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ORIGINAL ARTICLE

EVI1 expression is associated with aggressive behavior in intrahepatic cholangiocarcinoma

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Abstract

Ecotropic virus integration site 1 protein homolog (EVI1), a well-known oncogenic transcriptional factor of hematopoietic cells, contributes to pancreatic cancer oncogenicity through increased expression of KRAS. Because EVI1 was upregulated in cholangiocarcinoma by referring The Cancer Genome Atlas, we investigated the importance of EVI1 in intrahepatic cholangiocarcinoma (ICC) which has been regarded as a heterogeneous group of cancers. Immunohistochemical analysis results demonstrated that EVI1 was overexpressed in about half of ICC (53/101, 52.5%). Moreover, all intraductal papillary neoplasms of the bile duct cases expressed EVI1 regardless of histological grading and subtypes such as gastric, intestinal, pancreatobiliary, or oncocytic (20/20, 100%). EVI1-positive ICC showed higher frequencies of aggressive pathological indicators such as periductal infiltrative growth ($p = 0.022$), hilar invasion ($p = 0.041$), advanced UICC stage ($p = 0.026$), major vascular invasion ($p = 0.026$), and perineural invasion ($p = 0.007$) than EVI1-negative ICC. Patients with EVI1-positive ICC showed worse overall survival and recurrence-free survival in all resected cases and in curative resected cases. Recently, we proposed type 1/2 (large/small duct types) classification of ICC based on mucin productivity and immunophenotypes (S100P, N-cadherin, and NCAM). Type 1 predominantly consisted of EVI1-positive ICC (33/42 cases, 79%), and the frequency was significantly higher than type 2 (18/55 cases, 32.7%) ($p < 0.0001$). EVI1-positive ICC was likely to express stomach-specific claudin CLDN18 (correlation coefficient $r = 0.55373$) and mucin MUC5AC ($r = 0.42718$). EVI1-positive ICC is an aggressive ICC showing both large-duct and/or gastric phenotypes. Consequently, a transcriptional factor EVI1 is associated with aggressive behavior in ICC and can be a therapeutic target molecule, while EVI1 might be a key molecule for the development of intraductal papillary neoplasms of the bile duct.

Keywords EVI1 · Intrahepatic cholangiocarcinoma · Type 1 · Type 2 · Gastric phenotype

Introduction

Prognoses of intrahepatic cholangiocarcinoma (ICC), the second most common liver cancer next to hepatocellular carcinoma, are poor because of its typical early invasion and widespread metastasis, and because of a lack of effective therapeutic modalities [1]. In contrast to hepatocellular carcinoma, no approved targeted molecular therapy exists for ICC. Biliary tract and pancreas progenitors develop from endoderm cells in the embryonic foregut [2].

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Pathological conditions are sometimes similar in these organs, especially in hilar, extrahepatic bile duct, and pancreas [3–5]. For example, several inflammatory biliary diseases exist, such as IgG4-related disease and primary sclerosing cholangitis, and several biliary neoplastic lesions such as biliary intraepithelial neoplasia (BilIN) and intraductal papillary neoplasm of the bile duct (IPN-B), with pancreatic counterparts showing similar features [3]. Regarding the relevant genetic background, *KRAS* mutations apparently occur as an early molecular event during the progression of pancreatic intraepithelial neoplasia (PanIN) to pancreatic ductal adenocarcinoma and BilIN to cholangiocarcinoma, whereas p53 overexpression was identified as a late molecular event in pancreatic and biliary carcinogenesis [6].

Cholangiocarcinomas arise from different anatomical locations. They are classified as intrahepatic, perihilar, or distal type according to the staging of the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) system [7]. Recently, several reports have described ICC as heterogeneous. Precise classification has been proposed, with ICC divided into large-duct (type 1) and small-duct (type 2) types [8, 9]. Moreover, recent studies have extracted the aggressive characteristic group of ICC. For example, the ICC cases expressing foregut or gastric epithelium-like molecules such as gastric-specific tight junction molecule, claudin-18 (CLDN18), or gastric mucin such as MUC5AC or MUC6 showed high incidence of metastasis and worse prognosis [10–14].

