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Expression of Insulin-like Growth Factor II mRNA-binding Protein 3 in Gallbladder Carcinoma

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Abstract. *Background/Aim: Emerging evidence suggests that Insulin-like growth factor II mRNA-binding protein 3 (IMP3) promotes tumor progression in several human malignancies. We investigated whether IMP3 expression has clinicopathological and prognostic significance in gallbladder adenocarcinoma (GBAC). Patients and Methods: We examined immunohistochemical IMP3 expression in 204 GBACs and its associations with clinicopathological parameters and patient outcomes. Results: The majority (87.7%) of GBACs exhibited at least focal cytoplasmic and membranous IMP3 immunoreactivity. Tumor-specific IMP3 expression highlighted proper muscle invasion, which was not detected in the corresponding hematoxylin and eosin-stained slides. This finding upgraded pathological tumor stage (pT) from pT1a to pT1b in four well-differentiated GBACs. High IMP3 expression was associated with high histological grade, advanced stage, and lymphatic invasion, as well as worse overall survival. Conclusion: Tumor-specific IMP3 expression in GBAC is helpful in determining the tumor extent, especially in well-differentiated tumors. High IMP3 expression reflects aggressive oncogenic behavior of GBAC. IMP3 expression may be used as a diagnostic and prognostic marker in GBAC.*

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Key Words: Gallbladder, carcinoma, IMP3, immunohistochemistry, prognosis.

Gallbladder adenocarcinoma (GBAC) accounts for 2.9% of all malignancies in the Republic of Korea and is the sixth most common cause of cancer-related death (1). Due to nonspecific symptoms, the diagnosis of GBAC is often delayed and usually made postoperatively for advanced-stage tumors. Despite aggressive treatment, including extensive cholecystectomy and systemic chemotherapy, the prognosis of patients with advanced GBAC remains poor, with 5-year survival rates below 5% (2-5). Conventional clinicopathological parameters, including histological grade, lymph node metastasis, and stage, have significant value in predicting the prognosis of GBAC patients (2). However, prognosis cannot be predicted solely based on conventional prognostic parameters in a subset of GBAC patients. Identification of novel prognostic biomarkers in GBAC would be valuable for the assessment of disease progression risk as well as adequate postoperative treatment in high-risk patients.

Insulin-like growth factor II mRNA-binding protein 3 (IMP3), an oncofetal protein, is expressed in the epithelium, muscle, and placenta during the early stages of human embryogenesis (6, 7). IMP3 plays an important role in the migration of cells forming the roof plate of the neural tube

and the subsequent migration of neural crest cells (8). IMP3 also promotes adhesion, invasion, and metastasis of tumor cells (9). Recent studies have documented the significant diagnostic and prognostic value of IMP3 expression status in many different types of human carcinomas (10-17). High IMP3 expression has been reported in several malignancies but not in benign tissues; thus, high IMP3 expression has been reported to be associated with tumor development and aggressive oncogenic behavior.

To the best of our knowledge, the clinicopathological significance and prognostic implications of IMP3 expression in GBAC have not yet been established. Emerging evidence supporting the important role of IMP3 in tumor progression and metastasis has prompted the examination of its expression in GBAC. In this study, we investigated the expression status of IMP3 in GBAC tissue samples using immunohistochemical staining and analyzed its association with clinicopathological parameters and outcomes of GBAC patients.

Patients and Methods

Patient and tissue samples. This study (2020-01-021) was reviewed and approved by the Institutional Review Board of the Kyung Hee University Hospital (Seoul, Republic of Korea). We searched GBAC cases in the surgical pathology database of the Department of Pathology at Kyung Hee University Hospital (Seoul, Republic of Korea) using a combination of the keywords "carcinoma" and "gallbladder". Between 1982 and 2017, 204 patients who underwent surgical resection for GBAC were included in the study. We also included 42 cases of dysplasia and 12 cases of chronic cholecystitis. Two board-certified pathologists reviewed all available hematoxylin and eosin-stained slides and selected the most representative slide for immunohistochemical staining.

