ cells evidenced a strong expression of ERK1/2, p-ERK1/2, EGFR, ERBB2 and ERBB3. Therefore, ERK1/2 and p-ERK1/2 seem to be important in the development of GBC and GB-d1, G415 and NOZ cell lines may be used as experimental models for further *in vitro* and *in vivo* studies that help to decipher the role of MAPK/ERK pathway in gallbladder carcinogenesis.

1. Introduction

Gallbladder cancer (GBC) is the fifth most common malignant neoplasm of the digestive tract and the most common malignancy of the biliary tree [1], representing 80%–95% of biliary tree cancers worldwide [2]. Early diagnosis of GBC is rare because symptoms are unspecific and difficult to differentiate from other more common pathologies, such as cholelithiasis or chronic cholecystitis (CC) [3]. Therefore, this malignancy shows an invariably course to death in the absence of medical treatment [4,5]. The overall mean survival rate for patients with GBC is only about 6 months, with a 5-year survival rate of 5% [6]. The incidence and mortality rates are extremely variable among geographic regions and ethnic groups. The highest mortality rates have been reported among the Chilean Mapuche people, Hispanics, Bolivians and the indigenous peoples of North America [3,7].

There are many reports focused on the genetic and epigenetic alterations in GBC, which involve modifications in the expression of tumor suppressor genes and oncogenes [8,9]. Advances in molecular profiling and genomics have revealed that the extracellular

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signal-regulated kinase (ERK) signaling pathway – also known as mitogen-activated protein kinase (MAPK) pathway – is a key cellular signal transduction axis that plays a crucial role in mediating multiple cellular functions by phosphorylating and inducing a large variety of downstream targets [10]. In this manner, the ERK/MAPK pathway can regulate cell differentiation, proliferation, growth, cell cycle progression, apoptosis, survival, gene expression, migration, invasiveness, metastasis, metabolism and angiogenesis in several types of cancer, including GBC [11–15].

Immunohistochemical expression of phospho-ERK1/2 (p-ERK1/2) in GBC is very fluctuating among different studies ranging between 33 and 87% [16–18]. In fact, only one study has shown relations between the p-ERK1/2 expression and some clinicopathological variables. However, in general, these studies agree that p-ERK1/2 is overexpressed in GBC compared to preneoplastic lesions or other biliary malignancies. In addition, in some cases a high p-ERK1/2 expression has been correlated to lower survival, representing an independent predictive factor in GBC [18].

The present study aimed to evaluate the involvement of the most important regulators of ERK/MAPK pathway (total ERK1/2 and p-ERK1/2) in gallbladder carcinogenesis in a high-mortality population for this malignancy, such as the Chilean population, which has not yet been studied. Interestingly, the patient cohort chosen for this study belongs geographically to the Region of La Araucania, where there is a high concentration of Mapuche people, one of the populations most affected by GBC in Chile and the world [7,19,20]. Additionally, the expressions of ERK1/2, p-ERK1/2, EGFR, ERBB2 and ERBB3 were evaluated in seven GBC cell lines (GB-d1, G415, NOZ, OCUG-1, TGBC-1, TGBC-2 and TGBC-24) in order to characterize experimental models for further studies focused on the role of the ERK/MAPK pathway in GBC development.

2. Material and methods

2.1. Clinical samples

A retrospective cohort of 123 formalin-fixed paraffin-embedded (FFPE) tissue samples of GBC and 37 FFPE samples of chronic cholecystitis (CC) were included in this study for immunohistochemical (IHC) analysis. These samples belonged to patients who were assisted in the Hernan Henriquez Aravena Hospital in Temuco, Chile between 1994 and 2004 and were obtained after surgery and histopathological diagnosis. These samples were in stage II (70 cases) and stages III–IV (53 cases) according TNM classification and were obtained as the patients were receiving surgery at the Hospital.