Recently, we reported that ecotropic virus integration site 1 protein homolog (EVI1), a well-known oncogenic transcriptional factor of hematopoietic cells, plays an important oncogenic role for pancreatic cancer through increased expression level of KRAS [15]. Actually, we confirmed that EVI1 was upregulated in cholangiocarcinoma by referring The Cancer Genome Atlas (http://firebrowse.org/viewGene.html?gene=MECOM, Supplementary Fig. 1). Therefore, we investigated the importance of EVI1 in ICC. Furthermore, EVI1 is expressed immunohistochemically by gastric epithelium and is associated with gastric-related genes in the mRNA expression pattern [15]. We also investigated the correlation of EVI1 expression with bile duct and gastric phenotypes.

Material and methods

Cases

From The University of Tokyo Hospital pathology archives of 1995–2013, we reviewed 101 consecutive treated cases of ICC and 20 cases of intraductal papillary neoplasms of the bile duct (IPN-Bs). We retrieved records for 101 ICC of the non-papillary and mass-forming type with or without periductal infiltrative growth, and we followed the Union for International Cancer Control (UICC) definition which differentiated ICC from perihilar cholangiocarcinoma at the second-order bile duct. No cases were received preoperative treatment. All cases were examined in our previous study [8]. The intrahepatic IPN-Bs included 3 low-grade dysplasia, 11 high-grade dysplasia, and 6 carcinomas with invasion. The histological diagnosis of each lesion was based on the World Health Organization classification [1].

All aspects of this study were approved by The University of Tokyo Ethics Committee. Pathology reports and tissue slides were reviewed along with medical charts when necessary. Recurrent cases and cases with neoadjuvant treatment that significantly affected tumor morphology were excluded.

As a control material, we retrieved normal and inflamed mucosa from surgical specimens of hepatolithiasis or primary sclerosing cholangitis. All specimens were reviewed independently by two pathologists (MT and JS). Consensus between these pathologists was reached in all cases.

Histopathological examination

For each case, all tissue slides were reviewed. The entire tumor of the surgically resected specimen was fixed in 10% formalin at room temperature and was sectioned at intervals of 0.5–1.0 cm, with all tumor-containing sections processed routinely and embedded in paraffin. Serial sections of each tumor were cut and were stained with hematoxylin and eosin (H&E).

Immunohistochemical staining was performed for surgically resected specimens. The following primary antibodies were used: EVI1 C50E12 (1:1000; Cell Signaling Technology Inc., Danvers, MA, USA), CLDN18 (ZMD395, 1:1000; Zymed Laboratories Inc. Thermo Fisher Scientific Inc., Waltham, MA, USA), MUC5AC (CLH2, 1:100; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), and MUC6 (CLH5, 1:100; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). Normal gastric epithelial cells were used as a control for MUC5AC, MUC6, CLDN18, and EVI1. For analyses, 4-μm-thick, paraffin-embedded tissue sections from the representative area of each case were used. Immunohistochemical staining in surgically resected specimens was conducted according to standard techniques for a Ventana Benchmark® XT Autostainer (Roche Diagnostics Corp, Basel, Switzerland).

EVI1 was expressed in the nucleus. EVI1 expression was evaluated according to the staining pattern and intensity. The immunostaining of EVI1 was evaluated as negative, weakly positive, moderately positive, or strongly positive. Labeling of EVI1 was regarded as (3+) if more than 50% of the neoplastic cells were labeled at a moderate to strong intensity; (2+) if 25–

50% of the neoplastic cells were labeled at moderate to strong intensity, (1+) if 10–25% of the neoplastic cells were labeled at moderate to strong intensity, or if 25–100% of the neoplastic cells were labeled at weak intensity; and (0) if fewer than 10% of the neoplastic cells were labeled at any intensity. Two pathologists blindly and independently evaluated the immunohistochemical specimens.