We reviewed the medical records and pathology reports of GBAC patients for the documentation of various clinicopathological parameters, including age at diagnosis, sex, histological grade, tumor size, pathological tumor stage (pT), lymph node metastasis, stage group, lymphatic invasion, vascular invasion, perineural invasion, resection margin status, disease recurrence, and survival status at the last follow-up. Histological grade was determined according to the fourth edition of the World Health Organization Classification of Tumours of the Digestive System (18). pT, lymph node metastasis, and stage group were determined according to the 8th edition of Cancer Staging Manual developed by the American Joint Committee on Cancer (19). Development of local recurrence and distant metastasis were revealed on imaging analyses, including computed tomography and magnetic resonance imaging. To analyze the progression-free survival (PFS), the primary end-point was defined as the time to local recurrence or distant metastasis, whichever occurred first.

The demographic and basic clinicopathological features of patients are summarized in Table I. The age of patients ranged from 32 to 92 years (median=66 years; mean=54.3 years). Of the included patients, 101 (49.0%) were male and 105 (51.0%) were female. The mean tumor size was 3.1 cm. Lymph node dissection was performed in 121 (59.3%) patients. The distribution of stage group at the time of diagnosis was as follows: stage I, 18.4%; stage II, 46.3%; stage III, 28.4%; and stage IV, 6.8%. None of the patients received neoadjuvant chemotherapy or neoadjuvant concurrent chemoradiation therapy.

Table I. *Relationship between insulin-like growth factor II mRNA-binding protein 3 (IMP3) expression status and the clinicopathological parameters of gallbladder adenocarcinoma patients*.

Parameter	Number of cases	IMP3 expression		*p*-Value
		No/low (%)	High (%)	
Gender				
Man	101	23 (23.0)	77 (77.0)	0.347
Woman	105	31 (29.8)	73 (70.1)	
Age (years)				
<66	86	28 (32.9)	57 (67.0)	0.082
≥66	120	26 (21.8)	93 (78.1)	
Histological grade				
1-2	178	51 (28.9)	125 (71.0)	**0.039**
3	28	3 (10.7)	25 (89.2)	
Tumor size (cm)				
<2.5	75	24 (32.0)	51 (68.0)	0.250
≥2.5	120	29 (24.2)	91 (75.8)	
Not applicable	11			
Pathological tumor stage (pT)				
pT1-2	166	45 (27.4)	119 (72.6)	0.298
pT3-4	40	9 (22.5)	31 (77.5)	
Lymph node metastasis				
Absent	80	19 (23.8)	61 (76.2)	0.388
Present	41	8 (19.5)	33 (80.5)	
Not applicable	85			
Stage group				
I-II	134	41 (31.0)	91 (69.0)	**0.021**
III-IV	72	13 (18.1)	59 (81.9)	
Lymphatic invasion				
Absent	135	43 (32.3)	90 (67.7)	**0.008**
Present	71	11 (15.5)	60 (84.5)	
Vascular invasion				
Absent	180	51 (28.7)	127 (71.3)	0.061
Present	26	3 (11.5)	23 (88.5)	
Perineural invasion				
Absent	173	48 (28.1)	123 (71.9)	0.286
Present	33	6 (18.2)	27 (81.8)	
Resection margin involvement				
Absent	182	49 (27.2)	131 (72.8)	0.484
Present	24	5 (20.8)	19 (79.2)	

Bold values indicate statistical significance ($p<0.05$).

Immunohistochemistry. IMP3 expression was assessed by immunohistochemistry using the Bond Polymer Intense Detection System (Vision Biosystems, Mount Waverly, Victoria, Australia) according to the manufacturer's instructions with minor modifications (20-29). Briefly, 4 μm-thick sections of formalin-fixed and paraffin-embedded tissue were deparaffinized with Bond Dewax Solution, and antigen retrieval was performed using Bond ER Solution for 30 min at 100˚C. Endogenous peroxidases were quenched by incubation with hydrogen peroxide for 5 min. The sections were incubated for 15 min at ambient temperature with a

monoclonal rabbit antibody against IMP3 (dilution 1:50, clone EP286, Cell Marque, Rocklin, CA, USA) using a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system. Nuclei were counterstained with hematoxylin. An appropriate positive control (normal palatine tonsil sample) was concurrently stained to validate the staining method. A negative control was prepared by substituting the primary antibody with a non-immune serum sample, which resulted in no detectable staining.

