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Original contribution

IMP3 expression is associated with poor outcome and epigenetic deregulation in intrahepatic cholangiocarcinoma☆,☆☆

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Summary IMP3 is a fetal protein not expressed in normal adult tissues. IMP3 is an oncoprotein and a useful biomarker for a variety of malignancies and is associated with reduced overall survival of a number of them. IMP3 expression and its prognostic value for patients with intrahepatic cholangiocarcinoma (ICC) have not been well investigated. The molecular mechanism underlying IMP3 expression in human cancer cells remains to be elucidated. Here we investigated IMP3 expression in ICC and adjacent nonneoplastic liver in 72 unifocal primary ICCs from a single institute by immunohistochemistry, immunoblotting, and real-time polymerase chain reaction. IMP3 was specifically expressed in cancer cells but not in the surrounding normal tissue, and 59 (82%) of 72 ICCs were IMP3 positive by immunohistochemistry. Among 35 cases with lymphovascular invasion, 26 (74%) showed IMP3 positivity in lymph node metastases. IMP3 expression was significantly correlated with tumor size, pathological grade, metastasis, and clinical stage. Kaplan-Meier analysis demonstrated an inverse correlation between IMP3 expression and overall survival rate. Multivariate analysis revealed that IMP3 was the only risk factor associated with survival. To further explore the mechanism of IMP3 expression in cancers, we identified 2 CpG islands at IMP3 proximal promoter. Interestingly, the IMP3 promoter was almost completely demethylated in ICCs in contrast to densely methylated promoter in normal liver tissues. IMP3 expression is a useful biomarker for ICCs and can provide an independent prognostic value for patients with ICC. To our knoweldge, this is the first direct evidence of epigenetic deregulation of IMP3 in human cancer.

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1. Introduction

IMP3 is a family member of insulin-like growth factor II messenger RNA (mRNA)–binding proteins, which consist of IMP1, IMP2, and IMP3 [1]. Similar to other family members, IMP3 plays an important role in RNA trafficking, stabilization, and localization during early embryogenesis [2]. IMP3 is expressed in many developing human tissues such as the epithelium, placenta, and muscle; however, it is not expressed in normal adult tissue [1,2]. Recent studies have shown that IMP3 is expressed in some malignant tumors including adenocarcinomas of the pancreas, kidney, lung, breast, esophagus, cervix, and endometrium [3-10]. Moreover, IMP3 has been implicated in promoting cell proliferation, adhesion, and invadopodia formation during cancer progression [11]. IMP3 has been recognized as an indicator for cancer progression and metastasis and a predictor of poor prognosis for many types of cancers [5,6,8-10].

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer after hepatocellular carcinoma, accounting for 5% to 15% of all primary liver malignancies [12]. The global incidence and mortality rate of ICC are increasing, in contrast to the decreasing trend of extrahepatic biliary tract cancers [13]. Surgical resection is currently the most effective treatment and the only therapy associated with prolonged disease-free survival. After surgical resection, the 5-year overall survival rate ranges from 14% to 40% for patients with ICC. The poor survival rate is mainly due to the advanced tumor stage with intrahepatic metastasis at presentation and early postoperative recurrence [14,15]. Identifying a new biomarker for early detection and to predict the outcome and to identify new therapeutic target is needed to improve the management for these patients. Jeng and coworkers [16] have reported that IMP3 was expressed in hepatocellular carcinoma, and Riener et al [17] have shown that IMP3 was expressed in high-grade dysplastic extrahepatic bile duct epithelium and can be a useful marker in biliary brushing specimens. However, the expression of IMP3 and its correlation with clinicopathological features remain to be elucidated in ICCs.

In this study, we investigated the expression of IMP3 in ICC, adjacent nonneoplastic liver, and normal-appearing liver away from ICC by immunohistochemistry (IHC) in 72 surgically resected primary ICCs from a single institute. We also investigated the correlation of IMP3 expression with tumor histologic grade, progression, metastasis, clinical stage, and postoperative survival rate. A recent study by Ueki et al [18] showed that treatment of an osteosarcoma cell line with a DNA methyltransferase inhibitor can increase IMP3 expression, suggesting that epigenetic mechanisms including DNA demethylation are involved in IMP3 activation in cancer cells. Using EMBOSS CpGPlot, we identified 2 CpG islands at its proximal promoter. To further investigate the epigenetic mechanisms by which the oncofetal protein IMP3 was aberrently expressed in cancer cells, we analyzed DNA methylation of the IMP3 promoter in ICCs and normal tissues.