To determine types 1 and 2, the methods were reported earlier [8], but they are briefly described as follows. We classified ICC into type 1 and type 2 based on an assessment of the following three factors: mucin productivity, S100P immunoreactivity score, and combined scores of N-cadherin and NCAM immunoreactivity. Typically, type 1 cases showed mucin production and diffuse immunoreactivity to S100P. Type 2 cases showed little mucin production and immunoreactivity to *N*-cadherin and/or NCAM.

Statistical analysis

Quantitative variables were compared using the Student *t* test. Categorical variables were compared using chi-square test or Fisher's exact test with software (JMP Pro 13.0; SAS Institute Inc., Cary, NC, USA). $p < 0.05$ was inferred as significant ($^*p < 0.05$). Overall and recurrence-free survival curves were constructed using the Kaplan–Meier method and compared using the log-rank test with software (JMP Pro 13.0). Univariate and multivariate Cox regression analyses were performed to ascertain the prognostic factors using EZR software (http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html Saitama Medical Center, Jichi Medical University). The factors of multivariate Cox regression analyses were generated using stepwise method using Bayesian information criterion. We analyzed the correlation coefficient using excel CORREL Function.

Results

EVI1 expression in non-neoplastic and neoplastic biliary tract lesions

Non-neoplastic intrahepatic biliary epithelium of any size was negative or only very weakly positive for EVI1 (Fig. 1a, b). Hepatocytes were also negative or only very weakly positive for EVI1. No vascular endothelial cells were immunoreactive for EVI1.

By contrast, many intrahepatic neoplasms exhibited frequent immunoreactivity for EVI1 in the nucleus. In about half of ICCs, EVI1 was expressed (53/101, 52.5%) (Fig. 1c, d). In intrahepatic IPN-Bs, all tumors were positive for EVI1 (20/20, 100%) (Fig. 1e, f).

Clinicopathological features of EVI1-positive intrahepatic cholangiocarcinoma

EVI1 expression was observed in 53 cases (53%) of ICC. To assess the clinical significance of EVI1 expression, we compared the clinicopathologic features of EVI1-positive and EVI1-negative cases (Table 1). No significant difference was found among patients' clinical backgrounds. Regarding macroscopic features, comparing EVI1-negative cases, EVI1-positive cases were often associated with periductal infiltrative growth ($p = 0.022$) and hilar invasion ($p = 0.041$). Microscopically, major vascular invasion, perineural invasion, and advanced UICC stage (stage III to IV) were observed more frequently in EVI1-positive cases ($p = 0.026$, $p = 0.007$, and $p = 0.026$, respectively). Results of survival analyses using Kaplan–Meier curves are depicted in Fig. 2. EVI1-positive cases were associated with poorer recurrence-free survival and overall survival than EVI1-negative cases in all resected cases ($p = 0.0287$ and $p = 0.0036$, respectively) and in curative resected cases ($p = 0.0327$ and $p = 0.0019$, respectively).

Univariate and multivariate Cox regression analyses were conducted to identify prognostic factors of ICC (Table 2). The prognostic factors for recurrence-free survival were EVI1 expression, HBV infection, tumor size, advanced stage, periductal infiltration on macroscopic appearance, hepatic hilar connective tissue invasion, major vessel invasion, perineural invasion, intrahepatic metastasis, lymph node metastasis, and surgical margin on univariate analysis. Regarding overall survival, EVI1 expression, advanced stage, periductal infiltration on macroscopic appearance, hepatic hilar connective tissue invasion, co-existing dysplasia, poorly differentiated histology, major vessel invasion, perineural invasion, intrahepatic metastasis, lymph node metastasis, and surgical margin were important for univariate analysis. We applied the multivariate analysis using stepwise method. Finally, the multivariate analysis revealed that HBV infection, advanced stage, major vascular invasion, and intrahepatic metastasis were important for recurrence-free survival, and that EVI1 expression, intrahepatic metastasis, and surgical margin were prognostic factors for overall survival.

