Virchows Archiv (2019) 475:49–58
https://doi.org/10.1007/s00428-018-02511-7

ORIGINAL ARTICLE

DLEC1 methylation is associated with a better clinical outcome in patients with intrahepatic cholangiocarcinoma of the small duct subtype

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Received: 10 October 2018 / Revised: 7 December 2018 / Accepted: 12 December 2018 / Published online: 4 January 2019

Abstract

Intrahepatic cholangiocarcinoma is a complex disease with three different histologic subtypes, the large duct, small duct, and bile ductular types. In the present study, we elucidated whether the three histological subtypes have differences in their methylation profiles and developed a DNA methylation marker that might help identify a subset of ICC with a different prognosis. We screened 113 promoter CpG island loci against 10 cases of intrahepatic cholangiocarcinoma and normal cystic ducts using the MethyLight assay and selected 30 CpG island loci with cancer-associated hypermethylation. Then, we analyzed 172 intrahepatic cholangiocarcinomas for the methylation state at these 30 loci. Six loci, including *DLEC1*, were more frequently methylated in the bile ductular type and small duct type, whereas six loci were more frequently methylated in the large duct type. Of these 30 loci, *DLEC1* methylation was found mainly in the bile ductular type and small duct type but rarely in the large duct type. *DLEC1* methylation was significantly associated with a better clinical outcome in intrahepatic cholangiocarcinomas of the small duct type but not of the bile ductular type. *DLEC1* methylation was an independent prognostic variable in both cancer-specific survival and recurrence-free survival. For patients with intrahepatic cholangiocarcinoma of the small duct type ($n = 68$), *DLEC1* methylation was found in 26 (38.2%) and was associated with a better clinical outcome for both cancer-specific survival and recurrence-free survival. Our findings suggest that *DLEC1* methylation can be utilized to identify a subset with a better prognosis in intrahepatic cholangiocarcinomas of the small duct type.

Keywords Biomarker · CpG island · DLEC1 · Histologic type · Intrahepatic cholangiocarcinoma · Methylation · Prognosis

Introduction

Cholangiocarcinoma (CC), the second most common malignancy of the liver, is classified into three categories according to its location within the biliary tree: intrahepatic, perihilar, and extrahepatic CCs. Intrahepatic cholangiocarcinomas (ICCs) are tumors occurring in the bile duct within the liver and are differentiated from perihilar CCs in that the latter occurs at the confluence of the right and left bile ducts. Unlike perihilar or extrahepatic CCs, ICCs have multiple cell types of origin, including bipotential hepatic progenitor cells at the canals of Hering, cholangiocytes lining the intrahepatic large bile ducts, interlobular bile ducts, or bile ductules, and peribiliary gland cells [2, 7, 14, 26]. Of the three gross types of ICCs, ICCs of the periductal infiltrating (PI) type and those of the intraductal growth (IG) type are hypothesized to arise from cholangiocytes lining the intrahepatic large bile ducts or peribiliary gland cells, whereas ICCs of the mass-forming (MF) type are predicted to develop from hepatic progenitor cells at the canals of Hering or cholangiocytes lining the intrahepatic small bile ducts or bile ductules. Although histological subtyping of ICC was not provided in the World Health Organization classification, ICCs can be classified into histological subtypes, large bile duct (LD) type, small bile duct (SD) type, and bile ductular (BD) type, based on the level

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00428-018-02511-7) contains supplementary material, which is available to authorized users.

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or size of the affected bile ducts and resemblance of tumor cells to bile duct cells or bile ductule cells [1, 15, 20]. ICCs of the PI or IG gross type are histologically of the LD type, whereas ICCs of the MF gross type are either of the SD or BD type [18, 35].

Depending on the histological subtype (LD, SD, and BD types), ICCs exhibit differential clinicopathological features, including gross type, mucin production, association with viral hepatitis, association with pre-existing biliary inflammation or intraepithelial neoplasia, and molecular alterations [2, 4, 18]. The SD and BD types show associations with the MF gross type, ambiguous or no mucin production, chronic liver disease, and *IDH1/2* mutations, whereas the LD type exhibits association with either PI or IG gross type, mucin production, and *KRAS* mutations [1]. Recent next-generation sequencing-based studies listed genetic alterations in ICCs but did not explore their relationships with the histological subtypes [8, 13, 19, 23]. Furthermore, these genome-wide profiling studies of genetic changes did not investigate DNA methylation changes, although epigenetic genes, including *IDH1/2*, *BAP1*, *ARID1A*, and *PBRM1*, were found mutated [13, 19].