Evaluation of immunohistochemical staining. The degree of immunohistochemical IMP3 expression was semi-quantitatively determined by assessing the proportion of positively stained cancer cells and the staining intensity. The staining intensity score was scaled as follows: 0, no expression; 1, faint expression; 2, moderate expression, which is equivalent to the intensity observed in the control tissue; and 3, strong expression, which is stronger than that observed in the control tissue. The proportion score was scaled as follows: 0, no staining; 1, <10% of tumor cells; 2, 10-33%; 3, 34-66%; and 4, >66%. Total immunostaining scores were calculated by multiplying the intensity and proportion scores: 0, 1, 2, 3, 4, 6, 8, 9, and 12. The optimal cut-off values for high and no/low IMP3 expression levels were chosen based on the distribution of the staining results as well as the extent of heterogeneity with respect to overall survival (OS), as determined using the log-rank test. All immunostained slides were examined and scored by two board-certified pathologists who were blinded to the clinicopathological data and patient identities. The degree of agreement between the two pathologists was almost perfect (κ=0.94). Disagreements between the two pathologists were resolved by consensus.

Statistical analysis. The Chi-squared test or Fisher's exact test was used to examine the association between IMP3 expression status and clinicopathological parameters. Univariate survival analysis was performed to examine the prognostic significance of IMP3 expression status and clinicopathological parameters with respect to PFS and OS. Multivariate survival analysis was performed for parameters that had p<0.1 in the univariate analysis using the Cox proportional hazards model (95% confidence interval) with the backward-stepwise elimination method. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corporation, Armonk, NY, USA). Statistical significance was defined as p<0.05.

Results

IMP3 expression in benign and malignant gallbladder lesions. IMP3 expression was negative in all specimens of dysplasia and cholecystitis. Representative photomicrographs show the lack of IMP3 expression in gallbladder dysplasia (Figure 1A and B). Non-neoplastic biliary epithelium adjacent to GBAC also showed no IMP3 expression. In contrast, the majority (179/204; 87.7%) of GBAC specimens showed at least focal cytoplasmic and membranous IMP3 immunoreactivity in the tumor cells (Figure 1C). Regarding staining intensity and proportion, we observed uniform and strong IMP3 expression in 25 (12.2%) cases (Figure 1D) and patchy and heterogeneous expression in the remaining 154 (75.4%) cases (Figure 1E).

In addition to tumor cells, IMP3 expression was observed in lymphoid tissues located in the subepithelial and perimuscular connective tissues of the gall bladder GB as well as the secondary lymphoid follicles of lymph nodes. Strong IMP3 immunoreactivity was observed in most germinal center lymphocytes and a few non-germinal center lymphocytes (Figure 1F). Furthermore, some vascular endothelial cells expressed IMP3 weakly in their cytoplasm. Irregularly shaped vascular lumina lined by IMP3-positive endothelial cells mimicked those of infiltrating tumor glands. Vascular endothelial cells were distinguished from tumor cells according to the following features: low nuclear-cytoplasmic ratio, lack of nuclear atypia, and weaker IMP3 staining intensity.

Revision of pT via IMP3 immunostaining. Based on tumor-specific IMP3 expression, we re-evaluated pT in IMP3-immunostained slides. In 4 of 59 (6.7%) well-differentiated GBACs, we identified IMP3-positive tumor glands that infiltrated into the proper muscle. These glands were initially interpreted as Rokitansky-Aschoff sinuses in the corresponding hematoxylin and eosin-stained slides. Consequently, we revised the pT in these cases from pT1a to pT1b. The tumor glands identified by IMP3 immunostaining were few in number and possessed low-grade nuclei, mimicking benign glands.

Clinicopathological significance of IMP3 expression in GBAC. The cases were divided into two groups according to the total immunostaining score: no/low expression (0-4) and high expression (6-12). The high-expression group, characterized by strong IMP3 expression in at least 10% of the total tumor cells or moderate IMP3 expression in at least one-third of the total tumor cells, included 150 cases (73.5%).

Table I summarizes the association between IMP3 expression status and clinicopathological parameters of GBAC. Significant differences in IMP3 expression were observed between when the cases were classified according to histological grade [1-2 (well-to-moderately differentiated) *vs*. 3 (poorly differentiated)], stage (I-II *vs*. III-IV), and lymphatic invasion (absent *vs*. present). In other words, high IMP3 expression was more frequent in tumors with higher histological grade (p=0.039), more advanced stage (p=0.021), and lymphatic invasion (p=0.008). Other clinicopathological parameters showed no significant associations with IMP3 expression status.