Relation between EVI1 expression and specific subtype/phenotype of intrahepatic cholangiocarcinoma

Recently, several reports have presented some classification or some extraction of a specific phenotype in ICC [8, 9] and ICC is divided into large duct type (type 1) and small duct type (type 2). Furthermore, some aggressive cases can be extracted using immunohistochemical analysis. For example, ICC expressing foregut or gastric epithelium-like molecules behaved

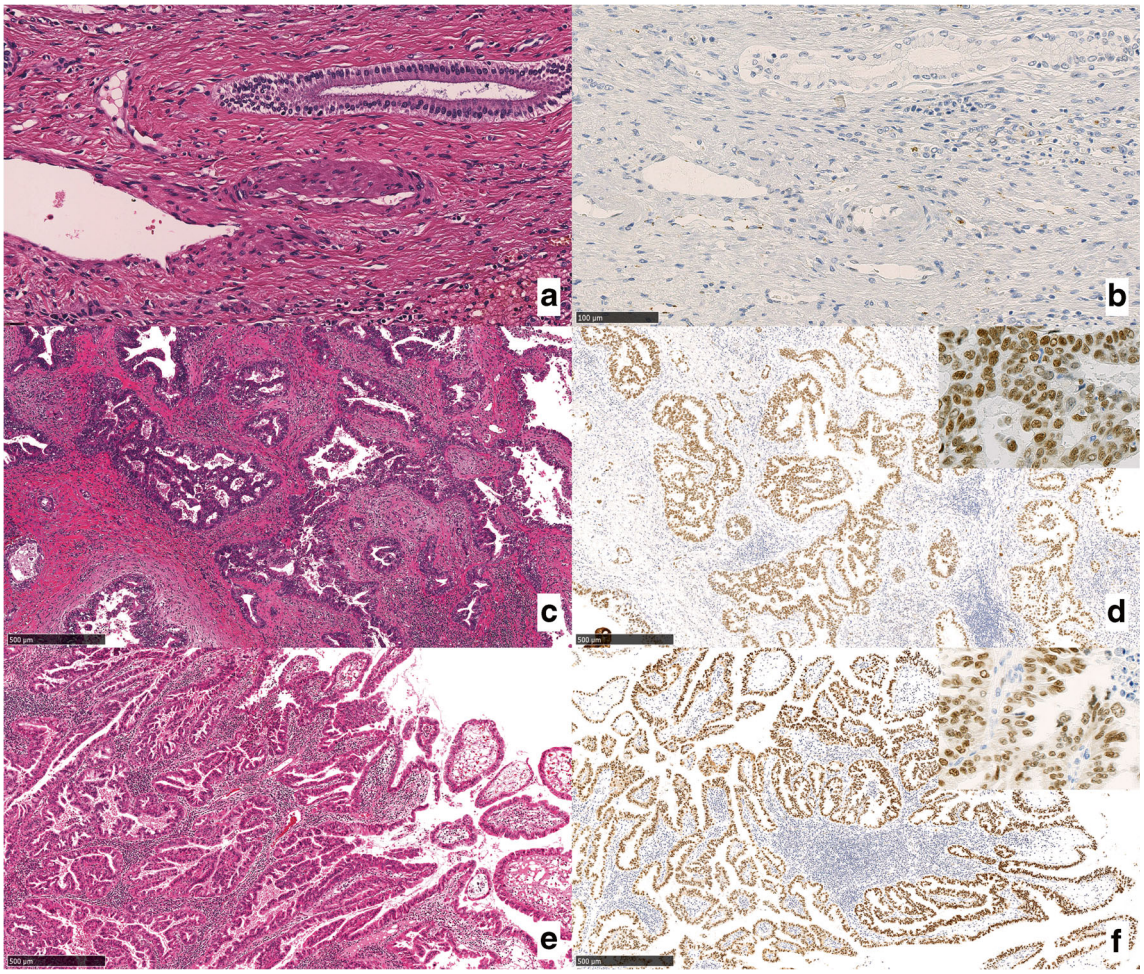

Fig. 1 EVI1 expression in non-neoplastic biliary tract epithelium and intrahepatic bile duct tumors. **a**, **b** Non-neoplastic biliary tract epithelium. Normal small bile ducts do not express EVI1 (**a** hematoxylin and eosin (H&E) staining; **b** EVI1 immunostaining, × 200). **c**, **d** Intrahepatic cholangiocarcinoma expressed EVI1 in the nucleus with strong intensity (**c** H&E; **d** EVI1, × 50 (inset, × 400)). **e**, **f** Intraductal papillary neoplasm of the bile duct expressed EVI1 in the nucleus with strong intensity (**e** H&E; **f** EVI1, × 50 (inset, × 400))