Promoter CpG island hypermethylation is found in virtually all tissue types of human cancers. For ICCs, the majority of studies analyzed the aberrant methylation of specific candidate genes, but few studies examined methylation profiles throughout the genome [10, 12, 33]. Regardless of the analysis scale, the relationship between aberrant methylation and histological subtypes was not investigated. Because ICC is a heterogeneous disease entity in terms of cell of origin and DNA methylation, signature for cell of origin might be stably maintained during carcinogenesis [3], differential methylation profiles are expected between histological subtypes. In the present study, we determined whether histological subtypes differ in their tendency towards aberrant promoter CpG island hypermethylation or whether a subset of CpG islands are differentially methylated between histological subtypes. In addition, we sought to identify DNA methylation markers which showed significant associations with survival of patients with ICC.

Material and methods

Patients

Formalin-fixed, paraffin-embedded tissue samples were obtained from 172 patients who underwent surgical resection for ICC at the Seoul National University Hospital between January 2005 and December 2012. Pathologic stages were determined using the 7th version of the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) tumor, node, metastasis (TNM) staging system. Gross types were determined using the 4th edition World Health Organization classification system for cholangiocarcinoma. Tumor was classified as mixed gross type when more than one gross type was found. For multiple tumors, the tumor with a larger size was considered a representative tumor. Tumor grades were determined at the invasive front as described previously [22]. Histological subtype, LD, SD, and BD types, was defined as described previously [1]. ICC was classified into SD and LD types according to the level or size of the affected bile ducts and BD type was further differentiated from the SD type by small tubular, acinar, or cord-like arrangement of small cuboidal cells in fine fibrous stroma, resembling reactive proliferating bile ductules [16]. ICCs were classified into the BD type when more than one-third of tumor area showed ductular reaction-like adenocarcinoma histology (Supplementary Fig. 1). Using microscopic examination, tissue slides were evaluated regarding perineural invasion, lymphatic invasion, venous invasion, accompaniment of biliary intraepithelial neoplasia (BilIN), or chronic liver diseases. Formalin-fixed, paraffin-embedded tissue samples from 10 patients who underwent cholecystectomy for chronic cholecystitis were included as non-neoplastic controls. The reason why cystic duct epithelia from cholecystectomy specimens were used as normal controls was that large bile ducts within the archival tissue blocks were usually denuded or degenerated by inflammation, thus hindering procurement of adequate number of normal epithelial cells for normal control. This study, including a waiver of informed consent, was approved by the Institutional Review Board (IRB No. 1804-168-942).

DNA extraction and bisulfite modification

Under microscopic examination, a tumor area that showed the highest tumor cellularity and representative histology was selected and marked. Cystic duct epithelia from cholecystectomy specimens were also marked. The marked area was scraped from the tissue slide with a scalpel blade and collected into a microtube containing tissue lysis buffer and proteinase K. After incubation at 55 °C for up to 2 days, the lysed tissue buffer solution was subjected to heat inactivation at 95 °C for 30 min. Bisulfite modification was performed using an EZ DNA methylation kit (Zymo Research, Orange, CA) according to the manufacturer's protocol.

MethyLight assay

For the initial screening of cancer-related methylation markers in ICCs, 113 promoter CpG island loci were analyzed. These 113 genes have been known to be inactivated by promoter CpG island hypermethylation in human cancers.

Oligonucleotide sequences of primers and probes for 113 genes are available upon request. Methylation analyses of 113 genes were conducted using the MethyLight assay as previously described [17] Briefly, bisulfite-modified DNA samples were subjected to the MethyLight assay for the quantitative measurement of methylation in CpG island loci. The percentage of the methylated reference (PMR) value was obtained for each CpG island locus as previously described. Hypermethylation of a CpG island locus was defined when the percentage of the methylated reference value was > 4.

Statistical analysis

Associations between methylation of individual genes and three histological subtypes were analyzed using a chi-square test. Associations between clinicopathological variables and hypermethylation of the *DLEC1* promoter CpG island locus were analyzed using a chi-square test or Fisher's exact test. Student's *t* test or ANOVA test was used to compare means between two subgroups or three groups, respectively. The Kaplan-Meier method was used to estimate cumulative survival rates, and differences in survival rates were assessed with a log-rank test. The cancer-specific survival (CSS) was measured from the date of surgical removal of the primary tumor to the date of ICC-related death or the most recent clinical follow-up. The recurrence-free survival (RFS) was calculated from the date of the primary surgery to the date of recurrence or the date of death (whichever came first). Variables predicted to be prognostic factors on the univariate analysis ($P < 0.05$) were entered into a multivariate analysis using a Cox proportional hazards regression model. SPSS ver. 25 (IBM SPSS Statistics, Armonk, NY, USA) was used for all statistical analyses. All *P* values were two-sided, and statistical significance was established at $P < 0.05$.