Prognostic implications of IMP3 expression in GBAC. Postoperative follow-up was performed in 184 of 204 (90.1%) patients. Median follow-up time was 27.5 months (range=2-225 months). Disease recurrence was detected in 34 of 184 (18.5%) patients. Seventy-five (40.8%) patients died by the last follow-up.

Table II summarizes the results of univariate survival analyses. Higher histological grade, higher pT, lymph node

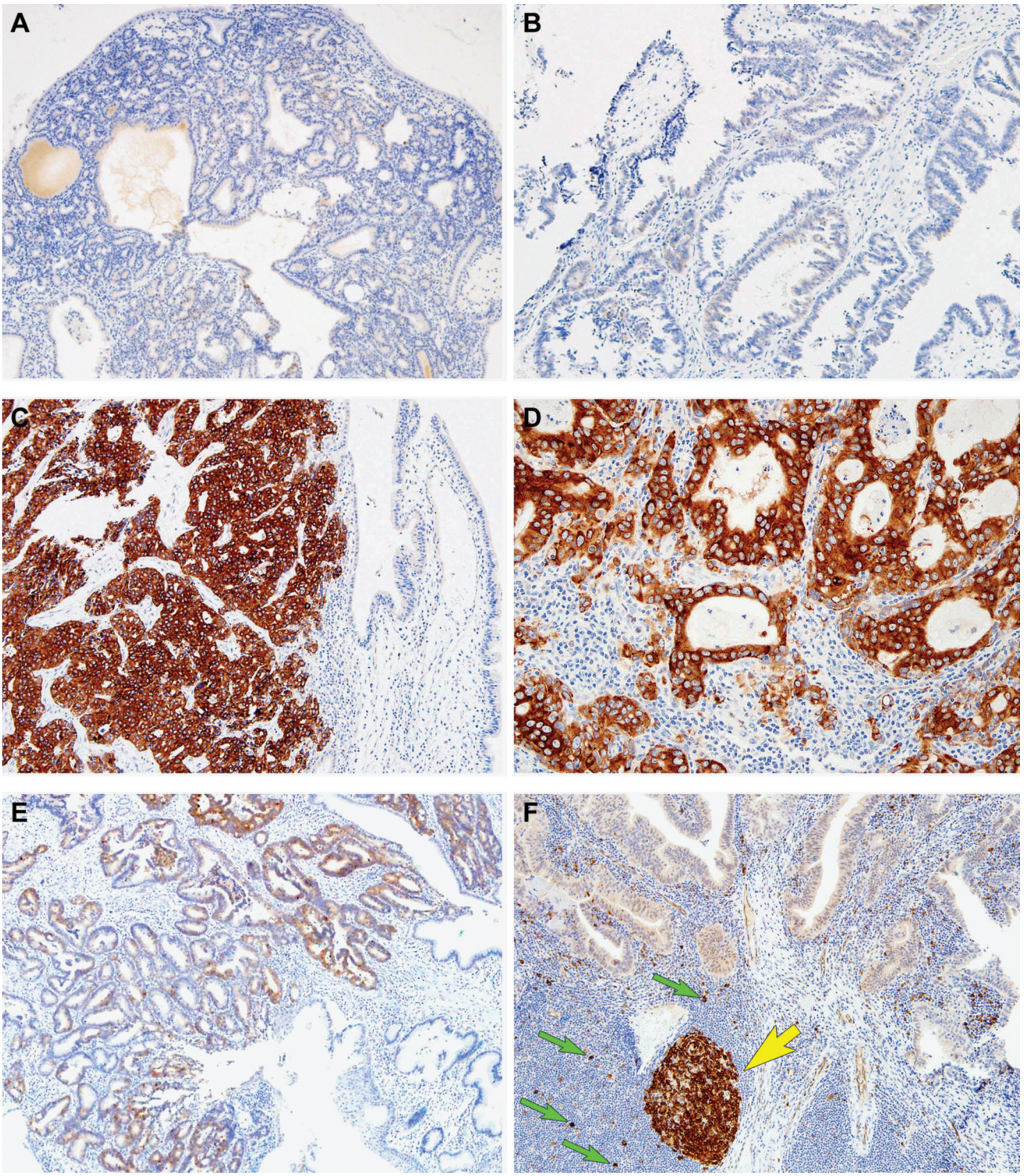

Figure 1. *Insulin-like growth factor II mRNA-binding protein 3 (IMP3) expression in benign and malignant gallbladder lesions. (A and B) Negative IMP3 expression in dysplasia. (C) Uniform IMP3 immunoreactivity in gallbladder adenocarcinoma (GBAC; left and middle one-thirds) and lack of IMP3 expression in the adjacent normal biliary epithelium (right one-third). (D) Diffuse and strong IMP3 expression in the membrane and cytoplasm of GBAC cells. (E) Heterogeneous IMP3 expression with variable staining intensity in GBAC cells. (F) Strong IMP3 expression in germinal center lymphocytes (short yellow arrow) and a few scattered non-germinal center lymphocytes (long green arrows). Original magnification, A, 40×; B, 100×; C, 40×; D, 100×; E, 40×; F, 40×.*

Table II. *Univariate survival analysis in patients with gallbladder adenocarcinoma.*

Parameter	Progression-free survival		Overall survival	
	Median (months)	*p*-Value	Median (months)	*p*-Value
Gender				
Man	65	0.640	73	0.587
Woman	68	Not applicable		
Age (years)				
<66	67	0.507	Not applicable	0.781
≥66	65		85	
Histological grade				
1-2	120	**<0.001**	149	**<0.001**
3	7		10	
Tumor size (cm)				
<2.5	132	0.754	149	0.843
≥2.5	78		85	
Pathological tumor stage (pT)				
pT1-2	120	**<0.001**	149	**<0.001**
pT3-4	10		12	
Lymph node metastasis				
Absent	Not applicable	**<0.001**	Not applicable	**<0.001**
Present	10		16	
Stage group				