aggressively. Then, we examined the correlation between EVI1 positive cases and subtype or phenotype (Fig. 3).

We divided these 101 cases of ICC into type 1 and type 2 based on mucin productivity and immunophenotype (S100P, *N*-cadherin, and NCAM) as reported previously [8]. Type 1 ICC included more EVI1-positive cases (33/42, 78.6%) than type 2 ICC (18/55, 32.7%) ($p < 0.0001$, Fig. 3a).

Regarding gastric phenotypes, EVI1 expression was correlated with gastric marker expression such as CLDN18 (correlation coefficient $r = 0.55373$) and MUC5AC ($r = 0.42718$) in ICC (Fig. 3).

Discussion

In this study, EVI1 was expressed in intrahepatic biliary neoplasms; 100% of intraductal papillary neoplasm of bile duct (IPN-B) and 53% of ICC cases, in contrast to negative expression in normal intrahepatic biliary tract epithelium. Importantly, EVI1-positive cases were associated with higher frequency of periductal infiltrative growth, major vascular invasion, and perineural invasion, and showing unfavorable prognosis. For them, EVI1 expression was an independent prognostic factor.

Recently, two subtypes of ICC were proposed. One type is thought to derive from small bile ducts or ductules consisting of tubular adenocarcinoma with scant mucus. The other type is thought to derive from the large bile ducts and is thought to be located at the perihilar location [8, 9, 16]. We proposed type 1 (almost all corresponding to perihilar, large duct type) and type 2 (almost all corresponding to peripheral and small duct type) as one classification [8]. The use of next-generation sequencing for ICC revealed recurrent actionable molecular alterations such as *TP53*, *IDH1*, and *KRAS* mutation [17–19] or amplification [20]. *KRAS* mutation was enriched in type 1 subtype, but *IDH1* mutation was enriched in type 2 subtype

Table 1 EVI1 expression and clinicopathologic features of intrahepatic cholangiocarcinomas

	EVI1-positive	EVI1-negative	*p* value
Age [> 65 years, ≤ 65 years]	30/23	24/24	0.506
Sex [M/F]	33/20	22/26	0.098
Hepatitis B virus infection [positive/negative]	5/48	4/43	1
Hepatitis C virus infection [positive/negative]	14/39	10/38	0.51
Tumor size [> 50 mm, ≤ 50 mm]	20/33	21/27	0.539
Periductal infiltrative growth [present/absent]	21/32	9/39	0.022*
Hilar invasion [present/absent]	26/27	14/34	0.041*
Co-existing dysplasia [present/absent]	6/47	3/45	0.493
Histology [poor/well to moderate]	6/47	3/45	0.493
UICC stage [III to IV/I to II]	22/31	10/38	0.026*
Major vascular invasion [present/absent]	12/41	3/45	0.026*
Perineural invasion [present/absent]	33 /20	17/31	0.007*
Intrahepatic metastasis [present/absent]	9/44	13/35	0.219
Lymph node metastasis [present/absent]	14/39	9/39	0.359

*$p < 0.05$

[8, 9]. Our results demonstrated that EVI1-positive cases were dominant in the type 1 subtype. Above all, it is possible that the type 1 subtype depends on the KRAS pathway and that some relation exists between EVI1 and KRAS. We reported recently that EVI1 overexpression and *KRAS* mutation converge on activation of the KRAS pathway in pancreatic carcinogenesis [15]. EVI1 might upregulate KRAS pathway and might contribute to carcinogenesis of type 1 subtype in ICC.