Results

Demographic and clinicopathological findings of 172 ICC cases are summarized in Table 1. The male to female ratio was 121:51 and the mean age of ICC patients was 62.8 years (median, 63 years; range, 38 to 80 years). Gross types were the MF type in 141 patients, PI type in 8 patients, IG type in 18 patients, and mixed type in 5 patients. Histologic subtypes were the BD type in 22 patients, SD type in 68 patients, and LD type in 82 patients. Cancer stages were as follows: 40 in stage I, 37 in stage II, 30 in stage III, and 65 in stage IV. Of the cases, 42 and 13 cases of ICC were accompanied by chronic liver diseases and chronic biliary diseases, respectively.

DNA methylation profiles of ICCs and their difference between histological subtypes

To identify cancer-associated DNA methylation markers, we analyzed a subset of ICC tissue samples ($n = 10$) and a control set of cystic duct tissue samples ($n = 10$) for their methylation states in 113 gene CpG island loci using the MethyLight assay. We selected 30 CpG island loci that were more methylated in ICCs than in cystic duct tissues, with a difference of 20% or more (Supplementary Fig. 2). Then, we analyzed 172 ICC tissue samples for their methylation statuses in the selected 30 cancer-specific CpG island loci and generated DNA methylation profiles of the 30 loci in the ICC cases (Supplementary Fig. 3). When we compared the number of methylated loci among three histological subtypes, there was no difference (16.8 for the BD type, 16.4 for the SD type, and 16.9 for the LD type). However, when the methylation frequency of individual loci was compared between histologic subtypes, 40% of the examined loci showed significant differences among the LD type, SD type, and BD type (Fig. 1). No significant difference was noted in methylation frequency of individual loci between the BD type and the SD type. Six loci were more frequently methylated in the BD type or SD type than in the LD type, including *DLEC1*, *RASSF1A*, *RIP3*, *SOCS3, PTGS2*, and *TNFRSF10C* (in decreasing order of statistical significance). Six loci were more frequently methylated in the LD type than in the SD or BD type, including *SH3GL3*, *ADAMSTSL3*, *CDH13*, *KCNQ5*, *TWIST1*, and *CPEB1* (in decreasing order of statistical significance). Of these 12 loci, the significant difference was the highest for *DLEC1*. *DLEC1* was methylated in 45% of the BD type and 38% of the SD type but in 4.9% of the LD type ($P = 2.85 \times 10^{-7}$) (Supplementary Table 1).

Survival analysis

When 30 methylation markers were analyzed for their prognostic values in ICCs using a univariate survival analysis of CSS and RFS (Supplementary Table 2), *DLEC1* was the only marker that was significant both in the CSS and RFS (Fig. 2a, b). Because *DLEC1* methylation was mainly found in ICCs of the MF gross type or ICCs of the SD or BD histological type, a univariate survival analysis was performed with restriction to ICCs of the MF gross type or ICCs of the SD or BD histological type. *DLEC1* methylation was strongly associated with a better prognosis of CSS and RFS in patients with ICC of the MF gross type (Fig. 2c, d) and in patients with ICC of the SD histological type (Fig. 2e, f), but not in the BD histological type (Fig. 2g, h). Although *DLEC1* methylation was frequent in ICCs of the BD type as much as in ICCs of the SD type, prognostic implication of *DLEC1* methylation was contrasted between ICCs of the BD type and SD type. To determine whether *DLEC1* methylation was independently significant in CSS, not only *DLEC1* methylation but also T category, N

Table 1 Clinicopathological features of intrahepatic cholangiocarcinoma with DLEC1 methylation