2.2. Tissue microarray construction and immunohistochemistry

Tissue microarrays (TMAs) were constructed with 2 mm cores of 2 different representative areas of each tumor and normal control mucosa. The IHC procedure was carried out according to Garcia et al. [21]. Briefly, 4-μm thick sections were cut from each TMA and de-waxed in xylene, rehydrated through graded concentrations of ethanol, and placed in an antigen retrieval solution (citrate buffer, pH 6.0) for 15 min at 95 °C. After cooling for 30 min, the tissue sections were incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. The slides were then washed thoroughly with phosphate-buffered saline and incubated (120 min, room temperature) with a dilution of 1:25 of each antibody: p44/42 MAPK (ERK1/2) and phospho-p44/42 MAPK (p-ERK1/2) (Threonine 202/Tyrosine 204) (Cell Signaling, USA). Labeling was detected with the Liquid DAB Substrate-Chromogen System (Dako, USA) according to the manufacturer's protocol. Sections were counterstained with hematoxylin, then dehydrated, cleared and mounted. A negative control was prepared by replacing the primary antibody with phosphate-buffered saline.

2.3. Evaluation of immunohistochemical staining

The expressions of ERK1/2 and phospho-ERK1 (p-ERK1/2) were independently evaluated in GBC and CC tissues by an expert pathologist (JCR) who was blinded to both the clinical and pathological data. IHC staining was evaluated using a previously described semi-quantitative scoring system [22] (Table 2). Staining intensity was scored as 1 (negative), 2 (weak), 3 (moderate), and 4 (intense). The percentage of positive cells was quantified as 0 (none), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). For statistical analysis, the total score of intensity and extent of staining was grouped into low expression (final score, 0–3) or high expression (final score, 4–7).

2.4. Cell line culture

Protein expression was also evaluated in seven GBC cell lines (GB-d1, G415, NOZ, OCUG-1, TGBC-1, TGBC-2 and TGBC-24). GB-d1 and G415 cells were grown in RPMI 1640 medium (Thermo Fisher, USA), the NOZ cell line was cultured in Williams' E medium (Invitrogen, USA) and OCUG-1, TGBC-1, TGBC-2 and TGBC-24 cells were grown in DMEM high glucose medium. Media were supplemented with 10% fetal bovine serum (FBS), 10 units/ml penicillin and 10 mg/ml streptomycin (1% P/S) (Thermo Fisher, USA). The exceptions were TGBC-1 and TGBC-2 cells, where the media were supplemented with 5% FBS and 1% P/S. All seven cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO2 and were subcultured during the logarithmic phase.

2.5. Western blot analyses

The seven GBC cell lines were lysed using a RIPA buffer (50 mM Tris, pH 7.2; 150 mM NaCl; 1% Triton X-100; and 0.1% SDS) containing protease (1:100, Roche, USA) and phosphatase (1:100, Sigma-Aldrich, USA) inhibitors. Protein concentrations were determined by a bicinchoninic acid assay (ThermoFischer, USA). Sixty micrograms of proteins were separated by SDS-PAGE on a 4–12% NuPAGE® Bis-Tris Precast Gel (Invitrogen, USA) and transferred to PVDF membranes (Millipore, USA). Protein expressions were quantified through the use of rabbit monoclonal antibodies against EGFR, ERBB2, ERK1/2 (1:1000, Cell Signaling Technology, USA) and p-ERK1/2 (1:2000, Cell Signaling Technology, USA). Also a rabbit polyclonal antibody against ERBB3 (1:500, Abcam, USA) was used. All antibodies were diluted in TBST-1% BSA solution (Cell Signaling Technology, USA). The expressions of these proteins were standardized to human β-actin using a rabbit monoclonal anti-β-actin antibody (1:5000, Cell Signaling Technology, USA). Primary antibodies were detected using goat anti-rabbit or goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000, Santa Cruz Biotechnology, USA). Immunoreactive bands were visualized through chemiluminescence in a MyECL image platform (ThermoFischer, USA).