2. Materials and methods

2.1. Case selection

A total of 72 surgically resected unifocal primary ICCs were collected from 2008 to 2011 at our institute, including 52 archived tissue blocks and 20 freshly frozen tumors. The study was approved and conducted according to the regulations of the ethics committee. The specimens were anonymized and analyzed in a blinded fashion. The patients included 40 men and 32 women with a mean age of 58.3 years (range, 28-75 years). All patients presented without jaundice. Serum hepatitis B surface antigen was detected in 23 patients, and anti–hepatitis C virus antibody was positive in 1 patient. Nineteen patients (26.4%) had hepatic cirrhosis. Fifty-nine were classified as Child-Pugh class A, and 13 were class B. None had received transhepatic arterial embolization or chemotherapy before the surgery. Follow-up time for survivors ranged from 3 to 46 months (median, 14.9 months). Fifty-four of the 72 patients were followed up for 1 year after surgery.

2.2. Histology and tumor staging

All specimens were formalin fixed and paraffin embedded. Histologic sections at 5-μm thickness were stained with hematoxylin-eosin and reviewed by experienced surgical pathologists to determine tumor grade and stage. The tumor was graded based on the criteria proposed by Edmondson and Steiner [19]. Tumors were staged according to the seventh edition of American Joint Committee on Cancer system [20], including 10 stage I (13.9%), 14 stage II (19.4%), and 48 stage III (66.7%) cases. All surgical margins were negative, and only completely resected specimens were included in this study.

2.3. Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue slides (5 μm) were deparaffinized and rehydrated. Antigen retrieval was peformed by incubating the tissue slides in 0.01 M citric acid buffer at 100°C for 10 minutes. After blocking with 3% H_2O_2 and 5% fetal bovine serum, the slides were incubated with a monoclonal antibody against IMP3 (1:100, ab109521; Abcam, Burlingame, CA) at 4°C overnight. The slides were then reacted with polymer–horseradish peroxidase reagent. The peroxidase activity was visualized with diaminobenzidine tetrahydroxychloride solution. The sections were counterstained with hematoxylin. Dark brown cytoplasmic staining of at least 1% tumor cells was defined as positive, and no staining or less than 1% cells stained was defined as

negative. As a negative control, we replaced the primary antibody with 5% fetal bovine serum. IMP3 positivity was graded based on the percentage of tumor cells with positive staining but not based on stain intensity, including strong (score 3+; ≥50%), moderate (2+; 10%-49%), and weak (1+; 1%-9%).

2.4. Immunoblotting

Twenty freshly frozen tissues were isolated for total protein extraction. An aliquot of protein extract (60 μg) was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P membrane; Millipore, Bedford, MA).The membrane-bound proteins were incubated with a primary antibody against IMP3 (1:1000; Abcam) or β-actin (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was washed 3 times and then incubated with a secondary antibody for 2 hours at 4°C. Finally, the immunoreactive signals were detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ).

2.5. Real-time polymerase chain reaction

Twenty fresh tumor samples were used to analyze the mRNA level of IMP3 by real-time polymerase chain reaction (PCR) performed with the ABI StepOnePlus PCR system (Applied Biosystems, Foster City, CA). Total RNA was extracted using TRIZOL reagent, and all primers were purchased from Invitrogen (Invitrogen, Carlsbad, CA). IMP3 mRNA was normalized with β-actin mRNA level, and results were presented as the ratio of IMP3 to β-actin. The primers were as follows: *IMP3* forward primer, 5′-ACGAAATATCCCGCCTCATTTAC-3′; *IMP3* reverse primer, 5′-GCAGTTTCCGAGTCAGTGTTCA-3′; β-actin forward primer, 5′-ACTGGAACGGTGAAGGTGAC-3′, β-actin reverse primer, 5′-AGAGAAGTGGGGTGGCTTTT-3′.