EVI1 was also expressed in IPN-B regardless of histological grading and subtypes such as gastric, intestinal, pancreatobiliary, or oncocytic. The finding is in parallel with that in EVI1 expression in pancreatic neoplasms, which is at high frequency in both pancreatic duct adenocarcinoma (PDAC) and precursor lesions, such as PanIN and pancreatic intraductal papillary neoplasm (IPMN). Since EVI1 was closely associated with aggressive behavior of ICC as described above, the role of EVI1 in precursor lesions may be different from that in overt carcinomas. *KRAS* mutation is a good example of the context-dependent role of oncogene. Additional mutations such as *CDKN2A*, *TP53*, or *SMAD4*

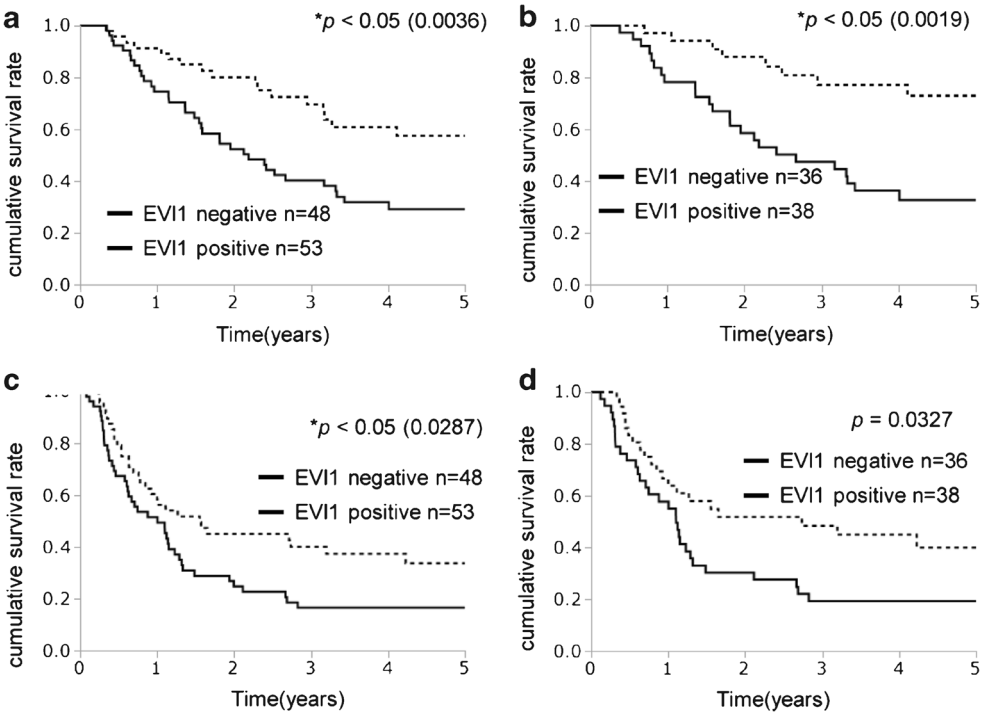

Fig. 2 Survival analysis of intrahepatic cholangiocarcinoma. **a** Overall survival of EVI1 +/− cases. **b** Overall survival of EVI1 +/− cases with curative resection. **c** Recurrence-free survival of EVI1 +/− cases. **d** Recurrence survival of EVI1 +/− cases with curative resection