2.6. Statistical analyses

All statistical analyses were performed using SPSS v.17.0 (SPSS, Inc.). The associations between IHC expression and clinicopathological variables were examined using the X^2 test as well as Fisher's exact test for categorical variables and the Student's *t*-test for continuous variables. Kaplan-Meier survival curves were plotted for cases with high versus low expression of ERK1/2 and p-ERK1/2. The difference between the survival curves was analyzed using

the log-rank test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. ERK1/2 expression in gallbladder tissues

For the 123 GBC cases the mean age was 65 years (range: 27–93 years) and for the 37 CC cases the mean age was 58 (range: 20–87 years). In case of GBC patients, 111 were women and 12 were men. In the case of CC patients, 32 were women and 5 were men (Table 1). Then, more clinicopathological features such as histological grade, infiltration level (pT), lymph node involvement (pN) and so forth, were analyzed. However, no significant associations were found between expression levels of ERK1/2 and p-ERK1/2 compared to any of these clinicopathological characteristics (Table 1). A significantly higher expression of ERK1/2 was found in GBC cases (73%) compared to chronic cholecystitis (CC) cases (13%), which served as controls ($p < 0.001$; Fig. 1A). Also, a significantly higher expression of p-ERK1/2 was also observed in GBC cases (38%) compared to CC cases (3%) ($p < 0.001$; Fig. 1B). Representative images of negative, weak, moderate and intense staining of ERK1/2 and p-ERK1/2 in GBC are shown in Fig. 1C.

Survival analysis showed no significant differences between the low expression and high expression groups for either protein ERK1/2 ($p = 0.852$) or p-ERK1/2 ($p = 0.749$) (Fig. 2).

3.2. Protein expression of ERK/MAPK pathway in gallbladder cancer cell lines

Protein expression analysis by western blot in the seven GBC cell lines is shown in Fig. 3. Immunoblotting showed that at least GB-d1, G415 and NOZ cells (three out of seven cell lines) evidenced expression of all the targets studied: ERK1/2, p-ERK1/2, EGFR, ERBB2 and ERBB3. Interestingly, EGFR and ERBB3 expression was markedly higher in GB-d1, G415, NOZ and OCUG-1 cells than in TGBC-1, TGBC-2 and TGBC-24 cells. Similar patterns were observed for ERBB2 and p-ERK1/2, where the only exceptions in the expression patterns were in TGBC-24 cells (for ERBB2) and in OCUG-1 and TGBC-1 cells (for p-ERK1/2).

4. Discussion

The ERK/MAPK family comprises a total of 14 different serine/threonine protein kinases including the most important pathway effectors such us the extracellular signal-regulated kinase 1 (ERK1), ERK2 and ERK3 [13,14]. The pathological activation of this pathway starts with gain-of-function mutations in upstream activator genes mainly in RAS and RAF, and less frequently in MEK1/2, which induce a constitutive activation [10,23]. Once the RAS protein is activated, it recruits RAF kinase family members to the plasma membrane and promotes their activation through their homodimerization or heterodimerization [24]. Then, activated