			DLEC1		*P* value
		n	Unmethylated	Methylated	
Sex	M	121	87 (71.9%)	34 (28.1%)	0.029
	F	51	45 (88.2%)	6 (11.8%)	
Age	< 64 years	87	65 (74.7%)	22 (25.3%)	0.590
	≥64 years	85	67 (78.8%)	18 (21.2%)	
Gross type	Mass forming	141	102 (72.3%)	39 (27.7%)	0.024
	Periductal infiltrative	8	8	0	
	Intraductal growing	18	18	0	
	Mixed	5	4 (80%)	1 (20%)	
Growth type	Expanding type	32	18 (56.3%)	14 (43.8%)	0.005
	Infiltrative type	140	114 (81.4%)	26 (18.6%)	
Differentiation	Well	23	21 (91.3%)	2 (8.7%)	0.028
	Moderate	94	75 (79.8%)	19 (20.2%)	
	Poor	55	36 (65.5%)	19 (34.5%)	
Histologic	Bile ductular type	22	12 (54.5%)	10 (45.5%)	< 0.001
subtype	Small duct type	68	42 (61.8%)	26 (38.2%)	
	Large duct type	82	78 (95.1%)	4 (4.9%)	
Mucin	Absent	78	50 (64.1%)	28 (35.9%)	0.002
Production	Present	94	82 (87.2%)	12 (12.8%)	
Lymphatic	Absent	102	81 (79.4%)	21 (20.6%)	0.36
emboli	Present	70	51 (72.9%)	19 (27.1%)	
Venous	Absent	95	75 (78.9%)	20 (21.1%)	0.472
invasion	Present	77	57 (74.0%)	20 (26.0%)	
Perineural	Absent	118	84 (71.2%)	34 (28.8%)	0.011
invasion	Present	54	48 (88.9%)	6 (11.1%)	
Chronic liver	Absent	130	106 (81.5%)	24 (18.5%)	0.012
disease	Present	42	26 (61.9%)	16 (38.1%)	
Biliary intraepithelial neoplasia*	Absent	102	64 (62.7%)	38 (37.3%)	< 0.001
	Present	65	63 (96.9%)	2 (3.1%)	
T category	T1	48	38 (79.2%)	10 (20.8%)	0.259
	T2a	38	24 (63.2%)	14 (36.8%)	
	T2b	14	11 (78.6%)	3 (21.4%)	
	T3	47	39 (83.0%)	8 (17.0%)	
	T4	25	20 (80.0%)	5 (20.0%)	
N category**	pN0	86	100 (75.2%)	33 (24.8%)	0.518
	pN1	39	32 (82.1%)	7 (17.9%)	
M category	pM0	161	123 (76.4%)	38 (23.6%)	1
	pM1	11	9 (81.8%)	2 (18.2%)	
TNM staging	Stage I	40	32 (80.0%)	8 (20.0%)	0.122
	Stage II	37	22 (59.5%)	15 (40.5%)	
	Stage III	30	24 (80.0%)	6 (20.0%)	
	Stage IVA	54	45 (83.3%)	9 (16.7%)	
	Stage IVB	11	9 (81.8%)	2 (18.2%)	

* 5 cases could not be assessed for biliary intraepithelial neoplasia

** 47 cases of surgically resected ICC did not have lymph nodes

category, lymphatic emboli, perineural invasion, gross type, histological type, and histological differentiation at invasive front were included in the multivariate analysis, which revealed that *DLEC1* methylation was an independent prognostic parameter heralding a better prognosis in patients with ICC (Table 2). Multivariate analysis of RFS also revealed that *DLEC1* methylation was independently associated with a better prognosis in patients with ICC (Table 3).

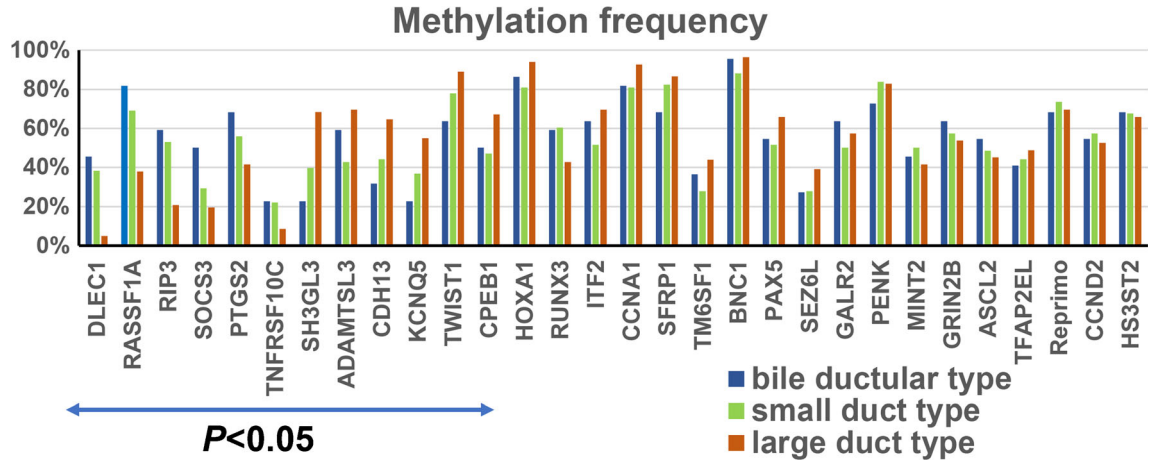

Fig. 1 Comparison of the methylation frequencies in 30 CpG island loci among ICCs of the bile ductular type, small duct type, and large duct type