I-II	Not applicable	**<0.001**	Not applicable	**<0.001**
III-IV	10		12	
Lymphatic invasion				
Absent	172	**<0.001**	Not applicable	**<0.001**
Present	10		14	
Vascular invasion				
Absent	85	**<0.001**	149	**<0.001**
Present	7		11	
Perineural invasion				
Absent	120	**<0.001**	149	**<0.001**
Present	13		14	
Resection margin involvement				
Absent	85	**<0.001**	149	**<0.001**
Present	10		10	
Insulin-like growth factor II mRNA-binding protein 3 expression status				
No/low	172	0.075	Not applicable	0.017
High	38		65	

Bold values indicate statistical significance ($p<0.05$).

metastasis, advanced stage, lymphatic invasion, vascular invasion, perineural invasion, and resection margin involvement were significantly associated with shorter PFS and OS. Median PFS was shorter in patients with tumors showing high IMP3 expression than in those with tumors showing no/low IMP3 expression, but the difference was not significant ($p=0.075$; Figure 2A). Median OS was significantly shorter in patients with GBACs showing high IMP3 expression than in those with GBACs showing no/low IMP3 expression ($p=0.017$; Figure 2B).

On multivariate analysis (Table III), the parameters that independently predicted shorter PFS and OS were higher histological grade ($p<0.001$ and $p<0.001$, respectively), more advanced stage ($p<0.001$ and $p<0.001$, respectively), and vascular invasion ($p=0.034$ and $p=0.006$, respectively). High IMP3 expression showed increased hazard ratios for PFS (1.070) and OS (1.491), but it did not independently predict survival ($p=0.301$ and $p=0.222$, respectively).

Discussion

In this study, we aimed to investigate whether the expression status of IMP3 has clinicopathological significance and prognostic implications in GBAC. Most (87.7%) GBAC specimens were found to show high IMP3 expression. According to previous studies, the positivity rate of IMP3 in