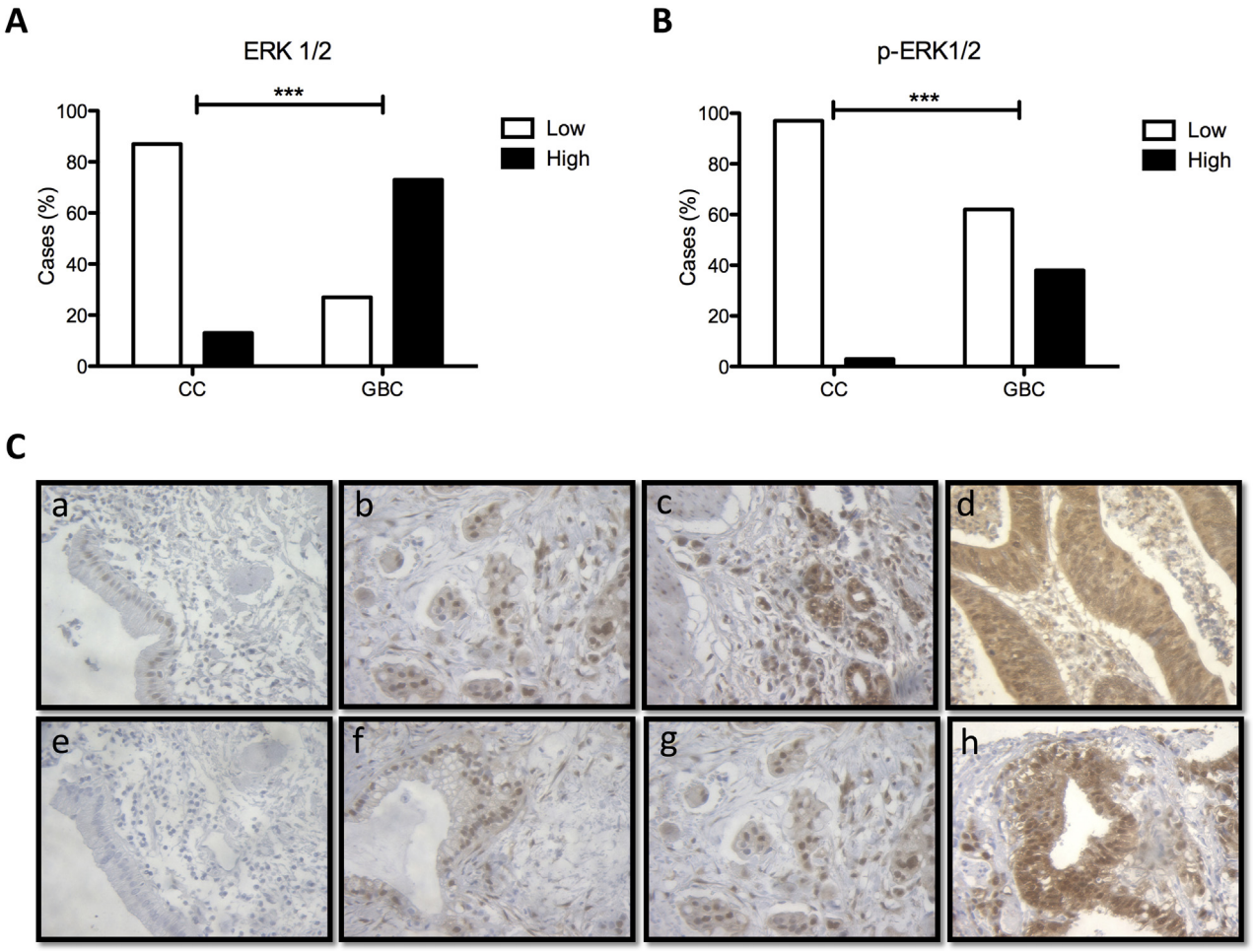

Fig. 1. Frequency distribution of ERK1/2 and p-ERK1/2 expression in biopsies of gallbladder cancer (GBC) and chronic cholecystitis (CC). (A) Higher ERK1/2 expression was observed in GBC tissues (73%) compared to CC (13%) (***$P < 0.001$). (B) Higher p-ERK1/2 expression was observed in GBC tissues (38%) compared to CC (only 3%) (***$P < 0.001$). (C) Representative immunostaining of ERK1 (a: Negative; b: Weak; c: Moderate; d: Intense); and p-ERK1 (e: Negative; f: Weak; g: Moderate; h: Intense) (40X).

Table 1
Clinicopathological features of studied patients.