DLEC1 methylation vs. clinicopathological features

Of 172 cases, 40 cases (23.3%) showed *DLEC1* methylation (Table 1). *DLEC1* methylation was more frequent in ICCs of male patients than in ICCs of female patients (28.1% vs. 11.8%, $P = 0.029$), in ICCs of the MF gross type than in ICCs of the other three gross types (27.7% vs. 3.3%, $P = 0.024$), in ICCs of the expanding growth type than in ICCs of the infiltrative growth type (43.8% vs. 18.6%, $P = 0.005$), in ICCs with poorly differentiated histology at the invasive front than in ICCs with moderately or well differentiated histology at the invasive front (34.5% vs. 20.2% or 8.7%, $P = 0.002$), in ICCs with no mucin production than in ICCs with mucin production (35.9% vs. 12.8%, $P = 0.002$), in ICCs with no perineural invasion than in ICCs with perineural invasion (28.8% vs. 11.1%, $P = 0.011$), in ICCs with no accompaniment of BilIN than in ICCs with accompaniment of BilIN (37.3% vs. 3.1%, $P < 0.001$), and in ICCs accompanied with chronic hepatitis than in ICCs not accompanied with chronic hepatitis (38.1% vs. 18.5%, $P = 0.012$). However, *DLEC1* methylation was not associated with T staging, N staging, M staging, lymphatic invasion, or venous invasion.

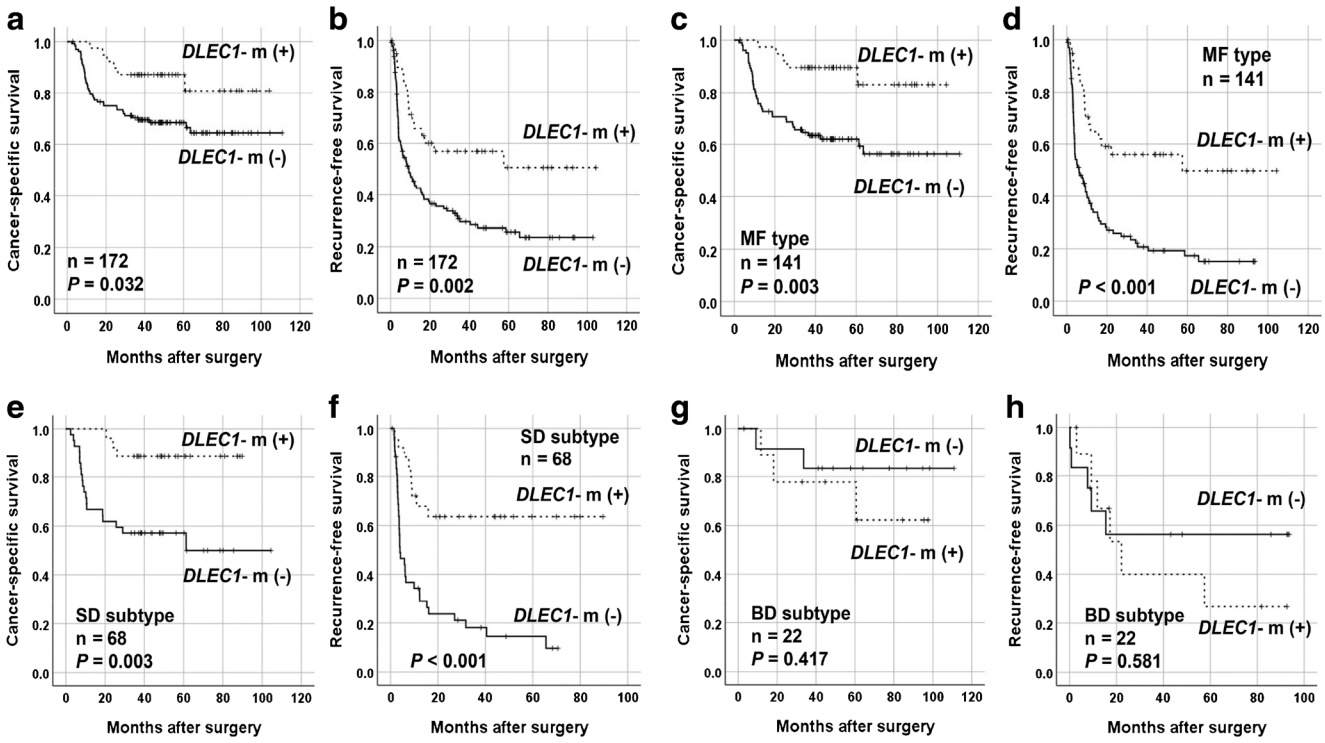

Fig. 2 Kaplan-Meier log-rank test of cancer-specific survival (**a**, **c**, **e**, and **g**) and recurrence-free survival (**b**, **d**, **f**, and **h**) in patients with ICC ($n = 172$) (**a** and **b**), patients with ICC of the mass-forming gross type ($n = 141$) (**c** and **d**), patients with ICC of the small duct subtype ($n = 68$) (**e** and **f**), and patients with ICC of the bile ductular subtype ($n = 22$) (**g** and **h**)