2.6. Promoter methylation analysis

The sequence from 2000 base pairs upstream to the transcription start site (+1) of transcript ENST00000258729 were extracted from Ensembl for analysis (gene ID, ENSG00000136231). CpG islands at IMP3 proximal promoter were predicted by EMBOSS CpGPlot. Two CpG islands were identified, designated as P1 (proximal) and P2 (distal) containing 29 and 19 CpG dinucleotides, respectively. Genomic DNAs (500 ng) extracted from 10 fresh tumors or 10 nonneoplastic normal-appearing liver tissues were treated with sodium bisulfite using the MethylCode Bisulfite Conversion Kit (Invitrogen) following the manufacturer's instruction. Bisulfite-converted DNAs (30 ng) were used as templates for PCR amplification of P1 and P2 CpG islands. The PCR primers were as follows: P1 forward primer: 5′-AGGTTTTYGAYGATTTTAGTTTT-3′ and reverse primer: 5′-AAAACCRCAAACACRTTTCTA-3′, P2 forward primer: 5′-TAGYGTTGAGGAATTGTTTGTTAG-3′ and reverse primer: 5′-AACRCAAAAAACRAAAAAAATC-3′. All PCR products were purified from 1.5% agarose gels using Gel Extraction Kit (Axygen, Union City, CA) and cloned into the pMD18-T vector (Promega, Madison, WI).

2.7. Statistics

The data analyses were peformed using SPSS 16.0 (SPSS, Chicago, IL) and GraphPad Prism v5.0 software (GraphPad, La Jolla, CA). Correlation between IMP3 expression and clinicopathological parameters was evaluated by χ^2 test and Fisher exact test. Survival rates were calculated using the unadjusted Kaplan-Meier method, and difference in survival curves was analyzed by the log-rank test. Multivariate analysis was used to evaluate the risk factors associated with postoperative survival. Two-tailed P values of .05 were considered statistically significant.

3. Results

3.1. IMP3 expression in ICCs and lymph node metastasis

We first evaluated the IMP3 expression by IHC in 72 cases of ICC. Fifty-nine (82%) of 72 cases were IMP3 positive, whereas 13 (18%) were negative for IMP3. Representative IMP3-positive and IMP3-negative tumors are shown in Fig. 1A and B. IMP3 was predominantly expressed in tumor nests but not in the surrounding tissue (Fig. 1C). For 35 cases (48%) with lymphovascular invasion, 26 (74%) of them had IMP3 expression in the lymph node metastases (Fig. 1D). To further confirm the expression of IMP3 in ICCs, we selected 20 cases to evaluate the IMP3 expression by 2 additional methods including real-time PCR and immunoblot using isolated fresh tumors, adjacent nonneoplastic liver, and normal-appearing liver away from ICC. As shown in Fig. 2A and B, both IMP3 mRNA and protein were exclusively detected in tumors but not or at very low level in nonneoplastic liver or normal-appearing liver away from ICC in all 20 cases. However, there was no significant correlation between IMP3 protein expression level and mRNA level detected by reverse transcriptase (RT) PCR.

3.2. IMP3 expression and clinicopathological features

IMP3 expression in ICC varies considerably in percentage of tumor cells stained or the intensity. Therefore, IMP3 positivity was categorized into 3 arbitrary levels based on the percentage of IMP3-positive tumor cells among the total tumor cell population, as described in Materials and