	N°	ERK		P*	p-ERK		P*
		Low expression	High Expression		Low expression	High expression	
Total	**123**	33 (26.8%)	90 (73.2%)		78 (63.4%)	45 (36.6%)	
Age (year; mean 60)							
<65	59	15 (25.4%)	44 (74.6%)	**0.839**	35 (59.3%)	24 (40.7%)	**0.454**
≥65	64	18 (28.1%)	46 (71.9%)		43 (67.2%)	21 (32.8%)	
Gender							
Female	111	32 (28.8%)	79 (71.2%)	**0.178**	69 (62.2%)	42 (37.8%)	**0.533**
Male	12	1 (8.3%)	11 (91.7%)		9 (75.0%)	3 (25.0%)	
Tumor invasion†							
pT2	91	22 (24.2%)	69 (75.8%)	**0.246**	54 (59.3%)	37 (40.7%)	**0.196**
pT3	31	11 (35.5%)	20 (64.5%)		23 (74.2%)	8 (25.8%)	
Lymph node metastasis							
NX	74	19 (25.7%)	55 (74.3%)	**0.902**	49 (66.2%)	25 (33.8%)	**0.104**
N0	19	5 (26.3%)	14 (73.7%)		8 (42.1%)	11 (57.9%)	
N1	30	9 (30.0%)	21 (70.0%)		21 (70.0%)	9 (30.0%)	
Metastasis							
MX	40	12 (30.0%)	28 (70.0%)	**0.754**	26 (65.0%)	14 (35.0%)	**0.942**
M0	68	18 (26.5%)	50 (73.5%)		43 (63.2%)	25 (36.8%)	
M1	15	3 (20.0%)	12 (80.0%)		9 (60.0%)	6 (40.0%)	
Stage by TNM							
Stage II	70	17 (24.3%)	53 (75.7%)	**0.539**	40 (57.1%)	30 (42.9%)	**0.130**
Stage III + IV	53	16 (30.2%)	37 (69.8%)		38 (71.7%)	15 (28.3%)	
Histologic grade							
Well differentiated	34	10 (29.4%)	24 (70.6%)	**0.551**	18 (52.9%)	16 (47.1%)	**0.150**
Moderately	52	14 (26.9%)	38 (73.1%)		38 (73.1%)	14 (26.9%)	
Poorly	28	5 (17.9%)	23 (82.1%)		17 (60.7%)	11 (39.3%)	
Infiltration							
Serosa	32	11 (3.,4%)	21 (65.6%)	**0.353**	23 (71.9%)	9 (28.1%)	**0.291**
Subserosa	91	22 (24.2%)	69 (75.8%)		55 (60.4%)	36 (39.6%)	

* Fisher's Exact test.
† One case with missing information was excluded from that analysis.