Table 2 Univariate and multivariate survival analyses of *DLEC1* methylation and the clinicopathological parameters with respect to cancer-specific survival

Parameter		Univariate analysis		Multivariate analysis	
		Hazard ratio (95% C.I.)	*P* value	Hazard ratio (95% C.I.)	*P* value
DLEC1 methylation	Absent ($n = 132$)				
	Present ($n = 40$)	0.405 (0.172–0.952)	0.038	0.280 (0.118–0.665)	0.004
N staging	pN0 ($n = 85$)				
	pN1 ($n = 39$)	2.207 (1.223–3.983)	0.009	2.125 (1.136–3.976)	0.018
Lymphatic emboli	Absent ($n = 102$)				
	Present ($n = 70$)	2.441 (1.380–4.318)	0.002	3.018 (1.650–5.519)	< 0.001
Perineural invasion	Absent ($n = 118$)				
	Present ($n = 54$)	0.395 (0.185–0.842)	0.016	0.206 (0.094–0.454)	< 0.001
pTNM staging			0.168		
	I ($n = 43$)	Reference			
	II ($n = 38$)	1.640 (0.741–3.629)	0.223		
	III ($n = 30$)	1.902 (0.173–1.710)	0.297		
	IV ($n = 61$)	2.956 (0.729–3.211)	0.261		
T staging			0.019		
	pT1 ($n = 48$)	Reference			
	pT2 ($n = 52$)	1.949 (0.982–3.869)	0.056		
	pT3 ($n = 47$)	2.379 (0.374–1.958)	0.713		
	pT4 ($n = 25$)	1.911 (0.123–1.522)	0.191		
M staging	pM0 ($n = 161$)				
	pM1 ($n = 11$)	1.516 (0.545–4.218)	0.425		
Multiplicity	Single ($n = 147$)				
	Multiple ($n = 25$)	1.289 (0.602–2.759)	0.513		
Gross type			0.073		
	Mass forming ($n = 141$)	Reference			
	Periductal ($n = 8$)	1.014 (0.001–99.999)	0.965		
	Intraductal growth ($n = 18$)	0.153 (0.019–0.989)	0.049		
	Mixed ($n = 5$)	0.882 (0.852–8.923)	0.090		
Histologic type			0.523		
	Bile ductular type ($n = 22$)	Reference			
	Small duct type ($n = 68$)	1.624 (0.614–4.296)	0.328		
	Large duct type ($n = 82$)	1.255 (0.475–3.318)	0.647		
Vascular invasion	Absent ($n = 95$)				
	Present ($n = 77$)	1.063 (0.603–1.875)	0.833		
Tumor border	Expanding ($n = 32$)				
	Infiltrative ($n = 140$)	2.668 (0.959–7.422)	0.060		
Tumor differentiation at invasive front			0.086		
	Well ($n = 23$)	Reference			
	Moderate ($n = 94$)	9.442 (1.288–69.208)	0.027		
	Poor ($n = 55$)	9.171 (1.219–68.986)	0.031		

Because *DLEC1* methylation was found mainly in the BD type and SD type but rarely in the LD type, a question was raised about the relationships between *DLEC1* methylation and clinicopathological features, whether the clinicopathological features associated with *DLEC1* methylation are attributed to the BD type or SD type. When we restricted the analyses to ICCs of the BD type and SD type, no significant associations with *DLEC1* methylation were found for sex, growth type, differentiation grade (at invasive front), mucin production, perineural invasion, or

Table 3 Univariate and multivariate survival analyses of *DLEC1* methylation and the clinicopathological parameters with respect to recurrence-free survival