Table 2 Univariate and multivariate analysis of intrahepatic cholangiocarcinoma

Recurrence-free survival	Univariate analysis		Multivariate analysis[a]	
Factors	HR (95% IC)	*p* value	HR (95% IC)	*p* value
EVI1 expression	1.670 (1.049–2.657)	0.031*		
Age	0.991 (0.626–1.568)	0.968		
Sex	1.088 (0.687–1.722)	0.721		
Hepatitis B virus infection	2.352 (1.122–4.928)	0.023*	3.465 [1.521–7.893]	0.003*
Hepatitis C virus infection	0.583 (0.320–1.062)	0.078		
Tumor size [> 50 mm]	1.738 (1.089–2.773)	0.020*		
Stage [III-IV]	2.926 (1.780–4.810)	< 0.001*	2.376 [1.370–4.121]	0.002*
Periductal infiltrative growth	1.638 (1.009–2.661)	0.046*		
Hilar invasion	1.794 (1.130–2.848)	0.013*		
Co-existing dysplasia	1.836 (0.875–3.855)	0.108		
Histology [poor]	1.836 (0.875–3.855)	0.108		
Major vascular invasion	3.890 (2.147–7.049)	< 0.001*		
Perineural invasion	2.355 (1.467–3.780)	< 0.001*	3.073 [1.569–6.015]	0.001*
Intrahepatic metastasis	2.818 (1.679–4.730)	< 0.001*	2.583 [1.456–4.582]	0.001*
Lymph node metastasis	2.755 (1.637–4.637)	< 0.001*		
Surgical margin	2.244 (1.283–3.926)	0.005*		
Overall survival	Univariate analysis		Multivariate analysis[a]	
Factors	HR (95% IC)	*p* value	HR (95% IC)	*p* value
EVI1 expression	2.228 (1.280–3.878)	0.005*	3.153 [1.659–5.992]	< 0.001*
Age	0.972 (0.575–1.645)	0.917		
Sex	1.115 (0.656–1.894)	0.689		
Hepatitis B virus infection	1.966 (0.837–4.614)	0.121		
Hepatitis C virus infection	0.865 (0.457–1.638)	0.656		
Tumor size [> 50 mm]	1.510 (0.890–2.561)	0.126		
Stage [III-IV]	2.879 (1.673–4.954)	< 0.001*		
Periductal infiltrative growth	2.225 (1.303–3.800)	0.003*		
Hilar invasion	1.728 (1.016–2.938)	0.043*		
Co-existing dysplasia	2.775 (1.246–6.180)	0.012*		
Histology [Poor]	2.775 (1.246–6.180)	0.012*		
Major vascular invasion	3.073 (1.594–5.924)	< 0.001*		
Perineural invasion	2.777 (1.600–4.818)	< 0.001*		
Intrahepatic metastasis	2.964 (1.692–5.190)	< 0.001*	3.066 [1.625–5.788]	< 0.001*
Lymph node metastasis	2.949 (1.641–5.301)	< 0.001*		
Surgical margin	3.281 (1.778–6.055)	< 0.001*	2.513 [1.326–4.764]	0.005*

*$p < 0.05$

[a] Only significant factors are presented in the table, since all significant factors by univariate analysis were evaluated by multivariate Cox regression analyses using stepwise method with Bayesian information criterion

are needed for the progression to adenocarcinoma in the *KRAS* G12D transgenic mouse model [21]. On the other hand, different mutations, such as *TGF alpha*, *Smarca4/Brg1*, *GNAS*, *Avcr1a*, or *PTEN* loss, drive ductal cells with the same *KRAS* mutation to IPMN [22, 23]. We speculate that EVI drives different sets of oncogene in precursor lesions and overt carcinomas of intrahepatic biliary epithelium.

Acquisition phenotypes of gastric epithelium are apparently important in several carcinogenic processes in pancreas and bile ducts. Some researchers have specifically examined gastric mucin such as MUC5AC or MUC6 [24, 25]. We have also taken notice of the gastric epithelial tight-junction molecules CLDN18 in biliary carcinogenesis [11]. Our results demonstrate that EVI1 was expressed in all cases of IPN-Bs and in about half cases of ICC. Regarding IPN-Bs, all cases were positive for CLDN18 as reported previously [11]. Moreover, as for ICC, correlation between the expression of EVI1 and the expression of gastric markers was confirmed.