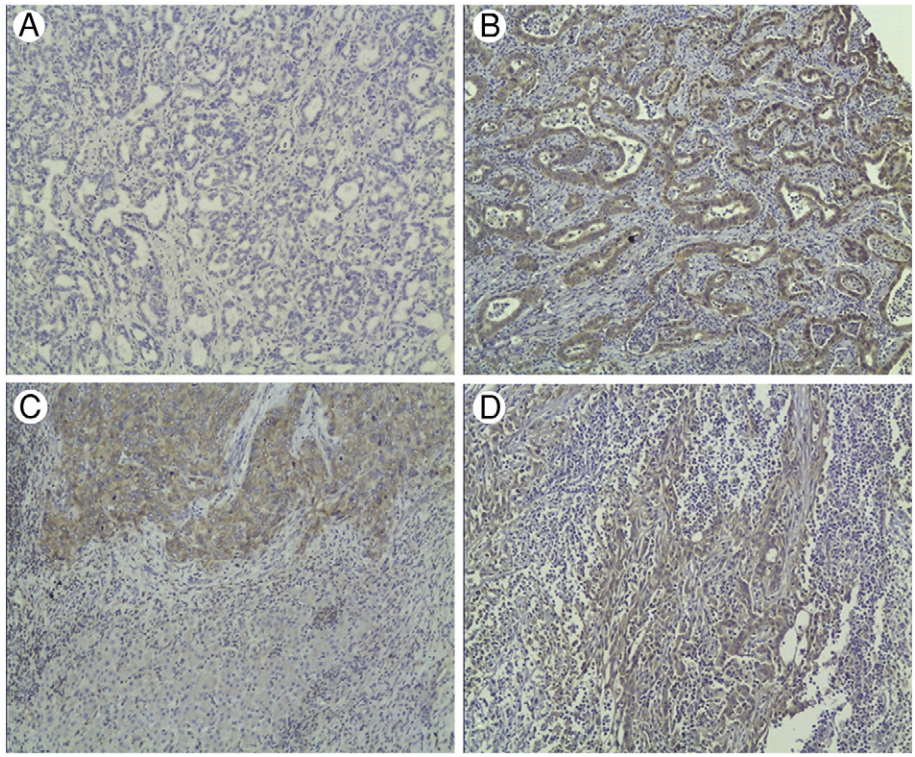

Fig. 1 IMP3 was exclusively expressed in primary and metastatic ICCs by IHC. Representative ICCs showing IMP3-negative tumor (A) and IMP3-positive tumor (B). IMP3-positive tumor surrounded by negatively stained normal tissue (C) and IMP3-positive staining in representative lymph node metastasis (D). Original magnifications ×100.

Methods: weak, moderate, and strong staining, corresponding to a semiquantitative score of 1+, 2+, and 3+, respectively. As demonstrated in Fig. 3, among the 59 IMP3-positive cases, IMP3 was assessed as strong, moderate, and weak in 22 (37%), 15 (25%), and 22 (37%) cases, respectively.

Next, the correlation between IMP3 expression and a variety of clinicopathological features was analyzed. As shown in Table 1, IMP3 expression was more frequently associated with metastasis (odds ratio [OR], 10.72; P = .001), higher stage (stages III-IV; OR, 10.71; P = .001), higher pathological grade (OR, 15.23; P = .002), and larger tumor size (>5 cm; OR, 2.33; P = .034). Expression of IMP3 was not associated with the patient's age (P = .44) and sex (P = .171).

3.3. IMP3 expression and clinical outcome

In 54 patients with at least 12 months of follow-up, Kaplan-Meier analysis showed that patients with IMP3 positivity had a shorter overall postoperative survival time than did IMP3-negative cases (Fig. 4; P = .010).

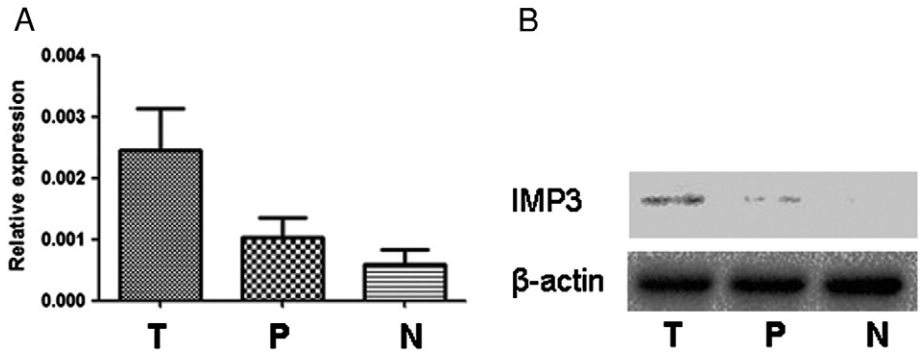

Fig. 2 IMP3 mRNA and protein expression in fresh ICC tissues. IMP3 mRNA levels detected in tumor (T), adjacent nonneoplastic liver (P), and surrounding normal liver away from ICC (N) by real-time PCR (A) and protein levels detected by immunoblot with β-actin serving as a loading control (B).