Parameter		Univariate analysis		Multivariate analysis	
		Hazard ratio (95% C.I.)	*P* value	Hazard ratio (95% C.I.)	*P* value
DLEC1 methylation	Absent ($n = 132$)				
	Present ($n = 40$)	0.456 (0.271–0.766)	0.003	0.406 (0.239–0.690)	0.001
N staging	pN0 ($n = 85$)				
	pN1 ($n = 39$)	2.272 (1.484–3.481)	< 0.001	1.508 (0.959–2.371)	0.075
M staging	pM0 ($n = 161$)				
	pM1 ($n = 11$)	5.416 (2.751–10.664)	< 0.001	4.304 (2.129–8.698)	< 0.001
Multiplicity	Single ($n = 147$)				
	Multiple ($n = 25$)	2.203 (1.346–3.605)	0.002	1.911 (1.150–3.154)	0.012
Vascular invasion	Absent ($n = 95$)				
	Present ($n = 77$)	1.421 (1.202–1.680)	0.001	1.512 (1.011–2.260)	0.044
Tumor differentiation at invasive front			0.001		0.002
	Well ($n = 23$)	Reference		Reference	
	Moderate ($n = 94$)	5.811 (2.113–15.979)	0.001	4.852 (1.731–13.599)	0.003
	Poor ($n = 55$)	7.138 (2.541–20.052)	< 0.001	6.428 (2.234–18.493)	0.001
pTNM staging			0.001		
	I ($n = 43$)	Reference			
	II ($n = 38$)	1.529 (0.819–2.852)	0.182		
	III ($n = 30$)	1.902 (1.019–3.548)	0.043		
	IV ($n = 61$)	2.956 (1.720–5.079)	< 0.001		
T staging			0.016		
	pT1 ($n = 48$)	Reference			
	pT2 ($n = 52$)	1.791 (1.037–3.093)	0.037		
	pT3 ($n = 47$)	2.379 (1.386–4.073)	0.002		
	pT4 ($n = 25$)	1.911 (1.083–3.802)	0.027		
Gross type			0.017		
	Mass forming ($n = 141$)	Reference			
	Periductal ($n = 8$)	1.014 (0.412–2.497)	0.976		
	Intraductal growth ($n = 18$)	0.153 (0.048–0.485)	0.001		
	Mixed ($n = 5$)	0.882 (0.279–2.787)	0.830		
Histologic type			0.284		
	Bile ductular type ($n = 22$)	Reference			
	Small duct type ($n = 68$)	1.677 (0.865–3.251)	0.126		
	Large duct type ($n = 82$)	1.642 (0.855–3.151)	0.136		
Lymphatic emboli	Absent ($n = 102$)				
	Present ($n = 70$)	1.750 (1.194–2.565)	0.004		
Perineural invasion	Absent ($n = 118$)				
	Present ($n = 54$)	1.893 (1.282–2.796)	0.001		
Tumor border	Expanding ($n = 32$)				
	Infiltrative ($n = 140$)	1.650 (0.955–2.850)	0.072		

chronic hepatitis (Supplementary Table 3). However, BilIN was not found in ICCs of the SD type or BD type with *DLEC1* methylation, which was contrasted with the frequency of BilIN (20%, 10 of 50) in ICCs of the SD type or BD type with no *DLEC1* methylation ($P = 0.019$).

Discussion

ICC is an uncommon cancer in the world and therefore has not gained much attention from researchers. ICC and hepatocellular carcinoma (HCC) are staged according to the same TNM

staging system before the 7th edition of the AJCC/UICC classification provided a separate TNM staging system for ICC. However, the recent increase in the incidence of ICC in the western world has led to an increased number of research efforts to identify the determinants of prognosis [5, 21, 31]. The 7th edition of the AJCC/UICC classification was flawed in its T classification, which was evidenced by a study in which patients with T2b ICC had worse survival compared with patients with T3 or T4 ICC [27]. Because the 7th TNM staging system lacks robustness in predicting the outcome of patients with ICC and because the 8th TNM staging system needs validation for its effectiveness of risk stratification, molecular markers that have a significant role in predicting clinical outcome are necessary. Although studies in ICC generated a number of molecular markers which were significantly associated with the prognosis of patients with ICC, these markers were not validated or gave contradictory results in further studies [6]. In the present study, we found that *DLEC1* methylation could be utilized as a molecular biomarker to identify a subset with better prognosis in patients with ICC.

DLEC1 is located at 3p22-3p21.2, which is a hot spot for deletion in variable tissue types of human cancers, including HCC, renal cell carcinoma, non-small cell lung cancer, colon cancer, and prostate cancer [9, 11, 24, 25, 28–30, 36–38]. 3p22 is also a chromosome region that shows frequent long-range epigenetic silencing of serial genes, including *DLEC1*, in colorectal cancers [34]. Although the DLEC1 protein resides mainly in the cytoplasm, a relatively small amount of DLEC1 protein is present in the nucleus [11]. DLEC1 plays the role of tumor suppressor by decreasing colony formation [25], inducing G1 arrest or apoptosis [25, 36], or inhibiting of cell migration [36]. *DLEC1* methylation was shown to be associated with a more advanced T stage in HCC [25] or renal cell carcinoma [37] and a poor prognosis in patients with non-small cell lung cancer [28]. However, a little progress has been achieved in elucidating the biological role of the DLEC1 protein since the first discovery of the gene [9].