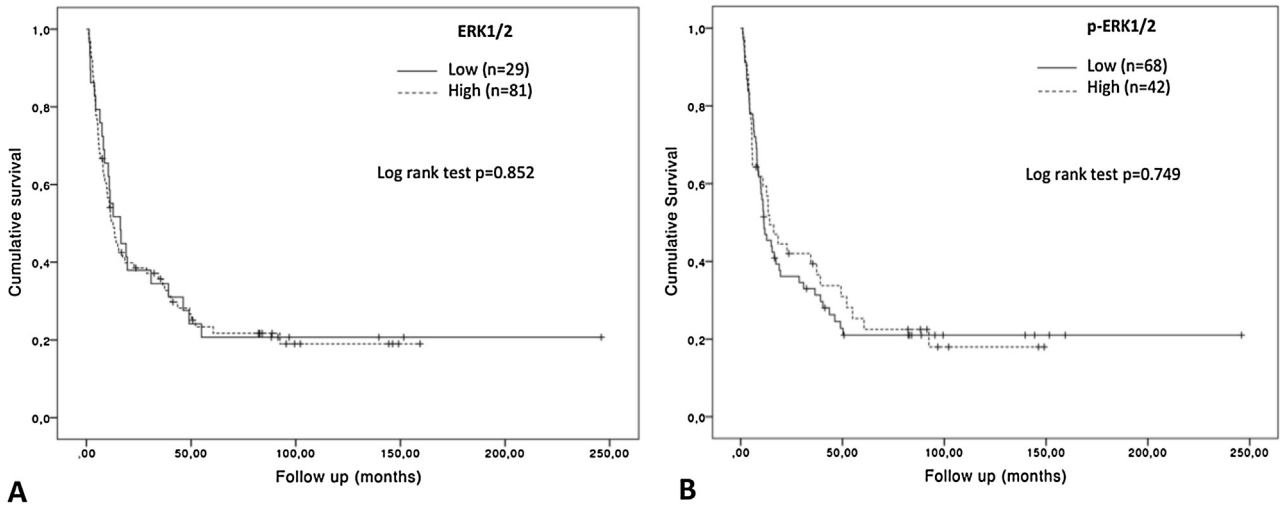

Fig. 2. Overall survival analyses of GBC patients according to ERK1/2 and p-ERK1/2 expression. (A) ERK1/2 and (B) p-ERK1/2. (p = 0.852 and p = 0.749, respectively) (Fig. 2). N = 110.

RAF(s) trigger the dual specificity MEK1/2 kinases (henceforth singular), which finally activates ERK1/2 through dual phosphorylation of the conserved T202-E203-Y204 (TEY) motif within its activation loop [25–29]. The consecutive activation cascade of RAS-RAF-MEK-ERK1/2 has already been described in several cancers, as this pathway has been found to be deregulated in about 30% of human neoplasias [10,25,30,31]. In cancer, this pathway can regulate cell differentiation, proliferation, growth, cell cycle progression, apoptosis, survival, gene expression, migration, invasiveness, metastasis, metabolism and angiogenesis in several types of malignancies [11–15].

The present work is the first study to report a high expression of total ERK1/2 protein and, additionally, an overactivated status of this protein (p-ERK1/2) in a cohort from a high-mortality population due to GBC, which the Chilean population is. Interestingly, the patients chosen for this study belongs geographically to La Araucania region, where there is a high concentration of Mapuche natives,

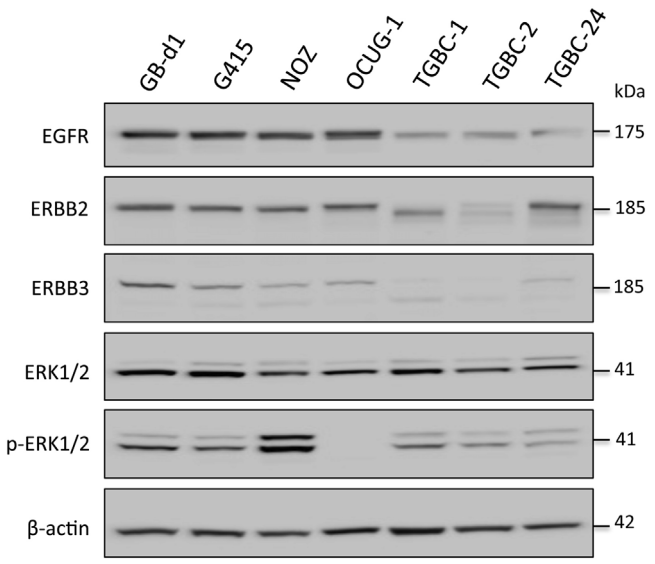

Fig. 3. Protein expression of the ERK/MAPK pathway in seven GBC cell lines. Total protein (60 μg) of each cell line was processed by immunoblotting using antibodies against EGFR, ERBB2, ERBB3, ERK1/2 and p-ERK1/2. Protein loading was normalized with β-actin and experiments were performed by triplicate.

Table 2
Parameters used to evaluate expression level according to IHC staining.

Staining Intensity		% Cells		Final Score	Expression
0	Negative	0	0	0–3	Low
1	Weak	1	1–25%		–
2	Moderate	2	26–50%	4–7	High
3	Intense	3	51–75%		
		4	76–100%		

one of the populations most affected by GBC in Chile and the world [7,19,20].