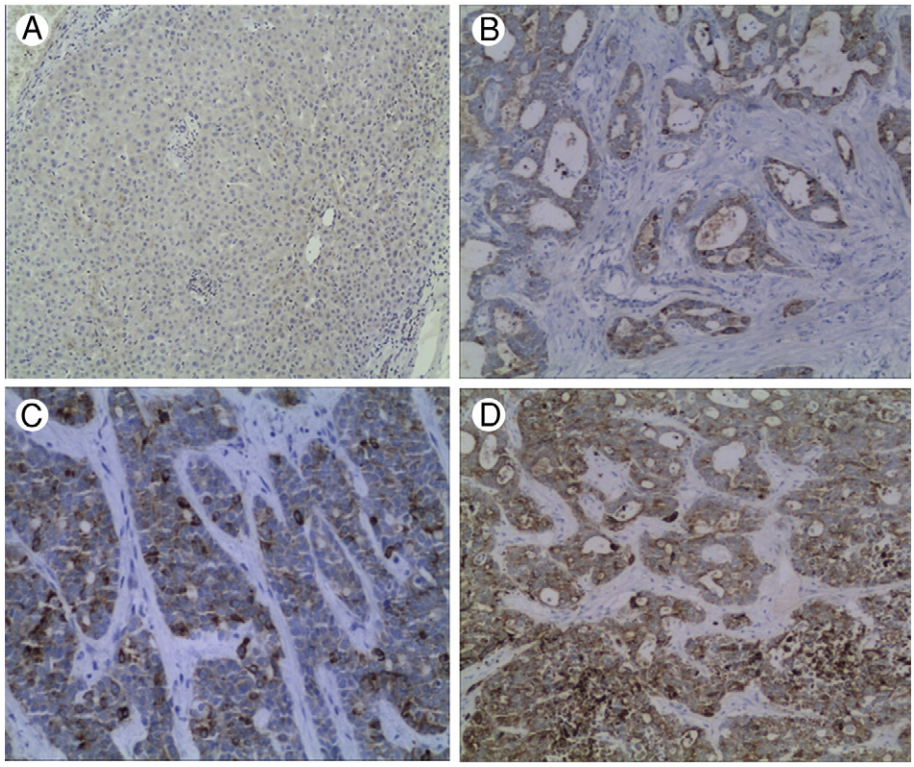

Fig. 3 Variable IMP3 expression levels in IMP3-positive ICCs. A, Normal-appearing liver tissue with negative IMP3 staining. As described above in Materials and Methods, IMP3 expression was semiquantitatively categorized into 3 groups as weak (1+; B), moderate (2+; C), and strong (3+; D), respectively. Original magnifications ×100.

Multivariate analysis revealed that IMP3 was the only independent risk factor associated with survival with a *z* value of 3.03 (Table 2; $P = .001$). Although tumor stage was the second most important risk factor, this association failed to reach statistical significance (Table 2; $P = .066$). We observed that all deaths were associated with disease recurrence, and there was no significant difference in the survival rate between the cirrhotic and noncirrhotic groups in this study. Interestingly, IMP3 expression level was inversely correlated with the 12-month survival rate and decreased from 90% in IMP3-negative patients to 70%,

Table 1 Univariate analysis of IMP3 protein expression and clinicopathological risk factors for ICC

	IMP3 protein expression			
	Total	Positive, n (%)	OR (95% CI)	*P*
Age (y)				
>55	43	34 (79.06)	1	.44
≤55	29	25 (86.20)	1.65 (0.46-5.98)	
Sex				
Female	32	24 (75.00)	1	.171
Male	40	35 (87.50)	2.33 (0.68-8.0)	
Sizes (cm)				
≤5	42	31 (73.81)	1	.034 *
>5	30	28 (93.33)	4.97 (1.01-24.38)	
Pathological grade				
Low	38	26 (68.42)	1	.002 *
High	34	33 (97.05)	15.23 (1.85-124.84)	
Tumor stage				
I-II	24	14 (58.33)	1	.001 *
III-IV	48	45 (93.75)	10.71 (2.58-44.45)	
Metastasis				
No	31	20 (64.51)	1	.001 *
Yes	41	39 (95.12)	10.72 (2.16-53.13)	

* Statistically significant.

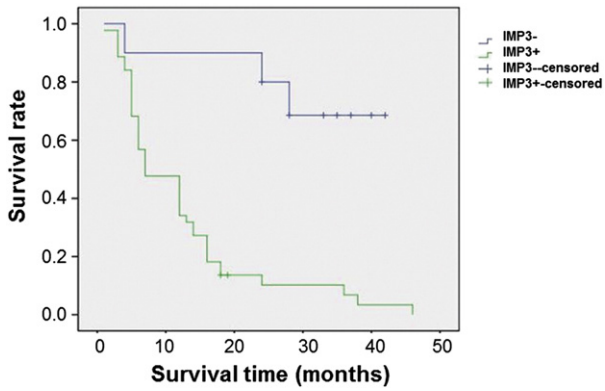

Fig. 4 IMP3 expression correlates with the prognosis of patients with ICC. The postoperative survival time and the IMP3 expression level of ICCs were evaluated by Kaplan-Meier in 54 patients with at least 12 months of follow-up. Patients with IMP3-positive ICC had a significantly lower survival rate than did those with IMP3-negative ICC.