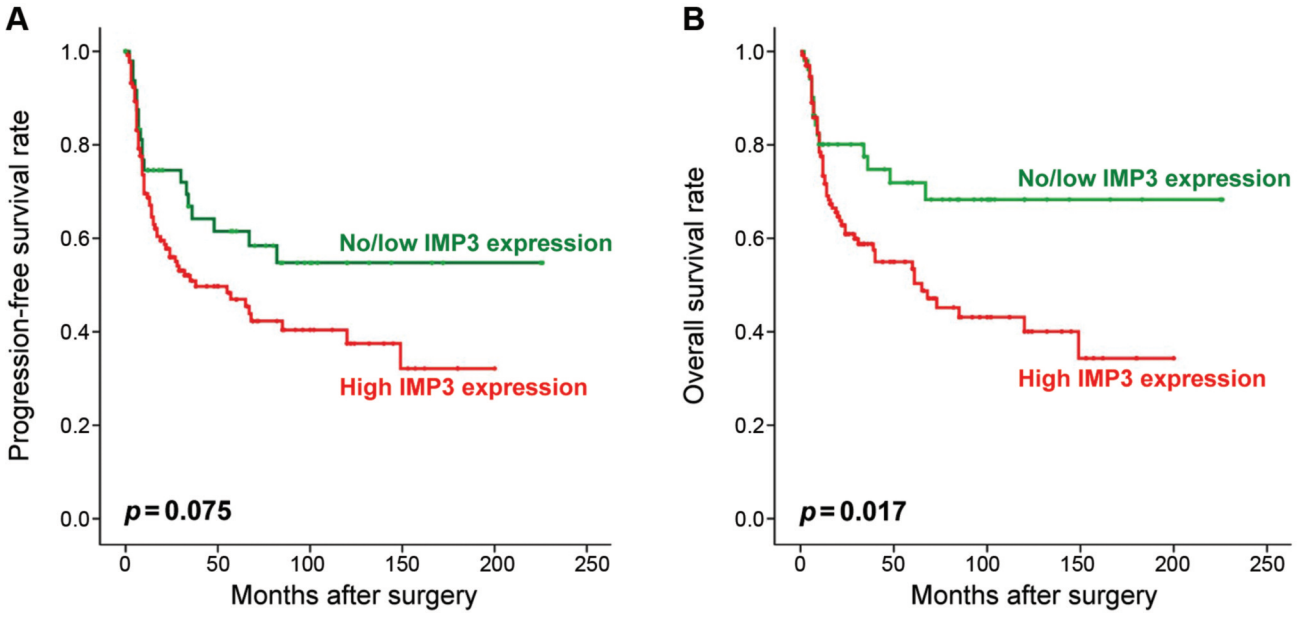

Figure 2. *Kaplan-Meier plots for (A) progression-free and (B) overall survival rates according to the expression status of insulin-like growth factor II mRNA-binding protein 3 (IMP3) in patients with gallbladder adenocarcinoma (GBAC). Red line, patients with GBACs showing high IMP3 expression; Green line, patients with GBACs showing no/low IMP3 expression. Overall survival rates are significantly lower in patients with GBACs showing high IMP3 expression than in those with GBACs showing no/low IMP3 expression (p=0.017).*

GBAC is variable, ranging from 50% to 92.7% (30-32). In contrast, IMP3 expression in non-cancerous tissues, including those with dysplasia and chronic cholecystitis, was negative, suggesting IMP3 immunostaining as a diagnostic marker for GBAC. Our observations are in agreement with previous studies documenting that IMP3 immunostaining has a significant diagnostic value in pancreatobiliary biopsy and fine needle aspiration specimens (33-38). Nevertheless, negative IMP3 immunoreactivity should be interpreted cautiously because some GBACs showed heterogeneous expression and partial negativity, resulting from intratumoral heterogeneity.

We revised the pT of four well-differentiated GBACs with the aid of IMP3 immunostaining. IMP3-positive tumor glands, which were not detected in the hematoxylin and eosin-stained slides but were observed in the IMP3-immunostained slides, were located within the proper muscle layer, resulting in upstaging from pT1a to pT1b. It is sometimes difficult to determine pT in well-differentiated GBACs because of the complex microanatomy of GB and deeply infiltrating Rokitansky-Aschoff sinuses. The observation of IMP3-expressing glands in such complex situations may help determine the nature of the glands more convincingly. In line with these findings, recent studies have shown that IMP3 immunostaining is helpful in detecting invasive foci of lung adenocarcinomas and papillary brain tumors (39, 40). We also noted IMP3 expression in some normal lymphocytes and vascular endothelial cells. In our experience, the staining pattern as well as cytomorphological findings can be used as distinguishing factors. Usually, IMP3 is expressed in only a few scattered lymphocytes located within clusters of IMP3-negative lymphocytes, whereas IMP3-positive tumor cells form glands or clusters. These tumor cells show homogeneous and uniform staining for IMP3, whereas endothelial cells display patchy staining with weak intensity along the vascular lumina.