Some previous reports have described the activation of the ERK/MAPK pathway in GBC within other populations or systems. For instance, Hori et al. [16] examined the expression patterns of p-ERK1/2 and p-AKT proteins in two bile duct cancer cell lines (Sk-ChA-1 and Mz-1), two GBC cell lines (TGBC2 and NOZ) and 20 frozen advanced extrahepatic biliary tract cancer specimens by western blot. Additionally, they analyzed 30 formalin-fixed specimens (15 GBC, 13 bile duct cancers and two ampullary cancers) by IHC. Considering only the GBC tissues they found that the p-ERK1/2 expression was observed in 87% of GBC cases (13 of 15) ($p = 0.03$). Mohri et al. [17] evaluated the expression of p-ERK1/2 and p-P70S6K1 by immunostaining in 30 GBC tissues. Expression of p-ERK1/2 expression was observed in 33% (10/30) of cases and, notably, all the cases with positive p-ERK1/2 expression also showed positive p-P70S6K1 expression with a significant positive correlation between both targets (p-value not shown). Li et al. [18] evaluated immunohistochemically the expression frequency of p-ERK1/2 and PI3K in GBC, peritumoral tissues, adenomatous polyps and chronic cholecystitis (CC) tissue samples. The positive staining for p-ERK1/2 was 63/108 (58.3%) in GBC, 14/46 (30.4%) in peritumoral tissues, 3/15 (20%) in adenomatous polyps and 4/35 (11.4%) in CC. Therefore, the positive rate of p-ERK1/2 in GBC was significantly higher compared to peritumoral tissues of adenomatous polyps and CC ($p < 0.01$ for all). The positive staining of p-ERK1/2 was significantly higher in poorly differentiated GBC, with lymph-node metastases and infiltration to tissues or organs ($p < 0.01$). Finally, the increased p-ERK1/2 expression was associated with poorer overall survival and is useful as an independent prognostic predictor of GBC in this study ($p = 0.045$) [18].

Our work is consistent with previous reports stating that the ERK1/2 protein is overactivated in this malignancy; however, the previous studies have only described a post-translational activation (phosphorylation) in specific residues of ERK1/2. Interestingly, our data have shown that total ERK1/2 is also overexpressed in GBC, suggesting an additional genetic or epigenetic mechanism regulating ERK1/2 expression. On the other hand, unlike Li's group, our data showed no significant associations between the expression of total and phosphorylated ERK1/2 and such clinicopathological features as age, gender, histological grade, infiltration level (pT) or lymph node involvement (pN). In our study, survival analysis showed no significant differences between the low expression and high expression groups for both ERK1/2 and p-ERK1/2 denoting that these two targets are prognosis-independent markers for GBC in our studying population. The most likely reason for the difference in survival outcomes between the Li et al.ís study and ours could be due to Liís group used incipient and advanced GBC cases [18], unlike us who only used advanced cases of GBC. This information is important to note because Roa et al. [32] have previously demonstrated that advanced GBC cases (from stage II of TNM onwards) have a poor survival due to the invasion of lesion upon reaching perimuscular connective tissue.

Due to difficulties in determining whether high or low of ERK1/2 and p-ERK1/2 expressions have a real impact on the development, survival or treatment of GBC by using only advanced GBC samples, it is necessary to establish *in vivo* and *in vitro* models in order to perform experiments that may help elucidate the biological role of the ERK/MAPK pathway in gallbladder carcinogenesis. Some previous reports have assessed the potential therapeutic uses of inhibitors against ERK/MAPK and mTOR pathways in TGBC-1 and NOZ cells and xenograft models, demonstrating an antitumor effect by inhibiting the signal crosstalk between both pathways that suggests a possible treatment for GBC [17]. This crosstalk interaction between mTOR and ERK/MAPK pathways has also been previously addressed by our group that studied mTOR pathway regulators in the same GBC tissues and cell lines [33] and in NOD-SCID mice models injected subcutaneously with G-415 or TGBC-2 cells [34].