Table 2 Multivariate analysis of risk factors associated with survival

	Estimate	SE	*z*	*P* > *z*	HR
Sex	0.10	0.33	0.31	.753	1.11
Age	0.00	0.02	0.01	.995	1.00
Grade	−0.02	0.35	−0.06	.952	0.98
Stage	0.79	0.43	1.84	.066	2.21
Metastasis	−0.76	0.77	−0.99	.322	0.47
IMP3	0.63	0.21	3.03	.001 *	1.87
Size	0.00	0.06	0.02	.987	1.00

* Statistically significant.

58%, and 32% in 1+, 2+, and 3+ IMP3 positivity cases, respectively (Table 3; $P = .009$).

3.4. DNA methylation status at the IMP3 promoter in ICC

Although IMP3 has been implicated in tumorigenesis and metastasis, the mechanisms of its deregulation during these processes have not been well studied. The temporal suppresion of IMP3 expression after embryogenesis and the presence of CpG islands at the proximal promoter prompted us to hypothesize that DNA demethylation contributes to *IMP3* gene activation in cancer cells. In fact, the RT-PCR results in Fig. 2A showed that IMP3 is transcriptionally activated in IMP3-positive cancer cells, although there was no significant correlation between the mRNA level and IMP3 expression observed. The next question raised was whether IMP3 expression is associated with its promoter demethylation. Ideally, to compare IMP3 promoter DNA methylation status in normal bile duct epithelium and ICC would address this question. Considering the technical challenge in harvesting the normal bile duct epithelium, we searched for benign bile duct lesions such as bile duct hamartoma as a control. However, no cases were available at this time. Because IMP3 is a fetal protein and not expressed in normal adult tissues, we decided to obtain an adjacent normal liver as a surrogate control that does not express IMP3 by IHC, immunoblot, or RT-PCR (Fig. 2). Using bisulfite conversion analysis, we found that both P1 and P2 were demethylated in 10 of 10 ICCs, as shown in Fig. 5A and C, respectively, in contrast to densely methylated P1 (10/10) and P2 (9/10) in the normal-appearing liver (Fig. 5B and D, respectively; $P = .0001$). One case of P2 region from the normal liver showed a demethylation pattern that is most likely due to a contamination. Interestingly, both P1 and P2 (data not shown) were also demethylated in IMP3-negative tumors by IHC. These data indicate that an epigenetic mechanism including DNA methylation at the *IMP3* gene promoter contributes to its temporal gene silencing in normal tissues and that DNA demethylation is necessary but insufficient in inducing IMP3 expression during cancer initiation or progression.

Table 3 The 1-year survival rate among differential expressions of IMP3 protein

IMP3 protein	Survival (mo)		n	1-y survival rate (%)	*P*
	≥12	<12			
Negative	9	1	10	90.00	.009 *
Weak (score 1+)	7	3	10	70.00	
Moderate (score 2+)	7	5	12	58.33	
Strong (score 3+)	7	15	22	31.81	

* Statistically significant.

4. Discussion

ICC is a highly malignant adenocarcinoma of the liver. Surgical resection is currently the only treatment that provides a potentially curative outcome. However, the 5-year survival rate for patients with resectable ICCs is low. For these reasons, identifying a reliable prognostic marker will be essential to improve the clinical management for these patients.

In this study, we demonstrate by 3 different methods that IMP3 is only expressed in cancer cells, with a sensitivity of 82% and specificity of 100%, consistent with the notion that IMP3 is a useful marker for malignancies. Furthermore, IMP3 expression is strongly correlated with tumor grade, tumor progress, and metastasis, as well as tumor clinical stage. In Kaplan-Meier analysis, we observed a significant inverse correlation between IMP3 expression level and 1-year survival rate. Multivariate analysis revealed that IMP3 is an independent risk factor associated with worse survival. These data suggest that IMP3 is not only an indicator for malignancy and metastasis but also a reliable poor prognosis marker, as previously reported for other types of cancers [3,7,9,10,16,21-27]. For example, IMP3 positivity is higher in more aggressive endometrial serous carcinoma than less aggressive types of endometrial adenocarcinomas [28]. In contrast, IMP3 would not be a good biomarker or prognostic factor for prostate adenocarcinoma because its expression is only slightly correlated with Gleason score and not with metastasis. [29]. Our data on ICCs strengthen the hypothesis that high IMP3 expression is prognostic for tumor aggressiveness and poor clinical outcome.