We demonstrated that high IMP3 expression was associated with parameters reflecting aggressive oncogenic behavior: higher histological grade, advanced stage, and lymphatic invasion. IMP3 has been shown to enhance tumor growth and invasion (41). Previous studies have suggested that IMP3 appears to resume its physiological functions in malignant cells, which not only contribute to the tumor progression but also participate in the tumorigenesis. In an *in vitro* study by Chen *et al*. (41), IMP3 expression was highest in $CD133^{+}/CD49f^{+}$ cells, which were isolated from mouse models and patients with hepatocellular carcinoma. These tumor-initiating stem-like cells were involved in the tumor development, progression, and resistance to chemotherapy of hepatocellular carcinoma. In addition, Hwang *et al*. (42) observed that down-regulation of IMP3 inhibited invadopodia formation, tumor growth, and invasiveness of oral squamous carcinoma, indicating that IMP3 is intimately correlated with tumor cell invasion of the tumor cells through invadopodia.

Table III. *Multivariate survival analysis in patients with gallbladder adenocarcinoma.*

Parameter		Progression-free survival		Overall survival	
		Hazard ratio (95% confidence interval)	*p*-Value	Hazard ratio (95% confidence interval)	*p*-Value
Histological grade	3	15.543 (1.925-7.024)	**<0.001**	13.957 (1.946-8.460)	**<0.001**
Pathological tumor stage (pT)	pT3-4	0.997 (0.382-1.367)	0.318	1.154 (0.331-1.381)	0.283
Lymph node metastasis	Present	0.035 (0.391-2.171)	0.852	1.094 (0.231-1.561)	0.296
Stage group	III-IV	19.090 (2.224-8.162)	**<0.001**	22.364 (3.091-15.273)	**<0.001**
Lymphatic invasion	Present	1.241 (0.758-2.733)	0.265	1.211 (0.747-.2.967)	0.271
Vascular invasion	Present	4.518 (1.062-4.361)	**0.034**	7.954 (1.380-6.754)	**0.006**
Perineural invasion	Present	0.314 (0.555-2.885)	0.575	0.005 (0.423-2.525)	0.943
Resection margin involvement	Present	1.326 (0.700-3.943)	0.250	0.655 (0.252-1.773)	0.418
Insulin-like growth factor II mRNA-binding protein 3 expression status	High	1.070 (0.355-1.377)	0.301	1.491 (0.272-1.353)	0.222

Bold values indicate statistical significance ($p<0.05$).

We also observed that patients with GBACs showing high IMP3 expression had significantly shorter OS than those with GBACs showing no/low IMP3 expression. High IMP3 expression has been reported in tumors with adverse prognostic parameters for other types of carcinomas (13, 17, 43-47). Kessler *et al*. (31) examined the relationship between patient outcome and the expression status of IMP1, IMP2, and IMP3 in GBAC and demonstrated that IMP2 expression was associated with worse prognosis, whereas IMP3 expression was not a significant prognostic factor.

Previous studies used tissue microarray blocks, whereas in this study, we used whole tissue sections to minimize the bias resulting from intratumoral heterogeneity. Considering that heterogeneous IMP3 expression patterns were observed in approximately 75% of the examined GBAC specimens, a major reason for the discrepancy with the results of previous studies may be the difference in type of tissue section used for immunostaining. Larger population-based studies would be valuable for confirming the prognostic implications of IMP3 expression in GBAC.

In conclusion, we demonstrated tumor-specific IMP3 expression in GBACs, which may help determine the tumor extent, especially in well-differentiated tumors. High IMP3 expression status in GBAC was significantly associated with adverse clinicopathological parameters, including higher histological grade, advanced stage, lymphatic invasion, and shorter OS. Our data suggest that IMP3 expression status may be used as a diagnostic and prognostic marker for GBAC.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

All Authors made substantial contributions to the conception and design of the study, acquisition of data, analysis and interpretation of the data, as well as drafting of the manuscript, in revising the article critically for important intellectual content, and providing final approval of the version to be published.

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