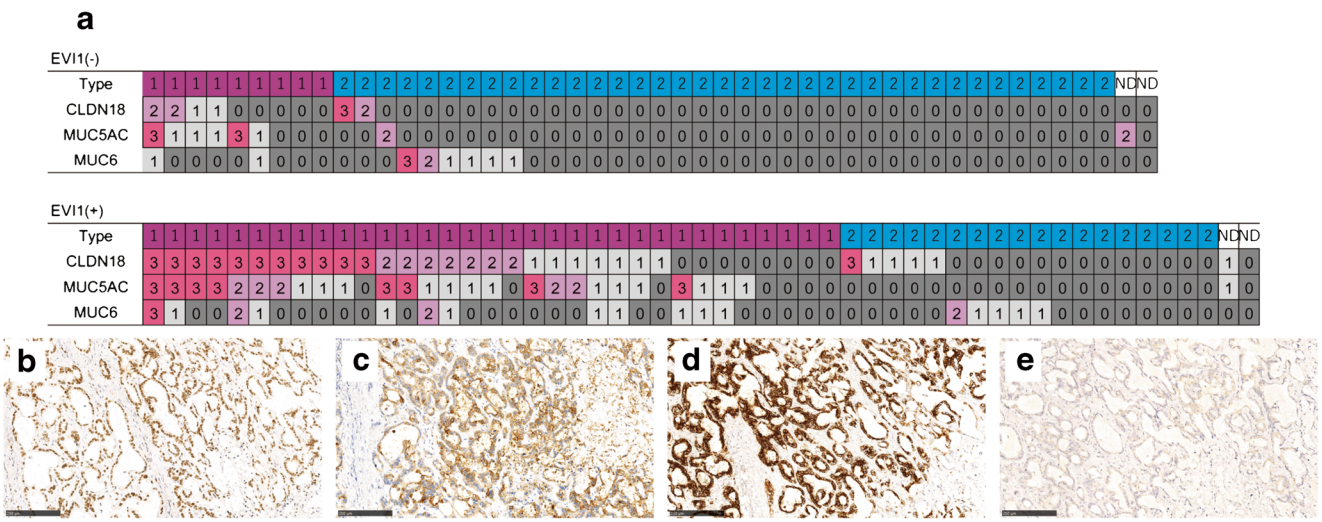

Fig. 3 Comparison among EVI1 and type1/type2 subtype and gastric marker expression in intrahepatic cholangiocarcinoma. **a** Type 1 intrahepatic cholangiocarcinoma included more EVI1-posivite cases than type 2 intrahepatic cholangiocarcinoma ($p < 0.0001$, **a**). ND not determined. **b** Gastric marker expression in EVI1-positive intrahepatic cholangiocarcinoma (**b** EVI1; **c** CLDN18; **d** MUC5AC; **e** MUC6, × 100). CLDN18, and MUC5AC strongly expressed and MUC6 weakly expressed in EVI1-positive case

These results may indicate EVI1 as an important factor for gastric metaplasia and subsequent carcinogenesis of the biliary tract.

Treatment of ICC is difficult: the great molecular target must be found. Recently, an EVI1-targeted molecule was produced [26]. It is a sequence-specific pyrrole-imidazole polyamide, PIP1, which can target specific base pairs of the REL/ELK1 binding site in the EVI1 minimal promoter. This designed PIP1 can inhibit EVI1 in vitro. EVI1 inhibitor such as EVI1-targeted PIP1 might be a useful therapy for EVI1-positive ICC.

In summary, we clarified EVI1 expression in ICC and found that EVI1 is associated with poor outcomes in ICC and EVI1-positive ICC showed specific pathological characteristics. It is a convenient marker for prognosis and is a potential key target to elucidate the mechanisms of this complicated cancer.

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Author contributions MT and MF contributed to the design and organization and conducted the study and wrote the manuscript. JS, AH, and KM created the pathological database. SI, TU, TM, AS, AT, and HK advised the direction of study and the interpretation of the data. KS helped in the immunohistochemical process. TK, YI, JA, YS, and KH contributed to provide surgical samples and clinical data. All the authors reviewed and accepted the manuscript.

Compliance with ethical standards

The study was approved by the institutional review board.

Conflict of interest The authors declare that they have no conflict of interest.

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