In this study, the GBC cell lines GB-d1, G415 and NOZ cells evidenced expression of all targets (ERK1/2, p-ERK1/2, EGFR, ERBB2 and ERBB3). Interestingly, EGFR and ERBB3 expression was remarkably higher in GB-d1, G415, NOZ and OCUG-1 cells than in TGBC-1, TGBC-2 and TGBC-24 cells. These results coincide with the data obtained by Subbannayya & Leal et al., which observed different invasiveness capabilities in the cell lines used [35]. For instance, OCUG-1, NOZ and GB-d1 have shown a varied invasive ability ranging from moderate to highly invasive. Conversely, the TGBC-24 cell line was described as non-invasive [35].

The binding of ligands of ErbB family receptors induces homo- and heterodimerization [36]. However, there is no a high affinity ligand for ERBB2 receptor, thus ERBB2 only can be activated by heterodimerization along with another ErbB receptor [37]. Furthermore, the homodimer of ERBB3 is inactive so the binding of ligand to ErbB3 induces receptor heterodimerization along with ERBB2 [36,38]. This phenomenon increases the complexity of signaling through the ErbB receptor family. Dimerization of receptors stimulates cytoplasmic kinase activity triggering self-phosphorylation and trans-phosphorylation of tyrosine residues [39,40], which serve in the binding to different adapter proteins and enzymes [41]. EGFR and ERBB3 receptors possess binding sites for different adapter proteins in their cytoplasmic domain. For instance, EGFR has multiple binding sites for Growth-factor-Receptor-Bound 2 (GRB2) and Src-homology-2-containing (SHC) that activate ERK/MAPK cascade, while ERBB3 has five binding sites for PI3K and only one site for SHC [41,42]. GB-d1, G415, NOZ and OCUG-1 cell lines showed a higher expression of both EGFR and ERBB2 receptors, which suggests a probable signaling

mediated primarily by ERK1/2 after heterodimerization induced between EGFR and ErbB2 receptors together. However, despite OCUG-1 expresses EGFR and ERBB2, this cell line showed lower levels of p-ERK1/2. On the other hand, TGBC-1 and TGBC-2 have previously exhibited a greater activation of AKT [33], which is concordant with the lower expression of EGFR and ERBB2 receptors shown in this study. This event indicates a probable dependence of the joint heterodimerization between EGFR and ERBB2 to induce the subsequent activation of ERK/MAPK pathway for triggering cell growth in these cells. A promising approach of this knowledge is the assessment of inhibitors against those overactivated ERK/MAPK pathway members such as EGFR (cetuximab and panitumumab), ERBB2 (trastuzumab), ERBB3 (KTN3379), BRAF (vemurafenib and dabrafenib), RAF (sorafenib) and MEK1/2 (trametinib and cobimetinib) [44–47], that could be tested in GB-d1, G415 and NOZ cell lines with the purpose of evaluating their potential usefulness in GBC treatment or studying the biological behavior of this pathway.

During the last decades, many researchers have focused on deciphering the role of the ERK/MAPK pathway in carcinogenesis due to the pleiotropic characteristics of the genes involved in this signaling axis. However, some studies in other cancers have presented contradictory findings [43]. This work emphasizes that ERK/MAPK pathway could be important in the gallbladder carcinogenesis in Chilean population. However, further studies are needed to elucidate the real role of this pathway in GBC. On this regard, some GBC cell lines (GB-d1, G415 and NOZ) could be recommended for conducting future experiments involving this signaling axis.

5. Conclusions

The clinicopathological relevance of ERK1/2 and p-ERK1/2 in GBC and CC samples was investigated. This work demonstrated that ERK1/2 and p-ERK1/2 expressions were significantly increased in GBC tissues but these expressions were not associated to clinicopathological features neither survival in Chilean population. On the other hand, GB-d1, G415 and NOZ cell lines evidenced, in general, a stronger expression of ERK1/2, p-ERK1/2, EGFR, ERBB2 and ERBB3, constituting the most recommended cell lines to be used in *in vitro* and *in vivo* experiments for studying the role of the ERK/MAPK pathway in gallbladder carcinogenesis and new inhibitors of this signaling axis.

Conflicts of interest

The authors have no conflicts of interest to declare.

Ethical statement

The study received institutional review board approval.

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