In the present study, the frequency of *DLEC1* methylation was significantly different between ICCs of the BD type or SD type and ICCs of the LD type (40.0% vs. 4.9%, respectively). In our previous studies, such a contrasting difference in the frequency of *DLEC1* methylation has been noted between HCCs ($n = 99$) and extrahepatic CCs (ECCs) ($n = 20$) (77.8% vs. < 5%, respectively) [17, 32]. Such findings raised the following question: whether the other 11 genes, which showed a significant difference in methylation frequencies between ICCs of the BD type or SD type and ICCs of the LD type, are differentially methylated between HCCs and extrahepatic CCs. To answer the question, we performed an additional study in which we analyzed 54 HCCs and 57 ECCs for their methylation statuses in 12 genes using the MethyLight assay (Supplementary Fig. 4). Of the six genes which were more frequently methylated in ICCs of the BD type or SD type than in ICCs of the LD type, five genes, including *DLEC1*, *RASSF1A*, *RIP3*, *SOCS3*, and *PTGS2*, exhibited more frequent methylation in HCC than in ECC. The six genes which were more frequently methylated in the ICCs of the LD type than in the ICCs of the BD type or SD type showed more frequent methylation in ECCs than in HCCs (Supplementary Table 4). To identify whether methylation in the five genes (*DLEC1*, *RASSF1A*, *RIP3*, *SOCS3*, and *PTGS2*) was related to the presence of chronic hepatitis in background liver, we compared the mean number of methylated genes between ICCs of the BD type or SD type with and without chronic hepatitis. The number of methylated genes was significantly higher in ICCs with chronic hepatitis than in ICCs without chronic hepatitis (2.5 vs. 1.8, $P = 0.009$, Student's t test). These findings led us to speculate that DNA methylation in these five genes might signify hepatic progenitor cell of origin in ICCs of the BD type or SD type.

In the present study, the histologic subtype was not a significant prognostic parameter in the univariate analysis. Instead of the histologic subtype, *DLEC1* methylation was found to be an independent prognostic parameter in both CSS and RFS, which suggests that *DLEC1* methylation may be used for the identification of a subset of ICC with a better clinical outcome. However, *DLEC1* methylation was not prognostic in ICCs of the BD type or LD type except for ICCs of the SD type. For ICCs of the LD type, *DLEC1* methylation was a rare event and thus, prognostic utility could not be determined. The reason why *DLEC1* methylation was not prognostic in ICCs of the BD type although frequency of *DLEC1* methylation was similar between ICCs of the BD type and SD type is unclear. At present, we could not provide a satisfactory explanation for the reason, which is partly attributed to the fact that biological role or function of DLEC1 protein is not well characterized. The result that *DLEC1* methylation was not prognostic in ICCs of the BD type was akin to the fact that *DLEC1* methylation is not prognostic in HCCs [17]. It is likely that regardless of *DLEC1* methylation status, ICCs of the BD type might originate from hepatic progenitor stem cells. However, ICCs of the SD type might differ in the cell origin depending on *DLEC1* methylation status and ICCs of the SD type with *DLEC1* methylation might originate from hepatic progenitor cells. Thus, clinical behavior is likely to be similar between ICCs of the SD type with *DLEC* methylation and ICCs of the BD type. When we analyzed ICCs of the BD type and SD type for their prognosis according to *DLEC1* methylation status, ICCs of the SD type with no *DLEC1* methylation showed contrasting clinical outcome compared with ICCs of the SD type with *DLEC1* methylation, ICCs of the BD type with and without *DLEC1* methylation.

In summary, because *DLEC1* methylation was found mainly in ICCs of the BD type or SD type which were heterogeneous for *DLEC1* methylation and ICCs of the SD type exhibited different clinical outcomes depending on the *DLEC1*

methylation status, *DLEC1* methylation could be utilized as a biomarker to identify a subset of ICCs of the SD type with a better clinical outcome in CSS and RFS. Future studies are needed to investigate whether *DLEC1* methylation is a signature of a hepatic progenitor origin in ICCs and whether ICCs with *DLEC1* methylation have distinct genomic alterations.

Contributions YK performed the analysis, collected part of the data, and wrote part of the paper; KL collected the data, contributed data, conceived the work, and performed the analysis; SJ, NYC and XW performed the analysis, collected the data, contributed to the interpretation of data; GHK conceived the work, wrote the draft, and interpreted the data. All the authors (YK, KL, SJ, XW, NYC, GHK) will participate in the final approval of the version to be published and express agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding sources This work was financially supported by grants from the National Research Foundation (NRF) funded by the Korean Ministry of Science, ICT and Future Planning (2016M3A9B6026921), a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute funded by the Korean Ministry of Health and Welfare (HI14C1277), and a grant from SNUH Research Fund (0320170070 (2017-1176)).

Compliance with ethical standards

This study was approved by the institutional review board of Seoul National University Hospital (IRB No. 1804-168-942). Under the condition of retrospective archival tissue collection and patient data anonymization, our study was exempted from the acquisition of informed consent from patients.

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

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