The mechanistic role of IMP3 in tumorigenesis or tumor progression remains to be investigated. A few studies have shown that IMP3 plays a key role in promoting invadopodia formation, cell proliferation and invasion, anchorage-independent growth, and chemoresistance in vitro [11,30-32]. Small interfering RNA knockdown of *IMP3* caused reduced migration and invasion in a cervical cancer cell line [8]. To our knowledge, there are no published functional data

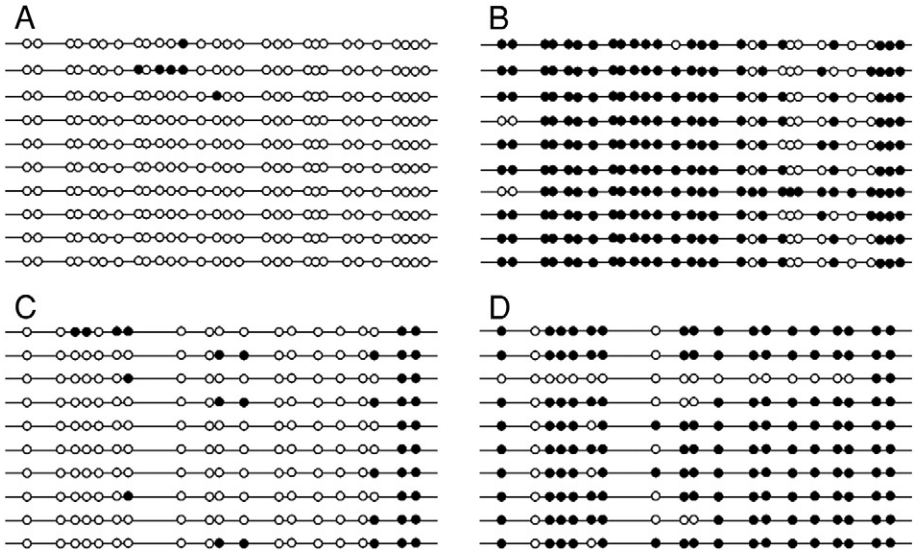

Fig. 5 IMP3 promoter was demethylated at P1 (A) and P2 (C) CpG islands in ICCs, in contrast to densely methylated P1 (B) and P2 (D) in normal liver tissues. Open circles represent demethylated CpG sites, and closed circles represent methylated CpG sites.

correlating the role of the CpG islands with IMP3 regulation in cancers. Our methylation studies suggest that epigenetic deregulation is involved in ICCs, although our study is limited by using a normal liver as a surrogate control mentioned above. However, promoter demethylation in IMP3-negative ICCs suggest that DNA demethylation is necessary but not sufficient for IMP3 expression in ICC. Additional factors including histone modifications may be also involved in this complex process. It would be interesting to expand IMP3 methylation analysis to other types of cancers to assess whether expression correlates with demethylation. Targeting epigenetic modifications including DNA methylation and histone modifications is emerging as a dynamic and efficacious mechanism for cancer therapy [33]. Interestingly, a phase II clinical trial of cancer vaccination targeting IMP3 for advanced esophageal cancer has been recently reported [34]. Hypermethylation of CpG island is generally linked to transcriptional silencing, whereas demethylation or hypomethylation is associated with transcriptional activation. Genome-wide hypomethylation is a well-known phenomenon in cancer biology [35]. However, it remains debatable whether hypomethylation is a cause or consequence of tumorigenesis. There are limited data regarding the epigenetic deregulation for IMP3 expression in carcinogenesis and cancer progression. Whether IMP3 is a key driver of carcinogenesis or it is merely a manifestation of genome-wide hypomethylation phenomenon remains to be investigated. Our data suggested that IMP3 is precisely silenced through epigenetic modifications including DNA methylation in normal adult tissues. DNA demethylation and aberrant IMP3 expression resulting in the aggressiveness of tumors, although the timing of DNA demethylation and coplayers during carcinogenesis or progression remain unclear. Our hypothesis is supported by a recent study showing that a global DNA methyltransferase inhibitor 5AZaD increased IMP3 expression in an osterosarcoma cell line [18].

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